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Influence of low oxygen tensions on expression of pluripotency genes in stem cells

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The stem cells are characterized by self-renewal ability and potential to differentiate into other cell types of the body. They are residuing in defined microenvironments - "stem cell niches". The embryonic stem cells (ESC) are derived from embryos which exist in 3-5 % oxygen condition. This environment is physiologically normal not only for ES cells but also for many other types of stem cells including neural stem cells (NSC). These observations suggest that low oxygen condition plays a very important role in the maintenance of cell stemness. Pluripotency is regulated by the family of hypoxia inducible factors (HIFs), which are dependent on oxygen tensions. HIF-2α is an upstream regulator of *Oct4*, which is one of the main transcription factors used to generate the first induced pluripotent stem cells (iPSCs). It has been shown that knock-down of HIF-2α but not HIF-1α, leads to a decrease in the expression of *Oct4*, *Nanog* and *Sox2*, which are important stem cells markers. The structure of hypoxia inducible factors as well as their behavior in hypoxia and normoxia was described. Therefore optimization of oxygen concentration seems to be crucial from the stem cell transplantation as well as iPS transplantation standpoint. Although many experiments with cell culture under low oxygen condition were performed, there is still much that is unknown. This short review presents some aspects on important issue of hypoxia induced regulation of stemness.

Key words: hypoxia inducible factors, oxygen tension, pluripotency, stem cells

INTRODUCTION

Conventional *in vitro* NSC cultivation similar to that of other stem cells is sustained under 21% oxygen tensions (ambient O_2 concentrations). This condition is defined as "normoxia". Whereas the results of many measurements from different stem cell niches showed the oxygen concentrations to be between 1% (and lower) to 8%, it is well below atmospheric oxygen tension (Mohyeldin et al. 2010). These conditions were defined as physiologic normoxia or normoxia in situ, in contrast to 20 or 21% oxygen concentration named as hyperoxic state (Ivanovic 2009).

Stem cells are residuing in defined microenvironments termed niches (Zhang and Li 2008). The oxygen tension seems to be an important element of this structure. Different oxygen conditions can give various cellular responses.

The first data on molecular consequences of oxidative damage to DNA during proliferation were demonstrated by Busettil and coworkers (2003). In their report mouse embryo fibroblasts (MEFs) from mice harboring a silent bacterial *lacZ* mutation reporter gene were cultured under 3% oxygen or the high O_2 tension of 20%. Thouse results indicated that the cells grown in higher oxygen tension (20% O_2) accumulated more mutations than cells in culture at 3% O_2. Most of mutation were transversions (G:C to T:A), which is a marker of mutation of oxidative DNA damage (Busettil et al. 2003).

The oxygen level in interstitial tissue of mammalian brain range from 1 to 5% (Studer et al. 2000). NSCs of mammalian central nervous system reside typically in

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subvetricular zone (SVZ) and in hippocampus (Alvarez-Buylla and Garcia-Verdugo 2002) in a relatively hypoxic environment (Mohyeldin et al. 2010) and sustaining undifferentiated state with the capacity to proliferate, differentiate and self-renew. These observations suggest that low oxygen level has influence on maintenance of pluripotential state of the stem cells, even those residing in the adult stem cell niches. *In vitro* cultured NSC, often used for transplantations to the animal brain in the pre-clinical studies of the ability of stem cells to treat neurological disorders (Kozlowska et al. 2009, Jablonska et al. 2010, Ali and Bahbahani 2010). Such transplantations were also shown to be dependent upon in vitro hypoxic preconditioning of transplanted stem cells (Zadori et al. 2011). Any effective stem cell replacement therapy would require the understanding of the mechanisms governing their early developmental process such as migration, proliferation and neural commitment (Buzanska et al. 2009, Szymczak et al. 2010) and their dependence from the surrounding oxygen level condition. Established functional relationship between hypoxia inducible factors and "stemness" transcriptional factors, together with the documented *in vivo* neural stem cell niche hypoxic conditions suggest strong dependence of the early developmental processes from the oxygen level.

