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RESEARCH ARTICLE | *Translational Physiology*

Impact of short- and long-term electrically induced muscle exercise on gene signaling pathways, gene expression, and PGC1a methylation in men with spinal cord injury

Michael A. Petrie,[1] Arpit Sharma,[1,2] Eric B. Taylor,[2] Manish Suneja,[3] and Richard K. Shields[1]

[1]*Department of Physical Therapy and Rehabilitation Science, Carver College of Medicine, The University of Iowa, Iowa City, Iowa;* [2]*Department of Biochemistry, Carver College of Medicine, The University of Iowa, Iowa City, Iowa; and* [3]*Department of Internal Medicine, Carver College of Medicine, The University of Iowa, Iowa City, Iowa*

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Petrie MA, Sharma A, Taylor EB, Suneja M, Shields RK. Impact of short- and long-term electrically induced muscle exercise on gene signaling pathways, gene expression, and PGC1a methylation in men with spinal cord injury. *Physiol Genomics* 52: 71–80, 2020. First published December 23, 2019; doi:10.1152/physiolgenomics.00064.2019.—Exercise attenuates the development of chronic noncommunicable diseases (NCDs). Gene signaling pathway analysis offers an opportunity to discover if electrically induced muscle exercise regulates key pathways among people living with spinal cord injury (SCI). We examined short-term and long-term durations of electrically induced skeletal muscle exercise on complex gene signaling pathways, specific gene regulation, and epigenetic tagging of PGC1a, a major transcription factor in skeletal muscle of men with SCI. After short- or long-term electrically induced exercise training, participants underwent biopsies of the trained and untrained muscles. RNA was hybridized to an exon microarray and analyzed by a gene set enrichment analysis. We discovered that long-term exercise training regulated the Reactome gene sets for metabolism (38 gene sets), cell cycle (36 gene sets), disease (27 gene sets), gene expression and transcription (22 gene sets), organelle biogenesis (4 gene sets), cellular response to stimuli (8 gene sets), immune system (8 gene sets), vesicle-mediated transport (4 gene sets), and transport of small molecules (3 gene sets). Specific gene expression included: oxidative catabolism of glucose including PDHB ($P < 0.001$), PDHX ($P < 0.001$), MPC1 ($P < 0.009$), and MPC2 ($P < 0.007$); Oxidative phosphorylation genes including SDHA ($P < 0.006$), SDHB ($P < 0.001$), NDUFB1 ($P < 0.002$), NDUFA2 ($P < 0.001$); transcription genes including PGC1α ($P < 0.030$) and PRKAB2 ($P < 0.011$); hypertrophy gene MSTN ($P < 0.001$); and the myokine generating FNDC5 gene ($P < 0.008$). Long-term electrically induced exercise demethylated the major transcription factor PGC1a. Taken together, these findings support that long-term electrically induced muscle activity regulates key pathways associated with muscle health and systemic metabolism.

epigenomic; exercise; genomic; muscle; paralysis

INTRODUCTION

The global epidemic of chronic noncommunicable diseases (NCDs) is due, in part, to lifestyle behaviors that exclude daily skeletal muscle activity (exercise). Healthy people are advised to capitalize on exercise as a form of medicine to attenuate the development and progression of chronic NCDs (4, 33). However, people with spinal cord injury (SCI) are unable to exercise the paralyzed muscle groups, which puts them at greater risk of developing chronic diseases during their lifetimes. Hence, the risk of developing cardiorespiratory impairment (6, 24), bone loss or osteoporosis (7, 16, 18, 19, 35, 63), and metabolic inflexibility or diabetes (32, 40) is pervasive among people with SCI (12, 27, 30). Recently, clinical scientists recommended that daily upper extremity exercise is important to promote the health of people with SCI (36); however, nearly 70% of the skeletal muscle of people with SCI is paralyzed, and there are no guidelines for regular use of electrically induced exercise of paralyzed muscle (36).

Prompted by scientific discovery, the healthcare field now recognizes that skeletal muscle is more than a simple force generator (54). Scientists realize that skeletal muscle plays a critical role in regulating metabolism (51) and that skeletal muscle releases small molecules via vesicle-mediated transport, like IRISIN, that influence the health of various tissues throughout the body, including the brain (28, 42, 51, 67).

In the months following SCI, paralyzed skeletal muscle atrophies, loses mitochondria causing a loss of oxidative capacity, and triggers the transformation of muscle fibers into a glycolytic, fast-fatigable phenotype (17, 53, 65). The transition of paralyzed muscle to a glycolytic phenotype is part of the cascade of events that contributes to metabolic inflexibility as the fast glycolytic skeletal muscle is associated with decreased insulin receptor sensitivity (60), decreased AMPK activation, and GLUT4 translocation of glucose into the skeletal muscle (13, 26). A central question is whether certain doses of electrically induced exercise can transform paralyzed, fast glycolytic muscle back into mitochondria-rich oxidative muscle and promote gene signaling pathways that may, someday, be used as molecular biomarkers of health in humans with disability (64).

Previous studies support that the downstream effects of electrically induced exercise mimic the bioenergetics and cell signaling of volitional exercise (2, 20, 21, 43–47). Exercise response genes, like peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1A), fibronectin type III domain containing protein 5 (FNDC5), protein kinase AMP-activated non-catalytic subunit beta 2 (PRKAB2), and myostatin (MSTN), are important regulators that support both muscle

Address for reprint requests and other correspondence: R. K. Shields, Dept. of Physical Therapy and Rehabilitation Science, 1-252 Medical Education Bldg., Univ. of Iowa, Iowa City, IA 52242-1190 (e-mail: richard-shields@uiowa.edu).

tissue as well as systemic health of other tissues. Specifically, these genes regulate mitochondrial biogenesis and metabolism pathways (31, 51), myokine development and release (irisin) (67), and hypertrophy pathways (5, 49), respectively. Importantly, many of these genes appear to be sensitive biomarkers for health in other populations (23, 29, 38, 41, 44) but have not been evaluated with long- and short-term electrically induced exercise in people with SCI.

