Chapter 5

Hypoxia in Mesenchymal Stem Cell

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Abstract

Mesenchymal stem cells (MSCs) are non-hematopoietic multipotent stem cells with self-renewal properties and ability to differentiate into a variety of mesenchymal tissues. This chapter overviews effects of hypoxia on MSCs, makes it promising therapy to various diseases. Cultivation of MSCs under hypoxic condition results in variety of outcome that is important to be noted in clinical use. In most studies, hypoxic condition appears to increase proliferation, differentiation, and immune regulatory performance of MSCs without affecting its characteristic. Those benefits are therefore utilized in clinical application. However, there are also studies that report on negative effects of hypoxia in MSCs such as chromosomal instability. Molecular mechanism of MSCs in hypoxic condition is provided for better understanding, which is crucial for further development with better outcome.

Keywords: mesenchymal stem cells, hypoxia

1. Introduction

In these days, stem cell therapy is becoming more believable in treating degenerative diseases compared to conventional medicine. Various diseases such as diabetes, myocardial infarction, spinal cord injury, stroke, and Parkinson's and Alzheimer's diseases have become more prevalent with increasing life expectancy. It has been estimated that in the United States alone, ~128 million individuals would benefit from regenerative stem cell therapy during their lifetime. Mesenchymal stem cells (MSCs) have been highly utilized to treat degenerative diseases among other stem cells. These cells are found in tissues such as bone marrow, adipose tissue, umbilical cord,

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and dental pulp. Self-renewal and multipotency are the key features of MSCs that make it promising tool. These properties have raised interest on researchers for finding appropriate method to optimize the genetic and environmental factors, which later enhance the biological activities of MSCs.

Many researches have been conducted in the last two decades to study the complex processes in stem cell maintenance. The role of hypoxic conditions (usually 2–9% O_2 concentration) on stem cell biology is very interesting subject due to its beneficial effects. Thus, cultivation of MSCs under hypoxia is currently studied to obtain better understanding, as well as further development to generate better outcome.

2. Mesenchymal stem cell

About 130 years ago, German pathologist Conheim proposed the presence of non-hematopoietic stem cells in the bone marrow that contributes to wound healing in numerous peripheral tissues. Later in the early 1970s, Friedenstein and colleagues demonstrated that the rodent bone marrow had fibroblastoid cells with clonogenic potential *in vitro* [1, 2]. In the study, after the non-adherent cells were removed a few hours later, spindle-like cells, which were morphologically heterogeneous, appeared to attach to the plastic, capable of forming colonies. These cells could also make bone and reconstitute a hematopoietic microenvironment in subcutaneous transplants. Moreover, they could regenerate heterotopic bone tissue in serial transplants, thus indicated their self-renewal potential. Over the years, many studies have investigated these findings and found that these cells were also present in the human bone marrow and could be sub-passaged and differentiated *in vitro* into a variety of the mesenchymal lineages such as osteoblasts, chondrocytes, adipocytes, and myoblasts [3–7]. It has been further renamed as "mesenchymal stem cell" or MSC [4].

MSCs or MSC-like cells are also found in fat, umbilical cord blood, amniotic fluid, placenta, dental pulp, tendons, synovial membrane, and skeletal muscle, yet the complete equivalency of such populations remains unclear [8–16]. Characteristic of MSCs according to The International Society for Cell Therapy [17] consists of (1) adherence to plastic in standard culture conditions; (2) expression of the surface molecules CD73, CD90, and CD105 in the absence of CD34, CD45, HLA-DR, CD14 or CD11b, CD79a, or CD19 surface molecules; and (3) a capacity for differentiation to osteoblasts, adipocytes, and chondroblasts *in vitro*. These criteria were established to standardize human MSC isolation but may not apply uniformly to other species. For instance, marker expression and behavior in murine MSCs were different compared to human MSCs [18]. Certain *in vivo* surface markers may no longer be expressed after Transplantation, although new markers are obtained during expansion. In study done by Jones et al., MSC uniformly expressed HLA-DR (a marker that should not be expressed on MSCs by the above definition) while also expressing CD90 and CD105, adhering to plastic in culture, and differentiating into osteoblasts, adipocytes, and chondroblasts [19]. Indeed, clear definition of MSC-specific characteristics is difficult to apply in both human and animal models.

