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The stabilization of hypoxia inducible factor modulates differentiation status and inhibits the proliferation of mouse embryonic stem cells

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ABSTRACT

Hypoxic conditions are suggested to affect the differentiation status of stem cells (SC), including embryonic stem cells (ESC). Hypoxia inducible factor (HIF) is one of the main intracellular molecules responsible for the cellular response to hypoxia. Hypoxia stabilizes HIF by inhibiting the activity of HIF prolyl-hydroxylases (PHD), which are responsible for targeting HIF-alpha subunits for proteosomal degradation. To address the impact of HIF stabilization on the maintenance of the stemness signature of mouse ESC (mESC), we tested the influence of the inhibition of PHDs and hypoxia (1% O_2 and 5% O_2) on spontaneous ESC differentiation triggered by leukemia inhibitory factor withdrawal for 24 and 48 h. The widely used panhydroxylase inhibitor dimethyloxaloylglycine (DMOG) and PHD inhibitor JNJ-42041935 (JNJ) with suggested higher specificity towards PHDs were employed. Both inhibitors and both levels of hypoxia significantly increased HIF-1alpha and HIF-2alpha protein levels and HIF transcriptional activity in spontaneously differentiating mESC. This was accompanied by significant downregulation of cell proliferation manifested by the complete inhibition of DNA synthesis and partial arrest in the S phase after 48 h. Further, HIF stabilization enhanced downregulation of the expressions of some pluripotency markers (OCT-4, NANOG, ZFP-42, TNAP) in spontaneously differentiating mESC. However, at the same time, there was also a significant decrease in the expression of some genes selected as markers of cell differentiation (e.g. SOX1, BRACH T, ELF5). In conclusion, the short term stabilization of HIF mediated by the PHD inhibitors JNJ and DMOG and hypoxia did not prevent the spontaneous loss of pluripotency markers in mESC. However, it significantly downregulated the proliferation of these cells.

1. Introduction

Currently, the biology of stem cells (SC) is under intensive study, as SC represent an important target for therapeutic application in the treatment of a wide range of human diseases. SC are suggested to be a valuable source of new cells for direct cell therapy or for supporting cell therapy [1,2]. Moreover, cancer SC are currently recognized as an important target in anti-cancer therapeutic strategies [3]. In spite of specific differences between particular SC populations, all SC are able to undergo self-renewal by symmetric division and differentiation by asymmetric division, which enable them to form an SC pool providing differentiating cells for tissue repair or, on the other hand, cancer development. SC reside in a specific niche, characterized by a low level of oxygen, nutrition, and metabolites, and a low turnover of other compounds.

The maintenance of embryonic stem cells (ESC) *in vitro* is based mainly on growth factors and the conditions repressing their spontaneous differentiation. Although mouse ESC (mESC) were isolated for the first time in 1981 [4] and human ESC in 1998 [5],

Abbreviations: ATP, adenosine triphosphate; DMOG, dimethyloxaloylglycine; EdU, 5-ethynyl-2′-deoxyuridine; ESC, embryonic stem cells; FIH, factor inhibiting HIF; HIF, hypoxia inducible factor; JNJ, JNJ-42041935; LDH, lactate dehydrogenase; LIF, leukemia inhibitory factor; mESC, mouse embryonic stem cells; ODD, oxygen dependent degradation; PHD, HIF prolyl hydroxylases; SC, stem cells.

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optimal culture conditions are still under development. Recent studies suggest that ESC differentiation status can be influenced by affecting signaling pathways mediating the response to hypoxia [6]. Interestingly, hypoxic conditions are suggested to be spontaneously established in aggregates formed during ESC differentiation *in vitro* [7,8]. It is, however, unclear whether hypoxia drives stem cells towards self-renewal or differentiation. Some authors suggest that hypoxic conditions applied in the range 1–5% O_2 support the maintenance of ESC in a pluripotent state, prevents their differentiation, and even reprograms the partially differentiated cells to a stem cell-like state [9–14]. On the other hand, other authors showed that hypoxic conditions applied in the range 0.1–1% O_2 contribute to the differentiation of ESC [8,15–17]. Overall, although hypoxia is suggested to be an important factor in determining stem cell fate, the effect of hypoxia on the maintenance of SC stemness under cell culture conditions *in vitro* is unclear.