Beyond single targeted gene analysis, high-throughput microarray analysis offers an opportunity to assess families of genes that may respond to electrically induced exercise. Gene set enrichment analysis (GSEA) allows us to compare the expression of an array of genes relative to predefined gene sets to discover the unique molecular signaling signatures and pathways activated with exercise (61, 62). For example, we used GSEA to explore a family of gene signaling pathways that others have used to show a relationship between skeletal muscle and diabetes (38). Within this study, we used a predefined gene set from the Reactome Pathway Knowledgebase (22) enabling us to explore gene pathways that have never been examined in relation to a dose of electrically induced muscle exercise in people with paralysis.

The purposes of this study were to explore predefined gene set signaling pathways from the Reactome Pathway Knowledgebase inventory; examine specific genes associated with metabolism, muscle hypertrophy, and skeletal muscle small molecule regulation; and determine if the major transcription factor, PGC1a, is epigenetically tagged with short- and long-term electrically induced exercise in humans with paralysis. We expected that novel gene signaling pathways will be discovered, that specific genes for metabolism (PGC1a and PRKAB2), skeletal muscle hypertrophy (MSTN), and skeletal muscle small molecule regulation (FNDC5) would be regulated and that the master transcription factor, PGC1a, will be epigenetically promoted with electrically induced muscle exercise.

MATERIALS AND METHODS

Subjects. We analyzed 15 paired muscle samples from males (36 ± 12 yr of age and 7.7 ± 5.8 yr postinjury) with a spinal cord injury (ASIA-A) who completed a unilateral exercise training program using electrical stimulation for a short and long duration (see Table 1 for participant characteristics). The opposite limb remained untrained to serve as a within-participant control for the exercised limb. Following exercise training, participants underwent bilateral percutaneous muscle biopsies of the trained and opposite, untrained muscle. Our previous studies support that the untrained limb maintains an untrained phenotype and serves as a stable control (2, 46). No participants reported any adverse events from the exercise training program. People with a history of lower limb fractures, peripheral nerve injury, active skin ulcerations, lower motor neuron injury, or myopathies were excluded from participation in this study. All participants provided written consent of the protocols that were submitted to, and approved by, the University of Iowa Institutional Review Board in compliance with the Declaration of Helsinki (ClinicalTrial.gov ID: NCT02622295).

Table 1. *Subject characteristics*

	Short-Term Training	Long-Term Training
Subjects, *n*	8	7
Age, yr	37.9 ± 16.1	32.3 ± 6.8
Years postinjury	6.3 ± 4.0	9.9 ± 7.8
Height, cm	187.3 ± 7.8	185.4 ± 7.3
Weight, kg	81.2 ± 17.1	72.4 ± 7.5
Training duration, wk	16.3 ± 2.4	212.9 ± 136.2
Compliance, %	87.8 ± 17.7	82.8 ± 16.4

Exercise training. Participants received unilateral exercise training using neuro-muscular electrical stimulation delivered through self-adhesive 7 × 13 cm rectangular carbon electrodes adhered to the skin above the muscle motor points (2, 46). For all participants, one limb was exercised while the opposite limb remained as a nonexercised control. All but two subjects trained the quadricep muscles, while the remaining two subjects trained the calf muscle (soleus). We included one subject in each group who trained the soleus muscle because we previously demonstrated that all paralyzed muscle becomes fast and fatigable and that these two individuals did not have assistance to gain access to their thigh muscles on a daily basis. The proximal electrode for the quadriceps muscle was placed close to the inguinal crease and over the most palpable border of the vastus lateralis, while the distal electrode was kept proximal to the musculotendinous junction of the vastus lateralis. The proximal electrode for the calf muscle was placed close to the popliteal fossa and over the most palpable border of the gastrocnemius, while the distal electrode was kept proximal to the musculotendinous junction of the soleus and gastrocnemius.

An electric stimulator with a pulse width of 200 μs and at a stimulus intensity between 200 and 500 mA was used for all exercise training sessions. Supramaximal stimulus intensity was determined during an in-laboratory session by increasing stimulus intensity until the force signal plateaued and no additional force was detected with further increases in stimulation current. This stimulus intensity was then used for remaining training sessions. Training at this supramaximal intensity ensured that nearly all muscle fibers were recruited during each muscle contraction. Each exercise training session delivered ~6,000 stimulus pulses, lasting ~1 h of total time for the session. A 1-on and 2-off ratio of contraction time to recovery time was repeatedly performed during training sessions using stimulation frequencies ranging from 5 to 20 Hz, depending on participant tolerance and the chronicity of their SCI. Because people with chronic SCI have significant musculoskeletal issues, the use of the lower frequency contractions and associated lower force levels permitted study participation while lowering the risk for musculoskeletal injuries (43, 48, 53, 55). All participants were asked to perform at least three exercise sessions per week for the duration of their exercise training program. We requested that half of the subjects train for a minimum of 3 mo (4.06 ± 0.5; 82.8% compliance) training dose, while the other half of the subjects were to train for a minimum of 6 mo (53.22 ± 34; 87.8% compliance) but were free to continue if they so desired. While no participants reported any adverse events during the duration of training, subjects in the latter group requested to continue with their training. This enabled us to track the long-term adaptations to skeletal muscle exercise in people with SCI.

Muscle biopsy and exon microarray procedure. Each participant underwent percutaneous muscle biopsies of the exercised and nonexercised limbs (vastus lateral or soleus) 72 h after no training (2, 45, 46). This 72 h time of no activity assured that we were examining the adaptations associated with the exercise rather than a short-term regulation of stress genes. Briefly, percutaneous muscle biopsies were taken from the exercised and nonexercised muscles via a Temno biopsy needle (T1420, Cardinal Health) under ultrasound guidance within a sterile field. Four passes of the needle were performed to assure a wide sampling range within the muscle and provide an adequate amount of tissue for gene expression analysis. Following harvest, muscle samples were immediately placed in RNALater (Ambion) for exon microarray analysis or flash-frozen for methylation analysis and stored at −80°C until further use. RNA was extracted with the RNeasy Fibrous Tissue Kit (Qiagen) based on the manufacturer's protocols. RNA samples were submitted to the Genomics Division of the Iowa Institute of Human Genetics (IIHG) core facility

at the University of Iowa RNA. All RNA samples met or exceeded quality and quantity standards established by IIHG with an RNA integrity number score range of 7.2–9.7 and an average of 8.6 ± 0.6. The IIHG facility performed the quantification and hybridization to Affymetrix Human Exon 1.0 ST microarrays according to manufacturer specification using a previously reported protocol (44). Microarrays were scanned with the Affymetrix Model 3000 scanner with a 7 G upgrade and the GeneChip Operating Software v1.4.

