Transcriptional Analysis of Normal Human Fibroblast Responses to Microgravity Stress

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To understand the molecular mechanism(s) of how spaceflight affects cellular signaling pathways, quiescent normal human WI-38 fibroblasts were flown on the STS-93 space shuttle mission. Subsequently, RNA samples from the space-flown and ground-control cells were used to construct two cDNA libraries, which were then processed for suppression subtractive hybridization (SSH) to identify spaceflight-specific gene expression. The SSH data show that key genes related to oxidative stress, DNA repair, and fatty acid oxidation are activated by spaceflight, suggesting the induction of cellular oxidative stress. This is further substantiated by the up-regulation of neuregulin 1 and the calcium-binding protein calmodulin 2. Another obvious stress sign is that spaceflight evokes the Ras/mitogen-activated protein kinase and phosphatidylinositol-3 kinase signaling pathways, along with up-regulating several G_1-phase cell cycle traverse genes. Other genes showing up-regulation of expression are involved in protein synthesis and pro-apoptosis, as well as pro-survival. Interactome analysis of functionally related genes shows that c-Myc is the "hub" for those genes showing significant changes. Hence, our results suggest that microgravity travel may impact changes in gene expression mostly associated with cellular stress signaling, directing cells to either apoptotic death or premature senescence.

Key words: microgravity, apoptosis, premature senescence, oxidative stress, subtractive hybridization

Introduction

Various primary mammalian cell strains have been utilized as models for space-related studies, including human fibroblasts (*1*), osteoblasts (*2*), and T lymphocytes (*3*). We have used the well-established normal fibroblast strain, the WI-38 cell line derived from human fetal lung, which has limited *in vitro* life span. In this strain, permanent exit from cell cycle traverse occurs at the G_1/S border, and is called replicative senescence due to the exhaustion of the ability to replicate further. Recently, serial passaging of cultures to achieve the state of permanent cell cycle arrest has been bypassed by treatment with oxidative stress (*4*), chromatin remodeling (*5*), ultra-violet exposure (*6*), or over-expression of Ras oncogenes or other members of the mitogen-activated protein kinase (MAPK) pathway (*7*, *8*); this state is called premature replicative senescence (*9*). This embryonic lung fibroblast culture has been widely used to study cellular aging, that is, replicative senescence (*10*, *11*). Likewise, we have used WI-38 normal human fibroblasts as our culture model to examine whether space-associated stress impacts their signaling transduction, and thus flew these cultures on the STS-93 space shuttle mission for a 5-day spaceflight. The WI-38 cell line was selected based on the fact that it is very sensitive to stress-induced growth arrest, in particular radiation, a dominant environmental hazard associated with space travel.

Previous spaceflight studies revealed alterations in gene expression of several regulatory elements in animal tissues (*12–14*) and cultured cells (*2*, *3*, *15*). However, these changes vary with tissue and cell types. For instance, in rats spaceflight reduces trans-

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forming growth factor β1 in hindlimb periosteum (*13*), but increases insulin-like growth factor 1 in the tibia (*12*). Recently, microarray-based genome-wide profiling analysis has demonstrated altered patterns of gene expression in cultured human renal cells (*15*) and T lymphocytes (*3*); most genes exhibiting space-associated changes are involved in regulating growth, signaling, adhesion, transcription, apoptosis, and tumorigenesis. In our previous microarray study, we identified expression changes in 10 genes belonging to either tumor necrosis factor- or interleukin-related gene families in space-flown human fibroblasts compared with ground controls; these genes are thought to be involved in regulating bone density and/or the development of pro-inflammatory status (*1*).

To more comprehensively analyze changes of gene expression in human fibroblasts, we constructed two cDNA libraries using RNA samples isolated from the space-flown confluent fibroblasts at early population doubling levels and from their ground-control counterparts. The two libraries were enriched specifically for either space-specific or ground-specific genes by suppression subtractive hybridization (SSH); the subtraction was performed from space-flown cells against ground-control cells, and *vice versa*. We report here that key genes related to oxidative stress and DNA repair are up-regulated by spaceflight, whereas energy metabolic patterns appear to be down-regulated compared with ground controls. Thus, we suggest that spaceflight stress may induce both pro-apoptosis signaling and pro-premature replicative senescence signaling, both of which are manifested by the experience of early and mid G_1 phase; however, the cells are triaged to their respective pro-survival or pro-death fates at the G_1/S border.

Results

Cell culturing conditions

In general, the selected WI-38 cell line of normal human fibroblasts reaches the limit of its replicative life span by approximately 54 population doubling levels (PDLs). For the STS-93 mission, we used WI-38 cultures at 20 PDLs, in order to avoid complication of any observed space-associated changes by gene expression changes due to replicative senescence. Along the same line, we used contact-inhibited quiescent cells of cultures at this PDL to avoid any confounding factors associated with replicating fibroblasts. High cell density (1.2×10^7 cells in 10 mL) was used in each cell culture module (CCM), and culture conditions were set to allow cells to attach to the hollow fibers within the modules. Eight individual CCMs of identical cell density were established with controlled constant medium flow and pH. These eight cultures were separated into two groups: four space and four ground CCMs. The four space CCMs were maintained for 4 d, in which 2 d in the laboratory and 2 d in the shuttle payload module, before the final shuttle lift-off. The four ground CCMs were processed in parallel in identical fashion. During the 4-day pre-flight period, both groups not only completed firm adherence to the hollow fiber substratum, but also attained the contact-inhibited quiescent state. Upon final retrieval of the cell samples after the flight, the two groups were processed in identical fashion for RNA isolation, as described in Materials and Methods. Thus, the gene expression changes described below are due to the putative effect of space travel, rather than possible culture permutation effects by the CCM unit.

