RESEARCH ARTICLE

Notch2 Signaling Regulates the Proliferation of Murine Bone Marrow-Derived Mesenchymal Stem/Stromal Cells via c-Myc Expression

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Abstract

Mesenchymal stem/stromal cells (MSCs) reside in the bone marrow and maintain their stemness under hypoxic conditions. However, the mechanism underlying the effects of hypoxia on MSCs remains to be elucidated. This study attempted to uncover the signaling pathway of MSC proliferation. Under low-oxygen culture conditions, MSCs maintained their proliferation and differentiation abilities for a long term. The Notch2 receptor was up-regulated in MSCs under hypoxic conditions. Notch2-knockdown (Notch2-KD) MSCs lost their cellular proliferation ability and showed reduced gene expression of hypoxia-inducible transcription factor (HIF)-1α, HIF-2α, and c-Myc. Overexpression of the c-Myc gene in Notch2-KD MSCs allowed the cells to regain their proliferation capacity. These results suggested that Notch2 signaling is linked to c-Myc expression and plays a key role in the regulation of MSC proliferation. Our findings provide important knowledge for elucidating the self-replication competence of MSCs in the bone marrow microenvironment.

Introduction

Mesenchymal stem/stromal cells (MSCs) have self-renewal and multipotent differentiation abilities *in vitro* and are an attractive candidate for regenerative medicine strategies [1]. Therefore, several clinical studies utilizing MSCs in degenerative diseases are underway all over the world. MSCs were identified in the bone marrow (BM) [2], dental pulp [3], adipose tissue [4],

synovium [5], and other tissues [6] based on their ability to form colony-forming unit fibro-blasts (CFU-Fs) and their surface markers [7, 8]. CFU-Fs have the potential to differentiate into osteoblasts, adipocytes, and chondrocytes [1, 9]. MSCs have been obtained from cell culture studies using normoxic conditions (an oxygen concentration of ~20%). However, the local oxygen concentration in murine BM is quite low, and several studies demonstrate that culturing BM stem cells under hypoxic conditions is more advantageous for cell proliferation [10, 11]. The mechanism underlying the effects of hypoxia on MSC proliferation and differentiation abilities remains to be elucidated.

Hypoxia regulates cell division and differentiation in stem cell populations [12]. Recent reports suggest that hypoxia regulates the quiescence of hematopoietic stem cells (HSCs) residing in the BM niche [11, 13]. Moreover, hypoxia-inducible transcription factors (HIFs) are increasingly recognized for their capacity to direct the homeostasis of other populations of stem cells without cellular senescence [12, 14]. Downregulation of either HIF-1a or HIF-2a dramatically affects MSC propagation and differentiation to adipocytes [10]. In addition, Notch signaling is also thought to play an important role in maintaining the undifferentiated status of stem cells [15–17]. Myogenic cell lines are maintained the immature state under hypoxic condition through crosstalk with Notch signaling [18]. Deletion of Notch signaling components in mesenchymal tissue reduces the number of MSCs in young mice [19]. HIF-1α and Notch signaling are closely related and induce cell proliferation [20]. Notch expression being activated through the HIF-1 under hypoxic condition [21]. Based on these results, hypoxic culture conditions and Notch signaling may be involved in maintaining MSC phenotype.

Prolonged culture of MSCs on plastic reduces their proliferative ability and causes them to change into mature phenotypes [22, 23]. MSC culture media has been supplemented with different growth factors and the culture conditions have been varied in an attempt to overcome this issue [24]. The use of hypoxic culture conditions is key methods for long-term culture of stem cells to promote proliferation and to maintain their multipotent state. In the present study, we aimed to explore whether Notch signaling is involved in the regulation of murine BM-MSC proliferation under hypoxic conditions. Using flow cytometry-based MSC isolation methods, we demonstrated that Notch2 signaling controls the proliferation of purified MSCs. Overexpression of the c-Myc gene in Notch2-knockdown (Notch2-KD) MSCs allowed the cells to regain their proliferation capacity. These data showed that Notch2-c-Myc signaling is a key factor in the regulation of MSC proliferation.

Materials and Methods

Animals

Adult C57BL6J wild-type mice (6–8 weeks old: female) were purchased from Sankyo Labo Service Corporation (Tokyo, Japan). All experimental procedures were approved by the Ethics Committee of Keio University (Tokyo, Japan) and were performed in accordance with the ARRIVE guidelines for reporting animal research. Animals were verified completely non-responsive to stimuli before euthanasia by cervical dislocation.

Detection of cellular hypoxia

To evaluate the environmental oxygen conditions for MSCs in the murine BM, BM-MSCs were isolated from adult C57BL6 wild-type mice after intraperitoneal injection of pimonidazole hydrochloride (Hypoxyprobe™; NPI Inc., Burlington, Massachusetts, USA), a marker of hypoxia [25–27]. The compound (1.5 mg/mouse in PBS) was injected into the tail vein, and mice were sacrificed by cervical dislocation after 90 min. For flow cytometric detection, cells were stained for anti-pimonidazole fluorescein isothiocyanate (FITC)-conjugated antibodies.

