Differentiation 84 (2012) 193–202

Contents lists available at SciVerse ScienceDirect

Differentiation

journal homepage: www.elsevier.com/locate/diff

Oxygen concentration modulates the differentiation of muscle stem cells toward myogenic and adipogenic fates

Zoe Redshaw*, Paul T. Loughna

The University of Nottingham, School of Veterinary Medicine and Science, Sutton Bonington Campus, Sutton Bonington, Leics LE12 5RD, United Kingdom

ARTICLE INFO

Article history:
Received 18 January 2012
Received in revised form
23 March 2012
Accepted 5 June 2012
Available online 11 July 2012

Keywords:
Myogenesis
Adipogenesis
Muscle
Porcine
Differentiation
Oxygen concentration

ABSTRACT

The physiological oxygen concentration of many tissues is far lower than that in which cells are typically cultured *in vitro* and this may inadvertently influence the proliferation and differentiation potential of many cell types. Muscle derived stem cells, known as satellite cells are responsible for the maintenance and repair of muscle tissue post-natally and *in vivo* would be exposed to oxygen concentrations of ~2–5%. Relatively few studies describe the function of these cells in large animal models and here we investigate the influence oxygen concentration has on modulating porcine muscle derived stem cell fate. We compared cells derived from two metabolically distinct muscles, the diaphragm and the hind limb semi-membranosus (SM) muscle. The two sub-populations responded differently to culture at atmospheric (~20%) and physiological (~5%) oxygen concentration. While myogenesis was enhanced in both populations at low oxygen, noticeably diaphragm derived cells exhibited greater myotube formation, than those from SM. The trans-differentiation of cells derived from these two sources was similarly affected, with considerable differences seen in adipogenic and neuronal tendencies. In addition to the effect of oxygen on cell phenotype, the expression of key signalling proteins varied between the two sub-populations during early time-points of induced differentiation, suggesting altered regulation of muscle specific stem cells under these conditions. While differences in muscle stem cell potential requires further investigation, the culture of cells in physiological oxygen concentration appears as fundamental to recreating the micro-environmental niche as routinely used factors such as cytokines, substrata and matrices.

1. Introduction

In vitro cell culture models are an essential tool for elucidating the mechanisms involved in a vast array of cellular pathways. Many adaptations to culture conditions such as defined media, extra-cellular matrix and environmental growth factors have evolved over the last 50 years, in an attempt to replicate cell behaviour *in vivo*. While the majority of work is carried out in enhanced carbon dioxide (CO_2, 5%), few studies alter the oxygen (O_2) concentration from atmospheric (20%) to more physiologically relevant levels and consequently, cells may not respond to proliferation and differentiation cues as they would *in vivo*. Muscle derived stem cells are responsible for maintenance and repair of skeletal muscle post-natally and are reportedly exposed to an O_2 environment *in vivo* of between 1–10% (Greenbaum et al., 1997; Richardson et al., 1998). The most predominant and well characterised of these stem cell populations are the satellite cells, so called because of their anatomical location (when quiescent), between the plasmalemma and basal lamina of the muscle fibre. Other adult stem cell populations have been associated with muscle differentiation including vascular derived pericytes (Dellavalle et al., 2007) and side population cells (Meeson et al., 2004). However, stem cell markers typically used for characterisation of such populations (in mouse), can differ in pattern of expression, or even existence of specific genes/proteins between small and large mammals, *e.g.* stem cell antigen-1 (Sca-1), which disappeared during speciation of mouse and rat (Holmes and Stanford 2007). There are considerably fewer studies describing the function of muscle stem cells in large animals.

