Examining Impacts of Spaceflight-Induced Cell Cycle Dysregulation on Skin Health and Wound Healing in Mice with Metabolic Profiling of IGF2

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ABSTRACT

Worsened skin health and wound healing have numerous implications amongst astronauts. Upon further investigation of the OSD-257 “Transcriptional analysis of dorsal skin from mice flown on the RR-7 mission” dataset, we identified down-regulation of genes involved in cell cycle regulation (*CDK1*, *CCNB1*, *CCNB2* [p-value < 0.05, log2FC < -1.5]) in female C57BL/6J *mus musculus* from their dorsal skin samples. This characteristic is also reflected in *homo sapiens*, where according to literature, microgravity induces dysregulation in cell cycle genes in human cells (Verma et al., 2015). Additionally, cell cycle genes have been established to be keystone in cellular proliferation, where upregulation of *CDK1*, *CCNB1*, and *CCNB2* have been found to be upregulated in various cancers. Essentially, when upregulated, these genes drive rapid cell division, contributing to tumor growth and progression. Thus, these genes with their connection to cell proliferation and development via regulation of the G2/M phases of the mammalian cell cycle are well established. To further explore this phenomenon and its impacts on skin health/wound healing in microgravity, we analyzed RNA-sequencing data from our dataset of mice dorsal skin samples, comparing mice in spaceflight conditions for 75 days to ground control. Based on our findings, we found *IGF2* (p-value < 0.05, log2FC > 1) to be involved in many cell growth and proliferative processes along with the three regulatory genes. Through further analysis, we found their direct cellular interactions within the MAPK pathway. We then identified *IGF2s* role in enhancing skin health (skin thickness/differentiated cell concentration) and wound healing (Ward et al., 2003) as well as metabolic characteristics. This led us to focus on identifying unique metabolic profiles characterized by *IGF2* expression in microgravity conditions. By designing an experiment utilizing immunofluorescence (IF), AO/PI flow cytometry assays, histological assessments, metabolomic assays, and numerous other potential procedures to examine impacts of cell cycle gene dysregulation and *IGF2* expression on mice skin health/metabolomic characteristics, we aim to understand underlying mechanisms of skin health and wound healing in microgravity. This knowledge will open doors to tailor solutions for microgravity impacts on skin health whilst identifying potential metabolic side effects.

Keywords: CDK1, CCNB1, CCNB2, IGF2, skin health, wound healing, cell cycle, metabolism, *mus musculus*, microgravity, space flight

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1 PROBLEM STATEMENT AND OBJECTIVES

1.1 Introduction

This proposal conducts an analysis of the NASA GeneLab dataset "OSD-254: Transcriptional analysis of dorsal skin from mice flown on the RR-7 mission." The RR-7 mission's primary aim was to observe possible changes in transcriptional expression of genes in the dorsal skin of mice exposed to space flown on the ISS. The launch of ten 11-week-old female C57BL/6J and ten 11-week-old female C3H/HeJ mice on 29 June, 2018 was performed to the ISS via SpaceX-15. The mice were kept within two Rodent Habitats on the ISS. Over time, frequent collection of samples of food swabs, living surfaces, and fecal pellets were collected for data generation. This included extended video monitoring and recording for studies on circadian rhythm and mass measurements. After 25 days in space, half of the mice from each strain were euthanized on the ISS using Ketamine/Xylazine/Acepromazine and cardiac puncture. The other half of the remaining mice were similarly euthanized after 75 days in space. In addition to the flight group, there were three ground control groups in this study: Basal (pre-launch state), Vivarium (standard vivarium housing for periods of time reflecting space flight), and Ground (habitat based on the ISS). Control groups had twenty mice with an equivalent division between the two strains and were euthanized with subsequent processing in parallel with the flight samples. GeneLab later received dorsal skin samples from forty C57BL/6J and C3H/HeJ strains of mice. These samples underwent RNA extraction, library generation, and sequencing to create a dataset aiming to contribute to the knowledge of transcriptional changes occurring in dorsal skin responding to spaceflight (NASA OSDR) (NASA GeneLab) (Open Data Network). This analysis includes five female C57BL/6J ground samples and a set of similar, five 75-day flight samples.

1.2 Hypothesis & Problem Statement

After conducting research and RNA-seq data analysis, we found cell cycle regulation genes *CDK1, CCNB1,* and *CCNB2* to be downregulated significantly in a microgravity environment. We also found *IGF2* being upregulated, potentially as a compensatory mechanism with its mitogenic and proliferative characteristics. With this upregulation, we would also expect its metabolic impacts to cause unique profiles in samples where IGF2 is being expressed.

Given this background, our hypothesis is based upon the fact that **when in microgravity conditions, cell cycle regulation genes (*CDK1, CCNB1, CCNB2*) are significantly downregulated, causing lowered rates of wound healing and worse skin health, measured by skin thickness and cell concentration.** By examining impacts of spaceflight-induced downregulation of cell cycle genes, we aim to identify impacts on skin health/wound healing. **We further connect *IGF2* as a potential compensatory mechanism due to its proliferative nature and role in rebalancing cell cycle dysregulation (Pavia et al., 2024).** Relative comparisons where expressions of IGF2 are higher (ie. wild-type vs knockout or spaceflight vs ground) will highlight unique metabolic profiles due to *IGF2s* involvement in metabolic/glycolytic pathways. **By examining *IGF2* and its impacts as a compensatory mechanism, we aim to open doors to potential solutions that can reduce negative impacts to skin health/wound healing while also identifying any potential side effects, such as unique metabolic characteristics.**

1.3 Aims

1. To analyze the impacts of the downregulation of cell cycle genes CDK1, CCNB1, and CCNB2 on skin in microgravity

a. **Working hypothesis #1**: We hypothesize that the downregulation of cell cycle genes in spaceflight conditions will lead to a decreased proportion of keratinocytes resulting from cell cycle dysregulation (G2/M phases) and lower levels of cyclins in these cells, leading to less cell concentration and skin thickness.

b. **How and significance #1:** Impacts will be assessed with the use of immunofluorescence (IF) with respective cyclin antibodies of targets (ie. CCNB1 and Abcam ab72, Keratinocytes and K10) and AO/PI flow cytometry assay to identify if spaceflight is causing cells to halt at a certain stage of the cell cycle.

