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Decreased expression of myogenic transcription factors and myosin heavy chains in *Caenorhabditis elegans* muscles developed during spaceflight

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Summary

The molecular mechanisms underlying muscle atrophy during spaceflight are not well understood. We have analyzed the effects of a 10-day spaceflight on *Caenorhabditis elegans* muscle development. DNA microarray, real-time quantitative PCR, and quantitative western blot analyses revealed that the amount of MHC in both body-wall and pharyngeal muscle decrease in response to spaceflight. Decreased transcription of the body-wall myogenic transcription factor HLH-1 (CeMyoD) and of the three pharyngeal myogenic transcription factors, PEB-1, CEH-22 and PHA-4 were also observed. Upon return to Earth animals displayed reduced rates of movement, indicating a functional defect. These results demonstrate that *C. elegans* muscle development is altered in response to spaceflight. This altered development occurs at the level of gene transcription and was observed in the presence of innervation, not simply in isolated cells. This important finding coupled with past observations of decreased levels of the same myogenic transcription factions in vertebrates after spaceflight raises the possibility that altered muscle development is a contributing factor to spaceflight-induced muscle atrophy in vertebrates.

Key words: spaceflight, muscle remodeling, *Caenorhabditis elegans*, myosin heavy chain, development.

Introduction

The neuromuscular system is recognized as one of the physiologic systems most affected by spaceflight (Fitts et al., 2001). When exposed to the microgravity of spaceflight, muscles developed on Earth have been shown to display changes in morphology, contractile function and myosin heavy chain (MHC) gene expression (Caiozzo et al., 1994; Caiozzo et al., 1996; Criswell et al., 1996; Day et al., 1995; Edgerton et al., 1995; Fitts et al., 2000; Harrison et al., 2003). Recently, it was shown that cultured embryonic avian muscle cells are directly responsive to spaceflight, undergoing atrophy as the result of decreased protein synthesis (Vandenburgh et al., 1999). The observation that MHC levels were decreased postflight relative to control cultures suggests that muscles developing in microgravity express less MHC.

Cultured muscle cells lack innervation, which is required for proper muscle development and to prevent muscle atrophy *in vivo* (Szewczyk and Jacobson, 2005). Therefore, studies of muscle development or atrophy in whole animals are required to confirm results obtained with cultured cells. Muscles of the nematode *Caenorhabditis elegans* have been studied extensively, and show significant similarity to vertebrate muscles. The principal muscles in *C. elegans* are the body wall and pharyngeal muscles (Epstein et al., 1974). Body wall muscle is analogous to vertebrate skeletal muscle and functions to allow locomotion. In body wall muscle, myogenesis appears to be controlled by the helix-loop-helix transcription factor HLH-1 (Chen et al., 1994; Krause, 1995), which controls the expression of two MHC isoforms [MHC A and B encoded by *myo-3* and *unc-54*, respectively (Dibb et al., 1985; Epstein et al., 1974; Karn et al., 1983; MacLeod et al., 1981; Miller et al., 1986)]. The pharyngeal muscles function rhythmically in feeding and possibly pseudocoelomic circulation. They are analogous to vertebrate cardiac muscle, and contain two MHC isoforms [MHC C and D, encoded by *myo-2* and *myo-1*, respectively (Ardizzi and Epstein, 1987; Miller et al., 1986)]. In developing pharyngeal muscle, these MHCs appear to be regulated by the cooperative action of

transcription factors PEB-1, PHA-4 and CEH-22 (Gaudet and Mango, 2002; Kalb et al., 2002; Okkema and Fire, 1994; Okkema et al., 1997). The transcriptional regulation of *C. elegans* myosin genes is in many respects similar to that of vertebrates. However, both body wall and pharyngeal muscle also contain the invertebrate paramyosin core protein encoded by *unc-15* (Epstein et al., 1985; Kagawa et al., 1989). The extensive similarities to mammalian muscle have allowed *C. elegans* to be developed as a small animal model for studies of a number of types of muscle atrophy, including muscular dystrophy (Grisoni et al., 2002), starvation (Zdinak et al., 1997), denervation (Szewczyk et al., 2000), growth factor alterations (Szewczyk and Jacobson, 2003), aging (Fisher, 2004) and altered function of myosin chaperones (Hoppe et al., 2004). *C. elegans* has also been developed as a model for studies of spaceflight effects on physiology (Hartman et al., 2001; Nelson et al., 1994a; Nelson et al., 1994b). In this study, we therefore employed space flown *C. elegans* to confirm and extend the findings made with cultured embryonic avian muscle cells.

In this report we demonstrate that muscles of *C. elegans* that developed in space, during the European Space Agency (ESA) DELTA mission, display decreased expression of the transcription factors controlling both body wall and pharyngeal muscle myogenesis as well as decreased expression of muscle-specific MHCs. These results demonstrate that the changes previously observed in cultured embryonic avian muscle cell development in space also occur *in vivo* in the nematod*e C. elegans*. Our results suggest that altered MHC expression is a highly conserved molecular response to spaceflight and can be studied in small genetic model organisms. Furthermore, decreased protein synthesis in developing muscle implies that, in space, a reduction in muscle repair and remodeling may underlie at least a portion of spaceflight-induced muscle atrophy.

