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The role of dissolved oxygen levels on human Mesenchymal Stem Cell culture success,
regulatory compliance and therapeutic potential

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Abstract

Most cells in the human body, including human mesenchymal stem cells (hMSCs), have evolved to survive and function in a low, physiological, oxygen (O₂) environment.

Investigators have become increasingly aware of the effects of O₂ levels on hMSC biology and culture and are mimicking the natural niche of these cells in vitro to improve cell culture yields. This presents many challenges in relation to hMSC identity and function and in the maintenance of a controlled O₂ environment for cell culture. The aim of this review is to discuss a “hMSC checklist” as a guide to establishing which identity and potency assays to implement when studying hMSCs. The checklist includes markers, differentiation potential, proliferation & growth, attachment & migration, genomic stability and paracrine activity. Evidence drawn from the current literature demonstrates that low O₂ environments could improve most “hMSC checklist” attributes. However, there are substantial inconsistencies around both the terminology and the equipment used in low O₂ studies. Therefore, “hypoxia” as a term and as a culture condition are discussed. The biology of short (acute) vs long-term (chronic) hypoxia is considered and a nascent hypothesis to explain the behaviour of hMSCs in long-term hypoxia is presented. It is hoped that by establishing an ongoing discourse and driving towards a regulatory recognisable “hMSC checklist”, we may be better able to provide the patient population with safe and efficacious regenerative treatments.

Introduction

Regenerative medicine is an active area of public health research. The definition of regenerative medicine can be extensive, covering a large spectrum of therapies spanning

across the generation and use of therapeutic cells to correct aberrant enzymic function, the generation of organic and inorganic scaffolds to promote wound healing, to the production of artificial limbs, joints and organs. Mason and Dunnill suggest a succinct definition including therapies incorporating the use of human cells whereby “Regenerative medicine replaces or regenerates human cells, tissues or organs, to restore or establish normal function” [1].

Stem cells are viewed by many as a primary raw material for the industry of regenerative medicine but even here lies debate around what crucially defines a stem cell (SC)[2,3]. Even on the backdrop of ongoing scientific discussion SCs are clearly a fundamental tool in the field and are presented as a long-term hope in the resolution of treatment refractory health problems. Yet to fully address the potential and hope associated with SC therapies we, as a scientific community, must continue to discuss and define what is sound science and to mean what we say when we report or collate results [3]. It is only then that we will provide the FDA [4,5], other regulatory authorities and above all patients with genuinely hopeful and safe and efficacious treatment(s) [6].

Stem cells commonly come from one of two main sources either being embryonic or adult (somatic) stem cells. Yet whatever the source of the cells and state of the debate around infinite asymmetric division they are commonly categorized by their potential to differentiate into other types of cells on a varying spectrum of potency. Human Mesenchymal Stem Cells (hMSCs) are multipotent SCs, which can be isolated with relative ease from various tissues including bone marrow, umbilical cord, adipose tissue and dental tissues [7]. As well as the relative ease of extraction and supply they can give rise to, minimally, specialized cell types such as osteoblasts, chondrocytes and adipocytes. In addition to their multipotent spectrum hMSCs have widespread immunomodulatory effects [8] as well as an angiogenic induction ability [9]. Taken together these characteristics give hMSCs a high potential of becoming a primary therapeutic option. There are 815 clinical trials listed by the US National Institute of

Health aiming to assess the effectiveness of MSCs in treating diseases such as Parkinson's, diabetes, liver, kidney and lung diseases as well as cardiovascular, bone and cartilage diseases [10]. Yet to date published clinical data does little to support the advancement of this therapeutic hope [4].

A significant delaying factor in the translation of hMSC therapies into the clinic is their low number in vivo; for example, they constitute about 0.001 to 0.01% of the bone marrow nucleated cell population [9]. After hMSC isolation there is a requirement for tremendous in vitro expansion to produce a therapeutic dose of cells; estimated at 10 to 50 million hMSC per treatment [11]. This process must be performed without compromising the viability, safety, purity or potency of the cells. The expansion step is challenging and requires a comprehensive understanding of cell culture conditions in order to produce validated therapeutic cells in large quantities.

Human MSC expansion has been studied widely across a variety of equipment types and culture condition combinations. The various cell culture systems available include uni-layered T-flasks, multi-layered T-flasks, hyper-stack cell culture vessels, cell factories, different bioreactor types with/without micro-carriers in suspension and manual versus automated systems. In terms of culture conditions, the variation is also extensive and involves serum-free medium [12,13], defined medium [14,15], human platelet lysate as fetal bovine serum replacement [16,17] and various serum (5-20%) [18] and oxygen levels (0.2-5%) [19] [18,20–26]. This backdrop is akin to having multiple synthetic routes for the generation of a single drug yet, in that instance as well as here, the ultimate goal from a manufacturing and patient perspective, is to produce a regulatory approved product able to deliver the desired “therapeutic function” with minimal side effects. This review aims to propose a “hMSC checklist” and then to discuss one influencing culture/manufacturing variable for the

production of therapeutic and regulatory quality hMSCs which is the level of dissolved oxygen (dO_2) in the medium.

"hMSC checklist" - a proposal to both enhance therapeutic potential and to navigate the regulatory terrain

Initially, three main criteria were used to define hMSCs; adherence to plastic, fibroblast-like morphology, and the presence of certain surface markers. However, relying solely on these criteria was problematic because they are not exclusive to hMSCs but shared with many primary mammalian cell types such as endothelial cells, fibroblasts, hematopoietic stem cells and B- and T- lymphocytes [27]. In 2006, the International Society for Cellular Therapy (ISCT) described a basic unified guideline on characterizing hMSC which consisted of:

- plastic adherence.
- expression of CD105, CD90 and CD73 with lack of expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules.
- ability to differentiate into osteoblasts, adipocytes and chondroblasts in vitro [28].

