SIMULATED-MICROGRAVITY INDUCED CHANGES IN GENE EXPRESSION IN ZEBRAFISH EMBRYOS SUGGEST THAT THE PRIMARY CILIUM IS INVOLVED IN GRAVITY TRANSDUCTION

Stephen J. Moorman\textsuperscript{1*}, Naoko Shimada\textsuperscript{1}, Gbalabo Sokunbi\textsuperscript{1}, and Cynthia Pfirrmann\textsuperscript{2}

\textsuperscript{1}Department of Neuroscience and Cell Biology, Robert Wood Johnson Medical School, Piscataway, NJ
\textsuperscript{2}Scotch Plains-Fanwood High School, Scotch Plains, NJ

ABSTRACT

Gravity has been a constant physical factor during the evolution and development of life on Earth. We have been studying effects of simulated-microgravity on gene expression in transgenic zebrafish embryos expressing \textit{gfp} under the influence of gene-specific promoters. We have looked at a number of different genes expressed in a variety of different organ systems. For instance, we have looked at beta-actin expression in the heart, eye, notochord and rohon bead neurons, hsp70 expression in the lens, alpha-A1 and beta-B1 crystallin expression in the lens, and fli1 expression in the heart and blood vessels. Different organs and cell types show periods of maximum susceptibility during developmental periods that coincide with specific developmental events. The organ-specific developmental events correlate with periods when primary cilia are playing organ-specific developmental roles. In the notochord, each primary cilium is positioned to function as a ‘strain gauge’ to monitor the stresses associated with bending of the notochord in response to forces such as gravity. Unloading the notochord by placing the embryos in a simulated-microgravity environment causes more dramatic changes in gene expression than those seen in any other tissue. The developing cardiovascular system loses its susceptibility to simulated-microgravity induced changes in gene expression as the primary cilium of the endothelial cell becomes a flow sensor in the lumen of the blood vessels. Rohon bead neurons show simulated-microgravity induced changes in the variability of gene expression levels that can be explained by a change in the balance between the canonical and non-canonical Wnt pathways, pathways that are influenced by the primary cilium. A search of the cilia-related proteome reveals a link between the primary cilium and hsp70 expression, which might explain the simulated microgravity induced change in hsp70 expression in the developing lens. The ubiquitous nature of the primary cilium as a cell organelle suggests that gravity sensing might be a general feature of all vertebrate cells where the primary cilium has not been co-opted for another sensory function.

INTRODUCTION

Gravity sensing is a well documented phenomenon in plant cells. It has been proposed that animal cells can also detect gravitational force. Despite an abundance of cellular and molecular data indicating that animal cells, both \textit{in vitro} and \textit{in vivo}, respond to changes in gravitational force, there have been very few, if any, testable hypothesis put forward to explain how these cells might detect and transduce gravitational force into meaningful intracellular signals.

The first mammalian cells studied in microgravity were HeLa cells. More recently, the activation of cellular responses during microgravity has been studied in human red blood cells, and in human and mouse immune cultures containing lymphocytes and macrophages (Claassen and Spooner, 1994). Normal T-lymphocytes on earth undergo activation on exposure to various mitogens such as concanavalin A (Con A) and stimulate T-cell receptor (TCR). In contrast, immune cells in microgravity showed little response to mitogen, indicating that the immune response was suppressed during space flight (Claassen and Spooner, 1994). This difference between the normal and microgravity exposed T-cells was due to a 97% reduction in the interleukin-2 (IL-2) production on mitogen stimulation (Cogoli et al., 1993; Walther et al., 1998, 1999). Microgravity has also been shown to affect expression of several cell surface markers (Sonnenfeld, 1999). These markers, including CD4 and CD8, play important roles in regulation of immune responses.

Exposure to microgravity is known to induce changes in the musculo-skeletal system in live animals. In addition to effects due to disuse atrophy, results from cells in culture suggest that direct effects of microgravity on gene expression are also possible. For instance, cultured osteoblasts exposed to microgravity show changes in mRNA levels for growth factor receptors (Akiyama et al., 1999; Kumei et al., 1999). C-fos levels in osteoblasts (Hughes-Fulford et al., 1998; Sato et al., 1999) and in cultured epidermal cells (Rijken et al., 1992) are also affected by exposure to microgravity. In addition, actin and myosin expression in skeletal muscle cells and cardio-myocytes change in microgravity (Thomason et al., 1992).