Materials and methods

Preparation of worms for spaceflight

The nematode *Caenorhabditis elegans* Bristol N2 strain was pre-cultured in *C. elegans* maintenance medium [CeMM (Szewczyk et al., 2003)] for 1 month at 20°C. Approximately 10 000 worms of mixed larval stage were transferred into 2.5 ml culture bags containing fresh CeMM. A total of 40 ml of culture bags were enclosed in a vented EC-1 (the Biorack Experiment Container type 1, developed by Professor Eberhard Horn from Ulm, Germany), which allows air exchange between the environment and the experimental culture bags. EC-1s were placed inside KUBIK, a modular incubator developed by the European Space Agency (ESA) for spaceflight experiments that can ensure a temperature rise from 6°C to 37°C by 1°C increments (http://www.esa.int/esaHS/SEMS9D638FE_iss_0.html). Nematodes in EC-1 containers were cultured in KUBIK at 12°C for 5 days and then at 12°C for the 2 days immediately preceding launch in the Soyuz spacecraft. After launch, the worms were kept at 20°C in KUBIK accommodated in the Soyuz spacecraft until the spacecraft had docked with the International Space Station (ISS). The flight worms were cultured in KUBIK at 20°C for 10 days, and after landing they were immediately frozen in liquid nitrogen. A full description of the ICE-FIRST experimental design and hardware has been submitted for publication elsewhere.

Extraction of total RNA

After thawing the samples, worms were washed twice with M9 buffer (Sulston and Hodgkin, 1988) to remove CeMM. The worms were repeatedly frozen and thawed five times. Total RNA from the space flown and ground control worms was extracted using an Isogen RNA purification kit (Wako Pure Chemicals, Osaka, Japan) following the manufacturer's protocol. The concentration and purity of extracted RNA were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The gene expression profile was analyzed using the Affymetrix GeneChip *C. elegans* Genome Array (22,150 gene species, 22,500 element array) (Affymetrix, Santa Clara, CA, USA) performed by the Dragon Genomics Center (TaKaRa Bio Inc., Shiga, Japan).

Real-time quantitative PCR

To measure the expression differences of muscle-related genes between ground control worms and space-flown worms, real-time PCR was performed. The preparation of cDNA was carried out using ExScript RT reagent kit (TaKaRa Bio) and iCycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Subsequently the preparation of the reaction mixture for measuring the cDNA quantity was performed using Premix EX Taq (TaKaRa Bio) according to the manufacturer's instructions. Reaction and fluorescence monitoring were done using a Smart Cycler real-time PCR system with Smart Cycler software version 2.0C (Cepheid, Sunnyvale, CA, USA). The following primer pairs were used for PCR amplification: *gpd-2* (5′-ACCGGAGTCTTCACCACCATC-3′ and 5′-ACGACT-ACGAGGTTACAAGCA-3′), *act-3* (5′-ACGAACATTG-GAGCATCAGCA-3′ and 5′-TCAATTGGGTACTTGAG-GGTAAGG-3′), *hlh-1* (5′-GAGCACACCAAATGCCAC-AGA-3′ and 5′-CTCCGCCAGCAGTAGACGTT-3′), *myo-3* (5′-AAAGGTCAAGCCAATGCTCAA-3′ and 5′-TCTTCA-ACCAAATCGGCAAC-3′), *unc-54* (5′-CCCGACTTGAG-GACGAACA-3′ and 5′-AGCCTTGGAACGGGATTGAC-3′), *peb-1* (5′-TGCGATTAGCGTGAGCAGTATG-3′ and 5′-TGTCTGGATTGTATCGGACAAATGA-3′), *pha-4* (5′-ATCTTGGCCTAATTGACCCATC-3′ and 5′-TCATCTGT-GCTTGCGTGGTT-3′), *myo-1* (5′-GGTCCGTCAAGAA-CAAGAGCA-3′ and 5′-ATCATGGCACGTTCAGCATC-3′), *myo-2* (5′-CAAGCAACGTCCACGTGAAGA-3′ and 5′-CTCCGAGTCAATTCCGAAGCA-3′) and *unc-15* (5′-CGC-CGATCTTGGATCACTCA-3′ and 5′-GGCACGCTCACG-TTCAACTC-3′). Each amplification was performed in triplicate. Values were statistically analyzed using PRISM software (GraphPad software Inc., San Diego, CA, USA). Significance was accepted at $P<0.05$.

