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Preconditioning of Hypoxic Culture Increases The Therapeutic Potential of Adipose Derived Mesenchymal Stem Cells

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Abstract

Stem cell-based therapy is one of the most promising strategies in promoting tissue regeneration. Mesenchymal stem cells (MSCs) derived from adipose are widely used as a source of stem cells. However, most donor stem cells die before their regenerative effects appear. Thereby, various strategies are carried out to enhance the therapeutic potential. It is hoped that the survival, engraftment, and function of implanted MSCs by preconditioning the cells will be improved. *In vitro* preconditioning strategies can improve the survival, engraftment, and paracrine properties of MSCs, thus optimize their reparative and regenerative capacities. One such attempt is to propagate these stem cells in a hypoxic environment, which mimics the natural tissue oxygen environment. Hypoxia preconditioning represents a comprehensive increase in regenerative gene expression in stem cell therapy. There are significant advantages in stem cell therapy with hypoxia preconditioning.

Introduction

Stem cell-based therapy is one of the most promising strategies in the medical field to promote tissue regeneration. Over the past few decades, stem cells have been widely used as regenerative therapy and mesenchymal stem cells (MSCs) emerged as a promising therapeutic option. Currently, stem cells are effective agents as therapeutic agents for several diseases due to their tissue protective and reparative mechanisms [1].

MSCs derived from adipose tissue are widely used as a source of stem cells. This is due to the fact they are accessible, abundant, and painless collection procedure in comparison to other sources. These cells can be maintained and cultured for long periods of time without losing their differentiation capacity [2].

Unfortunately, several recent studies have shown that donor stem cells die before their regenerative effects appear. Most of the stem cells did not survive the first 24 h *in vivo*, mainly due to the hypoxia-induced apoptosis of stem cells [3].

For this reason, various strategies are carried out to increase the therapeutic potential. Thus, it will improve the survival, engraftment, and function of implanted MSCs by conditioning the cells prior to implantation, known as preconditioning. To obtain optimal therapeutic potential, an adipose-derived MSCs (AdMSC) with maximum regenerative capacity is required and *in vitro* initial conditioning strategy can optimize its therapeutic potential [4].

One such attempt is to propagate these stem cells in a hypoxic environment prior to implantation (hypoxia preconditioning), which mimics the natural tissue oxygen environment (1–7%), unlike standard culture conditions (21%). This will increase the viability of these cells and will stimulate the secretion of growth factors required in the regenerative process [5], [6].

The following review aims to provide an overview of hypoxia preconditioning to enhance the therapeutic potential of AdMSCs.

Characteristics of MSCs

According to the MSC and Tissue Committee of the International Society for Cellular Therapy, MSCs have both self-renewing and differentiating capabilities. These cells showed positive expression for stromal markers CD73 and CD 105 and negative for the hematopoietic markers CD14 or CD 11b, CD34 and CD45; CD79a or CD19 and HLA class II. These cells have the ability to be plastic adherent and possess fibroblast-like morphology. These cells can be maintained in culture for a long period of time. Nevertheless, their potential for osteogenic, adipogenic, and chondrogenic differentiation must be demonstrated [2], [7].

The most useful characteristic reported for MSC is the non-immunogenic profile. MSCs are reported to exhibit Major Histocompatibility Complex (MHC) I, but deficiency of MHC II results in T-cell inactivation and immunosuppressive properties. MSCs secrete a variety of growth factors, chemokines, cytokines, and extracellular matrix (ECM) proteins that are involved in various biological processes including hematopoiesis, angiogenesis, leukocyte multiplication, immunity, and inflammatory responses. Multiple growth factors, including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1, hepatocyte growth factors (HGF), and transforming growth factors (TGF)-β1 are also secreted. This evidence holds a great promise in MSC-based therapies which allow allogenic transplantation without the need for immunosuppression [8], [9], [10].

AdMSCs

Stem cells in adipose tissue, called AdMSC, have been shown to have broadly the same biological capabilities as bone marrow MSC (BM-MSCs) [11]. The advantages of AdMSC over BM-MSCs and other types of adult stem cells are that they are relatively easy to obtain. It can be obtained in large quantities through liposuction performed under local anesthesia. These cells are able to be maintain long-term phenotype and plasticity of *in vitro* culture. These cells also possess low immunogenicity characteristic. As a result, AdMSC was able to attract greater interest and is the most preferred cell type for tissue engineering and regenerative medicine [12], [13], [14].

Stem cells found in adipose tissue have a higher number than those in bone marrow. One gram of adipose tissue yields approximately 3.5×10^5 to 1×10^6 AdMSC, whereas isolation from one gram of bone marrow aspiration yields only 5×10^2 to 5×10^4 BM-MSC. In addition, AdMSC has advantages in terms of proliferation and differentiation, as well as the fact that age and location of origin do not have a significant difference in the therapeutic effect of these adipose-derived stem cells [9], [15].

