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Comparative functional genomics of adaptation to muscular disuse in hibernating mammals

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Abstract

Hibernation is an energy saving adaptation that involves a profound suppression of physical activity that can continue for 6-8 months in highly seasonal environments. While immobility and disuse generate muscle loss in most mammalian species, in contrast, hibernating bears and ground squirrels demonstrate limited muscle atrophy over the prolonged periods of physical inactivity during winter suggesting that hibernating mammals have adaptive mechanisms to prevent disuse muscle atrophy. To identify common transcriptional programs that underlie molecular mechanisms preventing muscle loss, we conducted a large-scale gene expression screen in hind limb muscles comparing hibernating and summer active black bears and arctic ground squirrels using custom 9,600 probe cDNA microarrays. A molecular pathway analysis showed an elevated proportion of over-expressed genes involved in all stages of protein biosynthesis and ribosome biogenesis in muscle of both species during torpor of hibernation that suggests induction of translation at different hibernation states. The induction of protein biosynthesis likely contributes to attenuation of disuse muscle atrophy through the prolonged periods of immobility of hibernation. The lack of directional changes in genes of protein catabolic pathways does not support the importance of metabolic suppression for preserving muscle mass during winter. Coordinated reduction of multiple genes involved in oxidation reduction and glucose metabolism detected in both species is consistent with metabolic suppression and lower energy demand in skeletal muscle during inactivity of hibernation.

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Author Contributions

VBF and BMB conceived the study. ØT collected physiological data for bears. AVG, NCS, CC, LCS, and MKS designed and conducted expression experiments. AVG, HW, VBF and JY analyzed data. VBF, AVG, BMB wrote the paper.

Data Accessibility

All microarray data series were submitted to NCBI Gene Expression Omnibus (GEO) with accession number GSE17154 at http://www.ncbi.nlm.nih.gov/geo/.

Keywords

protein biosynthesis; hibernation; black bear; arctic ground squirrel; functional genomics; gene expression

Introduction

Hibernation is an adaptive strategy of reduced metabolic demand and greatly limited mobility that conserves energy during periods of low food availability in highly seasonal or unpredictable environments (Boyer and Barnes, 1999; Carey et al., 2003). In humans and most mammals, physical inactivity leads to loss of muscle strength and mass. In contrast, hibernating bears and ground squirrels demonstrate very limited muscle atrophy over the prolonged periods (6-8 months) of physical inactivity of winter hibernation suggesting that hibernating mammals have evolved natural mechanisms that prevent disuse muscle atrophy. Although attenuation of atrophy in muscle over the hibernation season in bears and ground squirrels has been documented (Harlow *et al*. 2001; Lohuis *et al*. 2007a; Gao *et al*. 2012), the molecular mechanisms underlying this protective musculoskeletal adaptation are not known.

Genome-wide transcriptional screening provides a means for the identification of the transcriptional changes and pathways that are potentially involved in the molecular mechanisms preventing disuse muscle atrophy in hibernating mammals. The first study of transcriptional changes in skeletal muscle of black bears using a pilot version of a custom microarray revealed elevated expression of genes involved in protein biosynthesis during hibernation, but no coordinated directional changes were detected for genes within catabolic pathways (Fedorov *et al*. 2009). Although genome coverage was limited, these results led to the hypothesis that elevated expression of genes involved in protein biosynthesis contributes to molecular mechanisms preserving muscle mass during periods of physical inactivity. The energy cost of increased anabolism was suggested as an important trade-off with the adaptive mechanisms that allow bears to maintain full musculoskeletal function and preserve mobility during and immediately after hibernation, thus promoting survival (Fedorov *et al*. 2012). Decrease in protein catabolism during hibernation due to metabolic suppression for conserving energy is an alternative hypothesis to explain the attenuation of muscle atrophy (Lee *et al*. 2008). We expect from this hypothesis coordinated decrease in expression level of genes involved catabolic pathways during torpor of hibernation.

The black bear (*Ursus americanus*) and arctic ground squirrel (*Urocitellus parryii*) are evolutionary distant species that demonstrate different modes of hibernation. Both species remain sequestered within hibernacula overwinter and show little or no locomotion for 5-7 months (Daan *et al*. 1991; Tøien *et al*. 2011). However, unlike bears hibernating with core body temperature 30 - 36°C and metabolic rate reduced by 50-75% (Tøien *et al*. 2011), small hibernators (<1 kg) such as the arctic ground squirrels enter deep levels of torpor with body temperatures near 0 °C and 98% reduction of metabolic rate. Torpor in small hibernators is periodically interrupted by spontaneous arousal episodes when animals raise their metabolism and body temperature returns to euthermic levels (>30°C) for less than 24

hours (Boyer & Barnes 1999). Similar to black bears, preservation of muscle mass has been reported in several species of ground squirrels (Gao *et al*. 2012; Andres-Mateos *et al*. 2013). The distant phylogenetic relation between black bears and ground squirrels suggests that mechanisms regulating gene expression rather than evolution of specific genes underlay their ability to prevent disuse muscle atrophy. Comparison of transcriptional profiles and pathways significantly enriched by co-regulated genes between these two evolutionary distant hibernating species is expected to identify general and species specific transcriptional changes important in protection from disuse atrophy in muscle.

In the present study, we used a more representative set of 9,600 cDNA probes on a microarray custom to black bears (Fedorov *et al*. 2011) to identify additional co-regulated functional groups of differentially expressed genes in muscle of hibernating bears as compared to summer active animals. To obtain the first insight into transcriptional changes in muscle of hibernating ground squirrels, we developed the genomics resources to fabricate a 9,600 probe microarray custom to arctic ground squirrels. We focused on pathway analysis identifying functional groups of co-regulated genes rather than on expression of individual genes to assess the biological significance of transcriptional changes in skeletal muscle of both hibernating species. To reveal general patterns in transcriptional profiles during hibernation, we compared functional groups of differentially expressed genes detected in each species. In addition, we conducted pathway analysis of differentially expressed genes that are common in hibernating black bears and arctic ground squirrels. Transcriptional changes are discussed in light of how disuse muscle atrophy is prevented in hibernating mammals.

