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Review Article

Benefits of hypoxic culture on bone marrow multipotent stromal cells

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Abstract: Cultivation of cells is usually performed under atmospheric oxygen tension; however, such a condition does not replicate the hypoxic conditions of normal physiological or pathological status in the body. Recently, the effects of hypoxia on bone marrow multipotent stromal cells (MSCs) have been investigated. In a long-term culture, hypoxia can inhibit senescence, increase the proliferation rate and enhance differentiation potential along the different mesenchymal lineages. Hypoxia also modulates the paracrine effects of MSCs, causing upregulation of various secretable factors, including the vascular endothelial growth factor and interleukin-6, and thereby promoting wound healing and diabetic fracture healing. Finally, hypoxia plays an important role in mobilization and homing of MSCs, primarily by its ability to induce stromal cell-derived factor-1 expression along with its receptor, CXCR4. After transplantation, an ischemic environment, that is the combination of hypoxia and lack of nutrition, can lead to apoptosis or cell death, which can be overcome by the hypoxic preconditioning of MSCs and overexpression of prosurvival genes like Akt, HO-1 and Hsp70. This review emphasizes that hypoxia is an important factor in all major aspects of stem cell biology, and the mechanism involved in the hypoxic inducible factor-1signaling pathway behind these responses is also discussed.

Keywords: Mesenchymal stem cells, hypoxia, hypoxic preconditioning, proliferation, differentiation potential, apoptosis, migration, engraftment, HIF-1

Multipotent stromal cells

Bone marrow contains several subpopulations of stem/progenitor cells that are capable of differentiating into various non-hematopoietic cells. Among the best studied subpopulations are the cells that are isolated by their adherence to tissue culture surfaces. Such cells are referred to as multipotent stromal cells (MSCs) [1-4]. MSCs have emerged as a promising tool for clinical applications such as tissue engineering and cell-based therapy. This is because they are readily isolated from a patient, can be expanded in culture with maintained differentiation potential and immune-modulating property, and have a limited tendency to form tumors.

Hypoxic niche of multipotent stromal cells

The bone marrow niche is vitally important to the survival of organism, which facilitates the maintenance of hematopoietic stem cells (HSCs) as undifferentiated, while supporting lineage commitment of the expanding blood populations [5]. MSCs, residing in the bone marrow, are associated with and maintain HSC functions, and give rise to mesenchymal committed cells such as osteoblast and adipocyte for maintaining bone structures. Thus, the roles of bone marrow niche in maintaining MSC properties and functions should be well investigated. Because bone marrow is hypoxic with the oxygen tension around 1 to 7% [6, 7], the usual culture condition of 21% O_2 is hyperoxic compared to the normal niche of MSCs. To know the effects of marrow niche on MSC properties and functions, MSCs should be cultured under hypoxic conditions and compared to that cultured under normoxic conditions (the air). It is not easy to recapture the in vivo hypoxic environment in

culture, unless maintaining the culture under hypoxic laminar hood combined with hypoxic incubator. We actually use a modified method of hypoxic culture by using the hypoxic incubator combined with pre-equalization of media under hypoxic conditions, rather than the use of hypoxic laminar hood. However, we need to complete medium change or subculture procedure within 30 to 60 minutes; otherwise transient reoxygenation caused by these procedures will eliminate the proliferation and differentiation properties of the cultured cells [8]. This article is aimed to discuss the benefits of hypoxic culture on MSCs. Notably, hypoxia has been reported to enhance proliferation, survival, and dopaminergic differentiation of central nervous system precursors [9]. In parallel, hypoxia also determines the cell fate of embryonic development, neural crest stem cells, and HSCs [10, 11]. These data suggest stem cells may exhibit a conserved response to reduced oxygen levels.