Zuk *et al*., were the first to investigate whether human adipose could be an alternative source of MSCs. They obtained human adipose from liposuction aspiration and used the enzyme collagenase to release stromal cells from the ECM by processing the stromal vascular fraction, which contains various cell types including AdMSC (Figure 1). Isolated adipose stromal cells are cultured with specific media to induce adipogenic, osteogenic, or chondrogenic differentiation. Adipose stromal cells are capable of developing intracellular lipid stores, markers representing adipose tissue, bone, and cartilage, respectively, as well as expressing alkaline

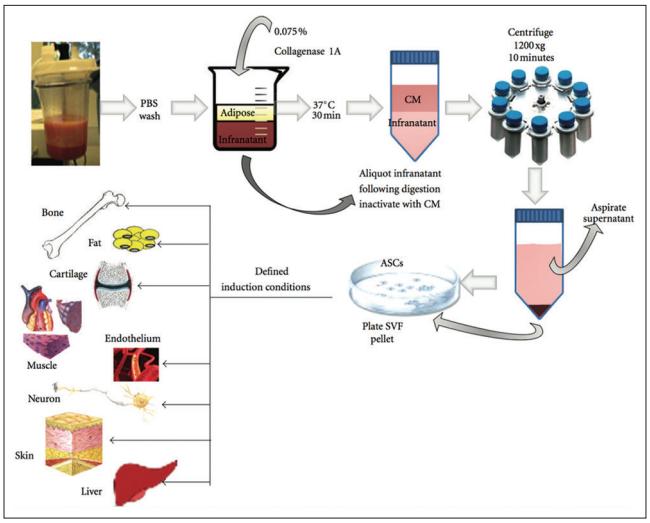

Figure 1: Isolation and utilization of adipose-derived mesenchymal stem cells [13]

phosphatase or proteoglycan [16]. To determine whether isolated adipose stromal cells were truly stem cells, Zuk *et al*., investigated surface antigen expression and differentiation capacity against clonogenic cultures [17].

Using flowcytometry, it was shown that clonogenic cells express a surface antigen similar to BM-MSCs. In addition to the differentiation of mesenchymal lineages, clonal cells are also able to differentiate into neuron-like cells, which are proven by morphology and expression of phenotypic markers. AdMSCs also tend to stimulate angiogenesis as an important feature for regenerative [18], [19]. Its neurotropic and angiogenic properties have been shown to be due to neural growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), VEGF-A, and angiopoietin-1 [20]. The secretome of AdMSC is very complex. AdMSC has the ability to secrete proteins involved in angiogenesis, injury healing, tissue regeneration, and immunomodulation [21]. In addition, AdMSC has the capacity to differentiate into Schwann cell [22], pancreatic beta cells [23], and hepatocytes [24]. AdMSCs have important healing properties in injuries and can be an attractive source of stem cells for tissue engineering as well as regenerative therapy for many medical and surgical applications.

A comparison of the yield and differentiation capacity of cells isolated from each tissue was performed to determine whether adipose tissue is a source of MSC bone marrow. De Ugarte *et al*., [25] found no significant difference in the number of cultured adherent cells between those of stromal cells obtained from bone marrow and those from adipose tissue. However, the average mass of adipose tissue (17 g) obtained from each patient was higher than the average mass of bone marrow (7 g). Cultured isolated cells in various differentiation media showed no difference in the number of cells developing lipid droplets (adipogenic cells), or osteogenic cell alkaline phosphatase activity. However, in terms of differentiation towards cartilage, adipose-derived cells were positively stained for chondrogenesis whereas bone marrow-derived cells were not.

Using a similar method, several other studies have compared the ability of bone marrow and adipose cells to differentiate along this lineage and demonstrated that cells from both tissues have the same capacity to become adipose, bone, and cartilage tissue [26], [27], [28]. Overall, this is the result of different culture conditions and/or isolation of different parts of MSC. Carbone *et al*. demonstrated that AdMSC cultured in conditioned medium from chondrocyte and osteocytes were capable of producing glycosaminoglycans and mineralized matrix, respectively [29]. These results suggest that AdMSC requires growth factor supplementation from the tissue environment in order to properly differentiate according to the mesodermic lineage. This evidence suggests that compared to bone marrow, a large number of MSCs capable of differentiating into multiple lineages can also be obtained from adipose tissue (Figure 2).

AdMSCs Precondition Concept

Therapy using MSCs is a therapy that provides hope for organ and tissue regeneration. The