The development of DNA microarray technology has spurred a renewed interest in the effects of microgravity on gene expression (Hammond et al., 2000). Using this technology, it is possible to measure the levels of thousands of different mRNAs simultaneously. This technique is particularly useful for comparing gene expression in the same tissue under different environmental conditions. Microarray data support the idea that microgravity influences expression of cytoskeletal genes (Lewis et al., 2001). Interestingly, recent microarray data suggest that there are differential effects of microgravity on different members of the same gene families. For instance, heat shock proteins (hsp)
represent a class of proteins whose expression is modulated by stress. Exposure to microgravity induces changes in hsp27 but not in hsp70 in cultured human lymphocytes (Cubano and Lewis, 2001). Similar experiments have been done using simulated-microgravity. Hepatoblastoma cells show changes in expression levels for 95 different genes (Khaoustov et al., 2001) when grown in a bioreactor NASA designed to simulate microgravity for cells in culture. In human renal cortical cells, more than 1,600 genes, including many transcription factors, showed dramatic changes in expression levels with exposure to microgravity and, to a more limited extent, with exposure to simulated-microgravity (Hammond et al., 2000).

For the past several years, we have been investigating the effects of simulated-microgravity on gene expression in zebrafish embryos with the hope of identifying common molecular mechanisms that might underlie the effects of microgravity on a variety of different cell and tissue types, and organ systems in vivo. We briefly summarize some of that work here and provide a potential unifying hypothesis, the primary cilium functions as a ‘gravity sensor’, to explain much of the work done to date.

**METHODS**

**Animals.** Zebrafish that express the gfp gene under the control of the promoter/enhancer of the zebrafish hsp70 gene (Halloran et al., 2000), the fli1 gene (Lawson and Weinstein, 2002), or the β-actin gene (Gillette-Ferguson et al., 2003) were used for these studies. Adult fish were maintained at 28°C on a 14 hour-light and 10 hour-dark cycle. Eggs were collected within 3 hours after their production and fertilization and maintained at the same temperature until 8 hours post-fertilization (hpf). Further incubation was carried out in 0.003% 1-phenyl-2-thiourea (PTU; Sigma, Louis, MO), an inhibitor of melanin pigment formation, at room temperature (20°C). We defined the stage of the embryos according to (Kimmel et al., 1995).

**Bioreactor.** A bioreactor (Synthecon, Houston, TX) that NASA designed to simulate microgravity for cells in culture (Jessup et al., 1993) was used to simulate many aspects of microgravity for zebrafish embryos (Moorman et al., 1999).

**Centrifuge.** To achieve net 2 g and 3 g forces, a custom built slow-speed centrifuge was modified to accommodate horizontally oriented, water-filled tubes containing zebrafish eggs.

**Exposure to Micro- and Hypergravity.** Embryos at different stages were placed in the bioreactor or on the centrifuge at different developmental times for specific durations (Figure 1). To account for the effect of vibration, tissue culture dishes with the same number of control eggs were placed on the support frame of the bioreactor and on the center of the centrifuge (a position that does not rotate). We have already published extensive experiments demonstrating that there is no difference between control embryos placed on the support frame of the bioreactor compared to embryos incubated in the bioreactor rotating around a vertical axis (Gillette-Ferguson et al., 2003; Moorman et al., 1999; Moorman et al., 2002; Shimada et al., 2005). Each experiment was repeated 4 times using 6 embryos, to obtain the images of GFP fluorescence.

![Figure 1. Developmental time (bars) that embryos were in the bioreactor. hpf = hours post-fertilization. N = 24 control and N = 24 experimental embryos for each exposure time.](image)