2. To examine the impact of IGF2 expression on skin health and wound healing

a. **Working hypothesis #2:** We hypothesize that wounded skin samples from IGF2 wild-type mice will yield greater Ki67 intensity (indicating higher cell proliferation in wound areas) and increased Keratin 10 levels (indicating keratinocyte maturation/differentiation and skin health) compared to IGF2 -/- mice.

b. **How and significance #2:** Wound healing will be assessed with the use of hematoxylin and eosin (H&E) staining for general histological assessment, providing information about the pattern, shape, and structure of cells within the sample. Ki67 immunohistochemical (IHC) staining will be done to measure rates of wound healing, where Ki67 marks dividing cells, accurately identifying proliferative cells. Skin health

3. To identify metabolic and glycolytic impacts through IGF2 upregulation in skin health and wound healing

a. **Working hypothesis #3:** We hypothesize that IGF2 positive mice in spaceflight will have enhanced glycolytic activity, causing increased glycolytic intermediates like lactate and increased Acetyl-CoA production along with elevated Acetyl-CoA use in unique mechanisms due to mitochondrial dysregulation from spaceflight compared to IGF2 -/- mice.

b. **How and significance #3:**

1.4 Proposed Significance

It has been found that the functions of wound healing in the broader immune system are suppressed in spaceflight, which threatens astronaut health because they are inevitably exposed to solar particle events, galactic cosmic radiation, and other sources that engender skin damage. By understanding the specific mechanistic roles of CDK1, CCNB1, and CCNB2, we can determine where, how, and to what extent the cellular processes affected by the downregulation of cell cycle genes are impaired during spaceflight. Moreover, considering the inverse regulation in IGF2, incorporating IGF2 as a standard supplement for astronauts could be key in mitigating skin damage and enhancing wound healing during space missions. Our study thus has the potential to improve the immediate health of astronauts **and** contribute to long-term solutions for maintaining skin integrity in extreme environments.

2 INTRODUCTION

3 SCIENTIFIC BACKGROUND AND RATIONALE

3.1 Preliminary Data Collection

We performed data analysis through Galaxy, ShinyGO, and cytoscape in order to understand the dataset and propose hypotheses based on certain genes and pathways of interest.

Figure 1: *GeneLab Galaxy Research Pipeline Utilized in the Investigation*

Main data sources. The analysis in this paper has been conducted on RNA-seq data from GLDS-254, "Transcriptional analysis of dorsal skin from mice flown on the RR-7 mission." GLDS-254 data comes from NASA GeneLabs' Open Science Data Repository. In the experiment, scientists studied the effects of spaceflight on the gut microbiota, specifically looking at its consequential effects on the immune system, metabolic system, and circadian rhythms in mice. Two strains of mice, including ten 11-week-old female C57BL/6J and ten 11-week-old female C3H/HeJ mice were flown on SpaceX-15 to the International Space Station, where they stayed in two Rodent habitats. They were monitored by 48-hour video segments, on-orbit mass measurement, and fecal pellets, food, and swabs from living surfaces were collected. Respectively, half the mice were euthanized after 25 days on-orbit and then the rest after 75 days on-orbit, collecting their dissected frozen carcasses on Earth. Their carcasses were preserved in RNA and sent back on SpX-15 and Spx-16. These mice were compared to three control groups: basal (pre-launch), vivarium (standard vivarium housing), and ground (International Space Station Environment Simulator), with 20 mice per group. (see Figure 1).

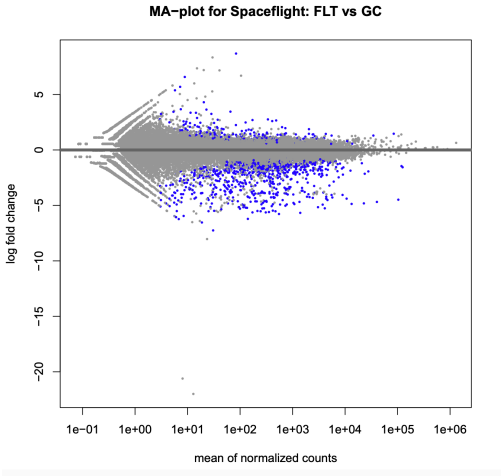
The dataset we analyzed was a subset of the original data, including the five C57BL/6J mice euthanized at 75 days on ground control and the five C57BL/6J mice euthanized at 75 days on spaceflight. In our data analysis, our group came to the decision to examine the effects of the space environment, specifically microgravity, on mus musculus dorsal skin.

Main tools. The main tool we used for analyzing our RNA-Seq data is Galaxy, developed by the Nekrutenko lab at Penn State, the Taylor lab at Johns Hopkins University, and the Goecks Lab at Oregon Health and Science University. It is an open-source computational analysis program, containing a multitude of omics analysis tools. These include FastQC, which we used for data quality control, Trim Galore to trim adapters and any reads above 20, RNA Star (or alignment) to align the sequences to a reference genome to see where the reads came from, featureCounts to count the number of reads assigned to a feature, multiQC to view the number of reads assigned to genes across all samples, DESeq2 for normalization and gene expression analysis, annotations to look at the gene IDs, volcano plot for a visual reference of top up- and down-regulated genes, GOSeq to analyze which pathways are affected by the treatment, and fgsea to perform geneset enrichment analysis. These tools help to both sequence and analyze our data for genes and pathways that were changed between ground control and spaceflight. We will be analyzing some graphs outputted by these tools to understand our data and process. (see Figure 2).

3.2 Preliminary Data Analysis

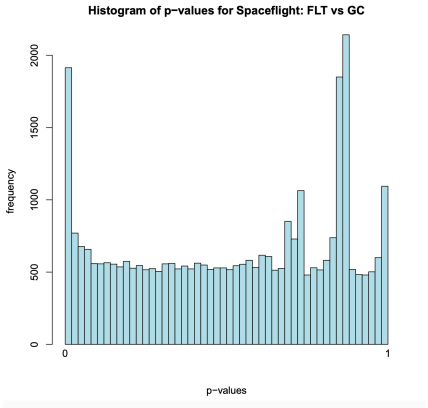
After procuring the featureCounts data, we then analyzed the GLDS-238 data using the DESeq2 Galaxy tool, yielding the plots below that proved helpful in our differential gene expression analysis.