Involvement of HIF-1α in hypoxia-mediated effects

Hypoxia has been known to regulate several cellular processes and signal transductions. Under hypoxic conditions, the α regulatory subunits of hypoxia inducible factors, HIF-1α and HIF-2α, are constitutively stabilized from oxygen-dependent and von Hippel-Lindau (VHL) tumor suppressor-mediated ubiquitylation and proteasomal degradation [12-15], leading to increased levels of HIF-1 and HIF-2 and an extensive range of hypoxia-inducible mRNAs including those involved in energy metabolism, angiogenesis and apoptosis (e.g. glucose transporter (GLUT-1) and vascular endothelial growth factor (VEGF). Most of the effects of HIF-1α have been investigated for cancer cells. Hypoxia or hypoxic state mimicked by using cobalt chloride ($CoCl_2$) and the iron chelator desferrioxamine (DFX) induced accumulation of wild-type p53 through HIF-1α-dependent association with and stabilization of p53 protein [16]. HIF-1α blocks neuronal and myogenic differentiation via recruitment to Notch-responsive promoters upon Notch activation under hypoxic conditions in neural stem cells and myogenic cells [17]. Recently, HIF-1α has been known to regulate MSC proliferation through the enhancement of TWIST expression, which downregulates the E2A-p21 pathway, and thereby inhibits senescence and increases proliferation [18]. Notably, HIF-1α upregulates CXCR4 and CX3CR1 in MSCs and thereby enhances their migration and engraftment after transplantation [19]. HIF-1α also plays a pivotal role in hypoxia-induced MSC mobilization into peripheral blood, possibly acting via its downstream genes VEGF and SDF-1α [20]. Recent studies also suggest that HIF-1α and hypoxia mimicking agents trigger the initiation and promotion of angiogenic-osteogenic cascade events and improve intraoral bone repair and regeneration procedures (reviewed by [21]). Besides, transcription factor networks related to HIF-1α and miR-124a in part control extensive changes of their global gene expression profile during the conversion of MSCs into neural stem cell-like cells [22]. Although, HIF-2α has been known to upregulate the expression of pluripotent gene such as Oct4 [23], thereby maintaining embryo development, however there is few studies mentioned the involvement of HIF-2α in regulating MSC properties. We have previously compared the protein levels of HIF-2α between MSCs cultured under hypoxic and normoxic conditions, and found there was no difference in the level of HIF-2α between these two conditions [18].

Effects of hypoxic culture on the apoptosis of multipotent stromal cells

Significant apoptosis in MSCs has been demonstrated in the conditions associated with serum depletion, especially in a prolonged condition [24]. However, most studies have demonstrated that MSCs cultured under hypoxia or absolute hypoxia do not undergo apoptosis or change of immunophenotypes [25, 26]. Moreover, combination of exposure to hypoxia partly inhibited apoptosis induced by serum depletion [24, 27]. Some of the mechanisms that MSCs mediate to survive under hypoxic conditions or ischemia include the use of the glycolytic pathway [28] and the production of glucose-6-phosphatase for the use of glucose [29]. These results suggest that MSCs are characterized by metabolic flexibility, which enables them to survive from hypoxic and ischemic stress and retain their multipotent phenotype. These results highlight the potential utility of MSCs in the treatment of ischemic diseases.

It has been documented that only 0.44% of MSCs survive 4 days after transplantation into the ischemia heart [30]. Hypoxic preconditioning enhances the capacity of MSCs to survive after transplantation to the ischemic heart [31-

Table 1. Comparisons of effect of hypoxia on MSC proliferation

MSC source	Effect on proliferation	O2 (%) or hypxia mimicking; seeding density (cells/cm^2); period tested	References
BM, murine	promotion	8%; 10,000; 7-8 days	[46]
BM, murine	inhibition	$CoCl_2$ or DFX; 10,000; 7-8 days	[46]
BM, human	inhibition	1 %; 50-10,000; 10 days	[19]
BM, human	inhibition	1-5 %; 6,250; 7 days	[43]
BM, human	promotion	1-7 %; 50; 2-7 passages	[18]
AT, murine	promotion	2 %; 10,000 ; 13 days	[101]
AT, human	promotion	2 %; 3,000; 14 days	[52]
BM & AT, canine	inhibition	1%, 5%; 5,000; 7 to 14 days	[44]
WJ, human	promotion	2%; 5,000; 10 passages	[51]
PDB, human	inhibition	1 %; 10,000; 2-4 days	[45]

