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Increased SCF/c-kit by Hypoxia Promotes Autophagy of Human Placental Chorionic Plate-Derived Mesenchymal Stem Cells Via Regulating the Phosphorylation of mTOR

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ABSTRACT

Hypoxia triggers physiological and pathological cellular processes, including proliferation, differentiation, and death, in several cell types. Mesenchymal stem cells (MSCs) derived from various tissues have self-renewal activity and can differentiate towards multiple lineages. Recently, it has been reported that hypoxic conditions tip the balance between survival and death by hypoxia-induced autophagy, although the underlying mechanism is not clear. The objectives of this study are to compare the effect of hypoxia on the self-renewal of bone marrow-derived mesenchymal stem cells (BM-MSCs) and placental chorionic plate-derived mesenchymal stem cells (CP-MSCs) and to investigate the regulatory mechanisms of self-renewal in each MSC type during hypoxia. The expression of self-renewal markers (e.g., Oct4, Nanog, Sox2) was assessed in both cell lines. PI3K and stem cell factor (SCF) expression gradually increased in CP-MSCs but were markedly downregulated in BM-MSCs by hypoxia. The phosphorylation of ERK and mTOR was augmented by hypoxia in CP-MSCs compared to control. Also, the expression of LC3 II, a component of the autophagosome and the hoof-shaped autophagosome was detected more rapidly in CP-MSCs than in BM-MSCs under hypoxia. Hypoxia induced the expression of SCF in CP-MSCs and increased SCF/c-kit pathway promotes the self-renewal activities of CP-MSCs via an autocrine/paracrine mechanism that balances cell survival and cell death events by autophagy. These activities occur to a greater extent in CP-MSCs than in BM-MSCs through regulating the phosphorylation of mTOR. These findings will provide useful guidelines for better understanding the function of SCF/c-kit in the self-renewal and autophagy-regulated mechanisms that promote of MSC survival. J. Cell. Biochem. 114: 79–88, 2013.

KEY WORDS: HYPOXIA; BONE MARROW-DERIVED MESENCHYMAL STEM CELLS (BM-MSCs); PLACENTAL CHORIONIC PLATE-DERIVED MESENCHYMAL STEM CELLS (CP-MSCs); STEM CELL FACTOR (SCF); SELF-RENEWAL; AUTOPHAGY

Additional supporting information may be found in the online version of this article.

Abbreviations: BM-MSCs, bone marrow-derived mesenchymal stem cells; CP-MSCs, chorionic plate-derived mesenchymal stem cells; HIF-1α, hypoxia-inducible factor 1a; LC3, microtubule-associated protein 1 light chain 3; MDC, monodansylcadaverine; MSCs, mesenchymal stem cells; mTOR, mammalian target of rapamycine; PDSCs, placenta-derived stem cells; PI3-kinase, phosphatidylinositol 3-kinase; SCF, stem cell factor.

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Mesenchymal stem cells (MSCs) were originally identified in bone marrow and have been found in several tissues, including adipose tissue, umbilical cord, placenta, and connective tissues of human adult [Pittenger, 1999; Zuk et al., 2002; Parolini et al., 2008]. It is well known that MSCs have multipotent potential and immunomodulatory effect can be modulated by inflammatory signals. For these reasons, MSCs are widely studied and have been applied for therapeutic trials in many fields of regenerative medicine [Ankrum and Karp, 2010]. However, the number of MSCs isolated from tissues are relatively low (0.001–0.01%), and the rate of self-renewal and the differentiating decrease with the age of the donor [Fehrer and Lepperdinger, 2005]. In addition, invasive procedures (e.g., bone marrow aspiration, liposuction) are essential for isolating MSCs from the tissues [Locke et al., 2009], and the senescence of MSCs by limited self-renewal activity restricts their application for clinical treatment [Fehrer and Lepperdinger, 2005]. The molecular mechanisms that determine MSC senescence and self-renewal activity are poorly understood, suggesting that an enhanced understanding of biological mechanisms in MSCs might overcome these limits. For instance, a new source of MSCs that exhibits strong multipotent potential and that are easy to cultivate is needed to overcome these drawbacks.

Recently, many scientists have reported that human placenta-derived stem cells (PDSCs) are an alternative to bone marrow-derived mesenchymal stem cells (BM-MSCs) because they are easy to collect in large numbers and exhibit multilineage potential, immunosuppression, and self-renewal abilities [Miao et al., 2006; Parolini et al., 2008]. Because the placenta is often discarded after the birth, there is also no ethical controversy for its clinical or experimental use. The placenta is an easily accessible, high-yielding source of stem cells, and various cell types can be isolated based on anatomical structures of placenta [Miao et al., 2006; Kim et al., 2011b]. In previous reports, we characterized chorionic plate-derived mesenchymal stem cells (CP-MSCs) isolated from the placental chorionic membrane, analyzed their potential for hepatogenic differentiation in vitro, and evaluated the therapeutic potential of CP-MSCs in a cirrhotic liver rat model [Lee et al., 2010; Shin et al., 2010].

Hypoxia is a state of low oxygen levels in an organism and causes cell death via oxidative stress in several cell types, including cancer cells. In addition, it has been reported that hypoxia can control the differentiation potential of a variety of stem cells [Mohyeldin et al., 2010]. The differentiation of embryonic stem cells (ESCs) is reduced when they are cultured under 3% or 5% oxygen, whereas other conditions promote the formation of embryoid bodies [Ezashi et al., 2005]. Hypoxia acts as a key component in the neural stem cell (NSC) niche, and the proliferation and survival of NSCs is significantly enhanced under hypoxic condition [Panchision, 2009]. Nekanti et al. [2010] reported that MSCs derived from the umbilical cord exhibit enhanced proliferative potential and maintain multipotent potential under hypoxic conditions. Other reports state that hypoxia appears to control hematopoiesis in the bone marrow and supports cell cycle control, metabolism, and survival of hematopoietic stem cells (HSCs) [Eliasson and Jonsson, 2010]. Thus, hypoxia-induced factor-1 alpha (HIF-1α) is one of the key molecules that act during hypoxia to induce the transcription of more than 200 molecules [Majmundar et al., 2010]. Overexpression of HIF-1α promotes the hypoxia-signaling pathway and is important for ESCs to maintain self-renewal and pluripotency [Ji et al., 2009]. Stem cells from HIF-1α-deficient mice lose their cell cycle quiescence, and the numbers of HSCs decline when they are exposed to various stress environments, including myelosuppression, bone marrow transplantation or aging [Takubo et al., 2010]. Thus, HIF-1α may be a key regulator of hypoxia-related mechanisms in various types of stem cells.