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3. Hypoxia in mesenchymal stem cell

Numerous *in vitro* studies have been conducted in the last two decades to observe the complex processes in stem cell maintenance. However, the role of physiologically hypoxic conditions (usually 2–9% O_2 concentration) on stem cell biology received very little attention. O_2 concentration is an environmental factor that plays a vital role on stem cell fate and function [20]. Stem cells are typically cultured under the ambient O_2 concentration without paying attention to the metabolic milieu of the niche in which they normally grow [21]. The effects of different O_2 levels in MSC culture were first studied in 1958, when Cooper et al. and Zwartouw and Westwood observed that some cells proliferated more rapidly under low O_2 tension levels compared to normal atmospheric levels [22, 23]. MSCs are present in perivascular niches in close association with blood vessels in virtually all tissues [11, 16, 24] and have been compared to pericytes [25]. Even though MSCs are located close to vascular structures, the different tissues where these stem cells are found exhibit low oxygen tensions [26–29]. Therefore, it is possible that maintaining MSCs in an undifferentiated state may require a hypoxic environment, in addition to other factors.

The higher O_2 concentration might cause environmental stress to the *in vitro* cultured MSCs. Recent studies have presented significant evidences regarding the negative outcome under ambient O_2 concentration on MSCs, including early senescence, longer population doubling (PD) time, DNA damage [30, 31], and poor engraftment following transplantation [32, 33]. These have shown the influential effect of O_2 concentration on MSCs biology and raised serious concern over its therapeutic efficiency and biosafety. Thus, the effect of different O_2 concentration on MSCs biology is further discussed based on recent research outcomes.

4. Characteristic of MSCs in hypoxic condition

As described above, MSC immunophenotype is characterized by the expression of CD73, CD90, CD105, CD106, CD146, and MHC class I molecules, and the absence of markers such as CD45 and CD34 or MHC class II molecules [17]. Many studies suggest that hypoxia has no effect on MSCs characteristic, indicated by surface markers. According to one study by Holzwarth et al., there were no significant differences in the expression of cell surface markers after 14 days of culture at 1% when compared to 20% of O_2 [34]. Referring to study carried out by Nekanti et al., WJ-MSCs cultured under both hypoxia and normoxia for 10 passages were positive for CD44, CD73, CD90, CD105, and CD166 and negative for CD34, CD45, and HLA-DR, and there was no significant difference between the two populations [35]. These results are also supported by study carried out by Widowati et al. The surface marker of WJ-MSCs of P4 and P8 both normoxic and hypoxic 5% O_2 were not significantly different. WJ-MSCs were positive for CD105, CD73, and CD90 and negative for CD34, CD45, CD14, CD19, and HLA-II [36].

Morphology changes are also documented in MSCs under hypoxia. Referring to Nekanti et al., WJ-MSC cultured under hypoxia showed a higher amount of large, flattened cells both at

early and late passages, compared to normoxic cultures. The enlargement in cell size under hypoxia might be due to a natural response to low oxygen, in which increased surface area would allow for an increase in oxygen diffusion rate [35].

5. MSCs proliferation in hypoxic condition

Capability for self-renewal is a key feature of stem cells. An increased proliferation rate is necessary for more efficient use of stem cells in regenerative therapies. Fehrer et al. demonstrated that bone marrow-derived MSCs (BM-MSCs) cultured in 3% O_2 concentration showed significant increased *in vitro* proliferative lifespan, with ~10 additional population doublings (PDs) (28.5 ± 3.8 PD in 20% O_2 and 37.5 ± 3.4 PD in 3% O_2) before reaching senescence compared to cells cultured in the ambient O_2 environment [31]. In addition, early passaged MSCs cultured in hypoxic conditions also exhibit increased proliferative lifespan along with significant difference in population doubling [30]. Furthermore, it is possible to harvest more than 1×10^9 MSCs from the first five passages cultured in 3% O_2, whereas in ambient condition only 2×10^7 cells can be obtained [30]. Higher *in vitro* expansion rate in hypoxic conditions has also been reported by other researchers [37–39]. Such *in vitro* culture environment also allows to maintain a higher proportion of rapidly self-renewing MSCs for a longer period of time [40]. Other study showed that the increased hypoxic (O_2 2.5%) condition was the best microenvironment for stem cell proliferation compared to normoxic and hypoxic (O_2 5%) for cells at a high passage (P7, P8) [41].

