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In Vitro Culturing of Adult Stem Cells: The Importance of Serum and Atmospheric Oxygen

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Abstract

Adult stem cells are undifferentiated cells found in many different tissues in the adult human and animal body and are thought to be important for replacing damaged and dead cells during life. Due to their differentiation abilities, they have significant potential for regeneration and consequently therapeutic potential in various medical conditions. Studies on in vitro cultivation of different types of adult stem cells have shown that they have specific requirements for optimal proliferation and stemness maintenance as well as induced differentiation. The main factors affecting the success of stem cell cultivation are the composition of the growth medium, including the presence of serum, temperature, humidity, and contact with other cells and the composition of the atmosphere in which the cells grow. In this chapter, we review the literature and describe our own experience regarding the influence of the presence of fetal bovine serum in the medium and the oxygen concentration in the atmosphere on the stemness maintenance and survival of adult stem cells from various tissue sources such as adipose tissue, muscle, brain, and testicular tissue.

Keywords

Adipose tissue · Atmospheric oxygen · Brain · Culturing · Differentiation · Muscle · Serum · Stem cells · Stemness · Testes

Abbreviations

ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
DMEM	Dulbecco's modified Eagle's medium
EGF	epidermal growth factor
FBS	fetal bovine serum
FGF	fibroblast growth factor
GDNF	glial cell line-derived neurotrophic factor
NGF	nerve growth factor

1 Introduction

Stem cells have enormous potential in health and medical research due to their regenerative potential. Different types of adult stem cells can be

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isolated from different tissues. These cells are multipotent, meaning that they are capable of differentiating into more than one cell type, but not all cell types (Fortier 2005). Mesenchymal stem cells, a special type of adult stem cells, have become increasingly important in the field of regenerative medicine in recent years for treating certain human diseases (Giordano et al. 2007; Trounson et al. 2011; Jossen et al. 2014). They are being investigated for their therapeutic potential in inflammatory, autoimmune, and degenerative conditions in preclinical and clinical studies (Inamadar and Inamadar 2013; Ratcliffe et al. 2013).

Species and strain variations in the properties of adult stem cells from different tissues and their requirements for optimal growth have been reported in numerous publications although in general, stem cells are difficult to isolate and maintain in vitro (Baddoo et al. 2003; Peister et al. 2004; Sung et al. 2008). In addition to growth medium composition and incubation temperature, partial oxygen pressure, extracellular matrix proteins, and contacts with other cells are known factors that affect stem cell viability, proliferation, function, and differentiation (Yoshida et al. 2009). In this review, we describe the importance of serum in the culture medium and the role of atmospheric oxygen in the growth and differentiation of adult stem cells from various sources. A broad literature review is accompanied by our own studies.

2 Sources of Adult Stem Cells

Mesenchymal stem cells, also called multipotent mesenchymal stromal cells, are found in adipose tissue, bone marrow, umbilical cord, and dental pulp (Klingemann et al. 2008). Muscles contain skeletal muscle stem cells and hematopoietic stem cells (Kawada 2001; Chen and Goldhamer 2003). Spermatogonial stem cells can be isolated from testes (Goossens and Tournaye 2006; Guan et al. 2006). Adult stem cells in the mammalian brain (neural stem cells) were discovered much later than in other tissues (Altman and Das 1965; Altman 1969), and it has been shown that these neural stem cells can differentiate into neurons and glia cells in vitro (Reynolds and Weiss 1992; Ma et al. 2009). Neural stem cells, mesenchymal stem cells, muscle stem cells, epidermal stem cells, and some others meet the basic criteria for stem cells (Prockop 1997; Gage 2000; Watt 2001) as they can proliferate and differentiate into various tissues in vitro, whereas corneal stem cells and endothelial stem cells are only capable of differentiating into a single type of differentiated cell (Daniels et al. 2001; Verfaillie 2002). The most studied adult stem cells are the hematopoietic stem cells, while other adult stem cells were defined much later and are therefore less studied (Verfaillie 2002). In this chapter, we focus on adipose-derived, muscle, neural, and spermatogonial stem cells.

2.1 Adipose Tissue-Derived Stem Cells

Adipose tissue is a rich source of mesenchymal adipose tissue-derived stem cells (Zuk et al. 2010), which can differentiate into mesodermal cells such as osteoblasts, chondrocytes, adipocytes, and muscle cells (Zuk et al. 2002; Lee et al. 2004; Guilak et al. 2004; Lin et al. 2008). Therefore, these cells have an important potential for cell therapy and are used for immunomodulation in pathologies such as Crohn's disease, regenerative medicine, and aesthetic medicine (Le Blanc and Ringden 2007; Ringden et al. 2007; Abdi et al. 2008; Mirotsou et al. 2011). Adipose tissue-derived stem cells offer several advantages over other cell types. Adipose tissue is easily accessible, requiring minimally invasive surgery for its harvesting. In addition, adipose tissue contains many more progenitor cells in comparison to other tissues (e.g., bone marrow) (Strem et al. 2005). Adipose-derived stem cells also have great expansion potential (Lee et al. 2004; Kern et al. 2006) which is important for cell therapies.

2.2 Muscle Stem Cells

Muscle stem cells belong to the satellite cell population and are responsible for skeletal muscle

growth and repair. They reside between muscle fibers within the basal lamina but outside the muscle fiber (Montano 2014). Studies of muscle stem cells play an important role in the development of novel treatments for muscular disorders (Pomerantz and Blau 2008; Wang et al. 2014; Relaix et al. 2021). However, these cells are difficult to isolate and purify, so alternative myogenic stem cells, including adipose-, bone marrow-, and umbilical cord-derived mesenchymal stem cells, as well as perivascular stem cells, are being investigated for their potential as possible cell sources for treating muscle disorders (Pantelic and Larkin 2018).