Several studies have looked at the effect oxygen concentration has on myoblast cell culture, using hypoxic (< 1%) and physiological (1–6%) conditions. They find that hypoxia tends to inhibit differentiation (Di Carlo et al., 2004; Yun et al., 2005), whereas physiological O_2 levels enhance cell proliferation and myogenesis (Chakravarthy et al., 2001; Csete et al., 2001; Kook et al., 2008; Martin et al., 2009), compared to atmospheric levels. In addition to myogenic differentiation, muscle stem cells exhibit plasticity in their ability to generate cells with adipogenic (Shefer et al., 2004; Singh et al., 2007; Yu et al., 2006), osteogenic (Asakura et al., 2001)

* Corresponding author. Tel.: +44 11 5951 6652; fax: +44 11 5951 6440.
E-mail addresses: zoe.redshaw@nottingham.ac.uk (Z. Redshaw), paul.loughna@nottingham.ac.uk (P.T. Loughna).

0301-4681/$ - see front matter © 2012 International Society of Differentiation. Published by Elsevier B.V. All rights reserved.
Join the International Society for Differentiation (www.isdifferentiation.org)
http://dx.doi.org/10.1016/j.diff.2012.06.001

and neuronal phenotypes (Romero-Ramos et al., 2002; Vourc'h et al., 2004). Few if any studies have looked at the effect of low O_2 on primary muscle stem cell plasticity. In muscle/mesenchymal cell lines however, conflicting evidence exists with regard to the role of low O_2 on adipogenic differentiation. In the mouse myoblast C2C12 cell line, adipogenesis was induced at 1% O_2 (Itoigawa et al., 2010), whereas in the C3H10T½ cell line, adipogenesis was inhibited at 2% O_2 (Csete et al., 2001). Increase in skeletal muscle adiposity with age and certain disease states, is currently of great interest, although whether this is due to *in vivo* adult stem cell transdifferentiation or infiltration of surrounding adipocytes remains to be determined. Our recent work has revealed that muscle origin of adult stem cells appears influential in cell potential, when coaxed towards different fates (Redshaw et al., 2010). In typical culture conditions at atmospheric O_2 concentration, we found that diaphragm derived cells had greater *in vitro* myogenic tendency than those from the hind limb semi-membransosus (SM) muscle and furthermore, the two populations differed in their plasticity. There is growing evidence for satellite cell heterogeneity (Li et al., 2011; Ono et al., 2010) as they contribute to a stem cell compartment encompassing a variety of tissue types with differing contractile and metabolic properties, in addition to developmental origin. Furthermore it is possible that the proportion of other stem cell populations may differ between such muscles. Typically small animal studies require pooling of cells from several muscles, for this work we wished to compare inter-muscular differences and examine the ability of O_2 to modulate porcine muscle stem cell potential and attempt to determine molecular events occurring during early differentiation.

2. Materials and methods

2.1. Isolation of muscle stem cells

For this work, satellite cells were isolated from diaphragm and hind limb semi-membranosus (SM) muscles, from two Large White Landrace pigs (4–6 weeks old), which were killed humanely via electrical stunning and exsanguination. For separate experiments, comparable cells were derived from the same animal, *i.e.* cells were not pooled from multiple animals (all studies except the Hispidin study utilised cells from the same animal). The isolation of cells was performed as previously described (Redshaw et al., 2010), following modification of techniques developed by (Mau et al., 2008; Qu-Petersen et al., 2002). Briefly, cells were isolated based on their adherence, with non-adherent cells being transferred to a new flask every 24 h. Our earlier work found that cells which attached between 2–48 h were better able to generate myotubes and consequently these cells were used for all future studies. Freshly isolated cells were cultured on type I rat tail collagen (0.01%, Sigma, UK) coated plastic, in Memα growth media (GM, 20% FBS (Invitrogen, UK), 2 mM L-glutamine (Sigma), 100 IU/ml penicillin, 100 μg/ml streptomycin, 3 μg/ml amphotericin B (Invitrogen)) and incubated at 37 °C in a humidified atmosphere of 5% CO_2 and air, or in 5% CO_2, 90% Nitrogen and 5% O_2 until approximately 60% confluent before cryogenic preservation in FBS and 10% DMSO (invitrogen).

