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Therapeutic potential of human adipose tissue-derived multi-lineage progenitor cells in liver fibrosis

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ABSTRACT

Introduction: Liver fibrosis is characterized by excessive accumulation of extracellular matrix. In a mouse model of liver fibrosis, systemic injection of bone marrow mesenchymal stem cells (BM-MSCs) was considered to rescue the diseased phenotype. The aim of this study was to assess the effectiveness of human adipose tissue-derived multi-lineage progenitor cells (hADMPCs) in improving liver fibrosis.
Methods and results: hADMPCs were isolated from subcutaneous adipose tissues of healthy volunteers and expanded. Six week-old male nude mice were treated with carbon tetra-chloride (CCl_4) by intraperitoneal injection twice a week for 6 weeks, followed by a tail vein injection of hADMPCs or placebo control. After 6 more weeks of CCl_4 injection (12 weeks in all), nude mice with hADMPCs transplants exhibited a significant reduction in liver fibrosis, as evidenced by Sirius Red staining, compared with nude mice treated with CCl_4 for 12 weeks without hADMPCs transplants. Moreover, serum glutamic pyruvate transaminase and total bilirubin levels in hADMPCs-treated nude mice were lower levels than those in placebo controls. Production of fibrinolytic enzyme MMPs from hADMPCs were examined by ELISA and compared to that from BM-MSCs. MMP-2 levels in the culture media were not significantly different, whereas those of MMP-3 and -9 of hADMPCs were higher than those by BM-MSCs.
Conclusion: These results showed the mode of action and proof of concept of systemic injection of hADMPCs, which is a promising therapeutic intervention for the treatment of patients with liver fibrosis.

1. Introduction

Various conditions such as viral hepatitis, chronic alcohol abuse, metabolic diseases, autoimmune diseases and bile duct epithelial injury can cause liver fibrosis [1,2]. Liver fibrosis is reversible, whereas cirrhosis, the end-stage result of fibrosis, is in general irreversible [3]. Liver fibrosis is characterized by excessive accumulation of extracellular matrix, with the formation of scar tissue encapsulating the area of injury [4]. The prognosis of patients with liver fibrosis is poor, but liver transplantation seems to improve the prognosis [5,6]. However, limited numbers of donor livers are available for the millions of patients who need them worldwide [7]. Thus, there is a need for novel therapeutic approaches.

Recently, cell therapy has been proposed as an attractive tool for treatment of patients with severe liver disease [8–13]. Stem/progenitor cells, which possess certain characteristics including self-renewal, proliferation, longevity, and differentiation, are valuable in cell therapy [14]. Several groups have demonstrated the effectiveness of bone marrow-derived mesenchymal stem cells (BM-MSCs) in animal models of liver fibrosis and cirrhosis [15–18]. However, others have reported the lack of any changes in the extent of liver fibrosis or liver function tests following the use of BM-MSCs in a rat model of severe chronic liver injury [19]. Thus, the therapeutic efficacy of BM-MSCs transplantation remains controversial at present [19].

Adipose tissue-derived progenitor/stem cells are an attractive cell source for cell therapy of liver fibrosis, based on several properties of these cells; (1) ample production of fibrinolytic enzymes and cytokines [20], (2) ease of obtaining stem cells compared to other tissue-specific stem cells including BM-MSCs, [21]. The use of human adipose tissue-derived multi-lineage progenitor cells (hADMPCs) supports the view that cytokine production could mediate the therapeutic actions of hADMPCs in liver fibrosis.

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In the present study, we investigated the efficacy of treatment using hADMPCs in nude mice with CCl_4-induced chronic liver dysfunction and the mechanism of their action in improvement of liver fibrosis.

2. Materials and methods

2.1. Adipose tissue

Adipose tissue samples were resected from 7 human subjects during plastic surgery (all females, age, 20–60 years) as excess discards. About 10–50 g subcutaneous adipose tissue was collected from the sample of each subject. All subjects provided informed consent. The protocol was approved by the Review Board for Human Research of Kobe University, Graduate School of Medicine, Osaka University, Graduate School of Medicine and National Institute of Biomedical Innovation, Japan.

