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Endurance training- induced changes in insulin sensitivity and gene expression.

Short Title:

Insulin Sensitivity and exercise training

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ABSTRACT

The beneficial effects of regular physical activity on insulin sensitivity (S_I) and glucose tolerance are well-documented, with considerable heterogeneity in responsiveness to exercise-training (ET). To find novel candidate genes for ET-induced improvement in S_I we used microarray technology. Total RNA was isolated from *vastus lateralis* before and after 20 weeks of exercise from individuals participating in the HERITAGE Family Study. S_I index was derived from a frequently sampled intravenous glucose tolerance test using the MINMOD Millennium software. Sixteen subjects were selected: eight showing no changes in S_I (low responders; $LS_{I}R$) and eight displaying marked improvement in S_I (high responders; $HS_{I}R$) with ET. The S_I increase was ~4 times greater in $HS_{I}R$ compared to $LS_{I}R$ ($+3.6 \pm 0.5$ vs. -1.2 ± 0.5 $\mu U/ml/min$, mean \pm SE), whereas age, BMI, % body fat, and baseline S_I was similar between the groups. Triplicate microarrays were performed, comparing pooled RNA from $HS_{I}R$ and $LS_{I}R$ individuals for differences in gene expression before (pre) and after (post) -ET using *in situ* generated microarrays (18,861 genes). Array data was validated by QRT-PCR. Almost twice as many genes showed ≥ 2 -fold differences between $HS_{I}R$ and $LS_{I}R$ after training compared to pre-training. We identified differentially expressed genes involved in energy metabolism and signaling, novel structural genes, and transcripts of unknown function. Genes of interest upregulated in $HS_{I}R$ include V-Ski oncogene, Four and half LIM domain 1, and Titin. Further study of these novel candidate genes should provide a better understanding of the molecular mechanisms involved in the improvement in insulin sensitivity in response to regular exercise.

Key words: microarray, exercise-training, insulin sensitivity, MINMOD

INTRODUCTION

The beneficial effects of regular physical activity on insulin sensitivity and glucose metabolism are well documented (26, 54). Regular physical activity has been shown to increase insulin mediated glucose transport in peripheral tissues and this effect is mediated through multiple adaptations in glucose and lipid metabolism (3, 19). Thirty to 50% of the *in vivo* insulin-mediated glucose disposal occurs in skeletal muscle and decreased insulin-stimulated muscle glucose uptake is one of the mechanisms responsible for the development of insulin resistance (3, 61). In fact, impaired capability of muscle for glucose disposal in response to normal levels of circulating insulin is the earliest detectable abnormality in patients with type 2 diabetes (12, 29, 47).

Exercise improves insulin action (14, 18, 19) and weight loss associated with regular exercise prevents the onset of diabetes (26, 54). Increased expression of the insulin-responsive glucose transport proteins has been observed in response to exercise training, and correlated with improved insulin action in skeletal muscle (20, 23, 44). Seven days of exercise have been shown to increase insulin sensitivity (9, 22). Exercise training has been also associated with increased oxidative capacity of skeletal muscle (7, 39, 48, 59). Even though skeletal muscle in older individuals seems to retain the ability to increase glucose transport into muscle with endurance training (9), recent data indicate that an age-associated decline in mitochondrial function contributes to the insulin resistance observed in the elderly (38, 50). It is not known whether exercise training-associated improvements in glucose uptake are limited primarily by increased expression of glucose transporters or by the improved muscle oxidative potential. Improvements in insulin sensitivity with exercise training may be related to coordinated changes in activity of

proteins involved in insulin signal transduction, fuel partitioning and metabolism, and cytoarchitecture of skeletal muscle.

How gene expression is modulated by acute and chronic exercise is important for the understanding of the mechanisms by which exercise training improves insulin action in skeletal muscle. The effects of exercise training on the expression of several genes involved on insulin signaling (IRS1, IRS2, PI3K, MAPK, AMPK), glucose transport (GLUT1, GLUT4), glycogen metabolism (GS, GSK-3), glycolysis (HKII, PFK), mitochondrial genes (COX4, ND4), mitochondrial biogenesis (PGC-1, NRF-1, NRF-2, TFAM), and fatty acid oxidation (CPTI, UCP3, NADH6) (13, 19, 50, 57, 60) have been investigated. These studies suggest that exercise training entrains a complex program of transcriptional changes in target tissues that has been associated with improved insulin sensitivity and glucose metabolism.

The microarray technology offers a powerful tool to characterize changes in transcript levels on a large scale. The array technology has been helpful in defining a set of insulin-regulated genes in human skeletal muscle of healthy individuals during a 3-h hyperinsulinemic euglycemic clamp (45). Several studies have provided a catalog of genes differentially expressed in skeletal muscle under diverse clinical conditions such as in the comparison of healthy non-diabetic subjects and individuals with type 2 diabetes mellitus (T2DM) (36), obese insulin-sensitive and obese insulin-resistant subjects (58), and old individuals with T2DM having good or poor glycemic control (52). However, none of these studies have focused on gene expression in skeletal muscle of healthy, previously sedentary individuals in response to an endurance exercise-training program taking into account their changes in insulin sensitivity (S_I). There is considerable variability for the changes in insulin sensitivity in response to exercise training, and several factors may account for this phenomenon. We recently showed that among

596 participants from the HERITAGE Family Study only 58% increased their S_I by 10% or more with a standardized 20-week training program. These changes in S_I were not associated with changes in body weight, waist circumference or physical fitness (6). In the current study, we sought to determine what differences in skeletal muscle gene expression patterns were present before and after 20 weeks of endurance exercise training in high and low S_I responders using a subsample from the HERITAGE Family Study subjects.

RESEARCH DESIGN AND METHODS

Subjects: The HERITAGE Family Study was designed to investigate the role of genetic factors in the cardiovascular and metabolic adaptations to 20 weeks of endurance training in White and Blacks (5). All subjects were required to be sedentary and in good health to participate in the HERITAGE Family Study and meet the inclusion criteria (5). None of the HERITAGE subjects were diabetics or taking hypoglycemic or hypertensive medication. Participants from the Québec Clinical Center were asked to volunteer for a muscle biopsy before and after the training program. Biopsies were obtained from 76 subjects and 16 volunteers with a mean (\pm S.E.) age of 30 ± 5 years and a body mass index of 23.7 ± 1.2 kg/m² were selected for the present experiment. The characteristics of these subjects are depicted in Table 1. The selection of these 16 subjects was based on their S_I responses to exercise training: eight subjects (4 males and 4 females) showed no changes in S_I (low responders; LS_IR) and eight subjects (3 males and 5 females) displayed marked improvements in S_I (high responders; HS_IR) with the exercise program. These subjects were matched for sex and baseline S_I. There were no statistical differences in baseline S_I among the whole sample of white individuals from the HERITAGE Family study (4.3 ± 2.9 μ U/ml/min, mean \pm SD, n=429), the subset of those who volunteered for a muscle biopsy (5.1 ± 3.7 μ U/ml/min, n=76), and the subsample selected for the array studies (4.1 ± 1.3 μ U/ml/min, n=16). Each Institutional Review Board of the HERITAGE Family Study research consortium approved the study protocol. Written informed consent was obtained from each participant.