Since 2006, to date the use of hMSCs has significantly increased [29]. In parallel, the criteria and indicators that can be used to define hMSCs and assess their quality have developed considerably [29,30]. By a working consensus in the scientific community these currently include (Refer also Figure 1):

Markers: These have become numerous and include the presence of CD105, CD73, CD90, CD102 and CD124 and absence of CD45, CD34, CD14, CD15 and CD18 (for expanded list see [9,31]). Ongoing discussion in the scientific community may yet widen the list of potential surface phenotypic markers for hMSCs [29,30] as the expression of even an ISCT guided set of surface markers has yet to guarantee hMSC homogeneity.

Differentiation potential: Human MSCs expanded in vitro are routinely assessed for their ability to differentiate into osteoblasts, adipocytes and chondrocytes. The protocols for inducing these specific lineages are well-established and are routinely performed in MSC-based studies [15,22,32,33] assessing MSCs for tri-lineage differentiation potential [33–35]. Also the differentiation of hMSCs into neural cells [33] and endothelial cells [36] has been achieved in vitro and the differentiation of hMSCs into cardio-myocytes in vivo and in vitro has also been discussed [37,38]. However, the differentiation assays commonly used are qualitative and not easy to quantify thus, making changes in differentiation open to some subjectivity.

Proliferation and growth: This can be evaluated via many methodologies including population doubling levels, population doubling times, cell mean viability, viable cell yield, and expansion yield. It has been demonstrated that hMSC proliferation rate can be enhanced by modifying culture conditions [11,19,22,24,26]. In parallel with proliferation rate are the determination of passages, or population doublings, up to which hMSCs can still be used for therapeutic purposes (i.e. before senescence or losing multipotency). The proteins that are more likely to be defected by population expansion are components of the cytoskeleton and those involved in stress response, metabolism, cycle regulation and apoptosis [39]. The number of passages may not be a concern for autologous therapies because usually one to four passages are enough to produce clinically relevant cell numbers [40]. However, for allogeneic banked cells, where an extensive in vitro expansion is required, growth without replicative senescence is an important factor.

Attachment and migration potential: Human MSC attachment and migration are vital for successful therapies. It is well documented that one of the main problems in the transplantation of stem cells in the human body is their loss or their navigation far from the desired therapeutic site, in other words, poor engraftment. MSC are often injected in injured

tissues where they must adapt to low oxygen and nutrients, oxidative stress and inflammatory and apoptotic signals [41]. This micro-environment affects the MSC's ability to adhere to the extracellular matrix resulting into cell death by anoikis [42]. Attachment and migration assays are established [43–48] and routinely used in hMSC studies but the protocols used can be subject to a high level of variation.

Genomic stability: Long-term hMSC in vitro expansion makes them vulnerable to accumulate genetic defects which may alter the potency of the cells and raise safety concerns. [49,50]. Cell expansion during the manufacturing process may be a cause of replicative stress, chromosomal abnormalities [39], reduced telomerase activity [51] and senescence [52]. Therefore, cells from early passages were recommended to be used in clinical settings [32,53,54]. However, there are conflicting reports on genomic stability in MSCs [55,56], suggesting that bone marrow MSCs may remain suitable for cell therapy even after extended ex-vivo expansion and that genomic stability may be a donor dependent variable [57]. A second level of genomic assessment may be a profile for the proteome of hMSCs listing highly expressed proteins involved in different defining aspects of stem cell activity such as metabolism, differentiation and structural components [58].

Paracrine function: This may become central to the assessment of hMSC function as there is growing evidence that in many cases it is not the hMSCs arriving at the site of injury alone which achieves tissue repair, but a significant part of their therapeutic potential relies on the growth factors and cytokines that they secrete (the hMSC secretome [59–63]). Human MSC secreted proteins include fibroblast growth factor 2 (FGF-2), hepatocyte growth factor (HGF) and epidermal growth factor (EGF) [30,64] among others. This secretome profile enables hMSCs to exert paracrine activity in key areas such as tissue regeneration, immunomodulation [65–70], and angiogenic induction [45,71–77], which in turn potentiates their therapeutic application. Although this functional capacity of hMSCs was not originally

listed in the ISCT 2006 criteria its emerging importance can be seen by the 2014 recommendation by the ISCT to assess human MSC immunomodulatory activity [78] and other ongoing discussions related to functional assay stabilisation for product development [30,67,79]. The strengthening of this facet of the “hMSC checklist” via the ISCT suggested assays shows the importance of defining these parameters for regulatory interaction.

It is currently relatively frequent practice to take into consideration the first three criteria (markers, differentiation potential and attachment & migration) from the “hMSC checklist” in most hMSC-based studies. However when we consider the production of hMSCs in the fast-evolving cell therapy industry, cell manufacturing for approved therapies will most probably follow two different routes: “on-site” [80] and “off-the-shelf” [19]. “On-site” or autologous therapies are more likely to be totally processed at the clinic i.e. harvesting, expanding, delivering and if required, storage of cells. In this case, assay implementation and attributes assessment will heavily rely on the nature of the treatment, on the facility and the staff [80]. “Off-the-shelf” or allogeneic therapies are more likely to be manufactured at a large scale, enabling mass production, banking and distributing. In such a setting, the implementation of many of the “hMSC checklist” attributes become even more important to enable product validation and regulatory compliance.

The translation from bench to bedside requires that hMSC efficacy to be proven both in vivo and in vitro. That is why more research and experimentation are required to develop potency assays that can define and quantify, at a regulatory level, hMSC efficacy in all facets especially paracrine effects such as angiogenesis and immunomodulation. While research works to discuss and reduce the inherent variability of these assays it is critical they effectively mimic the physiological niche of the cells to improve in vitro-in vivo correlation. One key culture condition that is suggested to be taken into consideration in developing these assays and in hMSC experimentation in general is low O₂ tension.

