Oxygen Regulation in Development: Lessons from Embryogenesis towards Tissue Engineering

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Abstract
Oxygen is a vital source of energy necessary to sustain and complete embryonic development. Not only is oxygen the driving force for many cellular functions and metabolism, but it is also involved in regulating stem cell fate, morphogenesis, and organogenesis. Low oxygen levels are the naturally preferred microenvironment for most processes during early development and mainly drive proliferation. Later on, more oxygen and also nutrients are needed for organogenesis and morphogenesis. Therefore, it is critical to maintain oxygen levels within a narrow range as required during development. Modulating oxygen tensions is performed via oxygen homeostasis mainly through the function of hypoxia-inducible factors. Through the function of these factors, oxygen levels are sensed and regulated in different tissues, starting from their embryonic state to adult development. To be able to mimic this process in a tissue engineering setting, it is important to understand the role and levels of oxygen in each developmental stage, from embryonic stem cell differentiation to organogenesis and morphogenesis. Taking lessons from native tissue microenvironments, researchers have explored approaches to control oxygen tensions such as hemoglobin-based, perfluorocarbon-based, and oxygen-generating biomaterials, within synthetic tissue engineering scaffolds and organoids, with the aim of overcoming insufficient or nonuniform oxygen levels and nutrient supply.

Introduction
The presence of oxygen in Earth’s early atmosphere allowed for the evolution of complex multicellular life and eventually our human species. Oxygen made abundant around 2.3 billion years ago [Canfield, 2005] provided a readily available energy source for many cellular functions, including metabolism, respiration, and proliferation. Thus, it is of no surprise that oxygen plays a vital role in development, cell and tissue maintenance/basic metabolism as well as cell and tissue repair. Beginning in the 1970s a substantial body of evidence has been constructed indicating the importance of oxygen availability in early development. During development, the levels of oxygen vary in each stage depending on the specific requirements of the developing embryo [Stamati et al.,...
Molecular oxygen is involved in most cell and tissue metabolic processes, and mitochondrial respiration accounts for the majority of O$_2$ consumption in humans. The citric acid cycle and β-oxidation of fatty acids are tightly coupled with the process of oxidative phosphorylation and production of ATP. These ATP molecules then provide the required energy for the majority of cell physiological processes. Thus, oxygen bioavailability is vital to all cell functions and asserts its influence from the beginning of conception, continuing throughout each phase of embryogenesis. Likewise, any attempt in tissue engineering towards the regeneration of a lost or damaged structure seems incomplete without considering the oxygen requirements and signaling during organogenesis and development.

The role of oxygen in early embryonic development

Oxygen regulation is crucial to embryonic development. At the very early stages of development, before the formation of the placenta, the embryo experiences a state of physiologic hypoxia. Physiologic hypoxia, by definition, is a microenvironment with oxygen levels ranging from 1 to 5% (8–38 mm Hg oxygen partial pressure [P$_{O2}$]), which is less than adult arterial pressures (10–13.15% oxygen, equivalent to 75–100 mm Hg P$_{O2}$). In extreme oxygen limitations, pathologic hypoxia occurs when oxygen levels drop below 1% or 8 mm Hg P$_{O2}$ [Fitzgerald et al., 1999; Ortega et al., 2017]. It is important to note that the exact definition of oxygen hypoxia varies between organs and is different in vitro and in vivo. However, in most tissues, a venous oxygen concentration <6% (approx. 46 mm Hg P$_{O2}$) creates mild hypoxic effects, whereas maximum hypoxic responses occur around 0.5–1% of oxygen (approx. 4–8 mm Hg P$_{O2}$) [Shao and Zhao, 2014].
In terms of the preferred microenvironment for embryonic stem cells (ESCs), low oxygen concentration (approx. 2% oxygen or 15 mm Hg P\(_{O_2}\)) is required to initiate ESC proliferation in the earliest stages of fetal development [Mohyeldin et al., 2010; Abdollahi et al., 2011]. Figure 1 provides a summary of oxygen levels available in each stage of embryonic development. This low oxygen tension is the result of limited connections with the maternal circulation during early development and is required for maintaining pluripotency in ESCs [Abdollahi et al., 2011]. In vitro studies also agree with these observations, where ESC cultures maintained at 3–5% oxygen (23–38 mm Hg P\(_{O_2}\)) preserve their embryonic nondifferentiated state versus cultures exposed to 21% oxygen (160 mm Hg P\(_{O_2}\)) [Stamati et al., 2011]. Likewise, Narva et al. [2013] have shown that reduced oxygen tension (4% oxygen or 30 mm Hg P\(_{O_2}\)) is crucial for maintaining the pluripotency of human (h)ESCs to encourage proliferation in an undifferentiated state in vitro.

The mammalian blastocyst forms during a crucial early stage of development and consists of an inner cell mass of ESCs inside a fluid-filled membrane (Fig. 1A) [Frankenberg et al., 2016]. The blastocyst serves as the niche and source for ESCs that maintains the required low oxygen microenvironment for this stage of development. Accordingly, experiments on bovine blastocysts developed in a hypoxic environment (2% oxygen or 15 mm Hg P\(_{O_2}\)) have shown more ESCs in their inner cell mass than under normoxic conditions [Harvey et al., 2004]. Further research supports that ESCs in the blastocyst must begin development in hypoxic conditions to successfully generate an embryo [Harvey et al., 2004; Ezashi et al., 2005; Shahbazi et al., 2016]. In parallel, during the very early stages of development, the human placenta forms from a
The independence of chick embryo development from maternal factors has led many investigators to focus on the influence of oxygen availability on chick embryogenesis. For instance, Metcalfe et al. [1981] conducted experiments on chicken eggs to investigate the effects of increased oxygen availability on the growth rate of chick embryos. Their experimental design consisted of 3 oxygen-generating regimes: (1) eggs covered with a neoprene membrane to serve as a diffusion barrier limiting oxygen transfer while being incubated with 21% oxygen (160 mm Hg \( P_{O_2} \)), (2) uncovered eggs exposed to 21% \( O_2 \) (160 mm Hg \( P_{O_2} \)), and (3) eggs incubated with 60% oxygen (456 mm Hg \( P_{O_2} \)). The results of this study indicated a significant (approx. 10%) delay in embryonic growth for the covered eggs with limited oxygen transfer, compared to the uncovered group. Furthermore, on day 18, the group incubated with 60% oxygen formed significantly heavier eggs (indicative of embryonic growth) in contrast to the control group exposed to 21% oxygen (160 mm Hg \( P_{O_2} \)), confirming that embryonic chick growth is regulated by oxygen availability. Similarly, Lourens et al. [2007] evaluated the effect of exposure to 3 different oxygen concentrations, low (17% oxygen or approx.129 mm Hg \( P_{O_2} \)), normal (21% oxygen or 160 mm Hg \( P_{O_2} \)), and high (25% oxygen or 190 mm Hg \( P_{O_2} \)) on the yolk-free egg masses. Increasing \( O_2 \) concentration was shown to increase the yolk-free mass and the length of the chick, especially at the 3-week time point. More recently, Giussani et al. [2007] studied the effect of oxygen availability on the fetal growth of chick embryos. In an interesting experiment, fertilized eggs laid by hens at sea-level were incubated at a high altitude (approx. 13% oxygen or 100 mm Hg \( P_{O_2} \)) and resulted in greater embryonic mortality than eggs incubated at sea level (21% oxygen or 160 mm Hg \( P_{O_2} \)). Decreased growth at the study end point (day 20) was also observed in the same conditions, as indicated by head diameter and body (crown-to-rump) length.