Immunofluorescence staining of BM sections

Frozen BM sections were prepared and immunostained according to the Kawamoto method, as previously reported [28]. Dissected femurs were embedded in Super Cryoembedding Medium (SCEM) and fixed using dry ice and hexane. Bones were cryosectioned (7 μm sections) using cryofilms and a CM3050s cryostat (Leica Biosystems Nussloch GmbH, Biberach, Germany). Immunofluorescence data were obtained and analyzed with a LSM710 confocal microscope (Carl Zeiss Japan, Tokyo, Japan). The markers and antibodies were as follows: Hoechst (a DNA marker used to detect cell nuclei), FITC-conjugated anti-mouse stem cell antigen 1 (Sca-1), phycoerythrin (PE)-conjugated anti-mouse cluster of differentiation (CD) 45, PE-conjugated anti-mouse TER-119, APC-conjugated anti-mouse platelet-derived growth factor receptor α (PDGFRα/CD140a) (eBioscience, San Diego, CA, USA), PE-conjugated anti-mouse Sca-1, and FITC-conjugated anti-pimonidazole hydrochloride.

Isolation and identification of mouse MSCs

Bilateral femurs, tibias, and ilia were dissected and crushed with a pestle. Bone and BM fragments were incubated with mixing for 1 h at 37°C in 0.2% collagenase (Wako, Osaka, Japan)/low-glucose Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan) containing 10 mM Hepes (Nacalai Tesque) and 1% penicillin/streptomycin (P/S). Next, the cell suspension was collected, and bone and BM fragments were again crushed. The suspension was filtered through a 70-μm cell strainer and collected by centrifugation at $280 \times g$ for 5 min at 4°C. The cells in the pellet were collected and soaked in 1 ml of water (Sigma Aldrich Co. LLC., Tokyo, Japan) for 5–10 s to lyse the red blood cells, after which time 1 ml of 2x phosphate-buffered saline (Nacalai Tesque) containing 4% fetal bovine serum (FBS; Biowest, Nuaillé, France) was added. The cells were resuspended in HBSS+ (Hanks balanced salt solution supplemented with 2% FBS, 10 mM Hepes, and 1% P/S), and the suspension was again filtered through the cell strainer. The filtered cells were then stained with the following reagents: PE-conjugated anti-mouse Sca-1, PE/cyanine 7 (Cy7)-conjugated anti-mouse CD45, PE/Cy7-conjugated anti-mouse TER-119 antigen, biotinized anti-mouse PDGFRα (eBioscience), and biotinized anti-mouse Sca-1 antibodies, as well as a streptavidin/allophycocyanin-cross-linked conjugate (Invitrogen™, Life Technologies Corporation, Carlsbad, CA, USA). Residual erythrocytes, debris, doublets, and dead cells were excluded by forward scatter, side scatter, and propidium iodide (PI) gating. Finally, PDGFRα$^+$Sca-1$^+$CD45$^-$TER119$^-$ (or PαS) cells were isolated by flow cytometry on a MoFlo XDP instrument (Beckman Coulter, Inc., Brea, CA, USA) [29, 30].

Cell culture and colony formation assay

CFU-F analysis was performed to investigate the MSC proliferation ability as described previously [29]. Briefly, freshly isolated PαS cells were cultured for 6 weeks under normoxic conditions (20% oxygen) or hypoxic conditions (1% oxygen) in culture medium (low-glucose DMEM containing 20% FBS, 10 mM Hepes, and 1% P/S). An oxygen monitoring incubator, Prescyto MG-70M (TAITEC CORPORATION, Tokyo, Japan) was used, which kept the oxygen concentration at 1%, as monitored by a sensor. In the colony formation assay, 1000 cells were seeded at the beginning of the culture in each independent experiment and were cultured for 10 days without passaging. The number of CFU-Fs (defined as colonies containing > 50 cells) was counted every week.

In vitro differentiation assay

After freshly isolated MSCs were cultured for 1, 2, or 4 weeks, adipogenic and osteogenic differentiation assays were performed as described previously [3, 5, 29]. Cells were plated onto 96-well plates and cultured for 7 days under normoxic conditions in differentiation medium containing the components included in the following kits: Adipogenic Maintenance Single-Quots Kit™ (Lonza Japan, Tokyo, Japan), Adipogenic Induction SingleQuots Kit™ (Lonza Japan), and Osteogenic SingleQuots Kit™ (Lonza Japan). The medium was changed every 3 days. Osteoblast differentiation was confirmed via alkaline phosphatase staining performed with the Histofine Assay Kit (Nichirei Biosciences Inc., Tokyo, Japan). Adipocyte differentiation was confirmed by staining with Oil Red O (Muto Pure Chemicals Co., Ltd., Tokyo, Japan). Quantitative analysis of the differentiation capacity entailed microscopic observation to determine the ratio of the number of differentiated cells to the number of nuclei in each field of view (500 cells).

Quantitative real-time PCR analysis

Cells were directly sorted into lysis buffer RLT (QIAGEN KK, Tokyo, Japan) and frozen at −80°C. RNA extraction and DNase I treatment were performed using an RNeasy Micro Kit (QIAGEN KK) according to the manufacturer's instructions (for samples containing $< 10^5$ cells). Eluted RNA samples were reverse transcribed using an AffinityScript cDNA Synthesis Kit (Agilent Technologies, Tokyo, Japan), according to the protocol supplied by the manufacturer. Quantitative real-time PCR was performed by mixing FastStart Essential DNA Green Master (Roche, Roche Diagnostics KK, Tokyo, Japan) with 5 μl of cDNA (final reaction volume, 20 μl). The reactions were performed in a LightCycler 96 System (Roche, Roche Diagnostics KK). The cycling parameters were as follows: preincubation at 95°C for 600 s, denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s. Melting was conducted at 95°C for 10 s, 65°C for 60 s, and 97°C for 1 s. Amplification proceeded for 55 cycles. The PCR primers are listed in S1 Table.