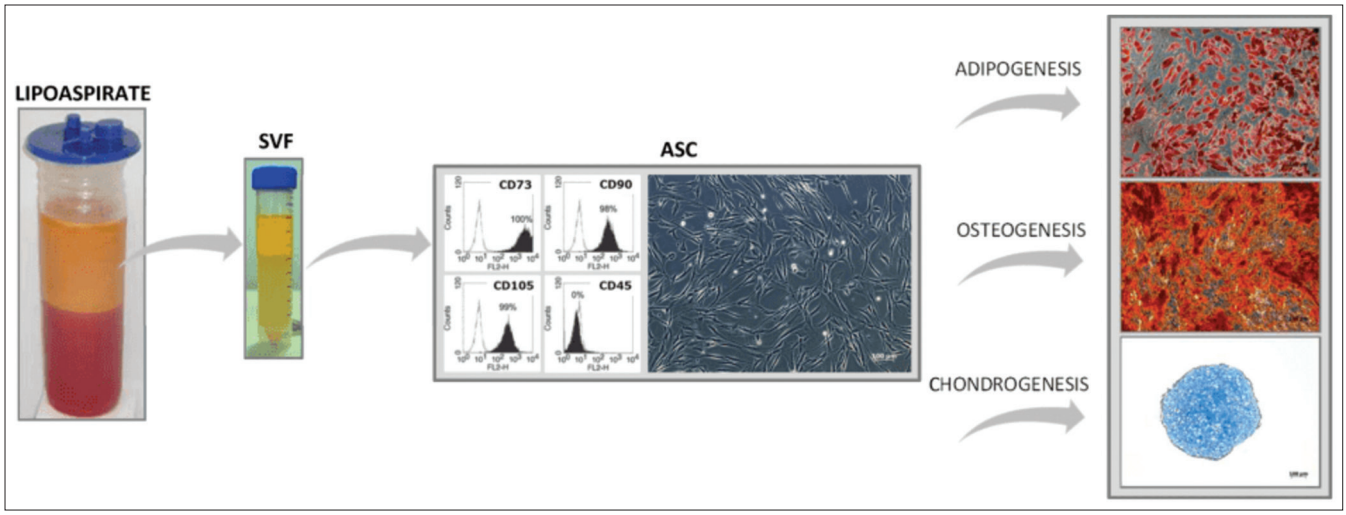

Figure 2: Adipose-derived mesenchymal stem cells: Origin, immunophenotype, morphology, and differentiation potential. Lipoaspirate can be easily obtained and processed to obtain a heterogenous cell population including stromal vascular fraction. Characterization of the cell was by mesenchymal stem cell markers (CD73, CD90, CD105) at the expense of hematopoietic stem cell markers (CD45) and their spindle-shaped morphology with their ability to differentiate into the adipogenic, osteogenic, and chondrogenic lineages. Histological staining was able to analyze the differentiation potential, Oil red O for adipogenic differentiation, Alizarin red for osteogenic differentiation, and Alcian blue for chondrogenic differentiation [30].

conditioned medium or MSCs-EVs exert a protective effect on peripheral nerve tissue and have been shown to promote regeneration in injured tissue. However, transplanting MSCs or administering MSCs-CM or MSCs-EV requires the MSC to have the maximum regenerative capacity. Thus, it is necessary to develop a new strategy to increase the regenerative efficiency of MSCs (Figure 3). *In vitro* preconditioning strategies can improve the survival, engraftment, and paracrine properties of MSCs so as to optimize their reparative and regenerative capacities [4].

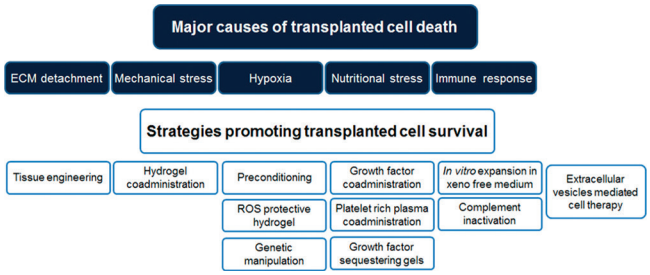

Figure 3: Main causes of transplanted cells death. The major factors limiting cell survival during transplantation and strategies to improve cell survival on cell-based therapy. ECM: Extracellular matrix, ROS: Reactive oxygen species [31]

Various *in vitro* preconditioning strategies have been implemented to increase the regenerative capacity of MSCs. Among them is modulation of culture atmosphere (hypoxia or anoxia), three-dimensional (3D) culture, addition of trophic factors (in the form of growth factors, cytokines, or hormones), lipopolysaccharides, and pharmacological agents. Factors secreted by MSCs in response to many different preconditions and exert anti-apoptosis, pro-angiogenic, and trophic or immunosuppressive immunomodulatory effects (Figure 4).

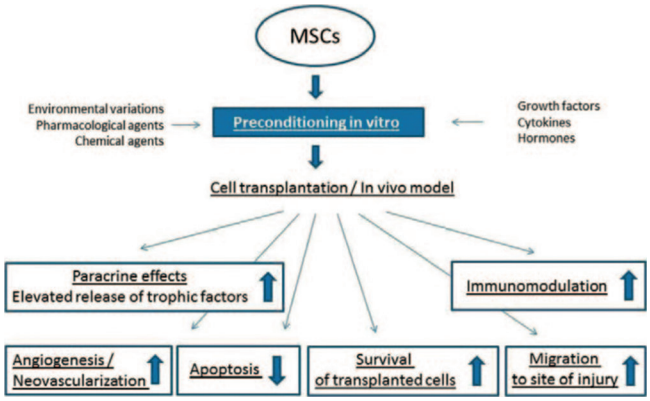

Figure 4: In vitro preconditioning. Several mechanisms are involved in enhancing the therapeutic potency of mesenchymal stem cells under prerequisite condition strategies [4]

The secretome (paracrine profile) of the pre-treated MSC varies according to the preconditioning method. Different preconditioning methods can activate or suppress molecular signals and different signal transduction cascades. The cellular response is very complex and the pre-treatment procedure affects not only one specific molecule or protein but also a number of factors [32].

Hypoxic Preconditions and Hypoxia Inducible Factor (HIF)-1α

Several strategies were carried out to increase the therapeutic potential of AdMSCs by increasing the survival, grafting, and function of the implanted MSCs by conditioning the cells prior to implantation. Implantation of MSCs into nutrient-deprived and oxygen-deprived environments, such as in recipient tissue, results in extremely low cell survival [32]. Among these strategies, hypoxia preconditioning represents a comprehensive increase in regenerative gene expression in stem cell therapy. Previous studies have shown the successful application of this strategy in several diseases. Furthermore, there are significant advantages in stem cell therapy in the precondition of hypoxia [33].