Another compelling case of oxygen involvement in embryogenesis is that in limb morphogenesis and the formation of growth plates. Considering the absence of vasculature in bone growth plates, the cartilaginous structures of new bone are under low oxygen levels. This physiological hypoxia acts as a signal to modulate chondrocytes to differentiate from a proliferative to a terminally differentiated state. It has been shown that, within the developing long bone, the oxygen tension varies from 21 mm Hg
in the areas further away from blood supply to 57 mm Hg in the hypertrophic regions close to the vasculature [Araldi and Schipani, 2010]. Focusing on each region of developing bone, the cell population present in the prehypertrophic areas, which exist at an oxygen tension of approximately 21 mm Hg, are more proliferative, which aligns with their more stem cell-like nature. The cell type, population, and oxygen level in the prehypertrophic region contrast with the hypertrophic region that is comprised of a population of terminally differentiated cells existing at a higher oxygen tension of around 57 mm Hg [Araldi and Schipani, 2010; Rankin et al., 2011; Stamati et al., 2011].

**Oxygen Homeostasis and Sensing Mechanisms**

After early embryonic morphogenesis, and later in development, oxygen-sensing mechanisms are required to both establish and maintain oxygen homeostasis. This allows the body to sustain oxygen gradients and maintain levels of oxygen within the range required for development. Oxygen homeostasis is defined as the mechanism through which cellular O$_2$ concentration is maintained within a narrow range, with an upper level of 21% oxygen (approx. 160 mm Hg P$_{O2}$) in the upper airway to the minimum of around 1% oxygen (approx. 8 mm Hg P$_{O2}$) at the corticomedullary kidney junctions [Semenza, 2010]. Homeostasis plays a critical role in the survival of all vertebrate species starting from early development and continuing to the later stages of adult life. Regulated and optimal oxygen delivery to all cells determines the physiological state of the organism. Sufficient tissue oxygenation depends on the precise development of the embryonic structures to provide a basis for oxygen homeostasis [Michiels, 2004]. At the cell level, defensive mechanisms have been developed to regulate oxygen levels to protect cells from extreme O$_2$ variations [Maltepe and Saugstad, 2009].

Generally, homeostasis pathways work to regulate oxygen levels within the body. Hypoxia-inducible factors (HIFs), prolyl hydroxylases (PHDs), factor-inhibiting HIF-1 (FIH-1), activator protein 1 (AP-1), nuclear factor (NF)-κB, p53, and c-Myc are the best-understood factors that influence oxygen regulation in vivo [Podar and Anderson, 2010]. Out of these, HIFs are typically considered as the body’s “master oxygen sensors” and belong to a family of transcriptional factors with many downstream actions [Bryant et al., 2018]. The mechanisms related to oxygen sensing and homeostasis through HIFs have been discussed in detail elsewhere and can be found in several reviews: Ivan et al. [2001], Maltepe and Saugstad [2009], Araldi and Schipani [2010], Semenza [2010], Zimna and Kurpisz [2015], Deng et al. [2016], and Graham and Presnell [2017].

**Oxygen-Mediated Embryonic Angiogenesis**

Angiogenesis is the developmental process that establishes the vasculature and is further regulated by the individual demands of tissues. These demands include a sufficient oxygen supply, the removal of excess carbon dioxide and other respiratory gases, and sufficient nutrients [Rouwkema and Khademhosseini, 2016; Yoon and Jones, 2016]. Embryonic angiogenesis initiates with the migration of mostly quiescent endothelial cells that are triggered by hypoxia (<25 mm Hg P$_{O2}$) or oxidative stress [Stamati et al., 2011]. Similarly, the same approximate oxygen tensions have been shown to stimulate the differentiation of varied progenitors derived from the embryonic mesoderm layer into hemangioblasts (hematopoietic or endothelial cells) in vitro [Fraisl et al., 2009]. The required energy for cell growth in the nonvascularized area is usually supplied through anaerobic glycolysis. Interestingly, similar to cancer cells, anaerobic glycolysis makes it possible for endothelial cells to tolerate a wide range of oxygen variations compared to other cell lineages [Fraisl et al., 2009]. It is worthy of mention that although hypoxia is often considered the driving force for endothelial cells to initiate the vascularization process [Krock et al., 2011], there is a growing body of literature suggesting that vessel growth is inhibited by both moderate and severe hypoxic conditions via a still-unknown mechanism [Faller, 1999; Hutton and Grayson, 2016]. Prior to the formation of the mammalian circulatory system, vascular development starts at a hypoxic condition with oxygen tensions of around 23 mm Hg. The HIFs largely mediate angiogenesis in hypoxia and, in the HIF family, HIF-1 target genes have been shown to play an essential role in angiogenesis [Schipani et al., 2009]. Aryl hydrocarbon receptor nuclear translocators (ARNTs)/HIF-1β and HIF-1α are both expressed in ESCs and are required for the regulation of hypoxia-responsive genes and angiogenesis. To test this hypothesis, Maltepe et al. [1997] used ARNT-deficient mice (i.e., lacking HIF-1β) to investigate the role of ARNT/HIF-1β in the regulation of the genes that are responsive to hypoxia under low oxygen conditions (1.5% oxygen and 12 mm Hg P$_{O2}$). The animals did not survive past E10.5, due to the arrested vasculature in the structure of their yolk sac, indicating the importance of HIF expression in embryonic angiogenesis. Furthermore, HIF-deficient mice embryos have been shown to develop improper placental structures.
as well as impaired vasculature [Dunwoodie, 2009]. Although low oxygen tensions are an activator signal for triggering angiogenesis, prolonged severe hypoxia can prevent the proliferation of blood vessels [Patel et al., 2005; Uno et al., 2007]. Thus, even though hypoxia is the preferred microenvironment to stimulate angiogenesis, low oxygen levels over a long time can be harmful to the process.

Oxygen-dependent homeostatic processes also play a role in angiogenesis, where hypoxia acts as a signal to trigger endothelial cells to begin the formation of new vasculature. In this regard, as soon as hypoxia is sensed by the endothelial cells, these cells activate their master oxygen sensors, the HIFs. HIF-α activates and translocates into the endothelial cell nucleus and attaches to HIF-β. The complex then enters the angiogenic signaling pathway, modulating the formation of capillaries and vessels [Pugh and Ratcliffe, 2003; Krock et al., 2011]. Accordingly, hypoxia (<5% oxygen or 38 mm Hg P O2) triggers endothelial cells to express angiogenic signaling factors, including vascular endothelial growth factor (VEGF)-A and endothelial nitric oxide synthase (eNOS), resulting in the subsequent migration and proliferation of endothelial cells [Cross et al., 2003]. Furthermore, the majority of additional angiogenic factors, such as transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF)-β, and angiopoietin AP-1 and AP-2, are direct transcriptional targets of HIFs [Deng et al., 2016]. Another contribution of oxygen in the embryonic angiogenesis process is through the modulation of cell-cell attachments to define the degree of vascular-bed permeability [Chan et al., 1984; Parks et al., 1984; van Wetering et al., 2002]. In terms of cell-cell adhesion and the permeability of the vascular bed, it has been shown that hypoxia (<1% O₂ or 8 mm Hg P O₂) can loosen endothelial cell junctions to increase the permeability within the arterial walls [Cerutti and Ridley, 2017].