Material and Methods

Animals

Animal protocols were approved by the University of Alaska Fairbanks Institutional Animal Care and Use and USAMRMC Animal Care and Use Review Office.

We sampled the skeletal muscle from black bears including animals reported on in a previous study (Fedorov *et al*. 2009). Bears (51 -226 kg) were captured May-July (two bears sampled in hibernation were captured in October) from the field in Alaska and transferred to Fairbanks. These were “trouble bears” captured by the Alaska Department of Fish and Game that were scheduled for euthanasia. In order to minimize intra-group variation in gene expression, only males > 2 years old were used in these experiments. Summer active bears that were still feeding and active and housed in an outdoor enclosure were euthanized and sampled for tissues between late May - early July (n = 5) and on October 2 (n = 2). Food was withdrawn 24 hours before these animals were euthanized. Bears in the hibernating condition were without food since October 27 and euthanized for tissue sampling between March 1 - 26 (n = 6), about 1.5 months before expected emergence from hibernation. For monitoring of physiological conditions, bears were instrumented as previously described (Fedorov *et al*. 2009; Tøien *et al*. 2011). Briefly, core body temperature, ECG and EMG were monitored with radio telemetry. Oxygen consumption and respiratory quotient were monitored with open flow respirometry by drawing air from the closed dens (Tøien 2013). Bears were euthanized by an intravenous injection of pentobarbital with death assessed by

termination of heart beats as determined with a stethoscope. Immediately prior to tissue sampling, the metabolic rate of anesthetized hibernating bears was 45.4 % of that of anesthetized summer bears, and body temperature was 3.5°C lower. Tissues collection from quadriceps followed immediately with samples frozen in liquid nitrogen within 10 minutes of death.

Arctic ground squirrels were trapped during July near Toolik Lake (68°N 149°W) and transported to the University of Alaska Fairbanks. Animals were housed at 20 ± 2°C with a 16:8-h light-dark photoperiod and provided with Mazuri rodent chow and water *ad libitum*. Hibernating animals were implanted abdominally with temperature-sensitive transmitters. Core body temperature (T_b) was monitored for stage of torpor by an automated telemetry system that records T_b every 10 min. Animals were transferred in September to a chamber with the temperature 2 ± 2°C and a 8:16-h light-dark photoperiod where they entered hibernation. Arctic ground squirrels in steady-state torpor and T_b 2.2+0.3°C (n = 10) were collected after 80-90% of the duration of the previous torpor bout (late torpor, 8-12 days). Squirrels sampled during hibernation had completed no less than two full-length torpor bouts and none were sampled after February (> 1 month of hibernation remained). Post reproductive, summer euthermic animals sampled in May and June 1-2 months after ending hibernation (n = 9) were used as non-hibernating controls. To decrease biological variation, all samples but one, were females in both groups. Torpid arctic ground squirrels were euthanized by decapitation without anesthesia, summer active animals were deeply anesthetized with isoflurane, euthanized with sodium pentobarbital and decapitated. Quadriceps skeletal muscle was rapidly dissected, frozen in liquid nitrogen and stored at −80°C until used.

RNA preparation

Skeletal muscle samples (approximately 250 mg) were homogenized in 2 ml Lysing Matrix D tubes containing specialized beads and RLT buffer using a mini-beater (FastPrep-FP120, Qbiogene, Inc., Carlsbad, CA, USA) for 1 min at 4800 rpm. Total RNA was isolated from the tissues using an RNeasy midi kit (Qiagen Inc., Valencia, CA, USA). All RNA samples were processed by DNase I (Qiagen) treatment. RNA quality was evaluated with an Agilent 2100 Bioanalyzer and concentration was measured by using Nanodrop ND-1000.

Developing genomic resources

Gene discovery and custom microarrays fabrication for the black bear are described in detail in Fedorov *et al*. (2009, 2011) as well as genomic analysis of the expressed sequencing tags (Zhao *et a*l. 2010). We used the same procedure for the arctic ground squirrel. In short, normalized, subtracted cDNA libraries were constructed from heart, brain, brown fat, skeletal muscle, and liver of hibernating and euthermic arctic ground squirrels. We used SMART template-switching protocol and primer extension PCR, normalization and subtraction as was previously described (Fedorov *et al*. 2009). Expressed sequencing tags (EST) were sequenced from the 5’-end with the universal M13 forward primer. After filtering off vector contamination and mtDNA inserts, a total of 24,371 high quality ESTs were mapped to the genome of the hibernating 13-lined ground squirrel, *Ictidomys tridecemlineatus* (Shao *et al*. 2010), a close relative of the arctic ground squirrel. Among

15,639 mapped sequences, 10,252 ESTs were annotated with 3,883 unique genes. All 24,371 ESTs were re-annotated by searching against human RefSeq sequences (http://www.ncbi.nlm.nih.gov/RefSeq/) (e-value <= 1E-10). For 20,477 ESTs with human accession number, we were able to annotate 6,647 sequences with Ensembl/biomart database (http://www.biomart.org/index.html) obtaining 5,944 different genes. A total of 9,600 cDNA inserts were PCR amplified and printed on nylon membrane with a Biorobotics arrayer in the Microarray core facility of the Wistar institute.

Hybridization

Hybridization of black bear and ground squirrel samples was carried out with a species specific 9,600 cDNA custom microarrays. Samples of total RNA were linearly amplified with Illumina TotalPrep RNA Amplification Kit (Ambion), and 1.6 μg of the amplified RNA was labeled with 65 μCi of [33P]dCTP as previously described (Kari *et al*. 2003). All RNA samples were amplified, labeled and hybridized in the same batch. The hybridization was carried out for 18 hours at 42°C in 3 ml of a hybridization buffer (6× SSC, 0.5% SDS, 6M Urea). Filters were rinsed at room temperature with 2× SSC/1% SDS to remove residual probe and MicroHyb solution and then transferred to preheated wash solutions in a temperature-controlled shaking water bath. Filters were washed twice for 30 min in 1.5 l of 2× SSC/1% SDS at 50°C and then once for 30 min in 1.5 l of 0.5× SSC/1% SDS at 55°C and once for 30 min in 1.5 l of 0.1× SSC/0.5% SDS at 55°C. Filters were then exposed to phosphorimager screens for four days and scanned at 50-μm resolution in a Storm Phosphorimager. Image analysis was performed with the ImaGene program (Biodiscovery).

