Changes in Ubiquitin Proteasome Pathway Gene Expression in Skeletal Muscle With Exercise and Statins

Maria L. Urso, Priscilla M. Clarkson, Dustin Hittel, Eric P. Hoffman, Paul D. Thompson

Objective—Statins are safe medications but have side effects including myalgia and rhabdomyolysis. How statins provoke muscle damage is not known, but this effect is exacerbated by exercise.

Methods and Results—Healthy subjects took Atorvastatin (80 mg/daily) or placebo for 4 weeks. Biopsies of both vastus lateralis muscles were performed 8 hours after eccentric exercise (known to result in muscle soreness and damage) of the left leg at baseline and the right leg after statin/placebo treatment. Gene expression was determined using Affymetrix GeneChips, and selected genes confirmed by polymerase chain reaction (qRT-PCR). Atorvastatin had little effect on gene expression at rest. When combined with exercise, 56 genes were differentially expressed with 18% involved in the ubiquitin proteasome pathway (UPP) and 20% involved in protein folding and catabolism, and apoptosis.

Conclusion—This is the first investigation to our knowledge to implicate involvement of the UPP in skeletal muscle in response to combined exercise and statin treatment, possibly explaining the onset of myalgia with exertion. Statins may alter the response of muscle to exercise stress by altering the action of the UPP, protein folding, and catabolism, disrupting the balance between protein degradation and repair. (Arterioscler Thromb Vasc Biol. 2005;25:2560-2566.)

Key Words: gene expression ■ exercise ■ cardiovascular pharmacology
problems that would limit leg exercise, bleeding problems, or the use of medications that could increase bleeding such as aspirin. Subjects underwent screening (medical history and physical examination) to detect unreported abnormalities.

Study Overview

After a fasting blood sample, subjects performed 300 eccentric contractions using the left leg. The right leg served as a nonexercise control. Bilateral vastus lateralis muscle biopsies were obtained 8 hours after exercise. Subjects were then randomly assigned to treatment with Atorvastatin 80 mg/d (N = 4) or placebo (N = 4) for 4 weeks in a double-blind manner. Subjects were contacted twice weekly to ensure compliance and to determine medication side effects. After 4 weeks of treatment, subjects returned to the laboratory and repeated the 300 eccentric contractions using the previously un-exercised control (right) leg. Bilateral biopsy samples of the vastus lateralis muscles were again obtained 8 hours after the exercise. Thus, 4 biopsy samples were taken from each volunteer, with exercised and unexerted biopsies both before and after treatment.

Eccentric Exercise

To perform the 300 1-leg, eccentric (muscle-lengthening) contractions,\(^\text{14}\) subjects lowered themselves into a mechanical chair using only the exercised leg. Once seated, the subject was assisted to the standing position by the mechanical chair. Each eccentric action took four seconds, and the entire exercise lasted \(~30\) minutes.

Blood Samples and Muscle Biopsy Procedure

Blood samples were taken from an antecubital vein after a 10-hour fast before and after statin or placebo treatment and analyzed for serum cholesterol, LDL cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, and CK levels. Muscle biopsies were performed using a Bergstrom 5-mm biopsy needle with applied nitrogen (LN\(_2\)), and stored at \(-80^\circ\text{C}\).

The time point of 8 hours after exercise was selected for biopsy because previous work has shown that alterations in mRNA expression levels are detectable at this time point, and these alterations represent acute changes in the muscle in response to exercise-induced injury.\(^\text{14}\) We have previously shown robust changes in gene expression 8 hours after exercise using a similar eccentric exercise model in the leg of human volunteers not treated with statins and sought to examine if statins altered this response.\(^\text{14}\)

