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Gene expression profile of aging in human muscle

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Welle, Stephen, Andrew I. Brooks, Joseph M. Delehanty, Nancy Needler, and Charles A. Thornton. Gene expression profile of aging in human muscle. *Physiol Genomics* 14: 149–159, 2003.4 First published June 3, 2003; 10.1152/physiolgenomics.00049.2003.—Studies of gene expression related to aging of skeletal muscle have included few subjects or a limited number of genes. We conducted the present study to produce more comprehensive gene expression profiles. RNA was extracted from vastus lateralis biopsies obtained from healthy young (21–27 yr old, $n = 8$) and older men (67–75 yr old, $n = 8$) and was analyzed with high-density oligonucleotide arrays. Of the ~44,000 probe sets on the arrays, ~18,000 yielded adequate signals for statistical analysis. There were ~700 probe sets for which *t*-tests or rank sum tests indicated a difference ($P \leq 0.01$) in mean expression between young and old and for which the estimated false discovery rate was <10%. Most of these differences were less than 1.5-fold in magnitude. Genes that encode proteins involved in energy metabolism and mitochondrial protein synthesis were expressed at a lower level in older muscle. Genes encoding metallothioneins, high-mobility-group proteins, heterogeneous nuclear ribonucleoproteins and other RNA binding/processing proteins, and components of the ubiquitin-proteasome proteolytic pathway were expressed at higher levels in older muscle. Expression of numerous genes involved with stress responses, hormone/cytokine/growth factor signaling, control of the cell cycle and apoptosis, and transcriptional regulation appeared to be affected by aging. More transcripts were detected in older muscle, suggesting dedifferentiation, an increased number of splice variants, or increased cellular heterogeneity. We conclude that in human skeletal muscle the expression of many genes tends to increase or decrease between the third and seventh decades. The changes are modest when averaged over all of the cells in the tissue.

microarray; mRNA; sarcopenia

IT IS WELL KNOWN that gene expression programs orchestrate the development and growth of complex organs, but the role of gene expression in the senescence of mature tissues is not understood. Studies have been done to search for changes in gene expression associated with senescence of muscle in mice (18), monkeys (16), and humans (15, 29, 40). Aging of muscle is an important issue because muscle accounts for approximately half of the cell mass of the human body, and loss of muscle bulk and performance (sarcopenia) is a key feature of age-related frailty. Our initial comparison of gene expression in young and old human muscle was based on the serial analysis of gene expression (SAGE) method (40). Only ~700 mRNAs were sufficiently abundant for statistical analysis of age-related differences. Both cDNA arrays and high-density oligonucleotide arrays can accurately quantify transcripts that yielded only 1–2 SAGE tags in that study (an insufficient number to detect effects of aging). However, in previous comparisons of gene expression profiles of young and old human muscle that were based on cDNA arrays, no more than 1,000 mRNAs were reliably detected (15, 29). More comprehensive gene expression profiles of normal aging in human muscle might provide leads for further research that were not detected in these earlier studies. Thus we have used the Affymetrix U133A and U133B high-density oligonucleotide arrays, which have >44,000 probe sets that target >33,000 mRNAs.

METHODS

Subjects. The subjects were eight young (21–27 yr) and eight older men (67–75 yr). They gave written consent after the procedures and risks were explained. The research was approved by the University of Rochester Research Subjects Review Board.

All subjects had normal neuromuscular function and were healthy according to history, physical examination, and laboratory tests. They were not engaged in any type of regular exercise program involving strenuous activity for more than 2 h per week, nor did they perform any high-resistance exercises involving the vastus lateralis muscle. Subjects were asked to assign their present level of physical activity to one of four categories: *1*) no weekly activities; *2*) only light activity most weeks; *3*) vigorous activity for at least 20 min once or twice each week; or *4*) vigorous activity for at least 20 min three or more times per week. Vigorous activity was defined as activity causing shortness of breath, rapid heart rate, and sweating. Two men in each age group were in *category 4*; four in each age group were in *category 3*; two young men and one older man were in *category 2*; one older man was in *category 1*.

Muscle biopsy procedure. Subjects were asked to refrain from any activity more strenuous than walking for 3 days before the muscle biopsy. They stayed at the University of Rochester General Clinical Research Center the night before the biopsy to minimize variability in the amount of activity performed on the day of the biopsy. They received a standard evening meal (12 kcal/kg body wt) and a standard breakfast (7 kcal/kg body wt) ~90 min before the muscle biopsy was taken. After breakfast, they rested until needle biopsies were obtained from vastus lateralis muscle of the left leg. The skin and muscle were anesthetized with lidocaine 2 min before

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tissue removal. Muscle samples were frozen in liquid nitrogen within 30 s of removal, then stored at −70°C until analysis.

Body composition. Total lean mass, percent body fat, and leg lean tissue mass were determined by dual-energy X-ray absorptiometry (Prodigy; Lunar, Madison, WI).

Muscle strength. Maximal voluntary isometric knee extensor strength was determined as follows. The subject was seated with the knee bent at 90° and the foot above the floor. An ankle strap was connected to a force transducer, which was attached to a rigid frame. Standard instructions were given, then subjects performed several contractions (at least 3, but more if trials were discarded because subject did not use proper form) during which they were instructed to exert maximal force against the ankle strap for 5 s. The force transducer was interfaced with a computer, which averaged values over 1-s intervals and provided audible feedback to the subject during the muscle contraction (higher force caused a higher pitch). The highest force (over a 1-s interval) of all trials was used as the index of strength.

Peak oxygen consumption. Subjects exercised on a cycle ergometer while oxygen consumption was measured with a MedGraphics CPX/D analyzer (Medical Graphics, St. Paul, MN). The workload was increased frequently until a subject asked to stop or until an increase in workload did not increase oxygen consumption more than 0.15 l/min. The rate at which the workload was increased was based on a formula that considers age, height, and weight, with the goal being to achieve peak oxygen consumption in ~10 min of exercise.

Plasma testosterone and IGF-I concentrations. Commercial radioimmunoassay kits were used to determine serum concentrations of free testosterone (Diagnostic Products, Los Angeles, CA) and total IGF-I (Diagnostic Systems Laboratories, Webster, TX).