Exercise training program. The exercise-training program has been described in detail previously (5). Briefly, the exercise intensity of the 20-week program was customized for each participant based on the heart rate-oxygen uptake relationship measured at baseline. During the

first 2 weeks, the subjects trained at a heart rate corresponding to 55% of the baseline VO_2 max for 30 min per session. Exercise duration was gradually increased to 50 min per session and intensity to the heart rate associated with 75% of the baseline VO_2 max, and these conditions were then sustained for the last 6 weeks of the training program. Training frequency was three times per week, and all training sessions were performed on cycle ergometers under supervision in the laboratory.

Measurement of glucose, insulin and insulin sensitivity (S_I). Fasting plasma glucose and insulin levels were determined at baseline and after the 20-week exercise-training program. The frequently sampled intravenous glucose tolerance test (FSIVGTT) as described by Walton *et al* (55) was administered in the morning after an overnight fast of 12 hrs. Data were collected at baseline and post-training 24 to 36 hours after an exercise session as described elsewhere (1). From the FSIVGTT data, S_I was derived using the recently released MINMOD Millennium software (4). S_I is a measure of insulin action and corresponds to the ability of a given increment in plasma insulin to accelerate glucose uptake and suppress glucose production (11).

Experimental Study Design: Muscle biopsies were taken from the middle of the vastus lateralis by the percutaneous needle biopsy technique (51). Each biopsy was partitioned into two pieces: one was immediately frozen in liquid nitrogen, and stored at -80°C until total RNA preparation and the other was frozen in isopentane cooled by liquid nitrogen and used for histochemistry and enzyme activity assays.

Total RNA Preparation. Frozen tissue samples were crushed in liquid nitrogen, and total RNA was extracted using the TRI Reagent (Molecular Research Center, Inc. Cincinnati, OH) followed by purification with Qiagen columns from the RNeasy kit (QIAGEN Inc, Valencia, CA). The concentration and quality of the RNA were determined spectrophotometrically. Once

the integrity of the RNA was confirmed in a non-denaturing agarose gel, RNA pools were prepared. Given the limited amount of material available, it was necessary to use RNA pools for the present study. In addition, using RNA pools provided an advantage by decreasing the variability that tissue heterogeneity might introduce in the gene expression profiles, reducing the “polymorphic noise” present in humans, and minimizing the effect of sex differences.

Furthermore, we were able to accumulate enough RNA in the pools, while still maintaining individual samples for validation of differentially expressed genes using QRT-PCR. Therefore, equal amounts of the individual samples were used to generate 4 RNA pools: baseline HS₁R, baseline LS₁R, post-training HS₁R, and post-training LS₁R. The samples from HS₁R and LS₁R were analyzed comparing pre-training and post-training RNA pools.

Array generation. High-density oligonucleotide microarrays were generated by the Pennington Biomedical Research Center Genomics Core Microarray Facility. Briefly, a human oligonucleotide library representing 18,861 genes was purchased from Compugen (Jamesburg, NJ). The 60-70mer oligonucleotides were diluted in 50% DMSO and spotted onto poly-L-lysine coated slides using an OmniGrid Microarrayer equipped with a Stealth SPH32 Micro Spotting Pin Matrix and SMP4 Micro Spotting Pins (Telechem International, Inc., Sunnyvale, CA).

RNA amplification and array detection. Microarray analysis was performed in triplicate comparing pooled RNA from the HS₁R and LS₁R groups to investigate the differences in gene transcript profiles prior to exercise training (pre-training arrays), deposited in [GEO](#) data base as Series: GSE1718, with samples id: GSM29507, GSM29523 and GSM29529. We also compared the HS₁R and LS₁R groups after exercise training (post-training arrays) in a second set of triplicate arrays, deposited in [GEO](#) data base as Series: GSE1718 with samples ids: GSM29533, GSM29535 and GSM29536. Dye switching was prepared in one slide of each set to account for

incorporation differences of Cy3 and Cy5. RNA was labeled and detected using a tyramide signal amplification (TSA) labeling and detection kit (Perkin-Elmer, Boston, MA). The TSA method (TSATM, NEN Life Science Products Inc., Boston, MA) is highly sensitive and allows for the use of small amounts (2 μ g) of total RNA. The TSA method has been shown to provide consistent and reproducible signal amplification across arrays (25). Probe labeling and array hybridization were performed as described in the instruction manual (MICROMAX TSA Labeling and Detection kit, Perkin-Elmer, Boston, MA). After hybridization to probes, slides were scanned using a GSI Lumonics ScanArray 5000 (Packard Biochip Technologies; Meriden, CT) and expression data analyzed with QuantArray V3.0 software (Packard Bioscience; Meriden, CT).

Array normalization. A uniform scale factor was applied to normalize signal intensities between Cy5 and Cy3. Locally weighted regression and scatter plot smoothing (LOWESS) was applied to account for dye variation between slides, and for signal intensity effects. For a given experiment, we assumed that most genes on our slides were present in equal amounts, such that their intensity ratios were equal to 1 (or 0 if data is in log form). However, since there are dye variations across a single slide, as well as between slides, a correction must be applied so that we get an average ratio of 1. Locally weighted regression and scatterplot smoothing (LOWESS) is one method to do this. LOWESS normalization is based on the concept that any function can be well approximated in a small neighborhood by a low-order polynomial (in our case, we use a linear fit). The LOWESS method fits a curve to a log-plot of intensity values and subtracts the fit values from the actual values to get the normalized values. An in-house software program was used to perform the analysis (Details of the algorithm can be found at <http://bioinfo.pbrc.edu>). Using the LOWESS method, several genes with significant differences

in expression across the microarray experiments were identified. The gene expression data was analyzed comprehensively using the software applications of Spotfire (DecisionSite, Somerville, MA) and GeneSpring (Silicon Genetics, Redwood City, CA). The nomenclature adopted for referencing gene names, symbols, and other description is that of the Nomenclature Working Group (http://archive.uwcm.ac.uk/uwcm/mg/docs//mut_nom.html).