In contrast, hypoxia also induces programmed cell death, including apoptosis and autophagy, via oxidative stress [Gustafsson and Gottlieb, 2008; Giansanti et al., 2011]. It triggers a feedback mechanism that inhibits apoptosis of cells through hypoxia-induced autophagy [Zhang et al., 2011]. Autophagy, also known as programmed cell death type II, is spotlighted as one of the key mechanisms of cell survival [Mathew et al., 2007]. The survival of cancer cells can be supported by autophagy mechanisms, and autophagy supports the stem cell characteristics of cancer cells, even under starvation or hypoxic conditions. Therefore, HIF-1α and autophagy induced by hypoxia can function as a programmed cell repair mechanism under harsh conditions, such as hypoxia and nutrient deprivation [Martinez-Outschoorn et al., 2010; Belibi et al., 2011]. However, the underlying mechanism for balancing cell survival and death by hypoxia-induced autophagy is not clear.

Stem cell factor (SCF), also known as mast cell growth factor, is a cytokine found in mast cells, HSCs, and cancer cells, and is controlled by HIF-1α. The membrane-bound form has been identified on the surfaces of bone marrow stem cells, and the soluble form can be detected in the serum. It promotes proliferation and differentiation of the cells by binding its receptor, c-kit [Ashman, 1999; Han et al., 2008; Krasagakis et al., 2011], which is a transmembrane protein with five immunoglobulin and tyrosine kinase domains. It is reported that c-kit is expressed in human brain, placenta, ovary, bone marrow, and spleen [Saito et al., 1994]. Activation of SCF/c-kit leads to the concomitant activation of downstream molecules. Recently, the overexpression of SCF/c-kit was implicated as a major cause of pancreatic cancer [Yasuda et al., 2006], gastrointestinal stromal tumors [Ali, 2007], lung cancer [Levina et al., 2010] and leukemia through the promotion of angiogenesis, cell growth, and metastasis [Han et al., 2008; Levina et al., 2010; Ma et al., 2011]. However, studies investigating the role of SCF/c-kit in stem cells in relation to specific microenvironments, such as hypoxia, are rare despite the proliferative similarities between cancer cells and stem cells.

Therefore, the objectives of this study are to analyze whether the expression of SCF/c-kit in MSCs derived from placenta and bone marrow is regulated by hypoxia and to further investigate the effect of SCF/c-kit activation on the regulation of self-renewal and autophagy mechanisms in each MSC population under hypoxic conditions.

MATERIALS AND METHODS

CELL CULTURE AND HYPOXIA TREATMENT

Placentas were collected from term pregnancies (≥37 gestational weeks) from women who were free of medical, obstetrical, and

surgical complications. CP-MSCs were harvested as described previously [Lee et al., 2010]. The collection of samples and their use in research was approved by the Institutional Review Board of CHA General Hospital, Seoul, Korea. All participants provided written, informed consent before the collection of samples. Briefly, CP-MSCs were separated from the inner-side of the chorioamniotic membrane of the placenta. Cells scraped from the membrane were treated with 0.5% collagenase IV (Sigma–Aldrich, St. Louis, MO) for 30 min at 37°C. Harvested cells were cultured in Ham's F-12 medium/DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 1% penicillin/streptomycin (P/S; Invitrogen), 10% fetal bovine serum (FBS; Invitrogen), 25 ng/ml FGF-4 (Peprotech, Inc., NJ) and 1 μg/ml heparin (Sigma–Aldrich). CP-MSCs were maintained in alpha-modified Eagle's medium (α-MEM; Invitrogen) with 1% P/S and 10% FBS. BM-MSCs were purchased (LONZA, Switzerland) and maintained in α-MEM with 2 mM L-Glutamate (Invitrogen), 1% P/S and 10% FBS at 37°C in a humidified atmosphere with 5% CO_2. For hypoxia treatment, the culture medium was exchanged with oxygen-free media containing 1% P/S and 10% FBS, and the cells were placed in an incubator with 1% O_2, 5% CO_2, and 94% N_2 for 3, 6, 9, 12, 24, 48, or 96 h.

RT-PCR ANALYSIS

Total RNA samples from experimental and control groups were extracted using TRIzol reagent (Invitrogen) following the manufacturer's protocol. Total RNA was extracted with 200 μl chloroform and was precipitated with 500 μl of 80% (v/v) isopropanol. The supernatant was removed, and the RNA pellet was washed with 75% (v/v) ethanol, air-dried, and dissolved in 0.1% (v/v) diethyl pyrocarbonate (DEPC)-treated water. The RNA concentration was measured using a NanoDrop ND-1000 (NanoDrop Technologies). A reverse transcription reaction was performed with 1 μg of total RNA and SuperScript™ III reverse transcriptase (Invitrogen). The cDNA was amplified using h-Taq DNA polymerase (Solgent, Seoul, Korea), following the manufacturer's protocol. First-strand cDNA was amplified in a final volume of 20 μl containing 0.5 U h-Taq DNA polymerase and 20 pmol of each human-specific target primer. The sequences and sizes of the primers are shown in Table I. Amplification reactions were performed according to the following conditions: denaturation at 95°C for 15 min, followed by 35–40 cycles of denaturation at 95°C for 30 s, annealing at 51–69°C for 40 s, and elongation at 72°C for 5 min. PCR products were mixed with 0.5 μg/mL Loading Star buffer (Dynebionic, Seoul, Korea) and analyzed by electrophoresis on a 1–1.5% (w/v) agarose gel (Cambrex Bioscience Walkersville, East Rutherford, NJ).

TABLE I. Sequence of Primers Used for RT-PCR and Length of Fragments

Gene	Sequence	Size (bp)
c-kit	F: 5′-TTC TTA CCA GGT GGC AAA GGG CAT GGC TTT CC-3′ R: 5′-CAT ACA TTT CAG CAG GTG CGT GTT CAG GGC-3′	388
gp130	F: 5′-TGT TGA CGT TGC AGA CTT GG-3′ R: 5′-TCT GGA GGC AAG CCT GAA AT-3′	380
HIF-1α	F: 5′- TGG ACT CTG ATC ATC TGA CC-3′ R: 5′-CTC AAG TTG CTG GTC ATC AG-3′	434
SCF	F: 5′-GAT GTT TTG CCA AGT CAT TGT TGG-3′ R: 5′-ACT GAC TCT GGA ATC TTT CTC AGG-3′	351
STAT3	F: 5′-TGC CTG GAG ACA GTT GAT GTG TCA-3′ R: 5′-TGG AAT TTG AAT GCA GTG GCC AGG-3′	414
28rRNA	F: 5′-TTGAAAATCCGGGGGAGAG-3′ R: 5′-ACATTGTTCCAACATGCCAG-3′	100

REAL-TIME PCR ANALYSIS

Real-time PCR analysis was used to quantify differences in gene expression. Target gene expression was normalized to that of an internal reference (GAPDH). Real-time PCR was performed with primers (Table II) and SYBR Premix Ex Taq (Takara, Japan) in an Exicycler 96 real-time PCR machine (Bioneer, Daejeon, South Korea). Target sequences were amplified by using the following thermal conditions: 2 min at 95°C, and 40 cycles of 5 s at 95°C and 30 s at 59°C. All reactions were performed in triplicate.

