Multi-Generational Culture of *C. Elegans* on a Long-Term Space Flight Revealed Changes in Expression of Genes Involved in Longevity, DNA Repair, and Locomotion

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ABSTRACT

Scientists are trying to determine the long term effects of exposure to microgravity and space radiation on various cellular and biological functions. We used *C. elegans* as the model organism to study the changes in gene expression. Wild type worms were grown in a liquid medium using the CHab hardware. The CHab was flown on the STS116 flight to the International Space Station (ISS) and returned to earth on the STS118 flight. Upon landing, surviving worms were extracted and RNAlater added. Ground controls were grown at Bioserve in Colorado and passaged synchronously. RNA was later extracted and mRNA gene expression was analysed using Affymetrix GeneChip® *C. elegans* Genome Array. The results revealed that 858 known genes were differentially expressed (p-value ≤ 0.05 and fold change ≥ ± 2); namely 608 genes were up-regulated and 250 genes down-regulated. The genes *dod-19* and *dod-3* which are the downstream effectors of the forkhead transcription factor *daf-16* were up-regulated. *Daf-16* regulates insulin/TGF signaling pathway that influence metabolic alterations, increased stress and microbial resistance. The glutathione S-transferase (*gst-1*), Flavin-containing MonoOxygenase (*fmo-3*) and radiation sensitive genes (*rad-51* and *him-6*) were all up-regulated suggesting responses to oxidative stress. The down-regulation of muscle-related genes (*mua-3*, *col-97*, *col-109*, and *col-113*) maybe due to reduced mechanical stress in muscle exposed to long-term microgravity. This was the longest exposure of a multi-generational cohort of *C. elegans* to microgravity which were passaged through at least 10 generations in space. The results suggest key changes in genes involved in ageing, DNA repair, oxidative stress and muscle growth.

INTRODUCTION

Due to increasing numbers of people spending longer time in space and with the possibility of long-term human travel to Mars in future, it is crucial to understand the long-term effects of exposure to the space environment like microgravity and space radiation on various cellular and biological functions. *Caenorhabditis elegans (C. elegans)*, a small nematode, provides a lot of advantages as the model organism to measure the long term exposure to microgravity as it is robust, easy to grow and is able to survive starvation. They have at least 60-80% of their genes homologous with humans (22, 27). *C. elegans* has previously been used in five spaceflights experiments, the first being onboard the STS-42 flight (1992) where it was observed that the male *C. elegans* mated successfully and the full life-cycle of *C. elegans* occurred in space without obvious abnormal developmental process (32). Two studies flown on the STS-42 (1992) and STS-76 (1996) flights, reported an 8-fold increased rate of mutation in the *unc-22* structural gene of the worms as well as a 3.3 fold higher rate of mutation in *fem-3* mutant strain (which is used to determine mutagenesis in worms) as the direct effect of cosmic radiation (15, 29). Another experiment carried onboard STS-95 (1998) yielded no results and both the ground control and flight animals died, probably due to anoxia. On the final flight (STS-107) in 2003 before ICE-FIRST, researchers from NASA established a new inflight culture system for *C. elegans* and using a chemically defined liquid medium for *C. elegans* growth. This medium was named the *C. elegans* maintenance medium (CeMM) (39). ICE-FIRST continued this and also demonstrated the potential utility of a model specimen approach for liquid culture of *C. elegans* to study biological changes in space

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(41). The ICE-FIRST study group reported that apoptosis level was normal with some increment of *ced-1* which demonstrated that the rate of apoptosis and the engulfment of cell corpses occurred normally during spaceflight. Similar gene expression on checkpoint and apoptosis related genes, *ced-1*, *-2*, *-3*, *-4*, *-9*, *egl-1*, *nuc-1*, *cep-1*, *atl-1*, *mrt-2*, *rad-5* and *rad-51/rdh-1* for both spaceflight and ground control animals suggested that there was no difference in apoptosis in response to spaceflight (19). This suggests that animals, perhaps human beings too, retain the ability to eliminate cells that failed to repair DNA lesions caused by cosmic radiation during short-term spaceflight (25). The gene transcription response to the 10-day spaceflight was examined and found that the bulk of these genes were thought to be regulated by two signaling pathways, insulin and TGF-beta pathways that the worm used to sense and also respond to the external environment (32). The results suggested that altered Insulin and /or TGF-beta signalling in space may underlie many biological changes seen in response to the short-term spaceflight (1).

In the short term exposure of *C. elegans* to microgravity and space radiation, there was no significant difference in the mutation rate of the *unc-22* gene and there was no chromosomal rearrangement detected between spaceflight and control samples (46). However, there was a decreased expression of myogenic transcription factors and myosin in spaceflight which suggested changes in the muscular development under microgravity (43). The genes coding F-actin also showed no changes after 10 days spaceflight. Their results demonstrated that the expression of *dys-1* increased significantly in body-wall muscles, while *hlh-1*, *myo-3*, *unc-54* and *egl-19* RNA levels showed a decrease. They postulated that the increased *dys-1* expression after flight implied that myocytes would accept more gravity signals by dystrophin-glycoprotein complex (DGC) in microgravity in order to keep mechanical balance within the cells. It was concluded that DGC was involved in the mechanical transduction in body-wall muscles of *C. elegans* when gravity varied, which potentially played a vital role in gravitational sensing. The changes of *hlh-1*, *myo*-3, *unc-54* and *egl-19* suggested that they had the effects of promoting microgravity-induced muscular atrophy both in structure and function (14, 43, 46).

