

## **PHYSIOLOGICAL EFFECTS OF MICROGRAVITY ON OSTEOBLAST MORPHOLOGY AND CELL BIOLOGY**

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### I. INTRODUCTION

### II. HUMAN DATA; IMPORTANCE OF STUDYING OSTEOBLASTS

- Findings on Gemini, Apollo and Skylab missions
- Findings on Soyuz, Shuttle and MIR missions
- Role of exercise, PGE<sub>2</sub> and diet on bone growth

### III. PHASES OF GROWTH AND STAGES OF OSTEOBLAST DIFFERENTIATION

- Osteoblast growth and phases of cell growth
- Stages of osteoblast development into bone
- Osteoblast growth in microgravity
- Changes in cell cycle in microgravity

### IV. CHANGES IN OSTEOBLAST MOLECULAR BIOLOGY IN MICROGRAVITY

- Importance of signal transduction in microgravity
- Changes in immediate early gene induction in space flight
- Osteoblast gene expression in microgravity
- Stem cell commitment to osteoblasts phenotype in microgravity
- Changes in differentiation and mineralization genes in microgravity

### V. CHANGES IN OSTEOBLAST MORPHOLOGY

- Changes in actin cytoskeleton in microgravity
- Changes in microtubules in microgravity
- Changes seen in cytoskeleton genes in microgravity

### VI. DISCUSSION

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## **Introduction**

When we think of modern space travel, we have images of the USS Enterprise outfitted with artificial gravity and all the comforts of home. But in the real world we do not flip a switch and turn on artificial gravity. The result is that during a long term spaceflight like the Mars Mission, humans will experience several physiological changes including: space adaptation syndrome (motion sickness), loss of muscle, reduced immune function and loss of calcium and bone (up to 19 percent loss of bone after 12 months of flight). Some of these changes may be due to systemic or hormonal changes in the body; others may be a direct result of lack of mechanical stress in the microgravity environment. In this chapter we will discuss bone and osteoblast physiology and the effect of microgravity on the osteoblast.

For the first three decades of spaceflight scientists studied the systemic changes in the body, which included loss of bone, increases of calcium, and changes in the endocrine system such as the increase in glucocorticoids. From these animal and human studies investigators found that loss of weight bearing bone is as high as 1% per month and this loss is primarily due to lack of new osteoblast growth in spaceflight. It is likely that studies of the osteoblast in microgravity will give us new target molecules for development of pharmaceutical agents that will stimulate bone growth. A projected bone loss of 20-30% in astronauts is one of the major physiological 'show stoppers' in the proposed 30-month manned mission to Mars.

Advances in cell and molecular biology over the past ten years have brought a new understanding of osteoblast cell physiology. Although flight opportunities have been limited, the volume of data about microgravity effects on cell proliferation and differentiation is accumulating. Altered gene expression, reduction in transcription factors and reductions in growth factor related proteins have been observed in microgravity. Specifically, it has been

demonstrated that cell growth and differentiation of osteoblasts is inhibited or altered in a microgravity environment. Cytoskeletal changes in actin intermediate filament and microtubule networks have been found in microgravity as well. These changes in the osteoblast physiology suggest that the decrease in bone formation in astronauts may be partly due to microgravity-induced alterations at the molecular level in osteoblast biology, gene expression and signal transduction.

### **Background human data: The importance of studying osteoblasts**

Skeletal changes and loss of total body calcium have been documented in both humans and animals exposed to microgravity from 7 to 237 days. During the Apollo and Skylab missions, photon absorptiometry was used to assess pre and post-flight bone mineral mass. For the 12 crew members of Gemini 4, 5, and 7, and Apollo 7 and 8, the average post-flight loss from the os calcis (heel) were 3.2 percent over an average of 8.5 days (1-4). Analysis of in-flight urine, fecal, and plasma samples from Skylab missions revealed changes in urinary output of hydroxy-proline indicating degradation of the collagenous matrix substance of weight bearing bones. Elevated concentrations of urinary calcium were noted in the early studies of Skylab astronauts starting during the first days of flight. In many of the astronauts urinary calcium concentrations remained at elevated levels throughout the mission. A direct effect of microgravity is the loss of mechanical stress on the skeletal system. Although in-flight exercise is used by astronauts as a countermeasure, the greatest bone mass losses in the US flight program (pre-Shuttle/Mir) occurred in the 84-day Skylab 4 mission even though exercise was regularly performed. Crewmembers using exercise as a countermeasure still lost an average of 4 percent of bone over the 84-day

mission period (5; 6). Additional evidence of bone loss was found in a 19.5 day flight on Cosmos 782 where rats formed significantly less periosteal bone than controls (7; 8).