Knockdown (KD) experiments under hypoxic conditions

Notch inhibition/KD experiments were performed in BM-MSCs under hypoxic conditions using DAPT, a gamma-secretase inhibitor (at 10, 20, 40 ng/ml) to directly inhibit Notch signaling (Sigma Aldrich Co. LLC.). The control group was treated with dimethyl sulfoxide (DMSO) vehicle (1:100; Merck KGaA, Darmstadt, Germany).

Knockdown of the Notch2 gene was performed with lentiviral vectors pLKO.1-puro expressing a short hairpin RNA (shRNA) targeting mouse Notch2 (TRCN0000340451) [31], which were purchased from MISSION shRNA Library (Sigma Aldrich Co. LLC.). MSCs (2×10^5) were cultured on a 35 mm dish. After 1 day, lentiviruses were added to the MSCs. Twenty-four hours later, the medium containing lentiviruses was removed and added the new culture medium. The transduced cells were selected by additional culture in the presence of puromycin. The scrambled shRNA control plasmid as well as pLKO.1 shRNA were purchased from the MISSION shRNA Library. CSII-EF-myc plasmids were cloned into a lentiviral vector generated in the laboratory of Dr. Miyoshi (Riken BioResource Center, Tsukuba, Japan) and used to overexpress c-Myc in BM-MSCs.

Statistical analysis

Data are expressed as mean ± standard error of the means (SEM; n = number of culture wells). The Tukey-Kramer test was used to compare the quantification of the efficiency and the size of

colonies generated among different groups. All reported p values were obtained using the SPSS software package (IBM® SPSS® Statistics version 22.0.0.0 for Macintosh), and p values less than 0.05 were considered significant.

Results

MSCs reside in the BM under hypoxic conditions

To investigate the oxidative environment of MSCs in the BM, we performed immunohistochemistry (IHC). In our previous reports, we demonstrated that the PDGFRα$^+$Sca-1$^+$ (PαS) cell population is significantly enriched for murine MSCs [29, 30]. We used these markers to check the *in vivo* location and surrounding oxygen concentration of MSCs using pimonidazole. Pimonidazole is a hypoxic probe marker, and a reducing agent under hypoxic conditions (incorporated to living cells under 10 mmHg). The IHC results showed that PαS cells were stained by pimonidazole (Fig 1A and 1B). Furthermore, quantitative analysis using flow cytometry revealed that the percentage of pimonidazole-positive cells was higher in the PαS population (P$^+$S$^+$: higher than 60%) than in any other cell fraction in hematopoietic lineage negative (CD45$^-$TER119$^-$): 13%, P$^-$S$^-$: 11%, P$^-$S$^+$: 16%, P$^+$S$^-$: 37%) (Fig 1C and 1D). These results suggested that PDGFRα$^+$Sca-1$^+$ MSCs (PαS-MSCs) reside under hypoxic conditions in the BM.

MSCs maintain proliferation and differentiation under hypoxic conditions

Under normal (normoxic) culture conditions, MSCs gradually lose the ability to proliferation over several passages as they become more senescent. To evaluate whether MSC stemness is maintained by hypoxia, PαS-MSCs were cultured under normoxic or hypoxic conditions, and then passaged once a week for six passages. Under normoxic conditions, the cells lost their ability to proliferate within four passages (Fig 2A, blue line). Under hypoxic conditions, the cells continued to proliferate over six passages (Fig 2A, red line). In the CFU-F assay, hypoxia-cultured PαS-MSCs generated a larger number of CFU-Fs than normoxia-cultured PαS-MSCs in primary cultures (Normoxia: 46±7 colonies, Hypoxia: 64±9 colonies) (Fig 2B). Next, we checked the differentiation capacity for adipocytes and osteoblasts in hypoxic conditions. Hypoxic PαS-MSCs maintained their capacity for both adipocytic and osteoblastic differentiation, whereas normoxic PαS-MSCs showed a low differentiation potential (Fig 2C and 2D). Furthermore, we analyzed the cell cycle under the different oxygen conditions by flow cytometry with bromodeoxyuridine (BrdU) and 7-aminoactinomycin C (7AAD) staining. The percentage of proliferating cells (i.e., cells at S or G2/M phase) was higher for the hypoxic PαS-MSCs than for the normoxic PαS-MSCs (Fig 2E). Senescence-associated β-galactosidase (SA-β-gal) staining strongly confirmed the reduced senescence in PαS-MSCs under hypoxic culture conditions (Normoxia: 97±2%, Hypoxia: 10±2%) (Fig 2F). These data suggested that MSCs maintain proliferation and differentiation capacity under hypoxic conditions with a reduction in cellular senescence.

