in vivo *29*: 423-434 (2015)

Genetic and Apoptotic Changes in Lungs of Mice Flown on the STS-135 Mission in Space

DAILA S. GRIDLEY[1], XIAO WEN MAO[1], JIAN TIAN[1], JEFFREY D. CAO[2], CELSO PEREZ[1], LOUIS S. STODIECK[3], VIRGINIA L. FERGUSON[4], TED A. BATEMAN[5] and MICHAEL J. PECAUT[1]

[1]*Department of Basic Sciences, Division of Radiation Research, Loma Linda University School of Medicine, Loma Linda, CA, U.S.A.;*
[2]*Department of Human Anatomy & Pathology, Loma Linda University and Medical Center, Loma Linda, CA, U.S.A.;*
[3]*BioServe Space Technologies, Aerospace Engineering Sciences, and*
[4]*Department of Mechanical Engineering, University of Colorado, Boulder, CO, U.S.A.;*
[5]*Department of Bioengineering, University of North Carolina, Chapel Hill, NC, U.S.A.*

Abstract. *Aim: The goal of the study was to evaluate changes in lung status due to spaceflight stressors that include radiation above levels found on Earth. Materials and Methods: Within hours after return from a 13-day mission in space onboard the Space Shuttle Atlantis, C57BL/6 mice (FLT group) were euthanized; mice housed on the ground in similar animal enclosure modules served as controls (AEM group). Lung tissue was collected to evaluate the expression of genes related to extracellular matrix (ECM)/adhesion and stem cell signaling. Pathway analysis was also performed. In addition, immunohistochemistry for stem cell antigen-1 (SCA-1), the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay for apoptosis, and staining for histological characteristics were performed. Results: There were 18/168 genes significantly modulated in lungs from the FLT group ($p<0.05$ vs. AEM); 17 of these were up-regulated and one was down-regulated. The greatest effect, namely a 5.14-fold increase, was observed on Spock1 (also known as Spark/osteonectin), encoding a multi-functional protein that has anti-adhesive effects, inhibits cell proliferation and regulates activity of certain growth factors. Additional genes with increased expression were cadherin 3 (Cdh3), collagen, type V, alpha 1 (Col5a1), integrin alpha 5 (Itga5), laminin, gamma 1 (Lamc1), matrix metallopeptidase 14 (Mmp14), neural cell adhesion molecule 1 (Ncam1), transforming growth factor, beta induced (Tgfbi), thrombospondin 1 (Thbs1), Thbs2, versican (Vcan), fibroblast growth factor receptor 1 (Fgfr1), frizzled homolog 6 (Fzd6), nicastrin (Ncstn), nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4 (Nfatc4), notch gene homolog 4 (Notch4) and vang-like 2 (Vangl2). The down-regulated gene was Mmp13. Staining for SCA-1 protein showed strong signal intensity in bronchiolar epithelial cells of FLT mice ($p<0.05$ vs. AEM). TUNEL positivity was also significantly higher in the FLT mice ($p<0.05$ vs. AEM), but no consistent histological differences were noted. Conclusion: The results demonstrate that spaceflight-related stress had a significant impact on lung integrity, indicative of tissue injury and remodeling.*

Astronauts on missions in space are subject to a variety of stressors, including high levels of radiation, which have potential to adversely affect the respiratory system. Most space-related studies to date have focused primarily on gravity-dependent changes in lung function (1-3) and some have concluded that the observed aberrations may not be a concern for missions lasting up to 6 months (4). The lungs, however, are very vulnerable to a variety of other environmental insults and are essential for survival.

Although low compared to doses commonly used in radiotherapy, radiation doses on board the International Space Station (ISS) are above those on Earth, especially during solar particle events (SPE). Ground-based studies have shown that radiation doses comparable to those found on board the ISS alter the distribution patterns and functions of protective immune cell populations (5-11), thus increasing the risk for infection due to airborne microbes that may have increased virulence in spacecraft (12) and greater resistance

Correspondence to: Daila S. Gridley, Ph.D., Department of Basic Sciences, Division of Radiation Research, Chan Shun Pavilion, 11175 Campus Street, Loma Linda University School of Medicine, Loma Linda, CA 92354, U.S.A. Tel: +1 9095588361, Fax: +1 9095580825, e-mail: dgridley@llu.edu

Key Words: Spaceflight, respiratory tract, gene expression, extracellular matrix, cell adhesion.

to antibiotics (13). After inhalation of bacteria (*Klebsiella pneumoniae*), impaired clearance and increased morbidity have been reported in mice that were hindlimb-unloaded and exposed to SPE-like radiation (14). Furthermore, aberrations in immune parameters due to exposure to the environment of low Earth orbit spaceflight have often been noted in both animals (15-21) and humans (22-24). Re-activation of latent viral infections, indicative of compromised immunity, have also been reported in astronauts (25-27). Interestingly, many of these reactivated viruses, *e.g.* members of the Herpesviridae family, are frequently detected in the lower respiratory tract of ventilated patients and are associated with adverse clinical outcomes (28). A recent study of rat lung epithelial cells exposed to doses ranging from 0.1 to 4 Gray (Gy) of high-energy protons, the most prevalent form of radiation in space, demonstrated dose-dependent radiation effects, namely enhanced expression of pro-apoptotic genes, increased DNA fragmentation and lower cell viability (29). A very recent study of mice exposed to space-relevant radiation at the age of 2.5 months found airspace enlargement and reduced systemic oxygenation at 26 months of age (30). In this latter study, mild inflammation and apoptosis in the lung parenchyma were among the abnormalities noted. Apoptotic cell death has, indeed, been reported in a variety of tissues by investigators conducting research under spaceflight conditions (31, 32). Collectively, it is clear that factors associated with missions in space have the potential to compromise respiratory tract function.

Knowledge of the molecular aspects of lung response to spaceflight condition is very limited. It is known, however, that lung repair mechanisms are initiated immediately following an insult and include inflammation, migration of epithelial cells to a provisional matrix and release of cytokines and other factors that regulate matrix metalloproteinases (MMP), cytoskeletal structures and adhesion molecules (33). During injury repair, progenitor stem cells, including local adult stem cells, undergo phenotypic differentiation so that destroyed cells are replaced and normal function is again achieved. Sometimes, however, normal parenchyma is replaced by mesenchymal tissue, leading to fibrosis (34). Lung fibrosis is characterized by a loss of epithelia and replacement by extracellular matrix (ECM) components and collagen.

We have previously studied lungs collected from mice within hours after Space Shuttle Endeavour (Space Transportation System 118, STS-118) returned from a 13-day mission to the ISS (35). There was more abundant collagen and enhanced expression of pro-fibrotic factors such as neural cell adhesion molecule (NCAM), connective tissue growth factor (CTGF) and transforming growth factor-β1 (TGFβ1) in lungs from the flight (FLT) group compared to the control group housed in similar animal enclosure modules (AEM) on ground, and many genes associated with adhesion molecules and ECM proteases were significantly modulated after flight. In addition, our previous ground-based investigations showed that simulated SPE radiation resulted in abundant accumulation of collagen and up-regulation of TGFβ1 and MMP2 in mouse lungs (36). Other studies conducted in our laboratories that included low-dose priming and acute radiation regimens, *i.e.* equivalent to background radiation in space followed by an SPE, also showed lung abnormalities, some of which suggested induction of apoptosis (37, 38).