In the 237-day Soviet Soyuz T-10 mission, the Cosmonauts lost bone in spite of 2-4 hours of daily exercise. Both compact and trabecular bone was lost from the os calcis during this mission. Bone loss appears to increase in general proportion to mission length, from 4 percent to 19.8 percent over an 84 to 184 day period (5; 6; 9; 10). Collet et al. showed that there was a slight decrease seen after one month of spaceflight and a marked loss of trabecular and cortical bone in the tibia after 6 months of microgravity exposure. Overall, the lower weight-bearing bones appeared to be more sensitive than the upper ones in terms of spaceflight-induced bone loss, moreover the recovery of the bone was not complete even 6 months after return to gravity (9). In studies conducted on Euro Mir 95, after 180 days in spaceflight bone formation and resorption biological markers in cosmonauts were measured. Parathyroid hormone (PTH) was found to decrease by almost half. Bone alkaline phosphatase (BAP), intact osteocalcin (iBGP) and type 1 procollagen propeptide (PICP) were decreased to approximately 30% of preflight ground control, showing for the first time that there is an uncoupling of bone remodeling between formation and resorption (11). Vico and colleagues measured bone mineral density (BMD) at the distal radius and tibia in 15 cosmonauts who spent 1, 2 or 6 months on the Russian MIR space station. They found that neither the cancellous nor cortical bone of the radius was significantly changed at any of the time points. In contrast, at the weight-bearing tibial site, cancellous BMD loss was seen after 2 months of microgravity. After 6-months, loss of cancellous bone was more pronounced than cortical bone. In some individuals, the tibial deterioration was great and BMD loss did not seem to depend on previous exposure to microgravity. Moreover, tibial bone loss persisted after return to earth and was not recovered during the post flight study period, suggesting the recovery time is greater than the

time spent in microgravity (12). Since microgravity-induced bone loss is not fully recovered after return to gravity this is a significant complication of long-term missions and must be addressed before a Mars Mission.

Exercise alone is not a total answer to the countermeasures, since paradoxically, excessive exercise aggravates bone loss. Stein et al. reported a negative energy balance in longer-term missions (13-17). This strongly suggests the need for a programmatic and efficient exercise program that would not result in a catabolic state. The cause of bone loss in response to reduction of mechanical stress is not yet known, but several ground studies have demonstrated that prostaglandins may be involved. Forwood et al. found that prostaglandins are released with exercise and are key regulators in exercise-induced bone growth *in vivo* (18). The specific cyclooxygenase-2 inhibitor, NS-389, completely blocked mechanical stress induced bone formation *in vivo*. Other studies have demonstrated that PGE<sub>2</sub> augments bone growth *in vivo* (19) and *in vitro* (20). Mechanical stress also causes increases in cytosolic Ca<sup>++</sup>, remarkably only *one cycle* of pressure can cause a 57% increase in proliferation demonstrating that very little force is needed to stimulate proliferation (21). It has also been demonstrated that 97% of exercise stimulation occurred during as little as 2% of a 24-hour period (22). The potential role of gravity and PGE<sub>2</sub> in microgravity-induced bone loss is strengthened by the findings of Stein et al. who discovered that PGE<sub>2</sub> synthesis is significantly decreased by 50% during a 12-day flight and remained significantly depressed by almost 50% until return to gravity. After landing, synthesis of PGE<sub>2</sub> rebounded to 140% of preflight values for the 6 days of post-flight testing (17) suggesting a direct correlation between gravity and PGE<sub>2</sub>.

### **Phases of growth and stages of osteoblast differentiation**

Bone development is regulated by sequential and stringently controlled gene expression of the osteoblast. Three principal stages of development are key elements of the temporal expression of genes that control cell growth and differentiation as illustrated in Fig. 1. In the proliferation phase, early immediate genes are upregulated along with cyclins, cyclin inhibitors and kinases associated with phases of the cell cycle. In addition, the synthesis of histone-4 and fibronectin are associated with the osteoblast growth stage. During the growth stage, osteoblast proliferation is divided into 4 phases, G1, S-phase (DNA synthesis), G2 and M (Mitosis). During G1, growth factors (or mechanical stress) activate MAPK pathways within seconds; immediate early genes are induced and transcription factors are activated within minutes. The cells then transition from G1 to S-phase, where PGE<sub>2</sub> and histone-4 synthesis peaks allowing osteoblast DNA replication. After DNA content is doubled, the cells then go into G2 where proteins necessary for cell replication and cell division are made. The cell then passes into mitosis where the osteoblast divides into 2 daughter cells. Key regulators in growth are the cyclins, (Fig. 2) which are induced in a temporal sequence of gene expression during the cell cycle. Once the osteoblasts have been replicated enough times to make a monolayer, the cells are ready for the second developmental stage of matrix maturation. During matrix maturation, genes for cell cycle and cell growth are downregulated and expression of genes needed for extracellular matrix maturation and organization begins. The third stage of bone development is the onset of extracellular matrix mineralization where osteoblasts mature into bone. These stages have been experimentally established (23) and defined as restriction points during osteoblast differentiation as depicted in Fig. 1. Throughout the sequential stages of osteoblast growth and differentiation there are specific requirements for proliferation; post-confluent proliferation accompanies osteoblast extracellular matrix biosynthesis. Post-confluent growth results in a monolayer of cells associated with type I collagen, which supports multilayering

of cells in developing bone nodules. Osteopontin and osteocalcin exhibit maximal expression during the maturation and mineralization period when bone tissue-like organization is ongoing (23). Establishing the basis of cellular competency for progression to the mature osteoblast necessitates the identification of the signaling pathways operative for each stage in order to understand the process. This is especially true of studies in microgravity, where investigators have used a range of models to study proliferation, matrix maturation and mineralization. For more details on osteoblast growth and bone development, see the review by Stein et.al. (23).