Microarray data analysis

Only non-empty spots with less than four flags across the samples were included into the analysis, the flagged signals were omitted. The background corrected signal was obtained by subtracting local background median density from signal median density. Background corrected signals were divided by their median on the array to obtain the normalized median densities representing the normalized expression values. The array data were transformed with quantile normalization (Bolstad *et al*. 2003) and a one-way ANOVA test was used to select genes that exhibited significant differences between hibernating and summer active animals. Similar to experiments with other tissues (Fedorov *et al*. 2011), a p-value<0.05 and $|\log_2 FC| > 0.5$ were set as cutoffs for significant differences in expressed genes, where FC is fold change (the mean expression value in the hibernating animals divided by the mean expression value in the summer active animals as the criteria for differentially expressed genes as previously reported for other tissues (Fedorov *et al*. 2009, 2011). The false discovery rate (FDR) was calculated using random permutation as described by Storey & Tibshirani (2003). The FDR was defined as the number of significant selected genes divided by the average of the number of significant genes under permutations. The genes demonstrating significant hybridization signal on the arrays were classified according to their Gene Ontology (GO) categories of the biological processes. If one gene had multiple probes on the array, we selected the probe with the smallest ANOVA p-value to represent the gene. Lists of all significant genes on the array and differentially expressed genes with cutoffs of p-value<0.05 and $|\log_2$ fold change$|>0.5$ were uploaded to GO miner (http://discover.nci.nih.gov/gominer/index.jsp). The enrichment in each GO category was

calculated as the proportion of differentially expressed genes relative to the expected proportion on the array. The significance of enrichment for each GO category was estimated by one-sided Fisher exact test. The false discovery rate was assessed by resampling all the significant genes on the array (Zeeberg *et al*. 2003; Zeeberg *et al*. 2005)

In addition to GO miner analysis, we verified enrichment in significant GO categories of the biological processes by using Gene Set Enrichment Analysis (http://www.broad.mit.edu/gsea/index.jsp). There are two advantages of this method. First, unlike GO miner, GSEA estimates enrichment by taking into consideration all of the genes with significant hybridization signals in an experiment, not only those above arbitrary cutoffs for significance of expression differences, false discovery rate and fold-change (Subramanian *et al*. 2005). Second, both over-expressed and under-expressed genes are analyzed in the same run to obtain integrative estimate of enrichment by the use of the following algorithm. Genes were ranked according to the correlation between their expression values and the phenotype class (hibernating and summer active phenotypes) distinction by using the signal to noise ratio. An enrichment score (ES) that reflects the degree to which genes involved in a category are overrepresented at the extremes (over-expressed genes at the top and under-expressed genes at the bottom) of the entire ranked list of genes was calculated. The ES was normalized to account for the size of the category gene set presented in the experiment, yielding a normalized enrichment score (NES). The positive values of the NES indicate enrichment by over-expressed genes and the negative values suggest elevated proportion of under-expressed genes. The statistical significance of the NES was estimated by using phenotype-based permutation test. A cutoff of 25% for false discovery rate of gene set enrichment was used as this value was suggested appropriate for exploratory studies (Subramanian *et al*. 2005). We also used GSEA to test enrichment in selected gene sets that are expected to be important for muscle homeostasis. These gene sets were obtained from Molecular Signatures Database www.broadinstitute.org/gsea/msigdb/index.jsp).

Quantitative real- time PCR

We validated the microarray experiments by quantitative real-time PCR (RT PCR) tests using the same total RNA samples. *Hint1* was selected as a reference gene for black bear skeletal muscle, and *Ywhaz* for ground squirrel, based on the stability of expression values across all samples obtained from the microarray experiments and then tested by RT PCR. All samples showed similar expression values for the correspondent reference genes with low standard deviation in multiple RT-PCR tests. Total RNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer, and cDNA was synthesized from 0.5 μg of total RNA from each sample. The reverse transcription was carried out with MiltiScribeTM reverse transcriptase (Applied Biosystems) with oligo d(T)16 primer in 25 μl reactions at 25° C for 10 min, 48° C for 30 min, and at 95° C for 5 min. The synthesized cDNA was diluted 4x with RNase-free water, and 4 μl of diluted cDNA was used in the 20 μl-volume real-time PCR. Primers were designed with the Primer3 software (http://frodo.wi.mit.edu/primer3/) using the black bear and arctic ground squirrel EST sequences (Table S1). Real-time PCR was performed in triplicates with Power SYBR Green PCR Master Mix (Applied Biosystems) on an ABI-7900 HT. Cycling parameters were 50° C for 2 min of incubation, 95° C for 10 min of Taq activation, and 40 cycles of 95° C for 15 sec and 60° C for 1 min.

Controls with no template were set to exclude contamination, and controls with no reverse transcriptase but all other components were taken to exclude nonspecific amplification from genomic DNA. Specificity of amplification was checked with the melting curve analysis and agarose gel electrophoresis. Four 10-fold dilutions of a sample with mixed cDNA from all samples were used for a standard curve for each primer set for calculating RT PCR efficiency. We tested a difference in gene expression between hibernating and summer active animals with $P < 0.10$ as cutoff according to Pfaffl (2001). We calculated the fold-change in level of expression of a target gene relative to a reference gene for each sample and then compared the values for each group using Student's t-test as described by Livak & Schmittgen (2001).

Results

Differentially expressed genes in black bears

Signals from 9,093 of 9,600 (95%) probes across all samples showed median intensities that were above background. A total of 415 unique genes (4.6% of all genes with significant signals) were differentially expressed in the skeletal muscle tissue of bears sampled during hibernation compared with in bears sampled in summer (Table S2). All but three differentially expressed genes (*Otud1, Col3a1, Ube2q2*) demonstrated changes in expression that differed less than four-fold ($|\log_2 FC| < 2$). Of the significantly differentially expressed genes, we identified 198 (47.5%) genes that were over-expressed and 217 (52.5%) genes that were under-expressed during hibernation.

