Chapter 5

Identification of Putative Major Space Genes Using Genome-Wide Literature Data

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Additional information is available at the end of the chapter

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1. Introduction

Microgravity in life sciences is an important field of study, not only because of our interest in exploring and living in space for extended periods, but also for the potential insights it gives on earthbound health problems. With genome-wide array technologies, the study of microgravity effects on living organisms can be examined in much greater detail at the cellular and molecular levels which is key to elucidating the molecular mechanisms of this environmental factor. Microgravity is a main environmental risk factor of spaceflight [1, 2] and the adverse effects of microgravity have much in common with earthbound health problems related to low physical activity or reduced mechanical loading. Bone loss and muscle atrophy as well as immune system dysfunction are some of the main consequences common to both extended spaceflight and physical inactivity such as that associated with premature aging and degenerative disorders [3, 4]. Remarkable similarities have been noted between the clinical presentation of spinal cord injury and prolonged gravity unloading including atrophy in muscle and bone, cardiovascular disturbances, and alterations in renal, immune and sensory motor [5]. Microgravity research also holds promise in the area of tissue engineering. Microgravity simulation devices such as Rotating Wall Vessel (RWV) have been increasingly explored to generate 3-D organ mimics for liver and pancreatic islet transplantations [6-9]. Continued effort in microgravity research will deepen our understanding of space adaptation response and improve our ability to treat health-related problems, such as spinal cord injury, diabetes, osteoporosis, and premature aging. A better understanding of microgravity effects at the molecular level could help in the development of countermeasures that will protect astronauts from the deleterious effects of living in space as well as lead to the development of treatments for human diseases here on Earth.

Cellular environmental changes such as sudden gravity change is likely to alter the fundamental activities of genes and any change in the physiological function of a cell or an organism is most likely the result of changes in certain genes' expressions. Genes from many cell types have been shown to be sensitive to the microgravity environments (reviewed by Clement 2012). With the advent of high-throughput genomic technology such as microarrays, large scale genome-wide studies have been performed to assess the mRNA levels of cultured cells and organisms exposed to microgravity. This is an effective approach because the control of mRNA abundance of genes is effectively adapted by cells through controlling transcription (especially transcription initiation), nuclear pre-mRNA processing, mRNA transport, mRNA stability, etc. The cellular abundance of mRNAs is critical to gene function and protein production, which is intriguingly fine-tuned by non-coding regulatory RNAs such as miRNAs. Since the turn of the century, microarray studies have been increasingly used in space life sciences to assess the abundance of mRNAs in response to microgravity. The microgravity biotechnologies combined with microarray technology have been successfully used to study microgravity effect on gene expression on a wide variety of cell types. In a previous review, data was combined from all retrievable microarray-based microgravity research to identify the most frequently altered putative "major space genes" [10]. At that time we identified 26 microarray based microgravity studies in mammalian cells or tissue that had some form of published gene lists. In addition, we included the then available results (published gene lists) from four Xenopus studies. Candidate major space genes were defined as genes that appeared to have significantly altered expression levels in at least four studies. The resulting list of merely eight potential space genes were CD44, CTGF, CYR61, FN1, MT2, MT1, MARCKS, TUBA4A [4]

Since 2011, substantially more progress has been achieved in the literature because significantly more studies have been published with retrievable gene lists. The combination of a greater number of studies and a general increase in the availability of published gene lists, has enabled us to greatly expand our list of putative "major space genes" from the initial number of eight [4] to the present number of 129 at the same initial level of stringency, a gene's expression was found to be altered by microgravity in four or more studies. Thus, this paper is an extended review and meta-analysis of gene expression profiles to identify major space genes, with emphasis on findings on mammalian cells. To accomplish this, we first defined the method and scope of the current literature-based study to identify the putative major space genes from published data on microarray based microgravity studies in the literature. We proceeded to obtain our novel data at three different confidence levels for the putative major space genes. We further refined the criterion for putative major space genes to only include genes that were found to have altered expression patterns in five or more studies or model cell lines. This higher stringency of selection yielded a more focused group of 35 putative major space genes. Furthermore, we identified 13 genes as the most likely candidates for the major space genes because they have been reported most frequently (≥ 6 studies) as microgravity sensitive genes. We then proceeded to perform bioinformatics analysis at each of the three confidence levels of the putative major space genes. We will present and discuss the lists of candidate major space genes that are most frequently altered by microgravity environments. We also review and discuss recent advances in the area of microarray based microgravity research.

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2. Methods and results

The scope of the current study includes all the microarray based microgravity studies on gene expression regulations that have been documented in the literature. For the initial data collection, we started by doing a PubMed search with the terms such as "microarray and microgravity", "space flight and microarray" and "gene expression and microgravity". From these searches, we were able to identify 48 mammalian microarray studies of microgravity effect. Of these 48 studies there was some form of published gene list from 38 different cell lines in 35 microarray publications of mammalian cells exposed to microgravity, which provide the initial "materials" that this current study is based on. In this Methods and Results section, we present the methods and results together since they are intimately linked in the current approach. We present the methods and results in the following stages: First, the scope of the study data collection is tabulated in Table 1; second, the compilation of the "Master" gene list; Third, identification of the putative major space genes at three different levels of stringency; Fourth, bioinformatics analysis of these putative major space genes using Database for Annotation, Visualization and Integrated Discovery (DAVID) and Search Tool for Interacting Gene/Proteins (STRING).