*Osteoblast growth and gene expression in microgravity*

Many human and animal studies have demonstrated that loss of bone formation (osteoblast proliferation) may be the root cause of space-osteoporosis. The problem of decreased osteoblast proliferation in microgravity is a key issue in cell biology that may determine if we can explore space. Over the past ten years evidence of changes in cell biology have been noted in a variety of cells. Specifically, it has been demonstrated that cell growth of lymphocytes (24-27) (see review chapter by Lewis in this volume) and osteoblasts (28-40) is inhibited or altered in a microgravity environment.

The first microgravity studies on mammalian cells were completed in 1973 on Skylab-3 using human embryonic lung cells (WI-38). Cells grew more slowly in microgravity, with the flight cells taking 2 hours longer to complete cell cycle. No changes were seen in the karyotype; however, glucose utilization was lower in the flight samples (41). A review of early cell biology can be found in publications by Hughes-Fulford (42) and Lewis and Hughes-Fulford (43).

*In vitro* osteoblast growth was first measured on STS-56 in December 1992. The MC3T3-E1 osteoblasts (a mouse line derived from calvarial cells) were launched in a quiescent

state (1% FCS) at launch. Growth was initiated by adding fresh media with 10% fetal calf serum in microgravity. Ground controls were treated in a parallel manner in identical equipment.

Samples were taken at 16 hours and fixed at four days. Microgravity grown cells grew slowly and cell number was reduced by 60% when compared to ground after 4 days of flight.

Prostaglandin synthesis was significantly increased at 16 hours after growth activation, however it returned to control levels after 4 days. Glucose utilization was significantly less than ground controls due to a lower number of cells. The cell viability was normal with glucose utilization being comparable on a per cell basis for flight and ground experiments (28). Confirming evidence was reported by Kumei et al. showing a 136 fold increase in PGE<sub>2</sub> synthesis in flight compared to ground controls. Moreover, they found that osteoblast growth was inhibited on day 4 by ( $9.2 \pm 2$  ug of DNA in flight vs. ground  $17.4 \pm 6$  ug DNA) and on day 5 ( $19.9 \pm 6$  ug DNA in flight vs. ground  $34.2 \pm 11$  ug DNA) (29). Studies by Vassy using MCF-7 cells demonstrated slowing of cell growth with mitosis being delayed as well as a lowering of phosphotyrosine signal transduction in microgravity. Table I shows more experimental details on microgravity growth and gene expression studies discussed in this section. Taken together, the data suggests that osteoblast growth is significantly inhibited in microgravity.

Studies in microgravity on lymphocytes have also shown a marked growth inhibition in lymphocytes (26; 44-46). This inhibition of lymphocyte growth is associated with an enrichment of cells in G<sub>2</sub>/M after four hours of flight. After 48 hours there were no significant cell cycle differences between microgravity and flight 1-g centrifuged cells (27). Recent studies by Vassy et al. have demonstrated that cell proliferation is reduced in breast cancer cells grown in microgravity as a consequence of a prolonged mitosis (47). Changes in cell cycle accompanied

by increased apoptosis have also been noted in osteoblasts ROS 17/ 2.8 cells grown in a vector averaged gravity clinostat (38) and in Jurkat cells grown in microgravity (27).

### **Changes in molecular biology osteoblast in microgravity**

#### *Importance of signal transduction in immediate early gene induction*

DeLaat's group reported the first study of the early gene expression in short duration microgravity. Studying signal transduction and gene expression in A431 cells in microgravity using sounding rockets, they found a reduction of response to Epidermal Growth Factor (EGF) after approximately 6 minutes of microgravity (48-51). Initiation and termination of the experiment was accomplished automatically allowing the study of microgravity effects in the early phases of EGF induced signal transduction. Several events of the EGF signal cascade were characterized and quantified under normal and microgravity conditions. An excellent endpoint of EGF signaling is the induction of immediate early genes. These genes are the first be transcribed after stimulation of cells with growth factors or stress; examples of the genes are *c-fos*, *c-jun*, *erg-1*, *c-myc* and *cox-2*. De Laet used analysis of *c-fos* and *c-jun* because of their prominent role in cell proliferation and differentiation. Quantitative analysis demonstrated that *c-fos* and *c-jun* expression was reduced by approximately 50% when compared to the normal gravity control samples. During normal growth stimulation of cells by EGF, there is dramatic redistribution of EGFR within 5 minutes resulting in receptor clustering. When the EGF binding and clustering was measured in microgravity, there was no change, suggesting a point downstream for signal transduction (52; 53). In testing the effect of the different pathways on EGF signal transduction in the A431 cells with PMA (PKC pathway), A23187 (Ca<sup>++</sup> pathway) and forskolin (PKA pathway), they found that only the PKC pathway was altered by microgravity

(53). It was concluded that the EGF signal transduction by microgravity occurred downstream of EGFR clustering, but upstream of *c-fos* transcription, and was most likely mediated by PKC-dependent signal transduction in the A431 cell, which is a human epidermoid carcinoma line.