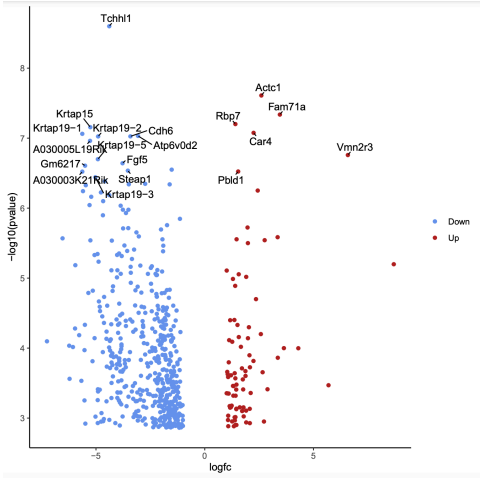
Principal Component Analysis. Principal component analysis (PCA) was performed to visualize the variance in gene expression data across samples. This PCA plot is derived from DESeq2, which demonstrates 2 dimensions from PCA run on normalized counts of sample. It helps to visualize the overall effect of experimental covariates and batch effects. The first dimension has a variance of 63%, showing strong variation between the ground control and spaceflight samples.



MA Plots. The MA plot was generated to visualize the log2 fold changes versus the mean expression levels of genes, highlighting differentially expressed genes with an adjusted p-value < 0.05 and expression patterns between the flight and ground samples. The horizontal axis (A) shows the average intensity while the vertical axis (M) shows the intensity ratio between the two samples for the same data point. The blue dots are representative of genes that passed the significance threshold (adj p-value < 0.1) and thus explain that there is biological difference in genes between ground and flight control. There also isn't much noise in our data, indicating good quality.

Histograms. Histograms were created to visualize the distribution of gene expression levels and log2 fold changes. The first two bars are deemed significant where the p-value is less than the significance threshold, 0.05. It seems there is a high frequency of genes with p-values below 0.05, showing statistical significance. We can be more confident that the change is due to biology rather than chance.

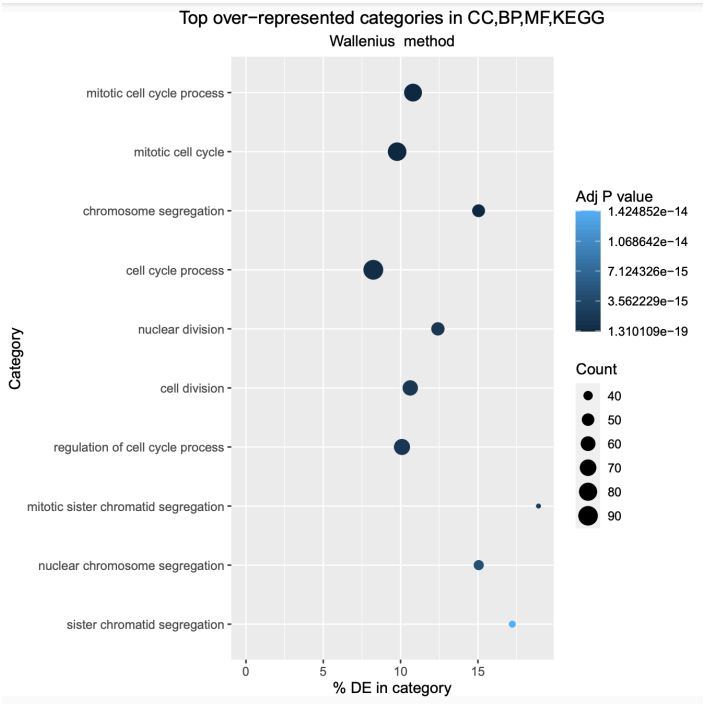




Volcano Plot. Volcano plots were generated to visualize the differentially expressed genes. The log2 fold changes and adjusted p-values from DESeq2 results were used to create volcano plots. Genes with an adjusted p-value < 0.05 and absolute log2 fold change > 1 were highlighted. We can see that Tchhl1 was significantly down-regulated, and Actc1 was significantly upregulated. It is also important to notice that many of the cell cycle regulator genes are downregulated in this graph, as circled with Cdk1, Ccnb1, and Ccnb2.

Goseq Analysis. Next, gene ontology (GO) enrichment analysis was performed to better understand changed pathways. The list of differentially expressed genes was used to identify enriched GO terms. The analysis accounted for gene length bias, and GO terms with an adjusted p-value < 0.05 were considered significantly enriched. In this analysis, we noticed there was a heavy dependence on cell cycle regulation, with prominent genesets being the mitotic cell cycle, cell cycle process, and regulation of cell cycle process.

In accordance with the data from this plot, we collected a set of genes from the following genesets relating to the over-represented categories: GO_……. The gene list derived from these genesets is Adra2b, Mmd2, Ltf, Igf2, Brca1, Lef1, Msx2, Gjb2, Ccna2, Skp2, Ccnb1, Ccnb2, Fhl2, Spo11, Pclaf, Exo1, Rad51ap1, Ticrr, Polq, Cdk1, Top2a, Clspn, Trip13, Rad51, Fanci, Fancd2, Fignl1, Neil3, Esco2, Mc1r, Fbxo5, Bard1, Wdhd1, Aunip, Eme1, Dtl, Mcm10, E2f8, Parpbp, Cdc25c, Hmgb2, Bub1, Bub1b, Sgo1, Nuf2, Ncaph, Aspm, Cks2, Smc2, Rad54l, Dmbt1, Fgb, Npas2, Fzd5, Parpbp, Adamts18, Bag2, Lrp8, Hmgb3, Ace2, Lhx8, Dmrta1, Slco4c1, Cip2a, Cenpi.



ShinyGO Platform. Then, after plugging in the curated geneset into ShinyGO, we found significant pathways that relate these genes and gained an understanding as to how the cell cycle pathways were interlinked with cell proliferation and wound repair. This led us to the focus of our aims: cell cycle regulation, cell growth, metabolism, and wound repair. Based on this analysis, we focused our attention on four genes: cyclin-dependent kinase 1, cyclin B1, cyclin B2, and insulin-like growth factor 2. Igf2 was a significant gene found due to its double role in cell cycle and metabolomics.

16	Regulation of developmental process	BRCA1 FBXO5 CDK1 MSX2 LEF1 LTF MMD2 FANCD2 ASPM HMGB2 ADRA2B CCNB1 DMBT1 IGF2 LRP8 HMGB3
14	Positive regulation of developmental process	BRCA1 FBXO5 CDK1 MSX2 LEF1 LTF MMD2 ASPM HMGB2 ADRA2B CCNB1 DMBT1 IGF2 LRP8
9	Cell population proliferation	CDK1 MCM10 LEF1 E2F8 ASPM CKS2 DMBT1 IGF2 FIGNL1
5	Growth	MSX2 ASPM CCNB1 DMBT1 IGF2
5	Regulation of growth	CDK1 LEF1 CCNB2 CCNB1 IGF2

3.3 Broader Biological Significance

Characterizing IGF2 and Cell Cycle Regulators

In order to further investigate the *IGF2* gene and its interaction with genes involved in cell cycling (*CDK1, CCNB1,* and *CCNB2*), we explored the different signaling pathways induced by the IGF2 gene. The gene, known to be responsible for cell growth and metabolism, secondarily initiates a set of alternative signaling cascades, which ultimately lead to cell proliferation and differentiation. While researching, we were specifically interested in its initiation of the MAPK pathway, which processes signals such as stress and promotes responses through expression changes of cell cycle regulator genes.