Western blotting

To determine the steady-state levels of myosin proteins, western blots were performed in triplicate. Total protein was extracted using two-dimensional (2D) extraction solution [7 mol l^{-1} urea (Wako Pure Chemicals, Osaka, Japan), 2 mol l^{-1} thiourea (Wako), 4% (w/v) CHAPS (Dojin, Osaka, Japan), 0.5% (v/v) carrier ampholyte (Bio-Rad), 40 mmol l^{-1} dithiothreitol (Nacalai Tesque, Kyoto, Japan)]. Total protein concentration was determined by using a 2D Quant Kit (Amersham Biosciences, Piscataway, NJ, USA). 110 μg of total protein was collected from approximately 40 000 worms of each ground control and space flown sample. The sample solution was prepared to contain 0.2 μg μl^{-1} total protein. An equal volume of 2× Laemmli sample buffer was added to the sample solution. The samples were boiled for 5 min and sonicated for 1 min. Samples containing 2 μg of protein per lane were electrophoresed on an SDS–polyacrylamide gel (5–10%, Bio-Rad) and blotted onto a Hybond-P membrane (Amersham) at 15 V for 60 min. The membrane was washed with blocking solution [5% (w/v) non-fat dried milk, 0.05% (w/v) Tween 20, 100 mmol l^{-1} Tris–HCl (pH 7.5), and 500 mmol l^{-1} NaCl]. MHC B and C were detected using an anti-MHC B and C mouse monoclonal antibody (5-12; 1:1,000 dilution), and paramyosin was detected using an anti-paramyosin rabbit polyclonal antibody (1:10 000 dilution). Both antibodies were kindly provided by Dr Kagawa. Anti-GAPDH antibody (1:1 000 dilution) (Imgenex, San Diego, CA, USA) was used to detect GAPDH as an internal standard. Anti-mouse IgG horseradish peroxidase-conjugated mouse anti-goat IgG (Pierce, Rockford, IL, USA) and anti-rabbit IgG horseradish peroxidase linked whole antibody from donkey (Amersham) were used as the secondary antibodies. Each secondary antibody was used at a 1:10 000 dilution. After treatment using and ECL Plus chemiluminescence detection kit (Amersham), immunoreactive proteins were detected using a VersaDoc chemiluminesence detection system (Bio-Rad). Values were statistically analyzed using PRISM software (GraphPad). Significance was accepted at $P<0.05$.

Movement assay

Live animals were video recorded at the landing site within 2 h of return utilizing a ProScope [Bodelin Technologies, Lake Oswego, OR, USA (http://www.theproscope.com)]. Animals were placed in a temperature controlled chamber at 20°C and allowed to equilibrate before videos were recorded. Bright-field illumination was achieved using a battery powered LED housed inside a 60 mm Petri dish. Samples were placed upon this Petri dish and recorded on a battery powered PC using the ProScope at high magnification (approximately 40× total magnification). Proper focal depth was achieved utilizing a custom plastic housing designed by Dr Conley for the ProScope, with oblique incident illumination. Videos were used to determine movement rates using the previously described swim test (Szewczyk et al., 2002). Ten randomly selected animals from each condition were evaluated, and the movement rate of each animal was measured 10 times. Sample videos can be found at http://weboflife.nasa.gov/celegans/questionsice.htm.

Results

Overall culture growth was similar between space flown and ground control worms

During the flight, population size more than doubled for both the space flown and matched control worms. From the result of statistical analysis, there was no significant difference between the number of ground control and flight worms ($P=0.083$). Approximately 20 μg of total RNA and approximately 110 μg of total protein were extracted from each population. Total RNA and total protein per worm were the same in flight and ground samples (Table 1). These results demonstrate that the populations of worms developed equally well, with no gross defects in growth due to this spaceflight.

Some muscle related genes show decreased expression after spaceflight by microarray analysis

DNA microarray analyses of muscle-related genes were performed using the Affymetrix GeneChip *C. elegans* Genome Array. Fig. 1 shows the full genome scatter plot of gene expression, comparing the flight worms and the ground controls. Colored symbols (triangles, open and closed squares) are genes involved in muscle development. Muscle-related genes that showed lower expression flags in flight worms rather than in ground control worms are indicated by closed square symbols. This group includes important genes involved in muscle development such as MHCs, troponins, tropomyosins. Muscle-related genes for which there was no difference in expression between control and flight worms are showed by open square symbols. Closed triangles indicate the genes of transcription factors involved in expression of MHCs. The expression change in muscle-related genes, as indicated by DNA microarray, are summarized in Table 2A. The expression

Table 1. *Summary of the numbers, proteins and total RNA of the worms in ICE-First*

Experimental group	Number of worms		Total proteins (μg)	Proteins/worm (ng/worm)	Total RNA (ng)	Total RNA/worm (ng/worm)
	Before launch	After landing*				
Ground	20 000	37 000 (±2400)	110	2.97	21.3	0.576
Flight	20 000	47 300 (±4300)	128	2.71	27.3	0.577

*The number of worms was statistically assessed by Student's *t*-test.