BM=bone marrow; AT=adipose tissue; PDB=placental decidua basalis; WJ=Wharton's jelly

Table 2. Efficiency of MSC expansion under different seeding densities and culture conditions; reference [18]

Hyp/ Nor	Seeding density (cells/cm^2)	Subculture periods (days)	Passages for 60 days	Cell fold increase for each passage	Expected cell fold increase for 60 days	Ratio to Nor/4000
Nor	4,000	5~7	10	5 for each passage	9,765,625	1
Nor	1,000	~10	6	18, 18, 16, 15, 14, 11	11,975,040	1.2
Nor	100	11~12	5	190, 121, 68, 45, 40	2,813,976,000	288.2
Nor	50	~12	5	252, 170, 80, 70, 48	11,515,392,000	1,179.2
Hyp	50	~12	5	~250 for each passage	976,562,500,000	100,000

33]. Similarly, hypoxic preconditioning of neural stem cells and MSCs also increases survival and decreases activation of caspase-3 after transplantation to the ischemic brain [34]. These data suggest short-term exposure of MSCs to hypoxia before transplantation improves their survival rates after transplantation. Although hypoxic preconditioning enhances survival, MSCs still cannot stay long in the ischemic tissues, conditions associated with hypoxia and lack of nutrition [24]. To these aims, several measures have been tested to help MSCs survive under these conditions, including transfection of MSCs with plasmids carrying integrin-linked kinase (ILK) [35], heme oxygenase-1 (HO-1) [36], angiogenin [37], angiopoietin-1 (Ang-1) and Akt [38], and Hsp70 [39] or pretreatment of MSCs with Cyclosporin A [40] or lysophosphatidic acid [41]. Most of these measures increased the survival ability of MSCs via downregualting the apoptosis-related pathways. Further, HO-1 may significantly affect the cell differentiation potential via suppressing miR-1, miR-133a, miR-133b, and miR-206 [42].

Effects of hypoxic culture on proliferation of multipotent stromal cells

The effects of hypoxic culture on MSC proliferation and expansion efficiency are still controversial. These effects depend on the sources of MSCs derived, the oxygen concentration, the seeding density, and the duration that were used to culture MSCs (**Table 1**). Some studies have found that hypoxia significantly inhibits proliferation [19, 43-46], and others have found that hypoxia increases the proliferation capacity or increases the life span [25, 46-52]. According to our previous results, the effect of hypoxia on proliferation was inhibitory within one passage of culture either at low-density or at high-density [19]. However, our recent experience with long-term culture has changed this conclusion (**Table 2**). We found that cell expansion efficiency at low-density culture decreased along with the increase of passage number under normoxic

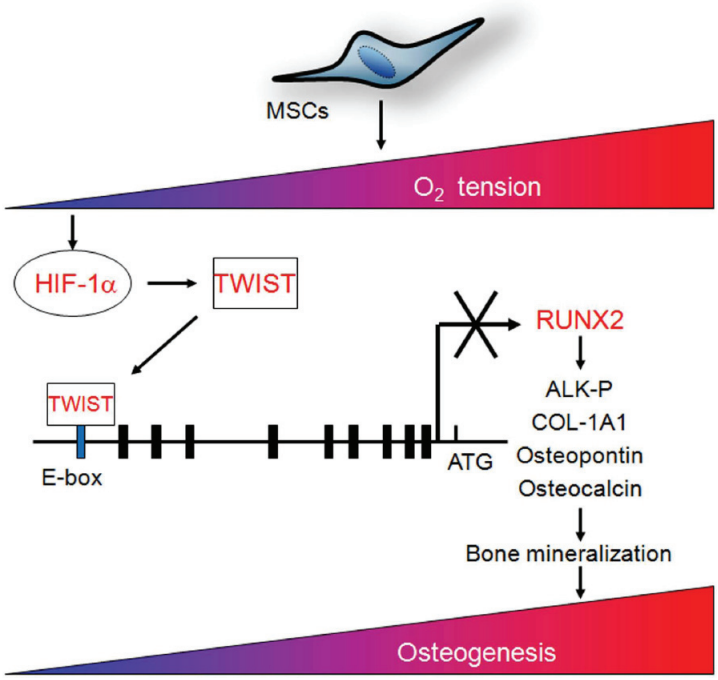

Figure 1. A schematic representation shows the underlying mechanism that hypoxia mediates to downregulate RUNX2 expression and inhibits osteogenesis in bone marrow MSCs [56].

