Molecular Effects of Spaceflight in the Mouse Eye after Space Shuttle Mission STS-135

Corey A. Theriot¹ and Susana B. Zanello²

¹Department of Preventive Medicine and Community Health, University of Texas Medical Branch, Galveston, TX; ²Division of Space Life Sciences, Universities Space Research Association, Houston, TX

ABSTRACT

Exposure to long-duration microgravity leads to ocular changes in astronauts, manifested by a variety of signs and symptoms during spaceflight that in some cases persist after return to Earth. These morphological and functional changes are only partly understood and are of occupational health relevance. To investigate further into the molecular basis of the changes occurring in ocular tissue upon exposure to spaceflight, eyes were collected from male C57BL/6 mice flown on STS-135 (FLT) on landing day or from their ground control counterparts maintained at similar conditions within the Animal Enclosure Module (AEM). One eye was fixed for histological sectioning while the contralateral eye was dissected to isolate the retina for gene expression profiling. 8-hydroxy-deoxyguanosine (8OHdG) staining showed a statistically significant increase in the inner nuclear layer of FLT samples

compared to AEM. Gene expression analysis in isolated retina identified 139 differentially expressed genes in FLT compared to AEM control samples. The genes affected were mainly involved in pathways and processes of endoplasmic reticulum (ER) stress, inflammation, neuronal and glial cell loss, axonal degeneration, and herpes virus activation. These results suggest a concerted change in gene expression in the retina of mice flown in space, possibly leading to retinal damage, degeneration, and remodeling.

INTRODUCTION

Space exploration involves challenges for an extended human presence. The space environment exposes the human body to a unique combination of physical and psychological stressors. Most noteworthy are the effects of microgravity and high charge/high energy (HZE) particle radiation with a potential for hypoxia (in off-nominal situations), occasional hyperoxia, dietary restrictions, and possible nutritional deficiencies as significant hazardous confounding factors (Sonnenfeld et al., 2003; Taibbi et al., 2013; Taylor et al., 2013; Sides et al., 2005; Mader et al., 1999; Planel et al., 1989; Reitz et al., 2009; Cucinotta et al., 2001; Cucinotta et al., 2008). Microgravity induces cephalad fluid shifts and physical unloading, leading to deleterious effects such as muscle atrophy, loss of bone mass, cardiovascular alterations, postflight orthostatic intolerance, and immunosuppression (Pietsch et al., 2011; Sundaresan and Pellis, 2009; Blaber et al., 2013). More recently, ocular changes

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Correspondence to: Susana Zanello
Universities Space Research Association –
Lyndon B. Johnson Space Center
Division of Space Life Sciences
2101 NASA Parkway
Mail Code SK
Houston, TX 77058
Telephone: 281-244-6779
Fax: 281-483-2888
E-mail: susana.b.zanello@nasa.gov

associated with spaceflight have been reported in astronauts exhibiting alterations in near visual acuity and other ocular-based signs and symptoms (Alexander et al., 2012). These medical data describe evidence of optic disc edema, choroidal folds and posterior globe flattening, hyperoptic shift in vision, optic nerve sheath distension, and in some cases, optic nerve kinking and empty sella (Kramer et al., 2012; Mader et al., 2011), possibly associated with intracranial pressure increase. Identifying causative factors and investigating microgravity's influence as a source of stress for the eye have acquired importance.

Previous basic research investigating the effects of spaceflight on eye structures and general ocular health is scarce and limited to basic histopathology on space-flown animal models (Marshall-Bowman et al., 2013). Neonatal rats flown for nine days onboard the Space Shuttle mission STS-72 displayed clear evidence of retinal degeneration (Tombran-Tink and Barnstable, 2006). In another study, adult rats flown onboard Russian Cosmos satellites for approximately 14 days exhibited cellular swelling in the retina and retinal disruption (Philpott et al., 1980).

Recently, our group investigated the effects of spaceflight on the eyes of mice flown onboard Space Shuttle mission STS-133 and, for the first time, reported molecular and cellular changes correlating to the histopathologic outcomes (Zanello et al., 2013). Flight samples from that study stained positive for β -amyloid and glial fibrillary acidic protein (GAP) in the optic nerve. Retina from mice flown on STS-133 also showed increased levels of 8-hydroxy-2'-deoxyguanosine (8OHdG), an oxidized form of guanosine that serves as a standard biomarker of oxidative DNA damage, and of caspase-3 immunoreactivity, suggesting an early step both in the extrinsic (death ligand) and intrinsic (mitochondria) apoptosis pathway. In addition, upregulated levels of oxidative and cellular stress response genes were detected. Herein, we report the findings of a subsequent animal experiment onboard STS-135 in which we expanded the molecular aspects of the impact of spaceflight on retinal biology by performing differential gene expression profiling between mice flown onboard STS-135 and their ground control counterparts.

MATERIALS AND METHODS

Animals

This work is the result of a tissue sharing effort that utilized specimens collected from a parent animal experiment onboard STS-135. Animal procedures were approved by the NASA Ames Research Center and Kennedy Space Center institutional animal care and use committees. Mice were flown onboard STS-135 that lifted off at 11:29 a.m. EDT on July 8, 2011 and landed at 5:57 a.m. on July 21, 2011. The mission duration was 12 days, 18 hours, and 28 minutes.

Male 9 to 11 week-old C57BL/6 mice were assigned to one of two experimental groups: Flight (FLT) and Animal Enclosure Module (AEM) ground controls. Both eyes from three animals for each group ($n=3$) were used in this study, one for gene expression analysis and the other for histology. The flight animals were housed in AEMs identical to the ground controls, and our analysis focuses on the comparison between these two conditions. The AEM is a self-contained habitat that provides ventilation, waste management, food, water, and controlled lighting (Naidu et al., 1995), and has previously been used in experiments studying rodent biology during spaceflight. The AEM was located in the middeck locker of the Space Shuttle, and the temperature was set at 3 to 8°C above the environmental middeck temperature. Lighting of 14 lux was set to a 12 hour day/12 hour night cycle for both FLT and AEM habitats.

After euthanasia at day one of landing, one eye of each mouse was enucleated and fixed for histologic examination. The contralateral eye was placed in RNA preservative (RNAlater®) until further dissection, which was performed by making an incision by scalpel to separate the posterior eye cup, removing the lens and vitreous and carefully scraping the retina. The retina was then placed again in RNAlater® and processed for gene expression profiling by microarray analysis as described below.