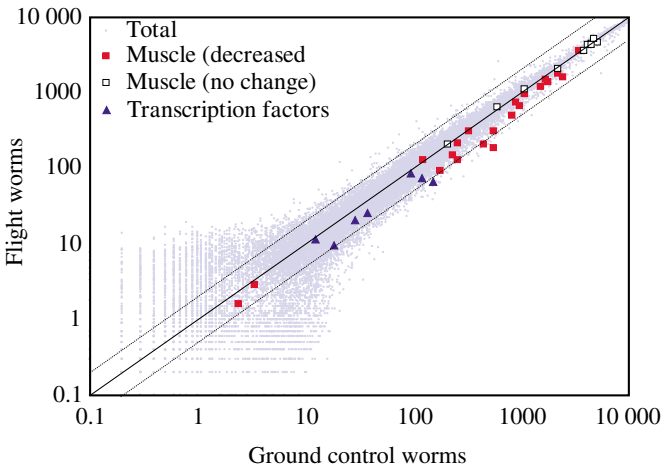


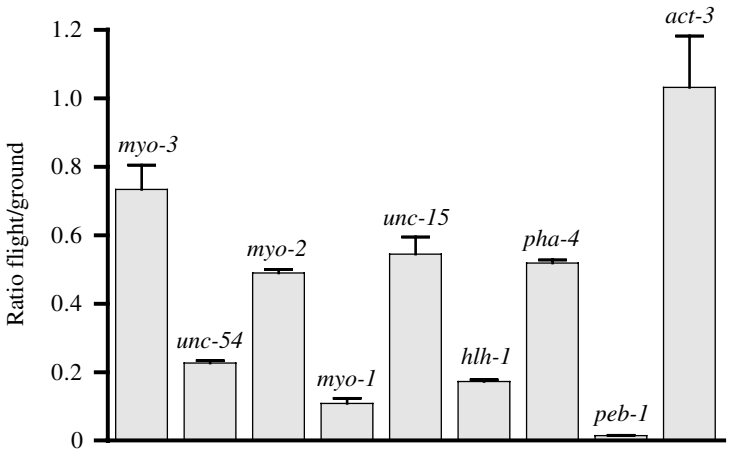
Fig. 1. Scatter plot of gene expression by DNA microarray analysis. The whole gene expression profile is plotted as gray spots. Squares and triangles indicate muscle-related genes and their transcription factors, respectively. The closed squares indicate significantly lower expression in response to spaceflight. These groups include myosin heavy chain (MHC) isoforms, troponins and tropomyosins whereas the open squares include actins and non-muscle myosins.

levels of *myo-1*, *-2*, *-3* and *unc-54* genes that encode myosin heavy chains (MHC D, C, A and B, respectively) and *unc-15* that encodes paramyosin, were all decreased in the space flown worms. Expression of the transcription factors controlling the expression of these genes was likewise decreased. Transcription of *myo-3* and *unc-54* in the body wall muscle is regulated by CeMyoD (HLH-1/*hlh-1*), which showed a modest decrease. Transcription of *myo-1* and *-2* in pharyngeal muscle is controlled by *ceh-22*, *peb-1* and *pha-4*, which decreased significantly in space flown worms (Table 2B). Statistically significant reductions in transcript levels of genes essential to muscle structure were not limited to the MHCs (Table 2A). The genes encoding troponins and tropomyosins were also significantly decreased, specifically, *tnc-2* (troponin C), *tnt-2*, *-3*, *-4* (troponin T) and *lev-11* (tropomyosin).

Not all myosin or muscle-related genes displayed statistically significant decreases (Table 2C). Non-muscle MHC and the actin-encoding genes *act-1*, *-2*, *-3* and *-4* did not show significant changes in gene expression. As a class, genes encoding molecules related to protein degradation, such as ubiquitin ligases, cathepsins and calpains, did not show significant changes in gene expression (Table 2D). However, two predicted ubiquitin ligases, *skr-6* and *skr-18*, show increased expression levels.

Real-time quantitative PCR confirms that myosin-related genes show decreased transcript levels after spaceflight

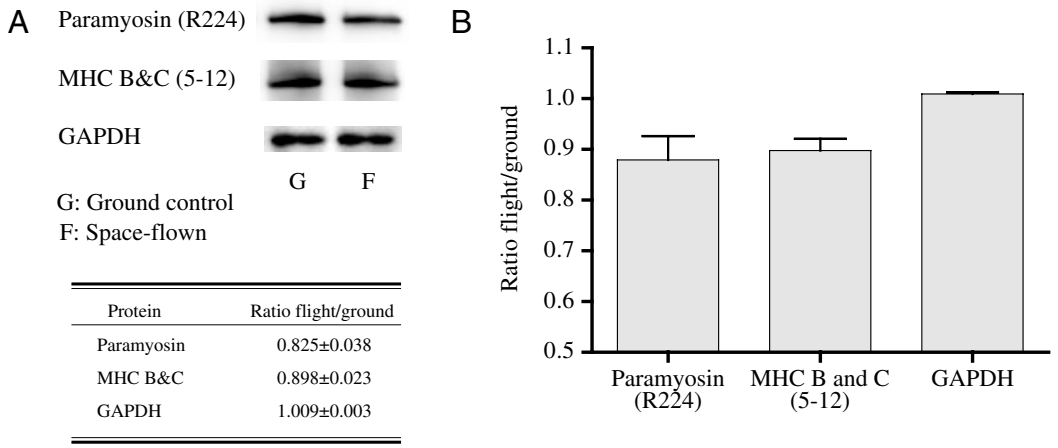
DNA microarrays are a semi-quantitative and an imprecise tool for examining expression differences. To confirm and evaluate more quantitatively the expression differences predicted by the DNA microarrays, real-time quantitative PCR was performed. The gene encoding one of three predicted glyceraldehyde-3-phosphate dehydrogenases, *gpd-2*, is expressed in muscle and is considered a 'housekeeping gene' that should be expressed at a steady level under most conditions, including spaceflight. We used *gpd-2* as an internal standard against which to normalize these quantitative PCR data. The body wall muscle-specific *myo-3* and *unc-54* were decreased 73.3% and 22.6%, respectively, in the space flown worms (Fig. 2). The myogenic transcription factor *hlh-1* was also decreased compared with that of ground control worms. The expression levels of pharyngeal muscle-specific *myo-2* and



Gene	Relative ratio	
	Real time PCR (F/G)	Microarray (F/G)
myo-3	0.733±0.072	0.801
unc-54	0.226±0.007	0.747
myo-2	0.490±0.010	0.329
myo-1	0.108±0.015	0.454
unc-15	0.544±0.051	0.878, 0.776, 0.799
hlh-1	0.172±0.006	0.927
ceh-22	–*	0.705, 0.522
pha-4	0.518±0.009	0.439, 0.594
peb-1	0.015±0.001	0.897, 0.660
act-3	1.031±0.150	0.917

* The primer set of *ceh-22* was not determined.

