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

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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.

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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)

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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$_2$0.

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

number, all of which are indicated in the figure legends. For immunocytochemical and Oil Red O quantification, data was generated for each replicate by taking 15 random fields of view (FOV) per well, to generate an average value for each replicate, or via a total count. Where applicable, student's t-tests were performed on data sets (unpaired, two-tailed), comparing differences between muscle or O_2 concentration. Error bars represent the standard error of the mean. Asterisks within figures denote degree of statistical relevance observed: $^* < 0.05$; $^{**} < 0.01$; $^{***} < 0.001$.

3. Results and discussion

3.1. Influence of oxygen on myogenic differentiation

The levels of O_2 are highly homeostatically regulated within mammalian tissues and numerous studies have shown that prolonged or even acute hypoxic or hyperoxic exposure can be deleterious to cells (Di Carlo et al., 2004; Yun et al., 2005). It has also been observed that tissue levels of O_2 in skeletal muscle of animals, while variable, are usually about 5% (Greenbaum et al., 1997). This physiological level is considerably below the 20–21% (atmospheric) employed in most cell culture studies.

We have recently shown that muscle stem cells isolated from the SM hind limb muscle and the diaphragm differ considerably in capacity to undergo myogenic and adipogenic differentiation, under atmospheric O_2 concentrations (Redshaw et al., 2010). Of note, the cells used in these studies, isolated using our current

methodology expressed both Pax7 and Pax3, which is indicative of them originating from a satellite cell pool. In the present study we further show the divide between the two populations and the influence of O_2 in governing cell fate. Following induction of myogenic differentiation, myoblasts fuse to form multinucleated myotubes which express functional proteins involved in muscle contraction (i.e. myosin heavy chain MHC) and maintenance of the cells structural integrity (i.e. desmin), which denote terminal differentiation. The expression of both of these proteins was changed in response to culture at differing O_2 concentrations, for diaphragm and SM derived cells. Myosin heavy chain expression was markedly increased in diaphragm cells at 5% O_2 ($P=0.001$) while expression in SM cells remained substantially lower at both concentrations (Fig. 1A). Similarly, expression of desmin was up-regulated at physiological O_2 concentration for both muscle types (SM $P=0.001$; diaphragm $P=0.02$), although as previously reported at 20% O_2 (Redshaw et al., 2010), diaphragm cells exhibited greater myogenic tendency in vitro than those from SM and continued to do so at 5% O_2 (Fig. 1B and C). Desmin expression was further quantified by SDS-PAGE during the early stages of differentiation (up to 48 h following induction), for cell populations undergoing myogenesis. Desmin was up regulated within 8 h of serum deprivation in both populations, increasing over the 24 h and 48 h time-points and noticeably more so at 5% than 20% O_2 (Fig. 1D and E, $P= < 0.002$ between O_2 concentration for each time-point, except undifferentiated cells).

For this work, the immunocytochemical quantification of myogenic differentiation was measured by the percentage surface area
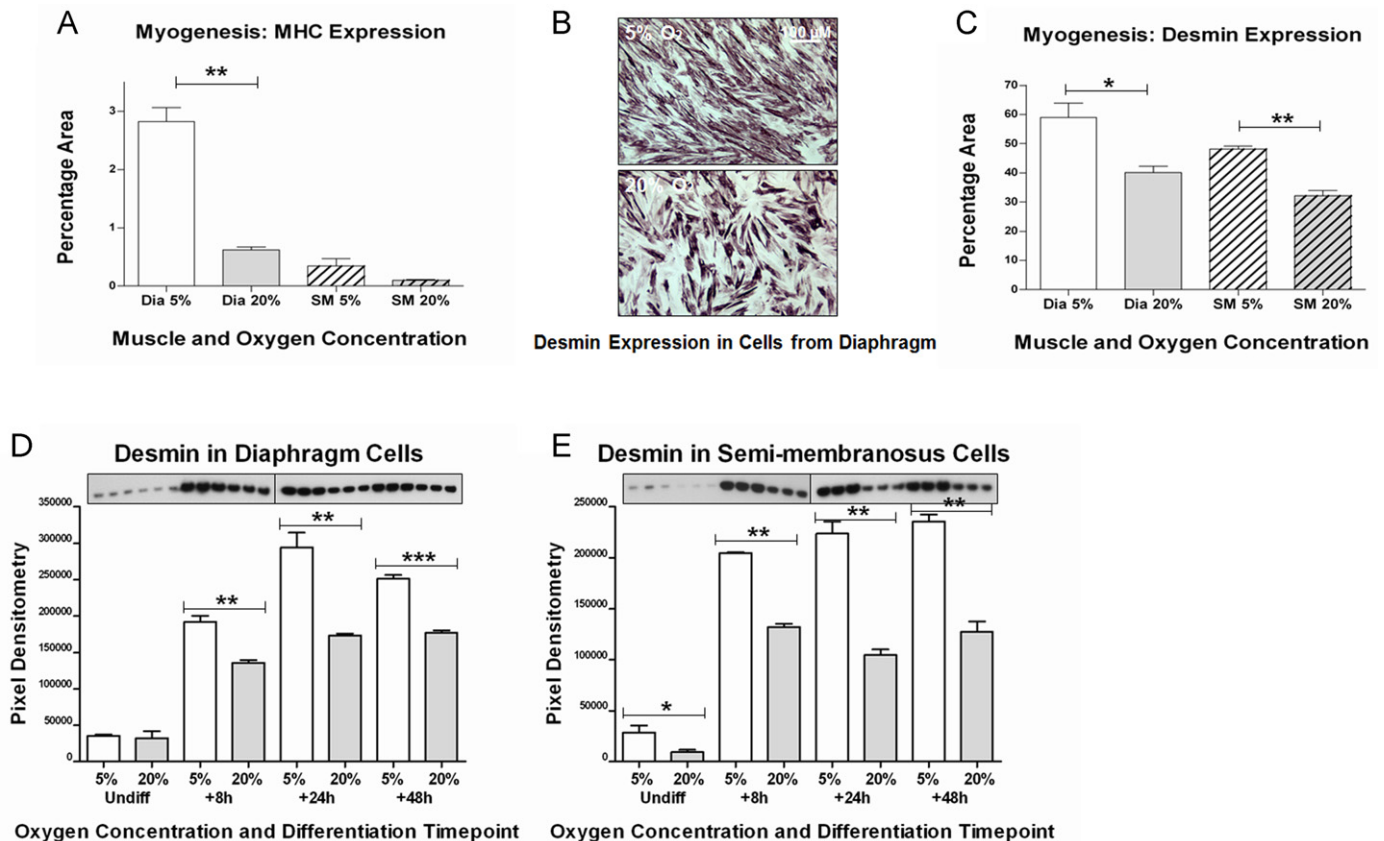