Quantitative reverse transcriptase PCR (QRT-PCR). We selected five genes from the microarray experiments to be validated by QRT-PCR. The genes were selected because they exhibited a minimum of 50% difference ($p < 0.05$) between HS₁R and LS₁R either at baseline or in the post-training experiments. These genes were: titin (TTN), pyruvate dehydrogenase kinase 4 (PDK4), V-SKI avian sarcoma viral oncogen homolog (SKI), C-terminal binding protein 1 (CTBP1), and four-and-a-half LIM domains 1 (FHL1). The QRT-PCR studies were performed using TaqMan probe and primer sets to measure the levels of mRNA (Applied Biosystems, Foster City, CA). The 4 RNA pools from the microarray experiments were also used for the QRT-PCR validation. Total RNA was reverse transcribed using the Reverse Transcription Reagents kit (Applied Biosystems) according to the manufacturer's instructions, for the two-step RT-PCR methodology. Briefly, cDNA was generated from 150 ng total RNA of each pool as the template, and a reference sample cDNA was obtained from a commercially available skeletal muscle total RNA source to generate the standard curves (Ambion, Austin, TX). PCR was performed using an ABI PRISM 7900 SDS instrument (Applied Biosystems). The primer-probe sets were either selected from the Gene Expression system database (Assays-on demand, Applied Biosystems) or designed using the Primer Express software (Applied Biosystems), and are available upon request. Reactions were carried out in a 384 well plate format with 12 μ l reaction volume. Two concentrations of each cDNA pool were used in triplicate reactions. We

also used a 1:50 dilution of the cDNA pools for normalization of the gene signals with 18S rRNA using the TaqMan Pre-Developed Assay Ribosomal RNA Control Reagents kit (Applied Biosystems). A standard curve was generated with serial dilutions of the cDNA reference sample and applied to each primer and probe set. The cycle threshold (Ct) value for each sample was then used to calculate relative expression. The expression level of each gene was normalized to 18S rRNA. Data are reported as arbitrary units (AU). Differential gene expression between the LS₁R and the HS₁R groups as measured by QRT-PCR was analyzed using one-tailed Student t-test, since we hypothesized that the differences between the groups would follow the same pattern as that measured by the oligonucleotide array analysis.

RESULTS

We report here only the results obtained following normalization by locally weighted regression and scatter plot smoothing (LOWESS), and only the expression ratios between HS₁R and LS₁R replicated among the 3 array experiments ($P < 0.05$). Before exercise training, there were 703 transcripts that met these criteria, and 1166 after exercise training. In addition, we used a cut-off of ± 0.5 value from a \log_2 scale to describe changes in expression. This corresponds to an increase of 1.4-fold or a decrease of 0.7-fold in the HS₁R/LS₁R expression ratio.

Pre-training arrays. At baseline, relatively few transcripts were > 1.4 -fold more abundant in the HS₁R compared to the LS₁R ($n=42$). Table 2 lists the HS₁R over expressed transcripts encoding proteins involved in glycolysis, nitrogen metabolism, signal transduction, transcription regulation, muscle contraction and development, and several other transcripts with unknown or unclassified function. In addition, 5 transcripts that were less abundant in HS₁R compared to the LS₁R (ratio < 0.7) are involved in cell growth/maintenance [1] and tRNA processing [1] or have an unknown function [3] (Table 3).

Post-training arrays. We identified 240 transcripts in the post-training samples that were at least 1.4-fold more abundant in the HS₁R compared to the LS₁R. Among these transcripts, 21 were upregulated by 2.0-fold or more in the HS₁R. Upregulated genes are involved in energy metabolism, muscle development and contraction, vesicle transport, signal transduction, cell growth regulation and differentiation, transcription regulation and protein synthesis, extracellular matrix structure and function, cellular defense response, or have unknown functions (Table 4). We also found 121 down-regulated transcripts (ratio < 0.7) (Table 5). The down-regulated genes participate in lipid metabolism, oxidative phosphorylation, ribosome biogenesis and transcription

regulation, proteolysis and peptidolysis, protein synthesis, GTPase mediated signal transduction, or have unknown functions.

Integration of pre and post-training arrays. A total of 38 genes were differentially expressed both at baseline and post-training experiments in the two sets of array experiments (Table 6). We developed a list of those genes based on ratios of post/pre-training experiments. Among them, 22 had a known function. Up-regulated genes [n=8], with ratios > 1.4 in post/pre-training experiments, are involved in cell adhesion/growth [4], signal transduction [2], and electron [1] and adenine transport [1]. Some of the down-regulated genes [n=5], with ratios < 0.7, participate in oxidative phosphorylation [2], transcription regulation [1] and protein biosynthesis [1].

QRT-PCR validation. The genes selected for validation exhibited a minimum of 50% difference ($p < 0.05$) in expression between HS₁R and LS₁R either at baseline or post-training. SKI and FHL1 are genes involved in cell growth and/or maintenance, TTN is a structural constituent of muscle and plays a role in sarcomere elasticity and the regulation of muscle contraction, PDK4 has protein kinase activity and participates in glucose metabolism, and CTBP1 is a functional dehydrogenase. The differences in expression levels among the 4 groups are depicted in Fig. 1 for PDK4, FHL1, SKI and TTN.

PDK4 levels were 1.8-fold greater in the HS₁R compared to the LS₁R group before exercise training by QRT-PCR; this is concordant with the difference observed between the two groups in the pre-training arrays (Fig. 1). There were no differences on PDK4 levels between the HS₁R and LS₁R with either the array data or QRT-PCR in the post-training arrays.

The differences between the HS₁R and LS₁R groups in the post-training arrays for the TTN gene were also validated by QRT-PCR. We found a 2.6-fold difference in TTN transcript

levels between the HS₁R and LS₁R groups after exercise training (Fig. 1). This difference is smaller than the average 4.7-fold difference observed in the post-training array experiments, but within the 2.6 - 6.9-fold range observed across the triplicate experiments.

The levels of FHL1, SKI and CTBP1 in the sedentary state were not different between the HS₁R and LS₁R groups by QRT-PCR, as we had found in the pre-training arrays (Fig 1). However, after exercise training the QRT-PCR results revealed significantly higher FHL1 and SKI levels in the HS₁R compared to the LS₁R group, as observed in the post-training arrays. The 1.7-fold difference on CTBP1 observed in the post-training arrays in the HS₁R vs. LS₁R group could not be replicated by the QRT-PCR. Instead, we observed only a 20% difference in CTBP1 levels favoring the HS₁R group after exercise training.

Therefore, differences in gene expression between HS₁R and LS₁R groups, either in the pre- or post-training states, were confirmed by QRT-PCR in four of the five genes identified by microarray analysis as differentially expressed.

DISCUSSION

The objective of this study was to identify genes that are differentially expressed in the skeletal muscle of individuals who markedly improved or failed to improve insulin sensitivity after 20 weeks of endurance training. The uniqueness of the present microarray study resides in the selection of previously sedentary individuals with similar baseline S_I but with a 4-fold difference in S_I response after 20 weeks of an endurance exercise program. The differences in gene expression observed between the $HS_I R$ and $LS_I R$ groups cannot be attributed to the influences of sex, age, BMI or body fat since high and low responders were carefully matched for them (Table 1). It has been recommended for the analysis of skeletal muscle gene expression in microarray experiments to use pools of individual samples in order to decrease the influence of inter-individual variability (2, 46). This strategy has been used successfully in other studies aimed at defining patterns of gene expression (2, 24, 46, 56). We pooled the individual samples of eight individuals in each of the high and low responder groups, before and after the exercise program.