Materials

A 4% paraformaldehyde-based histologic fixative was obtained from Excalibur Pathology, Inc., Oklahoma City, OK. Goat polyclonal antibody to 8-hydroxy-2'-deoxyguanosine (8-

OHdG) (ab10802) and rabbit polyclonal antibody to activated caspase-3 (ab52181) were purchased from Abcam Inc., Cambridge, MA. Paraffin embedding and histological sectioning were contracted from Excalibur Pathology. RNA isolation reagents were purchased from Qiagen Inc., Valencia, CA and BioRad, Hercules, CA. Tissue samples were assigned a different number for immunohistochemistry evaluation and gene profiling in order to perform a masked analysis.

Histology and Immunohistochemistry

Fixed eyes were paraffin embedded, sectioned at 5 μ m thickness, and stained with standard hematoxylin-eosin (H&E) for histologic examination. Two immunohistologic stains were performed: 8OHdG to detect oxidative-related DNA damage and activated caspase-3 to study apoptosis. For 8OHdG and caspase-3 staining, sections were equilibrated in water after deparaffinization and treated sequentially in 3% hydrogen peroxide, 1% acetic acid, and 2.5% serum (Vector Labs, Burlingame, CA) before incubating with the diluted primary antibody for either two hours at room temperature or overnight at 4°C. After washing, the specimens were incubated with Vector ImmPress detection kit, corresponding to the primary antibody's host and counterstained with hematoxylin.

Caspase-3 Detection

A qualitative approach was used to compare apoptosis in the retina. Activated caspase-3 positive cells were identified for the retinal ganglion cell (RGC) layer in a retina section for each sample. The histologic sections were cut in the proximity of the optic nerve head for better comparison.

8OHdG Immunoreactivity Quantification

Estimation of the 8OHdG immunoreactivity was done using ImageJ software (masked for specific study groups) on 8OHdG stained slides. Briefly, digital color images of the retina were processed using NIH ImageJ ver.1.68 (NIH) and converted to an 8-bit inverted grayscale image for analysis. Regions of interest were selected from each retinal section, corresponding to the RGC layer, inner nuclear layer (INL), and outer nuclear layer (ONL), as well as nearby areas without immunoreactivity for background measurements.

Five sections were analyzed for each sample, for which the mean density per unit area (minus mean background density) was measured. A 2-tailed *t* test with an alpha of $p < 0.05$ was used to determine statistical significance.

Gene Expression Analysis

Mouse retina was microdissected and isolated from the rest of the eye and then placed again in RNAlater®. Total RNA was then isolated from the retina using the AllPrep DNA/RNA Micro kit (Qiagen, Valencia, CA) and analyzed for quality using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). All samples used reported a RNA Integrity Number (RIN) >6.0 and were confirmed to have 2 sharp bands/peaks representing the 28s and 18s RNA. Some variability in the RIN among samples was observed, presumably due to a handling delay in sample processing that derived from reasons out of our control, which caused the samples to remain in RNAlater® at 4°C for four months before dissection of the retina. Microarray gene expression analysis was performed at the University of Texas Medical Branch (UTMB) at Galveston Molecular Genomics Core using Affymetrix Mouse 430 2.0 GeneChip (Affymetrix, Inc., Santa Clara, CA) with Affymetrix Expression Console and Transcriptome Analysis Console for secondary analysis. An Ingenuity iReport (Ingenuity Systems, Redwood City, CA) was then generated for canonical pathway identification. Two-way (gene and sample) hierarchical cluster analysis was performed on log-transformed, median-centered, and normalized signals from the FLT and AEM ground control samples. Normalized expression values were obtained using the Robust Multichip Analysis (RMA) algorithm. Results were visualized with the Transcriptome Analysis Console software 1.0.0.234 (Affymetrix, Inc.) using a cut-off *P* value of 0.05 and a minimum fold-change of 1.5.

RESULTS

Caspase-3 Analysis of Retina Specimens

Caspase-3 immunoreactivity was moderate and localized mostly in some RGCs. Caspase-3 positive cells in the RGC layer showed intense cytoplasmic and neurite staining, particularly in

the FLT samples. Representative images are present in Figure 1 (Panels A and B) with arrows pointing to localized Caspase staining. Higher magnification views are shown for the AEM

(panel C) and FLT (panel D) in Figure 1. We observed signs of retinal disruption at the level of the photoreceptors and INL, with vacuolization and cell swelling.

Figure 1. Activated caspase-3 immunoreactivity in mouse retina. Representative images of histological sections stained for caspase-3 are presented for AEM ground controls (Panel A) and FLT samples (Panel B). Individual cells staining positive for caspase-3 and undergoing various morphological changes are denoted by arrows (→) in Panels A & B. Panels C and D are higher magnifications of A and B, respectively. Retina cell layers are labeled between Panels C and D as ONL, INL, and RGC.

8OHdG Analysis of Retina Specimens

To quantify oxidative DNA damage in the retina, densitometric quantification of 8OHdG immunohistochemistry was performed on eye sections ($n=3/\text{group}$), as presented in Figure 2. A statistically significant increase in 8OHdG was detected in the INL between the FLT and AEM ground control groups. 8OHdG intensity levels were not shown to be statistically different in the ONL and the RGC between FLT samples and AEM ground controls.

Microarray Analysis of Retina Specimens

Microarray analysis was performed using the Mouse Expression Set 430 microchip, which allows the independent multi-probe detection of over 39,000 transcripts on a single array. Using the analytical parameters specified in the Methods section, 139 differentially expressed genes were identified in FLT retina samples compared to AEM controls (at least 1.5-fold expression change, $P<0.05$), of which 107 gene probesets

were upregulated and 32 gene probesets were downregulated (Table 1). The 139 differentially expressed genes were analyzed with a 2-way hierarchical cluster analysis and presented in Figure 3. The samples analyzed formed two groups on the horizontal axis, clearly separating the three FLT samples (right three lanes) from the three AEM samples (left three lanes) (Figure 3, Panel B). Figure 3 also shows two examples of downregulated clusters that contain numerous genes involved in transcription regulation and histone maintenance (Panel C).

