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Research Article

Culture in low levels of oxygen enhances in vitro proliferation potential of satellite cells from old skeletal muscles

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Abstract. The proliferation ability of satellite cells (considered the 'stem cells' of mature myofibers) declines with increasing age when cultured under standard cell culture conditions of 21% oxygen. However, actual oxygen levels in the intact myofiber in vivo are an order of magnitude lower. No studies to date have addressed the issue of whether culturing satellite cells from old muscles under more 'physiologic' conditions would enhance their proliferation and/or differentiation ability. Therefore, we analyzed satellite cells derived from 31-month-old rats in standard cultures with 21% O_2 and in lowered (~3%) O_2. Under the lowered O_2 conditions, we noted a remarkable increase in the percentage of large-sized colonies, activation of cell cycle progression factors, phosphorylation of Akt, and downregulation of the cell cycle inhibitor $p27^{Kip1}$. These data suggest that lower O_2 levels provide a milieu that stimulates proliferation by allowing continued cell cycle progression, to result ultimately in the enhanced in vitro replicative life span of the old satellite cells. Such a method therefore provides an improved means for the ex vivo generation of progenitor satellite cell populations for potential therapeutic stem cell transplantation.

Key words. Hypoxia; stem cell; cellular senescence; aging; cell cycle; Akt.

The use of ex vivo-expanded stem cells can improve several aspects of autologous stem cell transplantation. For example, allowing the limited numbers of stem cells to expand or differentiate ex vivo into specific subtypes by chemical induction or gene transfer greatly enhances the function of the end organ [1]. On the other hand, normal diploid cells when cultured have a limited proliferation potential prior to undergoing replicative senescence [2]. Therefore, better expansion of the stem cell pool ex vivo might enhance the efficacy of cell transplantation for therapeutic repair of tissues [3]. Understanding the methods to enhance proliferation and the potential therapeutic application of these stem cells for use in skeletal muscle degenerative diseases, such as muscular dystrophy and sarcopenia (age-associated involuntary loss of muscle mass) has become a major focus of research [4, 5]. Unfortunately, primary cultures of skeletal muscle precursor cells isolated from these degenerative conditions retain a limited ability for self-renewal, most likely due to the development of replicative senescence [6–9]. Therefore, these cells require some effector to maintain and enhance their proliferation in culture. Such an enhancement strategy would generate larger numbers of ho-

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mogenous myogenic stem cell progeny for possible therapeutic transplantation, since satellite cells are absolutely required for the growth and repair of mature skeletal muscles [10].

Remarkably, recent studies by Morrison et al. [11] and Studer et al. [12] demonstrated that the lowering of oxygen to more physiological levels in cultured immortalized neural crest and central nervous system stem cells increased their proliferation, reduced cell death, and enhanced dopamine neuron generation. Thus, these studies hinted toward the possibility that similar methods may also be employed to enhance the proliferative potential of mature non-immortalized skeletal muscle stem cells. To date, there are no data documenting the effects of decreased oxygen levels on the proliferation of this cell type. All previous studies of satellite cell proliferation in culture have been performed under standard tissue culture conditions of a 5% CO_2/95% air mixture (21% oxygen). However, studies by Richardson et al. [13] revealed that the intracellular myofiber PO_2 values are about 6–7 Torr (~1%). Therefore, to generate a more physiological environment, we cultured satellite cells from skeletal muscles of 31-month-old aged rats in decreased oxygen levels (~3% oxygen). More importantly, since the underlying biochemical mechanisms by which reduced oxygen levels promote satellite cell proliferation are currently unknown, our objective was also to discern the effect(s) of decreased oxygen on some of the key cell cycle regulatory pathways in the cultured, nearly senescent skeletal muscle satellite cells. Given that replicative senescence is biochemically characterized by a cell cycle arrest at the G_1/S boundary [14, 15], we therefore hypothesized that decreased oxygen tension (~3%), as compared to standard 21% O_2, would increase the proliferation of these old satellite cells by increasing G_1/S cell cycle progression factors [cyclin D1, cyclin E, cyclin A, and cyclin-dependent kinase 2 (cdk2)], as well as by decreasing the levels of cell cycle inhibitors, such as $p27^{Kip1}$, a critical G_1/S inhibitor of cdk 2 [16, 17]. Previous studies had implicated activation of the Akt signaling pathways as one of the mechanisms by which neuronal cells adapt and survive under hypoxic conditions [18]. Given that activation of Akt via its phosphorylation is associated with G_1/S cell cycle progression in a variety of different cell types [19, 20], we further hypothesized that low oxygen levels would also enhance Akt phosphorylation in the old skeletal muscle satellite cells.