HYPOXIA INDUCIBLE FACTORS (HIF) – HETERODIMERIC TRANSCRIPTION FACTORS

The crucial role in the cellular responses to changes at oxygen concentrations in environment plays hypoxia inducible factors (HIFs). They are heterodimeric transcription factors composed of the alpha and beta subunits. HIF-1 is a heterodimer consisting of 120 kDa (826 amino acids) HIF-1α subunit which complex with HIF-1ß subunit 91 to 94 kDa (two isoforms of 774 and 789 amino acids; Wang et al. 1995). There are three isoforms of alpha subunits (HIF-1α, HIF-2α and HIF-3α) and two isoforms of beta subunits (HIF-1β and HIF-2β; Zagorska and Dulak 2004). Wang and others (1995) also showed, that both HIF-1 α and β subunits (ARNT) are basic helix-loop-helix (bHLH) proteins containing a PAS (Per-AHR-ARNT-Sim) domain. Where Per stands for *Drosophila* period clock protein, ARNT stands for aryl hydrocarbon receptor nuclear translocator gene in mammals and Sim is *Drosophila* single-minded protein (Hewitson et al. 2003, Tian et al. 1997).

HIF-2α is also called Endothelial PAS domain protein 1 (EPAS1), HIF-1 like-factor (HLF), HIF-1 related-factor (HRF) and it shares 48% sequence identity with HIF-1α and heterodimerazes with ARNT for transcriptional activation in target genes which are expressed in endothelial cells (Tian et al. 1997).

HIF-1α and HIF-2α are structurally similar in their DNA binding and dimeryzation domains (Hu et al. 2003), but HIF-1α which was discovered earlier (as being responsible for expression of erythropoietin; Wang and Semenza 1995) have more universal expression pattern than HIF-2α, thus most studies have been focused on HIF-1 (Ema et al. 1997, Dery et al. 2005).

Human HIF-1α and HIF-1β genes are located on chromosome 14(14q21-q24) and chromosome 1(1q21) respectively (Dery et al. 2005).

STRUCTURE OF HYPOXIA INDUCIBLE FACTOR

The N-terminal part of HIF-1α contains bHLH domain, next is PAS (PAS-A and PAS-B), then the ODD domain (oxygen–dependent degradation) (Fig. 1). ODD domain includes two PEST- like sequences. The presence of PEST (P - proline, E - glutamic acid, S - serine, T -threonine) was shown to be connected to short life-time of HIF-1α (in 21% O_2 half life-time is about 5 min) and is followed by degradation (Wang et al. 1995, Huang et al. 1998). HIF-1α and HIF-2α contain two trasactivation domains TAD which are responsible for transcriptional activity. One is located in the N-terminal part of heterodimer termed N-TAD (NAD) and the second one is in the C-terminal part called C-TAD (CAD). The activity of CAD can be inhibited by FIH (Factor Inhibiting HIF) - asparaginyl hydroxylase. C-transactivation domain of HIF which contains asparagine is hydroxylated under normoxic conditions by asparaginyl hydroxylase. This results in the silence of CAD domain (Lando et al. 2002a). Wood and colleagues (1996) demonstrated that in HIF-1β / ARNT only the basic HLH and PAS, domains are necessary for response to hypoxia and the C- terminal part does not play any important role in the process (Wood et al. 1996, Huang et al. 1998, Dery et al. 2005).

HIF-1α as a transcriptional factor has to be imported into the nucleus. Transport of protein to nucleus requires nuclear localization signals (NLSs). HIF-1α contains two: N-NLS and C-NLS with the latter more important in translocation of HIF-1α to the nucleus

(Kallio et al. 1999). HIF-1β contains only one nuclear localization signal at the N-terminal end (Dery et al. 2005)

HIF-1α DEGRADATION PATHWAY IN STEM CELLS – PROCESS IN NORMOXIA

Hypoxia inducible factor-1 is regulated by oxygen sensitive hydroxylation of HIF-1α subunits (Tuckerman et al. 2004). HIF-1α contains many Pro (proline amino acid) in ODD domain that are recognized and hydroxylated by PHD (prolyl hydroxylase domain). PHD catalyses this reaction by adding an oxygen to Pro 402 and / or Pro 564 and converting them to 4-hydroxyproline (Maxwell 2005, Berra et al. 2006). The oxygen and 2-oxoglutarate are required for the PHD activity. The human PHD has three isoforms: PHD1, PHD2 and PHD3; their activities reveal similar oxygen dependence (Tuckerman et al. 2004).

