Original article

MOLECULAR METABOLISM

Global mRNA sequencing of human skeletal muscle: Search for novel exercise-regulated myokines

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ABSTRACT

Objective: Skeletal muscle is an important secretory organ, producing and releasing numerous myokines, which may be involved in mediating beneficial health effects of physical activity. More than 100 myokines have been identified by different proteomics approaches, but these techniques may not detect all myokines. We used mRNA sequencing as an untargeted approach to study gene expression of secreted proteins in skeletal muscle upon acute as well as long-term exercise.

Methods: Twenty-six middle-aged, sedentary men underwent combined endurance and strength training for 12 weeks. Skeletal muscle biopsies from *m. vastus lateralis* and blood samples were taken before and after an acute bicycle test, performed at baseline as well as after 12 weeks of training intervention. We identified transcripts encoding secretory proteins that were changed more than 1.5-fold in muscle after exercise. Secretory proteins were defined based on either curated UniProt annotations or predictions made by multiple bioinformatics methods.

Results: This approach led to the identification of 161 candidate secretory transcripts that were up-regulated after acute exercise and 99 that where increased after 12 weeks exercise training. Furthermore, 92 secretory transcripts were decreased after acute and/or long-term physical activity. From these responsive transcripts, we selected 17 candidate myokines sensitive to short- and/or long-term exercise that have not been described as myokines before. The expression of these transcripts was confirmed in primary human skeletal muscle cells during *in vitro* differentiation and electrical pulse stimulation (EPS). One of the candidates we identified was macrophage colony-stimulating factor-1 (CSF1), which influences macrophage homeostasis. CSF1 mRNA increased in skeletal muscle after acute and long-term exercise, which was accompanied by a rise in circulating CSF1 protein. In cultured muscle cells, EPS promoted a significant increase in the expression and secretion of CSF1.

Conclusion: We identified 17 new, exercise-responsive transcripts encoding secretory proteins. We further identified CSF1 as a novel myokine, which is secreted from cultured muscle cells and up-regulated in muscle and plasma after acute exercise.

Keywords Exercise; Myokine; Colony stimulating factor 1; RNA sequencing; Skeletal muscle secretome

1. INTRODUCTION

Skeletal muscle was recognized as a secretory organ about 15 years ago [1]. Proteins and peptides produced by and released from skeletal muscles are termed myokines, and several myokines play important roles in muscle physiology as well as in tissue cross talk [2–4]. Thus, interest in the secretory function of the skeletal muscle has increased markedly during the last decade. Physical activity alters the secretion of many myokines, several of which may play a role in mediating beneficial health effects of physical activity.

Interleukin 6 (IL6) is the most extensively studied myokine, and is secreted from skeletal muscle during acute physical activity [5]. IL6 may act as an energy sensor in skeletal muscle during exercise, promoting increased hepatic glucose output and enhanced glucose uptake in muscle cells [6].

More than 100 myokines have been identified [7], and the skeletal muscle secretome is predicted to include more than 300 proteins [8]. In several proteomics studies, scientists have identified peptides in medium conditioned by cultured human [9–12] or murine myocytes [13–16]. To explain some of the positive effects of physical activity, several studies focused on identifying myokines that are elevated in response to exercise or muscle contraction [11,17–20].

The aim of this study was to identify novel myokines regulated by acute or long-term exercise. Many myokines, such as IL6 and other cytokines, have a low abundance in basal conditions, and are therefore hard to detect with untargeted proteomics. We used global mRNA sequencing to detect all genes expressed in human skeletal muscle

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biopsies. In addition, we used cultures of primary human skeletal muscle cells to monitor the expression of novel myokine candidates during differentiation and after electrical pulse stimulation (EPS).

2. METHODS

2.1. Human trial

A human exercise intervention trial (NCT01803568) was performed as described before [21]. The National Committee for Research Ethics North (Tromsø, Oslo, Norway) approved the trial. This study adhered to the standards set by the Declaration of Helsinki.

Briefly, 26 sedentary (<1 bout of exercise/week during the previous year) men aged 40–65 y with BMI 26 ± 4.0 kg/m^2, were included in the trial. They were initially recruited to a control group (n = 13) with normal glucose metabolism and a dysglycemic group (n = 13) with fasting serum glucose ≥ 5.6 mmol/L and/or 2 h glucose ≥ 7.8 mmol/L based on an oral glucose tolerance test. Two subjects had normal glucose levels at screening, but both had a glucose infusion rate of 4.4 mg min^{-1} kg^{-1} during the euglycemic hyperinsulinemic clamp and were included in the dysglycemic group. Here we did not investigate group differences, and all subjects were therefore included (n = 26).

The participants underwent 12 weeks of supervised exercise training with two interval sessions (bicycle) and two whole-body strength-training sessions per week [21]. Bicycle tests (45 min at 70% of VO_2max) were conducted before and after the long-term training intervention [21]. Blood samples and biopsies from *m. vastus lateralis* were collected before, immediately after, and 2 h after the acute bicycle tests (Figure 1A).

2.2. High throughput mRNA sequencing

RNA was isolated from muscle biopsies and reverse-transcribed into cDNA. RNA integrity was determined using Agilent RNA 6000 Nano Chips and a Bioanalyzer 2100. Deep sequencing was performed with the Illumina HiSeq 2000 system with multiplexed design [22]. The cDNA was fragmented, and cDNA fragments with 51 bp nucleotides were selected and amplified. Tophat 2.0.8 with Bowtie 2.1.0 was used (with default settings) to align the RNA-seq reads against the UCSC hg19 annotated transcriptome and genome [23,24]. EdgeR v3.4.2 [25] was used for gene filtering, normalization, and calculation of p-values using a negative binominal generalized linear model in R v3.0.3 (R Core Team 2014). Correction for multiple testing was performed by using Benjamini-Hochbergs false discovery rate (FDR) control [26], set at FDR < 10%. The dataset generated from RNA-seq has been used in several other publications, including one study where gene expression data for extracellular matrix (ECM) genes were reported [27]. To compare our data on CSF1 with other published data sets on skeletal muscle and exercise, we analyzed two data sets [28,29]. Arrays were analyzed using the R package Oligo v1.36.1 following standard procedures for quality checks and calculation of normalized expression values using robust multi-array average. For differential gene expression analyses we used the LIMMA v3.20.9.

2.3. Identification of exercise-regulated transcripts encoding secretory proteins

We selected all transcripts of single genes that were up- or down-regulated more than 1.5-fold after acute or long-term exercise training. "Fast-responsive transcripts" were up/down-regulated just after the acute bicycle test (A2/A1 and/or B2/B1, Figure 1A–C), whereas "slow-responsive transcripts" were regulated after 2 h (A3/A1 and/or B3/B1, Figure 1A,D,E). The effect of long-term exercise training was assessed as the mRNA expression at B1 vs. A1 (Figure 1A,F).

To identify transcripts encoding secreted proteins, we used the MetazSecKB knowledgebase [30]. MetazSecKB identifies secretory proteins based on either curated evidence of secretion (annotated and reviewed in the UniProtKB/Swiss-Prot dataset) or being "highly likely" to be secreted based on computationally predicted secretory protein sequences, without containing transmembrane domains or endoplasmic reticulum (ER) retention signals, by several tools (SignalP4, Phobius, TargetP and WoLF PSORT).

2.4. Cell culture

Biopsies from either *m. obliquus internus abdominis* or *m. vastus lateralis* from three male donors (age 33–62 y) were used to isolate primary human satellite cells [31]. Myoblasts were proliferated to passage 4–5 and differentiated [27]. EPS (1 Hz, 2 ms, 11.5 V) was applied to the cells after 5 days of differentiation for 24 h or after 6 days of differentiation for 1–6 h. EPS should mimic muscle contraction similar to *in vivo* exercise [32]. Supernatants were collected and RNA isolated for further analysis.

2.5. Protein analyses

We measured CSF1 concentration in the supernatant of cultured myotubes and plasma samples using a human CSF1 ELISA Kit (DMC00B; R&D Systems Minneapolis; Minnesota, United States). Total protein concentration of plasma was measured by BC Assay (Protein assay kit, Uptima, Montlucon, France).

2.6. RNA isolation, cDNA synthesis and gene expression analyses

Total RNA was isolated from cultured cells using the RNeasy Mini kit (Qiagen, Hilden, Germany). RNA was reversely transcribed into cDNA using the High Capacity cDNA Revers Transferase kit (ThermoFisher, Foster City, California, United States). Quantitative real-time PCR was performed using the CFX96™ Real-Time System (Bio-Rad, Hercules, California, USA). The following pre-designed primers and probe sets were used (TaqMan assays; ThermoFisher, Foster City, California, United States) RPLP0 (Hs99999902_m1), MTRNR2L4 (Hs04276154_s1), SMPDL3A (Hs00378308_m1), FAM20C (Hs00398243_m1), WNT9A (Hs00243321_m1), TEK (Hs00945155_m1), FLT1 (Hs01052961_m1), CSF1 (Hs00174164_m1), C8G (Hs01113922_g1), CHSY1 (Hs00208704_m1), IL4R (Hs00166237_m1), LCN10 (Hs01596612_g1), STC2 (Hs00175027_m1), THBS4 (Hs00170261_m1), IGFBP2 (Hs01040719_m1), KAZALD1 (Hs00368867_g1), TNFRSF25 (Hs00600930_g1), and SCT (Hs00360814_g1). Relative target mRNA expression levels were calculated as $2^{-\Delta Ct}$ by normalizing to the expression of *RPLP0*.