The goal of the present study was to further evaluate lung tissue response in mice after return from a 13-day mission to the ISS; the animals were housed within the Space Shuttle Atlantis during the entire mission. We hypothesized that parameters associated with lung injury, *e.g.* apoptosis, and remodeling would be clearly evident. Lungs were collected in the Space Life Sciences Laboratory (SLSL) at the Kennedy Space Center (KSC) within a few hours after the landing of Space Shuttle Atlantis (STS-135). This was the last historic flight of the Space Shuttle Program executed by the National Aeronautics and Space Administration (NASA).

Materials and Methods

Animals and experimental conditions. The STS-135 mouse study was sponsored by Amgen, Inc. and its primary goal was to evaluate a proprietary drug against bone loss. However, as part of NASA's Biospecimen Sharing Program, we were allowed access to lungs from a limited number of placebo-treated mice that did not receive the experimental drug, *i.e.* FLT n=4 and AEM n=7. The placebo was A5SuT, a commonly known formulation consisting of 10 mM sodium acetate and 9% sucrose pH 5 to which 0.004% Tween 20 was added; the placebo was administered subcutaneously. C57BL/6 adult female mice were purchased from Charles River Laboratories, Inc., Wilmington, MA, USA. The FLT mice and AEM ground controls were kept under similar conditions, including temperature, humidity and CO_2 using 48-h delayed telemetry data from the FLT group. For example, temperature for all mice was kept at 26-28˚C, humidity was generally between 30-40% and CO_2 levels ranged from 2,150-3,480 parts per million in the shuttle during the mission. Gravity levels were below 0.01 at all times and below 0.0001 most of the time. In addition, based on an average of ~9 mRad/day, the radiation dose received during flight was likely to be around 115-120 mRad (http://spacemath.gsfc.nasa.gov). Water and a specially designed food bar diet, pretested and approved for rodents by NASA (39), were provided *ad libitum*. Since only tissues obtained after euthanasia (no live animals) were processed at our institution, no approval from the Loma Linda University Institutional Animal Care and Use Committee (ACUC) was required. However, it should be noted that all NASA activities involving vertebrate animals are carried-out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf). The overall Principal Investigator, Dr. V. L. Ferguson, obtained approval from the University of Colorado at Boulder ACUC (protocol 1104.11) that is her home institution. Approvals were also obtained from the NASA

Ames Research Center ACUC (protocol NAS-11-002-Y1) and the NASA Kennedy Space Center ACUC (protocol FLT-11-078). As noted, lungs for the current study were made available to us under the guidance/organization of NASA's Biospecimen Sharing Program. All three ACUCs mentioned above reviewed and approved our protocols described here.

Sample collection and processing. Within 3-5 hours after landing of Space Shuttle Atlantis, the mice were euthanized in the SLSL at KSC using 4% isoflurane followed by cardiac puncture/exsanguination and the lungs were excised. Right lungs were quickly frozen in liquid nitrogen and left lungs were preserved in 4% paraformaldehyde in phosphate-buffered saline (PBS) and then shipped overnight by courier to Loma Linda University. For immunohistochemistry and histology, fixed tissues were then embedded in paraffin according to standard procedures and stored at 4˚C until analysis. The same procedures were used for collecting lungs from AEM ground controls. All mice were approximately 11 weeks of age at the time of euthanasia.

Gene expression. Frozen lung tissue was thawed shortly before quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed. Samples were available from a total of 11 mice: n=4 from the FLT group and n=7 from the AEM control group. The two selected arrays, both purchased from SABiosciences/Qiagen Corp. (Frederick, MD, USA) were: RT^2 Profiler™ PCR Array Mouse Extracellular Matrix and Adhesion Molecules (PAMM-013A) and RT^2 Profiler™ Array Mouse Stem Cell Signaling (PAMM-047Z). The procedure was performed at the SABiosciences Technical Core and has been previously described (40). Briefly, RNA was extracted from the samples using standard procedures and its integrity was confirmed based on 18s and 28s rRNA peaks and RNA integrity number (RIN). Profiles generated on the Agilent 2100 Bioanalyzer allowed a visual inspection of RNA integrity. RIN >7 was translated as high-quality RNA. Our samples had a RIN range of 9.7-10. PCR was performed using a cycler (Bio-Rad Laboratories, Hercules, CA, USA) and RT^2 Real-Time™ SYBR Green PCR Master Mix PA-011 (SABiosciences/Qiagen). The amount of RNA converted to cDNA was 1,000 ng; cDNA was synthesized using the RT^2 First Strand Kit (Qiagen, Cat. No. 330401). The amount of cDNA analyzed was 10 ng/PCR reaction. Thereafter, the fold-regulation was calculated for each gene using the ΔΔC threshold cycle method. Expression levels for a total of 168 genes (84 genes/array) were determined. There were five housekeeping genes: glucuronidase, beta (*Gusb*), hypoxanthine guanine phosphoribosyl transferase (*Hprt*), heat shock protein 90 alpha (cytosolic), class B member 1 (*Hsp90ab1*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and actin, beta (*Actb*); the average of these genes was used to normalize the target genes. These are select genes whose expression often, but not always, remains constant under most experimental conditions. RT controls and positive PCR controls were also included during the analyses. Gene-expression profiles for lungs from the FLT mice were compared with those of the AEM ground controls.

Stem cell antigen-1 (SCA-1) immunohistochemistry. Fluorescein isothiocyanate (FITC)-labeled rat monoclonal antibody against SCA-1 (also known as Ly6A/E) was purchased from Abcam Inc., Cambridge, MA, USA. This antibody (ab25031) is specific for Ly6A.2 and Ly6E.1 that are allelic members of the Ly6 multigene family. Lung sections (6 μm thickness) from four mice per group were stained according to standard procedures. Two sections of the left lung of each mouse were imaged using an Olympus BX61 microscope (Olympus America Inc., Center Valley, PA, USA). To obtain the resulting SCA-1 immunoreactivity, fluorescence intensity was measured on three randomly selected fields on bronchiolar epithelium in each section and calculated using ImageJ 1.41 software (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/Java). Fluorescence intensities from the areas of interest were measured using the integral/density feature in the ImageJ program and data were extracted and averaged within the group. Fluorescence was averaged across four lung samples per group.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Immunohistochemistry was performed on lung tissue sections (6 μm thickness) from four mice per group using an antibody specific for TUNEL as previously described (17, 41). DNA fragmentation, indicative of apoptosis, was detected by incorporating fluorescein-12-dUTP at 3'-OH DNA ends using the recombinant terminal deoxynucleotidyl transferase enzyme (rTdT). The reagents in the DeadEnd™ Fluorometric TUNEL system kit (Promega Corp., Madison, WI, USA) were used. The sections were examined using an Olympus BX61 microscope. TUNEL-positive cells were identified by green fluorescence; the nuclei were counterstained with diamidino-2-phenylindole (DAPI, blue). Three fields from two sections per lung were randomly selected and imaged for analysis. For each lung section, the number of TUNEL-positive cells and total nuclei/mm^2 were counted in three randomly selected fields on bronchiolar epithelium and alveolar cells (because both were positive for TUNEL staining) using ImageJ 1.41 software. Cell densities were averaged within the group.

Histopathological analysis. A portion of the lungs from four mice per group were preserved in 10% neutral-buffered formalin for approximately 6 hours at room temperature. The tissues were then dehydrated through ethanol and embedded in paraffin. Lung sections at 6 μm thickness were stained with hematoxylin and eosin (H&E) using standard techniques and examined using a light microscope. Two sections from each mouse were used for histological analysis.

