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Transcriptional profiling in mouse skeletal muscle following a single bout of voluntary running: evidence of increased cell proliferation

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Choi, Sangdun, Xuebin Liu, Ping Li, Takayuki Akimoto, Sun Young Lee, Mei Zhang, and Zhen Yan. Transcriptional profiling in mouse skeletal muscle following a single bout of voluntary running: evidence of increased cell proliferation. *J Appl Physiol* 99: 2406–2415, 2005. First published August 4, 2005; doi:10.1152/japplphysiol.00545.2005.—Skeletal muscle undergoes adaptation following repetitive bouts of exercise. We hypothesize that transcriptional reprogramming and cellular remodeling start in the early phase of long-term training and play an important role in skeletal muscle adaptation. The aim of this study was to define the global mRNA expression in mouse plantaris muscle during (run for 3 and 12 h) and after (3, 6, 12, and 24 h postexercise) a single bout of voluntary running and compare it with that after long-term training (4 wk of running). Among 15,832 gene elements surveyed in a high-density cDNA microarray analysis, 900 showed more than two-fold changes at one or more time points. K-means clustering and cumulative hypergeometric probability distribution analyses revealed a significant enrichment of genes involved in defense, cell cycle, cell adhesion and motility, signal transduction, and apoptosis, with induced expression patterns sharing similar patterns with that of peroxisome proliferator activator receptor-γ coactivator-1α and vascular endothelial growth factor A. We focused on the finding of a delayed (at 24 h postexercise) induction of mRNA expression of cell cycle genes origin recognition complex 1, cyclin A2, and cell division 2 homolog A (*Schizoccharomyces pombe*) and confirmed increased cell proliferation by in vivo 5-bromo-2′-deoxyuridine labeling following voluntary running. X-ray irradiation of the hindlimb significantly diminished exercise-induced 5-bromo-2′-deoxyuridine incorporation. These findings suggest that a single bout of voluntary running activates the transcriptional network and promotes adaptive processes in skeletal muscle, including cell proliferation.

adaptation; exercise; high-density complementary deoxyribonucleic acid microarray; transcriptional reprogramming; cellular remodeling

MAMMALIAN SKELETAL MUSCLES comprise the majority (~55%) of the body mass and serve as the source of power for respiration, locomotion, and other physical activities that are essential for survival. More importantly, skeletal muscles play a vital role in overall metabolism. The biological importance of skeletal muscles is reflected by the remarkable plasticity, an ability to change the phenotype in response to external and internal stimuli. Physical inactivity, a decreased use of the musculoskeletal system associated with the modern lifestyle, impairs skeletal muscle contractile and metabolic functions, contributing to an epidemic emergence of the metabolic syndrome and other devastating chronic diseases (8, 27, 42). Accumulating evidence indicates that regular exercise has profound beneficial effects on these diseases due to phenotypic adaptations in skeletal muscles, including transformation of type IIb to IIa myofibers (17) and increased mitochondrial (21, 47) and capillary densities (43). Thus knowledge of the molecular and cellular mechanisms underlying the skeletal muscle plasticity may not only define the potential of adaptation in performance and metabolism, but also foster discovery of novel drug targets for the diseases associated with impaired skeletal muscle functions.

Extensive research in the past decades has significantly improved our understanding of exercise-induced skeletal muscle adaptation. It is now believed that an orchestrated transduction of signals from neuromuscular activity, mechanical stress, and metabolic factors (6) to the regulatory machinery of the genes in the mature myofibers plays a central role in mediating skeletal muscle adaptation. To date, several independent signal transduction pathways and components of the transcriptional control machinery have been implicated to regulate skeletal muscle fiber-type specialization, including the calcineurin (15, 31), the Ca^{2+}/calmodulin-dependent protein kinase (52, 53), the Ras-MAPK (29), and the p38 MAPK pathways (1), and peroxisome proliferator-activated receptor-δ and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (28, 48). These prior works have set a stage for further investigation of all possible signaling and molecular mechanisms of skeletal muscle adaptation and merited a comprehensive approach to study the transcriptome in skeletal muscle in response to exercise.

Recent development in mRNA profiling technology (oligo-based and cDNA-based microarrays) has emerged as a powerful tool for "genomewide" assessment of gene regulation in model systems, ranging from cultured cells to live animals. This technology has been employed to examine gene expression differences between fast-twitch and slow-twitch muscles (10) and define the effects of acute exercise (7, 11–13) and long-term training (20, 50, 51). The studies have provided evidence that long-term exercise-induced skeletal muscle adaptation is associated with altered expression of metabolic genes and contractile proteins, whereas acute exercise results in altered expression of a large set of genes in various functional categories (transcription factors, heat shock proteins/factors, cytokines/chemokines, DNA damage and repair factors, etc.). To date, there has been no comprehensive analysis of mRNA profiling in skeletal muscle in response to a single bout of physiological exercise, such as voluntary running.

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In this study, we expanded the expression profiling analysis in skeletal muscle by investigating a time course following a single bout of voluntary running in wild-type sedentary mice to identify genes that are differentially regulated in the early phase of skeletal muscle adaptation. Our premise was that important signaling and transcriptional events occur in response to early bouts of exercise training and play important functional roles in skeletal muscle adaptation, although the ultimate phenotypic changes are likely the results of cumulative and sequential regulatory events. The results from this study support a notion that active transcriptional reprogramming and cellular remodeling occur in response to an acute bout of voluntary running. Of particular interest was the finding that voluntary running induced cell proliferation in adult skeletal muscle, raising an intriguing question regarding the functional role of cell proliferation in endurance exercise-induced skeletal muscle adaptation.