conditions while maintaining the same under hypoxic conditions [18]. For long-term expansion (up to 60 days) of MSCs at low-density, hypoxia significantly increased the expansion efficiency, inhibited senescence, increased proliferation and enhanced the in vitro and in vivo differentiation potential [18]. Bases on our and other studies, the effects of hypoxia on short-term MSC proliferation varied and depended on the oxygen tensions, seeding densities and MSC sources used (**Table 1**). However, the beneficial effects of hypoxia on long-term expansion of MSCs are consistent even with different culture conditions or MSC sources [18, 25, 51].

Differentiation of multipotent stromal cells under hypoxic conditions

It is well documented that hypoxic culture inhibits the osteogenic and adipogenic differentiation of MSCs [25, 43, 53]. The same effects have also been demonstrated in adipose-derived MSCs [54]. Compared to normoxic culture, hypoxic cultures of MSCs induced for osteogenic differentiation show decreased expression of osteogenic genes, such as Runx2 and osteocalcin, and reduced alkaline phosphatase activity as well as mineralization ability [25, 26, 43, 53, 55]. The mechanism is mediated through the downregulation of Runx2 transcription factor by the HIF-TWIST pathway (**Figure 1**) [56]. Notably, hypoxia or activation of HIF-1α also enhanced in vivo osteoclastogenesis both in malignant and non-malignant conditions [57, 58]. Similarly, MSCs induced in adipogenic medium under hypoxic conditions show decreased expression of adipogenic genes, such as PPARγ and LPL, and reduced ability to accumulate fat droplets [25, 43]. However, MSCs induced for chondrogenic differentiation under hypoxic conditions show increased expression of chondrogenic genes and proteins, such as Sox5, 6 and 9, aggrecan and type II collagen [59-63], decreased expression of genes associated with osteogenesis and endochondral ossification, such as Col-1a1 and Col-10a1, and suppression of IL-1β-induced loss of extracellular matrix proteins [64]. Moreover, hypoxic culture as well as hypoxia-mimicking agents have been demonstrated to increase ectodermal differentiation such as neuron-like cells [65] and skin-regenerative potential [66]. A prolyl hydroxylase inhibitor, FG-0041, in combination with a ROCK inhibitor, Y-27632, has been demonstrated to initiate differentiation of MSCs into neuron-like cells [67].

Effects of hypoxic culture on maintaining stem cell properties of multipotent stromal cells

"Stemness" or stem cell properties, referred to as self-renewal and differentiation potential, is a prerequisite for success in stem cell application. Consistent with previous studies [68], we found that low-density culture provides a method for rapid expansion of MSCs. However, MSCs expanded at low-density culture gradually lost proliferation capacity and underwent senescence under normoxic conditions [18, 69], while maintaining proliferation as well as differentiation potential, increasing the expression of pluripotent genes, such as Oct4, Nanog and Sox2 [70], and inhibiting senescence under hypoxic conditions [18]. Further, long-term expansion of MSCs under normoxic conditions adversely affects stem cell function when it comes to pro-

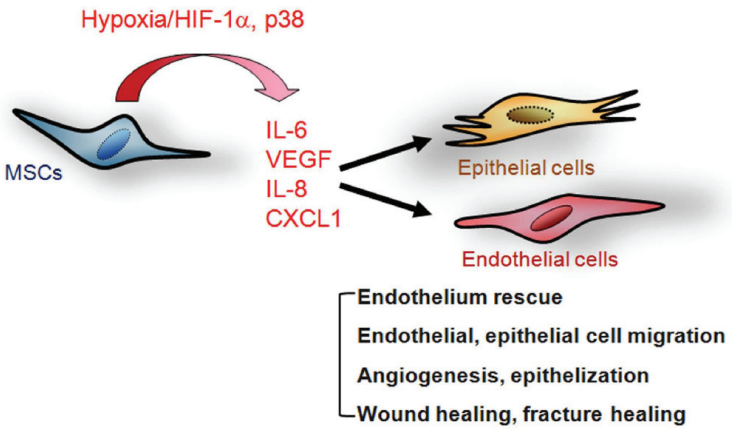

Figure 2. A schematic representation summarizes the effects of hypoxic culture on enhancing secretion of paracrine factors and promoting therapeutic effects in bone marrow MSC [27, 80, 82].