Statistical analysis. Fold-regulation in gene expression data was analyzed by the SABiosciences/Qiagen technical core using Student's *t*-test, a method widely accepted for data obtained after relative quantification with RT-PCR. Other quantitative data were also analyzed using the *t*-test (SigmaStat™ software, version 3.1, SPSS Inc., Chicago, IL, USA). A value of $p<0.05$ was considered as significant.

Results

ECM and adhesion genes. Table I presents the 12 out of 84 analyzed genes that were significantly modulated in lungs from the FLT mice ($p<0.05$ *vs*. AEM group). The expression of all genes except one was increased, especially *Spock1* which had a fold up-regulation of 5.14. Other genes with a more than 2-fold increase in expression were *Ncam1, Thbs1* and *Thbs2*. Expression levels for *Cdh3, Col5a1, Itga5, Lamc1, Mmp14, Tgfb1* and *Vcan* ranged from 1.43- to 1.97-

Table I. *Fold-regulation for genes associated with extracellular matrix and adhesion in lungs from the mice of the flight group (FLT) vs. the control group of mice in the animal enclosure module (AEM). Data were obtained using quantitative reverse transcriptase-polymerase chain reaction. Lung samples from a total of 11 mice were evaluated (n=4 in the FLT group and n=7 in the AEM group).*

	Gene	Fold-regulation	Description
$p<0.05$	*Cdh3*	1.97	Cadherin 3
	Col5a1	1.43	Collagen, type V, alpha 1
	Itga5	1.88	Integrin alpha 5 (fibronectin receptor alpha)
	Lamc1	1.73	Laminin, gamma 1
	Mmp13	−2.37	Matrix metallopeptidase 13
	Mmp14	1.70	Matrix metallopeptidase 14 (membrane-inserted)
	Ncam1	2.68	Neural cell adhesion molecule 1
	Spock1	5.14	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan 1
	Tgfbi	1.49	Transforming growth factor, beta induced
	Thbs1	2.43	Thrombospondin 1
	Thbs2	2.99	Thrombospondin 2
	Vcan	1.56	Versican
$p=0.05$ to <0.1	*Ecm1*	1.74	Extracellular matrix protein 1
	Fn1	2.40	Fibronectin 1
	Hapln1	2.32	Hyaluronan and proteoglycan link protein 1
	Mmp2	1.36	Matrix metallopeptidase 2
	Selp	4.17	Selectin, platelet
	Timp2	1.50	Tissue inhibitor of metalloproteinase 2

Table II. *Fold-regulation for genes associated with stem cell signaling in lungs from mice of the flight group (FLT) vs. the control group of mice in the animal enclosure module (AEM). Data were obtained using quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Lung samples from a total of 8 mice were evaluated (n=4 in the FLT group and n=4 in the AEM group).*

	Genes	Fold-regulation	Description
$p<0.05$	*Fgfr1*	1.65	Fibroblast growth factor receptor 1
	Fzd6	1.51	Frizzled homolog 6 (Drosophila)
	Ncstn	1.71	Nicastrin
	Nfatc4	2.45	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4
	Notch4	1.91	Notch gene homolog 4 (Drosophila)
	Vangl2	1.66	Vang-like 2 (van gogh, Drosophila)
$p=0.05$ to <0.1	*Crebbp*	1.55	CREB binding protein
	Fgfr3	1.43	Fibroblast growth factor receptor 3
	Rbl1	−1.68	Retinoblastoma-like 1 (p107)
	Smad2	−1.27	MAD homolog 2 (Drosophila)
	Tcf7l1	1.26	Transcription factor 7-like 1 (T-cell-specific, HMG box)

fold above that for the AEM control mice. The only gene in this array that was significantly down-regulated was *Mmp13*, by 2.37-fold compared to the AEM group ($p<0.05$).

Stem cell signaling genes. Table II shows that lungs from FLT mice had significantly modulated expression of six out of the 84 analyzed genes and that all were up-regulated compared to the AEM control group ($p<0.05$). The greatest effect was on *Nfatc4* and *Notch4*, but only the former was up-regulated by more than 2-fold. Values for *Fgfr1, Fzd6, Ncstn* and *Vangl2* ranged from 1.51 to 1.71 ($p<0.05$ *vs.* AEM).

SCA-1 immunohistochemistry. The top panels in Figure 1 show representative images of lung tissue sections from AEM and FLT mice after staining with FITC-labeled antibody against SCA-1 that is expressed by stem-like/progenitor cells. In the AEM ground control samples, bronchial epithelium showed little or no staining. In contrast, SCA-1 immunoreactivity in bronchiolar epithelial cells in the FLT lung samples was very strong. Flat alveolar capillary endothelium was also positive, but the vein wall showed no staining. The bar graph in Figure 1 shows that SCA-1 positivity in the FLT group was significantly higher compared to that of the AEM control group ($p<0.05$).

Apoptosis based on TUNEL assay. The top panels in Figure 2 are representative examples of lung sections from AEM control and FLT mice stained using the TUNEL assay. Immunochemical analysis showed that spaceflight induced significant apoptosis in FLT lungs compared to AEM controls; increased positivity was noted in both the bronchial epithelium and alveoli. The bar graph in the lower portion of Figure 2 shows the mean and standard error of the mean for each group. Our quantitative assessment revealed that TUNEL-positive cell density was significantly higher in the FLT mice compared to the AEM controls ($p<0.05$).

Histology based on H&E staining. The stained lung samples from FLT mice were compared with those from the AEM control group. Two sections from different levels of the tissue samples from the lung of each mouse similarly showed no significant abnormalities with respect to inflammation, necrosis, vascular changes, metaplasia, edema or cellular atypia (Figure 3). Two lung samples, AEM-32 and FLT-66, also included cross sections of the esophagus that also showed none of these anomalies. Specimen FLT-66 also included a section of the trachea which was normal.

Discussion

The results show that of the genes quantified, all but one of the significantly modulated ECM/adhesion genes were up-regulated in the lungs of the FLT mice. These included *Spock1* that was the most affected gene of all that were evaluated; a greater than 5-fold increase in expression was noted compared to the AEM ground control group. *Spock1* encodes a protein known as secreted protein acidic and rich in cysteine (SPARK), also referred to as osteonectin. The end product is a counter-adhesive glycoprotein expressed during tissue repair (42). This glycoprotein is expressed by many different cell types and has long been associated with cell turnover and tissue remodeling, as well as wound repair (43). Members of the SPARK family are important regulators of ECM assembly and are also involved in protease activities and cytokine signaling pathways (44). Based on these and other reports, the high expression of *Spock1* in lungs from the FLT mice suggests that some tissue remodeling due to spaceflight condition (either through damage or ECM perturbation) did occur.

Expression of *Ncam1* (also known as cluster of differentiation 56 or CD56) was increased by approximately 2.7-fold in the lungs from FLT mice. The NCAM isoforms are members of the immunoglobulin superfamily and many studies have focused on the role of these glycoproteins in neuron-immune cell interactions (45). In the lungs, polysialylated NCAM may serve as a protective mechanism to reduce tissue damage during inflammation (46).

Thbs1 and *Thbs2*, both significantly up-regulated by greater than 2-fold in FLT mice, encode thrombospondins 1 and 2. In spite of considerable structural homogeneity, these two matrix glycoproteins have distinct, as well as overlapping, functions. Processes in which either one or both of these TSPs have been implicated include wound healing, thrombosis, anti-angiogenesis, synapse formation and septic shock related to infection and fibrosis (47-50). Although the mechanisms regulating the expression of these TSPs remain unclear (51), their up-regulation in the current study is consistent with lung tissue remodeling due to spaceflight condition. It is also important that TSP1 has potent pro-apoptotic activities for both endothelial and non-endothelial cell populations (52).

