From the cover: Hatchling Bobtail squid Euprymna scolopes used as a model organism to assess the role of microgravity in symbiosis-induced animal development. From: “Potential of the Euprymna/Vibrio symbiosis as a model to assess the impact of microgravity on bacteria-induced animal development.” J. Foster, et al., p. 45.
GENERAL INFORMATION

*Gravitational and Space Biology* (ISSN 1089-988X) is a journal devoted to research in gravitational and space biology. It is published by the American Society for Gravitational and Space Biology, a non-profit organization whose members share a common goal of furthering the understanding of the biological effects of gravity and the use of the unique environment of spaceflight for biological research. *Gravitational and Space Biology* is overseen by a steering committee consisting of the Publications Committee, the Editor, the President, and the Secretary-Treasurer of the ASGSB.

The American Society for Gravitational and Space Biology was created in 1984 to provide an avenue for scientists interested in gravitational and space biology to share information and join together to speak with a united voice in support of this field of science. The biological effects of gravity have been acknowledged since Galileo’s time, but only since the 1970s has gravitational biology begun to attract attention. With the birth of the space age, the opportunity for experimentation over the full spectrum of gravity finally became a reality, and a new environment and research tool became available to probe biological phenomena and expand scientific knowledge. Space and spaceflight introduced new questions about space radiation and the physiological and psychological effects of the artificial environment of spacecraft.

The objectives of ASGSB are:

- To promote research, education, training, and development in the areas of gravitational and space biology and to apply the knowledge gained to a better understanding of the effect of gravity and space environmental factors on the flora and fauna of Earth.
- To disseminate information on gravitational and space biology research and the application of this research to the solution of terrestrial and space biological problems.
- To provide a forum for communication among professionals in academia, government, business, and other segments of society involved in gravitational and space biological research and application.
- To promote the study of concepts and the implementation of programs that can achieve these ends and further the advancement and welfare of humankind.

**MEMBERSHIP:** The American Society for Gravitational and Space Biology welcomes individual, organizational, and corporate members in all of the basic and applied fields of the space and gravitational life sciences. Members are active in the fields of space medicine, plant and animal gravitational physiology, cell and developmental biology, biophysics, and space hardware, and life support system development. Membership is open to nationals of all countries. Members must have education or research or applied experience in areas related to the Society’s purposes, and student members must be actively enrolled in an academic curriculum leading toward a career related to the Society’s purposes. Membership applications may be obtained at the society website (http://www.asgsb.org).

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Gravitational and Space Biology

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Gravitational and Space Biology

Instructions to Authors

Brief Overview:

The journal of the American Society for Gravitational and Space Biology (ASGSB), *Gravitational and Space Biology*, publishes quality, peer reviewed manuscripts in several categories. Manuscripts should be self-contained, and all conclusions substantiated and supported by results in the form of figures and/or tables. Authors are held to standards of writing (American English) for clarity and material appropriate for the *Gravitational and Space Biology* (GSB) journal. Subject matter can include any topic within the following broad categories: the impact of gravity and changes in the gravity vector on biology, spaceflight research (ISS and Shuttle), satellite payloads, advanced life support, planetary and orbital analog research, suborbital research, parabolic flight, sounding rockets, high altitude balloons, astrobiology, plus hardware development, mechanobiology, and other disciplines exploring the interface of biology and engineering technology. Brief summaries of manuscript types and guidelines for each category are below; detailed instructions and templates follow.

I. Short Communications.

Short communications are submissions typically 2 - 3 formatted pages in length (1000 – 2000 words, excluding references). These submissions are to be comprised predominantly of preliminary data for a larger study or a brief report to support work of a larger nature. It may be beyond the scope of these submissions for further experimentation, but a reviewer may request additional explanation of the presented data and hold the authors to appropriate conclusions for those data.

II. Methods papers.

Methods papers are manuscripts typically 3 - 6 pages in length. These manuscripts are comprised of data and protocols that support flight experiments or ground control experiments, of protocols in support of fundamental studies exploring biological responses to altered gravitational environments, and to biological responses to space and planetary analogs. The manuscripts should contain sufficient detail to enable a reader to replicate the protocol. Reviewers should particularly address shortfalls of detail, validation of protocols, and inconsistencies in any aspect of presentation. Figures may include illustrations of procedures and set-up and should include data that verify the efficacy of the procedures.

III. Research papers.

Research papers are manuscripts of typically 8 - 15 pages in length and, although there is no strict limitation to size, a reviewer may address extremes of brevity or length as appropriate to conveying the information. These manuscripts present original research of interest to the gravitational and space biology community

IV. Review articles.

Review articles are typically 10 - 15 pages in length. These manuscripts are often solicited from symposium speakers at the annual ASGSB meeting, but they are not limited to those solicitations. Any author may approach the editorial board with a suggestion or request to submit a review article, to be peer-reviewed as any other paper. A review article will be judged principally for accuracy of information and citation and appropriate scope and relevance of the subject of the article.

Detailed Instructions:

Format

The same basic format is used for each type of article. Consult a current issue of *Gravitational and Space Biology*, as well as the instructions below, for guidance on formatting, organizing, and preparing references, figures, tables, and legends. An article must have a brief abstract that summarizes the principal conclusions of
the paper. Manuscripts are submitted electronically as single column, double spaced Word documents, and figures as separate, individual documents. Details are provided below.

**Arrangement**

Arrange the manuscript in the following order, with all pages numbered consecutively in the footer of the lower right corner. The last name of the first author should precede each page number.

**Cover page** – In a separate page, include the Title, suggested Running Head (not to exceed 60 characters, including spaces), the full names and affiliations of all authors, and detailed Contact Information for the Corresponding Author: name, address, e-mail, telephone number. Also, provide a list of about 10 Key Words or short phrases; avoid generic terms, and terms already present in the Title.

**The remaining sections proceed without page breaks**

**Title** – Use a descriptive title (not to exceed 200 characters, including spaces).

**Authors** – Provide the complete names and affiliations of all authors; indicate the corresponding author.

**Abstract** – Summarize the principal approach and conclusions of the paper. Abstracts are not to exceed 150 words in Short Communications and 250 words in all others.

**Body of paper** – For Research Papers, the body of the paper should be arranged into subsections for Introduction, Materials and Methods, Results, and Discussion. Review Papers should be organized in a manner appropriate to the subject. Methods papers should include a short Introduction and also a Discussion of the application addressing the significance of the method being described. The Short Communication papers are not required to contain subdivisions, other than a short abstract, but may be organized into subsections at the discretion of the authors.

**References and Citations** – Cite each reference in the text by author(s) name(s) and the publication date: Examples: Smith, 1989 (one author) Smith and Jones, 2001 (two authors) Smith et al., 2010 (more than two authors).

- Alphabetize the reference list by authors' last names.
- List only published or in-press articles. Unpublished results, including personal communications and submitted manuscripts, should be cited as such in the text.
- References formatted as follows: author(s): last name(s) comma followed by initial(s) and a period comma before next author; year of publication followed by a period; article title in sentence case, followed by a period; journal title (italicized), followed by volume number, issue number in parenthesis (if applicable), a colon, and page numbers. Previous issues can be used as a guide, and an EndNote™ style template can be downloaded at the website. Two examples are provided below:

  **Journal Article:**

  **Book:**

**Figures** – Figures are submitted as separate graphic files. Resolution must be 300dpi

- Number Figures consecutively as they are used in the text.
- The first time a figure is discussed, refer to it actively rather than parenthetically.
- Provide enough information in the Figure Legend such that the reader can understand the figure without significant input from the text. For submission, provide Figure Legends at the end of the body of the manuscript, following the Reference section.
- Designate figure sections with letters and explain all symbols and abbreviations that are used in the figure.
Cover Art  – GSB welcomes the submission of artwork for the cover of the issue in which the manuscript will be published. Artwork must be submitted as a graphic file of 300 dpi or greater.

Tables – Tables are submitted on separated pages at the end of the manuscript.

- Number Tables consecutively as they are used in the text.
- The first time a table is discussed, refer to it actively rather than parenthetically.
- Give each table a concise title, followed by a legend that makes the general meaning of the table comprehensible without reference to the text. For submission, provide Tables and Table Legends at the end of the body of the manuscript, following the Figure Legends.
- Tables should be constructed in Word or Excel with the general format:

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Atmospheric pressure relative to altitude.</th>
</tr>
</thead>
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<td>Pressure (kPa)</td>
<td>Altitude (m)</td>
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<td>101 – 70</td>
<td>0 – 3000</td>
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<td>70 – 50</td>
<td>3000 – 5500</td>
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<td>5500 – 9000</td>
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<tr>
<td>30 – 5</td>
<td>9000 – 27000</td>
</tr>
</tbody>
</table>


Abbreviations or Other Standards

- Do not use abbreviations other than those that are standard for international usage.
- Use SI units as far as possible.
- Use $g$ (italicized) for unit gravity, to distinguish it from the standard abbreviation $g$ (not italicized) for gram.
- Use spaceflight (one word) rather than space flight (two words).
- Any acronyms that are used in the manuscript must be defined at first mention.

Manuscript Review and Preparation of Final Version

Prior to publication, manuscripts are reviewed by the editor assigned to an author’s article and, generally, by two scientific reviewers.

If reviewers recommend only minor textual changes, the editor may choose to make these changes and accept the manuscript essentially as submitted. The editor then sends the accepted manuscript to the journal’s publishing editor. Page proofs are provided to the authors for review prior to the journal going to press.

Deadlines

Editors will inform authors of their deadlines. Deadlines will have limited flexibility, but under no circumstances will the publication of the journal be delayed to accommodate late manuscripts.
Authorship Statement

Authorship of articles implies that an individual has made a substantial contribution to the article both in terms of the design of the study or collection/evaluation of data and with regard to the intellectual content of the manuscript.

Conflict-of-Interest Statement

Reviewers recruited for the evaluation of manuscripts being considered for publication in *Gravitational and Space Biology* will be held to Conflict of Interest standards comparable to those required of NSF and NASA panelists. You may be considered in conflict if:

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Journal Policies

indicate whether the institutional and national guide for the care and use of laboratory animals was followed. For research using Recombinant DNA, physical and biological containment must conform to National Institutes of Health guidelines or those of a corresponding agency.

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The general requirements for informed consent conform to guidelines and requirements outlined by the National Science Foundation [http://www.nsf.gov/bfa/dias/policy/docs/45cfr690.pdf] and Health and Human services [http://answers.hhs.gov/ohrp/categories/1566]. No investigator may involve a human being as a subject in research covered by this policy unless the investigator has obtained the legally effective informed consent of the subject or the subject's legally authorized representative. An investigator shall seek such consent only under circumstances that provide the prospective subject or the representative sufficient opportunity to consider whether or not to participate and that minimize the possibility of coercion or undue influence. The information that is given to the subject or the representative shall be in language understandable to the subject or the representative. No informed consent, whether oral or written, may include any exculpatory language through which the subject or the representative is made to waive or appear to waive any of the subject's legal rights, or releases or appears to release the investigator, the sponsor, the institution or its agents from liability for negligence.

Basic elements of informed consent.

In seeking informed consent the following information shall be provided to each subject:

- A statement that the study involves research, an explanation of the purposes of the research and the expected duration of the subject's participation, a description of the procedures to be followed, and identification of any procedures which are experimental;
- A description of any reasonably foreseeable risks or discomforts to the subject;
- A description of any benefits to the subject or to others which may reasonably be expected from the research;
- A disclosure of appropriate alternative procedures or courses of treatment, if any, that might be advantageous to the subject;
- A statement describing the extent, if any, to which confidentiality of records identifying the subject will be maintained;
- For research involving more than minimal risk, an explanation as to whether any compensation and an explanation as to whether any medical treatments are available if injury occurs and, if so, what they consist of, or where further information may be obtained;
- An explanation of whom to contact for answers to pertinent questions about the research and research subjects' rights, and whom to contact in the event of a research-related injury to the subject; and
- A statement that participation is voluntary, refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled, and the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.
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In Memoriam

Remembering Mary

This August, the American Society of Gravitational and Space Biology lost one of its founding members, Mary E. Musgrave. Members of the community lost a friend, a colleague, a mentor, a role model and much more. But what we will never lose is her spirit; it infuses so many aspects of this society, and has been shared and reflected on countless levels by us all. It has been said that “…Mary never forgot that the real goal for space research was adventure.” Thank you Mary, we will remember. – Anna-Lisa Paul

What follows is a set of remembrances contributed by several who knew Mary well. We discussed as a group how best to compile these thoughts, how to synthesize them into a cohesive tribute that did justice to her memory, particularly in the context of the society. In the end, it was clear that each tribute deserved its own voice, and each was preserved as it was contributed. Further, we beg the understanding of all of you who would have liked to contribute in kind … we know you share these thoughts.

Marshall Porterfield. Every fall we in academia welcome the start of a new academic calendar, and the new freshmen who represent rebirth in the next generation. This year those feelings are tempered by the loss of a great research leader, mentor, and teacher. Mary grew up in an academic environment as her father was a renowned professor at Cornell. She didn’t stray far from her roots, and pursued graduate degrees and an academic career track that culminated in administration. During her career Mary represented the pinnacle of success because she mastered all aspects of teaching, discovery, and service.

As my PhD advisor Mary had a tremendous impact on my career, philosophical approach, and personal outlook. As a mentor and role model, I am extremely grateful and hold her in my heart at the highest level of reverence, respect and affection. Beyond my short time with her in graduate school she continued to be supportive and provide advice and encouragement. She served as the shining example of what could be achieved through hard work, and innovative science. Leading by example is the most powerful and direct way to provide mentorship, and Mary provided this at all levels, to all she encountered and worked with.

In the field of Gravitational and Space Biology, there are only a handful of individuals who have had as much impact as Mary. Her research program innovated and pioneered the application of stress physiology and fundamental biophysical approaches in order to advance our understanding, and ability to successfully grow plants beyond the limits of earth. Her work focused on plant reproduction, but has had tremendous impact at all levels of the science as well as current and future engineering efforts. The impact of her work and pioneering efforts will play a critical role in the development of advanced biomimetic life support technologies that will support the future of manned long duration exploration.

Our society also continues to benefit from the years of hard-work and dedication to the society that she demonstrated. She served on numerous committees, the governing board, and eventually served as President of the society. Her leadership and vision in these roles continues to benefit and reverberate within our community. She also served as Publications Editor for the ASGSB, and dedicated countless hours of work in that role. Beyond her scientific impact we as a society owe Mary an incalculable
Remembering Mary

debt for her dedication and leadership to the society.

In every conceivable way Mary exemplifies the definition of scientist and professor. Beyond her role as mentor, discoverer, colleague, administrator, pioneer, advocate, and leader she was also a dedicated mother and spouse. So in our remembrance and in our sorrow and anguish of our loss we should try to find solace in this season of academic rebirth. Mary lives on in her work but also in each of us that she touched. It is now our obligation to pass that along to the next generation in the classroom, laboratory, and community. We all owe Mary a tremendous gift of gratitude, and I am most thankful here today standing on the shoulder of a giant… and the view is pretty good up here.

Stan Roux. As a charter member of the ASGSB I am keenly aware that Mary Musgrave was among the top luminaries who guided and contributed most to the Society during its first three decades. She was one of those rare persons who combined intelligence, eloquence, clarity of vision, energy and loyalty into a force for good, and it was by some remarkable good fortune that she chose to channel so much of this force for the benefit of ASGSB and for the gravitational and space biology science the society promotes. Many of her gifts to ASGSB were quietly given and often unnoticed, but certainly her more obvious gifts were most impressive: I would like to especially note her classic, oft-cited publications in the field and her invaluable contributions as president of the Society and as Editor of the ASGSB Newsletter and Science issues. I and others in the Society sought her counsel often and we were never disappointed. Her passing is a great loss to the Society, but an even greater loss to those who experienced the impact of her gifts even more personally and powerfully than we. Mary, we are grateful that we knew you, and you will be sorely missed.

Joan Allen. I had the rare good fortune to work with Mary on her NASA funded experiments on seed development in hypogravity as a research assistant from 2005 through 2008. Her professionalism, intelligence, kindness, sense of humor and enthusiasm were outstanding traits that come to mind. She was a mentor and a friend. She was generous in sharing both her knowledge and credit and recognition for our work. The one thing I would like to share with all those who knew, respected and loved Mary is how apparent it was to me that she deeply cared for and respected those she worked with and the fine institutions that you all represent. At UConn, she was a devoted advocate for individuals, our department, and the university. She was devoted to her family as well. I will miss her very much.

Lanfang Levine. There are no words that can adequately express my sorrow and the hole left in my heart by the loss of Dr. Mary Musgrave. There is no space that is sufficient to accommodate my gratitude to her. The best I can do is to relate some of my memories of her. These are personal memories for me, but may resonate with some of you.

I got to know Mary through her former graduate, Lindsey Tuominen, who came to my lab as a Planetary Biology Intern in 2004. Since then, we started a productive collaboration. To this date we still have an active project. I feel privileged to have had the opportunity to work with Mary, and I was constantly amazed at the breadth and depth of her knowledge and interests, clarity of her thoughts, lucidity of her writing and contagiously sunny and positive altitude. These traits and her sense of humor were also displayed vividly in a feature article titled “Reflections on Relevance” (Musgrave 2008).
Because she could seem reserved and inaccessible at times, it was fun to discover the personal side of her. It is not a stretch to call Mary a champion of edible landscaping. If you ever set foot on her property, you could feel the life on every inch of the soil (corn, turnips, onions, grains, black currants, red currants). During one of my visits, she invited me to dine with her and her husband John. Almost everything served came from their garden or was raised by them. It was a surprise and delight to find how tasty a simple free range oven baked chicken could be. It demonstrated that something could be simple and exquisite at the same time. Mary was a practitioner of many causes, especially conservation. She was also a great seamstress and used it to relax between her research proposals.

I admired her most for her personal integrity and was deeply touched by her thoughtfulness. You could always count on Mary to deliver what she said she’d do. Four months ago, I visited her for the first time since her cancer diagnosis. I arrived at Mary’s lab on a rainy April New England day at 8:30 am. Knowing of her three surgeries and months of chemotherapy, I did not expect to see her at such an early time, yet she was already in the lab to greet me and had supplies autoclaved so that we could dive into the experiment immediately. In spite of the toll taken on her physical strength by the cancer and medical procedures, she was still fully committed to our research project. Literally speaking, she was the honeybee, hand pollinating hundreds flowers to ensure synchronized development of siliques for our experiments. She patiently explained the value of the education aspect of the project and details of experimental procedures under development. She even arranged to have a key piece of equipment loaned to me and seeds shipped. I will never forget the last words she said to me “This is such an interesting project, I just love it.” She and those moments we shared will live in my heart forever. As Helen Keller articulated: "What we have once enjoyed we can never lose. All that we love deeply becomes a part of us.”

**Rebecca Darnell.** I didn’t know Mary through the ASGSB Society, but rather, I was a visiting scientist in her lab at UConn for eight months. I was interested in doing a sabbatical in an area I knew nothing about, and Mary kindly and generously accepted me into her lab. I fully expected that Mary would be very busy with both her research and her administrative responsibilities, and so was much surprised when I arrived at her office the first day and she personally escorted me to all the places I needed to go to for my ID card, parking permit, library privileges, etc. This generosity of spirit (and time) set the tone for my working with Mary. Mary was gentle, kind, respectful of others, and ferociously passionate about science. We spent several weeks working long hours together at the NASA Ames Research Center and it was there I really discovered that, along with all the other wonderful attributes Mary exhibited, she had a wonderfully low key sense of humor. I enjoyed those trips immensely and will always be grateful to Mary for allowing me to share her world.

**References and Links**


Xerces Society [http://www.xerces.org/give/](http://www.xerces.org/give/)
Autonomous Gravity Perception and Responses of Single Plant Cells
Mari L. Salmi¹, Thomas J. Bushart², and Stanley J. Roux²
¹Morehouse School of Medicine, 720 Westview Dr SW, Atlanta GA, 30301; ²The University of Texas at Austin, Section of Molecular Cell and Developmental Biology, Austin, Texas 78712-0183

ABSTRACT
Although multicellular plants are favored experimental subjects for most studies of gravitational responses in plants, there are single cells that can both sense and respond to gravity, independent of any input from other cells. This review focuses on what is known about these single-cell phenomena. Highlighted are studies in algae and moss that relate to the statolith hypothesis, studies in Euglena on gravitaxis, and studies in fern spores on gravity-induced calcium signaling and gene expression changes.

INTRODUCTION
Multicellular plants are favored experimental subjects for most studies of gravitational responses in plants, and the most studied of these responses is gravitropism (Morita, 2010). In multicellular plant gravitropisms, such as the downward curvature of roots and upward curvature of shoots, diverse tissue interactions are involved. Specifically in roots, chemical communication between the cap and the elongation zone, including the transport of the growth hormone auxin, appears to be crucial for the gravity signal to be transduced into downward growth (Harrison et al., 2007). Of course, in all these instances the initial response to gravity must occur in individual cells, even though, as in the case of root-cap cells, the graviresensing cells themselves may not show a growth change. However, there are single cells that can both sense and respond to gravity, independent of any input from other cells. This review will focus on what is known about these single-cell phenomena.

Statolith Model of Gravity Sensing: Single-cell Results
While perhaps not applicable to all plant species, the statolith model of gravity sensing in multicellular plants is well established. The model is based on the observation that certain organelles move downward (sediment) when the position of the cell is changed. In Arabidopsis columella cells the statoliths are dense starch-containing amyloplasts. Reduced-starch mutants have both decreased statolith sedimentation ability (MacCleery and Kiss, 1999) and reduced gravitropic responses (Kiss et al., 1996). These and subsequent studies favor the hypothesis that sedimenting organelles help initiate gravity sensing in multicellular tissues (Strohm et al., in press). Evidence that sedimenting organelles initiate gravity responses in single cells has also been published in

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multiple reports, most of which used cells of moss and algae as experimental subjects.

In gametophytes of the moss *Ceratodon purpureus*, amyloplast sedimentation results in negative gravitropic curvature of the protonemata, which act autonomously in their response to gravity. Conveniently, there is a wrong way response (wwr) mutant which exhibits positive gravitropic growth. The kinetics and directions of growth of these wwr mutant plants is exactly opposite that of the wild type. The mutated gene product in wwr plants seems to function downstream of amyloplast sedimentation since the position of sedimenting amyloplasts is unaltered in the mutant plants after gravity reorientation (Wagner et al., 1997). Kuznetsov et al. (1999) utilized strong magnetic fields to manipulate amyloplasts independent of the gravity vector to cement the connection between statolith movement and gravity response (Figure 1, A-D). In two instances the protonemata were rotated in a clinostat while exposed to a high-gradient magnetic field (HGMF) while in the third case they were not rotated. The clinostat served to nullify the sedimenting effects of gravity, and indeed the untreated protonemata showed neither sedimentation nor curvature (Figure 1D). However, the amyloplasts under clinorotation would sediment with an applied magnetic vector. The wild-type protonemata, as predicted, would sediment with an applied magnetic vector forces (opposite the direction of HGMF-induced sedimentation) as seen in Figure 1A. This was true regardless of the shape of the applied magnetic field. The wwr mutants showed the expected inverted growth pattern in relation to the HGMF displacement of their amyloplasts (Figure 1B). The inclusion of wwr mutant plants in this study demonstrates the importance of amyloplast sedimentation location in this system, regardless of differences in the signaling process downstream of that sedimentation. The researchers also noted a positive correlation between both field intensity and amyloplast size and the degree of the curvature response (Kuznetsov et al., 1999). They also noted that the responses support the statolith hypothesis over the pressure model. While these data directly tie the location of sedimenting statoliths to the detection of the vector of gravity, they do little to define the nature of this receptor mechanism. Still, this dovetails neatly with their previous work in *Arabidopsis* roots showing that magnetic field alterations to amyloplast sedimentation alter gravitropic curvature of the roots in a similar fashion (Kuznetsov and Hasenstein, 1996).