MATERIALS AND METHODS

Animals. Adult (8 wk of age) male C57BL/6J mice (Jackson Laboratory) were housed in temperature-controlled quarters (21°C) with a 12:12-h light-dark cycle and provided with water and chow (Purina) ad libitum. Mice were randomly assigned to sedentary control (Con) and running groups and housed individually in cages equipped with locked running wheels (4, 49) for 3 days for acclimation. Mice in the voluntary running groups were allowed to run by unlocking the wheels at the beginning of a dark cycle for 3 h (R3h), 12 h (R12h), or for 12 h followed by 3 (P3h), 6 (P6h), 12 (P12h), or 24 h (P24h) of resting periods, or for 4 wk followed by a 24-h resting period (R4w) ($n = 5$ for each time point). Sedentary mice ($n = 5$) were used as Con. The running activity for each mouse was recorded every 5 min to calculate the total distance per dark cycle (12 h). The active running time is the cumulative time of each 5-min period during which any running activity had been detected, and the average speed is the total distance divided by the total running time. At the designated time points, the mice were killed by an overdose injection of pentobarbital sodium (250 mg/kg ip), and the plantaris muscles were harvested and processed for microarray, RT-PCR, indirect immunofluorescence, and Western blot analyses. In a separate experiment, 15 mice were randomly assigned to a Con group and two running groups (run for 1 and 3 days) for quantification of cell proliferation in plantaris and gastrocnemius muscles. Twenty-four hours before muscle harvesting, 5-bromo-2′-deoxyuridine (BrdU; 500 mg/kg) was injected intraperitoneally to label DNA replicating nuclei. To evaluate the effects of X-ray irradiation on cell proliferation in skeletal muscle following exercise, mice ($n = 6$) were anesthetized with pentobarbital sodium, and the right hindlimbs were exposed to a single dose of 2,500 rad ionizing irradiation in 23 min. The remaining part of the animals was shielded from the radioactive source by a 2-cm-thick lead attenuator. The mice were randomly assigned to running and sedentary groups and allowed to recover for 3–4 days, and the mice in the running group were subject to voluntary running for 3 days. All animal protocols were approved by the Duke University Institutional Animal Care and Use Committee.

Agilent cDNA array fabrication and annotation. A total of 15,494 cDNA probes, representing 10,615 unique genes, were printed on 15,832 spots (with redundancy) on custom-made arrays. A majority (96%) of the probes came from the Riken Fantom collection, and the rest from the National Institute on Aging, Research Genetics, and Genome systems. The probes were PCR amplified at the California Institute of Technology and were inkjet printed on the array by Agilent Technologies. The annotation for all of the gene elements on the array chips are presented at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo/) under platform number GPL260.

Microarray hybridization. Total RNAs were extracted from plantaris muscles by using TRIzol (Invitrogen), according to the manufacturer's instructions. Florescence-labeled cDNAs for the microarray hybridizations were generated by using 3 μg of pooled total RNA from five mice of the same time point, as described previously (54). Microarray hybridization was performed by using a Cy5-labeled cDNA of exercised sample and a Cy3-labeled cDNA of the Con sample. To minimize the possibility of experimental variance, all array experiments (7 hybridizations for different time points) were performed simultaneously. Parallel hybridizations were carried out, and one amplified control was used for all comparisons against each test sample. The arrays were scanned by using Agilent Scanner G2505A (Agilent Technologies), with the scan resolution set to 10 μm and the laser intensity adjusted so that both the maximum red and green (Cy5 and Cy3) fluorescence intensities were ~20,000 units.

The image files were extracted with background subtraction (the Local background subtraction method) and dye normalization (the Rank consistent filter and the LOWESS algorithm) using the Agilent G2566AA Extraction software, version A.6.1.1. The entire raw data sets are available at GEO (Gene Expression Omnibus: http://www.ncbi.nlm.nih.gov/geo/) with a series entry number of GSE2001. For features that were saturated (with Agilent "glsSaturated" and "rlsSaturated" flags), nonuniform (with Agilent "gIsFeatNonUnifOL" and "rIsFeatNonUnifOL" flags), or below background (with Agilent "gIsWellAboveBG" and "rIsWellAboveBG" flags), their Cy5 and Cy3 fluorescence intensity and $\log_2$ (Cy5/Cy3) value were set to blank. The cutoff level was set to twofold, as has been used in previous studies in skeletal muscle (26, 46). We repeatedly (5 times) tested the reproducibility of the microarray hybridization between control samples and showed only an average of 0.6 ± 0.4 gene elements (0.004% of the total) with changes greater than twofold ($r = 0.989 \pm 0.003$).

Identification of gene expression signatures by K-means and hierarchical clustering analyses. The Cy5-to-Cy3 ratio (running/sedentary) was converted to $\log_2$ value first. To identify gene expression signatures in response to voluntary running, we conducted K-means clustering of gene expression change following the running to group genes, according to their expression patterns with GeneCluster 2.0 (http://www.broad.mit.edu/software/genecluster2/gc2.html), and generated a 4×4 self-organization map (44) after optimization for unique expression pattern (in timing and magnitude) for each of the clusters.

Determination of statistical significance for functional category enrichment. The hypergeometric distribution was used to obtain the chance probability of observing the number of genes from a particular functional category within each cluster, as described previously (45). P values $>3.3 \times 10^{-3}$ are not reported, since their total expectation within the cluster would be >0.05.

Semiquantitative RT-PCR analysis. Semiquantitative RT-PCR analysis was performed as described (54) to measure endogenous mRNA expression in plantaris muscle in response to voluntary running. The data were normalized by GAPDH mRNA and expressed as fold change to the Con muscle. The primers and reaction conditions used for semi-quantitative RT-PCR analysis are listed (Table 1).

Indirect immunofluorescence. To determine the changes in type IIa fibers and CD31-positive endothelial cells in plantaris muscles in the Con mice and after 4 wk of voluntary running, indirect immunofluorescence was performed, as described previously (49). To detect BrdU incorporation, frozen muscle sections were stained with anti-BrdU antibodies, as described previously (54) with modification. Briefly, muscle sections were fixed for 10 min in 4% paraformaldehyde on ice and stained for myosin heavy chain 2a, as described (49). The sections were then fixed in 4% paraformaldehyde on ice for 10 min followed by treatment with 2 N HCl for 60 min at 37°C to denature the DNA and neutralization in 0.1 M borate buffer, pH 8.5. The nonspecific binding sites were blocked with 5% normal goat serum (NGS)/PBS for 30 min at room temperature. Muscle sections were incubated with monoclonal mouse anti-BrdU antibody (Roche) diluted 1:25 in 5% NGS/PBS overnight at 4°C, followed by incubation with fluorescein-conjugated goat anti-mouse IgG diluted 1:25 in 5% NGS/PBS for 30 min at room temperature. The sections were then