Analysis of gene expression by high-density oligonucleotide arrays. RNA was extracted from the muscle samples as described previously (40). RNA was reverse transcribed to cDNA with oligo-dT-[T7] as the primer. Double-stranded cDNA was transcribed with T7 polymerase to produce biotin-labeled cRNA. The cRNA was probed with Affymetrix (Santa Clara, CA) HG-U133A and HG-U133B high-density oligonucleotide arrays. The HG-U133 set has ~44,000 probe sets that measure expression of ~33,000 genes. After hybridization and washing, arrays were stained with phycoerythrin-streptavidin, which binds to the biotinylated cRNAs hybridized with the probes. The initial scan detected the fluorescence of the phycoerythrin-streptavidin. After the initial scan, signals were amplified by staining the array with biotin-labeled anti-streptavidin antibody followed by phycoerythrin-streptavidin. In this report we present the data from the better scan for each probe set, which was selected before considering differences between groups. The better scan was selected with an algorithm based on the detection statistics (provided by Microarray Suite 5.0), which are derived from the ratios of specific to nonspecific hybridization signals and consistency across probe pairs within a set. For most probe sets (91% of those used in the statistical analyses), the antibody-enhanced scan yielded better results. Note that although data from either *scan 1* or *scan 2* were used for different probe sets, for any particular probe set the same scan was always used for all 16 samples.

Microarray data were analyzed as described in detail elsewhere (41). Briefly, the procedure involved using the standard Affymetrix normalization procedure (Microarray Suite 5.0), which forces the trimmed mean (excludes top and bottom 2%) of all signals on an array to the same value (500 arbitrary units). This normalization was accomplished by multiplying raw signals by a scaling factor. All pairwise comparisons of each normalized array with all other normalized arrays, based on the Affymetrix comparative analysis algorithm, were averaged to determine the relative expression ratios for a particular subject relative to the mean of all subjects. This method reduces within-group variance and improves statistical power relative to the Affymetrix method for analyzing arrays individually (41).

The present study was exploratory, with the goal being to generate leads for further research rather than to demonstrate with a high level of statistical certainty that there was differential expression for any particular mRNA. Thus we wanted to minimize false discovery (type 1 error rate) to the extent possible without using such stringent criteria that statistical power was severely compromised (high type 2 error rate). To this end, several strategies were employed. First, for a probe set to be included in the analysis, its detection P value ($P_{detection}$) from the Affymetrix detection algorithm (which is based on a Wilcoxon test comparing signals from the perfect-match and mismatch probes within a probe set) had to be <0.1 for at least four arrays within the same age group. This step limited the analysis to 18,245 probe sets. Second, transcripts were considered to be candidates for differential expression only if the difference between old and young was significant at $P \leq 0.01$ according to either a t-test or a rank sum test. Because 18,245 comparisons were made, the nominal P value from a t-test or rank sum test for any particular mRNA understates the probability of a type 1 error (declaring a difference that does not exist). A Bonferroni correction would be too conservative for an exploratory study (no difference would have been significant with a Bonferroni-corrected $P < 0.05$). The type 1 error rate (false discovery rate) can be determined only by examining a large number of the candidate genes in a prospective study, but it can be estimated based on statistical principles. The significance analysis of microarrays (SAM) method provides one approach for estimating the false discovery rate (34). The third criterion for a gene to be included on the list of candidates for differential expression was that the false discovery rate according to the SAM method (denoted as q) had to be <10%.

Quantitative PCR. The most abundant mRNAs are not precisely quantified by the microarrays because of saturation of the scanner. Therefore, relative levels of the mRNAs encoding the major adult isoforms of myosin heavy chain (MyHC 1, 2a, and 2x) were determined by quantitative RT-PCR with competitive internal standards as described before (39).

One of the mRNAs that was differentially expressed in young and old muscle according to the microarray data, p21 mRNA (2.9-fold difference), was examined by semi-quantitative RT-PCR. The same cDNA that was used for the MyHC mRNA determinations was amplified with primers specific for p21 mRNA. Sequencing confirmed the identity of the PCR product. Each tube contained the cDNA produced by reverse transcription of an identical amount of total RNA. After PCR amplification, products were separated on a polyacrylamide gel, which then was soaked in a solution of SYBR Green (Molecular Probes). The fluorescence of the bands was determined with a FluorImager (Molecular Dynamics).

The same cDNA samples that were used to generate the biotinylated cRNA for the microarray analyses were used to validate the hybridization results for five other transcripts that were differentially expressed in young and old muscle: follistatin, cysteine-rich motor neuron 1, FEZ2, metallothionein 1L, and GADD45-γ. A reference gene, proteasome subunit-β type 7, was chosen based on the fact that its expression level was consistent across all subjects according to the

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microarray results. The method employed a fluorogenic chemistry with probes and primers designed using Epoch Biosciences MGB Eclipse software (http://www.syntheticgenetics.com/products/MGBEclipse_Software.htm). The MGB Eclipse probe system incorporates minor groove binders, dark quenchers, and modified bases which work in concert to generate probes for difficult templates. In a nonhybridized state in solution, the MGB eclipse probe has a random coil structure so that the fluorescence of the 3′ reporter fluor (FAM for genes of interest, TET for reference gene) is suppressed by the 5′ quencher (BHQ1). When the probe hybridizes to a complementary target at the annealing temperature, the quencher and the reporter are separated and fluorescence is emitted. Following hybridization, the MGB folds into the minor groove of the probe-target duplex and stabilizes it. The cDNA samples were diluted to 1 ng/μl, and 2 μl were used for each 10-μl PCR reaction containing 400 μM dNTPs, 1× MGB Eclipse buffer, 1× primer mix, 1× MGB Eclipse probe mix, and 0.4 U JumpStart Taq (Sigma). Reactions were performed in triplicate in an ABI 7900 HT sequence detection system with the following cycle parameters: 1 cycle of 95°C (2 min), followed by 40 cycles of 95°C for 5 s, 56°C for 20 s, and 76°C for 20 s. Fluorescence was recorded during the annealing phase. Cycle thresholds (Ct, the cycle number at which fluorescence achieved a level above background noise) were determined using Sequence Detection Software (ABI, Foster City, CA). The difference in Ct between the reference gene and the gene of interest (ΔCt) is a measure of relative expression levels of the two genes within the same sample. These values were compared with the microarray-based relative expression levels for the same genes. To estimate mean fold changes from the differences in mean ΔCt, we used an amplification efficiency (E) of 0.9 (concentration ratio = $[1 + E]^{\Delta Ct}$); for known concentration ratios, based on dilution curves for five different cDNAs, the mean value of E was 0.9 (standard error 0.02).