Fig. 1. Myogenic differentiation of cells derived from diaphragm and SM muscles, cultured at atmospheric ($\sim$20%) and physiological ($\sim$5%) oxygen concentrations. (A) Quantification of Myogenic differentiation (at day 7) by surface area expression of Myosin Heavy Chain, (B) Representative images used for quantification of differentiation: Desmin expression (at day 7) for cells from diaphragm at both oxygen concentrations, (C) Quantification of Desmin expression (at day 7) by surface area expression. Change in Desmin expression during early differentiation, via SDS-PAGE densitometry for (D) diaphragm and (E) SM cells. ($n=3$, 15 FOV/replicate. Original image magnification 100 $\times$). Error bars represent SEM.

of antibody staining, rather than as the percentage of myotube nuclei in relation to the total number of nuclei, which is often used. Our reason being that many such studies (including porcine (Mau et al., 2008; Wilschut et al., 2010)) use desmin as the marker of differentiation to calculate the fusion index and while we have shown an increase in desmin expression with differentiation (Fig. 1D and E), we find that not all desmin-expressing cells fuse to form myotubes *in vitro*. *In vitro* differentiation of primary myoblasts is typically less efficient, with limited fusion observed in many models, particularly large mammals, compared with mouse cell lines such as C2C12. Even in studies which report a relatively high fusion index, non-fusing cells are always observed, some of which surprisingly proceed to fuse following removal of existing tubes (Wilschut et al., 2010). Therefore, we wished to illustrate the effect of O_2 on commitment to differentiate (desmin) and ability to fuse (MHC), using antibody staining of myotube surface area preferably, to represent these data. Our results show that cells derived from the same animal, prepared and treated in identical manner, consequently exhibit different capabilities in myogenic commitment and ability to fuse and form multinucleated myotubes.

3.2. Myogenic response to physiological oxygen levels

Based on the greater *in vitro* differentiation ability of diaphragm derived cells, we further quantified the morphological aspect of improved myogenesis at low O_2. An enhanced degree of fusion was observed at 5% O_2, with myotubes on average significantly longer ($P=0.005$, Fig. 2A), covering a greater range of length (denoted by the bars: 5% O_2 173.8–4934 μm; 20% O_2 20–2102 μm, Fig. 2B) and surface area ($P=0.01$, Fig. 2C), as determined via MHC expression.

Of note, the average number of tubes per field of view did not alter significantly between the two O_2 concentrations (data not shown). However, the number of nuclei expressing myogenin, a transcription factor involved in myogenic determination post-natally and which is transiently switched on during commitment to differentiate, was significantly higher at 5% O_2 ($P=0.02$, Fig. 2D and E). This suggests that cells are more susceptible to *in vitro* differentiation cues under physiological rather than atmospheric O_2 concentration and that these cells orchestrate myotube formation, with more nuclei contributing to tubes at 5% O_2, which then relates to subsequent myotube dimension.