Cell growth is regulated by both intracellular and signal transduction and it has recently been shown that MAPK is one of the key proteins involved in signal-transduction via growth factor mediated phosphorylation. This is illustrated in Fig. 3 where we see that many of the growth factors, G- proteins, and integrins act through a common MAPK pathway. Once phosphorylated, MAPK (ERK1/2) translocates to the nucleus in late G1 (54-57). When mitogens are removed there is no MAPK phosphorylation or translocation and cell cycle progression is inhibited (56). Taken together, these studies suggest that microgravity may inhibit growth by blocking signal transduction and inhibiting cell cycle in G1 or the G2/M phase.

The effect of microgravity on *c-fos* gene expression in the osteoblast (MC3T3-E1) was first studied by Sato et al. (58). The cells were initially studied in simulated microgravity induced by clino-rotation. They found that EGF induced *c-fos* expression was reduced in both the MC3T3-E1 osteoblast and HeLa carcinoma cells, with the osteoblast being more sensitive to gravity than the HeLa cell. In a second set of experiments, the effect of EGF on osteoblasts was studied in short-term microgravity condition in the sounding rocket (~ 6 minutes). Cell lysates from flight and ground groups were analyzed for *c-fos* induction and MAPK phosphorylation. The induction of *c-fos* was reduced in microgravity, while no significant difference in MAPK phosphorylation was seen between the flight and ground samples. It is not known which pathway is associated with inhibition of *c-fos* induction.

Several reports have described gravity-specific changes in mRNA levels following exposure of a variety of cultured cells and whole animals to varying periods of microgravity in

experiments performed in spaceflight, sounding rockets and clinostats. Studies by Hammond et al. using microarray have demonstrated that gene expression in human renal cells is altered in microgravity (59; 60) with the expression of transcription factors showing the largest changes. Studies by Lewis et al. also using micro array showed that the expression of cytoskeleton related genes in T-cells were changed in microgravity (61).

Here on Earth, serum activation of the osteoblast causes induction of the immediate early gene *c-fos* at 30 minutes and cyclooxygenase (*cox-2*) and cytosolic phospholipase A2 (*cpla2*) mRNA at three hours schematic for (Fig. 5) (62). In microgravity experiments completed on STS 76, 81 and 84, early gene activation by sera growth factors failed to fully up regulate *cox-2* and *cpla2* (Hughes-Fulford, unpublished data). The importance of signal transduction in *cpla2* protein transcription has been demonstrated with data from the Boonstra laboratory showing that *cpla2* activity is dependent upon MAPK phosphorylation in early G1 (57). Our laboratory examined quiescent cells for gravity-dependent changes in mRNA levels for several genes involved in bone cell growth and maturation. We asked the question: Does gravity affect the gene expression of *cox-2* in flight? This is an essential question because the *cox-2* enzyme is directly responsible for the synthesis of PGE<sub>2</sub>, which is directly linked to exercise induction of bone formation (18; 63; 64). In our experiments on STS-76, 81 and 84 we studied osteoblast gene expression in microgravity and have found a reduction of *cox-2* mRNA in microgravity; *cox-2* expression was recovered in 1-g centrifuge flight samples. Expression of EGFr, 18S and cyclophilin were constitutive and unchanged by microgravity. We found that *cox-2* mRNA is decreased in the 0-g samples when compared to 1-g controls. The copy number for *cox-1* mRNA was too low to detect. Finally, the lack of induction of *cox-2* in microgravity suggests that the cells subjected to the 0-g environment did not enter the growth phase of the cell cycle

(G1-S transition). This agrees with previous studies showing reduced cell growth in microgravity.

*Alteration in stem cell commitment to osteoblast phenotype in microgravity*

While calvarial-derived osteoblasts support exploration of mechanisms associated with osteoblast growth and differentiation, marrow cell cultures permit evaluation of mechanisms regulating recruitment of stem cells to the osteoblast lineage (23). Microgravity studies by Kumei et al. using primary femur marrow cells demonstrated that marrow cells had increased prostaglandin E<sub>2</sub> synthesis in flight compared to ground. These cells were grown in differentiating medium ( $\alpha$ MEM with  $\beta$ -glycerol phosphate and ascorbic acid). Analysis demonstrated up to a 9-fold increase in cox-2 and IL-6 mRNA in differentiating femur marrow cells after 5 days microgravity (29). This suggests increased stem cell recruitment to the osteoblast phenotype in microgravity and low levels of dexamethasone to facilitate recruitment to osteoblast phenotype.