FACS ANALYSIS

For the analysis of surface c-kit (CD117) expression, BM-MSCs and CP-MSCs were collected using dissociation buffer (Sigma–Aldrich) and washed with phosphate-buffered saline (PBS; Sigma–Aldrich). Cells were stained with APC-conjugated mouse anti-human CD117 (1:100, BD Biosciences, BD Biosciences, San Jose, CA) or negative control APC-conjugated mouse IgG (1:100, BD Sciences). Staining was performed at room temperature (RT) for 30 min in the dark, and cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) in the range of 660/20 nm.

For cell cycle analysis, BM-MSCs and CP-MSCs were collected and fixed in 70% EtOH at RT for 10 min followed by two washes in PBS. All samples were then treated with 0.5 μg/mL RNase A (Sigma–Aldrich) for 15 min at RT, washed with PBS and then stained with propidium iodide (PI) solution (1:200, Sigma–Aldrich). The cells were analyzed on a FACSCalibur flow cytometer in the range of 660/20 nm.

IMMUNOFLUORESCENCE ANALYSIS

Cells were fixed with 100% methanol for 10 min and washed with PBS. The cells were then incubated with blocking solution (DAKO, Carpinteria, CA) for 30 min at RT to inhibit internal factors in cells followed by an overnight incubation with mouse anti-human LC3 (1:200, Novus Biologicals, Littleton, CO) at 4°C. The samples were then stained with secondary antibody (Alexa 488-conjugated chicken anti-mouse IgG, 1:500, Invitrogen) for 1 h at RT and PI staining for 1 min as a counterstaining. Images were detected using a Zeiss Axioskop2 MAT microscope (Carl Zeiss MicroImaging).

TABLE II. Sequence of Primers Used for real-time PCR

Gene	Sequence
Oct4	F: 5′-CCTCACTTCACTGCACTGTA -3′ R: 5′-CAGGTTTTCTTTCCCTAGCT -3′
Nanog	F: 5′-TTCTTGACTGGGACCTTGTC-3′ R: 5′-GCTTGCCTTGCTTTGAAGCA -3′
Sox2	F: 5′-CCCAGCAGACTTCACATGT -3′ R: 5′-CCTCCCATTTCCCTCGTTTT -3′
GAPDH	F: 5′-CTCCTCTTCGGCAGCACA -3′ R: 5′-AACGCTTCACCTAATTTGCGT -3′

MONODANSYLCADAVERINE (MDC) STAINING FOR AUTOPHAGOSOMES

CP-MSCs and BM-MSCs were incubated with a 0.05 mM final concentration of MDC (Sigma–Aldrich) for 30 min at 37°C. Cells were then washed with PBS 3 times to remove excess MDC. After that, cells were fixed with 4% (v/v) paraformaldehyde for 30 min. Images were obtained on a Zeiss Axioskop2 MAT microscope.

WESTERN BLOT ANALYSIS

Cells were harvested and lysed in protein lysis buffer (Sigma–Aldrich). Proteins were quantified using a BCA assay kit (Pierce, Rockford, IL), and 30–80 μg of proteins were separated in 6–15% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) and transferred for 1.5 h at 100 V onto PVDF membranes (Bio-Rad Laboratories, Richmond, CA). Membranes were blocked in 5% nonfat dry milk (BD Biosciences) at RT for 1 h and were then incubated overnight at 4°C with the following antibodies: rabbit anti-human HIF-1α (1:1,000, kindly provided by Dr. Jong-Wan Park, Seoul National University, Seoul, Korea), mouse anti-human β-actin (1:1,000, Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit anti-human SCF (1:2,000, Santa Cruz Biotechnology), mouse anti-human phosphatidylinositol 3-kinases (PI3K p85; 1:300, BD Biosciences), mouse anti-human ERK (1:5,000, Santa Cruz Biotechnology), mouse anti-human phosphorylated ERK (p-ERK; 1:5,000, Cell Signaling Technology, Danvers, MA), rabbit anti-human mammalian target of rapamycine (mTOR; 1:1,000, Abcam, Cambridge, UK), rabbit anti-human phosphorylated mTOR (p-mTOR; 1:1,000, Abcam), rabbit anti-human Bcl2 (1:100, AbFrontier, Seoul, Korea), rabbit anti-human Bax (1:200, Santa Cruz Biotechnology), rabbit anti-human ATG5-12 (1:500, Santa Cruz Biotechnology), rabbit anti-human GAPDH (1:1,000, AbFrontier), and rabbit anti-human LC3 (1:1,000, Novus Biologicals). The membranes were washed and then incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:5,000, Bio-Rad Laboratories), anti-rabbit IgG (1:2,500, Bio-Rad Laboratories) or anti-goat IgG (1:2,500, Santa Cruz Biotechnology) secondary antibodies for 40 min at RT on an orbital shaker. After washing, bands were detected using enhanced chemiluminescence reagent (Pierce).

HIF-1 TRANSCRIPTION FACTOR ACTIVITY ASSAY

The activation of HIF-1 transcription factor was measured using a TransAM™ HIF-1 kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. The cell extracts (5 μg of each sample) were added to 96-well plates coated with specific double-stranded oligonucleotide. The plate was incubated for 1 h at RT. HIF-1α primary antibodies were then added to each well and incubated for 1 h at RT. After the binding of primary antibody, HRP-conjugated antibodies were added to the wells and then incubated for 1 h at RT. Each well was washed with wash buffer four times prior to development and was then measured on an E-MAX machine (Molecular Devices) at 450 nm. All samples were assayed in duplicate.

CASPASE ACTIVITY ASSAY

Caspase 3/7 activities in CP-MSCs and BM-MSCs were measured with a Caspase-Glo 3/7 assay kit (Promega, Madison, WI). The proluminescent substrate containing either the DEVD, the LETD, or the LEHD sequence (which are represented here in single-letter amino acid code) is cleaved by caspase 3/7. After caspase cleavage, a substrate for luciferase is released. The cells were lysed with protease inhibitor-free lysis buffer (Culture Lysis Reagent; Promega). An equal volume of reagents and protein (100 μg) were added to each well of a white-walled 96-well plate and incubated at RT for 2 h. The luminescence of each sample was measured in a plate-reading luminometer (TECAN). All samples were assayed in duplicate.

