# CELLULAR AND MOLECULAR RESPONSES OF HUMAN SKELETAL MUSCLE EXPOSED TO HYPOXIC ENVIRONMENT

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**The effects of a hypobaric, hypoxic environment and exercise performed under extreme conditions, such as at high altitudes, are intriguing physiological aspects that need to be investigated directly on human climbers. Their skeletal muscle is one of the main tissues that can suffer from hypoxia and physical challenges, which will both define the muscle adaptation and the molecular signature of regenerative capacity. We investigated the muscle regenerative capacity characterizing satellite cells. Our study shows that satellite cells are altered by hypobaric, hypoxic environments and exercise performed at high altitudes. Of note, in human skeletal muscle after this 5,000 m a.s.l. expedition, SCs showed a significantly lower ability to regenerate skeletal muscle, in respect to before this high-altitude expedition. This impairment appears to be due to reduced satellite cell activity, consistent with their decreased myogenicity and fusion ability. Furthermore, at the transcriptional level several pathways, such as cell cycle, myogenesis, oxidative metabolism, proteolysis and sarcomeric protein synthesis, were found dysregulated.**



High altitudes and the associated hypoxia pose physiological challenge to ensure organism survival. The term "acclimatization" is used to describe phenotypic alterations as a response to exposure to high altitudes. Little is known about functional adaptations to chronic hypobaric exposure of unconditioned populations (1). High altitude living produces physiological changes, including skeletal muscle (SM) characteristic and functionality, as consequences of adaptation to chronic hypobaric-hypoxemic environment (HHE). The scientific community agree that severe altitude exposure determines a loss of body weight (5%-10%) (2), while contrasting results have been reported about the effect on loss of SM mass (3) and changes in fiber cross-sectional areas (4). Physical activity may counteract the muscle loss. Thus, several parameters such as total time of exposure, the degree of hypoxia, and physical activity levels may markedly influence the adaptation of SM to hypoxia. In response to high altitude exposure, other compensatory changes also occur in the muscle tissues (5). Hypoxia stimulates

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**DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.**

vascular endothelial growth factor (VEGF) production via hypoxia-inducible factor-1α (HIF1) promoting angiogenesis and blood flow and supplying more oxygen to the tissues (6); mitochondria number and myoglobin increase facilitating movement of $O_2$ into the tissues and its utilization (5). The tissue content of cytochrome oxidase also increases (7). Environmental stimuli induce muscle adaptation and, in turn, muscle regeneration. In adults, muscle regeneration depends on satellite cell (SC) activation. SCs are undifferentiated quiescent mononucleated cells found in muscle with properties of stem cells residing close to the basal lamina. In response to a stimulus, SCs are activated to proliferate as myoblasts that migrate to the targeted region of a muscle fiber to differentiate and fuse to form myotubes via a similar process to that of myogenesis. Myogenesis is highly regulated by muscle-specific transcriptional regulators. SCs express characteristic proteins but are heterogeneous for Pax7 protein. Upon activation, SCs proliferate and up-regulate MyoD. Depending on the expression levels of MyoD, Pax7 and myogenin, proliferating myoblasts can be committed to differentiation or remain in a proliferative state or exit the cell cycle to form a new SC pool (8). In a study of climbers exposed to high altitude, an increased volume density of SCs and lipofuscin levels were shown after exposure to high altitudes, suggesting a failed activation of SCs and uncertain results in term of muscle regeneration (9). To date, very few studies have investigated the effects of hypoxia in human SCs *ex vivo*. It has been demonstrated that molecular tools are useful to study muscle plasticity mechanisms and also to distinguish the molecular signature of an expressional adaptation when hypoxic stress is added to an exercise protocol in climbers (10). In the present study, we investigated the adaptation mechanisms in the *Vastus lateralis* (VL) SM induced by physical activity while under hypoxia at high altitudes. Using VL muscle needle biopsies (pre- and post-expedition), cellular features and gene expression profiles were analyzed.