Gene annotation. Known or putative gene functions were ascertained from gene ontology (GO) classifications (provided at http://www.NetAffx.com), Online Mendelian Inheritance in Man (OMIM; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM), or publications cited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) entries. The tables provided in this report refer to the Affymetrix probe set identification numbers, which can be used at http://www.NetAffx.com to find the oligonucleotide sequences of the probes and links to UniGene and other descriptive information.

RESULTS

Subject characteristics (Table 1). As expected, older men had less muscle mass per kilogram body weight, reduced peak oxygen consumption, and reduced levels of IGF-I and free testosterone. Isometric strength tended to be less in the older men, but there was a high degree of variability and the difference was not statistically significant.

Myosin heavy chain mRNAs (Table 1). Quantitative RT-PCR indicated a trend toward a reduced ratio of type 2a to type 1 MyHC mRNA in older muscle, but the difference was not statistically significant (22% difference in mean ratio, $P = 0.40$). Expression of type 2x MyHC mRNA, the least abundant of the three major isoforms expressed in vastus lateralis, was highly variable in both younger and older muscle as observed in a previous study (40). Neither 2a/1 expression ratios nor 2x/1 expression ratios correlated significantly with expression of the genes probed by the microarrays (all $q \geq 20\%$ according to SAM method).

Number of expressed and differentially expressed genes. The microarray data have been deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo/; series accession number = GSE362). Many mRNAs probed by the microarrays are not expressed in muscle or are expressed at a level too low to be quantified accurately. The results described in this report refer to 18,245 probe sets (~40% of all probe sets) for mRNAs that were considered present by the detection algorithm (at $P_{\text{detection}} < 0.10$) in at least four of the samples from either young or old muscle. Regardless of the $P_{\text{detection}}$ value used to decide whether a transcript is present, and regardless of the minimum number of subjects achieving this threshold, older muscle expressed a few hundred more genes than young muscle (Fig. 1; $P < 0.001$ for all comparisons by χ^2 tests). However, this difference did not appear to introduce any systematic bias into the normalization procedure (signals were normalized against the mean signal of all genes expressed in muscle), as indicated by the regression of mean signals from old muscle against the mean signals from young muscle (Fig. 2). The slope of the regression line was ~1 and the intercept was ~0, as

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Table 1. *Body composition, muscle performance, hormone levels, and MyHC mRNA expression*

	Young	Old	*P*
Weight, kg	74.4 ± 9.5	79.2 ± 13.1	0.41
Lean body mass, kg	59.2 ± 5.1	53.6 ± 6.9	0.083
LLLM, kg	10.0 ± 0.9	8.6 ± 1.4	0.031
LLLM/weight, %	13.6 ± 1.6	10.9 ± 0.9	0.001
Peak isometric force (left leg), N	468 ± 104	412 ± 64	0.21
Peak V_{O_2}, ml/kg lean body mass per min	45.2 ± 6.0	31.3 ± 6.0	0.0003
IGF-I, ng/ml	402 ± 127	229 ± 106	0.01
Free testosterone, pg/ml	19.3 ± 5.1	9.9 ± 2.2	0.0003
MyHC1 mRNA, % mean value in young	100 ± 34	130 ± 48	0.17
MyHC2a mRNA, % mean value in young	100 ± 23	93 ± 29	0.57
MyHC2x mRNA, % mean value in young	100 ± 58	140 ± 101	0.35

Values are means ± one standard deviation. MyHC, myosin heavy chain; LLLM, left leg lean mass (excluding bone). *P* was determined by *t*-test (two-tailed).

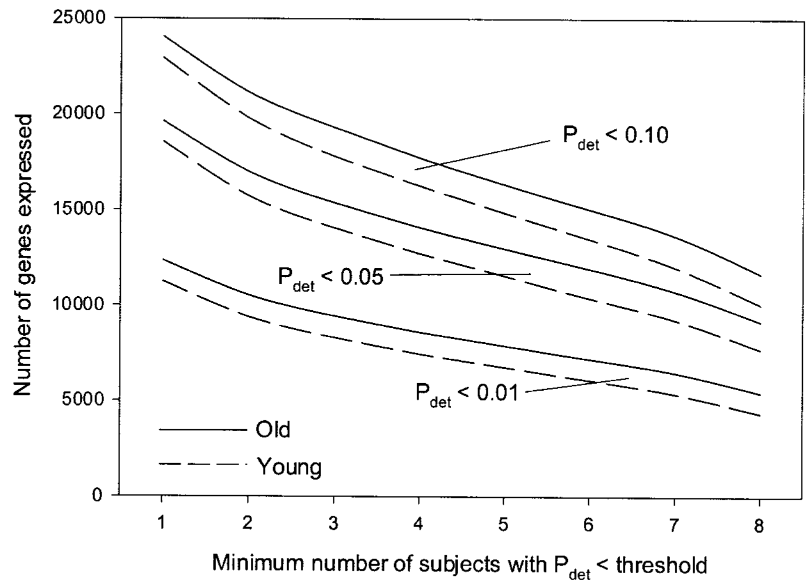

Fig. 1. Increased number of different transcripts in older muscle. Affymetrix Microarray Suite 5.0 program determined how many target transcripts were detected on each array with a probability lower than a threshold ($P_{\text{detection}} < 0.10$, < 0.05, < 0.01). The left end of each curve represents the number of genes defined as being expressed when using the least stringent filter, i.e., the mRNA had to be detected ($P <$ threshold) in one or more samples. The right end of each curve represents the number of genes defined as being expressed when using the most stringent filter, i.e., the mRNA had to be detected ($P <$ threshold) in all samples. Solid lines represent older subjects; broken lines represent young subjects. The estimate of the number of expressed genes declined as the filter became more stringent, but the difference between younger and older muscle was evident regardless of filter stringency.

expected for the ideal normalization. The variance in gene expression was similar among younger and older men (Fig. 3). For the 18,245 probe sets included in the statistical analyses, the average coefficient of variation was 21% among young subjects and 19% among older subjects.