2.3 Testicular Stem Cells

Spermatogonial stem cells reside inside the seminiferous tubules within the testes. They are capable of self-renewal and producing daughter cells to give rise to terminally differentiated cells, the spermatozoa. These cells are therefore responsible for the lifelong maintenance of spermatogenesis (Nagano 2003; Kubota and Brinster 2018). Since the number of spermatogonial stem cells decreases with aging, aging of their niche is a critical factor for the maintenance of these cells. Dysfunction of the niche leads to a decreased number of spermatogonial stem cells in older men (Zhang et al. 2006). Spermatogenesis can be impaired due to various congenital disorders, resulting in male infertility (Matzuk and Lamb 2008). Spermatogonial transplantation can be used to restore fertility in infertile men and to elucidate the mechanism of genetic defects in spermatogenesis (Shinohara et al. 2000).

2.4 Neural Stem Cells

The adult mammalian brain has a low regenerative capacity and is limited in its ability to replace neurons that become dysfunctional or atrophic due to acute or chronic injury. The discovery of neural stem cells opened the possibility of harnessing them for endogenous brain repair (Gage 2019). Neural stem cells are not distributed throughout the brain but are found in specific locations, especially in the ventricular-subventricular zone along the walls of the lateral ventricles and in the subgranular zone of the dentate gyrus of the hippocampus (Alvarez-Buylla and Lim 2004; Ma et al. 2005; Obernier and Alvarez-Buylla 2019). Lower number of neural stem cells is also found in the striatum, septum, and spinal cord (Palmer et al. 1995; Weiss et al. 1996b). Furthermore, the subcallosal zone, located between the white matter and the hippocampus, has also been reported to contain adult neural stem cells (Kim et al. 2016). The generation of neurons for cell therapy is promising for the treatment of neurodegenerative diseases and brain injuries.

3 The Effect of Serum on Stem Cell Growth

For the successful proliferation of adult stem cells in vitro and the maintenance of their stemness, the balance of nutrients and microenvironmental conditions is of paramount importance. The culture medium is one of the most important single factors in cell culture as it provides all essential nutrients (Butler and Jenkins 1989; Brunner et al. 2010). Serum is the most commonly used supplement in cell culture. Fetal bovine serum (FBS) is a common choice because it contains high concentrations of growth factors and other important signaling molecules such as adhesion proteins, nutrients, carrier proteins, cytokines, and hormones that, along with its buffering capacities, are required for cell survival, proliferation, and/or differentiation. FBS can be added to the medium in varying amounts from 1% to 20% to promote cell attachment and provide growth factors and vital nutrients (Harmouch et al. 2013; Forcales 2015). However, the use of FBS in cell culture can be problematic as FBS contains xenogeneic proteins and potentially pathogenic microorganisms, which presents a risk for the induction of an immunological response and the transmission of pathogens. FBS also poses a problem for optimizing cell culture conditions because its composition is not defined and

exhibits batch-to-batch variability (Heiskanen et al. 2007; Sundin et al. 2007). On the other hand, defined serum-free media have an accurate composition but are often less successful in supporting cell growth than media containing FBS (Jossen et al. 2014). This was also confirmed in our study as various adult stem cells from BALB/c mice grew significantly better in the FBS-containing media. Since serum deprivation can slow or even stop stem cell proliferation and increase cell death (Hasan et al. 1999; Cooper 2003; Shin et al. 2008), growth media should be supplemented with FBS for at least a defined period of time until stem cells reach confluence (Nonnis et al. 2016). In most cases, stem cells have been successfully cultured in FBS with no serious side effects reported (Le 2003; Berger et al. 2006; Mannello and Tonti 2007). Alternatives to FBS, including autologous or allogeneic serum, platelet lysate, and thrombin-activated platelet-rich plasma, are also in use in clinical treatments to avoid the aforementioned drawbacks of FBS (Duggal and Brinchmann 2011). In our laboratory, we performed a direct comparison between two media, commercial MesenCult medium and newly developed A20 medium, based on DMEM and containing 20% FBS.

3.1 Muscle Stem Cells

Several studies confirmed that muscle stem cells require high concentrations of FBS for their optimal growth. Most often, they are grown in medium containing 20% FBS and a high glucose content (Motohashi et al. 2014; Syverud et al. 2015; Mozzetta 2016; Čamernik et al. 2019; Boscolo Sesillo et al. 2020). Also in our laboratory, adult stem cells isolated from the muscle of a BALB/c mouse and cultured in a newly developed A20 medium containing 20% FBS grew significantly better than in a commercial serum-free MesenCult medium. In A20, cell cultures reached 80% confluence in 9 days, whereas cells in a serum-free medium did not proliferate. The cells in A20 were transplanted in several additional passages where they successfully proliferated.

3.2 Adipose Tissue-Derived Stem Cells

Like adult muscle stem cells, adipose tissue-derived mesenchymal stem cells grow best in medium containing FBS. Mouse, rat, pig, and human mesenchymal stem cells from adipose tissue are most commonly cultured in medium containing 5–15% FBS (Yamamoto et al. 2007; Arana et al. 2013; Alstrup et al. 2018). The importance of FBS in the cultivation of adipose-derived stem cells was confirmed in our laboratory. We tested the growth of adipose-derived stem cells from the BALB/c mouse in MesenCult medium without FBS and found a very slow growth and early loss of stemness. Interestingly, adipose tissue-derived stem cells also proliferated very slowly in A20 medium supplemented with FBS until hypoxic atmospheric conditions were applied. Our results point to an important fact that not only serum and media content but also atmospheric conditions play an important role in the cultivation of adult stem cells, which will be discussed in further sections.

3.3 Testicular Stem Cells

Long-term cultivation of murine spermatogonial stem cells in vitro is difficult due to low survival rate. Typically, only 10–20% of cells survive after 1 week in a culture. Medium supplements such as cytokines and growth factors, as well as serum (1–10%) are used to achieve greater proliferation over longer periods of time (Nagano et al. 1998; Kanatsu-Shinohara et al. 2003). We compared the efficiency of culturing spermatogonial stem cells from the BALB/c mouse in serum-free medium MesenCult and in A20 medium with FBS. The importance of FBS was confirmed as proliferation of cells in serum-free medium was slow and unsuccessful (Fig. 1). On the other hand, spermatogonial stem cells were efficiently cultured in A20 medium with 20% FBS, reaching 80% confluence around day nine after isolation. The cells were transplanted and grew successfully in the first passage as well.