3.3. Influence of oxygen on muscle stem cell plasticity

Following exposure to adipogenic inducing reagents, the two stem cell populations exhibited further variation in their potential, with contrasting adipogenic and neuronal tendencies (initially discovered at 20% O_2, Redshaw et al., 2010). In cells isolated from SM, we observed greater lipid accumulation than those from diaphragm at both O_2 levels ($P=0.001$, Fig. 3A), however the number of these cells was significantly reduced at 5% O_2 for SM ($P=0.02$), while remaining virtually unchanged and very low, for diaphragm (viewed by Oil Red O staining, Fig. 3A). The ability of muscle stem cells to differentiate towards an adipogenic fate has previously been shown (Asakura et al., 2001; Shefer et al., 2004), however such studies usually always use cells derived from lower limb muscles and so little is known of the ability of cells from non limb muscle to undertake this alternate mesenchymal fate. Furthermore, very little has been reported on the influence of O_2 concentration on the plasticity of muscle cell populations. Two studies which support our findings of reduced adipogenesis at
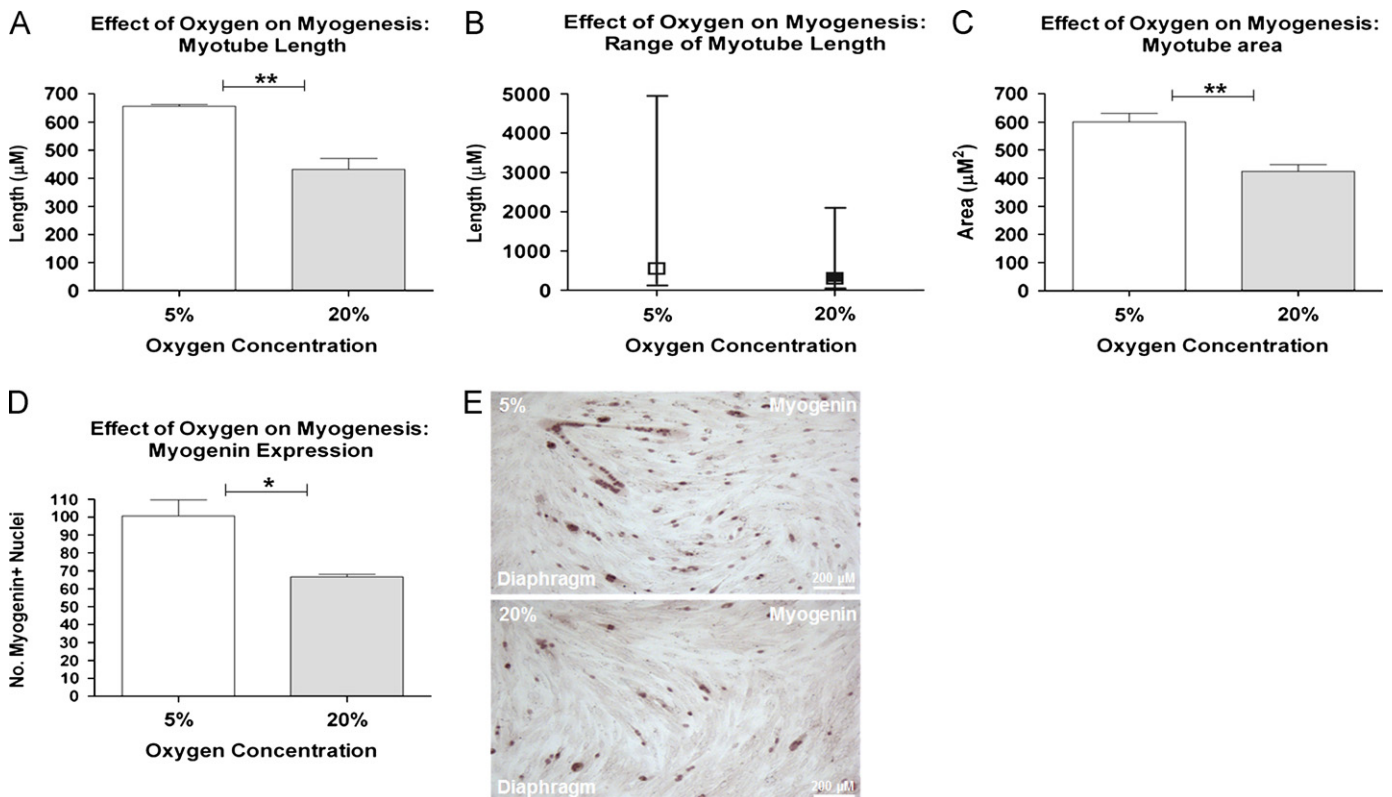

Fig. 2. The effect of oxygen concentration on the myogenic potential of diaphragm derived stem cells. Quantification by Myosin Heavy Chain expression for the comparison of oxygen concentration on: (A) average myotube length, (B) range of length and (C) average myotube area. Commitment towards a myogenic fate was measured by (D) Myogenin expression. ($n=3$, 15 FOV/replicate). (E) Representative immunocytochemistry of myogenin expression (at day 7) for cells grown at $\sim$5% and $\sim$20% oxygen. (Original image magnification 100 $\times$). Error bars represent SEM.
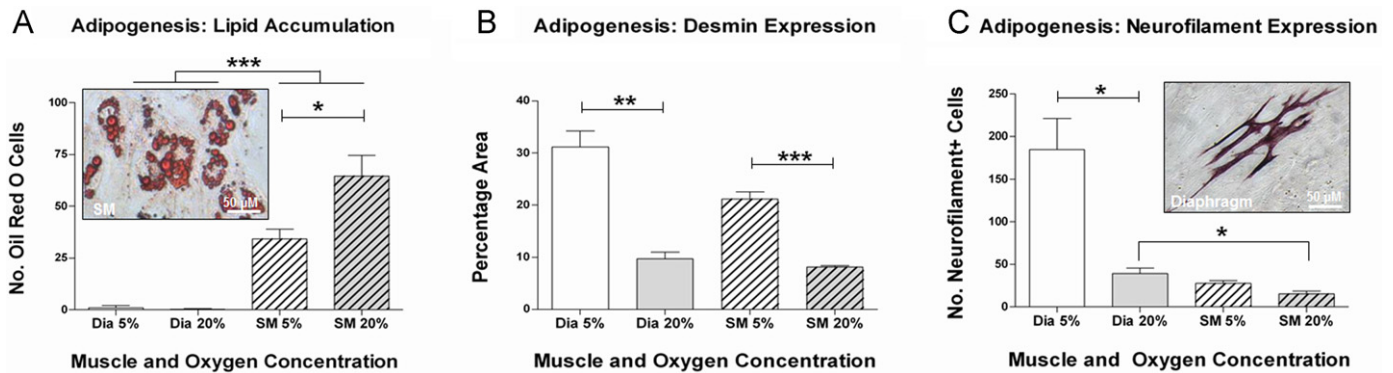
Fig. 3. Adipogenic differentiation at atmospheric ($\sim$20%) and physiological ($\sim$5%) oxygen concentrations. Lipid accumulation was measured by (A) Oil Red O staining (at day 6 of differentiation, $n=6$, 15 FOV/replicate). (B) Desmin expression after adipogenic differentiation (at day 6). (C) Expression of the neuronal protein Neurofilament under adipogenic differentiation conditions. ($n=3$, 15 FOV/replicate. Original image magnification $100\times$). Error bars represent SEM.