There were 718 probe sets that met the criteria set forth in METHODS for inclusion in the list of candidates for differential expression (394 genes with higher expression in older muscle, 324 with lower expression in older muscle). Most of the age-related differences were less than 1.5-fold in magnitude (Fig. 4). There were only 10 differences ≥2-fold in magnitude that were statistically significant at $P \leq 0.01$ by t-test or rank sum test and at $q < 10\%$. These are listed in Table 2.

Differential expression by broad functional categories. All of the genes mentioned in this section and in Tables 2–7 met the statistical criteria set forth in METHODS for being considered strong candidates for differential expression.

Table 3 lists differentially expressed genes encoding proteins involved in energy metabolism (glycolysis, mitochondrial electron transport, ATP synthesis, mitochondrial protein synthesis, and tricarboxylic acid cycle activity). The transcripts of these genes were ~15–25% less abundant in older muscle.

Five metallothionein mRNAs (1F, 1H-like, 1L, 1X, and 2A) were expressed at higher levels (~50–90% more abundant) in older muscle. Four mRNAs encoding high-mobility-group proteins (HMG2L1, HMGB1, HMGB2, HMGN2) were ~25–40% more abundant in older muscle. The mRNAs encoding several heterogeneous nuclear ribonucleoproteins (hnRNPs) were expressed at higher levels (~20–35%) in older muscle (Table 4). Several other mRNAs encoding RNA binding/splicing proteins were ~15–65% more abundant in

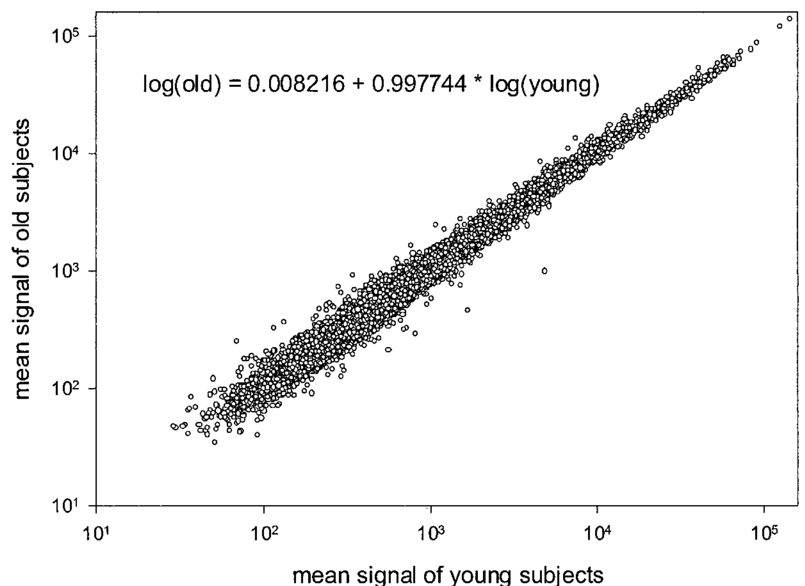

Fig. 2. Mean signals for eight older men vs. mean signals for eight young men. The 18,245 probe sets represent transcripts present ($P_{\text{detection}} < 0.1$) in at least four RNA samples in one or both age groups.

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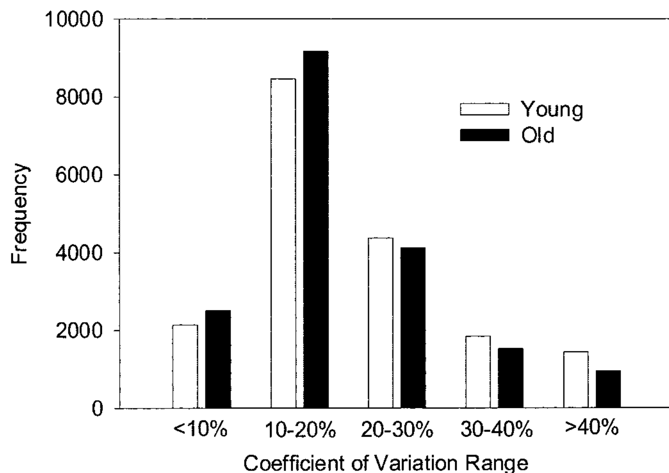

Fig. 3. Frequency distribution of coefficients of variation for expression levels of 18,245 genes in young muscle (open bars) and older muscle (solid bars).

Table 2. *Genes with ≥2-fold differences in expression that were significant at P ≤ 0.01 by t-test or rank sum test and at q < 10% by SAM*

Gene	Probe Set	Expression Ratio (old/young)
Follistatin	226847_at	3.56
Cyclin-dependent kinase inhibitor-1A (p21)	202284_s_at	2.87
Perinatal myosin heavy chain	34471_at	2.66
GenBank AI090487	242287_at	2.23
G protein-coupled receptor 49	213880_at	2.14
GenBank BF968482	236860_at	0.50
GenBank AI767727	229815_at	0.46
Calcium channel, voltage-dependent, γ subunit 4	62987_r_at	0.45
GenBank BF247552	224579_at	0.35
GenBank BF510813	229275_at	0.20

SAM, significance analysis of microarrays.

older muscle (Table 4). Several mRNAs encoding proteasome components, ubiquitin-like proteins, and proteins related to ubiquitin function were present at higher levels (~10–70%) in older muscle (Table 5). Not all genes related to proteasome proteolysis were overexpressed in older muscle. Thus one of mRNAs encoding a proteasome subunit was used as a reference gene for RT-PCR validation studies because there was no trend for an age-related difference (see METHODS).

Several stress response genes (loosely defined here as genes involved in immune or inflammatory responses, defense against oxidative or environmental stresses, or repairing damaged DNA) were differentially expressed in young and old muscle. Table 6 shows those for which the mean difference in expression was ≥1.3-fold. However, no clear pattern emerged to support the concept that aging is associated with a general activation of stress response genes. For example, GADD45-α mRNA, which is induced by DNA damage, was more abundant in older muscle, but GADD45-γ mRNA was less abundant in older muscle.