Magnetic fields are not the only way of experimentally manipulating statolith position in single cells. *Chara* algae contain large (diameter up to 30 µm), tube-like rhizoid cells that exhibit positive gravitropism (grow downward), and protonemata that exhibit negative gravitropism (grow upward), offering an elegant system for examining both gravitropic responses in single cells within the same species. Both of these gravity-sensing cell types contain structures that have been reported to serve the role of statoliths: i.e., vesicles filled with barium sulfate crystals (BaSO₄) that sediment in the direction of the gravity vector. The rhizoids and protonemata of characean algae have been manipulated using “optical tweezer” laser trapping. Laser-assisted displacement of 2-3 statoliths against the side of a horizontally oriented rhizoid for at least 5 minutes was sufficient to cause curvature towards that side (Figure 1, A’-D’). Similarly, the same conditions in a protonema lead to negative-gravitropic-like curvature away from the site of the repositioned statoliths (Braun, 2002). The manipulation of statoliths into various regions of *Chara* cells was an especially valuable approach due to two key observations. First, *Chara* cells appear to have a limited, belt-like region where gravity perception takes place (Braun, 2002) in both protonemata and rhizoids. Other gravi-responsive plant cell types are able to detect a change in orientation in any direction. Second, the actual movement of the statoliths does not appear to be the key to responsiveness (Braun, 2002). Rather, a cell requires contact, or pressure, from the statolith at the regions of sensitivity.

Findings using the *Chara* model system to study gravity perception in plant cells were recently reviewed by Braun and Limbach (2006). As they reiterate, it is clear that the position of the statolith near the plasma membrane within the region of graviperception of the rhizoid or protonema is integral to the cell’s gravity response and to its change in direction of growth. However, this positional requirement could be acting through simple contact or by secondary conditions of the contact (e.g. applied pressure). While the contact model has strong evidence, the pressure model cannot be discounted by the data thus far.

In the first mechanism, the “gravity receptor” of *Chara* cells could act as a classical hormone, lock-and-key type receptor that specifically detects the location of these statoliths and either directly or indirectly opens ion channels. This model has some of the strongest support in *Chara* rhizoids. Limbach and colleagues observed that individual statoliths lose contact with the cell cortex in as little as 2 s after inverting *C. globularis* rhizoids. Inverting for repeated intervals as short as 10 s could impact gravitropic growth (Limbach et al., 2005). Further, the quantitative decrease in a rhizoid’s angle of...
Curvature appears to directly relate to the total time of inversion, and logically, therefore, to the total time interval statoliths were not in contact with the cell cortex. Perhaps the most compelling data supporting the contact model was that a decrease in gravitational forces achieved during parabolic flight experiments did not alter rhizoid curvature in any statistically significant manner (Limbach et al., 2005). The rhizoids were pre-stimulated by being rotated horizontally for 10 minutes before the first parabola, meaning that the statoliths would already have sedimented and achieved cortex contact. Further, 2 g conditions achieved through centrifugation did not affect rhizoid curvature in any detectable manner (Limbach et al., 2005). Therefore it appears that graviperception in Chara rhizoids depends almost entirely on physical contact between organelles.

Figure 1. Single-cell responses of single cells to various gravity-related manipulations. A-D: Ceratodon purpureus cells were exposed to various magnetic fields (directional force indicated by Fm) while rotated in a clinostat to reduce the effects of gravity. Arrow heads indicate amyloplasts displaced by the High Gradient Magnetic Field (HGMF). A. WT protonema after 12 h exposure to HGMF. B. wwr protonema after 6 h exposure to HGMF. C. wwr protonema exposed to uniform magnetic field (non-HGMF). D. WT protonema away from HGMF. Bars = 50 µm. (Adapted from Kuznetsov et al., 1999, Copyright American Society of Plant Biologists, http://www.plantphysiol.org) A'-D': Growth changes over time of a single Chara globularis rhizoid manipulated by laser-assisted (circle) displacement (arrows) of statoliths (st). Bars = 10 µm. (Adapted with kind permission from Springer Science+Business Media: Protoplasma, 219, 2002, 150-159, Braun, M., figure 3, copyright Springer-Verlag 2002). Circular histograms: Histogram of directional swimming of Euglena gracilis as determined by motion analysis software. Upper left. Control cells. Lower right. RNAi against CaM.2, 15 days post-electroporation. Solid black arrow (theta) represents main movement direction of cells in the culture. r value represents precision of movement (0 = random, 1 = precise). (Adapted with kind permission from Springer Science+Business Media: Planta, 231, 2010, 1229-1236; Daiker, V. et al., figure 5, copyright Springer-Verlag 2010.) Line graph: Representative plot of calcium flux changes of a single Ceratopteris richardii spore in relation to changing gravity intensities from a parabolic flight. Vertical grey bars represent time of transition between hyper- and micro-g conditions. Arrows indicate time of change in calcium current (adapted with kind permission from Springer Science+Business Media: Planta, 233, 2011, 911-920; Salmi, M. et al., figure 4, copyright Springer-Verlag 2011).
These results may be related to ultrastructural observations in multicellular, statolith-dependent gravitropic responses such as in *Zea mays* roots. There, ER is largely absent in the central regions of the cells but enriched at the cell cortex (Yoder et al., 2001). A sedimentsing statolith, then, would pass through ER-devoid regions to settle at ER-rich points at the cell membrane. It is likely not a coincidence that the ER is a main storage organelle for Ca\(^{2+}\), an ion implicated in gravity perception and responses across multiple species. In animal systems, membrane contacts between the ER and PM are important for the phenomenon of store-operated calcium entry mediated by certain calcium-release activated calcium (CRAC) channels. To our knowledge, homologs of Orai1 and STIM1 have not been identified in plant systems, but calcium-induced calcium release has been documented with slow vacuolar (SV) channels participating in calcium participation of the whole protoplasm as a sensor, integrated by the cytoskeleton to function as a single mass, cannot be ruled out, particularly in systems lacking sedimenting organelles or particles.

Single spore cells of the fern *Ceratopteris richardii* offer another system for examining the statolith hypothesis. Here gravity directs the downward migration of the nucleus, which occurs about 24 h after the spore is induced to germinate by light. That the downward migration of the nucleus is, indeed, directed by gravity was demonstrated in Shuttle mission STS93, during which video microscopy revealed that the migration still occurred in microgravity, but its direction was random instead of being polarized toward one end of the cell (Roux et al., 2003). The nuclear migration toward the bottom of the cell at 1 g both localizes the site of the first cell division and the subsequent downward emergence of the rhizoid. These cytological effects of gravity on cell polarity are already set about 15 h before the nuclear migration begins, so the downward movement of the nucleus is part of the response to gravity rather than being part of the perception event (Edwards and Roux, 1994; Edwards and Roux, 1998). Most of the plastids in the spore are closely appressed around the periphery of the nucleus, and separate movements of these plastids prior to when the polarizing effects of gravity are set were not observed. Thus a clear statolith candidate has not yet been identified for the fern spore response to gravity.

**Single-cell Taxic Response to Gravity**

Unlike the other plant systems discussed here, the unicellular ciliate alga *Euglena gracilis* has a slightly different relationship to gravity. Since they are both aquatic and motile they exhibit a number of taxic responses in order to optimize their ability to carry out photosynthesis in a body of water. Light is scattered by water so light intensities inversely relate to depth. For proper photosynthesis *E. gracilis* needs to remain near the surface of a water column. The most obvious response, therefore, would be to light, and they do exhibit strong, intensity-dependent phototaxis. However, as light is not continuous throughout a 24 hr cycle, gravity is also an important
stimulus for *E. gracilis* to remain near the surface. To that end, *E. gracilis* exhibit a negative gravitaxic response.

Spaceflight experiments have demonstrated that the direction of swimming becomes random when gravity is less than 0.16 of normal (Häder and Hemmersbach, 1997). It is also thought that gravity is mainly sensed through differential pressures (dictated by the gravity vector) on cells. This idea is supported in *E. gracilis* by experiments manipulating media density. Directional movement was impaired without affecting taxis rate by increasing the surrounding densities. Even higher densities reversed the direction of normal taxis, effectively resulting in a situation where the algae appear to be positively gravitaxic (Häder and Hemmersbach, 1997). These outcomes parallel those we see in other plant systems. So while these protozoan-like algae differ in response (movement instead of asymmetric growth), they appear to share a similar mechanism for detection of the gravity vector.

More recent work with *E. gracilis* has expanded the molecular understanding of the gravitaxic sensing and response system(s). Like other systems, calcium is thought to be a main component of the relay between perception (orientation to gravity) and response (flagellar beating). Specifically, the gravitaxis of the ciliates *Stylonychia mytilus* (Krause et al., 2010) and *Euglena gracilis* clearly involves calcium signaling (Streb et al., 2001). To investigate whether the calcium-binding protein, calmodulin, was involved in the response, Daiker et al. (2010) sequenced five distinct cDNAs of calmodulin family proteins, designated CaM.1 through CaM.5. The sequences were unique enough that specific dsRNAs could be developed against each to knock them out/down to examine the effects. RNAi against CaM.3, CaM.4, and CaM.5 had no significant impact on gravitaxis. RNAi against CaM.1 was shown to have alterations in morphology and transient alterations in gravitaxis. Early after treatment the cells exhibited random, nonprogressive (euglenoid) movement, but by 15 days post-electroporation directed, negative gravitaxis was restored. It was noted that the change in morphology did not include noticeable alterations to the flagellum itself. Suppression of CaM.2 through RNAi similarly had alterations to morphology and induction of euglenoid movement. In this case, free swimming behavior was recovered within several days to a week. Also unlike the suppression of CaM.1, RNAi against CaM.2 did not show a return to normal gravitaxis for extended periods post-electroporation (Figure 1, circular histograms). The RNAi affects appear to be maintained by these cells, as clonal *E. gracilis* out to 30 days post-electroporation still showed free swimming, but in a generally randomized vector in contrast to the strongly negative gravitaxis of the control cells.

Taken in total, it appears that not only does *E. gracilis* contain calmodulins with distinct functions, but at least one of them, CaM.2, acts as a main component in the gravitaxis response. The current model positions CaM.2 as an intermediary between gravity-induced [Ca\(^{2+}\)] changes and alterations of flagellar beating via the cAMP output of a calmodulin-dependent adenyl cyclase. So while *E. gracilis* exhibits an overall different end response to the gravity vector when compared with other non-ciliate plants, it utilizes similar transduction and/or amplification steps involving calcium.

**Modulating Responses in Single Cells by Changing the g-Force**

As described above, after light induces single-cell spores of *Ceratopteris richardii* to germinate, gravity directs the polarization of these cells, including the downward migration of the nucleus. This migration occurs some 15 h after gravity has set the polarity of the cell, so there must be some gravity-induced event that occurs earlier. Because trans-cell calcium currents, driven by influx channels on one end and pumps at the other end, were known to induce cell polarization in tip-growing systems such as pollen tubes (Weisenseel et al., 1975), Chatterjee et al. (2000) used a self-referencing microelectrode to test whether such currents occurred in *C. richardii* spores, and, if so, whether gravity could influence the direction of these currents. They found that within two hours after the spores were irradiated, a calcium influx occurred at the spore bottom, and a 20-fold larger efflux emerged from the spore top. When the spore was rotated, the bottom-to-top calcium current rapidly realigned parallel to the vector of gravity. This initial study could not resolve how quickly the rotation-induced realignment of the current occurred, but recently Salmi et al. (2011) answered this question using a silicon microfabricated sensor array that could simultaneously measure calcium currents in real time from multiple cells arranged on a microchip. Their assay showed that on earth rotating the spores on the chip reverses the trans-cell calcium current within 25 sec.

Another key finding of Salmi et al. (2011) was that a change in g-force could modulate the magnitude of the current (Figure 1, line graph). When the spores were assayed during parabolic flight on the NASA C-9 aircraft, the trans-cell current increased in hyper-g and decreased to near baseline in micro-g. The current began to rise within 2 seconds as the g-
force increased during the aircraft’s transition out of the micro-g segment of its flight. Very likely the gravity-directed trans-cell calcium current, which peaks about 9-10 h after light-induced germination (Chatterjee et al., 2000; Salmi et al., 2011) is needed for cell polarization, because at 1-g, the calcium-channel blocker nifedipine blocks the current and randomizes the direction of rhizoid emergence (Chatterjee et al., 2000, Salmi et al., 2011). In the moss Funaria, the direction of nuclear migration is also toward the site of calcium entry, and blocking calcium entry also blocks this migration (Saunders and Hepler, 1983). In contrast to the nifedipine results, the calcium-pump inhibitor eosiin yellow suppressed the current, but not gravity-induced cell polarization. Taken together, the results of this study were consistent with the conclusion that gravity-induced calcium entry through channels at the bottom of the spores is one of the earliest responses of C. richardii spores to gravity, and that gravity can rapidly (probably post-translationally) modulate the activity of channels and pumps that drive a trans-cell current in these cells.

Other studies in single-cell model systems also show that gravity perception occurs in response to gravity forces less than 1 g. Data from TEXUS sounding rocket missions indicate that E. gracilis responds to as low as 0.12 g (Häder et al., 1998) and MAXUS-5 sounding rocket missions have provided evidence that Chara rhizoids perceive 0.14 g (Limbach et al., 2005). Although the precise gravity force necessary to activate gravity receptors has not been studied in the spores of C. richardii, an obvious hysteresis of de-activation was observed on NASA parabolic flight missions that may be explained by cellular perception of sub-earth gravity (in the realm of 0.1 g) (Salmi et al., 2011). The evolutionarily conserved role of calcium, as well as the kinetics of gravity signaling in many cell types, suggests that a likely candidate for the gravity receptor in plant cells is a stretch activated calcium channel.

**Gene-expression Changes That Occur in Single Cells in Microgravity**

Recently several genes have been added to the list of those involved in various steps of the higher plant gravitropism signaling cascade (Agee et al., 2010; Fortunati et al., 2008; Nakamura et al., 2011; Yang et al., 2011). Data from microarray analysis of gene expression changes induced by changes in orientation (Kimbrough et al., 2004; Moseyko et al., 2002) or, perhaps even more significantly, development in microgravity (Paul and Ferl, 2002; Salmi and Roux, 2008) provide a very useful starting point for a more targeted forward genetics approaches to identify genetic components of gravity perception signaling. Signaling components that have been implicated in gravity perception and response in several studies, in different plants, or even other organisms are an obvious target for this type of evaluation. For instance, those genes identified in the study of root Arabidopsis gravitropism (Kimbrough et al., 2004) that have likely homologs identified the spore microgravity study (Salmi et al., 2008), such as Catalase 3, Fibrillarin 2, and Protodermal factor 1, could be evaluated in the commercially available Arabidopsis insertion mutant lines. A comprehensive evaluation of knockout lines of these genes might result in the identification of new and valuable mutants in gravity perception and/or gravitropism.

It is important to integrate these high throughput data with previously identified aspects of gravity perception and gravity directed polarity development, like the well established involvement of calcium as a component of the gravity response mechanism. In C. richardii spores, numerous genes that likely encode proteins involved in calcium mediated signal transduction have been identified. A study of gene expression changes during the germination and early development of spores (Salmi et al., 2005) found several genes which likely encode calcium binding proteins that are up-regulated during early development. None of these genes have changes between microgravity and 1g development in any of the developmental time points analyzed (Salmi et al., 2008). Of course, the most important molecular changes induced by gravity to alter the polarity of growth could all be post-transcriptional, or even post-translational. There is one gene however, likely to encode a protein with a C2 domain, that is down regulated during normal spore early development, and is also up regulated in spaceflight samples at the time point coincident with its highest abundance during normal development. The C2 domain is a calcium dependent, membrane binding feature of many proteins involved in membrane trafficking. Proteins with the C2 domain have recently been implicated in the viability and elongation of tip growing pollen tubes of angiosperms and seed germination (Lee et al., 2009; Li et al., 2007; Yang et al., 2008), two plant systems highly regulated by gravity. The expression profile of this particular gene in C. richardii spores indicates that it is more abundant early on in germination, when the gravity-directed calcium current of the spore is likely creating localized regions of high cytosolic calcium (Chatterjee et al., 2000; Salmi et al., 2011). As yet there has been no test to determine whether this gene encodes a protein essential for converting an elevated localized calcium concentration into downstream cellular events to
establish polarity. In general all the data evaluating gene expression changes should be translated into systems where targeted genetic manipulations are possible in order to directly evaluate the role of candidate genes in the cellular perception and earliest responses to gravity.

CONCLUSION

As indicated throughout this review there are significant similarities between the gravity responses found in single cells and those in other plant cells that perceive gravity (i.e. angiosperm root columella “statocytes”). Of course, there are other similarities besides those highlighted in this review. For example, cytoskeletal elements significantly influence gravity responses in single cells (e.g., in the stability and movement of Chara statolith vesicles) (Braun, 2002; Limbach et al., 2005), and in multicellular tissues (Blancaflor and Masson, 2003). To the extent that the cellular responses to gravity found in single plant cells are fundamental to cellular gravity sensing and responding, one could expect to find at least some of them, such as the activation of mechanosensitive channels and rapid calcium changes, also in animal cells. Future studies will clarify the extent to which the response mechanisms of cells to gravity are evolutionarily conserved not only in single-cell and multicellular plants, but also in animals.

REFERENCES


Space Radiation and Bone Loss

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ABSTRACT

Exposure to ionizing radiation may negatively impact skeletal integrity during extended spaceflight missions to the moon, Mars, or near-Earth asteroids. However, our understanding of the effects of radiation on bone is limited when compared to the effects of weightlessness. In addition to microgravity, astronauts will be exposed to space radiation from solar and cosmic sources. Historically, radiation exposure has been shown to damage both osteoblast precursors and local vasculature within the irradiated volume. The resulting suppression of bone formation and a general state of low bone-turnover is thought to be the primary contributor to bone loss and eventual fracture. Recent investigations using mouse models have identified a rapid, but transient, increase in osteoclast activity immediately after irradiation with both spaceflight and clinically-relevant radiation qualities and doses. Together with a chronic suppression of bone formation after radiation exposure, this acute skeletal damage may contribute to long-term deterioration of bone quality, potentially increasing fracture risk. Direct evidence for the damaging effects of radiation on human bone are primarily demonstrated by the increased incidence of fractures at sites that absorb high doses of radiation during cancer therapy: exposures are considerably higher than what could be expected during spaceflight. However, both the rapidity of bone damage and the chronic nature of the changes appear similar between exposure scenarios. This review will outline our current knowledge of space and clinical exploration exposure to ionizing radiation on skeletal health.

INTRODUCTION

The effects of microgravity and space radiation on astronaut bone health represent two of the most serious challenges present within the spaceflight environment. Loss of bone strength resulting from a reduction in bone mass or architectural stability can increase the risk of a serious fracture, threatening mission success. Countermeasures capable of both limiting the loss of bone and permitting the eventual recovery of bone strength post-flight are necessary to ensure astronauts’ long-term skeletal health and productivity of extended space missions.

Bone damage is a known chronic (late) effect of exposure to therapeutic radiation. While animal models have long documented a persistent decline in bone volume after exposure, recent animal studies have demonstrated an early and transient increase in bone resorption followed by suppressed bone formation following exposure to spaceflight-relevant doses and qualities of radiation (Kondo et al., 2009; Willey et al., 2010). This initial bone loss can be prevented using the osteoporosis therapeutic risedronate (Willey et al., 2010), further demonstrating the role of osteoclastic bone resorption.

Skeletal unloading, due to extended periods of bed rest (Spector et al., 2009) or the microgravity environment of space (Lang et al., 2004), is a well-established cause of bone loss in both humans and rodent models (Bikle and Halloran, 1999). The bone
loss that occurs as a result of unloading is characterized by both an increase in bone resorption and a decrease in bone formation. This mechanism stands in contrast to traditional forms of bone loss, such as post-menopausal osteoporosis, during which bone resorption and formation move in parallel, albeit with the former increasing to a relatively greater degree. The combination of microgravity unloading and spaceflight radiation may interact to enhance bone loss (Alwood et al., 2010; Hamilton et al., 2006; Kondo et al., 2010). As the mechanisms leading to radiation-induced bone loss are less well established than unloading-induced bone loss, this review will focus on the current state of knowledge regarding the influence of spaceflight and clinical radiation on the development of osteoporosis and bone health in the adult skeleton.

Radiation and Biological Damage

Energy absorbed from radiation sources, such as from electromagnetic or charged particles, has the ability to excite atoms to the point of generating an ion. Non-ionizing radiation will not eject electrons from an atom, while ionizing radiation can sufficiently excite electrons enough to eject the electron, creating an ionization event. The generation of these charged particles can potentially break molecular bonds and lead to biological damage, such as DNA strand breaks or membrane damage. Bonds can be broken directly by radiation, or indirectly through radiation-generated reactive oxygen species that result from the ionization of water molecules. Cells have a remarkable ability to repair radiation damage, but some cells inevitably die or propagate damage to progeny. Humans on Earth are exposed to natural, background sources of radiation (e.g., environment, space, medical imaging, internal radioisotopes) at doses that do not affect long-term health at a measurable level. However, as exposures increase, damage can lead to greater levels of cell death or radiation-induced phenotypic changes that may be heritable.

Absorbed dose of radiation (D) is measured in energy per unit mass with the SI unit being Gray (Gy = Joule/kg). It is also common to see dose expressed in the unit of rad, where 100 rad = 1 Gy (1 rad = 1 cGy). The degree of biological damage can vary depending on the quality of radiation exposure, which itself is influenced by factors such as linear energy transfer (LET); type of radiation, such as alpha particles, beta particles, gamma rays (X-rays), neutrons, or heavy ions; and energy. For example, exposure to 10 cGy of neutrons will be more damaging than exposure to 10 cGy of X-rays. To account for these varying degrees of damage to humans, Sievert (Sv) is the SI unit for absorbed dose equivalent (H), which is the quality factor (Q) (relative biological effectiveness: RBE) times the dose (H = D*Q). Generally, 1 Gy of X-rays is equal to 1 Sv (100 rad = 100 rem) (Hall, 2000).