In other studies, responses of primary rat marrow cells to 1,25(OH)<sub>2</sub>D<sub>3</sub> were tested after 4 and 5 days of microgravity exposure on STS-65. These studies examined alterations of insulin-like growth factor expression in microgravity. After 20-hours treatment with 1 $\alpha$ , 25-dihydroxyvitamin D<sub>3</sub>, conditioned media were harvested and cellular DNA and/or RNA were fixed onboard. Insulin-like growth factor binding protein-3 (IGF BP-3) levels in the media were three- and ten-fold higher than ground controls on the fourth and fifth flight days, respectively, as quantitated by Western ligand blotting and radioimmunoassay. The increased IGF BP-3 protein levels correlated with two to threefold elevation of IGF BP-3 mRNA levels, obtained by reverse transcription-polymerase chain reaction (RT-PCR). The IGF BP-5 mRNA levels in flight

cultures were 33-69% lower than in ground controls. The IGF BP-4 mRNA levels in flight cultures were 75% lower than in ground controls on the fifth day but were not different on the fourth day. There was no change in the EGFr in flight, however PDGFr, and *c-fos* were significantly inhibited in microgravity. The glucocorticoid receptor mRNA levels in flight cultures were increased by three to eightfold on the fourth and fifth days compared with levels in ground controls (65). These studies suggest an altered response of marrow derived osteoblast cells in microgravity.

Differentiation studies by Carmeliet et al. examined the effect of microgravity using the osteosarcoma MG-63 cell line to analyze gene induction related to matrix formation and maturation both at the protein and mRNA levels. Cells that were untreated and hormone-treated with 1, 25 dihydroxyvitamin D<sub>3</sub> [1,25 (OH)<sub>2</sub>D<sub>3</sub>], at a 10<sup>-7</sup> M concentration and transforming growth factor beta 2 (TGF-β<sub>2</sub>), 10 ng/ml) were cultured for 9 days under microgravity conditions aboard the Foton-10 satellite. In-flight data was compared with ground and in-flight unit-gravity cultures. The expression of alkaline phosphatase (ALP) activity following treatment in microgravity increased only by a factor of 1.8 compared with the 3.8-fold increase at unit-gravity ( $p < 0.01$ ), whereas no alteration was detected in the production of collagen type I between unit-gravity and microgravity. In addition, gene expression for collagen Iα I, ALP, and osteocalcin following treatment at microgravity was reduced to 51, 62, and 19%, respectively, of unit-gravity levels ( $p < 0.02$ ). The lack of correlation between collagen type I gene and protein expression induced by microgravity is most likely related to the different kinetics of gene and protein expression observed at unit-gravity. Following treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> and TGF-β<sub>2</sub>, collagen I-alpha 1 mRNA increased gradually during 72 h, but collagen type I production was already maximal after treatment for 48 hours (66). Other studies by Carmeliet et al. again

using the osteosarcoma line MG-63 examined the effect of gravity on osteoblast differentiation. Using competitive RTPCR in untreated and hormone treated cells, they found that collagen I $\alpha$ I was reduced to 51% of normal treated. In addition osteocalcin message was reduced to 19% of 1-g in-flight control (31).

Changes in gene expression in human fetal osteoblast (hFOB) SV-40 transformed cells were examined in microgravity. The hFOB cells were cultured on Cytodex 3 beads<sup>TM</sup> inside hollow fiber cartridges that were continually fed media with peristaltic pumps with 10% FCS media. Glucose utilization was essentially the same for flight and ground cells. Samples taken in-flight on days 8 and 12 showed very little PGE<sub>2</sub>, most probably due to the fact that there was no refrigeration available for the samples. The cells were in microgravity for 17 days. Media samples were taken in-flight showing no change in glucose utilization. RNA samples were taken 8 hours (day 18) and 32 hours (day 19) after landing, there was no significant change in PGE<sub>2</sub> content in the flight and ground controls. Harris et al. demonstrated that 8 hours after return to earth, the hFOB cells had reduced expression of cytokines IL-1 $\alpha$  and IL-6 and skeletal growth factor TGF- $\beta$  message. Levels returned to non-flight levels after 24 hours. It is unknown if the forces of re-entry or the sheer flow of the medium through the hollow fiber cartridges had an influence on the post-flight analysis of the cells.

Landis and coworkers flew normal embryonic chicken calvaria grown on Cytodex 3 beads<sup>TM</sup> and maintained in hollowfiber cartridges in microgravity on STS-59. They demonstrated that flight osteoblasts had a less extensive extracellular matrix with reduced collagen gene expression and reduced collagen protein compared to controls. Osteocalcin was expressed in all cells showing a progressive differentiation of both flight and control osteoblasts, however the message levels of osteocalcin were also reduced in flight cells (32). Collectively,

these differentiation studies show that the message levels for several matrix and maturation genes are decreased under microgravity conditions, while stem cell commitment studies show enhanced recruitment to the osteoblast phenotype. Taken together, these studies support the conclusion that microgravity alters the activity of osteoblasts in vitro, in particular the growth stages I and differentiation stages III of osteoblasts in microgravity.