## MATERIALS AND METHODS

*Subjects and expedition*

Six healthy, male, non-smoking climbers with an average age of 42±6 (± Standard Error, SE) were enrolled in the study. Each climber was identified with an alphanumerical code (C as climber followed by a number). Health evaluations indicated that none of these subjects suffered from cardiovascular, respiratory, metabolic, or SM diseases. The health conditions of climbers were assessed at the time of each muscle biopsy. Written consent was obtained from each participant, and the study was designed in accordance with the recommendations of the Declaration of Helsinki and approved by the Ethics Committee of the Gabriele d'Annunzio University of Chieti-Pescara, Italy (protocol no. 1634/08 COET). The climbers took part in the INTERAMNIA 8000 - MANASLU 2008 expedition that started from Rome (at sea level) on 8 September, 2008, and ended again in Rome on 20 October, 2008. The subjects had not experienced high-altitude conditions within a period of at least 6 months prior to this expedition. After landing in Kathmandu, Nepal (~1,350 m a.s.l.) on 9 September, 2008, the climbers began the ascent phase on 11 September, 2008, to reach the Manaslu Base Camp (5,000 m a.s.l.) on 21 September, 2008. During this 10-day period, the climbers covered approximately 25 km/day and averaged about 7 h of trekking each day. The climbers remained at the base camp for 21 days, during which time they showed very few symptoms of acute mountain sickness, which decreased after a few days; no climber needed any medication. Throughout the expedition, a balanced food intake and *ad-libitum* fluid ingestion was allowed. When the meteorological conditions were favorable, the climbers reached Camp 1 (5,900 m a.s.l.) and Camp 2 (6,400 m a.s.l.), covering approximately 8-10 km/day, an average of 6 h of trekking per day. During the period at the base camp, the climbers attended to their personal needs, re-arranged their tents, and maintained the camp. Nepalese Sherpa packers and base-camp personnel supported the expedition; however, no data were collected from these Nepalese citizens. On 19 October, 2008, the subjects reached Kathmandu, and left on their return flight the following day. After landing in Rome on 20 October, 2008, the climbers were transferred to Chieti (Italy) for health evaluation and muscle biopsy.

*Muscle needle biopsy*

Muscle biopsies were taken before the expedition, after their return from Kathmandu and for C5 2 years after the expedition using a tiny percutaneous needle biopsy as described in Pietrangelo et al, 2009 (11). One sample was collected in HAM's Nutrient Mixture F10 (#ECB7503SL, Euroclone, Milan, Italy) supplemented with 1% gentamicin (#ECM0011B, Euroclone) and stored 1 day at 4°C, until used to isolate SCs. Another sample of about 10 mg was collected in RNA Later (#AM7020, Ambion, Milan, Italy), and stored at -80°C until used to perform transcriptional profile analysis. Another sample

was collected in ice cold skinning solution with 50% (v/v) glycerol (12) and stored at –20°C until used to perform the count of myonuclei and SCs by immunofluorescence.

*Cell culture*

The VL muscle needle biopsies were processed according to the procedures of Fulle et al. (2005). All of the muscle biopsies obtained from all of the climbers before and after the expedition were used to isolate SCs. The first mononucleated cells migrated from the explants at 7-13 days from the beginning of the culture (independent of donor age). Cell cultures were considered senescent when they did not reach one population doubling level (PDL) after three weeks of re-feeding (13). To induce the differentiation of mononucleated cells into multinucleated myotubes, myoblasts were plated at a confluence of 10,000 cells/cm$^2$ and maintained for two days in growth medium. At 2-3 days after plating, the medium was replaced with differentiation medium: DMEM high glucose (#ECB7501L, Euroclone) supplemented with 5% horse serum (#ECS0091L, Euroclone), 50 µg/ml gentamicin (#ECM0011B, Euroclone), 10 µg/ml insulin (#I-0516, SIGMA Chemical Co.; St. Louis, MO, USA) and 100 µg/ml apo-transferrin (#T-2036, Sigma). We followed the differentiation process until day 7. The efficiency of differentiation was determined by counting the number of nuclei in seven-day-differentiated myotubes as a percentage of the total number of nuclei. The ratio of the two values was defined as the fusion index. At least 1,500-2,000 nuclei were counted in 25-30 different randomly selected fields.

*Immunocytochemistry*

The myogenic purity of SC populations was calculated using desmin as a marker. Desmin expression was determined using the D33 antibody (Dako, dilution 1/50) and immunostaining with the biotin streptavidin complex method (Dako), as described by Decary et al. (13).

