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Hypoxia and Stem Cell-Based Engineering of Mesenchymal Tissues

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Abstract

Stem cells have the ability for prolonged self-renewal and differentiation into mature cells of various lineages, which makes them important cell sources for tissue engineering applications. Their remarkable ability to replenish and differentiate in vivo is regulated by both intrinsic and extrinsic cellular mechanisms. The anatomical location where the stem cells reside, known as the "stem cell niche or microenvironment," provides signals conducive to the maintenance of definitive stem cell properties. Physiological condition including oxygen tension is an important component of the stem cell microenvironment and has been shown to play a role in regulating both embryonic and adult stem cells. This review focuses on oxygen as a signaling molecule and the way it regulates the stem cells' development into mesenchymal tissues in vitro. The physiological relevance of low oxygen tension as an environmental parameter that uniquely benefits stem cells' expansion and maintenance is described along with recent findings on the regulatory effects of oxygen on embryonic stem cells and adult mesenchymal stem cells. The relevance to tissue engineering is discussed in the context of the need to specifically regulate the oxygen content in the cellular microenvironment in order to optimize in vitro tissue development.

Keywords

embryonic stem cells; adult stem cells; mesenchymal stem cells; oxygen tension; hypoxia; 3D

Introduction

Stem cells are currently used in clinical applications to augment the healing of orthopedic tissue defects. Their applicability to multiple other therapeutic situations has also been investigated. One approach to eliciting the therapeutic benefits of stem cells is to inject them into the defect site in suspension or in a delivery gel.[1] However, the therapeutic potential of stem cells may

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best be realized via tissue-engineering approaches to develop biological tissue substitutes which, through in vitro cultivation, can be functional at the time of implantation. The use of stem cell-based approaches in combination with scaffolds and bioreactor systems has resulted in engineered tissues of various mesenchymal lineages including bone,[2,3] cartilage,[4] fat,[5] and ligament,[6] among others.

Stem cells produce all multicellular tissues in the body through proliferation and differentiation in tightly controlled in vivo environments. Because of their plasticity, they are particularly sensitive to their immediate environments. In vivo they are thought to reside in specific "niches," which maintain their pluripotent or multipotent capabilities. Consequently, differentiation along specific lineages is thought to coincide with their migration out of their specific niche into an environment that provides appropriate differentiation cues.[7] The in vitro conversion of unspecialized cells to immature, but functional tissues depends on establishing the cellular microenvironment, regulated so that key in vivo stimuli, which guide cellular organization and development, are recapitulated.[8] As the field of tissue engineering has matured, new technology has been employed to regulate the application of mechanical as well as biological factors to developing tissue constructs. However, one developmentally important stimulus that is still rarely accounted for during in vitro culture is the oxygen tension. The role of oxygen as a metabolic substrate for cells in three-dimensional (3D) organization has now been investigated extensively[9-12] and others have reviewed techniques utilized to mitigate mass transport limitations and avoidance of anoxic regions and steep oxygen gradients in thick 3D tissue constructs.[13,14] However, much less studied (but gaining increasing attention) has been of the utilization of oxygen in its role as a signaling molecule that influences stem cell survival, proliferation, and differentiation in culture.

The effect of oxygen tension on stem cell physiology has been studied for over 30 years beginning with the haematopoietic system.[15-17] For haematopoietic stem cells (HSCs), it has been found that cultivation under low oxygen tensions maintained a significantly higher number of long-term colony initiating cells (LTC-ICs) relative to cultures under ambient (20%, v/v) oxygen concentrations.[18-20] Recently, it has also been seen for several other stem and progenitor cell populations that cultivation under hypoxic conditions resulted in enhanced proliferation and maintenance of their naïve states.[21] In vivo studies have shown that mesenchymal stem and progenitor cells home specifically to hypoxic events and function as therapeutic agents, enabling limited regeneration to damaged tissues.[22-24] In particular, the stem cells have demonstrated the potential to organize themselves into vascular structures as well as secrete angiogenic growth-factors in response to hypoxic challenges. Several in vitro studies have also shown that both bone marrow- and adipose-derived stem cells upregulate VEGF expression during hypoxia,[25-28] which along with other factors, may be responsible for the "cyto-protective" effects on neighboring cells.[26-29] Although the identity of cellular oxygen sensor is being debated,[30,31] emerging evidence indicate that some of the effects of hypoxia on stem cell function are directly regulated by hypoxia-inducible factor (HIF) proteins. The role of HIFs in regulating stem cells' response to hypoxia has been recently reviewed by Keith and Simon[32] and is not included in this review.

This review focuses on the role of oxygen tension on the stem cells' development into mesenchymal tissues in vitro. As a result, oxygen's influence as a signaling molecule (rather than metabolic substrate) on the proliferation, differentiation, and tissue development is discussed. Although there have been studies on numerous types of stem and progenitor cells, we discuss only embryonic stem cells (ESCs) and adult mesenchymal stem cells (MSCs) derived either from the bone marrow or adipose tissues in terms of their expansion and terminal differentiation into tissues of mesenchymal lineage. Cells from various mammalian species are included as they share many properties. The physiological relevance of low oxygen tension as an environmental parameter that uniquely benefits stem cells' expansion and maintenance is

described. This provides a context for reviewing the results of in vitro studies of stem cells cultivated under hypoxic conditions. Finally, we discuss the impact of these findings on tissue engineering approaches and the need to specifically regulate the oxygen content of the cellular microenvironment in order to optimize in vitro tissue development is discussed in the last section. It is noted that hypoxia refers to the condition when oxygen tension is below physiological level but is used in this review to describe O_2 lower than 21% for consistency with conventional terminology.