Exon microarray analysis. Hybridization probe intensities were imported, normalized using a robust multiarray average, transformed into a log2 hybridization signal with Partek Genomic Suites (v6.6; Partek Inc., St. Louis, MO). Gene symbols with multiple exon probes were collapsed into mean signal intensity for each muscle sample. Paired exercised and nonexercised samples were imported into the desktop implementation of the GSEA algorithm developed by the Broad Institute (Fig. 1) (38, 61, 62). We used the GSEA algorithm to compare 1,925 gene sets defined by the Reactome Pathways Knowledgebase, which categorized 26 primary pathway families (22, 37). There is an average of 53 genes defined within a gene set with a range of 1–2,348 genes. We used the GSEA recommended false discovery rate (FDR) cutoff of 25% to define gene sets that are significantly up- or downregulated (62, 63). The 25% FDR is recommended when searching for new signaling pathways as it limits the inclusion of gene sets with higher variations and larger potential for a false-positive result, while permitting exploratory results of gene sets with smaller effect sizes. The GSEA report highlights enrichment gene sets with an FDR of <25% as those most likely to generate interesting hypotheses and drive further research. A connectivity network map of the gene sets was developed (Fig. 2*A*) using Cytoscape 3.5.1 (52) from the gene set relationships defined within the Reactome Knowledgebase. Each node represents a gene set and the node color indicates the pathway family the gene set is defined within. Node size is relative to the number of genes defined within the gene set, therefore, gene sets with more genes defined are larger, while gene sets with few genes defined are smaller. The straight lines connecting two nodes represent the hierarchical relationship as defined by the Reactome Knowledgebase.

In addition to using the GSEA algorithm, we compared the normalized gene expression intensity and fold-change magnitude of the exercised limb to the nonexercised limb for a subset of genes with known roles in the regulation and maintenance of skeletal muscle metabolic and contractile processes. We selected pyruvate dehydrogenase subunits B and X (PDHB and PDHX) and mitochondrial carrier protein 1 and 2 (MPC1 and MPC2) as representatives of the oxidative catabolism of glucose. We selected succinate dehydrogenase A and B (SDHA and SDHB) and NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 1 and alpha subcomplex subunit 2 (NDUFB1 and NDUFA2) to represent the tricarboxylic acid and oxidative phosphorylation pathways. Finally, we selected the transcription factors PGC1A and myostatin (MSTN) and myokines fibronectin type III domain containing 5 (FNDC5) and protein kinase AMP-activated noncatalytic subunit beta 2 (PRKAB2) for their roles in signaling systemic tissues in people with diabetes. We used a least square regression and calculated the Pearson correlation coefficient between the fold-change of these select genes and the dose (training duration in weeks) of exercise training received. We repeated our GSEA with the two training subgroups. Our previous work has validated our microarray analysis with RT-PCR (2, 43, 44, 46). Additionally, we validated our methodology using muscle RNA samples analyzed on 2 distinct platforms (Affymetrix microarray platform and HTG Molecular HTG EdgeSeq oncology biomarker panel) and obtained a 0.6072 ± 0.0643 correlation coefficient ($P < 0.001$) from these 2 technologies. All microarray data sets have been submitted to the Gene Expression Omnibus (GEO) repository (accession #: GSE142426).

Epigenetic tagging: methylation analysis. We conducted a methylation analysis of a major transcription factor that regulates mitochondria biogenesis, PGC1A, on a subset of six paired samples from the long-term training cohort (49.3 ± 31.9 mo). Frozen muscle tissue samples were submitted to the University of Minnesota Genomics Center for DNA extraction, bisulfate conversion, and methylation analysis. DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen N.V., Venio, Netherlands) using the manufacturer's recommended protocol. The bisulfate conversion was performed using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA). Methylation levels were detected using the Illumina Infinium MethylEPIC BeadChip (illumine, San Diego, CA). The resulting IDAT files were imported into software using R programming language (v3.5.1) and Bioconductor (3.9) to obtain a beta value for all CpG sites for the major transcription factor PGC1α and calculated a median beta value. The specific CpG sites are: cg00767442;cg21543179;cg02946878;cg18744982; cg24716152;cg08658750;cg09427718;cg14591390;cg14757717; cg07317529;cg07744449;cg27365602;cg20723350;cg10619248; cg19701879;cg18253885;cg08550435;cg09082664;cg06772578; cg27182172;cg02868321;cg05158538;cg14892468;cg24160354; cg27514608;cg11270806;cg12691631;cg02896941;cg27461259.

Significance testing for methylation level was performed with a paired-sample *t* test between the long-term trained limb and the untrained (baseline) limb.

RESULTS

Pathway signal analysis with muscle activity. Using GSEA we discovered 223 Reactome Knowledgebase Pathways (gene sets) out of 1,925 analyzed that were significantly upregulated (FDR < 0.25) for the long-term training group. There were no gene sets that met the FDR cutoff of 0.25 for the short-term training group.

We depict the base connectivity map for the 1,925 gene sets used for the GSEA in Fig. 2*A*. The GSEA results from the long-term trainers were mapped to the base connectivity map (Fig. 2*B*). Of the 223 gene sets (1,291 genes) upregulated with long-term training, 123 of the gene sets fit under the broad family categories consisting of Metabolism (38 gene sets), Cell Cycle (36 gene sets), Disease (27 gene sets), and Gene Expression and Transcription (22 gene sets), while smaller numbers of gene sets fit under the important broad categories of Organelle Biogenesis (4 gene sets), Cellular Response to Stimuli (8 gene sets), Immune System (8 gene sets), Vesicle-Mediated Transport (4 gene sets), and Transport of Small Molecules (3 gene sets) (Fig. 2*C*). The extensive upregulation of gene sets for metabolism, disease, cell cycle, immune system, vesicle-mediated transport, and transport of small molecules was present only for the long-term training group. These findings prompted us to analyze the relationship between training time and key gene expression as well as direct gene comparisons between the long-term versus short-term training programs.

