Transient transcriptional events in human skeletal muscle at the outset of concentric resistance exercise training

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Running head: Resistance training and muscle transcriptional events

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Abstract

We sought to ascertain the time-course of transcriptional events that occur in human skeletal muscle at the outset of resistance exercise (RE) training in RE naïve individuals, and determine if the magnitude of any response was associated with exercise induced muscle damage. Sixteen RE naïve males were recruited, 8 underwent 2 sessions of 5x30 maximum, isokinetic knee extensions ($180°.s^{-1}$) separated by 48 hrs. Muscle biopsies of the *vastus lateralis*, obtained from different sites, were taken at baseline and 24 hrs after each exercise bout. Eight individuals acted as non-exercise controls with biopsies obtained at the same time intervals. Transcriptional changes were assessed by microarray, and binding of HSP27 and αB-crystallin to insoluble proteins by immunohistochemistry as a measure of muscle damage. In control subjects, no probesets were significantly altered (FDR<0.05) and HSP27 and αB-crystallin binding remained unchanged throughout the study. In exercised subjects, significant inter-subject variability following the initial bout of RE was observed in the muscle transcriptome, with greatest changes occurring when HSP27 and αB-crystallin binding was elevated. Following the second bout of RE, the transcriptome response was more consistent among subjects revealing a cohort of probesets associated with immune activation, the suppression of oxidative metabolism and protein ubiquitination as differentially regulated. The results reveal that the initial transcriptional response to RE is highly variable in RE naïve volunteers, is associated with muscle damage, and unlikely to reflect longer-term adaptations to RE training. These results highlight the importance of considering multiple time-points when determining the transcriptional response to RE and associated physiological adaptation.

Key words: Resistance exercise; muscle transcriptome; muscle biopsies; training adaptation; exercise-induced muscle damage

Introduction

It is universally acknowledged that resistance exercise training, when performed for a sufficient duration and intensity, will promote a change in the underlying morphology of the exercised muscle and culminate in gains of muscular strength and mass. The underlying adaptations elicited by chronic strength training are diverse and not yet fully understood, but include the preferential increase in type-II fibre cross sectional area (6), enhancement of basal rate of mixed muscle protein synthesis (11), an increase in type-II fibre-specific force production (peak force/cross sectional area) (6) and the activation and proliferation of satellite cells (28). Given the spectrum of changes elicited by chronic resistance exercise training, a host of transcriptional events must occur at various stages preceding the long-term morphological and metabolic adaptations.

With the widespread adoption of microarray technology, it is now feasible to interrogate the transcriptional changes elicited by resistance exercise on a genome wide scale in a relatively straightforward manner. While microarray technology has been utilised successfully by a number of researchers, it has almost invariably involved the comparison of pre-training muscle biopsy specimens with samples obtained following weeks of resistance exercise training (21), or alternatively, utilised to study acute changes following a single bout of resistance exercise (9, 13, 21). As a consequence, when a pre- versus post-training biopsy approach has been exclusively adopted, by which time the morphological and metabolic changes in muscle would be firmly established, important transient transcriptional events underpinning the physiological adaptation to resistance exercise would have been missed. Likewise, we predict that the transcriptional response observed after a single bout of resistance exercise would not reflect the long-term adaptations that occur in response to resistance exercise training. In contrast, we hypothesised that the

transcriptional changes important in the adaptation of skeletal muscle to resistance exercise would be dynamic and develop over the first few bouts of resistance exercise in previously untrained individuals.

Forcible muscle contractions as performed during resistance exercise are known to elicit muscle damage, with the degree of resultant damage in part dependent upon the underlying muscle fibre architecture (8). Moreover, muscle damage and the ensuing inflammation that is often associated, have notable effects on many muscle metabolic and signalling processes (19). HSP27 and αB-crystallin are known to be elevated following a single bout of eccentric exercise and accumulate around areas of myofibrillar disruption, particularly the z-disks and intermediate desmin-like structures (18). It has been suggested that they provide a protective role following myofibrillar damage and have been identified in a number of situations where muscle damage has been reported (18). Their association with myofibrillar disruption therefore makes them a quantifiable surrogate of muscle damage. Despite this, an assessment of changes in the binding of HSP27 and αB-crystallin to insoluble muscle proteins following resistance exercise and how this relates to changes in the muscle transcriptome have yet to be described.

A secondary aim of the study was to assess the effect of serial muscle biopsies on the muscle transcriptome. The vast majority of studies investigating the transcriptional changes that occur in skeletal muscle following an intervention, whether that be exercise, feeding, or pharmacological, rely upon the sequential taking of muscle biopsy specimens. This in turn is dependent upon the premise that the procedure used to obtain the muscle biopsy specimens does not result in transcriptional changes in neighbouring muscle tissue; yet the effect of muscle biopsying on the muscle transcriptome remains unknown. This is despite arguments

115 that damage and immunological changes occur in skeletal muscle as a result of
116 multiple biopsy sampling (1, 16).
117
118
119 Here we demonstrate, for the first time, that muscle sampling via the Bergström
120 needle-biopsy technique, one of the most widely cited methods for obtaining muscle
121 samples from humans under experimental conditions, does not affect the muscle
122 transcriptome. Furthermore, we demonstrate that the initial transcriptional response
123 to an unaccustomed bout of resistance exercise is highly variable among subjects
124 and dependent on whether muscle damage has occurred. However, we show that
125 the early exercise-induced muscle damage seen in some volunteers following
126 unaccustomed maximal-intensity resistance exercise does not influence the
127 transcriptional response to subsequent exercise bouts. Lastly, we describe a set of
128 transcripts that appear important in the adaptation of skeletal muscle to the
129 resistance exercise stimuli.

Methods

Subjects

Sixteen healthy non-smoking men (age 22 ± 2 yr; body mass index 23.0 ± 2.0 kg.m^{-2}) volunteered to take part in the study. Subjects were recreationally active but had never taken part in lower body resistance exercise training. Each of the volunteers underwent a routine medical screening, provided written informed consent to participate and was free to withdraw from the experiment at any point. The study was approved by the University of Nottingham Medical School Ethics Committee and conducted in accordance with the Declaration of Helsinki regarding the use of human subjects in medical research.

Experimental protocol

Subjects reported to the laboratory on the morning (~08:00 h) of 4 consecutive days in a fasted state (days 0 – 3), having abstained from food from 22:00 h the night before. Twenty-four hrs preceding the start of the experimental protocol and during the 4-day period of the investigation, subjects abstained from alcohol and any exercise outside of the laboratory setting. On day 0, measurements of lean mass of the isolated thigh region of the dominant leg were determined by dual-energy x-ray absorptiometry (Lunar Prodigy, GE Healthcare). To determine the transcriptional changes that occur in response to the exercise protocol, a baseline needle biopsy sample was taken at rest from the *vastus lateralis* muscle of the dominant leg (4) and the muscle sample immediately frozen in liquid nitrogen prior to microarray analysis. Following muscle sampling, subjects were randomly assigned to either exercise or control groups for the remainder of the study (n=8 per group).

Following group assignment, the maximal isometric strength of the knee extensor muscles were determined in the dominant leg of subjects to be exercised using an

158 isometric strain gauge coupled to a digitised recorder, with the subjects secured in a
159 supine position and the knee flexed at 90º. Following isometric strength
160 determination, exercise subjects performed 5 bouts of 30 maximal isokinetic knee
161 extensions from a position of 90º to 180º knee flexion and at an angular velocity of
162 $180º.s^{-1}$, with each bout separated by 1 min of rest. Subjects performed the exercise
163 in the seated position on an isokinetic dynamometer (Eurokinetics, UK) and were
164 given verbal encouragement. Both legs of the exercise subjects were exercised
165 using the above protocol. Isometric strength determination and the isokinetic
166 exercise protocol were repeated by subjects on day 2. Additional muscle biopsies
167 were obtained from the dominant legs of subjects on the morning of days 1 and 3
168 (i.e. 24 hrs after each exercise session), with each biopsy taken 2.5 cm distal to the
169 previous biopsy.
170
171 To control for the effect of serial muscle biopsies on the muscle transcriptome,
172 control subjects attended the laboratory for an equivalent amount of time as the
173 exercised subjects and muscle biopsies were obtained at the same time point and in
174 the same manner as the exercised subjects; the control subjects did not undergo any
175 exercise testing or training throughout the period of examination. Furthermore, to
176 eliminate the effect that nutrition can have on muscle metabolism and performance,
177 all subjects were provided with standardised meals to be consumed the evening
178 preceding exercise visits (4500 kJ with 12.6% from protein, 47.7% from
179 carbohydrates and 32.2% from fat) and immediately following the completion of
180 exercise (3300 kJ with 8.3% from protein, 54.2% from carbohydrates and 37.0% from
181 fats). Control subjects received the same meals and they were consumed at the
182 same time of day as the meals provided to the exercise subjects. With the exception
183 of the prescribed meals described above and the requirement to attend the facilities
184 in a fasted state, subjects were free to continue with their usual dietary behaviour.
185

Affymetrix Microarray

Total RNA was extracted from muscle biopsy specimens using TRIzol (Invitrogen, USA) and quantified spectrophometrically (DU 730 spectrophotometer; Beckman-Coulter, USA). Hybridisation, washing, staining and scanning of the Human Genome U133 Plus 2.0 Array chips (hgu133plus2) were performed according to the manufacturer's instructions (Affymetrix Inc., USA). Sample integrity and microarray data quality were confirmed by generating histograms of perfect-match probe intensities, RNA degradation plots and boxplots for each individual array, with each array passing our QC criteria. Eight muscle samples were not analysed by microarray due to either inadequate muscle sample size or insufficient RNA sample following extraction. This resulted in 5 - 8 samples remaining per group. Further verification of the microarray raw data was performed by examining average background intensity, scale factors, percentage present calls and 3'/5' ratios of present housekeeping genes, with all arrays conforming to Affymetrix's published guidelines. After satisfying the quality control criteria, arrays were background corrected by applying the robust multi-array averaging (RMA) methodology on the perfect-match probe data, followed by quantile normalisation and summarisation via the median polish regression method, resulting in the generation of Log_2 transformed expression values.