*Counting of myonuclei and SCs by immunofluorescence*

Single muscle fibers were manually dissected from the biopsy samples collected from each subject before and after expedition and analyzed by immune fluorescence techniques to determine the nuclear domain and to count the SCs. Single fibers were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. The fibers were permeabilized with 0.1% Triton X-100 in PBS at RT, and then incubated in 10% normal goat serum for at least 30 min to block non-specific antibody binding. Mouse anti Pax-7 (R&D System, Milan, Italy) monoclonal antibody (1:400) was applied at RT o/n at 4°C in PBS. After 3 washes (10 min each), fluorescent secondary Alexa-568 anti-mouse, (Molecular Probes) antibody was incubated for 2 h at room temperature. For visualization of nuclei, single fibers were stained with Hoestch (25 µg/ml; SIGMA) for 10 min. The fibers were viewed with a confocal microscope (VICO; Nikon) and number of nuclei and of Pax-positive nuclei were determined in segments of fibers where length and diameters were measured and volume was calculated. The reciprocal of the nuclear domain was determined as number of nuclei in a given fiber volume, while the SC density was expressed as the percentage of Pax7 positive nuclei.

*Statistical analysis*

Data were expressed as means ±SE. Statistical significance was set at $p<0.05$ and was calculated using the unpaired Student's *t*-test, with Welch's correction. Prism5 GraphPad software (Abacus Concepts GraphPad Software, San Diego, CA, USA) was employed for statistical analyses.

*Gene profiling of muscle biopsies*

The transcriptional profile analysis was performed as described by Mancinelli et al. (2011) using high quality RNA obtained from 5 of these 6 subjects for the microarray experiments (14). The statistical analysis was performed using values corresponding to the $\log_2$ ratios of the normalized intensities (e.g., $\log_2 I_{post\text{-}expedition}/I_{pre\text{-}expedition}$), where positive numbers corresponded to RNA over-expressed in muscle after the expedition. Differentially expressed genes were identified using the significant analysis of microarrays (SAM) permutation test procedure (STANFORD software, http://www-stat.stanford.edu/ tibs/SAM), which defines genes with a computed score greater than the threshold value as significant. The false discovery rate (FDR) associated with a given threshold was also calculated from the permutation data. We assessed the genes obtained from the SAM analysis according to their Δ and FDR as reported in Table I. The total number of common differentially expressed genes (common to at least two of the subjects) was 19, although we did not include genes for which a function was not known. Several differentially expressed (up-regulated and down-regulated) genes coding for proteins implicated in different pathways are shown in Table II.

## RESULTS

Muscle regeneration depends on SCs. Reduced numbers and/or decreased myogenicity of these cells can impede correct muscle maintenance and contribute to a decline in muscle mass and repair capacity. To determine whether muscle adaptations to an extreme environment can alter the SC

**Table I.** *Statistical parameters used for the analysis of significance of the microarray permutation test procedures.*

| Statistical parameter | Subjects | | | | |
|---|---|---|---|---|---|
| | C1 | C3 | C4 | C5 | C6 |
| Δ | 0.04 | 0.54 | 0.12 | 0.19 | 0.16 |
| False discovery rate (median) | 0.55 | 0.052 | 0.14 | 0.04 | 0.20 |
| Known genes (n) | 130 | 139 | 135 | 147 | 152 |

*Each climber was identified by an alphanumerical code (C as climber followed by a number).*