3. RESULTS

3.1. Fast exercise-responsive transcripts

To identify myokines up-regulated by acute exercise, we evaluated gene transcription in skeletal muscle biopsies immediately after two acute, 45 min bicycle tests (Figure 1A). After the first bicycle test (A2/A1), 551 transcripts were more than 1.5-fold up-regulated, and 97 of these were classified as secretory (Figure 1B, Supplementary Table 1). After the second bicycle test (after the exercise intervention; B2/B1), 587 transcripts were enhanced (>1.5-fold), and 108 of these encode secretory proteins (Figure 1C, Supplementary Table 2). In total, we identified 117 secretory transcripts that were increased immediately after acute exercise (>1.5-fold at A2/A1 and/or B2/B1, Figure 2A, Table 1). There was extensive overlap in expression pattern between

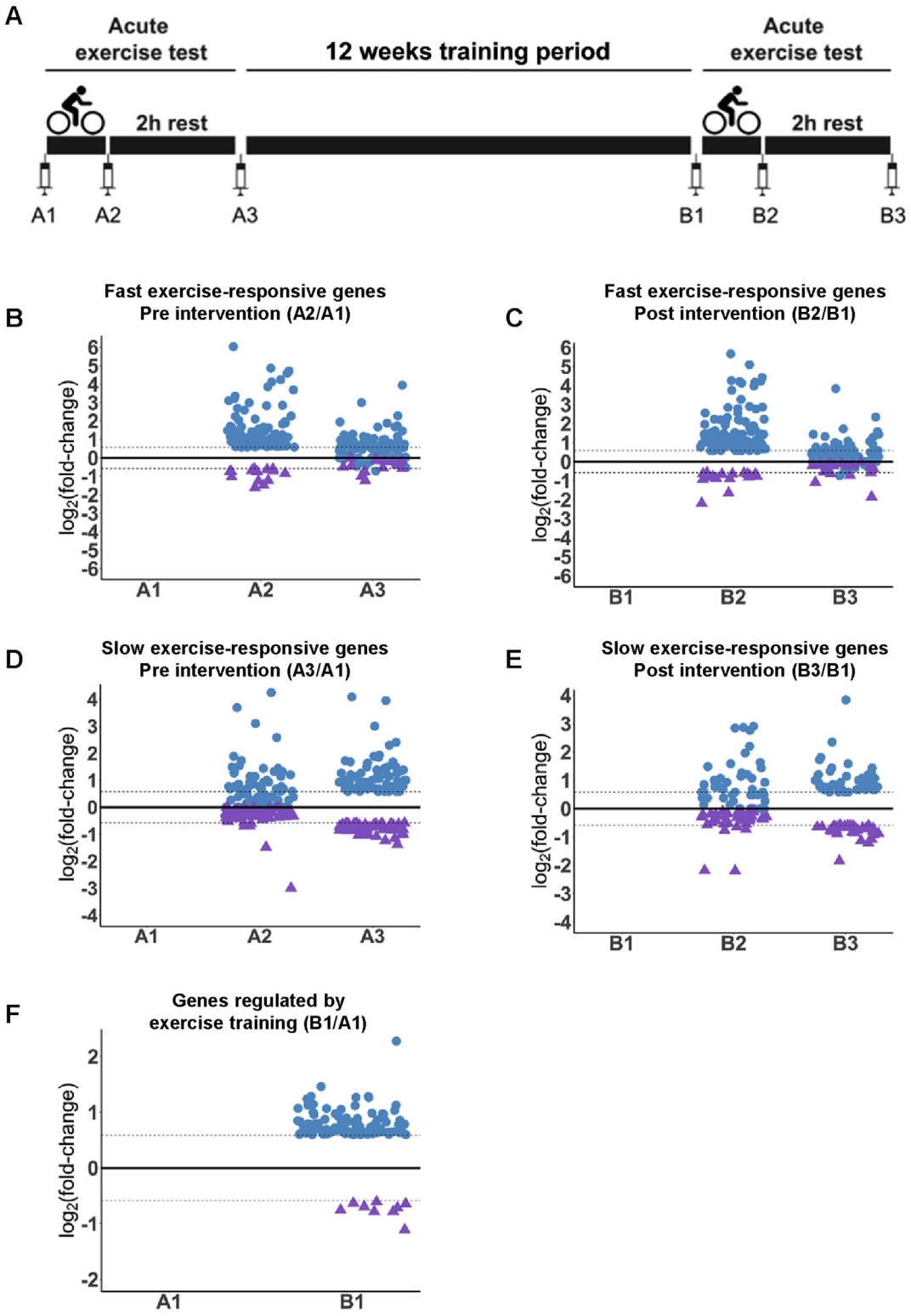

Figure 1: A) Overview of the study design. Skeletal muscle biopsies and blood samples were harvested before (A1, B1), immediately after (A2, B2) and 2 h after (A3, B3) the end of the bicycle sessions. B–F) Secretory genes up- or down-regulated >1.5-fold at one or several time-points after acute or long-term exercise. Log2 (FC) from baseline (A1 or B1). Blue dots represent up-regulated genes, purple triangles represent down-regulated genes. B) Genes up- or down-regulated >1.5-fold at A2/A1. C) Genes up- or down-regulated >1.5-fold at B2/B1. D) Genes up- or down-regulated >1.5-fold at A3/A1. E) Genes up- or down-regulated >1.5-fold at B3/B1. F) Genes up- or down-regulated >1.5-fold after 12 weeks exercise training (B1/A1).

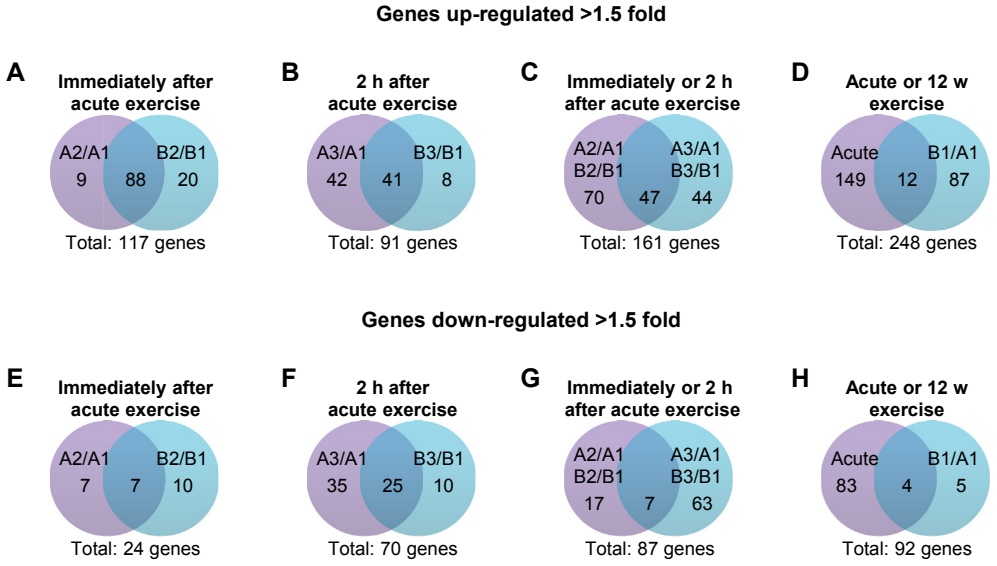

Figure 2: Venn diagrams showing the number of secretory genes that were up- or down-regulated >1.5-fold at different time points after acute and/or long-term exercise.

the two bicycle tests; 88 transcripts were detected after both tests (Figure 2A). Furthermore, 95 of the 97 genes detected after the first test were also up-regulated after the second test (FC > 1, p-value < 0.05), although not necessarily to above the 1.5-fold cut-off (Supplementary Table 1).

Many of these fast-responding transcripts encode cytokines or chemokines, with *IL6* being the most up-regulated gene. Other examples were *IL1B, CXCL1, CXCL2, CXCL3* and *CXCL8, CCL2*, and *CCL8*. These cytokines and chemokines were typically expressed at very low levels at baseline, but were markedly increased after exercise (Table 1). Furthermore, most of them returned to basal levels after 2 h rest.

Other fast-responding transcripts encode growth factors (*CTGF, FGF6, FGF18, PDGFA, PDGFB, TGFB3, VEGFA*, and *VEGFC*) or proteases and protease inhibitors involved in ECM remodeling (*ADAMTS4, ADAMTS1, PLAU, MMP19, ADAM8,* S*ERPINE1, SERPINH1, SERPINA3*, and *TIMP1*). We have previously published and discussed results related to ECM [27].

Only a few secretory transcripts were down-regulated after acute exercise; 14 and 17 transcripts were identified after the first (A2/A1) and second (B2/B1) bicycle test, respectively (Figure 2E, Supplementary Tables 3 and 4).

3.2. Slow exercise-responsive transcripts

Two hours after the first bicycle test (A3/A1), 501 transcripts were increased >1.5-fold, and of these, 83 were classified as secretory (Figure 1D, Supplementary Table 5). Furthermore, 274 transcripts were up-regulated 2 h after the second bicycle test (B3/B1), and 49 of these were secretory (Figure 1E, Supplementary Table 6). In total, we detected 91 secretory transcripts that were increased 2 h after either of the two tests (Table 2, Figure 2B). Some of these slow-responsive transcripts encode cytokines and growth factors (*INHBE*, *TGFB3*, *CLCF1*, *VEGFA* and *CCL2*) and proteases and protease inhibitors (*PLG, SERPINA1, SERPINA3, SERPINF2, ADAMTS8, ADAMTS9* and *ADAM8*). The gene expression response was generally stronger after the first bicycle test as compared with the second; a higher number of transcripts were identified after the first bicycle test (Figure 2B). Furthermore, of the 91 secretory transcripts detected, 77 increased more after the first test as compared with the second test (Table 2). Still, there was a substantial overlap between the results; 71 of the 83 transcripts detected after the first bicycle test were also significantly increased after the second test (FC > 1, p < 0.05, Supplementary Table 5).

Several secretory transcripts were down-regulated 2 h after bicycling; 60 and 35 secretory transcripts were decreased (>1.5-fold) after the first (A3/A1) and second (B3/B1) bicycle test, respectively (Figures 1D,E,2F, Supplementary Tables 7 and 8).

3.3. Transcripts regulated after long-term training

After 12 weeks exercise intervention, 289 transcripts were increased >1.5-fold in skeletal muscle (B1/A1). Of these, 99 were classified as secretory (Figure 1F, Table 3, Supplementary Table 1). The transcript encoding matrix-remodeling associated protein 5 (*MXRA5*) had the highest relative increase (2.8-fold). *SPARC* exhibited the highest expression level at baseline (71.5 FPKM) and was increased 1.8-fold after the intervention.

A large proportion of the up-regulated transcripts after long-term training were related to ECM (Table 3). We identified 10 collagens (e.g. collagen type I, III, IV), proteoglycans (e.g. *AGRN*, *LUM* and *BGN*) and a variety of ECM glycoproteins (e.g. *LAMB1*, *SPARC*, *NID1* and *ELN*). Some of these data were recently reported and discussed in another publication [27].

Only 9 transcripts encoding secretory proteins were decreased >1.5-fold in skeletal muscle after 12 weeks of intervention (B1/A1) (Figure 1F, Supplementary Table 10). One of these transcripts encodes *MSTN* (FC = −1.7), which is a negative regulator of muscle growth. This result was recently reported and discussed in another publication [33].