However, various responses of stem cells under hypoxia have been reported [42]. Those differences in cellular responses on hypoxia might be associated with degrees and durations of hypoxia, as well as other cell conditions. Oxygen tension in the stem cell niche for MSCs is suggested to be various from 1 to 7% [43]. A study by Holzwarth et al. showed that rates of MSCs proliferation were reduced after 7 days of culture under hypoxia at 21, 5, 3, and 1% O_2. In their study, only 1.37% of the cells entered the G2/M phase in hypoxic cultures (1% O_2) after 7 days, compared to 2.50% at hyperoxic culture (21% O_2). Reduced O_2 concentrations were therefore confirmed to inhibit cell proliferation as indicated by reduced number of cells in the G2/M phase [34].

6. Chromosomal stability of MSCs in hypoxic condition

Some recent studies have found that human mesenchymal stem cells (hMSCs) retained chromosomal stability following long-term culture *in vitro* [44–46]. Hypoxic environments have shown to increase mutation frequencies in cancer cell lines and trigger genomic rearrangements [47, 48]. It is suggested that oxygen concentration has a major impact on karyotypic aberration. Referring to study of Ueyama et al., chromosomal instability is associated with repeated cell division. A high frequency of chromosomal abnormality breakpoints in common fragile sites (CFSs) was detected by karyotypic analysis (e.g., 2q33, 7q11, 7q36, 8q22.1, 8q24.1,

11p15.1, 19q13) [49]. Generally, chromosomes have fragile sites that are prone to exhibiting gaps and breaks during metaphase [50], in which chromosome rearrangement occurs in cultured cells. Fragile sites are categorized into two main classes, common and rare, according to their frequency in the population [51]. In Ueyama study, several genes involved in regulation of the cell cycle, transcription and cell adhesion, are located in that region with a frequency of 6, 5, and 2%, respectively. In particular, the 11p15.5 domain known as an important tumor suppressor gene region such as tetraspanin 32 (TSPAN32) and tumor-suppressing subtransferable candidate 4 (TSSC4) is present in this region. Alterations in this region have been associated with some neoplasia. It is suggested that the deletion of contiguous genes may induce a multisystem developmental disorder and that these alterations might influence normal functioning and cell survival.

Sex chromosome aneuploidy was also one of the most observed aberrational karyotypes. Frequency of sex chromosome in cultured lymphocytes was significantly higher in females than in males, and that loss of Y chromosomes correlated with age in human bone marrow cells [52, 53]. There are several factors influencing karyotypic stability such as hypoxic culture conditions, donor age, and multiple passages. Karyotypic aberrations increased with passage number and hMSCs undergo spontaneous transformation with tumorigenic potential, especially in later passages under hypoxic culture conditions in hMSCs of elderly donors [49]. Shortly, monitoring of chromosomal stability in culture expanded hMSCs is required prior to exposure to human beings, in order to detect mutations and potentially immortalized clones and to prevent transplant-associated tumor formation.

7. MSCs plasticity in hypoxic condition

The multilineage potential of MSCs is one of the reasons underlying their use in regenerative medicine [54]. Results of MSC differentiation into other lineages diverse according to several studies [34, 55, 56]. Some *in vitro* studies have shown that cultures with low O_2 concentrations stimulated cells to differentiate into adipogenic, osteogenic, or chondrogenic cells. Previous study showed that Rat mesenchymal stem cells (rMSCs) cultured in 5% oxygen produced more bone than cells cultured in 20% O_2 throughout their cultivation time, as indicated by increased markers of osteogenesis, including alkaline phosphatase activity, calcium content, and von Kossa staining. These markers were usually elevated above basal levels when cells were switched from control to low oxygen at first passage and decreased for cells switched from low to control oxygen [57]. Hypoxia appears to exert a potent lipogenic effect independent of PPAR-γ2 maturation pathway [58]. The level of differentiated antigen H-2Dd and the number of G2/S/M phase cells increased evidently under 8% O_2 condition. Also, the proportion of wide, flattened, and epithelial-like cells increased significantly in MSCs. When cultured in adipogenic medium, there was a fivefold to sixfold increase in the number of lipid droplets under hypoxic conditions compared with that in normoxic culture. Oct4 was downregulated under 8% O_2 condition but still expressed after adipocyte differentiation in normoxic culture and treated with hypoxia-mimicking agents, cobalt chloride ($CoCl_2$) and deferoxamine mesylate

(DFX). These findings indicate that hypoxia enhances MSC differentiation and hypoxia and hypoxia-mimicking agents generate different effects on MSC differentiation [59].