tecting myocardium [71]. On the other hand, MSCs with hypoxic preconditioning have been shown to increase in the lineage differentiation into bone [72], fat and cartilage [73] both in vitro and in vivo [18]. Increased telomere length and telomerase activity, normal karyotyping and chromosome integration have also been observed [18]. Moreover, MSCs with long-term hypoxic preconditioning do not form tumors when transplanted into immunedeficient mice. These results suggest hypoxic culture provides a method for efficiently expanding MSCs without losing stemness [70, 74] and increasing tumorigenecity. Hypoxic culture may also be applied for future designs of expansion conditions for the clinical application of MSCs.

Effects of hypoxic culture on secretion of paracrine factors by multipotent stromal cells

The changes of MSC properties upon exposure to hypoxia or inflammation have been studied in the endogenous bone marrow MSCs of mice with a recent myocardial infarction (MI). Wang et al. demonstrate that recent MI impaired bone marrow cell therapeutic efficacy. MI led to myocardial inflammation and an increased inflammatory state in the bone marrow, changing the bone marrow cell composition and reducing their efficacy. Injection of a general anti-inflammatory drug or a specific interleukin (IL)-1 inhibitor to donor mice after MI prevented this impairment [75]. In contrast, in vitro hypoxic preconditioning enhances the capacity of MSCs to repair infarcted myocardium or diabetic cardiomyopathy. This is attributable to reduced cell death and apoptosis of implanted cells, increased angiogenesis/vascularization, and paracrine effects [31-33]. It has been demonstrated that the conditioned medium (CM) from Akt-MSCs markedly inhibits hypoxia-induced apoptosis and triggers vigorous spontaneous contraction of adult rat cardiomyocytes in vitro [76]. Similarly, CM from MSCs has also been noted to reduce apoptosis or enhance tube formation in human endothelial cells [27]. Moreover, the effects were more obvious in a CM from hypoxic MSCs when compared to one from normoxic MSCs. This is partly due to its higher content of antiapoptotic and angiogenic factors, such as IL-6, VEGF [27], fibroblast growth factor 2 (FGF2), insulin-like growth factor 1 (IGF-1), or hepatocyte growth factor (HGF) [77]. A gene expression profile analyzed by a microarray study also demonstrated that hypoxic MSCs increase the expression of several growth factors involved in cell proliferation, apoptosis and angiogenesis [78]. Also, a CM from hypoxic MSCs activated the PI3K-Akt pathway in endothelial cells and thereby inhibited hypoxia-induced endothelial apoptosis and increased angiogenesis of endothelial cells [27]. The in vivo cardioprotection of CM from hypoxic MSCs was tested in a model of MI induced in Wistar male rats by permanent left coronary occlusion. Intramyocardial injection of 25× concentrated CM three hours after coronary occlusion was able to promote a significant reduction (35%) in left ventricular end-diastolic pressure and improvement of cardiac contractility (15%) and relaxation (12%) compared to non-conditioned medium 19-21 days after medium injection [79]. These results suggest that soluble factors released in vitro by MSCs are able to promote cardioprotection in vitro and improve cardiac function in vivo. Moreover, a CM from human hypoxic MSCs also enhanced healing of skin wound [80] as well as radiation-induced small intestine injury in mice [81], and promoted fracture healing in diabetic rats [82]. These results together suggest that the administration of hypoxic MSCs or their secreted factors may provide a therapeutic method for enhancing angiogenesis, epithelilization, wound healing and fracture healing (**Figure 2**). In addition to the increase in the secretion of paracrine factors, hypoxic MSCs have also been shown to