Differentially expressed genes in arctic ground squirrels

Signals from 9,147 of 9,600 (95%) probes across all 19 samples showed median intensities that were above background. In total, 548 unique genes (6.0% of all genes with significant signals) were differentially expressed in arctic ground squirrel skeletal muscle tissue during late torpor (Table S4). All but three differentially expressed genes (*Ankrd1, Acaa2 , Abra*) demonstrated changes in expression that differed less than four-fold ($|\log_2 FC| < 2$). Of the significantly differentially expressed genes, 349 (63.7%) genes were over-expressed and 199 (36.3%) genes were under-expressed in ground squirrel skeletal muscle sampled during late torpor compared to summer.

Quantitative RT PCR validation of microarray results

To validate results from microarray, we conducted quantitative real-time PCR tests for 30 randomly selected genes that showed differences in expression at $P < 0.05$ in the array hybridization for the black bear. Twenty-four (80%) tested genes showed significant changes in the same direction as the array results (Table S3, Fig. 1). Similar to in the bear, a total of 20 (80%) out of 25 tested genes showed significant changes consistent with the array results for the arctic ground squirrel (Table S5, Fig. 1). The observed experimental false discovery rate of 20% only slightly exceeds the experimental value (15%) for the commercial whole human genome microarray platform (Agilent; Wang *et al*. 2006), thus this is reasonable for an exploratory study in a non-model species. Further support for microarray results comes from the highly significant positive correlations (black bear: r =

0.93, $P < 0.0001$; ground squirrel: $r = 0.84$, $P < 0.0001$) between the amplitude of fold changes for true positive genes in RT PCR and microarray experiments.

Differentially expressed genes common for both species

A total of 2138 genes with hybridization signals on the microarrays were shared between the two species. Among 66 differentially expressed genes shared between species, 46 genes changed in the same direction in both species (Table 1). In total 30 genes were over-expressed and 16 genes were under-expressed with a significant correlation ($r = 0.72$, $P < 0.0001$) between fold changes in both species during hibernation (Table 1). Notably, gene *Ndrg2* was over-expressed during torpor of hibernation in both species (Table 1) which is consistent with elevated expression of this gene previously detected by RNA-seq in muscle of torpid 13-lined ground squirrels (Hampton *et al*. 2011).

Functional gene sets enriched by differentially expressed genes

Genes with a significant hybridization signal on the arrays were classified according to their Gene Ontology (GO) categories of biological processes by using GO miner. GO categories with less than five differentially expressed genes detected were excluded from the analysis (Zeeberg *et al*. 2003). Significant enrichment of categories of biological processes by differentially expressed genes was validated by the results of gene set enrichment analysis (GSEA; Table 2, 3). To estimate enrichment, GSEA ranks all genes with significant hybridization signals in the experiment; thus, its results do not depend on the selection of genes above cutoffs for significance of expression differences and false discovery (Subramanian *et al*. 2005). For muscle in hibernating black bears, the proportion of over-expressed genes was significantly elevated for two related gene sets involved in translation and ribosome biogenesis (Table 2, Fig. 2). Biological process categories of glucose metabolism and oxidative reduction were significantly enriched by under-expressed genes (Table 2). The same GO categories with the addition of pyruvate metabolism and cellular respiration as part of oxidation reduction were enriched by under-expressed genes in skeletal muscle of the arctic ground squirrel during late torpor (Table 3, Fig 2).

Apart from validation of the GO miner result, we also used GSEA to test enrichment in selected gene sets (Molecular Signature data base) known to be important for muscle homeostasis (Table 4). In both species, gene sets separately involved in three stages of translation (initiation, elongation and termination) were enriched by over-expressed genes during hibernation. Except for reduction of the ubiquitin proteasome pathway in the bear muscle, no protein catabolism categories (proteasome, proteolysis and lysosome) demonstrated enrichment by differentially expressed genes during hibernation. The muscle contraction gene set expression was decreased in both species during hibernation.

To directly compare results of differential gene expression analysis in the black bear and arctic ground squirrel, we conducted the GO miner pathway analysis of genes common for both species including results from the previous study in black bear muscle (Fedorov *et al*. 2009). A total of 2138 genes with significant signals on the microarrays were shared between the two species. Similar to species specific results, the two GO categories were enriched with over-expressed genes common for bears and ground squirrels: translation and

ribosome biogenesis (Fig. 3). Under-expressed genes shared between the two species were over represented in oxidation reduction GO category (Fig. 3).

Discussion

Our comparative study reveals common transcriptional programming in skeletal muscle of two hibernating mammals that includes the coordinated induction of protein biosynthesis (translation) genes and suppression of genes involved in oxidation reduction and glucose metabolism. The relatively high proportion of over-expressed genes in muscle of torpid arctic ground squirrels (60.9%) and black bears (47.5%) suggests the importance of regulatory changes at the transcriptional level during torpor of hibernation. The similar proportion of over-expressed genes detected in muscle of two species with different modes of hibernation suggests that regulation at the transcriptional level is largely independent of T_b during hibernation, since bears maintain a relatively high T_b (30-36° C), compared to very low levels in torpid arctic ground squirrels (near 0° C) (Tøien *et al*. 2011; Barnes 1989).

Protein metabolism

Animals used as models of muscle atrophy due to disuse show a profound loss of protein from their muscle tissue (Lecker & Goldberg 2002). We recently showed that the coordinated induction of protein biosynthesis genes is a prominent feature of the transcriptome in liver, heart and muscle of hibernating black bears (Fedorov *et al*. 2009, 2011). Those results are now supported by the present wide-coverage study that identifies the elevated expression of 31 protein biosynthesis genes, in addition to the 16 translation genes over-expressed in skeletal muscle of hibernating bears (Fedorov *et al*. 2009). The coordinated over-expression of protein biosynthesis genes implies that translation of proteins is induced in skeletal muscle during hibernation, and this is supported by physiological evidence. Except for a moderate (10%) decrease in muscle protein concentration reported specifically for lactating female bears (Tinker *et al*. 1998), no muscle protein loss indicative of muscle atrophy was detected in hind limb muscles of black bears over prolonged periods of inactivity and fasting during hibernation (Lundberg *et al*. 1976; Koebel *et al*. 1991; Harlow *et al*. 2001; Lohuis *et al*. 2007a, b). It has been suggested that hibernating bears may maintain the potential for protein biosynthesis by recycling nitrogen lost in urea through hydrolysis by bacteria and hepatic synthesis of amino acids back into structural protein (Nelson 1980; Barboza *et al*. 1997). Transcriptome profiling revealed coordinated under-expression of genes involved in amino acid catabolism (Fedorov *et al*. 2011) and amino group utilization through the urea cycle (Fedorov *et al*. 2009) in the liver of hibernating bears. These findings imply reduction in amino acid catabolism and suggest, besides possible urea recycling, redirection of amino acids from catabolic pathways to the enhancement of protein biosynthesis.