2.1. Compilation of published gene expression data into a "Master" gene list

We collected information on microgravity sensitive genes from the literature into a tabular format so that the source of the reference, the model cell types, the types of microgravity, the duration of exposures, the platform of the gene expression analysis, magnitudes and directions of gene expression regulation, etc. were all included in the "Master" gene list. The source of data contributing publications used as the subject for our current study is shown in Table 1. The first step in the analysis of the collected data pool was to convert the collected published gene expression data into a format that can be compared directly. Since much of the comparison was across species, we chose to use gene symbols rather than accession numbers. This is mainly because accession numbers are different across species, but the gene symbols are typically the same. In addition, some of the gene lists included accession numbers and gene symbols, others included accession numbers and no gene symbols, and still others included gene symbols and no accession numbers. Therefore, we choose to use gene symbol for all further comparative analysis of these published data. Specifically, we used the DAVID Gene ID Conversion Tool [11] to convert all the differentially expressed genes in microgravity into the same format for comparison. To do this, we copied the accession numbers from each study and uploaded them into DAVID Gene ID Conversion Tool. Once uploaded, the accession numbers are automatically converted into the format chosen. In this case, we chose official gene symbols.

We then were able to assemble a "master" gene list from the 38 published gene lists (PGL) using gene symbols for direct comparison. Our main interest was to determine if a gene was differentially expressed in microgravity. For biological and technical repeats, the data were already averaged in the initial publications and the averaged data were presented in the PGL. There are also a few time-course studies using microarray profiling microgravity effects on

gene expression. If a gene was differentially expressed at any time point in a time-course study, it was included in the master list with its magnitude and direction of differential regulation. Even if a gene was differentially regulated in different directions among different time points, we counted it as a differentially regulated gene. Some of these differences in expression are discussed later in this paper.

This "Master" gene list is by no means a complete gene list since many of the publications in the scope of our current study do not include the full list of differentially regulated genes. Significantly, this master gene list provides the data necessary for the identification of putative major space genes at relatively high confidence levels.

Organism/Cell or Tissue Type	Microgravity	Array type	Citation
Human			
Renal	RWV/STS	Incyte	[12]
Renal	RWV/STS	Incyte	[13]
Liver	RWV	6K Human Array	[14]
Jurkat	STS	GeneFilter 20k array	[15]
Fibroblast	STS	in house	[16]
T-Cells	B	unknown	[17]
T-Cells	RPM	Affymetrix Human Genome Focus Array	[18]
T-Cells	RWV	Affymetrix Human U133A Array	[19]
Muscle	BR	Human AceGene Chip	[20]
Endothelial	RPM	unknown	[21]
Liver	RWV	Agilent 22k Human Microarray V2	[22]
Osteoblast	RPM	Atlas Glass Human 3.8 Microarray	[23]
Skin	RWV	Agilent 22k Human Microarray V2	[24]
Muscle	BR	MWG human 23k oligo array_version 3	[25]
Osteoblast	DL	Affymetrix Human U133 Plus 2.0 Array	[26]
Muscle	ULLS	unknown	[27]
Lymphoblastoids	ISS	Agilent 44k Whole Genome Microarray	[28]
Stem Cells	RWV	Affymetrix Human U133 Plus 2.0 Array	[29]
Lymphoblastoids	RWV	Illumina HumanWG-6 V4 BeadChip/RT2 miRNA PCR Array	[30]
T-Cells	RWV/ISS	Affymetrix Human U133 Plus 2.0 Array	[31]
Lymphoblastoids	ISS	Panorama Ab Microarray	[32]
Thyroid Cancer	RPM	Illumina HumanWG-6_V2_0_R3_11223189_A array	[33]
Endothelial	P	Illumina HumanWG-6_V2_0_R3_11223189_A array	[34]
Endothelial	RPM	Illumina HumanWG-6_V2_0_R3_11223189_A array	[35]

Organism/Cell or Tissue Type	Microgravity	Array type	Citation
Endothelial	ISS	Affymetrix Human Gene 1.0 ST arrays	[36]
Thyroid	RPM	Illumina HumanWG-6_V2_0_R3_11223189_A array	[37]
Lymphocytes	RWV	Agilent Whole Genome Microarray/Agilent Human miRNA Microarray V2	[38]
Mouse			
2T3	RPM	Amersham CodeLink Uniset Mouse I Bioarray	[39]
2T3	RPM	Affymetrix GeneChip Mouse 430 2.0	[40]
Muscle	HLS	Agilent Mouse Oligo Array	[41]
Brain	HLS	AECOM Mouse 27k cDNA array	[42]
Muscle	STS/HLS	Affymetrix Mouse Expression 430 A Array	[43]
Osteoblast	RWV	Agilent Mouse Oligo Array	[44]
Osteoblast	DL	Affymetrix Mouse Genome 430 A 2.0 Array	[45]
Stem Cells	RWV	Roche Nimblegen	[46]
Stem Cells	ISS	Affymetrix Mouse Gene 1.0 ST	[47]
Thymus	STS	Affymetrix Mouse Gene 1.0 ST	[48]
Osteoclasts	RWV	Agilent whole genome 4X44K	[49]
Liver	RWV	Affymetrix Mouse Genome 430 2.0 Array	[7]
Fibroblast	RWV	Affymetrix Mouse Genome 430 A 2.0 Array	[50]
Glial	HLS	Illumina MouseRef-8 v.2 BeadChips	[51]
Rat			
Muscle	HLS	Atlas Rat 1.2 cDNA Array	[52]
Muscle	HLS	Affymetrix U34A Rat Genome Microarray	[53]
Muscle	STS	Atlas Rat 1.2 cDNA Array	[54]
Gastrocnemius	STS/HLS/D	Affymetrix U34A Rat Genome Microarray	[55]
Muscle	HLS	Atlas Rat cDNA Expression Array	[56]
PC12	RWV	In house	[57]
Stem Cell	RWV	CapitalBio Rat Genomic Array	[58]