**GFP-Fluorescence Imaging.** All images were collected using a Leica DMRE microscope (Leica Microsystems Inc., Bannockburn, IL) equipped with a Ludl BioPrecision motorized stage (Ludl Electronic Products Ltd., Hawthorne, NY) and a Hamamatsu Orca-ER camera (Hamamatsu Photonics, Hamamatsu City Japan). The microscope, stage, and camera were controlled using OpenLab software (Improvision, Lexington, MA) running on an Apple Dual-processor G4 computer. Prior to collecting fluorescence images, a bright-field image of each embryo/larva was acquired using a 5x objective. This image was used to measure rostral-caudal length as an indication of the age of the embryo/larva. After the brightfield image was acquired, a complete Z-series of fluorescence images was acquired. For this series, images were collected at 3µm intervals using a 10x objective. The entire stack of images was saved to disk. The camera gain, offset, and exposure time were kept constant for all homzygous embryos/larvae. Because heterozygous embryos/larvae had approximately half the fluorescence intensity as homzygous embryos/larvae, a second set of camera settings was used for all of the heterozygous embryos/larvae. No post-acquisition image processing was done on any of the images. Embryos older than 24 hpf were anesthetized using tricaine (0.04% 3-amino benzoic acid ethylester: Sigma, St. Louis) during imaging to prevent movements of the embryo.

**GFP Intensity Measurements.** To measure the average intensity of fluorescence for the entire embryo/larva, an image was selected from the middle of the z-series stack and the average intensity for the entire image was...
calculated using the OpenLab software. An image was then selected where the outer edge of the organ of interest was in focus (this yields a focal plane mid way through the organ), a region of interest was drawn around the organ and the software automatically calculated the average intensity within the region.

**Data Analysis and Statistics.** For data analysis, individual measurements were normalized and the mean, standard deviation, and standard error were then calculated for each group. For statistical analysis, the means for the control and experimental groups were compared using a t-test.

**RESULTS**

**Notochord (β-actin).** As reported previously (Shimada et al., 2005), the notochord showed significant increases in β-actin:gfp expression in response to simulated-microgravity with two periods of susceptibility one at approximately 24 hours post-fertilization and a second at approximately 72 hours post-fertilization (Figure 2). These correspond to the period when spontaneous movements of the embryo begin and to the period when the movements peak at hatching. β-actin:gfp expression in the notochord was not measured after exposure to either 2 g or 3 g.

**Heart (β-actin).** As reported previously (Shimada et al. 2005), the heart showed significant increases in β-actin:gfp expression in response to simulated-microgravity with a peak of susceptibility around 48 hours post-fertilization (Figure 3). This corresponds to the period after the zebrafish heart has looped and is beginning to remodel into atrium and ventricle. β-actin:gfp expression in the heart was not measured after exposure to either 2 g or 3 g.

**Blood Vessels (flh).** The developing dorsal aorta and the developing blood vessels of the pharyngeal arches both showed significant changes in flh:gfp that were inversely related to exposure to changes in gravitational force, i.e. a decrease in gravitational force induced an increase in expression and an increase in gravitational force induced an decrease in expression (Figure 4). However, this was only true for the developmental period ending around 24 hours post-fertilization. After this period, changes in gravitational force induced changes in flh:gfp expression but the changes were not related to the magnitude or direction of the change in gravitational force (Figure 4). Between 20 and 24 hours post-fertilization, blood begins to flow through the dorsal aorta and the pharyngeal arch vessels.

**Rohon beard neurons (β-actin).** As reported previously (Shimada et al., 2005), Rohon Beard neurons showed increases in β-actin:gfp expression during the developmental time period beginning at 24 hour post-fertilization and ending at 80 hours post-fertilization (Figure 5A). Rohon Beard neurons enter an apoptotic pathway beginning at 72-80 hours post-fertilization and ultimately die as their sensory function is assumed by the developing dorsal root ganglion cells.

These data consisted of measurements of β-actin:gfp expression level in one Rohon Beard neuron in each of 4 spinal cord segments in 24 embryos in each group. As part of the original analysis, the coefficient of variation for each group of pooled (96) individual Rohon Beard neuron expression levels in each group was calculated. There was no significant difference between any of the coefficients of variation for any of the groups (Figure 5B). These data were reanalyzed separately calculating the coefficient of variation for the 4 Rohon Beard neurons in each embryo. When analyzed in this manner, the coefficients of variation for the simulated-microgravity groups where significantly higher than for the other groups (Figure 5C). This indicates that β-actin:gfp expression levels in Rohon Beard neurons are
Figure 4. Percent changes in fluorescence intensity of fli1:gfpp in the dorsal aorta (A) and the blood vessels of the pharyngeal arches (B) that resulted from exposure to simulated microgravity for the developmental times indicated on the x-axis. s-ug=simulated-microgravity; hpf=hours post-fertilization

much more variable when embryos are exposed to simulated-microgravity.