Materials and methods

Materials

Cell culture media and reagents were purchased from Gibco Life Technologies (Rockville, Md.). Anti-desmin (D3) and anti-sacromeric myosin (MF20) monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank, Iowa.

Animals

Twelve pathogen-free 31-month-old Fisher 344 × Brown Norway (FBN) F1 generation male rats were obtained from the NIH aging program from the National Institute on Aging. Pairs of animals were housed in previously autoclaved bedding in microisolator cages and provided with autoclaved food and water *ad libitum*, in a room maintained at 21 °C with a 12:12 h light:dark cycle. The animals were allowed to acclimatize to their new surroundings for ~2 weeks before any experiments were done. All experimental protocols and surgical procedures were approved by the Institutional Animal Care and Use Committees.

Harvesting of muscles and satellite cell culture conditions

Animals were euthanized by cervical dislocation following an overdose with anesthetic cocktail consisting of 0.84 mg/kg ketamine hydrochloride, 0.54 mg/kg xylazine, and 2.2 mg/kg acepromazine. Gastrocnemius muscles from both hindlimbs were then immediately removed, trimmed of excess connective tissue and fat, and weighed to determine wet weight. Gastrocnemius muscles from three sets of four animals were pooled to ensure an adequate amount of tissue for satellite cell isolation; the methods for their isolation have been described in detail previously [6]. After isolation, the cells were passaged before reaching confluency, and cultivated using Ham's F-10 nutrient mix containing 20% fetal bovine serum, 1% chicken embryo extract, with 1% penicillin-streptomycin antibiotic mix, and 1% L-glutamine as the culture medium (referred to as 'proliferation-rich medium') in standard humidified tissue culture incubators with a 5% CO_2/95% air mixture (21% O_2). Cells were grown on rattail collagen-coated tissue culture plates, as described previously [6].

Immunocytochemistry

Cultures were monitored for myogenic purity by using an antibody against desmin, an intermediate filament protein expressed only in myogenic cells. The methods are described in detail elsewhere [6, 21]. Apoptotic cells in culture were identified by condensed and fragmented chromatin after Hoescht 33342 staining (1:1000), and terminally differentiated cells were identified by staining with anti-sarcomeric myosin antibody, using previously published methods [6, 9].

Low-oxygen culture conditions

To achieve low oxygen tensions, tissue culture plates with satellite cells were inserted into gas-tight modular incubator chambers (Billups-Rothenberg, Del Mar, Calif.)

that were flushed with a custom gas mixture containing 1% O_2, 5% CO_2, with balance N_2, modified from the methods of Morrison et al. [11] and Studer et al. [12]. Based on their methods, we also noted that the precise O_2 levels in the chamber atmosphere, and hence within the culture media, depended on the length of flush with the custom gas mixture (90 s at 15 l/min achieved 6% O_2, and a 6-min flush achieved ~1% O_2). This was subsequently verified by direct oxygen concentration measurements via microelectrode (Animus Corp. Frazer, Pa.), which revealed an actual ambient concentration within the chamber of 3 ± 2% (denoted ~3%) oxygen, which in turn approximates physiologic intra-myofiber O_2 levels [13]. The entire chamber was then housed inside standard tissue culture incubators to maintain 37°C temperature. Once cultures were established in the reduced-oxygen chambers, opening of the gas-tight compartment was minimized to avoid contamination with room air. These cultures were refed every 7 days with proliferation-rich medium for those experiments where prolonged growth periods (4–5 weeks) were utilized (see below for colony size distribution assays). For experiments with cultivation periods less than 7 days (see below for immunoblotting and differentiation assays), the seals of the flushed incubators were left undisturbed until the termination of the experiment. Further, to minimize O_2 fluctuations at the time of medium changes, media were pre-equilibrated at 37°C at the decreased oxygen conditions in separate chambers in the incubator, and the chambers were thoroughly flushed with the custom gas mixture as detailed above.

Colony Size Distribution Assays

Prior to experimentation, satellite cells were trypsinized after one passage in culture following their isolation, and counted using a hemacytometer to ensure a single-cell suspension and to determine cell number. The cells were subsequently inoculated into several 60-mm collagen-coated tissue culture dishes at clonal density (~50–100 cells per dish), and placed in standard tissue culture incubators (21% O_2, control cultures) or in the modular incubator chambers (~3% O_2). At this plating density, individual colonies were still well separated, even after proliferating for 4–5 weeks. Determination of the proliferative behavior of single cells in these cultures was accomplished by colony size distribution assays as described previously [6, 22]. This method has been shown in numerous studies to validly assess the inherent heterogeneity present in the growth of such cultures. After a 5-week growth period, the dishes were fixed in 1% gluteraldehyde and stained with 0.5% crystal violet, and the number of cells in each colony (defined as one or more attached cells) up to a maximum of 256 cells was determined with a dissection microscope, and a total of 200–250 colonies were scored for each group. Parallel sets of cultures from the 21% O_2 control and low-O_2 groups at the end of the 5-week period were stained with anti-sarcomeric myosin, anti-desmin, and Hoescht 33342 as described above to ensure that the cells constituting the colonies were undifferentiated, myogenic, and non-apoptotic, respectively.