### **Possible role of the cytoskeleton in microgravity**

There is a large body of evidence that mechanical loading plays a critical role in cytoskeletal re-arrangement and cell growth and differentiation (67-69). Cytoskeletal integrity is required throughout the cell cycle, when the cytoskeleton is disrupted, cell cycle progression and expression of cyclins is also interrupted at the G1/S interface (67; 70; 71). Alterations in cytoskeleton actin, intermediate filament and microtubules have been noted when there is a significant load reduction on the cell in microgravity (27; 28; 72; 73). In addition, several investigators have noted that changes in specific gene expression is associated with microgravity exposure (31-34; 49; 50; 54; 59; 61; 74-77).

Rijken first noticed changes in cell shape in response to changed gravity in sounding rocket experiments in 1991 (73). Changes were also noted by Guignandon et al. examining cells in parabolic flight microgravity who found cytoplasmic retraction and membrane ruffling in ROS/17/2.8 cells (72). Increased PGE<sub>2</sub> was also found in flight medium accompanied by significant flight-induced changes that included a decrease in cell area and irregular shape in some cells. Indomethacin reduced the irregular shape changes, but not loss of cell area.

Studies performed by this laboratory demonstrated a gravitational effect on osteoblast proliferation and nuclear morphology on multiple spaceflights. Studies on STS-56 from our laboratory examining growth activation of serum deprived osteoblasts activated in flight

demonstrated that microgravity caused a decrease in cell proliferation and changes in cytoskeleton with a portion showing a spindle shape over a four day period (Fig. 4). We also found changes in nuclear shape after 4 days of spaceflight while glucose metabolism per cell was unchanged (28). As mentioned previously, the *cox-2* enzyme was not induced in microgravity, but paradoxically, media PGE<sub>2</sub> content was significantly increased in flight in both the static and 1-g samples. This could be due to increased release of PGE<sub>2</sub> from the membrane during the 18 hours in a microgravity environment before the start of the experiment or to increased half-life of the enzyme, or to residual *cox-2* activity present at activation. Inflight studies by Stein have demonstrated a reduction in PGE<sub>2</sub> in astronauts (78). Normally, PGE<sub>2</sub> is cleared by the kidneys within seconds, and must be made continually to maintain high sera/urine levels. In isolated osteoblasts, PGE<sub>2</sub> is degraded by the enzyme 15-hydroxyprostaglandin dehydrogenase; it is possible that the activity of the degrading enzyme or alterations in the degradation process may be inhibited in microgravity.

These data point to an inhibition of anabolic stimuli in the absence of gravity. There are several anabolic signals that are regulated by mechanical stress and these signals seem to be downregulated in microgravity. Figure 3 shows the potential anabolic transducers tyrosine kinase and serine/threonine kinase growth factor receptors, seven domain transmembrane receptors (such as the prostaglandin E<sub>2</sub> receptors), integrins and calcium channels. As illustrated, the receptors and the integrins have a common stimulation of the MAPK (Mitogen Activated Protein Kinase) pathway. Mechanical stress can also activate the MAPK pathway, possibly through the G-proteins or the cytoskeleton.

Growth factors and stress forces cause changes in cell function in a sequenced manner over seconds, minutes and hours as illustrated in Table III. If the cells are unable to recognize

growth factors, or to have a normal 1-g vector, alterations in the signal transduction would occur. More recently we have presented preliminary data showing that gene expression, cytoskeleton and nuclear structure is changed when compared to on flight 1-g and ground controls (33; 75; 79). Studies by Lewis et al. found that there were significant changes in the cytoskeleton gene expression and increased apoptosis in the Jurkat T cell line (27). Researchers examining the effect of weightlessness on breast cancer cells found that microtubules were changed in altered gravity. In addition, they noted that the cyokeratin network was loosely woven in some of the cells modified by microgravity (47). This laboratory recently found that osteoblast cyclin E mRNA was down regulated in microgravity (unpublished data). This finding correlates well with other reports of inhibition of cyclin E when actin cytoskeleton is disrupted by dihydrocytochalasin B accompanied by cell cycle arrested at late G1 (80). We do not think the shape change is due to changes in fibronectin matrix since we found that fibronectin mRNA, protein synthesis and extracellular matrix organization (Fig. 7) is not changed in microgravity (75).

The cause of the elongation of nuclei and elongation of cytoskeleton as shown in Figs. 4 and 6 is not known, but it is possible that it is a result of a microgravity-induced inability of anabolic stimulation. The only example of a similar nuclear elongation was caused by limiting cell access to mitogens. Studies by Wang and Walsh demonstrated that when monocytes undergo growth factor withdrawal and start to differentiate, a portion of the cells form elongated nuclei after 3 days, a shape much like those seen in spaceflight after 4 days. In addition, there was spindle shaped elongation of the myocytes accompanied the elongation of the nucleus (Fig. 8). Other studies using anti-integrins demonstrated an elongation of the actin cytoskeleton, suggesting that a deficit of focal adhesions or integrins could be causing the alteration of cytoskeleton (81). This is

supported by the observation of Guignandon et al. showing that intermittent effect of gravity causes flight induced shape changes that included focal contact plaque reorganization. When cells were blocked in G2/M with nocodazole the flight-induced decrease in adhesion was ameliorated (30) suggesting a role for the microtubule in gravity induced changes.