lower oxygen were carried out in human bone-derived mesenchymal stem cells (MSC, Fehrer et al., 2007) and the mouse embryo-derived mesenchymal cell line C3H10T½ (Csete et al., 2001), in which few or no lipid filled cells were observed at 2 and 3% O_2 respectively, compared with those cultured at 20% O_2. These findings are not directly comparable, but allude to the role of O_2 in the regulation of cell fate and/or transdifferentiation.

Under adipogenic culture conditions, we wished to ascertain the capability of muscle stem cells to adopt a myogenic fate and looked at desmin expression to ascertain this. At 20% O_2, few cells stained positive for desmin, with similar levels observed for both diaphragm and SM (Fig. 3B). At 5% O_2, desmin expression more than doubled for both muscles (diaphragm $P=0.003$, SM $P=0.0008$, Fig. 3B), although the percentage area remained approximately half of that seen under purely myogenic conditions (Fig. 1C). These results suggest that despite culture of stem cells under adipogenic inducing conditions, cells had a greater myogenic tendency under physiological O_2 conditions, which was then inhibited at higher atmospheric O_2 levels. Under such adipogenic conditions at 20% O_2, the inhibition of myogenesis may be ascribed to the presence of a key regulator of adipogenesis - peroxiosome proliferator-activated receptor gamma (PPARγ). Expression of the transcription factor PPARγ, is up-regulated at 20% O_2 (Csete et al., 2001) and has been shown to actively repress the promoter activity of a key myogenic regulatory factor, MyoD (Hunter et al., 2001). This balance between myogenesis/adipogenesis may relate to events which occur in muscle with aging, whereby an increase in reactive oxidative stress correlates with a decrease in muscle mass and increase in muscle fat deposition. There is an accumulating body of evidence that muscle stem cells both in vivo and in vitro have a greater propensity to differentiate along an adipogenic lineage when exposed to less optimal environmental conditions, such as occur during ageing, obesity, diabetes or in response to reactive oxygen species (ROS) (Scarda et al., 2010; Taylor-Jones et al., 2002). To extrapolate to the present study would suggest that 5% O_2 exposes the cells to a more optimum environment, with regard to reducing adipogenesis, when compared to atmospheric O_2 levels.

We have previously reported that both of these muscle stem cell populations were able to generate small numbers of cells with dentritic-like processes, which expressed a mature neuronal marker, neurofilament (NF), following exposure to either myogenic or adipogenic reagents (Redshaw et al., 2010). More of these neuronal-like cells were found in diaphragm derived cultures than SM and numbers substantially increased for diaphragm at low O_2 ($P=0.03$, Fig. 3C). Oxygen concentration did not appear to significantly alter the proportion of NF positive cells present in the SM samples and further highlights subtle differences in potential between these subpopulations of satellite cell. While it may be surprising that muscle stem cells spontaneously adopt a neuronal-like phenotype when grown in a basic, low serum non-neurobasal medium, work by Studer et al. 2000 have shown that neuronal differentiation increases at low O_2, suggesting a similar oxygen-dependent response. The functional significance of these cells is currently unclear.

3.4. Myogenic differentiation and MAPK phosphorylation

To examine further the mechanisms by which these muscle cells respond to O_2 conditions, we looked at the activity-related phosphorylation of the mitogen activated protein kinases (MAPKs). Recent studies in muscle cells have shown MAPKs to be modulated by altered O_2 levels (Osorio-Fuentealba et al., 2009; Ren et al., 2010). The MAPK family is considered a fundamentally important, ubiquitous intracellular signalling system, involved in the regulation of cell growth, differentiation and survival.

Following induction of myogenic and adipogenic differentiation (up to 48h), we looked at the activity-related phosphorylation of extra-cellular signal regulated kinase (pERK1/2), c-Jun N-terminal kinase (pJNK) and p38, via SDS-PAGE densitometry. Following myogenic induction, activation of pERK increased substantially by 24 h and 48 h in cells from diaphragm and significantly more so at 5% than 20% O_2 (24 h $P=0.01$; 48 h $P=<0.0001$, Fig. 4A). There were no changes in total ERK in response to O_2 (data not shown). The expression of pJNK also increased in diaphragm within 8 h of induction, again significantly elevated at the lower O_2 concentration (8 h $P=0.04$; 24 h $P=0.001$; 48 h $P=0.009$, Fig. 4C). Results for cells from SM were surprising, in that while pERK expression increased with time point as for diaphragm, contrastingly a greater degree of phosphorylation occurred at 20% rather than 5% O_2 (8 h $P=0.0001$; 24 h $P=0.03$; 48 h $p=0.006$, Fig. 4B). In undifferentiated SM cells, the basal level of pJNK was greater and more was present at 20% O_2 ($P=0.005$, Fig. 4D). Levels peaked at 8 h following induction, however more pJNK protein was then expressed at 5% compared with 20% O_2 (24 h $P=0.009$, Fig. 4D). The expression of p38 remained unchanged for both muscles (data not shown).