Dissolved Oxygen levels in hMSC culture

The ability of mammalian cells to survive limited O₂ is reflective of an earlier evolutionary stage where unicellular organisms were exposed to a low O₂ atmosphere [81]. A few human cell types have adapted to ambient atmospheric 21% O₂, such as those found in the lining of the oral cavity, trachea and the lungs; whereas the majority of the others in tissues and organs including stem cells, remained adapted to low O₂ supply. This is illustrated by the enhanced proliferation that primary cells, including hMSC [20], display in low O₂ environments and the widespread transcriptional alterations low O₂ induces via the evolutionarily conserved HIF family of transcription factors [81–84].

Human MSCs reside in tissues such as bone marrow, adipose, articular cartilage, brain, dental, skin and perinatal organs [7] where the levels of O₂ are precisely tuned. Inspired air has an O₂ concentration of 21% dropping to approximately 12% at the alveolar level. As O₂ diffuses across the Type I pneumocytes in the lungs it then circulates in the capillaries, complexed to haemoglobin, to reach organs and tissues. O₂ concentration drops dramatically to reach approx. 1-6% in the bone marrow, 2-8% in the adipose tissue and 4-14% in the heart [85]. This means that hMSC physiological niche is a low O₂ environment. In other words, it can be said that the “normal” environment for hMSC is limited in O₂ supply.

Due to the variation in oxygen concentrations in the human body it is important to cast light upon the terminology used in cell biology and regenerative medicine with regards to O₂. In the past twenty years, it has become more evident that culturing stem cells in conditions like their natural niche or similar to the niche where they are intended to be engrafted is more reliable when drawing conclusions about the cells’ potencies. Over the years, the terminology has settled on the use of “normoxic” culture condition when the cells are exposed to 20% O₂ level in culture headspace and the use of “hypoxic” culture condition when the cells are

exposed to 0.5-10% O₂ levels. According to the medical dictionary, “normoxia” can be defined as: “A state of oxygen normalcy; normal levels of oxygen in tissue or blood” [86] while “hypoxia” can be defined as: “A decrease below normal levels of oxygen in inspired gases, arterial blood, or tissue without reaching anoxia” [87].

From these definitions, taken together with their commonly applied terminology, it becomes clear that confusion and inaccuracy can easily and commonly prevail. Zoran Ivanovic [81] in his article titled “Hypoxia or in situ normoxia: The stem cell paradigm” considered that the term “normoxia” was transferred from physiology to cell biology and to cell culture with scant regard to the prevalence of “low” yet normal O₂ level in tissues and that hypoxia itself is often linked to disease. In addition, this oversight goes beyond the terminology itself to include a number of vital biological processes reliant upon a limited O₂ supply including erythropoiesis, angiogenesis, brain development, and regulation of gene expression. This historical oversight has resulted in the majority of cell culture being performed in atmospheric 21% O₂ which for virtually all tissues is considered to be a high O₂ level or “hyperoxia” (excess supply of O₂). Rafiq et al. [88] estimated that normoxia referring to a 20% O₂ level in the head space of a cell culture vessel is equivalent to a 100% dO₂ in the medium (physiologically hyperoxic) while hypoxia referring to an O₂ level between 0.5% and 10% oxygen in the head space is equivalent to 10-25% dO₂ in the medium (approaching physiologically normoxic when referencing 21% atmospheric oxygen).

To lend clarity to this discussion (to try and “mean what we say” [3]) what follows is an explanation of widely used O₂ terminology in relation to the physiochemical properties of oxygen. The amount of O₂ is always referred to as a percentage. In particular, the percentage of O₂ in the atmosphere, as aforementioned, is about 21%. However, O₂ percentage does not always correspond to the O₂ concentration which is measured in ppm or mg/L. The correspondence between the percentage and the concentration depends on the altitude which

subsequently affects O_2 partial pressure [89]. Measuring O_2 concentration in a cell culture system is complicated because O_2 exists in two phases: gaseous in the headspace and dissolved in the liquid media. The concentration of O_2 in the two phases is not the same. O_2 diffuses from the headspace into the liquid and this diffusion is ruled by several laws including Henry's law [90] and Fick's law [91]. Moreover, O_2 solubility is affected by several factors including temperature, salinity and pressure. In fact, dissolution reduces with increasing temperature, salinity and pressure. For example, in a hypoxic tissue culture workstation set at 5% O_2 level, a liquid medium can hold a maximum of X mg/L O_2 at temperature T1. At temperature T2, the same liquid medium at 5% O_2 will hold a maximum of Y mg/L O_2 . The difference between X and Y is of high importance when considering the amount of O_2 available for the cells in each scenario (refer Figure 2). Doran [92] explains in great details all the steps involved in calculating the dO_2 level in a cell culture system namely fermenters, bioreactors and shake flasks. However, monitoring dO_2 in small scale plastic cell culture flasks is less common but methods have been published on how to perform it [93,94].

So, when we come to consider cell culture the following terminology is often used with “normoxia”, referencing a 20% O_2 level in the head space and “hypoxia”, referring to an O_2 level between 0.5% and 10% O_2 in the head space. These terms are broad and only refer to O_2 in the gaseous phase without taking into consideration the temperature, salinity, pressure of the liquid phase, cell growth phase, media composition and the subsequent O_2 dissolution. Therefore, monitoring dO_2 must be one of the main steps when culturing cells in low O_2 . It is recommended that the terminology used in research articles to describe O_2 culture condition is defined early and kept constant.