2.2. Isolation and expansion of hADMPCs

hADMPCs were prepared as described previously [8–10]. Briefly, the resected excess adipose tissue was minced and then digested at 37 °C for 1 h in Hank's balanced salt solution (HBSS, GIBCO Invitrogen, Grand Island, NY) with Liberase (Roche Diagnostics, Germany) as indicated by the manufacturer. Digests were filtered through a cell strainer (BD Bioscience, San Jose, CA) and centrifuged at 800×*g* for 10 min. Red blood cells were excluded using density gradient centrifugation with Lymphoprep ($d = 1.077$; Nycomed, Oslo, Norway), and the remaining cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO Invitrogen) with 10% defined fetal bovine serum (FBS, Biological Industries, Israel) for 24 h at 37 °C. Following incubation, the adherent cells were washed extensively and then treated with 0.2 g/l ethylenediaminetetraacetate (EDTA) solution (Nacalai Tesque, Kyoto, Japan). The resulting suspended cells were replated on retronectin (RN)-coated dishes (Takara, Kyoto, Japan) in SteMedis (Nipro, Osaka, Japan), 1× insulin-transferring selenium (Nipro, Osaka), 1 nM dexamethasone (MSD, Tokyo, Japan), 100 μM ascorbic acid 2-phosphate (Sawai Pharmaceuticals Co., Osaka), 10 ng/ml epidermal growth factor (EGF, PeproTec, Rocky Hill, NJ), and 5% FBS (FBS, Biological Industries, Israel). The culture medium was changed twice a week and then the cells were applied for the experiments after 5–6 passages.

2.3. Flow cytometric analysis of hADMPCs

hADMPCs were characterized by flow cytometry. Cells were detached and stained with anti-human CD31, CD34, CD44, CD45, CD56, CD73, CD90, CD105 or CD166 antibodies (BD Lyoplate™ Screening Panels, BD Bioscience, San Jose, CA). Isotype-identical antibodies served as controls. After washing with Dulbecco's phosphate-buffered saline (PBS, Nacalai Tesque), cells were incubated with PE-labeled goat anti-mouse Ig antibody (BD PharMingen) for 30 min at 4 °C. After three washes, the cells were resuspended in PBS and analyzed by flow cytometry using a guava easyCyte flow cytometry systems (Merck Millipore, Darmstadt, Germany).

2.4. Adipogenic, osteogenic and chondrogenic differentiation procedure

Tri-lineage differentiation was examined as described previously [22]. Briefly, for adipogenic differentiation, the cells were cultured in Differentiation Medium (Zen-Bio, Inc.). After three days, half of the medium was replaced with Adipocyte Medium (Zen-Bio, Inc.) every two days. Five days after differentiation, characterization of adipocytes was confirmed by microscopic observation of intracellular lipid droplets after Oil Red O staining. Osteogenic differentiation was induced by culturing the cells in DMEM containing 10 nM dexamethasone, 50 mg/dl ascorbic acid 2-phosphate, 10 mM β-glycerophosphate (Sigma), and 10% FBS. Differentiation was examined by Alizarin red staining. For chondrogenic differentiation, 2×10^5 cells of the hADMPCs were centrifuged at 400×*g* for 10 min. The resulting pellets were cultured in chondrogenic medium (α-MEM supplemented with 10 ng/ml TGF-β, 10 nM dexamethasone, 100 M ascorbate, and 10 μl/ml 100× ITS Solution) for 14 days. For Alcian Blue staining, nuclear counter-staining with Weigert's hematoxylin was followed by 0.5% Alcian Blue 8GX for proteoglycan-rich cartilage matrix.

2.5. Animal model of liver fibrosis and cell administration

Chronic liver fibrosis was induced in nude mice using the procedure described previously [23,24] with some modification. Briefly, 6-week-old male nude mice (body weight of 20–30 g purchased from CLEA, Tokyo) were treated with a mixture of CCl_4 (Wako Pure Chemicals, Osaka) (0.3 ml/kg) and olive oil (Wako Pure Chemicals) (1:1 vol/vol) by intra-peritoneal injection twice a week for 6 weeks, and this was followed by a tail vein injection of hADMPCs (1.0×10^6 cells/kg body weight, $n = 4$) or placebo control ($n = 5$), and followed by 6 more weeks of CCl_4 treatment.

2.6. Liver function tests and histological analysis

Blood specimens were collected by cardiac puncture at the end of the experiment. Measurement of serum albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total-bilirubin levels by routine laboratory methods was outsourced to Oriental Yeast Co. (Shiga, Japan).

Hematoxylin and eosin (H&E) staining and Sirius Red (SR) staining were performed to determine the extent of liver inflammation and fibrosis. The stained slides were viewed on a BioZero laser scanning microscope (Keyence, Osaka). The area of liver fibrosis was quantified with SR staining. Briefly, the fibrotic area (red staining) was assessed at 40× magnification using computer-assisted image analysis with All-in-One analysis software (Keyence, Osaka). Sixteen fields were randomly selected for each group.

