Using cell biology in planning the Mars Mission

by Millie Hughes-Fulford

Space, the final frontier

These are familiar words, giving an image of modern space travel with artificial gravity and all the comforts of home. But in the real world this is not the case. We cannot flip a switch and turn on artificial gravity. The result is that during a Mars Mission humans will experience several known physiological changes: (i) space-adaptation syndrome (motion sickness), (ii) loss of electrolytes, (iii) increased levels of glucocorticoids in extended flight, (iv) loss of muscle, (v) reduced immune function and (vi) loss of calcium and bone (up to 19% loss of bone after long-term flight). Some of these changes are probably due to systemic or hormonal changes in the body; others may be a direct result of lack of mechanical stress in the microgravity environment. For this article, we will only consider the causes of bone loss. We know from medical studies on the Mir space station that loss of weight-bearing bone is as high as 1% per month and we know that the loss is due primarily to a lack of new osteoblast growth. Bone loss is one of the major physiological 'show stoppers' in the proposed 30-month manned mission to Mars. Bone loss in astronauts results in elevated serum calcium levels, which could cause kidney stones during a mission, and space osteoporosis could lead to serious fractures after return to Earth[1,2].

It is unknown if the decrease in bone formation in astronauts is due directly to the lack of mechanical stress (1 G force, the gravity experienced on Earth) in microgravity or to alterations in signal transduction at the molecular level. For the first two decades of space flight, the scientific approach to the problem was to investigate general systemic changes in the body, such as an increase in glucocorticoids, not basic cellular responses to microgravity[3,4]. During the last 10 years, basic scientists have been investigating changes in osteoblast cell metabolism with and without gravity. Early findings demonstrated that mammalian cells grow more slowly in microgravity and that an early response to zero gravity (0 G) includes alterations in prostaglandin signalling in bone[3–5].

Why do bone cells need gravity?

Contrary to popular belief, bone is a dynamic and responsive organ. Bone responds to mechanical stress (exercise) by making new bone. That is why athletes have strong bone. Lack of mechanical stress is one reason why bed-rest patients and the inactive elderly suffer bone loss. Studies of osteoporosis in young healthy astronauts in space flight help our understanding of osteoporosis here on Earth.

Which cellular responses change in space flight?

In order to answer this question, tissue-culture dishes are adapted to the 0G environment. 'OSTEO' experiments used the European Space Agency (ESA) BioRack. BioRack is a multi-user facility consisting of incubators with variable-gravity centrifuges, a cooler, a freezer and a sealed glovebox. Two identical BioRack modules were used: one ground model remained on Earth, the other was flown in SpaceHab,

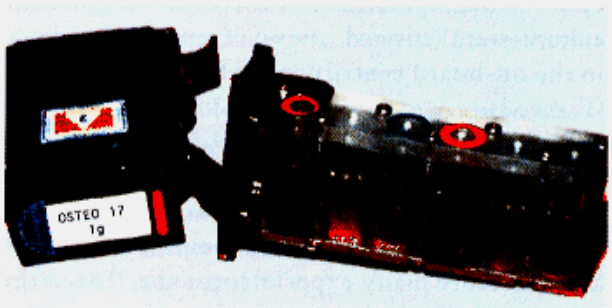

Figure 1. Black type-I container is on the left. To the right is the plungerbox with six reservoirs. The green ring is placed over the activator-medium (10% fetal calf serum) reservoir and the red ring denotes the fixative reservoir.

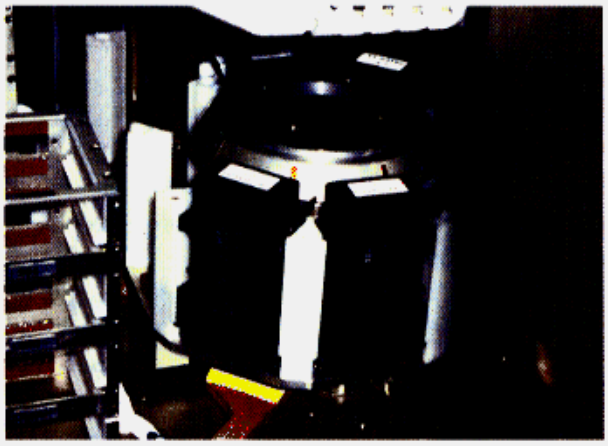

Figure 2. BioRack incubator with a 1 G centrifuge, on the right, loaded with type-I containers. Static storage for 0 G samples is on the left.