Identifying Specific Pathways of Interest

Using bioinformatics databases and prior research, we identified possible pathways of interest that *IGF2* and its related cell cycle regulators were involved in: cell proliferation, cell differentiation, metabolism, and glycolysis. It is through this research that we established the MAPK pathway and its close connection to our gene expression analysis. Through its protein interactions, the pathway eventually affects *CDK1*, *CCNB1*, and *CCNB2*. To understand how the genes link with the MAPK signaling pathway, we will have to gain insight into the biological mechanisms moving these interactions.

MAPK Signaling Pathway

The MAPK signaling pathway is a very notable cellular pathway, transmitting signals from the cell surface to the nucleus. The pathway participates in a series of cellular events, including growth, differentiation, and survival. Signaling involves essential elements that play different roles in the process of transduction. **Figure 9** shows a linear progression of these components, while **Figure 10** provides a more detailed diagram of the cellular pathway.

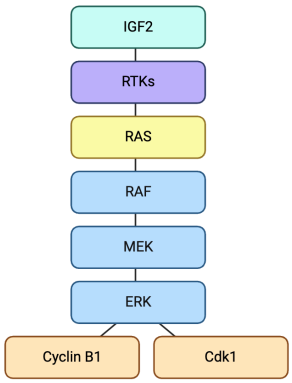


Figure 9: Proteins involved in the MAPK signaling pathways

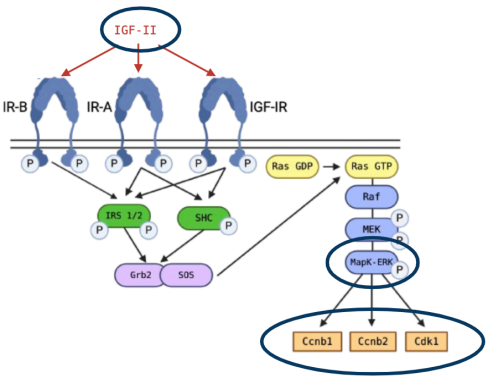


Figure 10: MAPK Signaling Pathway Diagram

Key Components and Detailed Functions

1. Activation of the MAPK signaling pathway begins with the IGF2 protein (coded for by the *IGF2* gene). IGF2 is a polypeptidic growth factor involved in the cellular growth and development process. This factor is secreted by cells and binds to its particular receptor present on the plasma membrane, known as IGF1R (Insulin-like Growth Factor 1 Receptor), which is a type of RTK receptor. The binding of IGF2 to IGF1R leads to a conformational change in the receptor that dimerizes it and induces autophosphorylation on specific tyrosine residues.
2. The cell surface receptors, RTKs, when activated, induce a downstream cascade of signaling events. Several docking sites are created for adaptor proteins and signaling molecules through the autophosphorylation of RTKs. Grb2 is one of the main adaptor proteins recruited to bind with the phosphorylated tyrosine residues on the RTK. Grb2 further recruits the guanine nucleotide exchange factor (GEF) SOS to the membrane.
3. RAS is a small GTPase that acts as a molecular switch, cycling between an active GTP-bound state and an inactive GDP-bound state. SOS acts as a catalyst for the exchange of GDP with GTP on RAS and, by doing so, activates RAS. Active RAS undergoes a conformational change and binds/activates the downstream effectors, one of which is RAF.
4. RAF is a serine/threonine-specific protein kinase that is activated by RAS. Active RAS recruits RAF to the cell membrane, where it is activated through several phosphorylation events. Active RAF phosphorylates and activates a dual-specificity kinase called MEK.
5. MEK is a dual-specificity kinase responsible for phosphorylating and activating ERK. MEK is phosphorylated and activated by RAF. The active MEK then phosphorylates ERK on specific threonine and tyrosine residues.
6. ERK itself is a protein kinase that, when activated, translocates into the nucleus to modulate gene expression. Activation of ERK through phosphorylation is mediated by MEK. On activation, ERK translocates from the cytoplasm into the nucleus. In the nucleus, ERK phosphorylates and activates different transcription factors. Transcription factors are proteins that associate with specific DNA sequences, regulating expression of their target genes. In the context of the MAPK signaling

pathway, ERK, which is phosphorylated itself, also phosphorylates such transcription factors as ELK1, c-Fos, and c-Jun.

7. The phosphorylated transcription factors then undergo conformational changes to make them more competent for DNA binding. Finally, the transcription factors are associated with the promoter regions of specific genes, which become cell-cycle target genes. Some of these target genes are CCNB1, CCNB2, and CDK1, coding for important cycle control proteins Cyclin B1, Cyclin B2, and Cyclin-Dependent Kinase 1, respectively.
    a. Cyclin B1 and Cyclin B2 are centrally involved in the progress of a cell through the cell cycle, predominantly at the G2-to-M transition. The phosphorylated transcription factors bind to the promoter sequences of CCNB1 and CCNB2 genes, giving the required signals for the increased transcription of these genes and the resulting synthesis of their proteins.
    b. The CDK1 protein is a cyclin-dependent kinase 1, which acts as the prime regulator of the cell cycle. The complex with Cyclin B1 or B2 is formed and is crucial for the initiation of mitosis. The ERK-mediated activation of transcription factors up-regulates the expression of *CDK1* genes at appropriate time points so that the cell-cycle progression proceeds normally.

The MAPK signaling pathway thus guarantees that, through the regulation of these cell cycle proteins, cells will smoothly transition between the G2 and M phase of the cell cycle.

Supporting Evidence: Literature Review

ERK and Cell Cycle Regulation: Several studies demonstrated that activation of ERK instigates the transcription of cyclin D1, involved in the G1/S transition. ERK can also modulate the activity of cyclin B1 and CDK1, key regulators of the G2/M transition.

Research has identified specific ERK-activated transcription factors that have been identified to coordinate cell cycle gene expression. For instance, the ERK-activated transcription factor ELK1 is known to activate the expression of cyclin D1. We further established that CCNB1 and CCNB2 are induced by ERK-activated transcription factors, generating complexes with CDK1. CCNB1-CDK1 and CCNB2-CDK1 complexes are key players in the G2/M transition of the cell cycle, driving the process of mitotic entry. One major reason this pathway is of interest is its implications for cancer research, with these genes being commonly upregulated in many cancers and driving the rapid, uncontrolled cell division contributing to growth and progression of tumors.