Spaceflight and Clinical Radiation Environments

The space radiation environment consists of a complex mix of ions from solar particle events (SPEs), which are large mass ejections from the sun commonly known as “solar flares,” and galactic cosmic radiation (GCR), a type of background radiation that originates from outside the solar system. The majority of GCR flux is from protons. While only 1% of GCR is composed of ions heavier than helium, due to the high LET of these high charge (Z) and energy (E) particles (HZE), approximately 41% of the dose equivalent is predicted to be from HZE particles with approximately 13% being from iron alone (Bandstra et al., 2009). LET is the rate of energy lost per length of a particle track and varies as the charge squared divided by the velocity squared. During extended missions in space, estimated tissue dose-rates from GCR would be about 0.4-0.8 mGy/day and 1-2.5 mSv/day, respectively (NCRP, 2000). SPE dose-rates may reach as high as 50 mGy/hr inside a shielded vehicle and between 250 mGy/hr for an astronaut exposed during extra-vehicular activity in deep space. The cumulative GCR doses on a deep space mission of 400 days to a near-Earth asteroid would be 0.16 to 0.32 Gy or 0.4 to 1.0 Sv. For a large SPE lasting 8-to-24 hours, the whole body cumulative doses could reach the 1- to 2-Gy level for protons (1.0 to 2.0 Sv) depending upon the tissue site. For comparison, cancer patients will receive daily doses (fractions) of 1.8 to 2.0 Gy targeted locally to the tumor and delivered over a period of a few minutes, with the total dose to the tumor ranging from 50 to 80 Gy or more.

Radiation is also absorbed by normal tissues surrounding a tumor during the course of radiation therapy (RT) for the treatment of cancer. The absorbed dose can be substantial. For example, treatment regimens for gynecological or pelvic (e.g., anal) tumors commonly prescribe the administration of thirty 1.8 Gy fractions over six weeks, for a total dose of approximately 54 Gy to the tumor. Throughout the course of treatment, normal skeletal tissue constituting each “hip” (e.g., femoral neck, sacrum, acetabular rim) can receive as much as half of each fraction (0.9 Gy), totaling 27 Gy. While the primary concern in radiotherapy has always been curative treatment of the tumor, the incidental irradiation of normal tissues is not insignificant. Primary consideration has always been given to radiation effects on normal nervous tissue, reproductive structures, and hollow organs (i.e.,...
esophagus, bowel), while concern for irradiation of bone was not a priority. Advances in surgical excision, concurrent chemotherapy, and modern RT modalities have afforded greater freedom to focus on minimizing the risk to bone and the potential for increased fracture risk.

**Fractures of Irradiated Bones After Clinical Exposure**

Ionizing radiation is an important and effective modality for the treatment of malignancies and has been a critical factor in the reduction of cancer mortality rates. With an increase in long term survivorship, however, the incidence of the long-term side effects caused by radiation damage to normal tissues near the tumor are of greater concern. Fractures at irradiated skeletal sites represent one such late effect. Bones within the irradiated volume exhibit a substantially increased fracture risk (Baxter et al., 2005; Brown and Guise, 2009; Florin et al., 2007; Guise, 2006; Oeffinger et al., 2006). Rib fractures have been documented in patients receiving treatment for breast cancer, with fracture rates ranging from 1.8% (Pierce et al., 1992) to as high as 19% (Overgaard, 1988). Similarly, patients receiving radiotherapy for various pelvic malignancies are at increased risk for hip fracture at the aforementioned pelvic skeletal locations that absorb dose (Baxter et al., 2005; Mitchell and Logan, 1998; Williams and Davies, 2006). A recent retrospective analysis of more than 6,400 postmenopausal women receiving RT for cervical, rectal, and anal cancers demonstrated an increased relative risk for hip (primarily femoral neck) fracture of 65%, 66%, and 214%, respectively, compared to women receiving non-RT cancer treatment such as surgery or chemotherapy (Baxter et al., 2005). These fractures were localized to the area that absorbed dose, and did not occur at distant skeletal sites, such as the wrist. Therefore, while systemic or non-targeted radiation effects cannot be discounted, clinically, the response seems limited to the irradiated volume.

**Deterioration of Bone Quantity and Quality After Irradiation**

Deterioration of bone quantity and quality following direct irradiation is thought to be associated with traumatic and spontaneous fractures of bone (Baxter et al., 2005; Ergun and Howland, 1980; Howland, 1975). Reduction in bone mass and overall bone quality is dependent on a variety of factors, including the dose absorbed, the energy of the radiation beam, the fraction size of the radiation dose, and the age and developmental stage of the patient (Mitchell and Logan, 1998; Overgaard, 1988; Williams and Davies, 2006). For cancer patients receiving doses considerably higher than spaceflight exposures, osteopenia (defined as reduced bone density) is frequently reported in patients one-year post-therapy, although the observed timing and degree of bone mass reduction can be variable (Hopewell, 2003; Mitchell and Logan, 1998). Overall, demineralization of bone, thinning of bones, sclerosis, and loss of trabecular connections has been characterized as a consequence of radiotherapy (Ergun and Howland, 1980; Hopewell, 2003; Howland, 1975; Mitchell and Logan, 1998). Thickenning of trabeculae can be observed within the irradiated volume (Williams and Davies, 2006). This coarsening of trabeculae is also observed from the marrow cavity of various animal models within weeks of exposure (Furstman, 1972; Sawajiri and Mizoe, 2003). Quantitatively, an approximately 30% reduction in bone mineral density from the third lumbar vertebrae was observed among a group of patients with uterine cervix carcinoma within five weeks following irradiation with either 45 Gy or 22.5 Gy total dose of high energy photons (Nishiyama et al., 1992). No subsequent recovery of bone mineral density was observed twelve months after RT.

While late observed bone loss has been documented for decades from irradiated animal models, recent studies have quantified rapid loss of bone that occurs following exposure to sub-clinical doses of both photon and particulate radiation. In particular, this bone loss can occur after exposure to doses and qualities of radiation relevant to long duration missions (Bandstra et al., 2008; Hamilton et al., 2006) and confirms long-term suppression of bone formation (Bandstra et al., 2008). A long-term reduction in trabecular bone quantity and quality occurs following a whole-body 2 Gy dose gamma-rays, protons, carbon, or iron ions (Hamilton et al., 2006). Bone loss persists at four months after irradiation with doses of protons as low as 1 Gy (modeling a solar flare), and at nine weeks after modeled galactic radiation of heavy ions <0.5 Gy (Bandstra et al., 2008; Bandstra et al., 2009). Functional bone loss has been identified as early as three days after a 2 Gy dose of gamma-rays (Kondo et al., 2009). In addition, an increase in osteoclast activity when mice are irradiated with a 0.5 Gy dose of iron ions during limb disuse has been observed, modeling the reduced loading of the spaceflight environment (Yumoto K, 2010).

**Loss of Bone Strength in Animal Models After Irradiation**

The primary concern regarding radiation-induced loss of bone mineral content or architecture is the weakening of the whole bone structure, leading to fractures. Such changes in bone strength following
irradiation would account for the increased fracture risk among RT patients. Animal models therefore have been used to identify a reduction in bone strength after exposure with time and at various locations within the bone. These studies generally use clinically-relevant, higher dose exposures, rather than spaceflight-relevant scenarios. Radiation has been shown to produce relatively greater damage to spongy trabecular bone compared to dense cortical bone (Bandstra et al., 2008), however, heavy ion radiation at relatively low doses (50 cGy) does cause an increase in cortical bone porosity, cortical area, and polar moment of inertia (Bandstra et al., 2009).

Bone quality will be significantly compromised with damage to both trabecular and cortical bone, resulting in a cascade of changes throughout the structure. For example, the loss of trabecular bone will result in a greater proportion of the loads placed upon the skeleton to be transferred to cortical bone. The decline in polar moment of inertia represents a reduced ability of this cortical bone to resist both torsional and bending loads. Additionally, any defect in the structure, such as a porous hole, will cause a disruption of the load distribution resulting in a stress concentration that further compromises structural competency. These changes do not mean a fracture is imminent, even when combined with microgravity-induced bone loss; however, the probability of fracture later in life is greater, and depends upon future regeneration or degeneration and the loads placed upon the skeleton.

It is also important to comment on the effects ionizing radiation has on the body mass of irradiated subjects, as this can affect loading and therefore skeletal strength. High doses can affect the health and well-being of mice, causing them to temporarily or permanently lose body mass and/or be inactive (Willey et al., 2007). Chronic bone loss has been reported in rats nine months after exposure to high dose iron radiation (2 and 4 Gy), and these changes were attributed primarily to a reduction in body mass (Willey et al., 2008a). However, spaceflight relevant doses of ionizing radiation (<2 Gy of X-rays or protons; <1 Gy of heavy ions) do not change the body mass, activity level, or nutritional status of mice (Bandstra et al., 2008; Bandstra et al., 2009; Hamilton et al., 2006; Lloyd et al., 2008; Willey et al., 2010; Willey et al., 2008b).

A 2 Gy dose of iron ions causes a loss of vertebral stiffness as tested by compression loading and calculated by finite element analysis (FEA) (Alwood et al., 2010). Compressive testing of mouse distal femora showed a reduction of strength at twelve weeks after 5 and 12 Gy acute doses of X-rays (Wernle et al., 2010). Compressive strength at two weeks was greater in the irradiated animals, which corresponded with an acute increase in cortical bone volume and bone mineral content, despite virtual elimination of trabecular bone parameters (Wernle et al., 2010). The subsequent loss of strength as determined by compressive testing and estimated by FEA occurred despite a sustained elevation of cortical bone mineral content. Thus, the bone appeared to be more brittle. Thus, strength changes in bone after irradiation may be influenced by both architectural and material properties.

**Radiation and Bone Cells**

**Vasculature**

Bone loss following radiotherapy has traditionally been thought to be a result of physiological changes within the vasculature and bone cells (Bliss et al., 1996; Ergun and Howland, 1980; Gal et al., 2000; Hopewell, 2003; Konski and Sowers, 1996; Mitchell and Logan, 1998; Rohrer et al., 1979). The first report of radiation-induced bone damage (termed “osteitis”) described reductions in bone vasculature following obliterator endarteritis (Ewing, 1926). Early loss of vascularization occurs as a result of swelling and vacuolization of endothelial cells within the vascular channels of the osteons (Ergun and Howland, 1980; Hopewell, 2003; Rohrer et al., 1979). Ultimately, this results in the deposition of sclerotic connective tissue within the marrow cavity. Fibrosis of the sub-intima and replacement of vascular smooth muscle cells with hyaline-like material occur as late injuries, resulting in constriction of the vessel lumen. Bony elements such as the skull and jaw are considered especially at risk for vascular injury due in part to the inherent paucity of vasculature and their rather superficial location (Williams and Davies, 2006). Ablation of vasculature has been identified within the bone, including marrow cavity and Haversian systems, in a variety of animal models following radiation exposure (Cao et al., 2011; Furstman, 1972; Rohrer et al., 1979).

**Osteoblasts and Osteocytes**

Damage to osteoblasts and osteocytes is thought to be a primary contributor to reduced bone mineral density following irradiation (Ergun and Howland, 1980; Hopewell, 2003; Mitchell and Logan, 1998; Sams, 1966). Most studies have examined high, clinically-relevant doses of X-rays, but spaceflight-relevant doses and types of radiation have been shown to negatively affect osteoblasts as well (Kondo et al., 2009; Willey et al., 2010; Yumoto K, 2010). A reduction in the overall number of osteoblasts occurs following irradiation, along with reduced matrix formation (Cao et al., 2011; Willey et al., 2010). Both in vitro and in vivo data suggest that radiation can impair bone formation by inducing a decrease in
osteoblast proliferation and differentiation, inducing cell-cycle arrest, reducing collagen production, and increasing sensitivity to apoptotic agents (Dudziak et al., 2000; Gal et al., 2000; Sakurai et al., 2007; Szymczyk et al., 2004). Radiation causes a decline in RUNX2 levels in osteoblast cultures stimulated with bone morphogenetic protein-2 (BMP-2), indicative of impaired osteoblast differentiation (Sakurai et al., 2007). Receptor activator of nuclear factor kappa-B ligand (RANKL) mRNA levels tended to increase in osteoblasts following exposure to gamma rays, but not to carbon ions (Sawajiri et al., 2006). Osteoblast precursors are likely damaged by radiation (Kondo et al., 2009). Mesenchymal stem cell (MSC) numbers and colony forming ability under osteogenic stimulation are also reduced in directly irradiated bone after exposure, which would likely delay recovery of osteoblast damage (Cao et al., 2011). Oxidative stress appears to contribute to this early damage to osteoprogenitors (Cao et al., 2011; Kondo et al., 2009). However, others have indicated no loss of MSC viability within irradiated bone, but rather suggested a later effect, at the stage of terminal differentiation into osteoblasts (Schonmeyr et al., 2008).

The effect of radiation on osteocyte numbers and health remain unclear. Several studies have identified loss of osteocytes within irradiated bone following exposure to high doses (Ergun and Howland, 1980; Mitchell and Logan, 1998; Rohrer et al., 1979). Osteocytes were shown to be killed within the irradiated field inside cortical lamellar and Haversian bone of monkey mandibles irradiated with 45 Gy, although their numbers were not affected within trabecular bone. Overall, however, reports suggest that osteocytes are relatively radioresistant, remaining viable for several months after a single high dose of radiation in mice and rabbits (Jacobsson, 1985; Rabelo et al., 2010; Sams, 1966; Sugimoto et al., 1991).

**Osteoclasts**

Recent studies show that irradiation results in an early increase in osteoclast number and activity, which likely contributes to radiation-induced osteoporosis (Willey et al., 2010). Serum tartrate-resistant acid phosphatase (TRAP5b), a marker for osteoclast activity, is elevated as early as twenty-four hours after exposure to a whole body dose of X-rays. An increase in osteoclast surface of >200% and activity have been identified to occur within rodent bones three days after exposure (Willey et al., 2008b), with subsequent loss of bone within a week of treatment (Kondo et al., 2009; Willey et al., 2010). Serum chemistry and histological analyses show significant increases in TRAP5b, as well as osteoblast number and surface, during the first week of treatment. However, in these rodent models, the majority of the bone loss occurs during a time when osteoblast numbers and bone formation are unchanged relative to control (Willey et al., 2010). Suppressing osteoclast activity with the bisphosphonate antiresorptive risedronate has been shown to completely block radiation-induced increases in osteoclast activity and subsequent deterioration of bone at multiple skeletal locations (Willey et al., 2010). Decreased osteoclast activity has been noted at time points greater than one week post-exposure, with conflicting reports of recovery (Margulies et al., 2003; Sawajiri et al., 2003). This subsequent decline in both osteoclast and osteoblast activity, if persistent, could sufficiently suppress bone remodeling and turnover and result in impaired material properties of bone tissue (Burr et al., 2003), as described in rodents (Wernle et al., 2010). Taken together, it can be seen that a combination of an early and acute loss of bone via increased osteoclast activity, as well as a persistent reduction in bone formation, contribute to osteopenia and bone deterioration following irradiation.

**CONCLUSION**

Radiation exposure represents a significant concern for skeletal health. During long duration spaceflight missions, astronauts could be exposed to low doses of radiation from cosmic and solar sources. These exposures could weaken bone and lead to mission critical fractures, especially combined with the significant bone loss associated with skeletal unloading in microgravity. From the oncological perspective, advances in cancer treatment, with improvements in radiation, chemotherapy and surgical modalities, have made concern for incidental radiation damage to non-tumor tissues a growing focus. Bone represents one such “normal” tissue concern. Clinicians are now considering normal tissue complication probabilities as part of the treatment planning process. One must note that radiation exposures outside the treatment volume may be substantially greater than those predicted for astronauts, and potentially at-risk volumes of bone need to be considered as critical structures rather than as neutral structures. Limited research has defined the magnitude and mechanisms behind bone loss-associated with radiation exposure. An inflammatory response does occur in response to radiation, and may represent a mechanism that induces early activation of osteoclast-mediated bone resorption. Ultimately, bone formation is also suppressed. Bone strength is thus compromised, which could leave individuals at a substantial risk for fractures, with accompanying
mortality as a late sequela, particularly in geriatric patients. A clear need exists for effective countermeasures to radiation-induced bone loss, both for the astronaut population and the ever growing population of individuals taking advantage of highly effective cancer radiotherapy. More work is required to adequately define the molecular and cellular mediators of this response and to define targets for novel therapeutics.

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Identification of Proteins Associated with Spatial-Specific Phytochrome-Mediated Light Signaling in *Arabidopsis thaliana* by Liquid Chromatography-Tandem Mass Spectrometry

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**ABSTRACT**

Photosensory phytochromes perceive mainly red and far-red light and utilize a linear tetrapyrrole chromophore, phytochromobilin, for their photoactivity in plants. Although phytochromes have been extensively studied for light-dependent regulation of numerous developmental processes, our understanding of the molecular mechanisms responsible for distinct organ- and tissue-specific phytochrome responses is still limited. Recent studies using transgenic *Arabidopsis thaliana* plants expressing a gene that encodes the biliverdin IXα reductase (BVR) enzyme, which reduces the biosynthesis and accumulation of phytochromobilin, and thus inactivates phytochromes, have led to advances in probing tissue-specific roles of phytochromes in plant development. We performed one-dimensional SDS-PAGE, followed by protein identification and peptide quantification using liquid chromatography-tandem mass spectrometry (LC/MS-MS) to identify proteins that accumulate differentially in transgenic *Arabidopsis* lines with mesophyll-specific phytochrome deficiencies (i.e., CAB3::pBVR2 plants) compared to wild-type (WT). We identified the large subunit of Rubisco (RbcL) and small subunit of Rubisco (RbcS), which accumulated to lower levels in CAB3::pBVR2 relative to WT under continuous far-red light. We found that Beta-glucosidase proteins (BGLUs) accumulated highly in the CAB3::pBVR2 line under these conditions. RT-PCR and microarray analyses showed a positive correlation between the expression of the target genes and the accumulation of their products in BVR lines. We conclude that RbcL, RbcS, and BGLU18 are targets of mesophyll-specific phytochrome-mediated light signaling under far-red conditions.

**INTRODUCTION**

As sessile organisms, plants have evolved several photoreceptors to monitor and respond to diverse light environments. Plants monitor several different properties of available light, including intensity, wavelength, duration, and direction. Phytochromes are an extensively-studied photoreceptor family, which mainly perceive red (R) and far-red (FR) light (Schepens et al., 2004; Franklin and Quail, 2010; Kami et al., 2010). It has been demonstrated that the subcellular localization of phytochromes is important for the associated light-dependent responses (Nagy et al., 2001; Nagatani, 2004). Phytochromes move from the cytosol into the nucleus in a light-dependent manner and in the nucleus they can interact with transcription factors to regulate gene expression.

**Phytochrome Functions in Plants**

Phytochromes are involved in light-dependent regulation of numerous developmental processes such as seed germination, hypocotyl elongation responses, hypocotyl gravitropism, shade avoidance, and flowering (Franklin and Quail, 2010; Kami et al., 2010). It has been demonstrated that the subcellular localization of phytochromes is important for the associated light-dependent responses (Nagy et al., 2001; Nagatani, 2004). Phytochromes move from the cytosol into the nucleus in a light-dependent manner and in the nucleus they can interact with transcription factors to regulate gene expression.
factors, including phytochrome-interacting factors (PIFs), resulting in the regulation of light-responsive genes (reviewed by Castillon et al., 2007). Phytochromes function through PIFs to regulate a number of light-dependent responses, including seed germination, chlorophyll accumulation, hypocotyl and cotyledon development and gravitropic responses (Shin et al., 2009). Specifically, phytochrome-dependent regulation of PIFs is involved in the control of hormone-dependent seed germination (Oh et al., 2009). Also, it has been demonstrated that phytochromes regulate hypocotyl gravitropism through PIF-dependent effects on endodermal amyloplast development (Kim et al., 2011). Phytochromes also appear to have roles in the cytosol, which include blue-light dependent negative gravitropism and red-light enhanced phototropism (Rössler et al., 2007). The diverse roles of phytochromes in various physiological responses in plants recently have been reviewed extensively (Franklin and Quail, 2010; Kami et al., 2010).

**Phytochrome Accumulation in Specific Plant Tissues**

In addition to light-dependent differences in subcellular phytochrome localization, the localization of phytochromes at the tissue and organ level also has been studied (for review see Nagatani, 1997). Early reports established the differential accumulation of phytochromes in distinct plant tissues, e.g., phytochromes accumulate differentially in the epicotyl hook, cotyledons, and root tips in pea seedlings (Furuya and Hillman, 1964). Such observations have been substantiated further by phytochrome promoter fusion studies, which indicate that distinct phytochrome isoforms display discrete tissue- and organ-specific patterns of expression in a range of plant species (Somers and Quail 1995a; 1995b; Adam et al., 1996; Goosey et al., 1997; Tóth et al., 2001), as well as by immunolocalization of phytochrome holoproteins (Sharrock and Clack, 2002). These observations began to implicate distinctive roles of phytochromes in specific tissues and organs.

**Phytochromes Regulate Discrete Responses in Different Tissues and Organs**

The spatiotemporal biochemical and physiological roles of phytochromes in light-dependent plant responses also have been explored extensively (for review see Bou-Torrent et al., 2008; Montgomery, 2008). At the physiological level, a number of early studies utilized detached or foil-covered plant parts or site-specific microbeam irradiation to photoactivate phytochromes in discrete plant tissues and to assay for distinct phytochrome-dependent responses in distal sites (Piringer and Heinze, 1954; Klein et al., 1956; De Greef et al., 1971; De Greef and Caubergs, 1972a; 1972b; De Greef and Verbelen, 1972; Tepefer and Bonnett, 1972; Black and Shuttleworth, 1974; Oelze-Karow and Mohr, 1974; Caubergs and De Greef, 1975; De Greef et al., 1975; Lecharny, 1979; Powell and Morgan, 1980; Mandoli and Briggs, 1982; Nick et al., 1993). Such studies, e.g., experiments using foil to cover specific portions of cucumber seedlings during irradiation (Black and Shuttleworth, 1974), led to the observation that activation of phytochromes in cotyledons could inhibit the elongation of hypocotyls through interorgan signaling in response to light. Other classical examples of cell- and tissue-specific, as well as interorgan, phytochrome-mediated responses emerged, including the perception of light by leaf-localized phytochromes that results in the regulation of the photoperiodic induction of flowering that produces a transition from vegetative to reproductive growth at the meristem (King and Zeevaart, 1973). Although such early physiological approaches have broadened our knowledge of spatial- and tissue-specific phytochrome responses, our understanding of the molecular mechanisms responsible for such tissue-specific phytochrome responses is still limited. These limitations persist partially due to a lack of molecular tools for probing the role(s) of phytochromes in a tissue-specific manner.