Similar to in the black bear, protein biosynthesis and ribosome biogenesis categories are significantly enriched by over-expressed genes in muscle of arctic ground squirrels during torpor. There are several lines of evidence for inhibition of translation, however, in liver of hibernating ground squirrels at the low T_b of torpor. Radioactively-labeled amino acids

show low rates of incorporation, and a significant portion of polyribosomes necessary for translation of mRNA are disassembled during torpor (Knight *et al*. 2000; van Breukelen & Martin 2001; Carey *et al*. 2003). Depression of protein biosynthesis in the liver is also indirectly supported by a large scale proteomic study that revealed no over-expressed proteins during torpor, as compared to summer active arctic ground squirrels (Shao *et al*. 2010). Protein synthesis in hibernating ground squirrels does occur during the brief (10-18 h) but regular interbout arousals when ground squirrels return to high, euthermic T_b each 1–3 weeks during the hibernation season (Knight *et al*. 2000; van Breukelen & Martin 2001; Carey *et al*. 2003). It has been shown that during torpor mRNA transcripts in liver of arctic ground squirrels are resistant to degradation, and this makes stabilized translatable transcripts available for elevated protein synthesis during the short arousal episodes (Knight *et al*. 2000; Pan & van Breukelen 2011). Transcriptional elevation of protein biosynthesis genes detected here in torpid squirrels likely facilitates induction of translation in muscle during arousals. Elevated expression of proteins involved in translation and ribosome biogenesis (Shao *et al*. 2010) as well as increases in incorporation rates of labeled amino acids (Zhegunov *et al*. 1988) during interbout arousals further support induction of protein biosynthesis at this stage of hibernation. There is a need for proteomic study in muscle of ground squirrels during interbout arousals to validate induction of protein biosynthesis during these episodes of hibernation. Elevated expression at protein level is expected for multiple translation genes that demonstrated transcriptional induction reported here in torpid muscle. Similar to bears, small mammalian hibernators such as ground squirrels and prairie dogs show resilience to muscle atrophy over prolonged periods of immobility and fasting as compared to non-hibernating mammals (Cotton & Harlow 2009; Gao *et al*. 2011; Andres-Mateos *et al*. 2013). Although moderate decreases in mass and protein concentration in some hind limb muscles but not others has been reported (Steffen *et al*. 1991; Wickler *et al*. 1991; Gao *et al*. 2011), no changes in mass or structure of quadriceps were detected over hibernation in ground squirrels (Andres-Mateos *et al*. 2013). Transcriptional induction of multiple protein biosynthesis genes reported here suggests that elevated translation during arousals likely contributes to protective mechanisms against muscle loss. Although decrease in protein synthesis is a prominent feature of muscle atrophy in mammalian disuse models (Rennie *et al*. 2010; Marimuthu *et al*. 2011), some increase in expression of several genes involved in ribosome biogenesis was detected in unloaded soleus of old rats (Wittwer *et al*. 2002; Dupont-Versteegden *et al*. 2008) and healthy women after 60 days of bed rest (Chopard *et al*. 2009). This increase in expression of genes encoding ribosomal proteins was considered as compensatory response to disuse atrophy (Dupont-Versteegden *et al*. 2008).

Under fasting conditions, elevated protein biosynthesis in skeletal muscle of hibernating mammals may be supplied with amino acids resulting from the catabolism of non-myofibrilar protein stored in other tissues (Harlow 1995). Collagen is an important source of essential amino acids, and increase in concentrations of metabolites of collagen catabolism in the blood plasma of hibernating bears implies its breakdown (Hissa *et al*. 1998; Lohuis *et al*. 2005). Small hibernating mammals such as prairie dogs demonstrate a 23% reduction in protein content of the liver overwinter (Cotton & Harlow 2009). Amino acids made available from the breakdown of labile protein reserves may support enhanced levels of

protein biosynthesis that prevent skeletal muscle loss and, thus, preserves locomotion ability through 6 months of winter hibernation.

In addition to the decrease in protein synthesis, induction of protein catabolism is an important mechanism that contributes to disuse muscle atrophy in non-hibernating mammals (Lecker & Goldberg 2002; Jackman & Kandarian 2004; Marimuthu *et al*. 2011). Consistently, a coordinated increase in expression of genes involved in proteolysis, ubiquitin-proteasome pathway, and lysosome proteolysis was detected in muscles of different mammalian models of disuse (Wittwer *et al*. 2002; Chopard *et al*. 2009; Bialek *et al*. 2011). In contrast, our study revealed no increase in transcriptional levels of proteolysis genes in muscle of hibernating mammals. The lack of induction of protein degradation pathways may be an additional factor contributing to preservation of muscles mass during hibernation. However, molecular mechanisms preventing elevation of muscle proteolysis remain obscure. The importance of suppression of protein catabolism was suggested by Fuster *et al*. (2007) who showed that hibernating bears likely produce a proteolytic inhibitor that is released into plasma that blocks proteolysis of skeletal muscle.

Decrease in protein catabolism during hibernation due to metabolic suppression for conserving energy has also been suggested to contribute to the attenuation of muscle atrophy (Lee *et al*. 2008). Our expectation from this hypothesis is a coordinated decrease in expression levels of genes involved in protein catabolic pathways. This decrease is specifically expected to be clearly defined in muscles of ground squirrels with a large (98%) reduction of metabolic rate during torpor. We did not find a reduction in the transcription levels of proteolysis genes, except for some down-regulation of the ubiquitin-proteasome pathway in bears.