RWV – Rotating Wall Vessel, HLS – Hind Limb Suspension

RPM – Random Positioning Machine, P- Parabolic Flight

D-Denervation, B-Balloon

STS-Space Shuttle, ULLS-Unilateral Lower Limb Suspension

DL-Diamagnetic Levitation

ISS - International Space Station

Table 1. Microarray Based Studies of Microgravity Effect on Mammalian Cells

2.2. Identification of putative major space genes with different levels of stringency

By compiling the published gene lists into the master list, it provided us with an accurate and convenient platform to identify putative major space genes at various levels of stringency using the simple "vote counting" method. At the very basic level, we identified 1199 genes that were differentially regulated in two or more of the documented studies. One level higher, we found 298 genes appeared to be affected by microgravity in three or more microarray-based microgravity studies. Furthermore, when we set the bar to four or more studies, we identified 129 genes (Table 2), which is in drastic contrast to the 8 genes found a few years ago using this same level of stringency. Because of the increase in the number of relevant studies, we were able to go beyond the level of four or more studies in the selection of putative major space genes which was the highest level possible in our previous report [4]. Just to reach one step further, we isolated 35 candidate major space genes in five or more studies (Table 2). Further still, we found 13 genes that were reported in six or more studies to be microgravity sensitive (Table 2). These two additional levels of higher stringency for the selection of putative major space genes enabled a significantly higher level of confidence. We performed further bioinformatics analysis on the differentially regulated genes of the top three stringency levels: gene lists of 129 genes (in ≥ 4 studies), 35 genes (in ≥ 5 studies), and 13 genes (in ≥ 6 studies), respectively.

Genes Differentially Regulated in 4 Studies				
ADAMTS1	CCT7	ETFA	MFNG	RPL29
ADORA2A	CD59	FOSL1	MMGT1	RPL9
ALDOA	CD9	FST	MMP1	RPLP0
ANPEP	CD93	GARS	MRPS35	SERPINE1
ANXA2	CDH1	GJB2	MX1	SGK1
ANXA3	CDV3	GNG10	NOTCH1	SLC16A3
AP1S1	CFLAR	GPNMB	NTN4	SNX7
AP3M1	CKS1B	HBEGF	PDGFRB	SPRY2
ASAP1	CLDN11	HERPUD1	PDIA4	SRGN
ASNS	CLIC3	ID1	PECAM1	TCP1
ATF3	CNBP	IGFBP6	PKIA	TFB2M
ATP5F1	CNIH	ITGAV	PLAT	TGM2
ATP6V0D1	COL8A1	JUNB	PLOD2	TLR4
BIRC3	CXCL2	KYNU	PLSCR4	TRIB3

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BNIP3L	DDIT3	LITAF	PRDX2	TXN
CAPN5	EEF1A1	LOC285741	PTX3	UQCRFS1
CBS	EFEMP1	LOC399942	RBM3	WISP2
CCL2	EGFL7	LOC643668	RPL10A	ZNF323
CCNC	EIF1B	LTBP2	RPL17	
Genes Differentially Regulated in 5 Studies				
ACP1	CD44	IGFBP3	LOX	TPM1
ADM	CDH5	IGFBP7	MMP10	TXNDC5
AKAP12	CMTM7	IL8	PHGDH	
CAV1	DDIT4	ITGA10	SFRP1	
CAV2	HSPA8	LIMCH1	TPI1	
Genes Differentially Regulated in 6 or more Studies				
CTGF	FN1	ITGB4	MT2	TXNIP
CYR61	FOS	KPNA2	MYC	
EGR1	HSPA1A	MT1	TUBA4A	

Table 2. Putative List of Major Space Genes differentially regulated in 4 or more studies

2.3. Bioinformatics analysis of the putative major space genes

In order to get a better understanding of the putative major space genes at the top three stringency levels, we subjected the genes listed in Tables 2 to further bioinformatics analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 [59, 60] and Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) [61, 62].

The DAVID analysis through gene enrichment allowed us to identify enriched Gene Ontology (GO) terms as well as statistically significant pathways. Each of the top three gene lists was uploaded to DAVID Functional tool (http://david.abcc.ncifcrf.gov) to identify the statistically significant KEGG Pathways as well as the frequency of genes belonging to a particular Gene Ontology. DAVID uses a modified Fisher Exact P-value for gene enrichment analysis and statistically determines the over-representation of functional gene categories in a gene list. P-values equal to or smaller than 0.05 are considered strongly enriched [59, 60]. We obtained the potential KEGG Pathways as well as enriched functional clusters as defined by DAVID [59, 60].

For the 129 genes differentially expressed in ≥ 4 microgravity studies, the pathway analysis resulted in eight pathways at P value ≤ 0.05. The KEGG Pathway analysis showed that largest number of enriched genes were in pathways directly related to various cancer. The 2nd largest pathway identified is focal adhesion (Table 3).