DETECTION OF SOLUBLE SCF CONCENTRATIONS

The concentrations of SCF in the culture supernatants of CP-MSCs and BM-MSCs were measured using a cytokine detection kit (Bio-Rad Laboratories). The assay was performed according to the manufacturer's protocol, except that all samples were first centrifuged for 20 s at 14,300 revolutions per minute to remove debris, and 50 μl of each sample was diluted at a ratio of 1:3 in sample diluent. All samples were assayed in duplicate and analyzed with a Luminex 200 Labmap system (Luminex). Data analyses were performed using Bio-Plex Manager software, version 4.1.1 (Bio-Rad Laboratories). SCF concentrations were interpolated from an appropriate standard curve.

SCF NEUTRALIZING ASSAY

CP-MSCs and BM-MSCs were cultured with or without 1 μg/ml of SCF-neutralizing antibody (R&D system, Minneapolis, MN) in hypoxia for 0, 24, 48, or 96 h. Cells were washed 5 times with PBS, and the cell pellets were then collected by trypsinization and analyzed by Western blot for p-mTOR and LC3.

STATISTICAL ANALYSIS

Results shown are the averages of three independent experiments ($n = 3$). The values are expressed as the means ± SD. The Student's *t*-test was used for analyses, and *P* values less than 0.05 were considered statistically significant.

RESULTS

To validate that CP-MSCs and BM-MSCs respond to hypoxia, the expression and the activity of HIF-1α were analyzed by Western blot and a TransAM™ HIF-1 kit assay, respectively. The expression of HIF-1α was increased in both CP-MSCs and BM-MSCs in a time-dependent manner according to their hypoxia exposure (Fig. 1A), although HIF-1α mRNA was consistently expressed (Supplement Fig. 1). However, the increase in HIF-1α expression was greater in CP-MSCs compared to BM-MSCs at 6, 9, and 12 h (Fig. 1A). In addition, the activity of HIF-1α increased until 12 h and then gradually declined after 12 h of hypoxia exposure; this pattern was equivalent in both CP-MSCs and BM-MSCs (Fig. 1B). These data show that both cell types successfully respond to hypoxia, that HIF-1α expressions and activities were increased by hypoxia in both CP-MSCs and BM-MSCs, and that the increased expressions was greater in CP-MSCs compared to BM-MSCs.

To analyze the effect of hypoxia on the self-renewing capacity of CP-MSCs and BM-MSCs, we assessed the expression of self-renewal

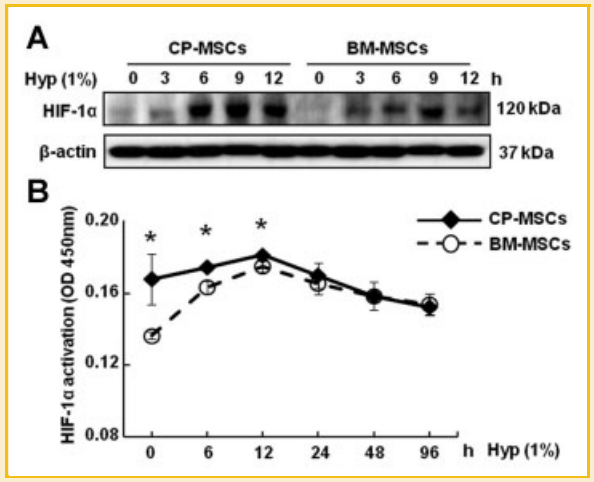

Fig. 1. The expression of HIF-1α in MSCs after exposure to hypoxic conditions. A: Western blot analysis of HIF-1α in CP-MSCs and BM-MSCs under hypoxia (1% O_2) for 0, 3, 6, 9, and 12 h. GAPDH was used as a loading control. B: The activity of the HIF-1α transcription factor was measured in CP-MSCs and BM-MSCs under hypoxia (1% O_2) for 0, 6, 12, 24, 48, and 96 h by ELISA. The closed circles indicate CP-MSCs, and the open circles indicate BM-MSCs. All reactions were performed in triplicate and the values are expressed as the means ± SD. *Significant differences between CP-MSCs and BM-MSCs at each time point ($P < 0.05$).

factors by real-time PCR and compared cell cycle analyses of cultured cells under hypoxic conditions. The key factors of self-renewal in stem cells, Oct4, Nanog, and Sox2, were expressed in both cell lines and increased after early exposure to hypoxia (3 and 6 h; Fig. 2A).

The expression of Oct4 was increased after 3 h of hypoxic exposure with more robustly induction in CP-MSCs than in BM-MSCs and decreased steadily. Nanog mRNA expression was significantly higher in CP-MSCs at 9–12 h of hypoxic exposure. Sox2 mRNA expression was increased in 3–9 h of hypoxic exposure with significantly high level at 9 h of hypoxia in CP-MSCs than BM-MSCs. In a flow cytometric analysis of cell cycle, the percentage of cells in S phase in CP-MSCs was altered from 6.03% to 10.26% by hypoxic cultivation, whereas the percentage of cells in S phase in BM-MSCs markedly declined from 6.20% to 3.75% after hypoxic cultivation (Fig. 2B). After hypoxic treatment, the sub G1 gate (i.e., the apoptotic fraction) in both CP-MSCs and BM-MSCs was increased to 15.55% and 11.72%, respectively. These findings suggest that the self-renewal activity of CP-MSCs is maintained under hypoxic conditions, as their turnover increased with exposure, whereas BM-MSCs exhibited reduced proliferation in the absence of morphological changes. We analyzed whether the balance of cell survival and apoptosis was altered in MSCs under hypoxia (Fig. 3). As shown in Figure 3A, the expression of p-mTOR versus total-mTOR, which is a known cell survival factor, increased until 24 h of hypoxia exposure and slightly reduced in CP-MSCs, and maintained until 12 h of hypoxia and markedly decreased after 24 h in BM-MSCs. PI3K p85 is involved in various cellular functions (e.g., cell growth, proliferation, differentiation, survival) and were increased in CP-MSCs over time. Meanwhile, PI3K p85 was sustained with some fluctuating expression in BM-MSCs exposed to hypoxia. The expressions of p-ERK and total-ERK were observed in CP-MSCs and BM-MSCs. The amount of p-ERK versus total-ERK in CP-MSCs increased over time by hypoxia, but decreased in BM-MSCs. Interestingly, the expression of SCF, a known regulator of self-renewal in stem cells, was markedly increased in CP-MSCs compared to BM-MSCs. The expression of SCF in CP-MSCs was maintained until 12 h and then increased after 24 h. In contrast, BM-MSCs exhibited very little SCF expression. These results show that the expression of survival factors in CP-MSCs and BM-MSCs were differentially regulated by hypoxia, respectively.