population, and thus their regenerative capacity, we isolated and characterized these cells. Consistent with the findings of Renault et al. (2000), the *in vitro* proliferative life span of myoblasts was limited and was related to donor age (15). In adult age, once the period of muscle growth ceases, the proliferative capacity of SCs tends to plateau, as reported Fig. 1. Cell cultures isolated from the VL biopsies of the climbers before the expedition were characterized according to their myogenic purity (% of desmin/$^{+ve}$ cells) and fusion index (%). The number of myogenic cells was as expected, according to our previous data (16). Moreover, their ability to fuse with each other into functional multinucleated cells (calculated as the fusion index in seven-day-differentiated myotubes) did not change across the cell cultures from these subjects (Table III). After exposure of the subjects to the HHE, in all except one of the samples, the SCs derived from the muscle biopsies did not migrate out of the explants. In fact, after the expedition, only one muscle sample obtained from the climber C6 did provide SCs (Table III, last row). However, the C6 POST SC myogenicity percentage was decreased by 40%, the fusion index by 50% and the positive desmin unfused cells by 95% (Table III). The proliferative capability of SC culture (C6) obtained after the expedition was compared to that obtained from the same climber before the expedition (Fig. 2). The graph shows that after the expedition the SCs slow down their proliferation rate despite them reaching the same number of division (PDL). Noteworthy, 2 years after the expedition, SC culture was obtained by C5 SM biopsy showing a 51.5% of miogenicity (Table III, C5 follow up, FU). To test whether the reduced myogenic activity could find a basis in a reduced number of SCs, myonuclei and SCs were counted in muscle fibers before and after the expedition. After the expedition, the total density of nuclei was greater than before the expedition: the mean ± SE was $140.5\pm7.4/10^6\mu m^3$ (N=15 fibers from 6 climbers), while the mean before expedition was $122.5\pm6.2/10^6\mu m^3$ (N=31 fibers from 6 climbers) indicating a reduction of nuclear domain. The percentage of Pax7 positive nuclei on 100 nuclei was significantly decreased ($p<0.05$) from 1.336±0.1877 (N=10) before the expedition and 0.5279±0.2061 (N=7) after the expedition (Fig. 3). Furthermore, we analyzed the muscle adaptations to HHE and exercise performed under extreme conditions using the transcriptional profile analysis of the VL muscle, comparing the gene expression profile after the expedition with that from before the expedition, from the same subjects (Table II). We assessed genes obtained from SAM analysis (see Materials and Methods section) according to Δ and false discovery rates reported in Table I. The total number of differentially expressed genes was on the average 140 per subject and we did not insert genes the function of which is unknown. Several differentially expressed (up- and down-regulated) genes coding for proteins implicated in diverse pathways were observed and we carefully analyzed and described those that we found to be differentially regulated in at least two subjects, as listed in Tables II, according to their common functions. It was found that exercise itself can induce dysregulation of genes involved in myogenesis. In the present study there was up-regulation of *myogenic factor 6 (herculin)*, the gene that codes for *MRF4*, which together with the myogenic basic helix-loop-helix family of

**Table II.** *Descriptions of differentially expressed genes (common at least to 2 climbers).*

| NCBI gene no. | Official symbol | Description | Mean $\log_2 I_{post}/I_{pre}$* | | | | |
|---|---|---|---|---|---|---|---|
| | | | C1 | C3 | C4 | C5 | C6 |
| ***Oxidative metabolism*** | | | | | | | |
| 549 | AUH | AU RNA binding protein/enoyl-Coenzyme A hydratase | 0.90 | | | | 0.58 |
| 522 | ATP5J | ATP synthase, $H^+$ transporting, mitochondrial F0 complex, subunit F6 | 0.68 | | 0.78 | | |
| 5507 | PPP1R3C | Protein phosphatase 1, regulatory (inhibitor) subunit 3C | -0.55 | | | -0.83 | |
| 3030 | HADHA | Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase | | | | 0.97 | 0.56 |
| 28965 | SLC27A6 | Fatty-acid-Coenzyme A ligase, long-chain 2 | | | | 0.86 | 0.56 |
| 2593 | GAMT | Guanidinoacetate N-methyltransferase | | | | -1.91 | -0.58 |
| 36 | ACADSB | Acyl-Coenzyme A dehydrogenase, short/branched chain | | 0.62 | | | 0.99 |
| ***Protein balance*** | | | | | | | |
| 4738 | NEDD8 | Neural precursor cell expressed, developmentally down-regulated 8 | | | -1.12 | -1.21 | |
| 55585 | NICE5 | NICE-5 protein | -0.93 | | | -0.96 | |
| ***Sarcomeric protein*** | | | | | | | |
| 9172 | MYOM2 | Myomesin (M-protein) 2 (165kD) | | | 1.15 | | 0.65 |
| 5339 | PLEC1 | Plectin 1, intermediate filament binding protein, 500kD | | | 0.92 | | 0.82 |
| 7140 | TNNT3 | Troponin T3, skeletal, fast | | | -0.77 | | -0.89 |
| 23336 | SYNM | Desmuslin | | | | 1.03 | 0.45 |
| 8048 | CSRP3 | Cysteine and glycine-rich protein 3 (cardiac LIM protein) | 0.84 | | 0.95 | | |
| ***Cell cycle*** | | | | | | | |
| 5774 | PTPN3 | Protein tyrosine phosphatase, non-receptor type 3 | | | 1.16 | | 1.23 |
| 5515 | PPP2CA | Protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform | | | 0.85 | 1.02 | |
| 9020 | MAP3K14 | Mitogen-activated protein kinase kinase kinase 14 | | | -0.94 | -1.03 | |
| ***Myogenesis*** | | | | | | | |
| 7123 | CLEC3B | Tetranectin (plasminogen binding protein) | -0.82 | | -0.83 | | |
| 4618 | MRF4 | Myogenic factor 6 (herculin) | | | 0.93 | 1.22 | |