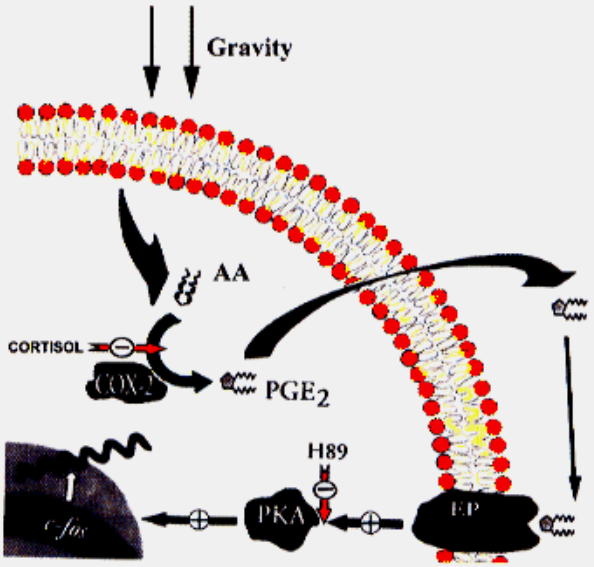

Figure 3. Proposed molecular mechanism for osteoblasts: the response to gravity begins with the stress stimulus. The first response is the release of arachidonic acid (AA) from the membrane, which is converted to prostaglandin E_2 (PGE_2) by the enzyme COX-2. The third action is the exportation of the prostaglandin, where it activates its own and neighbouring EP1 and EP2/4 prostaglandin receptors. The EP1 receptor activates the protein kinase C pathway, stimulating growth and new mRNA synthesis of *cox*-2. The EP2/4 receptor stimulates protein kinase A (PKA) activity and induces c-*fos* expression. Our data suggest inhibition of signalling from both the EP receptors in microgravity. H89 is a specific inhibitor of the enzyme protein kinase A (PKA).

a mini-lab that is sometimes manifested on the NASA Space Shuttle. BioRack has an important advantage over other microgravity facilities in that it provides a small-radius (78 mm) slow-rotating (107 ± 0.5 revs./min) centrifuge. This allows a 1 G on-board control. Because of proximity, both the 0 G and 1 G samples experience identical launch vibrations, accelerations, cosmic radiation and other unknown conditions in the flight. The only difference between the flight groups is the gravity parameter. Flight experimental hardware was designed by Centrum voor Constructie en Mechatronica (Nuenen, The Netherlands) for our experiment, OSTEO, which was flown as part of the BioRack payload on Space Shuttle flights STS-76, -81 and -84. The hardware consists of two separate units: a plungerbox unit type HM 2/3 and a type-I container (Figure 1). The plungerbox unit contains two cell chambers, each holding two 22 mm × 11 mm glass coverslips on which the cells grow. The plungerbox is placed into a type-I container, which provides a secondary containment for biological samples during the experiment. RNA samples were isolated from the cell lysates of two coverslips and each sample represented one data point. Once the cultures were activated, one set of samples was placed in the on-board centrifuge for flight 1 G controls (Figure 2).

Due to flight restrictions, most previous cell-culture experiments sent into space were actively growing at the time of launch. Moreover, lack of refrigeration limited on-board sample preservation and therefore many experiments experienced the stress of re-entry and normal Earth gravity for several hours before being harvested. Even for the few experiments that had the opportunity for on-board collection, the data was compromised by flight-imposed limitations, including lack of sufficient sample numbers, addition of supplements directly affecting gene expression, lack of fresh media changes and lack of on-board 1 G controls. OSTEO experiments were fortunate not to have had any of the above limitations.