Many of the genes that appear to be differentially expressed in younger and older muscle encode proteins that regulate transcription, including hormones, cytokines, growth factors, receptors, and proteins involved in signal transduction pathways. Table 7 lists those for which the mean difference in expression was at least 1.3-fold.

There were age-related differences in expression levels of genes in many other functional categories, including mitosis, apoptosis (genes involved in mitosis and apoptosis often overlap with other functional categories such as stress responses, signal transduction, transcription), small molecule transport (ion and amino acid), cell adhesion, protein folding, protein and vesicle trafficking, cytoskeleton and microtubule formation, cell size and shape determination, RNA and protein transport across the nuclear membrane, extra-

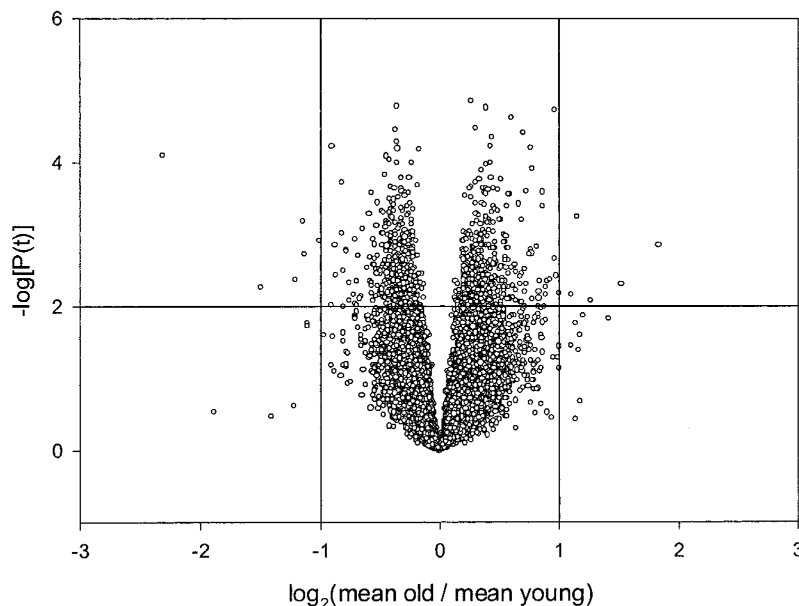

Fig. 4. Volcano plot of statistical significance (−log[*P*], with *P* based on *t*-tests), as a function of the average expression ratio (mean expression in old/mean expression in young). Horizontal axis is $\log_2$ scale, so vertical lines represent twofold differences. Horizontal line represents *P* = 0.01, the cutoff used to define differential expression.

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Table 3. *Differentially expressed genes ($P \leq 0.01$ by t-test or rank sum test and $q < 10\%$) encoding proteins involved in energy metabolism*

Category/Gene	Probe Set	Expression Ratio (old/young)
Mitochondrial electron transport/ ATP synthase		
BCS1-like (assembly of complex 3)	207618_s_at	0.83
Cytochrome *c* oxidase 7c	213846_at	0.78
Cytochrome *c*-1	201066_at	0.85
NADH dehydrogenase flavoprotein 3	226616_s_at	0.78
Succinate dehydrogenase subunit 3	202004_x_at	0.87
ATP synthase subunit 9, isoform 1	208972_s_at	0.83
Mitochondrial ribosomal proteins		
Mitochondrial ribosomal protein L12	203931_s_at	0.79
Mitochondrial ribosomal protein L41	227186_s_at	0.83
Mitochondrial ribosomal protein L48	218281_at	0.75
Mitochondrial ribosomal protein S5	224333_s_at	0.82
Mitochondrial ribosomal protein S24	224948_at	0.84
Mitochondrial ribosomal protein S25	224869_s_at	0.78
Glycolysis/TCA cycle		
Pyruvate dehydrogenase α1	200979_at	0.71
6-Phosphofructo-2-kinasefructose-2,6-biphosphatase 1	207537_at	0.83
Phosphoglucomutase 5	231024_at	0.85
Malate dehydrogenase 2	213333_at	0.84
Fumarate hydratase	203033_x_at	0.85
Aspartate aminotransferase 2	200708_at	0.80
Succinate dehydrogenase subunit 3	202004_x_at	0.87

cellular matrix formation, angiogenesis, chromatin formation, amino acid and oxysterol metabolism, and posttranslational processing of proteins. The complete list of candidates for differential expression is available as a Microsoft Excel file (see the Supplementary Material, available at the *Physiological Genomics* web site).[1] This file has two worksheets, one with named genes and one with uncharacterized genes.

Evidence for validity of microarray data. Although there undoubtedly are some false positives among the candidate genes identified in this study, three lines of evidence support the general validity of the microarray data. First, the reduced expression in older muscle of several genes involved in mitochondrial energy production is consistent with our previous data (derived from a separate group of subjects by SAGE and quantitative RT-PCR), with microarray data showing similar effects in older muscle of mice and monkeys (16, 18), and with physiological, biochemical, and histochemical data showing a reduced capacity for energy metabolism and mitochondrial protein synthesis in older human muscle (6, 22, 28). Second, there was internal consistency in the microarray data. Several genes within a number of functional categories (metallothioneins, high-mobility-group proteins, heterogeneous nuclear ribonucleoproteins, mitochondrial ribosomal proteins, mitochondrial ATP generation) showed similar age-related increases or decreases in expression. Third, quantitative RT-PCR validations were done for six candidates for differential expression, five of which never had been shown to be differentially expressed in young and old tissues. The microarray-based expression ratios for the genes of interest relative to the reference gene (selected empirically for lack of difference between young and old and for a relatively small intersubject variance) were consistent with ratios quantified by PCR (Table 8). This consistency was evident both at the level of group means and at the level of correlation coefficients based on 16 individual subjects.

[1]The Supplementary Material (complete list of candidates for differential expression) for this article is available online at http://physiolgenomics.physiology.org/cgi/content/full/00049.2003/DC1.