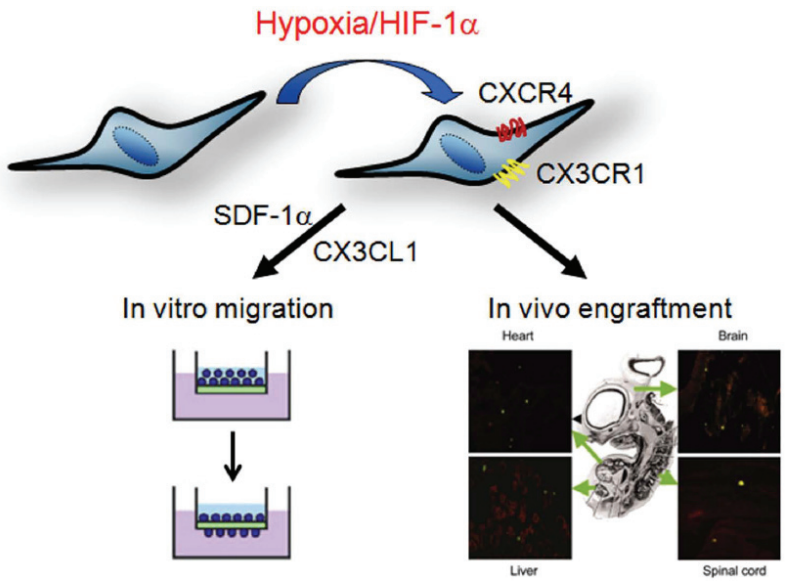

Figure 3. A schematic representation summarizes the effects of hypoxic exposure on enhancing the expression of chemokine receptors and increasing in vitro migration and in vivo engraftment in bone marrow MSCs [19].

increase total protein levels as well as different fibronectin expression patterns throughout the culture period, suggesting that oxygen levels can significantly affect tissue-development patterns [83]. The mechanism that hypoxic MSCs mediate to increase the release of angiogenic factor, such as VEGF is partly through the HIF-1α-mediated pathway [84]. Moreover, the advantages of using CM rather than cells include: (1) CM-based therapy circumvents some of the concerns and limitations in using viable replicating cells and does not compromise some of the advantages associated with using cells; (2) CM-based therapy is an ideal therapeutic approach because the complex cargo of proteins and genetic materials has the diversity and biochemical potential to participate in multiple biochemical and cellular processes, an important attribute in the treatment of complex disease; and others [85].

Effects of hypoxic culture on expression of chemokine receptors, migration and engraftment of multipotent stromal cells

The ability of stem/progenitor cells to migrate and engraft into host tissues is the key to their potential use in cell-based therapy. Homing and engraftment of cells have been detected in rapidly growing embryos, including mouse [86], chick [87] and sheep [88], and following tissue injury, such as ischemic damage to heart [89, 90] and brain [91]. However, various studies have shown that the degree of engraftment of MSCs in naive adult animals is very low [92-94]. Interestingly, it has been demonstrated that a 1-day exposure of MSCs to 1% O_2 increased expression of the chemokine receptors CX3CR1and CXCR4 and promoted SDF-1α and Fractalkine -dependent and independent migration [19]. Similarly, MSCs cultured under hypoxic conditions have also shown increased VEGFR1 expression and VEGF- or PLGF-dependent migration [95]. Similarly, hypoxia-induced increase in migration has also been demonstrated in human umbilical cord blood MSCs [96]. Moreover, xenotypic transplantation into early chick embryos demonstrated that MSCs from hypoxic culture engraft more efficiently than cells from normoxic culture and generate a variety of cell types in host tissues [19] (**Figure 3**). Preconditioning with oxygen and glucose depletion also increased the survival of Sca-1+ cells via PI3K/Akt-dependent caspase-3 downregulation and thereby increased engraftment rate [97]. In contrast to the migration and engraftment of transplanted MSCs, it has also been demonstrated that mobilization of MSCs from bone barrow to the circulating blood in rats is consistently and dramatically increased (by almost 15-fold) when animals are exposed to chronic hypoxia [98]. In addition to the increase in migration and survival, MSCs with hypoxic preconditioning have also been shown to enhance revascularization after transplantation for hind limb ischemia [99]. These results suggest that short-term hypoxic preconditioning of MSCs may provide a general method of enhancing their survival, migration, angiogenesis, and engraftment in vivo into a variety of tissues.