Immunoblotting

After the first passage following their isolation, satellite cells from the 31-month-old skeletal muscles were seeded into 100-mm collagen-coated tissue culture dishes at a density of $1 \times 3\%\ 10^5$ cells/dish and grown to about 40–50% confluency under standard tissue culture conditions (5% CO_2/95% air), after which one set of plates was transferred to the modular incubator chambers to be cultivated in ~3% O_2, while the other set remained in the standard incubator (21% O_2 control cultures). Pilot experiments had revealed that undisturbed cultivation for 3–4 days allowed these aged cells to reach ~80% confluency. Following exposure to low oxygen or 21% oxygen for 3–4 days, cultures were immediately lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 10% glycerol, 200 mM NaF, 20 mM sodium pyrophosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 200 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate) on ice for 30 min, and total protein from whole-cell extracts of satellite cells from each of the groups was prepared as detailed previously [19]. Aliquots of 50–75 μg of total protein from each cell lysate were subjected to Western blot analysis using antibodies against $p27^{Kip1}$ (06445), cdk2 (06-505), cyclin E (06-459), cyclin A (06-138), and cyclin D1 (5D4) (all from Upstate Biotechnology, Lake Placid, N.Y.). A phospho-specific antibody that recognizes phosphorylation of Akt specifically on the Ser^{473}-residue and a pan-Akt antibody (9270; New England Biolabs, Beverly, Mass.) were also used as described previously [19]. Bound antibodies were detected using horseradish peroxidase-linked sheep anti-mouse or donkey anti-rabbit antibodies (Amersham Pharmacia Biotech, N.J.), and visualized by enhanced chemiluminescence (NEN Life Science, Mass.) after exposure to X-ray film (Kodak X-OMAT AR). Scanning densitometry (Molecular Dynamics, Ore.) was then used to quantitate the protein bands on the film.

Differentiation

Satellite cells were plated at a density of 1×10^7 cells/100-mm dish following their first passage after harvesting from the 31-month-old skeletal muscles, and were maintained in a standard tissue culture incubator (21% O_2) until they reached >90% confluency. Subsequently, the proliferation-rich medium was removed and the cells were maintained in differentiation medium (DMEM/2% horse serum/1% penicillin-streptomycin antibiotic mixture/1 × insulin-transferrin-selenium mixture) for 5–6

days, either in 21% O_2 or in lowered-O_2 conditions. This was the minimum period required to observe fusion in these old satellite cells under standard tissue culture conditions, based on our previous studies [6]. Following this undisturbed cultivation period in the respective incubators, the cells were fixed and processed for staining with anti-sarcomeric myosin antibody, as described previously [6–9].

Statistical analysis

Data are presented as mean ± SE. Differences in cell proliferation and biochemical analyses between the two groups (21% vs ~3% O_2) were analyzed using a two-tailed Students t test (Statistica software package). For all statistical tests, the 0.05 level was used as statistical significance.

Results

Low oxygen enhances proliferation ability and myoblast differentiation of 31-month-old satellite cells

Wet weights of the 31-month-old gastrocnemius muscles and their total protein contents (both normalized to body weight), and the in vitro proliferation potential of the cultured satellite cells have all been documented previously [6, 7 ,23]. All of these parameters demonstrated a characteristic age-associated decline, compared to young (3-month-old) FBN rats. Remarkably, when the 31-month-old satellite cells were cultured at decreased oxygen tension (~3% O_2), they had a significantly greater ability to survive and form colonies compared to the age-matched control cultures (21% O_2). About 54% of the cells added to the culture plates formed colonies in decreased oxygen versus only 17% of cells in 21% oxygen ($p < 0.01$); a representative of the culture plates following staining with crystal violet is presented in figure1A. Concurrent staining of parallel sets of plates with anti-desmin antibody to determine the percentage of myogenic colonies revealed that 97.1 ± 1.8% and 96.4 ± 1.1% of the total colonies stained positively for desmin in the 21% and ~3% O_2 groups, respectively, which is similar to our previously documented results [6]. The remaining 2.9 ± 0.8% and 3.6 ± 0.5% of the non-desmin staining colonies in the 21% and ~3% O_2 groups, respectively, were deemed to be primarily fibroblastic based on their characteristic flattened morphology [6–9]. Additionally, parallel sets of colonies in each of the groups examined at the end of the 5-week growth period in serum-rich proliferation medium had minimal numbers of apoptotic and differentiated cells (data not shown). The number of colonies per dish did not change from week 1 through week 5, indicating that the migration of cells or reseeding of clones did not alter the colony size distributions, which is consistent with previous reports [6, 24].