*The first column specifies NCBI gene number, the second column, the official symbol, the third column the description of the different genes up- or down-regulated in the VL muscle, when post- and pre-expedition expression levels were compared. The other columns report changes in expression levels of genes for each climber analyzed as means of the log base-2 of the ratios ($\log_2 I_{post\text{-}expedition}/I_{pre\text{-}expedition}$).*

** Up-regulated genes have positive values, and down-regulated genes have negative values.*

**Table III.** *Myogenicity, fusion index and desmin-$^{+ve}$ non-fused cell characteristics of the cell cultures isolated from the VL biopsies of the climbers before the expedition, including the single sample obtained after the expedition (C6).*

| Cell Culture | | Myogenicity % (desmin$^{+ve}$) | Fusion Index (%) | Desmin$^{+ve}$ Unfused (%) |
|---|---|---|---|---|
| C1 | PRE | 62.5 | 44.0 | 33.4 |
| | POST | n.d. | n.d. | n.d. |
| C2 | PRE | 58.2 | 69.5 | 22.6 |
| | POST | n.d. | n.d. | n.d. |
| C3 | PRE | 51.7 | 67.6 | 21.0 |
| | POST | n.d. | n.d. | n.d. |
| C4 | PRE | 40.4 | 57.6 | 12.1 |
| | POST | n.d. | n.d. | n.d. |
| C5 | PRE | 61.3 | 68.9 | 10.8 |
| | POST | n.d. | n.d. | n.d. |
| | FU | 51.5 | n.d. | n.d. |
| C6 | PRE | 64.8 | 58.6 | 12.5 |
| | POST | 39.3 | 29.8 | 0.6 |

*Two years after the expedition cell culture was obtained by C5 biopsy (C5 follo- up, FU). Each climber was identified with an alphanumerical code (C as climber followed by a number).In all except one of the post samples, the SCs derived from the muscle biopsies did not migrate out of the explants; biopsy that was obtained from the only one climber (C6) did provide SCs; biopsy that was obtained from the only one climber (C5 FU) did provide SCs 2 years after the expedition. n.d. means not detected.*



transcription factors (MyoD, Myf5, myogenin), can activate the muscle differentiation program (8). *Tetranectin (plasminogen binding protein) (CLEC3B)* was down-regulated after exposure to the HHE, and it codes for a protein marker for myogenesis during embryonic development, muscle regeneration, and muscle cell differentiation *in vitro* . The down-regulation of tetranectin could be related to the fibrinolytic state after exercise training, as recently demonstrated (17). Another pathway that is significantly modified when SM is exposed to HHE and exercise is performed involved the regulation of the cell cycle. The protein encoded by the *protein tyrosine phosphatase, non-receptor type 3 (PTPN3)* gene is a signaling molecule that regulates a variety of cellular processes, including cell growth and differentiation, and the mitotic cycle. This gene was up-regulated, together with *protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform (PPP2CA)*, which codes for the phosphatase 2A catalytic subunit, one of the four major Ser/Thr phosphatases implicated in negative control of cell growth and division, through dephosphorylation and inactivation of ribosomal protein S6 kinase, 70-kD, polypeptide 1 (p70 kinase 1). Inactivated p70 kinase 1 fails to oppress the activity of glycogen synthase kinase 3 beta (GSK3β). In this case, GSK3β phosphorylates cyclin D, making it accessible to subsequent ubiquitination and proteosomal degradation (18). In addition, there was down-regulation of *mitogen-activated protein kinase kinase kinase 14 (MAP3K14)* gene, which codes for a Ser/Thr protein kinase. This kinase binds to TRAF2 and stimulates NF-kappaB activity (19). Other genes found dysregulated are involved in the oxidative metabolism pathway. Genes dysregulated in the VL muscle samples after the expedition showed a shift towards oxidative metabolism, in respect to the expression before the expedition. Indeed, we found up-regulation of the *AU RNA binding protein/ enoyl-coenzyme A hydratase (AUH)*, a gene that codes for a mitochondrial protein that binds to the AU-rich element, a common element found in the 3'-UTR of rapidly decaying mRNA, such as for c-fos, c-myc and granulocyte/ macrophage colony stimulating factor. These AU-