Genes identified by SSH and their functional categorization

Human fibroblasts of the WI-38 cell line cultured in the above CCM-designed bioreactor system were flown in space for 4 d and 23 h on the STS-93 space shuttle mission. After the return of the space shuttle, space-flown and ground-control cells were immediately dissolved and fixed in Trizol reagent for total RNA extraction (Figure 1). One portion of the extracted RNA was used for microarray studies (*1*), while the other was used to construct cDNA libraries enriched either for space-flown or ground-control cells through SSH. For SSH, both forward and reverse subtractions were performed. A total of 315 independent clones (147 and 168 clones from space-flown and ground-control cell populations, respectively) were identified and subsequently sequenced. The sequences of all the clones were searched against the human expression sequence tag (EST) database from the National Center for Biological Information (NCBI) with the BLAST program. A total of 159 sequences had no significant similarity hits, and therefore were considered as unknown human ESTs, whereas 5 sequences represent empty or "vector only" clones. Among those with significant similarity to known human ESTs, 65 are repeated sequence clones. Thus, only 82 genes (50 up-regulated and 32 down-regulated by the spaceflight) were identified as unique known human genes (Figure 2; Table 1). Based on

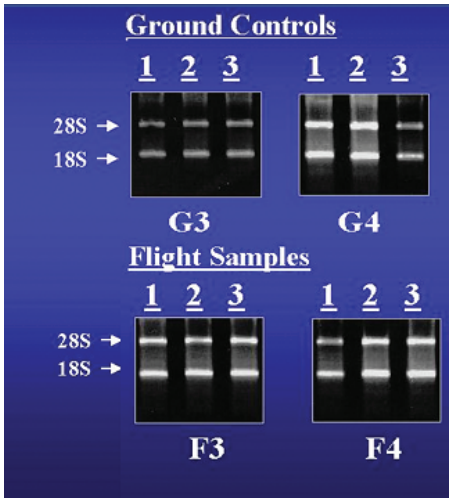

Fig. 1 Three total RNA samples independently extracted from equal amounts of culture derived from each of four independent cartridges. Shown in this figure are two examples of the four CCMs of three such extracted samples, either space-flown (F3 and F4) or ground controls (G3 and G4), using Trizol reagent. The quality of the total RNA samples was visualized on 1% agarose gel, stained with ethidium bromide and exposed to ultra-violet light. Note that more total RNA was extracted from space-flown than from ground-control cells.

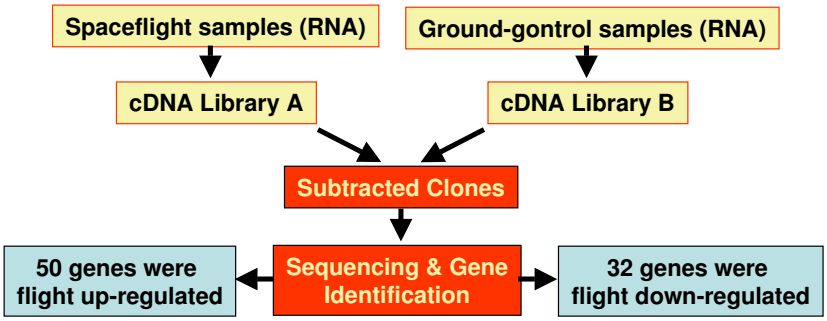

Fig. 2 Schematic flow diagram of SSH procedure and identification of genes either up- or down-regulated by spaceflight.

Table 1 All genes identified by SSH in normal WI-38 fibroblasts exposed to spaceflight stress

No.	Gene ID	Gene symbol	Gene product	Expression
1	Hs.39056	ABHD6	abhydrolase domain containing 6	up
2	Hs.145741	ANXA5	annexin A5	up
3	Hs.135056	C20orf139	chromosome 20 open reading frame 139	up
4	Hs.401703	C20orf52	chromosome 20 open reading frame 52	up
5	Hs.425808	CALM2	calmodulin 2 (phosphorylase kinase, delta)	up
6	Hs.458314	COBW	COBW-like protein	up
7	Hs.17377	CORO1C	coronin, actin binding protein, 1C	up
8	Hs.439777	CPT1B	carnitine palmitoyltransferase 1B	up
9	Hs.458302	DLL4	delta-like 4 (Drosophila)	up
10	Hs.439552	EEF1A1	eukaryotic translation elongation factor 1 alpha 1	up
11	Hs.211823	EIF4EL3	eukaryotic translation initiation factor 4E-like 3	up
12	Hs.374477	EWS	Ewing sarcoma protein	up
13	Hs.469126	FLJ20420	hypothetical protein FLJ20420	up
14	Hs.433670	FTL	ferritin, light polypeptide	up
15	Hs.808	HNRPF	heterogeneous nuclear ribonucleoprotein F	up