2.7. Measurement of MMP-2, -3 and -9 production by hADMPCs

One million cells of hADMPCs and BM-MSCs (DS Pharma Biomedical, Osaka) were seeded onto 6 well plates and then cultured for 24 h. The supernatants were harvested, centrifuged, and frozen at −80 °C until analysis. MMP-2, MMP-3 and MMP-9 were measured by enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN) using the instructions supplied by the manufacturer.

2.8. Statistical analysis

Serum parameters and fibrotic area are presented as mean ± SD. Differences between groups were assessed for statistical significance by the Student's *t*-test, with $p < 0.05$ considered statistically significant.

3. Results

3.1. Characterization of hADMPCs

Flow cytometry was used to assess markers expressed by hADMPCs (Fig. 1A). The cells were negative for markers of

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hematopoietic lineage (CD45) and hematopoietic stem cells, CD34 and CD133. They were also negative for CD31, an endothelial cell-associated marker, and c-Kit (CD117), a cell surface antigen. However, they stained positively for several surface markers characteristic of mesenchymal stem cells, but not embryonic stem (ES) cells, such as CD29, CD44 (hyaluronan receptor), CD73 and CD105 (endoglin).

Next, we examined the adipogenic, osteogenic and chondrogenic differentiation potentials of hADMPCs. Adipogenic differentiation was confirmed by accumulation of intracellular lipid droplets stained with Oil Red O (Fig. 1B). Differentiation and induction of hADMPCs was associated with increase in the amount of Oil Red O-stained lipid droplets, indicating that hADMPCs can differentiate into adipocytes. Osteogenic induction was examined by Alizarin red S staining (Fig. 1B). Induction of hADMPCs for osteogenesis was associated with Alizarin red S staining and appearance of mineralized nodules. The chondrogenic potential of hADMPCs is shown in Fig. 1B. Induction of chondrogenesis by pellet culture resulted in staining of extracellular matrices of hADMPCs-derived pellet-cultured chondrocytes for Alcian Blue, indicating the chondrogenic differentiation potential of hADMPCs. These results confirmed the tri-lineage differentiation potential of hADMPCs and the mesenchymal stem cell properties of hADMPCs.

3.2. Effects of hADMPC on CCl_4-induced chronic liver dysfunction in nude mice

We adopted the CCl_4-induced chronic mouse fibrosis model in this study rather than the CCl_4-induced acute model because CCl_4-induced acute liver fibrosis resolves spontaneously [25]. For this purpose, 9 male nude mice were injected intraperitoneally with CCl_4 twice weekly for 6 weeks, and then divided into two groups, 4 animals received hADMPCs transplantation via the tail vein and the other 5 vehicle control received Ringer's solution with 1/30 volume of heparin. All animals were followed for 6 weeks after the last injection (a).

H&E staining of liver sections showed reduced hepatocyte vacuolar degeneration in hADMPC-transplanted CCl_4-injured mice compared with the control (Fig. 2B). The peri-lobular regions were the main areas affected by CCl_4 hepatotoxicity while the centrilobular regions seemed to be the least affected. These findings suggest intact albumin secretion, which was confirmed by Sirius Red (SR) staining of control liver sections. SR staining of sections from hADMPC-transplanted mice showed mild liver fibrosis, while that of sections from control group mice showed moderate fibrosis (Fig. 2B). Quantitative image analysis of the fibrotic area in SR-stained sections confirmed the efficacy of hADMPC-transplantation on liver fibrosis. The mean fibrotic area was significant lower in hADMPC-transplanted CCl_4-injured mice ($1.8 \pm 1.1\%$ of fibrotic areas) than control mice ($10.9 \pm 3.9\%$ of fibrotic areas) ($p < 0.05$), indicating that hADMPC-transplantation ameliorated liver fibrosis and increased the area containing hepatocytes (c).

3.3. Functional recovery of liver damage following transplantation of hADMPCs

We next evaluated the effects of cell transplantation on the extent of liver injury and liver function. Serum transaminase levels (AST and ALT) were significantly higher in mice with liver damage (control), but the increase was attenuated by hADMPCs transplantation (Fig. 3A and B). These results confirmed the effectiveness of hADMPCs in the treatment of liver damage associated with fibrosis.