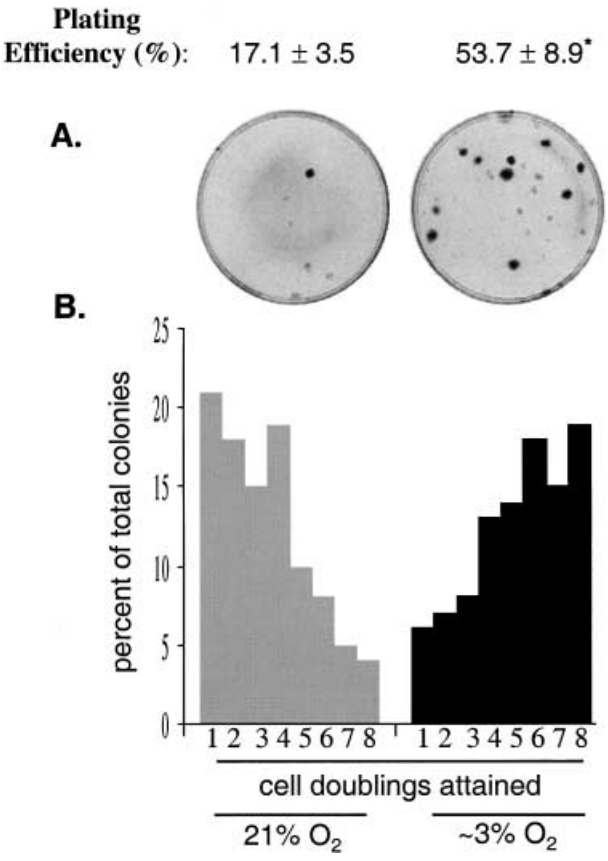

Figure 1. Culturing in lowoxygen tension increases the colony-forming capacity of satellite cells from 31-month old rats. (*A*) Representative set of plates of satellite cell colonies cultivated in 21% and ~3%. Colonies were stained with 0.5% crystal violet after allowing cells to grow for 5 weeks in serum-rich proliferation media. Plating efficiency indicates the percentage of cells inoculated into culture that subsequently went on to form colonies 5 weeks later. Data are presented as the mean ± SE from five independent experiments in triplicate in which an average of 200–250 colonies were counted per treatment per experiment. Results were compared using Student t tests: *denotes statistical significance ($p < 0.01$). (*B*) Colony size distributions (CSD) were obtained by counting the number of cells within each colony up to a maximum of 256 cells (representing eight cell doublings). The percentage of total colonies (y-axis) able to attain a specified size, denoted by the number of cell doublings attained (x-axis) in culture over the 5-week growth period is shown. Solid gray and black bars denote CSD from 21% or ~3% oxygen groups, respectively. Each of the groups was cloned in triplicate and the colony sizes attained were averaged. A total of 200–250 colonies were counted for each group, and the results are representative of five independent experiments for each of the two groups.

Decreased O_2 levels not only produced a greater number of colonies, but also resulted in larger colony sizes, thereby indicating a greater doubling capacity. Colony sizes were quantitated 5-weeks after seeding by determining the distribution of the colonies of different sizes as the percentage of colonies containing at least a specified number of cells. Satellite cells cultivated in decreased O_2 conditions had markedly larger colony sizes (fig. 1B) as evidenced by an approximately fivefold

higher ($p < 0.01$; fig. 1B) percentage of colonies consisting of ≥ 256 cells (or ≥ eight cell doublings) relative to age-matched cells cultivated in 21% O_2 (19 vs 4%, respectively). Indeed, there was a progressive shift to the right noted in the colony size distribution of cells cultivated in ~3% O_2, indicating an overall increase in the in vitro proliferation potential of the old satellite cells when cultured under these conditions. The colony size distribution of the corresponding age-matched cells in 21% O_2 was similar to previous observations [6], and hence confirmed previous reports of decreased satellite cell proliferation capacity with age under standard tissue culture conditions of 5% CO_2/95% air [7, 9]. Examination of the fibroblastic colonies revealed that lowering oxygen tension proportionally increased their colony sizes to an extent similar to that observed in the myogenic colonies an approximately four- to fivefold increase in the percentage of colonies with >256 cells in ~3% O_2 compared to 21% O_2), thereby corroborating the studies of Saito et al. [25] in human fibroblasts. However, the numbers of such non-myogenic colonies in both the 21% and ~3% oxygen cultures were significantly lower (<4%), and thus the colonies that were scored in the colony size distribution assays shown above predominantly represent the proliferative behavior of myogenic cells in the different oxygen environments.