Other studies by Guignandon et al. demonstrated that microgravity induced changes in cell shape after 4 days of microgravity, with osteoblasts becoming rounder and covered with microvilli. At the end of the flight, cells had mixed morphological types, including stellar shapes. In addition, alkaline phosphatase (ALK) was increased 2-fold in microgravity when compared to controls (82). Finally work of Vassy working with breast cancer MCF-7 in culture in microgravity found that microtubules were altered in microgravity, this observation was also made in the test tube, when Papaseit et al. demonstrated that isolated tubulin auto-polymerization is sensitive to the absence of gravity (83) during parabolic flight. Taken together these studies suggest that cell cytoskeleton may be involved in the changes in gene expression and function seen in osteoblasts in reduced gravity.

## DISCUSSION

Cell growth and differentiation are fundamental in bone development, and as such they act as master regulators of bone development and remodeling. In spaceflight there are numerous effects on tissues and cells, some of which, such as bone and muscle loss, could be related to cell proliferation and development anomalies in microgravity. More specifically, preliminary data from our own spaceflight gene expression experiments in osteoblasts (33; 75; 79) as well as others suggest that proliferation-related genes are downregulated in microgravity (48-51; 54; 58; 59; 61). Development is also affected with many of the matrix genes downregulated in microgravity (30-32; 66; 72; 74; 82).

Bone proliferation and mineralization are two of the most tightly regulated processes in the cell and multiple checkpoints during the cell cycle and development ensure that cells have normal development. Among those checkpoints are growth inhibitors that cause DNA transcription arrest and programmed matrix organization. Studies have demonstrated that cell proliferation is not constitutive, it requires promoters, such as the extracellular matrix and serum growth factors that activate mitogenic kinase signaling pathways. In many cases, such as in skin fibroblasts, cells are normally quiescent until serum exposure due to wounding or another factor causes proliferation. In bone and muscle tissues, however, there is continuous balanced cell proliferation and death to allow for dynamic remodeling. The dynamic nature of skeletal tissue allows it to quickly grow stronger with exercise and mechanical stimulation and to self-repair when bone breaks occur. Weight bearing and other mechanical stresses of earth gravity modulate the constant remodeling of these tissues. Therefore bone tissues will also dynamically decrease in mass if normal mechanical stimulation at 1-gravity is interrupted, such as during human bed rest, animal hind limb unloading (84), and spaceflight. Mechanical stimulation in skeletal tissues, therefore, appears to be accompanied by increased cell proliferation, and thus we hypothesize that force and proliferation are causally related. As a consequence we expect to observe decreased proliferative rates in musculoskeletal tissues in microgravity. Conversely, increased gravity might promote cell proliferation. Separate ground studies that address this question using centrifugation are already underway (unpublished data Hughes-Fulford).

Several investigators have reported reductions in glucose utilization in microgravity (28; 41; 85), however, this may be due to a reduced cell number caused by a reduction in cell growth and slowing of the cell cycle (25; 27-29; 41; 44; 46; 47). The causes of reduced growth may relate directly to the reduction of induction of the immediate early growth genes in the cell cycle,

namely reduction of *c-fos*, *c-myc*, and *cox-2* induction in microgravity (33; 34; 48; 49; 51; 54).

Some authors reported no change in osteoblast cell growth, but most of these exceptions were examining mineralization or were using culture systems with continual media flow over the cells, the resulting shear, even at low levels of flow, can stimulate cells (32; 47; 86).

Growth factor receptor EGFr was reported to be unchanged in several cell lines by several investigators (34; 52; 54; 87). There is a general consensus that the majority of changes are caused not due to loss of receptor number, rather to a change in signal transduction in microgravity. Several investigators have suggested that there is a microgravity induced alteration in signal transduction with altered gene expression being a major portion of the evidence. At the molecular level we have already discovered that mechanical stimulation by centrifugation of osteoblasts promotes the MAPK pathway (62). The MAPK pathway is a key growth factor and integrin linked signaling pathway, well known for its ability to promote proliferation and cell survival. The fact that mechanical stimulation from increased G levels also activates these molecules is suggests that gravity promotes cell growth through the MAPK pathway.

The role of the cytoskeleton in growth and its relationship to cell cycle and the MAPK pathway is being actively investigated in many laboratories. Results from several investigators suggest that the actin cytoskeleton is a necessary component of the cell cycle, especially needed for transition from G1-S phase and for anchorage dependent DNA synthesis (88; 89).

Restrictions to the cytoskeleton formation inhibit growth and can induce differentiation (90-92). The obvious changes in the cytoskeleton in microgravity may be a key component in regulation of growth and differentiation in microgravity as well as on Earth. The increase in PGE2 seen by several investigators may be a result of the altered cytoskeleton. Reports from Sawyer et al.

reported release of 6-11 fold increase of PGE<sub>2</sub> when the actin microfilament cytoskeleton is disrupted by cytochalasin D or latrunculin A in human umbilical vein endothelial cells (HUVEC). Disruption of microtubules did not cause a change in prostaglandin release (93).