Physiological Basis of Hypoxic Environments

In vivo microenvironments

Stem cells' unique ability to replenish themselves during the development, maintenance of tissue homeostasis, and repair of many tissues through self-renewal and differentiation are regulated by both intrinsic programming and input from their local environment, often referred to as the "stem cell niche or microenvironment."[7,33] As a basic unit of tissue architecture, the stem cell microenvironment constitutes specific molecular, cellular, and physiological components and is also subject to physical and mechanical stimuli. Replicating the essential components of the stem cell in vivo microenvironment has become an important approach in stem cell tissue engineering to develop functional constructs. Although stem cells can reside in markedly different local microenvironment and have distinctly different developmental paths, low oxygen tension seems to be a common in vivo feature shared by embryonic stem cells and many types of adult stem cells at early stages of development. Indeed, increasing evidence demonstrates that oxygen tension is not only a metabolic substrate but also a powerful signaling molecule that regulates stem cell proliferation and differentiation. In recent years, the requirements for replicating stem cell microenvironment have evolved from the initial effort of supplying sufficient oxygen to support tissue growth to understanding and utilizing oxygen in its more complex signaling role to regulate migration, differentiation, and development. The physiological basis for this is discussed in the context of the in vivo cellular microenvironment:

Embryonic microenvironment

Oxygen tension in the mammalian reproductive tract is in the range of 1.5–8%.[34] For example, the measured oviductal oxygen tension in rhesus monkeys was about 8%, whereas the intrauterine oxygen tension was only about 2%.[34] It is to this low oxygen environment that naturally conceived embryos are exposed during the early stages of development (Figure 1A). After implantation of embryos into the uterine wall, the trophoblast shell that surrounds the embryo excludes oxygenated maternal blood and forms a hypoxic environment until the onset of vascularization. At 8–10 weeks of gestation, the mean oxygen tensions at the uterine surface and within the endometrium were estimated to be ~2.3% and 5.2%, respectively.[35] The role of oxygen tension in modulating proliferation and differentiation of human placenta cells has been demonstrated using placental organ culture systems, in which 2% O_2 induces cytotrophoblast mitosis and 20% O_2 induces cell cycle arrest and differentiation.[36] The low oxygen environment at the range of 4–5% O_2 lasts throughout the fetal developmental process even after access to the maternal vasculature (normal pO_2 at ~10 and ~13% in venous and arterial, respectively) with dissolved O_2 in fetal circulation rarely exceeding 5%.[35] Human embryonic stem cells that are derived from the inner cell mass of early stage blastocytes adapt to grow in this low oxygen environment until about the 11th week when the cytotrophoblasts traverse the arterial walls and the local partial pressure of oxygen increases from ~2.3% to 7.8% O_2.[35] Thus, low oxygen environment is physiologic for most mammalian embryos, and ambient oxygen tension of 20% is in fact a "nonphysiological" condition to which the embryo is never exposed.

Bone marrow microenvironment

The oxygen distribution within the bone marrow has been studied almost exclusively in the context of hematopoietic stem cells (HSCs) and to a much lesser extent in the context of MSCs. However, bone marrow has a hierarchical structure, in which the haematopoietic compartments are bound by stromal elements[37]—mainly mesenchymal stromal or stem cells (MSCs)—and such that the two cell types form an integral part of each other's niche (Figure 1B). The oxygen concentrations in the MSCs' in vivo microenvironment may therefore be discussed in terms of what is known for HSCs. It has long been proposed that hematopoietic progenitors exist at high concentration at the endosteal surface and release via the central venous sinus as they differentiate and mature.[38] Although the importance of understanding the spatial progenitor cell distribution with respect to oxygen supply from blood vessel has long been recognized, direct, noninvasive in vivo measurement of spatial oxygen gradient in bone marrow has been a major technical hurdle. Early direct measurement revealed that bone marrow in general is hypoxic, where some regions are as low as ~1–2% O_2.[39,40] Results from recent in vivo studies, however, provided direct experimental evidence that long-term repopulating HSCs in the mouse reside in a hypoxic environment[41] and hypoxia may in fact be an essential part of the microenvironment that maintains them in an undifferentiated state. These measurements were further supported by modeling analysis of in vivo oxygen distribution in bone marrow indicating that HSCs exist within an extremely hypoxic region within bone marrow.[42] Results from in vitro studies of HSCs under hypoxic conditions strengthen this hypothesis: At 5% O_2, there is enhanced production of erythroid, megakaryocytic, and granulocyticmonocytic progenitors[18,43,44] and substantial increases in the number and frequency of colony-forming cells.[45]

Adipose stem cell microenvironment

Adipose tissue is readily available through the high abundance of elective surgeries for fat removal. This tissue is typically highly vascularized and is a rich source of adipose-derived mesenchymal stem cells (ADSCs) having the potential to differentiate into adipogenic, osteogenic, chondrogenic, myogenic, endothelial, hepatic, and neuronal lineages.[46,47] ADSCs are typically harvested from the stromal vascular fraction of lipo-aspirated tissues[47,48]; however, the position of ADSCs relative to other cell types (adipocytes, preadipocytes, etc.) and structures (blood vessels) is still unknown. Despite the high degree of vascularity, in vivo measurements of oxygen concentrations in mouse adipose tissues have shown the oxygen concentration to be in the vicinity of 3%[49] and so hypoxic cultures may still be beneficial to ADSC in vitro proliferation and differentiation characteristics. However, studies investigating the role of reduced oxygen tension on ADSC in vitro characteristics have not reported increased maintenance of an undifferentiated state or drastic changes in proliferation in an analogous manner with what has been reported with ESCs and bone marrow-derived MSCs, but hypoxia has been shown to affect the ADSC differentiation potential as discussed below.