The other ECM/adhesion genes that were significantly up-regulated in the lungs from the FLT group were all modified by less than 2-fold and will not be discussed here at length. Briefly, the proteins encoded by these genes are: cadherin 3 (*Cdh3*), a transmembrane protein important in cell adhesion; collagen, type V, alpha 1 (*Col5a1*), an alpha chain for one of the fibrillar collagens; integrin alpha 5 (*Itga5*), an alpha chain of integrins that function in cell–cell and cell–matrix interactions; laminin, gamma (*Lame1*), a subunit of laminins that form non-collagenous portions of basement membranes; matrix metallopeptidase 14 (*Mmp14*), an enzyme involved in ECM breakdown; transforming growth factor, beta induced (*Tgfbi*), a protein that binds to certain types of collagens and inhibits cell adhesion; and versican (*Vcan*), a large ECM protein often referred to as being anti-adhesive. These descriptions are based on information available at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/gene). Up-regulation of these genes, although relatively minor, is consistent with tissue remodeling.

In contrast to all other significantly affected genes in the ECM/adhesion array, Mmp13 was down-regulated (by 2.37-fold) in FLT lungs. MMP13, also known as collagenase-3, is part of a large group of proteases that regulate ECM turnover and participate in tissue remodeling. Deficiency in MMP13 has been reported to increase inflammation after acute hyperoxic lung injury in mice (53). However, after shock-induced lung injury in rats, use of inhaled hypertonic saline inhibited MMP13 accumulation in bronchoalveolar lavage fluid and reduced the degree of injury (54). In our study, histological analysis of H&E-stained lung samples from FLT mice did not reveal any obvious damage or differences in leukocyte composition compared to the AEM control group.

It must be noted that the pattern of ECM/adhesion gene expression in the current study differs markedly from the pattern we previously found in lungs from mice after the STS-118 flight (35). The same array (PAMM-013) was included in both studies and many of the conditions were

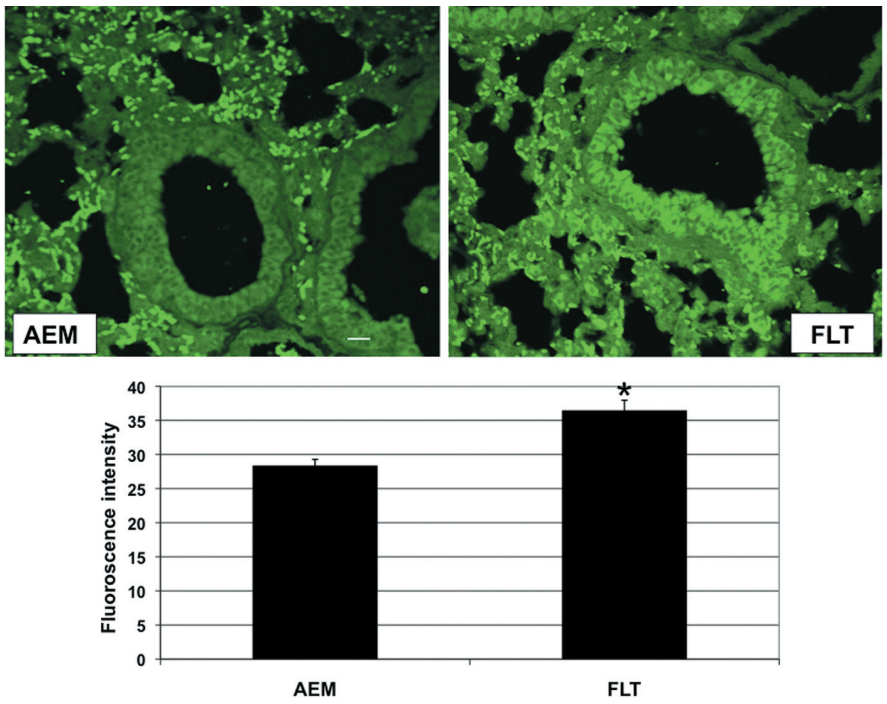

Figure 1. *Stem cell antigen-1 (SCA-1) protein expression in lungs from ground control mice housed in animal enclosure modules (AEM) and flight (FLT) mice. Top panels show representative images after staining with fluorescein isothiocyanate-labeled antibody against SCA-1. The signal in bronchiolar epithelial cells in the spaceflight samples was strong, while the immunoreactivity for this marker was negative in AEM samples. Magnification is ×40; magnification bar is 10 mm. The bar graph shows means and standard errors for n=4 mice/group. *p<0.05 vs. AEM.*

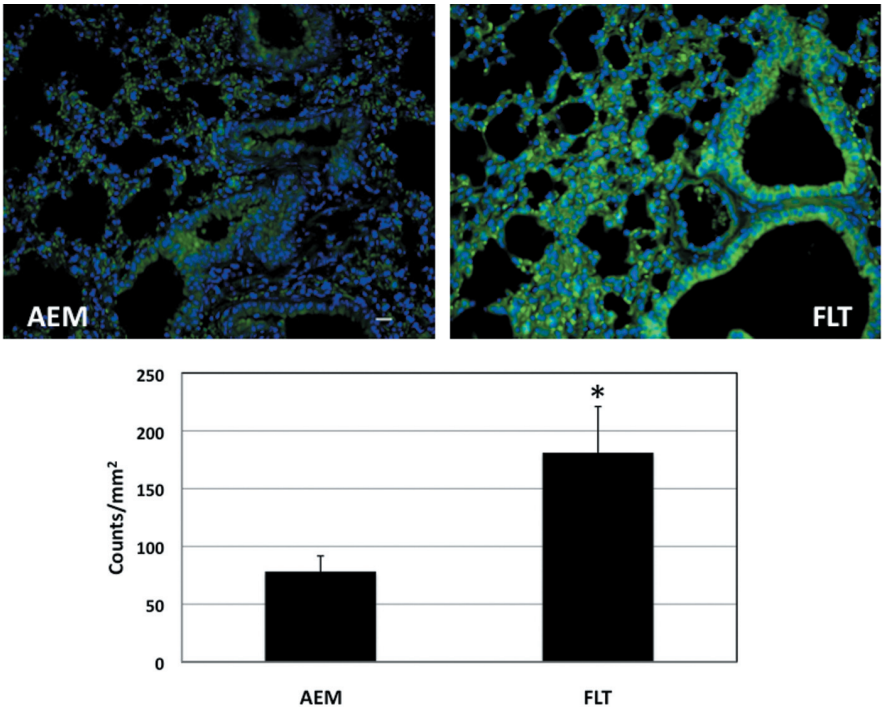

Figure 2. *Apoptosis in the lungs from ground control mice housed in animal enclosure modules (AEM) and flight (FLT) mice. Representative examples of sections stained using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay are shown in the top panels. TUNEL-positive cells: green; nuclei: blue. Magnification is ×40; magnification bar is 10 mm. The bar graph presents the means and standard errors for n=3-4/group. *p<0.05 vs. AEM.*