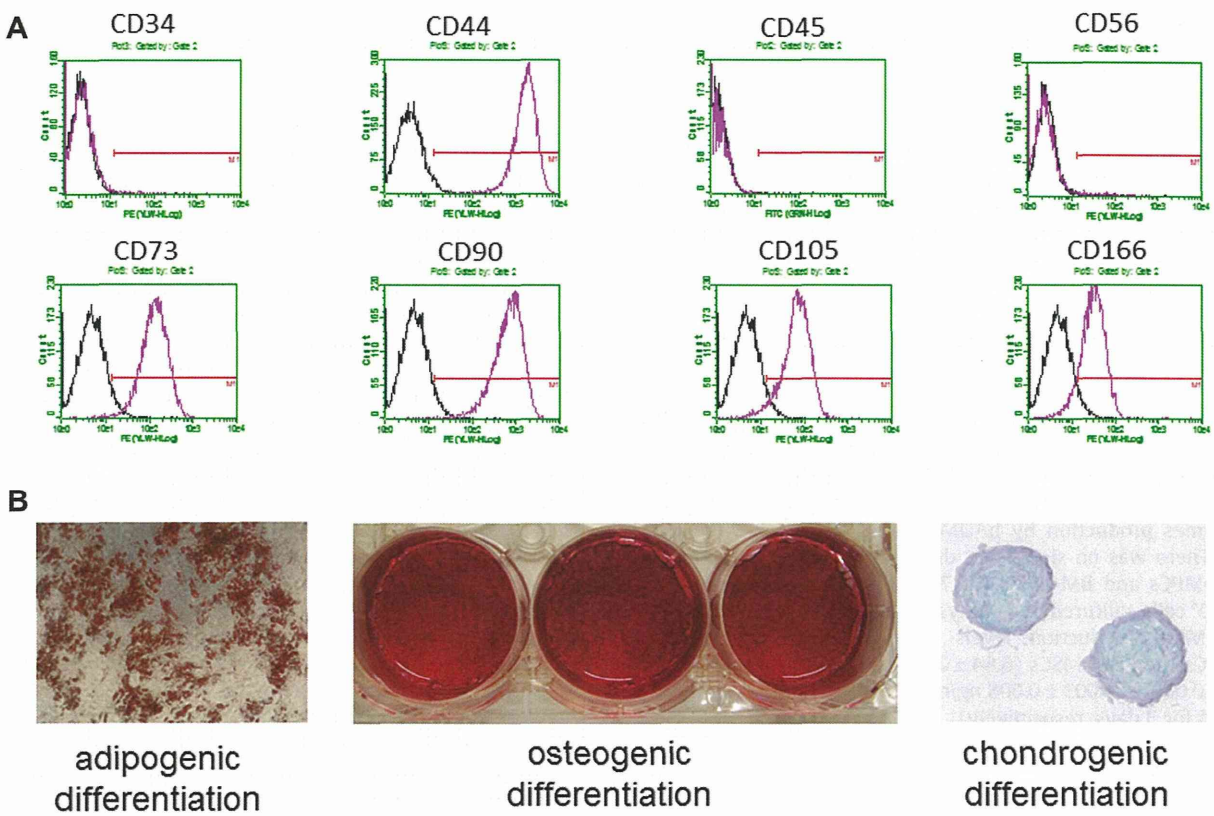

Fig. 1. Characterization of hADMPCs. (A) Flow cytometric characterization of hADMPCs. (B) An isotype-matched negative control indicated as red curve. (C) Adipogenic, osteogenic and chondrogenic differentiation potentials of hADMPCs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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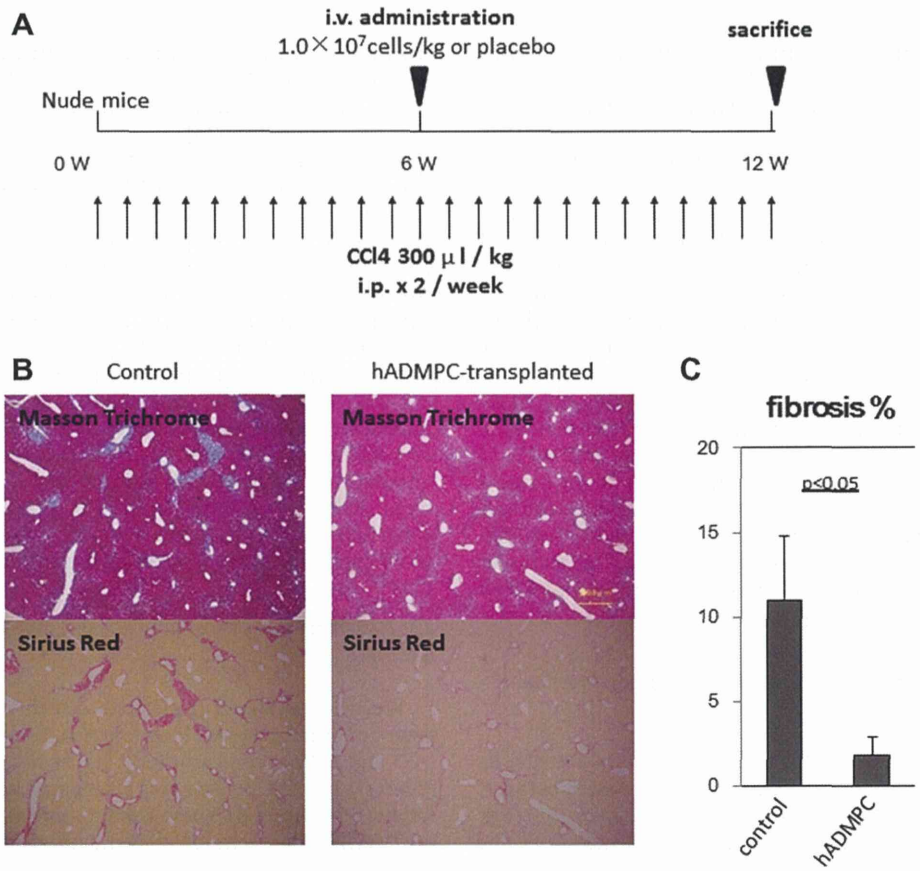