Biopsy Analyses

Microarray analysis was performed using Affymetrix GeneChip technology (Human Genome U133 Plus 2.0 Array) with standard operating procedures and quality control as recently described.\(^\text{15–17}\) Total RNA was extracted from biopsies using TRIzol reagent (GIBCO BRL, Invitrogen, Carlsbad, Calif). Ten \(\mu\text{g}\) of total RNA from each biopsy was converted into double-stranded cDNA by using SuperScript Choice system (GIBCO BRL, Invitrogen, Carlsbad, Calif) with an oligo-dT primer containing T7 RNA polymerase promoter (Genset). The double-stranded cDNA was purified by phenol/chloroform extraction, and then used for in vitro transcription using ENZO BioArray RNA transcript labeling kit (Affymetrix, Santa Clara, Calif). Biotin-labeled cRNA was purified by RNeasy kit (QIAGEN, Valencia, Calif), and fragmented randomly to \(~200\) bp (200 mmol/L Tris-acetate, pH 8.2, 500 mmol/L KOAc, 150 mmol/L MgOAc). cRNA samples of each biopsy were hybridized to a Human Genome U133 Plus 2.0 Array for 16 hours. Each microarray was washed and stained on the Affymetrix Fluidics Station 400, using Affymetrix (Affymetrix, Santa Clara, Calif) instructions and reagents.

Microarray Data Analysis

Absolute analysis of Affymetrix “raw” data were conducted using dCHIP. Scanned chip images were first scaled to a target intensity of 150 to account for variations in cRNA target hybridization efficiencies. Background and scaled noise were similarly averaged for all chips before analysis. The 18 400 transcripts represented on the Affymetrix Human U133 oligonucleotide array describe 14 500 well-characterized genes and 400 expressed sequence tags. In all, 22 000 probe sets were analyzed using 22 oligonucleotide probes (11 distributed probe pairs) for each probe set, producing multiple independent measurements for each gene. Comparison of the hybridization signal from the perfect match (PM) and mismatch (MM) probes allows for a specificity measure of signal intensity and elimination of most nonspecific cross-hybridization signal. Values of intensity differences as well as ratios of each probe pair were used to determine (statistically) whether a gene was called present (P) or absent (A). Data analysis required \(\geq30\%\) of profiles to show a present call for that gene to be carried into the next analysis. GeneSpring 5.1 (Silicon Genetics, Redwood City, Calif) was then used to filter potential candidate genes for statistical significance and using a paired t-test, only genes with \(P<0.005\) were retained for further analysis. All statistical analyses were performed using a longitudinal design, in which each subject served as his/her own control. This approach removes inter-individual variation because of genetic heterogeneity. Combined, these criteria increase the reliability of our microarray analysis allowing us to reduce false-positives.

Quantitative Real Time–Polymerase Chain Reactions

Total RNA was isolated and purified according to manufacturer’s instructions using an RNAeasy kit (Qiagen, Valencia, Calif). Total RNA was then reverse-transcribed into double-stranded cDNA using a SuperScript III First-Strand Synthesis kit (Invitrogen, Carlsbad, Calif). LUX™Fluorogenic Primers (Invitrogen) were used for all qRT-PCRs. FAM-labeled primers for FBX32 (Atrogen-1), UBE2M, FBXO3, and RING128 (Invitrogen) were designed using the Primer Express program v 2.0 (Table 1) (Applied Biosystems). Serial dilutions were performed on each sample (1/1 to 1/243). Quantification of mRNA was directly related to fluorescence of the respective probe after 40 cycles of amplification on the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif). Thermal cycling conditions were as follows: 50°C for 2 minutes for the UDGR reaction; 95°C for 2 minutes for UDGR inactivation/template denaturation; 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 30 seconds for denaturation, hybridization, and elongation, respectively. A melting curve analysis was performed at the end of each reaction.

JOE-labeled TATA box binding protein (TBP) (Invitrogen cat#104H-02) was first validated in muscle biopsy samples from placebo and statin subjects before being used as a reference standard. TBP is a constitutively expressed housekeeping gene that has been

### Table 1. Primer Sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (3’ to 5’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBX32</td>
<td>GACTGTCGTTCAAAATCCAGAGGTGAC</td>
<td>GCTAAAGGTGCTACGTCGTC</td>
</tr>
<tr>
<td>UBE2M</td>
<td>CGTCCTCCATCTCTCATC</td>
<td>CGTCCTCCATCTCTCATC</td>
</tr>
<tr>
<td>FBXO3</td>
<td>GTAACCTTTGACCGGACCTGTTGGATAC</td>
<td>TCCGACCAACAGTTGAAAA</td>
</tr>
<tr>
<td>RING128</td>
<td>CACTGATGGATGAGGATGGATCAGG</td>
<td>ATACCCGAGGAGGAGTACTTCTT</td>
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</table>
validated in previous human studies. Relative quantitation of amplified mRNA was normalized to JOE-labeled TBP, compensating for variations in quantity as well as for differences in RT efficiency. Quantitation of mRNA for FBX32 (Atrogin-1), UBE2M, FBXO3, and RING128 was calculated using the standard curve method.