Table 1. *Primers used for semiquantitative RT-PCR reaction*

Gene	Accession No.	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Size, bp
Pgc-1a	NM_008904	aaacttgctagcggtcctca	tttctgtgggtttggtgtga	428
Vegfa	NM_009505	ctttctgctctcttgggtgc	gcattcacatctgctgtgct	382
Fos	NM_010234	atgggctctcctgtcaacac	ggctgccaaaataaactcca	480
Dscr1	AK003336	ctaacctgtgggagagcagc	acacacacgatgactgggaa	456
Tnfrsf12a/Fn14	AK005530	cgagccagactctttcaacc	ccctcccctccaaacattat	440
Cdkn1a/p21Cip1	NM_010234	atgggctctcctgtcaacac	ggctgccaaaataaactcca	480
Cc16/Scya6	AK008547	cctaagcaccctgaagcaag	acaactgggaaccacaaagc	428
Csrp3/Crp3	AK011624	cacaagcaacccttggaaat	gctacaaaggaggctgttgg	367
Orc1	NM_011015	cgaggtggtcaaagaagagc	acacgagaagtacggatggg	373
Cdc2a	BG064846	tccatccagagggctacatc	ctcggctcgttactccactc	377
Ccna2	NM_009828	tccttgcttttgacttggctg	ttgactgttgggcatgttgtg	324
Gapd	NM_008084	gtggcaaagtggagattgttgcc	gatgatgacccgtttggctcc	290

incubated with 200 μl of 10 μg/ml propidium iodide in H_2O with 100 μg/ml RNase A at 37°C for 25 min before being protected with VECTASHIELD mounting media and examined under epifluorescent or confocal microscope. All BudU-positive cells were counted for each muscle section and normalized by the cross-sectional area.

Statistics. All data, except for the microarray data, are expressed as means ± SE. Statistical significance ($P < 0.05$) was determined by Student's *t*-test for any comparison between two groups, or by ANOVA followed by the Dunnett test for comparisons with a control mean (designated in the figure legends) to each other group mean.

RESULTS

A single bout of voluntary running induces differential gene regulation. Male (8 wk of age) wild-type mice (C57BL/6J) were subjected to voluntary running (Fig. 1*A*). Sedentary mice ran an average of 6.8 ± 0.3 km per dark cycle (12 h), and, after 4 wk of training, the total running distance per dark cycle doubled, not due to lengthened active running time but due to increased speed (Fig. 1*B*). Consistent with our laboratory's recent findings (1, 2, 49), 4 wk of voluntary running induced IIb-to-IIa fiber-type switching with concurrent increases in capillary density and increased PGC-1α and myosin heavy chain 2a protein expressions in the plantaris muscles (not shown), suggesting that this exercise regime resulted in reproducible skeletal muscle adaptation.

To define global gene expression in skeletal muscle in response to a single bout of voluntary running, we performed cDNA microarray hybridization for the plantaris muscles during (R3h and R12h) and following (P3h, P6h, P12h, and P24h) an overnight voluntary running and compared it with that from trained mice after 4 wk of voluntary running. Among 15,832 elements screened, 9,504 elements (60%) displayed detectable signals, and 900 elements (5.7%) were altered more than twofold in at least one time point (Fig. 2*A*). Approximately 200–360 elements (1.3–2.3%) showed more than twofold changes during voluntary running (R3h and R12h) and within 12 h postexercise (P3h–P24h), and a further increase in the number of changed elements (540 elements, 3.4%) occurred at 24 h postexercise. The total number of changed gene elements decreased to 94 (0.6%) after 4 wk of voluntary running.

K-means clustering and self-organization map (44) were used to analyze and assemble the results, which group together cDNA elements on the basis of their similarity in the temporal expression. Sixteen cluster groups (4 × 4) were chosen in this study (Fig. 2*B*) after optimization for a distinct pattern (in timing and magnitude) for each of the clusters. Complete results are presented as supplemental data (http://jap.physiology.org/cgi/content/full/00545.2005/DC1). In most cases, redundant cDNA probes were found in the same cluster or in clusters with similar expression profiles. For examples, four 8-oxoguanine DNA-glycosylase 1 (*Ogg1*) elements and three tumor necrosis factor receptor superfamily member 12a/fibroblast growth factor-inducible 14 (*Tnfrsf12a/Fn14*) elements were clustered in *cluster 15*, five cytoplasmic dynein light chain 1 were clustered in *cluster 14*, and seven β_5-tubulin elements were clustered in *cluster 3*. Clustering of redundant gene elements indicated the fidelity of the microarray analysis in this study.

Statistical analysis of the cumulative hypergeometric probability distribution was performed to determine whether any cluster was significantly enriched for genes with similar functions (45). *P* values were calculated for each cluster based on the frequencies of genes in particular functional categories. In this case, a statistically significant enrichment of a group of functional genes would indicate a coordinated regulation of a cellular process in skeletal muscle following the acute bout of running. As shown in Table 2, there was significant grouping of genes within the same functional class. Genes involved in metabolism (energy, lipid, carbohydrate, and protein metabolisms) and transport were significantly enriched in *clusters 1*, *4*, and *13*, with immediate or delayed patterns of reduced expression. In contrast, genes involved in defense and remodeling (cell cycle and growth, cell adhesion and motility, morphogenesis, signal transduction and apoptosis) were significantly enriched in *clusters 2*, *11*, *14*, and *15*, with induced expression patterns.

Multiple signaling and cellular processes are activated in skeletal muscle following a single bout of voluntary running. To validate the findings of the microarray analysis, we employed semiquantitative RT-PCR for six randomly selected genes in *cluster 15* (Fig. 2*C*), among which Down syndrome critical region homolog 1 (*Dscr1*) and FBJ osteosarcoma oncogene (*Fos*) mRNAs had been previously shown to be upregulated by endurance exercise (32, 37). As shown in Fig. 2*D*, there was a significant correlation between the microarray and the RT-PCR results for the genes tested. *Fos*, *Dscr1*, cyclin-dependent kinase inhibitor 1A (*Cdkn1a/p21Cip1*), and *Tnfrsf12a/Fn14* were annotated to function in mitogenic growth, calcium signaling, cell cycle, and apoptosis, respectively. These findings indicate a simultaneous activation of multiple signaling and cellular processes. The expression pattern of these genes