It is well known that hypoxic conditions trigger apoptosis in various cell lines. Thus, we investigated whether hypoxia induces apoptosis in CP-MSCs and BM-MSCs. As shown in Figure 3B, there were obvious differences in the expression of Bcl2 between CP-MSCs and BM-MSCs. Bcl2 expression was certainly low in CP-MSCs, while BM-MSCs exhibited a robust expression, furthermore the expressions were increased at 12 and 24 h of hypoxia on BM-MSCs (Fig. 3B). The expression of Bax was observed in both CP-MSCs and BM-MSCs under hypoxic condition. Furthermore, the activities of caspases 3 and 7 were significantly increased by hypoxia in a time-dependent manner and were more vigorously induced in CP-MSCs compared to BM-MSCs (Fig. 3C). These findings indicate that hypoxia induced apoptosis more strongly in CP-MSCs, suggesting that it promotes apoptosis and self-renewal in CP-MSCs through the dynamic regulation of survival and apoptotic factors.

We hypothesized that hypoxia could regulate the balance between survival and apoptosis in MSCs by promoting autophagy mechanisms because hypoxia has been shown to induce autophagy [Gustafsson and Gottlieb, 2008]. Therefore, to investigate whether autophagy is activated by hypoxia in CP-MSCs and BM-MSCs, we performed an immunofluorescence assay evaluating LC3, which is an early marker for autophagosomes. Additionally, we employed MDC, which is a fluorescent compound that becomes incorporated into the autophagosomal vacuoles. As shown in Figure 4A, LC3 was detected in the cytoplasms of CP-MSCs and BM-MSCs, and the expression of LC3 gradually increased during hypoxia exposure. However, LC3 expression was induced earlier and to a greater extent in CP-MSCs compared to BM-MSCs. Immunofluorescence also revealed the presence of many cup-shaped and ring-like structures in both cell types, which represent autophagosomes. We confirmed the formation of autophagosomes with the MDC incorporation assay (Fig. 4B). Autophagosomes are formed rarely under normoxic conditions. However, many autophagosomes were detected in CP-MSCs and BM-MSCs under hypoxic conditions, and they increased in a time-dependent manner. Notably, LC3 and MDC signals were more strongly observed in CP-MSCs compared to BM-MSCs at the same time of hypoxia exposure. From these results, we confirmed that the formation of autophagosomes in MSCs was induced by hypoxia but that the formation progressed faster in CP-MSCs compared to BM-MSCs.

To confirm the expression of autophagy-related proteins in the cells exposed to hypoxia, Western blot analysis was performed. As shown in Figure 4C, Beclin1 was strongly expressed and dropped at 96 h of hypoxia in CP-MSCs, although the total amount in BM-MSCs was less than in CP-MSCs. In addition, PI3K III expression increased

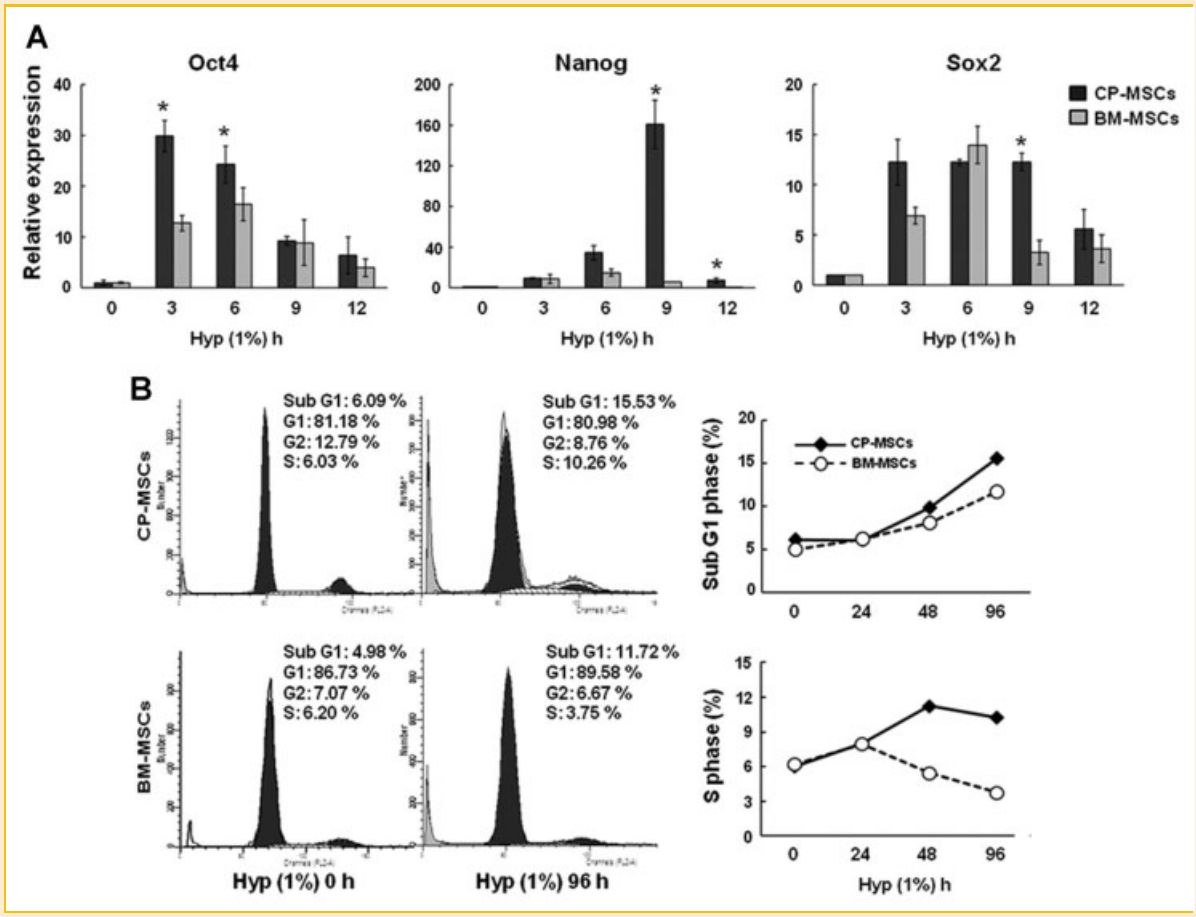

Fig. 2. Self-renewal marker expressions and an analysis of the proliferation activity in CP-MSCs and BM-MSCs after hypoxia treatment. A: Real-time PCR analysis of Oct4, Nanog, and Sox2 in CP-MSCs and BM-MSCs following hypoxic treatment. Target gene expression was normalized to that of an internal reference, GAPDH. All reactions were performed in triplicate and the values are expressed as the means ± SD. *Significant differences between CP-MSCs and BM-MSCs ($P < 0.05$). (B) Cell cycle analysis in CP-MSCs and BM-MSCs by FACS analysis. To analyze the cell cycle, CP-MSCs and BM-MSCs were cultured for 96 h under hypoxic conditions (1% O_2). The frequencies of cells in each phase of the cell cycle are indicated as percentages (%).