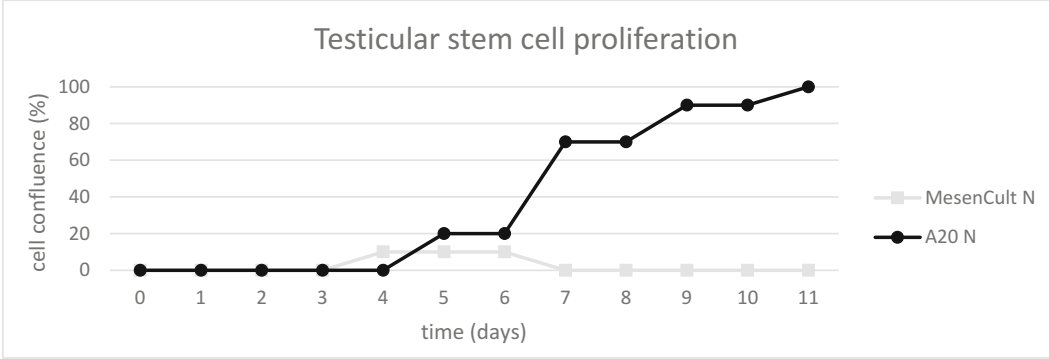

Fig. 1 Testicular stem cell growth in initial passage using serum-free MesenCult medium and A20 medium with 20% FBS. Legend: N – normoxia

3.4 Neural Stem Cells

Standard methods for culturing neural stem cells were developed in the early 1990s and include the neurosphere method and adherent monolayer culture (Reynolds and Weiss 1992; Palmer et al. 1995; Ray et al. 1995). Protocols for isolation and in vitro cultivation of neural stem cells from adult mouse whole brain in the form of neurospheres or monolayers use a serum-free medium supplemented with growth factors like epidermal growth factor and fibroblast growth factor, which are needed for the survival of cells (Walker and Kempermann 2014; Deshpande et al. 2019). However, in our study, very few neural stem cells were viable when cultured in a serum-free MesenCult medium, and they did not proliferate. Greater cultivation potential was achieved when FBS was available to the cells. Neural stem cells from all three brain regions (anterior, medial, and posterior) proliferated well in A20 medium (Fig. 2). However, we observed a much slower cell proliferation in comparison to other stem cell types. It took 2 weeks for the neural stem cells to become about 80% confluent. They were then transplanted into the next passage, where they continued to proliferate successfully.

4 The Effect of Hypoxia on Stem Cell Growth

Standard cell culture systems typically use environmental oxygen levels (20%) although the actual oxygen content in tissues is much lower. Interestingly, improved stem cell survival and reduced apoptosis have been reported when using low-oxygen partial pressure (Morrison et al. 2000; Studer et al. 2000). Several previous studies have shown improvement of stem cell culture when cells were grown in oxygen concentrations below 10% (Guyton and Hall 1996; Carreau et al. 2011). This is believed to better simulate in vivo conditions as oxygen concentrations in the brain have been reported to be around 0.5% in the midbrain, 2–5% in the cortex, and up to 8% in the pia mater (Mannello et al. 2011), about 3.8% in muscle (Carreau et al. 2011), and about 3% in the testes (Klotz et al. 1996). Interestingly, the oxygen concentration in adipose tissue varies from 4.5% to 5% in lean mice to about 1–2% in obese mice (Ye et al. 2007; Rausch et al. 2008; Netzer et al. 2015). It has been suggested that local oxygen concentration may directly affect stem cell proliferation, self-renewal, and differentiation. Stem cells seem to benefit from residing in hypoxic niches where oxidative DNA damage can be reduced (Keith and Simon 2007). When stem cells are cultured at oxygen concentrations that do not match those inside the niche microenvironment, cells undergo a number of changes such as metabolic turnover, oxidative stress, impaired motility, altered differentiation potential, and loss of stemness potential (Mas-Bargues et al. 2019).

When cells are cultured at low oxygen concentration, any available oxygen diffuses into the mitochondria, creating a hypoxic environment in

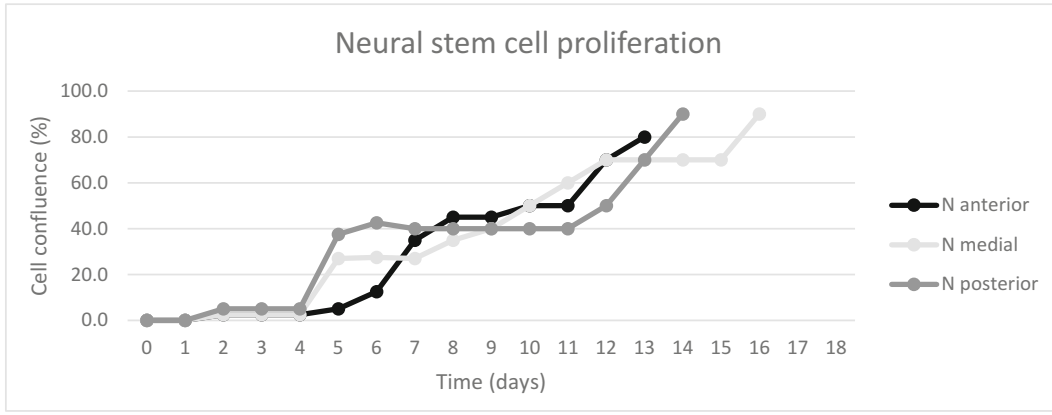

Fig. 2 Proliferation of neural stem cells from different parts using A20 medium with 20% FBS. Legend: N – normoxia

the cytosol. Hypoxia inhibits the activity of prolyl hydroxylases that regulate the activation of hypoxia-inducible factors. The original role of hypoxia-inducible factors, angiogenesis, has recently been expanded by new studies showing that they are also involved in self-renewal, stemness, and differentiation of stem cells. Under hypoxic conditions, hypoxia-induced factors are not hydroxylated and are thus stabilized to initiate their transcriptional activity (Bell and Chandel 2007). Hypoxia-induced factors are also important for the regulation of stem cell metabolism. Cells from hypoxic niches rely on anaerobic glycolysis to support ATP production. When exposed to atmospheric oxygen levels, cells are forced to decrease glycolysis and increase oxygen consumption through mitochondrial oxidative phosphorylation. This metabolic switch affects cellular function as it promotes senescence, genomic instability, and shortening life span (Estrada et al. 2012).