3.4. Transcripts up-regulated after both acute and long-term exercise

In total, we detected 161 unique secretory transcripts that were up-regulated after acute exercise (post-immediate and/or after 2 h; Figure 2C) and 99 transcripts that were increased after 12 weeks exercise intervention. We detected 12 genes that were up-regulated >1.5-fold after both acute and long-term exercise (Figure 2D). However, a large number of acute genes were also significantly increased

Table 1 — Transcripts up-regulated just after 45 min acute exercise of 70% of VO_2max.

Symbol	Gene name	FPKM A1[a]	FPKM B2	A2/A1		B2/B1		Metaz-SecKB[d]	Detected in CM
				FC[b]	q-Value[c]	FC	q-value		
IL6	Interleukin 6	0.09	0.12	65.4	9E-28	50.8	2E-20	Curated	Antibody array[g]
CXCL8	Interleukin 8	0.03	0.04	29.1	3E-21	34.4	7E-12	Curated	Antibody array[g]
CXCL1	Chemokine (C-X-C motif) ligand 1	0.05	0.08	26.2	3E-59	18.9	1E-34	Curated	Antibody array[g]
CCL8	Chemokine (C-C motif) ligand 8	0.07	0.13	24	5E-33	18.1	6E-40	Curated	Antibody array[g]
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif 4	0.38	0.58	18.9	3E-64	19.2	1E-32	Curated	MS Murine
PTGS2	Prostaglandin-endoperoxide synthase 2	0.02	0.03	17.3	2E-32	18.5	2E-25	Highly likely	—
CXCL2	Chemokine (C-X-C motif) ligand 2	1.21	2.14	14.5	8E-35	9.7	8E-23	Curated	—
CCL2	Chemokine (C-C motif) ligand 2	2.25	3.31	12.9	5E-16	13.5	3E-18	Curated	Antibody array[g]
CXCL3	Chemokine (C-X-C motif) ligand 3	0.09	0.15	10.1	1E-21	5.9	6E-13	Curated	—
IL1B	Interleukin 1, beta	0.05	0.04	9.3	2E-19	21.5	9E-21	Curated	Antibody array[g]
THBS1	Thrombospondin 1	0.4	0.75	8.6	6E-21	7.5	1E-12	Highly likely	MS hSkMC
CYR61	Cysteine-rich, angiogenic inducer, 61	9.29	12.53	8	9E-38	4.6	2E-30	Curated	MS Murine[h]
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	5.87	6.86	7.2	1E-82	6.9	7E-55	Curated	MS hSkMC[i]
PLAUR	Plasminogen activator, urokinase receptor	0.32	0.46	7.1	2E-54	7.3	2E-26	Curated	MS Murine[h]
LIF	Leukemia inhibitory factor	0.06	0.09	6.5	4E-13	5	3E-08	Curated	Antibody array[g]
STC1	Stanniocalcin 1	0.12	0.22	6	3E-28	4.7	3E-12	Curated	MS Murine[h]
SERPINE1	Serpin peptidase inhibitor, clade E, member 1	0.78	1.33	4.8	5E-26	4.3	2E-13	Curated	Antibody array[g]
F2RL3	F2R like thrombin/trypsin receptor 3	0.18	0.29	4.7	1E-20	3.2	3E-08	Highly likely	—
LDLR	Low density lipoprotein receptor	1.31	1.66	4.3	9E-83	4.5	9E-37	Highly likely	MS Murine[h]
INHBB	Inhibin, beta B	0.49	0.70	4.1	2E-46	4.7	9E-37	Curated	MS Murine[h]
ICAM1	Intercellular adhesion molecule 1	2.1	2.56	3.9	2E-20	3.7	2E-14	Highly likely	MS hSkMC[i]
ANGPTL4	Angiopoietin-like 4	0.52	0.55	3.7	1E-12	7.2	1E-24	Curated	MS hSkMC[i]
PLAU	Plasminogen activator, urokinase	2.58	3.38	3.6	2E-19	4.3	2E-20	Curated	MS hSkMC[i]
CHGB	Chromogranin B	0.25	0.31	3.3	7E-10	3.1	6E-11	Curated	—
LRRC32	Leucine rich repeat containing 32	4.11	5.59	3.2	8E-67	2.7	6E-47	Highly likely	—
FUT1	Fucosyltransferase 1 (H blood group)	0.74	0.92	3.2	1E-74	2.5	3E-31	Highly likely	—
TNFAIP6	TNF alpha induced protein 6	0.5	0.62	3.2	7E-13	3.5	2E-12	Highly likely	—
CTGF	Connective tissue growth factor	6.58	10.37	3.1	6E-26	2.4	6E-19	Curated	MS hSkMC[i]
SERPINH1	Serpin family H member 1	6.71	11.42	3	2E-41	2.3	8E-28	Highly likely	MS hSkMC[i]
FGF6	Fibroblast growth factor 6	7.46	6.00	2.9	2E-22	2	8E-07	Curated	Antibody array[g]
PTX3	Pentraxin 3, long	0.09	0.12	2.8	4E-03	3.8	2E-04	Curated	MS hSkMC[i]
FCGR3B	Fc fragment of IgG, low affinity IIIb, receptor (CD16b)	0.13	0.13	2.8	2E-06	6.8	6E-15	Curated	—
METRNL	Meteorin, glial cell differentiation regulator-like	1.37	1.68	2.7	1E-28	2.7	3E-17	Curated	MS Murine[h]
SEMA4C	Semaphorin 4C	3.11	3.38	2.7	1E-77	2.5	7E-51	Highly likely	MS Murine[h]
INHBE	Inhibin, beta E	0.42	0.66	2.7	4E-33	2.2	1E-19	Curated	—
CLCF1	Cardiotrophin-like cytokine factor 1	0.22	0.32	2.6	7E-15	2.8	1E-15	Curated	—
TNFRSF12A	Tumor necrosis factor receptor superfamily member 12A	6.53	10.35	2.5	1E-18	2.3	3E-14	Highly likely	—
SAA1	Serum amyloid A1	1.64	4.05	2.5	7E-09	2.1	1E-02	Curated	—
S100A9	S100 calcium binding protein A9	1.97	2.23	2.5	2E-07	3.9	5E-10	Curated	—
CX3CL1	Chemokine (C-X3-C motif) ligand 1	3.87	5.19	2.4	6E-15	2.2	4E-13	Curated	Antibody array[g]
SAA2	Serum amyloid A2	0.21	0.26	2.4	5E-11	2.2	3E-05	Curated	—
FGF18	Fibroblast growth factor 18	0.22	0.16	2.3	1E-08	2.8	2E-08	Curated	—
SECTM1	Secreted and transmembrane 1	0.34	0.40	2.3	2E-12	2.7	4E-08	Curated	MS hSkMC[i]
S100A8	S100 calcium binding protein A8	1.01	1.24	2.3	6E-05	4.6	2E-10	Curated	—
MMP19	Matrix metallopeptidase 19	0.25	0.35	2.2	2E-05	3	3E-09	Curated	MS Murine[h]
SRGN	Serglycin	7.81	11.94	2.2	1E-50	2	4E-18	Curated	MS hSkMC[i]
CCL21	Chemokine (C-C motif) ligand 21	0.35	1.21	2.2	5E-02	0.5	2E-01	Curated	Antibody array[g]
CSF3R	Colony stimulating factor 3 receptor (granulocyte)	0.32	0.49	2.2	9E-06	3	1E-07	Curated[e]	—
RELT	RELT tumor necrosis factor receptor	3.3	2.90	2.2	5E-28	2.1	3E-19	Highly likely	—
ITGA5	Integrin subunit alpha 5	4.42	6.48	2.2	3E-46	1.9	2E-21	Highly likely	MS Murine[h]
IL4R	Interleukin 4 receptor	1.17	1.73	2.2	1E-34	2.1	6E-14	Curated	MS Murine[h]
EPHA2	EPH receptor A2	1.58	2.08	2.1	1E-39	1.9	9E-16	Highly likely	MS Murine[h]
CHSY1	Chondroitin sulfate synthase 1	1.4	1.82	2.1	2E-31	2.3	2E-20	Curated	—
VEGFA	Vascular endothelial growth factor A	47.33	50.63	2	2E-42	1.8	2E-24	Curated	Antibody array[g]
IL7R	Interleukin 7 receptor	0.09	0.12	2	2E-08	2.5	7E-10	Curated	—
SERPINA1	Serpin peptidase inhibitor, clade A, member 1	0.16	0.22	1.9	3E-05	2.3	4E-05	Curated	—
SDC4	Syndecan 4	16.5	15.64	1.9	8E-27	1.9	9E-20	Curated	MS hSkMC[i]
GDNF	Glial cell derived neurotrophic factor	3.98	3.36	1.9	4E-12	1.8	2E-05	Curated	Antibody array[g]
GFPT2	Glutamine-fructose-6-phosphate transaminase 2	1.04	1.15	1.9	5E-09	1.8	8E-05	Highly likely	—
PDGFA	Platelet-derived growth factor alpha polypeptide	5.54	5.41	1.9	4E-21	2	5E-24	Curated	MS Murine[h]
GLA	Galactosidase alpha	2.94	3.10	1.9	2E-11	2.2	1E-12	Highly likely	MS Murine[h]
SEMA3F	Semaphorin 3F	2.01	2.57	1.9	6E-43	1.8	2E-15	Curated	—
PRSS42	Protease, serine, 42	1.54	1.45	1.8	6E-10	1.9	1E-08	Curated	—
CRISPLD2	Cysteine-rich secretory protein LCCL domain containing 2	3.31	3.91	1.8	2E-23	2	7E-13	Curated	—
C8G	Complement component 8, gamma polypeptide	1.79	2.00	1.8	3E-08	1.7	9E-10	Curated	—
LCN10	Lipocalin 10	0.42	0.60	1.8	5E-08	1.5	3E-04	Curated	—
PVRL2	Nectin cell adhesion molecule 2	2.3	3.25	1.8	2E-43	1.7	1E-18	Highly likely	MS Murine[h]
HAPLN3	Hyaluronan and proteoglycan link protein 3	0.89	1.14	1.8	2E-10	1.9	2E-07	Curated	—

Table 1 (*continued*)