Hypoxia Influences Stem Cells' In Vitro Characteristics

Embryonic stem cells

The exciting potential of embryonic stem cells (ESCs) in tissue repair and regeneration depends on the ability to maintain their pluripotency during expansion in culture and to direct their differentiation under controlled conditions. Recapitulating the physiological conditions that regulate these cellular events in vivo has emerged as an important criterion to achieve this goal. One challenge inherent in ESC culture is their tendency to differentiate spontaneously. Low oxygen tension has been explored as a strategy to maintain human ESC pluripotency and minimize spontaneous differentiation.[50-52]

Low O_2 environments have long been used by embryologists to culture embryos[53,54] and the blastocytes produced under low O_2 have significantly more inner cell mass compared with those generated under higher O_2.[55] The benefits have been demonstrated for rabbit,[56] mouse,[57] sheep,[53] cow,[53] and human[58] embryos. Physiological oxygen tension of 5% instead of 20% was also found to improve the establishment of mouse embryonic stem cell line by reducing oxidative stress.[59] A low oxygen tension of 2% resulted in enhanced human ESC (hESC) clonal recovery and reduced chromosomal abnormalities without inducing hESCs to a more differentiated phenotype.[60] When maintained at 1 or 3% O_2, hESCs showed less signs of overt morphological differentiation than the normoxic colonies (Figures 2A–D) and produced lower amounts of chorionic gonadotrophin (hCG) and progesterone.[50] The hypoxic condition also enhanced hESC formation of embryoid bodies and did not prevent subsequent differentiation of hESCs when they were exposed to biochemical cues. Manipulating oxygen tension has also been shown to direct ESC differentiation.[62] Systemic studies are required to provide in-depth understanding of the dynamic interactions between oxygen tension and ESC fate and to facilitate the development of robust stem cell expansion strategies.

Bone marrow-derived mesenchymal stem cells

Low oxygen tension studies have been carried out with mesenchymal stem cells (MSCs) from a variety of mammalian species. In general, MSCs exhibited greater colony-forming potential,[63,64] proliferated faster,[65,66] and longer,[64,61,67,68] and maintained their undifferentiated characteristics better under low oxygen conditions.[61,69] Our group demonstrated that hMSCs grown in 3D scaffolds under extended hypoxic conditions (2% O_2) increased their expression of pluripotent genes Oct-4 and Rex-1 and had elevated CFU-F ability while maintaining their ability to differentiate along osteogenic or adipogenic lineages.[64] In two-dimensional (2D) culture studies, hMSCs under hypoxic conditions exhibited 30-fold greater expansion potential over a 6-week period than normoxic cells, homogenously maintained their spindle morphology, and formed multiple cell-layers with high expression of connexin-43[61] (Figures 2E–H). Hypoxia (3% O_2) has also been used to isolate a population of marrow-isolated adult multilineage inducible (MIAMI) cells from human bone marrow with pluripotent characteristics.[70] Hypoxia affects the differentiation characteristics of MSCs in ways that may be correlated with the physiological oxygen requirements of the differentiated cells (e.g., chondrocytes in avascular cartilage have low oxygen requirements while osteoblasts in vascular bone require 20% O_2 for optimal differentiation). However, it is very difficult to arrive at a consensus even after collating and analyzing the results of published studies: Differences in cell isolation methods, experimental parameters, growth factors, oxygen tensions, and specific evaluation techniques highlight the challenges in determining the role of oxygen in stem cell differentiation. Therefore, it has been reported that hypoxia enhanced the in vitro and in vivo bone-forming potential of rat MSCs[63] but inhibited the in vitro osteogenic potential of MIAMI cells[69] and induced down-regulation of osteoblastic genes in hMSCs in vitro.[25] Likewise, late-passage murine MSCs grown in Matrigel under 2% O_2 tension exhibited increased angiogenic properties and formed tube-like structures[71] while immortalized murine mesenchymal cells incubated in 1% oxygen expressed increased levels of chondrogenic markers Sox-9, aggrecan, and Col IIa.[72] Other studies involving hMSC have found that hypoxia (1% O_2) induced lipid-droplet formation within 1 day of exposure to a hypoxic atmosphere but without actually upregulating the expression of adipocytic genes.[73] Table 1 is provided to give a more detailed (though not exhaustive) summary of the findings for the effects of in vitro hypoxia on the stem cells of interest.

Adipose-derived stem cells

Much less investigations have been carried out concerning the effects of hypoxia on adipose-derived stem cells. The first published study done by Wang et al.[74] on human ADSCs found that when they were grown in alginate beads at 5% O_2 there was no effect on their proliferation

when cultured in normal expansion medium, but that they had lower proliferation rates at 5% O_2 compared with 20% O_2 when cultured in chondrogenic medium. However, under these conditions they exhibited enhanced chondrogenic differentiation markers including collagen II, glucosaminoglycan, and chondroitin-4-sulfate production.[74] The influence of hypoxia on ADSCs is strongly dependent on cultivation conditions. For this reason, there are conflicting reports in the literature regarding chondrogenic gene expression during induction under hypoxic conditions.[76,77] In other studies of human ADSCs, it was found that hypoxia also affected their potential to differentiate along muscle lineages.[75] The effect of hypoxia on the differentiation potential of ADSCs was also reported for murine cells. It was found that murine ADSCs exhibited decreased chondrogenic and osteogenic potential when differentiated at 2% O_2 compared with normoxic conditions.[78] However, a subsequent study by this group reported that if the murine ADSCs were first expanded at 2% O_2 and then differentiated under normoxic conditions to either osteoblasts or chondrocytes, they still exhibited decreased osteogenesis, but increased their expression of chondrogenic markers relative to cells that were both expanded and differentiated under normoxic conditions.[79] This occurred even if cells were reoxygenated and cultured at 21% O_2 prior to chondrogenic differentiation, suggesting that hypoxic culture may have selected specifically for chondroprogenitors. The interpretation of the combined results is complicated by other studies from the same group demonstrating that HIF-1α deletion inhibited chondrogenesis in murine ADSCs[80] suggesting that upregulation of HIF-1α under hypoxic conditions should in theory enhance chondrogenic differentiation. In combination, these studies underscore the importance of understanding the effects of oxygen signaling in the context of the culture conditions and other growth factors present.