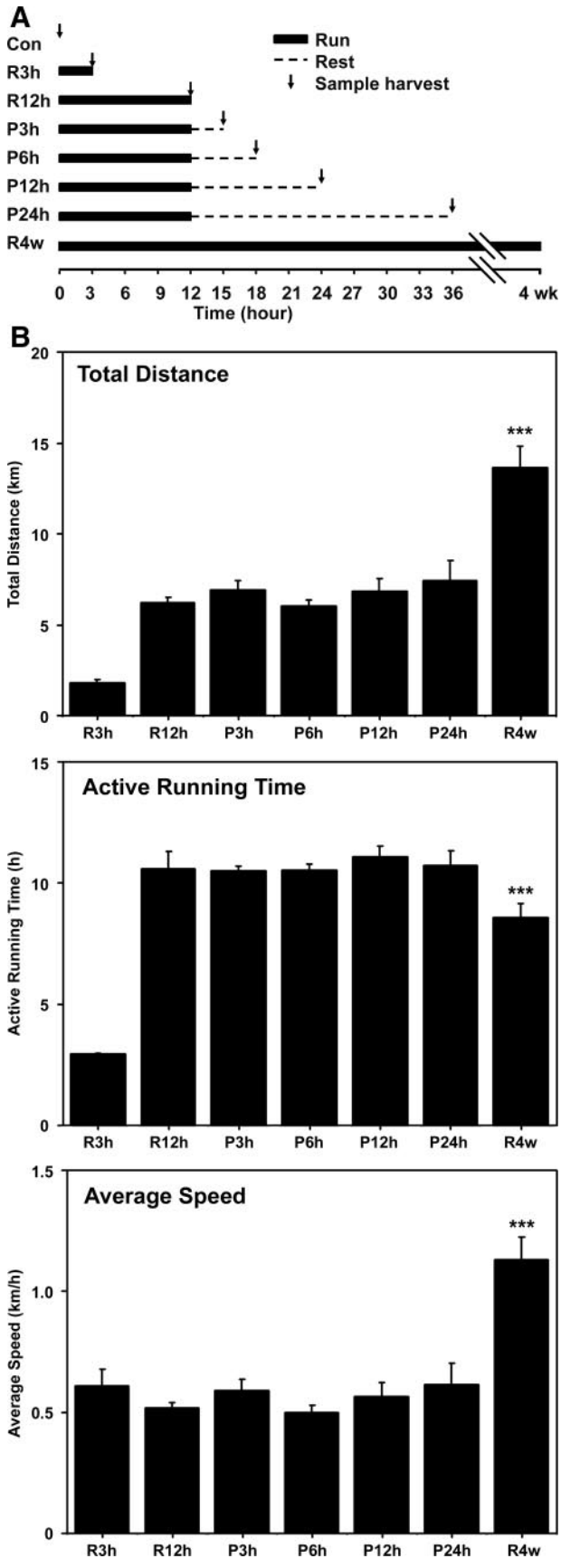
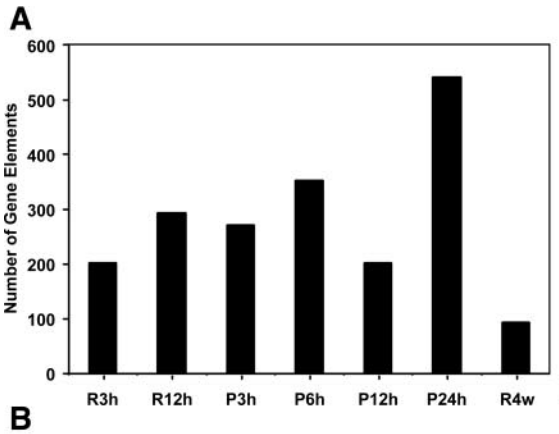


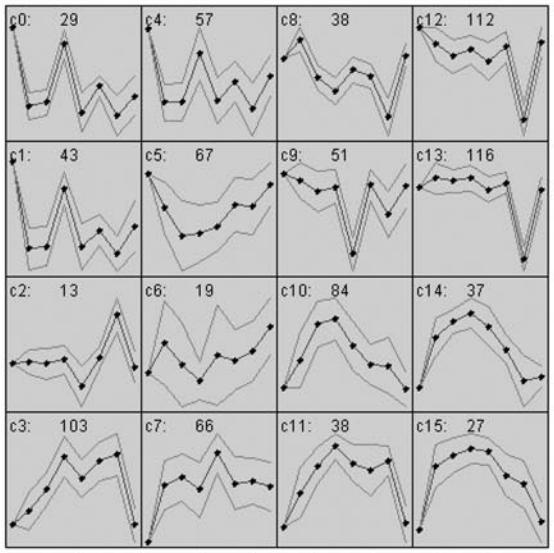
Fig. 1. Experimental design of microarray analysis in mouse skeletal muscle following a single bout of voluntary running. A: schematic presentation of the experimental design. Young adult (8 wk of age) male wild-type (C57BL/6J) mice were subject to voluntary running (thick solid line) followed by various lengths of resting period (thin dashed line). Con, sedentary control; R3h and R12h, run for 3 and 12 h, respectively; P3h, P6h, P8h, P12h, and P24h: rest for 3, 6, 8, 12, and 24 h after 12-h running, respectively; R4w, voluntary running for 4 wk. *B*: running parameters for the exercise groups. Running activity of each mouse was recorded every 5 min to calculate the total distance, active running time, and average speed. Values are means ± SE. ***$P < 0.001$ vs. R12h ($n = 5$). R3h group was not used for statistical analysis as running of the mice in this group was interrupted for sample harvesting during the acute exercise bout.

was similar to that of *Pgc-1α/Ppargc1a* and *Vegfa* (Fig. 2*E*), which are known to have potential functional roles in endurance exercise-induced mitochondrial biogenesis/fiber-type switching (3, 5, 19, 36) and angiogenesis (9, 49), respectively. Consistent with the induction of *Ogg1* mRNA following running, a transient induction of *Gadd45a* mRNA (*cluster 7*) indicates a possible activation of the p53 pathway related to DNA damage and repair (16). We have also observed an activation of the JAK/STAT pathway based on enhanced mRNA expression of interleukin-4, interleukin 4 receptor-α, and matrix metalloproteinase 10 (14).