Conversely, some others have reported suppressive effects of low O_2 tension levels on the plasticity of MSCs. Differentiation capacity into adipogenic progeny was diminished, and no osteogenic differentiation was detected at 3% oxygen. In turn, MSC that had previously been cultured at 3% oxygen could subsequently be stimulated to successfully differentiate at 20% oxygen [31]. Temporary exposure of MSCs to hypoxia resulted in (i) persistent (up to 14 days postexposure) downregulation of cbfa-1/Runx2, osteocalcin, and type I collagen and (ii) permanent (up to 28 days postexposure) upregulation of osteopontin mRNA expressions [60]. Another study by Widowati et al. showed both nor-WJ-MSCs and hypo-WJ-MSCs differentiated to osteocytes, chondrocytes, and adipocytes, although there was no significant difference among treatments [36]. Study conducted by Georgi et al. showed that molecular fingerprints of human MSCs, primary chondrocytes, and MSC/primary chondrocytes coculture differ when cultured in either normoxic (21% O_2) or hypoxic (2.5% O_2) conditions [61]. In the study, cartilage formation increased in cocultures of MSCs and primary chondrocytes was lost when the cells were cultured under hypoxia which was associated with a decrease in the mRNA expression of the chondrogenic marker SOX9 and FGF-1. This coincided with a significant decrease in lipids. Lipid profiles of normoxic and hypoxic cultures are different. The improved cartilage formation in cocultures of MSCs and chondrocytes may employ soluble factors, including small molecules, lipids, or proteins [62]. Lipids such as phospholipids, cholesterol, and diacylglycerols play significant roles in cellular signaling, membrane integrity, and metabolism [63]. Recent study described that short-term changes in sphingolipid metabolism resulted in long-term effects on the chondrogenic phenotype, and the stimulation of chondrocytes with acylceramidase improves cartilage repair and MSC differentiation [64].

8. Immunomodulatory effects of MSCs in hypoxic condition

One of the key factors of MSC in therapeutics development is their known anti-inflammatory/immunomodulatory properties. Clinical studies showed efficacy of MSC at inhibiting lethal, immune-based condition of graft versus host disease [65–70]. It has been reported that MSCs derived from adipose, bone marrow, and placenta have the capability to recover ischemic injury by increasing vascularization and reducing inflammation in ischemia-injured hindlimb, lung, heart, and brain [71–73]. Thus, these cells have been used in clinical trials to treat ischemic disease [74]. MSCs produce a broad variety of cytokines, chemokines, and growth factors that may potentially be involved in tissue repair. Hypoxia increases the production of several of these factors, although different responses are also noted in few studies. Referring to Chang et al., hypoxic preconditioning enhances the capacity of the secretome obtained from cultured human MSCs to release several of these factors and the therapeutic potential of the cultured MSC secretome in experimental TBI [75].

One of the most studied mechanisms of inflammation-induced MSC activity is treatment with interferon gamma (IFN-γ). This cytokine is usually secreted during inflammatory Th1 immune

responses that are associated with autoimmunity mediated by cellular means, such as CD8 T cells and NK cells, which commonly occur in multiple sclerosis, diabetes type 1, and rheumatoid arthritis [76]. Treatment of IFN-γ in MSC has been reported to enhance the immunosuppressive activity through stimulation of the enzyme IDO [77–80]. MSC expression of the tryptophan-catabolizing enzyme indolamine 2,3 deoxygenase (IDO) was markedly upregulated under hypoxia [81]. IDO is critical in immune regulation by MSC through induction of T cell anergy [82] and stimulation of T regulatory cells (T-regs) [83, 84].