Myogenic precursor cells are programmed to differentiate into myotubes when growth factors are withdrawn, e.g., when switching the serum-rich proliferation medium to low-serum differentiation medium [25]. As an independent test of myogenic potential for the cells cultured in low O_2, we initiated differentiation in the 31-month-old satellite cell cultures using low-serum conditions (2% horse serum) in either 21% or ~3% oxygen. After 5–6 days in the differentiation media, the cultures were stained with sarcomeric myosin to visualize the extent of myogenic differentiation (fig. 2). Culturing in ~3% O_2 (fig. 2B) compared to standard 21% O_2 (fig. 2A), produced robust myogenic differentiation with large myotubes. Since myotube formation is one of the important functional endpoints in the culturing of primary muscle precursor cells, and given that the ability to form myotubes is a distinguishing feature of all myogenic cells, these results further validate the high yield of myogenic purity obtained from our isolation procedures, and therefore additionally corroborates the assessment of myogenic purity of these cultures as determined via desmin staining.

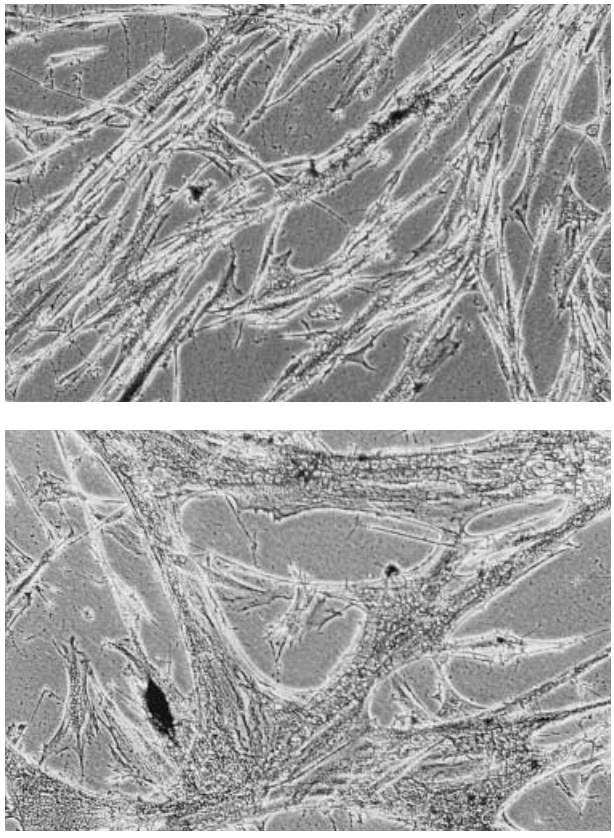

Figure 2. Low oxygen enhances myogenic differentiation by producing enlarged myotubes. Representative photomicrographs of myotubes stained with a differentiation marker, a monoclonal antibody specific for sarcomeric myosin (MF20, 1:5 dilution in PBS) using biotinylated horse anti-mouse antibodies and avidin-biotin-peroxidase complex (Vector), both diluted 1:100 in PBS, with 3-amino-9-ethyl carbazole as the chromogen (Sigma). Cells were photographed at ×10 magnification. The cultures allowed to differentiate in 21% oxygen (*A*) demonstrate relatively thin myotubes, in contrast to the cultures cultivated in ~3% oxygen, (*B*) which show robust formation of myotubes with hypertrophy.

Enhanced cell cycle progression and activation of Akt are induced by decreased oxygen