It is probable that a longer exposure of *C. elegans* in space may give a clearer view of the changes either in terms of mutations or gene expression. Our study was the first opportunity to culture *C. elegans* for more than 6 months in space and it has allowed the worms to multiply through successive generations including adaptation to the space conditions. The premise here was that the surviving worms at the end of the experiment would give us an insight to the cumulative adaptative changes occurring in and transferred from one generation to another. This study has enabled us to study the long term exposure of microgravity and space radiation on the genome-wide gene expression of the worms. Results yielded from this study would help us better understand the cellular adaptation mechanism to microgravity which can be extrapolated to other organisms including humans. Our experiment was flown on the Space Shuttle Discovery STS-116 on 9th December 2006 to the International Space Station (ISS). The worms, in a specially constructed CHab hardware, were incubated in the commercial generic processing apparatus (CGBA) and were passaged every month from one opticell to the next. Ground controls were grown at Bioserve and passaged synchronously. The CHab returned to earth on the STS118 flight (Space Shuttle Endeavour) on 21st August 2007. After landing, surviving worms were extracted and RNAlater® added. Both ground controls and space-flown samples were transported to our laboratory in Malaysia whereby the RNA was later extracted and mRNA gene expression was analysed using Affymetrix GeneChip® *C. elegans* Genome Array. Three technical replicates from each sample were used in the hybridization experiments.

MATERIALS AND METHODS

PREPARATION OF WORMS FOR SPACEFLIGHT

The wild type *C. elegans (CC1 strain)* was from a batch of 2,000 worms grown at the Jacobson Laboratory in the University of Pittsburgh's Department of Biological Sciences and later cultured in the *C. elegans* maintenance medium (CeMM) as previously described (39, 40) using the CHab hardware at the Bioserve Space Technology, University of Colorado, USA).

EXTRACTION OF TOTAL RNA

After thawing the samples, CeMM was removed by washing twice with M9 buffer (37). The worms were repeatedly frozen and thawed five times (18). Total RNA was extracted from the space flown and ground control worms using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) following the supplied protocol. The RNA was purified using RNeasy Mini kit and DNase 1 Solution (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The average concentration and purity were assessed by micro capillary electrophoresis Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and an RNA Integrity Number equal to or higher than 6 were selected for microarray genomic profiling.

3' IVT EXPRESSION ANALYSIS

RNA samples were processed following the two-cycle eukaryotic target labeling protocol by published methods from Affymetrix. Biotinylated targets were hybridised to the Affymetrix GeneChip® *C. elegans* Genome Array (Affymetrix, Santa Clara, CA, USA). This array represents 22,500 transcripts from *C. elegans*. Sequence information used to select these probe sets came from the Sanger Center

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database release and GenBank® release 121 (December 05, 2000). All sequences were functionally re-annotated by Affymetrix for improved descriptions (Affymetrix, Santa Clara, CA, USA). Hybridization results were scanned using the GeneChip Scanner 3000. The scanning process was conducted according to the fluidics protocols FS450_0007. Each probe set was treated as a separate gene. Internal controls of housekeeping genes and a test chip trial were run prior to test samples. The quality control process was done using the GeneChip Operation Software.

GENE CHIP SCANNING AND PRELIMINARY ANALYSIS

The quality of all GeneChip expression data were within the ‘‘good sample’’ limits according to preliminary data analysis parameters (GeneChip Operation Software) such as background and noise averages, percentage of present calls, presence of internal hybridization controls in increasing signals, presence of poly-A controls as decreasing signals and GAPDH to beta actin 30/50 signal ratios.

MICROARRAY DATA ANALYSIS

Expression profiles were analyzed using Partek® Genomics Suite™6.4 Software (Partek Inc, St. Louis, USA). After background correction, normalization for each array and gene was performed. Data were filtered based on both signal intensity and detection call. Genes with signal intensities < 50 under two conditions [Spaceflight (n = 3) vs. Ground Control (n = 3)] were filtered, as were those genes for which data were absent from six or more of 12 compared samples. Gene expression was considered different between spaceflight and ground control for P-value < 0.05 when compared by the unpaired Student’s t-test (called as ANOVA in Partek® Genomics Suite™6.4 Software) using multiple testing correction (Benjamini and Hochberg False Discovery Rate) and cut off fold change less than -2 and more than 2 were applied. Average linkage hierarchical clustering on both the genes and the samples was built. The general functional classes by Gene Ontology classification were identified.

QUANTITATIVE REAL-TIME PCR

Full-length gene sequences were extracted from WormBase (Release WS190) and primers were designed by the Primer3 software and tested for specificity using NCBI BLAST. Quantitative PCR was carried out using the DyNAmo™ SYBR® Green 2-Step qRT-PCR Kit (Finnzyme, Finland). The RNA was reverse transcribed into cDNA followed by subsequent qPCR which were performed separately (two-step qRT-PCR) according to manufacturer instructions. The samples were amplified separately using the RotorGene 6000 (Corbett Life Science). The RHO GTPase, *cdc-42* gene was used as reference (20). The following primer pairs were used for PCR amplification: *mua-3* (5’-TCCAACACTCAAAGGAAGAG-3’ and 5’-TGGGCAGTCACATTCATAAG-3’), *rad-51* (5’-CACATCATCGCTCACATGTCT-3’ and 5’-GCGTGCGTCCTCAATACCAT-3’), *him-6* (5’-CACCTCATCGCTCGCATGTCT-3’ and 5’-GCGTGCGTCCTCCATTCCAT-3’), *dod-19* (5’-GAGAAAGGCCCATGCCAGAA-3’ and 5’-CCTCGACTTTCGGGTACAG-3’) and fmo-3 (5’-GAGAAAGGCCAATGCCAGAA-3’ and 5’-CCTCGACATTCGGGTACAG-3’). Each reaction included a no-template control and was performed in duplicates. The cycling conditions were as follows: 50°C for 2 min, initial denaturation at 95°C for 15 min, followed by 40 cycles of 10 s at 94°C, 25 s at 60°C, and 30 s at 72°C (*gain set at 8 for SYBR Green*). Following the final cycle, melting curve analysis was performed to examine the specificity in each reaction tube (absence of primer dimers and other nonspecific products). Upon completion of amplification, the program continued directly to a melting-curve analysis in which temperature was ramped from 72°C to 95°C in steps of 1°C increments with a 5 s hold at each degree. A single melt peak for each reaction confirmed the identity of each PCR product.