Relevant gene expression changes were organized and filtered biologically and functionally by pathway analysis tools based on the Ingenuity Knowledge Base, a repository of curated biological interactions and functional annotations. The pathways were generated through the use of Ingenuity® iReport (Ingenuity® Systems, <http://www.ingenuity.com>) and are summarized in Table 2.

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Figure 2. 8OHdG immunoreactivity in neuronal retina layers. Each individual neuronal cell layer was analyzed for 8OHdG specific immunostaining intensity. Results shown are mean intensities from analysis of at least 5 sections per sample (n=3) for each group. Statistical significance is noted in the graph.

Figure 3. Hierarchical cluster analysis of AEM control and FLT samples using the 139 differentially expressed probesets identified by gene expression profiling. Hierarchical cluster analysis of microarray results of AEM and FLT samples (Panel A). Expanded view of the dendrogram showing AEM and FLT sample clustering (Panel B). Expanded view of two representative downregulated gene clusters (Panel C).

Table 1. Summary of the 139 differentially expressed genes in FLT compared to AEM ground controls. Genes are listed according to fold change and its associated known molecular function.

Symbol: Name	Fold Change	Molecular Function
DSP: desmoplakin	4.01	other
LY6E: lymphocyte antigen 6 complex, locus E	2.95	other
LCN2: lipocalin 2	2.75	transporter
SLC44A2: solute carrier family 44, member 2	2.55	transporter
CIRBP: cold inducible RNA binding protein	2.48	translation regulator
POU4F1: POU class 4 homeobox 1	2.44	transcription regulator
SGMS2: sphingomyelin synthase 2	2.36	enzyme
Defb9: defensin beta 9	2.36	other
RIPK4: receptor-interacting serine-threonine kinase 4	2.32	kinase
RPRD2: regulation of nuclear pre-mRNA domain containing 2	2.15	other
PAQR8: progesterin and adipoQ receptor family member VIII	2.04	other
1700019G17Rik/Cml2: camello-like 2	1.99	other
CUL2: cullin 2	1.98	enzyme
ALCAM: activated leukocyte cell adhesion molecule	1.96	other
ZBTB16: zinc finger and BTB domain containing 16	1.95	transcription regulator
CLVS1: clavesin 1	1.93	other
ZC3H6: zinc finger CCCH-type containing 6	1.92	other
B4GALNT1: beta-1,4-N-acetyl-galactosaminyl transferase 1	1.92	enzyme
GNA13: guanine nucleotide binding protein (G protein), alpha 13	1.89	enzyme
CTSH: cathepsin H	1.87	peptidase
WFS1: Wolfram syndrome 1	1.84	enzyme
HNRPDL: heterogeneous nuclear ribonucleoprotein D-like	1.84	other
PARP12: poly (ADP-ribose) polymerase family, member 12	1.83	other
EHD4: EH-domain containing 4	1.82	enzyme
ACOT9: acyl-CoA thioesterase 9	1.81	enzyme
AGXT2L1: alanine-glyoxylate aminotransferase 2-like 1	1.80	enzyme
SH3YL1: SH3 domain containing, Ysc84-like 1	1.80	other
2210403K04Rik: RIKEN cDNA 2210403K04 gene	1.79	other
INPP1: inositol polyphosphate-1-phosphatase	1.79	phosphatase
SLC39A4: solute carrier family 39 (zinc transporter), member 4	1.79	transporter
ZSCAN21: zinc finger and SCAN domain containing 21	1.79	transcription regulator
CMAS: cytidine monophosphate N-acetylneuraminic acid synthetase	1.77	enzyme
KALRN: kalirin, RhoGEF kinase	1.76	kinase
RUFY2: RUN and FYVE domain containing 2	1.76	other
TRIM25: tripartite motif containing 25	1.76	transcription regulator
ANGPTL4: angiopoietin-like 4	1.74	other
CHST15: carbohydrate (N-acetyl)galactosamine 4-sulfate 6-O) sulfotransferase 15	1.73	enzyme
PEX13: peroxisomal biogenesis factor 13	1.72	transporter
MKRN1: makorin ring finger protein 1	1.72	other
A2M: alpha-2-macroglobulin	1.72	transporter

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Symbol: Name	Fold Change	Molecular Function
MRPL15: mitochondrial ribosomal protein L15	1.71	other
FABP4: fatty acid binding protein 4, adipocyte	1.71	transporter
AI451250: expressed sequence AI451250	1.69	other
KRT23: keratin 23 (histone deacetylase inducible)	1.69	other
FKBP5: FK506 binding protein 5	1.69	enzyme
FAM194A: family with sequence similarity 194, member A	1.66	other
MFSD6: major facilitator superfamily domain containing 6	1.66	other
Gm9776: predicted gene 9776	1.66	other
SDR42E1: short chain dehydrogenase/reductase family 42E, member 1	1.66	enzyme
A930041H05Rik: RIKEN cDNA A930041H05 gene	1.65	other
TXNRD3: thioredoxin reductase 3	1.65	enzyme
Pax6os1: Pax6 opposite strand transcript 1	1.65	other
SLC1A1: solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter), member 1	1.64	transporter
COL19A1 (includes EG:12823): collagen, type XIX, alpha 1	1.63	other
GPCPD1: glycerophosphocholine phosphodiesterase GDE1 homolog (S. cerevisiae)	1.62	other
LGI1: leucine-rich, glioma inactivated 1	1.62	other
DCXR: dicarbonyl/L-xylulose reductase	1.62	enzyme
SBSN: suprabasin	1.62	other
NIPSNAP1: nipsnap homolog 1 (C. elegans)	1.61	enzyme
SMC6: structural maintenance of chromosomes 6	1.61	other
REPS2: RALBP1 associated Eps domain containing 2	1.60	other
HIST2H2AA3/HIST2H2AA4: histone cluster 2, H2aa3	1.60	other
CRISPLD2: cysteine-rich secretory protein LCCL domain containing 2	1.59	other
SORL1: sortilin-related receptor	1.59	transporter
WWC1: WW and C2 domain containing 1	1.59	transcription regulator
DHCR24: 24-dehydrocholesterol reductase	1.59	enzyme
ACSL5: acyl-CoA synthetase long-chain family member 5	1.59	enzyme
SMOX: spermine oxidase	1.58	enzyme
CHCHD7: coiled-coil-helix-coiled-coil-helix domain containing 7	1.58	other
NT5C2: 5'-nucleotidase, cytosolic II	1.58	phosphatase
CPNE8: copine VIII	1.58	other
TCTA: T-cell leukemia translocation altered gene	1.58	other
PLEKHH1: pleckstrin homology domain containing, family H (with MyTH4 domain) member 1	1.57	other
Rbpms: RNA binding protein gene with multiple splicing	1.57	transcription regulator
TTL11: tubulin tyrosine ligase-like family, member 11	1.57	other
FEM1C: fem-1 homolog c (C. elegans)	1.57	transcription regulator
PLIN4: perilipin 4	1.57	other
CDA: cytidine deaminase	1.57	enzyme
IFI35: interferon-induced protein 35	1.56	other
UGT1A6: UDP glucuronosyltransferase 1 family, polypeptide A6	1.56	enzyme