Selective gene expression analysis with long-term muscle activity. The training time was moderately correlated with gene expression (fold change) for the transcription factors, myokine small molecule transport, catabolism of glucose, tricarboxylic acid/oxidative phosphorylation genes as a result of the exercise training. Specifically, the Pearson Correlation Coefficients for signature genes associated with transcription factors and myokines were 0.79 ($P < 0.001$), −0.68 ($P = 0.005$), 0.35 ($P = 0.20$), and 0.75 ($P = 0.001$) for PGC1A, MSTN, FNDC5, and PRKAB2, respectively; signature genes associated with oxidative catabolism of glucose were 0.74 ($P = 0.002$), 0.61 ($P = 0.017$), 0.62 ($P = 0.013$), and 0.65 ($P = 0.009$) for PHDA, PDHB, MPC1, and MPC2, respectively; and signature genes

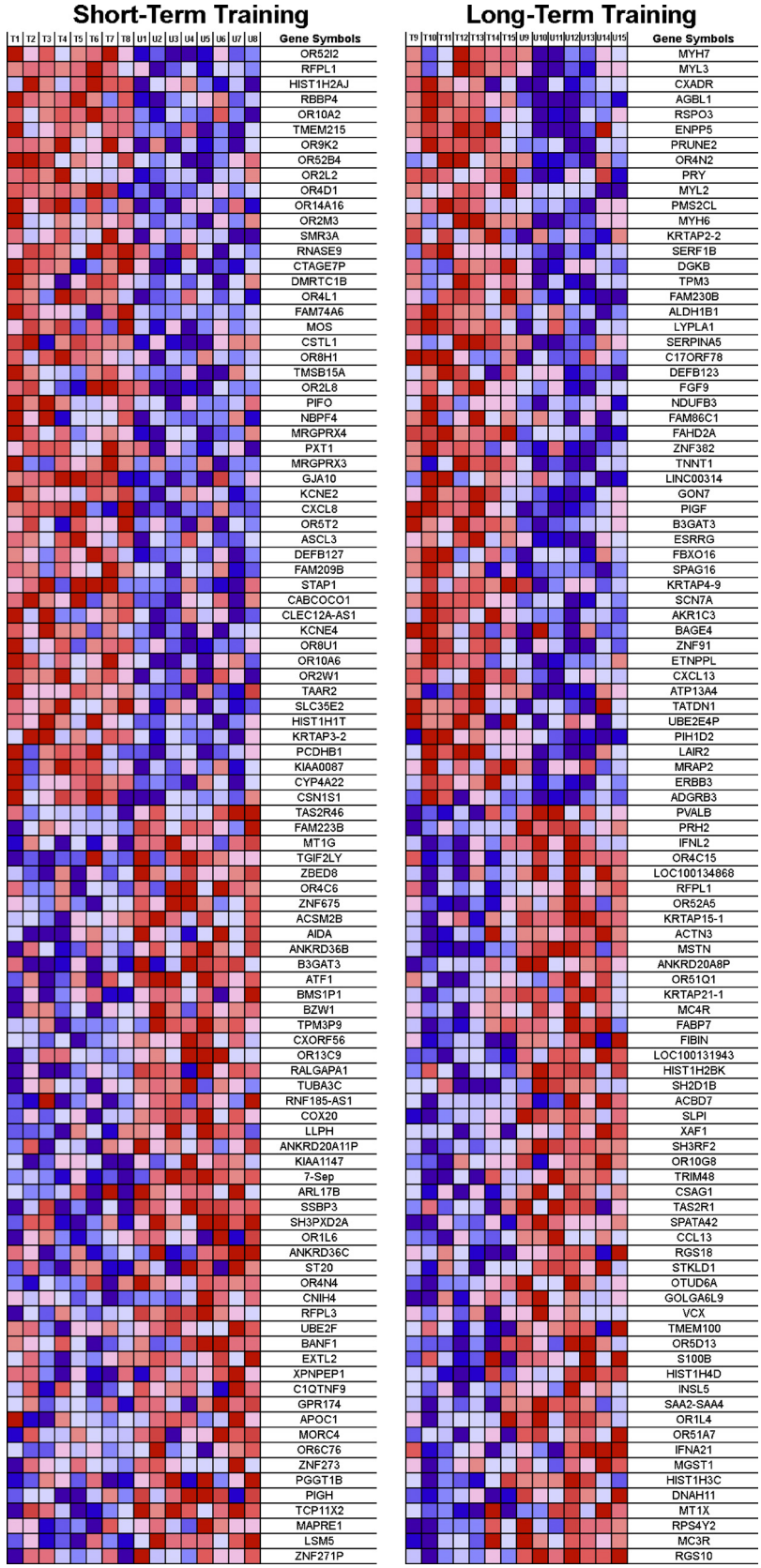

Fig. 1. Heat maps for the top 50 increased and top 50 decreased genes as determined by their enrichment score from gene set enrichment analysis (GSEA) for the short- and long-term training cohorts. The enrichment score is created from a combination of the *P* value statistic for the fold-change between the exercised and non-exercised limbs and the rank order expression intensity for each phenotype. The heat map represents the relative gene expression intensity for each subject represented as colors (red and blue). Red indicates a relative increased level of expression; while blue indicates a relative decreased level of expression for a given gene. The darker the color (red or blue), the larger the relative increase or decrease in gene expression, respectively. Among the top 50 genes the fold-changes varied across cohorts with a maximum of near 50 for the trained limb relative to the control limb, and a decrease for the control limb relative to the trained limb representing decreased gene expression (blue).