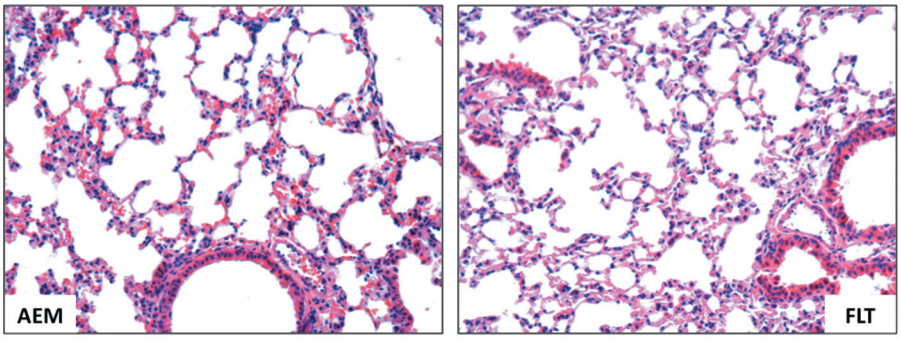

Figure 3. *Representative examples of lung sections from ground control mice housed in animal enclosure modules (AEM) and flight (FLT) mice stained with hematoxylin and eosin. No significant abnormalities were noted in lungs of FLT mice with respect to inflammation, necrosis, vascular changes, metaplasia, edema or cellular atypia. Magnification is ×20.*

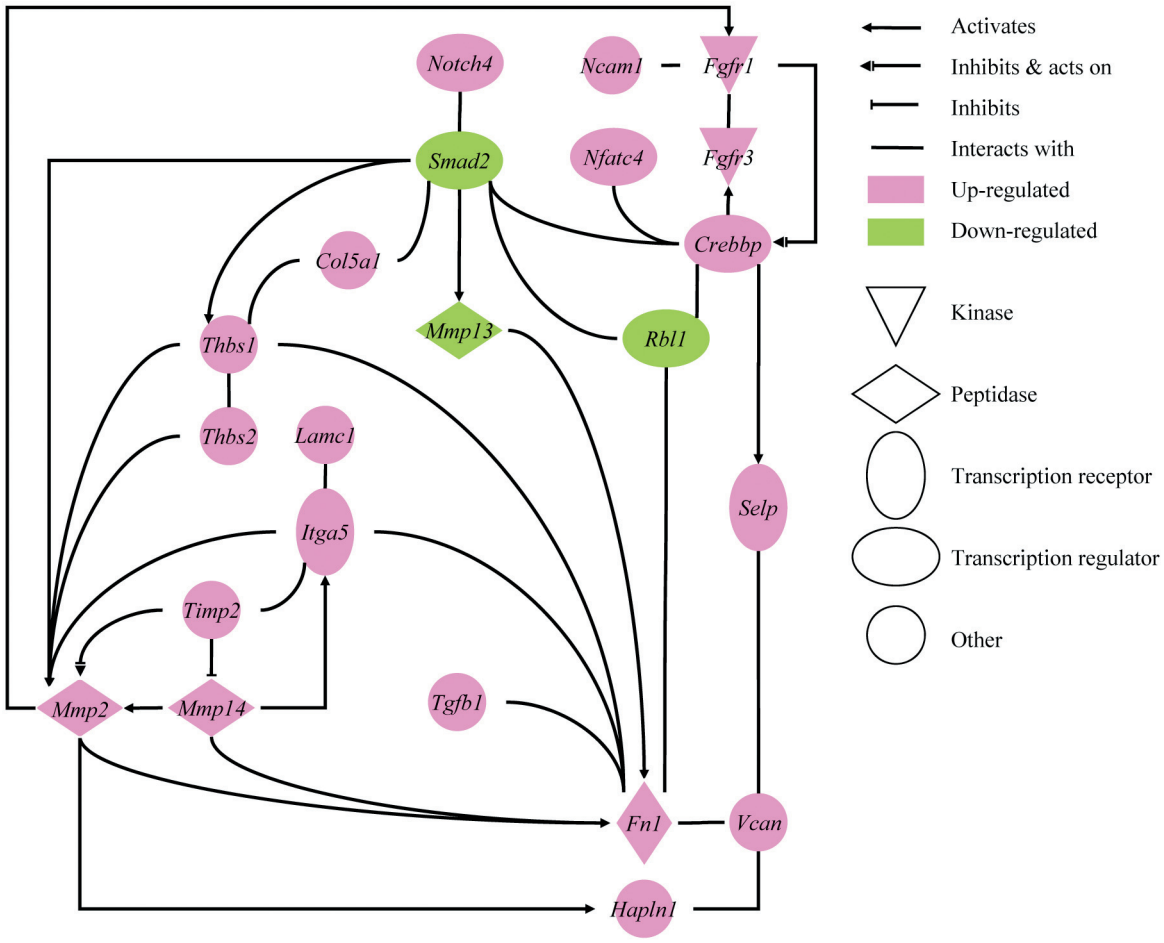

Figure 4. *Local network of functionally related genes. The figure was generated using Ingenuity Pathway Analysis software (www.ingenuity.com). Only genes in the lungs from the flight (FLT) mice that differed in expression from the control mice housed in animal enclosure modules (AEM) with a significance of p<0.1 and that directly interacted with the other measured genes were included. Col5a1: Collagen, type V, alpha 1; Crebbp: CREB binding protein; Fgfr1: fibroblast growth factor receptor 1; Fgfr3: fibroblast growth factor receptor 3; Fn1: fibronectin 1; Hapln1: hyaluronan and proteoglycan link protein 1; Itga5: integrin alpha 5; Lamc1: laminin, gamma 1; Mmp2: matrix metallopeptidase 2; Mmp13: matrix metallopeptidase 13; Mmp14: matrix metallopeptidase 14; Ncam1: neural cell adhesion molecule 1; Nfatc4: nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4; Notch4: notch gene homolog 4; Rbl1: retinoblastoma-like 1; Selp: selectin, platelet; Smad2: MAD homolog 2; Tgfbi: transforming growth factor, beta induced; Thbs1: thrombospondin 1; Thbs2: thrombospondin 2; Timp2: tissue inhibitor of metalloproteinase 2; Vcan: versican.*

similar, *i.e.* lungs were collected from adult female C57BL/6 mice within a few hours after return from a 13-day flight to the ISS and there were no obvious differences in food, water and housing. Although some overlap was noted, there were many more significantly up-/down-regulated genes in the previous study. This discrepancy is likely due to the fact that mice in the previous study were subjected to whole-body, *in vivo* nuclear magnetic resonance body composition measurements and muscle strength testing (55), and nuclear magnetic resonance body composition measurements by investigators prior to euthanasia and collection of samples for further testing at our institution. The extent to which these procedures may affect gene expression in the lungs is unknown.

The stem cell array that was used included genes that are well known to be important in signal transduction pathways for embryonic stem cell and induced pluripotent stem cell maintenance and differentiation. The induced pluripotent stem cells can be generated directly from adult cells and function as a repair system after injury. However, it is important to note that the genes that were affected in our study are not entirely specific for stem cells. Analysis showed that all six genes that were significantly modulated in FLT lungs were up-regulated. The most affected was *Nfatc4* (2.45-fold *vs.* the AEM group). The protein encoded by this gene is nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4 (NFATC4). NFATC4 is a member of the NFAT family of transcription factors that exerts pleiotropic functions in both adaptive and innate immune cell populations (56, 57). In addition, NFATs are well-known to facilitate maturation of precursor cells. It has been demonstrated that calcineurin-dependent NFATs are important in lung maturation and in regulation of genes involved in surfactant homeostasis (58).