Moreover, IFN-γ induced secretion of other inhibitors of inflammation by MSCs, including the complement inhibitor factor H [85], as well as the immunomodulatory molecules TGF-β and HGF [86]. At a functional level, Noone et al. demonstrated that IFN-γ pretreatment of MSC resulted in protection of MSCs from NK-mediated killing via upregulation of prostaglandin E (PGE)-2 synthesis [87]. IFN-γ, along with necrosis factor-alpha (TNF-α), IL-1α, and IL-1β, induces Gal-9 in MSC [88].

Another inflammatory mediator known to induce regenerative activities in MSC is the macrophage-derived cytokine TNF-α. Pretreatment of TNF-α in MSCs provided superior angiogenic activity *in vitro*, as indicated by expression of VEGF, as well as *in vivo* in an animal model of critical limb ischemia, as compared to untreated MSCs [89]. In other study, TNF-α preconditioning increased proliferation, mobilization, and osteogenic differentiation of MSCs and upregulated bone morphogenetic protein-2 (BMP-2) protein level. Osteogenic differentiation of MSC induced by TNF-α was partially inhibited after BMP-2 knockdown by siRNA [90]. Lipopolysaccharide and toll-like receptor (TLR) agonists, as activators of innate immunity, are also responsible for regenerative activity of MSCs by inducing paracrine factors secretion such as VEGF [91]. IFN-γ and TLR also upregulate the glucocorticoids production, which decreases T cells stimulated by radiotherapy in colonic mucosa [92].

Akiyama et al. reported that MSCs induced T cell apoptosis via the Fas/FasL pathway [93]. Telomerase improved immunomodulatory properties of MSCs by upregulating FasL expression [94]. Dental follicle cells and cementoblasts have been reported to trigger apoptosis of ameloblast-lineage cells, as well as Hertwig's epithelial root sheath (HERS)/epithelial rests of Malassez (ERM) cells, via the Fas/FasL pathway during tooth development [95]. FasL regulated the immunomodulatory properties of Human gingiva-derived mesenchymal stem cells (hGMSCs), which is promoted by hypoxia. However, the underlying pathways of such event remain unclear. Further studies regarding the pathways involved in hGMSC-mediated immunomodulation are encouraged.

9. Molecular mechanism of MSCs in hypoxic condition

O_2 concentration in the stem cell niche (usually 2–9% O_2) is considered a driver of cell function [20]. Hypoxia plays a vital role in maintaining homeostasis within the body from the early stage of embryonic development. It facilitates proper embryonic development, maintains stem cell pluripotency, induces differentiation, and regulates the signaling of multiple cascades, including angiogenesis [96]. In hypoxic conditions, these functions are regulated by several

transcription factors such as hypoxia-inducible factors (HIFs), prolyl hydroxylases (PHDs), factor-inhibiting HIF-1 (FIH-1), activator protein 1 (AP-1), nuclear factor (NF)-κB, p53, and c-Myc [97]. Although interaction among all of the transcription factors is required for cellular response, HIFs (especially HIF-1) are the key regulators of cellular response to hypoxia [98]. The discovery of HIF-1a by Greg Semenza provided profound insight into the cellular mechanisms that control hypoxic adaptation [99–101].

Generally, under hypoxic conditions, low O_2 level suppresses the prolyl hydroxylation that leads to HIF-1α accumulation and nuclear translocation [102]. After nuclear translocation, it binds with HIF-1β to form the heterodimer. Then, the HIF-1 heterodimer binds to a hypoxia-response element (HRE) in the target genes, associated with coactivators such as CBP/p300, and regulates the transcription (**Figure 1**) of as many as 70 genes involved in metabolism, angiogenesis, invasion/metastasis, and cell fate [103].