What changes occur in osteoblast growth activation in microgravity?

First we must understand 'normal' mechanical stress responses of bone. As seen in Figure 3, the osteoblast responds to gravity stress by releasing the membrane-bound arachidonic acid. This essential fatty acid is converted to prostaglandin by the enzyme cyclo-oxygenase 2 (COX-2). The prostaglandin is then released from the cell and stimulates the EP receptors [prostaglandin E_2 (PGE_2) receptors], which turns on the protein kinase C-pathway and the protein kinase A-pathway signals. Blocking either of these signals can block new bone growth. MC3T3-E1 osteoblasts were used to study bone cell growth in space flight. Osteoblasts were launched on the Shuttle in a

quiescent state and were activated by adding fresh medium with 10% fetal-calf serum in microgravity (10^{-6} to 10^{-9} G).

Growth is reduced

Flown osteoblasts grew slowly in microgravity, with the total cell number significantly reduced in the microgravity samples; 55±6 cells per microscopic field in 0 G versus 141±8 in 1 G after 4 days in microgravity.

Morphology is changed

The cytoskeleton of the flight osteoblasts had a reduced number of stress fibres and abnormal morphology. Nuclei in the ground-based controls were large and round with bright spots. Flight samples were elongated, as shown by Hoechst staining of the DNA nucleosomes in Figure 4[3,4].

Protein synthesis is slower during the first 3 h in microgravity

Significant decreases were seen in total protein synthesis in the microgravity condition[4]. However, synthesis of some specific proteins, like fibronectin, remained unchanged in microgravity. There were no changes in overall microgravity after 24 h.

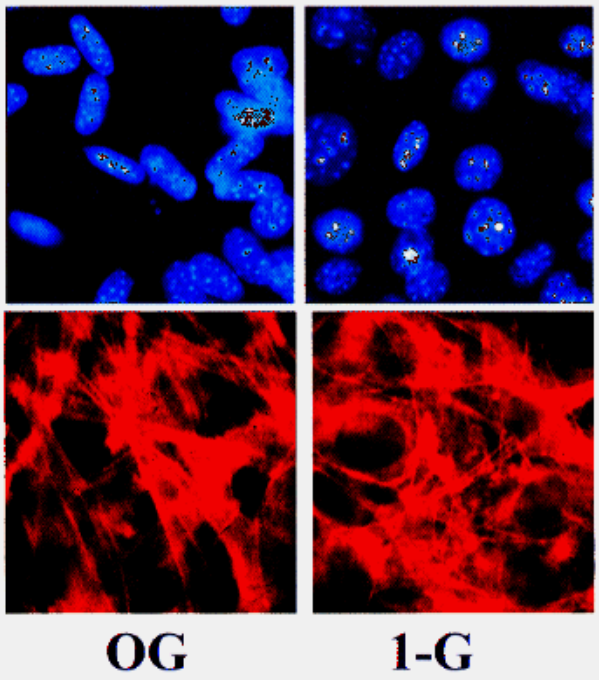

Figure 4. Osteoblasts grown in 0 G for 27 h have altered cell shape. Cells were launched in a quiescent condition. Once in orbit, cells were activated with 10% fetal-calf-serum containing media. Then, 27 h later, cells were fixed in formaldehyde while still in microgravity. The nuclei (blue) were stained with Hoechst 33258 and the actin cytoskeleton (red) was stained with Rhodamine-phalloidin. Note elongation of the nuclei and actin cytoskeleton in microgravity conditions.

Overall mRNA synthesis is not altered at 3 and 27 h, but specific transcriptional gene expression is altered

The mechanisms by which mammalian cells respond to gravitational signals are unknown. Several reports have described gravity-specific changes in mRNA levels following exposure of cultured cells and whole animals to varying periods of microgravity in experiments performed in space flight, sounding rockets and clinostats. We examined quiescent cells for gravity-dependent changes in mRNA levels for nine genes involved in bone cell growth and maturation. We asked the question: does gravity affect the gene expression of *cox-1* and *cox-2* in flight? This is an essential question because the COX-1 and COX-2 enzymes are directly responsible for the synthesis of prostaglandin E_2 (PGE_2). For the samples collected in flight with guanidinium thiocyanate (GITC), we found a difference in the 0 G and 1 G flight samples (see Table 1).