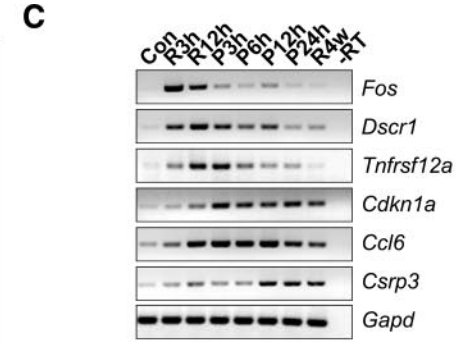
Voluntary running induces cell cycle gene expression and enhanced BrdU incorporation in skeletal muscle. A unique pattern of delayed induction was observed for genes in *cluster 2*. Two genes with sole function in the control of the cell cycle, cyclin A2 (*Ccna2*) and cell division 2 homolog A (*Schizosaccharomyces pombe*) (*Cdc2a*), were in this cluster, along with atrial natriuretic peptide precursor, corin (a cardiac pro-atrial natriuretic peptide convertase), and cardiac α-actin (*Actc1*) mRNAs. This delayed expression pattern of the cell cycle and cardiac genes is consistent with an activation of the cell cycle from quiescence and initiation of an embryonic muscle program. We performed semi-quantitative RT-PCR and confirmed induced mRNA expression of three genes directly involved in the cell cycle control. As shown (Fig. 3*A*), origin recognition complex 1, *Cdc2a*, and *Ccna2* mRNAs increased significantly at 24 h postexercise and returned to the basal level in 4-wk trained muscles. In addition to the induced cell cycle genes, the type III procollagen-α1, and the myogenic differentiation 1 (*Myod1*) gene were shown to be induced by a single bout of voluntary running, both of which were implicated in myogenic stem cell proliferation in vivo (18).

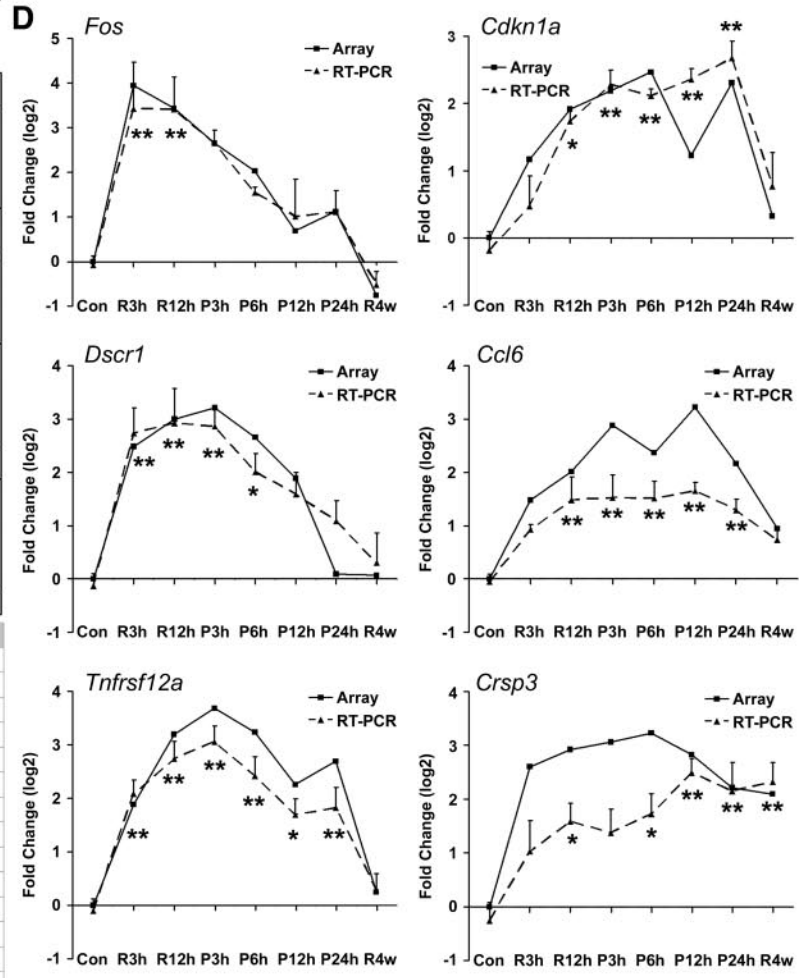
To confirm that voluntary running induces cell proliferation in skeletal muscle, we performed indirect immunofluorescence for plantaris and gastrocnemius muscles following in vivo BrdU labeling after 1 day, 3 days, or 4 wk of voluntary running. As shown in Fig. 3, *B* and *C*, there were few BrdU-positive cells in the Con muscles, and there was a trend of increase following 1 day of running (R1d). The number of BrdU-positive cells increased three- to fivefold ($P < 0.01$) in mice after 3 days of voluntary running (R3d) and returned to the control level after R4w. Induction of cell proliferation was also observed in soleus muscle (not shown). All of the BrdU-positive nuclei detected by pulse labeling were in single nucleated cells, not in multinucleated myofibers. Thus we have obtained direct evidence that voluntary running induces a significant increase of DNA replication and cell proliferation in skeletal muscle. The induced cell cycle gene expression before





Cluster	Con	R3h	R12h	P3h	P6h	P12h	P24h	R4w
c0	0	-3.24	-3.10	-0.73	-3.48	-2.41	-3.57	-2.79
c1	0	-1.85	-1.81	-0.56	-1.83	-1.45	-1.95	-1.37
c2	0	-0.02	0.01	0.21	-0.47	0.17	1.06	-0.15
c3	0	0.19	0.51	1.00	0.67	0.92	1.03	-0.01
c4	0	-1.09	-1.08	-0.38	-1.06	-0.80	-1.21	-0.72
c5	0	-0.53	-0.95	-0.91	-0.81	-0.48	-0.51	-0.17
c6	0	0.38	0.07	-0.10	0.13	0.16	0.32	0.57
c7	0	0.69	0.78	0.65	1.08	0.73	0.75	0.67
c8	0	0.51	-0.52	-0.87	-0.32	-0.47	-1.56	0.08
c9	0	-0.09	-0.26	-0.19	-1.18	-0.16	-0.60	-0.18
c10	0	0.42	0.92	0.98	0.61	0.33	0.33	0.00
c11	0	0.78	1.35	1.73	1.42	1.22	1.43	0.13
c12	0	-0.20	-0.37	-0.28	-0.44	-0.25	-1.22	-0.19
c13	0	0.15	0.10	0.14	-0.05	0.06	-1.22	-0.05
c14	0	0.92	1.26	1.42	1.15	0.71	0.12	0.19
c15	0	2.28	2.67	2.96	2.82	1.98	1.65	0.29