Overall, oxygen gradients, which are detected and maintained via O₂ homeostasis mechanisms, are the initiating stimuli for the angiogenesis process. Angiogenesis occurs through a sequence of stages starting with the angiogenic stimulus, the sprouting of endothelial cells, elongation and branching, the formation of the lumen, and finally regression. From a fundamental perspective, angiogenesis in adults is different from the embryonic process, in the sense that an embryo needs this process for organ development when oxygen levels are not enough to satisfy developmental requirements. In contrast, in an adult, angiogenesis commonly only occurs as a response to insufficient blood and oxygen supply, in instances such as wound-healing, reproduction, and exercise. The vascular system of a mature mammalian organism is formed by a large number of vessels providing highways for blood transportation, gas/waste exchange, and oxygen delivery as well as inflammatory cells and progenitors [Pugsley and Tabrizchi, 2000]. The circulatory system of a mature mammal is mainly comprised of the cardiovascular and lymphatic systems, within which arteries, capillaries, veins, and lymphatic vessels are the main components [Logsdon et al., 2014]. Local oxygen pressures are different within vascular channels, based on their function and location, and the metabolic requirements of the adjacent tissue.

Figure 2 summaries oxygen levels throughout the adult organism and the oxygen gradients that are established at the completion of embryonic development, which should also ultimately be achieved in any tissue engineering or organoid strategy. To summarize, low oxygen levels are considered a driving factor for angiogenesis during development in vivo. After formation of the blastocyst and prior to the maturation of the placenta, levels of oxygen and other essential nutrients are insufficient to support the formation of new embryonic structures, which triggers the system to form connections with the maternal vasculature. The inflowing maternal blood provides a route for the exchange of oxygen and other nutrients to the fetal circulatory system [Wang and Zhao, 2010]. Therefore, without supplying the required oxygen and nutrients to the developing organism, the process cannot proceed normally to form a fully developed embryo.

**Tissue Oxygen Monitoring and Measurement Techniques**

In order to satisfy the oxygen requirements of a tissue, a tissue-engineered construct, or an organoid, it is equally important to be able to both monitor and measure the oxygen levels in situ. Currently, challenges such as the thickness and structure of the scaffold and the tissue limit the effectiveness of oxygen monitoring and measuring techniques within 3-dimensional (3D) living structures [Weyand et al., 2015]. As mentioned before, oxygen levels directly correlate with cell growth, so extracellular matrix synthesis and the success of a tissue engineering approach during growth are essential to monitor. Despite this importance, currently available techniques to directly measure the oxygen levels both in vitro and in vivo are often limited to the surface layer of the developing tissue or the...
surrounding aqueous environment [Weyand et al., 2015]. Some of the most common techniques to directly measure oxygen levels include delayed fluorescence of endogenously overproduced protoporphyrin IX (PPIX), intravascular (cell-impermeable) phosphorescent probes, cell-penetrating small-molecule phosphorescent nanosensors, and solid-state phosphorescent sensors, all of which can be used for imaging and to quantitatively measure cell and tissue oxygenation. A detailed discussion of each of these methods as well as their advantages and limitations can be found in the review recently published by Papkovsky and Dmitriev [2018].

Direct optical sensing of oxygen is a common method also known as endogenous delayed fluorescence, which uses a metal-free endogenously produced precursor of heme, called PPIX, as an oxygen-sensing reporter. However, the optical fluorescence signals of the endogenous PPIX reporter is weak and need to be enhanced in animal or human tissues to enable oxygen measurement. This enhancement is usually done by treating tissues with 5-aminolevulinic acid (ALA) to increase mitochondrial PPIX levels and the specific optical signal. Despite the diverse applications of this method, it presents moderate selectivity and resolution, especially for quantitative oxygen measurement.

Another common type of oxygen measurement is performed indirectly via oximetry, integrating methods such as pH measurement, lactate concentration, or glucose consumption. One common indirect approach is to use the localization and quantification of a drug that undergoes oxidative metabolism, such as EF5/2-nitromidazole and (2-[2-nitro-1H-imidazole-1-yl]-N-[2,2,3,3,3-pentafluoropropyl]acetamide), as has been employed to study hypoxic tumor environments [Evans et al., 2000]. Reductive metabolism activates EF5, which leads to covalent bonding with macromolecules. Increased oxygen availability in the environment causes more inhibition in the reductive mechanism and activation [Koch, 2002]. The intracellular adducts of EF5 can subsequently be detected by using antibodies, quantified on immunohistochemistry, and correlated with oxygen availability [Evans et al., 2004]. Similar to EF5, pimonidazole has also been used clinically as a hypoxia probe to indirectly measure levels of tumor hypoxia [Nordsmark et al., 2003]. Pimidazole

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Fig. 2. Oxygen levels in adult mammals.
can bind to thiol-containing proteins present in hypoxic cells, which can then be quantitatively detected and converted indirectly to oxygen concentration using point-counting and image analysis software methods [Varia et al., 1998]. Among all the indirect methods, polarographic (Clark) electrodes are considered the oximetry gold standard, presenting advantages such as good reproducibility and accuracy as well as the low deviation between each sensor and the high detection resolution [Park et al., 2007]. The polarographic method uses an electrode made of platinum cathodes and silver anodes linked by an electrolyte bridge. While oxygen is reduced at the cathode surface, the amount of O$_2$ diffusing through the oxygen-permeable membrane increases, which closes the circuit and sends out a current proportional to the amount of oxygen at the measurement site [De Santis and Singer, 2007]. The polarographic method uses an electrode made of platinum cathodes and silver anodes linked by an electrolyte bridge. While oxygen is reduced at the cathode surface, the amount of O$_2$ diffusing through the oxygen-permeable membrane increases, which closes the circuit and sends out a current proportional to the amount of oxygen at the measurement site [De Santis and Singer, 2007]. The polarographic method uses an electrode made of platinum cathodes and silver anodes linked by an electrolyte bridge. While oxygen is reduced at the cathode surface, the amount of O$_2$ diffusing through the oxygen-permeable membrane increases, which closes the circuit and sends out a current proportional to the amount of oxygen at the measurement site [De Santis and Singer, 2007]. The polarographic method uses an electrode made of platinum cathodes and silver anodes linked by an electrolyte bridge. While oxygen is reduced at the cathode surface, the amount of O$_2$ diffusing through the oxygen-permeable membrane increases, which closes the circuit and sends out a current proportional to the amount of oxygen at the measurement site [De Santis and Singer, 2007]. The polarographic method uses an electrode made of platinum cathodes and silver anodes linked by an electrolyte bridge. While oxygen is reduced at the cathode surface, the amount of O$_2$ diffusing through the oxygen-permeable membrane increases, which closes the circuit and sends out a current proportional to the amount of oxygen at the measurement site [De Santis and Singer, 2007]. The polarographic method uses an electrode made of platinum cathodes and silver anodes linked by an electrolyte bridge. While oxygen is reduced at the cathode surface, the amount of O$_2$ diffusing through the oxygen-permeable membrane increases, which closes the circuit and sends out a current proportional to the amount of oxygen at the measurement site [De Santis and Singer, 2007]. The polarographic method uses an electrode made of platinum cathodes and silver anodes linked by an electrolyte bridge. While oxygen is reduced at the cathode surface, the amount of O$_2$ diffusing through the oxygen-permeable membrane increases, which closes the circuit and sends out a current proportional to the amount of oxygen at the measurement site [De Santis and Singer, 2007]. The polarographic method uses an electrode made of platinum cathodes and silver anodes linked by an electrolyte bridge. While oxygen is reduced at the cathode surface, the amount of O$_2$ diffusing through the oxygen-permeable membrane increases, which closes the circuit and sends out a current proportional to the amount of oxygen at the measurement site [De Santis and Singer, 2007]. The polarographic method uses an electrode made of platinum cathodes and silver anodes linked by an electrolyte bridge. While oxygen is reduced at the cathode surface, the amount of O$_2$ diffusing through the oxygen-permeable membrane increases, which closes the circuit and sends out a current proportional to the amount of oxygen at the measurement site [De Santis and Singer, 2007].