Previous literature examines the role of CDK1, CCNB1, and CCNB2 in cell cycle regulation and the analogous effects of IGF2 on the skin, providing a baseline to understand the genes' interrelation. Of the many papers reviewed, two were of particular interest:

In the paper "Structural-proliferative units and organ growth: effects of insulin-like growth factor 2 on the growth of colon and skin," downregulated (K:Igf2) and upregulated (Igf2 knockout) IGF2 in strains of mice are used in comparison with wild type to study the effect of IGF2 on the growth of structural-proliferative units (SPUs) in the epidermis. SPUs consist of a stem cell together with its progeny; the stem cells & the transit cells lie in the basal layer of the epidermis, while cells in suprabasal layers are postmitotic and differentiating. Researchers found that distinct doses of IGF2 altered the number of cell divisions and controlled the multiplication of the SPUs. K:Igf2 mice showed overgrowth of the affected organs and DNA content in contrast to IGF2 knockout, which showed a considerable growth deficit (60% of natural size).

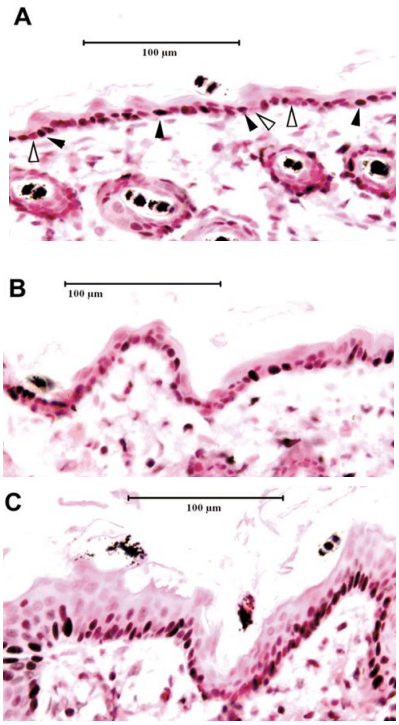


FIgure 11: PCNA Labeling of Transverse Section

Hypothesized that because of the increase of overall skin area, SPU numbers increased

Conclusion:

Considering this information, we concluded that IGF2 up-regulates *CDK1, CCNB1,* and *CCNB2* expression, thus impacting the G2/M transition of the cell cycle. However, expressions of *CDK1, CCNB1,* and *CCNB2* are also found to be impacted by other variables, such as downregulation caused by ERBB2 via phosphorylation. These confounding variables are why *CDK1, CCNB1,* and *CCNB2* expressions are not directly increasing in spaceflight though *IGF2* is upregulated, even if *IGF2* may be increased to compensate and to balance cell cycle regulation. In general, this upregulation is via the MAPK pathway, which processes signals like stress and promotes physiological responses such as cell proliferation and differentiation. The broader cellular growth and developmental role for *IGF2* is well proven, especially with respect to its role in metabolism and glycolysis pathways. Coupled with our genetic analysis, such knowledge may provide new implications for cell cycle regulation and wound healing research.

4 EXPERIMENT AND OUTCOMES

4.1 Methods

A General Overview (Figure 11). Our experimental design includes a total of 80 female C57BL/6j mice as outlined in **Figure 11**. These mice samples are stratified by two factors: *IGF2* knockout treatment vs *IGF2* positive treatment and Ground Control vs Flight. Additionally, half of these mice will receive 5 mm incisions on their dorsal skin, where these samples will be removed after euthanization post-25 days on either ground control and flight. The mice without 5 mm incisions will be left in their respective habitats for 75 days until being euthanized. This design aims to assess the true impacts of cell cycle gene dysregulation on skin health, *IGF2* expression on skin health and wound healing, and *IGF2* expression on unique metabolic profiles, each stratified by ground control vs flight environments.

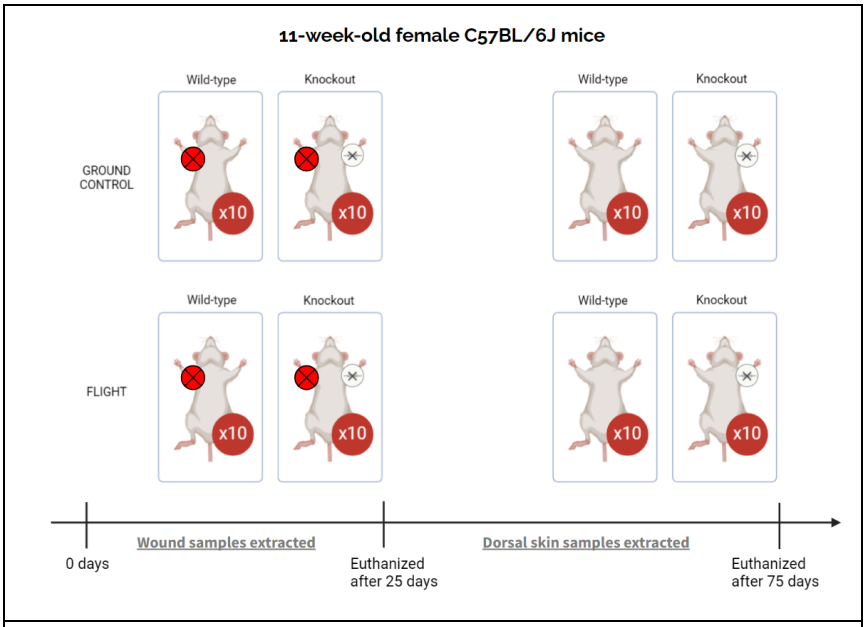


Figure 11: Respective layout of mice for this experimental design

Stratification by *IGF2*. Understanding the reasoning why the experiment's design stratifies by *IGF2* is paramount. Through literature review, we identified *IGF2* to be an essential gene related to skin thickness [Ward et. al, 2003], cell concentration (including differentiative cells [Hardouin et al., 2017]), proliferation, balance of cell cycle dysregulation [Pavia et al., 2024], and other crucial cell related functions. In order to identify *IGF2*'s function on skin in spaceflight, we decided to perform knockouts to draw comparisons between samples with and without the gene in space. Comparisons to terrestrial samples can also be made to identify *IGF2*'s unique impacts metabolically caused by spaceflight-induced mitochondrial dysregulation [Beheshti et al., 2024, where crucial metabolites resulting from *IGF2* take large roles in. Additionally, by performing *IGF2* knockouts in our incision population of mice, we believe we would be able to compare impacts of *IGF2* in wild type samples to spaceflight/knockout samples to further understand the gene's role in skin proliferative functions both on earth and in space.