To reduce the influence of false negatives in down-stream analysis caused as a consequence of the large number of probesets included on the array platform (>50k), the probeset data was filtered in an attempt to restrict the dataset to transcripts thought important in muscle processes. This was accomplished by only selecting probesets for further analysis that fulfilled the following two criteria. 1) Classified as present on ≥50% of arrays according to the MAS 5.0 implementation. 2) Identified by Ingenuity Pathways Analysis software (IPA v.8.0 build 82437) as involved in immune

cells, skeletal muscle, uncategorised tissue or primary cell processes. Filtering using the above criteria resulted in a subset of 9781 probesets for further analysis.

Linear modelling for microarray data (22), in conjunction with the Benjamini-Hochberg procedure for the control of the false discovery rate (3), were utilised to determine differentially expressed transcripts across the various time points and treatments. Transcripts were denoted as differentially expressed if the false discovery rate was ≤ 0.05 and the fold-change in expression greater than 1.5. The hierarchical ordered partitioning and collapsing hybrid (HOPACH) clustering method was subsequently employed to identify probesets with similar expression profiles at each time point examined. All analysis of the microarray data was performed using the freely available Bioconductor (v2.5) and R (v2.10.1) statistical computing software (http://www.bioconductor.org and http://www.r-project.org respectively). The original microarray .CEL files have been submitted to the Gene Expression Omnibus repository database (accession number: GSE45426; http://www.ncbi.nlm.nih.gov/geo/).

Quantitative real-time RT-PCR

To validate the microarray data, two muscle specific transcripts were selected for real-time RT-PCR analysis. Total RNA was extracted from biopsy samples and cDNA synthesised as described in detail elsewhere (17). Three previously designed Assay-on-Demand Taqman® primer and probe sets were purchased (Applied Biosystems, USA), Myostatin (#Hs00976237_m1), MAFbx/atrogin-1 (#Hs01041408_m1) and to act as an endogenous control, hydroxymethylbilane synthase (#Hs00609297_m1). Myostatin and MAFbx/atrogin-1 were selected on the basis that both are implicated in the control of muscle mass, and furthermore, appear differentially regulated in human muscle 24 hrs following resistance exercise performed under the same conditions as utilised in the current study (10). Following

validation of the primer/probe pairs, samples were run under standard conditions (17). To compare the data generated by the real-time RT-PCR and microarray methodologies, the resultant cycle threshold values of the two muscle transcripts minus the corresponding housekeeping gene Ct values, were compared with Log_2 transformed probeset expression values obtained by microarray analysis.

Pathway analysis

To associate biological function to the identified probesets, transcripts were uploaded to the Ingenuity Pathway Analysis (IPA) software for determination of significantly altered canonical pathways. To maximise the amount of pathway information gleaned from the transcriptional changes observed, probesets were first examined according to the primary clusters to which the individual transcripts associated following HOPACH, after which, pathways associated with transcripts at each time point independent of clustering result were assessed. The association between probesets in the dataset and canonical pathways were analysed using the Benjamini-Hochberg multiple testing correction unless otherwise stated. Significance was assumed when the criteria $p \leq 0.05$ was met.

Immunohistochemistry

To determine expression of αB-crystallin and HSP27 in muscle sections, serial muscle cross sections (8 μm) were mounted on superfrost+ slides (Gerhard Menzel GmbH, Germany), transferred to PBS and subsequently blocked in 5% horse serum in TBS for 2 hrs at room temperature. This was followed by overnight incubation at 4°C in αB-crystallin monoclonal (Enzo Life Sciences, UK; ADI-SPA-222; 1:500) and HSP27 polyclonal (Enzo Life Sciences, UK; ADI-SPA-803; 1:50) primary antibodies diluted in 5% horse serum in TBS. After subsequent washing in TBS, fluorochrome-labelled secondary antibodies were applied for 2 hrs at room temperature (1:200 each: Alexa Fluor® 568 rabbit anti-mouse IgG (#A11061) and Alexa Fluor® 488

donkey anti-rabbit IgG (#A21206); Invitrogen, UK). Afterwards, sections were washed and mounted in 1,4-diazabicyclo[2.2.2]octane (DABCO) reagent and coverslips applied (1.5 thickness, Gerhard Menzel GmbH, Germany).

Images were captured using appropriate filters with a digital camera (Hamamatsu 1394 ORCA-285) connected to an IRE2 fluorescence microscope (Leica) fitted with a motorised XY stage (Proscan). Individual pictures were obtained across the muscle section with a 20X objective and stitched together with a 40% overlap to create a mosaic image using the commercial Volocity software (Perkin Elmer). Any areas of physical overlap of individual muscle sections on the glass slides were discarded from further analysis.

Quantification of the percentage area of myofiber expressing αB-crystallin or HSP27 was determined using the freely available ImageJ platform (NIH). This was accomplished by measuring the area of each muscle section that was above a specified intensity threshold value expressed relative to the total area of the muscle section, which in turn was determined by assessing background staining of the myofibrils. Thresholds were set at the same value across all sections, where a linear relationship was observed between threshold value and the percentage area of the section above the defined threshold criteria.

To investigate the co-localisation of αB-crystallin with changes in muscle ultrastructure following resistance exercise, 15 μm muscle cryostat sections were thawed onto superfrost+ slides and stained with anti-αB-crystallin and anti-laminin (Sigma, UK) as described above. Additionally, nuclei in muscle sections were stained with the use of DAPI before embedding in a solution containing DABCO (2mg/ml) in PBS:glycerol (1:1). Once immunostaining was complete, sections were imaged as described above. Afterwards, the coverslips were removed from the slides and

allowed to equilibrate in PBS (2 x 5min) followed by 0.1M sodium-cacodylate solution (2 x 5min). Subsequently, sections were fixed overnight in 2.5% glutaraldehyde in 0.1M sodium-cacodylate before staining with 1% osmium tetroxide in 0.1M sodium-cacodylate and 0.5% uranyl acetate in 50mM malate (pH 5.0) for 1h each. This was followed by the samples being dehydrated in a series of increasing ethanol concentrations before they were incubated in stepwise increasing amounts of Araldite® CY212 resin in acetone, beginning at a 1:3 ratio of resin:acetone for 30 minutes, followed by incubation in resin:acetone at a 1:1 ratio overnight, and two final incubations in resin alone for 2h each. Afterwards the resin was polymerised by heating to 70°C for 24h and the section correctly orientated in the resin prior to cooling in liquid nitrogen. From the previously 15 µm sectioned sample, a single 0.5 µm thick slice was obtained, stained with toluidine blue, and photographed by light microscope to aid in the identification of specific fibres under the electron microscope. Once acquired, successive 80 nm sections were obtained, placed on formvar coated and carbon stabilised EM slot grids, and contrasted with osmium tetroxide and lead citrate.

The sections were analysed with a Philips 400 electron microscope, initially at low magnification (360x) to allow identification of regions of interest highlighted in the fluorescent staining micrographs. Once regions of interest had been located, higher magnification images were acquired (2800x to 10000x) to identify ultrastructural anomalies associated with the αB-crystallin positive signals. Neighbouring areas devoid of αB-crystallin served as a negative control. Eight αB-crystallin positive regions were followed up in this manner.

Statistical analysis

Correlation between real-time RT-PCR and microarray data was determined by linear regression analysis using the statistical software SPSS (v16.0) for Macintosh

(IBM, New York, NY.). Significant difference in HSP27 and αB-crystallin protein binding within groups was determined using one-way ANOVA with the Tukey's multiple comparison post-hoc test used to locate differences. Significance was accepted when the criteria $p \leq 0.05$ was fulfilled.