Inducing hypoxia in an experimental setting

Environmental hypoxia induction techniques vary hugely among laboratories ranging from home-made systems, hypoxic incubators and chambers to hypoxic workstations [19]. In multi-user facilities using hypoxic incubators prone the cells to periods of re-oxygenation every time the incubator door is opened (i.e. every time flasks are taken out for microscopic examination, medium change or passaging) [95]. This oxygen level fluctuation accompanied by the lack of direct oxygen monitoring in media may lead to unpredicted or conflicting results. Hypoxic workstations remain the current ultimate option for creating and maintaining a low oxygen environment throughout an experiment. These stations are usually equipped with humidity, temperature, oxygen and carbon dioxide controls that are connected to a real-time feedback system. They can be large enough to house a microscope and to host all activities so that flasks are not removed or exposed to ambient air at any point. In terms of hypoxia better experimental designs that allow close oxygen level monitoring and maintenance should enable current literature disparities to be resolved in the move towards a standardized and validated therapeutic product. Indeed, commercial systems are now entering the marketplace which describe the controlled modulation of dO_2 within culture media, to desired levels, prior to its use in cell culture. Environmental hypoxia induction techniques (equipment) are still surrounded by various challenges such as cost and stabilisation of low O_2 levels throughout experiments (Table 1). Alternatively, chemical induction of hypoxia using mimetic agents such as Cobalt Chloride ($CoCl_2$) and Deferoxamine Mesylate (Desferrioxamine; DFO) can be deployed. Both $CoCl_2$ and DFO are chelating agents which act to stabilise Hypoxia Inducible Factor (HIF) at atmospheric oxygen levels. Chemical induction of hypoxia is cheap and easy to perform and circumvents the main limitation of environmental hypoxia, re-oxygenation, but chemical induction of hypoxia has its own limitations as well (Table 1) [96].

Culturing hMSC in hypoxic conditions

If we accept some of the variation with O₂ (detailed above) as the current scientific paradigm and adopt a starting perspective that low O₂ reflects the hMSC endogenous environment, we can reflect on a volume of research from recent years (2006 to date) investigating the effect of low O₂, in percentage terms, on hMSC performance in culture. There is a mounting evidence that low O₂ affects all aspects of hMSC biology listed on the “hMSC checklist” and from the data collated from human cell-based studies it is revealed that environmental hypoxia generally enhances hMSC performance (refer Table(s) 2-7 and Figure 3).

aFrom the data presented in Table 2 it can be concluded that hypoxic culture has no significant effect on hMSC cellular phenotype (defined as defined as the presence of CD105, CD73, CD90 and absence of CD45) regardless of cell source. This conclusion aligned to the tripartite release criteria for MSC-like cellular products, and the physiological hypoxic niche of hMSCs, should enable some confidence that hypoxic culture conditions could become the “norm” for the generation of these cellular and gene therapy (CGT) based products. When examining the effects of hypoxia on hMSC population expansion (Table 5), again the data for hypoxic conditioning is mainly positive showing that hMSC yield is likely to be greater when the cells are cultured, across a number of days, under low O₂. Thus, hypoxia would enable a faster, larger-scale manufacturing platform for this CGT product. Hypoxia also appears to generally enhance the ability of implanted hMSCs to migrate which may be essential for certain therapeutic applications [158,159] (Table 4). With respect to favourable hMSC characteristics [98] which are not yet formally required for product authentication, the current literature strongly supports the maintenance of multi-potency [22,24,107,111,123], genetic stability [11,101,103,132] and paracrine activity [98,99,134,135] (refer Tables 6&7). Table 7, although directed to paracrine activity, indicates the influence of hypoxia upon microRNA (miRNA) release [136,137]. MiRNAs are one of several epigenetic regulators in hMSCs that alter in response to hypoxia and/or oxidative perturbations [160,161]. Epigenetic changes

produce alterations in gene expression that are not hard coded in the hMSC genome [162], encompassing mechanisms such as DNA methylation and histone modification as well as the non-coding RNAs (ncRNAs). Most data support the maintenance of “stemness” by hypoxic epigenetic pathways [130,163,164] and that these hypoxic pathways may involve epigenetic plasticity akin to that identified in a tumour microenvironment [160,165,166]. An epigenetic profile desired in a hMSC CGT product has yet to be fully elucidated but as knowledge develops the ambition would be to examine the full effects of hypoxia on the agreed epigenetic genotype.

Despite the high level of accord generated by environmental hypoxia on the “hMSC checklist”, differentiation potential is still seen as varied in low O₂ (Table 2). It is generally accepted that low O₂ promotes chondrogenic differentiation via hypoxic downregulation of RUNX2 [167]. Yet bidirectional variability exists with respect to osteogenic and adipogenic differentiation, which may be reflective of the different O₂ concentrations in in vivo niches for these different cells, the subsequent variation exerted on epigenetic regulation of differentiation, variability at the assay level or donor mediated variability [103,168–170]. Although hypoxia’s effect on hMSC differentiation potentially introduces variation to the ISCT criteria and the “hMSC checklist”, there is an accumulating body of evidence that hMSC’s paracrine activities are improved by environmental hypoxia (Table 7). So the potential endorsement of hypoxic culture conditions for hMSC product generation would depend on the relative importance placed on the secretion of trophic factors compared to the capacity to differentiate into various lineages by the scientific community [78,79].

The switch to chemical hypoxia leaves a slightly more complex landscape than environmental hypoxia in terms of the “hMSC checklist”. Tables 8 summarise studies (from 2006 to date) which investigate the effects of chemical hypoxia, mediated via CoCl₂ and DFO on hMSCs. It is apparent that both CoCl₂ and DFO at various concentrations and

incubation times upregulate HIF-1 α and increase the release of angiogenic mediators.