Fig. 2. Real-time quantitative PCR analysis of myosin heavy chain (MHC)-related genes in space flown worms. Expression levels of MHCs (*myo-1*, *-2*, *-3* and *unc-54*), paramyosin (*unc-15*), transcription factors (*hlh-1*, *pha-4*, *peb-1*, *ceh-22*) and actin (*act-3*) are shown. The mRNA levels of each gene were adjusted to that of GAPDH (*gpd-2*) mRNA, which was used as the internal standard. All MHC-related genes decreased in the space-flown worms. Actin levels remained unchanged in response to spaceflight. A summary of the PCR and DNA microarray values is shown in Table 2. Values in the column of real-time PCR are means ± s.e.m. (P<0.05). F, space-flown worms; G, ground controls.



Protein	Ratio flight/ground
Paramyosin	0.825±0.038
MHC B&C	0.898±0.023
GAPDH	1.009±0.003

Fig. 3. (A) Western blot analysis of myosin heavy chains (MHCs) and paramyosin. MHC B and C were detected by anti-MHC B and C mouse monoclonal antibody (5-12), and paramyosin was detected by anti-paramyosin rabbit polyclonal antibody (R224). The protein product of *gpd-2*, GAPDH, was again employed as an internal standard, but the measured levels are *not* adjusted relative to the standard. Both MHCs and paramyosin in the flight worms show a 10% decrease in the steady-state protein levels compared to that of the ground control worms (B). The table in A summarizes the expression level. Values are means ± s.e.m. (P<0.05).

myo-1 were decreased, 49.0% and 10.8%, respectively. The transcription factors controlling expression of these MHC genes, *pha-4* and *peb-1*, also decreased in the space flown worms (51.8% and 16.7%, respectively). These results qualitatively validate and confirm the observations made using the DNA microarray analysis, and give a quantitative evaluation of the extent of decreased mRNA expression.

MHC and paramyosin protein levels are reduced after spaceflight as measured by western blot

To establish whether the observed changes in mRNA level are reflected in the steady-state levels of myosin-associated proteins, we quantified the amount of MHC B, C and paramyosin by 2D (not shown) and triplicate western blot analysis using GAPDH as an internal standard (see Materials and methods). GAPDH is the protein product of *gpd-2,* which was used as an internal standard for the real-time PCR. The density of the MHC and paramyosin bands decreased 10% in the space-flown worms relative to the control worms, but GAPDH levels were not significantly altered (Fig. 3). These results both confirm the observations made by DNA microarray and real-time PCR analyses, and document that those changes are in fact reflected at the protein level.

Space flown C. elegans display reduced movement after landing

The observed decreases in MHC expression might be predicted to produce defects in muscle contractility. To evaluate whether the returned animals displayed any defects in movement, we examined the video footage recorded immediately after landing. Ten animals randomly selected from one sample moved with a velocity of 90±9 waves min^{-1} [mean ± standard deviation for 100 observations (10 animals each observed 10 times)]. Ten animals randomly selected from the matched ground controls had a velocity of 112±8 waves min^{-1}; not significantly different than animals under standard culture conditions (120±10 waves min^{-1}). These results demonstrate that space flown *C. elegans* displayed a small but significant movement defect.

Discussion

Altered muscle development in response to spaceflight

Our results demonstrate that four distinct MHC isoforms (MHC A, B, C and D), expressed in a tissue-specific manner, all display decreased expression in *C. elegans* developed in space relative to ground controls. MHC A and B, encoded by *myo-3* and *unc-54* respectively, are components of thick filaments in body wall muscle and are required for locomotion (Epstein et al., 1985; Epstein et al., 1974; MacLeod et al., 1981; Miller et al., 1986; Waterston et al., 1977). MHC B localizes in the terminal portion of thick filaments (Miller, 3rd et al., 1983; Miller et al., 1986), and is the major MHC isoform as measured by molar proportion (Honda and Epstein, 1990). MHC C and D, encoded by *myo-2* and *myo-1* respectively, localize to pharyngeal muscle and are required for eating (Ardizzi and Epstein, 1987; Miller et al., 1986). Despite the differences in tissue expression and function, the results from DNA microarray, quantitative PCR, and western blot analyses all indicate that expression of MHC genes is decreased in response spaceflight. As expected for methodological reasons (microarrays are subject to more quantitative variability), the DNA microarray and quantitative real-time PCR results agree qualitatively but not quantitatively. These data suggest that MHC gene expression is influenced by microgravity at the level of transcription. Given the different cellular locations and functions of these four MHCs, our results suggest that MHC gene expression is influenced by microgravity independent from MHC localization or function.

