

RESEARCH REVIEW

The Role of Hypoxia in Stem Cell Differentiation and Therapeutics

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Stem cells differentiate into a variety of cell lines, making them attractive for tissue engineering and regenerative medicine. Specific microenvironmental cues regulate self-renewal and differentiation capabilities. Oxygen is an important component of the cellular microenvironment, serving as both metabolic substrate and signaling molecule. Oxygen has been shown to have a variety of effects on embryonic and adult stem cells. This review examines the role of hypoxia in regulating stem cell biology, specifically focusing on growth, maintenance of pluripotency, differentiation, and production of growth factors. Particular attention is paid to hypoxia and stem cells in relation to therapeutic angiogenesis. We conclude that further study is needed to optimize the use of hypoxia as a stimulus for various stem cell functions, including its potential role in therapeutic angiogenesis. © 2009 Elsevier Inc. All rights reserved.

Key Words: hypoxia; stem cells; differentiation; therapeutic angiogenesis.

INTRODUCTION

Stem cells either self-renew or differentiate depending upon various microenvironmental cues, including soluble growth factors, extracellular matrix, and mechanical forces. Our laboratory has used these various stimuli *in vitro* to differentiate adult stem cells derived from adipose tissue (ASC) into endothelial cells for the purpose of creating autologous blood vessels/vascular grafts [1–3]. While ASC can acquire several important characteristics of endothelial cells, differentiation appears incomplete as evidenced by minimal expression

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of eNOS. This led us to evaluate the effect of another key microenvironmental component, oxygen tension, on the differentiation and function of these cells.

Molecular oxygen serves as a metabolic substrate and signaling molecule for cells both *in vitro* and *in vivo*. Its effect on stem cell self-renewal, differentiation, and ultimate function *in vitro* is incompletely understood, largely because the differential effects of this cue depend on oxygen concentration and cell type. In certain types of adult stem cells, low oxygen concentration *in vitro* promotes proliferation and maintenance of a multipotent state [4, 5]. This increase in proliferation and prolongation of the pluripotent state is important when dealing with cells that often are found in limited quantities and difficult to maintain for prolonged periods of time. Conversely, other investigators have demonstrated hypoxia to be a potent stimulus for differentiation into specific cell lines [6, 7]. For example, low oxygen has been shown to be a potent stimulus for chondrogenesis in stem cells, which is an important aspect toward engineering functional cartilage and applying it to clinical processes. Alternatively, hypoxia can stimulate cytokine production, thereby potentially playing a role in therapeutic angiogenesis [8–10]. Numerous studies have shown a significant increase in growth factor production, especially vascular endothelial growth factor (VEGF), when stem cells are exposed to hypoxia. Participation in angiogenesis by stem cells may occur directly *via* differentiation of cells that participate in angiogenesis, or indirectly *via* cytokine production stimulated by hypoxia [11, 12]. Hypoxic preconditioning of stem cells will optimize their potential for therapeutic angiogenesis and will have a direct clinical impact on the treatment of peripheral vascular disease and ischemic heart disease.

This review focuses on the known effects of hypoxia on stem cells. Stem cells are a unique population of cells



111 that have the innate ability to create or repair tissue
 112 through differentiation and self-renewal. Our review
 113 focuses on the effect of low oxygen on both embryonic
 114 and adult stem cells, including mesenchymal stem cells
 115 derived from bone marrow (BM-MSC) and adipose tis-
 116 sue (ASC). We explore the currently known roles of
 117 low oxygen concentration on maintenance of self-
 118 renewing properties, differentiation, and production
 119 of angiogenic growth factors. Finally, we expound
 120 upon the potential clinical benefits of hypoxia on stem
 121 cells in promoting therapeutic angiogenesis.