Table 1 ***Continued***

No.	Gene ID	Gene symbol	Gene product	Expression
16	Hs.433553	Kf-pending	similar to Kf-pending protein	up
17	Hs.190389	KIAA0266	KIAA0266 protein	up
18	Hs.200596	LCMT2	leucine carboxyl methyltransferase 2	up
19	Hs.293884	LOC150678	helicase/primase complex protein	up
20	Hs.411358	MORF4L2	mortality factor 4 like 2	up
21	Hs.79110	NCL	nucleolin	up
22	Hs.172816	NRG1	neuregulin 1	up
23	Hs.163724	OSTM1	osteopetrosis associated transmembrane protein 1	up
24	Hs.15250	PECI	peroxisomal D3,D2-enoyl-CoA isomerase	up
25	Hs.298229	PFDN2	prefoldin 2	up
26	AY339593	PL56	isolate F192 (PL56) mitochondrion	up
27	Hs.281117	RAB22A	member of RAS oncogene family	up
28	Hs.10842	RAN	member of RAS oncogene family	up
29	Hs.388918	RECK	reversion-inducing-cysteine-rich protein	up
30	U13369	RIBIN	rRNA promoter binding protein	up
31	Hs.406300	RPL23	ribosomal protein L23	up
32	Hs.419463	RPL23A	ribosomal protein L23a	up
33	Hs.380953	RPL38	ribosomal protein L38	up
34	Hs.416566	RPL6	ribosomal protein L6	up
35	Hs.412370	RPL9	ribosomal protein L9	up
36	Hs.356572	RPS3A	ribosomal protein S3A	up
37	Hs.443914	SOD1	superoxide dismutase 1	up
38	Hs.35052	TEGT	testis enhanced gene transcript	up
39	Hs.439911	TERT	telomerase reverse transcriptase	up
40	Hs.66744	TWIST1	twist homolog 1	up
41	Hs.369037	ZNF236	zinc finger protein 236	up
42	AK098165		unknown	up
43	AL135914		unknown	up
44	AP003439		unknown	up
45	AC008131		unknown	up
46	BX088689		unknown	up
47	AC079329		unknown	up
48	AC090764		unknown	up
49	AC068056		unknown	up
50	AC011586		unknown	up
51	Hs.75313	AKR1B1	aldo-keto reductase 1B1 (aldose reductase)	down
52	Hs.12152	APMCF1	APMCF1 protein	down
53	Hs.89474	ARF6	ADP-ribosylation factor 6	down
54	Hs.250882	BDKRB2	bradykinin receptor B2	down
55	Hs.7001	C14orf9	chromosome 14 open reading frame 9	down
56	Hs.440961	CAST	calpastatin, a cysteine protease calpain inhibitor	down
57	Hs.411515	CTCF	CCCTC-binding factor (zinc finger protein)	down
58	AF145469	CTCF	transcriptional repressor CTCF	down
59	Hs.129673	EIF4A1	eukaryotic translation initiation factor 4A, isoform 1	down
60	Hs.169919	ETFA	electron-transfer-flavoprotein, alpha	down
61	Hs.167344	FTH1	ferritin, heavy polypeptide 1	down
62	Hs.15265	HNRPR	heterogeneous nuclear ribonucleoprotein R	down
63	Hs.166463	HNRPU	heterogeneous nuclear ribonucleoprotein U	down

Table 1 ***Continued***

No.	Gene ID	Gene symbol	Gene product	Expression
64	Hs.283437	HTGN29	HTGN29 protein	down
65	Hs.348515	KIAA0601	KIAA0601 protein	down
66	Hs.407909	LGALS1	lectin, galactoside-binding, soluble, 1 (galectin 1)	down
67	Hs.376719	MAGED2	melanoma antigen, family D, 2	down
68	Hs.51299	NDUFV2	NADH dehydrogenase (ubiquinone) flavoprotein 2	down
69	Hs.14606	PALMD	palmdelphin	down
70	Hs.41270	PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase) 2	down
71	Hs.380964	POM121	POM121 membrane glycoprotein (rat)	down
72	Hs.33818	RECQL5	RecQ protein-like 5	down
73	Hs.410817	RPL13	ribosomal protein L13	down
74	Hs.416801	RPL7A	ribosomal protein L7a	down
75	Hs.356502	RPLP1	ribosomal protein, large, P1	down
76	Hs.436687	SET	SET translocation (myeloid leukemia-associated)	down
77	Hs.435468	SQRDL	sulfide quinone reductase-like (yeast)	down
78	Hs.423854	SURF1	surfeit 1	down
79	Hs.310640	T2BP	TRAF2 binding protein	down
80	Hs.300772	TPM2	tropomyosin 2 (beta)	down
81	Hs.9568	ZNF261	zinc finger protein 261	down
82	AC087257		unknown	down

Table 2 Functional categorization of all identified genes

Function	Gene
Anti-aging	EWS, MORF4L2, SOD1, TERT
Pro-aging	BDKRB2
Anti-oxidant	FTL, SOD1
Pro-oxidant	BDKRB2, FTH1
Anti-apoptosis	TEGT, TWIST1, APMCF1, CAST, FTH1, POM121, SET, T2BP
Pro-apoptosis	ANXA5, NCL, OSTM1, LGALS1
Anti-tumor	RECK, AKR1B1, CTCF (Hs.411515), CTCF (AF145469), MAGED2, PLOD2, RECQL5
Pro-tumor	CALM2, EEF1A1, EIF4EL3, EWS, Kf-pending, LOC150678, NCL, RAB22A, RAN, TERT, APMCF1
Anti-division	CTCF (Hs.411515), CTCF (AF145469), FTH1, LGALS1
Pro-division	CALM2, NCL, NRG1, RAN
Differentiation	CORO1C, NRG1, OSTM1, SOD1, TWIST1, ARF6, PALMD, PLOD2, TPM2
Detoxification and repair	ABHD6, FTL, LOC150678, PFDN2, RAN, SOD1, TERT, AKR1B1, FTH1, HNRPU, PLOD2, RECQL5, SQRDL
Energy metabolism	CPT1B, Kf-pending, PECI, ETFA, NDUFV2, SQRDL, SURF1
Protein synthesis	CALM2, EEF1A1, EIF4EL3, NCL, RAN, RIBIN, RPL23, RPL23A, RPL38, RPL6, RPL9, RPS3A, RPL13, RPL7A, RPLP1
Signaling	ANXA5, CALM2, DLL4, LCMT2, NRG1, OSTM1, T2BP
Transcription regulation	HNRPF, MORF4L2, NCL, ZNF236, CTCF (Hs.411515), CTCF (AF145469), EIF4A1, HNRPR, ZNF261
Function unknown	C20orf139, C20orf52, COBW, FLJ20420, KIAA0266, PL56, AK098165, AL135914, AP003439, AC008131, BX088689, AC079329, AC090764, AC068056, AC011586, C14orf9, HTGN29, KIAA0601, AC087257