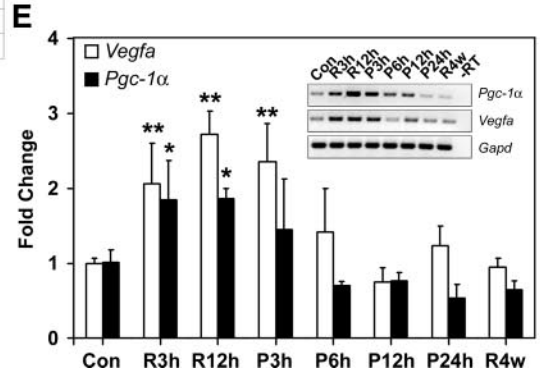


Table 2. *Enrichment of clusters for gene elements within functional categories*

Cluster	Number of Elements (n)	Functional Category (Total Elements)	Elements in Functional Category (κ)	P Value
1	43	Energy metabolism (31)	7	3×10^{-4}
		Lipid metabolism (39)	7	1×10^{-3}
2	13	Cell cycle and growth (48)	4	3×10^{-3}
		Cell adhesion and motility (55)	5	6×10^{-4}
4	57	Carbohydrate metabolism (31)	7	2×10^{-3}
8	38	Neuronal process (43)	8	2×10^{-4}
		Transport (73)	9	2×10^{-3}
11	38	Defense (70)	9	1×10^{-3}
13	116	Protein metabolism (161)	36	9×10^{-5}
		Morphogenesis and development (43)	9	2×10^{-5}
14	37	Neuronal process (43)	7	1×10^{-3}
		Signaling (82)	9	3×10^{-3}
15	27	Apoptosis (21)	4	3×10^{-3}
		Defense (70)	11	1×10^{-6}

P values were calculated by using the cumulative hypergeometric probability distribution for finding at least κ elements from a particular functional category within a cluster of size n. P values $> 3.3 \times 10^{-3}$ are not reported, as the total expectation within the cluster would be >0.05.

the increased BrdU incorporation is consistent with their functions in the control of skeletal muscle cell proliferation in vivo (54). To confirm that the increased BrdU incorporation in skeletal muscle was due to DNA replication, not DNA damage-induced DNA repair, we subjected mouse hindlimb to X-ray irradiation, which is known to sterilize somatic stem cells (35, 54) due to DNA damage (30). BrdU labeling in exercised plantaris muscle was reduced by 76% compared with the nonirradiated exercised muscle after 3 days of running (Fig. 3*D*). This finding strongly argues against the notion that exercise-induced oxidative DNA damage was responsible for the observed increase in BrdU staining in this study.

DISCUSSION

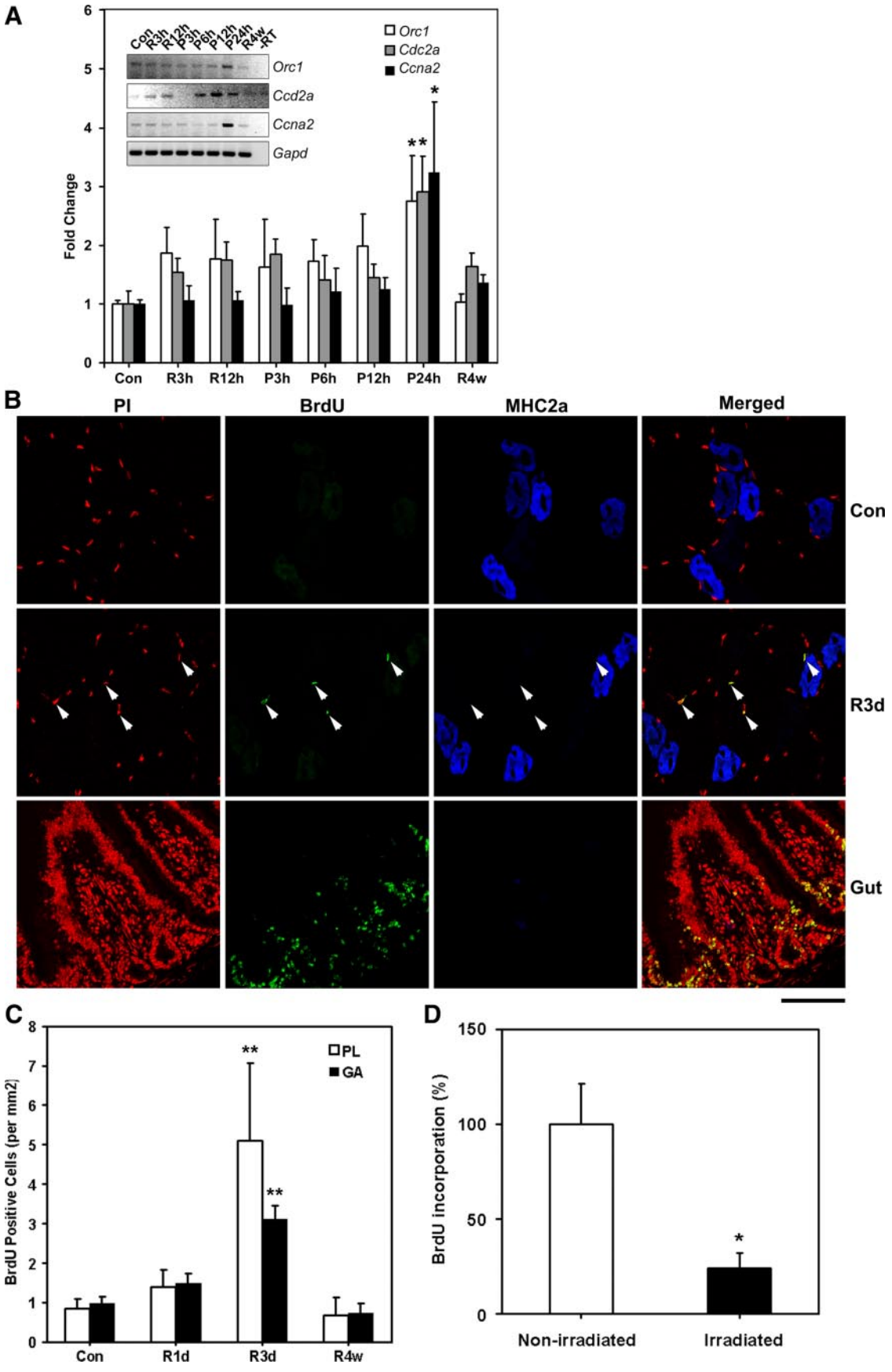
To improve our understanding of the molecular mechanisms underlying exercise-induced skeletal muscle adaptation, we performed mRNA expression profiling in mouse skeletal muscle following a single bout of voluntary running. Our premise was that important signaling and genetic events occur early during adaptation, although the ultimate phenotypic adaptation may depend on cumulative and sequential regulatory events. Consistent with this notion, the findings in this study suggest that skeletal muscle undergoes a phase of active cellular remodeling following a single bout of voluntary running, involving multiple signaling pathways and regulatory modules of the signaling-transcription network. A unique and intriguing finding is that voluntary running promotes cell cycle gene expression and cell proliferation in mouse skeletal muscle, raising an intriguing question regarding the functional role of cell proliferation in endurance exercise-induced skeletal muscle adaptation.