Optimizing Oxygen Tension for Stem Cell-Based Tissue Engineering

Oxygen consumption characteristics may be distinct in 2D vs. 3D

The creation of 3D functional tissue constructs using stem cells and 3D scaffolds depends on stem cells' innate ability to proliferate and differentiate in response to environmental cues. Hence, these processes are strongly influenced by the in vitro cultivation conditions and the scaffold properties for stem cell maintenance or differentiation. The localized physiological microenvironment is determined by the interplay between supply and demand. Oxygen supply to cells in 3D constructs is more complex than that of 2D cultures as it is influenced by the presence of scaffolding materials and extracellular substances as well as by the mechanism(s) of transport, i.e., diffusion or convection. To this end, modeling has served as a powerful tool to provide insight into the oxygen profile of the in vivo tissue and the 3D constructs (see a recent review by Sengers et al.[81]). Sophisticated modeling approaches have been developed to account not only for matrix formation and cell proliferation and differentiation but also variations of scaffold geometry and culture environment.

An important barrier to estimating the oxygen profile in the 3D stem cell constructs, however, is the reliability of input data—particularly, the rates of oxygen consumption at various developmental stages. The Michaelis-Menten-like constitutive equations for oxygen consumption are based on the assumption that the rate constants are independent of time and material, and the variation of oxygen profiles are primarily the results of cell density.[82,83] However, these assumptions are being challenged. Recent studies have shown that there may be a strong dependence of cellular metabolic activity and oxygen consumption rate on the scaffolding material and geometry, presumably due to the altered cell-material interactions in the scaffold.[84,85] In fact, the changes in regulatory effects of 3D cellular organization on the morphology, gene expression, growth, morphogenesis, and differentiation of cells are being established, and the signaling mechanisms are being identified.[86,87] The role of 3D scaffolds may be more pronounced for the stem cells that not only exhibit plasticity but also display an extensive ability to modulate their microenvironment. For example, hMSCs in 3D scaffolds developed an extensive ECM network and changed their integrin profile and cell adhesion

apparatus compared with 2D counterparts.[88] Thus, the role of oxygen tension in stem cell development needs to be addressed in the context of the 3D microenvironment that includes matrix properties, cell–cell and cell-matrix interactions, distribution of growth factors as well as the local physiological and biomechanical properties.

It is important to not only recognize the dynamic interplay between the developing stem cell microenvironment in the 3D construct and the biosynthetic activity that actively modulates the microenvironment, but also the varying developmental requirements over an extended period. For example, per cell oxygen demand decreased over a 40-day period when hMSC were grown in a perfusion bioreactor due to the variation in cellular microenvironment.[83] As a result, oxygen depletion that would otherwise occur due to high cell density was not observed at the later stage of the construct development. These results highlight the importance of obtaining temporal and spatial measurements of oxygen concentration in developing constructs in order to validate oxygen distribution profiles. Furthermore, these findings underscore the complexity associated with regulating the microenvironment and the need to further integrate experimental-computational approaches to understand the developmental dynamics and to identify the key regulatory mechanisms.

Regulating O_2 tension in developing tissue constructs

As a result of stem cells' natural adaptation to the “hypoxic conditions” and sensitivity to ambient oxygen concentrations, there is a need to carefully consider the role of oxygen in the microenvironment of stem cell-based tissue-engineered grafts. In contrast to cultures of terminally differentiated cellular phenotypes, maintaining hypoxic regions throughout stem cell-based constructs may be desirable if it allows cells to proliferate longer and give rise to constructs with higher cell densities. Furthermore, hypoxia appears to either support or “select” a more primitive population of cells, which maintain the potential to be directed along various lineage pathways.[62,64] Directing subsequent stem cell differentiation along particular lineage pathways may require modulation of the prevailing oxygen concentrations in conjunction with providing signaling stimuli from appropriate growth factors.

Given the strong dependence of stem cell behavior on oxygen tension, it is conceivable to employ a strategy of varying oxygen tension over the course of construct development to stimulate rapid cell proliferation and direct functional differentiation. Engineering bone and cartilage tissues for example may require distinctly different conditions of oxygen tension for optimal differentiation into either lineage. Empirical clues to validate this may even be based on the physiological example of endochondral ossification (Figure 3). During this process, undifferentiated mesenchymal cells condense and undergo chondrogenic differentiation in hypoxic, avascular environments. Similar conditions may stimulate hMSC chondrocytic differentiation within 3D scaffolds or gels: both human chondrocytes and ADSCs[74] maintained in chondrogenic media at 5% O_2 exhibited improved chondrogenic gene expression and phenotypic characteristics. However, subsequent endochondral bone formation is preceded by vascular invasion with a consequent increase in oxygen concentrations. In vitro bone formation also appears to require higher oxygen tensions to stimulate proper osteoblast differentiation and function.

Another scenario of varying oxygen tension to aid construct development is in the creation of vascularized tissue constructs. One approach to vascularize the implanted construct is to allow the native blood vessels to infiltrate the construct and establish a vascular network.[90] Although the delivery of multiple growth factors showed promising results,[91] controlled release at physiological dosage remains a major technical hurdle for regenerating clinically relevant thick constructs. MSCs are a known source of angiogenic factors that promote blood vessel development. A recent in vitro study has shown that the conditioned media from hypoxic MSCs are more effective than normoxic MSCs in inhibiting endothelial cell apoptosis, increasing

their survival, and enhancing tube formation.[92] Thus, a strategically controlled oxygen gradient in the 3D construct may potentiate MSC angiogenic activity and enable MSC to function as a factory of growth factors that promote blood vessel invasion and construct vascularization. These strategies, however, depend on the precise knowledge of oxygen demand and the ability to regulate oxygen microenvironment in 3D constructs.