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Symbol: Name	Fold Change	Molecular Function
D130037M23Rik: RIKEN cDNA D130037M23 gene	1.56	other
SLC7A1: solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	1.56	transporter
SNRPD3: small nuclear ribonucleoprotein D3 polypeptide 18kDa	1.55	other
TSC22D3: TSC22 domain family, member 3	1.55	other
KAZN: kazrin, periplakin interacting protein	1.55	other
SH3BP5: SH3-domain binding protein 5 (BTK-associated)	1.55	other
CHL1: cell adhesion molecule with homology to L1CAM	1.54	other
HNRNPD: heterogeneous nuclear ribonucleoprotein D	1.54	transcription regulator
PLIN2: perilipin 2	1.54	other
PTGIS: prostaglandin I2 (prostacyclin) synthase	1.53	enzyme
ARMCX5: armadillo repeat containing, X-linked 5	1.53	other
TNFAIP2: tumor necrosis factor, alpha-induced protein 2	1.53	other
BRD8: bromodomain containing 8	1.53	transcription regulator
BTBD11: BTB (POZ) domain containing 11	1.53	transcription regulator
PHYHIP: phytanoyl-CoA 2-hydroxylase interacting protein	1.53	other
RBM3: RNA binding motif (RNP1, RRM) protein 3	1.52	other
HAGHL: hydroxyacylglutathione hydrolase-like	1.51	enzyme
MARVELD2: MARVEL domain containing 2	1.51	other
WWP2: WW domain containing E3 ubiquitin protein ligase 2	1.51	enzyme
DIS3: DIS3 mitotic control homolog (S. cerevisiae)	1.51	enzyme
ZBTB22: zinc finger and BTB domain containing 22	1.51	other
CYB561: cytochrome b-561	1.51	enzyme
RNF208: ring finger protein 208	1.51	other
FAM122B: family with sequence similarity 122B	1.51	other
UPK3B: uroplakin 3B	1.51	other
C6orf115: chromosome 6 open reading frame 115	1.50	other
MARCH7: membrane-associated ring finger (C3HC4) 7	1.50	other
HSPH1: heat shock 105kDa/110kDa protein 1	-1.51	other
LOC100505793/SRSF10: serine/arginine-rich splicing factor 10	-1.51	other
LIMA1: LIM domain and actin binding 1	-1.53	other
KDM2A: lysine (K)-specific demethylase 2A	-1.53	other
CREBZF: CREB/ATF bZIP transcription factor	-1.54	transcription regulator
CRELD2: cysteine-rich with EGF-like domains 2	-1.54	other
BCAS2: breast carcinoma amplified sequence 2	-1.54	other
DYNLL1: dynein, light chain, LC8-type 1	-1.55	other
BB116930: expressed sequence BB116930	-1.55	other
SRSF6: serine/arginine-rich splicing factor 6	-1.56	other
CHORDC1: cysteine and histidine-rich domain (CHORD) containing 1	-1.57	other
PUM2: pumilio homolog 2 (Drosophila)	-1.59	other
HIST3H2A: histone cluster 3, H2a	-1.61	other
TRPM1: transient receptor potential cation channel, subfamily M, member 1	-1.62	ion channel

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Symbol: Name	Fold Change	Molecular Function
TRIM47: tripartite motif containing 47	-1.62	other
4931428L18Rik: RIKEN cDNA 4931428L18 gene	-1.63	other
XBP1 (includes EG:140614): X-box binding protein 1	-1.65	transcription regulator
HIST1H3A: histone cluster 1, H3a	-1.66	other
HSPA5: heat shock 70kDa protein 5	-1.69	enzyme
6530402F18Rik: RIKEN cDNA 6530402F18 gene	-1.70	other
ARSI: arylsulfatase family, member I	-1.70	enzyme
NECAB1: N-terminal EF-hand calcium binding protein 1	-1.73	other
CPSF6: cleavage and polyadenylation specific factor 6, 68kDa	-1.73	other
BTG1: B-cell translocation gene 1, anti-proliferative	-1.74	transcription regulator
HIST2H3C (includes others): histone cluster 2, H3c	-1.79	other
CA8: carbonic anhydrase VIII	-1.79	enzyme
C430048L16Rik/Rbm12b: RIKEN cDNA C430048L16 gene	-1.89	other
TRA2A: transformer 2 alpha homolog (Drosophila)	-2.10	other
MALAT1: metastasis associated lung adenocarcinoma transcript 1	-2.15	other
S1PR3: sphingosine-1-phosphate receptor 3	-2.26	G-protein coupled receptor
SFPQ: splicing factor proline/glutamine-rich	-2.33	other
SLC5A3: solute carrier family 5 (sodium/myo-inositol cotransporter), member 3	-2.64	transporter

Table 2. Potential molecular pathways, processes, and diseases states triggered by spaceflight in the mouse retina.