Similarly, magnetic resonance imaging (MRI) techniques using fluorine contrast agents have also been used to quantitate tissue oxygen levels [De Santis and Singer, 2015; Papkovsky and Dmitriev, 2018]. This method is based on the fact that dissolved oxygen molecules have paramagnetic properties that can affect the relaxation rate of fluorine nuclei which is linearly proportional to oxygen concentration. MRI can then use the $^{19}$F relaxation rate to either monitor the P$_{O2}$ levels or create an organ oxygen distribution map. Various perfluorinated compounds can be used as contrast agents, the distribution of which reveals the tissue oxygenation levels. For instance, MRI has been used to visualize tumor oxygenation in live animals after injection of hexafluorobenzene in the target tumor tissue [Ruiz-Cabello et al., 2011]. The high cost of this technique as well as requiring the patient’s cooperation can be considered as main drawbacks that limit its practical application [Aydogdu et al., 2012].

As described before, oxygen bioavailability plays a vital role, starting from development and later in tissue maintenance and regeneration. In order to be able to mimic any natural tissue regeneration process in vitro, it is of great importance to investigate oxygen levels in the native microenvironment, which is not possible without accurate and efficient tissue oximetry techniques. This, in turn, helps to maximize the efficiency of any oxygen modulation strategy, so it would be ideal to draw the required clues from the natural developmental processes such as oxygen gradients [Jaenisch et al., 2018]. However, this field is still lacking effective, accurate, and straightforward tools to provide us with quantifiable measures of oxygen, especially within a developing tissue, to then translate to a tissue engineering setting [Barinaga, 1997; Lovett et al., 2009]. Therefore, more research and advancements are required in the field of direct tissue oximetry to increase the outcomes of tissue engineering and regenerative medicine.

**Oxygen Delivery Approaches for Tissue Engineering**

From studying the role oxygen plays in the process of embryonic development, it is clear that the formation of tissues is tied closely to oxygen availability within the microenvironment. Thus, not only does this principle apply to embryogenesis, but the same conditions should also be considered in any tissue engineering setting. However, as...
opposed to embryonic development, during the formation of engineered tissue, there is no maternal circulation or vasculature to provide an enhanced oxygen and nutrient supply for developing engineered tissues [Stamati et al., 2011]. Unless adequately addressed, the lack of sustained and prolonged oxygen supply via oxygenating strategies can cause primary limitations in tissue-engineered constructs to create large, self-sustaining structures. One strategy to overcome this is via the application of engineered oxygen carriers. These carriers have shown the potential to provide a sufficient and prolonged supply of oxygen for tissue-engineered approaches, both in vitro and in vivo. More specifically, oxygen-controlled release strategies use 2 primary mechanisms, either via diffusion or by oxygen-generating reactions [Gholipourmalekabadi et al., 2016]. In an implantable strategy, a sustained and controlled level of oxygen allows enough time for early neovascularization to occur in vivo to support the engineered tissue, allowing the maintenance of cellular metabolic activity and signaling [Lovett et al., 2009]. Furthermore, engineered oxygen carriers can provide long-term oxygen sources within the tissue-engineered construct, as would be required in in vitro approaches where no vasculature is present.

The next sections deal with introducing novel oxygen-releasing technologies as well as their potential applications in the emerging field of organoid formation (summarized in Fig. 3).

Even if a large-enough construct can be created in vitro, an implanted construct often fails to integrate with the host and survive in vivo due to insufficient blood vessels and capillary networks [Griffith and Naughton, 2002]. Oxygen regulates angiogenesis using biological oxygen sensors, such as HIF signaling, resulting in the formation of vascular networks and capillaries [Lovett et al., 2009]. In the earlier part of this review, we discussed the role of oxygen in HIF signaling and hypoxia regulation. As discussed above, oxygen is a key regulator of many signaling pathways, which affect essential cellular processes such as differentiation, proliferation, and apoptosis. Thus, every tissue engineering strategy should consider the role of oxygen in both the formation/maturation of constructs and the successful integration and restoration of function after final in vivo implantation. Important knowledge can be gained from studying how oxygen gradients and levels change during different stages of development, as we have discussed above. With this knowledge, oxygen delivery strategies can be specially formulated to modulate oxygen microenvironments at prescribed levels and promote specific differentiation/developmental processes in tissue engineering. Currently available platforms for oxygen delivery are broadly classified as Hb-based, PFC-based, and oxygen-generating materials, as well as a few other recently introduced classes (Table 1). A general theme can be observed, which is to encapsulate or directly apply the formulations of these
oxygenation strategies in tissue-engineered scaffolds to supply the tissue-specific oxygen-microenvironment requirements.

**Hb-Based Oxygen Carriers**

One strategy to improve in vitro oxygenation is to isolate native Hb from red blood cells and then modify it to improve its oxygen-carrying function. Hb is an iron-containing metalloprotein existing in tetramer form in red blood cells [Jensen et al., 1998]. An Hb tetramer can be conjugated with Hb-based oxygen carriers (HbOCs) by various cross-linking and encapsulation techniques, as discussed later in this section [Pin et al., 1982; Bianconi et al., 1985]. With such an approach, HbOCs can be used to carry and maintain physiological oxygen levels in tissues, similar to their native function.

The earliest HbOC strategies mainly focused on Hb harvested from blood without any modification for use as a blood substitute. Unfortunately, this unmodified cell-free Hb, or meth-Hb, blocks renal tubules and leads to kidney failure while also acting as a proinflammatory molecule that induces oxidative toxicity in the kidneys, liver, central nervous system, and cardiac tissue [Harri-son et al., 1947; Kumar and Bandyopadhyay, 2005]. These adverse outcomes are largely a result of the high affinity of HbOCs for oxygen [Buehler et al., 2010], their short half-life, and their dissociation into dimers from tetramers [Bunn et al., 1969]. To improve the half-life and stability of HbOCs, strategies such as cross-linking [Hathazi et al., 2014; Romagnoli et al., 2015], polymerizing [Espes et al., 2015], coating with polydopamine and conjugating with dextran [Wang et al., 2017], and complexation with superoxide dismutase [Bian and Chang, 2015] have been studied. The underlying theme behind these strategies is to increase the size and reduce reactivity to diminish toxicity and renal filtration. The most significant improvement in the application of Hb as an oxygen carrier is the use of bacterially synthesized recombinant Hb. Mutagenesis of HbOCs can: (i) adjust the dioxygen affinity of Hb >100-fold, (ii) reduce NO scavenging >30-fold without compromising dioxygen binding, (iii) slow down the rate of auto-oxidation, (iv) impede subunit dissociation, and