2.2. Comparison of oxygen concentration on cell differentiation

For differentiation studies, early passage (P1 or P2) Dia and SM derived cells were seeded at a density of 2.6×10^4 cells/cm^2 on collagen coated plastic and initially cultured in GM at either atmospheric (~20%), or physiological (~5%) O_2 concentrations (all other culture parameters remained the same).

Myogenic differentiation: Once cells became ~80% confluent, GM was replaced by low serum myogenic differentiation media (DM, DMEM, 2% horse serum (Invitrogen), 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 3 μg/ml amphotericin B) and cells cultured for up to 7 day, with media changes every 48 h.

Adipogenic differentiation: As for myogenic differentiation, at ~80% confluence, GM was replaced by adipogenic differentiation media (Ad), which was DM supplemented with 1 μM dexamethasone (DEX, Sigma), 50 μM IBMX (Sigma), 10 μU insulin (Eli Lilly & Co Ltd, UK) for 3 day, followed by 3 day in Ad media minus DEX and IBMX. Following differentiation, all cells were initially fixed in 70% ethanol as previously described (Redshaw et al., 2010).

Hispidin study: As for adipogenic differentiation, at ~80% confluence, GM was replaced by Ad, with the addition of 4 μg/ml Hispidin (Sigma) dissolved in a final volume of 0.02% DMSO. Control cells were treated with Ad and 0.02% DMSO or Ad media alone. Media was changed as for adipogenic differentiation, with addition of fresh Hispidin on day 0 and day 3 only. Cells were fixed as previously described.

2.3. Immunocytochemistry and oil red O staining

Immunocytochemistry of cells was visualised using Vectastain® (Elite ABC (biotinylated anti mouse IgG) kit, Vector Labs ltd, UK) VIP peroxidase substrate (Vector labs Ltd), as per the manufacturers guidelines. The primary antibodies used were mouse monoclonal antibodies: Desmin (1:500, Abcam, UK), Myogenin (1:100, Abcam), Myosin Heavy Chain (MHC, 1:500, Abcam), pan Neurofilament (1:3000, Abcam). Prior to both Neurofilament and Oil Red O staining, cells were additionally fixed in 10% buffered formalin for 20 min and washed twice with PBS. Lipid was visualised via Oil Red O (0.5%, Sigma) staining for 15 min and washed twice and stored in dH_20.

2.4. Image analysis

All images used for the quantification of immunocytochemistry and Oil Red O staining, were analysed using Image Pro version 6.3. Phase contrast images were prepared as tiff documents for 100x and 400x magnification. For individual staining analyses, a macro was created to include the range of colour intensities denoting a positive result and applied to all grouped images. Error bars represent standard error of the mean.

2.5. SDS-PAGE/western blotting

Cell lysates of early myogenic and adipogenic differentiation were prepared in Ripa buffer (Invitrogen) with addition of protease (Mini Complete, Roche, UK) and phosphatase inhibitors (Roche). Four timepoints, undifferentiated (70% confluent), +8 h, +24 h and +48 h following initiation of differentiation were prepared in triplicate for each treatment. Protein lysates (5 μg/lane) were electrophoresed on 10% SDS-PAGE gel (Expedion, Uk) and transferred to nitrocellulose (GE Healthcare, UK) via wet transfer. Membranes were blocked in ~5% milk before probing with antibody. Primary antibodies used were Desmin (1:1000, Abcam), pERK (1:2000, Cell Signalling, UK) pJNK (1:1000, Cell Signalling) and α-Tubulin (1:500, Abcam). Secondary antibodies were anti-mouse HRP (1:1000, Cell Signalling) and anti-rabbit HRP (1:1000, Cell Signalling). Quantification of protein expression was analysed via densitometry using ImageQuant TL (version 2005, Amersham Biosciences).

2.6. Statistical analysis

All experiments were carried out in triplicate ($n=3$) as the minimum number of replicates, however some had a greater