Fig. 2. Assessment of liver fibrosis in hADMPC-transplanted nude mice and controls. (A) Diagram of the treatment protocol. (B) Extracellular deposition of collagen fibers stained with Sirius Red. (C) Quantification of collagen by image analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Interestingly, serum albumin level remained high after hADMPCs, similar to the control (Fig. 3C). These results could be explained by damage of the centrilobular region, the main site of albumin production. Considered together, the results suggest the beneficial effects of hADMPCs in attenuating liver damage and recovery of liver function.

3.4. hADMPCs-induced functional recovery is mediated by MMP release

Finally, we analyzed the mechanism of the hepatoprotective effect of hADMPCs. For this purpose, we measured the amount of the fibrinolytic enzymes, MMP-2, MMP-3 and MMP-9, secreted by hADMPCs by ELISA (Fig. 4). After 3-day culture, the amounts of enzymes production by hADMPCs and BM-MSCs were measured. There was no significant difference in MMP-2 production by hADMPCs and BM-MSCs (59.7 ± 2.3 vs 58.3 ± 0.0 ng/ml from 1.0×10^4 cells cultured for 3 days). On the other hand, MMP-3 and MMP-9 production levels were significantly higher in hADMPCs than BM-MSCs (6.84 ± 2.3 vs 0.03 ± 0.0 ng/ml, $p < 0.05$, 0.462 ± 0.015 vs 0.003 ± 0.008 ng/ml, $p < 0.05$, from 1.0×10^4 cells cultured for 3 days, respectively).

4. Discussion

The major finding of the present study was improvement of liver fibrosis in CCl_4-induced mice after systemic administration of hADMPCs, and that this effect was mediated, at least in part, through the production of fibrinolytic MMP-2, -3, and -9, from hADMPCs, suggesting that these cells could be particularly effective in resolving liver fibrosis.

Liver transplantation is an established treatment for severe liver cirrhosis, although the number of patients who could benefit from such treatment is small due to the limited number of donors [5]. Cell therapy has been proposed as an alternative and attractive tool for treating patients with severe liver disease [8–13]. Among the cell therapy tested so far, hepatocyte replacement therapy had been examined. Isolated hepatocytes from human liver [13], regenerated hepatocyte-like or –progenitor cells from embryonic, induce pluripotent [26,27], or hepatic progenitor cells [11], and *in situ* reprogrammable cells (9, 10) have been tested for their efficacy in animal models. The strategy has also been successful in clinical trials involving patients with certain inherited diseases [28]. Although large numbers of hepatocytes or hepatocyte-like cells are needed for meaningful cure and there should be no room for the cells in fibrotic hepatic parenchyma to engraft, such replacement therapies, however, do not seem to be clinically fruitful for liver fibrosis. We hypothesized that fibrolytic enzymes produced by hADMPCs could be useful for treatment of liver fibrosis, and therefore shifted the treatment strategy to improvement of liver fibrosis with cell-based fibrinolytic enzymes delivery. In this strategy, hADMPCs derived-MMPs should produce lysis of excess extracellular matrices and make room for the patient's own proliferative hepatocytes.