122 OXYGEN CONCENTRATIONS IN THE STEM CELL 123 MICROENVIRONMENT

124 Most tissue cultures are maintained *in vitro* at oxy-
 125 gen levels of approximately 20%. Ironically, natural
 126 cell microenvironments appear to contain much lower
 127 oxygen tensions: the mean oxygen concentration of
 128 arterial blood approximates 12%, and that of tissue is
 129 3%, with considerable variation based on location [5].
 130 Optimization and understanding stem cell growth and
 131 function requires knowledge of the specific microenvi-
 132 ronmental conditions *in vivo*. Similarly, one needs to
 133 be mindful of oxygen tension when interpreting exper-
 134 iments involving stem cells *in vitro*.

135 Embryonic stem cells, in particular, live at low
 136 oxygen concentrations beginning at implantation and
 137 continuing through fetal development. During implan-
 138 tation of the embryo, the lack of access to maternal cir-
 139 culation results in a hypoxic environment [13]. The
 140 uterine surface typically has oxygen concentrations of
 141 2% during early pregnancy. Even after the embryo
 142 establishes connection to the maternal vasculature,
 143 placental oxygen levels only increase to approximately
 144 8% [14, 15]. Hence, the normal physiologic environ-
 145 ment of embryonic stem cells is one of relative hypoxia
 146 compared with traditional *in vitro* culture conditions.

147 Adult stem cells similarly live in hypoxic conditions
 148 *in vivo*. The most direct evidence arises from research
 149 on hematopoietic stem cells, which share an environ-
 150 ment with bone marrow-derived mesenchymal stem
 151 cells (BM-MSC) [16]. In a study evaluating bone mar-
 152 row aspirates of volunteers, specimens were found to
 153 be consistently hypoxic, with some levels as low as
 154 1%–2% [17,18]. Similarly, oxygen tension in the bone
 155 marrow of mice is significantly lower than other tissues
 156 [19].

157 While the exact anatomical location of ASC within fat
 158 is not precisely known [20], it is postulated that they
 159 surround the capillaries where they interact with endo-
 160 thelial cells and provide structural vascular stability
 161 [21]. Given that lower oxygen concentrations are ob-
 162 served in adipose tissue compared with other tissues,

163 ASC likely live in a hypoxic environment despite their
 164 presumed proximity to the vasculature [22].

165 Most tissue cultivation employs ambient oxygen con-
 166 centration of 21% O₂ for cell culture. This environment
 167 may be suboptimal for stem cells whose natural micro-
 168 environment consists of a much lower oxygen concen-
 169 tration. Proven benefits of maintaining stem cells at
 170 their physiologic oxygen concentration include an in-
 171 crease in growth kinetics and prolonged maintenance
 172 of pluripotency. Furthermore, hypoxic preconditioning
 173 can increase cell differentiation toward certain cell lin-
 174 eages and up-regulates a variety of important cyto-
 175 kines. It is important to study stem cells within their
 176 physiologic norm to better understand their full thera-
 177 peutic potential and possible clinical impact.

178 EFFECT OF HYPOXIA ON STEM CELL GROWTH AND SELF- 179 RENEWAL

180 Given hypoxic conditions are the physiologic norms
 181 for a variety of stem cell niches, more research has in-
 182 corporated hypoxia into tissue culture technique. A
 183 variety of studies demonstrate significant benefit in
 184 terms of cell proliferation using low oxygen tensions
 185 [23, 24]. Embryonic stem cells, in particular, appear to
 186 grow more efficiently under low oxygen concentrations
 187 compared to room air. For example, bovine blastocysts
 188 demonstrate significantly more inner cell mass when
 189 cultured in hypoxia [25, 26]. Similar benefits occur in
 190 a variety of other species, including human embryonic
 191 cells [16, 27].

192 Further support to the benefits of hypoxia on cell
 193 growth and expansion arises from research on adult
 194 stem cells, mainly human BM-MSC. In a study by Gray-
 195 son *et al.* low oxygen culture resulted in a 30-fold in-
 196 crease in the expansion of cells compared with
 197 normoxic conditions [4]. Studies involving ASC, how-
 198 ever, have yielded conflicting results regarding the ben-
 199 efits of low oxygen concentration in tissue culture [8,
 200 28]. Further study is needed to delineate the effects of
 201 hypoxia on ASC proliferation.