**Light Perception by Photoreceptors Regulates Gene Expression in a Tissue-Specific Manner**

Through recent studies, insight into light-dependent regulation of gene expression at the organ- and tissue-specific level has increased. Tissue-specific expression of photoreceptors identified distinct tissues in which cryptochromes (Endo et al., 2007) and phytochromes (Endo et al., 2005) are required for the regulation of specific responses. Also, distinct subsets of genes have been shown to be controlled by light in different tissues, e.g. cotyledon, hypocotyls and roots in both rice and Arabidopsis (Jiao et al., 2005; 2007). Thus, it appears that specific factors are regulated in distinct tissues and organs downstream of light perception by photoreceptors. Furthermore, reverse genetic studies of genes regulated in response to light resulted in the identification and characterization of factors implicated in hypocotyl-localized phytochrome responses (Khanna et al., 2006).
Phytochrome Depletion in Transgenic Plants

Recently, great advances have been made in the elucidation of the molecular mechanisms responsible for tissue- and organ-specific phytochrome responses using transgenic plants expressing a gene that encodes the mammalian enzyme biliverdin IXα reductase (BVR). Constitutive expression of BVR in transgenic Arabidopsis and tobacco plants using a strong plant promoter, i.e., the promoter of the 35S RNA from Cauliflower mosaic virus (i.e., CaMV 35S; Odell et al., 1985), resulted in the inactivation of the precursors of the phytochrome chromophore, and alteration of light-dependent responses associated with reduced phytochrome activity (Lagarias et al., 1997; Montgomery et al., 1999; 2001). These responses mirrored the responses that have been observed in natural phytochrome chromophore-deficient mutants. Experiments with transgenic plants selectively expressing the BVR gene using tissue-specific promoters, and consequently in which the function of phytochromes have been inactivated in a tissue-specific manner, have allowed successful probing of the spatial-specific roles of phytochromes in plant development (Montgomery, 2009; Warnasooriya and Montgomery, 2009; Warnasooriya et al., 2011; Costigan et al., submitted). BVR expression in plastids of mesophyll tissues of Arabidopsis was achieved using the CAB3 promoter (i.e., CAB3::pBVR) and results in the disruption of FR-dependent responses, including the inhibition of hypocotyl elongation and the stimulation of anthocyanin accumulation, suggesting roles for mesophyll-localized phytochrome A (phyA) in the regulation of FR-dependent responses (Warnasooriya and Montgomery, 2009). CAB3::pBVR lines also have disruptions in red- and blue-light-dependent inhibition of hypocotyl elongation and anthocyanin accumulation responses (Montgomery, 2009; Warnasooriya and Montgomery, 2009; Warnasooriya et al., 2011). Inactivation of phytochromes in the shoot apical meristem in transgenic Arabidopsis plants, i.e. plants expressing BVR under the control of the MER15 promoter, results in an enlarged leaf phenotype and an increased leaf number specifically under short-day photoperiods, suggesting a photoperiod-dependent role of meristem-specific phytochromes in the regulation of leaf initiation and growth (Warnasooriya and Montgomery, 2009).

Identifying the Molecular Effectors that Mediate Tissue- and Organ-Specific Phytochrome Responses

Using microarray analysis to identify genes which were differentially expressed in FR-light-grown 35S::pBVR seedlings relative to CAB3::pBVR seedlings, we identified several genes involved in mesophyll-specific phytochrome responses (Warnasooriya, Oh, and Montgomery, unpublished data). These studies are providing insight into the molecular mechanisms underlying spatial-specific phytochrome responses during Arabidopsis seedling development. In this study, we report parallel experiments to identify specific proteins that accumulate differentially in No-0 wild-type (WT) relative to 35S::pBVR, CAB3::pBVR, or MER15::pBVR transgenic lines using SDS-PAGE analysis followed by enzymatic digestion, liquid chromatography-tandem mass spectrometry (LC-MS/MS) and database searching. We found that the large subunit of Rubisco (RbcL) and small subunit of Rubisco (RbcS) accumulated to lower levels in CAB3::pBVR lines than in No-0 WT or other BVR lines, whereas Beta-glucosidase proteins (BGLUs) accumulated highly in CAB3::pBVR lines relative to No-0 WT or other BVR lines. Using microarray data and RT-PCR analysis, we confirmed a positive correlation of protein accumulation with the accumulation of mRNA of the genes encoding the identified proteins in CAB3::pBVR lines. These data suggest that RbcL, RbcS, and BGLUs are targets of mesophyll-specific, phytochrome-mediated light signaling and potentially involved in tissue-specific phytochrome responses during seedling development.

Identification of Proteins Differentially Accumulated in BVR Lines

To identify proteins responsible for tissue- and organ-specific-phytochrome responses, we performed profiling of proteins differentially accumulated in BVR lines. Total soluble proteins were extracted from No-0 WT (hereafter WT), 35S::pBVR3 (35S), CAB3::pBVR2 (CAB3), and MER15::pBVR1 (MER15) seedlings grown on 1× MS medium containing 1% sucrose at 22 °C in FR light at 5 µmol m⁻² sec⁻¹ for 14 days. Soluble proteins (25 µg) were resolved on 8 % or 12 % (w/v) polyacrylamide SDS-PAGE gels. At least three distinctive protein bands were detected visually: Proteins from band 3 accumulated highly in CAB3::pBVR lines, whereas proteins from band 1 or 2 accumulated to lower levels in CAB3 lines, whereas proteins from band 3 accumulated highly in CAB3 lines (Figure 1). These altered patterns of protein accumulation that were observed on gels were reproducible in at least three biological replicates. The
three protein bands labeled 1 through 3 in the gels from WT and CAB3 samples (Figure 1) were excised and subjected to tryptic digestion followed by LC-MS/MS and database searching for homology to known proteins. All procedures for LC-MS/MS were performed at the Research Technology Support Facility (RTSF) at Michigan State University. The Mascot search engine (Matrix Science, Inc., Boston, MA) was used to match MS/MS spectra to peptide sequences. Proteins identified with more than 99.7% of protein identification probability by using Scaffold software (Proteome Software, Inc., Portland, OR) are listed in Table 1. Spectral counts (i.e., number of spectra) have been used as a good measure of relative abundance among different samples (Liu et al., 2004). We assessed the number of spectra, % of spectra, and % of coverage for the comparison of candidate proteins in WT relative to CAB3 (Table 1).

Consistent with data from SDS-PAGE gel analyses, the large subunit of Rubisco (RbcL), which is labeled protein band 1, was less abundant in CAB3 relative to WT when comparing spectral counts, i.e., 106 vs. 176, respectively (Table 1). The LC-MS/MS data for RbcL was reproducible when tested using two biologically independent samples. As predicted based on the protein gel, the molecular mass of RbcL was 53 kDa (Figure 1). Proteins from band 2 were identified as the small subunit of Rubisco (RbcS; Table 1). Two RbcS proteins encoded by At1g67090 and At5g38410 accumulated to lower levels in CAB3 relative to WT – i.e. spectral counts for WT vs. CAB3 of 83 vs. 48 for At1g67090 and 13 vs. 8 for At5g38410. Data from mass spectrometry analysis for RbcS was reproducible in two biologically independent replicates. Whereas the predicted molecular mass of RbcS was 20 kDa (Table 1), the apparent molecular mass of RbcS on the protein gel was approximately 10 kDa (Figure 1). This observation indicates post-translational modification of Rubisco proteins, e.g. proteolytic processing of the N-terminal portion as previously described (Houtz et al., 2008).

Proteins from band 3, which were highly accumulated in CAB3 relative to WT as observed on SDS-PAGE gels (Figure 1), were identified as BGLUs in our LC-MS/MS analysis (Table 1). The Arabidopsis genome encodes at least 48 Beta-glucosidase proteins (BGLUs) and BGLU23, BGLU22, BGLU21, and BGLU18 are most closely related (Xu et al., 2004; Ogasawara et al., 2009). All of the closely related BGLU23, BGLU22, BGLU21, and BGLU18 proteins were found to be more abundant based on spectral count in CAB3 than in WT—112 vs. 68 for BGLU23, 6 vs. 0 for BGLU22, 3 vs. 0 for BGLU21, and 5 vs. 2 for BGLU18 (Table 1).

Figure 1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of Arabidopsis total soluble proteins from wild-type (WT), 35S::pBVR3 (35S), CAB3::pBVR2 (CAB3), and MERI5::pBVR1 (MERI5) lines. Total soluble proteins were extracted from 14 day-old entire seedlings of WT, 35S, CAB3, and MERI5 lines grown under constant far-red light using 2× protein sample buffer. Proteins were resolved on either (A) 8 % or (B) 12 % polyacrylamide gels by SDS-PAGE electrophoresis. Protein bands (labeled 1, 2, or 3) were excised from gels for LC-MS/MS analysis. Reproducible SDS-PAGE gels were obtained from three independent experiments.
Table 1. Identification of proteins differentially accumulated in wild-type (WT) and CAB3::pBVR2 (CAB3) lines using liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. Proteins listed were identified with probabilities of ≥ 99.7% after spectral searches with the Mascot database search engine (Matrix Science, Inc.) to identify peptides and analysis with Scaffold software (Proteome Software, Inc.).

<table>
<thead>
<tr>
<th>Band No.</th>
<th>AGI No.</th>
<th>Annotation (Mass)</th>
<th>Samples</th>
<th>No. of spectra</th>
<th>% of spectra</th>
<th>% of coverage</th>
</tr>
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<td>0</td>
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<td>10.0</td>
</tr>
<tr>
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<td>(BGLU18)</td>
<td></td>
<td>CAB3</td>
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</tr>
</tbody>
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Expression Patterns of Genes Encoding Differentially Accumulated Proteins in BVR Lines Are Also Altered

We previously performed comparative gene expression profiling studies using BVR lines grown on MS medium for 7 days in FR light (Warnasooriya, Oh, and Montgomery, unpublished data). Using our microarray data, which was produced in triplicate and subjected to per chip normalization, the expression of genes encoding the candidate proteins identified from our LC-MS/MS analysis has been explored (Figure 2). AtCg00490 encoding RbcL was downregulated in CAB3 lines compared to 35S and WT – i.e., 3.5- and 6.4-fold downregulated, respectively (Figure 2A). The two genes encoding RbcS proteins, i.e., Atg67090 and Atg38410, were moderately downregulated by 1.6 fold in CAB3 compared to WT or 35S lines, which were not significantly different from each other (Figure 2B). Genes encoding the four identified BGLUs tended to be upregulated in BVR lines compared to WT (Figure 2C). Most strikingly, BGLU18 was highly upregulated in CAB3 lines relative to 35S and WT, i.e., 4.6- and 7.5-fold, respectively.

To verify the expression of the genes queried in our microarray data set, we performed RT-PCR analyses (Figure 3). Also, in some cases microarray data did not provide the expression of specific genes. For example, the two RbcS-encoding genes and the genes encoding BGLU21 and BGLU22 could not be distinguished. Thus, it was very important to verify the expression of those genes using RT-PCR analysis. Primer pairs for candidate genes and a control gene encoding an ubiquitin-conjugating enzyme 21 (UBC21) were designed using AtRTPrimer (Han and Kim, 2006). RT-PCR analyses confirmed the expression patterns of our candidate genes: AtCg00490 and Atg67090 encoding RbcL and RbcS, respectively, were downregulated in CAB3 lines compared to WT or 35S lines; whereas Atg52400 encoding BGLU18 was highly expressed in CAB3 relative to WT (Figure 3). Altogether, the results from microarray and RT-PCR analyses indicated a positive correlation between gene expression and associated protein accumulation in BVR lines.

Expression of Candidate Genes in Various Tissues and Organs

To obtain more insight into the expression of the candidate genes in different tissues and at distinct developmental stages, we constructed heat maps using AtGenExpress public Arabidopsis microarray data set. RBCL was not represented in this data set. Notably, the expression pattern of RBCS and BGLU18

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**Table 1. Identification of proteins differentially accumulated in wild-type (WT) and CAB3::pBVR2 (CAB3) lines using liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.** Proteins listed were identified with probabilities of ≥ 99.7% after spectral searches with the Mascot database search engine (Matrix Science, Inc.) to identify peptides and analysis with Scaffold software (Proteome Software, Inc.).

<table>
<thead>
<tr>
<th>Band No.</th>
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<th>Annotation (Mass)</th>
<th>Samples</th>
<th>No. of spectra</th>
<th>% of spectra</th>
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<td>CAB3</td>
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in various tissues was quite similar (Figure 4). \textit{RBCS} and \textit{BGLU18} are expressed abundantly in aerial tissues, including hypocotyls, cotyledons, and leaves. Unlike \textit{BGLU18}, \textit{BGLU23} and \textit{BGLU22/21} are upregulated highly in roots (Figure 4). \textit{BGLU23} is also abundant in hypocotyls and the shoot apex (Figure 4). These observations correspond well to \textit{BGLU18} being affected more significantly at the level of expression than the other \textit{BGLU}-encoding genes in CAB3::pBVR lines where phytochromes are inactivated in cotyledons and leaves—i.e. the site of \textit{BGLU18} upregulation.

Potential Roles of RBCL, RBCS, and BGLU18 in Mesophyll-Specific Phytochrome Responses

In the stroma of chloroplasts, Rubisco catalyzes the oxygenation of ribulose-1,5-bisphosphate in the photorespiratory pathway and carboxylation of the same substrate during the Calvin cycle (reviewed by Jensen and Bahr, 1977). Rubisco is one of the most abundant proteins comprised of RbcL and RbcS to form a massive hexadecameric protein structure (Baker et al., 1975). In \textit{Arabidopsis}, RbcL protein is encoded by a single chloroplast-localized gene, \textit{AtCg00490} (Bedbrook et al., 1979), whereas RbcS proteins are encoded by a nuclear multigene family, including \textit{At1g67090} and \textit{At5g38410} (Dean et al., 1989).

We found that the level of accumulation of RbcL and RbcS proteins in CAB3 was lower than in WT, 35S, or MERI5 lines (Figure 1). In CAB3 lines, the genes encoding those two proteins were also downregulated, suggesting a role of mesophyll-specific phytochromes in the positive regulation of \textit{RBCL} and \textit{RBCS} genes (Figures 2, 3). It has been demonstrated that phytochromes are involved in the induction of the expression of \textit{RBCL} and \textit{RBCS} at the transcriptional level in several species (e.g. Sasaki et
Oh and Montgomery -- *Arabidopsis* Phytochrome Protein

Furthermore, Antipova et al. (2004) demonstrated that FR light induces the accumulation of RbcL protein and antisense suppression of *PHYA* in transgenic tobacco plants results in a reduced level of accumulation of RbcL protein, indicating a role for phyA in the FR light-mediated photoregulation of the synthesis of RbcL proteins. Here, we further define the mesophyll-specific pool of phyA as critical for regulation of RbcL accumulation in *Arabidopsis*. It also has been shown that *At1g67090*, which encodes a RbcS protein, is highly expressed in mesophyll tissues and upregulated in cotyledons and hypocotyls in response to blue, red, and far-red light in *Arabidopsis* (Sawchuk et al., 2008). Consistent with reduced levels of RbcS protein in CAB3 lines (Figure 1), expression of *At1g67090* was downregulated in those lines (Figures 2 and 3). Striking phenotypes observed in CAB3 lines include defects in FR-dependent inhibition of hypocotyl elongation and anthocyanin accumulation, indicating roles of mesophyll-localized phyA in FR responses (Warnasooriya and Montgomery, 2009). Here, we highlight another mesophyll-localized, FR-dependent phenotype regulated by phyA—the regulation of expression of *RBCS* and *RBCL* genes and accumulation of their proteins.

We found that BGLU proteins were highly accumulated in CAB3 lines and the expression of *BGLU18* gene was correlated with the observed increased protein accumulation (Figures 1, 2 and 3), suggesting a role of mesophyll-specific phytochrome in the negative regulation of the *BGLU18* gene. BGLUs catalyze the hydrolysis of cellobiose, a unit of cellulose, or other disaccharides with release of glucose and play important roles in many biological processes, including chemical defense against pathogens (Morant et al., 2008), lignification (Dharmawardhana and Ellis, 1998), degradation of cell wall materials (Leah et al., 1995), and hydrolytic hormone release (Lee et al., 2006). Interestingly, it has been demonstrated that an oat BGLU protein, also named Avenacosidase, co-purifies with phytochrome (Gus-Mayer et al., 1994; Parker et al., 1995). However, there has been no further evidence reported of a functional role of BGLUs in phytochrome-mediated light signaling.

The *Arabidopsis* genome encodes 48 BGLUs and eight of them, i.e., *BGLU18* – *BGLU25*, are placed in the same phylogenetic clade (Xu et al., 2004). Identification of *BGLU23*, *BGLU22*, *BGLU21*, and *BGLU18* in our mass spectrometry analysis suggests a functional redundancy of those proteins in spatial-specific phytochrome-mediated light signaling (Table 1). Among them, only the expression *BGLU18* gene was heavily upregulated (~5 fold) in CAB3 compared to 35S lines (Figures 2 and 3), indicating a regulation of *BGLU18* by mesophyll-specific phytochromes. Interestingly, the expression pattern of *BGLU18* in different *Arabidopsis* tissues was roughly correlated with that of *RBCS*, i.e., *RBCS* and *BGLU18* were upregulated in mesophyll-abundant organs such as cotyledons and leaves, whereas *BGLU23*, *BGLU22*, and *BGLU21* were abundantly expressed in roots (Figure 4), suggesting a distinct mesophyll-specific
regulation of BGLU18 by phytochrome-mediated light signaling.

It has been shown that BGLU18 is induced by herbivory, methyl jasmonate, and wounding and its protein accumulates in endoplasmic reticulum bodies formed directly at the wounding site on cotyledons, indicating a role of BGLU18 in plant defense responses (Stotz et al., 2000; Ogasawara et al., 2009). Since a positive correlation between the specific activity of BGLU and the rate of cell wall elongation has been observed in pea seedlings (Murray and Bandurski, 1975), we propose that defense-related BGLU18 could also be involved independently in the regulation of cell elongation (e.g., inhibition of hypocotyl elongation) during seedling development. This involvement could be through the impact of BGLU18 on the cell wall, or perhaps through light-dependent regulation of the hydrolytic release of a hormone impacting cellular elongation. Interestingly, inactivation of phytochrome in CAB3 lines resulted in a defect in the inhibition of hypocotyl elongation (Warnasooriya and Montgomery, 2009) and in the present studies, BGLU18 proteins accumulated highly in CAB3 lines, compared with other BVR lines (Figure 1). Thus, it appears that an increased accumulation of the BGLU18 enzyme is associated with an elongation of hypocotyls – thus in normal seedling development expression of BGLU18 may be regulated negatively by mesophyll-specific phytochromes during the light-dependent inhibition of hypocotyl elongation.

SUMMARY AND CONCLUSION

We applied SDS-PAGE gel analysis coupled with mass spectrometry to identify key protein components regulated by mesophyll-specific phytochromes. Our mass spectrometry-based protein analysis identified three proteins differentially accumulated in BVR lines. In CAB3, transcript abundance of RBCS and RBCL and associated protein abundance were low relative to WT and other BVR lines with distinct tissue-specific disruptions in phytochrome accumulation. However, BGLUs accumulated to higher levels and transcript accumulation of BGLU18 was upregulated in CAB3. Since mesophyll-specific phytochromes are inactivated in CAB3 lines, we hypothesize that the genes encoding RbcS, RbcL, and BGLU18 proteins are targets of mesophyll-specific phytochrome signaling pathways. Further investigations using genetic approaches to characterize mutants for genes encoding these proteins and/or two-dimensional gel electrophoresis coupled with mass spectrometry analysis to identify additional candidate proteins differentially accumulated in various BVR lines will allow a better understanding of the molecular mechanisms underlying spatial-specific phytochrome-mediated light signaling and the associated regulation of distinct phytochrome-dependent responses.

ACKNOWLEDGEMENTS

We would like to thank Dr. Sankalpi Warnasooriya for conducting the microarray analyses and for reading and critically commenting on the manuscript. This work was supported by the National Science Foundation (grant no. MCB-0919100 to B.L.M.) and the Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, Office of Science, U.S. Department of Energy (grant no. DE-FG02-91ER20021 to B.L.M.).

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induced polymerization rapidly increases active pools of abscisic acid. *Cell* 126: 1109-1120.


Bone Marrow Stem Cells Differentiated Into Cartilage by Manipulation of Gas Phase

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INTRODUCTION

A continual need for bone in craniofacial repair exists; one solution to the problem of procuring enough bone for this purpose lies with tissue engineering (Doan et al., 2010). In most bone tissue engineering studies, osteoblasts are cultured on a scaffold with or without growth factors and then implanted (Doan et al., 2010). Because of concerns about rejection of implants, embryonic stem cells and stem cells from bone marrow have also been placed on scaffolds, differentiated into bone cells and implanted (Jukes et al., 2008). In a few instances, stem cells have been differentiated into bone-forming cartilage for bone repair (Oliveira et al., 2009; Jukes et al., 2008; Doan et al., 2010).

The rationale for using cartilage for bone repair is that during growth and development, the bony skeleton forms primarily through endochondral ossification, involving mineralization of a cartilaginous template, then its vascularization and replacement by bone (Duke et al., 1993). Fracture healing also includes a cartilaginous stage.

Previously, mouse embryonic limb bud cells were differentiated into cartilage in NASA’s rotating bioreactor and used to heal defects in the skulls of mice (Montufar-Solis et al., 2004; Doan et al., 2009). The purpose of the current study was to isolate mouse bone marrow stem cells (BMSCs), expand the cells in culture and differentiate them into cartilage, creating a flat cartilaginous implant. To grow cartilage from BMSCs, we used hardware developed for a 1992 space-flight, Flexcell (Flex I®) plates and 35 mm culture plates.

MATERIALS AND METHODS

Isolation of BMSCs was carried out according to Masoud and Samad (2009) for isolation and culture of MSC from mouse bone marrow. Isolated bone marrow cells from 7-9 day old C57BL mice were expanded in culture. After 2 passages, 0.5 ml differentiation medium (see below) with 88,000 cells was inoculated onto Silastic® membranes supported by hardware casings or in assembled hardware units that were placed in Petri dishes for culture. In other experiments, Flex 1® plates (Flexcell International) or 35 mm culture dishes (Falcon BD) were used. In these experiments the cell number inoculated onto the plate ranged from 7.55 x 10⁶ cells to 34.2 x 10⁶. Cultures were incubated at 37°C with or without 5% CO₂ for two hours; 5% CO₂ was used thereafter. After medium addition, cells were cultured for 3-7 days with ½ the medium changed every other day, then fixed with 10% buffered neutral formalin and stained.

Medium for BMSC differentiation was BGJb-Fitton-Jackson modification (GIBCO) supplemented with 10% fetal bovine serum (Fischer), 150 μg/ml ascorbic acid, and 1% Penicillin-Streptomycin solution (Sigma). BGJb-FJM is a chondrogenic/osteogenic medium. In one experiment, DMEM medium replaced the BGJb-FJM.

Cells were stained in situ with methylene blue and cresyl violet, both of which stain neuronal Nissl bodies, and either Toluidine blue, a metachromatic dye specific for cartilage matrix, or Alcian blue at low pH, again a cartilage specific stain (Huang et al., 2010). Flex 1® aggregates were sectioned and stained with Toluidine blue.

Hardware used in these experiments was the BEX unit designed for our CELLS experiment on the International Microgravity Laboratory-1 mission in 1992 (Duke et al., 1995). The hardware (Figure 1) consists of a two-welled polycarbonate chamber into which is inserted a gas exchanging membrane of a Silastic® compound (Dow-Corning 5150+360) in the shape of two domes or “bubbles” which allow for gas exchange, and inflate or collapse as medium is added or removed. Deflector rings inside the bubble control fluid forces related to medium removal or addition.
The complete assembly also includes a silicon rubber gasket, a polycarbonate bottom plate, and a stainless steel support plate. For flight, cells were cultured on a cover slip between the gasket and the bottom plate. For some of these experiments, complete hardware units were used, and for some, only Silastic membrane bubbles were used. Cells were cultured on the interior surface of the bubble and the units placed in a Petri dish.

![Figure 1. BEX hardware. A. Position used to culture prechondrocytes on coverslips for spaceflight. B. Position used to culture BMSCs on membrane.](image)

To form a flat piece of tissue, cells were cultured on Flex I™ plates which look like regular culture dishes, but have a bottom surface of Silastic. For controls, 35 mm culture dishes were used.