In vertebrates, MyoD, a basic helix-loop-helix protein

(bHLH), has been identified as a myogenic factor playing a critical role in the determination and differentiation of skeletal muscle (Davis et al., 1987). This transcription factor has been proposed as a putative target for degradation in response to intramuscular atrophy inducing signaling events on Earth (Szewczyk and Jacobson, 2005). *C. elegans* has a body wall muscle-specific transcription factor, CeMyoD, that is also a helix-loop-helix type transcription factor (Chen et al., 1994; Krause, 1995). Pharyngeal muscle-specific transcription factors are *peb-1*, *pha-4* and *ceh-22* (Gaudet and Mango, 2002; Kalb et al., 2002; Okkema and Fire, 1994; Okkema et al., 1997). Transcripts encoding all of these transcription factors were significantly decreased in the space flown worms, consistent with our hypothesis that microgravity is influencing MHC protein expression at the level of transcription. Based upon decreased mRNA levels, the regulation of these transcription factors also appears to be at the level of transcription, even more so given that mRNA may be more, not less, stable in response to spaceflight. Elucidating the mechanism for microgravity depression of myogenic transcription factors is an important area for future research.

In our DNA microarray, two ubiquitin ligases show increased

Table 2. *DNA microarray analyses of muscle related genes*

Gene	Encoded protein	Probe ID	Base signal (ground control)	Experimental signal (space-flown)	Exp/base log ratio
(A) Muscle related genes (decrease in the space-flown worms)					
myo-3	Myosin A	CEG_20137	265.2	213.4	–0.3
unc-54/myo-4	Myosin B	CEG_20207	1843.9	1376.5	–0.5
myo-2	Myosin C	CEG_20321	570.8	188	–1.1
myo-1/let-75	Myosin D	CEG_20622	463.7	210.5	–1.2
unc-15	Paramyosin	CEG_03849	1097.9	964.1	–0.2
		CEG_18717	1544.7	1198.5	–0.4
		CEG_22451	2280.4	1822.1	–0.3
mup-2	Troponin T	CEG_21254	927.7	744.1	–0.2
tnt-2		CEG_13924	994.2	676.8	–0.4
tnt-3		CEG_02096	180.9	94.2	–0.6
tnt-4		CEG_12457	266.2	130.4	–1.2
pat-10, tnc-1	Troponin C	CEG_18940	3593.2	3536.8	–0.2
tnc-2		CEG_17171	566.2	308.6	–0.8
lev-11	Tropomyosin	CEG_00278	837.5	492.6	–0.7
		CEG_00282	1752.3	1479.2	–0.3
		CEG_04015	127.2	129.1	0.4
		CEG_04571	2.4	1.6	–0.8
		CEG_04677	240	151.5	–0.9
		CEG_04705	3.4	2.9	–0.4
		CEG_04706	335.6	315.6	–0.2
		CEG_22538	2543	1627.8	–0.5
(B) Transcription factors of myosin heavy chains					
hlh-1	HLH-1	CEG_22040	12.4	11.5	0.4
ceh-22	CEH-22	CEG_03982	29.5	20.8	–0.6
		CEG_21974	18.6	9.7	–0.9
pha-4	PHA-4	CEG_22243	154.3	67.7	–0.8
		CEG_22244	123.9	73.6	–0.9
peb-1	PEB-1	CEG_02310	97.1	87.1	–0.4
		CEG_16296	38.5	25.4	–0.4
(C) Muscle related genes (no change)					
act-1	Actin	CEG_18324	5296.9	4624.1	–0.2
act-2		CEG_01097	4673.2	4551.7	–0.1
act-3		CEG_22586	4867	5166.3	0.1
		CEG_22587	4267.5	4346.3	0
		CEG_02273	2271.9	2082.7	–0.2
act-4		CEG_03076	3901.1	3557.2	–0.1
		CEG_05204	4613	4379.4	–0.1
nmy-1	Nonmuscle myosin	CEG_04032	1099.9	1112.3	0
	heavy chain	CEG_20094	612.7	633.3	0
nmy-2		CEG_18023	211.6	204.6	0.1