We sought to determine part of the underlying biochemical basis for the dramatic enhancement in proliferation ability observed in the 31-month-old satellite cells cultivated in low oxygen. We had previously observed [6] that 31-month-old satellite cells were near-senescent (i.e., had fewer doublings left before reaching a state of irreversible growth arrest), termed replicative senescence, and which is characteristic of all normal diploid cells [2]. Replicative senescence is biochemically marked by a distinctive arrest at the G_1/S boundary of the cell cycle: many of the molecules (cyclin A, cdk2, $p27^{Kip1}$) regulating this transition point are dysregulated in these 31-month-old satellite cells [Chakravarthy et al., unpublished observations]. Therefore, we investigated whether a decreased oxygen stimulus would modulate these same regulatory factors so as to promote G_1/S cell cycle progression and, consequently, allow these cells to continue doubling. Low O_2 (~3% O_2), compared to room air, induced a dramatic increase in the protein abundance of cyclin D1, cyclin E, cyclin A, and their kinase partner, cdk2 (fig. 3A–D). Since cell cycle inhibitors are known to bind and hence inactivate cyclin-dependent kinases [16],

we also examined the protein abundance of the well-characterized inhibitor, $p27^{Kip1}$. This molecule has been shown to bind specifically to cdk2 [17], and to play a central role in the control of cellular senescence in a variety of other cell types [19, 20, 26]. Low oxygen markedly downregulated the total protein abundance of $p27^{Kip1}$ (fig. 3E). Thus, lowering oxygen levels may be a potent inducer of cellular proliferation by positively modulating critical G_1/S regulatory factors to promote cell cycle progression, ultimately resulting in the observed enhancement of the in vitro replicative life span of these cultured cells.

Activated Akt is one of the central regulators of cellular proliferation, especially via its interaction with several of the G_1/S cell cycle molecules [19, 20, 26]. Phosphorylation of Akt on the Ser^{473} residue situated in its catalytic site has been shown necessary for the induction of its activation [28]. Hence, we examined the effects of decreased oxygen on phosphorylation of Akt by immunoblotting with an antibody specific for Ser^{473}-phosphorylated Akt. Thirty-one-month-old satellite cells exposed to low oxygen tension (~3% O_2) for 4 days demonstrated a dramatic enhancement in phospho-Akt immunoreactivity compared to that seen in cells exposed to 21% oxygen (fig. 3F). This effect was not due to changes in the total amount of Akt protein, since similar levels of total protein were present in both groups (fig. 3G). No change in Akt phosphorylation was observed after 24–48 h of exposing cells to decreased oxygen levels (data not shown), but phosphorylation was significantly enhanced after the 3rd day of low oxygen. Indeed, the effect of decreased oxygen on phosphorylation of Akt in these 31-month-old satellite cells was robust, as it was similar to the high levels of Akt phosphorylation seen with IGF-I-stimulated satellite cells at 1 month [19], as well as IGF-I-stimulated satellite cells at 31 months (data not shown). The decreased level of Akt phosphorylation seen in the 31-month-old satellite cells cultivated in 21% oxygen is consistent with previous observations noted in a variety of senescent cell types [20, 27], including senesced satellite cells [19].

Since this effect on Akt phosphorylation was noted only after 3 days of undisturbed exposure to decreased oxygen levels, the effects of exogenous drugs such as wortmannin could not be effectively tested given the toxicity of this drug when used for prolonged periods of time (after 2 days of 50 nM wortmannin or 20 nM LY294002, cells were rounded and detached from the substrate; data not shown). Furthermore, to ensure a constant low oxygen level, we had to avoid repeated opening and closing of the modular incubator chamber and, consequently, adding exogenous agents during the course of this experiment was not possible. This was an inherent limitation of this system.