Term	Count	PValue	Genes
Bladder cancer	4	0.02	IL8, CDH1, MYC, MMP1
Focal adhesion	8	0.02	CAV2, CAV1, ITGAV, ITGB4, PDGFRB, ITGA10, BIRC3, FN1
Small cell lung cancer	5	0.028	CKS1B, ITGAV, BIRC3, MYC, FN1
ECM-receptor interaction	5	0.028	CD44, ITGAV, ITGB4, ITGA10, FN1
Ribosome	5	0.031	RPL17, RPL9, RPLP0, RPL10A, RPL29
Pathways in cancer	10	0.035	CKS1B, FOS, IL8, ITGAV, PDGFRB, CDH1, BIRC3, MYC, MMP1, FN1
Pathogenic Escherichia coli infection	4	0.043	LOC399942, TUBA4A, CDH1, TLR4
NOD-like receptor signaling pathway	4	0.053	CCL2, IL8, CXCL2, BIRC3

Table 3. KEGG pathway analysis of 129 putative space genes

We also conducted DAVID functional cluster analysis to determine functionally enriched gene sets from the list of 129 genes differentially regulated in ≥ 4 studies. We set the Stringency at the Highest and used a P-Value cut-off of ≤ 0.05 for inclusion of a term on the list. Based on these criteria, we generated a list with 40 functionally enriched GO categories (Table 4). Some of the top functional categories (based on P-Value) were regulation of apoptosis (17.8%), ion homeostasis (8.5%), cell motility (7.75%), and insulin-like growth factor binding (4.6%).

Term	Count	%	PValue
GO:0005520~insulin-like growth factor binding	6	4.65	1.85E-06
GO:0042981~regulation of apoptosis	23	17.8	4.54E-06
GO:0043066~negative regulation of apoptosis	14	10.9	2.57E-05
GO:0043065~positive regulation of apoptosis	13	10.1	6.73E-04
GO:0016477~cell migration	10	7.75	0.001106
GO:0051674~localization of cell	10	7.75	0.002297
GO:0048870~cell motility	10	7.75	0.002297
GO:0006873~cellular ion homeostasis	11	8.53	0.002595
GO:0007596~blood coagulation	6	4.65	0.002624
GO:0055082~cellular chemical homeostasis	11	8.53	0.002909
GO:0007599~hemostasis	6	4.65	0.00336
GO:0050801~ion homeostasis	11	8.53	0.004886
GO:0030005~cellular di-, tri-valent inorganic cation homeostasis	8	6.2	0.005339
GO:0032496~response to lipopolysaccharide	5	3.88	0.005751
GO:0002237~response to molecule of bacterial origin	5	3.88	0.008466
GO:0006469~negative regulation of protein kinase activity	5	3.88	0.008811
GO:0051412~response to corticosterone stimulus	3	2.33	0.009483

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Term	Count	%	PValue
GO:0030003~cellular cation homeostasis	8	6.2	0.009652
GO:0033673~negative regulation of kinase activity	5	3.88	0.009902
GO:0042325~regulation of phosphorylation	11	8.53	0.011735
GO:0051348~negative regulation of transferase activity	5	3.88	0.012339
GO:0030324~lung development	5	3.88	0.013689
GO:0051385~response to mineralocorticoid stimulus	3	2.33	0.014654
GO:0030323~respiratory tube development	5	3.88	0.01513
GO:0051174~regulation of phosphorus metabolic process	11	8.53	0.015164
GO:0019220~regulation of phosphate metabolic process	11	8.53	0.015164
GO:0055080~cation homeostasis	8	6.2	0.017558
GO:0060541~respiratory system development	5	3.88	0.018291
GO:0048754~branching morphogenesis of a tube	4	3.1	0.022669
GO:0006874~cellular calcium ion homeostasis	6	4.65	0.02823
GO:0009165~nucleotide biosynthetic process	6	4.65	0.029991
GO:0055074~calcium ion homeostasis	6	4.65	0.031203
GO:0001763~morphogenesis of a branching structure	4	3.1	0.031688
GO:0034654~nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic process	6	4.65	0.034365
GO:0034404~nucleobase, nucleoside and nucleotide biosynthetic process	6	4.65	0.034365
GO:0006875~cellular metal ion homeostasis	6	4.65	0.036355
GO:0005840~ribosome	6	4.65	0.036807
GO:0045859~regulation of protein kinase activity	8	6.2	0.042448
GO:0055065~metal ion homeostasis	6	4.65	0.042745
GO:0043549~regulation of kinase activity	8	6.2	0.049406

Table 4. GO categories for the 129 space genes. Processed through DAVID with stringency set at highest

Next, we submitted the list of 35 genes that were differentially regulated in five or more studies to DAVID for bioinformatics analysis. The KEGG Pathway analysis identified focal adhesion and Extracellular Matrix (ECM)-receptor interaction pathways were the largest number of enriched genes (Table 5).

Term	Count	%	PValue	Genes
ECM-receptor interaction	4	11.43	0.004	CD44, ITGB4, ITGA10, FN1
Focal adhesion	5	14.29	0.007	CAV2, CAV1, ITGB4, ITGA10, FN1
Hypertrophic cardiomyopathy (HCM)	3	8.571	0.0432	ITGB4, ITGA10, TPM1
Dilated cardiomyopathy	3	8.571	0.0498	ITGB4, ITGA10, TPM1

Table 5. KEGG Pathways associated with 35 genes that are differentially regulated in 5 or more studies

We processed the same list of 35 genes through the DAVID Functional Clustering Tool using the highest stringency setting and generated a list with 31 enriched GO categories (Table 6). Some of the top categories are cell adhesion (22.9%), biological adhesion (22.9%), response to steroid hormone stimulus (20%), response to hormone stimulus (20%), response to endogenous stimulus (20%), regulation of apoptosis(20%), regulation of programmed cell death (20%), regulation of cell death (20%), and insulin-like growth factor binding (11.4%).