In our laboratory we compared the growth of adult stem cells from the BALB/c mouse in a serum-free and a serum-supplemented medium in normoxic (5% carbon dioxide, 20% oxygen, 75% nitrogen) and hypoxic atmospheres (5% carbon dioxide, 2% oxygen, 93% nitrogen). Cells from testis and muscle proliferated slightly better under hypoxic conditions when grown in MesenCult medium, but cells from adipose tissue grew only under normoxic conditions with this medium, yet not as successfully as in A20 medium with FBS. Cells from all tissues (adipose tissue, muscle, testes, and brain) grew much better in A20 medium than in MesenCult medium regardless of atmospheric conditions. However, for cells grown in A20 medium, atmospheric conditions affected only proliferation of cells from adipose tissue and brain, while cells from testis and muscle tissue proliferated at similar rates under both atmospheric conditions. Interestingly, the effect of hypoxia was the opposite for neural- and adipose tissue-derived cells. Cells from adipose tissue grew better under hypoxic conditions, while neural cells grew better under normoxic conditions.

4.1 Adipose Tissue-Derived Stem Cells

Many studies have observed low proliferation rate at the environmental oxygen concentration in adipose-derived stem cells (Efimenko et al. 2011; Kim et al. 2012, Mas-Bargues et al. 2019). Our studies correspond to these findings as the isolation of adult stem cells from the adipose tissue of the BALB/c mouse using A20 medium containing FBS was successful only in the hypoxic atmosphere, with cells visible as early as 24 h after tissue plating. Cell culture reached 80–90% confluence by day 12 (Fig. 3). The cells were then transplanted and proliferated faster in the first passage than in the passage zero,

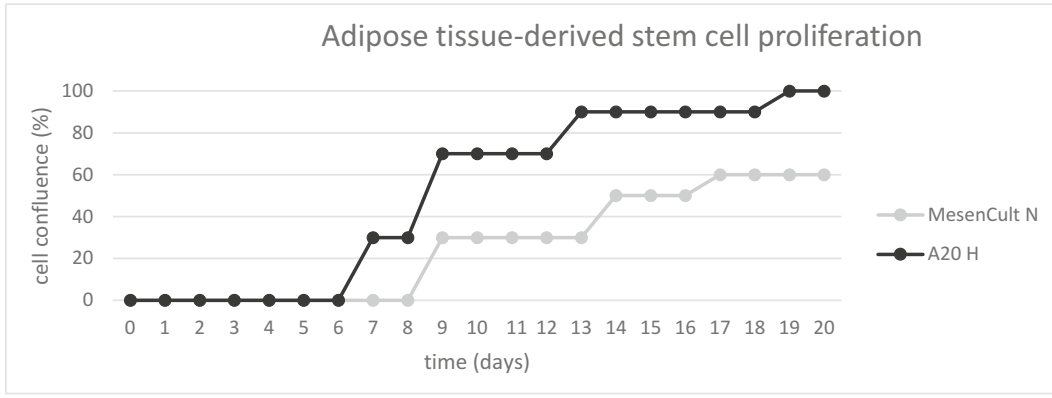

Fig. 3 Adipose tissue-derived stem cell growth curves in zero passage using MesenCult and A20 mediums. Legend: N – normoxia, H – hypoxia. Cells did not grow in A20 media in normoxic conditions and in MesenCult media in hypoxic conditions

reaching 80% confluence in 4 days. The cells were also transplanted in the second passage and reached 80% confluence in 3 days. Similar results for adipose tissue-derived stem cells have been reported previously. Hypoxia has been shown to promote stemness, proliferative capacity, and viability of adipose tissue-derived stem cells and to prevent adipogenic differentiation through negative gene regulation (Lin et al. 2006; Ye et al. 2007; Weijers et al. 2011; Yamamoto et al. 2013; Choi et al. 2014; Kakudo et al. 2015). This likely reflects the adaptation of cells to low oxygen concentrations in adipose tissue in vivo.

4.2 Muscle Stem Cells

Unsuccessful proliferation of muscle stem cells under normoxic conditions has been reported in some studies (Csete et al. 2001; Lees et al. 2008). Ambient oxygen concentration affects cell cycle regulation as p53 phosphorylation increases in cultures grown at 20% oxygen, resulting in cell cycle arrest (Chen et al. 2007). A similar effect of atmospheric conditions was observed in our laboratory. Muscle stem cell isolation and proliferation in a serum-free MesenCult medium was successful only under hypoxia (Fig. 4), with cells initially proliferating rapidly. However, their proliferation slowed considerably after reaching 50% confluence, and they never reached 80% confluence. Spontaneous differentiation occurred. On the other hand, oxygen concentration did not affect the proliferation of cells from muscles cultured in a serum-supplemented A20 medium as the cells grew at a similar rate under both normoxic and hypoxic conditions. Proliferation was successful, and cell cultures reached about 80% confluence in 9–11 days (Fig. 4). Cells were transplanted in several more passages where they proliferated successfully under both atmospheric conditions. This suggests that various factors, including growth media, affect the proliferation and self-renewal of stem cells from different tissues, and cells from different tissues show different sensitivity to oxygen concentrations. Although oxygen concentration affects proliferation of some cell types in vitro, other factors, such as media and unknown tissue-specific factors, seem to modulate the sensitivity of cells to atmospheric conditions. Since we still do not know all the factors that influence adult stem cells viability and stemness, future studies need to focus on identifying factors that influence stem cells in culture in order to develop optimal growth media and optimal atmospheric and other conditions for culturing adult stem cells from different tissues.