Notch4 is the other stem cell signaling gene that was up-regulated in FLT mouse lungs by almost 2-fold compared to the AEM group. This gene encodes neurogenic locus notch homolog 4 (NOTCH4), a Notch receptor on endothelial cells that functions in the formation and morphogenesis of the vasculature (59). Notch signaling is also an important feature in development of smooth muscle cells (60). It has been reported that components of Notch pathway signaling are down-regulated shortly after injury (61), but up-regulated during pulmonary hypertension (62).

A brief description of the proteins encoded by the other genes in the stem cell signaling array that were significantly modified (all up-regulated by <2-fold) in the FLT group is as follows: fibroblast growth factor receptor 1 (*Fgfr1*), also known as CD331, serves as a receptor tyrosine kinase that binds to members of the FGF family, thereby triggering signals related to mitogenesis and cell differentiation; frizzled homolog 6 (*Fzd6*) is a transmembrane domain protein that serves as a receptor for certain signaling proteins; nicastrin (*Ncstn*) is a component of the gamma secretase protein complex; and vang-like 2 (*Vangl2*) is a membrane protein important in establishing cell polarity. This information can be found at the NCBI website (http://www.ncbi.nlm.nih.gov/gene).

Because of the relatively limited number of genes analyzed in the study, a full pathway analysis of gene expression was not warranted. However, many of the assessed genes interact directly, as indicated in Figure 4, *i.e.* there was a direct connection between 22 out of 29 genes modified in the FLT mouse lungs (see Tables I and II). Generated through Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, Redwood City, CA, USA; www.ingenuity.com), this Figure shows a network of mostly up-regulated gene expression related to the ECM and cell adhesion. Direct interactions between these genes and those involved with stem cell signaling can influence overall lung status under spaceflight conditions.

Considering the up-regulation of genes involved in remodeling, we opted to determine the level of SCA-1 protein that is expressed by stem cells. The results showed a significantly enhanced production of SCA-1 in lungs from the FLT group. Especially strong SCA-1 staining intensity was noted in the bronchial epithelium after flight. The cause of the seeming discrepancy between relatively little effect on stem cell signaling genes and high presence of SCA-1 remains to be determined, but may be related to the low abundance of stem cells in the lungs. The lungs do, indeed, contain undifferentiated stem/progenitor cells with ability to regenerate specific cell types after damage (63). In the normal adult lung, these cells are relatively quiescent and have diverse characteristics that are dependent on the microenvironment in which they reside. Studies of lung injury have reported that a variant subset of Clara cells express SCA-1 (64). Other studies have found that SCA-1-positive cells can have either epithelial or mesenchymal commitment (65). To our knowledge, the current study is the first to demonstrate that spaceflight condition significantly enhances SCA-1 expression in the lungs.

To further support the premise that spaceflight condition resulted in lung injury, the TUNEL assay was performed. The results showed that DNA fragmentation, typical of apoptosis, was more than two-times greater in lungs from FLT mice compared to those from AEM ground controls. This is consistent with the up-regulation of *Thbs1*, a pro-apoptotic gene, in FLT lungs. Previous studies in mice subjected to repeated radiation exposure and hyperoxia that mimics pre-breathe protocols in preparation for extravehicular activity in space found changes in lung tissue that included DNA damage based on the TUNEL assay (31). An *in vitro* study with pulmonary microvascular endothelial cells reported increased apoptosis, *i.e.* high caspase-3 mRNA expression, after microgravity simulation with a rotating clinostat (32). Increased levels of DNA

fragmentation have also been noted in proton-irradiated rat lung epithelial cells; the doses were within a range that could occur during space travel (29). Spaceflight-related apoptosis has been reported in other body compartments. In a study of ocular tissue from the same mice that we received the lungs from, apoptosis was noted in the retina (41). A study of astrocytes under simulated microgravity conditions (rotation in clinostat) found evidence of apoptosis (66).

The changes in expression of genes associated with ECM/adhesion and stem cell signaling, as well as increased SCA-1 expression and apoptosis, in lungs from the FLT mice indicate that spaceflight condition had a significant impact. It must be noted, however, that only 18 out of the 168 analyzed genes (10.7%) were significantly modulated in the FLT group ($p<0.05$); expression of 5% of these could have been different based on chance alone. However, IPA analysis indicated that many of these genes were clustered together (Figure 4), interacting among a functional related pathway, suggesting that this is indeed a real response. The presented data are unique and support further investigation of lung response to stressors encountered during space travel. Although the observed changes may not pose an immediate threat, much more research is needed. Future studies should include both on-flight and long-term monitoring after flight for any adverse health consequences. A better understanding over stress-induced response in the lungs could also lead to improvements in treatment strategies for pulmonary pathologies diagnosed in the general population.

Acknowledgements

We would like to thank Amgen, Inc. and their support team for the opportunity to participate in this study and BioServe Space Technologies at the University of Colorado at Boulder for organizing and overseeing this project. The valuable contributions of all team members of the Ferguson and Bateman laboratories who participated in the tissue processing at KSC, Paula Dumars and Vera Vizir of the BSP and Ames Research Center, and the KSC SLSL support staff are very much appreciated. The technical assistance of Tamako A. Jones and Erben J. M. Bayeta, as well as the support of Dr. James M. Slater at Loma Linda University Medical Center for space-related research, were essential for performance of this study and is greatly appreciated.

References

1 Paiva M: Contribution of weightlessness in respiratory physiology. Bull Mem Acad R Med Belg *161*: 479-485; discussion 485-486, 2006.

2 Prisk GK: The lung in space. Clin Chest Med *26*: 415-438, 2005.

3 Prisk GK: Microgravity and the respiratory system. Eur Respir J *43*: 1459-1471, 2014.

4 Prisk GK, Fine JM, Cooper TK and West JB: Lung function is unchanged in the 1 G environment following 6-months exposure to microgravity. Eur J Appl Physiol *103*: 617-623, 2008.

5 Gridley DS, Luo-Owen X, Rizvi A, Makinde A, Pecaut MJ, Mao XW and Slater J.M: Low-dose photon and simulated solar particle event proton effects on Foxp3+ T-regulatory cells and other leukocytes. Technol. Cancer Res. Treat *9*: 637-649, 2010.

6 Gridley DS, Rizvi A, Luo-Owen X, Makinde AY, Coutrakon GB, Koss P, Slater JM and Pecaut MJ: Variable hematopoietic responses to acute photons, protons and simulated solar particle event protons. In Vivo *22*: 159-169, 2008.

7 Gridley DS, Rizvi A, Makinde AY, Luo-Owen X, Mao XW, Tian J, Slater JM and Pecaut MJ: Space-relevant radiation modifies cytokine profiles, signaling proteins and Foxp3+ T-cells. Int J Radiat Biol *89*: 26-35, 2013.

8 Pecaut MJ and Gridley DS: The impact of mouse strain on iron ion radio-immune response of leukocyte populations. Int J Radiat Biol *86*: 409-419, 2010.

9 Rizvi A, Pecaut MJ, Slater JM, Subramaniam S and Gridley DS: Low-dose γ-rays modify CD4+ T-cell signalling response to simulated solar particle event protons in a mouse model. Int J Radiat Biol *87*: 24-35, 2011.

10 Sanzari JK, Wan XS, Krigsfeld GS, Wroe AJ, Gridley DS and Kennedy AR: The effects of gamma and proton radiation exposure on hematopoietic cell counts in the ferret model. Gravit Space Res *1*: 79-94, 2013.