Statistical Analysis
In addition to the statistical analysis of the microarrays described, a paired t test was used to compare changes in blood levels and to compare qRT-PCR gene expression results between statin and placebo subjects. Statistical significance was set at \( P < 0.05 \).

Results

Subjects
Placebo and statin subjects (mean±SEM) were of similar age (22.75±0.53 versus 24.50±1.57 years), height (1.82±0.04 versus 1.80±0.02 m), and body weight (83.98±0.78 versus 77.61±2.36 kg), respectively.

Blood Measures
There were no differences between baseline CK levels in the placebo and statin group, respectively (161.3±45.2 IU versus 166.3±38.1 IU). CK values did not change with treatment in the placebo group (153.8±34.8 IU) and were not significantly higher with treatment in the statin subjects (256.5±86.1 IU).

Total cholesterol, HDL cholesterol, LDL cholesterol, and triglyceride values were not different between the placebo and statin group at baseline. With treatment, total cholesterol decreased in the statin group (151.8±30.2 mg/dL to 100.5±25.11 mg/dL; \( P = 0.005 \)), whereas there were no changes in the placebo group (204.5±51.7 mg/dL to 180±31.4 mg/dL; \( P = 0.2 \)). HDL cholesterol decreased in the statin group (50±4.8 mg/dL to 47.8±3.9 mg/dL; \( P = 0.04 \)) with no change in the placebo group (50.8±10.4 mg/dL to 46.5±11.6 mg/dL; \( P = 0.3 \)). LDL cholesterol levels decreased in the statin group (83.1±25.1 mg/dL to 37.1±20.6 mg/dL; \( P = 0.005 \)) with no change in the placebo group (119±37.1 mg/dL to 115.0±30.1 mg/dL; \( P = 0.7 \)). There were no differences in triglyceride values in the statin or placebo group before or after treatment (\( P = 0.2 \)).

Effects of Time/Limb on Gene Expression
We compared the right (nonexercised; baseline) versus the left (nonexercised; after 4 weeks of treatment) leg in the placebo-treated subjects. Of the 38,500 genes present on the Affymetrix U133 Plus 2.0 Array, only 2 genes were differentially expressed between the right and left nonexercised leg using threshold criteria (\( P < 0.005 \), fold-change >1.5). These results suggest low physiological variation of gene expression over time and between the right and left legs. Expression of phosphoinositide-3-kinase regulatory subunit (PIK3R1), an intracellular signaling molecule, increased 1.87-fold, and thyroid hormone receptor-associated protein (TRAP95), a transcription factor, increased 1.92-fold.

Effects of Exercise Alone, Statin Alone, and Statin Plus Exercise on Gene Expression
Results for the effects of exercise alone in the placebo group showed that 80 genes were differentially expressed in the eccentrically exercised leg as compared with the nonexercised leg of the placebo subjects (\( P < 0.005 \), fold-change >1.5). These genes were most frequently involved in cell cycling/growth and transcription according to their gene ontology biological function classification as determined using the gene ontology database (http://www.NetAffx.com; Figure 1a). These genes were also found to be altered by eccentric exercise in a previous study from our laboratory.

Results for the effects of statin treatment alone (no exercise) on gene expression showed that only 5 genes were differentially expressed in the statin treated leg as compared with the nonstatin-treated leg indicating that statin treatment without an exercise provocation has little effect on skeletal muscle gene expression. Two genes were downregulated and 3 were upregulated (Table 2).

Results for the effects of statin treatment plus eccentric exercise on gene expression showed that 56 genes were
Differentially expressed (Table I, available online at http://atvb.ahajournals.org). These genes are grouped into 11 biological function categories as determined using the gene ontology database. Statin treatment plus eccentric exercise had the greatest effect on transcription factors and genes involved in the UPP (Figure 1b).