The genes differentially expressed between HS_I and LS_I responders in the post-training arrays likely include a distinctive set of genes involved in the divergent S_I response to the training program. Moreover, since there were no differences in mean S_I at baseline, the genes differentially expressed in the pre-training arrays between $HS_I R$ and $LS_I R$ groups are likely to represent another set of genes that contribute to S_I response to the exercise training program.

Several microarray studies have provided a catalog of differentially expressed skeletal muscle genes under diverse clinical conditions associated with insulin resistance such as TD2M, obesity and aging (36, 50, 52, 58). A direct comparison of these reports with our study is,

however, difficult since the selection of the individuals in the HS_IR and LS_IR groups was based on S_I responses to an exercise-training program. Our study contrasted the differences in gene expression between low and high S_I responders and not the effects of exercise training as such on gene expression patterns. Nonetheless we searched intentionally our data for results on genes that, according to the literature, are modified by regular exercise or insulin resistant states. Interestingly, there were no remarkable effects before or after ET on HS_IR/LS_IR expression ratios for genes involved in pathways of insulin signaling (IRS1, IRS2, PI3K, MAPK, AMPK), glucose transport (GLUT1, GLUT4), glycogen metabolism (GS, GSK-3), glycolysis (HKII, PFK), mitochondrial function (COX4, ND4), mitochondrial biogenesis (PGC-1, NRF-1, NRF-2, TFAM), and fatty acid oxidation (CPTI, UCP3, NADH6). These findings are undoubtedly due to the uniqueness of the experimental design upon which the current study is based.

Differences in S_I responses are expected to involve the transcriptional reprogramming of sets of skeletal muscle genes. Differences in gene expression, particularly in clinical studies, are represented by modest changes in groups of related genes such as those associated with key metabolic pathways (13, 21, 49). Such small differences are often difficult to identify in microarray studies, but can be readily evaluated using QRT-PCR methodologies.

Genes Up-Regulated Before Exercise Training.

Given the matching criteria between the HS_IR and LS_IR groups, we did not expect to find dramatic differences in gene expression before the exercise training intervention. Nevertheless we verified whether there were differences in gene expression that could predispose the HS_IR to increase S_I by regular exercise more than the LS_IR group. None of the differences were greater than 2.0-fold, and the differentially expressed transcripts encode proteins involved in glycolysis,

nitrogen metabolism, signal transduction, transcription regulation, muscle contraction and development, plus several new sequences with unknown or unclassified functions.

Among the novel candidate genes upregulated in the HS_IR before exercise training we identified: sorting nexin 10 (SNX10), inositol (myo)-1(or 4)-monophosphatase 2 (IMPA2), diacylglycerol kinase delta (DGKD) and PDK4. SNX10 contains a phosphoinositide binding domain and belongs to a family of proteins involved in intracellular trafficking. IMPA2 share similar enzyme activity to enzymes of the inositol phosphate second messenger signaling pathway. DGKD is a cytoplasmic enzyme that phosphorylates diacylglycerol to produce phosphatidic acid that in turn acts as second messenger in signaling cascades; therefore, DGKD is thought to play an important role in cellular signal transduction. Whether these genes play a role in the regular exercise – induced insulin sensitivity response will require additional studies.

We further validated the increased PDK4 expression in the HS_IR group before exercise training. Skeletal muscle PDK4 expression increases in situations where carbohydrate availability and insulin levels are decreased and free fatty acids are increased, such as in fasting, high fat/low carbohydrate diets and acute exercise (37, 40, 41). In skeletal muscle, PDK4 is induced during metabolic states in which there is a perceived deficit in whole body glucose availability (i.e. insulin resistance). A shift in substrate utilization in exercising muscle is driven by the increased delivery and oxidation of fatty acids, eliciting a progressive rise in acetyl-CoA that allosterically inhibits pyruvate dehydrogenase (PDH) activity (43). The induction of PDK4 suggests that PDK4-mediated inhibition of PDH in muscle represents a mechanism for conserving carbohydrate substrates by gradually limiting the entry of glycolytic products into the mitochondria for oxidation and thereby conserving tricarboxylic acids for gluconeogenesis. This function may be particularly important in muscle fibers that rely heavily on glycolytic

metabolism, such as *vastus lateralis*. We speculate that a persistent elevation in PDK4 expression may ensure that glucose entering the cell is preferentially used for muscle glycogen resynthesis, reflecting the high metabolic priority given to the replenishment of energy reserves in the HS_IR even in the sedentary state. This could represent one of the biological markers of the HS_IR predisposition to benefit more from a training program in terms of S_I increment than the LS_IR. Interestingly, there was no difference in PDK4 expression levels between the HS_IR and LS_IR groups after exercise training. Further studies will be required to more thoroughly examine if the expression of PDK4 or other PDK isoforms, like PDK2, PDH phosphorylation and/or activity contribute to the changes in S_I observed in certain individuals with exercise training.

Genes Up-Regulated After Exercise Training

More transcripts were upregulated in the HS_IR group after exercise training than in the sedentary state. These upregulated transcripts relate to genes involved in energetic and signal functions, muscle development and contraction, cell growth regulation and differentiation, as well as sequences with unknown functions (Table 4). Some of the upregulated genes were validated by QRT-PCR methodologies including SKI, TTN and FHL1.

SKI is a nuclear proto-oncoprotein that can induce both oncogenic transformation and terminal muscle differentiation (OMIM *164780). SKI could function either as a positive or negative regulator of transcription, depending on its tissue expression and/or the promoter context of the genes which use SKI as a transcription factor. In transgenic mice, Ski overexpression induces muscle differentiation in embryo fibroblasts and causes postnatal hypertrophy of type II muscle fibers. The *ski*-null mice show a marked decrease in skeletal muscle mass in addition to other abnormalities (for review see (31)). The human SKI gene has

been associated with congenital fiber-type disproportion type myopathy (CFTD), hypotonia and craniofacial dysmorphism (1p36 deletion syndrome) (35). Severe hyperinsulinemia and insulin resistance has described in patients with CFTD (OMIM 255310). It remains to be clarified whether the increased expression of the SKI gene we observed in the high S_I response group is causally related to the improved S_I in muscle.