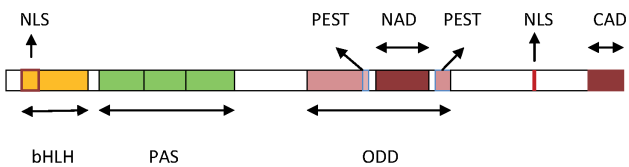

Fig. 1. Hypoxia inducible factor-1α. NLS - nuclear localization signal, bHLH- basic helix-loop-helix, PAS- Per-AHR-ARNT-Sim, PEST – P (proline) E (glutamic acid) S (serine) T (threonine), ODD- oxygen dependent degradation domain, NAD (N-TAD), CAD (C-TAD) N and C – terminal transactivation domain

In normoxic conditions HIF-1α is ubiquitinated and degraded via 26S proteasome (Kallio et al. 1999). Von Hippel–Lindau (pVHL) tumor suppressor protein plays an important role in ubiquitination process by specific ubiquitin E3 ligase complex. pVHL is bound to hydroxylated HIF-α subunit (Fandrey et al. 2006).

HIF-1α STABILIZATION PATHWAY IN STEM CELLS – PROCESS IN HYPOXIA

In low oxygen tensions, α subunits of hypoxia inducible-factors are stabilized and accumulated because prolyl hydroxylation (Ivan et al. 2001) as well as the factor inhibiting HIF (FIH identified as asparaginyl hydroxylase) are blocked. Both require molecular O_2 as a substrate. When asparagine is nonhydroxylated, then transcriptional co-activators such as p300/