in CP-MSCs over time, while PI3K III was only induced at early time points in BM-MSCs, followed by a gradual down-regulation of its expression. The ATG5-12 complex consists of well-known molecules that induce autophagy by elongating the phagophore into the autophagosome in cells. Additionally, LC3 is the representative marker of autophagy. LC3 is cleaved to generate LC3 I (the cytosolic form) and LC3 II (the active form attached to the autophagosome membrane). Altered expression of ATG5-12 and LC3 II were observed in CP-MSCs and BM-MSCs by hypoxia in a time-dependent manner. Interestingly, the expression of ATG5-12 was strongly increased in CP-MSCs after 12 h and was sustained to 96 h of hypoxia. In BM-MSCs, however, it was maintained from 6 to 48 h and diminished after 96 h of hypoxia. In addition, LC3 II expression was increased by hypoxia in both cell types. Therefore, these results suggested that hypoxia altered autophagy-related molecules and triggered autophagy in MSCs, and that this induction is more dynamically activated in CP-MSCs compared to BM-MSCs.

A variety of cell-signaling pathways (e.g., ERK pathway, PI3K/STAT3 pathway) have been shown to be activated by the binding of soluble SCF to the cell surface c-kit receptor [Krasagakis et al., 2011]. Although, we already observed increased expression of SCF in CP-MSCs by hypoxia (Fig. 3A), the levels of soluble SCF were further examined because SCF can be secreted, as mentioned above. To investigate whether SCF expression is affected by hypoxia, secreted SCF in MSC cultures was analyzed by ELISA. In addition, the surface expression of c-kit, which is known as a receptor for SCF, was analyzed by FACS. As shown in Figure 5A, CP-MSCs expressed more SCF than BM-MSCs under normoxia, which gradually increased with hypoxic culture conditions. In contrast, the expression of SCF in BM-MSCs was maintained, showing significant differences compared to CP-MSC ($P < 0.05$). Further, the surface expression of c-kit was not altered in either CP-MSCs or BM-MSCs (Fig. 5B). We performed an SCF/c-kit-neutralizing assay to investigate whether autophagy was regulated by hypoxia-induced soluble SCF. CP-MSCs and BM-MSCs were cultured in hypoxic conditions with or without 1 μg/ml of SCF-neutralizing antibody for 24 and 48 h, and the expressions of p-mTOR and LC3 I and II proteins were detected by Western blot analysis (Fig. 5C). Blocking of SCF with SCF-neutralizing antibody treatment induced the phosphorylation of mTOR more markedly in CP-MSCs compared to BM-MSCs at 24 h of

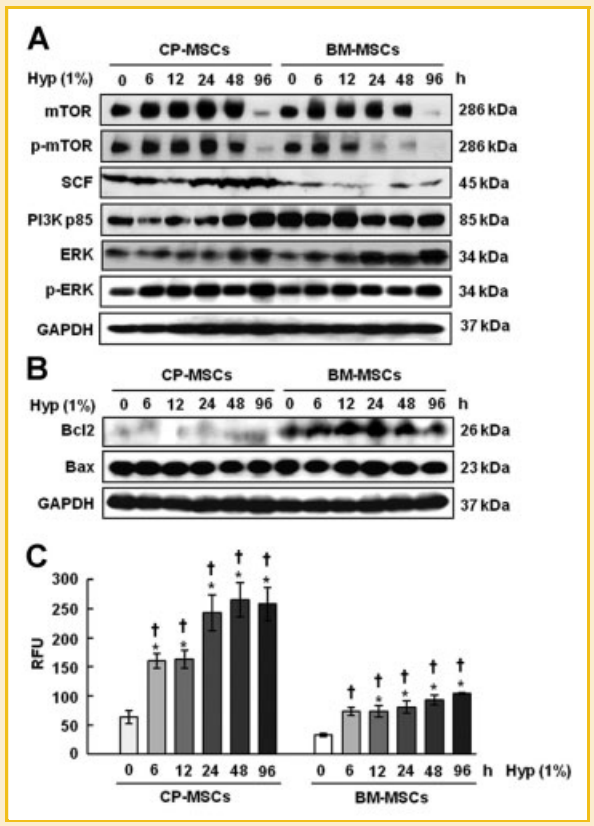

Fig. 3. Analysis of survival and apoptosis in CP-MSCs and BM-MSCs after hypoxia treatment. A: Expression of proliferation-related factors in CP-MSCs and BM-MSCs under hypoxic conditions for 0, 6, 12, 24, 48, and 96 h. GAPDH was used as a loading control. B: Expression of apoptosis-related factors in CP-MSCs and BM-MSCs under hypoxic conditions for 0, 6, 12, 24, 48, and 96 h. GAPDH was used as a loading control. C: Activities of caspases 3 and 7 in CP-MSCs and BM-MSCs under hypoxic conditions for 0, 6, 12, 24, 48, and 96 h. *Significant differences between 0 h of hypoxia and other time points in each cell group ($P < 0.05$). †Significant differences between CP-MSCs and BM-MSCs at each time point ($P < 0.05$).