Symbol	Gene name	FPKM A1[a]	FPKM B2	A2/A1		B2/B1		Metaz-SecKB[d]	Detected in CM
				FC[b]	q-Value[c]	FC	q-value		
ADM5	Adrenomedullin 5 (putative)	0.3	0.39	1.7	4E-06	1.6	9E-07	Curated	—
STC2	Stanniocalcin 2	0.31	0.41	1.7	8E-08	2	2E-12	Curated	MS hSkMC[i]
IFI30	Interferon, gamma-inducible protein 30	2.02	2.43	1.7	4E-15	1.9	6E-08	Curated	MS hSkMC[i]
SERPINA3	Serpin peptidase inhibitor, clade A, member 3	0.51	0.53	1.7	3E-04	2.7	4E-05	Curated	—
NFAM1	NFAT activating protein with ITAM motif 1	0.17	0.22	1.7	1E-06	1.9	1E-08	Highly likely	—
SEMA7A	Semaphorin 7A, GPI membrane anchor	0.57	0.64	1.7	1E-15	1.9	3E-11	Curated	MS hSkMC[i]
GABRE	Gamma-aminobutyric acid type A receptor epsilon subunit	0.63	0.77	1.7	4E-17	1.8	1E-21	Highly likely	—
IL1R1	Interleukin 1 receptor, type I	2.49	2.86	1.7	7E-12	2.1	2E-12	Curated	—
FCGR2A	Fc fragment of IgG receptor IIa	0.38	0.61	1.6	2E-04	1.6	2E-05	Highly likely	—
QPCT	Glutaminyl-peptide cyclotransferase	0.37	0.68	1.6	1E-05	1.3	3E-03	Curated	MS hSkMC[i]
TNC	Tenascin C	0.37	0.60	1.6	1E-04	2	3E-04	Curated	MS hSkMC[i]
SLC39A14	Solute carrier family 39 member 14	1.61	2.25	1.6	5E-15	1.7	8E-12	Highly likely	—
VWA1	von Willebrand factor A domain containing 1	1.52	2.80	1.6	2E-19	1.3	2E-14	Curated	MS Murine[h]
GBP1	Guanylate binding protein 1, interferon-inducible	3.33	3.63	1.6	7E-13	1.8	3E-11	Curated	MS hSkMC[i]
SMPDL3A	Sphingomyelin phosphodiesterase, acid-like 3A	10.06	10.15	1.6	1E-17	1.5	1E-16	Curated	MS Murine[h]
LILRB3	Leukocyte immunoglobulin like receptor B3	0.26	0.36	1.6	1E-06	1.9	5E-08	Highly likely	—
NPTX2	Neuronal pentraxin II	1.09	0.90	1.6	4E-10	1.7	5E-07	Curated	—
VEGFC	Vascular endothelial growth factor C	0.78	1.24	1.6	2E-08	1.2	2E-02	Curated	Antibody array[g]
TFPI2	Tissue factor pathway inhibitor 2	0.62	0.96	1.6	4E-03	1.6	5E-04	Curated	—
TGFB3	Transforming growth factor, beta 3	2.91	4.05	1.6	5E-19	1.6	2E-13	Curated	Antibody array[g]
TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	3.26	4.32	1.6	2E-09	1.9	2E-11	Curated	MS Murine[h]
FAM57A	Family with sequence similarity 57 member A	0.59	0.87	1.6	4E-11	1.4	9E-07	Highly likely	—
ADAM8	ADAM metallopeptidase domain 8	0.3	0.40	1.5	2E-05	1.7	3E-05	Highly likely	—
APLN	Apelin	1.61	2.74	1.5	3E-06	1.5	7E-10	Curated	—
TIMP1	TIMP metallopeptidase inhibitor 1	6.83	9.72	1.5	2E-09	1.6	3E-06	Curated	Antibody array[g]
CSF2RB	Colony stimulating factor 2 receptor beta common subunit	0.34	0.53	1.5	7E-04	1.7	1E-04	Highly likely	—
FLT1	Fms-related tyrosine kinase 1	3.59	4.40	1.5	1E-25	1.5	1E-12	Curated[e]	—
PDGFB	Platelet-derived growth factor beta polypeptide	5.5	8.35	1.5	2E-11	1.3	4E-09	Curated	Antibody array[g]
POSTN	Periostin, osteoblast specific factor	0.45	0.75	1.5	2E-03	1.1	4E-01	Curated	MS hSkMC[i]
LILRA6	Leukocyte immunoglobulin like receptor A6	0.21	0.26	1.4	2E-04	1.5	6E-06	Highly likely	—
PLOD2	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	0.94	1.22	1.4	4E-07	1.6	3E-08	Highly likely	MS hSkMC[i]
CSF1	Colony stimulating factor 1 (macrophage)	1.84	2.17	1.4	5E-04	1.7	6E-06	Curated	Antibody array[g]
CD200	CD200 molecule	0.6	0.95	1.4	4E-04	1.5	5E-07	Highly likely	MS Murine[h]
LYZ	Lysozyme	3.53	5.51	1.4	5E-04	1.6	3E-04	Curated	—
FCN1	Ficolin (collagen/fibrinogen domain containing) 1	0.63	0.55	1.4	7E-02	2.4	1E-05	Curated	—
DNAJB9	DnaJ heat shock protein family (Hsp40) member B9	9.39	9.01	1.3	7E-09	1.5	2E-09	Highly likely	MS Murine[h]
CXCL10	Chemokine (C-X-C motif) ligand 10	1.33	1.07	1.3	2E-01	1.6	2E-03	Curated	Antibody array[g]
MMP25	Matrix metallopeptidase 25	0.31	0.48	1.2	1E-01	1.5	1E-02	Curated	—
HLA-G	Major histocompatibility complex, class I, G	0.21	0.28	0.8	6E-01	1.6	1E-01	Highly likely	—
IL1RN	Interleukin 1 receptor antagonist	0.03	0.04	f	f	15.8	9E-21	Curated[e]	MS Murine[h]
CGA	Glycoprotein hormones, alpha polypeptide	0.06	0.05	f	f	17.5	1E-14	Curated	—
TREM1	Triggering receptor expressed on myeloid cells 1	0.03	0.03	f	f	5.9	2E-07	Curated	—
LIPG	Lipase, endothelial	0.06	0.11	f	f	2.9	3E-07	Curated	—
VNN2	Vanin 2	0.14	0.17	f	f	2.9	7E-08	Highly likely	—
TNFRSF10C	Tumor necrosis factor receptor superfamily member 10c	0.12	0.19	f	f	2.8	3E-05	Highly likely	—
LRG1	Leucine-rich alpha-2-glycoprotein 1	0.37	0.40	f	f	1.8	1E-05	Curated	—
CFP	Complement factor properdin	0.24	0.33	f	f	1.7	1E-03	Curated	—
RNF24	Ring finger protein 24	0.23	0.28	f	f	1.5	4E-07	Highly likely	—
SERPINF2	Serpin peptidase inhibitor, clade F, member 2	0.25	0.28	f	f	1.5	9E-05	Curated	—

[a] Fragments per kilobase of transcript per million mapped reads.
[b] Fold change.
[c] False discovery rate.
[d] Annotation in MetazSecKB.
[e] Annotated as secreted in Swissprot, but not in MetazSecKB.
[f] Expression level below EdgeR threshold for quantification.
[g] Detected in conditioned medium from human skeletal muscle cells with antibody array [9,10,18,19].
[h] Detected in conditioned medium from murine muscle cells or explants with mass spectrometry analysis [13–16,20,34–36].
[i] Detected in conditioned medium from human skeletal muscle cells with mass spectrometry analysis [9–12].

after 12 weeks training, although below the 1.5-fold cut-off. For instance, of the transcripts that increased >1.5-fold after the first bicycle test (A2/A1), more than half were significantly increased (FC > 1, p-value < 0.05) after 12 weeks training (B1/A1, Supplementary Table 1).

Some transcripts encode well-known myokines, whereas others have never been studied in skeletal muscle. Approximately half of these myokines have previously been detected by mass spectrometry or antibody arrays in medium conditioned by cultured human or murine skeletal muscle cells [9–20,34–36]

Table 2 — Transcripts up-regulated 2 h after 45 min acute exercise of 70% of VO_2max.