330 **Results**

331 We used Affymetrix® microarrays to determine the transcriptional changes that occur
332 in the skeletal muscle of resistance training naïve, healthy young male adults
333 following concentric resistance exercise or in response to serial muscle biopsy
334 sampling. A workflow relying upon established methods to background correct,
335 normalise and summate probe level data to probeset expression values was
336 employed. Comparison of the Log_2 transformed probeset expression values of two
337 prominent muscle proteins, MAFbx and myostatin, with amplification data generated
338 from real-time RT-PCR analysis of the same samples, revealed a linear relationship
339 between the two methods (Figure 1). This provided confidence that the selected
340 microarray workflow was appropriate and could be trusted to generate reliable data.
341
342 To determine the effect of performing repeated muscle biopsies on the muscle
343 transcriptome response to exercise, microarray data generated from control subjects
344 was examined. As each volunteer had undergone the muscle biopsy procedure on
345 three separate occasions within a 72 hr period, it represented a valuable insight into
346 the compounding effects of muscle sampling by needle biopsy on transcriptional
347 processes. Comparison of the array data obtained from the initial biopsy with those
348 associated with either the second or third biopsies obtained 24 and 72 hrs later
349 respectively, failed to identify any probesets that met the pre-determined false
350 discovery rate criteria for differential regulation (FDR < 0.05). This strongly suggests
351 that the biopsy procedure as described did not result in transcriptional changes that
352 can be ascribed to the procedure *per se*.
353
354 To investigate if inter-subject variability was responsible for any failure to discriminate
355 between differentially regulated probesets as a consequence of the biopsy
356 procedure, the microarray data from control subjects was visualised using principal
357 component analysis (PCA). PCA is a widely used technique for visualising the high-

dimensional data obtained in microarray studies, enabling the identification of large-scale changes in gene transcription. Inspection of the PCA plot obtained from the microarray data generated from control subjects, demonstrated that any effect of the biopsy procedure on gene transcription when considered on a global scale, was smaller in magnitude than the natural variability that existed in the transcriptome between subjects at each time point examined (Figure 2A).

In contrast to the control subjects, the exercise protocol resulted in detectable changes in the muscle transcriptome. 1953 probesets satisfied the pre-determined criteria for differential regulation 24 hrs following the second exercise session (FC > 1.5; FDR<0.05), but no probesets were identified as differentially regulated 24 hrs following the first exercise session. The observed discord in the transcriptional response between the two exercise sessions was substantial and the biological relevance of such disparate responses difficult to discern. Examination of the PCA plot generated from the microarray data obtained from exercised subjects revealed a high degree of variability in the transcriptomic response to resistance exercise, particularly after the first exercise session (Figure 2B). In comparison, the response following the second exercise session was more uniform in nature, demonstrating a lower degree of variability in the transcriptomic response, although one subject still failed to show any transcriptional response to the exercise stimuli. This would explain the discrepancy in the number of probesets identified as differentially regulated between the two timepoints examined and the failure to detect significantly altered probesets following the first exercise session. Furthermore, the largely conserved nature of the response following the second exercise session would suggest it represents important transcriptional changes that underpin the adaptive events that occur in response to resistance exercise *per se*.

From the PCA plot (Figure 2B) it is apparent that with some of the examined individuals, large scale changes in the muscle transcriptome occurred following the first exercise session, but the basis for why this was not universally observed across all volunteers is unclear and is not the result of a discrepancy in the volume of work performed. Subjects were instructed to exercise at maximum self-sustainable intensity and received verbal encouragement; given the exercise protocol it is likely that all populations of muscle fibre types were recruited and that observed differences were not due to recruitment of different populations of fibres with differing metabolic characteristics. Furthermore, on inspection of the workload performed, the volume was equivalent between subjects with identifiable changes in the muscle transcriptome by PCA analysis 24 hrs following the first session of resistance exercise (n=2) compared to those subjects without identifiable changes (n=5; Figure 3). Likewise, no discernible difference in thigh lean mass could be detected regardless of whether changes in the muscle transcriptome were or were not evident following the first exercise session (6.0 ± 0.4 vs. 5.6 ± 0.4 kg, respectively).

It is feasible that the variable response in the transcriptomic response observed following the first exercise session was the result of differing degrees of myofibrillar damage elicited. To ascertain whether exercise-induced muscle damage may be responsible for the changes observed in the muscle transcriptome, two heat shock proteins associated with muscle damage were assessed by immunohistochemistry staining, HSP27 and αB-crystallin. As would be anticipated, no change in either the binding of HSP27 or αB-crystallin to the muscle sections could be observed in control subjects throughout the period of investigation (Figure 4). In contrast, a discord in the amount of HSP27 and αB-crystallin bound to insoluble structures occurred between exercised individuals following a bout of unaccustomed resistance exercise, the pattern of which appeared to mirror whether changes had been observed in the muscle transcriptome according to interpretation of the PCA plot. Specifically, in

individuals where transcriptional changes appeared evident in response to the initial exercise session, a concomitant increase in bound HSP27 and αB-crystallin was observed. Conversely, in individuals where large-scale transcriptional changes were not detectable by PCA, no increased binding of either heat shock protein was observed. Twenty-four hours following the repeat bout of resistance exercise, HSP27 and αB-crystallin binding to insoluble proteins in muscle sections were equivalent between subjects and did not differ from baseline values.

Since αB-crystallin has previously been shown to bind to myofibrillar structures following eccentric exercise (18), we wished to determine whether αB-crystallin positive regions as determined by immunohistochemistry staining, co-localised with regions of myofibrillar microtrauma. Imaging by electron microscopy of nine regions previously identified as areas of high αB-crystallin expression, consistently revealed evidence of anomalies in the myofibrillar structure in the form of electron dense strips in the vicinity of αB-crystallin expression (Figure 5). These structural anomalies are consistent with those described previously following eccentric damage [REF ####]. Furthermore, the electron dense stripes could not be observed in areas devoid of αB-crystallin.

Discovery of an association between heat shock protein binding to cellular structures and the degree of change in the muscle transcriptome following resistance exercise, presented the requirement to perform subsequent analysis based on whether a subject displayed elevated HSP27 and αB-crystallin binding following an acute bout of resistance exercise. Utilising such an approach would allow the delineation of transcriptional changes that occur in response to resistance exercise independent of muscle damage, potentially identifying transcripts important in the adaptation process. Exercised subjects were subsequently partitioned based on either high (n=2) or low (n=6) levels of heat shock protein retention in response to an

441 unaccustomed bout of resistance exercise prior to linear modelling of the microarray
442 data. In individuals with high heat shock protein binding, 3081 and 1735 probesets
443 were identified as differentially regulated (FC > 1.5; FDR < 0.05) following the first
444 and second resistance exercise sessions, respectively. In concordance with the
445 PCA, exercised subjects displaying low levels of heat shock protein retention in
446 response to an unaccustomed bout of resistance exercise experienced more modest
447 transcriptional changes, with 40 and 830 probesets significantly altered over the
448 same timeframe.
449
450 Next, to examine the biological function of the transcripts highlighted and their
451 temporal association with the exercise protocol, probesets previously determined as
452 significantly altered at any of the stages examined were clustered using the
453 HOPACH algorithm and the associated canonical pathways of the top-level clusters
454 identified by use of the Ingenuity Pathway Analysis software (Table 1). Five of the 8
455 top-level clusters were associated with known biological processes according to the
456 software's proprietary database. Visual representation of the expression profile of the
457 5 individual clusters over the period of study revealed a dynamic network of changes
458 occurring in response to the exercise protocol (Figure 5). In individuals where an
459 unaccustomed bout of resistance exercise resulted in enhanced heat shock protein
460 binding to insoluble structures, the most prominent changes 24 hrs following the first
461 exercise session were an increase in transcripts associated with translation initiation
462 (cluster A) in parallel to a fall in mRNA levels of transcripts associated with oxidative
463 metabolism (cluster F). In stark contrast, expression of the 5 individual clusters
464 appeared unaltered following the first exercise session in participants where no
465 robust increases in bound heat shock proteins were found (Figure 5).
466
467 Following the second exercise session, the differences in cluster expression between
468 exercised individuals based on prior classification of heat shock protein binding were

less obvious. Transcriptional changes in signalling processes associated with translation initiation decreased to near basal levels following the repeat bout of resistance exercise in individuals where high heat shock protein binding had previously been reported (cluster A). Furthermore, below basal mRNA expression levels for components of oxidative metabolism and the protein ubiquitination pathway occurred irrespective of previous heat shock protein association (clusters F and H respectively).

Following the repeat bout of resistance exercise, 629 probesets fulfilled the criteria for differential regulation in subjects regardless of the heat shock protein response to an unaccustomed bout of resistance exercise, with a remarkably similar magnitude of change and direction between individuals (Figure 6B) which was more prominent than that observed following the initial exercise session with the same probesets (Figure 6A). The existence of a subset of probesets that are transcriptionally regulated by resistance exercise independent of the initial response to an unaccustomed exercise bout, suggest that they are an important feature in the adaptation to resistance exercise. Assessment of the associated canonical pathways suggests that the conserved cohort of probesets represent elements spanning a diverse set of themes encompassing the maintenance of the actin cytoskeleton, extracellular matrix and the induction of the immune response and/or pro-inflammatory state (Table 2).

Discussion

While the transcriptional changes that occur in response to chronic resistance exercise training have received significant attention (9, 13, 21), in the initial stages of training, where transcriptional changes are likely to be most pronounced and underpin the physiological adaptations that ultimately occur, the effect on the muscle transcriptome appears less well defined. Furthermore, it remains unclear if the taking of repeated muscle biopsies, a common approach to the study of temporal transcriptional changes in human skeletal muscle, has a measurable impact on the muscle transcriptome. Our results demonstrate for the first time that serial needle muscle biopsy sampling, where repeated biopsies are taken 2.5 cm distal to each other, does not elicit confounding changes in the muscle transcriptome when analysed by microarray technologies. In addition, our results demonstrate clearly that the transcriptional responses elicited at the very outset of resistance exercise training are transient. Moreover, while exercise-induced muscle damage has a profound impact on the muscle transcriptome, damage is not universally observed and does not appear to regulate transcriptional events that occur following this initial bout of training, which may be more important in the longer term adaptation to resistance exercise.