Metabolic processes

A coordinated under-expression of genes involved in the broad category of oxidation reduction and glucose metabolism was found in skeletal muscle of both torpid arctic ground squirrels and black bears. The decrease in energy producing oxidation reduction processes is consistent with metabolic suppression during hibernation which overall is an energy saving physiological adaptation. Transcriptional reduction of multiple genes responsible for key oxidation reduction processes such as glycolysis, cellular respiration and electron transport was previously reported for the liver of hibernating bears (Fedorov *et al*. 2011) and arctic ground squirrels (Yan *et al*. 2008). Although transcriptional induction of several genes involved in glycogenesis in the liver of both hibernating species may act to provide a source for glucose that is likely an important metabolic fuel for the brain, heart, and other glucose dependent tissues and functions (Fedorov *et al*. 2011; Yan *et al*. 2008; Buck & Barnes 2000), glycogenic genes were under-expressed in skeletal muscle. Consistent with this, decreased quantities of proteins involved in glucose metabolism in skeletal muscle of torpid ground squirrels (Hindle *et al*. 2011) implies a limited role of carbohydrate fuel in muscle metabolism during hibernation. Notably, under-expression of oxidation reduction and glucose metabolism genes was also detected in several mammalian disuse models (Calura *et al*. 2008; Bialek *et al*. 2011), corresponding to lower energy demand in skeletal muscle during physical inactivity.

Conclusions

Our comparative study represents the first research effort to elucidate transcriptional changes for thousands of genes in skeletal muscle during torpor of hibernation in two evolutionary distant mammalian species. The novelty of our study is the use of two diverse hibernating models to identify common gene expression programs underlying natural adaptation to musculoskeletal disuse with potential relevance to muscle atrophy prevention in non-hibernating mammals.

The common transcriptional program includes a coordinated increase in transcriptional levels of anabolic genes involved in protein biosynthesis that implies an induction of translation at different hibernation states. Anabolic transcriptional changes likely contribute to the attenuation of disuse muscle atrophy through prolonged periods of immobility and fasting. This adaptive mechanism allows hibernating mammals to maintain full musculoskeletal function and preserve mobility during and immediately after hibernation, thus promoting survival. The lack of directional changes in genes of protein catabolic pathways does not support the importance of metabolic suppression for preserving muscle mass.

The comparative transcriptome analyses reported here identify candidate pathways and generate an impetus for follow-up studies that include validation of expressional changes of translation genes at protein level in hibernating mammals. Candidate genes showing differences in expression consistent at transcriptional and protein levels in hibernating mammals need to be selected for protein expression analysis in non-hibernating mammalian models of disuse. It is expected that genes important for muscle loss prevention in hibernating mammals will demonstrate expression changes to opposite direction or no changes under conditions of disuse in non-hibernating models as compared to hibernating black bears and ground squirrels. The next step will be to test whether experimental altering of candidate gene expression is sufficient to reduce muscle atrophy in non-hibernating mammals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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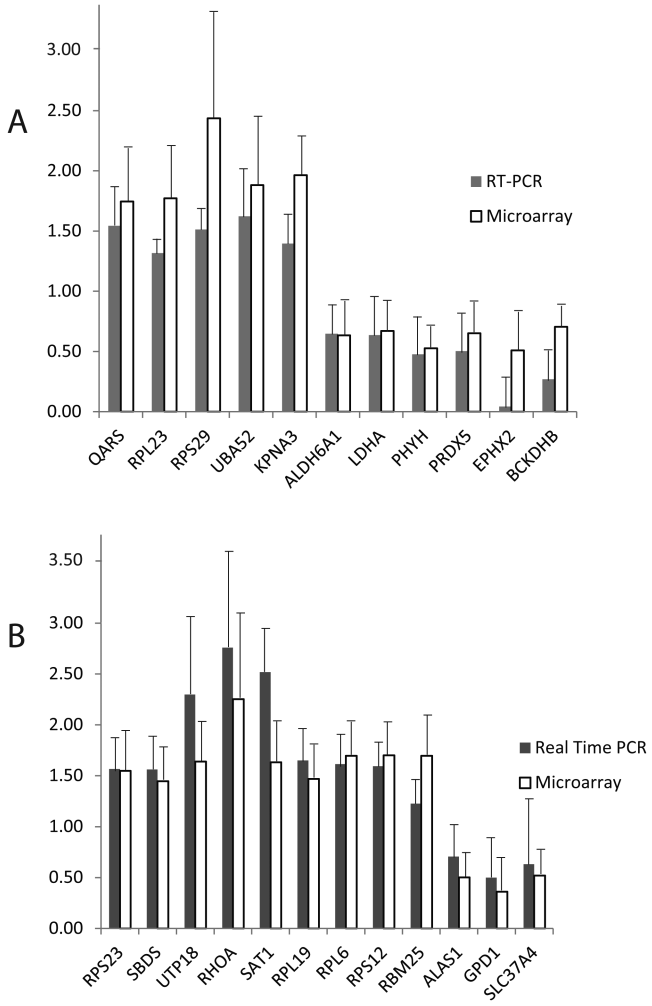

Figure 1.

Differentially expressed genes in skeletal muscle tissue confirmed with real-time PCR: A – black bear, B - arctic ground squirrel. Solid and open bars with standard deviation bars show normalized expression values obtained in real-time PCR and microarray experiments, respectively. The values were normalized to the mean in summer active animals.