To establish the cell-based fibrinolytic enzyme delivery therapy as first line next to liver transplantation, some challenging issues

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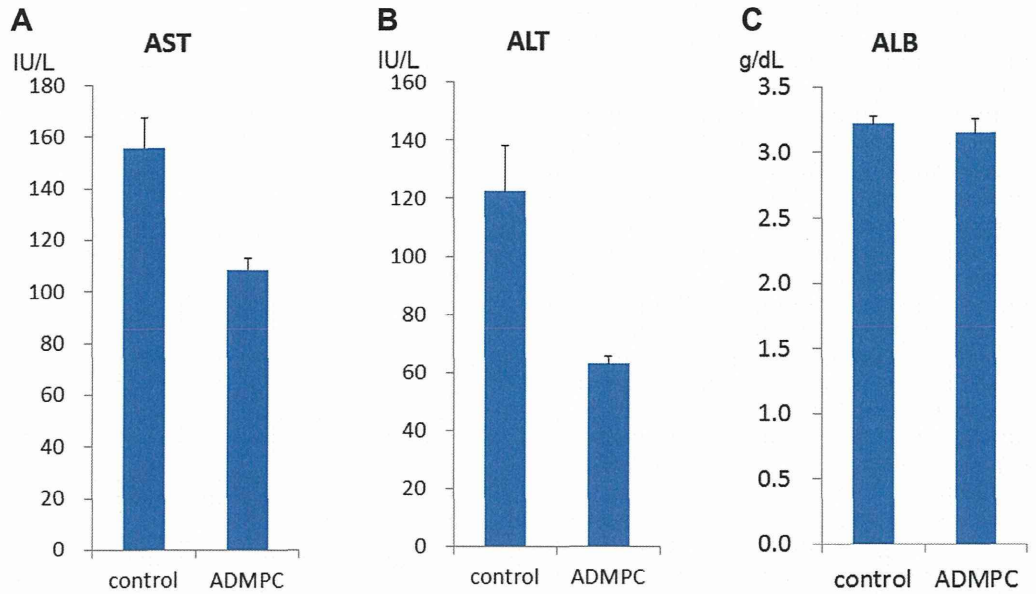

Fig. 3. Examination of serum parameters. (A, B) Transaminase (AST and ALT), (C) Serum albumin. Data are mean ± SD. C: control mice; T: mice transplanted mice with hADMPCs.

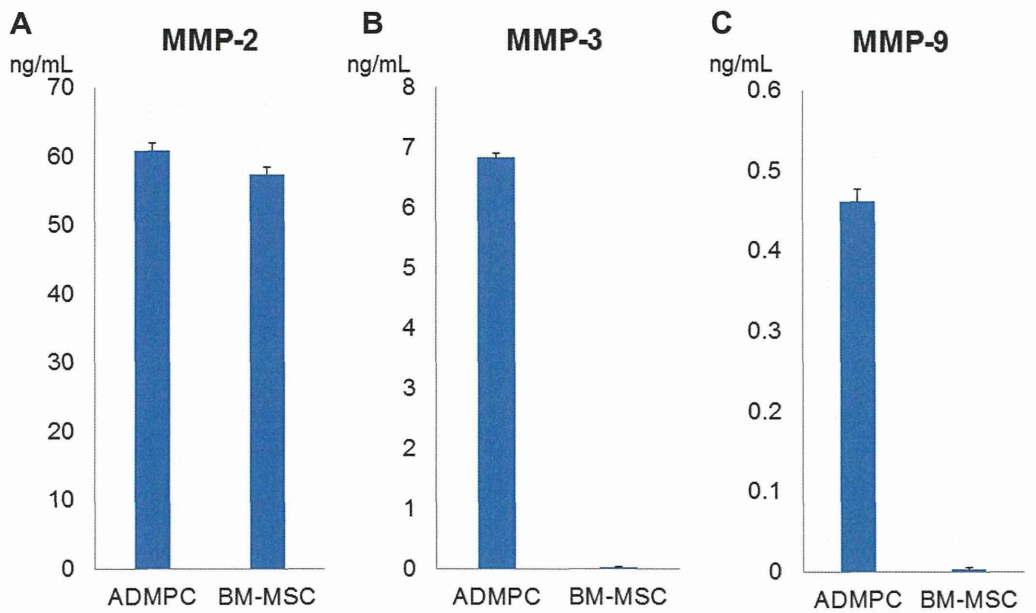

Fig. 4. Quantitative analysis of MMP-2, MMP-3 and MMP-9 level produced by hADMPCs and BM-MSCs. The amount of MMP-2 (A), MMP-3 (B) and MMP-9 (C) after 3 days of culture.

should be dealt with; (1) the cells should be obtained easily and ethically in large quantities, (2) the cells should improve liver fibrosis and liver panel, and (3) the cells act as vehicle for the delivery of MMPs.