202 In addition to the improvement of cell growth and ex-
 203 pansion on various stem cells, some investigators note
 204 that hypoxic culture conditions allow maintenance of
 205 potency. Embryonic stem cells remain undifferentiated
 206 in hypoxia for up to 4 wk, with one study showing pro-
 207 longed pluripotency up to 18 mo duration [29, 30]. This
 208 finding is corroborated by normal morphologic appear-
 209 ance and preservation of OCT-4. In adult BM-MSC, cul-
 210 ture in hypoxic conditions (2% O₂) for up to 6 wk,
 211 increases the expression of embryonic markers such
 212 as OCT-4 [4, 31]. Some investigators observe that hyp-
 213 oxia halts differentiation of ASC, allowing prolongation
 214 of the dedifferentiated state [32].

221 Although these studies suggest that hypoxia results
 222 in maintenance of potency, often small shifts in oxygen
 223 tension stimulate differentiation. Furthermore, this re-
 224 sponse to hypoxia may depend on various culture condi-
 225 tions. To better delineate the effect that low oxygen
 226 concentration has on the variety of stem cells types,
 227 standardized experiments are needed to specifically
 228 examine different oxygen tensions and their effect on
 229 each specific stem cell source. This standardized
 230 approach would provide a better understanding of
 231 how different stem cells behave under different oxygen
 232 concentrations.

233 234 235 **HYPOXIA AS A STIMULUS FOR STEM CELL** 236 **DIFFERENTIATION** 237

238 While research demonstrates a beneficial role of hyp-
 239 oxia in maintaining an undifferentiated stem cell, some
 240 researchers have explored the possibility of using hyp-
 241 oxia to stimulate differentiation. Manipulation of oxy-
 242 gen tension shows promising results in driving stem
 243 cells towards specific cell lines, particularly chondro-
 244 cytes and cardiomyocytes [6]. The optimal oxygen
 245 concentration to stimulate differentiation *versus* main-
 246 tenance of stemness is unknown, however, and is likely
 247 affected by other culture conditions.

248 The majority of evidence for hypoxia's effect on differ-
 249 entiation involves formation of cartilage. As a tissue,
 250 cartilage is avascular and receives nutrients and oxy-
 251 gen mainly from the surrounding synovial fluid [6].
 252 Oxygen concentration within cartilage is reported to
 253 Q1 be between 1% and 8% [33]. In a study by Kaoy *et al.*,
 254 human embryonic stem cells cultured in 2% oxygen con-
 255 centration significantly increase production of cartilage
 256 matrix proteins, most notably collagen II [6]. This find-
 257 ing has been confirmed in other stem cell lines, includ-
 258 ing bone marrow-derived MSC and ASC [7, 34, 35].

259 Similarly, osteocytes live at low oxygen tensions (4%–
 260 7%) *in vivo* [18, 36, 37]. Unlike chondrogenesis, how-
 261 ever, the results for stimulation of osteogenesis appear
 262 mixed. Research demonstrates both a beneficial and
 263 deleterious response to hypoxia for stimulating osteo-
 264 genesis. In a study of rat bone marrow-derived MSC,
 265 culture in 5% oxygen produces significant increases in
 266 bone production markers and proliferation of the differ-
 267 entiated cells [38]. Conversely, Malladi *et al.*
 268 demonstrate that 2% oxygen inhibits osteogenic differ-
 269 entiation in ASC [39]. It appears that the ultimate
 270 effect of hypoxia depends upon several factors, includ-
 271 ing stem cell line, degree and duration of hypoxia, as
 272 well other culture conditions.