However more conflicting data exists related to cell morphology, viability and proliferation rate. This increased variation could be explained by the different mode of hypoxic induction (chemical v environmental), variation in hypoxic mimetic concentration, incubation time and/or cell source. In this respect, environmental hypoxia appears superior as the data regarding hMSC characteristics is slightly less variable and mainly positive (the mixed pattern of data is further reinforced when including other species such as mice and rats, yet this species extension is beyond the scope of this review).

The biology of acute and chronic hypoxia

As cited above O₂ sensing plays a role in genetic stability, survival, differentiation and proliferation of cells. The mechanism behind this powerful control is the tight regulation of gene expression. O₂ enters the cells by diffusion. Once in the cytoplasm its level regulates the function of a transcription factor called Hypoxia Inducible Factor 1 (HIF1) which is a dimer composed of two subunits HIF α and HIF β [82–84]. It is important to note that the production of the two subunits is O₂-independent. This means that they are produced in the cells regardless of the level of O₂. It is the dimerization of the two subunits that is O₂-dependent. In a normoxic culture condition where hMSCs are exposed to a high percentage of O₂, the enzymes HIF-Prolyl-Hydroxylases (HPHs), whose function relies on O₂, hydroxylate the proline residues 402 and 564 on the alpha subunit of HIF1 [85,171]. As a result, HIF1 α becomes tagged for ubiquitination and subsequently degraded. So, in a high level of cellular O₂ HIF1 is not functional. In a setting where hMSCs are exposed to a physiological O₂ level, HPHs become unable to perform the hydroxylation process allowing for the HIF1 α subunit to accumulate in the cytoplasm. This enables the HIF1 subunits to dimerize and translocate to the nucleus where they bind to specific DNA regions of gene promoters called Hypoxia Response Elements (HRE) [85,171]. This binding results in the regulation of hundreds of

genes involved in various cellular functions such as angiogenesis, migration and metabolism [18,19]. So, in a low level of cellular O₂ HIF1 is stabilised and functional and is considered a key regulator of cells' response [85,172]

Hypoxic conditions can be maintained across both the short and long term (Refer Figure 4) often with differing end points in terms of cellular health. When hMSCs are cultured in hypoxia for a short-time, the cells become prone to apoptosis which is translated as a decrease in proliferation. Alternatively, an extended duration under hypoxic conditions gives the cells some time to adapt and reprogram so survival is promoted, and proliferation increases after a lag period [22,100,123,173]. One can argue here that the process is not an adaptation but a selection process since an MSC population is not homogeneous, or a combination of both adaptation and selection.

A closer look at HIF could offer an additional biological explanation to the MSC response to long-term exposure to hypoxia which appears biphasic. There are three types of HIF namely HIF1, HIF2 and HIF3 whose composition and production are similar. HIF1 is well studied and its role in controlling the cellular response to hypoxia is well established [111,142,174–177]. The role of HIF2 in hMSC response to hypoxia is not as well assessed, however, it is more studied in several cancer types as well as in embryology [178]. HIF3 role in hypoxia is the least investigated with reports on its supportive [179] and/or inhibitory effects [180] on HIF1 [82]. Further to the variable role of HIF3 in response to oxygen when interacting with HIF1 differential expression of HIF3 has been documented in hMSCs related to epigenetic changes elicited by pro-inflammatory cytokines in an oxygen-independent manner [181]. In a review published by Mei Yee Koh and Garth Powis [171]) entitled “Passing the baton: the HIF switch”, the authors discuss that between two of the types of HIF, HIF-1 and HIF-2, gene regulation is not completely overlapping with each HIF having unique genes that they regulate as well as some common ones.

The modulation of gene expression in hMSCs via HIFs has been reported and is summarised in Table 9, and although the dissection to the level of individual HIFs in hMSCs has yet to emerge, in some cell lines HIF-1 drives the initial response to hypoxia while HIF-2 drives the chronic response to hypoxia [171] see Figure 5 [182]. One can suggest here that a switch from HIF1 to HIF2 or HIF3 occurs during hMSC response to chronic hypoxia. The mode of action of these two types of the transcription factor HIF could then be correlated to the two-phase events that occur in MSC long-term hypoxia. That is the lag phase at the beginning of the exposure could be mainly driven by the expression of HIF1 which stimulates metabolic switching to enhance glycolysis [11,26,141]. As the switch from HIF type 1 to type 2 occurs, MSC multi-potency and cell cycle progression are promoted in hypoxia, with examples of this HIF switching seen in other stem cell types [178,183,184]. The correlation suggested here is a novel suggestion, and the pathways potentially involved in this time course are complex [102], which means like many other facets of MSC biology it requires further research and investigation but hopefully could become a final addition on the “hMSC checklist”.

Conclusion and Future Perspectives

This review shows that culturing hMSCs in low oxygen is beneficial for most of their characteristics in terms of the “hMSC checklist” (proposed) and should be used in therapy development. Understanding the biology of hMSC hypoxia in more detail along with improved experimental design and reporting would offer a better basis for successful translation into the clinic and enable the stem cell community to provide the “scientific evidence” that the FDA [6] and other regulators require. The consensus through open discussion we may be able to afford our “hMSC checklist” experimental assays may also help with the ongoing quest “to mean what we say” as scientists as well as gaining regulatory acceptance. Attributed to Dostoevsky “Intelligence alone is not nearly enough when it comes

to acting wisely.” So maybe we need to be organised as well as intelligent to get MSCs to the point of regulatory compliant therapies of choice for both public and health care providers.

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Figure legends

Figure 1: Human MSC checklist. ‘Critical to quality’ therapy-specific attributes that could be defined. The above list could serve as a checklist when deciding which assays to implement when conducting a study to produce regulatory compliant hMSCs.