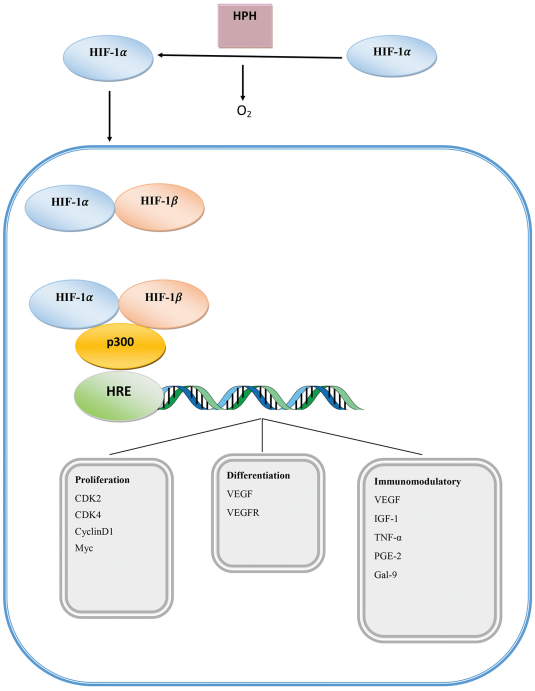


Figure 1. Regulation of hypoxia in MSCs. HIF, hypoxia-inducible factor; HPH, HIF-prolyl hydroxylases; HPE, HIF-prolyl hydroxylases; HRE, hypoxia-response element; CDK, cyclin-dependent kinase; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; IGF, insulin-like growth factor; TNF, tumor necrosis factor; PGE-2, prostaglandin E-2; Gal9, galectin-9. The prolyl hydroxylation process is suppressed due to lack of O_2 that leads HIF-1α accumulation and nuclear translocation after nuclear translocation, and it binds HIF-1β to form the heterodimer. Then, the HIF-1 heterodimer binds HRE in the genes target, associated with coactivators such as CBP/p300, and regulates the transcription of as many as 70 genes involved in proliferation, differentiation, and immunomodulatory.

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In 2007, iPSCs were discovered by Shinya Yamanaka and colleagues, and the subsequent identification of the necessary transcriptional programs was required to maintain stem cells in a pluripotent state [104, 105]. The measurement of low partial pressures of oxygen in various stem cell niches raises question whether HIF-1a and iPSCs pathways were converged. It was described initially in Embryonic stem cells (ESCs) [106], hematopoietic stem cells (HSCs) [107], neural stem cells (NSCs) [108], and cancer stem cells (CSCs) [109], which now further expanded to include iPSCs [110]. Remarkably, Yamanaka first reprogrammed fibroblasts to iPSCs using only four transcription factors (Oct4, Sox2, c-Myc, and Klf4) [105] in the same year that Oct4 was shown to be a specific target gene of HIF-2a [111]. The correlation between HIF-2a and Oct4 has been proposed as underlying mechanism of stem cells response to hypoxic conditions in their niche and direct modification of stem cell function by low O_2. HIF-2a expression has recently been investigated in several stem cell lineages, and Oct4 expression is tightly regulated throughout embryogenesis. Loss or even decrease in Oct4 expression leads to differentiation [112]. Oct4 works in concert with Nanog and Sox2 to maintain stem cell identity and repress genes that promote differentiation [113]. The recent identification of HIF-2a upregulation by Oct4 in CSCs and ESCs underscores the importance of this axis in maintaining stemness in both development and disease.

It is known that phosphorylation of protein kinase B (Akt), a downstream gene of phosphatidylinositol 3-kinase (PI3K) signaling pathway, is an important step in signaling pathways that mediate cell proliferation [114, 115]. In PI3K/Akt pathway, a large number of substrates are phosphorylated, including HIF-1 [116]. Referring to study done by Rosová et al., the preculture of MSCs in hypoxia prior to injection activated the PI3K/Akt signaling pathway while maintaining their viability and cell cycle rates [117].

Hypoxia-mediated MSC differentiation by reducing apoptosis via activating the PI3K/Akt/FoxO pathway. Referring to Wang et al., MSCs underwent apoptosis upon induction for chondrogenic differentiation [118]. Apoptosis has been demonstrated as a general phenomenon that occurs during endochondral differentiation of chondrocytes [119]. One study demonstrated that chondrocytes progression to endochondral ossification employed higher FAS receptor and caspase protein as indicators of apoptosis [120]. Other studies showed that both the Wnt/beta-catenin and Indian hedgehog (Ihh) signaling pathways play important roles in endochondral ossification. Beta-catenin is needed at upstream of Ihh signaling for chondrocyte survival and inhibition of apoptosis [121]. The expression of Sox9, col2a1 and aggrecan in prechondrogenic cells 30 and chondrocytes 14 is regulated by PI3K/Akt pathway. It has also been demonstrated that PI3K/Akt regulated col2a1 and aggrecan by modulating Sox9 expression and transcriptional activity in nucleus pulposus cells 31.