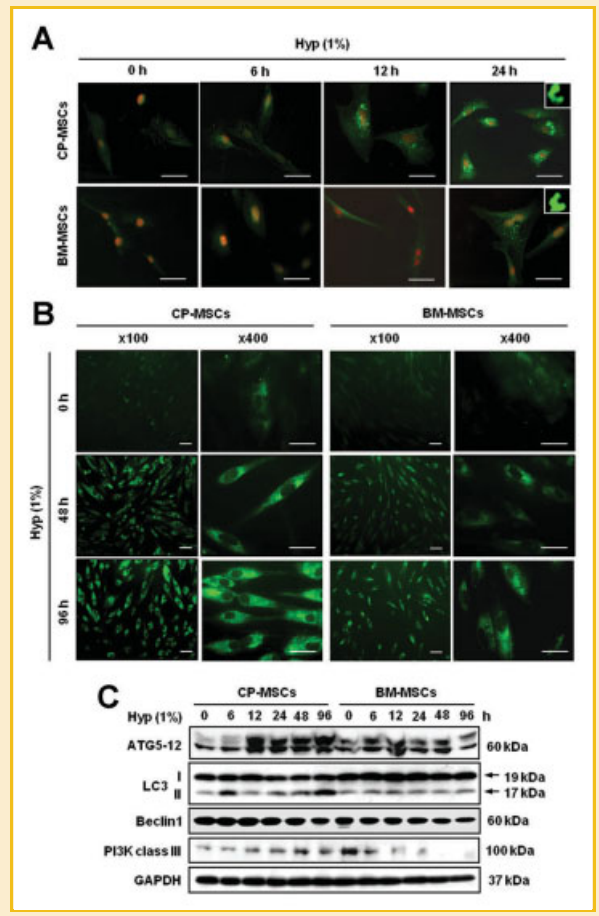

Fig. 4. Activated autophagy in CP-MSCs and BM-MSCs by hypoxia. A: Cellular LC3, which is the final molecule induced during autophagy, was detected by immunofluorescence in CP-MSCs and BM-MSCs after 0, 6, 12, and 24 h of hypoxia treatment. The upper right side of each picture shows cup-shaped autophagophores for both CP-MSCs and BM-MSCs at 24 h. B: Monodansylcadaverine (MDC) staining to detect autophagosomes. Scale bar = 100 μm. C: The expression of ATG5-12, LC3 I and II, Beclin 1, and PI3K III in CP-MSCs and BM-MSCs after 0, 6, 12, 24, 48, and 96 h of hypoxia treatment. GAPDH was used as a loading control.

hypoxia. Furthermore, SCF blocking led to decreases in LC3 II at 24 and 48 h of hypoxia in both CP-MSCs and BM-MSCs. These findings show that hypoxia-induced increases in soluble SCF enhanced autophagy in CP-MSCs and BM-MSCs through regulating the phosphorylation of mTOR to the regulation of cell proliferation via autophagy, in part because of their high level of SCF expression.

DISCUSSION

Scientists have been studying stem cells as useful tools for regenerative medicine, continued research, or clinical use because of their unlimited self-renewal capacity. However, working with ESCs also has many drawbacks; they harbor genetic mutations, can form teratomas [Knoepfler, 2009], involve difficult ethical considerations and may induce immune rejection. For these reasons, MSCs have been studied as an alternative source for cell therapy despite many deficiencies, such as decreases in yield and function with donor age and the invasive procedure required to obtain them [Fehrer and Lepperdinger, 2005; Locke et al., 2009]. Nonetheless, work with MSCs requires no ethical controversy, and they have the potential for multipotency, though it is limited compared to pluripotent ESCs [Yen et al., 2005]. Finally, MSCs exhibit immunosuppressive properties [Noel et al., 2007; Parolini et al., 2008] and can home to damaged tissues [Khaldoyanidi, 2008].

Recently, PDSCs have been spotlighted for their capacity overcome drawbacks of BM-MSCs. PDSCs are MSCs derived from the placenta, which is a temporary organ originating from the fetus, and can be immunomodulatory, exhibit self-renewal and are multipotent [Yen et al., 2005; Chang et al., 2006; Miao et al., 2006]. Furthermore, PDSCs have more advantages than adult MSCs, as they are readily available and are easily procured without invasive procedures and because sufficient numbers can be obtained for cell

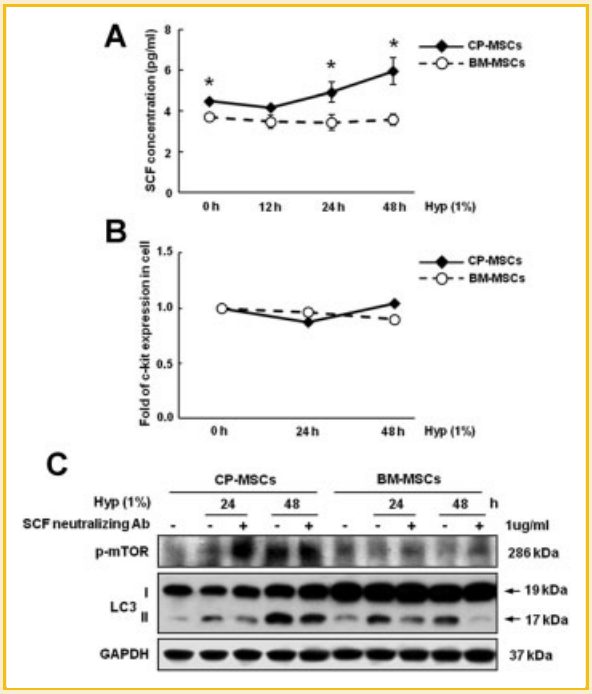

Fig. 5. Soluble SCF and surface c-kit expression in CP-MSCs and BM-MSCs under hypoxic conditions. A: Soluble SCF protein was detected in the supernatants of CP-MSC and BM-MSC cultures under hypoxic conditions. All reactions were performed in triplicate and the values are expressed as the means ± SD. *Significant differences between CP-MSCs and BM-MSCs at each time point ($P < 0.05$). B: Expression of surface c-kit was detected by binding of the CD117 antibody followed by flow cytometric analysis in CP-MSCs and BM-MSCs after 0, 24, and 48 h of hypoxia treatment. C: Expression of phosphorylated mTOR and LC3 I and II in CP-MSCs and BM-MSCs after hypoxic culture for 0, 24, and 48 h with or without 1 μg/ml of SCF-neutralizing antibody was detected by Western blot analysis. GAPDH was used as a loading control.