At 3 h after activation in 0 G, c-*fos* mRNA was decreased in the 0 G samples when compared with the 1 G control. This would suggest that the cells subjected to the 0 G environment did not enter the growth phase of the cell cycle, since c-*fos* is depressed, and that the cells had begun to enter a differentiation phase, as shown by an increase in osteocalcin mRNA. These data are statistically significant and are consistent with other investigations[3–8], showing that mechanical loading (or lack thereof) alters mRNA levels and cytoskeleton. There is no increase in *cox-2* mRNA, suggesting a lack of signalling through the EP1 receptor. In addition, prostaglandin EP1 receptor mRNA levels are overexpressed in the flight samples, a phenomenon that can occur when signalling is abnormal and the cells try to compensate by making more message. There was not enough RNA available to examine *cox-1* or EP2/4 mRNA.

Table 1. Effect of gravity on gene expression during flight.

Gene mRNA	0 Gravity	1 Gravity
c-*fos*	⇓	⇑
cox-2	⇓	⇑
EP-1	⇑	⇑
Osteocalcin	⇑	⇓
β-Actin	–	–
Cyclophilin	–	–

Changes in signal transduction

We have shown previously[3] that PGE_2 is increased in flown cells after activation at early time points followed by a decrease in synthesis later in flight. We have confirmed these results in our STS-76 experiment. At 29 h after activation, the PGE_2 content of cells in flight is increased both in the 1 G and 0 G flown samples. The puzzling question is: why isn't the *cox-2* gene expression up-regulated in the 0 G cells? COX-2 is a feed-forward enzyme and should be up-regulated by increased levels of PGE_2 in the media. This did not happen in OSTEO microgravity flight samples. One explanation may be a change in signalling at the level of prostaglandin receptors in the 0 G activated cells. It has been noted previously that other receptor signalling functions are modified by 0 G[5,8].

The reason for reduced growth activation by sera in microgravity is not fully understood; however, the work of de Groot et al. in other cell types has given us a basis for a hypothesis. They have demonstrated that a number of responses of A431 cells to epidermal growth factor (EGF) are affected by microgravity. The responses include decreased c-*fos* and c-*jun* induction and reduced serum-response-element activity[8]. In a sounding rocket, they demonstrated that EGF-induced expression of the proto-oncogenes c-*fos* and c-*jun* was reduced almost 4-fold by microgravity. The same reduced response was seen with serum-response element in microgravity. The decreased gene inductions were attributed to possible alterations in the c-*fos* promoter–enhancer region's response. The transcription factors c-*jun* and c-*fos* are known regulators of DNA synthesis and are required for entry into S-phase DNA synthesis. The EP2 and EP4 receptors are responsible for c-*fos* induction in the osteoblast.

In later studies, de Groot et al.[9] also demonstrated that the nuclear responses to protein kinase C (PKC) signal transduction were sensitive to gravity changes. In these studies, they demonstrated that EGF- and phorbol ester (PMA)-induced gene expression of c-*fos* and c-*jun* was affected by microgravity, whereas gravity changes mediated by the PKC calcium response had no change, thus implicating the diacylglyeride portion of the protein kinase C signal-transduction pathway. This would correspond to EP1 signal transduction in the osteoblast.