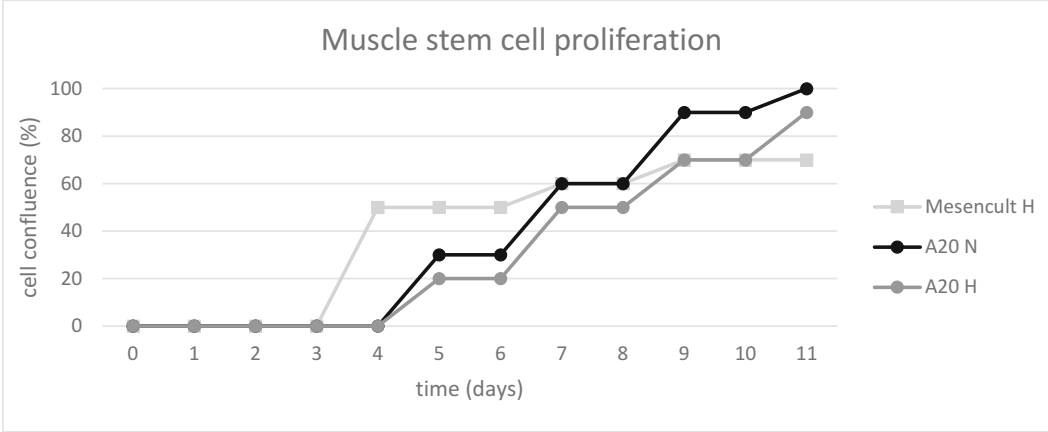

Fig. 4 Muscle stem cell growth in initial passage in both atmospheres using MesenCult and A20 mediums. Legend: N – normoxia, H – hypoxia. Cell did not grow in MesenCult media in normoxic conditions

4.3 Testicular Stem Cells

Previous studies have shown that culturing spermatogonial stem cells can be improved by reducing the atmosphere oxygen concentration. Kubota et al. (2009) and Helsel et al. (2017) used 10% of oxygen and achieved successful long-term culturing. Interestingly, however, no difference was observed in the proliferation rate under both atmospheric conditions in our laboratory. We compared the growth of adult murine spermatogonial stem cells in a serum-free and a serum-supplemented media in normoxic and hypoxic atmospheres containing 20% and 2% oxygen, respectively, and the results were similar with both media. Cells in serum-free media grew very poorly under normoxic and hypoxic conditions, whereas cells in A20 medium grew successfully regardless of atmospheric conditions (Fig. 5).

4.4 Neural Stem Cells

The effect of oxygen concentration in the atmosphere on culturing neural stem cells remains unclear as there are many controversial studies published. Some studies of culturing neural stem cells report that ambient oxygen concentration decreases their proliferation and promotes differentiation, while some report the opposite (Vieira et al. 2011; Mas-Bargues et al. 2019). We found that neural stem cells grew faster in serum-supplemented A20 medium at 20% oxygen in comparison to conditions with 2% oxygen. With A20 medium, we obtained viable cells from all brain regions studied (anterior, medial, and posterior parts of the brain). However, overall, cell proliferation of neural cells was slower in comparison to other tissues. Cells grew in both atmospheres, but proliferation was much faster under normoxic conditions (Fig. 6). Under normoxic conditions, cells were about 80% confluent in 2 weeks, whereas under hypoxic conditions, cells reached only about 40% confluence in the same time when isolated from the medial and posterior parts of the brain. This may reflect the higher requirement of neuronal cells for continuous oxygen supply. It is well known that neurons require a constant supply of oxygen and are the first cells in the body to die in hypoxia. Therefore, it would be intuitive to expect neuronal cells to grow better in an atmosphere with higher oxygen concentration.

Indeed, this has already been shown as some studies reported that optimal growth of neural stem cells occurs under normoxic conditions (Kilty et al. 1999; Kang et al. 2010). In addition to the medial part of the brain, which contains the main regions known to harbor stem cells, we have

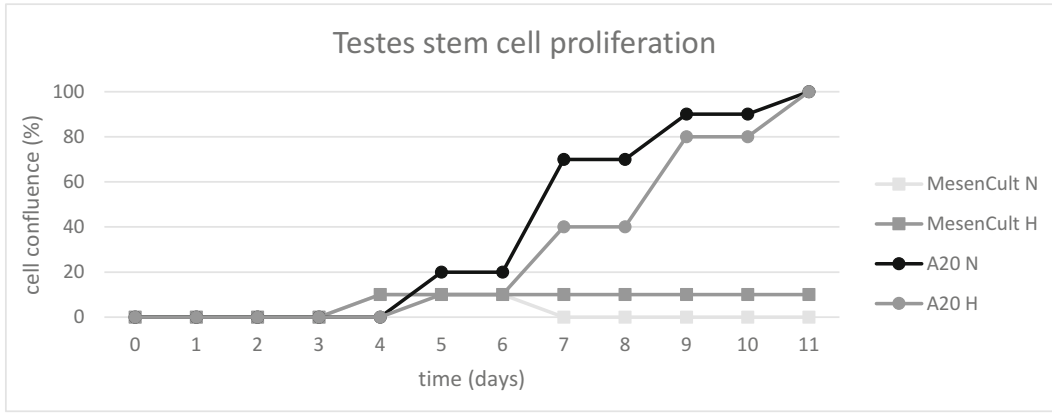

Fig. 5 Testicular stem cell growth in initial passage in both atmospheres using MesenCult and A20 mediums. Legend: N – normoxia, H – hypoxia