Although the pattern of pERK contrasted between cells from diaphragm and SM during myogenic differentiation, at any one time point or oxygen concentration, a considerable difference in the level of pERK was observed between muscles. Comparing a single time point and O_2 concentration, densitometry results for pERK/α-Tubulin ratio at the 24 h time point and 5% O_2 show that cells from SM exhibited a significantly greater degree of expression than those
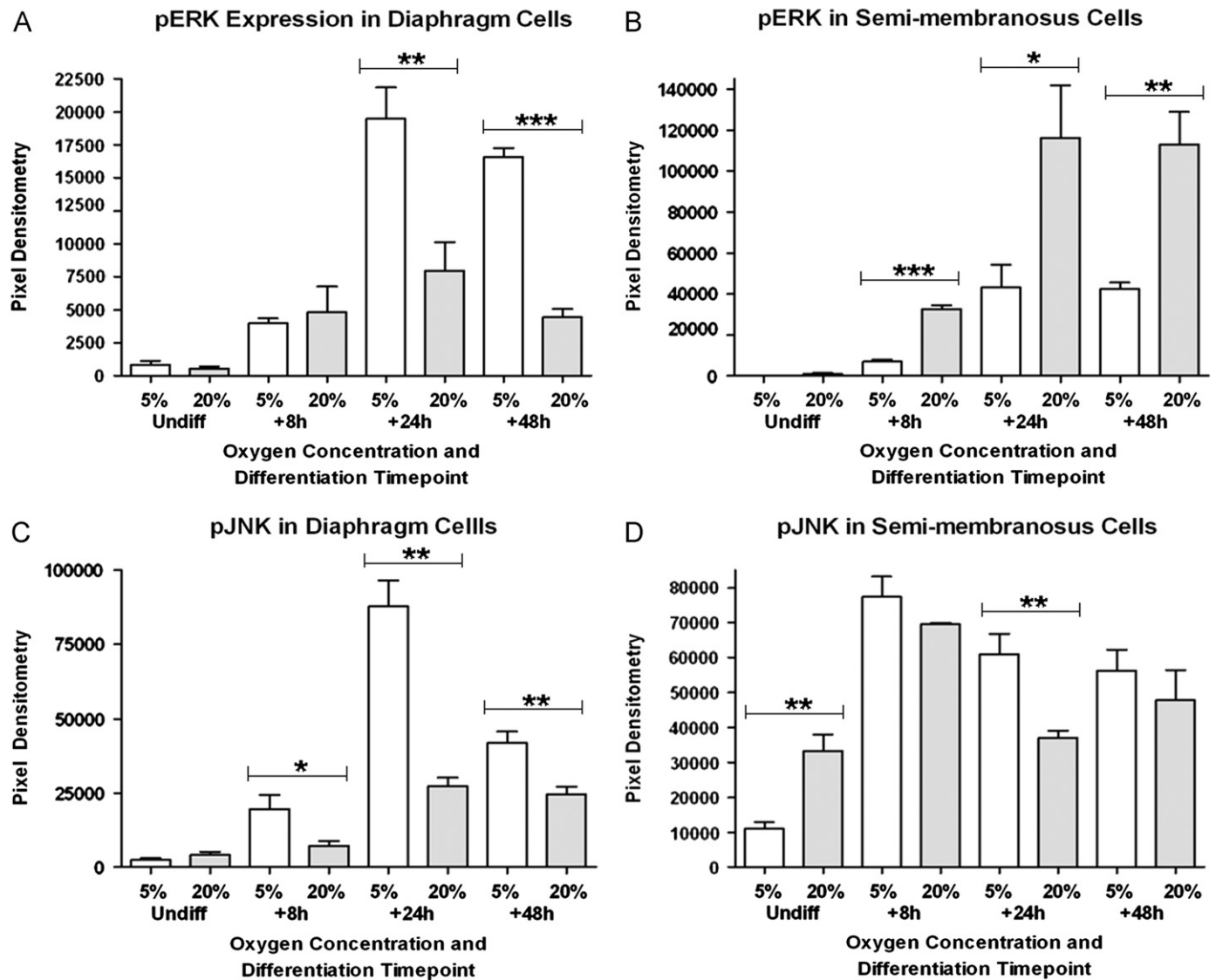
MYOGENESIS

Fig. 4. SDS-PAGE densitometry for signalling factors during myogenic differentiation. Expression of pERK during early myogenic differentiation in (A) diaphragm cells and (B) SM cells. Expression of pJNK during early myogenic differentiation in (C) diaphragm cells and (D) SM cells. Time points taken were for cells at: undiff −70% confluent, +8 h, +24 h and +48 h of differentiation ($n=3$). Error bars represent SEM.

from diaphragm ($P=0.008$, Fig. 5A). This difference in level of expression between diaphragm and SM cells and the contrasting pERK expression pattern is further illustrated at the 48 h time point by Western Blot (Fig. 5B).