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<tr>
<th>Class</th>
<th>Application</th>
<th>Oxygen carrier strategy</th>
<th>Reference</th>
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<tbody>
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<td>Hemoglobin</td>
<td>Artificial red blood cells</td>
<td>Hemoglobin encapsulated in liposomes</td>
<td>Sakai [2017]</td>
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<td>Bone and islet cells (pancreas)</td>
<td></td>
<td>HEMOXCell®</td>
<td>Le Pape et al. [2018]; Rodriguez-Brotons et al. [2016]</td>
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<td>Hepatocytes (liver)</td>
<td>Cardiomycocyte proliferation</td>
<td>Hemoglobin-albumin microspheres</td>
<td>Lai et al. [2015]</td>
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<td></td>
<td>Hemoglobin/gelatin/fibrinogen scaffolds</td>
<td>Ravichandran et al. [2013]</td>
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<td>Oxygen-generating biomaterials</td>
<td>Skin tissue, preserves skeletal muscle homeostasis</td>
<td>Sodium percarbonate</td>
<td>Harrison et al. [2007]; Ward et al. [2013]</td>
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<td>Mesenchymal stem cells</td>
<td></td>
<td>Calcium peroxide</td>
<td>Steg et al. [2017]</td>
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<td>Oxygen delivery to tumors and the urinary tract</td>
<td></td>
<td>Calcium oxide</td>
<td>Huang et al. [2016]; Lv et al. [2016]</td>
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<td>Pancreatic islets</td>
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<td>Oxsyte®</td>
<td>Coronel et al. [2017]</td>
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<td>Pancreatic β cells</td>
<td>Perfluorotributylamine (FC-43) emulsion</td>
<td>Goh et al. [2010]</td>
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<td>Liquid ventilation</td>
<td></td>
<td>Perfluorodecalin (Fluosol) emulsion</td>
<td>Waxman [1986]</td>
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<td>Bone</td>
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<td>Perfluor-octane emulsion</td>
<td>Lee et al. [2015]</td>
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<td>Bis(perfluorobutyl)ethane emulsion</td>
<td>Lowe et al. [1998]</td>
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<td>Covalently immobilized perfluoro-octanoyl chloride (MACF)</td>
<td>Li et al. [2014]</td>
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<td>MACF</td>
<td>Wijekoon et al. [2013]; Patil et al. [2016]</td>
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<td>Photosynthetic-engineered microalgae</td>
<td>Lode et al. [2015]; Schenck et al. [2015]; Chavez et al. [2016]</td>
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<td>Armstrong et al. [2015]</td>
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<td>Microtanks</td>
<td>Cook et al. [2015]</td>
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<td>Endoperoxides</td>
<td>Benz et al. [2013]</td>
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Oxygen in Development and Tissue Engineering

(v) diminish irreversible subunit denaturation [Varnado et al., 2013]. Additionally, recombinant Hb helps to eliminate the chance of cross-contamination from mammalian donors [Pishchany et al., 2010]. These improvements in HbOCs have been somewhat successful in overcoming limitations presented during previous HbOC blood substitute applications.

Despite the considerable time and effort spent on developing HbOCs, it is important to highlight that, to date, artificial, cell-free HbOCs have not been proven safe in humans, which is of concern because their application poses the risk of myocardial infarction [Natanson et al., 2008]. These HbOCs can induce vascular thrombosis of the heart and other organs by scavenging NO rapidly leading to vasoconstriction [De Caterina et al., 1995; Lin et al., 2001; Rother et al., 2005]. Clinical trials and preclinical studies using HbOCs as artificial blood oxygen carriers have not shown any significant benefits of their use, especially considering the reported side effects [Terraneo et al., 2017; Kao et al., 2018]. Thus, careful clinical evaluation of newly developed promising strategies is required, or else HbOCs may be better suited for purely in vitro endeavors such as the first phases of tissue engineering applications.

Recent advances in the field have shown promise for assisting tissue engineering strategies by improving respiratory gas transport. Specifically, a combination of protein-based scaffolds with Hb has been formulated to create tissue engineering scaffolds and shown some recent success. Specifically, scaffolds of Hb blended with gelatin/fibrinogen have been shown to improve O2 diffusivity and exhibit the potential to promote cardiomyogenic differentiation of mesenchymal stem cells (MSCs) [Ravichandran et al., 2013]. In a different approach, for liver tissue engineering, Hb-albumin microspheres increased hepatocyte cell viability, depending on the P50 value [Lai et al., 2015], defined as the PO2 when oxygen saturation reaches 50% in the oxygen dissociation curve. These microspheres were found to have a P50 of up to 12 mm Hg. HEMOXCell® is a product created using the extracellular Hb M201 with a P50 of 35 mm Hg at 37 °C; it has been explored in islet-cell preservation and bone reconstruction. HEMOXCell has shown the ability to reduce cell hypoxia while restoring functions in encapsulated islet cells in an in vitro model [Rodriguez-Brotons et al., 2016]. In a bone tissue engineering application, HEMOXCell improved oxygenation to the hypoxic regions and promoted the proliferation of human bone marrow-derived MSCs [Le Pape et al., 2018]. As discussed earlier in this review, stem cells are known to preserve their undifferentiated state and proliferate in hypoxia (<5% oxygen or PO2 <38 mm Hg). The proliferation of human bone marrow-derived MSCs likely favors a PO2 of mild hypoxia, while preserving the differentiation potential of the cells. Oxygenating biomaterials or carriers such as HEMOXCell can be used to overcome chronic hypoxia (approx. 1% PO2 or PO2 8 mm Hg) and sustain a mild hypoxic environment. Thus, it is important to study the oxygen tensions required to preserve function and differentiation potential during proliferation in all types of cells.

Oxygen-Generating Biomaterials

As an alternative to chemical or enzymatic means of oxygen generation and delivery, oxygen-generating approaches have been formulated to meet tissue-specific oxygen demands. Oxygen-generating chemicals, like sodium percarbonate, calcium peroxide, magnesium peroxide, and hydrogen peroxide, are decomposed in the biological environment to produce oxygen and, in some cases, byproducts [Camci-Unal et al., 2013; Wang et al., 2017]. Biomaterial strategies typically incorporate an oxygen generation mechanism through these chemical species where the ability to generate oxygen is encapsulated within the scaffolds. The advantage of delivering oxygen this way is that it can be generated in situ instead of via an external source or reservoir [Camci-Unal et al., 2013]. Oxygen-generating biomaterials provide flexibility and can be used in the form of microspheres [Steg et al., 2017], scaffolds [Coronel et al., 2017], films [Harrison et al., 2007], and electrospun nanofiber mats [Wang et al., 2011].

Recent tissue engineering-based reports have revealed the potential benefits of an oxygen generation approach. One such approach is achievable by calcium peroxide hydrolysis that forms hydrogen peroxide, which, in turn, reacts with water to form oxygen in the biological environment [Huang et al., 2016]. Coronel et al. [2017] introduced OxySite®; generating oxygen in situ through calcium peroxide hydrolytic decomposition, and showed its ability to mitigate anaerobic glycolysis and also preserve and stimulate insulin release in rat pancreatic islet culture (an oxygen gradient of 0.8 × 10−4 mm [0.1 mm Hg PO2] on the cell side to 0.3 mm [214 mm Hg PO2] in OxySite). In a similar approach, calcium peroxide particle-embedded silk fibroin scaffolds (a PO2 saturation of 10–11 mm Hg over 21 days) were shown to enhance repair in a dog urethra model, suggesting potential for application in urinary tract reconstruction [Lv et al., 2016]. Finally, the beneficial effects of a poly(trimethylene carbonate) matrix with calcium peroxide particles (PTMC/CaO2 0.05–
prefer this oxygen microenvironment to maintain their about 75–100 mm Hg; it can thus be concluded that CDCs gery. Normal arterial and ventricular oxygen tensions are lar biopsy specimens of patients undergoing heart sur-

ertherapy after myocardial injury. Cardiosphere-derived cell (CDC) therapy after myocardial injury [Li et al., 2012]. The oxygen-releasing sys-
tem improved the survival of cells for 7 days and pre-
served their differentiation potential even under hypoxic conditions (<8 mm Hg oxygen environment). CDCs are stem cell-like phenotypes derived from atrial or ventricu-

tary biopsies of patients undergoing heart surgery. Normal arterial and ventricular oxygen tensions are about 75–100 mm Hg; it can thus be concluded that CDCs prefer this oxygen microenvironment to maintain their functions. Accordingly, strategies like H2O2-releasing microspheres and bovine liver catalase provide a platform to maintain preferred oxygen tensions to preserve the differentia-
tion potential of CDCs after myocardial injury.