To analyze the effect of hypoxia on MSCs in more detail, we next quantified the reactive oxygen species (ROS) expression. Under the normoxic condition, cell proliferation was increased by the addition of N-acetylcysteine (NAC), a glutathione precursor that removes excess ROS (S1A Fig). Conversely, cell proliferation was decreased by the addition of buthionine sulfoximine (BSO), which obstructs glutathione production under hypoxic conditions (S1A Fig). Using flow cytometer, ROS levels in the cell were reduced under hypoxic condition (S1B Fig). Furthermore, electron transmission microscopy showed that the number of mitochondria increased in the cell body under normoxic conditions, but not under hypoxic conditions (S2 Fig). Taken together, hypoxic conditions might promote mitophagy, preventing the
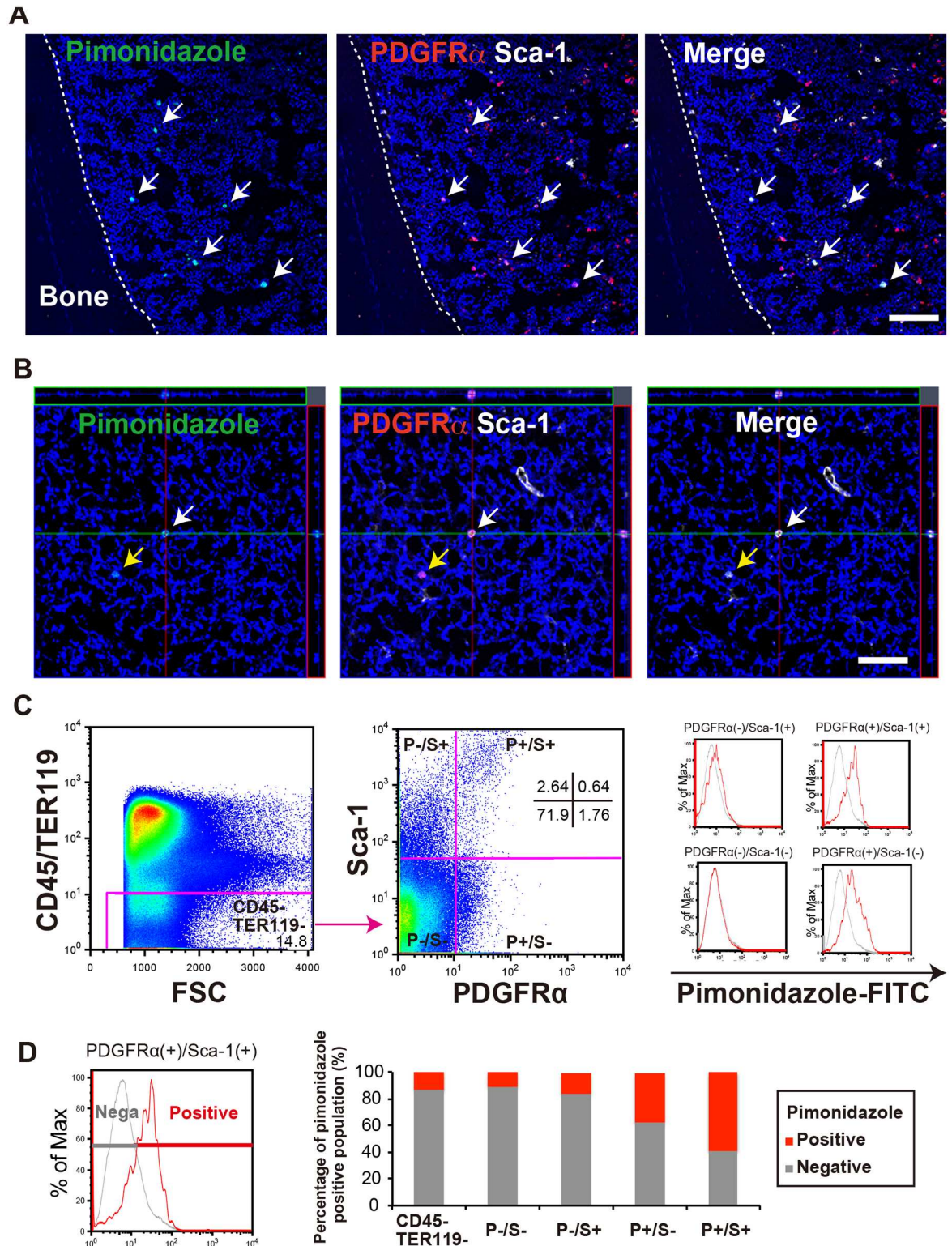
Fig 1. MSCs maintain a low oxidation level in the murine BM. (A) Representative IHC results for pimonidazole (green), PDGFRα (red), and Sca-1 (white), as well as Hoechst (blue) staining, in mouse BM. Scale bar = 100 μm. White arrows indicate cells co-expressing pimonidazole, PDGFRα, and Sca-1. (B) Confocal fluorescence microscopy (z-axis) images of pimonidazole-positive cells. Arrows indicate cells co-expressing the markers (white: pimonidazole high, PDGFRα, and Sca-1, yellow: pimonidazole low, PDGFRα, and Sca-1). Scale bars = 50 μm. (C) Flow cytometric analysis of pimonidazole-stained cells in the BM compartment. BM cells were split into positive-pimonidazole (Red) and negative -pimonidazole (Gray) populations, with a negative control as an index. The hematopoietic lineage negative (CD45⁻TER119⁻) and PDGFRα/Sca-1