FHL1, also known as Skeletal Muscle LIM 1 (SLIM1) protein, is mainly expressed in skeletal muscle; FHL proteins are new members of the LIM-only protein family. FHL1 plays an important role in muscle development (28, 34) and its expression is more abundant in oxidative fibers (30). FHL1 is also thought to function as a scaffold for protein assembly in the actin-based cytoskeleton; it is located in an integrin-dependent manner to the nucleus, focal adhesions, and stress fibers. It has been suggested that one of the FHL1 LIM domains interacts with α -actinin, a component of the Z discs, whereas interactions of the other LIM domains with actin filaments and spectrin have been demonstrated for FHL3 (8). FHL1 was one of the genes identified by Roth (46) as being upregulated in response to a 4 month strength training program. It was recently reported that FHL1 might play a role during the early stages of skeletal muscle differentiation, specifically in the $\alpha 5\beta 1$ -integrin-mediated signaling pathway (32). In addition, FHL proteins have been implicated in one of the downstream mechanisms by which the extracellular signal-regulated kinase 2 (ERK2) signaling pathway affects the regulation of differentiated growth (42). Interestingly, FHL2 (a cardiac muscle FHL protein) binds TTN and one of its domains may couple the metabolic enzymes creatine kinase, adenylate kinase and phosphofructokinase to the sarcomeric structures (27). The binding partners for the homologous FHL1 and FHL3 proteins have not been defined.

TTN is a giant protein, the largest known polypeptide (3 MDa), that contributes to the maintenance of sarcomere organization and myofibrillar elasticity (for review see (53)). TTN may also participate in myofibrillar cell signaling. Tissue-specific expression of various TTN isoforms results in differential myocyte elasticity. Mutations located in the Z-disc binding region of TTN have been identified in tibial myopathies (17). Disruption of TTN has been reported to cause impaired sarcomerogenesis and results in thin, poorly contractile muscle cells. More than half of the TTN molecule is attached to the thick filament, where it appears to control the exact assembly of myosin and other filament components. From the end of the thick filament and the Z-line, TTN forms elastic connections. The Z line domain of TTN interacts with telethonin/T-cap, which in turn interacts with the muscle growth factor myostatin and the muscle LIM protein (16). Near the middle of the thick filament, TTN has a kinase domain, but its functions and substrate(s) are not fully understood. Both ends of the molecule have potential phosphorylation sites that may be involved in protein signaling. For example, the COOH terminus of a TTN molecule is integrated in the myosin lattice and contains a Ser/Thr kinase domain whose absence leads to sarcomeric disassembly (15). The main functions of the TTN family seem to be to interconnect myosin and actin filaments axially and provide passive elasticity. TTN also makes it possible for equal forces to be distributed by myosin in both halves of the sarcomere (53).

Since FHL1 and TTN were upregulated after exercise training in the HS₁R group, it is tempting to speculate on the potential role of these genes in the improved S₁. The mechanisms by which exercise training improves S₁ in healthy skeletal muscle involve enhanced insulin action and signaling, glucose transport, and overall metabolic capability (10). The adaptive response to exercise training requires the sensing of biomechanical signals involving the interface between the contractile cytoskeleton (myofibrils) and the sarcolemma at specialized

cell-cell junctions (intercalated discs) and cell-substrate adhesion complexes (costameres). New evidence suggests that complexes associated with the TTN protein sense myocyte stretch, and TTN has been proposed recently as an ideal biomechanical sensor (33). If one of the complexes associated with TTN is FHL1, which in turn could interact with metabolic enzymes, it could explain how increased TTN and FHL1 contribute to an improved S_I response with exercise training. Thus far, there is little direct evidence to support this hypothesis and experimental data will be required to validate this concept.

In conclusion, we have identified a unique profile of genes that are differentially expressed between individuals with high S_I response to exercise training versus others carefully matched who showed absolutely no improvement. Among the genes identified with this approach we found that, before exercise training, PDK4 is over expressed by 80% in the high S_I responders to regular exercise. In those who expressed the highest S_I improvement, SKI, FHL1 and TTN were over expressed by 50% to 470% compared to those whose S_I remained stable. The proteins encoded by these novel genes could play a role through either cell-matrix interactions or enhanced signaling pathways involved in the S_I response. Moreover, our studies have identified other transcripts of unknown functions that could participate in the regulation of S_I response to regular exercise. The data presented here offer new candidate genes to account for human variation in the ability to improve insulin sensitivity in response to regular exercise.

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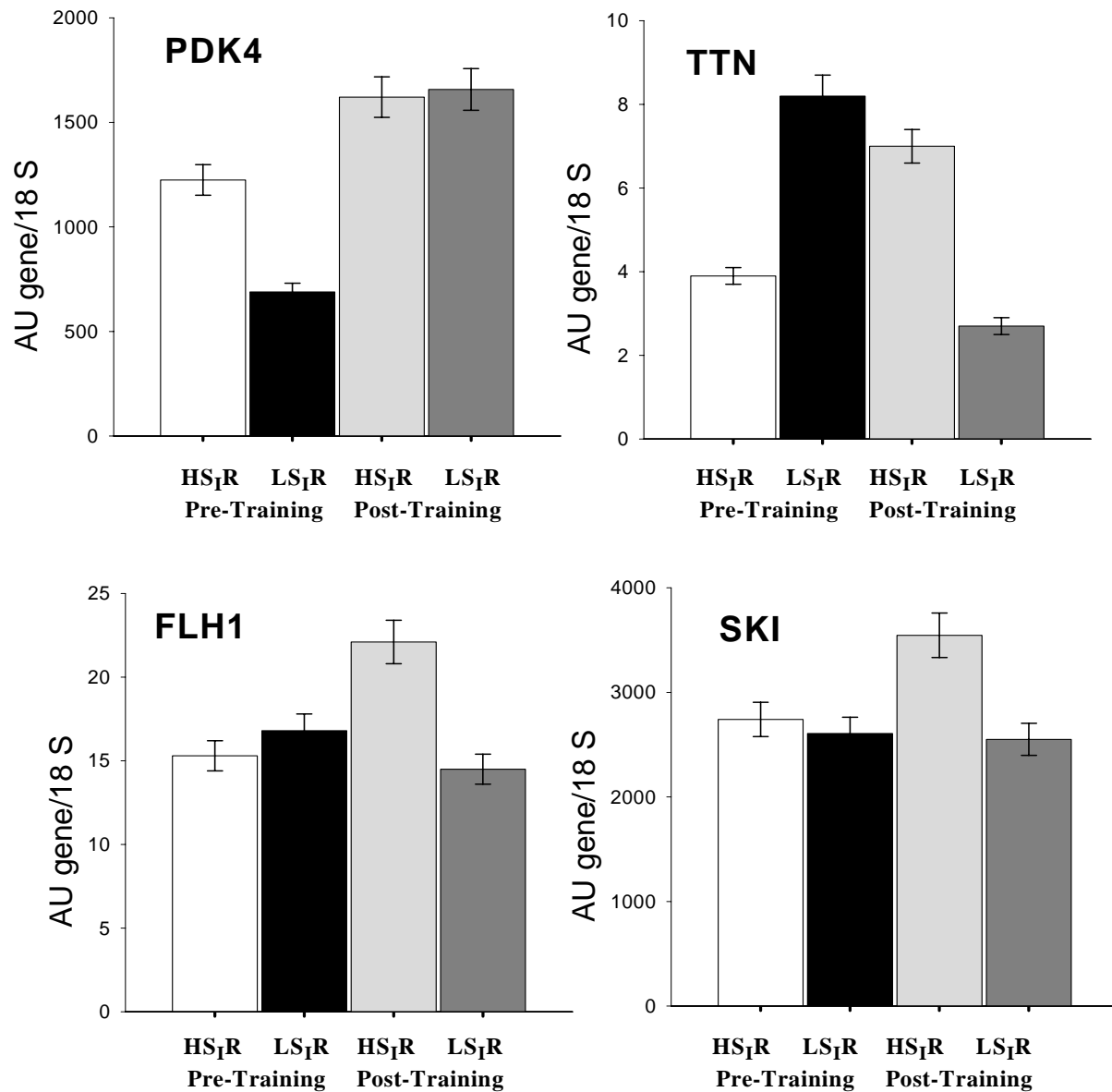