their GenBank accession numbers, we annotated all the identified genes using the web-based DAVID program and Medline literature review. Functional categorization of each annotated gene was performed according to the key words in the gene function description. The result shows that 10 genes are of unknown function; the other 72 genes are represented at least by a gene symbol or name, and have a putative product encoded by the gene with minimal function description (Tables 1 and 2). Based on the annotation, most identified genes encode regulatory factors and signal elements primarily allied to cell death, survival, and growth activities; another group codes for enzymes involved in metabolic reactions related to stress responses (Figure 3).

Imposed oxidative stress and altered metabolism

We detected an expression pattern change related to energy metabolism in normal WI-38 fibroblasts exposed to space microgravity and irradiation. Specifically, we identified two fatty acid oxidation genes, encoding muscle carnitine palmitoyltransferase (CPT1B, Hs.439777) and peroxisomal enoyl-CoA isomerase (PECI, Hs.15250), and three detoxification genes, encoding Cu/Zn superoxide dismutase (SOD1, Hs.443914), ferritin light polypeptide (FTL, Hs.433670), and α/β hydrolase (ABHD6, Hs.39056), all of which were up-regulated by spaceflight (Tables 1 and 2). In contrast, genes involved in respiration redox reactions, such as ferritin heavy polypeptide (FTH1, Hs.167344), electron-transfer-flavoprotein alpha (ETFA, Hs.169919), NADH dehydrogenase (NDUFV2, Hs.51299), surfeit (SURF1, Hs.423854), and sulfide quinine reductase (SQRDL, Hs.435468), were all down-regulated as a result of spaceflight (Tables 1 and 2). Since the expression of Cu/Zn superoxide dismutase gene *Sod1* is a hallmark of cellular response to oxidative stress (*16*), the up-regulation of this gene evidently implies that space travel imposes oxidative stress on normal WI-38 fibroblasts. This discovery is consistent with the report of increased spaceflight-related lipid peroxidation in human erythrocyte membranes (*17*). Therefore, to alleviate oxidative stress and/or reduce endogenous free radical production, our space-faring WI-38 cells are responded by both up-regulating detoxification genes like *Sod1*, *Abhd6*, and *Ftl*, and down-regulating aerobic respiration-related genes such as *Fth1*, *EtfA*, *Surf1*, and *Ndufv2*.

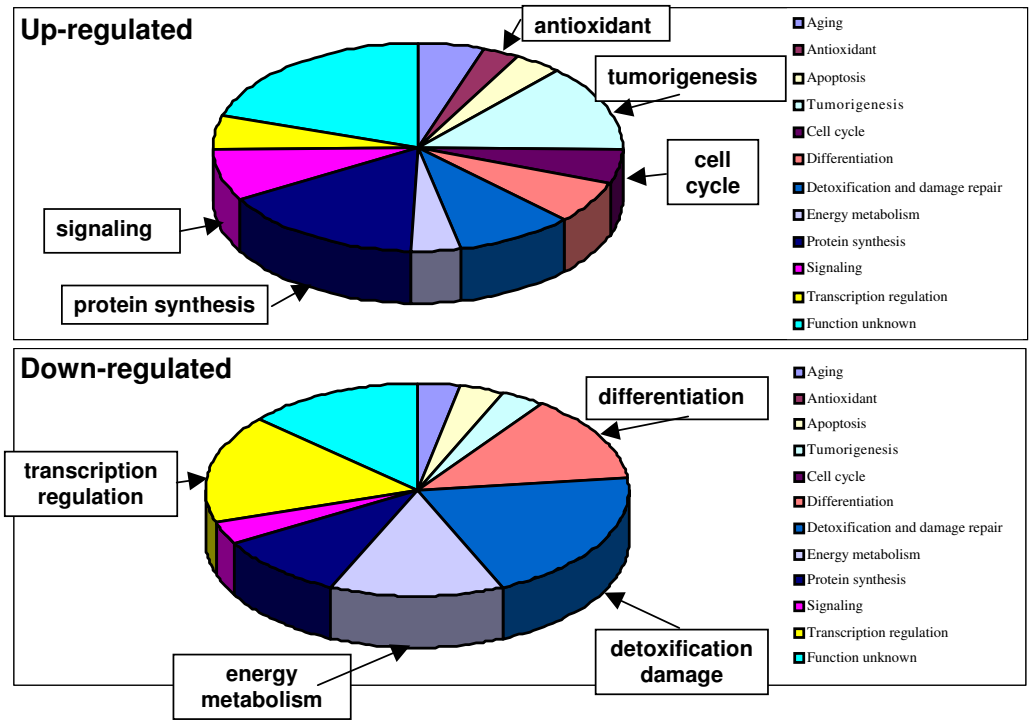

Fig. 3 Functional categories of genes either up- or down-regulated by spaceflight compared with ground controls.