Testing Cell Cycle Gene Dysregulation on Skin Health. In studies conducted on human fibroblasts, we found that the Heparan Sulfate Proteoglycan Pathway operates largely within the fibroblast cells of the body (Kleinman et al., 1975). Considering that researchers recently determined a strong homology between fibroblast cells in mice and in humans through studying "230,000 fibroblasts across 17 tissues, 50 datasets, 11 disease states and 2 species," we are confident that our tests will be able to affect the execution of this pathway (Buechler et al., 2021). In other words, we are confident that changes in expression of the collagen-creating COL1A1 and COL1A2 genes will affect the activity of the integrin-alpha-2 receptor in the heparan sulfate proteoglycan pathway (Staatz et al., 1990).

Testing *IGF2* Impacts on Skin Health/Wound Healing. In studies conducted on human fibroblasts, we found that the Heparan Sulfate Proteoglycan Pathway operates largely within the fibroblast cells of the body (Kleinman et al., 1975). Considering that researchers recently determined a strong homology between fibroblast cells in mice and in humans through studying "230,000 fibroblasts across 17 tissues, 50 datasets, 11 disease states and 2 species," we are confident that our tests will be able to affect the execution of this pathway (Buechler et al., 2021). In other words, we are confident that changes in expression of the collagen-creating COL1A1 and COL1A2 genes will affect the activity of the integrin-alpha-2 receptor in the heparan sulfate proteoglycan pathway (Staatz et al., 1990).

Testing *IGF2* Impacts on Metabolic/Glycolytic Behavior. To investigate the metabolic and glycolytic effects of

IGF2 upregulation, we used two groups of mice: one with IGF2 upregulation and the other with IGF2 knockout. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and conducted according to National Institutes of Health guidelines. Skin tissues from both IGF2 overexpressing and IGF2 knockout mice were resected, snap-frozen in liquid nitrogen, and stored at –80°C until further processing. Metabolites were extracted from the skin tissues using a methanol-chloroform-water extraction method. The tissues were homogenized in methanol and chloroform, followed by the addition of water. The samples were then centrifuged to separate the aqueous and organic phases. The polar metabolites in the aqueous phase were collected, dried under vacuum, and analyzed by tandem mass spectrometry. The dried metabolite extracts were reconstituted in an appropriate solvent and injected into a liquid chromatography-tandem mass spectrometry (LC-MS/MS) instrument. The LC-MS/MS analysis was conducted on a high-resolution mass spectrometer equipped with an ESI source. Metabolites were identified and quantified based on their mass-to-charge ratio (m/z) and retention time using both targeted and untargeted approaches. For targeted metabolomics analysis of Acetyl-CoA and its derivatives, a standard solution was prepared, and a calibration curve was generated. The targeted analysis was conducted in multiple reaction monitoring mode on the LC-MS/MS system. Metabolite levels were determined by comparing the peak areas of the samples with those of the calibration curves. To determine the distribution of Acetyl-CoA and its derivatives in cellular compartments, subcellular fractionation was performed. Skin tissues were homogenized, and differential centrifugation was used to separate the cytoplasmic and mitochondrial fractions. Metabolites in these fractions were extracted and analyzed by LC-MS/MS as described above. Peak detection, alignment, and quantification of data were performed using appropriate software. Statistical analysis was used to identify metabolites that were significantly different between the IGF2 upregulated mice and their IGF2 knockout littermates. Pathway enrichment analysis was then conducted to identify significantly altered metabolic pathways.

Scientific Breakdown of Procedures. To test whether COL1A1 and COL1A2 genes accelerate wound healing mechanisms in the cell tissue cultures, we will apply two distinct, cost-effective testing procedures—scratch assays and western blots.

Creating Scratch Assays (Figure 13***).*** To test the rate of fibrotic cell migration in closing an allotted wound test site, we plan to use a monolayer scratch assay applied to 36 out of the total 72 cell cultures (18 male and 18 female). Our scratch assay methodology is heavily modeled after the methodology of Sano et al. (2018). To begin, we would create the cell cultures by seeding mice skin fibroblast cells (0.05×10^6) in 12 of the available 96 wells for each of the 3 well plate arrays for 8 hours with standard culture media. For cell cultures subjected to our FOS studies, we would supplement the cell media in those cell culture wells with 10% FOS (see methodology & materials in the "Collaborations" section). The well plates would then be kept in an incubator at 37° C and 5% CO_2 until the formation of a confluent monolayer. To perform the scratch assay tests, we would scratch the monolayer of cells using a P200 micropipette tip. Then, the cells would be washed three times using phosphate buffered saline (PBS) to remove the cells that were scratched and we would thereby measure the width of the scratch in μm upon its creation. Finally, based on the particular cell culture condition assigned to each well array, we would then subject the wells to different gravity conditions (simulated microgravity in a 3D clinostat, simulated partial gravity (0.16) in a 3D clinostat, and 1g in an incubator as the control). Intermittently at noon of each research day, the scratched wells would be removed from the clinostat for imaging, until cells had completely migrated into the wound and recreated the confluent monolayer. Using video stitching methods to create "time-lapse microscopy," we would measure and image the time it takes for the new confluent monolayer to form and we would record the final width of the wound. Lastly, we'd calculate the rate of cell migration using the equation below.

Preparing Cell Cultures for Western Blots (Figure 13***).*** To create a normal cell culture used for the western blot analyses rather than the scratch assay, we would seed mice skin fibroblast cells (0.05×10^6), acquired from CellBiologics in the other in 12 of the available 96 wells for each of the 3 well plate arrays for 8 hours with standard culture media. For the cells within each gender condition that are given FOS compounds, we would aim to give the wells a 10% FOS supplemented culture media. The well plates would then be kept in an incubator at 37° C and 5% CO2 until the formation of a confluent monolayer. From there, we'd conduct the western blot analysis.