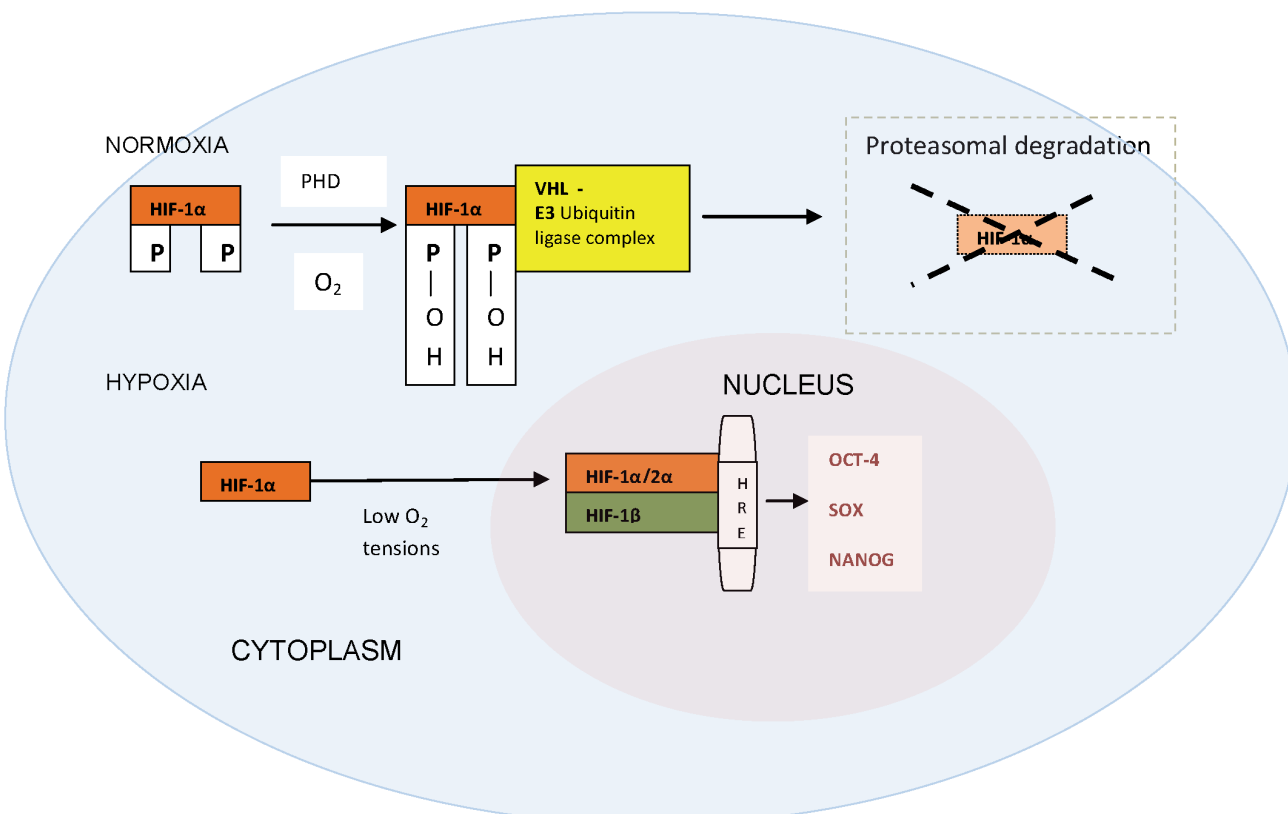

Fig. 2. Activation of the pluripotency genes in stem cells

CBP - CREB (cAMP-response-element-binding-protein), can interact with CAD. The full activity of hypoxic response requires stabilization of HIF-α transcription factor and activation of CAD (Lando et al. 2002b). HIF-1α after stabilization is translocated from cytoplasm into the nucleus and dimerization with HIF-1β occurs. HIF-1ß (ARNT) can heterodimeraze with HIF-1α, AHR and Sim (Wang et al. 1995). The bHLH domain and some part of PAS domain are required for dimerization of HIF-1α with HIF-1ß and bHLH domain is very important for the binding of the complex HIF-1α + HIF-1ß to DNA (Bardos and Ashcroft 2005). The complex HIF-1 (HIF-1α + HIF-1β) binds to the specific DNA sequences named HRE (Hypoxia response elements 5'-TACGTG-3'). The recognition of HRE is done through the basic domain (N-terminal end) of HIF-1α and HIF-1β (Fandrey et al. 2006). The hypoxia response elements may be located within either promoter or enhancer regions of target genes (Zagorska and Dulak 2004).

RELATION OF HYPOXIA INDUCIBLE FACTOR WITH OTHER INTRACELLULAR PATHWAYS

Hypoxia inducible factor promotes or represses the activity of many genes involved in different cellular functions, such as cell survival, oxygen homeostasis, proliferation, angiogenesis, glucose metabolism and apoptosis (Semenza 2003).

In addition, there is interaction between hypoxia-responsive transcription factors and other transcription factors that play important role in the cellular processes, of the NF-*κ*B, AP-1 (activator protein 1), p53 and c-Myc etc. (Kenneth and Rocha 2008). NF-*κ*B is involved in the immune system, inflammatory responses as well as cancer (Perkins and Gilmore 2006). Van Uden and coworkers (2008) demonstrated that NF-*κ*B can directly modulate HIF-1 transcriptionally. AP-1 activation has been associated with proliferation, apoptosis as well as tumorigenesis. Interaction between AP-1 and HIF-1 has been shown to occur in hypoxia (Michiels et al. 2001). Hypoxia induces a G1 arrest in the cell cycle through HIF-1-dependent mechanism (Gordan et al. 2007). Several studies demonstrated that hypoxia-induced tumor suppressor p53 is both dependent and independent of HIF-1 (reviewed in Hammond and Giaccia, 2006). Genes of the *Myc* family of transcription factors are involved in a multitude of cellular processes such as cell growth and proliferation, inhibition of cell differentiation, promotion of angiogenesis and genomic instability (reviewed in Adhikary and Eilers 2005). MYC and HIF complexes can compete for binding sites at the promoters of target genes to alter their expression profile. This has been shown in the regulation of the cyclin-dependent kinase inhibitor p21 (Koshiji et al. 2004). HIF and c-Myc are involved in inducing shared target genes *VEGF* (vascular endothelial growth factor), *HK2* (hexokinase 2) and *PDK1* (pyruvate dehydrogenase kinase 1; Kim et al. 2007). The above-mentioned examples show a complex interplay between the HIF and many other transcription factors, which are probably cell type-dependent.

Hypoxia is not the only method to activate HIF-1: other stimuli include growth factors, cytokines, vascular hormones and viral proteins that can increase the rate of HIF-1α mRNA transcription (Dery et al. 2004).

DEVELOPMENTAL ASPECT OF HYPOXIA INDUCIBLE FACTORS RELATED PROCESSES

The lack of HIF-1 activity results *in vivo* in serious defects of the circulatory system and death at midgestion (Semenza et al. 2005).

Developmental effects in HIF-2α deficient animals were tested in mice model, in iron absorption contexts. Results showed that deletion of HIF-2α decreased the level of iron in serum and liver (Mastrogiannaki et al. 2009). HIF-2α knockout also resulted in bradycardia, vascular defect, pancytopenia, retinopathy and global postnatal deletions (Patel and Simon 2008). Since HIF-2α is an upstream regulator of many genes and it absence may be analyzed also by a lack of HIF-2α target genes.