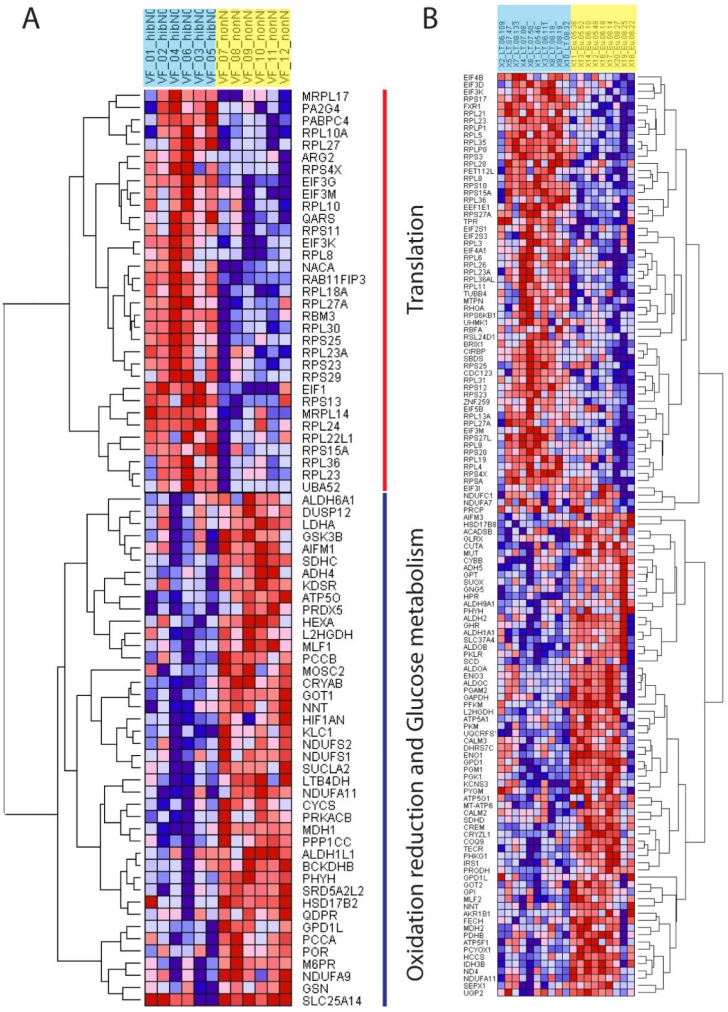

Figure 2.

Clustering and heat maps of expression values of genes involved in translation, glucose metabolism and oxidation-reduction GO category in skeletal muscle in black bears (A) and arctic ground squirrels (B). On the top hibernating animals are in blue and summer active individuals in yellow. Over-expressed genes are in red, under-expressed genes are in blue.

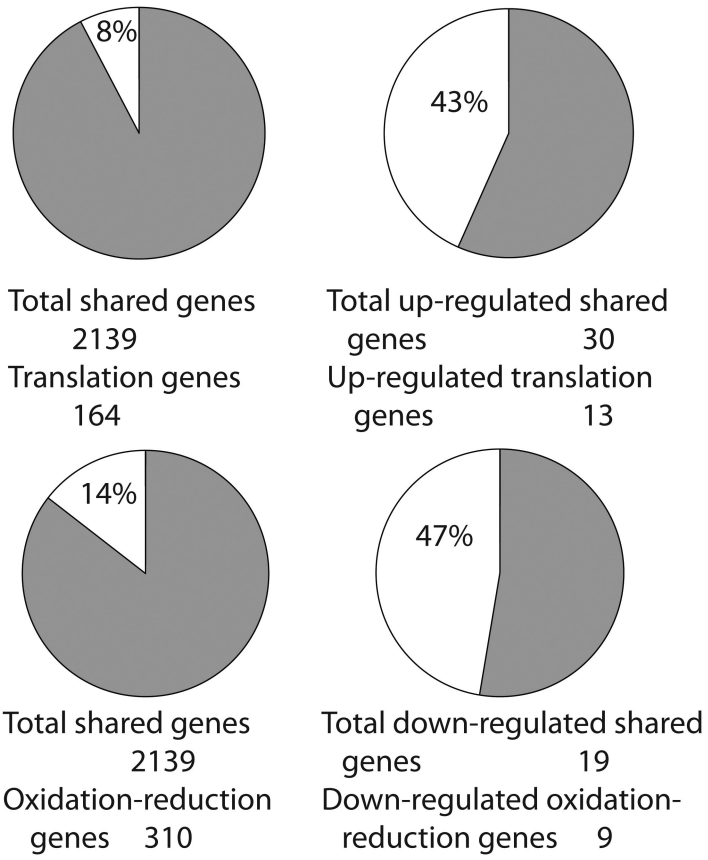

Figure 3.

Gene Ontology (GO) enrichment analysis of common genes differentially expressed in the black bear and arctic ground squirrel skeletal muscles during torpor of hibernation. A – translation GO category, enrichment is 5.625 ($P<0.001$), B – oxidation reduction GO category, enrichment is 2.904 ($P<0.05$). Numbers show a percent of genes in the correspondent GO category among the total number of common genes with significant hybridization signals on the microarrays for both species (left pie), and percent among the total differentially expressed genes shared between two species (right pie).

Table 1. Differentially expressed genes changed in the same direction in skeletal muscle of both the black bear and the arctic ground squirrel during torpor of hibernation.

Gene Symbol	Gene Name	Black Bear		Arctic Ground Squirrel	
		P	Log_2FC	P	Log_2FC
Translation (13 genes)					
Eif3k	Eukaryotic translation initiation factor 3 subunit K	0.006	0.995	0.012	0.630
Eif3m	Eukaryotic translation initiation factor 3 subunit M	0.020	0.876	0.030	1.021
Fxr1	Fragile X mental retardation syndrome-related protein 1	0.008	1.649	<0.001	0.871
Rpl23	60S ribosomal protein L23	0.013	0.826	<0.001	0.641
Rpl27	60S ribosomal protein L27	0.011	0.990	0.012	0.485
Rpl27a	60S ribosomal protein L27a	0.012	0.931	<0.001	0.619
Rpl8	60S ribosomal protein L8	0.021	0.389	0.005	1.354
Rps12	40S ribosomal protein S12	0.008	1.534	<0.001	0.767
Rps13	40S ribosomal protein S13	0.010	0.856	0.036	0.335
Rps15a	40S ribosomal protein S15a	0.011	0.713	<0.001	0.553
Rps23	40S ribosomal protein S23	0.008	1.099	0.004	0.630
Rps24	40S ribosomal protein S24	0.012	1.738	0.034	0.495
Rps27	40S ribosomal protein S27a	0.008	1.592	0.003	0.831
Oxidation Reduction (7 genes)					
Adh4	Alcohol dehydrogenase 4	0.004	−0.505	0.022	−0.346
Aldh9a1	4-trimethylaminobutyraldehyde dehydrogenase	0.032	−1.538	0.015	−0.706
Atp5f1	ATP synthase subunit b, mitochondrial	0.030	−0.415	0.034	−0.727
L2hgdh	L-2-hydroxyglutarate dehydrogenase, mitochondrial	0.011	−0.622	0.003	−0.832
Ndufs1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	0.009	−0.514	0.041	−0.450
Nnt	NAD(P) transhydrogenase, mitochondrial	0.007	−0.756	0.007	−0.873
Phyh	Phytanoyl-CoA dioxygenase, peroxisomal	<0.001	−0.926	<0.001	−0.802
Other GO Categories (26 genes)					
Acyp1	Acylphosphatase	0.014	0.794	0.002	0.827
Ankrd1	Ankyrin repeat domain-containing protein 1	0.020	1.580	0.005	3.273
Atp6v0d1	V-type proton ATPase subunit d 1	0.015	1.505	0.008	0.665
Caprin1	Caprin-1	0.012	0.533	0.010	0.390
Cebpz	CCAAT/enhancer-binding protein zeta	0.021	0.477	0.017	0.760
Cmya5	Cardiomyopathy-associated protein 5	0.026	1.48	0.016	0.512
Gbe1	1,4-alpha-glucan-branching enzyme	0.027	0.698	0.004	1.189
Gnb1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	0.040	0.598	0.042	0.347
Gnb2l1	Guanine nucleotide-binding protein subunit beta-2-like 1	0.013	0.353	<0.001	0.802