Table 4. *Differentially expressed genes ($P \leq 0.01$ by t-test or rank sum test and $q < 10\%$) encoding hnRNPs and other RNA binding/splicing proteins*

Gene	Probe Set	Expression Ratio (old/young)
hnRNPs		
A1	200016_x_at	1.26
	214280_x_at	1.28
A3	211929_at	1.38
	211931_s_at	1.34
C (C1C2)	200014_s_at	1.24
	212626_x_at	1.17
	214737_x_at	1.25
D	200073_s_at	1.23
	221481_x_at	1.10
	229129_at	1.25
H2	201132_at	1.29
K	200097_s_at	1.27
M	200072_s_at	1.20
R	208766_s_at	1.21
U	200594_x_at	1.24
Other RNA binding/splicing		
RNA binding motif protein 3	208319_s_at	1.65
Splicing factor proline glutamine rich	201586_s_at	1.27
Splicing factor CC1.3	208720_s_at	1.17
Ataxin-1	203232_s_at	1.23
Muscleblind-like	201152_s_at	1.29
	201153_s_at	1.28
Ewing sarcoma breakpoint region 1	209214_s_at	1.21
RNA binding protein HQK7B	212636_at	1.24
RNA binding motif protein, X chromosome	213762_x_at	1.18
Splicing factor, arginine/serine-rich 11	200686_s_at	1.28
	225356_at	1.23
Splicing factor, arginine/serine-rich 12	212721_at	1.21
Cold-induced RNA binding protein	200810_s_at	1.37
DEADH box polypeptide 9	202420_s_at	1.24
Regulator of nonsense transcripts 3A	214323_s_at	1.29
DEADH box polypeptide 24	200694_s_at	1.14
U5 snRNP 116 kDa	222398_s_at	1.19
Step II splicing factor SLU7	231718_at	1.34
RNA binding motif, single-stranded interacting protein 1	209868_s_at	0.81
RNA binding protein (autoantigenic, hnRNP-associated with lethal yellow)	201271_s_at	0.81

hnRNP, heterogenous nuclear ribonucleoproteins.

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Table 5. *Differentially expressed genes ($P \leq 0.01$ by t-test or rank sum test and $q < 10\%$) encoding proteasome components, ubiquitin-like proteins, and proteins related to ubiquitin function*

Gene	Probe Set	Expression Ratio (old/young)
Proteasome 26S subunit, non-ATPase, 5	203447_at	1.70
Proteasome 26S subunit, ATPase, 6	201699_at	1.27
Proteasome subunit, beta type, 4	202243_s_at	1.11
Ubiquitin specific protease 1	202413_s_at	1.24
Ubiquitin specific protease 6	206405_x_at	1.35
F-box and leucine-rich repeat protein 11	208988_at	1.23
Ubiquitin conjugating enzyme E2B	202334_s_at	1.19
Ubiquitin-like 5	225256_at	1.27
Sentrin 2	213881_x_at	1.17
SUMO-1 activating enzyme subunit 2	201177_s_at	1.23

DISCUSSION

Summary of previous studies comparing gene expression profiles of young and old muscle. There have been several exploratory studies to search for differences in gene expression between young and old skeletal muscle. Lee et al. (18) examined gastrocnemius muscles of three young adult and three old mice. Several genes involved in energy metabolism were underexpressed in old muscle, whereas several involved in responses to oxidative stress and reinnervation of muscle fibers were overexpressed in old muscle. We used SAGE to compare RNA pools from eight young and eight old men (40) and found that older muscle had reduced expression of genes involved in energy metabolism. Kayo et al. (16) examined RNA from vastus lateralis muscles of three young adult, three middle-aged, and three old rhesus monkeys. Aging was associated with reduced expression of genes involved in mitochondrial electron transport and ATP synthesis, and increased expression of several genes involved in oxidative stress responses, reinnervation of muscle fibers, and inflammatory or immune responses. Jozsi et al. (15) examined 588 mRNAs with filter arrays to compare pooled RNA from muscle of 11 young men with pooled RNA from muscle of 12 old men. Four stress response genes appeared to be overexpressed in older muscle, although it is not clear whether these effects were statistically significant when individual samples were evaluated by quantitative RT-PCR. Roth et al. (29) used filter arrays to evaluate expression of ~4,000 genes (~1,000 of

Table 6. *Some differentially expressed genes ($P \leq 0.01$ by t-test or rank sum test, $q < 10\%$, and ≥ 1.3-fold difference) encoding proteins involved in stress response, DNA repair, immunity, and inflammation*

Gene	Probe Set	Expression Ratio (old/young)
GADD45-α	203725_at	1.95
Interleukin 6 receptor	226333_at	1.85
Heat shock transcription factor 2	209657_s_at	1.42
MAPKKKK3	218311_at	1.38
Cold-inducible RNA binding protein	200810_s_at	1.37
Paired immunoglobulin-like receptor-β	225321_s_at	1.31
CD80 antigen	207176_s_at	0.75
ECSIT (intermediate in Toll/IL-1 signal transduction)	218225_at	0.75
Glutathione reductase	225609_at	0.75
MAPKKKK1	214339_s_at	0.73
Chemokine binding protein 2	206887_at	0.72
GADD45-γ	204121_at	0.55

Table 7. *Some differentially expressed genes ($P \leq 0.01$ by t-test or rank sum test, $q < 10\%$, and ≥ 1.3-fold difference) encoding hormones, growth factors, cytokines, receptors, signal transduction proteins, and proteins involved in transcription*