Conducting Western Blots (Figure 13***).*** After the cell cultures are set up, we will then utilize western blots to quantify the average change in activity of integrin receptors during fibroblast inflammation response pathways. The general methodology for our proposed western blot is largely based on the NCBI research paper "Western Blot: Technique, Theory, and Troubleshooting" (Mahmood & Yang, 2012). We will initially conduct gel electrophoresis to separate out the activated integrin-alpha-2 receptors, transfer those proteins to

a stable support frame, and then use various antibodies (especially those that can produce a colorimetric or illuminating signal that can be picked up) to detect the integrin receptor activity ("Bio-Rad," 2015). As a side note, from our literature review, we noticed that it is possible to determine whether an integrin-alpha-2 is active or not based on how collagen causes a conformational change in the ADMIDAS binding region whereby an inhibitory calcium ion binds to manganese and changes the structure of the rest of the integrin (Adorno-Cruz & Liu, 2018). Thus, after being able to successfully separate & transfer out the activated integrins, we could use two antibodies of interest in the "detection" stage of our western blot, including:

- C-9 (Anti-Integrin α2 Antibody) which is a monoclonal antibody with specificity for one of the main specific binding regions, or epitopes, on the Integrin Alpha 2, that has been used in 21 western-blot oriented publications to detect and chemilluminate integrin Alpha 2 in cells of mouse, rat and human origin. We believe that the range of usage spanning across multiple different organisms and research papers in the past would lend credence to using C-9 as our primary antibody for our western blotting (SCBT, 2019).
- P1H5, which is another monoclonal antibody with specificity for the main epitope on Integrin Alpha 2. This would be used as a back-up if C-9 did not work.

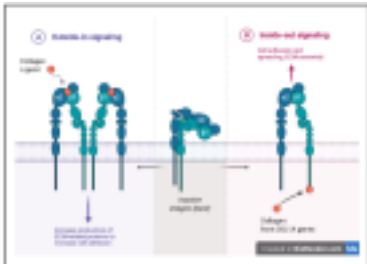


Figure 14: *When no C-9 antibodies are present, collagen can freely bind to the integrin alpha 2 receptors (Stautz et al., 1990).*

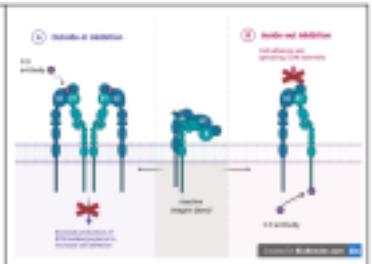


Figure 15: *When C-9 bodies are present, they competitively compete for the ability to bind to the receptors, thus showing up more in the western blots (SCBT, 2019; Tuckwell, 1995).*

Because collagen and antibodies like C-9 and P1H5 generally compete for the same binding spot, these antibodies will only be able to bind to integrin-alpha-2s in great concentration when there is less collagen concentration in the cell (Figure 14 & 15; Tuckwell, 1995). In turn, since FOS decreases the amount of collagen, we believe that the C-9 antibody will be more expressed and the western blots will be much, much brighter when FOS is supplemented in the cell culture. In the very unlikely chance that this method of western blotting does not work, we could instead use antibodies to investigate the proteins involved in the pathways activated by the integrin-alpha-2 following collagen binding, including the FAK, Cas, SRC, or PI3K proteins mentioned earlier (Adorno-Cruz & Liu, 2018).

4.2 Results

Results: Cell Cycle Assays. In the aftermath of conducting the western blot, we predict seeing reduced activity of integrin-alpha-2 receptors in cell cultures inundated with FOS, and thus a reduced stiffness in the extracellular matrix. By this logic, if this result is verified, that means that the cell adhesion to other cells will accordingly decrease based on Figure 16, and so this would lead to the discovery of a new link between FOS and a protein involved in the wound healing processes. We expect to see no difference in the trials between males and females

Results: Histological Analysis. In the aftermath of conducting the western blot, we predict seeing reduced activity of integrin-alpha-2 receptors in cell cultures inundated with FOS, and thus a reduced stiffness in the extracellular matrix. By this logic, if this result is verified, that means that the cell adhesion to other cells will accordingly decrease based on Figure 16, and so this would lead to the discovery of a new link between FOS and a protein involved in the wound healing processes. We expect to see no difference in the trials

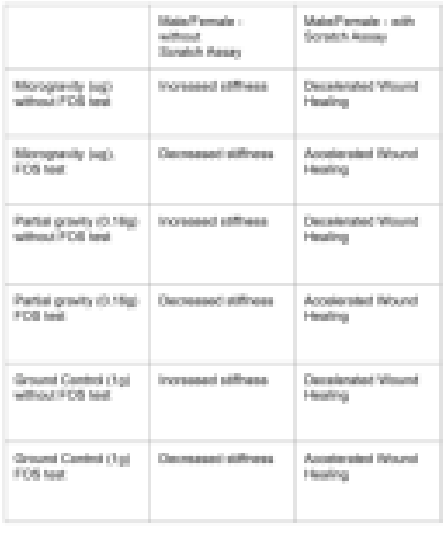


Figure 16: *Expected results, sorted by trial type in terms of diet, gravity condition, and scratch test analysis.*

between males and females

Results: Metabolomics Assays. We expect that up-regulation of IGF2 to result in distinct metabolic features in the skin tissues of mice. Particularly, we expect to see considerable changes in the metabolic pathways associated with IGF2, including glycolysis. Thus, quantitation of glycolytic intermediates is expected to show increased levels of glucose-6-phosphate, fructose-1,6-bisphosphate, and pyruvate in IGF2 upregulation mice compared with the IGF2 knockout mice. This would be an indication of high activity of the glycolytic activity attributed to up-regulation of IGF2. Finally, using targeted metabolomics, we will also show that IGF2 upregulation mice have increased acetyl-CoA levels in the cytoplasm, for it will then be shunted away from being used by mitochondria for other cellular processes. This could be due to mitochondrial dysregulation. This will result in variation of other metabolites, which may further signal conditions like increased lactate levels in the cytoplasm. Such pathway enrichment analysis would be expected to yield a number of metabolic pathways that are significantly changed in the IGF2 upregulation mice, such as fatty acid synthesis, amino acid metabolism, and nucleotide biosynthesis. These results would therefore detail more general effects of IGF2 on cellular metabolism beyond glycolysis and underline the potential of targeted metabolic interventions in conditions where IGF2 expression becomes misregulated.

Results: Western Blots. In the aftermath of conducting the western blot, we predict seeing reduced activity of integrin-alpha-2 receptors in cell cultures inundated with FOS, and thus a reduced stiffness in the extracellular matrix. By this logic, if this result is verified, that means that the cell adhesion to other cells will accordingly decrease based on Figure 16, and so this would lead to the discovery of a new link between FOS and a protein involved in the wound healing processes. We expect to see no difference in the trials between males and females.