It has been postulated that endurance exercise elicits multiple signals transduced through specific pathways to regulate genes in various functional categories, ensuring the fidelity of the transcriptional reprogramming. Adding to this complexity is a cumulative regulation of gene expression in response to repetitive exercises. Findings in this study suggest that, following a single bout of voluntary running, multiple signaling pathways are activated. For example, a rapid induction of *Dscr1* mRNA expression is consistent with an activation of the calcineurin pathway, which has been strongly implicated in fiber-type specialization (40). The exact anatomical location and the physiological function of induced *Dscr1* mRNA expression in skeletal muscle in response to exercise remains to be determined. We also observed enhanced mRNA expression of target genes indicative of activation of the mitogenic, apoptotic, heat shock, p53, and JAK/STAT pathways with coordinated regulation of mRNA expression of various modules in the signaling transcription networks, such as secreted humoral factors (*Ccl6*), plasma membrane receptors (*Tnfrsf12a/Fn14*), intracellular signaling molecules (*Dscr1*), and transcription factors (*Fos*). Simultaneous activation of the apoptotic genes and cell cycle genes strongly supports an active remodeling in the exercised muscle. These findings reveal a dynamic and complex adaptive process in the signaling-transcription network in skeletal muscle following a single bout of voluntary running.

The transcriptome analysis was employed with an attempt to define early events in transcription in response to an acute bout of exercise. The transcriptional changes may or may not be related to an adaptive change that occurs later during adaptation. It requires a combination of bioinformatics analysis and genetic manipulation in a cell culture or animal model to ascertain the functional importance of a candidate gene in the future. We have focused on cell proliferation in this study, because cell proliferation is a cellular event that can be monitored with greater certainty, and the relationship between cell cycle gene expression and cell proliferation is well defined. The finding of voluntary running-induced cell proliferation in skeletal muscle is relatively novel and may bear greater health implications. We are currently investigating the functional role of other regulatory factors identified by this study.

Compared with previous findings in acute resistance exercise mimicked by high-frequency nerve stimulation in rats or eccentric exercise in humans, both of which exert potent stimuli for muscle growth (12, 13), our findings shared some similar transcriptional responses. At least 8 of 56 genes that

Fig. 2. Single bout of voluntary running induces changes in global gene expression in skeletal muscle. *A*: number of gene elements that showed more than twofold change at various time points. *B*: self-organization map clusters using the 900 regulated expression profiles. The number of clusters was specified as 16, and an algorithm grouped them into discrete clusters. c0 to c15: *clusters 0–15*. The number in the *top middle* of each box indicates the number of gene elements in each cluster. Time points are Con, R3h, R12h, P3h, P6h, P12h, P24h, and R4w, designated by solid dots from *left* to *right*, where Con is the assumed 0 in $\log_2$ ratio between control samples. Thick lines represent the mean expression values, and thin lines represent standard deviation. The mean expression values of each time point are also presented at the bottom of the figure in $\log_2$ ratios. *C*: semiquantitative RT-PCR for genes encoding *Fos*, *Dscr1*, *Tnfrsf12a*, *Cdkn1a*, *Ccl6*, *Csrp3*, and *Gapd*. Representative images are presented for each gene with reactions without reverse transcriptase (−RT) as a negative control. *D*: quantitative data ($n = 5$ for each time point) are presented with a direct comparison to the microarray data. Mean values are statistically different from Con: $^*P < 0.05$ and $^{**}P < 0.01$. *E*: semi-quantitative RT-PCR for *Vegfa* and *Pgc-1α* mRNA. Representative images are presented as an *insert*. Quantitative data ($n = 5$ for each time point) are presented. Statistically different from Con: $^*P < 0.05$ and $^{**}P < 0.01$.



were reported to be altered after high-frequency nerve stimulation (13) showed the same directional changes at one or more time points after an acute bout of voluntary running. These genes include *Fos*, *Jun*, *Gadd45a*, *Myod1*, cardiac muscle ankyrin repeat domain 1, four and a half LIM domain 4, myosin regulatory light chain A, smooth muscle homolog (*Rattus norvegicus*), and hexokinase 2 (*Hk2*). Similarly, at least 6 of 25 genes that are upregulated in human skeletal muscle following eccentric exercise (12) increased after voluntary running, including *Fos*, *Gadd45a*, cardiac α-actin, *Carp*, and DnaJ (heat shock protein 40) homolog subfamily B member 4. Increased mRNA expression of *Fos*, *Jun*, *Cdkn1a*, *Ccna2*, and *Myod1* has been shown to be associated with cell proliferation. It is important to be aware of the possibility that circadian rhythms might have impacted our findings, since we had to collect the samples at different time points after the nocturnal running activities. Circadian rhythm regulated genes in skeletal muscle have been reported previously (55). Nevertheless, it is worth further investigating the voluntary running-induced cell proliferation in skeletal muscle.

The cumulative hypergeometric probability distribution analysis determines whether genes with similar functions are significantly clustered in groups with similar expression patterns. The analysis revealed that genes involved in energy, lipid, carbohydrate, and protein metabolisms were significantly enriched in one of *clusters 1*, *4*, and *13* with reduced expression patterns. We noticed that virtually all of the genes involved in carbohydrate metabolism in this category showed a delayed (24 h postexercise) decrease in mRNA expression, in line with an attenuation of glycolysis during recovery from acute exercise. Only three genes, *Hk2*, phosphoglycerate mutase 1, and *Ogg1*, showed peak induction at the cessation of exercise. The induced expression patterns of phosphoglycerate mutase 1 and *Hk2* mRNAs was consistent with their function in promoting glucose utilization for ATP production during exercise. Induced *Hk2* mRNA expression has been previously reported in skeletal muscle following an acute bout of exercise (33, 36).