Technological requirements for regulating the in vitro oxygen microenvironments

New technologies are being developed to directly measure local oxygen concentration in 3D constructs. An optical fiber-based oxygen sensing system was developed for in situ, real-time oxygen monitoring for human dermal fibroblasts in 3D collagen constructs.[93] Needle microelectrodes have been used to monitor local O_2 and cellular activity in several tissues and have potential for oxygen monitoring in regenerating tissue.[94] A novel microsystem was also developed that was able to generate and impose 1D or 2D oxidative gradients on tissue in culture.[95] Although these technologies are important in controlling in vitro oxygen microenvironment, challenges remain in monitoring oxygen tension in the developing 3D constructs with high spatial resolution over the course of construct development. To this end, noninvasive imaging technology allows repetitious measurement in both in vitro and in vivo settings and offers many advantages. Local oxygen tension in the vicinity of implants was measured by EPR oximetry using the microparticles of an acta-*n*-butoxy derivative of naphthalocyanine neural radicals as implantable oxygen sensors. The oxygen sensor microparticular compounds can be implanted in the extravascular space or internalized by cells and thereby provide high accuracy, noninvasive local oxygen measurement of the implants for up to 10 weeks.[96] The ability to measure local oxygen tension also eliminates the disparity between ambient and pericellular oxygen tension that has been reported.[73] Significant disparities exist between ambient and pericellular oxygen concentrations, thus increasing oxygen concentrations by 3% (say from 2–5% O_2) does not correspond to a 3% increase in the oxygen concentrations in the cellular region. The use of bioreactors may be of considerable interest in overcoming these challenges. Innovations in bioreactor design that address mass transfer limitations can be extended to improve control of oxygen profiles in the 3D constructs. The effort to determine the optimal mass transfer conditions for the developing 3D constructs can also be supported by simulation methods. Several recent reviews have summarized the development of bioreactors for stem cell expansion[97] and modeling of tissue regeneration in 3D scaffolds.[81]

Summary

Oxygen concentration is an important component of stem cell “niche,” where it plays a fundamental role in maintaining the stem cells' proliferation and plasticity. Recapitulating the critical environmental components that control stem cell proliferation and differentiation in 3D constructs is therefore an important aspect of stem cell-based tissue engineering strategies. Recent progress has provided invaluable insight into the regulatory mechanisms of oxygen tension on stem cells' phenotype. Given the sensitivity of stem cells to their physiological microenvironment, it is imperative to evaluate the role of oxygen tension (and oxygen delivery strategies) in developing stem cell-based 3D constructs in the context of its roles as a signaling molecule as well as a metabolic substrate. Future challenges include getting more insight into the oxygen-related complex cell behavior and the interactions between the cells and their evolving environment, through integrating experimental and modeling approaches. Novel 3D culture systems coupled with advanced monitoring technology will help uncover the dynamic interplay between the stem cells in the developing tissue constructs and their microenvironment. Computational modeling is expected to help delineate the contributions of various factors and to identify the key regulatory mechanisms. Integrated experimental and modeling approach will be necessary for the optimization of tissue engineering protocols.

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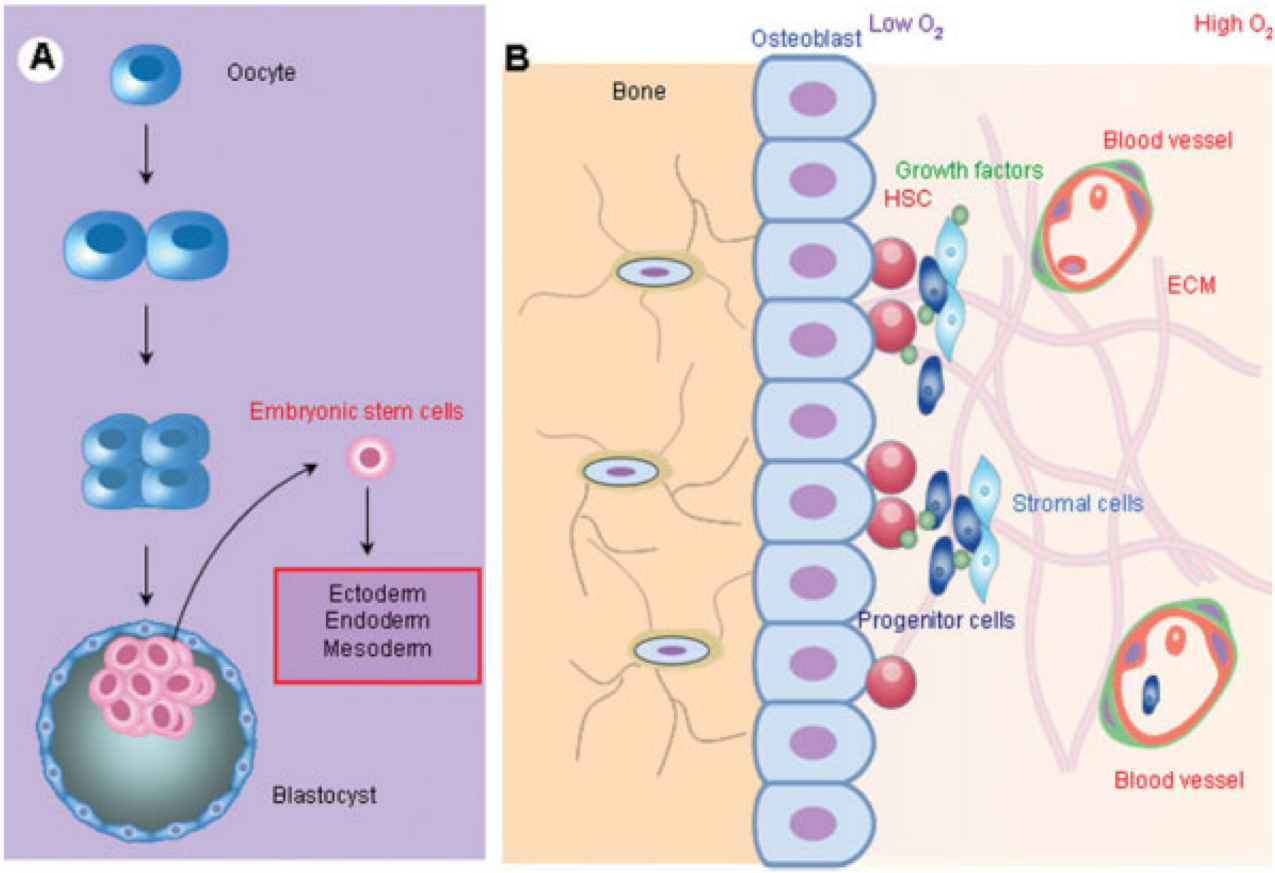