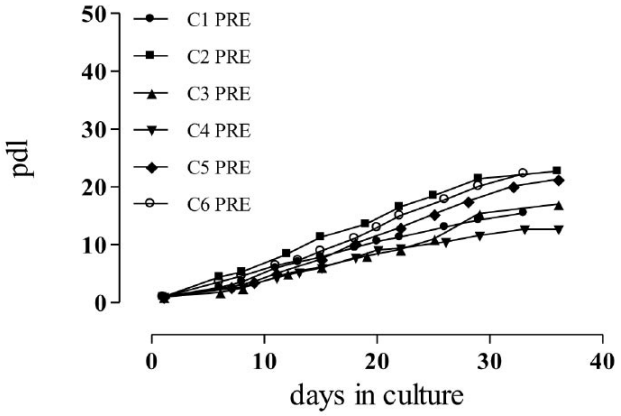


**Fig. 1.** *Proliferative life span of human SCs. Cell populations isolated from VL biopsies of the climbers before the expedition were serially passaged and cultured until their cell division ceased. At each passage, the population doubling level was calculated by counting cell numbers.*

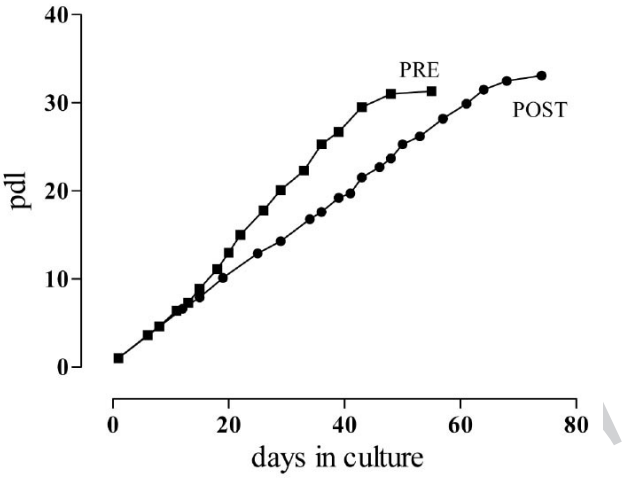


**Fig. 2.** *The two curves represent the life spans of the SC cultures isolated from VL biopsies obtained from climber C6 before (PRE) and after the expedition (POST).*

rich elements are involved in directing the RNA to rapid degradation and de-adenylation. *AUH* is also homologous to enol-CoA hydratase, an enzyme that is involved in fatty-acid degradation and that has been shown to have intrinsic hydratase enzymatic activity. *AUH* is thus a bifunctional chimera, between RNA binding and metabolic enzyme activity (20). *ATP synthase, $H^+$ transporting, mitochondrial F0 complex, subunit F6 (ATP5J)*, which codes for the mitochondrial ATP synthase that catalyses ATP synthesis, was up-regulated. Moreover, the up-regulated *hydroxyacyl-coenzyme A dehydrogenase/ 3-ketoacyl-coenzyme A thiolase/ enoyl-coenzyme A hydratase (HADHA)* gene codes for the alpha subunit of the mitochondrial trifunctional protein, which catalyses the last three steps of mitochondrial beta-oxidation of long-chain fatty acids. Linked to this last gene, the *fatty-acid-coenzyme A ligase, long-chain 2 (SLC27A6)* gene codes for a member of the fatty-acid transport protein family, and it was also up-regulated. The fatty-acid transport protein family is involved in the uptake of long-chain fatty acids and its members show unique expression patterns (21). *Acyl-coenzyme A dehydrogenase, short/ branched chain (ACADSB)* was found to be up-regulated after the expedition, as compared to before. This gene codes for an enzyme that catalyses