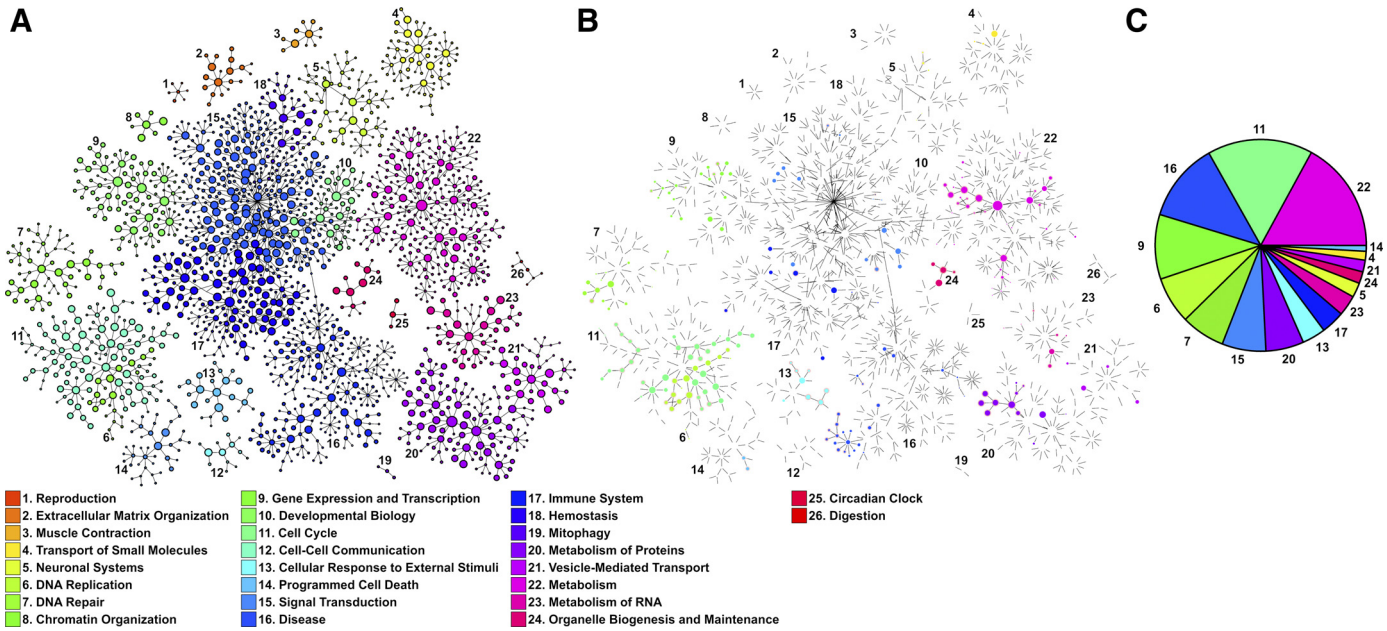

Fig. 2. *A*: base connectivity map for the 1,925 gene sets used for the gene set enrichment analysis (GSEA). Each node represents a gene set pathway defined within the Reactome Knowledgebase, which defines various biological processes categorized into 26 primary families, as indicated by node color. The size of each node depends on the number of genes defined within the gene set; therefore, larger nodes have more genes defined compared with the smaller nodes. Nodes with direct relationships are connected by a black line. *B*: the GSEA results mapped the connectivity map for the people in the long-term exercise training cohort. Only the 223 gene set pathways that met the <25% false discovery rate (FDR) cutoff are visible. The darkness of the red border color for each node indicates whether the significance of upregulation detected in the exercise limb compared with the nonexercised limb. Node transparency indicates the relative FDR from the GSEA; therefore, darker nodes were more significantly regulated than lighter nodes. Nodes that did not meet an FDR of 25% are fully transparent, but their relative position in space and connections to neighboring nodes are preserved. *C*: there were 223 gene sets found to be significantly regulated for people in the long-term exercise training cohort from 16 of the 26 gene families. Of note is that 39, 13, and 8 were gene sets associated with the Metabolism, Metabolism of Proteins, and Metabolism of RNA families. Additionally, the Gene Expression and Transcription and Cell Cycle families had 34 and 23 pathways significantly upregulated with long-term exercise training.

associated with tricarboxylic acid/oxidative phosphorylation were 0.66 ($P = 0.007$), 0.75 ($P = 0.001$), 0.20 ($P = 0.468$), and 0.71 ($P = 0.003$) for SDHA, SDHB, NDUFB1, and NDUFB2, respectively.

There was increased expression of the potent transcription factors PGC1α ($P = 0.030$) and PRKAB2 ($P = 0.011$) and the myokine-generating FNDC5 ($P = 0.008$) for the long-term training group, while the skeletal muscle atrophy transcription factor MSTN ($P < 0.001$) was decreased with long-term training, as directionally expected for upregulation of hypertrophy pathways (Fig. 3). Genes related to the oxidative catabolism of glucose including PDHB ($P < 0.001$), PDHX ($P < 0.001$), MPC1 ($P = 0.009$), and MPC2 ($P = 0.007$) were all upregulated with long-term training, but not short-term training, as compared with baseline (Fig. 4). Finally, there was an increase with long-term training for the genes associated with the tricarboxylic acid and oxidative phosphorylation pathways: SDHA ($P = 0.006$), SDHB ($P < 0.001$), NDUFB1 ($P = 0.002$), and NDUFA2 ($P < 0.001$) (Fig. 5).

Epigenetic tagging with long-term muscle activity. Methylation levels of the major gene transcription factor PGC1α was assessed on a subset of the long-term trained subjects (Fig. 6). PGC1α was significantly demethylated ($P = 0.03$). This finding supports that long-term training was associated with promoting a major transcription factor that promotes mitochondrial biogenesis and skeletal muscle hypertrophy. In the presence of periodic lapses in training, epigenetic tagging may offer a memory to promote sustained muscle oxidative capacity or promote a faster return to an oxidative state upon resuming a training protocol.

DISCUSSION

Long-term electrically induced muscle exercise appears to be a feasible technology that influences novel gene signaling pathways; regulates specific genes related to skeletal muscle metabolism, hypertrophy, and small molecule myokines; and epigenetically regulates a major transcription factor for mitochondria biogenesis. The findings suggest that long-term electrically induced muscle activity regulates paralyzed skeletal muscle gene signaling in a similar direction as that observed for volitional exercise (4).

Long-term training studies in humans with disability are complex. We employed a protocol that allowed participants to train beyond their prescribed dose of 6 mo. When participants choose to continue with a training protocol, it supports that the intervention is pragmatic, has real life compliance, and may be reasonably implemented, if deemed effective. We captured the effect of long-term training, for the first time, in people with paralysis, showing a strong relationship between training duration and regulation of several genes and gene signaling pathways. We intervened on only one leg, offering a method to control for other epigenetic and/or lifestyle factors such as diet (1), sleep (10), circadian rhythm (25), and hormonal factors (50).