It has recently been observed that hypoxia (1% O_2) causes an elevation in activity-related ERK phosphorylation in murine neonatal satellite cells when compared to cells at 20% O_2 (Osorio-Fuentealba et al., 2009). Furthermore, in the murine satellite cell derived C2C12 cell line, it has been observed that under hypoxic conditions (1% O_2), that insulin growth factor (IGF) induced an enhanced activation of ERK but inhibited p38 activation, when compared to atmospheric O_2 levels (Ren et al., 2010).

In this study we examined the effects of physiological (5%) O_2 levels compared to atmospheric (20%) in cells derived from the porcine SM and diaphragm. Although we did not observe any effects on the activity related phosphorylation of p38, we found significant difference in ERK and JNK phosphorylation between the two O_2 levels, both in myogenic and adipogenic differentiation media. The most surprising observation was seen in cells

under myogenic conditions, where phosphorylation of ERK was dramatically higher at 5% compared to 20% O_2 in the diaphragm cells, whereas the opposite was the case for the SM cells. A few studies have shown that different skeletal muscles can exhibit opposing responses, with regard to expression of transcription factors, contractile proteins and metabolic enzymes, when treated to the same stimulus such as stretch or denervation (Brownson and Loughna, 1996; Walters et al., 2000). To our knowledge however, the opposite responses of pERK in cells from these two muscles to O_2, is unprecedented with regard to satellite cells derived from different muscle sources. The picture is further complicated nevertheless by the fact that at both O_2 concentrations, the basal level of activity related ERK phosphorylation is far greater in the SM than in the diaphragm. Interestingly however, it has been shown that basal pERK levels are much lower in the slow oxidative soleus muscle of the rat than in fast phasic muscles (Shi et al., 2007). Furthermore, the Shi et al. (2007) study shows that pERK was not affected by beta-agonist administration in the soleus but was dramatically up regulated in fast muscles. Another rodent
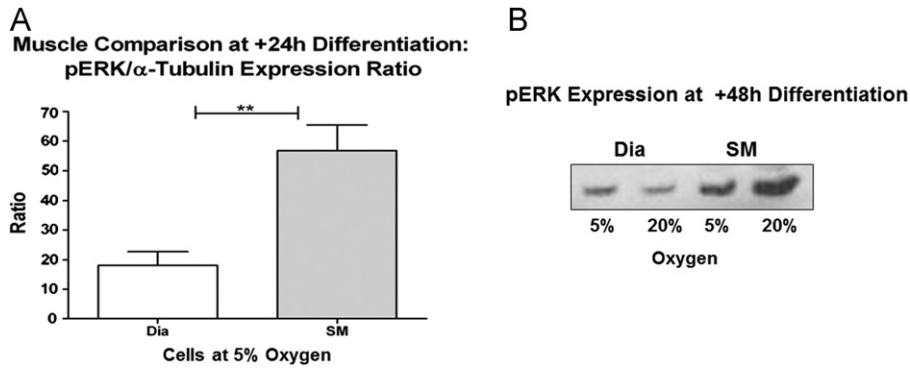
Fig. 5. Comparison of pERK expression between stem cell sub-populations. (A) Expression ratio of pERK/α-Tubulin at +24 h myogenic differentiation ($n=3$). (B) Representative image of pERK expression by SDS-PAGE at +48 h myogenic differentiation ($n=3$).
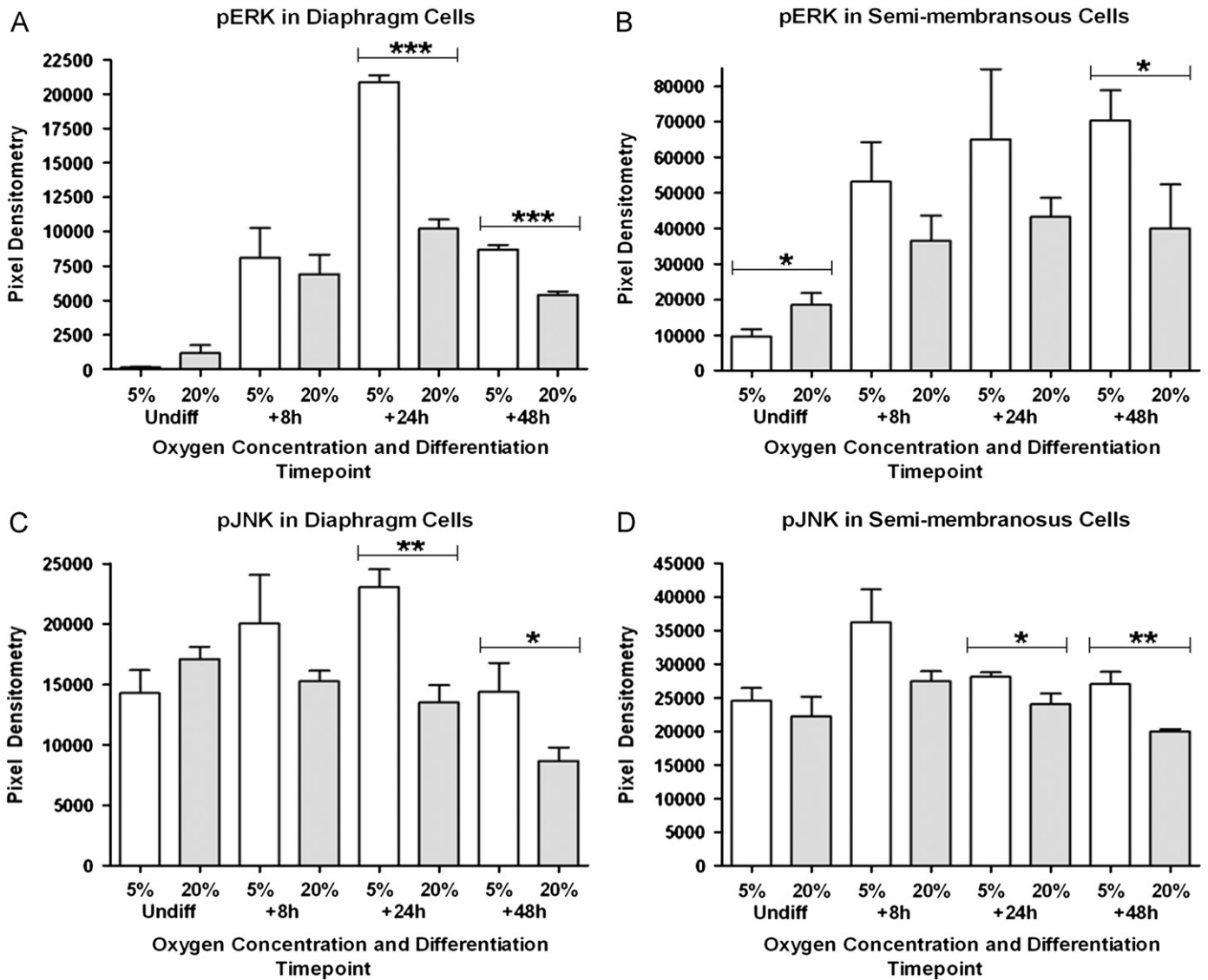