Lee et al. [122] showed novel pathway for hypoxia-induced proliferation and migration in human mesenchymal stem cells that employ HIF-1α, FASN, and mTORC1O. Hypoxia treatment stimulates UCB-hMSC proliferation, along with the expression of two lipogenic enzymes: fatty acid synthase (FASN) and stearoyl-CoA desaturase-1 (SCD1). FASN is a key enzyme in UCB-hMSC proliferation and migration. Hypoxia-induced FASN expression was regulated by the HIF-1α/SCAP/SREBP1 pathway. Mammalian target of rapamycin (mTOR) was phosphorylated by hypoxia, whereas inhibition of FASN by cerulenin suppressed

hypoxia-induced mTOR phosphorylation, as well as UCB-hMSC proliferation and migration. Hypoxia-induced proliferation and migration are significantly inhibited by raptor small interfering RNA. Hypoxia-induced mTOR also regulates CDK2, CDK4, cyclin D1, cyclin E, and F-actin expression as well as c-myc, p-cofilin, profilin, and Rho GTPase. Moreover, hypoxia-induced FASN stimulates FFA production as well as proliferation and migration. Several studies reported that FAS and FA derivatives inhibited and uncoupled oxidative phosphorylation of various cells [123–125]. Palmitic acid treatment rescues inhibition of mTOR phosphorylation as well as restriction of UCB-hMSC proliferation and migration. Change in cellular metabolite ratios may be another pathway, in addition to the HIF1a/SCAP/SREBP1 pathway, involved in the regulation of lipid metabolism in UCB-hMSCs. Some studies reported that alteration of cellular metabolite ratios, such as NADP/NADPH, by hypoxia has also an important role in the regulation of various stem cell functions such as cell cycle and self-renewal activities [126, 127].

10. Hypoxic MSCs in clinical application

MSCs possess anti-inflammatory/immunomodulatory properties, which are utilized in therapeutics development. Clinical studies on efficacy of MSCs have been shown to inhibit lethal, immune-based condition of graft versus host disease [65–70]. Moreover, MSCs derived from adipose, bone marrow, and placenta have the capability to recover ischemic injury by increasing vascularization and reducing inflammation in ischemia-injured hindlimb, lung, heart, and brain [71–73, 128]. These cells have been used in clinical trials to treat ischemic disease [74], and the safety of MSCs has been evaluated [129, 130]. There are several modified approaches, which have been proposed to improve the effect of MSCs on ischemia-related disease, such as over expression of angiogenesis-related genes such as bFGF on MSCs [131], combination with other cells such as endothelial cells [84], antioxidants such as melatonin [132], serum deprivation [72], and cell spheroids [133].

From isolation to engraftment, the MSCs usually pass through two different phases, consisting of *in vitro* culture condition (from isolation to transplantation) and *in vivo* or physiological condition (before isolation and after transplantation). At present, most of the expansion procedures of MSCs are performed under ambient O_2 concentration, where cells are exposed to 20% O_2, which is ~4–10 times more than the concentration of O_2 in their natural niches [134, 135]. Maintaining genetic stability has been a challenge during *in vitro* expansion of MSCs. Increased rates of aneuploidy, double-stranded DNA breakdown, and faster telomere shortening have been reported for MSCs cultured in ambient condition [30]. According to recent review, major causes behind aneuploidy were defective spindle assembly checkpoint, centrosome amplification, and merotelic attachments [136], which are caused by ROS [137]. ROS also acts in acceleration of telomere shortening and DNA breakdown [138, 139]. Correlation between telomere shortening and aneuploidy in embryonic and hepatocellular carcinoma cells has also been reported in recent studies [140, 141]. The higher ROS production due to the increased mitochondrial respiration during expansion of MSCs in ambient O_2 concentration might be the cause behind genetic instability in them. However, cells undergo anaerobic

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respiration during hypoxia, which lowers the ROS concentration within the cells. This might reduce the DNA damage, telomere shortening, and aneuploidy which in return may increase the biosafety of stem cell-based therapy.