therapy. However, it is still important to sustain their self-renewal activities [Fehrer and Lepperdinger, 2005; Miao et al., 2006]. The self-renewal capacity of stem cells is a key characteristic that distinguishes them from other cells in an organism; this capacity may extend for the lifetime of the organism. For cell therapy, transplanted MSCs must be well engrafted and consistently sustain their renewal capacity to obtain a good prognosis. Though the control of self-renewal in stem cells is a key strategy for overcoming these problems, the mechanisms of self-renewal have not received much attention in the literature, primarily because the number of MSCs from an adult patient is too low to fulfill the needs of cell therapy [Fehrer and Lepperdinger, 2005]. Many researchers have tried to identify the "stem cell niche," which consists of various micro-environmental factors that regulate the balance of self-renewal and differentiation [Moore and Lemischka, 2006]. Several cellular and non-cellular factors have been defined, such as BMP, WNT, Notch signaling, matrix glycoproteins, blood vessels, and the three-dimensional space [Scadden, 2006]. Hypoxia has been proposed as one of the micro-environmental factors that control self-renewal and has recently been reported to enhance specific stem cell qualities of a variety of stem cells, including NSCs, ESCs, HSCs [Eliasson and Jonsson, 2010; Mohyeldin et al., 2010; Nekanti et al., 2010]. It has also been reported that HIF-1α induced by hypoxia plays a role as a mediator to maintain stem cell properties and to inhibit MSC senescence. Furthermore, hypoxic conditions have been advantageous in maintaining stem cell characteristics of HSCs. Danet et al. [2003] demonstrated that HSCs from human bone marrow cultured under hypoxic conditions enhanced graft success in immunodeficient NOD/SCID mice []. Additionally, hypoxia increased the proliferation, migration and angiogenesis of MSCs [Kim et al., 2011a]. In our study, we demonstrated that CP-MSCs and BM-MSCs steadily expressed stem cell markers and exhibited self-renewal under hypoxic conditions (Fig. 2).

Interestingly, our data show a time-dependent increase in the expression of SCF in CP-MSCs compared to BM-MSCs under hypoxic conditions. SCF is a well-known molecule that enhances cell proliferation of hematopoietic progenitors, mast cells and germ cells. In addition, SCF functions to enhance cell proliferation in stem cells [Ashman, 1999; Krasagakis et al., 2011] and correlates with bromodeoxyuridine (BrdU) incorporation into proliferating cells from the rat forebrain under hypoxia [Jin et al., 2002]. Further, HIF-1α, a key hypoxia-induced molecule, directly increased the transcription of SCF [Han et al., 2008]. Many scientists have reported that the SCF/c-kit pathway promotes proliferation and migration of cells via autocrine and paracrine mechanisms [Han et al., 2008; Krasagakis et al., 2011]. There are two types of SCF: a soluble form and membrane-bound form. SCF is initially synthesized as a membrane-bound polypeptide that is proteolytically cleaved to release the soluble protein [Ashman, 1999]. In our study, both the soluble form and membrane-bound form exhibited increased expression under hypoxia to similar extents in CP-MSCs (Figs. 3A and 5A). As oxidative stress continued under low-oxygen conditions, CP-MSCs expressed more SCF, which could help CP-MSCs maintain their self-renewal capacity. However, the expression of c-kit was constant, as shown in Figure 5B. These findings indicate that increased SCF by hypoxia could tip the balance between survival and apoptosis via autophagy mechanisms in CP-MSCs, which exhibit physiological differences compared to BM-MSCs following exposure to hypoxic stress. As shown in Figure 3, the amount of p-ERK and p-mTOR altered, which might indicate regulation via the autocrine or paracrine effects of SCF. Previous reports have shown that blocking the autocrine SCF/c-kit loop led to the inhibition of cell proliferation [Ali, 2007; Levina et al., 2010; Krasagakis et al., 2011]. These data were consistent with our own that showed that blocking of SCF with neutralizing antibodies leads to a decrease in LC3 II expression at 24 and 48 h of hypoxic conditions in CP-MSCs (Fig. 5C). This result implies that SCF-enhanced self-renewal is closely related to autophagic events.

As suggested above, SCF and p-ERK were maintained to promote the self-renewal activities of CP-MSCs, which were greater in CP-MSCs compared to BM-MSCs (Fig. 3). However, we also observed that the apoptosis rate in CP-MSCs was more aggressive than it was in BM-MSCs through the cell cycle and caspase activity assays (Figs. 2B and 3C). Therefore, hypoxia induced the expression of proliferation-related proteins, which led CP-MSCs to promote self-renewal activities and simultaneously enhanced the activity of

caspase 3/7 in response to oxidative stress. Finally, the turnover rate of CP-MSCs under hypoxia was higher than that of BM-MSCs due to the competing forces of enhanced survival by increased SCF and cell death by autophagosomal mechanisms. It is hypothesized that self-renewal activities of CP-MSCs are modulated via the regulation of a greater or lesser induction of proliferative and apoptotic events and through autophagy signaling.

An important aspect of this study is whether the mechanism of autophagy is related to cell maintenance and thereby promotes the self-renewal activities of MSCs. Autophagy is a cellular degradation pathway activated in response to various stressors, such as starvation and oxidative stress. It has been considered an alternative to non-apoptotic programmed cell "death" [Tsujimoto and Shimizu, 2005]. However, recent studies show that autophagy is not just for "death" but that it also plays a role in "survival" [Baehrecke, 2005; Kang et al., 2007; Ma et al., 2011] because degradation by autophagy may promote cell death or may provide reusable materials for cell survival. Thus, "cell death with autophagy" and "cell death by autophagy" are not easily distinguished. Autophagy mechanisms reduce genome damage and chromosomal instability in epithelial cells [Mathew et al., 2007]. In our data, autophagy was induced by hypoxia, which is consistent with previous reports [Bellot et al., 2009; Mazure and Pouyssegur, 2010]. The formation of the autophagosome in CP-MSCs was induced at an earlier time point than in BM-MSCs (Fig. 4). This early induction of autophagy by hypoxia may be strongly linked to the self-renewal activities of CP-MSCs. Autophagy may be an effective way to sustain a healthy population of stem cells via balancing abnormal cell clearance with normal cell proliferation, which may eventually contribute to maintaining self-renewal activities. Based on our results, we hypothesized that hypoxia-induced autophagy signaling allows MSCs to activate their self-renewal capacity (Fig. 6).

Taken together, our results clearly show that hypoxia induced the expression of SCF in CP-MSCs. Specifically, the SCF/c-kit pathway promotes the self-renewal activities of CP-MSCs via an autocrine/paracrine mechanism that balances cell survival and cell death events by autophagy. Further, these activities occur to a greater extent in CP-MSCs than in BM-MSCs through regulating the phosphorylation of mTOR. These findings will provide useful guidelines for better understanding the function of SCF/c-kit in the self-renewal and autophagy-regulated mechanisms that promote of MSC survival.

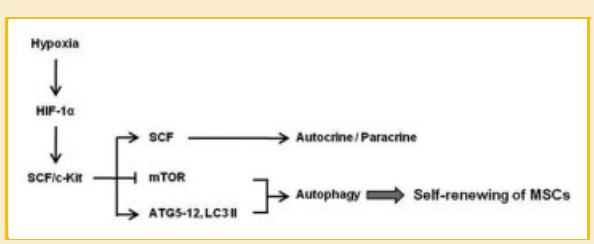

Fig. 6. Model summarizing the hypoxia-induced signals observed in self-renewing MSCs, including SCF/c-kit, apoptosis and autophagy.

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