Figure 1. Oxygen tensions in stem cell microenvironments

Embryonic stem cells and adult bone marrow-derived stem cells reside in low oxygen environments. (A) Embryonic stem cells are derived from the inner cell mass of early stage blastocytsts and maintain a totipotent state that forms ectoderm, endoderm, and mesoderm. The intrauterine environment in which the embryonic stem cells growth and development occur has an oxygen tension ranging from 1–5% through the first trimester. (B) Oxygen tension in bone marrow is well below ambient oxygen environment in the range of 3–7% O_2 with oxygen concentrations decreasing with distance away from sinuses. Recent in vivo studies provided direct experimental evidence that HSCs are distributed predominantly at the lowest end of an oxygen gradient within the bone marrow and away from sinuses.

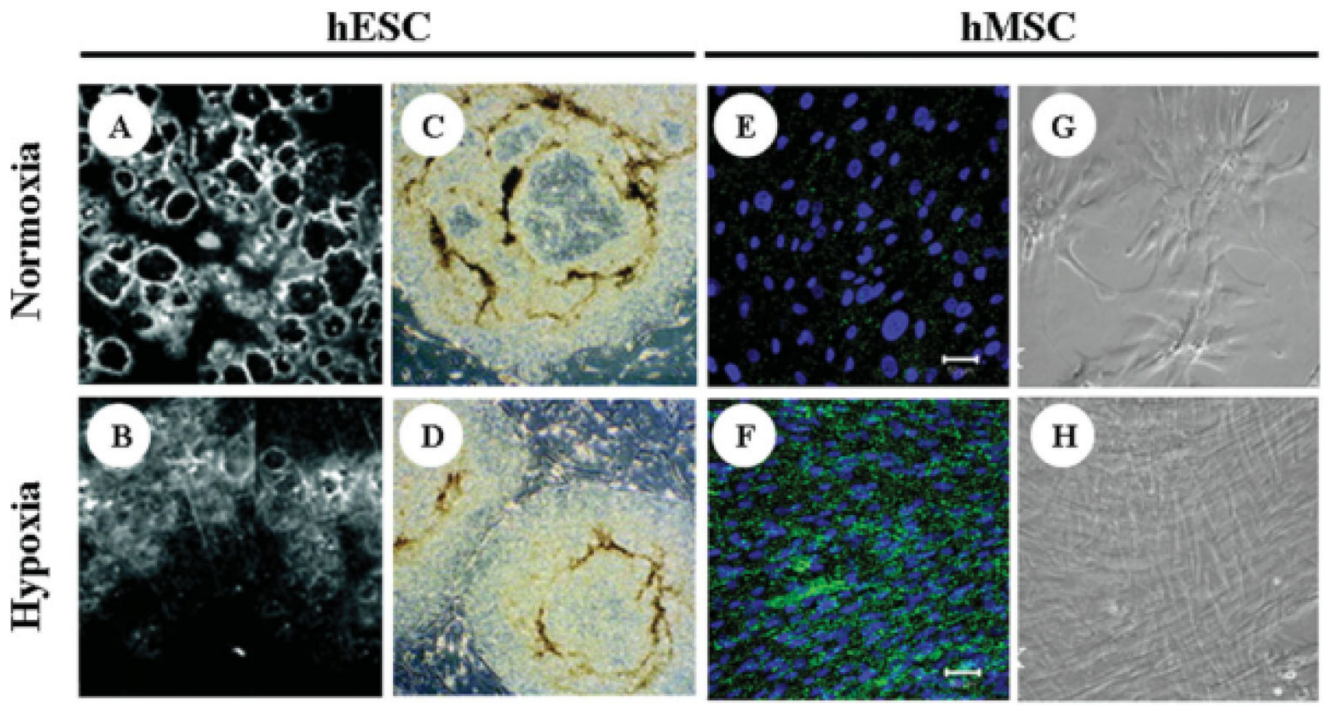

Figure 2. Effect of hypoxia on in vitro stem cell phenotype

(A and B) Human ESCs (H1) cultured on MEF feeder layers under normoxic (21% O_2) and hypoxic (5% O_2) conditions. Normoxic cells display extensive regions of differentiation (dark circles surrounded by bright rings) compared with hypoxic cultures. (C and D) Further comparison of H1 hESCs grown at 21% O_2 and 3% O_2, respectively, showing fewer differentiated (darker, nonuniform) regions under hypoxic conditions. Images adapted from Ref. 50, with permission from National Academy of Sciences. (E–H) Comparisons of bone marrow-derived hMSCs grown under normoxic (21% O_2) or hypoxic (2% O_2) conditions: (E and F) Significantly higher connexin-43 expression is observed in hypoxic hMSCs grown in monolayer culture for 11 days using expansion medium. (G and H) Phase contrast images demonstrate the maintenance of uniform spindle morphology at late stages of passage under hypoxic conditions. Images adapted from Ref. 61, with permission from Academic Press.