Hypoxia preconditioning ensures the cells to adapt to the ischemic environment [34]. Preconditioning MSCs can be done by cultered them in a hypoxic environment, which mimics the natural tissue oxygen environment (1–7%) unlike the standard culture conditions (21%). This increases the survival of these cells via HIF-1α and through Akt-dependent mechanisms. In addition, the hypoxia preconditioning stimulates the secretion of pro-angiogenic growth factor, increases the expression of chemokines stromal cell-derived factor-1 (SDF-1) and its receptor chemokine receptor type 4 (CXCR) - CXCR7 and increases engraftment of MSCs *in vivo* [5], [6], [35]. Hypoxia preconditioning has also been shown to enhance the paracrine effect of MSCs and has been applied to several diseases, such as myocardial infarction and diabetes-related erectile dysfunction [36].

The *in vivo* oxygen concentration is about 0.4%, significantly lower than the oxygen concentration conventionally used *in vitro*, 21% [37]. Most stem cells die within the first 24 h *in vivo*, mainly due to stem cell apoptosis induced by hypoxia [3]. Research by injecting bone marrow stem cells into mice with ventricular myocardial infarction found that 99% of bone marrow stem cells died 4 days later. These results indicate that these stem cells are highly susceptible to ischemia and hypoxia [38]. Based on these results, many studies have used hypoxic state methods to improve stem cell viability. Greijer and van der Wall showed that the severity of hypoxia affects the rate of cell apoptosis versus cell survival during hypoxia. The 0.5% O_2 condition was shown to initiate apoptosis in some cells. In order to prevent the accumulation of hypoxia-induced genetic mutations, there is a balance between pro-apoptotic and anti-apoptotic factors. HIF-1α as shown in Figure 5, plays an important role in maintaining that balance [39].

Sun *et al*. simulated a hypoxic environment to induce hypoxic precondition in bone marrow stem cells and found that after hypoxia preconditioning, the bone marrow stem cells overexpressed HIF-1α, leading to decreased apoptosis and minimizing loss

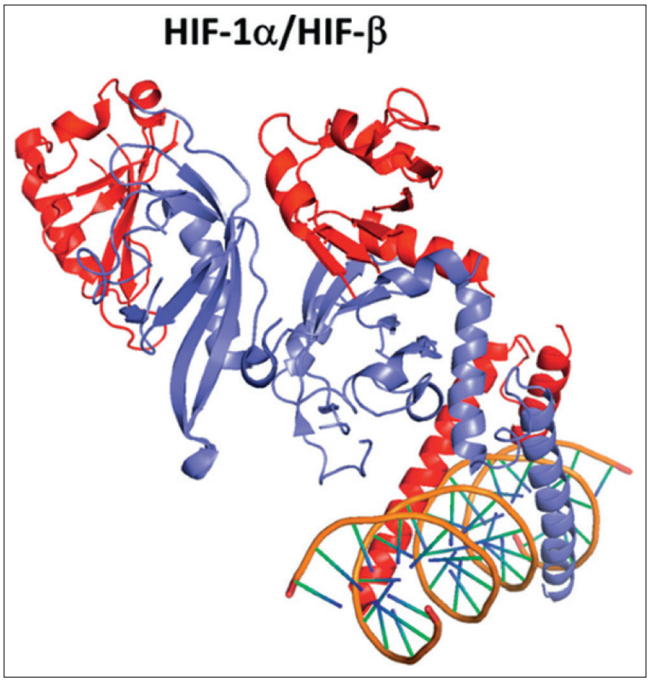

Figure 5: The three-dimensional structure of hypoxia-inducible factor (HIF)-1α and HIF-ß [40]

of mitochondrial membrane potential. These results suggest that hypoxia preconditioning has a protective effect on the survival of BM-MSCs *in vivo* under ischemic and hypoxic conditions. Once MSCs are exposed to the precondition of hypoxia (Figure 6), the hypoxia-induced apoptosis pathway is rapidly activated thereby inhibiting feedback from this pathway and then reducing apoptosis in subsequent ischemic and hypoxic conditions [41].

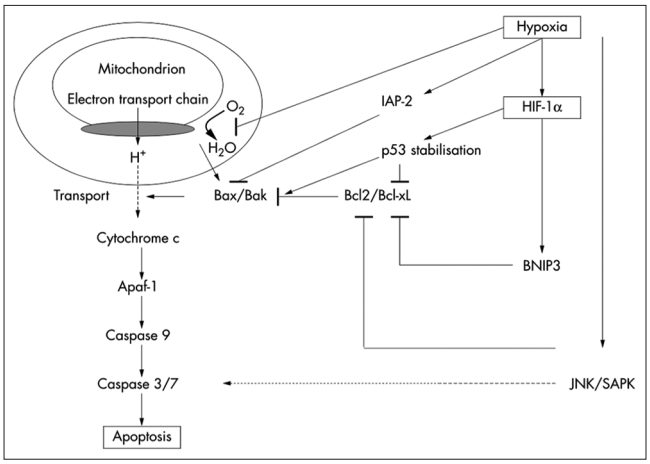

Figure 6: Hypoxia-induced signaling pathways with HIF-1α Involvement. Schematic depiction of signaling pathways induced by hypoxia leading to apoptosis suggesting the involvement of HIF-1α. Solid lines represent direct interactions, dotted lines represent indirect interactions. Apaf-1: Apoptotic protease activating factor-1, BNIP3: BCL-2/adenovirus E1B 19 kDa interacting protein 3, HIF-1: Hypoxia-inducible factor 1, IAP-2: Inhibitor of apoptosis protein 2, JNK: c-Jun NH2 terminal kinase, SAPK: Stress activated protein kinase [39]

HIF-1α is a subunit of a heterodimeric transcription factor HIF-1α encoded by the HIF-1α gene. It is the basic domain of the PAS helix-loop-helix-containing proteins and is considered to be the primary transcription regulator of cellular responses and cellular development responses to hypoxia. Dysregulation and overexpression of HIF-1α caused by hypoxia or genetic alteration are involved in cancer biology, a number of pathophysiology in the fields of vascularity and angiogenesis, metabolism energy, cell survival, and tumor invasion [42].