4.3 Management and Cost Plan

As a part of our experiment, we would like to use collaborations involving mainly the procurement of the key elements for our proposal. The most important collaboration will be with The Jackson Laboratory, where we will procure wild-type, IGF2 knockout, IGF2-/-, and IGF2 overexpressing mice. We would also make use of resources and expertise at NASA Ames Research Center. The Principal Investigators (Azim, Shreya, Yash, and Pakhi) will be involved in the general management of the project, experimental design, data analysis, and manuscript preparation.

- Research Scientist: Engaged in conducting experiments, collection, and initial analysis of data
- Lab Technician: Assist in the preparation of samples and reagents and routine maintenance of the lab
- Bioinformatician: Handle data analysis from flow cytometry and mass spectrometry and statistical analysis and aid in data interpretation.

CREATE DIAGRAM FOR MANAGEMENT PLAN?

Cost Plan

Our experiment draws on established methodologies, and the experiment is performed onsite at NASA; The cost plan for the project includes various components. For analyzing cyclin levels in keratinocytes, primary antibodies for cyclins will cost $500, secondary antibodies (HRP-conjugated) $200, cell culture reagents $300, and flow cytometry reagents $150. The flow cytometer is a shared resource and incurs no additional cost. The AO/PI Flow Cytometry Assay will require Acridine Orange (AO) and Propidium Iodide (PI), each costing $100, along with $150 for flow cytometry reagents. Histological staining for wound healing will involve a H&E staining kit ($100), primary antibodies for IGF2 and Ki67 ($500), secondary antibodies (HRP-conjugated) ($200), IHC reagents ($150), and tissue processing reagents ($200). Similarly, histological staining for skin health will require a H&E staining kit ($100), primary antibodies for K10 and Involucrin ($500), secondary antibodies (HRP-conjugated) ($200), IHC reagents ($150), and tissue processing reagents ($200). The microscope is a shared resource and incurs no additional cost. For tandem mass spectrometry and metabolomics analysis, sample preparation reagents will cost $300, mass spectrometry reagents $500, and metabolomics analysis reagents $500. The mass spectrometer is a shared resource and incurs no additional cost. The cost for mice, adjusted for 10 mice each, includes $1,000 for wild-type mice, $2,000 for IGF2 knockout (IGF2 -/-) mice, and $2,000 for IGF2 overexpressing mice. The total cost for the project is estimated to be $10,000.

“Overall, with this cost analysis completed, we contend that our proposal is extremely competitive as it addresses a maximum amount of knowledge gaps for a minimal cost.”

INCLUDE IMAGE?

Timeline & Next Steps

The project timeline is divided into several phases. In the preparation phase (Month 1-2), reagents and antibodies will be ordered, coordination with The Jackson Laboratory for mouse breeding and shipment will be done, and cell culture and flow cytometry reagents will be prepared. The experimental phase (Month 3-4) will involve conducting cyclin level analysis in keratinocytes, performing the AO/PI flow cytometry assay, and conducting histological staining for wound healing and skin health. In the mass spectrometry and metabolomics analysis phase (Month 5-6), samples will be prepared for tandem mass spectrometry, and metabolomics analysis will be conducted. The data analysis and interpretation phase (Month 7-8) will involve analyzing flow cytometry and histological staining data, as well as mass spectrometry and metabolomics data, with the help of a bioinformatician. Finally, Months 9-10, the results will be compiled, and then submitted to a peer-reviewed journal for publication.

*MAKE A TIMELINE AND THEN MAKE THIS LESS BLOCKY

4.4 Future Additions to Experimental Plan

5 SIGNIFICANCE AND RELEVANCE TO NASA

5.1 General Biology Research

Fibroblast Research. Despite fibroblasts playing an incredibly significant role in inflammatory response, **"few studies have investigated the effects of existing or novel therapies on fibroblast function;"**, there haven't been major research papers that discuss novel therapies to address existing skin diseases concerning fibroblasts (Murray et al., 2009). By leveraging the strong homology of fibroblast function in both humans and mice, we plan to address the knowledge gap surrounding fibroblasts in humans (Buechler et al., 2021).

Extracellular Matrix (ECM) Research. Quite similarly, despite the role that the extracellular matrix plays in cell adhesion and inflammatory response, and despite the fact that a uniquely-stiff collagen composition within the ECM can promote significant tumor progression, researchers at the University of Texas indicate that "**the relationship between the mechanical properties of the ECM and the intracellular mechanical properties that influence cell migration is [still] not well understood"** (Seewaldt, 2014; Baker et al., 2009). Considering that integrins connect the ECM to the internal signaling pathways and considering that "rogue fibroblasts" with overactive integrins can contribute to ECM stiffness, uncovering the biological dynamics at this key flashpoint in the cell membrane of fibroblasts in mice and in humans would undoubtedly give us a better understanding as to how skin cells interact with each other; thus, by specifically targeting integrin-alpha-2 and its related protein pathways for our research, we aim to fill in the knowledge gaps in regards to the ECM (Gkretsi & Stylianopoulos, 2018; Najafi et al., 2018).

5.2 Human Factors Research

Diet Research. According to Whelan (2017), foods that are high in FOS include blue agave, garlic, chicory root, Jerusalem artichokes, asparagus, bananas, and others. Some of these foods are already included into the diet of astronauts aboard the International Space Station; for example, garlic has made its way to the ISS in the form of garlic bread, and bananas and asparagus have also been on the ISS (Space Food and Nutrition, n.d.). However, many of the more obscure foods that are high in FOS have not been introduced into the regular diets of astronauts, especially since limited research has been performed on the beneficial effects of FOS on wound healing and immune function in spaceflight. In this way, our research paper would be able to further this diet research and improve the menus of astronauts as well as those regularly affected by skin ailments on Earth.

Space-Related Skin Cancer Research. Finally, the largest implication for our research is developing safer deep-space missions for astronauts heading to the Moon, Mars, and beyond. At the moment, astronauts are already subjected to radiation that damages the skin—specifically, astronauts experience 1.12 skin rashes per flight year,

compared to 0.044 cases per year on Earth (a **2445.45% increase in skin disease occurrences**). Furthermore, in one study, 46% of long-duration ISS crew members reported notable health afflictions while in space, 40% of which were rashes (Dunn et al., 2018). With this in mind, we find it **imperative to better investigate the therapeutic properties of FOS** on wound-healing and tumor formation to minimize the net risk that astronauts face (Moreno-Villanueva et al., 2017; Myung-Hee et al., 2006). Similarly, we find it extremely important to investigate how FOS could contribute to wound-healing for astronauts heading to the Moon in the Artemis Mission, which is exactly why our novel partial gravity studies would give us an unparalleled look into diet research for Moon-travelers!

6 ACKNOWLEDGMENTS

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