RESULTS

Cells in units placed in CO₂ for the attachment period spread on the membrane assuming a neuronal-like appearance. Staining with methylene blue revealed numerous dark bodies (Nissl bodies) in the cytoplasm (Figure 2A). Cells in units not exposed to CO₂ during the attachment period did not spread, and the cell layer consisted of rounded cells with areas of aggregation typical of early cartilage formation (Figure 3). When cells in these layers were stained with Toluidine blue after 7 days, the entire layer was metachromatic, indicating the presence of sulphated glycosaminoglycans (data not shown). Alcian blue staining identified the aggregated cells as chondrocytes (Huang et al., 2010). When a non-differentiating medium (e.g. DMEM) was used, only neuronal-like cells formed (Figure 2B). With increased initial cell densities, the Flex I™ group formed aggregates that, when sectioned and stained with Toluidine Blue, resembled hypertrophied cartilage with metachromatic matrix (Figure 4). Aggregates formed in the Petri dishes stained with Alcian blue at low pH, confirming the presence of cartilage matrix (Figure 5; Huang et al., 2010).

![Figure 2. Neuronal like cells. A. Stained with methylene blue. B. Stained with cresyl violet. Bars = 50 µm.](image)

![Figure 3. Aggregates of rounded cells interspersed with neuronal-like cells. Bar = 100 µm.](image)

![Figure 4. Aggregate from Flex I™ plate, sectioned and stained with Toluidine blue. Pink indicates cartilage matrix. Bar = 50 µm.](image)
DISCUSSION

This study shows that BMSCs can be differentiated into cartilage or neuronal-like cells by manipulating the gas environment during the initial attachment phase. The exposure to CO₂ during this initial stage of culture changed the pH of the medium, making it more alkaline as indicated by the phenol red indicator in the medium. The exposure to CO₂ using a non-differentiating medium (e.g. DMEM) did not result in chondrocyte-like cells being present; instead all the cells were spread with numerous processes. The same result was seen in all culture vessels.

To advance these studies toward the ultimate goal of developing a cartilage implant for humans, the ability to grow more cartilage must be developed. One way this could be done is by the use of TGFβ. The medium used for human studies must be serumless, so that the implant is not exposed to animal products during culture. First, BMSCs must be grown in serumless medium, differentiated into cartilage which will be implanted into defects in the skulls of mice to determine its bone-forming capabilities.

Another major problem arising in this study was the tendency of the cartilage to come off the plate because of the cartilage’s loss of contact with the surface as matrix develops. Using Cell-Tac as in the spaceflight experiment could solve this problem.

The phenomenon of forming bone from a cartilaginous template continues to ignite hope for patients with skeletal and craniofacial disorders, offering a promising alternative approach in the realm of bone regeneration and reconstruction.

ACKNOWLEDGEMENTS

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REFERENCES


Fluid Motion for Microgravity Simulations in a Random Positioning Machine

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BACKGROUND

To understand the role of gravity in biological systems one may decrease inertial acceleration by going into free-fall conditions such as available on various platforms. These experiments are cumbersome and expensive. Thus, alternative techniques like Random Positioning Machines (RPM) are now widely used to simulate the micro-gravity environment (Yuge et al., 2003; Borst and van Loon, 2009; Pardo et al., 2005). These instruments generate random movements so that cumulative gravitational effects cancel out over time. However, comparative studies performed with the RPM and culture cells were unable to reproduce the spaceflight results (Hoson et al., 1997). These differences may be explained by stresses acting on the culture cells in an RPM whereas these stresses are not present in microgravity conditions. They may be caused by internal fluid motion, originating from instationary rotation. The aim of this study is to quantify fluid flow behavior and wall shear stresses (as they are relevant to cells cultured at the flask wall), and internal shear stresses as they are relevant to suspended (free-floating) cells in an RPM container. We do this both experimentally using Particle Image Velocimetry (PIV) and numerically using 3D Direct Numerical Simulation (DNS) of the flow.

METHODS

A dual-axis rotating frame machine is used to reproduce the motion of a real RPM in a controllable way according to a pre-programmed motion protocol. A flask filled with fluorescent tracer particles (d=13 μm, ρ=1150 kg/m³) and water with sodium chloride of the same density as the particles is positioned at the center of rotation, where it is surrounded by a co-rotating 2-component planar PIV system (Adrian and Westerweel, 2011), see Figure 1. A green light dye-laser of 534 nm wavelength is used to create a light sheet inside the flask. We use a double-frame camera to record the particles inside the flask. The motor motion is generated with a servo-control system up to a maximum angular speed of 30°/s, and acceleration up to 60°/s². Images are recorded at a maximum frequency of 15 Hz for a duration of typically 30s corresponding to 6 motion cycles.

Figure 1. Left: Particle Image Velocimetry system mounted on a two-axis rotating frame. Right: Detailed view of the container. A light sheet is created in the XY plane at a distance ∆z from the container wall.

The same experiment is simulated numerically using an existing DNS model (Pourquie, 2009) to which the effect of rotation is added by implementing extra body forces (angular acceleration, centrifugal, Coriolis) to the momentum equation, including the motion protocol as applied to the experiment. The simulation has sufficient spatial and temporal resolution to fully resolve the flow field. The (rectangular) flask geometry equals the one used for the experimental study (65 x 40 x 20 mm³).
RESULTS

For a periodic and sinusoidal rotation around a single axis, fluid motion induced by inertia is observed parallel to the wall; see Figure 2 and 3. The RMS-average difference between the simulated and measured velocity field is equal to 0.5 mm/s, or 6% of the maximum velocity. The quantitative result demonstrates the good agreement between the simulated and the measured velocity profiles.

In Figure 3, measured and simulated velocities at the center of the measurement plane are depicted as a function of time. It can be seen that a periodic state is reached after only one period. The time evolution of the velocity profiles is shown on Figure 4 for the velocity in the y direction $V_y$. The increase and decrease of $V_y$ along the y direction due to the circulation in the container can be seen. Along the x-axis of rotation, the profile is flat (i.e. 2D flow), except for end-effects (boundary layers) near the flask wall. Since the PIV measurements provided the velocity field only over the XY plane at a (fixed) distance of 3.3 mm from the wall, the 3-D simulated results are used to study the velocity profile along the z axis. The evolution of the boundary layer can be seen near the flask wall. A maximum wall shear stress of 6.2 mPa is obtained with an angular phase shift of 54 degree with respect to the angular velocity of the rotating axis.

For spin-up about 2 axes according to the instationary angular velocity used in a commercial RPM (see Figure 5), more complex patterns are obtained in the DNS. A maximum velocity up to 70 mm/s near the wall and a maximum stress at the container wall of 62 mPa are derived.
Figure 4. Comparison between the simulated and measured velocity profiles at several time steps as a function of Y position, at the intersection of the S$_{xy}$ and S$_{yz}$ plane (left), and as a function of the x position at the intersection between the S$_{xy}$ and S$_{zx}$ planes (right).

Figure 5. Angular velocity patterns for both axes (top), maximum wall shear stress (bottom) according to the angular velocity used in an RPM. Box-size: $6 \times 4 \times 2$ [cm].
CONCLUSION

For sinusoidal rotation, quantitative agreement between simulated and measured velocity field has been found which is considered as a validation of the numerical model. Considering motions as are employed for an RPM, the numerical study suggests that stresses induced by the fluid are particularly high during changes of rotation velocity, i.e. acceleration. In the near future, we aim to provide a quantitative comparison between experimental and numerical results for dual axis motion. Furthermore, we are developing a user protocol for the RPM users.

REFERENCES


Plants in an orbital environment experience conditions that are distinctly unlike the earth-bound environments that have directed their evolution on Earth. This presents a unique opportunity to examine biological responses, particularly those involved in integrating gravity as a force shaping biological systems. One means of measuring these adaptive responses is to monitor the expression of genes that allow survival in those peculiar environments. In a recent series of spaceflight experiments conducted on the International Space Station (ISS) we utilized Arabidopsis thaliana (Arabidopsis) plants engineered with specific gene promoters driving to Green Fluorescent Protein (GFP). These biological sensor plants are referred to as TAGES, an acronym for Transgenic Arabidopsis Gene Expression System. One component of the TAGES Spaceflight experiment was the use of several different GFP reporter gene constructions to record tissue-specific changes in the patterns of gene expression in real time. Another component was the evaluation of genome-wide changes in these plants, a goal that required the use of the nucleic acid preservative RNAlater™ (Ambion).

The utility of GFP gene reporters is well established (Haseloff & Amos, 1995; Manak et al., 2002; Paul et al., 2003; Paul et al., 2004; Sheen et al., 1995). The application to spaceflight is especially appealing as gene expression data can be collected telemetrically, thereby placing minimal demands on crew time.

The use of RNAlater™ also offered the opportunity to evaluate the preservation of GFP for postflight analysis. In a series of preliminary experiments prior to launch, the TAGES plants were utilized in ground-based studies as part of a Payload Verification Test (PVT) to test the operations and science before the experiment was launched. One aspect that was evaluated was the responsiveness of the biosensor plants to environmental stress and the efficacy of using RNAlater™ to preserve evidence of GFP expression long after the stress response was incurred, in addition to its general use as an effective means to preserve the integrity of RNA.

In the PVT, plates of TAGES plates were prepared with two or three genotypes and a variety of methods used to collect GFP expression data. Figure 1 shows GFP reporter gene plants grown vertically on nutrient agar plates. The TAGES ISS experiments utilized a variety of plant lines engineered to report on several aspects of the spaceflight environment, and among the plants shown in Figure 1, the left-hand side of each plate contains Adh::GFP plants, which respond to reduced levels of oxygen. The plants on the far right-hand side of the plate are used as positive controls (CaMV35s::GFP) and continuously express GFP throughout their cells. In the experiment shown...
in Figure 1A and 1B, the roots of the plants were covered with a thin layer of solid agar, which reduced the flow of oxygen to those tissues. As seen from the faint green glow in the roots of those plants, that thin blanket of agar was sufficient to induce the sensitive Adh::GFP reporter gene. Figure 1A was photographed with a standard digital camera in white light, and that same camera was used to photograph 1B through an Illumatool™ (Light Tools Research) GFP imaging device. The image in Figure 1C was captured by the TAGES GFP Imaging System (GIS) developed by Kennedy Space Center, which was later launched on STS-129 and installed on the ISS for the subsequent TAGES spaceflight experiments.

Figure 1A shows the faint green glow in the roots of those plants, Figure 1B was photographed with a standard digital camera in white light, and that same camera was used to photograph 1B through an Illumatool™ (Light Tools Research) GFP imaging device. The image in Figure 1C was captured by the TAGES GFP Imaging System (GIS) developed by Kennedy Space Center, which was later launched on STS-129 and installed on the ISS for the subsequent TAGES spaceflight experiments.

Although the resolution of the GIS is excellent for general patterns of gene expression, it was our hope that RNAlater™-preserved material returned from the ISS could be utilized to capture higher resolution tissue-specific data. Figure 2 shows images of a representative plant returned from orbit in TAGES experiment 2B, which was launched on STS-131, and spent several weeks on the ISS as part of the APEX-TAGES payload. The plants were grown for 12 days on the ISS, and then harvested to RNAlater™ in a Kennedy Space Center Fixation Tube (KFT) (Ferl et al., 2011). The KFT was allowed to equilibrate at ambient temperature for 12-18 hours, and then placed in the MELFI freezer on the ISS. The samples were recovered on the return flight of STS-132, photographed and then prepared for subsequent biochemical assays (analyses in progress). The RNAlater™ fixed plant in Figure 2 is a constitutive GFP line (CaMV35s::GFP). This line was used as a positive control for GFP expression in the imaging portion of the TAGES experiment. Figure 2A shows a white light image 2A and 2B provides the corresponding fluorescent image of the plant. 2C shows a tangle of roots from the collection of harvested material before the individual plants were separated. The GFP expressing root can be seen clearly on the backgrounds of the non-GFP roots.

Ground-based experiments were also conducted to calibrate GFP reporter gene utility for gene expression assays in RNAlater™ fixed material. Figure 3 shows the hypocotyl-root junction of Adh::GFP plants. The top row shows un-stressed plants, while the bottom row shows the pattern of GFP expression in hypoxic plants (flooding for 48 hours). Panels 3A and 3D are white light image, 3B and 3E are the corresponding fluorescent microscope images, and panels 3C and 3F are fluorescent microscope images of the same regions of plants after preservation in RNAlater™. The exposure times for 3B and 3E is 5 seconds (Olympus S2X12, GFP long-pass filter); however, since preservation in RNAlater™ diminishes the fluorescence of both chlorophyll (red) and GFP (green), the exposure time for 3C and 3F was extended to 10 seconds. In 3C the autofluorescence of plant cell wall is noticeable, and in 3F it can be seen that the GFP fluorescence that was masked by chlorophyll in the hypocotyl can now be seen with better clarity. The phenomenon of diminished GFP fluorescence in RNAlater™ preserved samples has also been demonstrated in flow cytometry applications (Zaitoun et al., 2010).
Cell and subcellular resolution of GFP is possible in RNAlater™ preserved tissue. Figure 4 shows the fluorescent microscopy used to collect subcellular data from a plant preserved in RNAlater™. The white light photograph in 4A shows the surface of the leaf, venation and a trichome. Figure 4B shows the same section of the leaf under fluorescent illumination to visualize GFP expression and 4C shows the same section after preservation in RNAlater™. Again, an extended exposure time was required to obtain similar signal strengths as seen in fresh tissue. In this case, the image in 4C was also subjected to post-capture processing. Minor adjustments of brightness and contrast can enhance the visualization of GFP. A comparison of the positions of the guard cells fluorescing in 4B compared to those in 4C confirm that the green fluorescence Fluorescent microscopy shows that GFP is distributed primarily among the guard cells of the epidermis, and is especially prominent in the nuclei of the guard cells and the trichome.

RNAmar™ preservation does not seem to be an impediment to at least a few other standard methods of subcellular labeling techniques. Figure 5 provides examples of DAPI nuclear staining (Figures 5A, 5B, 5C) and calcofluor white cell wall staining. DAPI and calcofluor white are both fluorescent stains (e.g. Kamo & Griesbach, 1993; Zhou et al., 2009). As seen in the figure, both stains are very effective in RNAlater™ preserved material. Figure 5A shows a freshly stained root next to a root stained after fixation in RNAlater™ (Figure 5B). Figure 5C shows the nucleus of a trichome stained with DAPI after fixing in RNAlater™. Figure 5D shows an RNAlater™ fixed root stained with calcofluor white.

In conclusion, plants containing GFP reporter genes can still be evaluated after being preserved in RNAlater™. This method of preserving GFP analyses may be important in situations where sophisticated imaging technology is not immediately available on orbit. It does appear that the level of GFP visualization is lessened in RNAlater™, but that the location of the signal is maintained even at subcellular levels. In addition, it is clear that plant material preserved in RNAlater™ can also be effectively used for other traditional sub-cellular labeling techniques. Given that RNAlater™ is a well-documented on-orbit fixative with a repeated and extensive deployment history within KFTs, it is likely that RNAlater™ could serve space biology science well beyond its originally intended purposes of nucleic acid preservation.

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Short Communication

Potential of the *Euprymna/Vibrio* Symbiosis as a Model to Assess the Impact of Microgravity on Bacteria-Induced Animal Development

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Long-duration spaceflight imposes numerous physiological challenges to astronauts working in the space environment. Some of these challenges have been well-documented including bone and tissue loss (Smith and Heer, 2002), dysregulation of the immune system (Crucian et al., 2008), and increased risk to infectious diseases (Wilson et al., 2007). However, relatively little is known regarding the impact of the space environment, specifically microgravity, on those mutualistic bacteria that interact directly with animal cells. To understand the effects of microgravity on beneficial microbes we propose using the model system between the bobtail squid *Euprymna scolopes* and the luminescent bacterium *Vibrio fischeri* strain ES114. For over 20 years the *Euprymna/Vibrio* system has provided key insight into the role that symbiotic bacteria play in the normal development of animal tissues (McFall-Ngai and Ruby, 1991; Weir et al., 2010). This mutualistic association has several advantages that render it highly amenable to experimental manipulation in the space environment. First, the symbiosis is monospecific with only one host and one symbiont making it easier to discern the effects of the symbiont on host cells. Second, both partners can be cultured independently in the laboratory and the symbiosis can be initiated at any point after the juvenile animal hatches. Lastly, the symbiosis can be activated and monitored using currently available flight hardware such as the Fluid Processing Apparatus (FPA; BioServe Space Technologies, Boulder, CO). Here, we present evidence from a pilot study that demonstrates the overall potential of the *Euprymna/Vibrio* model system to examine the impact of microgravity on bacteria-induced development of host animal tissues.

In the *Euprymna/Vibrio* model system the mutualism is initiated when symbiosis-competent strains of *V. fischeri* colonize a specialized host structure called the light organ (Figure 1A). On either side of the incipient light organ there are two appendage-like structures comprised of ciliated epithelial cells (Figure 1B). The function of these ciliated cells is to entrain bacteria from the environment into the vicinity of pores found on the surface of the light organ (Figure 1C). If the symbiosis-competent strain of *V. fischeri* is not present in the environment then these ciliated epithelial appendages are retained and the light organ does not undergo morphogenesis (Figure 1C, D).

However, if symbiosis-competent *V. fischeri* is present then the bacteria enter the light organ through one of the surface pores and travel to an epithelial-lined crypt space where the bacteria then begin to divide. Upon reaching a critical cell density the bacteria begin to luminesce within the light organ. A few hours after initial colonization the bacteria trigger a series of developmental remodeling events that includes an apoptotic cell death event of the ciliated epithelial appendages (Figure 1E). Over a period of 4 d the light organ undergoes complete morphogenesis resulting in the loss of the ciliated appendages (Figure 1F). Several of these bacteria-induced events, such as the onset of luminescence, occur at discreet time intervals within the first 24 h after the initiation of symbiosis. These early developmental events can be readily monitored to assess the progression of the natural symbiosis. To assess the tractability of the *Euprymna/Vibrio* symbiosis in the space environment, we first determined whether the animals could survive under microgravity conditions. To simulate microgravity we used eight 10-ml volume high aspect ratio vessels with a rotary culture system (HARV; Synthecon, Houston, TX) at 13 rpm. These HARV have been successfully used in numerous microgravity studies to reproduce space-like conditions (Hammond and Hammond, 2001) and have been shown to be directly comparable to real
Figure 1. The morphology of the *Euprymna scolopes* light organ. A. Juvenile animal with light organ (arrow) in the center of mantle cavity. Bar = 0.5 mm. B. Micrograph of nascent light organ depicting fields of ciliated epithelial appendages (cea) Bar = 100 μm. C. One half of 24 h aposymbiotic light organ with arrow pointing to pores. Bar = 50 μm. D. Micrograph of 96 h aposymbiotic light organ showing no signs of morphogenesis in the absence of competent bacteria. E. Symbiotic light organ at 14 h stained with acridine orange, a fluorescent molecule that intercalates into cells undergoing cell death. F. Symbiotic animal at 96 h showing full morphogenesis of the light organ and the loss of the cea.

Figure 2. Effects of simulated microgravity on *Euprymna/Vibrio* symbiosis. A. Comparison of the survivability of aposymbiotic (Apo) and symbiotic (Sym) host squid under gravity (+) and simulated microgravity (-). B. Impact of microgravity (-) on the luminescence of host squid compared to gravity (+) conditions.
microgravity conditions (for review Nickerson et al., 2004). Animals were collected within 1 h of hatching, rinsed in filtered seawater (FSW) and either maintained aposymbiotically (i.e., without *V. fischeri* ES114) or were rendered symbiotic by inoculating with *V. fischeri* at a concentration of 1 x 10^5 cells per ml of aerated FSW. Animals were placed in the HARV chambers and incubated for up to 72 h at 23°C. A subset of control animals was maintained under normal gravity conditions in 5 ml of aerated FSW in borosilicate vials at 23°C. The results of five replicate experiments (n = 8 animals per treatment) are depicted in Figure 2A. All of the animals maintained aposymbiotically under both normal gravity (n = 40) and simulated microgravity (n = 40) conditions survived to 72 h. However, in the symbiotic animals there was a difference in the survival rate between gravity and simulated microgravity treatments. In normal gravity conditions all symbiotic animals survived throughout the experiment, whereas in symbiotic animals exposed to simulated microgravity there was a gradual increase in animal death rate over the course of the three-day experiment. At 24 h, an average of 98% of the animals exposed to symbiotic bacteria survived in each of the five replicate experiments. However, the mean survival rate decreased to 90% and 75% by 48 and 72 h, respectively. The variability between experiments also increased over time (Figure 2A) and may be the result of differences between host squid size (2-3 mm), or may reflect changes in the symbiont under microgravity conditions. Despite the gradual decrease in survival, most symbiotic squid survived the HARV experiments during the critical time window (0 - 24 h) when many of the early symbiosis phenotypes are being initiated. Additional testing of symbiotic animals using the HARV chambers perpendicular to the axis of rotation in the microgravity treatments showed that the survival rate was 100% (n = 15) after 72 h. These results coupled with the 100% survival of the aposymbiotic controls in the HARV chambers under microgravity conditions indicate that the HARV chamber itself has a minimal impact on the squid survival. Lastly, animals tested with flight approved FPA hardware under normal gravity conditions indicated that the animals survived up to five days in aerated FSW (data not shown); well beyond the four-day time window of light organ morphogenesis.

In addition to basic survival under microgravity conditions we examined the onset of luminescence, an early phenotype of the symbiosis and bacterial growth rate in the HARV chambers. In the experiments described above, the luminescence of the host squid was checked by temporarily removing the aposymbiotic and symbiotic animals from the HARV chamber for ~5 min, placing them in borosilicate vials and inserting the vial into a luminometer to measure luminescence (GloMax 20/20, Promega, WI). The results indicated that at 24 h there was no statistical differences between luminescence levels between symbiotic animals maintained under normal gravity and simulated microgravity (Figure 2B). However, by 48 h there was a significant decrease in the luminescence output of microgravity-exposed symbiotic animals. In addition to luminescence there were also differences detected in the growth rate of the symbiont under microgravity conditions. *V. fischeri* cultures within the HARV were examined and shown to have a 2-fold increase in growth rate at 24 h compared to gravity controls (data not shown). These results indicate that although microgravity does not impact the initiation of the symbiosis, microgravity does affect the persistence and maintenance of the symbiosis compared to the normal gravity controls. These results may reflect the health of the host organism, or the flow of oxygen into the light organ crypt spaces, as luminescence requires aerobic conditions. However, the bacteria were viable despite the loss of luminescence. Plating of dissected, homogenized light organs on sea water tryptone agar plates revealed no significant loss in cell numbers between animals exposed to microgravity and gravity conditions (data not shown).

Together these results suggest that the *Euprymna/Vibrio* model system can be successfully manipulated under simulated microgravity conditions and that the symbiotic association may be negatively impacted by microgravity. Further work is required to elucidate the precise effects that microgravity has on the symbiotic partners and the overall developmental timeline of the host squid. Understanding the effects that spaceflight has on the animal/human microbiome may provide critical insight into maintaining the health of crew members and decrease their potential risk during the exploration of space.