Lens (hsp-70). As reported previously (Shimada and Moorman, 2006), the lens showed changes in hsp70:gfpp expression (Figure 6A) and changes in hsp70 mRNA levels (Figure 6B) in the developing lens during the developmental period that brackets lens cell differentiation in the zebrafish.

DISCUSSION

The results presented here typify the results of space biology experiments conducted over the past decade – although they reinforce the message that microgravity can affect cells, tissues, and organs systems, they don’t appear to tell us much about the underlying mechanisms. The major differences between these experiments and most experiments exposing cells, tissues, embryos, or adult animals to the microgravity environment of space is that we have careful control of the timing and duration of exposure to simulated-microgravity and we can repeat the experiments on a regular basis. This allows us to expose embryos to simulated-microgravity during developmental time periods when the functional states of specific cells, tissues, or organs are changing. Analyzing the data from

Figure 5. (A) Percent changes in fluorescence intensity of β-actin:gfpp in Rohon Beard neurons for the developmental times indicated on the x-axis (note: measurements at 3 g were made only for the 4 groups indicated). (B) Variation in gene expression in a population of Rohon Beard neurons in a group of zebrafish embryos. The coefficient of variation of β-actin:gfpp expression in 96 Rohon Beard neurons (4 neurons in each of 24 zebrafish embryos) in each group was calculated and plotted. (C) Variation in gene expression in the Rohon Beard neurons within individual zebrafish embryos. The coefficient of variation of β-actin:gfpp expression in the 4 Rohon Beard neurons in each zebrafish embryo was calculated independently. These coefficients were averaged for the 24 embryos in each group and plotted. s-ug=simulated-microgravity; hpf=hours post-fertilization
this perspective has led to identifying the primary cilium as a putative cellular gravitational force sensor.

The primary cilium is a recently rediscovered, nearly ubiquitous, cell organelle that serves specific sensory functions on specific cell types. For instance, the primary cilium is a flow sensor on kidney tubule epithelial cells and vascular endothelial cells (see Pan et al., 2005), a key modifier of the hedgehog signaling pathway (Corbit et al., 2005), and a key determinant for left-right asymmetry (Ferrante et al., 2006; Tamakoshi et al., 2006). The primary cilium has also been suggested to act as a strain gauge for osteocytes in vivo (Whitfield, 2003) and a molecular switch between the canonical and non-canonical WNT pathways (Otto et al., 2003). Interestingly, the balance between canonical and non-canonical WNT pathways has been postulated to play a role in reducing the signal to noise ratio in gene expression resulting in less variable gene expression levels (Arias and Hayward, 2006). If this is true, the primary cilium might be a key regulator of transcriptional noise during gene expression.

**Heart and Blood Vessel Primary Cilia.** In the developing chick heart, primary cilia are found on endocardial cells predominantly in areas of low shear forces (van der Heiden et al., 2006). This is probably due to the active remodeling of the heart that takes place in regions of high shear forces. In order for the heart to add endocardial cells as the chambers remodel, the primary cilium would have to be resorbed for the cells to actively divide (Alieva and Vorobjev 2004). If the zebrafish heart is similar to the chick heart, then the primary cilium on the endocardial cells would be resorbed as the tubular heart begins to remodel into definitive atrium and ventricle around 48 hours after fertilization. This timeframe coincides with the period of susceptibility for simulated-microgravity induced changes in gene expression (Figure 3). We have recently been investigating the effects of simulated-microgravity on expression of *fli1* in zebrafish embryos. *Fli1* is the earliest known endothelial cell marker in zebrafish (Kidd and Weinstein 2003). Simulated–microgravity causes dramatic changes in *fli1:GFP* expression in endothelial cells (Figure 4), but only prior to the initiation of blood flow through the developing vessels. This suggests that either endothelial cells lose their susceptibility to simulated-microgravity or that the effects of blood flow through the capillary outweigh the effects of simulated-microgravity. An interesting change in the endothelial cell coincides with the change in susceptibility to simulated-microgravity – the primary cilium on the endothelial cell begins to function as a flow sensor monitoring blood flow through the lumen of the blood vessel.