Moreover, Hammond, Kaysen and colleagues have cultured human renal cells in rotating-wall vessels in centrifuged bags and in bags flown aboard the Shuttle[10], and have compared these data with 1 G static, non-adherent, bag cultures. They did not have an on-board centrifuge, so there were no flight controls for this study. However, they reported a plethora of gene steady-state-level changes occurring in space (1632 of 10000 genes), and these changes were unrelated to the gene pattern in the best ground-based rotating-wall vessel. Shear-stress response elements and heat-shock proteins showed no changes in steady-state gene expression in the flight culture. Specific transcription factors underwent large changes during flight. These changes were not due to a general down-regulation of mRNA production, since there are reports that other gene synthesis is not changed during flight; for instance, recent studies have demonstrated that osteoblast fibronectin RNA expression, protein synthesis and matrix do not

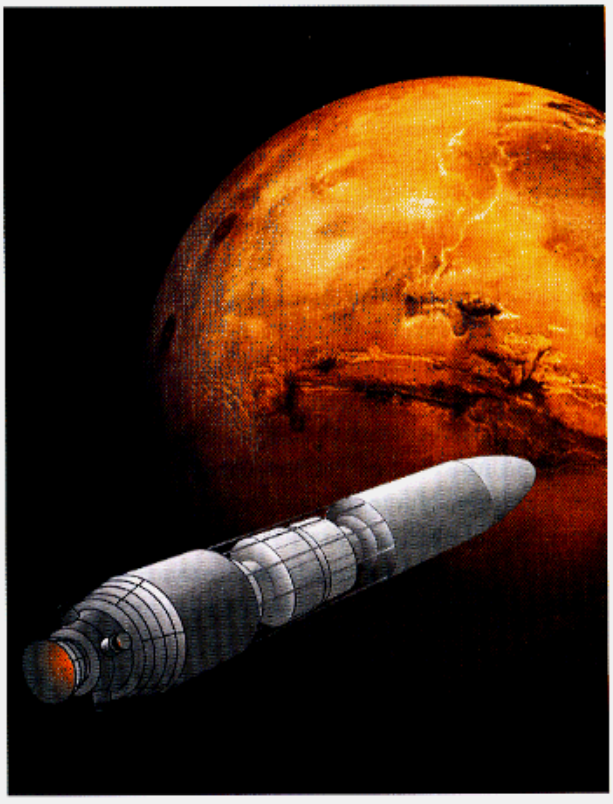

Figure 5. Mars Vehicle approaching the planet Mars. The vehicle is divided into four sections: two large and two small rotating compartments. The forward large compartment contains flight controls and living quarters. The aft compartment contains supplies and fuel. Between the two large compartments is a connecting passage. The two rotating compartments are entered from the passageway. Once inside the forward compartment, astronauts will start the rotation and exercise in a gravity field. It is possible that a short duration of gravity will be sufficient to maintain bone mass. If extended gravity force is needed, these rotating compartments could be used as sleeping quarters. The planet Mars is a composite mosaic of 102 images taken from the Viking Orbiter. Illustrator: Paulo Chang, San Francisco.

change in microgravity[11]. Studies on Earth have demonstrated that gravity or vibrational fields increase expression of certain transcription and growth factors, such as c-*fos*, c-*myc* and transforming growth factor β[6,7].

With multiple cell biology experiments showing many specific changes in gene expression in the absence of gravity, there is little hope that a single pill to 'cure all woes' could be devised for the Mars Mission. Instead, we will need to provide gravity for the astronauts, perhaps in a rotating chamber, in the form of a large on-board 1 G centrifuge. A simulated gravity field could be created with counter-rotating chambers, such as those shown in the illustration of a manned Mars Vehicle (Figure 5). This engineering design solution may be the best answer to the physiological problems of long-term space flight until we can flip that imaginary switch to activate an artificial gravity field ●

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Dr Millie Hughes-Fulford was the first civilian female science astronaut. In 1991 she flew on NASA's first medical mission. Today she is a Professor at the University of California and the Associate Chief of Staff for Education at the Veterans Affairs Medical Center, San Francisco. As the Director of the Laboratory for Cell Growth, she continues her studies of gene expression in osteoblasts in order to understand the molecular mechanisms of bone growth activation by mechanical stress.

More information on space osteoporosis can be found at http://www.spacedu.com. Millie Hughes-Fulford's e-mail address is milliehf@spacedu.com