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Effects of Slow Clinorotation on Growth and Yield in Field Grown Rice

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Several studies concerning with the effects of microgravity conditions on plant growth have already been carried out using spacecrafts (Abilov et al., 1986; Aliyev et al., 1987; Kordyum, 1994). Clinostats have been used to compensate for the unilateral influence of gravity for a long period of time in the field of plant physiology. Several experiments have been carried out on a slow rotating (ranging from 0.25 – 3 rpm) clinostat to study the effects of clinorotation also termed as simulated microgravity on plant growth and development.

Plants, especially cereal crops, are the basis for human being to accomplish the food requirement; hence, it is of great importance to study the influence of microgravity on growth and development in food grain crops. Earlier studies carried out on the effects of clinorotation on plants showed that plant growth, morphology, chlorophyll content was affected by slow clinorotation (Sievers et al., 1996; Sack 1997; Jagtap et al., 2010). Several reports have described the effects of clinorotation on physiology of plants and cell biology (Hilaire et al., 1995a), protein expression (Piastuch and Brown, 1995), carbohydrate metabolism (Brown and Piastuch, 1994, Obenland and Brown, 1994), calcium distribution (Hilaire et al., 1995b) and the cell cycle (Legue et al., 1992). Colla et al. (2007) showed that morphological and growth characteristics such as growth, yield, and quality of the dwarf tomato variety ‘Micro-Tom’ were modified during clinorotation treatment. They also showed that clinorotation-exposed seeds were viable and subsequent generations might be obtained in microgravity (Colla et al., 2007). In most of the studies, seeds were germinated and grown under clinorotation. As per our knowledge, there are no reports on the effects on changes in growth, yield, and soluble protein content of seeds from plants that were grown from clinorotated seeds. In the present investigation, effects on growth, various yield attributes as well as seed nutrient contents such as starch, carbohydrates and protein content in plants raised from rice seeds (Oryza sativa L) exposed to slow clinorotation (2 rpm) for 12 days and grown in the field at normal (1 g) condition were studied.

Authentic seeds of rice (variety PRH-10) were procured from National Seeds Corporation, New Delhi, India. Total 600 mature seeds of uniform size were selected and surface sterilized by using 0.5% fungicide (Uthane M-45® manufactured by United Phosphorus Limited), washed 4-5 times with distilled water (D/W) to remove traces of fungicide, and soaked in D/W for 24 h. After 24 h soaking, seeds were removed from D/W and blotted dry with blotting paper to prevent germination during clinorotation. For clinorotation treatment, 300 seeds were placed in cavities made in disc shaped thermacol on a periphery of a circle of radius 1.5 cm. On each piece of

Figure 1. Plant growth in control and clinorotated samples. The Control plants are shown on the left and the plants grown from clinorotated seeds on the right.
thermacol 25 seeds were placed. About 12 such thermacol discs containing 300 seeds separated with cotton were placed in two Perspex® beakers and then clinorotated for 12 days continuously at 2 rpm resulting in \( g \sim 7 \times 10^{-5} \) (Jagtap et al., 2010) in dark under ambient conditions of temperature (23±5 °C) and humidity (50±10 %). Remaining 300 seeds at same environmental conditions but without clinorotation were used as control. After the treatment, clinorotated and control seeds packed in polythene bags were stored at room temperature, and sent to Agricultural Research Station, Shirgaon, District-Ratnagiri (Maharashtra) India. On 15th day from the date of clinorotation treatment, seeds were sown in the field at Agricultural Research Station, Shirgaon. Seedlings were transplanted on 40th day after sowing (DAS). All the measurements such as plant height, number of productive tillers per plant, length of panicle, number of tassels, number of filled grains per panicle, weight of 1000 filled grains, biological yield per plant, and harvest index were recorded at the time of harvest, i.e. on 80th day after transplanting. The results of present study revealed that the exposure of seeds to slow clinorotation for 12 days had caused significant improvement in paddy yield and protein content over control.

The appreciable changes were observed in various yield attributes. Number of productive tillers per plant, height of tiller from soil level (Figure 1), length as well as number of panicle (Figure 2), number of tassels, number of filled grains, total grain weight per plant, weight of 1000 grains, biological yield per plant, and harvest index were recorded at the time of harvest, i.e. on 80th day after transplanting. The results of present study revealed that the exposure of seeds to slow clinorotation for 12 days had caused significant improvement in paddy yield and protein content over control.

In comparison with these observations the earlier studies have shown that the ‘Micro-Tom’ plants grown under simulated microgravity exhibited a spreading growth and an increase in internodal length. The number of flowers per plant was significantly increased by 32% in clinorotated plants in comparison to those grown in absence of clinorotation. However, total fruit yield, small fruit yield, leaf area, leaf dry weight, fruit dry weight, total dry weight were decreased significantly in the clinorotated tomato plants than those grown in the control treatment (Colla et al., 2007). Also the studies carried out by Hilaire et al. (1996) confirmed that slow clinorotation had influenced morphology and ethylene production, i.e. metabolic process in soybean seedlings. However, the present study differs in one respect; that

**Table 1. Effects of clinorotation on different yield attributes in field grown rice**

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Control</th>
<th>Clinorotated</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of productive tillers / plant</td>
<td>14.5±0.7</td>
<td>18.0±0.7*</td>
</tr>
<tr>
<td>Height of tiller from soil (cm)</td>
<td>40.0±1.7</td>
<td>42.4±1.3**</td>
</tr>
<tr>
<td>Number of tassels</td>
<td>8.0±0.7</td>
<td>10.4±0.6*</td>
</tr>
<tr>
<td>Length of panicles (cm)</td>
<td>18.7±0.3</td>
<td>23.3±0.3*</td>
</tr>
<tr>
<td>Number of grains per panicle</td>
<td>71.6±0.5</td>
<td>105.4±0.6*</td>
</tr>
<tr>
<td>Filled grains per panicle</td>
<td>34.6±0.8</td>
<td>46.2±0.1*</td>
</tr>
<tr>
<td>Number of filled grains per plant</td>
<td>493.6±12.9</td>
<td>828.5±17.3*</td>
</tr>
<tr>
<td>Total grain weight per plant (g)</td>
<td>14.4±0.2</td>
<td>23.3±0.1*</td>
</tr>
<tr>
<td>Weight of 1000 grains (g)</td>
<td>20.4±0.01</td>
<td>20.8±0.02*</td>
</tr>
<tr>
<td>Biological yield per plant (g)</td>
<td>18.0±0.6</td>
<td>24.3±0.9*</td>
</tr>
<tr>
<td>Harvest Index</td>
<td>80.1±3.4</td>
<td>95.9±3.3*</td>
</tr>
<tr>
<td>Total Chl content (3rd leaf)</td>
<td>5.71±0.9</td>
<td>7.97±1*</td>
</tr>
<tr>
<td>Protein content</td>
<td>11.10%</td>
<td>11.80%</td>
</tr>
<tr>
<td>Carbohydrate (Total sugars)</td>
<td>66.90%</td>
<td>66.40%</td>
</tr>
<tr>
<td>Starch</td>
<td>54.60%</td>
<td>51.60%</td>
</tr>
<tr>
<td>Embryo viability (harvested seeds)</td>
<td>~ 97%</td>
<td>~ 97%</td>
</tr>
</tbody>
</table>

**Figure 2. Number of panicles in control (left, blue bowl) and the plants grown from clinorotated seeds (right, red bowl).**

**Table 1. Effects of clinorotation on different yield attributes in field grown rice**

In this study, the exposure of seeds to slow clinorotation (2 rpm) for 12 days and grown in field which is highly important and having a dietary significance (Table 1). However, there was ~ 1% and ~ 5 % reduction in carbohydrate and starch content respectively. It is well documented that plants with high chlorophyll concentration exhibit higher rates of photosynthesis under low light relative to plants with less chlorophyll pigment concentration (Bjorkman, 1981; Boardman, 1977). Therefore increase in growth and various yield attributes in this study may be due to an increase in chlorophyll content in clinorotated sample.
is, the seeds were exposed to clinorotation and plants were grown under normal (1 g) condition. Hence the observations in the present study show a marked difference from the studies reported earlier.

Also the results of tetrizolium test performed on rice seeds from harvested plants showed ~ 97 % embryo viability in control as well as clinorotated seeds suggesting that these seeds have mature embryo and can be used further for sowing to raise new crop. Similar results were obtained by Colla et al. (2007) showing that seeds formed under simulated microgravity proved to be biologically and functionally complete, showing that 'Micro-Tom' plants could realize complete ontogenesis, from seed to seed in microgravity (Colla et al., 2007).

In the present investigation seeds exposed to clinorotation before germination were sowed under normal (1 g) conditions fifteen days after the exposure, and compared directly to seeds with identical heritage that had not been subjected to clinorotation. Comparisons of the development of seeds exposed to clinorotation to the development of the control seeds showed statistically significant differences in the two sets as the plants matured. These data suggest that the physiological differences between the two sets of mature plants are related to the exposure of the seeds to clinorotation, as all other aspects of the seed treatment and culture were controlled between the two sets. This is a novel observation and may have future benefit for improving crop productivity of various cereals, including rice.

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A Retrospective Analysis on Gender Differences in the Arterial Stiffness Response to Microgravity Exposure

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BRIEF SUMMARY

Several studies indicate that the incidence of orthostatic intolerance (OI) among female astronauts is significantly higher than compared to their male counterparts. Our lab has previously demonstrated that astronauts who are orthostatically tolerant after spaceflight develop an increase in central arterial stiffness during spaceflight. We hypothesize that the arterial stiffness of female astronauts does not increase, or increases insufficiently, during spaceflight. This may explain, in part, the gender difference in the incidence of OI. We explored this hypothesis by using previously collected hemodynamic data from astronauts as well as from subjects subjected to -6° head-down tilt bedrest. Female astronauts had a non-significant decrease in arterial stiffness following spaceflight (0.08 ± 0.066 mmHg/ml, N=11, P=0.2405). In contrast, male astronauts demonstrated a significant increase in arterial stiffness (0.10 ± 0.04 mmHg/ml, N=46, P=0.0145). Female bedrest subjects had an insignificant increase in arterial stiffness during bedrest, (0.17±0.059 mmHg/ml, p=0.0973, N=3); however, it was not as great as the increase in arterial stiffness developed by the male subjects (0.33±0.056 mmHg/ml, p=0.01, N=4). Thus, it is apparent that there is a gender difference in the arterial stiffness response to microgravity exposure. This gender-based differential arterial stiffness response may explain, in part, the disparity of microgravity-induced OI incidence between the genders.

INTRODUCTION

It has been reported that the incidence of post-spaceflight orthostatic intolerance (OI) is between 35-100% for women compared to 7-20% for men (Fritsch-Yelle, et al., 1996; Waters, et al., 2002). There is a large disparity between males and females in terms of microgravity induced OI. It is well known that OI is due to a collection of maladaptive responses to spaceflight involving the cardiovascular system. These responses have been noted in both men and women. However, gender studies among the astronaut population have highlighted that female astronauts have further depressed adrenergic responsiveness, a higher dependence on plasma volume status, and a decreased ability to increase total peripheral resistance, in response to a gravitational stress following microgravity compared to male astronauts (Meck, et al., 2004; Waters, et al., 2002). Although these findings are all likely significant contributors to the gender disparity in OI, they alone may not entirely account for the difference.

We have recently demonstrated that orthostatically tolerant (OT) astronauts had an increase in central arterial vascular stiffness following spaceflight while orthostatically intolerant (OI) astronauts showed either no change or a slight decrease in this parameter (Tuday, et al., 2006). We hypothesized that the increase in vascular stiffness was beneficial for the astronauts in terms of OI based on the inverse relationship of arterial stiffness and the resistance to venous return (RVR). The OT astronauts were able to decrease their RVR through an increase in arterial stiffness which allowed for the maintenance of cardiac output and arterial blood pressure during an orthostatic stress. We contend that females experience little to no change in arterial stiffness while males exhibit large increases in vascular stiffness as a result of microgravity exposure. This would predispose females to OI, and may represent an underlying cause in the gender disparity in microgravity induced OI.
The goal of this study was to determine whether there is a gender difference in the vascular stiffness response to microgravity. We hypothesize that female vascular stiffness does not increase to the same degree as men in response to actual (spaceflight) or simulated (bedrest) microgravity. We have undertaken this study utilizing previously collected astronaut and bedrest data.

METHODS

Human astronaut data was obtained and analyzed as previously described (Tuday, et al., 2006). Briefly, all human astronaut data was from previously published studies, with permission from the lead researcher, conducted by Meck, et al. (2004) The data set consisted of supine hemodynamic measures taken from 57 short duration flight astronauts (11.5 +/- 1 days for females and 12.1 +/- 0.4 days for males, range 5-18 days; p=0.22) usually ten days before launch, on landing day, and three days after landing. Detailed methods are available from Meck, et al. (2004) and Fritsche-Yelle, et al. (1996). Briefly, blood pressure was measured using an automated arm cuff. Aortic cross-sectional area, determined using two-dimensional echocardiography, and ascending aortic flow, sampled with pulsed-Doppler, were used to determine the stroke volume. Arterial stiffness was estimated as the arterial pulse pressure (systolic minus diastolic blood pressure) divided by the stroke volume. The data was then pooled into male and female groups.

The human bedrest protocol was approved by the Johnson Space Center Institutional Review Board, and all subjects gave their written informed consent. Seven healthy subjects (four males, three females) underwent 45-60 days (49.5 +/- 3.6 days for males and 57.7 +/- 2.3 days for females; p=0.5039) of –6° head-down bed rest. Hemodynamic data was collected in the same manner as was done for the astronauts. Arterial stiffness was estimated as described.

RESULTS

Male astronauts demonstrated a significant increase in arterial stiffness after spaceflight (0.57±0.024 mmHg/ml pre-flight vs. 0.67±0.032 mmHg/ml at landing day, p=0.0145 N=46) (Figure 1).

Female astronauts did not respond in the same manner to microgravity as did males despite the fact they both had similar pre-flight values (Figure 2). The female astronauts demonstrated a trend toward a significant decrease in arterial stiffness (0.56±0.060 mmHg/ml pre-flight vs. 0.48±0.027 mmHg/ml on landing day, N=11). Both male and female blood pressures and stroke volumes are presented in Table 1.

Table 1. Astronaut mean arterial pressure (MAP) and stroke volumes (SV) ten days before, immediately after, and three days after a mission. Data are reported as means +/- SE.

<table>
<thead>
<tr>
<th></th>
<th>Preflight</th>
<th>Landing</th>
<th>Post Landing</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP</td>
<td>89 (7)</td>
<td>94 (10)*</td>
<td>90 (9)</td>
</tr>
<tr>
<td>SV</td>
<td>86.6 (20.4)</td>
<td>78.8 (21.4)</td>
<td>84.9 (24.9)</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP</td>
<td>75 (6)</td>
<td>82 (6)*</td>
<td>77 (6)</td>
</tr>
<tr>
<td>SV</td>
<td>79.3 (15.7)</td>
<td>77.1 (10.6)</td>
<td>79.2 (17.8)</td>
</tr>
</tbody>
</table>
Male bedrest subjects had a significant increase in arterial stiffness following bedrest (0.61±0.11 mmHg/ml pre-bedrest vs. 0.94±0.10 mmHg/ml post-bedrest, p=0.01, N=4) (Figure 3). Female bedrest subjects had a small increase in arterial stiffness, though it was less than that of the male subjects (0.61±0.055 mmHg/ml pre-bedrest vs. 0.78±0.10 mmHg/ml post-bedrest, p=0.0973, N=3). Both male and female blood pressures and stroke volumes are presented in Table 2.

Table 2. Bedrest (BR) mean arterial pressure (MAP) and stroke volumes (SV) before and after bedrest. Data are reported as means (+/- SD).

<table>
<thead>
<tr>
<th></th>
<th>Pre BR</th>
<th>Post BR</th>
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<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP</td>
<td>86 (8.5)</td>
<td>96 (4.8)</td>
</tr>
<tr>
<td>SV</td>
<td>80.2 (31.4)</td>
<td>68.4 (17.3)</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP</td>
<td>80 (5.8)</td>
<td>76.7 (3.1)</td>
</tr>
<tr>
<td>SV</td>
<td>59.7 (4.5)</td>
<td>57.7 (3.6)</td>
</tr>
</tbody>
</table>

DISCUSSION

It is apparent from the data that there is indeed a gender difference in the arterial stiffness response to microgravity exposure. This was evidenced by the significant increases in male vascular stiffness during both actual and simulated spaceflight compared to unmatched changes in their female counterparts. As compared with males, the failure of females to increase vascular stiffness during spaceflight impairs their ability to lower their RVR. This in turn makes it more difficult to maintain adequate venous return, cardiac output, and arterial blood pressure during an orthostatic stress. Finally, our data present a strong case for actual vessel stiffness changes rather than simply vessel distension; though the mean arterial pressure was elevated in both male and female subjects at landing, stroke volume was decreased (though not significantly) indicate a greater pressure response to less volume. Furthermore, both groups had parallel changes which would not explain the gender discrepancy in stiffness. However, caution must be used in interpreting these results as they are estimates and not direct measures of stiffness. In conclusion, the observed gender-based differential stiffness response may explain, in part, the large disparity of OI incidence between the genders. The physiologic and molecular mechanisms underlying this observation remain to be explored.

ACKNOWLEDGEMENTS

We appreciate the help of the NASA cardiovascular team. This research was supported in part by a grant from the NSBRI (CA00405), by a NIH grant (ROI HL105296), and by a grant from NASA (BRC-2003-0000-0543).

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Metabolic Control as a Strategy for Payload Cost Reduction and Mitigation of Negative Space Environmental Factors

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As human presence in space will likely extend to the Moon and beyond, such exploration missions will involve significant risks of accidents, system failures, and various life-threatening emergency situations. The ability to put space explorers into a temporary hypo-metabolic state using metabolic control technologies would enhance our ability to manage these risks. Reversible arrest of essential life processes would radically reduce astronaut needs for life support and produce extraordinary resistance to environmental stress, such as radiation exposure and low-gravity. It can also solve many medical problems including diseases and physical injury over a long period in space.

Despite promising avenues of these enquiries, the mechanism of inducing such reversible hypometabolic state in non-hibernated animals is not yet fully understood (Blackstone, et al., 2005; Tøien, et al., 2011; Haouzi, et al., 2008). We developed the methodology to achieve metabolic control, which allows the metabolism of non-hibernated animal model system (mice) to be reduced to a minimum level for a significant time and subsequent restoration to normal level with no apparent effect on physiology or neurological function. In order to demonstrate the potential application of the metabolic control technology we have engineered a hypometabolic chamber with a range of life-monitoring equipment.

Figure 1. Schematic presentation of the hypometabolic system used in this study.
for high-throughput testing of hypometabolic parameters and conditions that enable reversible induction of a state of suspended animation in mice (Figure 1). The automated monitoring system is responsible for initiation, maintenance, and termination of the stasis through the administration of hypometabolic agents and providing immediate short-term monitoring and control.

C57BL/6J male mice from Jackson Laboratories were used in this study. The animals were 6 weeks of age at the time of experimentation and weight 20-30 g. Two groups of 5 animals (control and experimental) were implanted with biotelemetry transmitters to continuously measure body temperature and cardiac activity. Induction of the hypometabolic state in mice was carried out by using a custom designed atmospheric chamber. Following short adaptation in air atmosphere mice was exposed to 80 ppm H₂S air mixture at a constant pressure and flow rate 1L/min to induce hypometabolic state (Blackstone et al., 2005). At this point the environmental temperature was decreased gradually using programmable temperature control software from ambient temperature to 15°C. Hypometabolic state was evident by reduction in metabolic rate and drop in oxygen consumption and carbon dioxide output. After 10 hours of exposure to H₂S the atmosphere in the chamber was substituted with air and mice were returned to room temperature. The recovery to the normal state was monitored by metabolic rate and core body temperature returning to normal. After this stage animals were placed in a standard vivarium cage for 30 days observation period. All animal experiments were performed in strict accordance with National Institutes of Health guidelines, and animal protocols were approved by the IACUC at Ames Research Center.

After exposure to the hypometabolic mixture, core body temperature drops from 37°C to 15°C over 8.5 hours of the gas exposure (Figure 2) leading to substantial reduction in rate of all biochemical reactions in organism.

The mouse initially started at a heart rate of 762 beats/minute, then dropped to as low as 120 beats/minute after 10 hours (Figure 3) – a 75% drop in heart rate.

![Figure 2](image)

Figure 2. Core body temperature of mice exposed to H₂S using the same protocol and recovered from the stasis at different times. Control mice were not exposed to H₂S. Each data point is average of three experiments.

![Figure 3](image)

Figure 3. Heart rate recorded at different body temperatures. (a) 37°C; (b) 15°C; (c) 37°C, after recovery from the stasis.

Our results show that the hypometabolic stasis can be significantly extended from 4 hours as demonstrated in previous study (Blackstone et al., 2005), up to 11, or even 18 hours (not shown) by optimization of the transform kinetics and environmental parameters. Under this condition the respiratory rates dropped from132 breaths per minute.
to less than 5 breaths per minute, demonstrating reduction in metabolism more than 94%. After recovery from the stasis, none of the hypometabolic animals displayed any behavior deficits over 3 months of observation. We have shown that recovery from the intentionally induced hypometabolic state may be 30% better than in natural hibernators (~47% lethality) when the kinetics of induction, extension and recovery from this state are optimized.

Our data provide basis for subsequent animal studies and novel approaches in further extension of this state and development of continuous external monitoring and modification of metabolic conditions. Despite the obvious limitations of H$_2$S for induction of hypometabolism in large animals, such as sheep (Haouzi et al., 2008), its application on small animals allows investigation of key underlying mechanisms and consequences of metabolic alteration in non-hibernated animals regardless of stasis protocol. Some other potential beneficial outcomes of the metabolic reduction, such as increase in life span, resistance to ionized radiation, low gravity and many pathogenesis are currently under investigation.

Investment in a program of research to define the optimum methods for achieving metabolic control in larger animals is needed at this time, in order to demonstrate application of a metabolic control system within deep space mission architecture as a potentially enabling technology.

REFERENCES


Short Communication

Iron Ion ($^{56}$Fe) Radiation Increases the Size of Pre-Existing Atherosclerotic Lesions in ApoE-/- Mice

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Epidemiological studies have demonstrated that radiation exposure from a number of terrestrial sources is associated with an increased risk for atherosclerosis. For example, for women with early breast cancer, the benefit of radiotherapy can be nearly offset by the increased risk of mortality from vascular disease (Early Breast Cancer Trialists' Collaborative, G., 2000). In fact, head and neck cancer patients who undergo radiation treatment are at significantly elevated risk of stroke, even in a relatively young patient population. Similarly, atomic bomb survivors have an increased incidence of coronary artery disease and stroke. Even radiation technologists working before 1950 (when occupational exposure was higher) had increased mortality due to circulatory diseases (Hauptmann et al., 2003).

The risk of radiation during deep-space travel, however, is difficult to predict. To date, the only travel beyond the protection of the Earth’s magnetic field by humans has been the moon missions. Since so few astronauts have been exposed to these high levels of radiation, there is a lack of epidemiological data available to estimate the cardiovascular risks involved. Furthermore, studies of the effects of cosmic radiation using animal atherosclerosis models have been lacking.