(P⁻S⁻, P⁻S⁺, P⁺S⁻, and P⁺S⁺) populations were analyzed according to pimonidazole labeling in each fraction (n = 3). (D) Quantification of pimonidazole-stained cells in the BM compartment (n = 3).
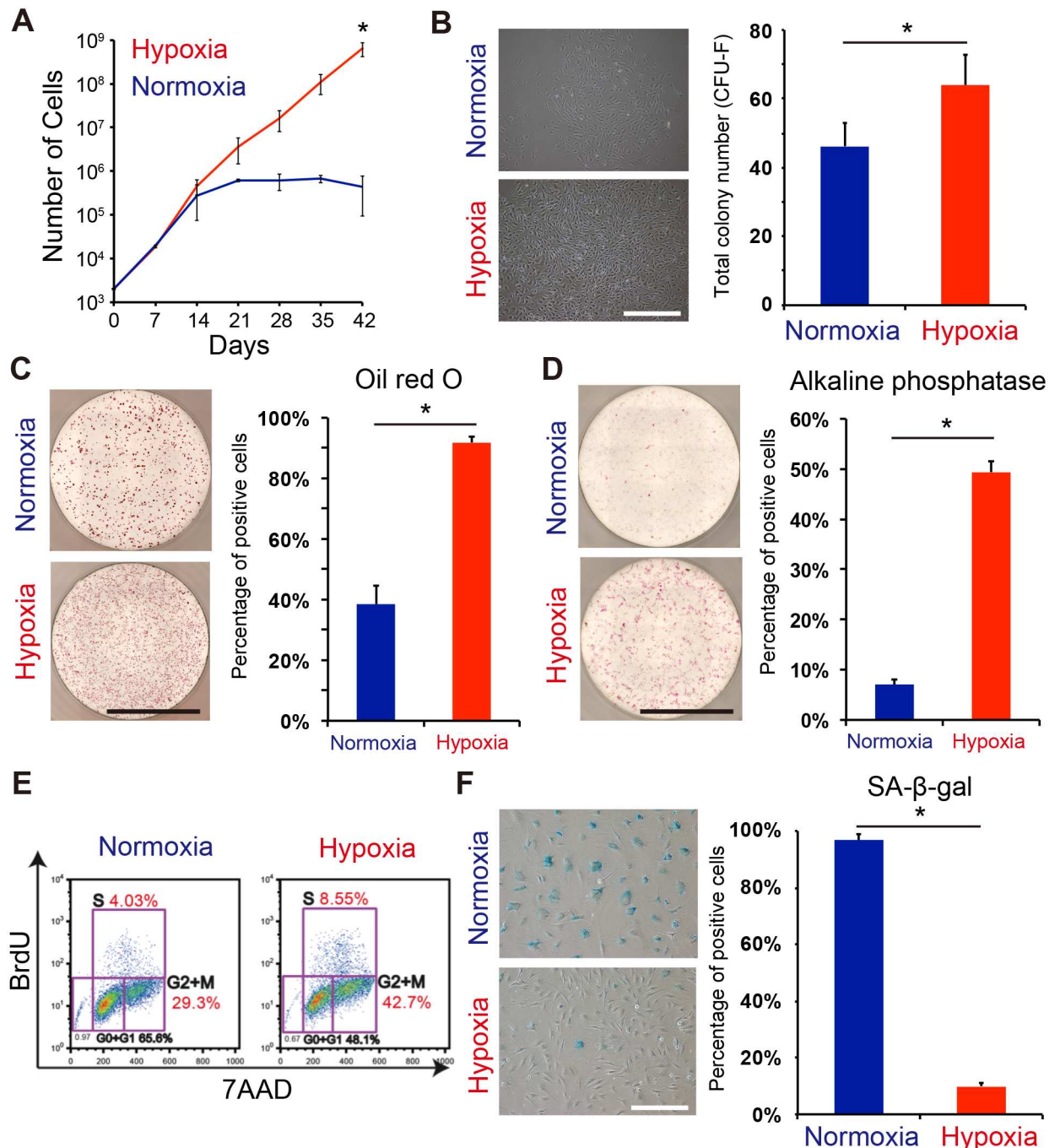
Fig 2. MSC potency is enhanced under hypoxic conditions. (A) Growth curve of MSCs (P⁺S⁺ cells) under normoxic (blue) and hypoxic (red) conditions. (B) Numbers of CFU-Fs detected at 14 days after plating 1000 cells (n = 3). Phase-contrast micrographs of MSC colonies are shown. The small box represents a high-resolution image (scale bar = 50 μm). (C, D) Capacity of MSCs to differentiate into adipocytes and osteoblasts (scale bar = 3.5 mm). Quantitative analysis of the MSC differentiation capacity at passage 4 (n = 3). (E) Flow cytometric analysis of BrdU/7AAD-stained P⁺S⁺ cells under normoxic (left) and hypoxic (right) conditions at passage 4. (F) SA-β-gal assay of cultured MSCs. Senescent cells were counted for quantitative analysis (n = 3, scale bar = 50 μm).

generation of ROS. These data also supported that the oxygen concentration affects the maintenance of MSC proliferation.