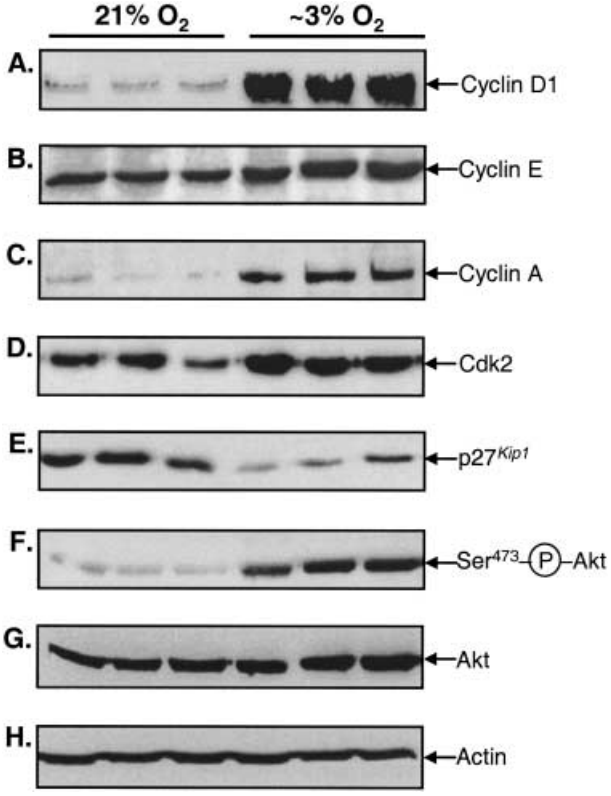

Figure 3. Decreased oxygen levels induce the activation of G_1/S cyclins, cdk2, and Akt phosphorylation, while downregulating the $p27^{Kip1}$ cell cycle inhibitor. Thirty-one-month-old satellite cells were maintained under standard tissue culture conditions of 5% CO_2/95% air (21% O_2) or exposed to low oxygen tension (~3%) for 4 days. Total protein from whole cell lysates of these satellite cells was extracted and resolved by SDS-PAGE and subjected to Western blot analysis using antibodies to cyclin D1 (*A*), cyclin E (*B*), cyclin A (*C*), cdk2 (*D*), $p27_{Kip1}$ (*E*), and Ser^{473}-phospho-specific Akt (*F*). The latter blot was subsequently stripped and reprobed with a pan-Akt antibody that recognizes total phospho- and dephospho-Akt (*G*). For normalization, each of these blots was subsequently probed with an antibody against actin (*H*) that recognizes all isoforms of cardiac, skeletal and smooth muscle actins. Immunoblots were performed with n = 12 dishes of cells for each condition (21% O_2 and ~3% O_2), performed in triplicate in four separate experiments. Representative blots from one such triplicate set from the series are presented.

Discussion

A novel observation of this study was the dramatic enhancement in in vitro proliferative potential of satellite cells derived from 31-month-old rat skeletal muscles when cultured in decreased oxygen tension. While previous studies had shown that 2–5% O_2 levels enhanced proliferation of neural crest stem cells [11], multipotent central nervous system progenitor cells [12], fibroblasts [25], and hemopoietic cells [29], the findings presented in this study are the first to document a role for lowered oxygen in extending the in vitro replicative life span of near-senescent muscle precursor cell cultures.

Thus far, most culture experiments measuring proliferation and hence replicative senescence in satellite cells have been performed at atmospheric oxygen levels (~21%), which are far above the O_2 concentration (~1%) estimated within skeletal muscle fibers of the human limbs in situ [13], or the O_2 levels reported in other tissues, such as those seen in alveolar air (14%), arterial blood (12%), and venous blood (5.3%) [30]. Morrison et al. [11] cautioned that while the kinetics of O_2 diffusion may vary between in vitro cultures and in vivo tissues, thereby making difficult precise comparisons between O_2 levels in culture and in vivo, a 2–5% O_2 concentration in culture is clearly much closer to physiological levels than the 21% oxygen used in most cell culture experiments. Given these facts, we opted to compare 21% and ~3% O_2 as the gas phase oxygen concentration in the current experiments. We speculated that the cultivation of the 31-month-old satellite cells in 21% O_2 may result in a dysregulation of their enzymatic/metabolic functions, resulting in a growth-arrested, senescent-like phenotype. This notion is supported by our intriguing finding that when these cultures were shifted to a lower O_2 environment, thereby restoring them to a more 'homeostatic' and physiologic milieu, these old cells were able to resume proliferation and continued to double, resulting in the observed increase in the number of large-sized colonies.

Furthermore, myogenic differentiation was significantly promoted in decreased-oxygen cultures, suggesting that such low-O_2 ('physiologic') conditions may also provide a milieu that induces 'hypertrophic' stimuli. To minimize effects based upon increased fusion due to continued myoblast proliferation in low O_2, we controlled not only for cell number by plating equal numbers of cells in both groups, but also for differences in growth rates. The latter was based on pilot experiments using BrdU incorporation which revealed that at the time point when the 31-month-old cultures were >90% confluent, their BrdU labeling index was <2–3%, and hence these cells could be assumed to be mostly growth arrested. Only after reaching this confluent stage in 21% oxygen, were a subset of these cultures inserted into the modular incubator chambers to study the effects of low oxygen on differentiation. Therefore, based on our experimental design, an effect of low O_2 on proliferation is likely not a major factor. Rather, low O_2 either improved the efficiency of satellite cell fusion, enlarged the mass of the myotubes, or both. Future experiments would have to test these possibilities. Taken together, these culture data not only confirm the observations of Ito et al. [31], who showed that mild hypoxia (10% O_2) induced hypertrophy in cultured neonatal rat cardiomyocytes, but also extend the *in vivo* observations of Takarada et al. [32], demonstrating that hypoxia (induced by vascular occlusion) significantly enhanced skeletal muscle hypertrophy in humans undergoing resistance exercise training.