Symbol	Gene name	FPKM A1[a]	FPKM B1	A3/A1		B3/B1		Metaz-SecKB[d]	Detected in CM
				FC[b]	q-value[c]	FC	q-value		
CGA	Glycoprotein hormones, alpha polypeptide	0.06	0.05	17	1E-05	f	f	Curated	—
ANGPTL4	Angiopoietin-like 4	0.52	0.55	15.36	2E-37	14.35	7E-26	Curated	MS hSkMC[i]
STC2	Stanniocalcin 2	0.31	0.41	7.97	3E-45	5.14	2E-27	Curated	MS hSkMC[i]
PLG	Plasminogen	0.09	0.09	5.34	1E-15	3.49	3E-17	Curated	—
FCGR3B	Fc fragment of IgG, low affinity IIIb, receptor (CD16b)	0.13	0.13	4.85	4E-07	3.28	7E-09	Curated	—
LCN10	Lipocalin 10	0.42	0.60	3.85	7E-18	2.68	8E-12	Curated	—
S100A8	S100 calcium binding protein A8	1.01	1.24	3.69	3E-06	3.0	4E-08	Curated	—
LCN6	Lipocalin 6	0.54	0.87	3.65	1E-11	2.25	2E-08	Curated	—
S100A9	S100 calcium binding protein A9	1.97	2.23	3.31	3E-05	2.71	2E-11	Curated	—
VEGFA	Vascular endothelial growth factor A	47.33	50.63	3.19	9E-50	1.94	4E-12	Curated	Antibody array[g]
C8G	Complement component 8, gamma polypeptide	1.79	2.00	3.18	4E-11	2.49	2E-18	Curated	—
IL6R	Interleukin 6 receptor	4.42	4.11	3.09	5E-49	2.31	3E-22	Curated	—
FCN3	Ficolin 3	0.56	0.95	2.84	5E-13	1.94	4E-12	Curated	—
WNT9A	Wingless-type MMTV integration site family, member 9A	4.9	5.58	2.76	1E-09	2.13	2E-11	Curated	—
SERPINA1	Serpin peptidase inhibitor, clade A, member 1	0.16	0.22	2.76	3E-05	1.73	1E-05	Curated	—
HBA2	Hemoglobin subunit alpha 2	5.6	5.80	2.69	1E-03	1.25	4E-01	Highly likely	—
DFNA5	DFNA5, deafness associated tumor suppressor	1.12	1.57	2.65	4E-19	1.96	2E-09	Highly likely	—
PTGIR	Prostaglandin I2 (prostacyclin) receptor (IP)	0.25	0.30	2.59	3E-08	2.31	7E-12	Highly likely	—
WDR81	WD repeat domain 81	2.29	2.58	2.54	2E-39	1.84	7E-24	Highly likely	—
TNFRSF8	Tumor necrosis factor receptor superfamily member 8	0.15	0.20	2.44	2E-11	2.05	5E-12	Highly likely	Antibody array[g]
CSF3R	Colony stimulating factor 3 receptor	0.32	0.49	2.43	2E-04	1.72	9E-06	Curated[e]	—
SERPINA3	Serpin peptidase inhibitor, clade A, member 3	0.51	0.53	2.36	6E-06	2.4	7E-06	Curated	—
ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif 9	1.41	1.90	2.35	2E-16	2.01	3E-11	Curated	—
VNN2	Vanin 2	0.14	0.17	2.34	2E-02	f	f	Highly likely	—
THBS1	Thrombospondin 1	0.4	0.75	2.33	8E-04	1.93	2E-03	Highly likely	MS hSkMC[i]
SERPINF2	Serpin peptidase inhibitor, clade F, member 2	0.25	0.28	2.29	1E-07	2.12	8E-12	Curated	—
CHGB	Chromogranin B	0.25	0.31	2.24	7E-03	1.62	9E-02	Curated	—
TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	3.26	4.32	2.23	9E-29	1.75	4E-09	Curated	MS Murine[h]
FGF6	Fibroblast growth factor 6	7.46	6.00	2.16	9E-06	1.35	1E-02	Curated	Antibody array[g]
ANGPTL2	Angiopoietin like 2	13.79	18.98	2.14	1E-25	1.73	7E-13	Curated	MS Murine[h]
SMPDL3A	Sphingomyelin phosphodiesterase, acid-like 3A	10.06	10.15	2.06	3E-10	1.59	4E-11	Curated	MS Murine[h]
FAM20C	Family with sequence similarity 20, member C	7.55	7.82	2.05	2E-28	1.57	2E-11	Curated	MS Murine[h]
SLC39A14	Solute carrier family 39 member 14	1.61	2.25	2.03	5E-10	1.79	4E-10	Highly likely	—
RELT	RELT tumor necrosis factor receptor	3.3	2.90	2.01	4E-12	1.8	8E-10	Highly likely	—
SDC4	Syndecan 4	16.5	15.64	2	8E-06	1.45	6E-04	Curated	MS hSkMC[i]
SAA1	Serum amyloid A1	1.64	4.05	1.98	5E-02	1.74	2E-01	Curated	—
POR	Cytochrome p450 oxidoreductase	4.25	5.27	1.95	1E-20	1.65	2E-10	Highly likely	MS Murine[h]
FCGR2A	Fc fragment of IgG receptor IIa	0.38	0.61	1.94	4E-06	1.43	1E-03	Highly likely	—
CES3	Carboxylesterase 3	8.67	7.90	1.9	6E-17	1.63	3E-09	Highly likely	—
VWA1	von Willebrand factor A domain containing 1	1.52	2.80	1.9	2E-15	1.44	1E-08	Curated	MS Murine[h]
INHBE	Inhibin, beta E	0.42	0.66	1.87	5E-05	1.34	2E-02	Curated	—
ADAM8	ADAM metallopeptidase domain 8	0.3	0.40	1.84	2E-04	1.38	2E-03	Highly likely	—
FCN1	Ficolin 1	0.63	0.55	1.83	1E-02	1.67	4E-03	Curated	—
PTTG1IP	Pituitary tumor-transforming 1 interacting protein	15.52	18.94	1.79	2E-20	1.42	2E-11	Highly likely	—
TOR3A	Torsin family 3 member A	5.87	6.83	1.77	6E-10	1.8	3E-16	Highly likely	—
ADM	Adrenomedullin	2.76	3.71	1.77	2E-05	1.19	2E-01	Curated	MS hSkMC[i]
PRG4	Proteoglycan 4	0.4	0.66	1.74	8E-02	1.05	9E-01	Curated	MS hSkMC[i]
PDE7A	Phosphodiesterase 7A	9.57	9.09	1.73	4E-17	1.65	9E-19	Highly likely	—
NFAM1	NFAT activating protein with ITAM motif 1	0.17	0.22	1.72	2E-05	1.26	5E-03	Highly likely	—
PFKP	Phosphofructokinase, platelet	2.44	3.11	1.71	3E-12	1.43	2E-06	Highly likely	MS Murine[h]
SEMA3G	Semaphorin 3G	4.88	7.13	1.7	4E-11	1.34	2E-05	Curated	MS Murine[h]
LPL	Lipoprotein lipase	20.1	25.10	1.69	5E-12	1.74	6E-20	Curated	MS Murine[h]
FKBP7	FK506 binding protein 7	1.14	1.24	1.69	5E-12	1.43	7E-11	Highly likely	MS Murine[h]
SRGN	Serglycin	7.81	11.94	1.68	1E-12	1.26	6E-04	Curated	MS hSkMC[i]
TGFB3	Transforming growth factor, beta 3	2.91	4.05	1.68	2E-14	1.59	5E-14	Curated	Antibody array[g]
SAA2	Serum amyloid A2	0.21	0.26	1.67	1E-01	2.13	5E-03	Curated	—
SFRP2	Secreted frizzled-related protein 2	0.33	0.73	1.67	3E-02	1.01	1E+00	Curated	MS Murine[h]
STC1	Stanniocalcin 1	0.12	0.22	1.67	6E-02	1.12	7E-01	Curated	MS Murine[h]
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4	0.38	0.58	1.65	2E-03	1.45	4E-02	Curated	MS Murine[h]
TEK	TEK tyrosine kinase, endothelial	3.71	5.23	1.63	6E-17	1.4	1E-07	Curated	—
CNTFR	Ciliary neurotrophic factor receptor	14.48	11.88	1.62	1E-08	1.45	5E-07	Highly likely	—
FAM57A	Family with sequence similarity 57 member A	0.59	0.87	1.61	1E-05	1.27	2E-03	Highly likely	—
SLC45A4	Solute carrier family 45 member 4	0.61	0.76	1.61	2E-07	1.32	4E-06	Highly likely	—
SLC6A8	Solute carrier family 6 member 8	41.86	38.87	1.6	6E-29	1.34	5E-07	Highly likely	—
SFRP1	Secreted frizzled-related protein 1	0.36	0.32	1.6	7E-03	1.5	9E-03	Curated	—
SLPI	Secretory leukocyte peptidase inhibitor	1.63	1.06	1.6	8E-03	f	f	Curated	MS Murine[h]
CD163L1	CD163 molecule-like 1	0.16	0.29	1.6	5E-03	1.76	6E-08	Curated	—
APLN	Apelin	1.61	2.74	1.58	8E-04	1.49	3E-03	Curated	—

Table 2 (*continued*)

Symbol	Gene name	FPKM A1[a]	FPKM B1	A3/A1		B3/B1		Metaz-SecKB[d]	Detected in CM
				FC[b]	q-value[c]	FC	q-value		
TNFRSF12A	Tumor necrosis factor receptor superfamily member 12A	6.53	10.35	1.58	6E-03	1.05	8E-01	Highly likely	–
CCL2	Chemokine (C-C motif) ligand 2	2.25	3.31	1.58	4E-02	1.35	1E-01	Curated	Antibody array[g]
GLA	Galactosidase alpha	2.94	3.10	1.57	3E-06	1.37	6E-05	Highly likely	MS Murine[h]
MTRNR2L6	MT-RNR2-like 6	19.29	22.12	1.56	8E-08	1.27	5E-03	Curated	–
IFI30	Interferon, gamma-inducible protein 30	2.02	2.43	1.56	9E-04	1.46	4E-04	Curated	MS hSkMC[i]
PLA2G15	Phospholipase A2, group XV	8.45	9.28	1.55	2E-12	1.39	4E-09	Curated	MS Murine[h]
LOXL2	Lysyl oxidase-like 2	1.1	2.64	1.55	2E-07	1.4	3E-06	Curated	MS hSkMC[i]
METRNL	Meteorin, glial cell differentiation regulator-like	1.37	1.68	1.54	5E-03	1.33	2E-02	Curated	MS Murine[h]
GFPT2	Glutamine-fructose-6-phosphate transaminase 2	1.04	1.15	1.54	3E-03	1.31	2E-02	Highly likely	–
ST3GAL1	ST3 beta-galactoside alpha-2,3-sialyltransferase 1	14.1	14.03	1.53	1E-14	1.41	3E-11	Curated	MS Murine[h]
ADIPOQ	Adiponectin, C1Q and collagen domain containing	0.29	0.28	1.52	1E-01	1.19	5E-01	Curated	–
NRG2	Neuregulin 2	0.41	0.40	1.51	5E-03	1.47	6E-06	Curated	–
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	0.62	0.69	1.51	3E-04	1.62	2E-08	Curated[e]	–
MTRNR2L4	MT-RNR2-like 4	68.77	74.18	1.5	3E-09	1.36	5E-15	Curated	–
CD300LG	CD300 molecule like family member g	4.55	7.60	1.5	2E-09	1.15	3E-02	Highly likely	–
NPTX2	Neuronal pentraxin II	1.09	0.90	1.5	1E-03	1.57	1E-04	Curated	–
TLR9	Toll like receptor 9	0.62	0.40	1.3	3E-02	1.71	3E-06	Highly likely	–
FCGR3A	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)	0.59	0.62	1.29	1E-01	1.52	2E-04	Curated	–
PLAUR	Plasminogen activator, urokinase receptor	0.3	0.46	1.4	3E-02	1.51	2E-03	Curated	MS Murine[h]
CLCF1	Cardiotrophin-like cytokine factor 1	0.22	0.32	f	f	1.98	9E-11	Curated	–
FJX1	Four jointed box 1	0.18	0.35	f	f	1.69	3E-05	Curated	–
HLA-G	Major histocompatibility complex, class I, G	0.21	0.28	0.57	2E-01	1.53	1E-01	Highly likely	–
LILRB3	Leukocyte immunoglobulin like receptor B3	0.3	0.36	f	f	1.51	2E-04	Highly likely	–

[a] Fragments per kilobase of transcript per million mapped reads.
[b] Fold change.
[c] False discovery rate.
[d] Annotation in MetazSecKB.
[e] Annotated as secreted in Swissprot, but not in MetazSecKB.
[f] Expression level below EdgeR threshold for quantification.
[g] Detected in conditioned medium from human skeletal muscle cells with antibody array [9,10,18,19].
[h] Detected in conditioned medium from murine muscle cells or explants with mass spectrometry analysis [13–16,20,34–36].
[i] Detected in conditioned medium from human skeletal muscle cells with mass spectrometry analysis [9–12].