DATA ANALYSIS OF REAL-TIME QRT-PCR NON TEMPLATE

For both calibration and quantification of reactions tenfold serial dilution (1x, 10x, 10^2x, 10^3x) of cDNA were applied including controls of NTC (no DNA-template control) and ddH_2O. Data were analyzed by relative quantification of the 2^{-DDCq} method (24). *Cq values (quantification cycle)* is the threshold of fluorescence value of the amplified PCR products was determined manually above the background of fluorescence signals. Standard curve correlating Cq values to log amount of DNA were plotted at high R2-ratio (0.9 – 1.0). ΔCq*:* ΔCq values were calculated as Cq(target) – Cq(reference) for sample and Cq(target) – Cq(reference) for control (14) ΔΔCq values: ΔΔCq values were determined as mean Cq(sample) – mean Cq(calibrator) and calculated the 2^{-DDCq} value (23).

RESULTS

The microarray data was analyzed and comparison made between spaceflight and ground control samples. Data from the three technical replicates from each sample were used in the analysis, allowing us to identify putative significant genes involved in cellular adaptation to spaceflight conditions.

WHOLE-GENOME TRANSCRIPTOME ANALYSIS, GENE LISTS AND EVALUATION

Out of 22,625 genes contained in the genome array which we used, 858 known genes were differentially expressed (p-value ≤ 0.05 with false discovery rate). Hierarchical

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clustering performed for these 858 genes revealed that the genes were clustered according to conditions (space and ground) [refer to Figure 1 for expression patterns and complete gene list in Table 1. The genes whose expression level changed in between 2- and -2-fold were considered as not significant (NS) when constructing the gene lists. Amongst the 858 genes, 608 of these increased by more than two fold, and 250 others decreased to less than negative two fold, in the spaceflight samples relative to the ground controls (P < 0.05) (Figure 1).