Figure 2: A schematic representation of the levels of gaseous (tissue culture head space) and dissolved (media dissolution of O₂) oxygen in tissue culture illustrating that at 5% O₂, any increase in; Temperature (T) and/or Salinity (S or mg/L) and/or Pressure (p) will see gaseous O₂ (culture headspace) will fall and dissolved O₂ (media) will rise. ¹Henry's law put into mathematical terms (at constant temperature) provides $p = kHc$ where p = the partial pressure of the solute in the gas above the solution; c = the concentration of the solute; k = the solubility of the substance; H = Henry's law constant (which depends on the solute, the solvent, and the temperature). ²Fick's Law essentially states that the rate of diffusion of a gas across a permeable membrane is determined by the membrane itself (material, thickness and surface area) the partial pressure gradient of the gas across the membrane: $V'_{\text{gas}} = D \cdot A \cdot \Delta P / T$ where V'_{gas} = Rate of gas diffusion across permeable membrane; D = Diffusion coefficient of that particular gas for that membrane; A = Surface Area of the membrane; ΔP = Difference in partial pressure of the gas across the membrane; T = Thickness of the

membrane.

Figure 3: A generalised schematic representation summarising the effects of hypoxic cell culture conditions on human mesenchymal stem cells. Closed (black) arrows represent a positive influence of hypoxia, open (white) arrows represent no significant impact of hypoxic conditions and the patterned (grey) arrows reflect a mixed response displaying both positive and negative outputs.

Figure 4: Summary of a review by Buravkova et al., [172] on the impact of chronic and acute hypoxia on hMSCs. Acute exposure (maximum duration of 72 hours to a level of atmospheric oxygen between 0 and 5%) and chronic exposure (minimally, one week to continuously, with a level of atmospheric oxygen between 0.5 to 10%). Analysis took into consideration MSC viability, proliferation, migration, metabolism, angiogenic activity and gene expression.

Figure 5: HIF1 and HIF2 mode of action. “HIF-1 drives the initial response to hypoxia while HIF-2 drives the chronic response to hypoxia” [85].

Table 1: Methods of induction of hypoxia with strengths and limitations

(*https://www.accessdata.fda.gov/drugsatfda_docs/nda/2004/076019_S000_DEFEROXAMI NE%20MESYLATE_PRNTLBL.pdf, accessed on 10/05/2018).

Method	Strengths	Limitations
Hypoxia induction using a hypoxic sub-chamber [19]	Cheap	Re-oxygenation periods during activities such as media change (unless done in hypoxia)
	Easy to operate	No oxygen monitoring (although some oxygen controllers are available)
	Efficient gas consumption	Size
	Transportable	Repeated re-flushing may be required to maintain low oxygen levels
		Gas leak could be a problem
		Size limitation and chamber to chamber variability
		Minimum of two air exposure events per week
Hypoxia induction using an hypoxic incubator [19]	Humidity, temperature, oxygen and CO ₂ levels of the incubator are monitored	Re-oxygenation of the incubator with every door opening
	Easy to operate	Re-oxygenation periods during activities such as microscopic examination
	Easier to incorporate controls /replicates into experimental plan	No oxygen monitoring when cells are out of the incubator.
	Reduced size constraints	The range of oxygen levels can be limited
		Gas consumption
		One air exposure event per week for single user
		Air exposure events per week for multi-users
Hypoxia induction using a hypoxic Glovebox or workstation [19]	No disruption to the hypoxic environment	Cost
	Robust oxygen, CO ₂ , humidity and temperature monitoring	Maintenance/servicing
	Better reflection of the in-vivo niche	Cleaning
	Workstation can be large enough to host all activities or equipment such as a microscope	Access to the chamber without disruption of the hypoxic environment
	Air exposure events per week	Gas consumption
Hypoxia induction using cobalt chloride	Cheap	Optimal dose and incubation time must be assessed
	Easy and accurate preparation	May cause cellular toxicity
	No need for oxygen monitoring	Intracellular accumulation with unknown effects
	Useful model to study hypoxia effects	Could be hazardous to user
Hypoxia induction using deferoxamine mesylate	FDA approved*	Optimal dose and incubation time must be assessed
	Utilised in clinical setting	May cause cytotoxic effects at longer exposure periods
	Cheap	
	Easy and accurate preparation	
	Not classified as hazardous	
	No need for oxygen monitoring	
	Useful model system	

Table 2: The consequence of hypoxia on hMSC cell surface phenotype. [Key to source of human Mesenchymal Stem Cells (hMSCs) column abbreviations: AD; Adipose tissue: BM; Bone Marrow: DP; Dental Pulp: WJ; Wharton's Jelly: UC; Umbilical Cord: UCB; Umbilical Cord Blood: Other sources written in full).

Effect of hypoxia	Hypoxia conditions (% O ₂ and duration)	Source of hMSC	Ref
Cell surface markers			
ADMSC cell surface markers unaltered	1%; 48h	AD	97
	1%; 48h	AD	98
	1%; 48h	AD	99
	2%; 7 days	AD	100
	2%; up to 21 days	AD	101
	5%; Early & late passage	AD	102
ADMSC cell surface markers slightly altered	5%; up to 14 days	AD	103
BMMSC cell surface markers unaltered	2 & 0.2%; 21 days	BM	19
	1%; up to 24h	BM	104
	<2%; 14 days	BM	105
	2%; 7-14 days	BM	106
	2%; 14 days	BM	26
	2%; up to 21 days	BM	107
	5%; up to passage 4	BM	108
BMMSC cell surface markers upregulated	1%; up to 90 days	BM	109
	2% & 5%; up to 21 days	BM	25
WJMSC cell surface markers unaltered	2-3%; up to passage 10	WJ	110

	5%; 14 days	WJ	111
UCBMSC cell surface markers unaltered	5%; 5 days	UCB	112

Table 3: The effect of hypoxia on hMSC multi-lineage differentiation. Hypoxia appears to assist in the maintenance of chondrogenic differentiation while it may improve or diminish osteogenic and adipogenic differentiation. [Key to source of human Mesenchymal Stem Cells (hMSCs) column abbreviations: AD; Adipose tissue: BM; Bone Marrow: DP; Dental Pulp: WJ; Wharton's Jelly: UC; Umbilical Cord: UCB; Umbilical Cord Blood: Other sources written in full).