(Tables 1–3). About 70% of the transcripts we identified encode curated, secreted proteins, whereas the rest encode proteins that are "highly likely" to be secreted. Some of these "highly likely" secreted proteins are mostly considered intracellular, but may have secreted isoforms.

3.5. Expression of novel myokines in primary human skeletal muscle cells

Several of the transcripts we identified have not been studied previously in skeletal muscle and may encode novel myokines. We selected 17 candidates exhibiting enhanced expression after acute and/or long-term training (Supplementary Figure 1). The candidates were chosen based on the expression level in skeletal muscle and the magnitude of change in response to exercise. We prioritized genes that may encode myokines with potential endocrine functions over ECM-related factors. We chose the following candidates: stanniocalcin 2 (*STC2*), insulin like growth factor binding protein 2 (*IGFBP2*), family with sequence similarity 20 member C (*FAM20C*), *CSF1*, secreted frizzled related protein 4 (*SFRP4*), tumor necrosis factor receptor superfamily member 25 (*TNFRSF25*), IL4 receptor (*IL4R*), chondroitin sulfate synthase 1 (*CHSY1*), kazal type serine peptidase inhibitor domain 1 (*KAZALD1*), angiopoietin-1 receptor (*TEK*), sphingomyelin phosphodiesterase acid like 3A (*SMPDL3A*), thrombospondin 4 (*THBS4*), complement component 8 gamma polypeptide (*C8G*), humanin-like 4 (*MTRNR2L4*), wnt family member 9A (*WNT9A*), Fms related tyrosine kinase 1 (*FLT1*), and lipocalin 10 (*LCN10*).

All mRNAs except *WNT9A*, *FLT1*, and *LCN10* were expressed in cultured muscle cells (Figure 3B). The relative expression levels of the investigated mRNAs showed differences between *in vitro* differentiated myotubes and skeletal muscle biopsies. *MTRNR2L4* and *THBS4* were highly expressed in biopsies, whereas in cultured myotubes the expression was low. In myotubes *STC2* was highly expressed, which was not the case in skeletal muscle biopsies.

To investigate mRNA expression during myogenic differentiation, myoblasts were differentiated to multinuclear myotubes for 7 days (Figure 3C–F). During myogenesis, *THBS4* (4-fold), *FAM20C* (2.3-fold), *CHSY1* (1.5-fold), *TEK* (2.5-fold), *IGFBP2* (3-fold), *TNFRSF25* (3.5-fold), and *KAZALD1* (2-fold) all increased significantly, whereas *STC2* was the only gene that was down-regulated (−3.3-fold; Figure 3C). *MTRNR2L4*, *SMPDL3, CSF1, C8G*, and *IL4R* were not significantly changed (data not shown).

To gain further insight into the exercise-related regulation of gene expression, cultured myotubes were subjected to EPS for 24 h to induce myotube contraction (Figure 3G). EPS increased the expression of *PPARGC1A* and *IL6*, 1.3- and 3-fold, respectively, as previously shown [27]. However, only *CSF1* was significantly up-regulated by 24 h EPS (1.3-fold, $p < 0.05$), whereas *FAM20C* expression was reduced −1.25-fold ($p < 0.05$).

3.6. CSF1 is secreted from human skeletal muscle cells

Because *CSF1* expression was enhanced in skeletal muscle after acute exercise (Figure 4A) and in cultured myotubes after 24 h EPS (Figure 3G), we further focused on CSF1. The expression of CSF1 was also slightly (9%) increased in muscle after 12 weeks. Interestingly, the expression of CSF1 receptor (*CSF1R*) in skeletal muscle increased after 12 weeks exercise training (Figure 4B). We also measured CSF1 concentration in plasma samples from participants, before ($n = 26$) and after ($n = 22$) the 12 weeks intervention. Plasma concentration of

Table 3 — Transcripts up-regulated after 12 weeks exercise intervention.

Symbol	Gene name	FPKM A1[a]	B1/A1		MetazSecKB[d]	Detected in CM
			FC[b]	q-value[c]		
SFRP5	Secreted frizzled-related protein 5	0.31	4.84	1E-01	Curated	–
MXRA5	Matrix-remodelling associated 5	0.48	2.75	3E-14	Curated	MS hSkMC[h]
THY1	Thy-1 cell surface antigen	1.7	2.44	3E-14	Highly likely	–
CPXM1	Carboxypeptidase X (M14 family), member 1	0.2	2.43	2E-04	Curated	–
COL1A1	Collagen, type I, alpha 1	4.92	2.4	8E-18	Curated	MS hSkMC[h]
COL3A1	Collagen, type III, alpha 1	16.73	2.4	4E-17	Curated	MS hSkMC[h]
COL4A1	Collagen, type IV, alpha 1	14.09	2.36	2E-26	Curated	MS hSkMC[h]
THBS4	Thrombospondin 4	21.74	2.21	1E-19	Curated	–
SFRP2	Secreted frizzled-related protein 2	0.33	2.18	1E-07	Curated	MS Murine cells[g]
LOXL2	Lysyl oxidase-like 2	1.1	2.18	3E-24	Curated	MS hSkMC[h]
COL4A2	Collagen, type IV, alpha 2	13.41	2.17	4E-25	Curated	MS hSkMC[h]
BGN	Biglycan	2.86	2.1	2E-15	Curated	MS hSkMC[h]
OGN	Osteoglycin	0.62	2.09	6E-07	Curated	MS Murine cells[g]
CCL21	Chemokine (C-C motif) ligand 21	0.35	2.07	6E-02	Curated	Antibody array[f]
COL6A6	Collagen, type VI, alpha 6	0.1	2.04	3E-07	Curated	–
AMICA1	Adhesion molecule, interacts with CXADR antigen 1	0.3	2.0	5E-09	Highly likely	–
IGF2	Insulin-like growth factor 2	0.7	1.98	3E-21	Curated	MS hSkMC[h]
LOX	Lysyl oxidase	0.3	1.96	4E-23	Curated	MS hSkMC[h]
TMEM119	Transmembrane protein 119	0.4	1.96	3E-13	Highly likely	MS Murine cells[g]
CDH24	Cadherin 24, type 2	0.27	1.95	3E-08	Highly likely	–
PXDN	Peroxidasin	3	1.94	5E-29	Curated	MS hSkMC[h]
VIPR1	Vasoactive intestinal peptide receptor 1	0.41	1.9	3E-10	Highly likely	–
WISP1	WNT1 inducible signaling pathway protein 1	0.14	1.86	3E-10	Curated	MS Murine cells[g]
ADAMTS7	ADAM metallopeptidase with thrombospondin type 1 motif, 7	0.52	1.85	9E-19	Curated	MS Murine cells[g]
ASPN	Asporin	2.45	1.83	1E-15	Curated	MS Murine cells[g]
NRP2	Neuropilin 2	0.53	1.83	3E-19	Curated	MS Murine cells[g]
ST8SIA2	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2	0.09	1.82	5E-03	Highly likely	MS Murine cells[g]
QPCT	Glutaminyl-peptide cyclotransferase	0.37	1.81	3E-07	Curated	MS hSkMC[h]
IL10RA	Interleukin 10 receptor, alpha	0.7	1.8	5E-12	Highly likely	Antibody array[f]
COL14A1	Collagen, type XIV, alpha 1	0.62	1.78	7E-10	Curated	MS hSkMC[h]
OMD	Osteomodulin	0.31	1.78	8E-06	Curated	MS Murine cells[g]
SFRP4	Secreted frizzled-related protein 4	2.51	1.77	5E-05	Curated	MS hSkMC[h]
F2R	Coagulation factor II receptor	1.5	1.77	2E-26	Highly likely	–
PTN	Pleiotrophin	0.62	1.77	1E-07	Curated	MS Murine cells[g]
COL1A2	Collagen, type I, alpha 2	16.05	1.76	1E-10	Curated	MS hSkMC[h]
SPARC	Secreted protein, acidic, cysteine-rich	71.53	1.76	5E-24	Curated	MS hSkMC[h]
ANGPTL7	Angiopoietin-like 7	0.34	1.74	4E-01	Curated	–
LAMB1	Laminin, beta 1	6.8	1.74	3E-16	Curated	MS hSkMC[h]
CTHRC1	Collagen triple helix repeat containing 1	1.34	1.73	2E-10	Curated	MS hSkMC[h]
PAMR1	Peptidase domain containing associated with muscle regeneration 1	0.86	1.73	7E-05	Curated	–
LAMA4	Laminin, alpha 4	2.95	1.72	1E-32	Curated	MS hSkMC[h]
MEST	Mesoderm specific transcript	1.24	1.71	2E-07	Highly likely	–
NID2	Nidogen 2 (osteonidogen)	2.27	1.71	3E-22	Curated	MS hSkMC[h]
VWA1	von Willebrand factor A domain containing 1	1.52	1.71	1E-15	Curated	MS Murine cells[g]
LAMC3	Laminin, gamma 3	0.09	1.7	1E-03	Curated	–
GPR162	G protein-coupled receptor 162	0.44	1.69	8E-10	Highly likely	–
ADCYAP1R1	Adenylate cyclase activating polypeptide 1 receptor type I	0.3	1.68	1E-11	Highly likely	–
KCP	Kielin/chordin-like protein	0.2	1.67	1E-07	Curated	–
ITIH3	Inter-alpha-trypsin inhibitor heavy chain 3	0.46	1.66	9E-05	Curated	–
HEPH	Hephaestin	0.26	1.66	2E-08	Highly likely	MS Murine cells[g]
IGFBP3	Insulin-like growth factor binding protein 3	3.48	1.66	5E-16	Curated	Antibody array[f]
VMO1	Vitelline membrane outer layer 1 homolog	1.54	1.65	3E-01	Curated	–
CD163L1	CD163 molecule-like 1	0.16	1.65	1E-05	Curated	–
CD300LG	CD300 molecule-like family member g	4.55	1.64	1E-23	Highly likely	–
FCGR2B	Fc fragment of IgG, low affinity IIb, receptor	0.44	1.63	3E-05	Highly likely	–
AGRN	Agrin	1.88	1.63	9E-19	Curated[e]	MS hSkMC[h]
ADAMTS15	ADAM metallopeptidase with thrombospondin type 1 motif, 15	0.78	1.63	3E-20	Curated	–
KAZALD1	Kazal-type serine peptidase inhibitor domain 1	2.08	1.62	7E-14	Curated	MS Murine cells[g]
APLN	Apelin	1.61	1.62	9E-07	Curated	–
EMILIN3	Elastin microfibril interfacer 3	0.35	1.61	1E-03	Curated	–
COL5A2	Collagen, type V, alpha 2	2.94	1.59	2E-11	Curated	MS hSkMC[h]
ECM2	Extracellular matrix protein 2	1.86	1.58	4E-15	Curated	MS hSkMC[h]
THBS1	Thrombospondin 1	0.4	1.58	2E-05	Highly likely	MS hSkMC[h]
FGFR3	Fibroblast growth factor receptor 3	0.13	1.58	2E-04	Curated[e]	–
ADAMTS8	ADAM metallopeptidase with thrombospondin type 1 motif, 8	0.26	1.58	1E-03	Curated	–
SERPINE1	Serpin peptidase inhibitor, clade E, member 1	0.78	1.57	7E-06	Curated	Antibody array[f]
ADAMTSL3	ADAMTS-like 3	0.84	1.57	2E-13	Curated	MS Murine cells[g]
NID1	Nidogen 1	4.32	1.57	4E-16	Curated	Antibody array[f]