Notch2 signaling is required for MSC proliferation

Notch signaling is crucial for stem cell maintenance [32–35]. To investigate whether Notch signaling affects the MSC proliferation ability, we analyzed the effect of DAPT (a proteolytic inhibitor of Notch) on PαS-MSCs. Inhibition of Notch signaling by DAPT treatment influenced proliferation under hypoxic conditions in a dose-dependent manner (Fig 3A). These results suggested that Notch signaling is required to induce MSC proliferation. To investigate the key signaling for MSCs, we next analyzed the expression of Notch receptors. The expression level of Notch receptor 2 was higher than those of other Notch family receptors (Notch1, 3, and 4) (Fig 3B) in freshly isolated PαS cells. The presence of PαS cells expressing Notch2 was confirmed by single cell sorting with immunocytochemical staining (Fig 3C). Notch signaling requires ligand-induce proteolytic release of intracellular domain. Cleaved Notch2 intracellular domain was also detected and increased in cultured PαS-MSCs under hypoxic
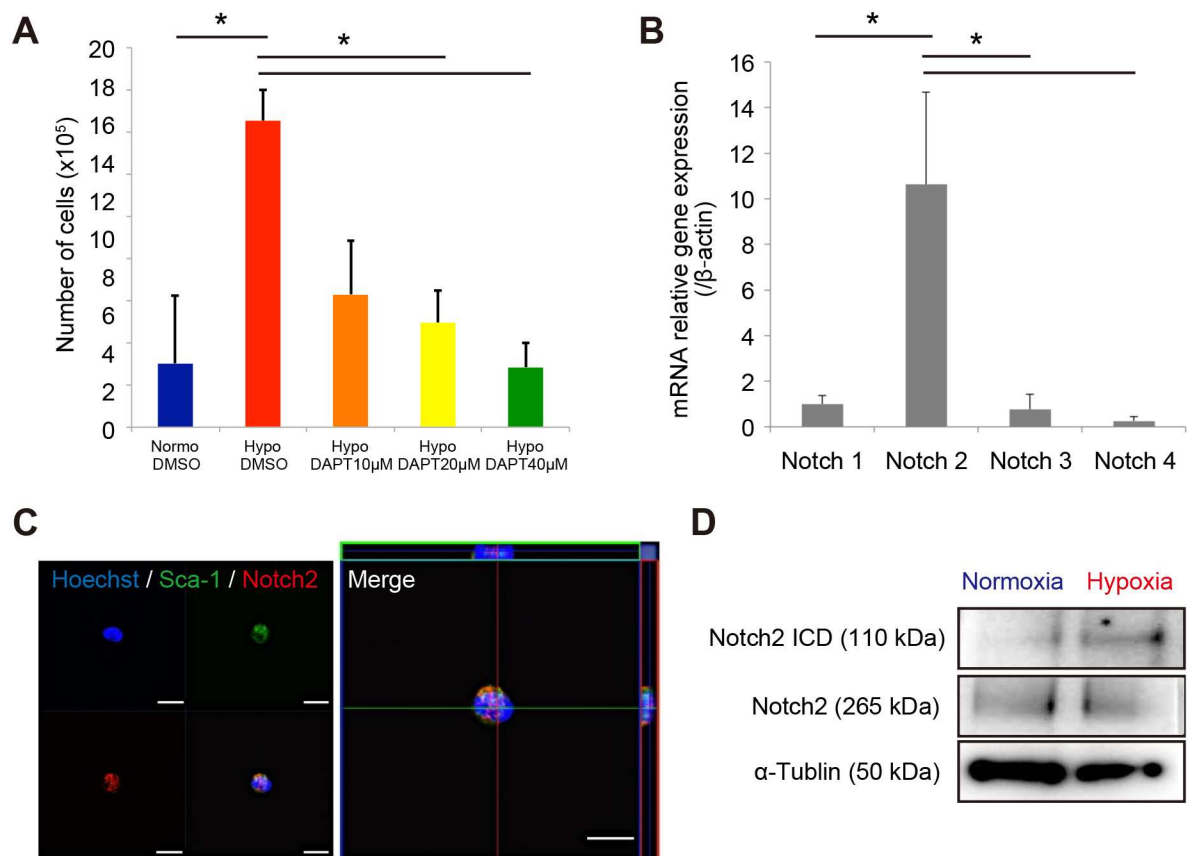
Fig 3. Notch signaling is upregulated in MSCs under hypoxic condition. (A) The effect of the Notch inhibitor DAPT on the MSC proliferation capacity. The normoxic group without DAPT treatment served as a control. (B) Quantitative real-time PCR of Notch family receptors (Notch1–4) in freshly sorted MSCs (P⁺S⁺ cells) (n = 3). (C) Immunocytochemistry staining of Sca-1 (green), Notch2 (red), and DAPI (blue) in freshly sorted P⁺S⁺ cells. (D) Western blotting of Notch2 ICD (ab52302, Abcam) and Notch2 full-length (D76A6, Sigma) expression in cultured P⁺S⁺ cells under hypoxic and normoxic conditions. α-tubulin (T9026, Cell signaling) served as a control. ICD, intracellular domain.

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condition ([Fig 3D](#)). These results suggested that Notch2 is one of the key signaling molecules for MSC proliferation.