Fig 1. QRT-PCR validation of genes for the insulin sensitivity response to exercise training identified in the microarray experiments. Data represent samples analyzed in triplicates using two-step QRT-PCR methodology. PDK4 (pyruvate dehydrogenase kinase 4), TTN (titin), SKI (V-Ski avian sarcoma viral oncogene homolog) and FHL1 (four-and-a-half LIM domains 1). Levels normalized over the corresponding 18S rRNA value.

Table 1. Subject characteristics

	LS_IR Mean ± SE (n = 8)	HS_IR Mean ± SE (n = 8)
AGE (yr)	30.3 ± 5.4	31.2 ± 4.9
BMI (basal, kg/m ²)	25.0 ± 1.9	23.4 ± 1.0
% body fat	24.4 ± 3.6	22.9 ± 4.5
Glucose (baseline) (mmol/L)	5.0 ± 0.2	5.1 ± 0.2
Insulin (baseline) (pmol/L)	72.6 ± 12.3	60.1 ± 6.0
S _I (μU/ml/min) at:		
Baseline	4.1 ± 0.6	4.0 ± 0.4
After training	3.1 ± 0.4	8.6 ± 1.1 ^{a)}
Response	-1.2 ± 0.5	3.6 ± 0.5 ^{b)}

a) P < 0.05 for difference between after versus baseline.

b) P < 0.05 for differences in response between LS_IR and HS_IR.

Table 2. Genes up-regulated pre-training. The fold difference represents the average ratio of HS₁R /LS₁R in 3 microarray experiments*

GENE	Unigene	GenBank identifier	Gene ontology	Fold difference	P-Value
H. sapiens fc fragment of iga, receptor for (FCAR)	Hs.193122	NM_002000	immune response	1.8	0.050
H. sapiens kelch-like 1 protein (KLHL1)	Hs.106808	AF252283	developmental processes	1.7	0.042
H. sapiens cDNA: FLJ21940 FIS, clone hep04512	Hs.104916	AK025593	developmental processes	1.6	0.025
H. sapiens sh3-domain grb2-like 3 (SH3GL3)	Hs.80315	NM_003027	central nervous system development	1.6	0.044
H. sapiens clone pp591 unknown	Hs.118666	AF218022	amino acid & derivative metabolism	1.5	0.034
H. sapiens kraken-like (BK126B4.1)	Hs.301947	NM_014509	nitrogen metabolism	1.5	0.036
H. sapiens unnamed herv-h protein (LOC51581)	Hs.274490	NM_015875	cell motility	1.5	0.052
H. sapiens aquaporin 2 (collecting duct) (AQP2)	Hs.37025	NM_000486	water transport	1.5	0.047
H. sapiens aldo-keto reductase family 7, member a2 (aflatoxin aldehyde reductase) (AKR7A2)	Hs.6980	NM_003689	aldehyde metabolism	1.4	0.048
H. sapiens inositol(myo)-1(or 4)-monophosphatase 2 (IMPA2),	Hs.5753	NM_014214	signal transduction	1.4	0.048
H. sapiens sorting nexin 10 (SNX10)	Hs.106260	NM_013322	endocytosis	1.4	0.006
H. sapiens lengsin (LGS)	Hs.149585	NM_016571	cell adhesion	1.4	0.018
H. sapiens cDNA: FLJ22662 fis, clone hsi08080	Hs.178470	AK026315	retrograde (Golgi to ER) transport	1.4	0.020
H. sapiens complexin 2 (CPLX2)	Hs.321567	NM_006650	neurotransmitter release	1.4	0.037
H. sapiens cDNA FLJ13448 fis, clone place1002993	Hs.288945	AK023510	nitrogen metabolism	1.4	0.050
H. sapiens cDNA: FLJ23360 fis, clone hep15172	Hs.161279	AK027013	cell death	1.4	0.053
H. sapiens ris (LOC51285),	Hs.27018	NM_016563	synaptic transmission	1.4	0.006
H. sapiens HSPC265 , partial cds	Hs.284207	AF161383	muscle contraction	1.4	0.009
H. sapiens tumor necrosis factor receptor superfamily, member 9 (TNFRSF9)	Hs.73895	NM_001561	signal transduction	1.4	0.001
Human clone h3		U03672	regulation of transcription	1.4	0.047
H. sapiens diacylglycerol kinase, delta (130kd) (DGKD)	Hs.115907	NM_003648	signal transduction	1.4	0.023
H. sapiens cDNA FLJ14356 fis, clone y79aa1002103, similar to zinc finger protein zfp-36	Hs.253193	AK024418	transcription regulation	1.4	0.008
H. sapiens cgi-127 protein (LOC51646)	Hs.184542	NM_016061	leading strand elongation	1.4	0.050
H. sapiens cDNA FLJ12576 fis, clone nt2rm4001032	Hs.193261	AK022638	muscle development	1.4	0.007

* This is a partial list of genes with known function. The complete list of genes can be accessed in the GEO database series GSE1718.

Table 3. Genes down-regulated pre-training. The fold difference represents the average ratio of HS₁R /LS₁R in 3 microarray experiments *

GENE	Unigene	GenBank identifier	Gene ontology	Fold difference	P-Value
H. sapiens fbj murine osteosarcoma viral oncogene homolog b (FOSB), mRNA	Hs.75678	NM_006732	cell cycle control	0.7	0.04
H. sapiens pre-b lymphocyte gene 3 (VPREB3), mRNA	Hs.136713	NM_013378	humoral defense mechanism	0.7	0.01
H. sapiens retinoic acid- and interferon-inducible protein (58kd) (RI58), mRNA	Hs.27610	NM_012420	immune response	0.6	0.04
H. sapiens creatine kinase, brain (CKB) mRNA	Hs.173724	NM_001823	transferase activity, phosphorous-containing groups	0.6	0.03

* This is a partial list of genes with known function. The complete list of genes can be accessed in the GEO database series GSE1718.