**Rohon Beard Neurons and Primary Cilia.** When we initially began to suspect the involvement of primary cilia in mediating the effects of simulated microgravity on gene expression, we were hard pressed to explain how primary cilia could be involved in the changes seen in Rohon Beard neurons and in lens cells. Rohon Beard neurons are the primary sensory neurons of the zebrafish prior to the development of the dorsal root ganglia cells.
Rohon Beard neurons are a single rostro-caudal row of large neurons in the dorsal spinal cord on each side. Each Rohon Beard neuron has a single axon that projects peripherally to segmentally innervate the same tissue that will ultimately be innervated by dorsal root ganglia cells. The data (Figure 5A) indicate that there is a pronounced increase in gene expression in simulated-microgravity between 24 and 72hrs. As the dorsal root ganglia develop, the Rohon Beard neurons degenerate. This process of degeneration begins at 72-80 hours post fertilization. This time frame coincides with the end of the period of susceptibility to simulated-microgravity induced changes in gene expression in Rohon Beard neurons (Figure 5A). The primary cilium has recently been shown to function as a molecular switch between the canonical and non-canonical WNT pathways (Oishi et al. 2006; Otto et al. 2003). The balance between canonical and non-canonical WNT pathways has been proposed to play a role in maintaining the consistency of gene expression levels by regulating transcriptional noise levels (Arias and Hayward, 2006). Within each zebrafish embryo, the β-actin:GFP expression levels in Rohon Beard neurons are more variable in those embryos exposed to simulated-microgravity (Figure 5C). This implicates the primary cilium in the simulated-microgravity induced changes in β-actin:GFP expression in Rohon Beard neurons.

**Hsp70 and Primary Cilia.** The cilia proteome was recently made available on-line (http://www.ciliaproteome.org/). This database represents a compendium of the repertoire of proteins required for ciliary biogenesis and function in eukaryotes (Gherman et al., 2006). Of the genes that we have assayed in zebrafish, only hsp70 is present in the cilia proteome. The only tissue in zebrafish embryos that expresses hsp70 in the absence of any environmental stressor is the developing lens. HSP70 probably functions in the lens to prevent the lens fiber cells from dying when their nuclei enter an apoptotic pathway (Ribeil et al., 2007). Since simulated-microgravity does not induce expression in any tissue of zebrafish embryos other than the lens, simulated-microgravity cannot be considered an hsp70-related environmental stressor. However, simulated microgravity does cause an increase in hsp70 expression in the lens (Figure 6) and a coincident decrease in TUNEL positive cells in the lens (Shimada and Moorman, 2006). This supports the idea that simulated-microgravity induced changes in gene expression are mediated through effects on the primary cilium.

**Other Primary Cilia Mediated Effects.** Many of the effects of microgravity mentioned in the introduction could be initiated at the primary cilium. For instance, many of the effects on cells of the immune system could be due to changes in the hedgehog signaling pathways (Benson et al., 2004; Chan et al., 2006) and these pathways are modulated by the primary cilium (Corbit et al., 2005). The primary cilium is also known to modulate c-fos levels (Takaoki et al., 2004) and to regulate the cytoskeleton (Satir and Christensen, 2007). In addition, hsp27 and hsp70 are among the repertoire of proteins required for ciliary biogenesis and function in humans (Ostrowski et al., 2002).

**CONCLUDING REMARKS**

If the primary cilium is involved in mediating effects of microgravity in vivo, issues associated with astronaut health can be raised. For instance, the primary cilium is involved in regulating the cell cycle. Because the primary cilium is physically linked to the mother centriole, the primary cilium must be withdrawn/resorbed in order for a cell to actively divide (Alieva and Vorobjev, 2004). Stimuli that cause changes in the primary cilia in cells in culture can induce the cells to enter the cell cycle and divide (Tucker et al., 1983). This suggests that microgravity affecting primary cilium could result in an increased incidence of cancers in a variety of different tissues in astronauts.

All in all, the data presented are consistent with the primary cilium playing a role in mediating the simulated-microgravity induced changes in gene expression seen in zebrafish embryos and support the hypothesis that the primary cilium acts as a gravitational force transducer in vertebrate cells. It should be emphasized that these data do not prove that the primary cilium is involved. Experiments that could help prove this hypothesis would include selectively inactivating the primary cilium and showing that the simulated-microgravity induced changes in gene expression no longer occur. These experiments are technically possible in zebrafish embryos using techniques such as morpholino knock-down of primary cilia related genes during development.

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