As an alternative to enzyme-catalyzed oxygen gener-
ation, endoperoxide-mediated approaches have been used. Endoperoxides generate oxygen upon contact with water in biological environments through simple reorganiza-
tion reactions. Methylated pyridone-derived endoperox-

ides underwent retro-Diels-Alder reactions in an aque-
ous environment releasing high yields of oxygen with a half-life of up to 13 h [Benz et al., 2013], but a fluores-
cence-based assay was used that provided only relative measures of oxygen abundance. These molecules, in combination with vitamin C, as a singlet oxygen quench-
er, were shown to significantly improve the survival of 3T3 fibroblasts and rat smooth muscle cells when chal-

gle the hypoxic environment.

Overall, as oxygen-generating materials evolve, it is important to realize their implications for tissue micro-

vironments and understand and overcome the limita-
tions presented by each approach. The chemical compo-
sition of oxygen-generating materials, such as metal ox-
ides or metal peroxides, determines their oxygenation characteristics. Several environmental factors, such as pH, temperature, buffer conditions, and the presence or absence of catalysts or inhibitors, affect oxygen genera-
tion [Oh et al., 2009]. These challenges present an oppor-
tunity to develop the next generation of oxygen-generat-
ing materials for improved tissue engineering applica-
tions. The oxygen-generating biomaterial technologies are interesting and are still evolving as a tool for producing tissue engineering constructs. They have great potential to evolve as tunable oxygenation strategies in tissue engineering.

PFC-Based Oxygen Carriers

PFCs can readily dissolve oxygen in aqueous condi-
tions. The mechanism and kinetics of oxygen binding to PFCs and the release are different from what is found with native Hb [Guzy et al., 2005]. Solutions of native Hb show sigmoidal O2 dissociation behavior [Lowe et al., 1998] whereas colloidal suspensions of PFCs, such as perflu-

orotributylamine, perfluorodecalin, and perfluoro-octane, show a linear relationship between oxygen content and P2O [Lowe et al., 1998; Spahn, 2000; Khattak et al., 2007]. At standard temperature and pressure, the solubility of O2 in water is 2.2 mM. This value can be much higher, up to 44 mM, for PFCs like bis(perfluorobutyl)ethene, repre-
senting a 20-fold increase over O2 solubility in water alone [Lowe et al., 1998]. There is considerable interest in using PFCs as oxygen carriers in a variety of tissue engi-

neering applications and as blood substitutes [Veen and Hunt, 2015; Santiesteban et al., 2016].

Owing to their ability to carry oxygen, some PFCs in the past have been approved by the FDA as blood substi-
tute products for specific applications. These products include Fluosol emulsions and perfluorodecalin emulsi-

fied primarily with the synthetic poloxamer Pluronic F-68, both of which were approved for use in coronary artery balloon angioplasty and liquid ventilation proce-
dures [Waxman, 1986]. Despite FDA approval, the pub-
lished literature demonstrates a limited understanding of biological interactions of PFCs, and not all PFCs have been studied for short- and long-term toxicity responses. One study showed a PFC emulsion of perfluorotributyl-
amine (FC-43) to be biologically inert and nontoxic and did not produce toxic metabolic products in rats [Chubb, 1985].

A few reports show that nonimmobilized, free PFCs, which are highly lipophilic, tend to exhibit toxic responses to cells and inhibit growth. However, this is contra-
dicted by the in vitro cell toxicity response observed by Khattak et al. [2007] when using >1% of the PFC, per-
fluoroocanoylbromide (PFOB), which is more lipophilic than FC-43. The toxicity most likely arose from the fact that they utilized a physical binding process that lacked stability over time, allowing the individual PFOB mole-

cules to dissociate and then diffuse into cells where they

0.1 mg/L; 0.55–1.7 mm Hg P2O above control) showed stepwise release of oxygen for up to 20 days. This biomati-
terial showed beneficial maintenance of human (h)MSCs under hypoxic conditions, which was confirmed by the increased mitochondrial activity and enhanced prolifera-
tion [Steg et al., 2017].
Oxygen in Development and Tissue Engineering

The wound-tissue oxygen microenvironment has been previously studied by researchers, and nonhealing chronic wounds can exhibit a $P_{O_2}$ of as low as 5 mm Hg, compared to healthy skin with a $P_{O_2}$ of 10–40 mm Hg [Mutluoglu et al., 2013]. Importantly, vital wound-healing processes such as collagen synthesis, angiogenesis, and epithelialization require local oxygen bioavailability with a $P_{O_2}$ ranging from 25 to 100 mm Hg [Tuderman et al., 1977; Edwards et al., 1984]. Biomaterial strategies such as MACF can be used to address this oxygen tension gap in chronic and healthy wound tissues to enhance wound-healing. However, the later stages of wound-healing and angiogenesis require oxygen to support cell proliferation and tissue growth. Thus, understanding the relationship between the effects of oxygen availability during the steps of angiogenesis, and modulating them by using biomaterials with different oxygenation potentials temporally, could provide a next-generation solution to the long-standing problem of tissue-engineered scaffold vascularization. In a tissue engineering study with the MACF materials described above, Li et al. [2014] encapsulated neural stem progenitor cells (NSPCs) in the 3 types of MACF hydrogels and studied the local oxygen concentrations and cellular responses. This study demonstrated that the oxygen concentration of MACF in a 3D hydrogel could be tuned based on the fluorine moieties, and oxygen tensions were higher (and the gradient less severe) when using the 3 available MACF formulations. At the center of the cell seed gels, the following oxygen tensions were reported: aromatic PFC (Ar5), 124 mm Hg, aliphatic short-chain (Ali5), 121 mm Hg, and aliphatic long-chain (Ali15), 130 mm Hg. Thus, the highest oxygen levels were seen when using the PFOC-modified version, which contained the most fluorines per substitution. Furthermore, the local oxygen concentration in MACF hydrogels leads to enhanced NSPC cell proliferation and neuronal differentiation, suggesting that the measured oxygen tensions translate to enhanced cellular functions. The role of oxygen tension in NSPC maintenance and fate decisions is supported by other studies, including one that demonstrated that, under high $O_2$ conditions (21% oxygen or 160 mm Hg $P_{O_2}$), NSPCs tend to prefer neuronal differentiation, and under low $O_2$ conditions (2%, 15 mm Hg $P_{O_2}$), they differentiate into glial cells [Xie et al., 2014]. Early populations of NSPCs expand their populations by self-renewal and proliferation at low oxygen tensions (1–5% oxygen or 8–38 mm Hg $P_{O_2}$) [Clarke and van der Kooy, 2009]. Thus, for neural tissue engineering, it is important to understand the stage of stem cell differentiation, lineage commitment, and tissue development,
and to utilize the appropriate oxygenating material strategy.