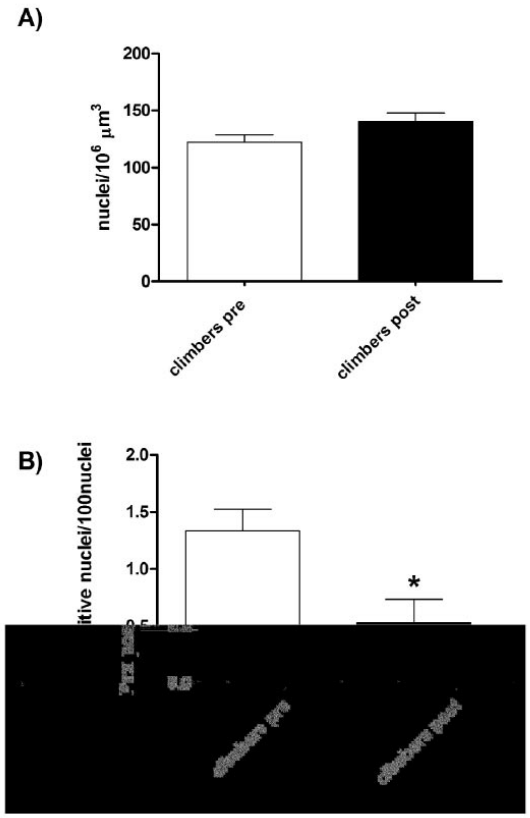


**Fig. 3.** *Total nuclear density and percentage of SCs in single muscle fibers before and after the expedition. Panel **A)** Number of nuclei in a fixed volume ($10^6$ µm$^3$); panel **B)** Percentage of Pax7 positive nuclei; white columns, before expedition, black columns, after expedition. The data are expressed as means and standard errors, * indicates the statistically significant decrease in Pax7 positive nuclei after expedition ($p<0.05$).*

dehydrogenation of acyl-CoA derivatives in the metabolism of fatty acids and branch-chained amino acids (22). In contrast, *protein phosphatase 1, regulatory (inhibitor) subunit 3C (PPP1R3C)*, which codes for a protein involved in glycolytic metabolism, and *guanidinoacetate N-methyltransferase (GAMT)*, which codes for a methyltransferase that converts guanidoacetate to creatine (23), were found to be down-regulated. Two genes linked to ubiquitin-dependent protein degradation processes were down-regulated after the expedition, compared with before: *neural precursor cell expressed, developmentally down-regulated 8 (NEDD8)*, which codes for a protein that activates ubiquitination through an increase in the efficiency of polyubiquitin chain assembly that is modulated by its covalent conjugation to cullin molecules (24); and *NICE-5 protein*, which codes for a protein member of the E2 ubiquitin-conjugating enzyme family. In agreement with the down-regulation of protein-degradation processes, genes that code for sarcomere-specific proteins were also up-regulated. *Myomesin (M-protein) 2 (MYOM2)* codes for titin, which interconnects the M bands and Z discs. *Plectin 1, intermediate filament binding protein (PLEC1)* codes for the plectin protein that can interlink different elements of the cytoskeleton in SM. The protein encoded by d*esmuslin (SYNM)* is an intermediate filament family member that has been shown to form a link between desmin and the extracellular matrix, which provides important structural support in muscle. *Cysteine and glycine-rich protein 3 (cardiac LIM protein) (CSRP3)* was also up-regulated. The protein encoded by this gene interacts with cytoskeletal actin-regulating F-actin depolymerization (25), whereby deregulation of this interaction leads to sarcomere dysfunction and disease. The gene *troponin T3, skeletal, fast (TNNT3)* codes for the skeletal troponin T protein, and this was also down-regulated.