In comparison to exercise alone, statin treatment plus eccentric exercise also produced a marked reduction in the number of genes involved in apoptotic and inflammatory processes and an increase in the number of genes involved in protein catabolism, independent of the UPP (Figure 1a and 1b).

**qRT-PCR Analysis**

Because of the limited amount of tissue, we carefully chose genes for qRT-PCR that would provide new insight regarding the effects of statin and exercise. We chose to study the UPP genes for qRT-PCR that would provide new insight regarding the effects of statin and exercise. We chose to study the UPP because the UPP is tightly regulated and responsible for the recognition and degradation of the majority of proteins in intracellular signaling,1.76 0.0036 Calmodulin 3 CALM3 Calcium ion binding 1568623_a_at

**TABLE 2. Effects of Statin Treatment Alone on Gene Expression**

<table>
<thead>
<tr>
<th>Category</th>
<th>Fold Change</th>
<th>P Value</th>
<th>Gene Title</th>
<th>Gene Symbol</th>
<th>Biological Process</th>
<th>Probe ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transport</td>
<td>1.86</td>
<td>0.0020</td>
<td>Hemoglobin, gamma G HBG2</td>
<td></td>
<td>Oxygen transport</td>
<td>202388_at</td>
</tr>
<tr>
<td>Intracellular signaling</td>
<td>1.76</td>
<td>0.0036</td>
<td>Calmodulin 3 CALM3</td>
<td></td>
<td>Calcium ion binding</td>
<td>1568623_a_at</td>
</tr>
</tbody>
</table>
| Metabolism                | −2.22       | 0.0050   | Glucosamine-6-phosphate isomerase GPNI Glucosamine catabolism 201149_s_at
| Ubiquitin proteasome path | 1.58        | 0.0043   | Putative tumor suppressor 101F6 Ubiquitin conjugating enzyme activity 205378_s_at
| Other                     | −2.64       | 0.0044   | myc-induced nuclear antigen MINA53 Other 223366_at

This study examined gene expression changes in response to exercise and statin treatment in eight volunteers. There are 4 primary findings from the microarray experiment. First, there was little difference in global gene expression between the nonexercised legs in placebo subjects. Second, eccentric exercise alone increased the expression of many genes, especially those involved in cell cycle, growth and transcription. These results are similar to a previous study in our laboratory that used a similar eccentric exercise model in the leg.15 Third, statin treatment without exercise produced little alteration in gene expression. And fourth, statin treatment plus exercise produced substantial changes in gene expression compared with statin treatment alone or exercise alone; this analysis showed that statin plus exercise considerably affected the UPP (Figure 1b). This finding is significant because the UPP is tightly regulated and responsible for the recognition and degradation of the majority of proteins in skeletal muscle. Because the UPP has not been identified as being altered by statin treatment, we chose to further explore this important muscle protein regulatory pathway using qRT-PCR.

In response to eccentric exercise alone, 34% of the gene alterations were related to cell cycle/growth and transcription, whereas only 8% were related to the UPP (Figure 1a). Eccentric exercise has previously been shown to induce the upregulation of genes involved in regeneration and repair and result in an overall gene expression profile indicative of protein accumulation. Thus, there is a distinct transcriptional profile in skeletal muscle in response to eccentric exercise, which is characterized by the expression of genes involved in the progression of the cell cycle. In contrast, in response to disuse, denervation, and muscle damage, previous studies have found that mRNAs for the expression of UPP components are increased 2- to 3-fold, reflecting an increased rate of protein breakdown.20,22,24–26

Surprisingly there were only small changes in genes affecting cholesterol metabolism. Expression of the LDL receptor gene decreased in exercised muscle with statin therapy, but <1.25-fold (P>0.01) and this change did not meet our strict criteria for inclusion. Also, although some have suggested that statin myopathy is produced by mitochondrial dysfunction,13,27 we found little change in genes encoding for mitochondrial proteins. The four mitochondrial genes that were differentially expressed in our dataset were all downregulated, although fold changes were modest (−1.1 to 1.4) and did not meet our strict criteria for inclusion.