To meet the energy requirement for surviving the stress, on the other hand, cells may need to mobilize reserved lipids, the molecules binding the most energy for respiration. Thus the shift to fatty acid oxidation, in which CPT1B and PECI are engaged, may be a strategy helping WI-38 cells adapt to the space environment. It is worth noting that the ferritin heavy chain (FTH) is a catalytic subunit of the enzyme, mainly functioning in redox reactions that oxidize iron and produce hydrogen peroxide, thus endogenously generating oxidative stress, whereas the ferritin light chain (FTL), the other subunit of this enzyme, mainly serves as storage space for the reduced iron, thus preventing it from being oxidized. Therefore, the combination of over-expression of *Ftl* and under-expression of *Fth1* reflects the coordinated efforts of WI-38 cells to reduce endogenous reactive oxygen species (ROS).

DNA repair response

Besides being stressed by endogenously produced ROS, WI-38 cells flown in space may be subject to elevated risk from irradiation by energetic heavy ions that induce biological effects. In general, high linear energy transfer (LET) radiation such as $^{56}Fe^{2+}$ can induce arrays of damages including cell growth arrest, chromosomal strand breaks, and intra-chromosomal aberrations, all of which have profound effects on gene expression changes; some of these are similar to those observed with exposure to oxidative stress. The space-imposed oxidative stress on the cultured cells is thus thought to arise mostly exogenously from irradiation-associated free radicals. Although cells may attempt to reduce their respiration rate to avoid producing more free radicals endogenously, as suggested by the above SSH data, this reduction may not be adequate for space-flown WI-38 fibroblasts to resist all macromolecular damage from the ionizing radiation in space. This suggestion is supported by the fact that specific DNA repair gene expressions such as helicase (Hs.293884), Ran GTPase (Hs.10842), stress response gene prefoldin 2 (PFDN2, Hs.298229), human telomere reverse transcriptase (hTERT, Hs.439911), and its transcription activator EWS (Hs.374477) (*18*), were all induced by the spaceflight (Tables 1 and 2). In fact, DNA damage caused by space radiation has indeed been detected in human cervical carcinoma (HeLa) cells (*19*), while spaceflight induction of hTERT expression (*20*) and activity (*21*) has been observed in KG1 and Jurkat cell lines, respectively. Taken together, these findings clearly state that space-flown WI-38 cells suffer DNA damage caused directly by the high LET radiation and indirectly by the ROS generated by the radiation. This is clearly reflected by the up-regulation of several key DNA damage signaling pathways.

Gene expression changes related to cell cycle traverse

Among genes up-regulated by spaceflight, a large group are cell cycle traverse-related genes, such as *Rab22a*, *Ran*, *Kf*, *Ews*, *Eif4el3*, *Eef1a1*, *Eife4l3*, *Tert*, *Ncl*, and *Calm2*, indicating a strong signal for reentry to the replicative cycle from quiescent status established pre-flight by contact inhibition. Conversely, specific signaling regulators that inhibit this traverse were down-regulated by spaceflight, among which are the newly identified transcription repressor CTCF (AF145469), FTH1, and the carbohydrate-binding protein galectin 1 (LGALS1, Hs.407909) (Tables 1 and 2). Down-regulation of CTCF and FTH1 should promote cell cycle traverse, because expression of both proteins correlates negatively with cellular proliferative activities (*22*, *23*). Likewise, galectin 1 has also been shown to restrain growth and proliferation of mouse embryonic fibroblasts and T lymphocytes, and induce cell cycle arrest (*23*). Interestingly, other anti-tumor genes such as *Fth1* (*24*), *Lgals1*, *Maged2* (*25*), *Akr1b1* (*26*), and *Plod2* were also down-regulated (Tables 1 and 2). Taken together, these gene expression changes indicate replicative activation of contact-inhibited, growth-arrested WI-38 cells, re-entering cell cycle traverse in response to spaceflight stress.

Up-regulation of pro-apoptosis signaling

We also identified activation of pro-apoptotic genes and repression of anti-apoptotic genes. Three apoptotic genes, encoding the RNA binding protein NCL (Hs.79110), osteopetrosis-associated transmembrane protein OSTM1 (Hs.163724), and calcium-dependent phospholipid-binding protein annexin A5 (ANXA5, Hs.145741) (*27*, *28*), were up-regulated by spaceflight in WI-38 cells, whereas six anti-apoptotic genes, encoding a nuclear pore membrane glycoprotein (POM121, Hs.380964)—a nuclear apoptotic target for caspases (*29*), a small G-protein (APMCF1,

Hs.12152) speculated to be involved in anti-apoptosis (*30*), an inhibitor of calpain—calpastatin (CAST, Hs.440961)—one of the two major cysteine protease families involved in apoptosis (*31*), a TNF receptor-associated factor binding protein (T2BP, Hs.310640) (*32*), a myeloid leukemia-associated SET translocation (SET, Hs.436687), and FTH1, were all down-regulated (Tables 1 and 2). It is reported that cultured human lymphocytes (Jurkat), after exposure to spaceflight, undergo cell cycle traverse; however, this activity is associated with a large number of apoptotic cells (*33*, *34*). Similar trends were also observed in experiments with mouse fibroblasts (*35*) and osteoblast cultures (*36*). This may suggest that the space environment stresses signal cells to experience cell cycle traverse, with some of the cells triaged to apoptotic death at the G_1/S border, the checkpoint for either entering the S phase or dying.

Effect on Ras/Raf-1/MAPK and PI3K signaling pathways

The induction of the epidermal growth factor (EGF)-like protein neuregulin 1 (NRG1, Hs.172816) (Tables 1 and 2) is an unprecedented sign suggesting that spaceflight stimulates growth factor synthesis. Binding of growth factors to their receptors activates receptor tyrosine kinase, which evokes the Ras/Raf-1/MEK/MAPK cascade (*37*). Clearly, strong stimulation was generated by space stress to activate this signaling pathway. A sustained, strong signal for this pathway is reported to induce cell cycle arrest, possibly at late G_1 phase, which is linked to apoptosis and premature replicative senescence in fibroblasts (*38*).