11 Sanzari JK, Wan XS, Wroe AJ, Rightnar S, Cengel KA, Diffenderfer ES, Krigsfeld GS, Gridley DS and Kennedy AR: Acute hematological effects of solar particle event proton radiation in the porcine model. Radiat Res *180*: 7-16, 2013.

12 Wilson JW, Ott CM, Honer zu Bentrup K, Ramamurthy R, Quick L, Porwollik S, Cheng P, McClelland M, Tsaprailis G, Radabaugh T, Hunt A, Fernandez D, Richter E, Shah M, Kilcoyne M, Joshi L, Nelman-Gonzalez M, Hing S, Parra M, Dumars P, Norwood K, Bober R, Davich J, Ruggles A, Goulart C, Rupert M, Stodieck L, Stafford P, Catella L, Schurr MJ, Buchanan K, Morici L, McCracken J, Allen P, Baker-Coleman C, Hammond T, Vogel J, Nelson R, Pierson DL, Stefanyshyn-Piper HM and Nickerson CA: Space flight alters bacterial gene expression and virulence and reveals a role for global regulator Hfq. Proc Natl Acad Sci USA *104*: 16299-16304, 2007.

13 Schiwon K, Arends K, Rogowski KM, Furch S, Prescha K, Sakinc T, Van Houdt R, Werner G and Grohmann E: Comparison of antibiotic resistance, biofilm formation and conjugative transfer of *Staphylococcus* and *Enterococcus* isolates from Internation Space Station and Antarctic Research Station Concordia. Microb Ecol *65*: 638-651, 2013.

14 Li M, Holmes V, Zhou Y, Ni H, Sanzari JK, Kennedy AR and Weissman D: Hindlimb suspension and SPE-like radiation impairs clearance of bacterial infections. PLoS One *9*: e85665, 2014.

15 Baqai FP, Gridley DS, Slater JM, Luo-Owen X, Stodieck LS, Ferguson V, Chapes SK and Pecaut MJ: Effects of spaceflight on innate immune function and antioxidant gene expression. J Appl Physiol *106*: 1935-1942, 2009.

16 Gridley DS, Slater JM, Luo-Owen X, Rizvi A, Chapes SK, Stodieck LS, Ferguson VL and Pecaut MJ: Spaceflight effects on T-lymphocyte distribution, function and gene expression. J Appl Physiol *106*: 194-202, 2009.

17 Gridley DS, Mao XW, Stodieck LS, Ferguson VL, Bateman TA, Moldovan M, Cunningham CE, Jones TA, Slater JM and Pecaut MJ: Changes in thymus and spleen after return from the STS-135 mission in space. PLoS One *8*: e75097, 2013.

18 Gridley DS, Nelson GA, Peters LL, Kostenuik PJ, Bateman TA, Morony S, Stodieck LS, Lacey DL, Simske S and Pecaut MJ: Genetic models in applied physiology: selected contribution: Effects of spaceflight on immunity in the C57BL/6 mouse, Part II: Activation, cytokines, erythrocytes, and platelets. J Appl Physiol *94*: 2095-2103, 2003.

19 Lebsack TW, Fa V, Woods CC, Gruener R, Manziello AM, Pecaut MJ, Gridley DS, Stodieck LS, Ferguson VL and DeLuca D: Microarray analysis of spaceflown murine thymus tissue reveals changes in gene expression regulating stress and glucocorticoid receptors. J Cell Biochem *110*: 372-381, 2010.

20 Ortega MT, Pecaut MJ, Gridley DS, Stodieck LS, Ferguson VL and Chapes SK: Shifts in bone marrow cell phenotypes caused by spaceflight. J Appl Physiol *106*: 548-555, 2009.

21 Pecaut MJ, Nelson GA, Peters LL, Kostenuik PJ, Bateman TA, Morony S, Stodieck LS, Lacey DL, Simske SJ and Gridley DS: Genetic models in applied physiology: selected contribution: Effects of spaceflight on immunity in the C57BL/6 mouse, Part I: Immune population distributions. J Appl Physiol *94*: 2085-2094, 2003.

22 Crucian BE, Stowe RP, Pierson DL and Sams CF: Immune system dysregulation following short- *vs*. long-duration spaceflight. Aviat Space Environ Med 79: 835-843, 2008.

23 Crucian B, Stowe R, Quiriarte H, Pierson D and Sams C. Monocyte phenotype and cytokine production profiles are dysregulated by short-duration spaceflight. Aviat Space Environ Med *82*: 857-862, 2011.

24 Crucian B, Stowe R, Mehta S, Uchakin P, Quiriarte H, Pierson D and Sams C: Immune system dysregualtion occurs during short duration spaceflight on board the space shuttle. J Clin Immunol *33*: 456-465, 2013.

25 Mehta SK, Cohrs RJ, Forghani B, Zerbe G, Gilden DH and Pierson DL: Stress-induced subclinical reactivation of varicella zoster virus in astronauts. J Med Virol 72: 174-179, 2004.

26 Mehta SK, Crucian BE, Stowe RP, Simpson RJ, Ott CM, Sams CF and Pierson DL: Reactivation of latent viruses is associated with increased plasma cytokines in astronauts. Cytokine 61: 205-209, 2013.

27 Pierson DL, Stowe RP, Phillips TM, Lugg DJ and Mehta SK. Epstein-Barr virus shedding by astronauts during space flight. Brain Behav Immun *19*: 235-242, 2005.

28 Lopez-Giraldo A, Sialer S, Esperatti M and Torres A: Viral-reactivated pneumonia during mechanical ventilation: Is there need for antiviral treatment? Front Pharmacol *2*: 66, 2011.

29 Baluchamy S, Ravichandran P, Periyakaruppan A, Ramesh V, Hall JC, Zhang Y, Jejelowo O, Gridley DS, Wu H and Ramesh GT: Induction of cell death through alteration of oxidants and antioxidants in lung epithelial cells exposed to high energy protons. J Biol Chem *285*: 24769-24774, 2010.

30 Cristofidou-Solomidou M, Pietrofesa RA, Arguiri E, Schweitzer KS, Berdyshev EV, McCarthy M, Corbitt A, Alwood JS, Yu Y, Globus RK, Solomides CC, Ullrich RL and Petrache I: Space radiation associated lung injury in a murine model. Am J Physiol Lung Cell Mol Physiol *308*: L416-L428, 2015.

31 Pietrofesa RA, Turowski JB, Arguiri E, Milovanova TN, Solomides CC, Thom SR and Christofidou-Solomidou M: Oxidative lung damage resulting from repeated exposure to radiation and hyperoxia associated with space exploration. J Pulm Respir Med 3. pii: 1000158, 2013.

32 Kang CY, Li T, Zou, Yuan M, Li TZ, Guo YH, Wang Y and Liu CT: Salidroside inhibits clinorotation-induced apoptosis in pulmonary microvascular endothelial cells. Nan Fang Yi Ke Da Xue Xue Bao *31*: 649-652, 2011.

33 Crosby LM and Waters CM: Epithelial repair mechanisms in the lung. Am J Physiol Lung Cell Mol Physiol *298*: L715-L731, 2010.

34 Phan SH: Genesis of the myofibroblast in lung injury and fibrosis. Proc Am Thorac Soc *9*: 148-152, 2012.

35 Tian J, Pecaut MJ, Slater JM and Gridley DS: Spaceflight modulates expression of extracellular matrix, adhesion and profibrotic molecules in mouse lung. J Appl Physiol *108*: 162-171, 2010.