The key regulatory factor involved in cell response to alternating oxygen levels is hypoxia inducible factor 1 (HIF-1) [18]. HIF-1 is composed of two subunits. The oxygen-dependent subunit HIF-1alpha (HIF-1α) dimerizes with constitutively expressed HIF-1beta (HIF-1β), also known as the aryl hydrocarbon receptor nuclear translocator [19]. HIF-1α subunits are regulated by oxygen on two levels – protein stability and transcription activity. HIF-1α contains two oxygen-dependent regulating domains: an oxygen-dependent degradation (ODD) domain hydroxylated by the prolyl-hydroxylase domain enzymes PHD1–3, and a C-terminal transactivation domain hydroxylated by factor inhibiting HIF (FIH) [19]. Both PHDs and FIH require HIF-1α, oxygen, and 2-oxoglutarate as substrates and an Fe^{2+} ion and ascorbate as cofactors. The ODD domain within HIF-1α controls its degradation by the ubiquitin proteasome pathway [19]. PHDs play the roles of specific cellular oxygen sensors. According to the availability of oxygen, PHDs catalyze the hydroxylation of proline residues at the ODD domain of the HIF-1α subunit. Thus, under normoxic conditions, HIF-1α is targeted for degradation through the ubiquitin proteasome pathway involving enzyme coding by the von Hippel-Lindau gene [20]. Under normoxic conditions, HIF asparaginyl hydroxylase FIH negatively regulates the HIF-1α transactivation domain function by preventing its interaction with the essential coactivator CBP/p300. In contrast, under hypoxia, HIF-1α translocates to the nucleus, where it binds to HIF-1β, forming heterodimer HIF. Then, after interaction with transcriptional coactivator CBP/p300, HIF binds to the hypoxia response element sequences in the promoter of hypoxia-responsive genes and regulates their expression [19]. Peet and Linke suggest the presence of an oxygen sensing cascade, in which HIF-1α is stabilized in moderate hypoxia; however, its transcriptional activity is still repressed by FIH, because of its higher affinity to oxygen. FIH-mediated repression is then released under more severe hypoxic conditions [21]. A further two HIF-α subunits are expressed in mammalian cells: HIF-2α, which is similarly regulated and has similar functions as HIF-1α; and HIF-3α, which lacks the transactivation domain and is suggested to play a role in the negative feedback loop inhibiting HIF-1α [16,19].

The pharmacological inhibition of PHD with consequent HIF stabilization is widely employed to mimic effects of hypoxic conditions. The most frequently used traditional PHD inhibitor is dimethyloxaloylglycine (DMOG), a 2-oxoglutarat mimetic, which competes with 2-oxoglutarat at the PHD's active site. However, the inhibitor DMOG is not specific only to HIF prolyl-hydroxylases [22,23]; thus, other enzyme inhibition related effects could also be involved in potential DMOG effects. Therefore, in this study, we uniquely employed a newly-developed PHD inhibitor, namely JNJ-42041935 1-(5-Chloro-6-(trifluoromethoxy)-1H-benzoimidazol-2-yl)-1H-pyrazole-4-carboxylic Acid (JNJ). JNJ was identified through structure-based drug design methods and is suggested to be highly selective for PHD relative to FIH, although it is not selective for a particular PHD isoform and exhibits potency with respect to all 3 PHDs [23]. The effects of this inhibitor on mESC and their physiological response had never been tested previously.

In this study, we compared the effect of HIF stabilization mediated either by hypoxia or by the inhibitors DMOG and JNJ on the loss of the spontaneous differentiation of mESC induced by the depletion of leukemia inhibitory factor (LIF), a key factor responsible for the maintenance of mESC [24,25]. The presented data show for the first time the stabilization of HIF-1α and HIF-2α in spontaneously differentiating mESC by the new inhibitor, JNJ. Interestingly, the stabilization of HIFs interfere with cell proliferation and the gene expressions of markers characterizing both the undifferentiated and differentiated phenotypes.

2. Material and methods

2.1. Cell culture, growth, and differentiation

Feeder-free adapted R1 line mESC were propagated in an undifferentiated state by culturing on gelatinized tissue culture plastic in Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal calf serum (ESC tested; both from Invitrogene, USA), 1x non-essential amino acid, 0.05 mM β-mercaptoethanol, 100 IU/ml of penicillin, and 0.1 mg/ml of streptomycin (all from PAA), supplemented with 1000 U/ml of leukemia inhibitory factor (LIF; Chemicon, USA) [26]. The cells were routinely incubated in 21% O_2 and 5% CO_2.

To analyze the effects of PHD inhibitors, ESC were seeded on gelatinized tissue culture plastic at a density of 5×10^4 per cm^2 in the medium with LIF for 24 h. After 24 h, the medium was replaced with fresh medium with LIF (below referred to as non-differentiating – ND ESC) and without LIF (below referred to as spontaneously differentiating ESC) [24]). Cells without LIF were treated as described under the particular experimental setup for 24 and 48 h or transferred to hypoxic conditions for 24 h and 48 h. The desired level of O_2 (1 or 5%) was maintained by increasing the relative N_2 level in a cell culture incubator (Sanyo, Japan) with 5% CO_2.

2.2. Transfection and luciferase reporter assays

For the luciferase reporter assay, cells were transfected using polyethyleneimine in a stoichiometric ratio of 4 μl per 1 μg of DNA 24 h after seeding. 0.5 μg of pT81/HRE-luc construct and Renilla luciferase construct (Promega, USA) were used per one well in a 24-well plate. pT81/HRE-luc [27] (generously provided by prof. L. Poellinger) contains three tandem copies of the erythropoietin HRE in front of the herpes simplex thymidine kinase promoter and the luciferase gene. The cell culture medium was changed 6 h after transfection and cells were treated with PHD inhibitors or cultivated in 1% or 5% hypoxia for the selected time points. Dual-Luciferase assay kit (Promega, USA) was used for the evaluation of luciferase activity according to the manufacturer's instructions. Relative luciferase units were measured using a ChameleonTM V plate luminometer (Hidex, Finland) and normalized to the Renilla luciferase expression.

2.3. Cell viability and the determination of cell proliferation

Two independent approaches were chosen to determine cell viability and proliferation. The first was based on the determination of the total amount of adenosine triphosphate (ATP) in whole cell lysates, while the second was based on the determination of the total amount of proteins in whole cell lysates [28]. Both parameters