Notch2 signaling promotes cell proliferation via c-Myc expression

To identify the mechanism underlying MSC proliferation, we next performed a KD assay under varying oxygen culture conditions. PαS-MSC proliferation was significantly disturbed by Notch2-KD under hypoxic and normoxic conditions ([Fig 4A](#)). Moreover, the c-Myc, HIF-1α, and HIF-2α (known as interaction molecules of Notch signaling) mRNA was decreased ([Fig 4B](#)). Overexpression of c-Myc in Notch2-KD MSCs recovered the proliferation capacity *in vitro* ([Fig 4C](#)). These results suggest that the Notch2 signaling pathway regulates cell proliferation and maintenance of MSCs via c-Myc expression ([Fig 4D](#)).

Discussion

The present study demonstrates that the Notch2 signaling pathway in BM-MSCs is essential during stem cell proliferation and maintenance. Recent reports suggest that hypoxia maintains
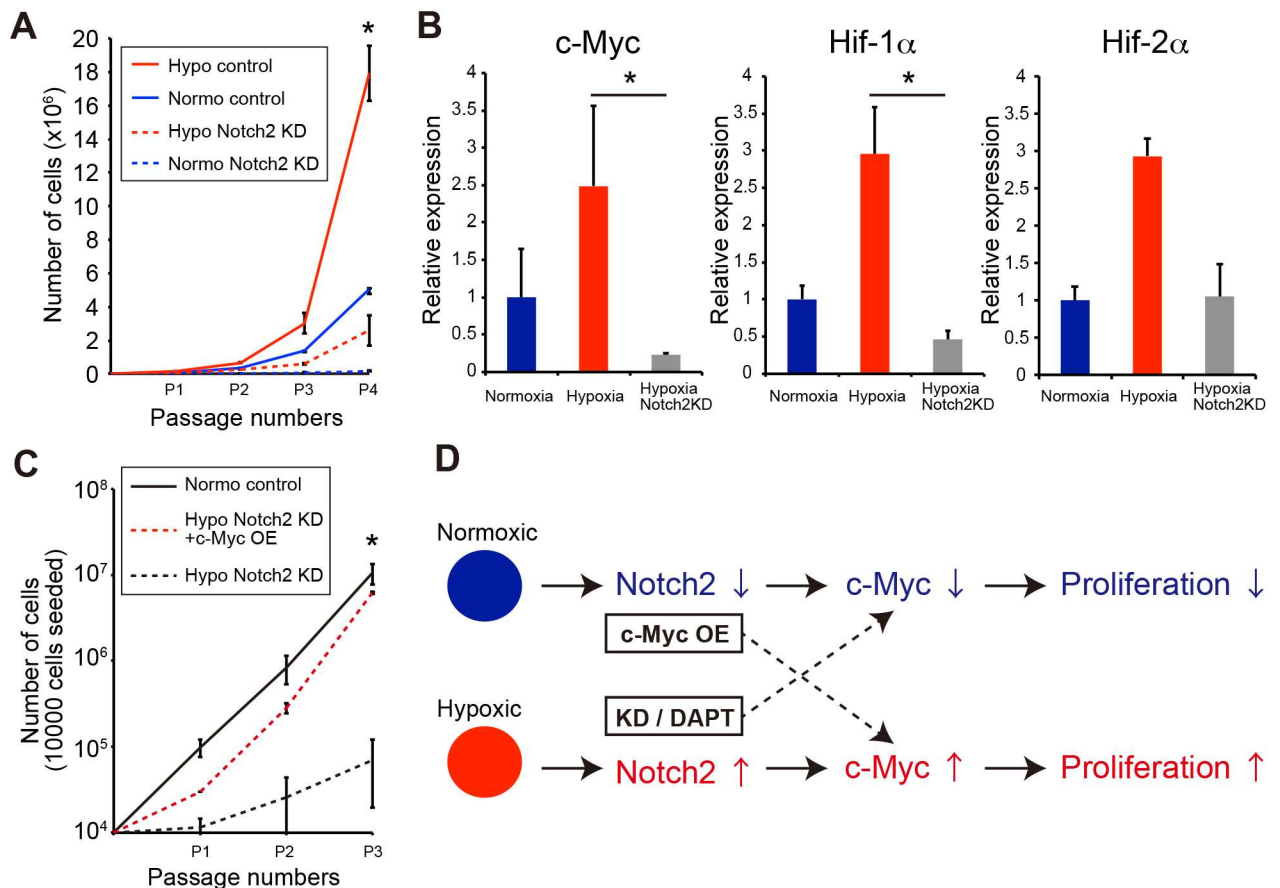
Fig 4. Hypoxia induces c-Myc expression via Notch2 signaling. (A) Proliferation assay of Notch2-KD MSCs. MSCs were cultured under low- or high-oxygen conditions. Notch2 KD was performed using shRNA methods. Control shRNA = scrambled shRNA. (B) Quantitative real-time PCR assay of c-Myc, HIF-1, and HIF-2 gene expression under hypoxic conditions. Normoxia served as a control. (C) Overexpression of c-Myc in Notch2-KD MSCs. Notch2-KD PαS-MSCs with or without c-Myc overexpression was cultured for three passages. (D) Schema of MSC proliferation under low- or high-oxygen conditions. Notch2 expression, together with the downstream expression of c-Myc, is higher under hypoxic conditions than under normoxic conditions. However, c-Myc expression is decreased by a Notch signaling inhibitor (DAPT), leading to the progression of senescence. Overexpression of c-Myc promotes cell proliferation and delays cell aging.