HIF-1α protein plays a major role in regulating the expression of many genes involved in angiogenesis and erythropoiesis, metabolic adaptation to hypoxia, epithelial-mesenchymal transition, extracellular matrix degradation, and chemotaxis through CXCR4 and the CXCL12/SDF-1 axis [43].

Exposure of MSCs to hypoxic conditions may enhance their immunomodulatory and regenerative properties by over-expression of cytoprotective genes and secretory factors. HIF-1α has a crucial role in upregulating these genes and factors [44]. HIF1α can promote MSC migration to ischemic and hypoxic sites by regulating the expression levels of molecules such as stromal cellderived factor1 (SDF1) in the microenvironment. Stem cells express the SDF1 receptor C- XC CXCR4 and CXCR7, and serve a key role in tissue repair [45].

Hypoxia activates the hypoxia signaling pathway, which is largely regulated by HIF stabilization (Figure 7). Under normoxic conditions, the proline residues from the HIF-1α subunit is hydroxylated by oxygen-dependent prolyl-4-hydroxylase (PHD). The Von Hippel-Lindau protein (pVHL), a ubiquitin E3 ligase, binds to the hydroxylated HIF-α and acts as a substrate recognition component of the ubiquitin E3 ligase complex, leading to proteosomal degradation of the HIF protein. Asparagine residue from the HIF-α subunit is also hydroxylated by

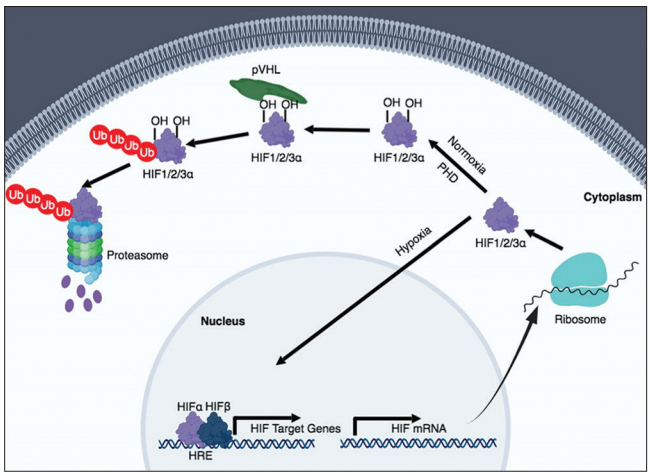

Figure 7: Regulation of hypoxia-inducible factor (HIF) during normoxic and hypoxic states. In the oxygenated state, HIF undergoes hydroxylation of the proline residue by PHDs and is polyubiquitinated by pVHL. This results in the degradation of the HIF by the 26S proteasome system. In a hypoxic state, HIF is stabilized and translocated into the nucleus, where HIF1α binds to the HIF1βdimerization partner and increases transcription of the HIF target gene [46]

factor inhibiting HIF (FIH), which inhibits the binding of HIF to the p300/CREB binding protein co-activator [46].

In a hypoxic state, PHD and FIH activity are suppressed, and the HIF-α subunit translocates into the nucleus to bind to HIF-1β. The heterodimeric complex of transcription factor HIF-1α: HIF-1β then looks for hypoxia-responsive elements of its target genes, which results in transcriptional upregulation (Figure 8). There are other HIF-independent signaling pathways that are activated under hypoxia, such as the factor-κB (NF-κB) pathway. Early studies reported that IκBα is phosphorylated during hypoxia and this results in degradation of IκBα and activation of NF-κB1. Other studies have shown that IκB kinase activity is increased via calcium/calmodulin-dependent kinase 2 (calcium/calmodulin-dependent kinase 2) during hypoxia and required kinase 1 activated by TGF-β [47].

(Nobel Prize in Physiology or Medicine 2019: How Cells Sense and Adapt to Oxygen Availability).

Hypoxia can cause tissue inflammation and HIF stabilization can reduce tissue inflammation and promote its repair. HIF elicits a variety of adaptive responses, particularly focusing on enhancing the transcription cascade which is important for tissue protection and adaptation. HIF-1α (HIF1A) is known to be associated with the upregulation of glycolytic gene such as phosphoglycerate kinase (CKD) and lactate dehydrogenase A, both of which function metabolically to adapt tissues to oxygen uptake and anaerobic ATP synthesis. HIF-2α (HIF2A) induces erythropoietin and VEGF, which are important for increasing oxygen supply to hypoxic areas [48].

The secretion of several cytokines associated with neuroprotective and vascular protection was significantly increased from MSCs after hypoxic conditions, namely VEGF, bFGF, BDNF, and GDNF [30]. Hypoxia was reported to increase the angiogenic and mitogenic paracrine activity of AdMSCs. Hypoxia (0.5–2% O2) significantly increases VEGF, HGF, bFGF, IL-6, angiogenin, NGF, IGFBP-1, IGFBP-2, and the PDGF-b receptors. In contrast, EGF and anti-angiogenic factors are inhibited by hypoxic condition. The increased paracrine factors improve survival, homing, and angiogenesis, which increases the regenerative potential of AdMSCs after transplantation [9], [49].