The taking of multiple muscle samples by the percutaneous needle-biopsy approach does not affect the muscle transcriptome

The muscle biopsy technique represents an invaluable tool to researchers concerned with investigating metabolic disorders, changes in muscle metabolism with age, or the effects of exercise on muscle processes. Therefore, reports that many popular biopsy techniques result in tissue damage, the effects of which can persist for weeks (23) and impact on important cellular and molecular signalling events (1, 16), is of

significant concern. Despite this, in an attempt to control costs and aid subject recruitment, the vast majority of human physiology studies fail to consider the impact of serial muscle biopsies on transcriptional processes as they rarely include a control group which undergoes the full set of serial muscle biopsies as dictated by the study protocol, but not the intervention under investigation. Within the field of resistance exercise research, it is particularly commonplace to observe longitudinal study designs which do not capture the effects of taking repeated biopsies on the muscle transcriptome. Our results, highlighting that the biopsy procedure as employed does not induce detectable changes in the muscle transcriptome or induce enhanced association of the heat shock proteins HSP27 and αB-crystallin with myofibrillar structures, is reassuring and highlights the validity of the technique.

While the taking of repeated muscle biopsies has been associated with muscle infiltration of neutrophils and macrophages, satellite cell activation (16), extracellular-regulated kinase (ERK) pathway activation (1) and appearance of atrophic and degenerating fibres (23), the discrepancy with our findings as reported here could be the result of differences in the muscle biopsy procedures employed. Where changes have been observed that can be directly attributable to the percutaneous needle biopsy method, the same incision site has been used during the biopsy procedure. Indeed, in the report by Aronson and colleagues (1), which is often cited as an example of the negative confounding effects of muscle biopsy sampling in humans, enhanced ERK phosphorylation was only observed when repeated percutaneous needle biopsy samples were obtained via the same incision site. When samples were obtained 5 cm distal to the original incision site, there was no detectable change in ERK phosphorylation between the original and repeat muscle biopsies. Likewise, mRNA levels of several genes involved in the response to endurance exercise were found to be unaffected by the taking of multiple percutaneous needle biopsy specimens when obtained through separate incision sites (15). These findings

548 in conjunction with our own, suggest that the percutaneous needle biopsy method as
549 pioneered by Jonas Bergström, is satisfactory for the study of muscle transcriptome
550 responses and that multiple biopsies can be employed in close succession when
551 obtained from different incision sites not in close proximity to one another (>2.5 cm
552 apart).
553
554 ***The initial transcriptional response to unaccustomed resistance exercise is***
555 ***highly variable among individuals and is dependent on whether exercise***
556 ***induced muscle damage has occurred***
557
558 At the outset of resistance exercise training skeletal muscle is subjected to both
559 mechanical and metabolic stress, particularly in those individuals naïve to this form of
560 exercise. Over time, the biochemical properties and architecture of the muscle fibre
561 adapt to accommodate these novel demands, a process termed remodelling. We
562 hypothesised that the transcriptional changes that occurred as a result of the
563 resistance exercise would therefore be dynamic in nature, reflecting the evolving
564 demands placed upon the exercising muscle. On initial inspection, our results
565 suggest that an unaccustomed bout of resistance exercise has no effect on the
566 muscle transcriptome when examined 24 hrs after the exercise stimulus. However,
567 on closer examination, it becomes apparent that the transcriptional response elicited
568 by unaccustomed resistance exercise is not universal, with the largest changes
569 observed when muscle damage has been purportedly induced, as assessed by
570 increased heat shock protein binding. In humans, significant variation exists in the
571 magnitude of accretion of muscle mass during resistance exercise training and it has
572 been postulated that age, nutritional support and genetic predisposition are all in part
573 responsible for the differences observed between individuals (26).
574

Following an unaccustomed bout of resistance exercise, increased heat shock protein binding was associated with a transient rise in transcripts linked to eIF2 and AKT signalling. AKT and eIF2 are responsible for the promotion of translation initiation, where translation initiation is considered the main point of control of muscle protein synthesis. This observation, in conjunction with the postulated role of HSP27 and αB-crystallin to facilitate the stabilisation of cytoskeletal and myofibrillar structures and aid in the restoration of damaged and malformed proteins (18), suggests that the muscle is undergoing a period of repair and/or remodelling following the unaccustomed bout of resistance exercise. This is in agreement with previous evidence that non-steroidal anti-inflammatory drug administration blunts the rise in muscle protein synthesis 24 hrs following high-intensity eccentric exercise (27), and suggests that the changes we observe in anabolic signalling are likely due to enhanced inflammation secondary to exercise-induced muscle damage. The failure of a repeat bout of resistance exercise performed 48 hrs after the initial bout to stimulate heat shock protein binding, and the uniformity of the transcriptional response to the exercise stimuli at this time-point, suggests that the repair and remodelling processes were effective.

The initial transcriptional response to an unaccustomed bout of resistance exercise has no bearing on the transcriptional response to a second bout of resistance exercise.

The association between heat shock protein and transcriptional responses to an unaccustomed bout of resistance exercise was found to dissipate when the transcriptional response to a repeated bout of resistance exercise was examined (Fig 6B). The fact that the repeated bout of resistance exercise resulted in a largely different transcriptional response confirmed our hypothesis that the response at the outset of a resistance exercise program is dynamic. Similar observations have

603 recently been reported when transcriptome changes to resistance exercise training
604 have been examined, with less than 2% of the transcriptional changes that occur
605 following chronic resistance exercise training also observed in response to acute
606 resistance exercise (20). However, the high degree of concordance between
607 subjects in the probesets differentially regulated in response to the exercise stimuli
608 (Fig. 6B, r^2 = 0.94) as reported here, suggests that they are an important feature in
609 the adaptive response to resistance exercise. In particular, we observed the
610 suppression of transcripts associated with mitochondrial oxidative phosphorylation
611 and ubqituin-mediate proteolysis in conjunction with increases in probesets
612 associated with the innate immune response.
613
614 While the suppression of muscle proteolysis may appear intuitive in the context of
615 promoting protein anabolism, resistance exercise is known to enhance muscle
616 protein breakdown rates to facilitate fibre remodelling (5) and pivotally, the ubiquitin
617 proteasome system appears central to this process. Reasons for the observed
618 suppression of transcripts associated with ubiquitin proteasome mediated proteolysis
619 in the current study are not possible to discern but it is not without precedent.
620 Twenty-four hours following a bout of unaccustomed leg extension resistance
621 exercise, a 50% decrease in MAFbx mRNA compared to pre-exercise values has
622 been reported (7), where MAFbx is a muscle-specific ubiqutin ligase thought central
623 to the proteolysis of muscle proteins in response to atrophying conditions (12). In
624 contrast, when examined at the earlier timeframe of 6 hrs post-exercise, a significant
625 increase in MAFbx mRNA levels had been observed in the same subjects. Similarly,
626 Louis and colleagues, detailing the time course of proteolytic gene expression
627 following an acute bout of resistance exercise in previously trained volunteers,
628 demonstrated a transient fall in MAFbx mRNA 8 to 12 hrs following the exercise
629 stimulus (14). In conjunction, these observations would seem to suggest that the
630 downregulation of ubiquitin proteasome system transcripts 24 hrs post-exercise may

represent one mechanism by which exercise-induced ubiquitin proteasome mediated proteolysis is reduced following a period of enhanced activity.

Another common theme revealed from the microarray analysis was the suppression of transcripts associated with mitochondrial function and oxidative metabolism. Following endurance exercise training, both an increase in mitochondrial mass and abundance of oxidative enzymes have typically been described (2), with the latter underpinned by transcriptional increases in mitochondrial oxidative genes (24). In comparison, the effects of resistance exercise training on transcripts regulating oxidative metabolism appear less well defined, and of the studies performed to date, conflicting results have been obtained. In one study comparing the muscle transcriptome of strength trained individuals (>8 years of regular training) at rest with samples obtained from untrained individuals under otherwise identical conditions, a lower abundance of mRNAs associated with mitochondrial oxidative capacity were observed in the trained subjects (24). In contrast, an alternative study examining the effects of an acute bout of resistance exercise pre- and post- 12-weeks of resistance exercise training, observed a suppression of transcripts associated with mitochondrial structure and oxidative phosphorylation only at the start of the training protocol (9).

In the present study, in subjects where damage was purported to have occurred following an unaccustomed bout of resistance exercise, a robust decrease in transcripts associated with energy production via mitochondrial oxidative processes was observed. These included genes encoding for components of the electron transport chain (cytochrome c and NADH dehydrogenase), the TCA cycle (succinate dehydrogenase and aconitase) and fatty acid metabolism (carnitine palmitoyltransferase 1, enyol-CoA isomerase and hydroxyacyl-CoA dehydrogenase). After examination of the muscle transcriptome following a repeat bout of resistance

exercise, transcripts associated with mitochondrial oxidative phosphorylation were suppressed in exercised subjects *per se*. These findings suggest that muscle damage following unaccustomed resistance exercise is associated with the suppression of mitochondrial oxidative phosphorylation but it is not a prerequisite for suppression of the same transcripts following successive repeat bouts of resistance exercise.