**Discussion**

This is the first study to examine the effects of statins and exercise on global gene expression using a microarray analysis of skeletal muscle. Statins and exercise have been shown to have additive effects on insulin resistance, cardiovascular health, and skeletal muscle.14–16 This study examined gene expression changes in response to exercise and statin treatment in eight volunteers. There are 4 primary findings from the microarray experiment. First, there was little difference in global gene expression between the nonexercised legs in placebo subjects. Second, eccentric exercise alone increased the expression of many genes, especially those involved in cell cycle, growth and transcription. These results are similar to a previous study in our laboratory that used a similar eccentric exercise model in the leg.15 Third, statin treatment without exercise produced little alteration in gene expression. And fourth, statin treatment plus exercise produced substantial changes in gene expression compared with statin treatment alone or exercise alone; this analysis showed that statin plus exercise considerably affected the UPP (Figure 1b). This finding is significant because the UPP is tightly regulated and responsible for the recognition and degradation of the majority of proteins in skeletal muscle. Because the UPP has not been identified as being altered by statin treatment, we chose to further explore this important muscle protein regulatory pathway using qRT-PCR.

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Although other probe sets in 3/H11032 but only one was included in our dataset after 0.005. These results suggest that the dose of statin used in the present study has little effect on resting skeletal muscle gene expression in healthy, asymptomatic subjects. However, these results should be interpreted with caution as there was insufficient power to rule out potentially important biological effects on gene expression that did not meet our 1.5-fold and P<0.005 criteria.

In contrast to the results of exercise alone or statin treatment alone, exercise plus statin treatment produced considerable changes in gene expression. Importantly, 18% of the alterations occurred in genes involved in the UPP (Figure 1b). We decided to explore 4 of these genes: FBX32, RING128, and UBE2M, which are involved in the UPP.

The first step in the pathway is the activation of the ubiquitin molecule by the ubiquitin-activating enzyme (E1). Once activated by the E1 enzyme, the ubiquitin molecule binds to the ubiquitin-conjugating enzyme (E2; eg, UBE2M), which is a ubiquitin carrier protein. E2 conjugating enzymes then interact with a 4-subunit complex of proteins called the “ubiquitin protein ligase.” Ubiquitin protein ligases (or E3 ligases) are called SCF complexes as they are composed of Skp1, Cul-1, and an F-box family protein. The F-box proteins confer substrate specificity to the ubiquitin protein ligase, and there are many forms that are both tissue- and condition-specific. The best characterized muscle F-box protein is FBX32, also called Atrogin-1, and this F-box is strongly induced by any stimulus that induces muscle atrophy (deneration, starvation, corticosteroids, inactivity) and is thought to be a key effector of muscle degradation during catabolic states.28

There are 6 different FBX32 probe sets on the U133 Plus 2.0 gene chip but only one was included in our dataset after screening for number of present calls and fold change. Although other probe sets in 3’ adjacent regions (225801_at, 225328_at, 225345_at) and alternatively spliced transcripts (225803_at) were downregulated, each provided a number of absent calls and low signals and probability values were high ranging from 0.02 to 0.3. The probe set which met our stringent criteria (241762_at) detects an intronic transcript of the FBX32 gene.

Likely more biologically significant is the 4-fold increase we observed in the ubiquitin ligase FBX3 (also called FBX3). FBX3 is also an SCF complex component. The significant upregulation of FBX3 after statin treatment and exercise implies that the ubiquitin ligase machinery is altered, possibly affecting the balance between protein degradation and repair. Because there are many known F-box proteins, and FBX3 has a high sequence homology to other F-box proteins, additional work needs to be performed to identify the particular role of the FBX3 protein in muscle. Furthermore, we need to identify specifically how changes in FBX3 expression levels with statin treatment and exercise affects protein degradation and repair in skeletal muscle.