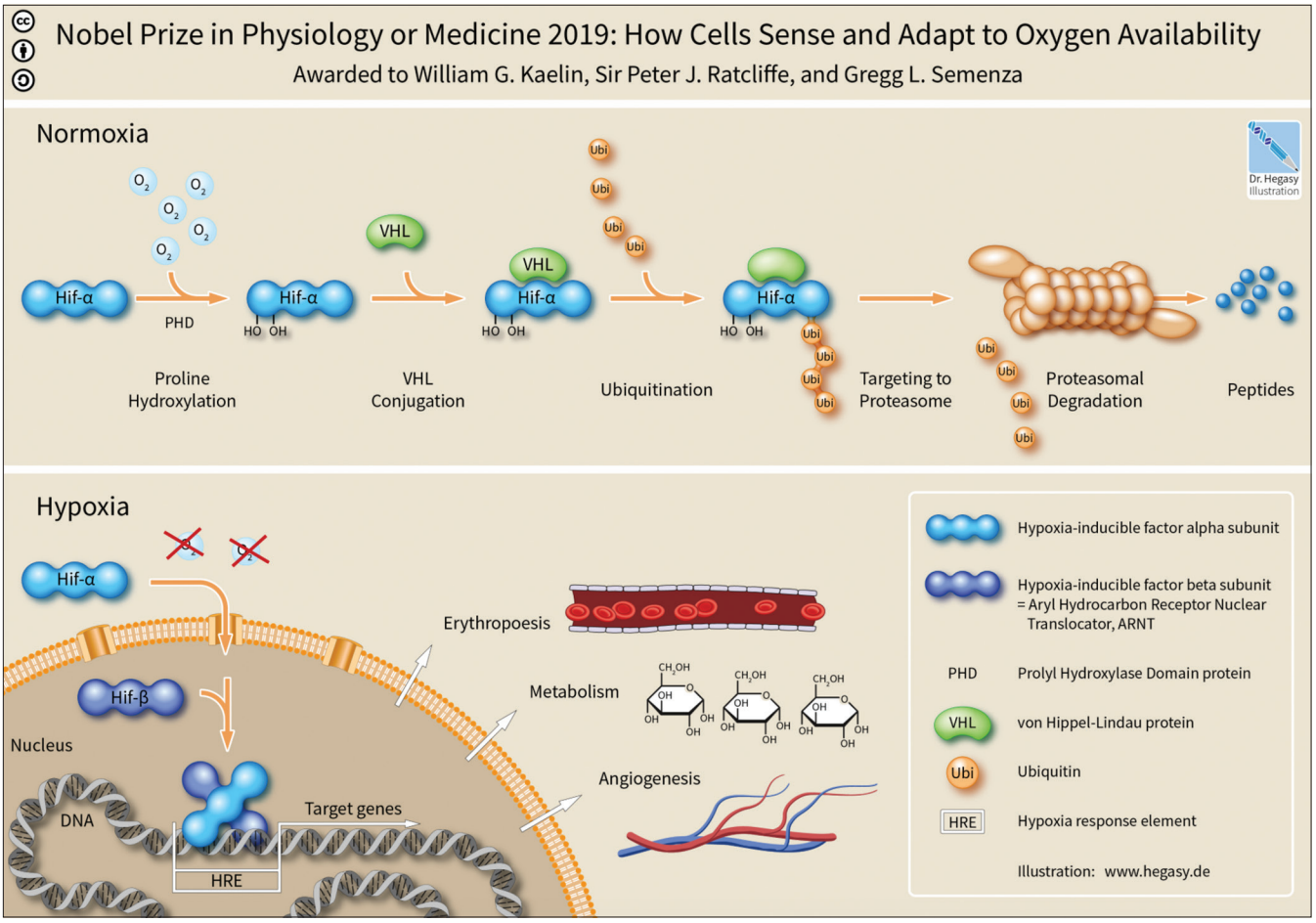

Figure 8: Hypoxia-inducible factor (HIF)-1α in Normoxia and Hypoxia State. Under normoxic conditions, HIF-1α is hydroxylated at two proline residues. The protein is then combined with VHL and then tagged with ubiquitin. After the ubiquitination process, it is transferred to the proteasome which is then undergone degradation. Under hypoxic conditions, no oxygen is available for the hydroxylation of proline. In this situation HIF-1α translocates to the cell nucleus (nucleus) and combines with HIF-1β, also called aryl hydrocarbon receptor nuclear translocator. This complex then binds to a region of DNA called the hypoxia-responsive element, which causes the target gene to be transcribed. This gene is involved in the regulation of many processes, including erythropoiesis, glycolysis, and angiogenesis

Flowcytometric analysis showed that the stem cell phenotype of adipose derivatives was not significantly affected by hypoxic conditions for 24 h. Hypoxic conditions can cause MSCs to remain undifferentiated for a certain period of time [50]. Several research reports indicate the existence of hypoxia-induced differentiation of MSCs, however, to grow MSCs in a hypoxic state and in differentiation condition it takes a longer time (can be more than 2 weeks). After transplantation, MSCs in the precondition of hypoxic state significantly reduced vascular and nerve damage compared with MSCs in the normoxic state [33].