It is remarkable that a cell cycle-related calcium-binding protein, calmodulin 2 (CALM2, Hs.425808), was also up-regulated in the space-flown WI-38 cells (Tables 1 and 2). The Ras/MAPK signaling pathway initiated by NRG1 may be suppressed by calmodulin (CaM), which is reported to bind to EGF receptor, Ras, and Raf-1 to inhibit their signaling activities in mammalian fibroblasts (*38*). In contrast, CaM interaction with the p85 regulatory subunit of phosphatidylinositol-3 kinase (PI3K) activates PI3K in Chinese hamster ovary (CHO) cells (*39*). Therefore, CaM activation may be either a response to the sustained MAPK signaling, attempting to down-regulate Ras, or an independent attempt to stimulate PI3K signaling in order to induce cell survival (*38*). Since CALM2 is also reported to participate in the re-entry into the cell cycle (*40*), the coordinated induction of *Nrg1* and *Calm2* leads us to suggest that there is a concordant effort to activate MAPK and PI3K signaling during spaceflight stress, in order to depart from the quiescent phase to enter into early and possibly mid G_1 phase.

Activation of chromatin remodeling signaling

The over-expression of mortality factor 4-like 2 protein (MORF4L2, Hs.411358) (Tables 1 and 2) is an additional sign of WI-38 cells' re-entry to G_1 phase. *Morf4l2* is a member of a family of seven transcription factor genes, of which only MORF4 and the MORF4-related genes (MRGs) *Morf4l1* and *Morf4l2* are expressed in human cells. MRGs are thought to transcriptionally modulate important cell growth genes because they activate *B-myb* (*41*), an essential transcriptional factor for cell growth, at the G_1/S boundary of the cell cycle. In the case of our space-flown WI-38 fibroblasts, up-regulation of MORF4L2, in combination with the aforementioned hTERT, EWS, and SOD1, reflects the underlying dramatic change in chromatin remodeling in accordance with activation of DNA repair signaling. Therefore, MORF4L2 can be used as a key biomarker, possibly for the main response to the ionizing radiation sustained by the cells during spaceflight.

Stress-induced protein synthesis

In WI-38 cells, spaceflight activates protein synthesis pathways, as inferred from the SSH data. The activated protein synthesis genes include a eukaryotic translation initiation factor 4E-like protein (EIF4EL3, Hs.211823), a eukaryotic translation elongation factor (EEF1A1, Hs.439552), and various ribosome proteins (RPL6, RPL9, RPL23, RPL23A, RPL38, and RPS3A) (Tables 1 and 2), which compose the main body of the translation machinery. Activation of EIF4E exerts a mitogenic and oncogenic effect through the activation of the Ras signaling pathway (*42*). Another important component in the translation machinery is EEF1A; increased expression of EEF1A is positively corrected with protein synthesis (*43*). In addition, the protein kinase C (PKC) and MAPK signaling pathways, which seem to be stimulated by spaceflight, may also greatly enhance the activities of both EIF4EL3 and EEF1A via phosphorylation by PKC or MAPK interacting with serine/threonine kinase, respectively (*43*, *44*).

Besides the main translational machinery, rRNA synthesis and post-transcriptional processes contribute considerably to protein synthesis. Nucleolin (NCL, Hs.79100), an RNA binding protein, is a major nucleolar protein in dividing eukaryotic cells and is directly involved in the regulation of ribosome biogenesis and maturation. Genes encoding NCL, CALM2, NRG1, RIBIN, HNRPF, and RAN are all highly expressed in normal WI-38 fibroblasts upon spaceflight (Tables 1 and 2), suggesting that the enhanced protein synthesis in human fibroblasts due to spaceflight may be supported accordingly by rRNA synthesis, process, and export activities.

Discussion

This report on changes in gene expression due to spaceflight can be summed up as follows: (1) up-regulation of stress response signaling, such as the redox oxidative defense and DNA repair pathways; (2) up-regulation of cell-cycle re-entry, as indicated by up-regulation of pro-cell cycle traverse genes and down-regulation of anti-cell cycle traverse genes, as well as up-regulated MAPK and PI3K pathways and activation of protein synthesis machinery; (3) up-regulation of pro-apoptotic signaling gene expression, as well as up-regulation of several key pro-survival factors; and (4) down-regulation of genes involved in energy metabolism. On the surface, these seem to be a lot of discordant signaling responses to spaceflight-associated stress, involving multiple parallel up-regulation of gene expressions exerting opposing functionalities, exemplified by up-regulation of both pro-apoptotic signaling and pro-survival gene expressions. Here, it is important to point out that re-entering cell cycle traverse, especially the early and mid G_1 phase, can be shared by cells that are engaged in either an apoptotic path or premature replicative senescence; that is, the G_1/S boundary is not only the checkpoint for entry to the S phase, but also a triage point to suicidal death or permanent exit from cell cycle traverse (*45*, *46*). With this in mind, the stress involved in spaceflight may have induced signaling responses such as DNA repair and redox defense, which in turn activated re-entry to cell cycle traverse, serving as early phases for either apoptosis or premature replicative senescence signaling. However, this traverse is abortive, since what is strikingly *not* seen in our study is the up-regulation of *late* G_1-phase gene expressions, notably the cyclin-dependent kinase family, as well as other gene expressions required for entry to the S phase. Therefore, we suggest that the fibroblasts' response to spaceflight and its associated stress is to re-enter cell cycle traverse by experiencing early and mid G_1 signaling, but then to halt at the G_1/S border, as documented by the lack of identified changes in late G_1-phase gene expressions such as thymidine kinase (TK), cdc2, cyclin A, cyclin B, cdk2, and cdk4. Future work with ground-based experiments simulating spaceflight-associated stress will reveal whether these cells indeed halt at the G_1/S checkpoint and are triaged either to premature replicative senescence or to apoptotic death.