In contrast, genes involved in defense and structural remodeling (cell cycle and growth, cell adhesion and motility, morphogenesis, signal transduction and apoptosis) were enriched in *clusters 2*, *11*, *14*, and *15* with induced expression patterns. The overall implication is that the skeletal muscle undergoes active remodeling following a single bout of exercise. The finding that cell cycle genes were upregulated by a single bout of voluntary running was later confirmed by in vivo BrdU labeling. Putman et al. (38) reported that chronic motor nerve stimulation, which induces fast-to-slow fiber-type switching, promotes satellite cell proliferation and suggested the increase in muscle nuclei of the fast-twitch fibers be a prerequisite for fiber-type transition. Irintchev and Wernig (22) have reported that voluntary running induces damage and repair in soleus and tibialis anterior muscles due to increased neuromuscular activity rather than passive stretch. Therefore, our findings extended the previous finding by providing comprehensive mRNA profiling in actively recruited plantaris muscle.

It is important to know whether increased BrdU incorporation is truly due to S-phase DNA replication. One argument against this notion is that increased BrdU staining may be caused by enhanced mitochondrial DNA replication. Because we observed a significant increase of BrdU-positive nuclei, not the cytoplasmic fraction of the myofibers, we can rule out the possibility of enhanced staining of mitochondrial DNA due to enhanced mitochondrial biogenesis. Another argument is that increased DNA repair by oxidative stress results in increased BrdU incorporation. This argument appears in line with our finding that some of the genes related to DNA damage and repair, such as *Gadd45a* and *Ogg1* mRNAs, were upregulated. However, we believe that the increased BrdU incorporation observed in this study was not due to DNA repair for two reasons. First, the detected nuclear BrdU staining was similar in intensity and extent to that observed in regenerating skeletal muscle (54), more robust than DNA repair-mediated BrdU incorporation (34). Most importantly, X-ray irradiation of the hindlimb, which sterilizes myogenic stem cells through DNA damage (30, 35, 54), did not induce BrdU incorporation. Instead, it attenuated voluntary running-induced BrdU incorporation (Fig. 3*D*).

Recent evidence supports the notion that endurance exercise induces muscle injury, which presumably promotes cell proliferation and muscle regeneration. Irintchev and Wernig (22) have obtained results indicating that acute muscle injury occurs upon onset of voluntary running and suggested that exercise-induced injury is a usual event in muscle adaptation to altered use. LaBarge and Blau (25) have reported the presence of donor-derived satellite cells in the skeletal muscle in mice with bone marrow transplantation and increased myogenesis in response to long-term voluntary running. They also attributed the enhanced myogenesis to exercise-induced injury. Induction of genes that were previously reported to be induced in regenerating skeletal muscle in vivo (18), including *Cdkn1a*, *Gadd45a*, type III procollagen α1, *Fos*, and *Myod1*, provides transcriptional clues for an injury-mediated activation of the myogenic program in mice following voluntary running. It would be important to know the functional implication.

The previous finding that chronic motor nerve stimulation promotes satellite cell proliferation (38) raised the issue of whether proliferation is a prerequisite to contractile activity-induced fiber-type switching and/or increases in microvasculature. The timing of enhanced cell proliferation observed in the present study (*day 3*) appears to be concurrent with enhanced angiogenesis, since increased CD31-positive endothelial cells occur around type IIb + IId/x fibers between *day 3* and *day 7* in the same animal model (49). Therefore, the cell proliferation induced by voluntary exercise could be directly

Fig. 3. Voluntary running induces cell cycle gene expression and DNA replication in skeletal muscle. *A*: semi-quantitative RT-PCR for genes encoding origin recognition complex 1 (*Orc1*), *Cdc2a*, and *Ccna2* mRNAs. Reactions without reverse transcriptase (−RT) were used as negative controls. Representative images are presented as an *insert*. *B*: indirect immunofluorescence staining of representative muscle sections from Con and R4w plantaris muscles, along with a duodenum section as a positive control for cell proliferation. All sections were stained for nuclear DNA with propidium iodide (PI), 5-bromo-2′-deoxyuridine (BrdU), and myosin heavy chain 2a (MHC_{2a}). Arrowheads point to the proliferating nuclei on a plantaris muscle section from mice after 3 days of voluntary running (R3d). The bar denotes a 100-μm scale. *C*: quantitative data for skeletal muscle ($n = 5$ for each time point) are presented. **Statistically different from Con, $P < 0.05$. *D*: quantitative data for skeletal muscle after 3 days of voluntary running, with and without X-ray irradiation. *$P < 0.05$ vs. nonirradiated plantaris muscle after 3 days of voluntary running ($n = 3$).

involved in, or play a permissive role for, fiber-type switching, mitochondrial biogenesis, angiogenesis, activity-dependent muscle growth, and/or repair of muscle injury. A more direct test of the function of cell proliferation in this exercise model could be achieved through physical or genetic ablation of the activity of certain muscle resident cell population in the future.

The most straightforward hypothesis is that contractile activity promotes muscle growth by stimulating somatic stem cell proliferation in skeletal muscle, which is agreeable with the findings that treadmill exercise induces enlargement of both fast-twitch and slow-twitch muscles in rats (23, 39, 41), and voluntary running with low resistance induces soleus muscle hypertrophy in mice (24). Our finding that voluntary running induced cell proliferation not only in fast-twitch plantaris and gastrocnemius muscles but also in slow-twitch soleus muscle is consistent with this notion, since slow-twitch soleus muscle does not generally have dramatic changes in fiber-type composition and angiogenesis following long-term training. Our finding that X-ray irradiation diminished exercise-induced cell proliferation provided evidence supporting this notion and laid ground to test the functional role of cell proliferation in skeletal muscle adaptation induced by voluntary running.

In summary, consistent with the notion that transcriptional reprogramming and cellular remodeling start in the early phase of long-term exercise training and play an important role in skeletal muscle adaptation, we have found significant and dynamic changes in mRNA expression profiling in skeletal muscle following an acute bout of voluntary running, involving multiple signaling pathways and regulatory modules of the signaling transcription network. Furthermore, we have obtained direct evidence that voluntary running promotes cell cycle gene expression and DNA replication in skeletal muscle. Future research should be focused on the functional roles of stem cell proliferation in exercise-induced skeletal muscle adaptation in this physiological exercise model with respect to changes in fiber-type composition, angiogenesis, and muscle growth.

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