Table 2. *Continued*

Gene	Encoded protein	Probe ID	Base signal (ground control)	Experimental signal (space-flown)	Exp/base log ratio
(D) Protein degradation related genes					
Ubiquitin ligase					
ari-1	ARI-1	CEG_03828	56.2	67.7	0
		CEG_13039	224.9	261.2	0.2
skr-1	SKR-1	CEG_22058	832.5	822.8	0
skr-2	SKR-2	CEG_22056	647.5	841.2	0.2
skr-3	SKR-3	CEG_16266	785.4	752	0
skr-4	SKR-4	CEG_16509	400.8	624.1	0.5
		CEG_16510	845.1	966.9	0.2
skr-5	SKR-5	CEG_16273	341.9	328.1	–0.1
skr-6	SKR-6	CEG_16248	5.2	6.3	0.9
skr-7	SKR-7	CEG_04899	14.5	11.1	–0.3
skr-8	SKR-8	CEG_00023	31.1	38.6	0.4
		CEG_01062	40.1	41.8	0.5
skr-9	SKR-9	CEG_01063	15.5	20.5	0.3
skr-10	SKR-10	CEG_16699	14.1	10.4	–0.3
skr-11	SKR-11	CEG_16353	22	23	0
skr-12	SKR-12	CEG_17470	8.2	6.3	–0.3
skr-13	SKR-13	CEG_01053	14.1	22.5	0.4
skr-14	SKR-14	CEG_04912	1	10.4	1.4
skr-15	SKR-15	CEG_16533	34	31.9	–0.2
skr-16	SKR-16	CEG_16446	63.4	68.9	0.1
skr-17	SKR-17	CEG_16549	203	232.1	0.2
skr-18	SKR-18	CEG_03762	0.7	2.6	1.4
		CEG_10998	49.5	42.6	–0.1
		CEG_10999	3.6	13.1	1.6
		CEG_16424	68.1	86.9	0.3
skr-19	SKR-19	CEG_22039	232.7	230.1	0
skr-20	SKR-20	CEG_22041	214.2	198.9	–0.1
skr-21	SKR-21	CEG_02240	40.3	42.6	–0.3
		CEG_22051	91.3	89.5	0
wwp-1	WWP-1	CEG_03315	291.6	257.7	–0.1
wwp-1		CEG_15444	570.8	602	0.1
Cathepsin					
asp-1	ASP-1	CEG_11751	6069.1	5242.5	–0.3
cad-1	cathepsin D				
cpl-1	CPL-1	CEG_00167	35	40.6	0
		CEG_02271	1893.6	1659.4	–0.2
cpr-1	CPR-1	CEG_21574	1492.9	880.4	–0.9
cpr-4	CPR-4	CEG_22239	2779.8	1750.4	–0.6
cpr-6	CPR-6	CEG_08403	1904.2	1716.9	–0.2
cpz-1	CPZ-1	CEG_18329	1515.7	1387.4	–0.2
cpz-2	CPZ-2	CEG_17861	1877.3	1993.6	0
F32A5.3	Serine carboxypeptidases	CEG_19556	306.1	335.5	0
R07E3.1	Cysteine proteinase Cathepsin F	CEG_18041	1786.4	1358.5	–0.3
Calpain					
clp-1	CLP-1	CEG_17007	502.3	433.8	–0.1
clp-2	CLP-2	CEG_18817	139.6	144.6	0.2
clp-3	CLP-3	CEG_03586	9.5	6	0.2
		CEG_13836	19.8	13.7	0.1
clp-4	CLP-4	CEG_15434	400.6	287.7	–0.2
clp-6	CLP-6	CEG_15512	13.5	13.6	0
clp-7	CLP-7	CEG_01518	239.3	247.1	0.1
		CEG_15514	163.9	144	0

level of expression. Each of these *skr* genes is predicted to encode a homolog of Skp1 in *S. cerevisiae*, a core component of the SCF (Skp1/Cullin1/F-box) ubiquitin-ligase complex that facilitates ubiquitin-mediated protein degradation (Nayak et al., 2002). Currently, the function of *skr-6* and *skr-18* remain to be established *in vivo.* Skp1-containing complexes can bind muscle-specific F-box proteins, for example atrogin-1, and induce muscle atrophy in vertebrates (Bodine et al., 2001; Gomes et al., 2001). In denervated muscle of rats, mRNA levels of MHC I were decreased whereas mRNA level of atrogin-1 was significantly increased, although a causal relation of these altered levels of expression was not examined (Horinouchi et al., 2005). It may be of interest to determine if *skr-6* or *skr-18,* for which RNAi results in altered movement (Simmer et al., 2003), contributes to muscle atrophy in *C. elegans*.

We have limited our detailed analyses to MHC expression in order to validate observations previously made with cultured cells. It seems clear from the microarray analysis that MHCs are not the only muscle genes that show altered expression when development takes place during spaceflight. It seems probable that development in space does not simply lead to smaller or 'atrophic' muscles, since actin expression is not significantly altered, but rather that development responds to the environment. Until such time as we are able to understand genomic (e.g. microarray) 'signatures' of muscle, and we have multiple replicate analysis from a number of flights (which means reproducibility both within one flight, as is the case here, and between identical or near-identical exposures on multiple flights), broader interpretation of the microarray data is difficult. It is clear that there are numerous changes but we currently lack the ability to model the functional consequences of multiple simultaneous changes in gene expression within muscle. For now all we can conclude is that muscle development is altered as the result of numerous changes in gene expression (Fig. 4), of which MHC gene expression is but one example.