Table 4. Genes up-regulated post-training. The fold difference represents the average ratio of HS₁R /LS₁R in 3 microarray experiments*

GENE	Unigene	GenBank identifier	Gene ontology	Fold difference	P-Value
H. sapiens for cardiac titin, clone ziss (TTN)		X98114	structural constituent of muscle	4.7	0.04
H. sapiens; cDNA dkfzp761m222 (from clone dkfzp761m222)	Hs.273186	AL137511	electron transport	3.9	0.03
H. sapiens corin (PRSC)	Hs.62794	NM_006587	proteolysis and peptidolysis	2.3	0.01
H. sapiens ras-gtpase activating protein sh3 domain-binding protein 2 (KIAA0660)	Hs.6727	NM_012297	RAS protein signal transduction	2.2	0.02
H. sapiens for KIAA1157 protein, partial cds	Hs.21894	AB032983	protein dephosphorylation	2.2	0.01
H. sapiens cyclin-dependent kinase inhibitor 1b (p27, kip1) (CDKN1B)	Hs.238990	NM_004064	cell cycle arrest	2.2	0.005
H. sapiens forssman glycolipid synthetase precursor rna, complete cds		AF163572	glycolipid biosynthesis	2.2	0.03
H. sapiens vesicle transport-related protein (KIAA0917)	Hs.27023	NM_016106	intracellular protein traffic	2.2	0.02
H. sapiens cug triplet repeat,rna-binding protein 2 (CUGBP2)	Hs.211610	NM_006561	RNA processing	2.1	0.02
H. sapiens neuronal protein (NP25)	Hs.169330	NM_013259	central nervous system development	2.0	0.02
H. sapiens biglycan (BGN)	Hs.821	NM_001711	histogenesis and organogenesis	2.0	0.03
H. sapiens nuclear factor of kappa light polypeptide gene (NFKBIL1)	Hs.2764	NM_005007	transcription factor	1.9	0.01
H. sapiens for unknown liver orphan	Hs.118463	X56789	leading strand elongation	1.9	0.01
H. sapiens delta-5 desaturase; delta-5 fatty acid desaturase (D5D)	Hs.132898	NM_013402	fatty acid desaturation	1.9	0.04
H. sapiens nitrilase 1 (NIT1)	Hs.146406	NM_005600	nitrilase	1.9	0.04
H. sapiens disrupted in schizophrenia 1 protein (DISC1) complete cds	Hs.26985	AF222980	cell growth and/or maintenance	1.9	0.02
H. sapiens progesterone binding protein (HPR6.6)	Hs.90061	NM_006667	cation transport	1.8	0.04
H. sapiens quinolinate phosphoribosyltransferase (QPRT)	Hs.8935	NM_014298	cell cycle	1.8	0.01
H. sapiens von willebrand factor (VWF)	Hs.110802	NM_000552	cell adhesion	1.8	0.02
H. sapiens neu1 protein (LOC51162)	Hs.91481	NM_016215	signal transduction	1.8	0.03
H. sapiens g-protein coupled receptor salpr (LOC51289)	Hs.170146	NM_016568	chemotaxis	1.8	0.04
H. sapiens c-terminal binding protein 1 (CTBP1)	Hs.239737	NM_001328	negative control of cell proliferation	1.8	0.03
H. sapiens zic family member 3 (odd-paired drosophila H.log, heterotaxy 1) (ZIC3)	Hs.111227	NM_003413	transcription regulation	1.7	0.04
H. sapiens tissue inhibitor of metalloproteinase 3 (TIMP3)	Hs.245188	NM_000362	developmental processes	1.7	0.01
H. sapiens cDNA FLJ12323 fis, clone mamma1002094	Hs.214979	AK022385	cell growth and/or maintenance	1.7	0.02
H. sapiens tubulin, beta, 4 (TUBB4)	Hs.159154	NM_006086	microtubule-based process	1.7	0.03
H. sapiens cDNA FLJ12688 fis, clone nt2rm4002534	Hs.13526	AK022750	iron homeostasis	1.7	0.02
H. sapiens udp-galbetaglcnac beta 1,4-galactosyltransferase, polypeptide 2 (B4GALT2)	Hs.206713	NM_003780	lactose biosynthesis	1.7	0.04
H. sapiens four and a half LIM domains 1 (FHL1)	Hs.239069	NM_001449	histogenesis and organogenesis	1.7	0.01

*This is a partial list of genes with known function. The complete list of genes can be accessed in the GEO database series GSE1718.

Table 5. Genes down-regulated post-training. The fold difference represents the average ratio of HS₁R /LS₁R in 3 microarray experiments*