PATHWAYS	PROCESSES	DISEASES
Endoplasmic Reticulum (ER) stress	RNA processing (mRNA splicing)	Molecular mechanisms of cancer
Pyrimidine metabolism	Cell death of sensory neurons and RGC	Neurodegeneration of nerves and nervous tissue
Cytokine production and signaling (IL-1, IL-6, IL17)	Apoptosis of microglia and neuronal cells	Degeneration of the optic nerve
Sphingosine-1-P signaling	Stabilization and assembly of desmosomes	Reactivation of herpes virus
Axonal guidance and actin cytoskeleton	Axon branching	

DISCUSSION

Our work explores the possible effects of the spaceflight environment on retinal biology, in an effort to shed light on the confounding factors and processes that may contribute to vision risks in space. Corollary to a previous report on the effects of spaceflight on the retina of BALB/cJ mice

(Zanello et al., 2013), we now report results of a similar experiment carried out on C57BL mice onboard STS-135. The main findings in this work demonstrate evidence of increased oxidative stress-DNA damage, histological changes, as well as potential effects of spaceflight on retinal biology based on microarray gene expression profiling. Similar results have been reported by an

independent group (Mao et al., 2013); however, the gene expression profiling performed in the cited work originated from non-dissected eyes that included all eye tissues and structures. The eye is a complex organ with a heterogeneous tissue composition due to its various structures: sclera, cornea, retina, lens, choroid, retina microvasculature, aqueous humor production, and drainage system. Therefore, gene expression derived from the entire eye globe does not precisely reflect the tissue of origin of the genetic responses. Our work presents, for the first time, comparative whole genome expression profiling of retinas dissected from mice exposed to spaceflight and their ground controls. Even in this case, we must bear in mind that the retina is composed of various cell types from both neural and vascular cellular populations.

A number of activated caspase-3 positive cells were observed, in particular, in the RGC layer of FLT samples, but due to the limitation in the number of sections, our examination was limited to a qualitative comparison. Overall, caspase-3 immunoreactivity in the FLT samples was observed to be localized at moderate levels in the RGC, with light levels seen throughout the photoreceptor inner segments. Of note, cytological alterations in the photoreceptor layer from FLT mice in this study were in line with previous reports in the literature of spaceflight induced damage to the ganglion cell layer and disruption of the plexiform layers and photoreceptors of rat neonates (Tombran-Tink and Barnstable 2006). Quantification of 8OHdG immunoreactivity showed statistically significant ($P<0.05$) increase in DNA damage in the INL of FLT mice compared to AEM controls. Similar findings were observed in the previous experiment in BALB/cJ mice; however, BALB/cJ mice showed larger damage in the photoreceptor layer upon exposure to spaceflight. The propensity of this strain to develop retinal degeneration due to light (Song et al., 2012; Hadziahmetovic et al., 2012; Whelan and McGinnis 1988; Chang et al., 2002) (and probably other kinds of electromagnetic radiation) may provide an explanation for this difference.

Pathway analysis derived from differential gene expression of microarray data revealed processes related to neuronal and glial cell loss, mechanisms of apoptosis, and ER stress. Evidence

supporting these processes included, among others, the downregulation (-1.65 fold FLT vs. AEM) of XBP1, a potent transcription factor involved in the unfolded protein response (UPR). Processing of XBP1 mRNA is an obligate step in the UPR pathway and the IRE1/XBP-1 pathway is required for efficient protein folding, maturation, and degradation in the ER (Calfon et al., 2002; Lee et al., 2002; Sriburi et al., 2004). In addition, Hspa5, a member of the Hsp70 family that also resides in the ER and participates in protein folding, was also downregulated -1.69 fold, in agreement with a possible impairment of the ER stress response, which other studies have linked to increased susceptibility to Alzheimer's disease (Hebert and Molinari 2007; Guzel et al., 2011; Hsu et al., 2008). A result of cellular insults that cause prolonged ER stress include apoptosis through caspase-7-mediated caspase-12 activation and lead to neuronal and glial cell loss (Nakagawa et al., 2000; Szegezdi et al., 2003).

Interestingly, XBP1 is also implicated in processes related to the reactivation of herpes virus (Lawson et al., 2008; Dalton-Griffin et al., 2009; Wilson et al., 2007). It is well known that virus reactivation is a common phenomenon associated with spaceflight, but the mechanisms leading to it are not understood (Brinley et al., 2013; Mehta et al., 2000; Mehta et al., 2004; Stowe et al., 2001). We must bear in mind that the retina is mostly composed of neural cells and that the herpes virus is neurotropic. A putative implication of XBP1 as a transcription factor crucial in the protection against viral reactivation in space is not implied, but could be further investigated to shed some light into possible targets for mitigation against the risk of viral reactivation in space.

Other differentially expressed genes between FLT and AEM retinas suggested pathways of neuronal cell loss and degeneration. SLC1A1, a member of the high-affinity glutamate transporters that play an essential role in transporting glutamate across plasma membranes, was upregulated +1.64 in FLT compared to AEM retinas. In the brain, these transporters are crucial in terminating the postsynaptic action of the neurotransmitter glutamate and in maintaining extracellular glutamate concentrations below neurotoxic levels (Sheldon and Robinson 2007; Deng et al., 2007; Arnold et al., 2006). It has also

been shown to be involved in RGC death (Martins et al., 2006; Sheldon and Robinson, 2007) and its upregulation in FLT samples may suggest that glutamate level increase may be associated with space environmental factors. Concurrently, the transporter lipocalin 2 (LCN2) showed a marked upregulation of +2.75 fold in the FLT group. LCN2 has been shown in the literature to be related to inflammation in the retina following ischemia-reperfusion injury (Abcouwer et al., 2013) and may serve a similar role in modulating an inflammatory response to spaceflight.

Beta-amyloid deposits have previously been suggested as related to RGC and optic nerve degeneration (Zanello et al., 2013). Alpha-2-macroglobulin (A2M) inhibits many proteases, including trypsin, thrombin, and collagenase and is implicated in Alzheimer disease due to its ability to mediate the clearance and degradation of A-beta, the major component of beta-amyloid deposits (Liao et al., 1998; Blacker et al., 1998). Our results show that A2M was induced +1.72 fold in FLT compared to ground AEM, possibly suggesting the triggering of clearance mechanisms to reduce an increase in any deposits.