Term	Count	%	P Value
GO:0048545~response to steroid hormone stimulus	7	20	6.09E-06
GO:0005520~insulin-like growth factor binding	4	11	2.47E-05
GO:0009725~response to hormone stimulus	7	20	2.28E-04
GO:0009719~response to endogenous stimulus	7	20	3.87E-04
GO:0007155~cell adhesion	8	23	0.00126
GO:0022610~biological adhesion	8	23	0.00128
GO:0019838~growth factor binding	4	11	0.00178
GO:0005539~glycosaminoglycan binding	4	11	0.00403
GO:0016477~cell migration	5	14	0.00435
GO:0030247~polysaccharide binding	4	11	0.00525
GO:0001871~pattern binding	4	11	0.00525
GO:0051495~positive regulation of cytoskeleton organization	3	8.6	0.00535
GO:0051674~localization of cell	5	14	0.00634
GO:0048870~cell motility	5	14	0.00634
GO:0042981~regulation of apoptosis	7	20	0.01206
GO:0043067~regulation of programmed cell death	7	20	0.01263
GO:0010941~regulation of cell death	7	20	0.01284
GO:0006916~anti-apoptosis	4	11	0.01357
GO:0010638~positive regulation of organelle organization	3	8.6	0.01736
GO:0030005~cellular di-, tri-valent inorganic cation homeostasis	4	11	0.01756
GO:0055066~di-, tri-valent inorganic cation homeostasis	4	11	0.02011
GO:0030003~cellular cation homeostasis	4	11	0.02357
GO:0030324~lung development	3	8.6	0.02416
GO:0030323~respiratory tube development	3	8.6	0.02554
GO:0060541~respiratory system development	3	8.6	0.02839
GO:0055080~cation homeostasis	4	11	0.03198
GO:0014706~striated muscle tissue development	3	8.6	0.03393
GO:0060537~muscle tissue development	3	8.6	0.03711
GO:0051493~regulation of cytoskeleton organization	3	8.6	0.04324
GO:0044087~regulation of cellular component biogenesis	3	8.6	0.04674
GO:0030246~carbohydrate binding	4	11	0.04741

Table 6. 31 enriched GO categories generated from the list of 35 putative space genes.

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For the visualization of the association between the genes in the network, we performed further bioinformatics analysis using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) [61, 62]. By using STRING we can examine co-occurrence, co-expression, and experimental evidence for relationships between the genes of interest. For our analysis, physical and functional interactions among the genes were determined using the high confidence score of 0.7. We uploaded the 129 genes that were differentially regulated in at least 4 of the studies to STRING and the resulting gene association network were shown in Figure 1. The blue lines indicate an association; the thicker the lines the higher the level of confidence. Most of the genes clustered near the center and with a strong association are among the 35 genes we identified as differentially regulated in five or more studies. For example, FN1 (identified in 6 or more studies) shows a strong association with MYC, EGR1 and CTGF all of which were also identified in 6 or more studies. FN1 also shows strong association with LOX, CD44, IGFBP3 and IL8 which are in 5 or more studies. FOS, which is another gene identified in 6 or more studies, shows strong association with MYC, EGR1, MMP10, and IL8 which are genes identified in 5 or more studies.

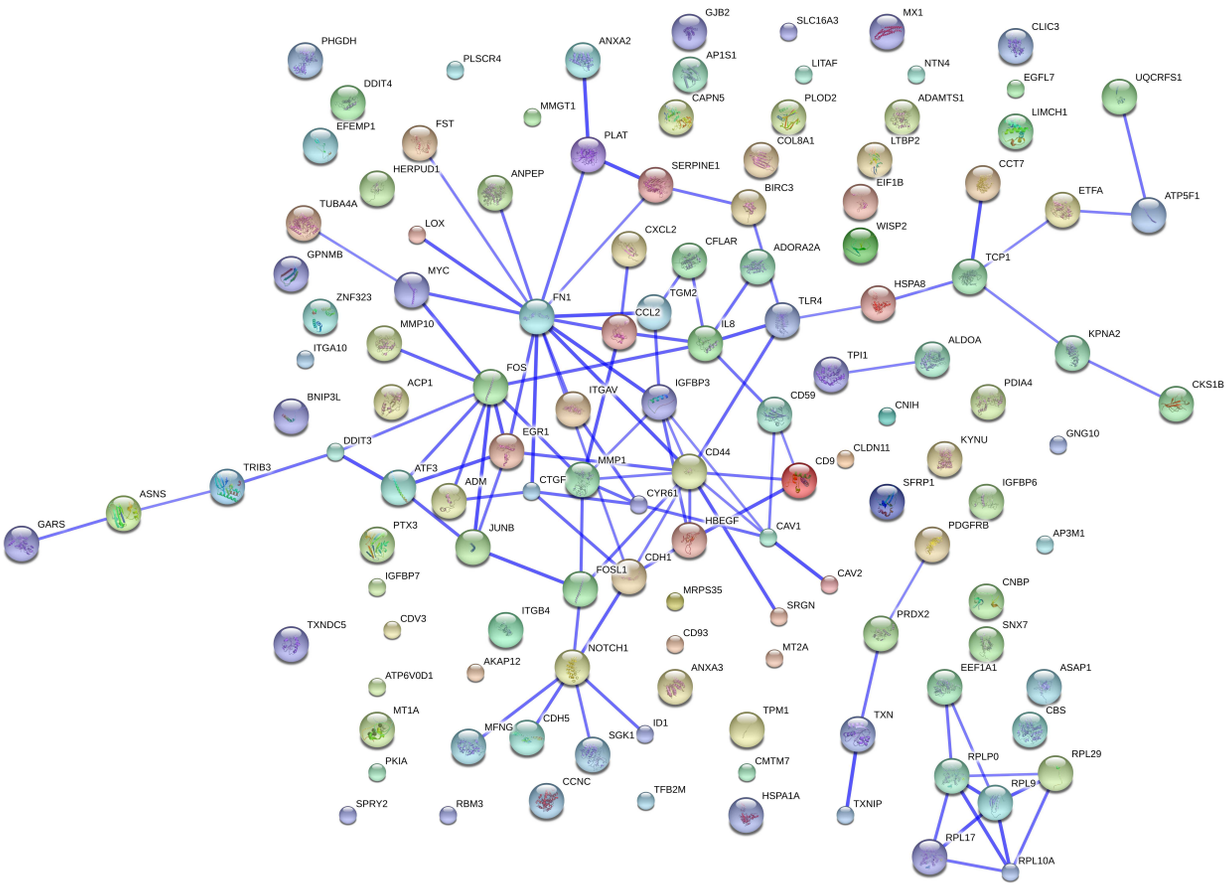

Figure 1. 129 genes that were differentially regulated in at least 4 of the studies were uploaded to STRING. This view shows the evidence of the association between genes. The thicker the line the higher the confidence level.