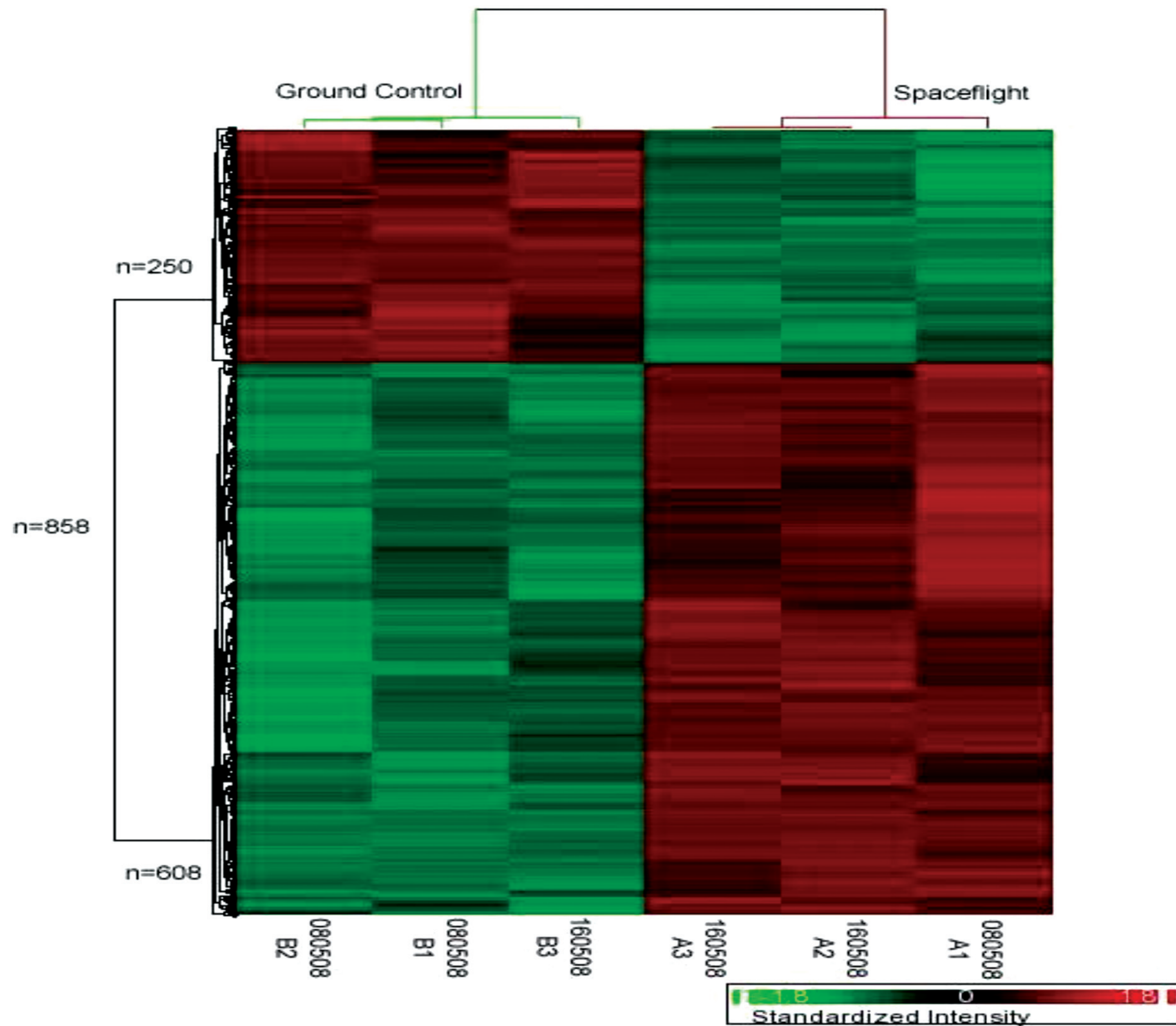

Figure 1: Hierarchical clustering of 858 differentially expressed genes which were differentially expressed (p-value ≤ 0.05, fold change ≥ ± 2) between space flown and ground control samples. Each row represents a single transcript, and each column an experimental sample. Red and green colours correspond to up and down regulation, respectively, with a darker colour denoting less differential expression. Transcripts were hierarchically clustered into those with similar profiles, which were showed to cluster according to the experiment conditions. Amongst these genes, 608 genes were up-regulated, while 250 genes were down-regulated under the same conditions.

GENE ONTOLOGY CLASSIFICATION

To further understand the changes observed in the gene expression of the worms grown in space especially in terms of biological functions, we used the GO classification for both the down-regulated and the up-regulated genes. Each gene was classified according to its ontology, by which genes are organized into hierarchical categories based on biological process (Partek) (4, 9, 10). The Chi-Square test was used; gene ontology categories were identified and the top enriched biological processes were ranked based on the Enrichment Score (Table 1). The list of genes involved in top 25 of the enriched biological processes (refer to Table 2) included genes like Mutator (*mut-2*), BaRD homolog (tumor suppressor gene Bard1) (*brd-1*), Zinc finger In Meiosis (*zim-3*), RADiation sensitivity abnormal/yeast RAD-related (*rad-51*), High Incidence of Males (increased X chromosome loss) (*him-6* and *him-8*), and this highlighted their involvement in the worms' overall adaptation and survival in spaceflight conditions, including

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Table 1: Gene Ontology: Top 25 enriched biological processes as classified by gene ontology biological process

GO category	Enrichment Score	% of genes in list	Genes in list
GO: 45132: meiotic chromosome segregation	44.06	25.42	30
GO: 7059: chromosome segregation	42.86	24.22	31
GO: 51301: cell division	27.82	19.29	27
GO: 910: cytokinesis	26.77	20.34	24
GO: 22402: cell cycle process	21.44	16.56	26
GO: 51383: kinetochore organization	20.78	100.00	4
GO: 70: mitotic sister chromatid segregation	17.35	55.56	5
GO: 819: sister chromatid segregation	17.35	55.56	5
GO: 51304: chromosome separation	16.15	66.67	4
GO: 51382: kinetochore assembly	15.96	100.00	3
GO: 33554: cellular response to stress	15.55	19.72	14
GO: 9790: embryonic development	13.47	6.77	174
GO: 34453: microtubule anchoring	13.46	75.00	3
GO: 51313: attachment of spindle microtubules to chromosome	13.46	75.00	3
GO: 22403: cell cycle phase	13.37	20.76	11
GO: 51716: cellular response to stimulus	13.17	17.50	14
GO: 6974:response to DNA damage stimulus	13.06	50.00	4
GO: 9792: embryonic development ending in birth or egg hatching	12.16	6.68	170
GO: 51052: regulation of DNA metabolic process	11.87	44.44	4
GO: 70193: synaptonemal complex organization	11.87	44.44	4
GO: 34984: cellular response to DNA damage stimulus	11.87	18.97	11
GO: 6281: DNA repair	11.40	19.61	10
GO: 51276: chromosome organization	10.82	17.74	11
GO: 6259: DNA metabolic process	10.31	23.33	7
GO: 1556: oocyte maturation	9.97	36.36	4

meiotic chromosome segregation. Up-regulation of *him-8* and *zim-3*, two genes encoded in the same operon and appearing in meiotic chromosome segregation biological functions, indicated that expression of these two genes is involved in the regulation of transcription factor activity (38). The expression of *him-6* and *rad-51* appeared together in biological functions such as embryonic development, cellular response to stress, cellular response to stimulus, cellular response to DNA damage stimulus and DNA repair suggesting that both genes were crucial for maintaining genome stability and survival of the worms in space. *Spo-11* (homolog to homolog of yeast SPOrulation gene) which is the upstream effector of *rad-51* and *him-6* is involved in the cell cycle, DNA metabolic process, embryonic development and chromosome segregation. Up-regulated expression of *msh-2* (which is homologous to the mismatch repair gene *MSH*), *xpc-1* (Xeroderma Pigmentosum group C DNA repair gene homolog) and *brd-1* BaRD homolog (tumor suppressor gene Bard1) suggested the cellular responses to stimuli (including DNA damage) and the activation of DNA repair mechanisms in the worms grown in space.

Genes involved in biological adhesion and muscle tissue integrity that were down-regulated included the MUscle Attachment abnormal (*mua-3*) gene and the collagen genes, *col-97*, *col-109* and *col-113*, indicating that biological adhesion in microgravity was less crucial. The MYOsin heavy chain structural (*myo-3*) gene which is involved in muscle tissue integrity was found to be up-regulated in the spaceflight samples. Expression of *daf-19* gene which is crucial for cilia formation was down-regulated, indicating adaptation to microgravity environment which requires less movement and detection of environmental signals.