The RNA used in this study originated from dissected retina containing a mixed cell type population. While the major representation is of neural cells of various types, it is expected that some relevant genes were represented due to their expression in other retinal cell types. Endothelial cells from the retinal microvasculature are one example. S1PR3 encodes a member of the EDG family of receptors, which are G-protein coupled receptors (Crousillac et al., 2009). This protein has been identified as a functional receptor for sphingosine 1-phosphate and likely contributes to the regulation of angiogenesis and vascular endothelial cell function (Sanchez et al., 2007; Daum et al., 2009). We observed a significant downregulation of -2.26 fold in FLT retinas versus AEM, a change that may suggest vascular remodeling could be taking place in conjunction with some degree of retinal degeneration and neural remodeling and should be investigated in future studies. Changes in the expression of genes related to axonal guidance were also observed in FLT compared to AEM such as GNA13 and KALRN (Lee and Luo, 1999; Mandela et al., 2012), in agreement with the concept of vascular and neural guidance as coordinated processes

(Suchting et al., 2006; Eichmann et al., 2005; Park et al., 2003). A significant upregulation of two key genes implicated in reorganization of the cytoskeleton, cell shape, and cell-cell adhesion by desmosomes, guanine nucleotide binding protein (G protein) alpha 13, and desmoplakin (+1.89 and +4.01 fold, respectively) also suggests that remodeling processes were taking place in the stressed retinas of FLT mice.

In summary, these results bring light to some of the molecular processes taking place in the retina when mice are exposed to spaceflight environmental conditions. While we have highlighted the pathways that may be biologically relevant and correlated with the changes observed in the histomorphology of retinal tissues of animals exposed to spaceflight, this work provides a catalog of differentially expressed genes that can be used to explore various hypotheses related to the pathophysiology of visual alterations in spaceflight and translated to possible ocular implications for human crews in long-duration space exploration missions.

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Molecular Effects of Spaceflight in the Mouse Eye after Space Shuttle Mission STS-135 -- Supplemental Table 1

Gene Symbol	Gene Name	Fold Change	p Value	Probe Set ID	Entrez Gene ID
Dsp	Desmoplakin	4.01	0.045	1435493_at	109620
Ly6e	Lymphocyte antigen 6 complex, locus E	2.95	0.044	1453304_s_at	17069
Lcn2	Lipocalin 2	2.75	0.000	1427747_a_at	16819
Slc44a2	Solute carrier family 44, member 2	2.55	0.008	1438559_x_at	68682
Cirbp	Cold inducible RNA binding protein	2.48	0.000	1416332_at	12696
Pou4f1	POU class 4 homeobox 1	2.44	0.006	1429668_at	18996
Sgms2	Sphingomyelin synthase 2	2.36	0.025	1428663_at	74442
Defb9	Defensin beta 9	2.36	0.017	1427612_at	246079
Ripk4	Receptor-interacting serine-threonine kinase 4	2.32	0.045	1418488_s_at	72388
		2.24	0.002	1442025_a_at	
Rprd2	Progesterin and adiponQ receptor family member VIII	2.15	0.001	1434817_s_at	75137
		2.11	0.001	1447223_at	
Pagr8	Progesterin and adiponQ receptor family member VIII	2.04	0.006	1428958_at	74229
1700019G17Rik	Camello-like 2	1.99	0.021	1436669_at	75541
Cul2	Cullin 2	1.98	0.003	1419765_at	71745
Alcam	Activated leukocyte cell adhesion molecule	1.96	0.029	1437466_at	11658
Zbtb16	Zinc finger and BTB domain containing 16	1.95	0.002	1419874_x_at	235320
Clvs1	Clavesin 1	1.93	0.020	1433906_at	74438
		1.93	0.003	1441430_at	
Zc3h6	Zinc finger CCH-type containing 6	1.92	0.011	1428682_at	78751
B4galnt1	Beta-1,4-N-acetyl-galactosaminyl transferase 1	1.92	0.006	1418655_at	14421
Gna13	Guanine nucleotide binding protein (G protein), alpha 13	1.89	0.000	1430295_at	14674

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Ctsh	Cathepsin H	1.87	0.006	1443814_x_at	13036
Hnmpd1	Heterogeneous nuclear ribonucleoprotein D-like	1.84	0.001	1428224_at	50926
Wfs1	Wolfram syndrome 1	1.84	0.043	1448411_at	22393
Pap12	Poly (ADP-ribose) polymerase family, member 12	1.82	0.027	1426774_at	243771
Ehd4	EH-domain containing 4	1.82	0.020	1449852_a_at	98878
Acot9	Acyl-CoA thioesterase 9	1.81	0.023	1418073_at	56360
Sh3yl1	SH3 domain containing, Ysc84-like 1	1.80	0.011	1449579_at	24057
Agxt2l1	Alanine-glyoxylate aminotransferase 2-like 1	1.80	0.020	1452975_at	71760
Slc39a4	RIKEN cDNA 2210403K04 gene	1.79	0.032	1451139_at	72027
Inpp1	Inositol polyphosphate-1-phosphatase	1.79	0.016	1442073_at	16329
2210403K04Rik	RIKEN cDNA 2210403K04 gene	1.79	0.001	1428562_at	100042498
Zscan21	Zinc finger and SCAN domain containing 21	1.79	0.004	1449732_at	22697
		1.78	0.033	1449968_s_at	
Cmas	Cytidine monophosphate N-acetylneuraminic acid synthetase	1.77	0.004	1426662_at	12764
Kalrn	Kalirin, RhoGEF kinase	1.76	0.016	1429796_at	545156
Rufy2	RUN and FYVE domain containing 2	1.76	0.030	1434347_s_at	70432
Trim25	Tripartite motif containing 25	1.75	0.016	1419879_s_at	217069
Angptl4	Angiopoietin-like 4	1.74	0.005	1417130_s_at	57875
Chst15	Carbohydrate (N-acetyl)galactosamine 4-sulfate 6-O) sulfotransferase 15	1.73	0.017	1452092_at	77590
Pex13	Peroxisomal biogenesis factor 13	1.72	0.012	1422472_at	72129
Mkml	Makorin ring finger protein 1	1.72	0.015	1451425_a_at	54484
A2m	Alpha-2-macroglobulin	1.72	0.019	1434719_at	232345
Mrp15	Mitochondrial ribosomal protein L15	1.71	0.002	1430798_x_at	27395
Fabp4	Fatty acid binding protein 4, adipocyte	1.71	0.009	1417023_a_at	11770