In similar approaches, other groups have immobilized linear as well as branched PFCs to hyaluronic acid (HA) [Palumbo et al., 2014], chitosan, and alginate [Gattas-Asfura et al., 2012], and showed benefits in an in vitro culture of mammalian or human cells. More researchers are now exploring the potential to immobilize PFCs on biomaterials in tissue engineering. In another recent study, 3-pentadecafluoroheptyl,5-perfluorophenyl-1,2,4-oxadiazole (FOX) molecules were immobilized to graphene oxide to form nanoplatforms [Maio et al., 2018]. These nanoplatforms can act as oxygen reservoirs, and at a dissolved concentration of 0.33 mg/mL, they show transient release of 431.0 ± 0.5 mm Hg oxygen over 200 s at 37°C. Even at low concentrations, the nanoplatforms demonstrated a high oxygen content at saturation and a high diffusion rate compared to the materials currently used as O2 reservoirs in tissue engineering or regenerative medicine. Another interesting approach for delivering oxygen in vitro and in vivo for diagnosis and treatment applications is using nanoscale technologies such as oxygen reservoir nanodroplets. Oxygen reservoirs created by PFC nanodroplets have been used to overcome tumor hypoxia to enhance cancer radiotherapy (RT). Polyethylene glycol (PEG) stabilized PFC nanodroplets were decorated with TaOx nanoparticles (TaOx@PFC-PEG) to form a multifunctional radiotherapy sensitizer to enhance the effectiveness of radiotherapy against tumors [Song et al., 2014]. TaOx@PFC-PEG nanodroplets with 20% (v/v) perfluorohexane increased tissue oxygenation by releasing 443 mm Hg P O2 dissolved oxygen. As a result, enhanced in vivo RT treatment efficacy was realized using TaOx@PFC-PEG as a multifunctional radiosensitizer, which could not only concentrate the effective irradiation dose inside the tumor but was also able to overcome hypoxia-associated radio-resistance.

Importantly, the formulation of PFCs plays an essential role in limiting or eliminating toxicity concerns. As we briefly discussed covalent immobilization, encapsulation in the biorthogonal/biocompatible matrices, coating emulsion droplets with biopolymers/lipids allow for the stabilization of PFCs and can drastically reduce bioaccumulation and toxicity. Proper toxicological studies are yet to be performed for some of the most promising compounds and strategies; this must occur before any clinical translation can ensue [Serex et al., 2014; Takahashi et al., 2014]. Thus, newer formulation strategies can potentially open doors for the application of PFC-based oxygen delivery systems in tissue engineering.

**Technological Advances in Oxygen Delivery**

Recently, novel technology has made new oxygen delivery applications possible. Techniques have evolved as an independent oxygen delivery class and are of importance considering their potential for application in tissue engineering. As summarized in Table 1, emerging technologies such as photosynthetic algae, myoglobin-polymer-surfactant complexes, microtanks, and endoperoxides show potential for supplemental oxygenating solutions [Farris et al., 2016]. Photosynthesis is plants’ preferred means to generate energy, and a byproduct of this process is oxygen. This concept was recently utilized to create oxygenating biomaterials using photosynthetic algae. Chávez et al. [2016] have shown that photosynthetic biomaterials can produce and provide oxygen (saturation P O2 ≥400 mm Hg; 18.0 mg/L) independently of blood perfusion by generating chimeric animal-plant tissues during dermal regeneration. They demonstrated the safety and efficacy of photosynthetic biomaterials in vivo after engraftment in a fully immunocompetent mouse skin defect model. In addition to oxygen generation, photosynthetic microalgae can be genetically modified to express the angiogenic recombinant protein VEGF, demonstrating its use as a versatile platform for photosynthetic biomaterials for tissue engineering.

The creation of a membrane-binding complex of myoglobin and an anionic polymer-surfactant is another such recently introduced and versatile system. Myoglobin is a protein which has a similar structure to Hb and thus binds oxygen to its structure [Endeward et al., 2010]. This novel polymer complex of myoglobin was delivered to the cytoplasmic membrane of hMSCs [Armstrong et al., 2015]. Pretreating MSCs with this complex was shown to improve tissue distribution and biochemical composition of hyaline cartilage. This direct cell oxygenation avoids the size limitations imposed by diffusion-limited mass transport and should enable cartilage, bone, and cardiac tissue engineering.

Another recent “microtank” approach utilized polymeric hollow microspheres which can be hyperbarically loaded with oxygen (total O2 1.04 µmol in 8.5 h) [Cook et al., 2015]. Polycaprolactone scaffold embedded with microtanks was shown to prolong the survival of human adipose-derived stem cells and human umbilical-vein endothelial cells (HUVECs) under hypoxic conditions. The results of this study suggest that the microtank approach may be a feasible means of maintaining cell viability in tissue-engineered scaffolds during the critical period of vascularization in vivo.
Overall, these novel tools are versatile and easily adaptable to the constantly evolving field of tissue engineering and associated methodologies. Interestingly, no recent reports of expansion of these technologies in tissue engineering applications were found. They bear great potential and hence must be explored in various tissue engineering applications, and perhaps eventually be extended to human studies.

**Oxygenating Strategies in Spheroids and Organoids**

Spheroids and organoids have recently been introduced as a 3D approach in tissue engineering as an attempt to better relate the results of in vitro studies to in vivo conditions. Spheroids and organoids are also potential tools to help improve the biological relevance of in vitro model platforms to human medicine [Fennema et al., 2013]. Spheroids (or multicellular aggregates) are building blocks of “microtissues” with a controllable composition and tailorable biological properties [Mironov et al., 2009]. Spheroids owe their diverse applications in tissue engineering to the 3D microenvironment they provide for cells, and they have been applied to many different cell types, such as stem cells, hepatocytes, and neuronal cells [Fang and Eglen, 2017]. In a 3D environment, cells are subject to a heterogeneous spatial distribution of oxygen diffusion and nutrients. Spheroids are great candidates for mimicking these physiologic in vivo conditions towards forming complex organ-like structures, and they offer the ability to use human cells to reproduce better and study human responses [Laschke and Menger, 2017].

Despite all the advantages of spheroids for tissue engineering, their application is often challenged by insufficient oxygen diffusion, especially in their cores (Fig. 4). The lack of vasculature in spheroids, which triggers the formation of HIFs, often results in cell apoptosis in the central oxygen and nutrient-deficient regions in prolonged cases of oxygen deficiency [Laschke and Menger, 2017; Lazzari et al., 2017]. To overcome insufficient oxygen diffusion and the formation of hypoxic cores, especially in cells with high metabolic activity, several in vitro strategies have been developed to prevent necrosis and improve spheroid functionality via direct oxygen delivery [Lou and Leung, 2018]. In line with this approach, Anada et al. [2012] utilized 3D culture chips made of gas-permeable polydimethylsiloxane (PDMS) to enable a direct oxygen supply to the cells within their spheroids, while maintaining cell viability and function with an equilibrium oxygen tension of 50–60 mm Hg P<sub>O<sub>2</sub> in the culture media after 5 days. Their histochemical analysis showed a significant reduction in hypoxic core formation.
In a different approach, Kamoya et al. [2016] developed an oxygen-permeable spheroid culture chip (Oxy chip) to enable direct oxygen delivery to murine MSCs. They observed a P\textsubscript{O}2 of 130 mm Hg in their culture system after 7 days using these chips versus a decreased to 70 mm Hg in non-Oxy chip controls. The oxygen delivery was also shown to assist the differentiation of MSCs into osteoblasts. Furthermore, spheroids cultured on the Oxy chip show viable nucleated cores whereas cell necrosis was observed at the center of the non-Oxy chip controls. Similarly, Pedraza et al. [2012] designed a PDMS-encapsulated solid calcium peroxide system (PDMS-Ca\textsubscript{O}2) as an oxygenating biomaterial to successfully overcome hypoxia-induced spheroid necrosis in islet spheroids. They showed that a single PDMS-Ca\textsubscript{O}2 disk could improve the oxygen concentration in the culture solution from 0.16 ± 0.017 mM (approx. 114 mm Hg P\textsubscript{O}2) in the first week to an average of 0.073 ± 0.007 mM (approx. 52 mm Hg P\textsubscript{O}2) over baseline controls of PDMS-only disks over 4 weeks. The enhanced and prolonged proliferation of β cells was observed when cocultured with their PDMS-Ca\textsubscript{O}2 disks.