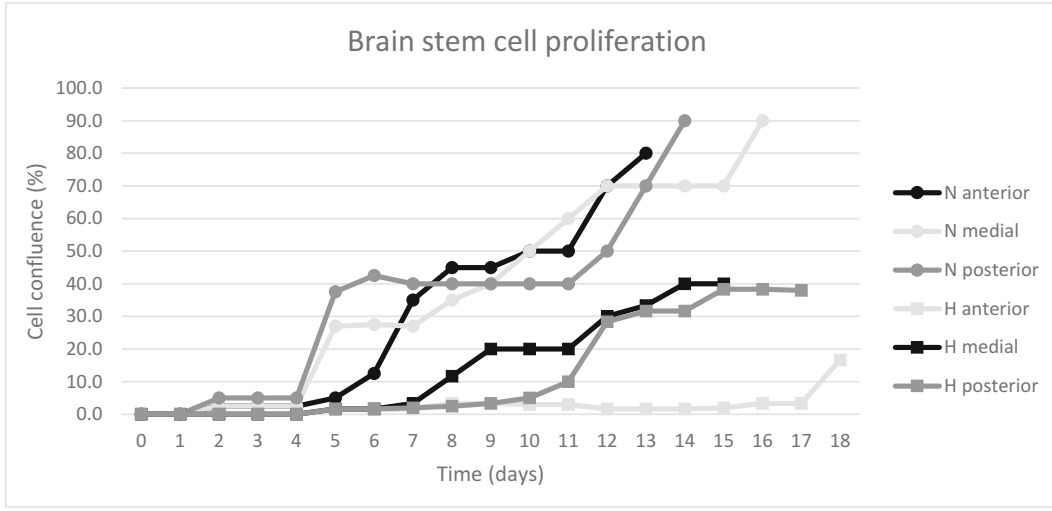

Fig. 6 Proliferation of brain stem cells from different parts in both atmospheres using A20 medium. Legend: N – normoxia, H – hypoxia

successfully isolated stem cells from the anterior and posterior parts of the brain as well. Interestingly, all three brain regions appear to be an equally good source of neural stem cells when cultured with A20 medium in normoxia. The stem cells from the anterior part of the brain most likely originated from the cortex and part of the optic nerve as these areas have been previously shown to contain stem cells (Palmer et al. 1999). Recently, it has been reported that neural stem cells are also located in the inferior colliculus, auditory cortex, and dorsal vagal complex (Li et al. 2003; Bauer et al. 2005; Volkenstein et al. 2013; Völker et al. 2019), which likely explains our positive results in obtaining stem cells from the posterior parts of the brain.

Although there are some reports suggesting that neural stem cells grow well also under hypoxic conditions, this could be a response to pathological conditions. Previous studies have shown that neural stem cell proliferation in the brain is stimulated in vivo by hypoxia as a consequence of stroke, asphyxiation, or other trauma as a

homeostatic mechanism that attempts neuroregeneration (Mannello et al. 2011; Wagenaar et al. 2018).

5 The Effect of Serum in Culture Medium and Atmospheric Oxygen Concentration on Stem Cell Differentiation

Some previous reports suggest that FBS may contain factors that could induce sensitive cells to lose their stemness and differentiate spontaneously (Brunner et al. 2010; Meenakshi 2013). However, in our study, we never observed spontaneous differentiation in the FBS-containing media even when cells grew in several passages. In contrast, cells from adipose tissue, muscle, and testis spontaneously differentiated when grown in serum-free MesenCult medium. These cells proliferated very slowly in MesenCult and appeared to have lost their stemness already during the first passage and showed signs of spontaneous differentiation into different cell types.

Apart from serum in the medium, ambient oxygen concentration in the atmosphere has been shown to have certain negative impacts on culturing adult stem cells as it can promote spontaneous differentiation of stem cells toward specialized cell lineages (Chen et al. 2007; Mas-Bargues et al. 2019). The important role of oxygen concentration in maintaining stemness was also observed in our laboratory. Interestingly, cells from different tissues often spontaneously differentiated when growing in the serum-free media but followed different differentiation pathways. In some cases, as with cells from the testes, oxygen concentration even affected the differentiation pathway of the cells.

Adult stem cells are sometimes induced to differentiate into specialized cells with the aim to use them for specific cell therapy. Usually, the addition of growth factors and neurotrophic factors into the medium is needed to direct the differentiation process into the desired cell lineage. The presence of serum also plays an important role in induced differentiation. To achieve differentiation into a particular cell lineage, some adult stem cells require FBS in the medium, together with additional inducing factors, while in some cases, a serum-free medium is required. A well-known example of heterogenous-induced differentiation demands are adult stem cells derived from adipose tissue, which can readily differentiate into osteocytes, chondrocytes, and adipocytes, depending on the presence of certain growth factors in the medium.

5.1 Adipose Tissue-Derived Stem Cells

The absence of serum in the growth medium may provoke early loss of stemness and spontaneous differentiation of isolated adipose tissue-derived stem cells. In our laboratory, morphological changes were observed in adipose tissue-derived stem cells cultured in serum-free medium within the first few days after plating when hypoxic conditions were used. Even after 3 weeks of cultivation, cells from adipose tissue grown in hypoxia with serum-free MesenCult medium remained in passage 0 because proliferation was very slow. From day nine, some spheroids with high confluency appeared. We used differential staining to investigate and confirm what type of cell differentiation occurred under the hypoxic conditions. Cells were stained with Alcian Blue and Alizarine Red S and were positive for both, indicating that spontaneous differentiation into chondrospheroids and osteocytes occurred in the cell culture under these conditions (Fig. 7).

Interestingly, proliferation of adipose tissue-derived stem cells grown in a serum-free medium at atmospheric oxygen concentration was slow and unsuccessful, but no spontaneous differentiation was observed. Similarly, no spontaneous differentiation was observed when cells were cultured in FBS-supplemented A20 medium. These results suggest that a combined effect of serum in the medium and hypoxic atmosphere is required for the best long-term proliferation of adipose-derived stem cells. The absence of serum in the medium immediately reduces the proliferation rate and triggers spontaneous differentiation, while a low oxygen concentration in the atmosphere promotes