STRING analysis of the 35 genes differentially regulated in 5 or more studies more clearly show the strong association between FN1, EGR1, CTGF, LOX, MYC, FOS, IGFP3, and CD44 (Figure

2). Note that the genes FN1, EGR1, CTGF, MYC, FOS are among the genes that were differentially regulated in six or more studies, and therefore were identified to be the candidate major space genes at the highest confidence level in the present study.

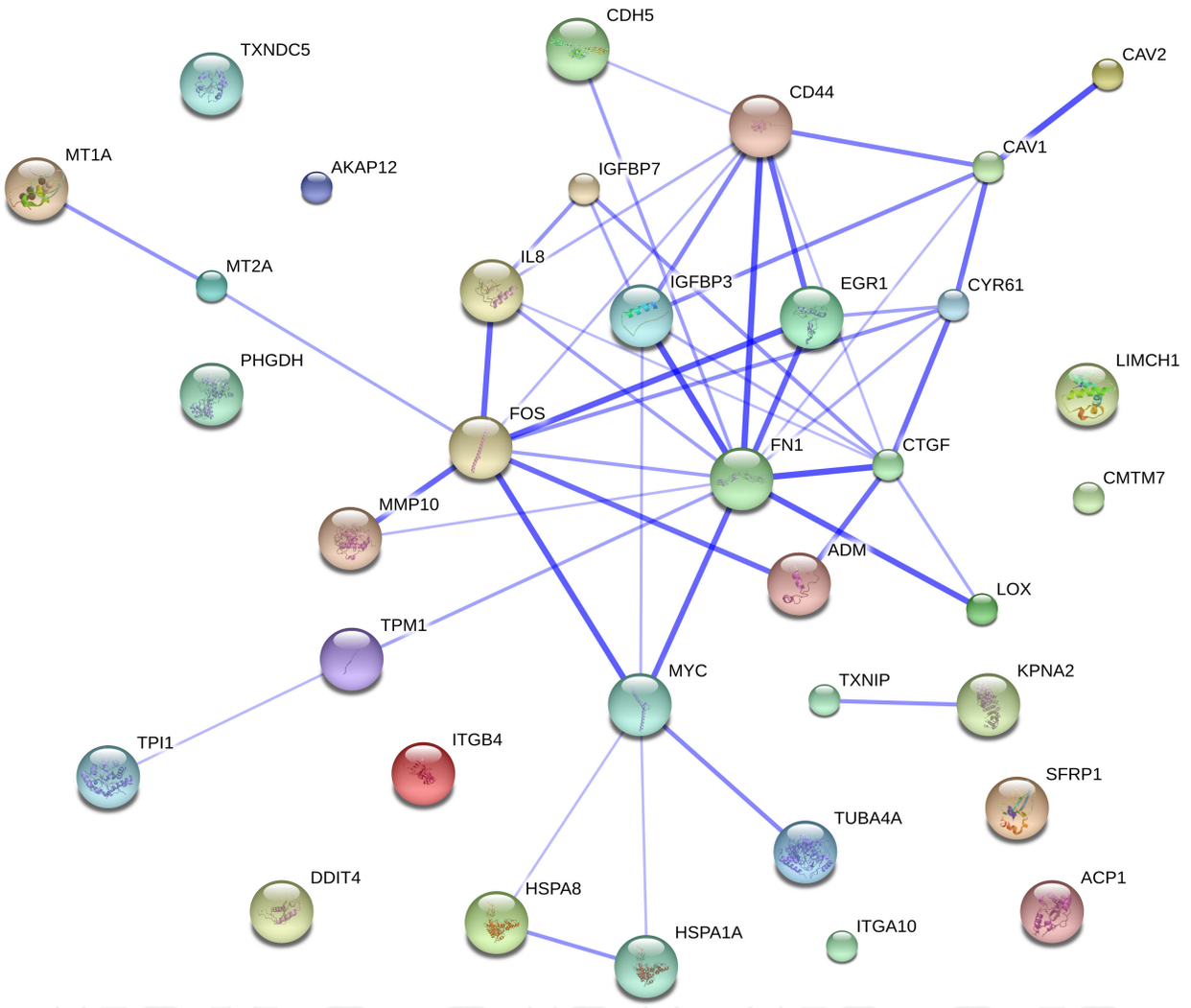

Figure 2. 35 genes that were differentially regulated in at least 5 of the studies were uploaded to STRING. This view shows the evidence of the association between genes. The thicker the line the higher the confidence level.

To further examine the nature of the top 13 genes that were identified in six or more studies to be gravity sensitive, we compiled them into a table which showed the species, cell types, types of microgravity, duration in microgravity and sources of references as well as the directions of the differentially regulated genes (Table 7). From this table we can see that none of the genes were consistently differentially regulated in the same direction. Genes that tend to co-express such as MT1 and MT2, CTGF and CYR61also seemed to co-express in the same direction in these studies. It is not clear why there is such a convergence in the expression patterns. Variables such as different cell types, different species, different forms of microgravity, duration of exposure, and different microarray platforms may be the contributing factors.