Other genes that were not from the top 25 GO list but were also involved in significant pathways included the Downstream of DAF-16 (*dod*) genes. There was up-regulated expression of *dod-19*, *dod-3* and the heat shock protein *hsp-16* gene which are all downstream effectors of DAF-16 of the TGF-beta pathway. The xenobiotic metabolizing enzymes such as cytochrome P450 (*cyp-13B1*, *-14A4*, *-34A9*, *-35B1*, *-35B2*, *-37B1 and -37B1*) were all up-regulated in spaceflight samples. The genes for another xenobiotic enzyme, UDP-glucosyl transferase,

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Table 2: List of significant genes related to cellular response to DNA damage and DNA repair mechanism, muscle-related genes, oxidative stress related genes and transcription factor related genes

Gene	Gene Ontology (Biological Function)	Probe ID	Fold change	P Value
(A) Cellular response to DNA damage and DNA repair mechanism related genes				
brd-1	Required for homologous recombination and DNA double-strand break repair	179791_s_at	2.49648	0.000135
msh-2	Involved in mismatch repair after exposure to radiation	193072_s_at	2.15574	0.000437
rad-51	Required for double stranded breaks repairs induced by irradiation	193054_s_at	3.29784	0.000538
xpc-1	Required for nucleotide excision repair induced by UV light	187080_at	2.02433	0.001346
him-6	DNA damage checkpoint signaling in response to ion irradiation	189916_at	2.16031	0.000825
mut-2	Involved in transposon silencing and RNA interference	184510_at	2.31091	0.000055
spo-1	Maintain genomic stability	188650_at	2.63585	0.000166
(B) Muscle related genes				
mua-3	Positive regulation of growth rate	189800_s_at	-2.49227	0.0002675
myo-3	Myosin A	191856_s_at	3.11556	0.0011562
col-97	Extracellular matrix or adhesion	174960_at	-4.73784	0.0008101
col-109		173235_at	-9.09213	0.0000085
col-113		173518_s_at	-4.46384	0.0014773
(C) Longevity related genes				
dod-19	Downstream effector of DAF-16/TGF beta signaling pathway	175170_s_at	2.41566	0.0028085
dod-3		173335_s_at	4.046	0.0006910
hsp-16		188282_at	2.28231	0.0000120
(D) Oxidative stress related genes				
fmo-1	Xenobiotic metabolizing enzyme	191850_s_at	2.62025	0.00068966
fmo-3		194135_at	-2.38922	0.0008818
gsto-1	Protects from long-term oxidative stress	175993_at	2.60862	0.0010937
gst-13	Involved in the oxidative stress response by detoxicification of xenobiotics	190852_at	4.05802	0.0000379
gst-17		190932_at	2.17239	0.0015696
gst-26		191431_at	2.39447	0.0004633
gst-27		191393_s_at	5.03714	0.0001351
gst-36		191221_at	2.39447	0.0011611
gst-22		190899_at	-2.3688	0.0032545
gst-29		191303_at	-3.46122	0.0000268
gst-31		191307_at	-2.42005	0.0017632
gst-39		191337_at	-2.61787	0.0008503
cyp-13B1	Involved in the oxidative stress response by detoxicification of xenobiotics	185599_s_at	2.39574	0.0034556
cyp-14A4		189218_at	2.18539	0.00113924
cyp-34A9		189457_at	3.23857	0.00000308
cyp-35B1		189366_at	4.51908	0.000230574
cyp-35B2		189256_at	3.50233	0.000877842
cyp-37B1		189221_at	3.9097	0.001077
cyp-43A1		189413_at	2.05028	0.00256461
ugt-9	Involved in the oxidative stress response by detoxicification of xenobiotics	191776_at	-2.39168	0.00188094
ugt-13		188934_at	2.01688	0.000466988
ugt-17		191689_s_at	2.53381	0.00210593
ugt-41		190849_at	-2.72725	0.000194547
ugt-44		191445_at	2.35085	0.000483495
ugt-45		175194_at	-3.43084	0.000135254
ugt-46		173204_s_at	3.14785	0.0014191
ugt-62		190886_at	3.1026	0.000286272
(E) Transcription factor activity				
zim-2	Involved in meiosis (sexual reproduction)	190765_at	4.30691	0.00120765
zim-3		190670_at	2.34034	0.0002350
him-8	Regulate ZIM family proteins	190648_at	2.77989	0.0005772

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showed up-regulation in the isoforms *ugt-13*, *ugt-17*, *ugt-44*, *ugt-46 and ugt-62*, whilst the isoforms *ugt-9*, *ugt-41 and ugt-45* were down-regulated. Flavin-containing MonoOxygenase family (*fmo-3)*, a recently discovered xenobiotic metabolizing enzyme (XME) orthologous to the same human gene was also up-regulated in the spaceflight samples. Various isoforms of oxidative stress-related genes such as (gluthathione S transferase) *gst* were significantly expressed, with increased expression of *gsto-1*, *gst-13*, *gst-17*, *gst-26*, *gst-27*, *gst-36* and decreased expression of *gst-22*, *gst-29*, *gst-31 and gst-39*.