Evidence supports that reduced skeletal muscle activity contributes to pervasive systemic metabolic disease. The chal-

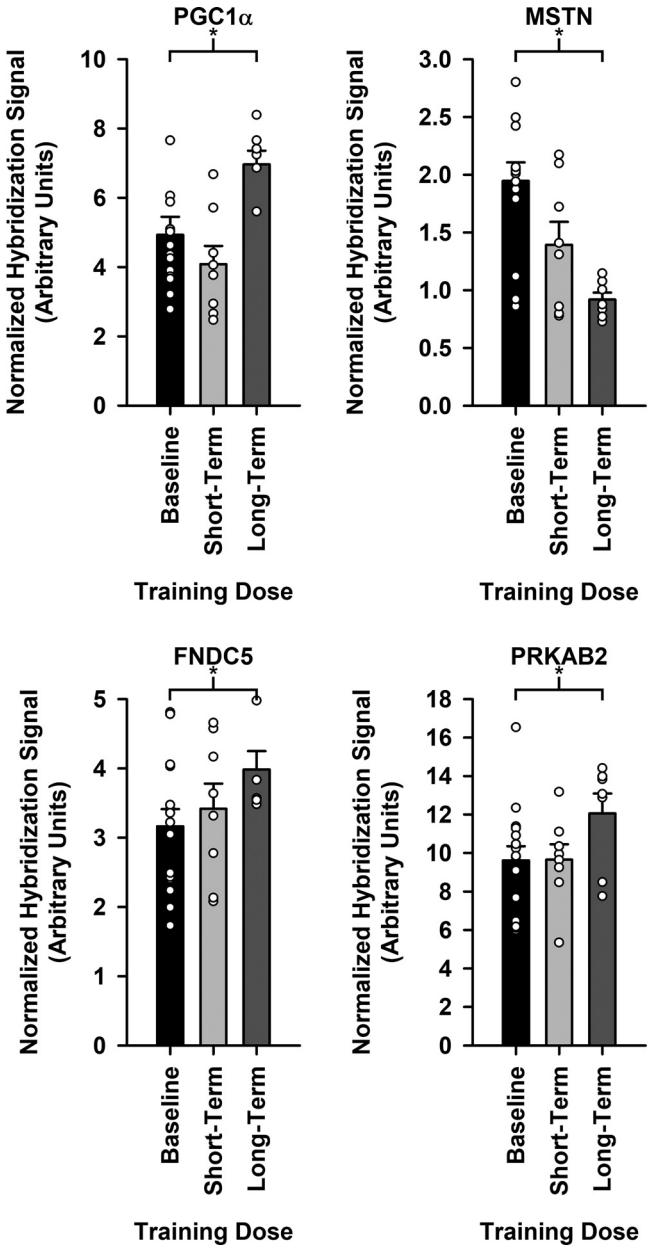

Fig. 3. Gene expression for genes with important transcriptional regulatory activity in skeletal muscle (PGC1α), regulation of muscle atrophy (MSTN), and myokine signaling to systemic tissues (FNDC5 and CTSB). The expression in the long-term exercise training cohort was significantly higher compared with those in the short-term exercise training cohort and at baseline (untrained limb) for PGC1α, FNDC5, and PRKAB2. Meanwhile, MSTN was significantly decreased after long-term exercise training compared with those in the short-term exercise training cohort and at baseline (untrained limb). *P value < 0.05 from a mixed-model ANOVA.

lenge for rehabilitation medicine scientists is to determine if small durations of skeletal muscle activity per day can regulate key gene pathways associated with improved systemic health, even if minimal activity is possible for the remainder of a day. The novelty of this study is that the activity level of people with SCI is much less than that of people without SCI who are sedentary. For example, most people who are sedentary stand up and sit down multiple times per day, unlike someone with a disability who is transferred into and out of their wheelchair without any weight-bearing opportunities. For these reasons, we designed our study to focus on genes linked to muscle hypertrophy, metabolism, and small molecule myokine production that are regulated with regular muscle activity.

We expected that electrically induced exercise would increase signaling pathways known to increase the size of the muscle; as increased capacity for work should increase muscle hypertrophy (59). The significant repression of the MSTN gene, along with our previous phenotype reports (2, 46, 57), supports that long-term electrically induced training triggers