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Species	Cell	µg	Duration	CTGF	CYR61	EGR1	FN1	FOS	HSPA1A	ITGB4	KPNA2	MT1	MT2	MYC	TUBA4A	TXNIP	Citation
True Microgravity																	
Human	T-Cell	ISS	1.5h			-								-			[31]
Human	TSCE5	ISS	8d								-						[32]
Human	WTK1	ISS	8d								-						[32]
Human	WTK1	ISS	8d									-	-				[28]
Human	HUVEC	ISS	10d						-							+	[36]
Mouse	gastrocnemius muscles	ISS	11d 19h	+	+							+	+		-		[43]
Mouse	Thymus	ISS	13d									+	+				[48]
Mouse	Mesenchymal Stem Cells	ISS	200h								-					-	[29]
Rat	gastrocnemius muscles	STS	16d				-					+	+				[55]
Human	EA.hy926	PF	22s						-	-							[34]
Microgravity Emulation																	
Human	MG-63	ML	24h		-		+										[26]
Human	EA.hy926 Adherent	RPM	5d	-	-	-		-		-	+			+		-	[35]
Human	EA.hy926 Adherent	RPM	7d	+	-	-		-		-	+			+		+	[35]
Human	EA.hy926 3D aggregate	RPM	5d	-	-	-		-		-	+			+		-	[35]
Human	EA.hy926 Adherent	RPM	7d	-	-			+		-	-			-		+	[35]
Human	FTC-133	RPM	24h	+	+	+		+	+	+	+			+	+	+	[33]
Human	FTC-133 MCTS	RPM	24h	-	-	+		+	+	+	-			+	-	+	[33]
Mouse	RAW 264.7 OCL	RWV	24h							+							[49]
Human	Mesenchymal Stem Cells	RWV	24h	-			-										[29]
Human	HepG2	RWV	3D												-		[22]
Human	HEK	RWV	3-4D						+			+	+	-			[24]
Human	T-Cells	RWV	24h						+								[19]
Human	Renal	RWV	6d		+							-	-				[13]
Mouse	hepatocytes	RWV	4h													+	[7]

Species	Cell	µg	Duration	CTGF	CYR61	EGR1	FN1	FOS	HSPA1A	ITGB4	KPNA2	MT1	MT2	MYC	TUBA4A	TXNIP	Citation
Mouse	hepatocytes	RWV	24h					+									[7]
Mouse	calvarial osteoblasts	RWV	5d	-			-		-						-		[44]
Mouse	Dermal Fibroblast	RWV	2d/7d					-									[50]
Human	Muscle	BR	60d	+			+								+		[25]
Mouse	Soleus Muscle	HLS	7d				-										[56]
Rat	gastrocnemius muscles	HLS	16d		+												[55]

BR= bed rest HLS = Hind limb suspension ISS = International Space Station ML = Magneto Levitation PF = Parabolic flight RPM = Random Positioning Machine RWV = Rotating Wall Vessel STS = Space Shuttle. "+" Indicates up-regulation; "-" Indicates down-regulation.

Table 7. The top 13 putative space genes organized according to types of microgravity. The columns under the gene symbols show the direction of differential regulation in each cell type and microgravity condition.

3. Discussion

The Space Shuttle and the International Space Station (ISS) are engineering miracles [63]. However, the biological importance of the ISS remains mired in controversy over academic and commercial priorities and funding. Do microgravity models provide specialized biological conditions that can be exploited for translational application in the commercial healthcare sector? This is a matter of critical importance to national funding priorities, international competitiveness of the United States, and the health status of Americans and our allies [64]. Not humble or simple questions.

To quote Gene Kranz, the Johnson Space Center Flight Director best known for his leadership during the Apollo13 crisis: "Let's look at this from the point of view of status"[65]. To plan strategically, we need to understand the data we have, the timing of samples, the models studied, and the specifics of the analysis.

This article aims to summarize the status of genome-wide microarray studies in models of microgravity and the true microgravity of space: the data available, the timing of samples, the models studied, and the specifics of the analysis. Specifically, identifying the genes and pathways that may be of central importance in microgravity, may direct areas of commercial and health translation.

With genome-wide array technologies, it becomes possible to study microgravity effects on living organisms at the cellular and molecular levels and in much greater detail, which is key

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to elucidation of the molecular mechanisms of this environmental factor. Since 2011, there has been both an increase in the number of relevant publications and an increase in the quality of retrievable data. This has allowed us to expand our list of putative "major space genes" from the initial number of eight (from our initial attempt toward the identification of the major space genes using literature data [4]) to the present number of 129 genes, at the comparable confidence level. Because of the increase in the number of relevant studies, we were able to go beyond the level of four or more studies in the selection of putative major space genes which was the highest level possible in our previous report [4]. We proceeded to go two levels higher to identify genes found differentially regulated in five or more and six or more studies to be 35 genes and 13 genes, respectively (Table 2). These two additional levels of higher stringency for the selection of putative major space genes enable a significantly higher level of confidence. Our further bioinformatics analysis on the differentially regulated genes showed interesting connections among many of the putative major space genes and several key pathways.

Perhaps the most important insights gleaned from this analysis are the limitations of the current data, which in turn suggests vectors for future analysis [64]. The animal and cellular ground control models are diverse, and incompletely characterized for advantages and limitations and the durations of exposure to true microgravity or simulations, are broadly spread. The analysis platforms vary, but are similar in scope and sensitivity. Despite these limitations, some themes emerge. The effects of radiation are apparent, as are changes in redox potential in the response to microgravity. These are both pathways relevant to tumors.