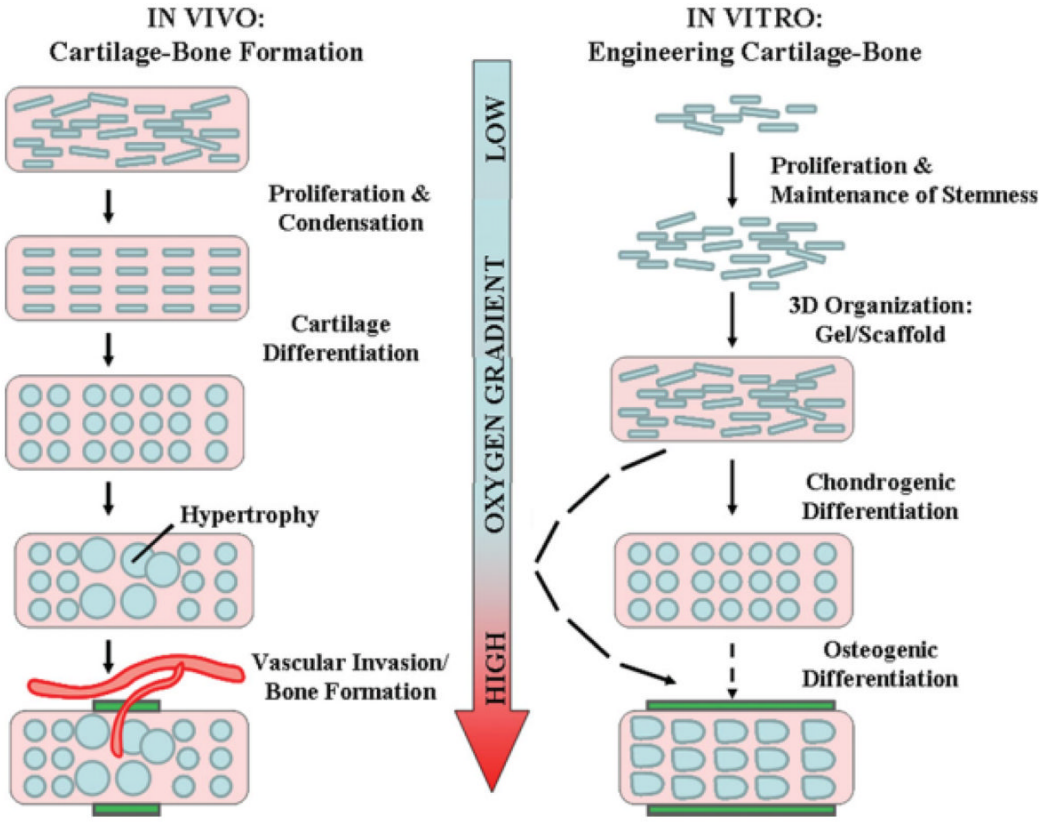

Figure 3. Learning from nature: The influence of oxygen tension during bone-cartilage tissue formation

In vivo: During development, cartilage forms via the processes of mesenchymal condensation and carefully regulated chondrogenic differentiation in very hypoxic environments. Long bone formation occurs via endochondral ossification where hypertrophic chondrocytes upregulate angiogenic genes triggering vascular invasion (with consequent increases in oxygen tension) followed by new bone formation (green rectangles). Images adapted from Ref. 89, with permission from Nature Publishing Group. In vitro: Hypoxia enables the expansion of stem cells while maintaining their undifferentiated states. Cells are then seeded into 3D organizations to facilitate functional tissue development. Chondrogenic differentiation is enhanced under hypoxic conditions relative to 20% O_2. Differentiation into osteogenic lineage and subsequent bone formation (green rectangles) in vitro is optimized at ambient (20%) O_2 tensions and inhibited under hypoxic conditions.

Table 1

Effect of Hypoxia on Proliferation and Differentiation Properties of ESC, hMSC, and ADSC