Fig. 6. SDS-PAGE densitometry for signalling factors during adipogenic differentiation. Expression of pERK during early adipogenic differentiation in (A) diaphragm cells and (B) SM cells. Expression of pJNK during early adipogenic differentiation in (C) diaphragm cells and (D) SM cells. Time points taken were for cells at: undiff −70% confluent, +8 h, +24 h and +48 h of differentiation ($n=3$). Error bars represent SEM.

study has also shown that diabetes and insulin administration can have opposite effects on pERK in the white and red regions of the gastrocnemius muscle (Ekladous et al. 2008).

The differential effects between satellite cells from the two muscle tissues, both in response to differentiation medium and O_2 concentration can only easily be explained in two ways. Firstly, all stem cells

within the diaphragm differ from those in the SM, in their differentiation properties and response to stimuli such as O_2. Secondly, these data could be explained by heterogeneity in cells present in all skeletal muscles. Evidence for the latter comes from studies in rodents that suggest that two sub-populations of satellite cells exist and that they differ in their proliferation and differentiation properties (Rossi et al., 2010; Wozniak et al., 2005). They show that one of the sub-populations has a greater myogenic capacity and the other is less differentiated and is more 'stem cell like' in its properties (Rossi et al., 2010). The presence of two such populations in the diaphragm and SM, but differing considerably in their relative proportions, could explain the observations in this study. Our own proliferation studies, originally reported for 20% O_2 (Redshaw et al., 2010) revealed that cells from SM underwent greater proliferation that those from diaphragm and this pattern was also observed at 5% O_2 ($P=<0.0001$, data not shown). Furthermore, while little difference between oxygen concentration was observed for cells from SM, diaphragm derived cells exhibited greater proliferation at 5% O_2 ($P=<0.0001$, data not shown). Alternatively, the 'two populations/relative proportions' suggestion may be an oversimplification, as recent studies have stated that satellite cells can only differentiate along a myogenic lineage and that adipogenic or ostoegenic differentiation is a property of a population(s) of stem cells resident in skeletal muscle but external to the fibre basil lamina and with a different developmental origin (Joe et al., 2010; Uezumi et al., 2010). These studies were based upon isolation of cells using FACS analysis based upon cell surface markers expressed in mice. As stated in one of these studies it is extremely difficult to promote adipose accumulation in muscle of mice even in pathological conditions (Uezumi et al., 2010) which is not the case in large animals and humans, suggesting potential species differences. Likewise, a recent comparative analysis of muscle growth and wasting has shown marked differences between rodents and humans (Rennie et al., 2010). The elucidation and characterisation of such populations (satellite and non-satellite cells) within the skeletal muscle of large animals is currently hampered however, due to the paucity of suitable markers.

3.5. Adipogenic differentiation and MAPK phosphorylation

As with myogenesis, we compared the expression of ERK and JNK at the same time points of adipogenic differentiation. The pattern of pERK expression in diaphragm was similar, with levels peaking at 24 h and significantly higher at 5% O_2 (24 h $P=0.0001$; 48 h $P=0.0008$, Fig. 6A). Likewise, pJNK levels peaked at 24 h, with more expressed at the lower O_2 concentration (24 h $P=0.005$, 48 h $P=0.05$, Fig. 6C). In SM cells undergoing adipogenesis, pERK increased with time, however in undifferentiated cells pERK expression was greater at 20% O_2 ($P=0.04$), but this switched to 5% O_2 from 8 h onwards (48 h $P=0.05$, Fig. 5B). Phosphorylated JNK expression was similar to that seen in myogenesis, with more protein present at 5% O_2 (24 h $P=0.04$; 48 h $P=0.008$, Fig. 6D).