In addition, astronauts will encounter not only X-rays and γ-rays, which are major components of most terrestrial radiation, but also cosmic radiation, which differs from radiation encountered on Earth in important ways. Cosmic radiation includes accelerated ions, which interact with tissues differently than photons. A small percentage of these ions are heavier elements such as $^{56}$Fe. This small component of cosmic radiation is particularly damaging, however, due to its high energy and propensity to interact with shielding to generate secondary particles.

Recently, we examined the effect of $^{56}$Fe on 10-week old apolipoprotein-E deficient (apoE-/-) mice, a well-established atherosclerosis model (Yu et al., in

Figure 1. Radiation increases atherosclerotic plaque development in the aortic arch of apoE-/- mice. ApoE-/- mice were anesthetized by intraperitoneal injection of ketamine and xylazine (150 mg/kg and 15 mg/kg body weight respectively), immobilized in custom-built mouse holders, and irradiated with 2 or 5 Gy of $^{56}$Fe. Thirteen weeks later, aortae were dissected out, cut open, pinned flat, and stained with Sudan IV. Images were acquired with a digital camera attached to a dissecting microscope. A-C are images of the aortic arch, cut open and pinned flat so that the entire inner surface is visible. Red = atherosclerotic plaques. (D) Experimental setup showing mouse in a holder. A collimator was used to restrict radiation exposure to a targeted area slightly smaller than the cutout in the holder. White boxes over image of dissected mouse (E) and en face aorta preparation (F) delineate the portion of the aortic tree exposed to radiation.
press). These mice spontaneously develop atherosclerotic lesions while on a normal diet, as do humans. Radiation was targeted to the aortic arch, avoiding exposure of other major organs. Thirteen weeks later, irradiated mice had significantly greater atherosclerosis than un-irradiated controls. It was not determined, however, whether this was due to increased production of new atherosclerotic plaques or accelerated development of existing lesions.

The current study was undertaken to address this question. Ten-week-old apoE −/− mice were irradiated at Brookhaven National Laboratory with 2 or 5 Gy of 600 MeV 56Fe, restricted to the area of the aortic arch. The mice were then shipped to the University of Alabama at Birmingham and maintained on a normal diet. At 13 weeks post-irradiation, the mice were euthanized and dissected to analyze atherosclerotic plaque development (Figure 1).

Images similar to those in Figure 1A-C were then analyzed to quantify the number and size of the aortic lesions for each radiation dose (Figure 2). The lesion size in mice exposed to 5 Gy 56Fe was significantly greater than in un-irradiated controls (measured as a percentage of total vessel surface area to control for differences in stretching of the tissue during preparation). This difference was not present in un-irradiated portions of the aorta. The number of atherosclerotic lesions, however, was not affected.

Therefore, exposure to 56Fe accelerates development of existing plaques to increase lesion size. This radiation-induced vessel wall damage might have serious consequences years after deep space exploration. Since asymptomatic atherosclerotic lesions are common in young, healthy adults (Velican and Velican, 1980), it is expected that exacerbation of pre-existing plaques may pose a significant threat for astronauts.

In this study, a brief exposure to radiation caused irreversible damage to the artery, resulting in atherosclerotic progression as the animal aged. This is consistent with human epidemiologic data, where a brief exposure to radiation from an atomic blast is known to increase cardiovascular risk years later. Interestingly, protracted exposure to relatively low level radiation, such as that experienced by early radiation technologists, carries similar risks.

The dose required to produce this effect was somewhat higher than what astronauts would encounter, for example, on a Mars mission. It has been estimated that astronauts would absorb a cumulative dose of 0.42 Gy of accelerated ions during a 1000-day mission to Mars and back (Cucinotta, F. and Durante, M., 2006). The mice in this study, however, were exposed to an acute dose of 56Fe over less than ten minutes, whereas astronauts will be exposed to a mixture of accelerated ions and high-energy photons over a prolonged period. It is likely that all components of radiation in the galactocosmic radiation spectrum, both photons and particles, will contribute to atherosclerosis.

In addition, mice are known to be relatively resistant to radiation-induced atherosclerosis. For example, in a similar study (in which radiation was similarly restricted to the upper aorta and parts of the carotid arteries), atherosclerotic changes in apoE −/− mice were seen following 14 Gy of X-rays (Stewart, F.A., et al., 2006). It is known from epidemiologic studies, however, that X- and gamma rays can be a risk factor for humans at doses as low as 1Gy. It is, therefore, quite possible that much lower doses of 56Fe might have significant effects on atherosclerosis in humans.

![Figure 2. Radiation increases lesion area but not lesion number. 10-week old apoE −/− mice were irradiated once with the indicated dose of 56Fe, then fed a normal diet for 13 weeks. Images of dissected aortas (as in Figure 1) were then analyzed to determine the area of atherosclerotic lesions as a percent of total vessel area. While the percent of the irradiated aorta occupied by atherosclerotic lesions (A) was significantly increased at 5 Gy (p = 0.012 by Mann-Whitney non-parametric test), the number of lesions (B) was not significantly different. N = 10 animals in each group.](image)
We conclude that the potential for $^{56}$Fe radiation to exacerbate early, subclinical atherosclerosis constitutes a major concern for astronaut health on prolonged deep-space missions. Further studies will explore the underlying pathological mechanisms in order to better estimate risk and develop appropriate countermeasures.

ACKNOWLEDGEMENTS

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REFERENCES


Short Communication

Cellular and Molecular Biology During Spaceflight

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While the International Space Station (ISS) offers the possibility of maintaining microorganisms, cells, and tissues, it does not possess the instrumentation and facilities required to perform modern (analytical) molecular biology techniques. In the near future and due to the lack of sample return capacity following shuttle retirement, state-of-the-art instrumentation enabling onsite acquisition of molecular data will be critically needed. Space-adapted technologies will also serve to develop in situ medical diagnostic tools for future manned spaceflights. The Canadian Space Agency has selected a few innovative technology platforms intrinsically endowed with a very high potential for supporting in situ biomolecular analysis and biodiagnostics. These systems were selected for their versatility, their potential as robust, portable and compact instruments, and their capacity to detect and quantify a large spectrum of macromolecules and biomarkers. This paper will focus on the development of a miniaturized fiber optic flow cytometer for space life science research and on-board medical diagnostic.

One of the most versatile analytical instruments in molecular biology is a flow cytometer. By using fluorescent dyes or fluorochrome-coupled antibodies, a flow cytometer can be used to perform immunophenotyping, intracellular staining, gene expression studies, signal transduction studies, and many assays such as calcium flux, proliferation, apoptosis, cytokine secretion, etc. (Bonetta, 2005). Once cell suspensions are labeled with fluorescent markers, they are excited by one or several light sources at specific wavelengths and the emitted light is collected and analyzed to perform quantitative molecular assessment. Alternatively by using microbead-coupled antibodies, concentration of multiple soluble analytes (hormones, microbial molecules, biomarkers) can be quantified. The availability of such an instrument in Low Earth Orbit (LEO) would significantly accelerate space life science research, reduce the requirement to bring samples back to investigators, decrease sample storage needs on the ISS and support crew medical monitoring (i.e. immunology, hematology sampling amongst others). A first attempt to determine whether a flow cytometer could function in microgravity was performed using NASA KC-135 reduced gravity research aircraft (Crucian and Sams, 2005). However, even if they could withstand launch, conventional flow cytometers would be restricted for use in ISS due to their need for manipulation by highly qualified personnel, and cumbersome optical and fluidic management requirements. The current flow cytometers available on the market, with their footprint, complexity and fragility are not compatible with the constraints of human spaceflight. Moreover, experience has shown that in particular, fluidic interactions in microgravity are highly affected. For instance, tiny air bubbles that naturally move to the top of a ground-based apparatus can coalesce in a side-mounted pump cavity in microgravity, preventing it to prime. In consideration of capillary and/or centrifugal fluidics forces, which are foremost at play, a miniaturized fiber-based and sheathless flow cytometer, would have substantial advantages in its acceptance as standard crew equipment. Another advantage of a small, portable system is that it can be battery operated, simplifying the interface restrictions and handling, while increasing the flexibility for operation locations within the ISS. A cytometer that can be independently used by the crew, without requirements for call down, specific time-dependant data transfers or long and complex operational procedure, for instance, will also gain valuable time as well as maximum utilization efficiency with the crew.

As shown in Figure 1, a new compact and portable flow cytometer (MicroFlow-1) is under development based on a fiber-optic flow cell (FOFC) technology designed at the ‘Institut National d’Optique’ (INO) in Canada. A special optical fiber has been designed and engineered, through which a square hole is transversally bored by laser micromachining. A capillary is fitted into that hole to flow analyte within
the fiber square cross-section for detection and counting (Figure 2).

Figure 1. Fiber optic flow cytometer: Microflow-1.

Figure 2. Fiber-optic flow cell (FOFC) concept.

Figure 3. Comparison between FACSarray (right panel) and Microflow-1 (left panel) using LinearFlow Deep Red beads.

A micro-flow cytometer would strongly enhance the capabilities of a diagnostic system designed to monitor spacecraft environment as well as crew health status (Cohen, 2008). Flow cytometers are now routinely used in clinics to perform cell-based assays for real-time determination of leukocyte counts, immune system activation, identification of abnormal DNA content due to the presence of tumor cells, as well as HIV infection based on CD4 T cell counts (Brown and Wittner, 2000). Beside medical diagnostic applications for astronauts, such an instrument will be invaluable to collect real-time data from gravitational biology experiments for instance using GFP reporter genes in transformed cell lines. The possibility to use microbead assays such as the Cytometric Bead Array (CBA) assay (BD Biosciences) further broadens the application potential of this platform to molecular detection. The principle of the CBA assay is to mix several populations of antibody-coupled fluorescent microbeads to a sample of 25 µl to 50 µl of biological fluid (lysate, serum, or cell supernatant), the beads playing the role of solid support in the suspension. Microbeads are linked to specific molecules within the sample through antibodies, and fluorochrome-conjugated antibodies are added to the sample to form complete sandwich complexes. For instance, the Human Th1/Th2 Cytokine assay (BD Biosciences) can be used to simultaneously quantify the concentration of six cytokines. Such assay can be customized by using antibodies against soluble factors of interest for space life science experiment and can then be performed in situ on the ISS or future spacecrafts.

The key feature sets of portability, affordability, and inherent ruggedness of the micro-flow cytometer concomitantly render it ideal as a point-of care (PoC) tool. This is mainly attributed to the usage of the core fiber-optic flow cell technology, and compatibility with low-cost optical sources (i.e. diode lasers,
LEDs). It also has the ability to translate to a handheld device for both absolute count and assessment of cellular and molecular biomarkers. One of the major commercial drivers for the development of such a device is to enable regular and continuous monitoring of immunologically compromised individuals. For example, patients requiring partial white blood cell differentials analysis, HIV and acute leukemia testing, or sepsis monitoring, can be accommodated within access restricted areas (i.e., war zones, economically challenged countries, where resources are scarce and test simplicity is highly valued). To this aim, we have conducted initial pilot benchmarking studies of our Microflow technology (comparing to commercial cytometers) for CD45 and CD4 cell staining in the context of PoC HIV testing and T-cell monitoring applications. Using standard methods and protocols in lysed whole blood samples, the outcome was very promising, indicating the ability to clearly distinguish monocyte, lymphocyte and granulocyte populations, based on CD45 expression and light scattering channels (Figure 4). The results compared well in terms of sensitivity and CV performances parameters to current commercial cytometer technologies.

The development of biomedical devices for PoC testing is a growing worldwide trend and is now a focus of the Canadian program in Space Life Sciences. Such instrumentation will support state-of-the-art experiments and accelerate research in gravitational biology by providing real-time data while decreasing the requirement for sample return.

**REFERENCES**


Differentiation of Dental Stem Cells in Simulated Microgravity

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Stem cell niches are present in many different tissues and organs, however, there are currently no reliable methods to induce stem cells to differentiate into specific cell types or to generate enough cells for transplantation, limiting their application in clinical therapy. Among these tissues, dental pulp, entrapped within the 'sealed niche' of the pulp chamber, is an extremely rich site for collecting stem cells. These stem cells are called DPSCs (dental pulp stem cells), which can differentiate into various cytotypes such as neural progenitors, adipocytes, myotubes, and pre-osteoblasts under particular conditions. The focus of this study was to optimize conditions for adult stem cell purification, proliferation, and differentiation.

The first two of these objectives were accomplished by culturing either stem cells isolated from dental pulp (DPSCs) or HEPM cells (pre-osteoblasts) purchased from ATCC in 2D monolayer. The second objective was accomplished by culturing the DPSCs and HEPM cells in the rotary cell culture (RCC) bioreactor (Synthecon, Houston, TX) for 72-hours to investigate the potential of osteogenic differentiation.

The RCC provides a 3D environment with simulated microgravity, which is optimal for stem cell differentiation. It has been previously shown that the RCC environment induces differentiation (Facer et al., 2005; Ko et al., 2007). Successful differentiation of adult stem cells was determined by investigating several osteogenic differentiation markers using flow cytometry. This novel technique for stem cell differentiation can potentially provide new therapeutic option for bone tissue regeneration.

INTRODUCTION

As the aging population increases, more people will become reliant on regenerative medicine. Likely situation of necessary regenerative medicine is bone augmentation for partially edentulous jaws, followed by the use of dental implants. For the best biologic, biomechanical, and aesthetic results of these implant rehabilitations, appropriate quality and quantity of bone available at the surgical site is essential for stable implant placement. The placement of an implant into a defective osseous site not only prevents adequate positioning of the final prosthetic restoration, but also results in poor osseointegration and subsequently, a poor prognosis for the therapeutic outcome. Current approaches in bone reconstruction use biomaterials, autografts, or allografts, although restrictions on all these techniques exist (Papa et al., 2005; Papa et al., 2009). These restrictions include donor site morbidity and donor shortage for autografts, immunologic barriers for allografts, and the risk of transmitting infectious diseases.

Artificial bone substitutes containing metals, ceramics, and polymers have been introduced to maintain bone function; however, each material has specific disadvantages, and none can adequately substitute for current autograft therapies.

A viable alternative to current autograft procedures is osseous tissue engineering for tissue regenerative medicine. Our laboratory (as well as other researchers) has been investigating the effects of simulated microgravity on osteoblast differentiation as a means to study cellular and molecular mechanisms associated with 3D osseous tissue growth (Ko et al., 2007). Several studies have analyzed the effect of simulated microgravity on osteoblast differentiation using established osteoblast cell lines (Ko et al., 2007). These studies demonstrated increased cellular aggregation and enhancement of osteoblast differentiation using the RCC bioreactor.

A theoretical way to circumvent problems associated to currently used autologous or allogeneic grafting materials could be to grow adult stem cells such as stromal cells isolated from adult human dental pulp (DPSCs) in a microgravity environment to enhance the rate of osteoblast differentiation and mineralization.

Thus, the objective of this current study was to test the hypothesis that simulated microgravity environments created by RCC would enhance osteoblast differentiation and osteogenic gene expression.
**METHODS**

**Dental pulp extraction and digestion**

Human dental pulp was extracted from teeth of healthy adult subjects aged 21–45 years. Before extraction, each subject was checked for systemic and oral infection or diseases. Only disease-free subjects were selected for pulp collection. Each subject was pretreated for a week with professional dental hygiene. Before extraction, the dental crown was covered with a 0.3% chlorhexidine gel (Forhans, New York, NY), for 2 min. Dental pulp was obtained by means of a dentinal excavator or a Gracey curette. The pulp was gently removed and immersed in a digestive solution: penicillin 100 U/ml/streptomycin 100 mg/ml, 0.6 ml clarithromycin 500 mg/ml in 4 ml PBS 1M, with 3 mg/ml type I collagenase, 4 mg/ml dispase for 1 h at 37°C. Once digested, the solution was filtered through 70 µm Falcon strainers (Becton & Dickinson, Franklin Lakes, NJ) (Graziano et al., 2008).

**Cell culture**

After filtration, the DPSCs were placed in D-MEM culture medium supplemented with 10% heat inactivated FBS, 2mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin (all purchased from Invitrogen, Carlsbad, CA) and placed in 75 ml flasks with filtered caps. Flasks were incubated at 37°C in 5% CO2 and the medium changed twice a week. As soon as cells became confluent, they were subdivided into new flasks. Several passages were performed in total. Stem cells were sorted using a FACsorter (Becton & Dickinson) and the mouse anti-human CD117 (c-kit) and CD34 antibodies.

As a control, HEPM 1486; ATCC pre-osteoblasts were cultured in EMEM supplemented with 10% FBS 2mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin (all purchased from Invitrogen, Carlsbad, CA) and placed in 75 ml flasks with filtered caps.

**Simulated microgravity cultures**

Cells were counted and 2x10⁷ cells were placed in a 50 mL Synthecon rotating vessel RCC for 72-hours. Rotation was set at 10 RPM for the first 24-hours and then was increased to 30 RPM for the next 48 hours. Cells were then removed, and dissociated from the formed 3D aggregate. Cells were counted again using trypan blue to determine cell viability and reacted with fluoresceinated antibodies for flow cytometric analysis.

**Flow cytometry analysis**

Cells were analyzed by flow cytometry using antibodies against Stro-1 (#sc-47733), RunX-2 (#sc-10758), Osteocalcin (#sc-30044), and Osteonectin (#sc-25574) purchased from Santa Cruz (Santa Cruz, CA), and against Osteopontin (#2671-1) purchased from Epitomix (Burlingame, CA), following standard protocols. Briefly, cells were detached using 0.02% EDTA in PBS and pelleted (10 min at 1,000 rpm), washed in 0.1% BSA in PBS at 4°C, and incubated in a solution of 1 µg antibody + 9 µl 0.1% BSA in 1X PBS. Cells were washed in the same solution once and were processed for sorting (FACS Aria, BD Bioscience, San Jose, CA).

**RESULTS**

Compared with pre-osteoblasts cultured in 2D tissue culture plastic environments, cells grown in 3D simulated microgravity environments (Figure 1) show a very distinctive pattern of cell growth. By 24-hours, cells cultured in the RCC started to aggregate. These 3D small aggregates coalesced into larger aggregates of approximately 7 mm in size by 72-hours (Figure 1).

Gross light microscopic analysis of aggregates isolated from 3D RCC revealed a mass that appeared tissue-like with a white, shiny, translucent surface, similar to that of cartilage or bone. The harvested 3-D cells were analyzed by flow cytometry to characterize osteogenic markers. The expression of Osteocalcin and RUNX-2, which have been described as osteogenic markers, were increased following culture of the dental stem cells in the RCC bioreactor as observed by flow cytometric analysis (Figure 2).

![Figure 1. 3-D RCC chamber aggregate at 72-hours showing a cell aggregate measuring 7 mm across.](image-url)
Interestingly, the expression of Stro-1, a marker of stemness, dramatically decreased, indicating that the dental stem cells underwent differentiation following the RCC treatment. The decrease of other markers of early differentiation such as osteopontin, also indicates that the cells were committed to differentiate toward the osteogenic lineage. The RCC derived 3D specimens will be further characterized by immunohistochemistry analysis and morphological staining.

REFERENCES


Another Go-Around: Revisiting the Case for Space-Based Centrifuges

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Please note that the following paper reflects the personal opinions of the listed co-authors. These opinions and recommendations are based on our discipline expertise in the areas of gravitational biology and artificial gravity. None of the following comments are official NASA or U.S. government positions.

The need for space-based centrifuges for both research applications and astronaut countermeasures has been articulated for decades. Key reviews and reports from NASA and the space life sciences community have long identified artificial gravity (AG) facilities as a top priority for the gravitational biology and aeromedical communities:

There was unanimity of opinion that any major adverse effects of spaceflight could probably be prevented by creating an artificial gravitational field within the spacecraft... The need for a centrifuge on future flights is of the highest priority (NRC, 1979).

Variable Force Centrifuge...is the single most important facility in any life sciences program...[and] should increase the scientific return from space experiments by orders of magnitude...A VFC is an essential instrument for the future of space biology and medicine (NRC, 1987).

Whether used in the near-term to facilitate human missions to Mars, or put off until developing missions to destinations farther away, artificial gravity will eventually be required to protect humans exploring space (IAA, 2009).

The cancellation of the Centrifuge Accommodation Module planned for ISS left life sciences researchers with no rotational or glovebox facilities for flight investigations, and reopened the call for such a core capability. As recently as 2009, approximately 10 percent of the nearly 150 position papers publicly submitted to the ongoing National Academies' Decadal Survey on Biological and Physical Sciences in Space included some mention of centrifugation or artificial gravity (National Research Council, 2010), including both a key position paper from the Aerospace Medical Association and a highly focused white paper representing the views of 18 senior investigators and engineers in the field. Together, these documents make specific recommendations for developing an effective artificial gravity regimen to protect astronaut health through a combination of ground and flight resources for studying cells, animals, and humans.

Although free-flyers, such as the Russian Bion/Biocosmos, can test some animals and provide for centrifugation, only the ISS will currently permit both animal and human testing of artificial gravity. Once the ISS ceases operation, our opportunities to explore the efficacy of AG as a human countermeasure will be limited to yet undefined opportunities on commercial or other spacecraft.

Clinical deconditioning associated with microgravity exposure is dramatic and progressive, with changes markedly faster than age-related declines and recovery in some systems incomplete after as long as a year postflight (Buckey, 2006). Serious commitment to exploration-class missions, whether or not they involve surface stays, requires more effective and efficient countermeasures, with better knowledge of gravity threshold requirements and clearer prescription of how much, how often, how long such loads must be applied. This requires understanding the role of gravity in biology and physiology, and will not be adequately met by current approaches based on ground-based trials. To do so requires the ability to test a range of living organisms, including humans, using space-based rotating facilities. Importantly, such experiments are also relevant to understanding the role of gravity in...
maintaining health on Earth, as well as the nature of life elsewhere in the Universe.

The needs of the life sciences community with respect to artificial gravity systems are not monolithic, nor is the appropriate solution likely to be. Core themes that could be addressed with flight centrifuges are enumerated below:

1. Countermeasure development and testing
   (a) Efficacy: Artificial gravity is the only known integrated countermeasure designed to address all physiological systems affected by microgravity exposures. However, while the efficacy of very large rotating vehicles operating at 1-g is generally accepted by the community, the effectiveness of short-radius intermittent exposures still needs substantial research (International Academy of Astronautics, 2009). Successful ground studies (Young and Paloski, 2007; Warren, et al., 2006) require flight validation.
   (b) Responses to rotation: Numerous studies have shown that short-radius centrifugation up to 30 rpm is acceptable on the ground (e.g. Iwasaki, et al., 2001); however, an in-flight human countermeasure feasibility test is needed, which can also answer fundamental questions about the role of gravity in vestibular function, serving the basic research community.

2. Fundamental gravitational biology
   (a) 1-g control: Among the greatest criticisms of microgravity research efforts to date has been the inability to accurately distinguish the effects of reduced gravity from other factors in the flight environment, including launch and entry loads, radiation, stress, etc. An appropriate 1-g control environment would substantially improve the quality of research conducted on ISS.
   (b) Partial-g responses: Understanding biological responses to partial gravity environments between microgravity and 1-g is a key unexplored area of both basic and applied research. Partial gravity data is vital for understanding the fundamental mechanisms of mechanosensing, and for designing potential countermeasures appropriate for Lunar and Mars exploration. Research into plant and animal response at partial gravity will be essential to planning long-term life support systems to support lunar and Mars bases, and animal results will provide an early indication of the magnitude of any problems for long-term human bases. This information is needed as early as possible in the design and planning for extended human stays on the Moon and Mars.