Intense muscle contraction as observed during resistance exercise has been associated with the promotion of proinflammatory cytokines, both at the localised and systemic level, and with macrophage and neutrophil infiltration of the exercised muscle itself (25). It has been postulated that the induction of the inflammatory response, and in particular the intravasion of the immune cells into skeletal muscle, is in part the consequence of muscle damage (25). Moreover, additional work utilising non-steroidal anti-inflammatories has shown this response to be beneficial in the promotion of muscle protein synthesis and the hypertrophic response (27). In keeping with these findings, we observed an over representation of transcripts associated with immune system function in parallel with transcripts associated with AKT/eIF2 signalling in muscle samples where heat shock protein binding was enhanced following unaccustomed resistance exercise. In a previous longitudinal study, 12-weeks of resistance exercise training was demonstrated to blunt increases in immune activation observed with acute resistance exercise (9). In contrast, following a repeat bout of resistance exercise, canonical pathways associated with immune activation were enhanced in exercised individuals independent of their response to an unaccustomed bout of resistance exercise. This suggests that the enhancement of the immune response to resistance exercise at the initial stages of training is an important feature of the adaption programme and is not necessarily the result of the muscle damage commonly observed.

Conclusions

We recognise that microarray studies by design carry certain limitations, principally encompassing small sample numbers and low sensitivity as a consequence of strategies to deal with high type-II error rates, however the strong correlation between our microarray and qPCR data gives a high level of confidence in the data generated. Furthermore, it is important to emphasise that early transcriptional changes do not necessarily predict the phenotypic changes that ultimately occur in response to resistance exercise training. However, our results clearly highlight the variability that exists in the transcriptional response to a single bout of maximal-intensity resistance exercise in young, healthy males. This variation needs to be considered when designing studies where muscle damage is likely to be elicited and, in particular, when only a single post-exercise biopsy sample is available. Furthermore, it appears that any early exercise-induced muscle damage seen following unaccustomed maximal-intensity exercise does not influence the transcriptional response to subsequent exercise bouts. Therefore, greater insight regarding chronic adaptation may be gleaned by considering changes occurring after the initial transient response. Collectively, these observations may be particularly relevant to patient based exercise intervention studies, where deconditioning is prevalent and single time-point biopsy sampling is common place. Lastly, we also report that the percutaneous needle biopsy procedure can be used in a manner that does not induce measurable transcriptional effects determined by microarray analysis confirming the suitability of the technique for detailed time-course studies.

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814 **Figure 1: Validation of microarray data by real-time RT-PCR.** Following
815 normalisation, summation, and Log_2 transformation of the microarray data, probeset
816 intensity values for the myostatin and MAFbx transcripts were compared with cycle
817 time obtained by real-time RT-PCR analysis (following correction for the endogenous
818 control gene, hydroxymethlbilane synthase). In both cases, a significant correlation
819 between microarray and real-time RT-PCR data was observed, as determined by
820 linear regression ($p<0.0001$).
821
822 **Figure 2: Principal component analysis of filtered probesets in individual**
823 **muscle biopsy specimens 24 hrs following an unaccustomed and repeat bout**
824 **of concentric resistance exercise.** Two-dimensional representation of the obtained
825 microarray data from control (A) and exercised (B) subjects with principal component
826 1 representing the greatest variance in the dataset. Microarray data generated from
827 baseline (day 0) samples represented by open-circles (◦), 24 hrs following an
828 unaccustomed bout of resistance exercise (day 1) by closed-circles (•), and 24 hrs
829 after a repeat bout of resistance exercise (day 3) by plus symbols (+).
830
831 **Figure 3: A) Total isokinetic work performed and B) isometric strength of the**
832 **quadriceps, for each individual during the exercise protocol.** Volume of
833 isokinetic work performed by each individual separated into the individual extension
834 and flexion components. Horizontal lines denote the mean values, with exercised
835 subjects grouped by the presence or absence of a large transcriptional response to
836 an unaccustomed bout of resistance exercise as determined by principal component
837 analysis (solid lines, absence of a large transcriptional response; dashed lines, large
838 transcriptional response in response to unaccustomed resistance exercise). No
839 differences were readily apparent between groups or time points for either measure.
840

Figure 4: Immunohistochemistry analysis of HSP27 and αB-crystallin protein levels in skeletal muscle 24 hrs following an unaccustomed or repeat bout of resistance exercise. Percentage area of the sectioned muscle samples above threshold binding levels of either A) HSP27 or B) αB-crystallin protein. Exercised subjects were subdivided depending on the presence (large response) or absence (small response) of large-scale global changes in the muscle transcriptome in response to an unaccustomed bout of resistance exercise as determined by principle component analysis. Bars represent means ±SEM. * denotes significantly different from baseline P≤0.05. C) Representative images of muscle sections from exercised subjects stained for HSP27 and αB-crystallin. Images are of the same magnification with the white bar denoting a length of 100 μm.

Figure 5: Co-localisation of αB-crystallin to regions of myofibrillar abnormalities 24 hrs following unaccustomed resistance exercise. Serial sections of skeletal muscle from exercised individuals demonstrating large transcriptional responses to unaccustomed resistance exercise were analysed in parallel by immunohistochemistry and electron microscopy. A) A representative image obtained after immunostaining for αB-crystallin (magenta) and laminin (yellow), with nuceli stained with DAPI (blue). The area denoted by the white box encapsulating a αB-crystallin positive region was followed up by examination under a higher objective (x63; panel B) and mapped to a corresponding image obtained by electron microscopy in an adjacent cryostat section (panels C and D). The presence of electron dense material indicative of [#######] appears restricted to the area of αB-crystallin expression. In these individuals 8 similar αB-crystallin expressing regions were followed up by electron microscopy and all displayed the same co-localisation pattern. The white bar in panel A represents 30 μm, while in panels B, C and D it denotes 5 μm.

Figure 6: Fold-change in probeset expression by cluster 24 hrs following an unaccustomed or repeat bout of resistance exercise. To determine transient changes in transcript levels in response to resistance exercise, the average fold-change normalised to baseline values was plotted for each of the five clusters where statistically significant canonical pathways were available. Subjects were subdivided based on whether enhanced heat shock protein binding was observed following an unaccustomed bout of resistance exercise. Arrows denote when resistance exercise was performed. Control subjects showed no changes over the period of study and are not included for clarity.

Figure 7: Comparison of probeset expression for a common set of transcripts differentially regulated 24 hrs following a repeat bout of resistance exercise in subjects showing an earlier discord in the transcriptional response to an unaccustomed bout of resistance exercise. Comparison of the summated and normalised probeset values of 629 differentially regulated transcripts between subjects with or without enhanced heat shock protein binding in response to unaccustomed resistance exercise. The same probesets were examined 24 hrs following either unaccustomed (A) or a repeated bout (B) of resistance exercise. In both cases, a significant positive correlation between subject groups based on heat shock protein response was observed as determined by linear regression ($p<0.001$).

890 **Table 1: Canonical pathways of HOPACH clustered transcripts significant**
891 **altered in skeletal muscle 24 hrs following either an unaccustomed or repeat**
892 **bout of resistance exercise.**
893

Canonical Pathways	p-value	Ratio	Genes
Cluster A			
NRF2-mediated Oxidative Stress Response	0.012	0.11	MAFK, BACH1, JUNB, GSK3B, DNAJB4, MAP3K5, HSPB8, HERPUD1, DNAJA1, MAFF, UBE2K, DNAJA4, MAP2K3, DNAJC5, NRAS, DNAJB9, NFE2L2, MAP2K1, GCLM, KRAS, DNAJB11
EIF2 Signaling	0.012	0.14	EIF1AX, GSK3B, EIF2C2, PAIP1, EIF3I, EIF4E, PPP1R15A, EIF5, NRAS, SHC1, MAP2K1, KRAS, EIF4G2
Aldosterone Signaling in Epithelial Cells	0.033	0.11	HSPD1, HSPB7, DNAJB4, HSPB8, DNAJA1, PDIA3, HSPH1, DUSP1, HSP90B1, DNAJB9, DNAJC5, MAP2K1, KRAS, DNAJB11, HSPA5, HSPA13, HSPA6
PAK Signaling	0.033	0.12	PDGFA, CFL1, ARHGAP10, CASP3, NRAS, MAP2K1, SHC1, PAK1IP1, ITGB1, MYL12A, KRAS, PAK2
PI3K/AKT Signaling	0.033	0.11	LIMS1, MAP3K5, GSK3B, RELA, NFKB1, EIF4E, PPP2CA, YWHAZ, HSP90B1, NRAS, MAP2K1, SHC1, ITGB1, KRAS

Oncostatin M Signaling	0.033	0.21	OSMR, PLAU, IL6ST, NRAS, MAP2K1, SHC1, KRAS
Regulation of eIF4 and p70S6K Signaling	0.033	0.11	EIF1AX, EIF2C2, PAIP1, EIF3I, EIF4E, PPP2CA, NRAS, SHC1, MAP2K1, ITGB1, KRAS, EIF4G2, MAPK13
SAPK/JNK Signaling	0.033	0.12	GNA12, ZAK, CRK, MAP3K5, GADD45A, NRAS, DUSP10, SHC1, HNRNPK, GNB1, KRAS, GNA13
Acute Phase Response Signaling	0.033	0.10	SERPINA1, IL6ST, MAP3K5, RBP1, IL6R, RELA, NFKB1, OSMR, SOCS3, MAP2K3, NRAS, MAP2K1, SHC1, NOLC1, HNRNPK, KRAS, MAPK13
Ephrin Receptor Signaling	0.045	0.09	PDGFA, CRK, ADAM10, ACTR3, GNA12, CFL1, ABI1, ARPC5L, NRAS, MAP2K1, SHC1, ITGB1, GNB1, PAK2, KRAS, GNA13, GNAI3
Cluster C			
Caveolar-mediated Endocytosis Signaling	0.000	0.18	ACTG1, ACTC1, ITGAM, HLA-C, HLA-A, COPG, ITGA5, ITGB2, ITGA6, FLNB, ACTB, FLNA, COPB1, HLA-B
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	0.000	0.17	ACTG1, ARPC1A, MYO5A, ACTC1, YES1, LYN, LCP2, AKT3, CBL, RAC2, TLN1, ACTB, ARPC5, ARPC1B, FCGR2A, EZR