The first issue is whether the cells could be obtained easily and ethically in large quantities. hADMPCs is favorable for the therapy because adipose tissue, from which ADMPCs are obtained, is easily and safely accessible and large quantities of the tissues can be obtained without serious ethical issues, since liposuction surgery yields from 100 ml to >3 L of lipoaspirate tissue [8–10]. Therefore, hADMPCs can potentially be applied not only for autologous but also allogenic cell-based enzyme delivery in the future. Based on the above advantages, hADMPCs represent a potentially promising source of cells for the therapy.

Second, we need to show that hADMPCs-administration results in improvement of liver fibrosis and liver panel, as proof-of-concept of therapy. As shown in Fig. 2, hADMPC significantly improved liver fibrosis in CCl_4-treated nude mice (a model of chronic liver cirrhosis). The treatment also resulted in improvement of serum transaminase levels. In this model, massive fibrosis was mainly noted in the peri-hepatic lobular region but not in the centrilobular regions surrounding the central veins. Albumin is known to be mainly produced by hepatocytes in the centrilobular region. This is the most likely reason for the lack of difference in serum albumin

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levels between hADMPC-transplanted animals and controls. These results indicate that hADMPCs transplantation showed the proof-of-concept to liver dysfunction associated with fibrosis.

Finally, an important issue in this kind of therapy is whether the cells secrete sufficient amount of MMPs. One study reported that matrix metalloproteinase gene delivery could decrease collagen fibers and reduce liver fibrosis [29]. The mode of action was considered to be the strong expression MMPs on the transplanted cells, indicating that MMPs-producing cells other than BM-MSCs [30] are suitable for use for the cell-based enzyme delivery. The present study showed that hADMPCs expressed MMP-2, -3 and -9 (Fig. 2). There was no significant difference in MMP-2 production between hADMPC and BM-MSCs. However, the production of MMP-3 and MMP-9 from hADMPCs was superior to that from BM-MSCs. MMP-3 and MMP-9 are known to lyse collagen types III and I, which are major compartment of liver fibrotic lesion [29]. These data highlight the potential effectiveness of hADMPCs in the treatment of liver fibrosis and the superiority of hADMPCs compared to other therapies.

In conclusion, the present study demonstrated that systemic administration of hADMPCs significantly attenuated liver fibrosis and improved liver function, and that the therapeutic effect of hADMPCs was in part due the secretion of fibrinolytic enzymes, MMPs. These proofs of concept and mode of action prompted us to choose hADMPCs for cell therapy of liver fibrosis. hADMPCs therapy, as cell-based enzyme delivery therapy, has the potential to be an effective source of inducers that support liver regeneration.

Declaration

The authors declare no conflict of interest.

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Role of Notch Signaling in the Maintenance of Human Mesenchymal Stem Cells Under Hypoxic Conditions

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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 the 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. Five percent 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 on 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 that are necessary for tissue regeneration [7–11]. In addition, due to 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

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intrinsic reactive oxygen species (ROS) production [14,18,19]. Since 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 iPSCs 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; for example, 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 on 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 at investigating 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]. Five percent oxygen activated Notch signaling, resulting in 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.

Materials and Methods

Adipose tissue samples

Subcutaneous adipose tissue samples (10–50 g each) were resected during plastic surgery from five female and two 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 [11,32–34] and maintained in a medium containing 60% DMEM low glucose, 40% MCDB-201 medium (Sigma Aldrich), 1× insulin-transferrin-selenium (Life Technologies), 1 nM dexamethasone (Sigma Aldrich), 100 mM ascorbic acid 2-phosphate (Wako), 10 ng/mL epidermal growth factor (PeproTech), 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) 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 twice 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 ROS 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) 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 six-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 [34]. Briefly, hADMPCs were harvested and resuspended 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) 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), or CD166 (Beckman Coulter). Nonspecific 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

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) accroding to the manufacturer's instructions. cDNA was generated from 1 μg of total RNA using the Verso cDNA Synthesis Kit (Thermo Scientific) and purified using the MinElute PCR Purification Kit (Qiagen). Quantitative polymerase chain reaction (Q-PCR) analysis was conducted using the SsoFast EvaGreen supermix (Bio-Rad) 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 on request.

Western blot analysis

Whole cell extracts were prepared by washing cells with ice-cold PBS and lysing them with M-PER Mammalian Protein Extraction Reagent (Thermo Scientific Pierce) 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 membranes (Immobilon-P; Millipore), and probed with antibodies against cleaved Notch1 (#2421; Cell Signaling Technology), HIF-1α (#610959; BD Bioscience), 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) using BZ Analyzer Software (Keyence).