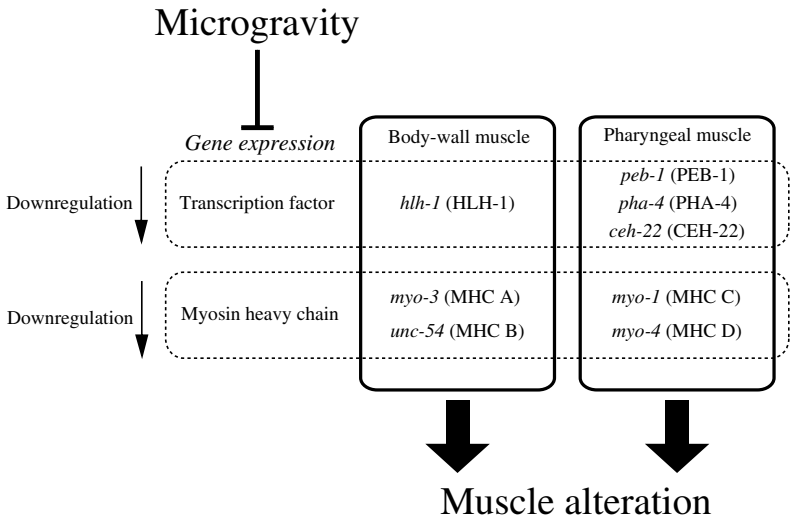


Fig. 4. Model for downregulation of myosin heavy chain (MHC) gene expression in space. This downregulation is due to a reduction in the level of transcripts encoding the transcription factors that regulate body-wall and pharyngeal MHC expression. Gene expression is influenced by microgravity at the level of transcription, independent of the differences between MHC isoforms and these transcription factors in tissue expression and function.

A functional consequence of decreased body-wall MHC or myogenic transcription factor gene expression, as determined by RNAi experiments, is decreased locomotion (Simmer et al., 2003). Therefore, our observation of decreased MHC levels is sufficient to explain the observed decreased locomotion, at the population level. However, alternative explanations such as decreased arginine kinase expression (the nematode orthologue of creatine kinase) or increased degradation facilitated by *skr-6* or *skr-18*, could also explain our observations. Data obtained at the level individual worms will be required to ask and answer questions regarding the specific nature of the relationship between MHC levels and decreased locomotion. Although future studies during spaceflight are required to answer such questions, our results suggest that small genetic model organisms, such as *C. elegans*, can be employed usefully in such studies.

Decreased contractile demand may not underlie the observed decrease in MHC expression

Past studies in vertebrates have shown shifts in MHC isoform-based fiber types in response to spaceflight (Kischel et al., 2001; Widrick et al., 1999). Here we report that spaceflight also influences MHC expression in developing *C. elegans* nematodes. As in vertebrates, *C. elegans* MHCs are essential and major molecules of sarcomeric thick filaments (Epstein et al., 1974). The observation that in *C. elegans*, as in vertebrates, MHC expression is altered in response to spaceflight suggests that the mechanisms monitoring MHC expression requirements are highly conserved.

Reduced workload is believed to be a primary cause of atrophy associated with spaceflight (di Prampero and Narici, 2003), and has resulted in many physiologists renaming postural muscles 'antigravity' muscles. However, two observations suggest there was not a decreased contractile demand in *C. elegans* muscles during development. First, there is a strong contractile demand place upon body wall muscle in order to molt and complete development successfully (Frand et al., 2005). Second, pharyngeal muscle contraction, like cardiac muscle contraction, is rhythmic and required throughout life. Defects in pharyngeal contraction disrupt developmental timing (Lakowski and Hekimi, 1998). Because developmental timing was unaffected in response to spaceflight (N.J.S. and C.A.C., unpublished observations) it seems unlikely that there was a large decrease in contractile activity in either body wall or pharyngeal muscle. At present, our results with *C. elegans* lacking 'antigravity' muscle suggest that reduced contractile activity, *per se*, is not the only underlying cause of spaceflight-

induced muscle alterations. Furthermore, the observation that atrophy in response to spaceflight is not limited to 'antigravity' skeletal muscle, but also occurs in vascular smooth muscle and cardiac muscle (Goldstein et al., 1998; Zhang et al., 2001), suggests that such non contractile activity-based mechanisms for muscle alteration may be highly conserved.

Altered muscle development in flight may contribute to spaceflight induced muscle atrophy

Muscle atrophy in response to space flight can become a serious medical condition, for which the development of countermeasures is essential. Current countermeasures (e.g. exercise) are known to be relatively ineffective (Adams et al., 2003). It may be that we have simply not yet developed the optimal exercise regimen to combat spaceflight-induced muscle atrophy. However, there is growing evidence that decreased use may not be the only cause of muscle atrophy in response to spaceflight. For example, past observations show that vascular and cardiac muscles also undergo atrophy (Zhang et al., 2001) and that cultured embryonic muscle is intrinsically sensitive to the effects of spaceflight (Vandenburgh et al., 1999). Here, we have shown that *C. elegans* muscles are also intrinsically sensitive to the effects of spaceflight, specifically, that developing nematode muscles decrease myogenic transcription factor and MHC expression during spaceflight. Our observations appear to complement the past observations of decreased MHC expression seen in developing chick muscle cells (Vandenburgh et al., 1999) and decreased MyoD expression seen in developing rat muscles (Inobe et al., 2002). These results suggest not only that decreased MHC expression observed in vertebrate muscles during spaceflight may be due to decreased levels of myogenic transcription factors, but also that muscle metabolism in flight (e.g. normal fiber turnover, repair) will produce 'atrophic' muscle as the result of decreased levels of myogenic transcription factors. The recent observation in rats that genes involved in regulation of muscle satellite cell reproduction are downregulated during spaceflight (Taylor et al., 2002) is consistent with this hypothesis. Although future studies to confirm this hypothesis are required, these transcription factors are likely to represent targets for countermeasure development. Such small-molecule countermeasures may also become more broadly useful in the treatment of muscle atrophy induced by bedrest, aging and a number of clinical conditions.

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