VALIDATION OF THE DIFFERENTIALLY EXPRESSED GENES USING REAL-TIME QUANTITATIVE PCR

To validate and evaluate more quantitatively the differences in gene expression as showed by the DNA microarrays, real-time quantitative PCR was performed. The RHO GTPase gene, *cdc-42*, is considered a 'housekeeping gene' that should be expressed at a steady level under most conditions, including spaceflight (20). We used *cdc-42* as an internal standard against the genes of interest to normalize the quantitative PCR data. Expression of the muscle-related genes *mua-3* decreased by 1.35 fold while the expression for DNA repair genes *rad-51* and *him-6* increased by 1.84 fold and 0.86 fold respectively. The expression of insulin/ TGF-beta signaling related genes, *dod-19* increased by 0.26 fold compared with ground control worms, while the expression for *fmo-3* genes decreased by 1.44 fold. The qRT-PCR correlated well with the microarray data ($R^2 = 0.917$) which qualitatively validated and confirmed the observations made using the DNA microarray analysis, and gave a quantitative evaluation of the extent of mRNA expression.

DISCUSSION

Whole genome transcriptome profiling has been used previously to investigate the changes in *C. elegans* cultured in the space environment (18, 19, 33); however their findings were attributed to the short term space exposure of 11 days. This was the first study to look at whole transcriptome profiling of *C. elegans* grown in space for more than 6 months and which had passed through at least 10 full generations in the ISS. The limitations of this study included the fact that the starting batch of worms was asynchronous and due to limitations of the hardware, we were not able to have biological replicates for the microarray study. Nonetheless, this study provides an insight on the adaptation of the worms through changes in gene expression as they grow and pass through multiple generation cycles in the space environment. The vital design here was the comparison of the survivors from the space flown worms with the ground controls which were passaged synchronously over the 6 months period.

We showed that expression of genes involved in gross development, apoptosis and DNA repair were consistent with the profiles reported by previous studies (19, 45). From the GO classification, it was noted that *him-6* and *rad-51* were highly expressed in the top 25 genes list, which also included genes involved in other major biological functions such as meiotic chromosome segregation, cellular response to stress, cellular response to DNA damage (refer to Table 2) highlighting that the worms' cellular adaptation to radiation in space. These 2 genes have been shown to be crucial in maintaining genome stability in the germ line by repairing double stranded DNA breaks induced by irradiation in normal proliferating germ cells (44). *Him-6* is the ortholog of the human Bloom's syndrome RecQ-like helicase genes (BLM) in which mutant genes cause increased genomic instability that can eventually lead to cancer (42). This study also showed that *him-6* functions downstream of *spo-11* and *rad-51*; while *him-6* is required for normal levels of recombination during meiosis (44). Another study confirmed this observation in *him-6* mutants and correlated the lost of *him-6* function to significantly reduced life span from genomic instability caused by increased number of irreversible DNA lesions (13). Damage caused by radiation treatment in late embryonic stages in animals depleted from *rad-51* resulted in several developmental defects in vulva and gonad formation (36). A dramatic increase in germ cell apoptosis was observed when *rad-51* is inactivated (36). Loss of DNA damage checkpoint function can lead to genome instability which is one of the driving forces towards carcinogenesis (36). Up-regulation of *him-10* in spaceflight also indicate involvement of this gene in the mitosis checkpoint, especially in the kinetochore organization in chromosome segregation of worms under microgravity condition, which is necessary to maintain normal cell cycle process of the worms (35). Up-regulation of h*im-8* together with *zim-2* and *zim-3* in space suggest increased transcription factor activity, chromosomal homologous pairing and synapsis during meiosis, which is crucial for the sexual reproduction of the worms (31, 38).

There have been many studies on *daf-16* downstream effectors because *daf-16* has been postulated to play a key role in the lifespan extension mediated by reduced Ins/ IGF-signaling (7). Stress response genes were found to act downstream of *daf-16* and microarray data on the up-regulation of stress response genes have been consistent (7). Wild-type *daf-16* appears necessary for up-regulation of many genes involved in cellular stress responses, including heat-shock proteins, super-oxide dismutases, and many cytochrome p450s (21). Our results showed that *dod-19* and *dod-3*, the downstream effectors of the forkhead transcription factor *daf-16* were up-regulated in the flight samples. *Daf-16* also regulates the insulin/TGF signaling pathway that influences metabolic alterations, increased stress and microbial resistance. Furthermore, up-regulated expression of *hsp-16* in this study also corroborated with a previous study that showed that expression of *hsp-16*, *hsp-70*, *hsp-90* were up-regulated by the DAF-16 pathway (26). There were also other genes associated with environmental

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stress which were up-regulated. All of the cytochrome P450 genes were up-regulated in space suggested that the different isoforms were activated by the same xenobiotics, probably due to the increasing excretion of toxic metabolites in the CeMM (28). The *fmo-1* and *fmo-3* gene encodes for a xenobiotic-metabolizing enzyme which is expressed hypodermally in *C. elegans* and is inducible by exposure to xenobiotic FMO substrate benzydamine (30), therefore the up-regulation of *fmo-1* gene and down-regulation of *fmo-3* genes in space suggested that activation of the different isoforms could be responses to environmental stress in space. Studies have shown that *gst-5*, *-6*, *-8*, *-10*, *-24* are involved in metabolizing 4-hydroxynon-2-enal (4-HNE), an eletrophilic aldehyde which act in parallel with reactive oxygen species (ROS) to cause lipid peroxidation (5, 16, 17). The studies also showed that these *gst* isoforms that are involved in 4-HNE metabolism belong to two distant phylogenetic branches. *Gst-26*, *-27*, *-29*, *-39* which were differentially expressed in spaceflight belong to the same phylogenetic branch. These *gst* isoforms were not found to be sensitive to 4-HNE (5), however, the role of these isoforms are still unclear in preventing oxidative stress. On the other hand, the *gsto-1* gene was shown to be crucial in protecting worms from environmental pro-oxidant-induced stress (11), thus the increased expression of *gsto-1* gene in spaceflight suggested that the space environment to be highly pro-oxidant and up-regulation of this genes, other DNA repair genes and stress-responsive genes could be part of the adaptative changes crucial for the survival of the worms in long-term space flight.