Table 3 (*continued*)

Symbol	Gene name	FPKM A1[a]	B1/A1		MetazSecKB[d]	Detected in CM
			FC[b]	q-value[c]		
ADAMTS2	ADAM metallopeptidase with thrombospondin type 1 motif, 2	0.34	1.57	1E-05	Curated	MS hSkMC[h]
OLFML2B	Olfactomedin-like 2B	2.82	1.57	2E-07	Curated	MS hSkMC[h]
IGFBP2	Insulin-like growth factor binding protein 2	4	1.56	5E-10	Curated	Antibody array[f]
KDR	Kinase insert domain receptor	2.34	1.56	1E-20	Curated	–
EDN1	Endothelin 1	0.66	1.56	2E-05	Curated	Antibody array[f]
GRIN2C	Glutamate receptor, ionotropic, N-methyl D-aspartate 2C	0.17	1.56	5E-05	Highly likely	–
SERPINH1	Serpin peptidase inhibitor, clade H, member 1	6.71	1.55	4E-18	Highly likely	MS hSkMC[h]
HSPG2	Heparan sulfate proteoglycan 2	12.38	1.55	3E-19	Curated	MS hSkMC[h]
SCT	Secretin	7.26	1.55	4E-03	Curated	–
LOXL3	Lysyl oxidase-like 3	0.44	1.55	9E-07	Curated	MS Murine cells[g]
TNFRSF25	Tumor necrosis factor receptor superfamily, member 25	2	1.54	8E-13	Curated	–
ACE	Angiotensin I converting enzyme	5.22	1.54	3E-17	Curated[e]	–
MMP14	Matrix metallopeptidase 14	2.66	1.54	1E-13	Highly likely	MS hSkMC[h]
CSF1R	Colony stimulating factor 1 receptor	1.84	1.54	3E-07	Highly likely	–
TYRP1	Tyrosinase-related protein 1	0.78	1.53	1E-02	Highly likely	–
IGSF10	Immunoglobulin superfamily, member 10	0.08	1.53	8E-04	Curated	MS Murine cells[g]
GREM1	Gremlin 1, DAN family BMP antagonist	0.74	1.53	1E-06	Curated	MS hSkMC[h]
CLEC11A	C-type lectin domain family 11, member A	1.37	1.53	1E-06	Curated	MS hSkMC[h]
CDH5	Cadherin 5, type 2	15.17	1.53	1E-28	Highly likely	MS Murine cells[g]
FAT1	FAT atypical cadherin 1	0.33	1.53	6E-09	Highly likely	MS Murine cells[g]
FGF9	Fibroblast growth factor 9	0.28	1.53	2E-06	Curated	Antibody array[f]
VEGFC	Vascular endothelial growth factor C	0.78	1.53	4E-09	Curated	Antibody array[f]
NOTCH4	Notch 4	0.93	1.53	2E-32	Highly likely	–
CCDC80	Coiled-coil domain containing 80	3.43	1.52	8E-05	Curated	MS hSkMC[h]
AEBP1	AE binding protein 1	2.14	1.52	2E-07	Curated[e]	MS hSkMC[h]
COL15A1	Collagen, type XV, alpha 1	21	1.52	2E-11	Curated	MS Murine cells[g]
ELN	Elastin	2.35	1.52	1E-07	Curated	MS hSkMC[h]
COL5A1	Collagen, type V, alpha 1	2.73	1.51	2E-09	Curated	MS hSkMC[h]
TYROBP	TYRO protein tyrosine kinase binding protein	2.81	1.51	1E-05	Highly likely	–
LUM	Lumican	9.96	1.51	2E-05	Curated	MS hSkMC[h]
ISLR2	Immunoglobulin superfamily containing leucine-rich repeat 2	0.41	1.51	1E-06	Highly likely	MS Murine cells[g]

[a] Fragments per kilobase of transcript per million mapped reads at baseline (A1).
[b] Fold change.
[c] False discovery rate.
[d] Annotation in MetazSecKB.
[e] Annotated as secreted in Swissprot, but not in MetazSecKB.
[f] Detected in conditioned medium from human skeletal muscle cells with antibody array [9,10,18,19].
[g] Detected in conditioned medium from murine muscle cells or explants cells with mass spectrometry analysis [13–16,20,34–36].
[h] Detected in conditioned medium from human skeletal muscle cells with mass spectrometry analysis [9–12].

CSF1 was significantly increased immediately after exercise, and was reduced to below baseline after 2 h recovery (Figure 4C). Acute exercise may influence plasma volume and protein concentration [37], however total protein concentration was measured and did not change significantly during acute exercise in our study. Interestingly, the concentration of CSF1 also increased after 12 weeks exercise training (33%, $p = 0.03$).

In addition to 24 h EPS, short-term EPS enhanced CSF1 expression 2.4-fold after 2 h (Figure 4D). Furthermore, we measured CSF1 concentration in cell culture medium collected from myotubes after 24 h with or without EPS (Figure 4E). Although the baseline concentration of CFS1 in supernatants of myotubes differed substantially between the donors ranging from 38 pg/mL to 310 pg/mL, we observed that EPS increased CSF1 concentration in all experiments, promoting an increase of 1.5-fold ($p < 0.05$). This suggests that CSF1 is an exercise-responsive myokine secreted from human skeletal muscle cells.

4. DISCUSSION

In the present study, we used mRNA sequencing as an untargeted screening to identify exercise-responsive myokines. We searched for secretory transcripts that were either up- or down-regulated, although we chose to focus on myokines that were increased by exercise. In total, we detected almost 250 genes encoding putative myokines that were up-regulated after acute and/or long-term training. About half of these proteins have not been detected by previous proteomics studies on skeletal muscle cell cultures. To our knowledge, there are no published studies that have used mRNA sequencing to search for new myokines, which is a useful approach to substantially expand our knowledge of the skeletal muscle secretome. Most transcripts followed different patterns of expression; some were "fast" or "slow" responders to exercise, whereas others responded to long-term exercise, or both. Moreover, several transcripts responded differently to acute exercise after long-term exercise.

Several other studies have focused on the transcriptional response in skeletal muscle to physical activity. Catoire et al. used microarray to measure gene expression after 1 h one-legged cycling or 12 weeks of combined exercise training [17]. The authors identified 52 putative myokines that were significantly ($p < 0.01$) up-regulated in the exercising leg after acute exercise and 66 that were induced after 12 weeks training. Interestingly, these data are in concordance with our RNA-seq data; of the 52 acute transcripts identified by Catoire et al., 50 were also increased in our dataset (A2/A1, $p < 0.05$). Furthermore, of the 66 putative myokines induced after 12 weeks, 62 were also significantly up-regulated in our participants (B1/A1).