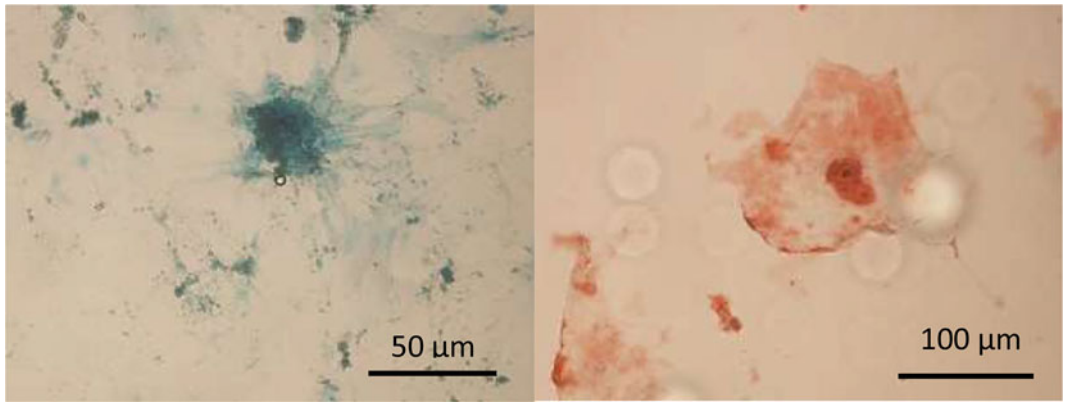

Fig. 7 Cells from adipose tissue, cultured for 3 weeks (hypoxia, MesenCult), spontaneously differentiated into chondrocyte-like cells (left) and osteocyte-like cells (right)

survival of the cells, resulting in increased spontaneous chondrogenesis and osteogenesis.

Adipose tissue-derived stem cells can be induced to differentiate into several different specialized cell types by altering the formulation of the growth medium (Zuk et al. 2002; Guilak et al. 2004). Apart from additional induction molecules, osteogenic and neural differentiation of adipose-derived stem cells require a medium without FBS, whereas adipogenic and chondrogenic differentiation media are supplemented with FBS (20% and 1–10%, respectively) (Bunnell et al. 2008). Adipocyte differentiation in vitro is induced with serum-supplemented medium and induction cocktails containing insulin, methylisobutylxanthine, hydrocortisone or dexamethasone, and indomethacin or thiazolidinedione (Halvorsen et al. 2001; Bunnell et al. 2008). In addition, stem cells from adipose tissue can be induced to differentiate into chondrocytes or the osteogenic lineage. Chondrogenesis can be promoted by adding transforming growth factor, ascorbate, and dexamethasone to a serum-supplemented medium and maintaining the cells in a three-dimensional, rounded morphology in a micromass pellet culture or within a hydrogel (Erickson et al. 2002; Awad et al. 2003). Differentiation into osteoblast-like cells is induced by the absence of FBS in the medium and by the addition of ascorbate, β-glycerophosphate, and dexamethasone (Heng et al. 2004; Bunnell et al. 2008). Adipose tissue-derived stem cells can also serve as a source of stem cells that can undergo neural differentiation. Neurospheres form when adipose-derived stem cells are cultured at high density using a neurogenic differentiation medium, which is usually a serum-free medium with the addition of antioxidants, indomethacin, insulin, and isobutylmethylxanthine (Safford et al. 2002, 2004).

5.2 Muscle Stem Cells

A high concentration of FBS in the medium is required for the maintenance of undifferentiated adult muscle stem cells in vitro, with some studies also recommending the use of hypoxic conditions (Lees et al. 2008). This was confirmed in our study as cell proliferation was arrested when cells were cultured in serum-free medium in an ambient oxygen atmosphere. Moreover, muscle stem cells cultured in serum-free MesenCult medium under hypoxia showed morphological changes after 9 days of cultivation with lipid vacuoles appearing inside the cells. Staining with Oil Red O was performed and confirmed that the cells accumulated lipid deposits and presumably spontaneously differentiated into adipose cells (Fig. 8). No such spontaneous differentiation was observed in cells grown in A20 medium containing 20% FBS under normoxic or hypoxic conditions.

For myogenic induction, cocultivation of muscle stem cells with primary myoblasts and

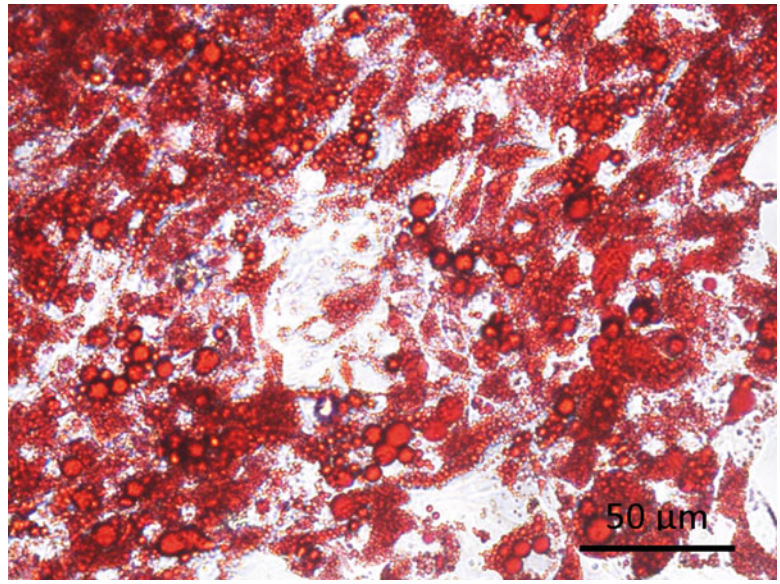

Fig. 8 Muscle-derived cells, cultured for 9 days (hypoxia, MesenCult), spontaneously differentiated into adipose-like cells with rich lipid deposits (stained with Oil Red O)

additional induction with dexamethasone and FGF is usually used (Eberli et al. 2009; Bitto et al. 2013). Stem cells isolated from adult muscle can also be a good source of autologous neural cells, useful for cell replacement in neurodegenerative and demyelinating diseases. When grown in a serum-free medium under nonadherent conditions, muscle stem cells can be induced to differentiate into neurospheres. Further cultivation under adherent conditions provokes differentiation into neurons and oligodendrocytes (Romero-Ramos et al. 2002).