Gene Symbol	Gene Name	Black Bear		Arctic Ground Squirrel	
		P	Log_2FC	P	Log_2FC
Maged2	Melanoma antigen, family D, 2	0.016	0.293	0.008	0.460
Ndrg2	NDRG family member 2	<0.001	1.223	<0.001	1.705
Nolc1	Nucleolar and coiled-body phosphoprotein 1	0.026	0.466	0.035	0.518
Peal5	Phosphoprotein enriched in astrocytes 15	0.029	0.342	0.014	1.202
Psmd13	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 13	0.012	0.570	0.047	0.609
Rb×1	Ring-box 1, E3 ubiquitin protein ligase	0.008	0.490	0.037	0.334
Sptbn1	Spectrin, beta, non-erythrocytic 1	0.008	1.318	0.027	0.608
Tomm20	Translocase of outer mitochondrial membrane 20 homolog (yeast)	0.009	0.617	0.008	0.656
App	Amyloid beta (A4) precursor protein	0.006	−0.556	0.042	−0.489
CdkS	Cyclin-dependent kinase 8	0.008	−0.579	0.005	−0.454
Ckm	Creatine kinase, muscle	0.029	−0.575	0.015	−0.889
Coq10b	Coenzyme Q10 homolog B (S. cerevisiae)	0.001	−0.664	0.023	−0.393
Dnm11	Dynamin 1-like	0.003	−0.483	0.013	−0.372
Kbtbd2	Kelch repeat and BTB (POZ) domain containing 2	0.015	−0.531	0.027	−0.452
Pdlim7	PDZand LIM domain 7	0.008	−0.642	0.006	−1.192
Tmeml26a	Transmembrane protein 126A	0.001	−0.436	0.004	−0.671
Usp10	Ubiquitin specific peptidase 10	<0.001	−0.805	0.001	−0.364

Table 2

Selected Gene Ontology categories of biological processes significantly enriched with differentially expressed genes in black bear skeletal muscle based on the data from two microarrays (Fedorov *etal.* 2009; this study). Arrows indicate direction of gene expression changes in hibernating animals. FDR is false discovery rate and NES is normalized enrichment score.

GO Category	Total genes on array	Changed genes	GO Miner		GSEA	
			Enrich-ment	FDR	NES	FDR
Translation (GO: 0006412)	365	47↑	2.999	<0.001	1.59	0.037
Ribosome biogenesis (GO: 0042254)	134	16↑	2.781	0.006	1.54	0.035
Glucose metabolic process (GO: 0006006)	131	18↓	2.579	0.030	−1.41	0.076
Oxidation reduction (GO: 0055114)	610	59↓	1.815	0.003	−1.66	<0.001

Table 3

Gene Ontology categories of biological processes significantly enriched with differentially expressed genes in the arctic ground squirrel skeletal muscle. Arrows indicate direction of gene expression changes in torpid animals. FDR is false discovery rate.

GO Category	Total genes on array	Changed genes	GO Miner		GSEA	
			Enrich-ment	FDR	NES	FDR
Translation (GO:0006412)	343	581↑	2.548	<0.001	1.81	0.006
Ribosome biogenesis (GO:0042254)	142	34↑	3.608	<0.001	1.98	<0.001
Oxidation reduction (GO:0055114)	589	59↓	2.657	<0.001	−1.24	0.123
Glucose metabolic process (GO: 0006006)	125	29↓	6.154	<0.001	−1.76	0.006
Glucose catabolic process (GO:0006007)	54	18↑	8.842	<0.001	−1.94	<0.001
Gluconeogenesis (GO:0006094)	38	13↓	9.075	<0.001	−1.89	0.012
Alcohol catabolic process (GO:0046164)	64	19↓	7.875	<0.001	−1.31	0.133
Pyruvate metabolic process (GO:0006090)	52	15↓	7.652	<0.001	−1.69	0.009
Cellular respiration (GO:0045333)	141	16↓	3.010	0.002	−1.55	0.063

Table 4

Gene Set Enrichment Analysis (GSEA) results for selected pathways. NES - normalized enrichment score, positive values indicate elevated expression and negative - under-expression in torpid muscle, FDR false discovery rate. Significant values are in bold.

Pathway	Black Bear		Arctic Ground Squirrel	
	NES	FDR	NES	FDR
Translation initiation	**2.49**	**<0.001**	**1.77**	**0.008**
Translation elongation	**2.83**	**<0.001**	**1.91**	**<0.001**
Translation termination	**2.78**	**<0.001**	**1.93**	**<0.001**
Proteosome	−0.85	0.691	1.03	0.503
Proteolysis	−1.08	0.321	−0.86	0.674
Lysosome	−0.90	0.619	1.10	0.289
Ubiquitin proteosome	**−1.31**	**0.065**	−0.81	0.709
Muscle contraction	**−1.61**	**0.016**	**−1.61**	**0.014**