It is interesting that we observed the up-regulation of both pro- and anti-apoptotic (pro-survival) gene expression in our flight-specific cell cultures. This suggests that, as mentioned above, space travel induces oxidative stress, which impacts on intracellular organelles such as endoplasmic reticulum, frequently manifested as activation of both pro- and anti-apoptotic genes. Our well-controlled CCM culturing conditions, as well as our comparative study between flight- and ground-based samples, would argue against that such stress is associated with poor nutrient conditions. Thus, the simultaneous activation of two groups of genes, both for and against the innate programmed cell death program, is indeed due to weightlessness and/or other space-associated stress such as radiation.

Due to the specific arrangements of the space shuttle mission, we were able to collect cells for analysis only after the conclusion of the entire flight; therefore our results reflect the cumulative effects of vibration associated with launching and re-entry, gravity changes (both acceleration and microgravity), and ionizing radiation. Dissecting specific gene expression changes due to each of these factors, or any combination among them, must await future experiments with ground-based conditions simulating the individual categories of stress associated with travel in space. Nevertheless, our work reveals that a global approach to identify coordinated patterns of change in gene expressions among diverse signaling pathways may provide insights that would not be possible by the single-gene reductionist approach. Furthermore, the gene-network analysis discussed below may reveal unexpected results, leading to the identification of "hubs" embedded in the stress response to spaceflight (Figure 4).

We have employed the recently popular "interactome" analysis to explicate the data mining of our

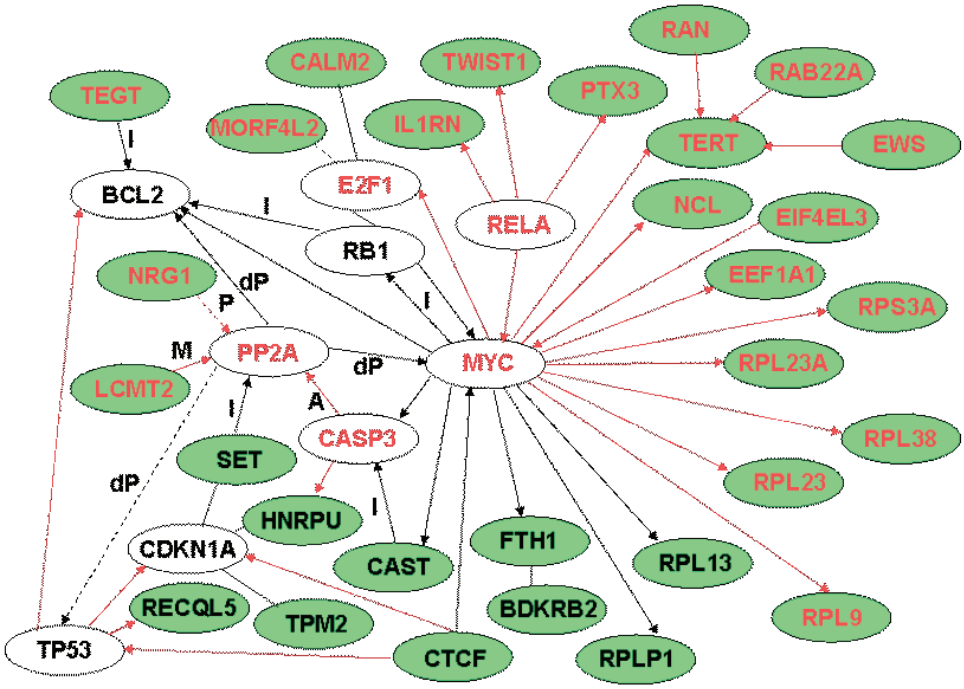

Fig. 4 A putative interactomic network of normal WI-38 fibroblasts after spaceflight. The directions of the arrows indicate one element acting on another. Without an attached abbreviation sign, red arrows denote "up-regulate", while black arrows mean "down-regulate". With attached signs, "P", "dP", "M", and "I" stand for "phosphorylate", "dephosphorlate", "methylate", and "inhibit", respectively. The solid lines illustrate a physical binding between two elements; dashed lines and arrows suggest a likely but unproven relationship. In addition, red- or black-lettered genes are expected to be up- or down-regulated by spaceflight, respectively. Finally, green- and blank-background genes are identified by our SSH experiment or by speculation, respectively.

results, beyond the obvious presentation of either up- or down-regulated gene expressions shown in Table 1. Based on our SSH data, together with known information, we have attempted to connect together 29 genes listed in Table 1 into an "interactome" network by virtue of their relationships at the transcriptional, translational, or post-translational levels. As shown in Figure 4, the major "hub" for this network is c-Myc, with secondary "hubs" represented by E2F1, RELA, PP2A, and Caspase 3. Several genes are connected to others such as p53, Bcl2, and CDKN1A, as shown on the left side of the interactome network. Interestingly, none of these extraneous "hubs" or "nodes" is detected by our subtraction screening as being significantly up- or down-regulated. This may be because our experiment was, as described above, unable to perform temporal and segmental changes of all spaceflight-associated stress responses; they may have experienced changes that our single time point and post-flight analysis was unable to detect. Alternatively, they may prove to be "master" switches, whose changes may be either transient or at modest levels, stimulating "ripple" signaling effects of which our observations are the "foot-prints".