## DISCUSSION

In the present study, we characterized SC cultures obtained from the VL needle biopsies of these climbers before and after the expedition. Noteworthy, after the expedition, only one (C6) out of six VL needle biopsies of the climbers provided SCs showing significantly decreased myogenicity percentage and fusion index of 40% and 50%, respectively, compared to cell cultures obtained from the same subject before the expedition. Furthermore, the failed coming-out of SCs from explants after the expedition could be related to the lower number of Pax-7 positive cells that we found. The reduction in the density of these myogenic precursor cells after expedition to about 30% of the value determined before expedition is therefore consistent with the decreased myogenic response. The delayed activation of SCs exposed to HHE when put in culture could be explained by two possible mechanisms: (1) lipofuscin accumulation in SCs (9) preventing their differentiation into functional cells, with the consequent damage and loss of maintenance of the stem cell pool (26); (2) the deregulation of genes involved in negative cell-cycle regulation. However, the SCs pool was only temporarily exhausted as demonstrated by collection of SC culture from a biopsy collected 2 years after the expedition. In several studies on mountaineering muscle atrophy with reduction of cross-sectional area and muscle mass has often been reported. Ubiquitin-proteasome system usually mediates the atrophic process and also HIF-α oxygen-dependent ubiquitination. Conversely, under hypoxia, HIF-α escape this mechanism activating numerous hypoxia-inducible genes (27).

The down-regulation found in our study of genes involved in the proteolytic pathway suggests the activation of HIF-mediated adaptation mechanisms to hypoxia. In general, genes that code for sarcomere-specific proteins were up-regulated. Furthermore, this result is in agreement with other data obtained from same climbers, demonstrating for the first time a significant increase in slow type fibers after the expedition (28). The up-regulation of sarcomeric genes confirms data already published demonstrating that the myofibrillar protein synthesis rate is markedly elevated after high altitude hypoxia (29). The down-regulation of the proteolytic pathway together with up-regulation of sarcomeric protein suggest an anabolic positive balance probably due to the exercise at high altitude. In fact, even though a reduction in anthropometric parameters was found, the development of force did not change, while the cross-sectional area increased with the absence of

atrophy following the expedition (28). The physical activity carried out by the climbers consisted of trekking that took them down to return to sea level providing lengthening contractions (eccentric muscle contractions). As previous studies have reported, exercise induces changes in expression of MRFs and IGF-1, at both the mRNA and protein levels. In agreement with previous reports, we found up-regulated genes involved in the process of muscle building. After high altitude expedition the overall picture that emerges shows a lack of action by the SCs to regeneration and/or muscle growth. In spite of this, our results show an increase of total density of fiber myonuclei. This result is also supported by data deriving from the same climbers (28) showing an increase in fiber cross-sectional area. It has been shown that hypertrophy can occur as a result of physical activity with enhanced transcriptional and translational activities (30) that we find increased based on deregulation of the protein balance and up-regulation of sarcomeric protein pathways. In this case, the role of SCs could be explained as described above. The participation of other myogenic cells in fusion events that induce changes in cross-sectional area and muscle dimension is also to be considered (31). With SM activity, the mitochondria have a pivotal role in ATP production, a pathway that requires oxygen. When cells are exposed to hypoxia, adaptive mechanisms mediated by AMPK and HIF1 and metabolic changes take place (32). Furthermore, Hoppeler (2001) reported a shift towards oxidative metabolism as a compensation mechanism during training under hypoxia (33). The larger oxidative capacity also necessitates adaptations in oxygen transfer system, normally detectable in subjects exposed to HHE. This effect was also induced by the eccentric contractions that occurred during the descending phase of the expedition. Indeed, it is well documented how training can induce fiber type transitions towards a slow-oxidative phenotype (34). The shift towards a slow-oxidative phenotype, in agreement with data obtained from Doria et al. (28), is supported by up-regulation of genes involved in oxidative metabolism. Furthermore, this shift is supported by the down-regulation of genes involved in glycolytic metabolism, regulated by HIF1 under hypoxia, revealing a novel metabolic adaptation of cells to hypoxia (35). Therefore, overall, the up-regulation and down-regulation of these genes lead us to hypothesize a metabolic shift to oxidative processes. Taken together, the results we obtained suggest that the exposure to HHE accompanied by physical activity lead to remodelling of SM in which loss of muscle mass (with an increase in cross sectional area) does not appear due to the participation of SCs. A key role seems instead be played by increasing sarcomeric protein synthesis and the decrease in proteolytic degradation with a positive muscle balance, or an absence of atrophy and muscle loss. These data were supported by the initial contribution of SCs to fuse with fibers in response to the stimulus represented by physical activity. Collectively, the stay at high altitude and the exercise carried out could suggest a rearrangement in SM fibers, rather than a regeneration.

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