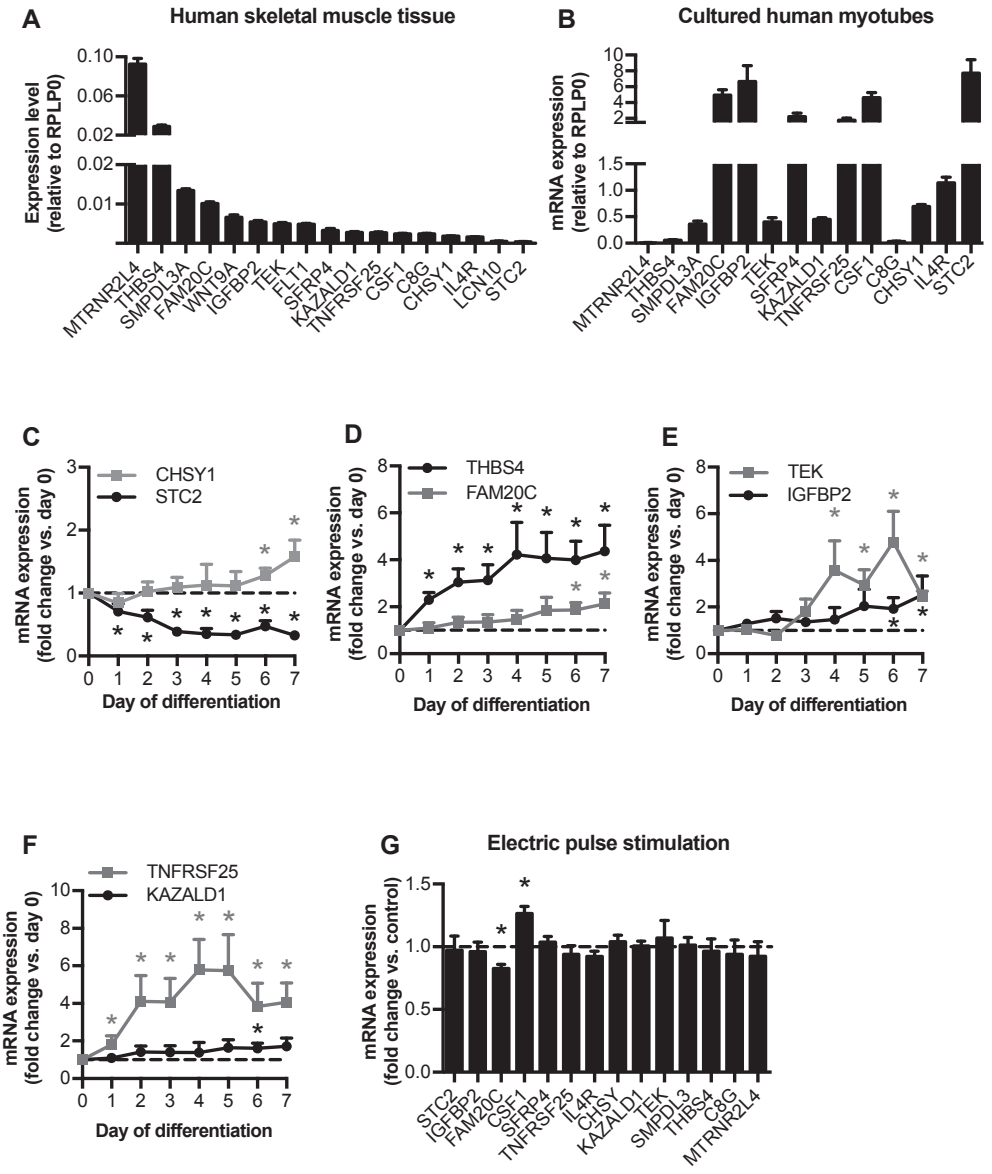

Figure 3: A–B) mRNA expression of selected genes in skeletal muscle biopsies (in A1, n = 26) or cultured human myotubes. mRNA expression in biopsies was determined with RNA-seq, and in myotubes by RT-PCR from 5–6 experiments using different donors. C–F) Primary human myoblasts were differentiated to multinucleated myotubes for 7 days. mRNA expression values are shown as fold change from the expression in myoblasts (day 0) and represent means + SEM from 3–4 experiments using different donors. *p < 0.05 vs. D0, students *t*-test. G) Primary human skeletal muscle cells were differentiated for 5 days and subjected to electrical pulse stimulation (EPS; 1 Hz, 2 ms, 11.5 V) for 24 h. Data are shown as fold vs. control and represent means + SEM from 5–7 experiments. *p < 0.05, students *t*-test. Gene expression values were normalized to *RPLP0*, bars depict means + SEM.

After acute exercise, several cytokines, chemokines, growth factors and ECM remodeling enzymes were up-regulated. Several of these are known contraction-regulated myokines, including IL6, IL8, LIF, CCL2, CX3CL1, SERPINE1, ANGPTL4, and VEGF [17,38]. Because many myokines and cytokines are difficult to detect by the use of mass spectrometry, antibody arrays have been used as a tool for myokine discovery. Raschke et al. used a cytokine antibody array to detect proteins released from cultured human muscle cells in response to electrical stimulation [19]. In total, they identified 45 proteins that were induced by EPS, and many of them were cytokines, chemokines, or growth factors. About half were up-regulated after acute or long-term exercise in our participants ($p < 0.05$, B1/A1).

After long-term training, a large proportion of the up-regulated transcripts were related to ECM. Induction of ECM related genes after exercise training has been reported by several others [17,39–41]. We have discussed the data related to ECM in more detail elsewhere [27]. We used primary human skeletal muscle cells as a model system for further investigation of 17 novel myokine candidates *in vitro*. Cultured human myotubes share many morphological and biochemical characteristics with skeletal muscle fibers *in vivo* [42,43]. They are multinucleated and have the ability to contract upon electrical stimulation. The relative expression levels of the 17 selected candidates were different in cultured cells as compared to muscle biopsies; some of the most highly expressed transcripts in biopsies were expressed at low levels *in vitro* and *vice versa*. *IL6, STC2*, and *CSF1* were all higher expressed in cultured myotubes than in muscle tissue (relative to the expression of *RPLP0*). Moreover, three transcripts were not expressed in cultured myotubes. Gene expression patterns in cultured myotubes

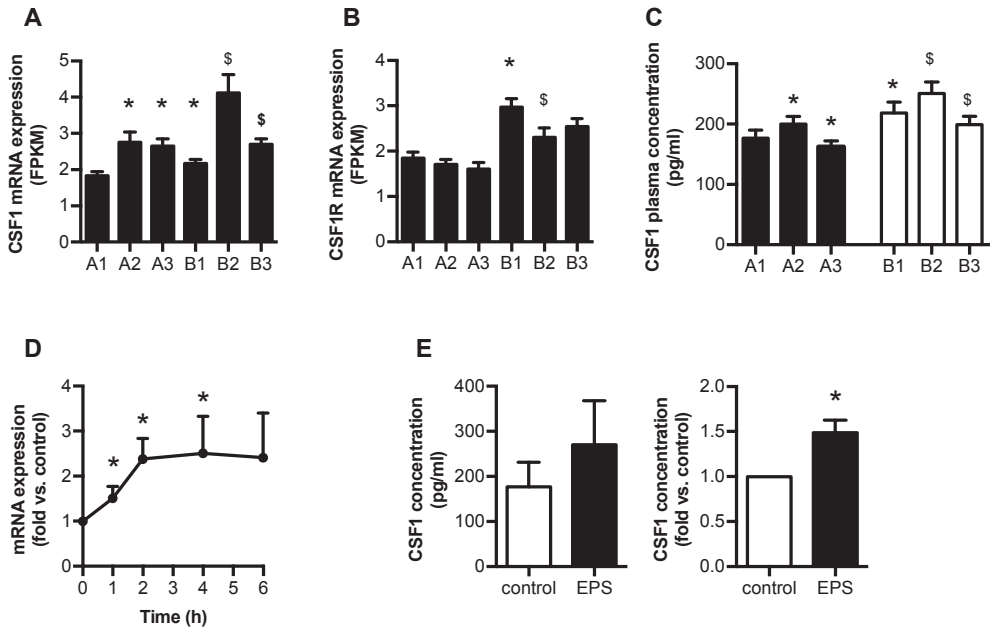

Figure 4: A) mRNA expression of CSF1 and B) CSF1 receptor (CSF1R) in skeletal muscle biopsies at baseline (A1—A3) and after 12 weeks (B1—B3), *p $<$ 0.05 vs. A1, $ p $<$ 0.05 vs. B1, p-values obtained using paired *t*-test. C) Plasma CSF1 before (n $=$ 26) and after (n $=$ 22) the 12 weeks intervention. Data are shown as absolute values, means $+$ SEM. *p $<$ 0.05 vs. A1, $ p $<$ 0.05 vs B1, paired *t*-test. D) Human skeletal muscle cells were differentiated for 6 days and subjected to acute EPS up to 6 h. mRNA expression was determined by RT-PCR, and gene expression values were normalized to expression of *RPLP0*. *p $<$ 0.05 vs. unstimulated cells (*t*-test), n $=$ 4—5. E) Myotubes were left unstimulated (control) or subjected to 24 h EPS, and supernatants were collected. CSF1 concentration was determined by ELISA. Data are shown as absolute values (left panel) and fold vs. control (right panel) and represent means $+$ SEM, *p $<$ 0.05 (*t*-test), n $=$ 5.

vs. skeletal muscle have been investigated previously. Raymond et al. reported lower expression of genes involved in metabolism, mitochondria, and muscle contraction, whereas genes related to ECM and apoptosis were more highly expressed [44]. Muscle cell cultures are isolated systems that lack *in vivo* microenvironment, innervation and communication with other cells and organs [43]. Furthermore, cultured myotubes are not as differentiated as muscle fibers *in vivo*. Although most cells in culture merge to form multinuclear myotubes, a fraction of undifferentiated and atrophic cells can be found [45]. Lastly, unlike cell cultures, skeletal muscle tissue contains many different cell types such as endothelial cells, neuronal cells, and fibroblasts. This may explain the difference in gene expression between muscle biopsies and cultured myotubes.

We focused on CSF1, because *CSF1* mRNA levels were increased after acute and long-term exercise in skeletal muscle and in cultured skeletal muscle cells after EPS. The concentration of CSF1 was increased in plasma after acute and long-term exercise and in medium conditioned by cultured myotubes after EPS. By investigating the expression of *CSF1* in publically available transcriptomic datasets from human skeletal muscle, we also know that CSF1 is influenced by several types of acute exercise. A single bout of endurance or strength training increased the expression of *CSF1* ($p < 0.05$) 2.5 h after the exercise bout (1.5- and 1.6-fold, respectively) [29]. At 5 h post-exercise the *CSF1* expression levels were returned to baseline. Furthermore, 3 h after an eccentric exercise bout *CSF1* expression was increased 1.4-fold [28].

CSF1, also known as macrophage-CSF, is a cytokine and an important hematopoietic growth factor [46], inducing differentiation of myeloid progenitors into monocytes, macrophages, dendritic cells and bone-resorbing osteoclasts [47]. CSF1 is a central regulator of macrophage numbers in tissues, and injecting mice with recombinant CSF1 induces a marked increase in number of blood monocytes. CSF1 may influence macrophage survival, proliferation, differentiation, and function, and CSF1 has been linked to diseases like arthritis, cancer, nephritis, pulmonary fibrosis, atherosclerosis, and vascular injury [48—50].

Based on our data, we hypothesize that CSF1 mediates cross-talk between skeletal muscle cells and immune cells and could be involved in exercise-induced immune responses. It is also possible that CSF1 may have other functions during exercise. Several of the previously described myokines are cytokines with immune-regulatory functions. For instance, IL6 was originally identified as a proinflammatory cytokine secreted from T-cells and macrophages, whereas exercise-induced IL6 is not associated with muscle damage and inflammation, but has been linked to metabolic regulation [5,6].

Alternative functions of CSF1 during exercise may be related to muscle adaptation. Incubation of skeletal muscle cells or monocytes with CSF1 promotes increased VEGF production and angiogenesis [51,52]. VEGF promotes angiogenesis by stimulating proliferation of endothelial cells [53]. Our data demonstrate increased expression of both *VEGFA* and CSF1 after exercise, which might be important for skeletal muscle vascularization.

Several lines of evidence suggest that CSFs may reduce serum lipids and cholesterol levels. Human CSF1 was injected to 7 boys with chronic neutropenia. After 7 days treatment, absolute neutrophil numbers increased in 4 patients, but serum cholesterol levels were reduced in all of them [54]. Shimano et al. injected rabbits with recombinant CSF1 for 7 days, lowering plasma cholesterol levels by 33% [55]. Moreover, CSF1 may modulate lipoprotein metabolism by promoting macrophages to produce lipoprotein lipase (LPL) [56]. Thus, enhanced plasma concentration of CSF1 after acute exercise may influence lipid metabolism and lower cholesterol levels.

In summary, we identified numerous transcripts that were regulated in skeletal muscle after acute and/or long-term exercise. These

transcripts encode potential myokines, which may play key roles in local and systemic adaptations to exercise. Furthermore, we identified CSF1 as a novel myokine, which was increased after acute and long-term exercise, and secreted from cultured human myotubes in response to EPS.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.01.007.

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