GENE	Unigene	GenBank identifier	Gene ontology	Fold difference	P-Value
H. sapiens mRNA; cDNA dkfzp434b1231 (from clone dkfzp434b1231);	Hs.35945	AL137493	cell adhesion	0.3	0.02
H. sapiens glucocorticoid receptor af-1 specific elongation factor mRNA,	Hs.181165	AF174496	protein synthesis elongation	0.3	0.01
H. sapiens cDNA FLJ13533 fis, clone place1006371	Hs.143671	AK023595	transcription regulation	0.4	0.003
H. sapiens collapsin response mediator protein 1 (CRMP1) mRNA	Hs.155392	NM_001313	neurogenesis	0.4	0.004
H. sapiens ribosomal protein s29 (RPS29) mRNA	Hs.539	NM_001032	protein biosynthesis	0.4	0.001
H. sapiens death-associated protein kinase-related 2 (DRAK2) mRNA	Hs.120996	NM_004226	protein phosphorylation	0.5	0.02
H. sapiens ribosomal protein l6 (RPL6), mRNA.	Hs.174131	NM_000970	protein biosynthesis	0.5	0.003
H. sapiens ribosomal protein l9 (RPL9), mRNA.	Hs.157850	NM_000661	ribosome biogenesis	0.5	0.03
H. sapiens ras-related associated with diabetes (RRAD) mRNA	Hs.1027	NM_004165	small GTPase mediated signal transduction	0.5	0.04
H. sapiens collagen, type xii, alpha 1 (COL12A1), mRNA	Hs.101302	NM_004370	cell adhesion	0.5	0.02
H. sapiens claudin 2 (CLDN2), mRNA	Hs.16098	NM_020384	pathogenesis	0.5	0.02
H. sapiens mRNA for MEGF7, partial cds	Hs.4930	AB011540	lipid metabolism	0.5	0.005
H. sapiens dynein light chain-a (LOC51143), mRNA	Hs.266483	NM_016141	cell growth and/or maintenance	0.5	0.05
H. sapiens ribosomal protein l10a (RPL10a), mRNA	Hs.252574	NM_007104	protein biosynthesis	0.5	0.005
H. sapiens microfibril-associated glycoprotein-2 (MAGP2), mRNA	Hs.300946	NM_003480	posterior midgut invagination	0.5	0.03
H. sapiens natural killer-tumor recognition sequence (NKTR), mRNA	Hs.241493	NM_005385	immune response	0.5	0.006
H. sapiens atp synthase, h+ transporting, mitochondrial f0 complex, subunit	Hs.85539	NM_007100	proteolysis and peptidolysis	0.5	0.02
H. sapiens cathepsin l2 (CTSL2) mRNA	Hs.87417	NM_001333	proteolysis and peptidolysis	0.6	0.05
H. sapiens hn1 protein (LOC51155), mRNA	Hs.109706	NM_016185	mesoderm development	0.6	0.02
H. sapiens cdc2-related protein kinase 7 (CRKRS), mRNA	Hs.123073	NM_016507	developmental processes	0.6	0.03
H. sapiens cd5 antigen-like (scavenger receptor cysteine rich family)	Hs.52002	NM_005894	cellular defense response	0.6	0.03
H. sapiens mitochondrial proteolipid 68mp H.log (PLPM) mRNA	Hs.109052	NM_004894	complex III (ubiquinone to cytochrome c)	0.6	0.02
Human hepg2 3' region mboi cDNA, clone hmd1c11m3		D17129	RNA splicing	0.6	0.0001
H. sapiens kaptin (actin-binding protein) (KPTN), mRNA	Hs.25441	NM_007059	actin filament organization	0.6	0.05
H. sapiens abcg5 (ABCG5) mRNA, complete cds	Hs.132992	AF320293	transport	0.6	0.05
H. sapiens cDNA: FLJ20896 fis, clone adka03527	Hs.26756	AK024549	signal transduction	0.6	0.03
H. sapiens karyopherin alpha 4 (importin alpha 3) (KPNA4) mRNA	Hs.302499	NM_002268	NLS-bearing substrate-nucleus import	0.6	0.01
H. sapiens ran binding protein 3 (RANBP3), transcript variant ranbp3-a,	Hs.176657	NM_003624	protein targeting	0.6	0.004
H. sapiens ribosomal protein l24 (RPL24) mRNA	Hs.184582	NM_000986	RNA processing	0.6	0.006

* This is a partial list of genes with known function. The complete list of genes can be accessed in the GEO database series GSE1718.

Table 6. List of genes that were consistently up- or down- regulated in the pre- and post-training experiments.

GENE	Unigene	GenBank identifier	Gene ontology	Pre- training HS ₁ R /LS ₁ R arrays		Post- training HS ₁ R /LS ₁ R arrays		Post/pre-training H/LS ₁ R arrays ratio
				Fold difference	P- Value	Fold difference	P- Value	
H. sapiens mRNA; cDNA DKFZp686K23233 (from clone DKFZp686K23233)	Hs.273186	AL137511	electron transport	0.9	0.03	3.9	0.03	4.2
H. sapiens clone flb4630		AF113688	transcription factor activity	0.7	0.03	1.7	0.02	2.4
H. sapiens von willebrand factor (VWF), mRNA	Hs.110802	NM_000552	cell adhesion	0.8	0.02	1.8	0.02	2.3
H. sapiens voltage-dependent anion channel 3 (VDAC3), mRNA	Hs.7381	NM_005662	adenine transport	0.8	0.03	1.5	0.01	1.8
H. sapiens toll-like receptor 4 (TLR4), mRNA	Hs.159239	NM_003266	signal transduction	0.8	0.05	1.4	0.05	1.7
H. sapiens colony stimulating factor 2 receptor, beta, low-affinity (csf2rb), mRNA	Hs.285401	NM_000395	signal transduction	0.8	0.02	1.3	0.03	1.6
H. sapiens 3-hydroxyanthranilate 3,4-dioxygenase (HAAO), mRNA	Hs.108441	NM_012205	cell adhesion	0.8	0.01	1.3	0.03	1.6
H. sapiens KIAA1018 protein (KIAA1018), mRNA	Hs.5400	NM_014967	cell growth and/or maintenance	0.9	0.05	1.4	0.05	1.5
H. sapiens LIM domain-containing preferred translocation partner (lpp), mRNA	Hs.180398	NM_005578	cell adhesion	0.9	0.002	1.3	0.01	1.4
H. sapiens mRNA for KIAA1160 protein, partial cds	Hs.33122	AB032986	ubiquinone metabolism	0.9	0.01	1.3	0.04	1.3
H. sapiens mitogen-activated protein kinase kinase kinase 11 (MAP3K11), mRNA	Hs.89449	NM_002419	protein kinase	0.9	0.03	1.2	0.04	1.3
H. sapiens complement c1q a chain precursor, (LOC51089), mRNA	Hs.9641	NM_015991	cell adhesion	0.9	0.03	0.9	0.04	1.0
H. sapiens interleukin 11 (IL11B), mRNA		NM_000881	mesoderm determination	1.3	0.04	1.3	0.01	1.0
H. sapiens mRNA for KIAA0226 protein, partial cds	Hs.141296	D86979	cytoskeleton organization and biogenesis	0.9	0.05	0.8	0.05	0.9
H. sapiens cDNA: FLJ23529 fis, clone lng06042	Hs.246306	AK027182	cell motility	0.9	0.01	0.8	0.05	0.9
H. sapiens complexin 2 (CPLX2), mRNA	Hs.321567	NM_006650	neurotransmitter release	1.4	0.04	1.2	0.03	0.9
H. sapiens cytochrome c oxidase subunit via H.log mRNA, complete cds		AF020589	energy pathways	0.9	0.004	0.7	0.05	0.8
H. sapiens map 19p13.3; 18.87 cr from 1b1264 repeat region, complete sequence	Hs.124161	AF064851	behavior	0.8	0.05	0.6	0.01	0.7
H. sapiens hypothetical protein FLJ11155 (FLJ11155), mRNA	Hs.176227	NM_018342	apyrase	1.2	0.01	0.8	0.05	0.7
H. sapiens POU domain, class 4, transcription factor 2 (POU4F2), mRNA	Hs.266	NM_004575	transcription regulation	0.9	0.01	0.6	0.0003	0.6
H. sapiens NADH dehydrogenase (ubiquinone) 1B subcomplex, 7 (ndufb7), mRNA	Hs.661	NM_004146	complex I (NADH to ubiquinone)	1.1	0.04	0.7	0.02	0.6
H. sapiens mitochondrial proteolipid 68mp H.log (PLPM), mRNA	Hs.109052	NM_004894	complex III (ubiquinone to cytochrome c)	1.1	0.03	0.6	0.02	0.5
H. sapiens ribosomal protein s21 (RPS21), mRNA.	Hs.1948	NM_001024	protein biosynthesis	1.1	0.01	0.2	0.05	0.1

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