In addition to the paracrine effect, AdMSCs have been shown to have the potential to differentiate into a wider range of cell types including endothelial cells, smooth muscle cells, Schwann cells, and neuron cells [51]. Engraftment and pluripotency of transplanted stem cells can contribute to the regeneration of blood vessels and nerves. Hypoxia preconditioning can increase the involvement and viability of MSCs in the host tissue (Figure 9). At 4 weeks after transplantation, the *in situ* differentiation of the transplanted cells was also analyzed by staining the tissue sections with vascular markers (CD31 and α-SMA). It can be concluded that hypoxic pretreatment increases the binding of AdMSCs to the tissue [36], [52].

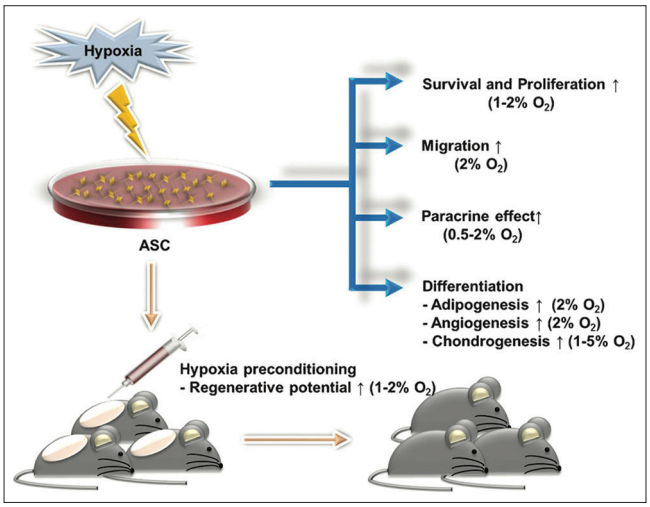

Figure 9: Functional change of AdMSCs in hypoxic state. Hypoxia induces the proliferation, migration, and secretion of growth factor AdMSCs in vitro. Hypoxia preconditioning increases the regenerative potential of AdMSCs in vivo. In addition, hypoxia regulates the differentiation of AdMSCs in an oxygen concentration-dependent manner [49]

Hypoxic conditions of 1–2% O_2 increase the survival or proliferation of AdMSCs. The hypoxic state of 2% O_2 increases the phosphorylation of mitogenic signaling molecules, such as Akt and ERK1/2, with the formation of Nox4-mediated reactive oxygen species (ROS); and 2% O_2 is recommended to increase survival, the proliferation rate of AdMSCs, and migration of AdMSCs. Weijers *et al*. (2011) stated that 1% O_2 increases the proliferation of AdMSCs, while 5% O_2 was controversial in terms of increasing or decreasing the proliferation of AdMSCs [53], [54], [55], [56]. Increased mobilization and adhesion to damaged tissue are important factors that have a positive effect on the therapeutic potential of AdMSCs. As in the case of proliferative AdMSCs, the formation of Nox4-mediated ROS is important in the migration of hypoxia-promoted AdMSCs [49], [57]. The table below summarizes cellular response of AdMSCs due to the hypoxic state *in vitro*.

Table 1: Cellular Response of AdMSCs *In Vitro* Hypoxic State [49]

O_2 level	Altered functions	Reference
0.1%	Increased cell death	(Follmar et al., 2006)
0.5%	Increased ROS level and enhanced paracrine effect	(De Barros et al., 2013)
1%	Increased growth factor secretion	(Rehman et al., 2004), (Liu et al., 2013), (Rasmussen et al., 2011)
	Increased proliferation, survival, and reduced aging	(Weijers et al., 2011)
	Differentiation arrest and maintain undifferentiation state	(Lin et al., 2006)
	Increased proliferation, colony formation	(Pilgaard et al., 2009)
	Upregulated leptin expression	
	Increased expression of pro-angiogenic factors and decreased expression of anti-angiogenic factors	(Efimenko et al., 2011)
2%	Increased growth factor secretion	(Lee et al., 2009), (Amos et al., 2008), (Przybyt et al., 2013), (Chaldakov et al., 2009)
	Increased myogenic potential	(Lee and Kemp, 2006)
	Increase proliferation and chondrogenesis	(Xu et al., 2007)
	Increased proliferation and decreased osteogenic differentiation	(Malladi et al., 2006)
	Increased proliferation and migration	(Kim et al., 2012), (Kim et al., 2011)
	Decreased ATP content and downregulation of DNA repair gene	(Oliveira et al., 2012)
	Increased adipogenic and osteogenic potential	(Valorani et al., 2012)
5%	Increased chondrogenesis	(Khan et al., 2007), (Jurgens et al., 2012), (Buckley et al., 2010)
	Increased chondrogenesis, but decreased osteogenic differentiation	(Merceron et al., 2010)
	Increased survival and enhanced paracrine effect	(Stubbs et al., 2012)
	Decreased proliferation and keep undifferentiated state	(Ranera et al., 2012)
	Slightly increased proliferation	(Yang et al., 2012b)

Several studies have shown that hypoxic or anoxic preconditions substantially increase the regenerative potential of MSCs (Figure 10). Exposure to reduced partial oxygen pressure to MSCs induces expression of genes involved in migration and homing (e.g. CXCR4 and SDF-1), regulated mainly by the activity of HIF-1α [58]. In addition, MSCs culture in a hypoxic environment resembles the presence of actual MSCs *in situ* than in the standard 'artificial' (21% O_2) culture conditions. Cellular response to hypoxia *in vitro* varies at different oxygen concentrations; 0.1–5% O_2 [4], 1–7% O_2 [5], [6], 1% O_2 [33], [59], 0.5–2% O_2 [49].