The ability of stem or progenitor cells to home and engraft into target tissues after transplantation is the key to succeed in clinical application. The degree of homing and engraftment of MSCs in adult recipients is very low [142–144]. Hypoxic culture conditions may also provide a solution for more efficient engraftment. Recently, early passaged mouse BM-MSCs showed better engraftment than late passaged mouse BM-MSCs in *in vivo* model [145]. In other study, hypoxic preconditioned murine MSCs also enhanced skeletal muscle regeneration and improved blood flow and vascular formation compared to normoxic condition [146]. Furthermore, hypoxic conditions cause MSCs to grow faster [30] while maintaining a higher number of rapidly self-renewing cells [40]. Hypoxic environment also upregulated chemokine receptors CXCR4, CXCR7 and CX3CR1 [147, 148], and they may facilitate tissue-specific trafficking of MSCs. Thus, sufficient numbers of MSCs with a higher fraction of rapidly self-renewing cells are suggested, and highly expressed chemokine receptors on their surface can be obtained from the early passages of hypoxic cultures, which could increase the efficiency of damaged tissue-specific migration and engraftment following transplantation.

MSCs cultured under hypoxic conditions also increased in vascular endothelial growth factor receptor 1 (VEGFR1) expression and VEGF- or placental growth factor (PLGF)-dependent migration (Okuyama et al., 2006). Preconditioning with oxygen and combined glucose depletion also increased the survival of stem cell antigen (Sca)-1þ cells via PI3K/Akt-dependent caspase-3 downregulation and thereby increased the engraftment rate [149]. In addition to the increase in migration and survival, MSCs with hypoxic preconditioning have also been shown to enhance revascularization after transplantation for hindlimb ischemia [117]. Therefore, culturing MSCs in hypoxic conditions can also be considered as a solution for tissue-specific engraftment.

Hypoxia-stimulated immune regulation of MSCs has been observed in the situation of allogeneic use of BM-MSCs for stimulation of therapeutic angiogenesis. Recent study showed hypoxia-conditioned BM-MSCs from B6 mice repair limb of Balb/c mice compared to normoxic MSCs. Engraftment in allogeneic recipients increased by decreasing NK cells cytotoxicity and the accumulation of host-derived NK cells when transplanted *in vivo*. These allogeneic hypoxia-treated BM-MSCs increased CD31+ endothelial cells and αSMA+ and desmin + muscle cells, thereby enhancing angiogenesis and restoring muscle structure. Moreover, anti-NK antibodies along with normoxic MSCs enhanced angiogenesis and prevented limb amputation in allogeneic recipients with limb ischemia [150].

Some studies have shown that MSC transplantation contributes to tumor formation *in vivo* [24, 151, 152], whereas Furlani et al. reported that cultured MSCs with spontaneous transformations had no functional effects after intracardiac transplantation [153]. Further studies regarding tumorigenicity and safety of the stem cell-based products are encouraged. However, complexity of cell therapy requires more standards for advanced medicinal products [154]. Thus, especially in the field of regenerative medicine, concrete and specific standards and governmental support systems are necessary to promote their production [154].

11. Perspective of hypoxic MSCs

Hypoxic condition has been confirmed to enhance MSCs proliferation, differentiation, and immune regulatory performance. However, some studies have also reported opposite and negative effects. Different outcomes in each study raise interest in availability of more appropriate methods for cell cultures, which require further study in standardizing the culture of MSCs for use in cell therapy. Optimal conditions for the culture of MSCs have not yet been clearly defined, and it is very crucial to precisely determine the effects of hypoxia on MSCs differentiation, proliferation, and morphology, among other aspects. Moreover, hypoxic MSC-based therapies require a complete understanding of stem cell molecular mechanism. The clarity in stem cell regulation is important for further development such as periodic monitoring of chromosomal stability in culture prior to exposure to human to detect mutations and to prevent transplant-associated tumor formation, and also genetic engineering of physiology of MSCs to acquire better outcome.

12. Conclusion

The growing interest in the potential application of MSCs in regenerative medicine was followed by the several studies measuring the effects of low O_2 levels on the behavior and function of MSCs. Hypoxic condition appears to enhance MSCs proliferation, differentiation, and immune regulatory performance in damaged tissues without affecting its characteristic. However, there are also studies that report on negative effects of hypoxia in MSCs.

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