3. Systems architecture trades
   Despite dozens of white papers and trade studies since the earliest days of human spaceflight, the concept of a large-radius rotating vehicle has yet to be demonstrated in flight. Hardware designs abound, from deployable tethers and booms to more traditional assembled structures. If such an architecture is to be seriously considered for exploration-class missions, a successful flight with living organisms using a free-flyer platform would be a critical first step towards future crewed flights.

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**Table 1. Alignment between research themes, model organisms and flight platforms**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Animal</th>
<th>Human</th>
<th>ISS</th>
<th>Free flyer*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-g control</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Partial-g responses</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Responses to rotation</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Efficacy</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systems architecture trades</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Free-flyers do not at this time permit human testing.

Interest from Japan, ESA, Russia, and China has been growing; we have recently visited ground centrifuges and discussed AG research needs with colleagues in Nagoya, Xi’An, Toulouse, Cologne, and Moscow. Separate ESA Topical Teams are at work on flight animal centrifuges and large radius ground centrifuges. Furthermore, JAXA, ESA, and NASA are reviewing the AGREE project (Iwase, et al., 2010) studying the requirements and practicality of the placement of a short-arm human centrifuge on the ISS. All participants appear convinced of the importance of international cooperation in the further research on both the scientific and countermeasure aspects of AG. This is consistent with NASA’s renewed commitment to ISS research, transformative technology and international cooperation.

We strongly recommend that NASA fund immediate technical evaluation of the readily available flight technologies, with the aim of a rapidly deployed flight system or systems designed to deliver near-term science results that can clearly recommend for or against the development of more expensive and complex systems.
A subset of such approaches, any of which we estimate could be readied for flight in less than three years, given appropriate resources, include:

- Requalifying the Neurolab Rotator previously flown for human research on STS-98;
- Design of a small, human-powered portable centrifuge for ISS, drawing on substantial ground experience (e.g. Clément and Buckley, 2007);
- Adaptation or relight of the Russian Biocosmos centrifuge being readied for animal flights in 2013;
- Possible salvage of elements of the Japanese CAM rotor and supporting hardware;
- Leveraging existing CAM experience and designs to prepare a double rack-scale small animal centrifuge for use on ISS or a free-flyer platform.

Depending on upmass, downmass, and volume availability, systems proposed for use on ISS could be flown in the existing Station volume, or mated to a docked transfer module such as an ATV or COTS platform. Free-flyer approaches could similarly leverage either a dedicated platform, or commercial orbital access, e.g., SpaceX DragonLab or Bigelow inflatable platforms. While it might be possible to mount biology research payloads to a human centrifuge, or even to mate a human platform to a biology centrifuge rotor, we see this added complexity as a barrier to rapid progress and support parallel evaluation of the strategic and scientific value of independent human and animal system designs.

We believe firmly that there is an engaged community, both domestically and across our ISS partner nations, ready to respond to opportunities aligned with any of the options presented above. A coordinated, collaborative approach could provide answers to pressing scientific and operational questions. Success is crucial to safe planning and execution of exploration programs, and holds substantial promise for basic research. Space agencies interested in human space exploration should not let this opportunity pass by again.

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Humans face daunting challenges to successful extra-terrestrial exploration and colonization, including adverse alterations in gravity and radiation. The Earth-determined biology of plants, animals and humans is significantly modified in space environments. One physiological requirement shared by larger plants with humans and animals is a complex, highly branching vascular system. Genetic programs for these vasculatures respond sensitively to alterations in cellular metabolism, immunological status, and other specialized cellular/tissue signals resulting from environmental effects.

VESSEL GENeration Analysis (VESGEN) software is being developed at NASA Glenn as a mature, user-interactive research computer code for mapping and quantification of the fractal-based complexity of vascular branching. Change in vascular branching pattern provides an informative read-out of alterations in complex regulatory signaling pathways. VESGEN has provided novel insights into the cytokine, transgenic, and therapeutic regulation of pathological and physiological angiogenesis, lymphangiogenesis, and other microvascular remodeling phenomena (Liu et al., 2009; McKay et al., 2008; Parsons-Wingerter et al., 1998, 2000a&b, 2006a&b, 2010). Vascular morphology is mapped and quantified by User-specified selection from the VESGEN Tree, Network and Tree-Network Composite Analysis Options. Innovative applications described in these studies include disease progression from ophthalmic clinical images of the human retina, experimental regulation of vascular remodeling in the mouse retina, avian and mouse coronary vasculature, and other experimental models in vivo reviewed recently by Vickerman et al. (2009).

As a feasibility study, we demonstrate here that alterations of venation pattern in the leaves of plants flown on ISS such as Arabidopsis thaliana can be

![Figure 1. Mapping of Developing Leaf Venation Pattern by VESGEN. Binary vascular patterns (a, d) extracted from microscopic images of terrestrially grown Arabidopsis thaliana leaves at Day 2 (D2) and Day 8 (D8) were analyzed by VESGEN software. To provide useful quantification (Table 1), the binary vascular patterns (a, d) were mapped as large structural (1°-2°) veins and small reticular veins (≥ 3°, b, e) according to established rules of leaf venation architecture. 10 Other venation groupings determined by branching orders are available to the User in VESGEN. Note the large increase in leaf size from Day 2 to Day 8 (e). By an alternative VESGEN representation (c, f), for example, distance mapping displays the local thickness of vessel diameter throughout the vascular tree-network composite. Black indicates avascular spaces enclosed by, and quantified for, the tree-network structures.](image-url)
analyzed successfully by VESGEN. Although venation patterning in plants such as *A. thaliana* has not yet been characterized in ISS experiments, such patterning is clearly important for plant health and applications in space. More generally, terrestrial leaf patterning is clearly important for plant health and was not yet been characterized in ISS experiments, such as those analyzed successfully by VESGEN. Although *A. thaliana* has been characterized in other studies, it remains important to study its patterning in microgravity on Earth to understand its effects on plant growth and survival in space.

Finally, the richness of terrestrial biodiversity was attributed recently to the sudden, increased complexity in leaf venation pattern during the Early Cretaceous Period (Pennisi, 2010).

We therefore assessed the suitability of VESGEN analysis for plant leaves (Fig. 1) by analyzing venation patterns at Day 2 and Day 8 from *A. thaliana* seedlings grown terrestrially (Kang and Dengler, 2004). Leaf size increased greatly during maturation from Day 2 to Day 8 (Fig. 1c). Venation patterns within the leaves were binarized by semi-automated image processing (Fig. 1) described previously for human ophthalmic clinical images and laboratory animal experiments (Parsons-Wingerter et al., 2010; Vickerman et al., 2009). The black/white vascular pattern and its region of interest (ROI) were analyzed automatically by VESGEN using the Vascular Tree-Network Composite option. Vessels within the branching tree-network composites were grouped into successively smaller generations of venous branching. VESGEN further mapped the vascular patterns into two groups of large structural veins (1°-2°) and small reticular veins (≥ 3°, Fig. 1 and Table 1).

Vascular groups were determined by VESGEN according to established rules for leaf venation patterning, in which the large structural, branching veins are of primary (1°) and secondary (2°) order and the smaller net-worked veins forming the reticulum, of tertiary or greater (≥ 3°) order (Ellis et al., 2009). In the ordering of large structural vessels for pinnate leaves such as *A. thaliana*, 2° (costal) veins branch from the single, central 1° vein. Veins of order ≥ 3° branch primarily from 2° veins to fill intercostal gaps between the roughly parallel 2° veins. Venation patterns in dicot leaves (pinnate and palmate) are therefore organized as tree-network composites, in which large structural veins form the hierarchical vascular branching tree and small reticular veins form the intercostal vascular network or net.

By VESGEN analysis, leaf venation pattern matured considerably in branching complexity during leaf development (Table 1). As a sensitive measure of branching complexity and space-filling capacity (Mandelbrot, 1983; Parsons-Wingerter et al., 1998), the fractal dimension ($D_f$) of the entire skeletonized branching pattern increased from 1.32 at Day 2 to 1.47 at Day 8. The ratio of vessel density ($A_v$) for small reticular vessels to large structural vessels increased from 1.16 at Day 2 to 1.51 at Day 8. From Day 2 to Day 8, the average diameter ($D_v$) also termed vein gauge) of large structural vessels increased from 14.0 µm to 100.9 µm and $D_v$ of small reticular vessels, from 9.3 µm to 70.0 µm. Other venation parameters measured by VESGEN but not reported here include vessel branch point density, vessel end point density, vessel length density and network analysis of avascular spaces (Vickerman et al., 2009).

### Table 1. Quantification of Venation Pattern in *A. thaliana* Leaves by VESGEN Analysis

<table>
<thead>
<tr>
<th>Day</th>
<th>Vessel Group</th>
<th>$D_f$</th>
<th>$A_v$ (µm²/µm²)</th>
<th>$D_v$ (µm)</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>All</td>
<td>1.32</td>
<td>0.208</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Large</td>
<td>–</td>
<td>0.096</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td>Small</td>
<td>–</td>
<td>0.111</td>
<td>9.3</td>
</tr>
<tr>
<td>8</td>
<td>All</td>
<td>1.47</td>
<td>0.276</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Large</td>
<td>–</td>
<td>0.110</td>
<td>100.9</td>
</tr>
<tr>
<td></td>
<td>Small</td>
<td>–</td>
<td>0.166</td>
<td>70.0</td>
</tr>
</tbody>
</table>

As a feasibility study, results of the VESGEN analysis generally correspond to the botanical rules for large structural (1°-2°) and small reticular (≥ 3°) vessels described above (Ellis et al., 2009). Discrepancies between these rules and our quantitative assessment may result from: 1) the immature state of the developing *A. thaliana* venation patterns, which is consistent with pattern irregularities in developing animal vasculature, 2) insufficient image resolution, and 3) current VESGEN mapping limitations. In the future, VESGEN mapping capabilities will be optimized for improved detection of specific dicot leaf venation attributes such as branching angle and vessel tapering that differ somewhat from human and animal vascular branching.

In conclusion, we recommend that mapping and quantification of leaf venation patterns by VESGEN of plants flying as experiments on ISS would provide new, helpful insights for successful space exploration and colonization. Interesting questions to be addressed include: What are the effects of microgravity on leaf venation pattern? Of radiation? What genetically engineered modifications in leaf venation pattern improve plant growth and survival in space environments?
REFERENCES


Heat stress has been found to attenuate atrophy of skeletal muscle in vivo and in vitro (Naito et al., 2000; Westerheide et al., 2004). This anti-atrophy effect is known to involve induction of heat shock proteins (HSPs) such as HSP72, an important molecular chaperone for protein quality control. For instance, hindlimb-unloaded rats whose body temperature was raised to 41.6°C by mild heat showed a significant retention of muscle mass, concurrently with significant upregulation of HSP72 expression, compared to the vivarium control. Muscle atrophy occurs when the proteoytic rate exceeds the anabolic rate (Hoffman and Nader, 2004). Because HSPs play a critical role in folding and repair of proteins as well as cytoprotection against external stresses (e.g., hyperthermia and physical stress) (Naito et al., 2000), it is reasonable to consider that upregulation of HSPs aid not only to elevate protein anabolism but also to reduce protein degradation. Moreover, since these proteins are crucial for organization and protection of myofilaments (Srikakulam and Winkelmann, 2004), structural integrity of myofibrils can be stabilized by upregulation of HSPs even in the unloaded state.

Muscle atrophy is an inevitable phenomenon in space microgravity where mechanical loading is nearly absent. Previous reports demonstrate that the astronauts lost muscle force by about ~3% per week despite continuous daily exercise (Adams et al., 2003). As upregulation of HSPs seems to parallel anti-atrophy effect on the skeletal muscle, it is therefore worth investigating an alternative means of countermeasure such as natural compounds that stimulate HSP expression. Celastrol (CEL), a quinone methide triterpene, was revealed to activate heat shock transcription factor 1 (HSF1) in various cell types (e.g., cancer and kidney cells) (Westerheide et al., 2004). This compound was also reported to have many functions including antioxidant activity, neuroprotection, and inhibition of proteasomal activity (Walcott and Heikkila, 2010). However, despite significant researches on these multiple functions of CEL, its anti-atrophy effect on muscle cells has not been elucidated to date. Here, we present our results of CEL effect on C2C12 muscle cell size in the presence or absence of dexamethasone (DEX), a synthetic form of glucocorticoid that is known to induce muscle atrophy (Zheng et al., 2010).

C2C12 mouse muscle cells were cultured in 100-mm culture dishes containing Dulbecco’s modified Eagle’s Medium (DMEM) (Welgene, Daegu, South Korea) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1% antibiotic-antimycotic (Gibco, Burlington, Ontario, Canada) until the cells reached 90% confluence. The confluent myoblasts were then differentiated to myotubes by differentiation media (2% horse serum in DMEM) for five days. All cells were maintained at 37°C in 5% CO2. Fresh medium was replaced every 2 days.

CEL (Cayman Chemical; Ann Arbor, MI) and DEX (Calbiochem; San Diego, CA) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) at a stock concentration of 25 mM and 200 mM, respectively. To elucidate potency of CEL on HSP72 expression, myotubes were treated with 0.5, 1.0, 1.2, and 1.5 μM of CEL and incubated for 24 hr. To determine dose-dependent effects of DEX on the myotube diameter, the cells were treated with 10, 25, 50, 100, 200, 400 μM of DEX and incubated for 24 hr. To test the anti-atrophy efficacy of CEL, the cell diameter was also determined under treatments of the following conditions: (1) vehicle (DMSO), (2) 200 μM DEX, (3) 1.0 μM CEL, and (4) both 200 μM DEX and 1.0 μM CEL (DEX+CEL).

To gauge HSP72 expression, the cells were harvested after 24 h of the treatments, and homogenized in RIPA buffer containing the protease inhibitor Complete Mini and phosphatase inhibitor...
cocktail (Roche, Mannheim, Germany). The samples were sonicated for 3 sec on ice and centrifuged for 15 min at 13,000 rpm at 4°C. The supernatants were collected as the whole-cell soluble lysate, and its protein concentration was determined using a Bradford assay kit. The protein samples were subject to SDS-PAGE to detect HSP72 bands, and were electrophoretically transferred from a gel to a nitrocellulose membrane. The membrane was incubated in a blocking buffer for 1 h at room temperature. The membrane was subsequently incubated overnight at 4°C with primary antibodies. The membrane was then incubated with HRP-conjugated secondary antibody for detection in 10 ml of blocking buffer with gentle agitation for 1 h at room temperature. Immune complexes were then detected with the ECL system (GE Healthcare, Fairfield, CT). The bands were quantified using a densitometer (Bio-Rad, Hercules, CA). Densities of the proteins were normalized to mouse monoclonal GAPDH (ab8245, Abcam, Cambridge, UK). The primary antibody used for the assays were mouse anti-HSP72 from Stressgen (C92F3A-5, Victoria, BC, Canada). The secondary antibody was HRP-conjugated anti-mouse IgG from Cell signaling Technology (#7076, Beverly, CA).

Data are presented as mean ± SEM (n = 3). Significance of differences in protein quantity or cell diameter among groups was examined by Kruskal-Wallis test and post-hoc comparisons as in Langley (1971).

Our data demonstrate that HSP72 expression levels increased 6-, 21-, 35-, and 42-fold at CEL concentrations of 0.5, 1.0, 1.2, and 1.5 μM, respectively, in comparison to the vehicle control (Fig. 1). As in other cell types (Westerheide et al., 2004), this data indicate that CEL functioned as a potent HSP inducer in the muscle cell.

Dexamethasone is a synthetic member of glucocorticoid that induces cell death and protein degradation via the proteasomal pathway (Zheng et al., 2010). Our result showed that the diameter of the C2C12 cells gradually decreased with the increase in dexamethasone concentrations (Fig. 2). At DEX concentrations of 50 – 400 μM, the diameter was 0.7–~ 0.77-fold that of the vehicle control (P < 0.05).

Finally, we attempted to examine whether CEL had an anti-atrophy effect on muscle cells even under the influence of DEX. As shown in Figure 3, the diameter of DEX-treated cells decreased 0.27-fold that of DMSO (P < 0.05), whereas the diameter of CEL-treated cells did not change significantly. More importantly, the atrophy-inducing effect of DEX was almost abolished when the cells were treated together with CEL.
Conclusively, our data demonstrate that CEL was a potent HSP72 inducer and diminished the atrophic effect of DEX in the muscle cell. Such efficacy seemed to be due to overexpression of HSP72 that might suppress the proteolytic signals involving the ubiquitin-proteasomal system (Walcott and Heikkila, 2010). Further studies on anabolic and catabolic signaling pathways will reveal a precise mechanism in which celastrol works for the anti-atrophy effect on the muscle cell.

Figure 3. The atrophy-inducing effect of DEX was abolished when the cells were treated simultaneously with CEL. Cell counts: 230 ~ 264. *, P < 0.05.

ACKNOWLEDGEMENTS

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Candida albicans is an opportunistic fungal pathogen responsible for a wide range of illnesses, including oral thrush, onychomycoses, vaginitis, and disseminated candidiasis. As an opportunistic pathogen, C. albicans is capable of sensing and responding to a variety of environmental stresses, perhaps even microgravity. Research has suggested that some, but not all, microbial pathogens respond to microgravity with increased virulence and virulence-associated phenotypes (Nickerson, et al., 2004; Rosenzweig, et al., 2010). This is troubling from the perspective of astronaut health, particularly as spaceflight missions become potentially longer and exposure to the extreme environment of microgravity increases. Furthermore, immunological research has indicated that spaceflight adversely affects the human immune system, possibly leaving astronauts more susceptible to infections that would otherwise be limiting in a healthy host (Kaur, et al., 2005). Fungal infections are difficult to treat, resulting in a high mortality rate from disseminated disease. Therefore it is important to better understand the response of C. albicans to microgravity from the perspective of maintaining astronaut health.

At the 2010 American Society for Gravitational and Space Biology Annual Conference, our laboratory described a variety of phenotypic changes observed in a eukaryotic pathogen (C. albicans) following exposure to low-shear modeled microgravity (LSMMG) (Searles, et al., submitted). The environmentally induced phenotypes and morphological alterations included filamentation, antifungal resistance, altered colony morphology, and biofilm formation (Altenburg, et al., 2008; Woolley, et al., 2010; Searles, et al., 2011, submitted). Notably, the morphologic changes occurred following relatively long-term LSMMG exposure (5-12 days). The physiological alterations are consistent with phenotypes associated with increased virulence and may be very useful in predicting C. albicans response to microgravity, but ultimately, flight studies are necessary for confirmation (Mitchell, 1998). Some genetic and phenotypic alterations have been detected following short-term spaceflight (25 hrs, unpublished data), but our research also indicates that long-term microgravity exposure elicits a more complex set of adaptations that are of particular importance, especially in consideration of the potential duration of future spaceflight missions to the moon, asteroids, or Mars.

Currently, spaceflight experiments without repetitive astronaut involvement are restricted to the time it takes for cells to reach stationary phase (approximately 1 day); therefore, additional exposure is required in order to detect long-term phenotypic and genotypic alterations. This report explores a possible method to circumvent inherent limitations associated with short-term spaceflight experiments without requiring additional astronaut assistance. We propose that cells can be pre-exposed (or “primed”) in LSMMG prior to being subjected to spaceflight, where they will continue on the adaptation trajectory initiated during preliminary LSMMG exposure. To test this hypothesis, cells were primed in a ground-based LSMMG bioreactor and stored in water to mimic conditions that may exist prior to launch. However, for priming to ‘extend’ spaceflight exposure, the LSMMG-induced phenotype must be maintained during water storage. The studies outlined in this report aim to determine the feasibility of utilizing this priming principle in spaceflight experiments in order to elicit a more robust response during limited zero-gravity exposure.

Two separate eight-day ground-based LSMMG exposure experiments were performed in HARV bioreactors. One experiment was inoculated with a standard overnight culture (standard), whereas the other was inoculated with cells that had been primed by a five-day LSMMG exposure and stored in water for seven days (primed). These two experiments were compared using previously characterized phenotypic outcome measures to evaluate the effect of priming on the environmental adaptive response.
Expression of Hwp1, a hyphal-specific gene, was evaluated following exposure of C. albicans to LSMMG for either one or eight days (Figure 1). In cells cultured in standard conditions, Hwp1 expression is increased nearly 1.6-fold by 8 days of LSMMG exposure, consistent with prior findings from our laboratory (Altenburg, et al., 2008). Hwp1 levels also increase following priming (1.75-fold by 8 days), but with considerably less variability than in standard LSMMG conditions. This data suggest that priming results in a more uniform transcriptional response to subsequent LSMMG exposure. In addition, when comparing the Hwp1 response between cells grown in primed relative to standard conditions, there is a modest trend towards a more rapid response at both 1 and 8 days. These data indicate that some genetic alterations underlying filamentation may be synchronized and stabilized following the priming process and water storage.

To further evaluate phenotypic stability, filamentation was quantified by microscopic analysis (Figure 2). Primed cells showed a more robust filamentation response to LSMMG as compared to cells prepared using standard procedures. These data suggest that the rate of filamentation was amplified by priming.

Colonial morphology phenotypic switching is another alteration induced by LSMMG. Over time, cells exposed to LSMMG convert from smooth to ‘hyper’ irregular wrinkled (HIW) colony morphology (Figure 3A and B, respectively). To evaluate colony morphology, cells cultured in LSMMG following standard or priming conditions were diluted and plated daily. The percentage of colonies demonstrating the HIW morphology was determined based on visual inspection of spread plates (Figure 3C). Under standard conditions, about six days of exposure to LSMMG are required before the appearance of HIW colonies. By eight days of standard LSMMG exposure, nearly 25% of colonies are HIW. Conversely, primed cells demonstrate altered colony morphology after as little as three days following LSMMG exposure. Within eight days of exposure to LSMMG, over 65% of colonies are HIW. Therefore, primed cells can be induced to undergo phenotypic switching following a relatively short-term exposure to LSMMG. These data suggest that the precursor adaptations necessary to trigger the HIW phenotype are stable during water storage.

Taken together these data suggest the priming concept outlined in this report may be useful to better exploit opportunities associated with spaceflight and may be effective at shortening the amount of microgravity exposure required to elicit comparable longer-term phenotypic alterations. A precedent for consistency in prokaryotic cellular physiology in LSMMG and true microgravity has been established.
(Wilson, et al., 2002); therefore, combining these methodologies to provide an extended analysis of yeast response to microgravity is logical. To better understand the changes induced by long-term exposure to microgravity, cells can be primed by exposure to LSMMG prior to launch. The ability of primed cells to respond more quickly to LSMMG than standard cells implicates a certain degree of phenotypic stability incurred by priming. The stability of the phenotype induced by LSMMG would permit primed cells to be stored in water prior to spaceflight and accommodate the timeline often associated with flight opportunities. Continued research on the effects of LSMMG and true microgravity on \textit{C. albicans} and other human pathogens is needed to prepare for eventual long-term space missions.

**ACKNOWLEDGEMENTS**

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