It is a known fact that microgravity in space resulted in muscle atrophy, which included muscle volume and

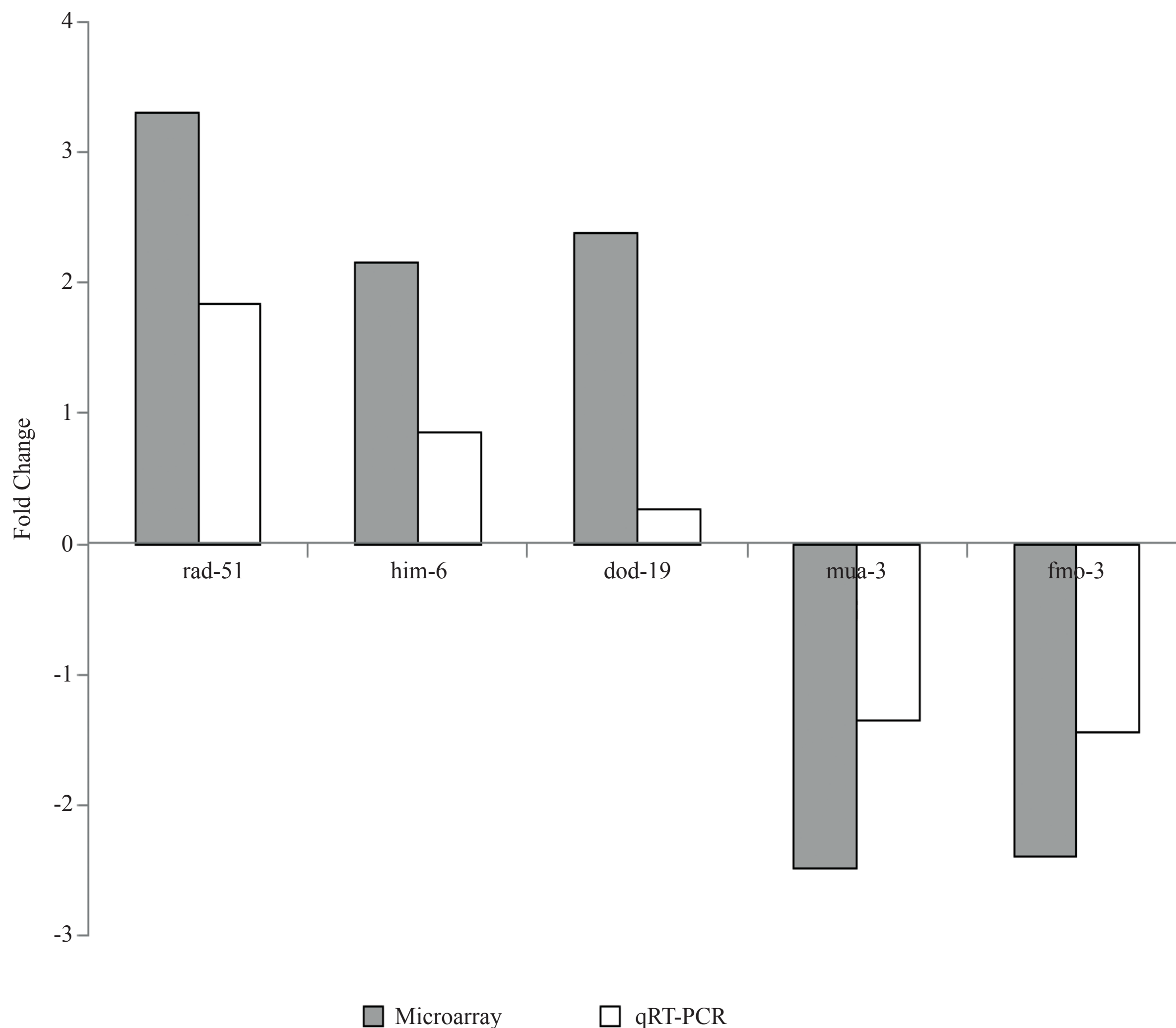

Figure 2: Comparison of expression of representative genes selected from microarray data with qRT-PCR. Comparison of fold change values from microarray data with expression ratios calculated from qRT-PCR. Values were determined using qRT-PCR and represents relative expression of genes between spaceflight and ground control sample. The relative expression of the target genes [*dod-19*: Downstream Of DAF-16 (regulated by DAF-16), *mua-3*: MUscle Attachment abnormal, *fmo-3*: Flavin-containing MonoOxygenase family gene, *him-6*: High Incidence of Males and *rad-51*: RADiation sensitivity abnormal/yeast RAD-related gene] normalized to *cdc-42*: Cell Division Cycle related gene and relative to the expression of control. Bars represent standard errors calculated from 3 replicates of each experiment. *Significant difference ($P < 0.05$) between qRT-PCR and microarray data.