Effect of hypoxia	Hypoxia conditions (% O ₂ and duration)	Source of hMSC	Ref
Differentiation potential			
Increased adipogenic differentiation	0.2%; 7-14d	BM	113
	1%; up to 3d	BM	114
	1%; up to 90 days	BM	109
	1%; 100d	BM	24
Decreased adipogenic differentiation	1%; 7 days	BM	115
	1%; 28 days	BM	116
	1%; 28 days	BM	117
	2%; 7-14 day	BM	106
	<2%; 14 days	BM	105
	3%; 28-31 days	BM	118
No significant effect on adipogenesis or osteogenesis	2%; 24h	BM	44
No significant effect on osteogenesis or adipogenesis	2%; 14 days	BM	26

Enhanced chondrogenic potential	1%; 100 days	BM	24
	2%; 14 days	BM	119
	2%; 42 days	BM	19
	2 & 5%; 21 days	BM	25
	3%; 21 days	BM	21
No significant effect on chondrogenesis	2% 7-14 day	BM	106
Enhanced osteogenic differentiation potential	1%; 28 days	BM	117
	1%; up to 90 days	BM	109
	2%; 3 day (preconditioning) then 21 days	BM	120
	<2%; 14 days	BM	105
Impaired osteogenic differentiation potential	0.2%; 7-14d	BM	113
	1%; 72h	BM	121
	1%; 7d	BM	115
	1%; 28d	BM	116
	2%; 21 days	BM	120
	2% & 5%; 21 days	BM	25
	3%; 28-31 days	BM	118
No significant effect on osteogenesis	1%; 100 days	BM	24
No significant effect on osteogenesis or chondrogenesis	2%; 7-14 day	BM	106
No effect on tri-lineage differentiation	1%; 48h	AD	122
	1%; 48h	AD	99
	1-3%; 21 days	AD	123
	2-3%; up to passage 10	WJ	110
Enhanced tri-lineage differentiation	5%; 21 days	AD	102

Table 4: The effect of hypoxia on hMSC attachment and migration. [Key to source of human Mesenchymal Stem Cells (hMSCs) column abbreviations: AD; Adipose tissue: BM; Bone Marrow: DP; Dental Pulp: WJ; Wharton's Jelly: UC; Umbilical Cord: UCB; Umbilical Cord Blood: Other sources written in full).

Effect of hypoxia	Hypoxia conditions (% O ₂ and duration)	Source of hMSC	Ref
Attachment and migration			
Higher migration rate	1-3%; 24h	BM	124
	1%; 7 days	BM	117
	2%; 72h	BM	107
	3%; 6h	BM	47
	5%; 4h	BM	125
	5%; up to 14 days	AD	103
	5%; 24h	DP	126
Increased expression of cell adhesion molecules	2%; 72h	BM	107
Increased motility and vascularisation and/or innervation of tissue	1-3%; 24h	BM	124

Table 5: The impact of hypoxia on hMSC proliferation and growth. [Key to source of human Mesenchymal Stem Cells (hMSCs) column abbreviations: AD; Adipose tissue: BM; Bone Marrow: DP; Dental Pulp: WJ; Wharton's Jelly: UC; Umbilical Cord: UCB; Umbilical Cord Blood: Other sources written in full).

Effect of hypoxia	Hypoxia conditions (% O ₂ and duration)	Source of hMSC	Ref
Growth and proliferation			
Enhanced MSC expansion	2%; 7 days	BM	127
	5%; up to passage 4	BM	108
	5%; up to 100 days	AD	102
Enhanced MSC expansion and proliferation	2%; up to 12 days	BM	26
Increased proliferation rate	1-3%; 72, 96 & 144h	AD	123
	1.5, 2.5 & 5%; 72h	UC	128
	1%; 7 days	BM	117
	1%; 7 days	AD	129
	1%; 2 passage (8 days)	BM	116
	1%; up to 84 days	BM	130
	2%; 24h	BM	44
	2%; 3-7 day	BM	127
	2%; up to 14 days	AD	100
	2%; up to 21 days	AD	101
	2-3%; up to passage 10	WJ	110
	5%; 5 days	UCB	112
	5%; up to 14 days	AD	103
	5%; up to passage 12	WJ	111
No effect proliferation	1-3%; 16h	BM	124
	1%; up to 33 days	BM	115
Higher population doublings	1%; 2 passage (8 days)	BM	116
	1%; up to 52 days	BM	115
	1%; up to 90 days	BM	109

	1%; up to 350 days	BM	24
	3%; up to 120 days	BM	118
	3%; up to passage 22	AD	11
	5%; up to 100 days	AD	102
	5%; up to passage 12	WJ	111
Increased number of CFU recovered	1%; 48h	AD	99
	1%; 14 days	BM	115
	2%; 14 day	BM	131
	3%; 14 day	BM	21
	5%; 14 days	BM	125
No significant alteration in CFU-F	2%; up to 21 days	AD	101
	2%; up to 21 days	BM	25
Decreased number of CFU-F	5% ; up to 21 days	BM	25

Table 6: The effect of hypoxia on hMSC genomic stability and expression. [Key to source of human Mesenchymal Stem Cells (hMSCs) column abbreviations: AD; Adipose tissue: BM; Bone Marrow: DP; Dental Pulp: WJ; Wharton's Jelly: UC; Umbilical Cord: UCB; Umbilical Cord Blood: Other sources written in full).