We found thioredoxin-interacting protein (TXNIP) to be one of the putative space genes that was most frequently differentially expressed in microgravity (Table 2). TXNIP was up-regulated by 10.5 fold in human umbilical vein endothelial cells (HUVECS) in the ISS, making it the most significantly altered gene expression in that study of true microgravity. In microgravity emulated by the random positioning machine (RPM), TXNIP in endothelial cells was down regulated by more than 4-fold after five days and slightly up-regulated after 7 days [35]. However, the same group found TXNIP to be up-regulated in thyroid cancer cells exposed to emulated microgravity [33]. TXNIP is a tumor suppressor in thyroid cells [66]. Up-regulation of this tumor suppressor gene may explain why Grosse et al found that thyroid cancer cells became less aggressive when grown in emulated microgravity [33]..

Metallotionin I and II (MT-I and –II) are also among the top 13 putative major space genes affected (Table 2). These isoforms function primarily in metal ion homeostasis, scavenging of ROS, redox status, immune defense responses, cell proliferation and cell death [67, 68].

Changes in redox-related genes were also identified using fitness profiling of yeast deletion series grown in spaceflight and ground [69] using next generation sequencing. Techniques such as next generation sequencing technology, offer the potential for far more nuanced and detailed analysis of the whole genome, and secondary pathway analysis of the sequence data generated [69]. The genome-wide sensitivity profiles obtained from spaceflight were queried for their similarity to a compendium of drugs whose effects on the yeast collection have been previously reported. The effects of spaceflight have high concordance with the effects of changes in redox state, suggesting mechanisms by which spaceflight may negatively affect cell fitness.

The redox state of tumor cells is frequently disrupted and this is difficult to reproduce in ground-based cultures [70-72]. Hence, redox-dependent drug metabolism in tumors may be uniquely modeled in microgravity. Many of the genes most commonly associated with microgravity-related changes have been identified to have roles in cell cycling, which is critical for both carcinogenesis and responses to radiation damage. EGR1 (Early growth response protein 1; also referred to as Zif268, zinc finger protein 225; and NGFIA, nerve growth factor-induced protein A) is a tumor suppressor transcription factor for differentiation and mitogenesis. MYC encodes for a transcription factor with roles in cell cycle progression, apoptosis and cellular transformation [73]. Karyopherin alpha 2 (KPNA2) promotes tumorigenicity through up-regulation of c-MYC [74]. In endothelial cells grown in microgravity KPNA2 was shown to up-regulated in both adherent cells and multicellular conglomerates at 5 days in simulated microgravity, but down-regulated in multicellular conglomerates at 7 days [35]. HSP70 family members such as HSPA1A have been found to be critical to cellular homeostasis and cancer cell survival[75]. Integrin, Beta 4 (ITGB4) is the receptor for laminin and has been found to be up-regulated in thyroid cancer cells and MCTS grown on RPM for 24 hours [33].

Similar to the results of the current meta-analysis, Nislow et al. 2014 found spaceflight has subtle but significant effects on core cellular processes including growth control via RNA and ribosomal biogenesis, metabolism, modification and decay pathways. Furthermore, significant roles for DNA repair and replication, response to pH signaling, control of gene expression, and mitochondrial function were observed. The yeast chemogenetic analysis of spaceflight samples strongly implicates DNA and RNA damage as the major ground based analogs of spaceflight stress. Given the unique, and substantial radiation exposure in space, this is consistent with major radiation-mediated effects which may mimic cancer related effects.

Suppression of the immune system has been thought to be an important side-effect of microgravity exposure [1, 2, 31, 76]. Recently, a global gene expression analysis of human T cells after 1.5 h of stimulation by Con A and anti-CD28 in the LEUKIN spaceflight has identified immediate early genes whose transcription are inhibited in microgravity [31]. The transcription of immediate early genes is inhibited in T cells activated in microgravity, which may be involved in the molecular basis of spaceflight immunosuppression. NF-κB is known to regulate transcription in most mammalian cells and plays a key role in immune responses to antigens, cytokines, UV radiation, oxidized LDL, free radicals, etc. [77-81]. CREB, a cAMP-responsive transcription factor, regulates immune genes including IL-2, IL-6, IL-10, and TNF-α. CREB also promotes survival and proliferation to T-cells, monocytes, and macrophages [82]. EGR1 and MYC, which are among the 48 most significantly down-regulated by microgravity in the T cell activation study are identified as the putative major space genes in the current study (Table 2).

This analysis shows a commonality of gene changes and pathways between different microgravity models. As data is systematically accumulated, this type of analysis will allow even more meaningful analysis. A key question is whether the unique environment of the ISS induces biological changes of commercial translational value to enhance ground-based health care? In which areas does ISS provide a specific advantage over ground-based biological simulations to direct strategic planning of space based biological science? Within and between ground-based microgravity simulations, can we identify areas where specific techniques are

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best suited for health care applications? This approach will place space-based science at the center of academic medical center activity [83], and translate to commercial applications.

Abbreviations / Glossary

DAVID: Database for Annotation, Visualization and Integrated Discovery

EGR1: Early growth response protein 1

GO: Gene Ontology

HARV: High Aspect Rotating Vessel

HUVEC: Human Umbilical Vein Endothelial Cells

ISS: International Space Station

KPNA2: Karyopherin alpha 2

MT-1, -2: Metallotionin-I and –II

RCCS: Rotating Cell Culture System

ROS: Reactive oxygen species

RPM: Random Positioning Machine

RWV: Rotating Wall Vessel

STRING: Search Tool for the Retrieval of Interacting Genes/Proteins

TXNIP: Thioredoxin-interacting protein

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