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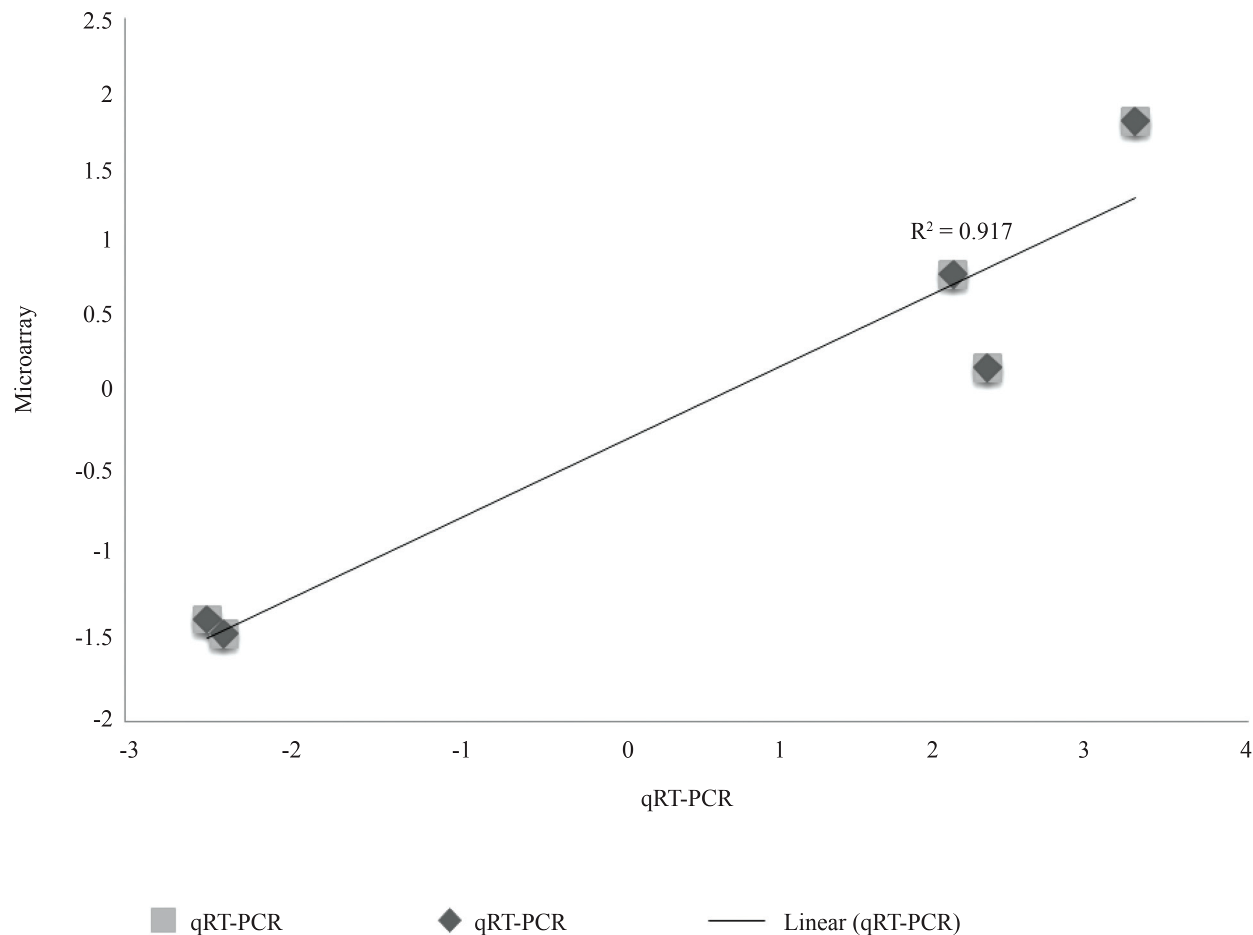

Figure 3: Correlation between the fold change values from microarray and the expression ratios calculated from qRT-PCR presented as level of gene expression. Correlation coefficient between the fold change values from microarray and qRT-PCR showed that mirroarray and qRT-PCR results are highly correlated indicated that the qRT-PCR validates the microarray data.

strength (12), while cosmic radiation caused reduction in muscle fibers (6). A recent study on the effects of spaceflight on murine skeletal muscle gene expression revealed that besides the physical signs of muscle atrophy, genes involved in extracellular matrix or adhesion such as collagen (*col-1a2*, *col-1a1*, *col-3a1*, *col-4a3bp*) were down-regulated (3). We observed similar changes in our study with the down-regulation of *col-97*, *col-109*, *col-113*. The *mua-3* gene codes for the MUA-3 protein, which is a novel transmembrane protein that localizes to hypodermal hemidesmosomes at the sites of skeletal muscle and to other epithelial sites where stress-resistant cuticular adhesion is required.

This MUA-3 protein is also required for muscle tissue integrity during normal locomotion of the worms (8). Down-regulation of this gene in the space flight samples could be an adaptative change due to the reduced locomotion in microgravity. We also showed that the expression of *myo-3* was up-regulated in space, whereas previous studies involving worms grown over 10 days of spaceflight showed reduced expression of myosin heavy chain in the absence or reduction of muscle activity (2, 14, 43, 46). The *daf-19*, gene, which is involved in environmental sensing and movement, was down regulated. This could be due to the decreased cilia formation and sensory detection in microgravity which could be crucial for the worms to respond to environmental signals such as food, dauer pheromone, or nose touch (34).

In conclusion, long term exposure and multi-generational culture of *C. elegans* in microgravity revealed key changes in the expression of genes involved in ageing, DNA repair, oxidative stress and muscle growth. These changes are most likely due to adaptation to the environmental signals as the worms divide and reproduce, over at least 10 generations, in space conditions. This study provided a preliminary insight of cellular and genetic adaptation to long term spaceflight environment through transcriptome regulation.

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