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the stemness of various types of cells, including adipose-derived stem cells [36], embryonic stem cells [37, 38], and cancer cells [39]. In the BM, HSCs maintain intracellular hypoxia and stabilize HIF-1α, where the latter is required for the preservation of HSC stemness [11, 13]. HIFs also regulate hematopoiesis in a non-cell autonomous manner by inhibiting activation of a latent program in mesenchymal progenitors that promotes hematopoiesis [10]. In our report, HIF-1α and HIF-2α mRNAs were downregulated in Notch2-KD MSCs, implying that Notch2 is located upstream of HIF. It was reported that mesenchymal progenitor proliferation *in vitro* is dependent on activation of the Notch cascade, whereas bone differentiation is enhanced by transiently suppressing this pathway [19]. These results support a model where Notch signaling in the BM maintains the stemness of a pool of MSCs by suppressing osteoblast differentiation.

Notch signaling pathways are important in MSC biology. Jagged1 (JAG1) is a Notch2 receptor ligand and most likely plays a role in activating this pathway [40]. For example, transient JAG1-mediated Notch signaling promotes the maintenance and expansion of human BM-MSCs, and concomitantly increases their skeletogenic differentiation capacity *in vitro* and *in vivo* [34]. The involvement of JAG1 in cell proliferation has also been described in breast cancer stem cells [41]. Furthermore, while Notch signaling appears to promote BM-MSCs proliferation, it also suppresses osteoblastic differentiation *in vivo* [19]. The clinical relevance of these BM-MSCs signaling pathways may be seen in two rare syndromes. Hajdu-Cheney syndrome is an autosomal dominant skeletal disorder characterized by facial anomalies, osteoporosis, and acro-osteolysis caused by truncating mutations in the last exon of Notch2 [42]. Alagille syndrome is characterized by developmental abnormalities in a variety of organs including the liver and heart [43]. The frequency of identifiable genetic mutations in patients with a clinically consistent diagnosis of Alagille syndrome is high, with JAG1 mutations identified in 94% and Notch2 mutations in 2% of affected individuals [43]. In a mouse model, Notch2 mutations disrupt osteogenesis and suppress the differentiation of functional osteoblasts [44]. Our current findings imply that Notch2-c-Myc signaling was required for proliferation by inhibiting cellular senescence under hypoxic condition *in vitro*. We also showed that MSCs (PaS cells) were resided in BM under the hypoxic condition. Although, further studies are required to examine, the pathway may prove to be a therapeutic target to treat- Hajdu-Cheney syndrome and Alagille syndrome.

Notch signaling is a key regulator of cell fate specification and cancer development. Lentivirus-mediated expression of NICD2 and c-Myc could promote proliferation of granulosa cells [45]. Other report shows that Notch2 is predominantly expressed in pancreatic intraepithelial neoplasia injuries and regulate the c-Myc signaling during tumor development [46]. Notch2 signaling strongly influenced the cell proliferation. However, overexpression of c-Myc had a limited effect on the recovery of MSC self-renewal. We speculate that the self-renewal capacity of MSCs is partially mediated by another unknown mechanism. Hypoxic condition read to remove the mitochondria and to reduce the ROS activity (S1 and S2 Figs). Our data also confirmed to link the cell proliferation (S1 Fig). The hypoxic condition might have massive effect for the MSC proliferation.

In conclusion, this study showed that c-Myc activation downstream of Notch2 is involved in the maintenance of MSC proliferation. Because c-Myc is an oncogene, modulation of Notch signaling in BM-MSCs cannot be directly exploited for regenerative medicine purposes. However, control of the Notch pathway can potentially be applied for expansion of other stem cells (e.g., HSCs, skeletal muscle stem cells, and cancer stem cells). Indeed, our culture method may provide a useful technique for stem cell expansion.

Supporting Information

S1 Fig. ROS expression in hypoxic and normoxic conditions. (A) Cell proliferation under hypoxic and normoxic conditions (blue bar: normoxic, red bar: hypoxic, orange bar: normoxic with NAC, and green bar: hypoxic with BSO). (B) ROS expression analysis by flow cytometry ($1\% \; O_2$: hypoxic, $20\% \; O_2$: normoxic, control: negative control, and NAC^+: normoxic with NAC). NAC; N-acetylcysteine, BSO; buthionine sulfoximine, ROS; reactive oxygen species. (TIF)

S2 Fig. Scanning electron microscope photographs of a cell under hypoxic and normoxic conditions. Electron transmission microscopy showed the number of mitochondria under normoxic (top) and hypoxic (bottom) conditions. The area enclosed by the black square is shown at a higher magnification in the image on the right side. (TIF)

S1 Table. List of gene-specific primers used for quantitative RT-PCR. (DOCX)

Acknowledgments

Author Contributions

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Methodology: YS Y. Mabuchi SM.

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Supervision: Y. Matsuzaki.

Writing – original draft: YS Y. Mabuchi DDH Y. Matsuzaki.

Writing – review & editing: YS Y. Mabuchi Y. Matsuzaki.

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