Gene	Probe Set	Expression Ratio (old/young)
Follistatin	226847_at	3.56
G-protein coupled receptor 49	213880_at	2.14
Basic transcription element-binding protein 2	209211_at	1.97
Interleukin 6 receptor	226333_at	1.85
Zinc finger protein homologous to murine Zfp-36	201531_at	1.81
Histone deacetylase 4	204225_at	1.76
Forkhead box O1A	202724_s_at	1.68
Phosphodiesterase 4B	203708_at	1.66
Inositol hexaphosphate kinase 3	231179_at	1.64
Ortholog of mouse myocytic induction/differentiation originator	214846_s_at	1.53
ELL-related RNA polymerase II, elongation factor	226982_at	1.52
SCAN domain-containing 1	231059_x_at	1.51
Zinc finger protein 36, C3H type-like 2	201368_at	1.46
RAB10 (ras oncogene family)	222981_s_at	1.45
IGF binding protein 6	203851_at	1.42
Brain protein 14-3-3 zeta	200639_s_at	1.39
Basic transcription element-binding protein 1	203543_s_at	1.39
Paired immunoglobulin-like receptor-β	220954_s_at	1.38
MAPKKKK3	218311_at	1.38
RAB21 (ras oncogene family)	226268_at	1.37
CREB binding protein	202160_at	1.37
Far upstream binding element 3	212824_at	1.37
Phosphatidylinositol-3-kinase regulatory subunit 1	212239_at	1.33
Thyroid hormone receptor interacting protein 12	201546_at	1.31
Transcription factor CA150	202396_at	1.31
TGF-β receptor III	226625_at	1.30
Vascular endothelial growth factor C	209946_at	0.76
PTPRF interacting	209011_at	0.75
ECSIT (intermediate in Toll/IL-1 signaling to NF-κB)	218225_at	0.75
β-Catenin-interacting protein ICAT	203081_at	0.75
Bone morphogenetic protein 5	205431_s_at	0.74
Kruppel-like factor 7	204334_at	0.74
MAPKKKK1	214339_s_at	0.73
Nuclear receptor subfamily 4, group A, member 3	207978_s_at	0.73
RAP1, GTPase activating protein 1	203911_at	0.72
Never in mitosis gene a-related kinase 11	219542_at	0.69
Retinoid X receptor gamma	205954_at	0.62
Albumin D-box binding protein	209782_s_at	0.59
STAT inhibitor 2 (SOCS2)	203373_at	0.57

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Table 8. *Quantitative PCR validation of microarray data*

Gene	Age Effect by Array	*P* (*t*-test)	Age Effect by PCR	*P* (*t*-test)	*P* for Correlation Across 16 Samples
Follistatin	↑ 3.5 fold	0.001	↑ 2.6	0.0004	0.0006
p21	↑ 2.9	0.005	↑ 3.9	0.0002	0.0002
CRIM1	↑ 1.7	0.0005	↑ 1.9	0.0003	0.007
Metallothionein 1L	↑ 1.6	0.025	↑ 1.6	0.035	0.004
FEZ2	↑ 1.5	0.012	↑ 1.4	0.178	0.036
GADD45-γ	↓ 1.8	0.003	↓ 2.4	0.003	0.00003

The expression level for each subject is relative to the signal from proteasome subunit, β-type, 7, which showed no age-related difference and low within-group variance according to microarrays (probe set 200786_x_at). The PCR for p21 mRNA was done with a different method than the others, so p21 expression is in relation to total RNA.

which were reliably detected) in 4 pooled RNA samples from human muscle: 5 young men, 5 young women, 5 older men, and 5 older women. Aging appeared to affect expression of ~50 genes in various functional categories.

These previous studies were limited by a small number of subjects or pooling of samples and evaluated a small proportion of the muscle transcriptome. The array studies used arbitrary cutoffs of the mean fold change (>1.7 or >2) to define differential expression. The preponderance of smaller effects (<1.5-fold) in the present study reflects a different approach to data analysis, i.e., the use of statistical significance rather than fold change cutoffs to define differential expression. A study of senescence in *Drosophila* also indicated that statistically significant differences in gene expression were often <1.5-fold (14).

Increased number of transcripts detected in older muscle. According to the Affymetrix algorithm for defining the presence or absence of a particular transcript, older muscle expressed several hundred more genes than young muscle. Note that these genes typically were not considered to be differentially expressed by other criteria, mainly because they tended to have relatively high within-group variance. This result is consistent with our SAGE study (40), in which 13% more mRNA species were detected in older muscle. The increased number of different transcripts in older muscle, which presumably would be associated with an increased number of transcription sites, therefore might have some relation to the elevated expression of genes encoding hnRNPs and other RNA binding/splicing factors. A similar effect of aging on expression of splicing factors was noted in murine muscle and brain (25). Another possible link between an increased number of mRNA species in older muscle and increased expression of RNA binding/splicing proteins would be misregulation of pre-mRNA splicing such that more splice variants are present (25). Aging of postmitotic cells may alter nucleosome composition such that there is a stochastic reversal of gene silencing (1). Such "dedifferentiation" might contribute to the detection of more mRNA species in older muscle. Another possible explanation for the presence of more mRNA species in older muscle would be an increased number of fibroblasts, vascular cells, or other mononuclear cells. However, there is no evidence that this occurs with normal aging. We previously noted that the ratio of mononuclear cells to myonuclei (~1:3) does not change with normal aging (38) but did not count the different types of mononuclear cells.

Genes related to stress and inflammation. The gene expression profiles of aging in muscles of mice and monkeys suggested increased oxidative stress (16, 18). Moreover, there is evidence for increased oxidative damage to DNA, lipids, and proteins in older human muscle (24, 27). In the present study, a few genes that are activated by oxidative stress were expressed at a higher level in older muscle. However, a few other stress response genes were expressed at a lower level in older muscle. Several genes that would be expected to be activated by oxidative stress (10, 12), including those encoding catalase, glutathione peroxidase, superoxide dismutase, and heme oxygenase 1, were not expressed at a higher level in older muscle according to the oligonucleotide arrays. This observation is consistent with the data of Pansarasa et al. (27), who found no increase in older human muscle of the activities of superoxide dismutase, glutathione peroxidase, and catalase.

Expression profiles of muscle samples taken from monkeys suggested that inflammatory pathways were more active in older animals (16). Increased inflammation in older muscle would be expected to be associated increased expression of many of the genes that are activated in patients with inflammatory myopathy (13), although quantitatively smaller effects would be expected with normal aging. However, in the present study only a few genes that are involved in inflammatory pathways were expressed at a higher level in older muscle, and others were expressed at a lower level.