5.3 Testicular Stem Cells

Serum in the growth medium is of high importance for promoting the proliferation of undifferentiated spermatogonial stem cells. In our laboratory, cells cultured in the absence of FBS spontaneously differentiated. Cells isolated from testes and grown in serum-free MesenCult medium under both normoxic and hypoxic conditions changed their morphology after only 4 days of cell culture. Interestingly, atmospheric conditions seem to influence the direction of the spontaneous differentiation. Cells grown under normoxic conditions at 20% oxygen in MesenCult medium accumulated cellular lipid inclusions, like differentiated cells from muscle tissue. Differentiation into adipocytes was confirmed by Oil Red O staining (Fig. 9, left). However, under hypoxic conditions with 2% oxygen and when grown in the same serum-free medium, cells appeared to spontaneously differentiate into chondrospheroids, which was confirmed by positive staining with Alcian Blue (Fig. 9, right). This was only observed in cells isolated from testicular tissue, and we do not know at the moment what causes these differences in spontaneous differentiation. The testis is composed of different cell types, and in vivo, these cells have different access to both oxygen and nutrients from the blood due to the composition of the testis and the testis-blood barrier. It is therefore possible that different cell types grow better under normoxic and hypoxic conditions and that these cells have different differentiation capacities. Alternatively, the same cells could respond differently to different atmospheric conditions, but this will have to be investigated in future studies.

Induced in vitro differentiation of spermatogenic cells seems to be a possible method for the treatment of male infertility. Studies of in vitro spermatogenesis have shown that FBS plays an important role and is essential to allow

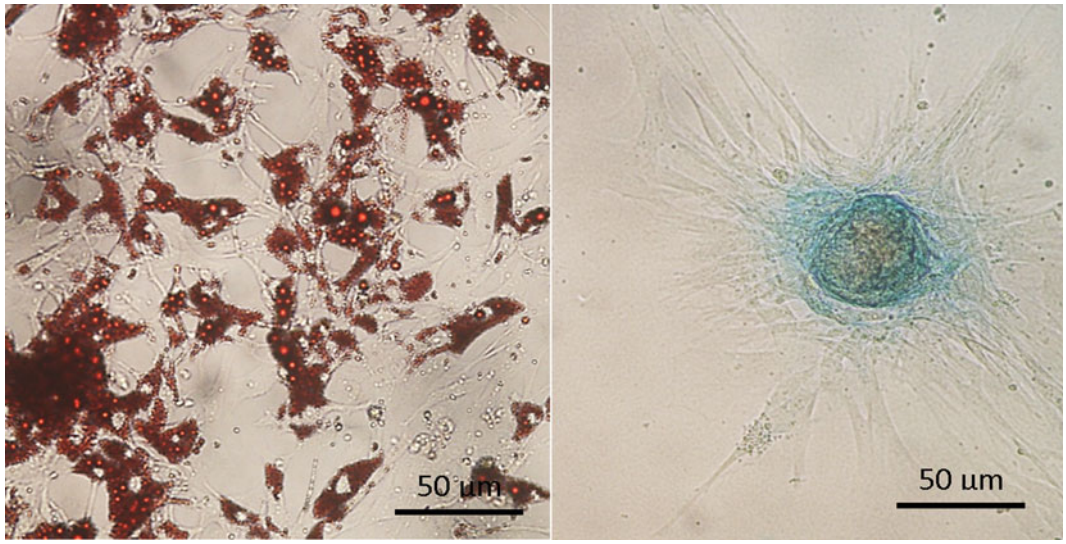

Fig. 9 Using MesenCult, testicular cells spontaneously differentiated in adipose (4 days, hypoxia, left) or chondrocyte-like cells (9 days, normoxia, right)

the progress of spermatogenesis, together with additional molecules like growth factors and hormones (Lee et al. 2006; Sato et al. 2011; Zhao et al. 2018).

5.4 Neural Stem Cells

A standard serum-free culture system for neural stem cells, also known as the neurosphere assay, allows selective growth of stem cells isolated from the adult brain. Undifferentiated neural stem cells survive and proliferate, while most other differentiated cell types die (Reynolds and Weiss 1992.) The use of certain growth factors such as EGF and FGF as mitogens can induce a consistent, renewable source of undifferentiated neural stem cells, which could be expanded into defined proportions of neurons, astrocytes, and oligodendrocytes (Gritti et al. 1995, 1996, 1999: Reynolds and Weiss 1996; Weiss et al. 1996b, 1996a). Removal of growth factors, present in stemness-promoting medium, and addition of FBS into the growth medium cause neurosphere-derived cells to differentiate (Rietze and Reynolds 2006; Liu et al. 2018). In general, undifferentiated neural stem cells express glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF). Neurotrophic factors and the presence of serum in the media are needed to induce differentiation. The regulatory factors in the serum can strongly influence the expression of neurotrophic factors. 1% FBS in the media reduces the expression of GDNF in differentiating neural stem cells, while 10% suppresses it completely (Niles et al. 2004).

A negative impact of ambient oxygen concentration on cell growth was shown also in the cultivation of neural stem cells. Cultivation under normoxic conditions can lead to spontaneous differentiation of neural stem cells toward the glial lineage (Chen et al. 2007). However, some studies report that normoxic conditions decrease proliferation and promote differentiation of neural stem cells, while some reports suggest that low oxygen concentration increases the differentiation potential of such cells (Vieira et al. 2011; Mas-Bargues et al. 2019). Controversial results have also been observed in some other types of adult stem cells and can be explained partially by the concentration of oxygen in the atmosphere and the duration of exposure used in the studies. Some of the studies used short-term hypoxia (less than 72 h), while others maintained the cells in

hypoxia permanently. Furthermore, the oxygen concentration in various studies varied between 0.1% and 5%, meaning some cells were exposed to a more anoxic environment than the others and therefore making such studies difficult to compare directly.

6 Conclusions

Many studies have shown that adult stem cells isolated from different tissues respond differently to different environmental conditions such as the content of growth media, in particular the presence or absence of FBS, and atmospheric composition. In general, serum in the media seems to have a positive effect on both cell growth and the prevention of spontaneous differentiation of stem cells. Interestingly, cells from different tissues often spontaneously differentiate when grown in serum-free media but follow different differentiation pathways. In some cases, such as cells from the testes, even atmospheric oxygen concentration can affect the differentiation pathway of cells. Apart from serum, the oxygen concentration in the atmosphere of the culture has a major impact on the stemness maintenance and survival of stem cells, so this is an essential factor to consider when culturing adult stem cells for tissue engineering and regenerative medicine.

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