273 Other studies, although few in number, examine the
 274 role of hypoxia adipogenic, cardiogenic, endothelial,
 275 Q2 and differentiation. Fink *et al.* demonstrate that

276 culture in 1% oxygen induced an adipose phenotype in
 277 bone marrow-derived MSC but no increase in adipo-
 278 cyte-specific genes [40]. Conversely, Lee *et al.* note
 279 that culture at 2% oxygen inhibits adipogenesis in
 280 ASC [41]. Again, general findings remain inconclusive
 281 and mandate further research. In a study using embry-
 282 onic stem cells, investigators reveal an increase in the
 283 number of cardiomyocytes in cultures at 4% oxygen ten-
 284 sion compared with those grown in normoxic conditions
 285 [42]. Cao *et al.* demonstrate significant neovasculariza-
 286 tion in an *in vivo* mouse hind limb ischemia model after
 287 treatment with ASC; given evidence of stem cell incor-
 288 poration into new vessels, they surmise that the stem
 289 cells differentiate into endothelial cells, presumably in
 290 these hypoxic conditions [11]. Another study suggests
 291 that hypoxia stimulates an endothelial phenotype in
 292 ASC, but specific endothelial cell markers are not pres-
 293 ent [8]. In culture conditions previously shown to
 294 induce endothelial differentiation in ASC [1], we found
 295 that hypoxia (2%–5%) over a 3-wk period inhibits the
 296 expression of both von Willebrand factor and CD31
 297 (unpublished data).
298
299

300 **EFFECT OF HYPOXIA ON THERAPEUTIC ANGIOGENESIS**

301
302 Oxygen is an important signaling molecule that
 303 impacts cellular activity. In stem cells, like others, hyp-
 304 oxia is known to increase expression of specific genes
 305 involving glycolysis, erythropoiesis, and angiogenesis
 306 (glut-1, Epo, and VEGF, respectively) [43]. Using hyp-
 307 oxia to stimulate angiogenesis *via* stem cells may there-
 308 fore hold promise for treating vascular occlusions in the
 309 coronary and peripheral circulations.

310 Independent of hypoxia, stem cells may promote an-
 311 giogenesis *via* several mechanisms, including differen-
 312 tiation into cells that participate in new blood vessel
 313 formation (e.g., endothelial cells) and production of
 314 growth factors. Although evidence exists for the former
 315 mechanism mentioned above [11], several studies sug-
 316 gest stem cells may influence angiogenesis *via* a para-
 317 crine mechanism. Both bone marrow-derived MSC
 318 and ASC produce a variety of angiogenic cytokines,
 319 including VEGF [9, 10, 44]. Additionally, when stimu-
 320 lated by hypoxia, ASC express a 5-fold increase in
 321 VEGF production [8, 10].

322 In a hypoxic environment, VEGF produced by stem
 323 cells directly impacts surrounding cells. When co-cul-
 324 tured with endothelial cells in hypoxic conditions,
 325 ASC produce VEGF, which stimulates increased capil-
 326 lary formation by the endothelial cells *in vitro* [8]. Sim-
 327 ilarly, other studies show that conditioned media from
 328 ASC or BM-MSC cultured in hypoxia increases endo-
 329 thelial cell growth and prevented apoptosis [10, 45,
 330 46]. The latter protective benefit is also seen in other

TABLE 1
Key References Investigating the Effect of Hypoxia on Stem Cell Differentiation and Function

Author	Ref	Stem cell source	Oxygen concentration	Major findings
Prasad	[30]	Human embryonic stem cells	5%	Promotion of stemness
Grayson	[31]	Human bone marrow derived mesenchymal stem cells	2%	Increase in expansion and prolonged maintenance of stemness
Lin	[32]	Mouse adipose derived stem cells	1%	Promotion of stemness
Koay	[6]	Human embryonic	2%	Increase in chondrogenesis
Khan	[7]	Human adipose derived stem cells	5%	Decrease in proliferation with an increase in chondrogenesis
Lennon	[38]	Rat bone marrow derived mesenchymal stem cells	5%	Increase in proliferation and osteogenesis
Malladi	[39]	Mouse adipose derived stem cells	2%	Increase in proliferation with decrease in osteogenesis
Rehman	[10]	Human adipose derived stem cells	1%	Increase in vascular endothelial growth factor (VEGF) production and survival of endothelial cells exposed to hypoxic conditioned media
Thangarajah	[8]	Mouse adipose derived stem cells	1%	Increase in proliferation and VEGF production.

cells; Sadat *et al.* note that co-culture with ASC decreases hypoxia-induced apoptosis in rat cardiomyocytes [9].