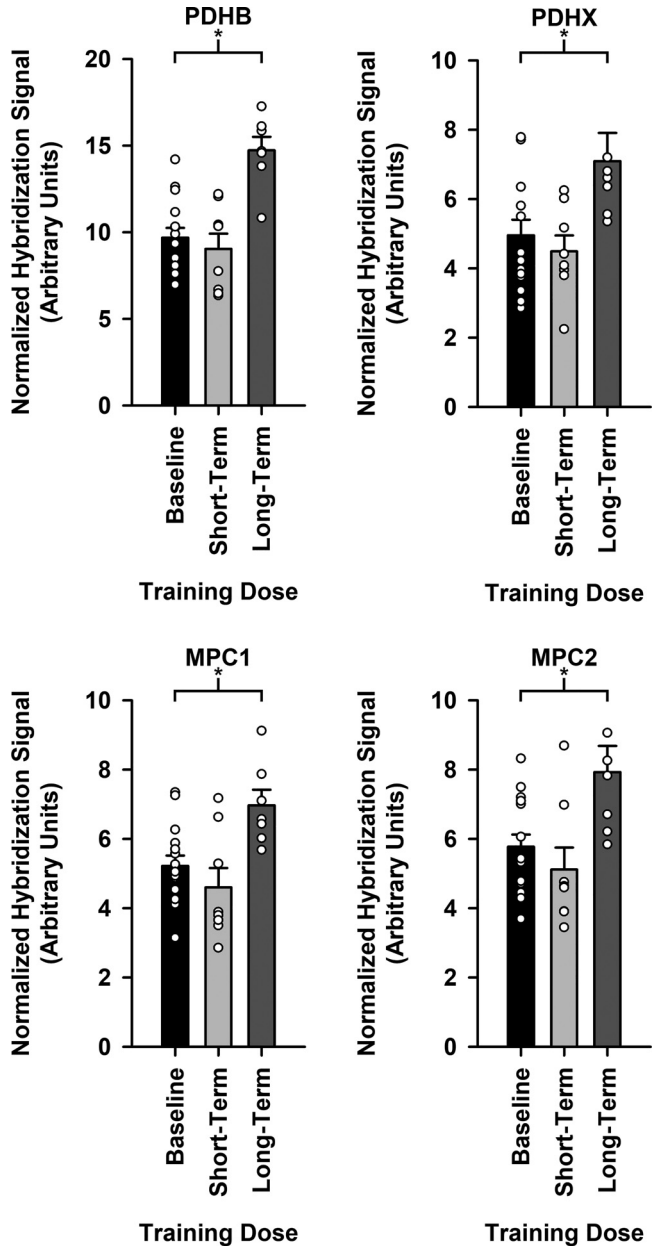

Fig. 4. Gene expression for genes important for glucose metabolism (PDHB and PDHX) and the transfer of pyruvate from the cytosol to the mitochondria for continued oxidation within the tricarboxylic acid cycle and oxidative phosphorylation pathways (MPC1 and MPC2). The expression in the long-term exercise training cohort was significantly higher compared with those in the short-term exercise training cohort and at baseline (untrained limb) for PDHB, PDHX, MPC1, and MPC2. *P value < 0.05 from a mixed model ANOVA.

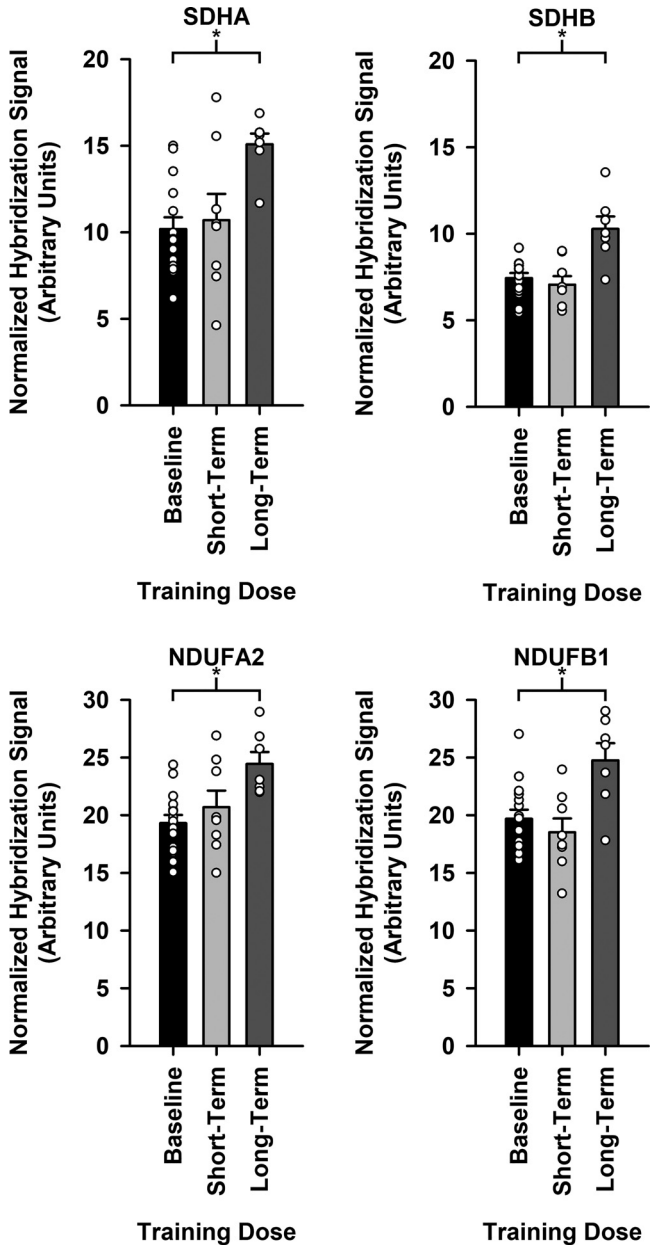

Fig. 5. Gene expression for genes important for tricarboxylic acid cycle (SDHA and SDHB) and oxidative phosphorylation (NDUFA2 and NDUFB1). The expression in the long-term exercise training cohort was significantly higher compared with those in the short-term exercise training cohort and at baseline (untrained limb) for SDHA, SDHB, NDUFA2, and NDUFB1. *P value < 0.05 from a mixed-model ANOVA.

muscle hypertrophy, even though the muscle is active for only a small part of each day. Only ~1 h of daily paralyzed muscle activity, as performed in this study, coupled with nearly 23 h of relatively no activity, significantly regulated hypertrophy gene signaling pathways, relative to the untrained opposite leg. This finding is important because studies for healthy people typically include other muscle activities outside of the training regimen (walking and activities of daily living). The notion that ~1 h of muscle activity per day, relative to the opposite limb that received no intervention, regulated these pathways suggest that this intervention holds promise, if it can be delivered to more muscles simultaneously. While this study is a "proof of concept" as to what is possible, it does not offer a strategy to deliver an intervention to multiple muscle groups simultaneously.

While many individuals with SCI have involuntary spasms, the relative contribution of spasticity to "normal" overall activity when compared with people without SCI has been estimated at <5% of a typical 24 h day (54). Our work showing that all people, with and without spasticity, transform their skeletal muscles to fast glycolytic phenotype illustrates this point (54–59). Importantly, the amount of activity lost with SCI does not approximate the limited activity observed in an able-bodied person who elects to be sedentary.

Consistent with our previous work, there was a 52% improvement in muscle endurance for the trained limbs as compared with the untrained limbs, supporting a change from fatigable, glycolytic fibers to a more fatigue resistant, oxidative phenotype (57). The increased expression of the major transcription factor PGC1α, as evident from the long-term training in this study and the demethylation, indicates that mitochondrial biogenesis was increased. Close examination of the data supports it took much longer to upregulate significant oxidative phosphorylation pathways in SCI compared with the weeks to months reported for several voluntary-based muscle exercise studies (34, 39, 66). This finding is likely attributable to the significant epigenetic repression in response to the limited use of paralyzed skeletal muscle for several years. Accordingly, the long-term protocol, delivered only 3 times/week, is necessary to attenuate repression and promote key transcription factors associated with oxidative metabolism.