Increased expression of metallothionein genes. Metallothionein mRNAs were more abundant in older muscle. Metallothioneins bind heavy metals, reducing their toxicity, and also may have other important roles (8, 9). Metallothionein genes are activated by Zn or by toxic levels of some other heavy metals. They also can be activated by cytokines, glucocorticoids, oxidative stress, and other stresses and under some conditions may protect cells from apoptosis (8). Many of the subjects, both young and old, stated during their physical examination that they consumed multivitamin supplements. However, we do not know whether these contained Zn, which regulates metallothionein gene ex-

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pression. Two older men who stated that they did not take supplements had high metallothionein mRNA levels. Moreover, there appears to be increased metallothionein gene expression in muscle of older monkeys, even though young and old monkeys were fed a defined diet (16). The increase in metallothionein gene expression in muscle of older monkeys was attributed to oxidative stress. However, an age-related difference in muscle concentrations of Zn or other heavy metals should be ruled out before increased metallothionein gene expression is designated as a response to oxidative stress.

Growth factors. Three differentially expressed genes have potential relevance to the activity of myostatin, an inhibitor of muscle growth (23, 45). It was recently reported that serum myostatin levels increase with aging (43). Although there was no age-related difference in myostatin mRNA levels in the present study, confirming a previous report (37), it is possible that aging influences myostatin protein expression, post-translational processing, or the myostatin signaling pathway. Myostatin overexpression induces expression of cyclin-dependent kinase inhibitor 1A, also known as p21, and suppresses expression of cyclin-dependent kinase 2 (cdk2) (32, 45). Thus the increased p21 mRNA levels and reduced cdk2 mRNA levels in older muscle could reflect increased myostatin activity, although other factors may be responsible for altered expression of these genes. The gene with the greatest increase in expression in older muscle (3.6-fold) was follistatin, which binds myostatin and inhibits its activity (19). Increased expression of follistatin might be a feedback mechanism to restrain the effect of increased myostatin activity.

Serum IGF-I levels tend to decline with aging because of reduced growth hormone secretion (7). The older subjects in the present study had an average plasma IGF-I concentration that was 43% lower than that of the young men. We recently reported (37) that older men also tend to have lower IGF-I mRNA levels in muscle (determined by RT-PCR, mean expression ratio = 0.75, $P = 0.002$). The microarrays revealed the same trend for the subjects examined in the present study (probe set 209541_at, expression ratio = 0.76, $P = 0.059$). There was a modest increase in growth hormone receptor mRNA in older muscle, perhaps a feedback mechanism to mitigate the effect of reduced growth hormone secretion. Another finding relevant to growth hormone/IGF-I signaling was reduced expression in older muscle of suppressor of cytokine signaling 2 (SOCS-2, also known as STAT inhibitor 2). SOCS-2 expression is enhanced by growth hormone, and SOCS-2 mRNA levels in liver (but not muscle) were reported to be reduced by aging in rats (33). SOCS-2 appears to be part of a negative feedback mechanism whereby high growth hormone levels diminish tissue sensitivity to growth hormone or IGF-I by suppressing the signaling pathways. Mice lacking SOCS-2 resemble those with excessive growth hormone or IGF-I levels (26).

Genes relevant to protein metabolism. Several studies have indicated that older human muscle has a slower rate of protein synthesis (30, 42, 44), although Volpi et al. (35) did not observe slower muscle protein synthesis in older men. We did not detect any age-related differences in expression of genes encoding cytoplasmic translation initiation or elongation factors, but we did observe reduced expression in older muscle of mRNAs encoding mitochondrial ribosomal proteins. Slower protein synthesis would have to be offset by slower proteolysis or else the rate of muscle wasting with aging would be much more rapid than observed. Thus the increased expression in older muscle of several genes involved in the ubiquitin-proteasome pathway of protein degradation was unexpected, although it must be emphasized that increased expression of a few of the genes related to this pathway does not prove that the rate of proteolysis was affected. This finding is, however, consistent with the slightly elevated rate of leg proteolysis in older men that was reported by Volpi et al. (35). Increased expression of genes in the ubiquitin-proteasome pathway might be related to impairment of proteasome function caused by accumulation of lipofuscin in old muscle (31). It is noteworthy that there was no change with aging (expression ratio = 0.9) in expression of the mRNA encoding atrogin-1/MAFbx (F-box 32). This ubiquitin ligase is highly overexpressed in several rodent models of muscle atrophy (11), and mice deficient in this protein are resistant to muscle atrophy (4).

Genes potentially relevant to denervation or reinnervation of muscle fibers. Aging is associated with a loss of motor neurons (36). Denervated muscle fibers can be reinnervated by sprouting of axons from other motor neurons, a process that results in fiber type grouping and enlarged motor units in old muscle. Old muscles of mice (18) and monkeys (16) have increased expression of a few genes that might be related to denervation or reinnervation, although the specific genes identified were not the same across species. We did not observe altered expression of these genes in older human muscle. However, there was increased expression in older muscle of a gene involved in axonal elongation and fasciculation, FEZ2 (3), and one involved in motor neuron differentiation and survival, cysteine-rich motor neuron 1 (CRIM1) (17). It is possible that increased expression of these genes influences the reinnervation of denervated fibers, although their specific roles within skeletal muscle have not been defined.

Myosin heavy chain isoforms. Vastus lateralis contains slow-twitch (type 1) and fast-twitch (type 2) muscle fibers in approximately equal proportions. Aging generally is associated with greater atrophy of type 2a fibers than type 1 fibers (20). Thus some of the age-related differences may be secondary to an altered ratio of type 1 fiber mass to type 2 fiber mass, since some genes are differentially expressed in type 1 and type 2 fibers (5). However, there was not a statistically significant effect of aging on the expression of any of the mRNAs encoding the isoforms of myosin heavy chain (types 1, 2a, and 2x) that define fiber types.

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Although a decline with aging in the ratio of MyHC-2a expression to MyHC-1 expression has been reported (2, 40), this finding is not universal (21, 38). Most importantly, the patterns of MyHC isoform expression (at the mRNA level) were not significantly correlated with the overall gene expression profiles according to the SAM quantitative response method ($q \geq 20\%$ for all genes).

Final remarks. We have shown that aging affects the relative abundance of hundreds of gene transcripts. When averaged over all of the cells in the tissue, the changes are modest, generally less than 1.5-fold. Gene expression profiles are exploratory tools that can generate false leads, so the apparent effect of aging on any particular transcript should be considered tentative until confirmed in a prospective study.

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DISCLOSURES

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