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Al451250	Expressed sequence Al451250	1.69	0.033	1447436_at	102524
Krt23	Keratin 23 (histone deacetylase inducible)	1.69	0.018	1418213_at	94179
Fkbp5	FK506 binding protein 5	1.68	0.000	1416125_at	14229
Fam194a	Family with sequence similarity 194, member A	1.66	0.046	1458320_at	545527
Mfsd6	Major facilitator superfamily domain containing 6	1.66	0.020	1424463_at	98682
Gm9776	Predicted gene 9776	1.66	0.010	1435723_at	328309
Sdt42e1	Short chain dehydrogenase/reductase family 42E, member 1	1.66	0.030	1427082_at	74032
A930041H05Rik	RIKEN cDNA A930041H05 gene	1.65	0.031	1442157_at	77834
Txnrd3	Thioredoxin reductase 3	1.65	0.006	1449623_at	232223
Pax6os1	Pax6 opposite strand transcript 1	1.65	0.014	1440445_at	402728
Slc1a1	Solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter), member 1	1.64	0.046	1425415_a_at	20510
Col19a1	Collagen, type XIX, alpha 1	1.63	0.009	1421698_a_at	12823
Gpcpd1	Glycerophosphocholine phosphodiesterase GDE1 homolog (S. cerevisiae)	1.62	0.004	1429144_at	74182
Lgil	Leucine-rich, glioma inactivated 1	1.62	0.045	1423183_at	56839
Dextr	Dicarbonyl/L-xylose reductase	1.62	0.001	1419456_at	67880
Sbsn	Suprabasin	1.62	0.003	1459897_a_at	282619
Nipsnap1	Nipsnap homolog 1 (C. elegans)	1.61	0.021	1456748_a_at	18082
Ctsh	Cathepsin H	1.61	0.044	1418365_at	13036
Smc6	Structural maintenance of chromosomes 6	1.61	0.004	1448829_at	67241
Reps2	RALBP1 associated Eps domain containing 2	1.60	0.026	1435389_at	194590
		1.60	0.011	1438815_at	
Crispld2	Cysteine-rich secretory protein LCCL domain containing 2	1.59	0.015	1434758_at	78892
Sorl1	Sortilin-related receptor	1.59	0.041	1453003_at	20660
Wwc1	WW and C2 domain containing 1	1.59	0.020	1420008_s_at	211652

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Dhcr24	24-dehydrocholesterol reductase	1.59	0.029	1451895_a_at	74754
Acs15	Acyl-CoA synthetase long-chain family member 5	1.58	0.008	1428082_at	433256
Chehd7	Coiled-coil-helix-coiled-coil-helix domain containing 7	1.58	0.029	1440158_x_at	66433
Smox	Spermine oxidase	1.58	0.022	1424268_at	228608
Nt5c2	5'-nucleotidase, cytosolic II	1.58	0.000	1425933_a_at	76952
Cpne8	Copine VIII	1.58	0.023	1431146_a_at	66871
Cpne8	Copine VIII	1.58	0.006	1430520_at	66871
Tcta	T-cell leukemia translocation altered gene	1.58	0.043	1420123_at	102791
Plekhh1	Pleckstrin homology domain containing, family H (with MyTH4 domain) member 1	1.57	0.039	1447807_s_at	211945
Ttll11	Tubulin tyrosine ligase-like family, member 11	1.57	0.038	1452964_at	74410
Rbpms	RNA binding protein gene with multiple splicing	1.57	0.038	1429359_s_at	19663
Fem1c	Fem-1 homolog c (<i>C. elegans</i>)	1.57	0.044	1438075_at	240263
Plin4	Perilipin 4	1.57	0.005	1418595_at	57435
Hnrpd1	Heterogeneous nuclear ribonucleoprotein D-like	1.57	0.008	1420093_s_at	50926
Cda	Cytidine deaminase	1.56	0.013	1427357_at	72269
Ifi35	Interferon-induced protein 35	1.56	0.029	1445897_s_at	70110
Ugt1a6	UDP glucuronosyltransferase 1 family, polypeptide A6	1.56	0.026	1426260_a_at	
D130037M23Rik	RIKEN cDNA D130037M23 gene	1.56	0.006	1457321_at	328330
Slc7a1	Solute carrier family 7 (cationic amino acid transporter, y ⁺ system), member 1	1.56	0.016	1454992_at	11987
Snrpd3	Small nuclear ribonucleoprotein D3 polypeptide 18kDa	1.55	0.016	1449629_s_at	
Tsc22d3	TSC22 domain family, member 3	1.55	0.010	1425281_a_at	14605
Kazn	Kazrin, periplakin interacting protein	1.55	0.029	1434169_at	71529
Sh3bp5	SH3-domain binding protein 5 (BTK-associated)	1.54	0.004	1421923_at	24056
Hnrpd	Heterogeneous nuclear ribonucleoprotein D	1.54	0.043	1457568_at	11991

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Chl1	Cell adhesion molecule with homology to L1CAM	1.54	0.037	1435190_at	12661
Plin2	Perilipin 2	1.54	0.043	1448318_at	11520
Ptgis	Prostaglandin I2 (prostaacyclin) synthase	1.53	0.034	1448816_at	19223
Armex5	Armadillo repeat containing, X-linked 5	1.53	0.002	1424481_s_at	494468
Brd8	Bromodomain containing 8	1.53	0.005	1456351_at	78656
Tnfrsf2	Tumor necrosis factor, alpha-induced protein 2	1.53	0.026	1438855_x_at	21928
Btbd11	BTB (POZ) domain containing 11	1.53	0.044	1428377_at	74007
Phyhip	Phytanoyl-CoA 2-hydroxylase interacting protein	1.53	0.030	1455463_at	105653
Btbd11	BTB (POZ) domain containing 11	1.53	0.022	1459838_s_at	74007
Hmrpd1	Heterogeneous nuclear ribonucleoprotein D-like	1.52	0.036	1449039_a_at	50926
Lgl1	Leucine-rich, glioma inactivated 1	1.52	0.030	1435851_at	56839
Rbm3	RNA binding motif (RNP1, RRM) protein 3	1.52	0.042	1422660_at	19652
Haghl	Hydroxyacylglutathione hydrolase-like	1.51	0.022	1428630_x_at	68977
Wwp2	WW domain containing E3 ubiquitin protein ligase 2	1.51	0.003	1448145_at	66894
Marveld2	MARVEL domain containing 2	1.51	0.002	1426914_at	218518
Dis3	DIS3 mitotic control homolog (S. cerevisiae)	1.51	0.005	1426609_at	72662
Cyb561	Cytochrome b-561	1.51	0.014	1417507_at	13056
Zbtb22	Zinc finger and BTB domain containing 22	1.51	0.011	1448705_at	81630
Rnf208	Ring finger protein 208	1.51	0.013	1435105_at	68846
Fam122b	Family with sequence similarity 122B	1.51	0.013	1452682_at	78755
Upk3b	Uroplakin 3B	1.51	0.014	1454881_s_at	100647
March7	Membrane-associated ring finger (C3HC4) 7	1.50	0.021	1420609_at	57438
3110003A17Rik	RIKEN cDNA 3110003A17 gene	1.50	0.008	1426964_at	73112
Sfpq	Splicing factor proline/glutamine-rich	-1.50	0.016	1423796_at	71514
HspH1	Heat shock 105kDa/110kDa protein 1	-1.51	0.004	1423566_a_at	15505