In all, identifying c-Myc as a major "hub" for interactome analysis further supports the notion that in responding to spaceflight stress, cells may engage in the attempt to re-enter cell cycle traverse, since it involves the key immediate early genes controlling the progress of early and mid G_1 phase. However, as suggested above, this traverse is abortive, halted at the G_1/S border, as indicated by the lack of late G_1 phase-specific gene expressions. What is revealed by our results is that not only genes observed to change are important, but more significantly genes not identified by our screening attempt are equally important, including late G_1-phase genes necessary for successfully entering the S phase, and all the key "hub" genes presented in our interactome network analysis. These unseen "embedded" links may be the "smart" keys to future functional studies of how spaceflight stress impacts normal signaling events in fibroblasts.

Conclusion

In conclusion, our comprehensive analysis of all gene expression changes with the interactome network construction shows that in response to spaceflight stress, contact-inhibited quiescent fibroblasts may attempt to re-enter cell cycle traverse by up-regulating genes involved in early and mid G_1 phase, as evidenced by up-regulated MAPK/PI3K signaling pathways, up-regulation of pro-cell cycle traverse genes, and down-regulation of gene expressions repressing this traverse, as well as mapping c-Myc, a key immediate early gene, as a key "hub" of our interactome network. However, this traverse may be abortive, halted prior to the G_1/S checkpoint, because late G_1 gene expressions, such as TK, cdc2, cyclin A, cyclin B, cdk2, and cdk4, show neither up- nor down-regulation. The absence of these changes leads us to suggest that cells may be triaged to either premature replicative senescence or apoptosis, two alternative cell fates besides entering the S phase and proceeding with the rest of cell cycle traverse. This suggestion is further supported by the fact that up-regulation of both pro-survival factors (possibly for those triaged to premature replicative senescence) and pro-apoptosis signaling (for those committed to die) is observed in our results.

Materials and Methods

Human fibroblasts and RNA extraction

Cultures of normal human WI-38 fibroblasts were maintained at the Life Sciences Support Facility of the Kennedy Space Center as previously described (*1*). CELLMAX cartridges (Cello, Rancho Dominguez, CA, USA) containing hollow cellulose acetate fibers were equilibrated with CCM under tissue culture conditions, and were inoculated with approximately 1.2×10^7 young cells (at a cumulative PDL of 20) in a volume of 10 mL minimum essential medium (Invitrogen, Carlsbad, CA, USA). This number of cells used for inoculation is to ensure that the cultures would reach confluence by the time they were flown. Cells were allowed to attach to the fibers for 4 h before switching on constant medium flow for 2 d. Then the cartridges, attached to a 110 mL medium bag, were assembled and kept in the CCM for 2 d. After that, they were transferred to and kept in the space shuttle payload for 2 d in preparation for the STS-93 mission. On Day 6, the bags of medium were changed once due to launch delay. On Day 7, the space shuttle Columbia was finally launched into orbit for a flight of 4 d and 23 h. Once the shuttle returned to the ground, the cartridges were immediately removed from the CCM and disassembled. Cells attached to the cellulose acetate fibers were resuspended in approximately 10 mL of Trizol reagent (Invitrogen) and stored at $-70°$C. Total RNA extraction was carried out according to the manufacturer's protocol. RNA concentrations were determined by absorbance at 260 nm; RNA quality was assessed by running a 1% agarose gel stained with ethidium bromide. RNA samples were subsequently treated with DNase and cleaned with RNeasy columns (Qiagen, Mississauga, ON, USA) according to the manufacturer's recommendations.

SSH

cDNAs were synthesized from 1 μg of total RNA extracted from either space-flown or ground-control cells, using the SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA). SSH was carried out with the PCR-Select cDNA Subtraction Kit (Clontech). We performed both forward and reverse subtractions using the space-flown cell-derived cDNAs first as a "tester", with the ground-control cell-derived cDNAs as a "driver", and *vice versa*. The tester cDNAs were digested with restriction endonuclease *Rsa*I to generate blunt-ended cDNA fragments. To select for transcripts over-expressed in the tester population but under-expressed in the driver population, two different polymerase chain reaction (PCR) adaptors were separately ligated to the tester cDNAs; tester cDNAs with the adaptors were subsequently hybridized to the excess driver cDNAs. After hybridization, suppression PCR, using the primer pair specific to the two ligated tester-adaptors, selectively amplified over-expressed transcripts in the tester pool. Amplified cDNA sequences were subcloned into a TA-cloning vector PCR II-TOPO (Invitrogen) as subtracted clones.

SSH clone sequencing and gene identification

The subtracted clones were sequenced and analyzed for homology with known genes in the GenBank database, using computer programs BlastN and BlastP from NCBI (http://www.ncbi.nlm.nih.gov/blast/). Sequences bearing significant similarity to ESTs were researched against the EST database of

GenBank. Overlapping sequences were then pooled by the program Gene Runner (version 3.00; Hastings Software Inc., Hudson, NY, USA). Finally, the representative sequences were used for homology searches to known genes.

Gene function classification and regulatory network construction

All putative genes identified by SSH were annotated by the DAVID annotation tool at the National Institute of Allergy and Infectious Diseases (NIAID) website (http://apps1.niaid.nih.gov/david/upload.asp). The DAVID annotation for each gene was supplemented with information obtained from Medline literature search. Key words in the putative description for an identified gene were used for biological function classification. The putative proteins encoded by the identified genes were then roughly network-connected by a web-based program Ingenuity Pathway (http://www.ingenuity.pathway.com/), followed by fine-tuning completion based on detailed, comprehensive Medline information.

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Authors' contributions

YL collected the datasets, conducted data analyses, and prepared the manuscript. EW conceived the idea of using this approach, supervised the project, and co-wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors have declared that no competing interests exist.

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