Our specific gene regulation studies support that the intervention impacted downstream genes to transport pyruvate across the inner membrane of the mitochondria in preparation for the pyruvate dehydrogenase reaction (conversion to acetyl CoA). The increased expression of the MPC1 and MPC2 genes

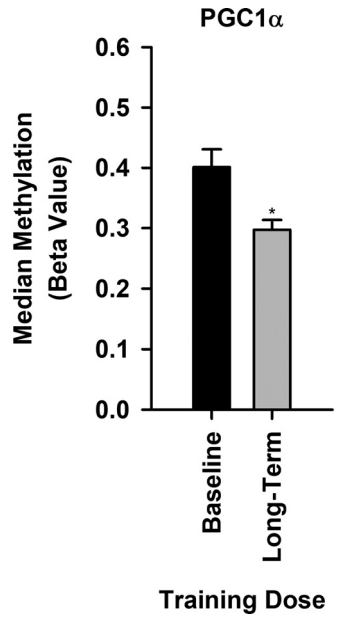

Fig. 6. The median demethylation after long-term exercise training across all CpG sites for the potent transcription regulator PGC1α in people with SCI at baseline and after long-term exercise training using electrical muscle stimulation. *P value < 0.05 from a paired t test.

(9) with long-term training supports that the capacity to transport pyruvate into the mitochondria was likely enhanced, along with the increased expression of the PDHA and PDHB genes whose encoded proteins are involved with the conversion of acetyl CoA for entry into the TCA cycle.

Downstream regulation of NDUFB1 and NDUFB2, both electron transport-encoding genes that transfer electrons from NADH to ubiquinone within the mitochondrial inner membrane as part of Complex I, were induced by the long-term training protocol. Taken together, the gene expression and extensive gene signal pathway analysis supports that approximately 1 h of exercise can positively influence skeletal muscle in people with paralysis, if it becomes part of a routine long-term lifestyle behavior, even if the muscle activity is induced by electrical stimulation and not by volitional activation. Importantly, the demethylation of the PGC1α gene is the first demonstration, to our knowledge, that a major transcription factor can be epigenetically tagged in humans with chronic paralysis.

A novel finding from this research is the connectivity network map developed from the Reactome Knowledgebase. From the total 1,291 genes that were regulated, 601 were tightly coupled with metabolism. We previously reported that paralyzed skeletal muscle becomes atrophied and converts to fast, fatigable, glycolytic muscle within 1 yr after paralysis (11, 53, 55, 56, 58). These muscle adaptations are thought to be associated with reduced insulin receptor sensitivity (3, 60), triggering systemic hyperinsulinemia (8, 15, 32, 40) and ultimately metabolic disease. The upregulation of gene sets, induced by the long-term training, for other categories like disease, immune system, vesicle-mediated transport, and transport of small molecules was not anticipated, given the minimal daily dose of activity. Accumulating evidence supports that long-term exercise triggers the development of small extracellular vesicles with microRNA as their cargo (14). Once in the circulating blood stream, small noncoding RNA may play pivotal roles in regulating tissues throughout the body, including the central nervous system and cognitive function and memory (67).

Methodological considerations. Executing a long-term training protocol in people with SCI offers several challenges and requires careful implementation and interpretation of the data. Because a muscle biopsy requires that participants stop training for at least 3 wk, we designed this study to only biopsy 3 days after the final training session. We designed the study with only single muscle biopsies because our experience supported that midtraining biopsies disrupt the training to the extent that we lose the effect of long-term training by damaging the paralyzed muscle tissue. In addition, forcibly stopping an exercise behavior creates an uncertainty as to whether participants will get back into the routine of training again. For these reasons, we utilized a within-subject control model that afforded us the opportunity to biopsy, at the same time, the opposite limb that received no training but had identical predisposing genetics.

One source of variance in our study could have been that the long-term training and short-term training each had one subject that trained the calf muscles rather than the quadricep muscles. These individuals did not have the personal assistance to gain daily access to their thigh muscles but wanted to participate in the study. We verified that 97% of the variance of gene expression in the trained vastus lateralis samples could be explained by the trained soleus muscle samples, supporting that the training induced was similar for both muscles studied. This was not surprising because we previously showed that the soleus muscle becomes a homogenous fast, glycolytic muscle as a result of paralysis from SCI (53). Furthermore, while our previous work demonstrated a high consistency between changes in gene expression detected by a microarray analysis and PCR analysis (2, 43, 46), the lack of a secondary analysis by PCR must be considered when interpreting these results.

Because we allowed those in the 6 mo groups to continue training, we ended up with two distinct groups; short-term and long-term trainers, but the identical number of training weeks was not consistent within those two broad categories. Despite these realities of long-term human-based trials, we obtained compliance with the protocol as determined by a sensor embedded in the electrical stimulator that provided the date, time, and the number of pulses delivered each day. Overall, our pragmatic outcomes are internally consistent and support that long-term electrically induced muscle activity may be an important future recommendation for rehabilitation after an SCI.

Summary and Conclusions

Our study shows that people with SCI who participate in long-term electrical stimulation-induced muscle exercise upregulate key genes or gene signaling pathways and promote epigenetic tagging of PGC1a, a major gene transcription factor. These findings suggest that a lifestyle of a little muscle activity each day may evolve into a recommended guideline to enhance the health of people with SCI. Future studies are warranted to refine the dose (number of muscles activated) and assess the impact on health outcomes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.K.S. conceived and designed research; M.P., M.S., and R.K.S. performed experiments; M.P., A.S., E.B.T., M.S., and R.K.S. analyzed data; R.K.S. interpreted results of experiments; M.P., A.S., E.B.T., M.S., and R.K.S. prepared figures; M.P., A.S., E.B.T., M.S., and R.K.S. drafted manuscript; M.P., A.S., E.B.T., M.S., and R.K.S. edited and revised manuscript; M.P., A.S., E.B.T., M.S., and R.K.S. approved final version of manuscript.

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