Leukocyte Extravasation Signaling	0.000	0.13	MSN, F11R, CXCR4, ACTG1, ACTN1, ARHGAP1, ACTC1, MLLT4, CYBB, ITGAM, VCAM1, JAM3, GNAI2, RAC2, MYL6, TIMP1, RAP1B, ITGB2, WIPF1, PECAM1, ACTN4, ACTB, GNAI1, EZR
Integrin Signaling	0.003	0.11	ACTG1, ACTN1, ARPC1A, ACTC1, ITGAM, ARF3, AKT3, RAC2, TLN1, RAP1B, WIPF1, ARHGAP26, ITGA5, ITGB2, CAPN2, ITGA6, ACTN4, ACTB, ARPC5, ARPC1B, RALB, ASAP1
Antigen Presentation Pathway	0.003	0.21	HLA-G, CANX, CALR, HLA-C, PSMB9, HLA-A, HLA-B, TAP2, NLRC5
N-Glycan Biosynthesis	0.003	0.22	RPN1, MGAT5, MGAT1, DDOST, RPN2, B4GALT4, MAN1A2, MGAT4A, ALG14
Clathrin-mediated Endocytosis Signaling	0.003	0.12	ACTG1, ARPC1A, ACTC1, AAK1, CBL, GAK, LDLR, FGF9, CD2AP, ITGA5, ITGB2, HIP1, SH3BP4, ACTB, ARPC5, DAB2, NUMB, ARPC1B, CLTC
Germ Cell-Sertoli Cell Junction Signaling	0.003	0.12	ACTG1, ACTN1, ACTC1, MLLT4, TUBB3, TGFBR1, IQGAP1, RAC2, TUBB2A, TGFB1, TUBA1C, MTMR2, TGFBR2, ITGA6, TUBA1B, ACTN4, TUBB6, ACTB, MAP3K8

Virus Entry via Endocytic Pathways	0.006	0.14	ACTG1, ACTC1, HLA-C, HLA-A, RAC2, ITGA5, ITGB2, ITGA6, FLNB, ACTB, FLNA, HLA-B, CLTC
Tight Junction Signaling	0.006	0.12	F11R, ACTG1, ACTC1, MLLT4, CPSF6, AKT3, TGFBR1, JAM3, MYL6, TGFB1, TJP2, TGFBR2, SMURF1, NUDT21, MYH9, ACTB, CPSF1, TNFRSF1B
p53 Signaling	0.008	0.14	PMAIP1, HDAC1, SNAI2, PRKDC, THBS1, SCO2, CCND1, AKT3, TNFRSF10B, APAF1, SERPINE2, TNFRSF10A, DRAM1
Inhibition of Angiogenesis by TSP1	0.011	0.23	TGFB1, TGFBR2, THBS1, AKT3, TGFBR1, KDR, CD47
Actin Cytoskeleton Signaling	0.011	0.09	MSN, CSK, ACTG1, ACTN1, ARPC1A, CYFIP1, ACTC1, IQGAP1, PIP4K2A, RAC2, GNG12, MYL6, FGF9, ITGA5, ACTN4, MYH9, ACTB, ARPC5, PFN1, ARPC1B, EZR
Notch Signaling	0.025	0.18	NOTCH1, JAG1, MFNG, RBPJ, NUMB, HEY1, PSEN1
Hepatic Fibrosis / Hepatic Stellate Cell Activation	0.028	0.11	VCAM1, IL1R1, TGFBR1, MYL6, TIMP1, TGFB1, EDNRB, TGFBR2, LY96, MYH9, IFNGR1, EDN1, KDR, TNFRSF1B, IGFBP3
Cluster D			
Complement System	0.001	0.26	C1QC, CFI, SERPING1, CR1, C1R,

			C3, C4B, C1QB
Hepatic Fibrosis / Hepatic Stellate Cell Activation	0.019	0.10	MYH10, PDGFRB, A2M, CTGF, MMP2, COL1A1, COL3A1, PDGFRA, IL10RA, MYL9, COL1A2, FN1, TIMP2, IGFBP4
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	0.019	0.15	HLA-DMA, B2M, HLA-DRB1, CASP6, HLA-F, HLA-DPB1, HLA-DQA1, HLA-DMB
Antigen Presentation Pathway	0.019	0.16	HLA-DMA, B2M, HLA-DRB1, HLA-F, HLA-DPB1, HLA-DQA1, HLA-DMB
OX40 Signaling Pathway	0.019	0.13	HLA-DMA, B2M, HLA-DRB1, TRAF5, HLA-F, HLA-DPB1, HLA-DQA1, HLA-DMB
Protein Kinase A Signaling	0.019	0.07	MYH10, ITPR2, GNG11, RAP1A, GLI3, ROCK1, ADD3, PRKAR2B, ANAPC4, PPP1R14A, ITPR1, CHP, ADCY7, TCF4, MYL9, KDELR1, AKAP11, MAP3K1, PRKACB, GNG2, PDE3B, TCF7L2
Allograft Rejection Signaling	0.039	0.12	HLA-DMA, B2M, HLA-DRB1, HLA-F, HLA-DPB1, HLA-DQA1, HLA-DMB
N-Glycan Degradation	0.039	0.20	MANBA, MAN2B1, MANEA, MAN2B2, FUCA2
Intrinsic Prothrombin Activation Pathway	0.039	0.19	COL1A2, PROS1, COL1A1, COL3A1, F13A1
Cluster F			

Mitochondrial Dysfunction	0.000	0.27	NDUFA10, NDUFB3, NDUFA3, NDUFB7, PINK1, MAPK12, COX5B, UQCRC2, UQCRH, SDHB, NDUFB10, SDHA, CAT, NDUFS2, NDUFS3, SDHD, CASP9, NDUFS7, ATP5C1, CYC1, NDUFA8, NDUFV2, NDUFS8, NDUFA6, TXN2, CPT1B, NDUFA7, NDUFS1, NDUFB5, COX7A2, NDUFA9, AIFM1, COX6A2, PDHA1, NDUFV1, NDUFA2
Oxidative Phosphorylation	0.000	0.26	NDUFA10, NDUFB3, NDUFA3, NDUFB7, NDUFC1, ATP5G1, COX5B, UQCRC2, UQCRH, SDHB, ATP5O, NDUFB10, SDHA, NDUFS2, NDUFS3, ATP5L, SDHD, NDUFS7, ATP5C1, CYC1, NDUFA8, NDUFS8, NDUFA1, UQCR10, NDUFV2, NDUFA6, NDUFA7, NDUFS1, NDUFB5, COX7A2, NDUFA9, COX6A2, NDUFV1, NDUFA2
Ubiquinone Biosynthesis	0.000	0.38	NDUFB3, NDUFA10, NDUFA3, NDUFB7, NDUFS7, NDUFC1, NDUFA8, NDUFV2, NDUFS8, NDUFA1, NDUFA6, COQ3, NDUFB10, NDUFA7, ALDH6A1, NDUFS1, NDUFB5, NDUFA9, BCKDHB, MGMT, NDUFS2, NDUFV1, NDUFS3, NDUFA2

Valine, Leucine and Isoleucine Degradation	0.000	0.25	BCAT2, PCCB, MCEE, ACAA2, HIBADH, MUT, ALDH6A1, PCCA, BCKDHB, ECH1, HMGCL, HADHA, ACADS, ALDH1A1
Propanoate Metabolism	0.000	0.24	MLYCD, PCCB, MCEE, MUT, ALDH6A1, PCCA, ECH1, ACADS, SUCLG1, ACSL1, HADHA, ACSS2, ALDH1A1
Glycolysis/Gluconeogenesis	0.000	0.19	FBP2, TPI1, PFKM, PDHX, PGM2L1, DLD, BPGM, DLAT, PDHB, PGAM2, ACSL1, PDHA1, ACSS2, ALDH1A1, GPI
Alanine and Aspartate Metabolism	0.000	0.27	GOT1, GOT2, CARNS1, DLAT, PDHB, ASPA, PDHX, CRAT, DLD, PDHA1
Pyruvate Metabolism	0.000	0.20	DLAT, PDHB, LDHD, GRHPR, BCKDHB, PDHX, ACSL1, HADHA, DLD, ACSS2, PDHA1, ALDH1A1
Butanoate Metabolism	0.000	0.21	BDH1, SDHA, PDHB, HMGCL, ECH1, ACADS, HADHA, PDHA1, ALDH1A1, SDHB, SDHD
Citrate Cycle	0.000	0.25	SDHA, CLYBL, SUCLG1, DLD, ACO2, SDHB, SDHD
Fatty Acid Metabolism	0.000	0.11	ECI2, CPT1B, ECH1, ACADS, ACSL1, HADHA, ECI1, GCDH, ALDH1A1, ACAA2, CPT2
Arginine and Proline Metabolism	0.001	0.13	GOT1, GOT2, CARNS1, GAMT, CKMT2, BCKDHB, CKB, NOS1,