Cell Type	Species	Oxygen Tension	Cultivation Conditions	Expansion (compared to 20% O_2)	Differentiation (compared to 20% O_2)	Reference
ESC	Human	1%, 3%, 5%	Monolayer culture of ES cells and embryoid body formation	3 and 5% : ○ proliferation rate of ES colonies 1% : Slightly ↓ proliferation of ES colonies	Better maintenance of undifferentiated state ↑ EB formation, ↑ SSEA-4, ↑Oct-4, ↓SSEA-1	Ezashi et al.[50]
		2%	Monolayer culture of ES cells	↑ (Increase in clone recovery)	Smaller and less granular cells ○ SSEA4, SSEA1, TRA-1-60, or TRA-1-81 ↓ chromosomal aberrations	Forsyth et al.[60]
	Mouse	1%	EB formation	No changes in first 5 days	↑ mRNA levels of Aldolase A and VEGF	Gassman et al.[52]
		5%, 20%, 40%	Monolayer culture of ES cells and EB formation	Suppressed proliferation of ES cells at 5% in comparison to 20 and 40% O_2	5% and 20%: ↓ specific AP activity in ES cells 40%: ○ AP activity in ES cells, ↑ Oct-4 expression in comparison to 5 and 20% (ES cells as well as EBs)	Kurosawa et al.[51]
Adipose-derived MSC	Human	5%	Expansion in monolayer or/and alginate beads in chondrogenic medium: up to 14 days	○ proliferation in expansion medium ↓ proliferation in chondrogenic cultures	Chondrogenesis: ↑ GAG synthesis at certain time points; ○ collagen type II ↑ protein content, ↑ total collagen synthesis	Wang et al.[74]
		2%	Monolayer; osteogenic and adipogenic differentiation	N/A	Adipogenesis: No intracellular lipid droplets Osteogenesis: No mineralization	Lee and Kemp[75]
		5%	Cell aggregate culture in chondrogenic medium, 14 days.	↓ cell proliferation	Chondrogenesis: ↑ GAG content, ↑collagen type II, ↑IX, ↑XI, ↑ SOX6, ↑ SOX5, ↑ SOX9, ↑ HIF2α not HIF1α	Khan et al.[76]
		5%	Elastin-like polypeptide scaffold; 7 days DMEM/F-12 supplemented with ascorbate 2-phosphate	N/A	Chondrogenesis: ↓ expression of Collagen type II, ↑ expression of SOX9, ↓ expression of Collagen X	Betre et al.[77]
	Mouse	2%	Micromass culture in chondrogenic medium; up to 12 days	↑ cell proliferation	Chondrogenesis: ↓ collagen type II, ↓ GAG content	Malladi et al.[78]
			Monolayer culture in osteogenic medium; up to 3 weeks		Osteogenesis: ↓ AP activity, ↓ extent of mineralization	
			Monolayer in basal medium Micromass culture in chondrogenic medium in normoxia; up to 6 days	↑ cell proliferation	Chondrogenesis: ↑ PG nodule formation, Micromasses rapidly condensed	Xu et al.[79]
			Monolayer; Osteogenic differentiation in normoxia; up to 3 weeks		Osteogenesis: ↓ AP activity, ↓ extent of mineralization	
BM-derived MSC	Human	2%	3D PET Scaffolds (4 weeks) Osteogenic and adipogenic differentiation at 20 % O^2	Slower proliferation during initial period but maintained expansion phase longer	↑ Oct-4, ↑ Rex-1 expression, ↑ CFU-F Osteogenesis: ↑ Mineralization (von Kossa) ↑ AP activity (max. levels) ○ Osteonectin Adipogenesis: ↑ LPL expression	Grayson et al.[64]
			Monolayer; Osteogenic and adipogenic differentiation at 20 % O_2	↑ (30 times greater expansion over 6 passages)	↑ Oct-4, ↑ HIF-2α, ↑ connexin-34 expression Osteogenesis: ○ mineralization (von Kossa), ○ AP activity Adipogenesis: ○ lipid vacuole formation	Grayson et al.[61]
		3%	Long term monolayer culturesfollowed by osteogenic, adipogenic differentiation.	↑ proliferative lifespan over 100 days. Delayed onset of senescent phenotype (↓ AGE)	○ expression of hypoxia related genes: HIF-1α, PH-4, HIF1AN, VHL, Hyou1, HIG1, HIG2. Osteogenesis: ↓ calcium, ↓ AP mRNA, ↓ IBSP mRNA. Adipogenesis: ↓ lipid-droplet containing cells, ↓ FABP4, ↓ LPL Osteogenic and adipogenic potential regained when cells induced in normoxia.	Fehrer et al.[67]
		1.5%	Monolayer cultures exposed to hypoxia for 24 h, followed by osteogenic (14 days), adipogenic (21 days) and chondrogenic (21 day-pellets) differentiation in normoxia	↑ rate of cell division and proliferation	↑HIF1α, ↑BNIP3, ↑VEGFA, ↑AK3L1/L2, ↑STC1, ↑EPHA3, ↓EDG1expression Osteogenesis: ○ mineralization, ○ RUNX2 expression Adipogenesis: ○ number of lipid	Martin-Rendon et al.[65]

Cell Type	Species	Oxygen Tension	Cultivation Conditions	Expansion (compared to 20% O_2)	Differentiation (compared to 20% O_2)	Reference
					droplets, ○ (PPAR)-α Chondrogenesis: ↑ pellet size, ↑SOX9 expression	
		4%	Monolayer cultures exposed to hypoxia for 48 h, followed by osteogenic differentiation (up to 28 days)	Cell survival not affected when lasted up to 72 h. Cell death (35%) occurred when cultured for 120 h.	↑ bFGF mRNA but ↓bFGF protein secretion, ↑VEGF mRNA, ↑VEGF protein secretion, ○ IL-6, MCP-1, TIMP-1, TIMP-2 Osteogenesis: ○ AP expression, ○ BMP-2, ○ BSP, slightly ↓ expression of cbfa-1/Runx2 and Osteocalcin (day 0 and 14); no changes at day 28, ↑ Osteopontin, ↓ Collagen type I expression	Potier et al.[25]
	Mouse	8%	Monolayer cultures in expansion medium (IMDM) and adipogenic differentiation	↑ cell proliferation (2.8 fold increase in cell number after 8 days)	↑ in number of AP positive, flattened cells, ↑HIF-1α, ↑ H-2Dd, ↑ CD44, ○ IA-d and CD13, ↓Oct4, ○ VEGF expression Adipogenesis: ↑ in number of lipid droplets (5.6 fold)	Ren et al.[66]
	Rat	5%	Monolayer culture, osteogenic differentiation; in parallel ceramic scaffolds implanted in vivo (up to 6 weeks)	↑ number of colonies	Osteogenesis in vitro: ↑ AP activity, ↑ calcium content, ↑ mineralization (von Kossa) Osteogenesis in vivo: ↑ bone formation	Lennon et al.[63]

AGE, advanced glycation endproducts; SSEA, stage-specific embryonic antigen; ES, embryonic stem; EB, embryoid body; VEGF, vascular endothelial growth factor; AP, alkaline phosphatase; GAG, glycosaminoglycans; PG, proteoglycan; HIF, hypoxia-inducible factor; BSP, bone sialoprotein; PPAR, peroxisome proliferator-activated receptor; BMP, bone morphogenetic protein; TIMP, tissue inhibitor of metalloproteinase; MCP, monocyte chemoattractant protein; IL, interleukin; bFGF, basic fibroblast growth factor; EDG, endothelial differentiation gene; STC, stanniocalcin; LPL, lipoprotein lipase; FABP4, fatty acid–binding protein; HIG, hypoxia-inducible gene; Hyou, hypoxia upregulated.

↑, increase;

↓, decrease;

○, unchanged (or change not quantified).