Despite the numerous reports [11, 12, 28, 29] demonstrating the hypoxia-induced enhancement of stem cell proliferation in a variety of other cell types, none of these studies revealed any of the underlying biochemical mechanisms by which reduced O_2 levels promote such proliferation. $p27^{Kip1}$ protein abundance was strikingly lower in the satellite cells cultured in a low-oxygen atmosphere in the current study, compared to its levels in cells cultured under standard 21% oxygen. Since lowering $p27^{Kip1}$ protein by other factors has previously been shown to restore the depressed cdk activities [16], coincident with a progression of the cell cycle through G_1 to S phase [19, 20, 27, 33], the association between lower O_2 and decreased $p27^{Kip1}$ protein is likely to play some role in the enhancement of the in vitro proliferation potential observed in the cultured satellite cells. Polyak et al. [17] suggested that the binding of $p27^{Kip1}$ to cdk2 interferes with the phosphorylation of its activating site (Thr 160). Additionally, $p27^{Kip1}$ has also been suggested to act as a threshold device controlling cdk2 kinase activity, because increased cyclin D1 accumulation sequesters free $p27^{Kip1}$, thereby decreasing $p27^{Kip1}$-cdk2 association, consequently allowing cdk2 activation [34]. Consistent with this notion, we observed a marked accumulation of cyclin D1 protein in the 31-month-old satellite cells cultured in lower O_2. We suggest that the increase in cyclin D1 and E proteins could indicate a greater number of cells entering G_1 in the low-O_2 cultures, while the increase in cyclin A under the low-O_2 conditions may demonstrate more progression through the late stages of G_1 into S phase, since cyclin A is a well-characterized late-G_1 gene whose upregulation results in enhanced G_1/S progression [14].

As a next step, we examined the signal transmission involving activation of Akt, since evidence is increasing that Akt-mediated signaling pathways are involved in the control of G_1/S cell cycle progression [28]. Furthermore, previous studies in satellite cells [19] and other cell types [20, 27] have indicated that $p27^{Kip1}$ is a downstream target of the phosphatidylinositol 3′-kinase/Akt pathway, the activation of which downregulates $p27^{Kip1}$ protein levels. Thus, the observed downregulation of $p27^{Kip1}$ protein in 31-month-old satellite cells exposed to lowered oxygen tension hinted at the possibility that one of the potential mechanisms for the mediation of such downregulation in $p27^{Kip1}$ may in fact be via activation of Akt. Low oxygen dramatically increased phosphorylation at the Ser^{473} residue of Akt, a site that has been shown to be critical for its kinase activity, and hence its subsequent activation for signaling further downstream events [28]. These results are consistent with those of Beitner-Johnson et al. [18], who showed that hypoxia (1% O_2) activated Akt in immortalized neural PC-12 cells. The present data demonstrate an intriguing association of increased Akt phosphorylation, decreased $p27^{Kip1}$ protein, and enhanced in vitro proliferative potential of old satellite cells when ex-

posed to low oxygen levels. Obviously, further experiments will be required to determine whether there is any causal relationship among these findings under low-O_2 conditions, since the regulation of cellular proliferation is known to be multifactorial.

Given the multiple cellular targets of Akt, activation of this molecule by lowering oxygen concentration suggests that the enhanced proliferative potential observed in the 31-month-old cultured satellite cells exposed to lower O_2 could be orchestrated via a multifaceted and highly complex regulatory process. Hypoxia-induced activation of the Akt signaling cascade has been shown to be associated with phosphorylation of GSK-3 [18], as well as phosphorylation of other transcription factors such as CREB and EPAS1 [18], suggesting that hypoxia regulates multiple signaling pathways, including both Akt-dependent and- independent systems. Furthermore, studies by Zhong et al. [35] and Zundel et al. [36] have suggested that activation of Akt leads to stabilization of the hypoxia-inducible transcription factor, HIF-1a. Interestingly, the HIF family of transcription factors are known to bind to hypoxia response elements localized within the promoters of several genes regulating the cell cycle, such as p21 and p53 [37]. Thus, activation of Akt may represent an important aspect of the proliferation response, and Akt likely has a pleiotropic role in various oxygen environments. Additional experiments would help clarify the potential roles (if any) of these other signaling pathways controlled by decreased oxygen that may be functioning in these old satellite cells.

Overall, our results suggest that culturing old skeletal muscle stem cells in physiologic concentrations of O_2 (~3%) is a powerful means to enhance precursor cell proliferation, a highly desirable quality that can be exploited for more efficacious ex vivo manipulation of stem cell cultures for gene therapy and/or myoblast transplantation procedures. Insights into additional biochemical mechanisms by which lowered O_2 levels affect key cellular processes such as proliferation would also help in delineating of fundamental aspects regarding the regulation of replicative senescence.

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