Effects of hypoxic culture on glucose metabolism and oxidative stress of multipotent stromal cells

It has been demonstrated that exposure to hypoxia or the hypoxia mimetic agents such as $CoCl_2$ induces the expression of HIF-1α and glu-

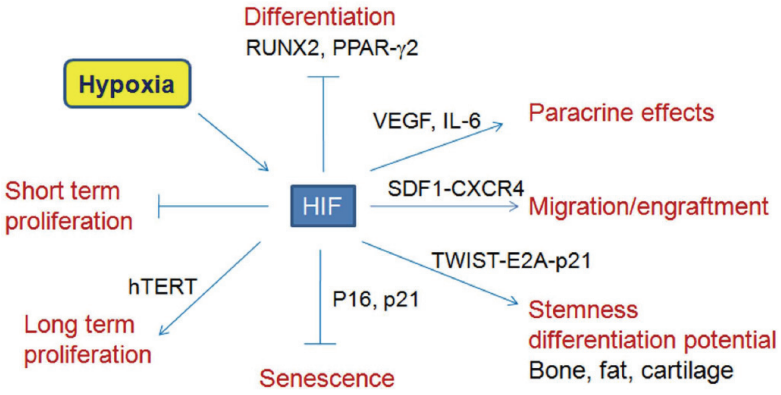

Figure 4. A schematic representation summarizes the effects of hypoxic culture on proliferation, expansion, differentiation, engraftment and stem cell properties in bone marrow MSCs [18, 19, 27, 56, 70, 80, 82].

cose-6-phosphate transporter (G6PT) in MSCs. Moreover, knockdown of HIF-1α inhibited G6PT expression in hypoxic MSCs, whereas constitutive expression of HIF-1α with a mutant oxygen-dependent degradation domain resulted in increased G6PT expression in normoxic MSCs, suggesting the involvement of the HIF pathway in the expression of G6PT [29]. Moreover, a potent G6PT inhibitor, AD4-015, specifically triggers cell death in MSCs constitutively expressing the HIF-1α mutant. Collectively, these data suggest that G6PT may account for the metabolic flexibility that enables MSCs to survive under conditions characterized by hypoxia [29]. MSCs cultured under hypoxia also induce higher specific consumption of nutrients, especially early in culture, but exhibit lower specific production of inhibitory metabolites. However, hypoxic culture of MSCs has been shown to increase cell division and favor expansion of the progeny of CFU-F, while maintaining MSC characteristics like immunophenotype and differentiation potential [100]. These data suggest hypoxia favors expanded MSCs increasing cellular metabolism efficiency, which leads toward the maximization of cell yield for application in clinical settings [100]. MSCs were also resistant to exposure to absolute hypoxia (0.5% O_2), as well as inhibition of mitochondrial respiration with 2,4-dinitrophenol [28], indicating that in the absence of oxygen, MSCs can survive using anaerobic ATP production. Further studies have demonstrated that MSCs are capable of surviving hypoxia because of their ability to rely on glycolysis rather than mitochondrial respiration [28]. All together, these studies suggest that MSCs are characterized by metabolic flexibility, which enables them to survive under conditions of hypoxic and ischemic stress, and allows them to function in a reparative or regenerative capacity in the treatment of ischemic diseases.

Summary

All together, these studies suggest that hypoxic cultures have multiple effects on MSCs, which affect short-term proliferation, long-term expansion efficiency, differentiation potential, stemness or maintenance of stem cell properties, paracrine secretion capacity, the expression of chemokine receptors, migration and engraftment ability (**Figure 4**). Most of the effects are mediated through the HIF-1α related pathways. Moreover, MSCs are able to survive under hypoxia because of their metabolic flexibility in the synthesis of G6PT, anaerobic production of ATP, and dependence on glycolysis.

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