36 Tian J, Pecaut MJ, Coutrakon GB, Slater JM and Gridley DS: Response of extracellular matrix regulators in mouse lung after exposure to photons, protons and simulated solar particle event protons. Radiat Res *172*: 30-41, 2009.

37 Tian J, Tian S and Gridley DS: Comparison of acute proton, photon and low-dose priming effects on genes associated with extracellular matrix and adhesion molecules in the lungs. Fibrogenesis Tissue Repair *6*: 4-14, 2013.

38 Tian J, Zhao WL, Tian S, Slater JM, Deng Z and Gridley DS: Expression of genes involved in mouse lung cell differentiation/regulation after acute exposure to photons and protons with and without low dose pre-irradiation. Radiat Res *176*: 553-564, 2011.

39 Sun GS, Tou JC, Liittschwager K, Herrera AM, Hill EL, Girten B, Reiss-Bubenheim D and Vasques M: Evaluation of the nutrient-upgraded rodent food bar for rodent spaceflight experiments. Nutrition *26*: 1163-1169, 2010.

40 Gridley DS, Coutrakon GB, Rizvi A, Bayeta EJM, Luo-Owen X, Makinde AY, Baqai F, Koss P, Slater JM and Pecaut MJ: Low-dose photons modify liver response to simulated solar particle event protons. Radiat Res *169*: 280-287, 2008.

41 Mao XW, Pecaut MJ, Stodieck LS, Ferguson VL, Bateman TA, Bouxsein M, Jones TA, Moldovan M, Cunningham CE, Chieu J and Gridley DS: Space flight environment induces mitochondrial oxidative damage in ocular tissue. Radiat Res *180*: 340-350, 2013.

42 Lane TF and Sage EH: The biology of SPARC, a protein that modulates cell-matrix interactions. FASEB J *8*: 163-173, 1994.

43 Yan Q and Sage EH: SPARC, a matricellular glycoprotein with important biological functions. J Histochem Cytochem *47*: 1495-1506, 1999.

44 Bradshaw AD: Diverse biological functions of the SPARC family of proteins. Int J Biochem Cell Biol *44*: 480-488, 2012.

45 Chavarria A and Cárdenas G: Neuronal influence behind the central nervous system regulation of the immune cells. Front Integr Neurosci *7*: 64, 2013.

46 Ulm C, Saffarzadeh M, Mahavadi P, Muller S, Prem G, Saboor F, Simon P, Middendorff R, Geyer H, Henneke I, Bayer N, Rinné S, Lütteke T, Böttcher-Friebertshäuser E, Gerardy-Schahn R, Schwarzer D, Mühlenhoff M, Preissner KT, Günther A, Geyer R and Galuska SP: Soluble polysialylated NCAM: a novel player of the innate immune system in the lung. Cell Mol Life Sci *70*: 3695-3708, 2013.

47 Adams JC and Lawler J: The thrombospondins. Int J Biochem Cell Biol *36*: 961-968, 2004.

48 Esemuede N, Lee T, Pierre-Paul D, Sumpio BE and Gahtan V: The role of thrombospondin-1 in human disease. J Surg Res *122*: 135-142, 2004.

49 Halper J and Kjaer M: Basic components of connective tissues and extracellular matrix: elastin, fibrillin, fibulins, fibrinogen, fibronectin, laminin, tenascins and thrombospondins. Adv Exp Med Biol *802*: 31-47, 2014.
50 Kyriakidaes TR and Maclauchlan S: The role of thrombospondins in wound healing, ischemia, and the foreign body reaction. J Cell Commun Signal *3*: 215-225, 2009.
51 Stenina-Adognravi O: Invoking the power of thrombospondins: Regulation of thrombospondins expression. Matrix Biol *37*: 69-82, 2014.
52 Mirochnik Y, Kwiatek A and Volpert OV: Thrombospondin and apoptosis: molecular mechanisms and use for design of complementation treatments. Curr Drug Targets *9*: 851-862, 2008.
53 Sen AI, Shiomi T, Okada Y and D'Armiento JM: Deficiency of matrix metalloproteinase-13 increases inflammation after acute lung injury. Exp Lung Res *36*: 615-624, 2010.
54 Wohlauer M, Moore EE, Silliman CC, Fragoso M, Gamboni F, Harr J, Accurso F, Wright F, Haenel J, Fullerton D and Banerjee A: Nebulized hypertonic saline attenuates acute lung injury following trauma and hemorrhagic shock *via* inhibition of matrix metalloproteinase-13. Crit Care Med *40*: 2647-2653, 2012.
55 Stodieck LS, Greybeck BJ, Cannon C, Hanson AM, Young MH, Simske SJ and Ferguson VL: *In vivo* measurement of hindlimb neuromuscular function in mice. Muscle Nerve *45*: 536-543, 2012.
56 Vaeth M, Schliesser U, Muller G, Reissig S, Satoh K, Tuettenberg A, Jonuleit H, Waisman A, Müller MR, Serfling E, Sawitzki BS and Berberich-Siebelt F: Dependence on nuclear factor of activated T-cells (NFAT) levels discriminates conventional T-cells from Foxp3+ regulatory T-cells. Proc Natl Acad Sci USA *109*: 16258-16263, 2012.
57 Vandewalle A, Tourneur E, Bens M, Chassin C and Werts C: Calcineurin/NFAT signaling and innate host defence: a role for NOD1-mediated phagocytic functions. Cell Commun Signal *12*: 8, 2014.
58 Davé V, Childs T, Xu Y, Ikegami M, Besnard V, Maeda Y, Wert SE, Neilson JR, Crabtree GR and Whitsett JA: Calcineurin/Nfat signaling is required for perinatal lung maturation and function. J Clin Invest *116*: 2597-2609, 2006.
59 Wu J, Iwata F, Grass JA, Osborne CS, Elnitski L, Fraser P, Ohneda O, Yamamoto M and Bresnick EH: Molecular determinants of NOTCH4 transcription in vascular endothelium. Mol Cell Biol *25*: 1458-1474, 2005.
60 Fouillade C, Monet-Leprêtre M, Baron-Menguy C and Joutel A: Notch signaling in smooth muscle cells during development and disease. Cardiovasc Res *95*: 138-146, 2012.
61 Kume T: Novel insights into the differential functions of Notch ligands in vascular formation. J Angiogenes Res *1*: 8, 2009.
62 Qiao L, Xie L, Shi K, Zhou T, Hua Y and Liu H: Notch signaling change in pulmonary vascular remodeling in rats with pulmonary hypertension and its implication for therapeutic intervention. PLoS One *7*: e51514, 2012.
63 Liu X, Driskell RR and Engelhardt JF: Stem cells in the lung. Methods Enzymol *419*: 285-321, 2006.
64 Reynolds SD, Giangreco A, Power JH and Stripp BR: Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration. Am J Pathol *156*: 269-278, 2000.
65 Hittinger M, Czyz ZT, Huesemann Y, Maneck M, Botteron C, Kaeufl S, Klein CA and Polzer B: Molecular profiling of single SCA-1^{+}/CD34^{+}- cells–the putative murine lung stem cells. PLoS One *8*: e83917, 2013.
66 Uva BM, Masini MA, Sturla M, Tagliafierro G and Strollo F: Microgravity-induced programmed cell death in astrocytes. J Gravit Physiol *9*: P275-P276, 2002.

Received April 3, 2015
Revised April 30, 2015
Accepted May 4, 2015