In our adipogenic differentiation studies, phosphorylated ERK levels were significantly higher at 5% O_2 than at 20%, for cells from both muscles. Typically, increases in pERK expression are a consequence of the classical signalling of insulin, but the difference in response to O_2 most likely involves the action of other factors. It has been shown that oxidative stress induced by hyperglycaemia, can induce transdifferentiation of muscle stem cells into adipocytes and that this process involves the action of p66Shc (Natalicchio et al., 2011). Furthermore, p66Shc has been shown to suppress phosphorylation of ERK and thus could explain the lower levels observed in cells at 20% O_2 (Natalicchio et al., 2011).

P66shc has a function that is distinctly different from other shc proteins in that in response to pro-oxidant stimuli, it translocates to mitochondria where it directly generates ROS, by

transferring electrons from cytochrome c to O_2 (Giorgio et al., 2005). The phosphorylation of p66shc and its subsequent mitochondrial accumulation has been shown to be through the action of Protein Kinase Cβ (PKCβ), which in turn is activated by oxidative conditions within the cell (Pinton et al., 2007). This pathway has subsequently been shown in a number of different cell types. Thus, if the increased adipogenesis observed for the SM in this study at 20% O_2 (Fig. 3A), is due to oxidative stress activating increased ROS production mediated by p66shc, then the inhibition of PKCβ should inhibit this difference. We therefore used the specific PKC inhibitor Hispidin to examine this in SM cells.

Following adipogenic differentiation, the action of blocking PKCβ with Hispidin significantly reduced the accumulation of lipid observed in cells at 20% O_2 ($P=<0.01$, Fig. 7), however no difference was seen at 5% O_2 (data not shown). As complete lipid ablation did not result, this suggests that the PKC pathway was only partially involved in the transdifferentiation of SM cells towards an adipogenic fate. However, the initial difference we observed in lipid accumulation for SM cells was ~50% greater at 20% O_2 than 5% (Fig. 3A) and following Hispidin treatment (at 20% O_2), we also observed ~50% greater lipid in untreated cells compared with those that were Hispidin treated (Fig. 7). This suggests that the increased adipogenesis, seen here at the higher O_2 concentration can be completely explained by the action of PKCβ and is probably a function of ROS produced in the mitochondria. A number of studies have shown that while short-term activation of specific ROS (such as nitric oxide) can be beneficial in the activation and proliferation of muscle stem cells, chronic exposure to ROS can be deleterious (Pani, 2010; Wozniak et al., 2005). Of note, the inhibition of the PKC pathway was only affected at the higher 20% O_2 concentration and not at physiological levels, supporting the role of oxidative stress involvement in pushing SM cells towards a non-myogenic fate.

One of the main conclusions of this study is that the O_2 levels in which cells are cultured *ex vivo* appears critical to the differentiation response of cells. Although the degree of differentiation varied between the two sub-populations of muscle derived stem cells, culture at a more physiological O_2 concentration enhanced myogenesis for cells from both SM and diaphragm. Conversely, culture of cells at atmospheric O_2 levels benefited adipogenic but impaired myogenic commitment. This alternate fate for myogenic stem cells
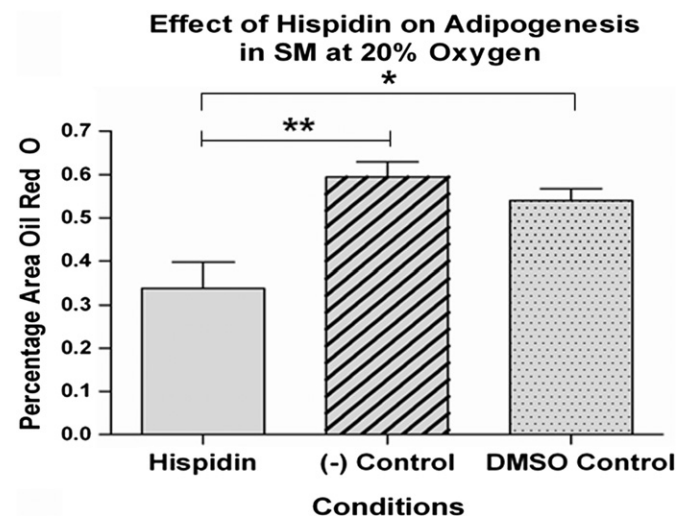

Fig. 7. Effect of Hispidin on adipogenesis/lipid accumulation in SM derived cells. Inhibition of protein kinase C pathway at 20% oxygen concentration following differentiation (at day 6, $n=4$, 15 FOV/replicate). (Original image magnification 100x). Error bars represent SEM.

appears triggered in response to elevated ROS and which seem likely to involve the PKC pathway, somewhat mimicking events which occur during ageing and disease. Interestingly, our two sub-populations of muscle stem cell exhibited some differences in the expression of key signalling proteins involved in differentiation, suggesting altered regulation depending on the muscle origin and perhaps metabolic requirement of that tissue. In summary, physiological O_2 concentration appears as fundamental to recreating the micro-environmental niche as routinely employed strategies such as substrata, matrices and addition of growth factors.

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Author contributions

Zoe Redshaw participated in the conception and design of the study, carried out all experimental work, collection of data, data analysis and manuscript writing. Paul T Loughna participated in the conception and design of the study, data analysis, manuscript writing and final approval of the manuscript.

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