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Srsf10	Serine/arginine-rich splicing factor 10	-1.51	0.003	1449121_at	14105
Kdm2a	Lysine (K)-specific demethylase 2A	-1.53	0.036	1438890_at	225876
Limal	LIM domain and actin binding 1	-1.53	0.013	1450629_at	65970
Crebzf	CREB/ATF bZIP transcription factor	-1.54	0.017	1434618_at	233490
Creld2	Cysteine-rich with EGF-like domains 2	-1.54	0.001	1452754_at	76737
Beas2	Breast carcinoma amplified sequence 2	-1.54	0.013	1437262_x_at	68183
		-1.55	0.020	1417339_a_at	
		-1.55	0.009	1456125_a_at	
Cpsf6	Dynein, light chain, LC8-type 1	-1.55	0.006	1428232_at	432508
BB116930	Expressed sequence BB116930	-1.55	0.017	1440130_at	98740
Srsf6	Serine/arginine-rich splicing factor 6	-1.56	0.047	1448454_at	67996
Chordc1	Cysteine and histidine-rich domain (CHORD) containing 1	-1.57	0.001	1460645_at	66917
Xbp1	X-box binding protein 1	-1.59	0.007	1420011_s_at	22433
Pum2	Pumilio homolog 2 (Drosophila)	-1.59	0.004	1418017_at	80913
		-1.60	0.023	1422404_x_at	
		-1.60	0.046	1424609_a_at	
Hist3h2a	Histone cluster 3, H2a	-1.61	0.035	1435866_s_at	319162
Trpm1	Transient receptor potential cation channel, subfamily M, member 1	-1.62	0.025	1437445_at	17364
Trim47	Tripartite motif containing 47	-1.62	0.007	1426784_at	217333
4931428L18Rik	RIKEN cDNA 4931428L18 gene	-1.63	0.017	1430898_s_at	70988
Xbp1	X-box binding protein 1	-1.65	0.007	1437223_s_at	22433
Hist2h3c2-ps	Histone cluster 2, H3c	-1.65	0.006	1422155_at	97114
Hspa5	Heat shock 70kDa protein 5	-1.69	0.014	1427464_s_at	14828
6530402F18Rik	RIKEN cDNA 6530402F18 gene	-1.70	0.003	1441725_at	76220

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Arsi	Arylsulfatase family, member I	-1.70	0.047	1427508_at	545260
Necab1	N-terminal EF-hand calcium binding protein 1	-1.73	0.046	1437156_at	69352
Cpsf6	Cleavage and polyadenylation specific factor 6, 68kDa	-1.73	0.006	1428233_at	432508
Btg1	B-cell translocation gene 1, anti-proliferative	-1.74	0.045	1426083_a_at	12226
Slpr3	Sphingosine-1-phosphate receptor 3	-1.78	0.019	1437173_at	13610
Hist2h3c1	Histone cluster 2, H3c	-1.79	0.002	1442051_at	15077
Car8	Carbonic anhydrase VIII	-1.79	0.019	1427482_a_at	12319
Rbm12b	RIKEN cDNA C430048L16 gene	-1.89	0.020	1435158_at	72397
		-2.03	0.014	1418188_a_at	
Tra2a	Transformer 2 alpha homolog (Drosophila)	-2.10	0.002	1454725_at	101214
Malat1	Metastasis associated lung adenocarcinoma transcript 1	-2.15	0.001	1418189_s_at	378938
Slpr3	Sphingosine-1-phosphate receptor 3	-2.26	0.016	1438658_a_at	13610
Sfpq	Splicing factor proline/glutamine-rich	-2.33	0.001	1423795_at	71514
Slc5a3	Solute carrier family 5 (sodium/myo-inositol cotransporter), member 3	-2.64	0.017	1435484_at	53881

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Gene Symbol	Gene Name	Fold Change	p Value	Probe Set ID	Entrez Gene ID
Dsp	Desmoplakin	4.01	0.045	1435493_at	109620
Ly6e	Lymphocyte antigen 6 complex, locus E	2.95	0.044	1453304_s_at	17069
Lcn2	Lipocalin 2	2.75	0.000	1427747_a_at	16819
Slc44a2	Solute carrier family 44, member 2	2.55	0.008	1438559_x_at	68682
Cirbp	Cold inducible RNA binding protein	2.48	0.000	1416332_at	12696
Pou4f1	POU class 4 homeobox 1	2.44	0.006	1429668_at	18996
Sgms2	Sphingomyelin synthase 2	2.36	0.025	1428663_at	74442
Defb9	Defensin beta 9	2.36	0.017	1427612_at	246079
Ripk4	Receptor-interacting serine-threonine kinase 4	2.32	0.045	1418488_s_at	72388
Rprd2	Progesterin and adipoQ receptor family member VIII	2.15	0.001	1434817_s_at	75137
		2.11	0.001	1447223_at	
Pagr8	Progesterin and adipoQ receptor family member VIII	2.04	0.006	1428958_at	74229
		-2.03	0.014	1418188_a_at	
Tra2a	Transformer 2 alpha homolog (Drosophila)	-2.10	0.002	1454725_at	101214
Malat1	Metastasis associated lung adenocarcinoma transcript 1	-2.15	0.001	1418189_s_at	378938
Slpr3	Sphingosine-1-phosphate receptor 3	-2.26	0.016	1438658_a_at	13610
Sfpq	Splicing factor proline/glutamine-rich	-2.33	0.001	1423795_at	71514
Slc5a3	Solute carrier family 5 (sodium/myo-inositol cotransporter), member 3	-2.64	0.017	1435484_at	53881