Effect of hypoxia	Hypoxia conditions (% O ₂ and duration)	Source of hMSC	Ref
Genomic stability and profiling			
Enhancement of a genetic profile that maintains the cells undifferentiated and multipotent	1%; up to 7 days	BM	117
	1%; up to 90 days	BM	109
	5%; 14d	BM	22

	5%; 24h	DP	126
Partial genetic profile that maintains the cells undifferentiated	5%; 24h	DP	126
Telomere maintenance	1%; up to 100 population doublings	BM	130
	2%; up to 21 days	AD	101
	2%; up to passage 5	AD	132
	2%; up to passage 5	BM	132
	3%; up to passage 15	AD	11
Reduced DNA damage	2%; up to 21 days	AD	101
Chromosomal variability and instability	5%; up to passage 7	BM	133
Reduced expression of tumor suppressing genes p16, p21, p53 and pRb	2%; up to 21 days	AD	101

Table 7: The influence of hypoxia on hMSC paracrine activity. [Key to source of human Mesenchymal Stem Cells (hMSCs) column abbreviations: AD; Adipose tissue; BM; Bone Marrow; DP; Dental Pulp; WJ; Wharton's Jelly; UC; Umbilical Cord; UCB; Umbilical Cord Blood; Other sources written in full).

Effect of hypoxia	Hypoxia conditions (% O ₂ and duration)	Source of hMSC	Ref
Paracrine activity			
Increased release of growth factors	1%; 24h	BM	134
	1%; 48h	AD	122
	1%; 48h	AD	99
	1%; 7 days	BM	117
	1%; 7 days	BM	115
	1%; 7 days	AD	129

	2%; up to 21 days	AD	101
	3%; 16h	BM	47
	3 & 5%; up to passage 5	DP	126
Increased release of chemotactic and angiogenic mediators	0.1%; 14 days	BM	135
Decreased release of chemotactic and angiogenic mediators	5%; 14 days	BM	135
Increased release of inflammatory/immunomodulatory mediators	5%; up to passage 5	DP	126
Altered microRNA (miRNA) release	0-1%; 12-24h	BM	136
	2%; 21 days	BM	137
Increased motility and vascularisation and/or innervation of tissue	1-3%; 24h	BM	124
Increased and altered activity of ERK pathway	1%; up to 24h	BM	104
	1%; up to 24h	AD	129
Increased release of angiogenic mediators (altered secretome profile)	1%; 48h	AD	98

Table 8: The effect of chemical hypoxia, mediated by CoCl₂ and DFO, on the “hMSC checklist” parameters.

Effect of hypoxia	Chemical Concentration and duration	Source of MSC	Ref
Cell morphology			
Altered cell morphology	CoCl ₂ : 100 µM for 4 days	UC	146
	DFO: 120µM for 4 days	UC	146
Unaltered cell morphology	DFO: 150µM or 400µM for 48h	AD	147

Growth and proliferation			
No effect on viability	CoCl ₂ : 100μM for 24h	Abdominal aortic aneurysm	148
Limited proliferation	CoCl ₂ : 50μM or 100μM up to 72h	UC	149
	DFO: 150μM or 400μM for 48h	AD	147
Decreased proliferation	CoCl ₂ : up to 100 μM up to 96h	UC	146
	CoCl ₂ : 500μM for up to 72h	Abdominal aortic aneurysm	148
	DFO: up to 120μM up to 96h	UC	146
Differentiation potential			
Enhanced osteogenic differentiation potential	CoCl ₂ : 50μM or 100μM for 10 days	UC	149
	CoCl ₂ : 100μM up to 21 days	UC	150
Decreased osteogenic differentiation potential	CoCl ₂ : 100μM for 7 days	BM	151
	CoCl ₂ : 100μM up to 21 days	AD, DP	150
Attachment and Migration			
Higher migration rate	DFO: 120μM for 24h	AD	152
	DFO: 120μM for 24h	BM	153
Increased expression of CXCR4	CoCl ₂ : 100μM for 24h	AD	152
	DFO: 120μM for 24h	AD	152
	DFO: 120μM for 24h	BM	153
Unaltered CXCR4 and CXCR7 expression	CoCl ₂ : 100μM up to 24h	BM	153

Paracrine activity			
Increased release of angiogenic mediators	CoCl ₂ : 50,100 or 150μM for 7 days	Periosteum-derived	154
	DFO: 150μM or 400μM for 48h	AD	147
	DFO: 15μM , 50μM or 100μM up to 5 days	BM	155
	DFO: up to 120μM up to 7days	AD	156
HIF-1α regulation			
Up-regulation of HIF-1α	CoCl ₂ : 250μM up to 24h	BM	136
	CoCl ₂ : 50 or 100μM for 48h	BM	151
	CoCl ₂ : 100μM for 6 to 48h	DP, UC	150
	CoCl ₂ : 100μM for 72h	BM	157
	DFO: up to 120μM for 12h	AD	156
	DFO: 150μM or 400μM for 48h	AD	147
No change in HIF-1α	CoCl ₂ : 100μM for 6 to 48h	AD	150

Table 9: Genes that are reported to be regulated by HIFs in human MSCs (* designates a mixed species example (human/mouse)).

	Reference
Up regulated	

VEGF	101, 115, 138, 139
HGF	115
FGF	100, 115
MIF	140
CHIP, Sox9	138
CFD, Leptin, LPL, PGAR	113
Wnt10b	116
G6PT	141*
HK1, HK2, PKM1, PDK1	142
TWIST	121
CXCL12	143
3BP2, MT1-MMP	
Down regulated	
E2A, p21	130
RUNX2	120, 145
PDK2	142