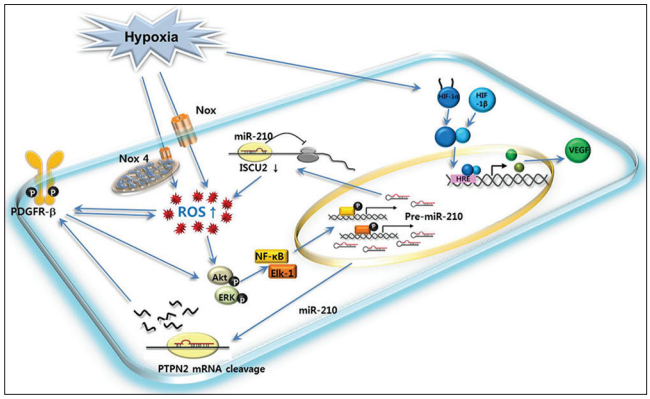

Figure 10: Signaling pathways and molecular changes in a hypoxic state. Hypoxia induces ROS formation primarily by Nox4. Acute increase in cellular ROS levels induces PDGFR-β activation and results in phosphorylation of Akt and ERK1/2. Activation of this signaling pathway induces expression of miR-210 via phosphorylation of NF-kB and Elk1; elevated miR-210 levels downregulate PTPN2 and ISCU2, thereby increasing the proliferation and migration of AdMSCs. On the other hand, inhibition of proline hydroxylase under hypoxic states stabilizes HIF-1α, which modulates angiogenic growth factor transcription after binding to hypoxia-responsive elements in the nucleus [49]

Hypoxia preconditioning *in vitro* has been shown to stimulate the secretion of growth factors, cytokines and other proteins, and the release of extracellular vesicles (exosomes and microvesicles) from MSCs [60]. Extracellular vesicles also carry various biomolecules such as growth factors, receptors, enzymes, transcription factors, signaling and immunomodulatory molecules, DNA, RNA transcripts, and non-coding RNA transcript including retrotransposons, vault RNA, long non-coding RNA, and long non-coding microRNA [61], [62].

Hypoxic preconditions have also been shown to increase survival and cell proliferation [63], [64], [65]. Pre-incubation of hypoxic MSCs induces metabolic changes resulting in higher *in vivo* cell survival after transplantation [63]. This suggests that culture in a hypoxic state increases the angiogenic potential of MSCs and improves their survival in both *in vitro* and *in vivo* studies [63], [64]. The signaling pathway involved translocation of HIF-1α to the nucleus by activation of gene expression (e.g. VEGF), as well as the formation of ROS and phosphorylation of Akt - MAPK ERK1/2 (Figure 11) [66].

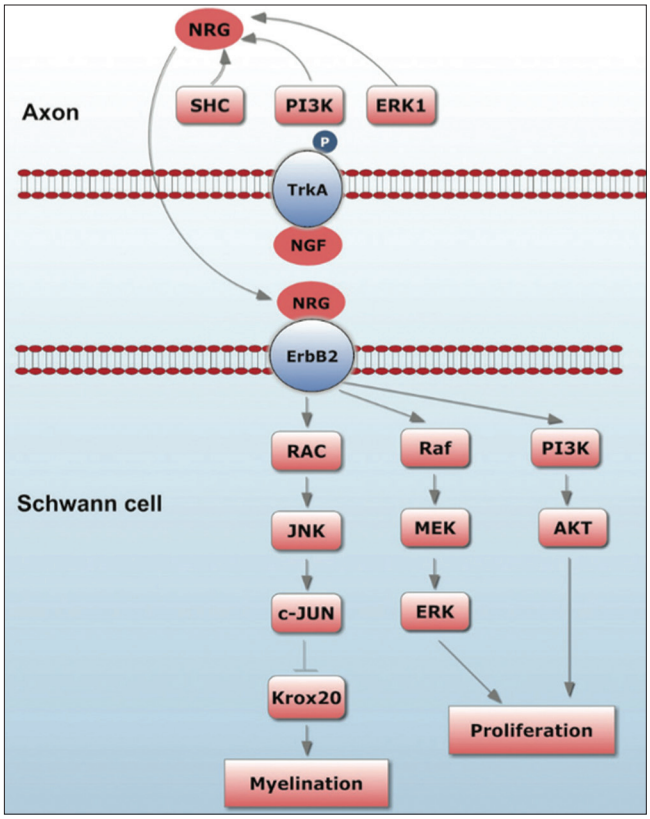

Figure 11: Axon-Schwann cell interactions and some of the molecules involved [66]

Conclusion

Transplantation of MSCs or administration of MSCs-CM or MSCs-EV requires MSC to have maximum regenerative capacity, so it is necessary to develop new strategies to increase the regenerative efficiency of MSCs. *In vitro* preconditioning strategies can improve the survival, engraftment, and paracrine properties of MSCs thus optimize their reparative and regenerative capacities. Among these strategies, hypoxia preconditioning represents a comprehensive increase in regenerative gene expression in stem cell therapy. There are significant advantages in stem cell therapy with hypoxia preconditioning.

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