			ALDH1A1
β-alanine Metabolism	0.001	0.16	ALDH6A1, CARNS1, MLYCD, ECH1, ACADS, HADHA, ALDH1A1
LPS/IL-1 Mediated Inhibition of RXR Function	0.008	0.07	MGST3, GSTM3, CPT1B, MGST2, ALDH6A1, GSTK1, GSTM4, MGMT, ALAS1, CAT, ACSL1, ALDH1A1, CPT2
Pentose Phosphate Pathway	0.010	0.17	FBP2, PFKM, PGM2L1, RGN, GPI
Urea Cycle and Metabolism of Amino Groups	0.010	0.17	GAMT, CKMT2, CKB, ASPA, CAT
Fatty Acid Elongation in Mitochondria	0.011	0.22	ECH1, HADHA, MECR, ACAA2
Glycine, Serine and Threonine Metabolism	0.011	0.11	GOT1, GAMT, SRR, GRHPR, ALAS1, GCAT, DLD
Synthesis and Degradation of Ketone Bodies	0.013	0.33	BDH1, HMGCL, HADHA
Glutathione Metabolism	0.013	0.12	MGST2, GSTK1, MGST3, GSTM4, OPLAH, GSTM3
Valine, Leucine and Isoleucine Biosynthesis	0.023	0.27	BCAT2, PDHB, PDHA1
Galactose Metabolism	0.027	0.13	FBP2, UGP2, GALT, PFKM, PGM2L1
AMPK Signaling	0.033	0.07	CPT1B, PRKACA, PFKFB1, MLYCD, MAPK12, PFKM, GYS1, PPM1J, CPT2
Phenylalanine, Tyrosine and Tryptophan Biosynthesis	0.049	0.20	PCBD2, GOT1, GOT2
Ascorbate and Aldarate Metabolism	0.049	0.20	BCKDHB, RGN, ALDH1A1

Cluster H			
Protein Ubiquitination Pathway	0.023	0.04	DNAJB5, PARK2, UBE2N, USP28, DNAJB1, DNAJC14, USP10, HSP90AB1, DNAJC11, USP25, DNAJC4
Aldosterone Signaling in Epithelial Cells	0.023	0.05	DNAJB5, DNAJB1, PIK3C2A, DNAJC14, HSP90AB1, DNAJC11, SOS2, DNAJC4

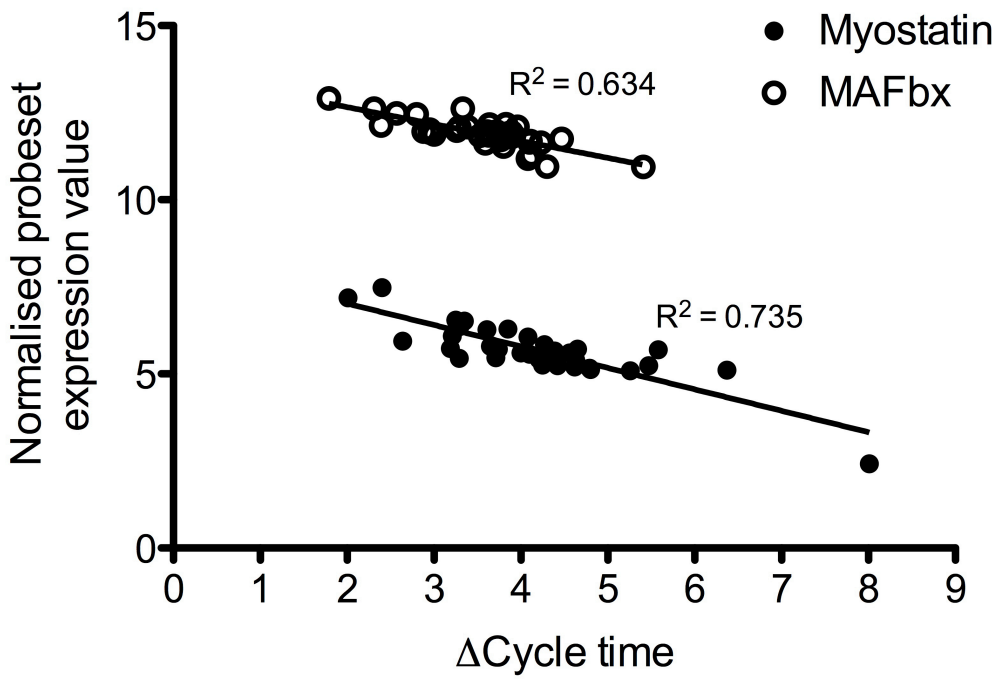
Statistically significant pathways presented for each top-level cluster as determined by HOPACH with the associated p-values, ratio of transcripts significantly regulated compared to the total number forming the pathway, and differentially regulated genes reported. Pathways identified for three clusters (B, E and G) failed to reach significance ($p \leq 0.05$).

Table 2: Canonical pathways associated with transcripts identified as significantly altered in skeletal muscle 24 hrs following a repeat bout of concentric resistance exercise.

Canonical Pathway	p-value
Hepatic fibrosis / Hepatic stellate cell activation	0.000
RhoA signalling	0.000
Actin Cytoskeleton signalling	0.001
Complement system	0.003
Lipid antigen presentation by CD1	0.004
Leukocyte extravasation signalling	0.004
Tight junction signalling	0.004
Germ cell-sertoli cell junction signalling	0.044
IL-1 signalling	0.044
Acute phase response signalling	0.044
N-Glycan degradation	0.044
Intrinsic prothrombin activation pathway	0.044

Significance accepted when $p \leq 0.05$.

907 **Figure 1:**



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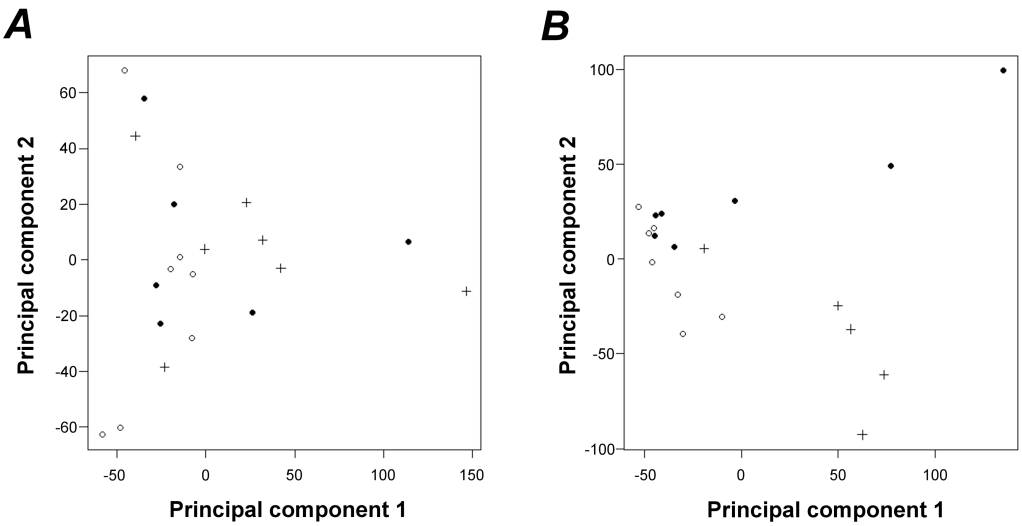
910 **Figure 2:**



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913 **Figure 3:**

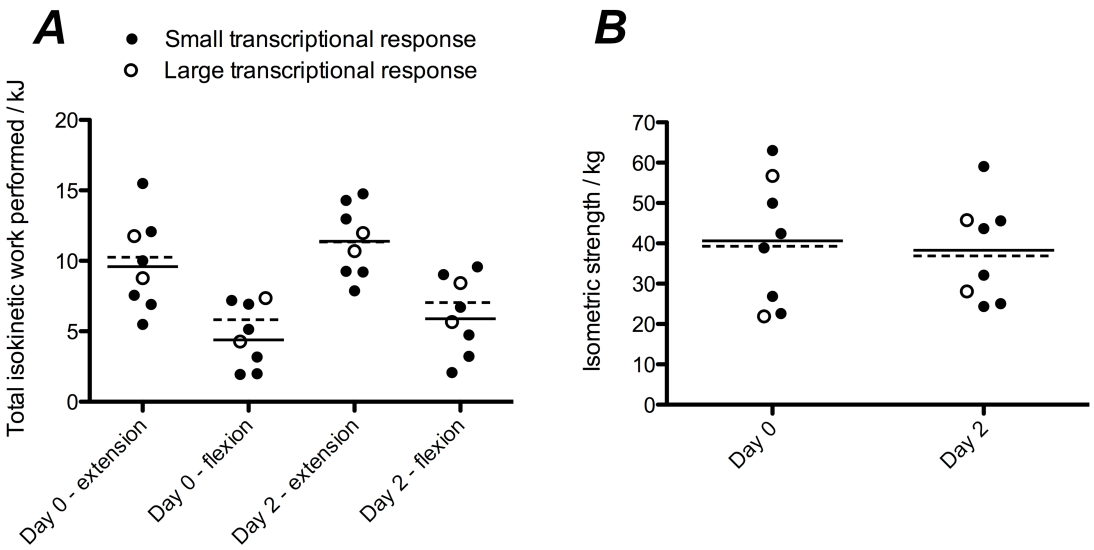

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Figure 4:

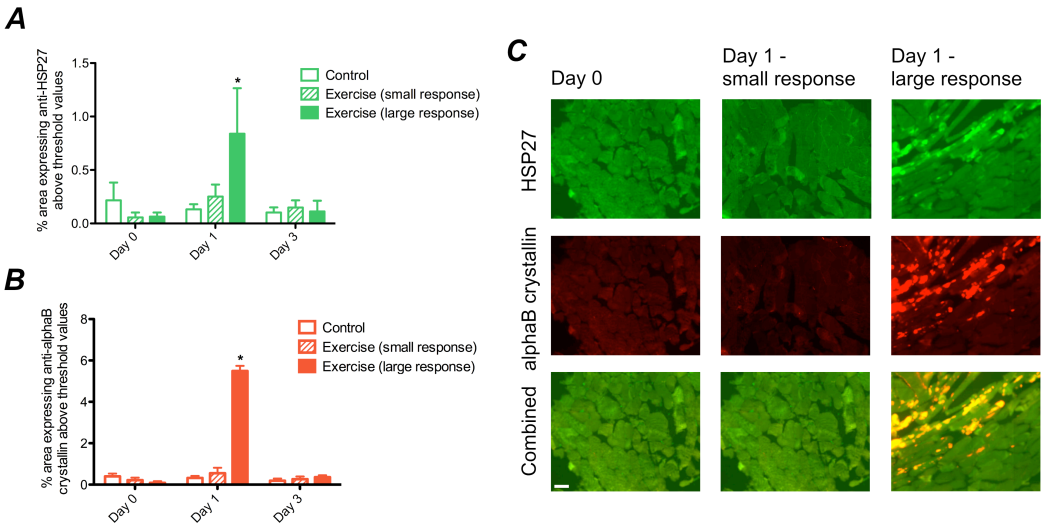
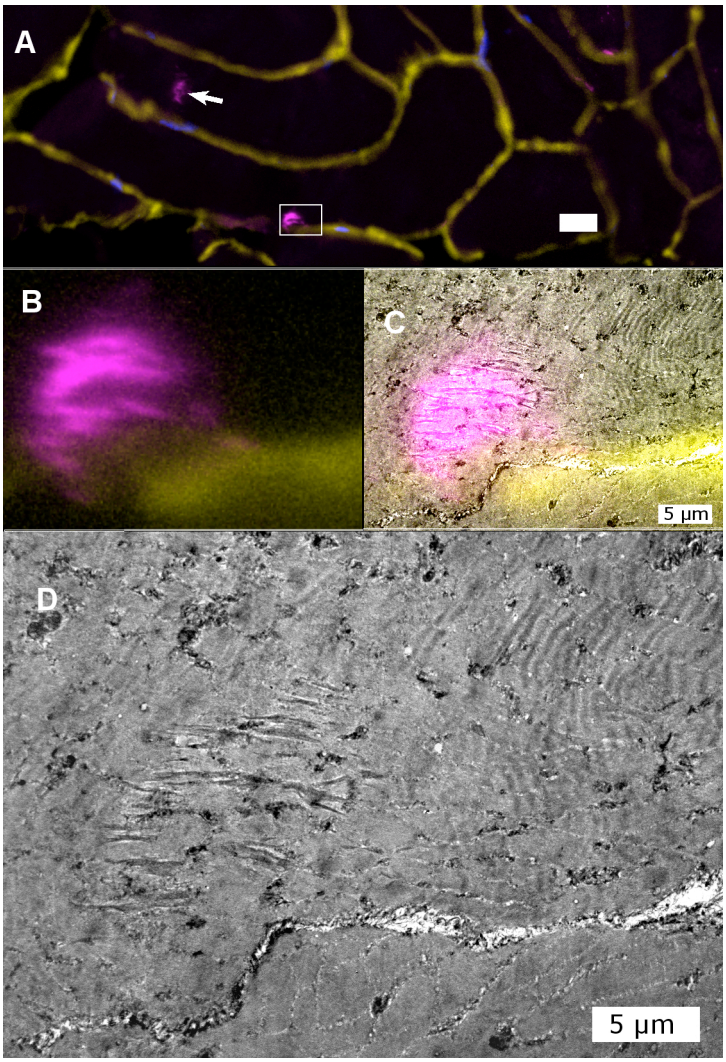
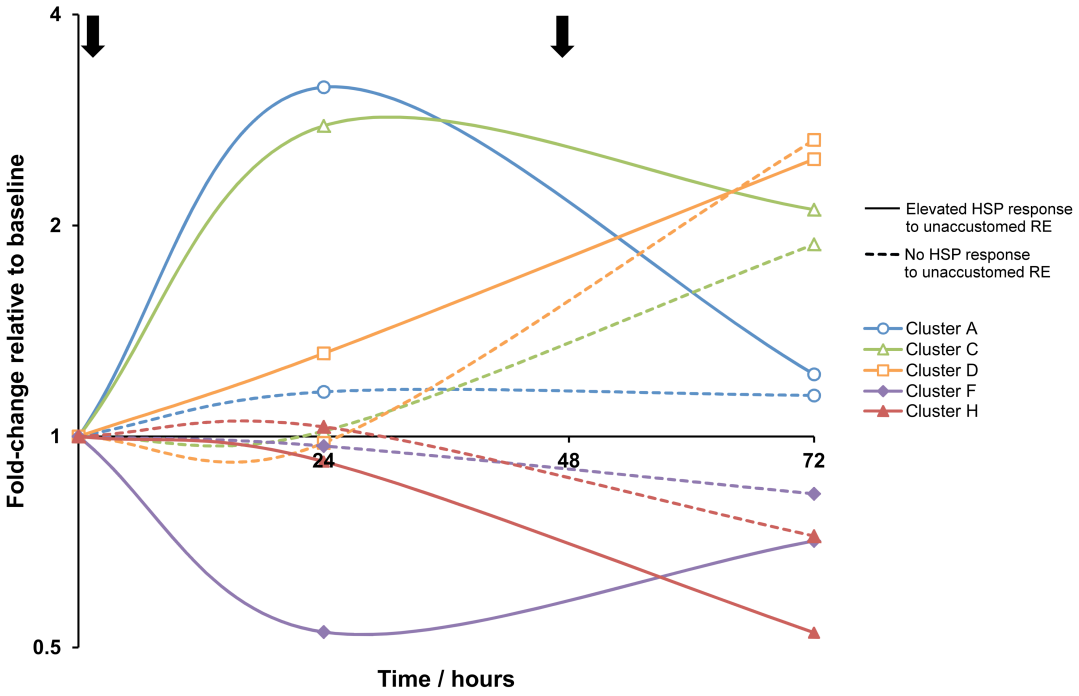

A
% area expressing anti-HSP27 above threshold values
1.5
1.0
0.5
0.0
Day 0
Day 1
Day 3
*
Control
Exercise (small response)
Exercise (large response)
B
% area expressing anti-alphaB crystallin above threshold values
8
6
4
2
0
Day 0
Day 1
Day 3
*
Control
Exercise (small response)
Exercise (large response)
C
Day 0
Day 1 - small response
Day 1 - large response
HSP27
alphaB crystallin
Combined

Figure 5:



921 **Figure 6:**



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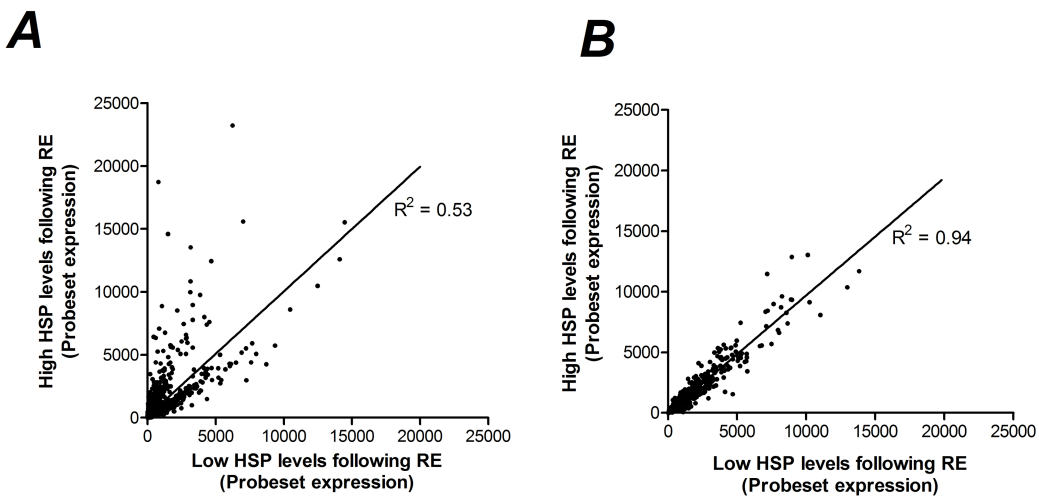
924 **Figure 7:**



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