In vivo models provide further support for the use of stem cells in promoting angiogenesis. Numerous studies show significant improvement in revascularization using ASC and BM-MSC in a mouse hind limb ischemia model [10, 36, 46]. In this model, improvements are demonstrated both histologically, where an increase in vascular density in the treated group is seen, and by laser Doppler testing. In a clinical trial of patients with claudication, there is a 3.7-fold increase in pain-free walking distance and improvement in ankle-brachial indices of patients receiving intra-arterial and intramuscular transplantation of autologous bone marrow-derived MSC [47]. Improvement in ejection fraction is noted in rats with ischemic cardiomyopathy after treatment with ASC [48]. In this study, no significant cardiomyocyte differentiation is demonstrated, leading the authors to conclude that the stem cell effect is derived from increase in growth factors. In studying the influence of BM-MSC on new blood vessel formation, Ziegelhoeffer *et al.* did not observe direct incorporation of the BM-MSC into new blood vessels; rather, they noticed strong localization of these cells around the nascent collateral arteries, suggesting an indirect mechanism of incorporation [49].

CONCLUSION

Oxygen concentration is an important factor in the maintenance, differentiation, and function of stem cells. The use of low oxygen concentration as a means to simulate the physiologic norm of the stem cell micro-

environment can be useful in maintaining and expanding a population of cells that may be limited in supply or difficult to cultivate. Under other conditions, hypoxia may be used as a stimulus to promote differentiation into various cell lines. Table 1 lists several key publications that serve to define the various effects hypoxia has on several stem cells lines. Future research should continue to define the differential effects of hypoxia in a standardized fashion.

Finally, hypoxia has a significant influence on the production of growth factors involved in promoting angiogenesis. Given the effect of stem cells on promoting angiogenesis *in vitro* and *in vivo*, including in clinical trials, it is possible that preconditioning of the cells in hypoxic culture, prior to clinical use, may further their

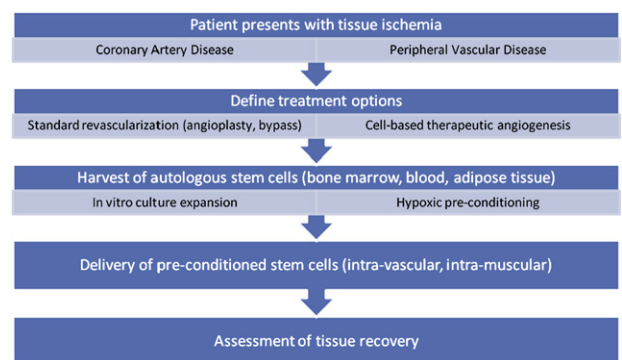


FIG. 1. Algorithm of treatment for patient presenting with coronary artery or peripheral vascular disease. If the patient is not a candidate for standard revascularization options, cell-based therapeutic angiogenesis may be an option. Areas of future research should define which tissue source is most optimal for promoting angiogenesis, the ideal preconditioning strategy using hypoxia prior to use, as well as the most efficacious route of administration. Hypoxia may prove to be a useful stimulus for both culture expansion and stimulating cytokine expression.

efficacy in clinical use. The figure poses a possible algorithm for incorporating hypoxic preconditioning into clinical usage (Fig 1). Areas of future research might include optimizing preconditioning in terms of timing, oxygen concentration, and autologous stem cell source to produce maximal amounts of growth factors, as well as investigating the most efficacious route to promote angiogenesis.

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