Despite of many similarities between HIF-2α and HIF-1α, each have different target genes. It was shown that HIF-2α but not HIF-1α is direct upstream regulator of *Oct-4*, transcription factor which is essential for maintaining pluripotency (Covello et al. 2006, Forristal et al. 2010). In addition to *Oct-4*, also *Sox2* and *Nanog* were shown to be regulated by HIF-2α (Forristal et al. 2010). *Oct-4* with *Sox2* and *Nanog* maintain stemness and repress genes that promote differentiation (Boyer et al. 2005). OCT-4 and SOX-2 were two of the four transcription factors that were introduced to cells by Takahashi and Yamanaka (2006) to get the first iPS

cells. The connection between hypoxia, HIF-2α and pluripotency genes is extremely crucial for generation of induced pluripotent stem cells, since the evidence was provided proving that hypoxia increases efficiency of reprogramming (Yoshida et al. 2009).

Hypoxia promotes undiffertiated cell state in various stem and progenitor cells. It was demonstrated that hypoxia blocks neuronal and miogenic differentiation in Notch –depend manner (Gustafsson et al. 2005). Additionally, differentiation ability of neural precursors expanded under hypoxia conditions was tested. The results show that dopaminergic differentiation is favored in lower oxygen and involves HIF-1α in the regulation of dopamineric differentiation of neural stem cells (Zhang et al. 2006). Different study confirm enhanced dopamine neuron generation in lowered oxygen but also show increased proliferation effect and reduced cells death (Studar et al. 2000). Zadori and others (2011) suggested that O_2 requirement and sensitivity to low oxygen condition depend on the developmental stage of neural stem / progenitor cells. *In vitro,* in non-committed neural stem cells hypoxia supports survival, but in neuronal precursor and neurons induces apoptotic cell death. This data remain in controversy with the above mentioned stimulation of neuronal maturation in hypoxic conditions (Studar et al. 2000, Zhang et al. 2006).

Forristal and coauthors (2010) presented the culture time–dependent concept of regulation of HIF α subunit localization and its effect on proliferation in hES cells. The HIF-1α may play a role in the initial adaptation of cells to hypoxia but after 48h under low oxygen, when expression is lost, HIF-3α is upregulated, translocates into the nucleus and upregulates HIF-2α, which is upstream regulator of *Pou5f1*(*Oct-4*) expression. The role of HIF-3α has previously been unknown, but some data suggests that HIF-3α4 (the alternatively spliced human HIF-3) negatively regulates HIF-1 by blocking binding to HER (Maynard et al. 2005). Meanwhile Forristal and colleagues (2010) demonstrated that HIF-3α is the regulator of HIF-1α and HIF-2α expression.

Taken into consideration time of expression HIF-1α (48h) and previously described role of HIF-1α as only initiator of response on hypoxia conditions in cells, additionally in concept Forristal and colleagues (2010) suggests very crucial role time under hypoxia condition. After HIF-1α, HIF-2α continuous adaptation to hypoxia environment. As showed HIF-1α and HIF-2α have different target genes. The conclusions is that not only level of oxygen, developmental stages cells, but maybe also time of cultivation in under lowered O_2 tensions it should be analysis depends on what kind of effect we will need.

Forristal and colleagues (2010) suggested sequential activation of different HIF transcription factors under hypoxia condition, depending upon the time of exposure. This was due to the observation, that HIF-1α is only the initiator of the cellular response to low oxygen conditions, since after 48 hours its activity is shut down and further on HIF-2α plays the crucial role. HIF-1α and HIF-2α have different target genes, thus in stem cell culture *in vitro* pluripotency genes such as *Oct4*, *Sox2* and *Nanog* are activated by HIF-2α after initial adaptation time. Thus the time of stem cell culture cultivation under lowered O_2 tensions together with developmental stage of the starting population should be taken into consideration while analyzing cell fate control due to oxygen level manipulation in the artificial *in vitro* stem cell niche.

CONCLUSIONS

The goal of this review was to demonstrate how important and luckily more noticeable the level of oxygen in the stem cell niche has become. As presented above, different concentrations of oxygen may in different ways affect many cellular processes and in doing so change the properties of cells. Given the increasing number of different stem cell transplantations, it is imperative to find out the best conditions for *in vitro* culture of stem cells. To preserve the unique properties of stem cells, the origin of these cells and the conditions of their culturing *in vivo* should be taken into account, therefore culturing under hypoxic and normoxic conditions should be carefully reexamined. Many studies demonstrate that low oxygen concentrations are more closely related to the physiological conditions compared to the atmospheric conditions. Speaking of hypoxia in conditions of low oxygen but not lower than the physiological state is a misconception and may involve the less than optimal design of experiments and even can be the cause of their failures.

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