There have been many recent publications of human muscle expression profiling in both volunteers and neuromuscular disease patients (see http://pepr.cnmresearch.org), and this typically affords comparative studies between different conditions. However, the alternatively spliced isoform of FBXO3 seen strongly upregulated and validated by qRT-PCR in the current study does not have probe sets on the commonly used U95A or U133A microarrays. We were able to query the FBXO3 refseq transcript (218432_at; http://www.genome.ucsc.edu) in our previous muscle projects, and we found that this transcript was highly expressed in most human muscle studies and was observed to significantly increase during a 96 hour de-training in women29,30 (http://pepr.cnmresearch.org). Four muscle biopsies were performed: 1 at study entry, after 9 months of aerobic training at high intensity (24 hours after last bout of training), and during 2 de-training time points (96 hours, 2 weeks).29,30 The FBXO3 expression levels were 593.5±92.6 (mean±SE) at study entry, 605.8±34.8 after 9 months of training, 751.4±56.4 at 96 hours of de-training, and 748.9±123.4 after 2 weeks de-training. The observed increase in FBXO3 in skeletal muscle in response to detraining, lends support to our speculation that the increase observed after statin treatment and exercise may be indicative of muscle protein breakdown via ubiquitin ligase machinery.

Excessive exercise stimulates adaptation of skeletal muscle via cytoskeletal remodeling and alterations in protein turnover. The selective degradation of proteins following eccentric exercise is regulated in part by the UPP, although more complex muscle structures such as actomyosin and myofibrils must first be cleaved by proteases not involved in the UPP.31 The increased expression and number of genes involved in protein catabolism we observed with eccentric exercise, exclusive from UPP genes, are consistent with previous reports that document alterations in gene expression after eccentric exercise.32 We hypothesize that instability of the skeletal muscle cell membrane may be an effect of statin treatment, and when the integrity of the membrane is challenged in response to a bout of eccentric exercise, proteolytic cascades are activated in muscle. This would explain the increase in the number of genes involved in protein catabolism, as well as lend support to the increase in UPP genes. Future work should explore the effects of statin treatment and exercise on proteolytic pathways in muscle independent of the UPP.

We chose to confirm the genes in the UPP because of the large number of genes that were changed in this pathway, and the novelty of this finding. Changes in expression of other genes were not confirmed by qRT-PCR because of tissue availability. There were marked reductions in response to exercise and statin treatment in the expression of genes encoding for proteins involved in inflammatory and apoptotic processes. These findings are consistent with previous observations that statins inhibit the synthesis of inflammatory mediators and may also reduce peripheral markers of inflammation when combined with exercise.33-35 The decline in apoptotic gene expression in the present study may result from the reduced inflammatory response and, thus, attenuated programmed cell death. We hypothesize that the reduction in the expression of inflammatory response and apoptotic genes that we report here with exercise and statin treatment, suggests an additive, suppressive effect of statin treatment and eccentric exercise on the inflammatory response. Further studies using appropriate direct techniques, such as qRT-
PCR, will be needed to confirm this proposed suppression of the inflammatory response.

In the present study, statin treatment did not produce myalgia, although there were marked alterations in gene expression with the stress of exercise. This is not surprising because the incidence of myalgia is low in most studies suggesting that the muscles of most individuals treated with statins can adjust to their effect.

There are several limitations to the present study. Only 4 subjects were included in the statin treated and placebo groups, but such sample sizes are typical of gene expression studies because of the complexity of the analysis,30,36–37 The longitudinal design and the use of each subject as their own control greatly reduces the inter-individual baseline heterogeneity.29,30 Moreover, our use of standard operating procedures and quality control metrics reduces variability to a negligible level.15,18 Our finding of virtually no difference in gene expression between the control legs (before versus after placebo treatment) supports the stringency of the microarray results. Also, genes of interest that were identified in the microarray data set were confirmed by qRT-PCR. None of the present subjects had myalgia or other signs of statin myopathy during the study either because of their general good health or the limited time of statin exposure. Consequently, we cannot state that similar expression changes occur in myopathic patients, although this issue is presently under examination.

In conclusion, our study suggests that FBXO3 and other members of the UPP are transcriptionally activated in response to statins plus exercise, but are not induced by statins or exercise alone. Further studies are needed to determine the specificity of upregulation of the alternatively spliced isoform of FBXO3 for statins plus exercise. However, our data suggest that this isoform of FBXO3 is a strong candidate gene for mediating statin plus exercise alterations of muscle ubiquitin ligase machinery. Moreover, the role of FBXO3, and its relationship to the better-studied FBX32 (Atrogin-1) protein requires detailed study.

Acknowledgments

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29. Clarke J, Mitchell-Lewis, who assisted with the qRT-PCR.

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