In an attempt to assess the oxygen tension profile within a spheroid system made of MSCs, Murphy et al. [2017] utilized oxygen-sensitive microelectrodes to measure the oxygen tension as a function of spheroid diameter, and correlated this with the existence of a hypoxic core. In their largest spheroids, containing 60,000 cells, the data showed a 10% decrease in oxygen gradients while moving from the outer spheroid diameter towards the inside core. In spite of low radial oxygen fluctuations, the cell metabolism decreased with increasing spheroid size, possibly due to the adaptive changes in matrix deposition and the packing density of the spheroids. These observations indicate that the function of MSC spheroids and their hypoxic cores are not oxygen-dependent.

Moving one step closer towards mimicking the actual properties of tissues, organoids have been introduced as an approach for culturing small fetal or adult organ-like structures for follow-on in vitro study or for in vivo implantation [Lou and Leung, 2018]. An organoid is defined as a group of organ-specific cells that are developed from stem cells or organ progenitors [Fang and Eglen, 2017]. Organoids hold promise for applications such as organ replacement, modeling of many diseases, drug discovery, and safety screening studies [Hu et al., 2018] (Fig. 4). So far, organoids have been formed for intestinal, stomach, liver, kidney, brain, and retinal tissue replacement [Xinarios et al., 2015]. However, the current organoid technologies face significant challenges, including managing the various cell types in each specific organoid system, while providing the appropriate extracellular matrix and improving the oxygen and nutrient requirements for each particular cell type within the same organoid [Lou and Leung, 2018]. In addition, oxygen diffusion issues lead to the formation of a necrotic core at the center of the organoid, preventing the normal development of the structure [Kelava and Lancaster, 2016]. Considering that the size of a cultured organoid depends highly on the maximum diffusion distance of nutrients, and oxygen in particular, tackling the oxygen diffusion challenge could result in significant advances in organoid approaches [Akkerman and Defize, 2017].

Bioreactor-Based Strategies and Microfluidics in Organoid Development

A straightforward strategy to enhance oxygen perfusion within an organoid system is to use higher oxygen levels in a culture setup [Akkerman and Defize, 2017]. However, this approach might cause severe toxicity if not appropriately controlled for oxygen radical formation [Halliwell and Gutteridge, 1984]. Another novel approach to tackle oxygen diffusion is to use spinning bioreactors to provide a driving force for diffusion. To this end, Qian et al. [2016] developed a spinning bioreactor and successfully cultured forebrain-specific organoids originated from human induced pluripotent stem cells (PSCs).

In a different approach, continuous perfusion bioreactors have been used to maintain the long-term viability of hepatocyte spheroids in a silicon wafer with an array of channels and cell adhesive walls. Bioreactor dimensions, with a channel width of 300 µm, were designed, such that the perfusion rate of 8.2 × 10\textsuperscript{–8} mol/cm\textsuperscript{3}/s was achieved at the expected rate of hepatocyte oxygen consumption (3.5 × 10\textsuperscript{–8} mol/cm\textsuperscript{3}/s). The results indicated long-term maintenance of the spheroids and the corresponding formation of tissue structures [Powers et al., 2002]. Perfusion bioreactors have also been used to develop intestinal [Kim et al., 2007], skeletal muscle [Chmiak et al., 1998], heart [Maidhof et al., 2012], lung [Ott et al., 2010], and liver [Baptista et al., 2011] organoids.

Recently, DiStefano et al. [2018] developed a rotating-wall vessel bioreactor to culture PSCs and differentiate them into 3D retinal organoids. They showed enhanced proliferation as well as the well-defined differentiation of these cells into neurons and S-cone photoreceptors. Miniaturized spinning bioreactors have also been used to develop region-specific brain organoids derived from PSCs [Qian et al., 2018]. Despite the lack of vasculature, these multiwell, spinning bioreactors have been able to success-
fully produce fore-brain, midbrain, and hypothalamus organoids over a period of 14–84 days, indicating an efficient regime of oxygen and nutrient transport.

Despite the advantages of spinning bioreactors, developing larger tissue structures needs more enhanced oxygen diffusion, especially because organoids cannot grow their own vasculature. To address this issue, microfluidics and bioprinting approaches are currently studying how artificial vessels can be added to tissue-engineered scaffolds to reduce oxygen/nutrient deficiencies in organoid cultures, thus allowing for the formation of larger tissue constructs [Zhang et al., 2017]. For example, Zhang et al. [2016] used a composite bio-ink to coprint endothelial cells inside a microfiber hydrogel scaffold. The fluorescent images of their multilayered scaffold cross-sections showed that the incorporated HUVECs were able to form a tubular pattern similar to that of the walls of blood vessels. Furthermore, they successfully produced an endothelialized myocardium organoid with controlled anisotropy, which indicated enhanced oxygen transport throughout the scaffold. To make an enhanced platform for cardiac toxicity studies, researchers have used microfluidics technology to form an endothelialized-myocardium-on-a-chip using a perfusion bioreactor. The design of their bioreactor was such that the perfusion rate of 50 µL/min provided a situation where an endothelialized myocardial construct could experience an oxygen concentration of 0.12 mM (approx. 85 mm Hg PO2) throughout the scaffold.

Future Directions and Concluding Remarks

In summary, variations in oxygen bioavailability affect embryonic development starting from the very early stages of blastocyst formation to morphogenesis and organogenesis, and then continuing to be important during angiogenesis and into adulthood. Oxygen contributes to development by signaling the processes of proliferation, differentiation, angiogenesis, morphogenesis, and organogenesis as well as providing the energy required via anaerobic glycolysis. Different levels of oxygen are required at each stage of embryonic development, and so an understanding of the specific in vivo microenvironment, as well as the levels of oxygen present at each stage during normal fetal development, helps instruct the environments needed for tissue engineering approaches where the fine-tuning of cell proliferation and differentiation are essential. These mostly in vitro tissue engineering approaches are a response to diseases and injuries after birth, where the formed embryonic tissues and organs might require repair or replacement. Tissue engineering applies the principles of normal development for the sake of secondary tissue development. Therefore, any success in the field of tissue engineering relies on a thorough understanding of the factors involved in the formation of new tissues during development. One of these major factors, the availability of oxygen during development and tissue formation in a growing embryo, provides clues as to how this process can be mimicked in a tissue engineering setting, and can also aid the development of new technologies, such as organoids, to better mimic native tissue microenvironments.

It is worth stating that the findings discussed here highlight that the field lacks agreement about accurate and reliable technologies for measuring the levels of oxygen both in vitro and in vivo. This is especially important at each stage of embryonic development and could translate to direct design criteria to satisfy the specific requirements of adult tissues to form appropriate tissue-engineered structures. This would also provide a direct means to recognize the amount of oxygen needed in vitro to form organ-like structures.

Angiogenesis occurs in response to insufficient levels of oxygen as required by a developing tissue or organ in vivo. However, in a tissue-engineered construct or organoid/spheroid, the lack of a vasculature component makes it difficult to entirely mimic the normal developmental process in vitro, and thus inhibits the growth of any newly formed tissue after a certain point. At such a tipping point, a supplemental oxygenating system might be beneficial to support the oxygen requirements to allow further growth and enhance ultimate functions. In this regard, oxygen-releasing biomaterials have recently emerged to help overcome the challenge of an inadequate oxygen supply within a tissue-engineered structure or organoid. Thus, expanding the research into engineered oxygen carriers will present valuable new tools to overcome this challenge. These approaches are still in their infancy as oxygen-specific design criteria for tissue engineering, and organoid systems are still being formulated, while new technologies are constantly emerging and evolving to meet all of these requirements.

Statement of Ethics

The authors have no ethical issues to disclose.
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