Adipogenic, osteogenic, and chondrogenic differentiation procedures

For adipogenic differentiation, cells were cultured in differentiation medium (Zen-Bio). 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 a 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) 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) 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), 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), 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 (Fig. 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, EdU, an alternative to 5-bromo-2′-deoxyuridine (BrdU), was incorporated into the

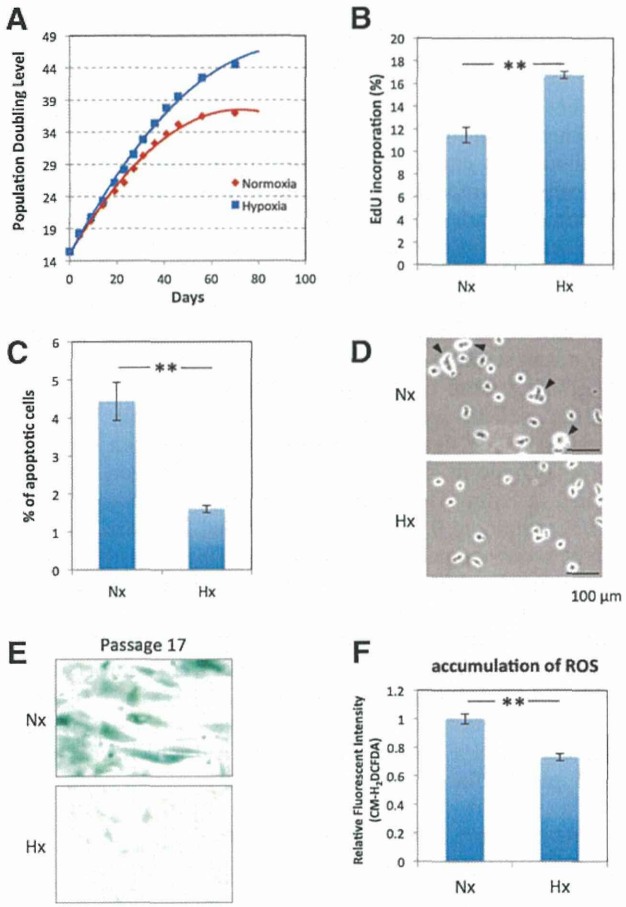

FIG. 1. Hypoxia increases proliferation capacity and decreases senescence in tissue-derived multilineage progenitor cells (hADMPCs). **(A)** Growth profiles of hADMPCs under normoxic (*red square*) and hypoxic (*blue square*) conditions. The population doubling level (PDL) was determined to be 0 when cells were isolated from human adipose tissue. Cells were maintained until they reached PDL13–15 (passage 3) and then split into four aliquots of equal cell densities. PDL was calculated based on the total cell number at each passage. **(B)** Detection of normoxic (Nx) and hypoxic (Hx) cells by flow cytometry after incorporation of EdU. **(C)** Percentages of apoptotic cells with sub-G1 DNA under Nx and Hx conditions. The results are presented as the mean of three independent experiments. **(D)** hADMPCs cultured under Nx and Hx conditions were harvested by trypsin-EDTA and then imaged using a phase-contrast microscope. *Arrowheads* indicate cells with a larger and more irregular shape. **(E)** Cells expanded under Nx and Hx conditions were stained with SA-β-gal. **(F)** Cellular reactive oxygen species detection by the oxidative stress indicator CM-H_2DCFDA in hADMPCs under Nx or Hx. Data are presented as the mean fluorescence intensity of three independent experiments. Error bars indicate SD. $^{**}P < 0.01$ indicates significant difference (independent *t*-test) between Nx and Hx. Scale bars; 100 μm. Color images available online at www.liebertpub.com/scd

genomic DNA of the hADMPCs, and the amount of incorporated EdU was quantified by flow cytometry. As shown in Fig.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 (Fig. 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 (Fig. 1D), which suggests that the Hx culture condition prevented hADMPCs from entering senescence [35]. To further investigate this phenomenon, cellular senescence was measured by staining for SA-β-Gal, which revealed that SA-β-Gal activity was increased in Nx-cultured hADMPCs at passage 17 (Fig. 1E). Since it has been hypothesized that senescence results from oxidative stress [20], accumulation of ROS in hADMPCs was detected using the nonfluorescent probe, CM-H_2DCFDA. Flow cytometry analysis revealed that ROS were generated at higher levels in hADMPCs when cultured in the Nx condition (Fig. 1F), suggesting that reduced production of ROS in the Hx condition may prevent the cells from entering replicative senescence.

Hypoxic culture maintains some MSC 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, and CD133 (Fig. 2 and data not shown). These data were consistent with previous reports describing the expression profiles of cell surface markers of hMSCs [36,37]. 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 (Fig. 3A, 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 maintained 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 (greater than twofold) in the Hx culture condition (Fig. 4A). 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