Continued studies of osteoblasts in microgravity will facilitate our understanding of mechanisms controlling anchorage-dependent anabolic signaling and regulation of bone growth in a gravity free environment. Since all terrestrial organisms evolved in a 1-g environment, understanding the effect of Earth's gravity on cytoskeleton, cell growth and regulation will give us insight to fundamental biological laws underlying gravity based life.

**TABLE I: Effects of Microgravity on osteoblast growth and gene expression**

Osteoblast Cell Type	Hardware, control, and Samples per data point	Flow	Media Type	Collection and storage	Change in growth or mRNA / Time in orbit	Ref.
Mouse metatarsal (STS-42)	Biorack; bags inside Type I Container	No	1% $\alpha$ MEM	Collected after 4 days in microgravity and stored at $-10^{\circ}$ C	Glucose utilization and mineralization were decreased under microgravity. Mineral resorption was increased. No change in percent length increase and collagen synthesis in microgravity. Authors suggested that bone loss was result of impaired mineralization.	(85)
MC3T3-E1 (STS-56)	CMIX Ground control N=4	No	1% $\alpha$ MEM, then activation with 10% $\alpha$ MEM	Collected in microgravity on days 1 and 4 Stored at $20^{\circ}$ C	Glucose utilization decreased per well, however, there was no change if calculated on a per cell basis. Reduced cell proliferation by day 4. Increased prostaglandin synthesis day 1 in microgravity, no difference day 4.	(28)
Primary femur marrow cells (STS-65)	Ground control	No	10% FCS aMEM with ascorbic acid, dexamethasone $\beta$ -glycerol-phosphate	Collected in microgravity on day 5 Stored at $-20^{\circ}$ C	Reduced cell growth (ug DNA) in microgravity. Increases in PGE <sub>2</sub> synthesis in flight samples compared to ground. Increases in <i>cox-2</i> enzyme message and IL-6 mRNA were increased up to 9.3 fold.	(29)
MG-63 (Foton-10)	Plunger box Ground and flight 1-g control n=4	No	10% FCS DMEM	Collected in microgravity day 9 stored at $+26^{\circ}$ C	Response to 1,25(OH) <sub>2</sub> D <sub>3</sub> and TGF $\beta$ 2 was significantly reduced. Expression of alkaline phosphatase and osteocalcin was decreased, while that of collagen 1a was not. Message expression for both ALK, osteocalcin and Col I was significantly reduced	(66)
Primary femur marrow cells (STS-65)	CCU Ground control n=2	No	10% FCS aMEM with ascorbic acid, dexamethasone $\beta$ -glycerol-phosphate	Collected in microgravity days 4 & 5 At $-20^{\circ}$ C	Response to 1,25(OH) <sub>2</sub> D <sub>3</sub> was altered in flight caused increased levels of IGF-BP3 in media; mRNA levels were also increased. mRNA of IGFBP-4 and IGF-BP5 were decreased in flight	(65)

MC3T3-E1 (STS-76, 81, 84)	Plunger box Ground control & flight 1-g controls n=4	No	1% $\alpha$ MEM; activation with 10% $\alpha$ MEM	Collected in ug @ 3 h and 24 h Stored at -20°C	<i>cox-2</i> message was decreased in the microgravity-activated cells compared to ground or 1-g flight controls. <i>Cox-1</i> was not detected in any of the samples. There were no significant differences in the expression of actin mRNA between the 0-g and 1-g samples. Increased PGE <sub>2</sub> was present in media at 3 and 24h	(33)
MG-63 (Foton-10)	Plunger box Ground and flight 1-g control n=4	No	10% FCS DMEM changed at 3 and 6 days	Collected Day 9, kept at 12°C until landing	Differentiation markers OC and collagen 1aI were decreased to 51% of 1-g control. Alkaline phosphatase mRNA was reduced compared to 1-g control. Osteocalcin mRNA was 19% of 1-g control.	(31)
MC3T3-E1 (sounding rocket)	CCEM	No	1% $\alpha$ MEM the n activation with 10% $\alpha$ MEM	Collected at t=0 and t=10 min	Response to EGF in sounding rocket experiment showed a reduced <i>c-fos</i> response in microgravity. No changes were seen in MAPK phosphorylation during the short period of microgravity.	(58)
MC3T3-E1 (STS-76, 81, 84)	Plungerbox Ground control & flight 1-g controls n=4	No	1% $\alpha$ MEM then activation with 10% $\alpha$ MEM	Collected in ug 3hours and 24 hours Stored at -20°C	Synthesis of FN mRNA, protein, and matrix were measured after activation in microgravity. FN mRNA synthesis was significantly reduced in microgravity (0-g) when compared to ground (GR) osteoblasts flown in a centrifuge simulating earth's gravity (1-G) field 2.5 h after activation. However, 27.5 h after activation there was no significant differences in mRNA synthesis. A small but significant reduction of FN protein was found in the 0-g samples 2.5 h after activation. Total FN protein 27.5 h after activation showed no significant difference between any of the gravity conditions, however, there was a fourfold increase in absolute amount of protein synthesized during the incubation.	(75)
Primary rat femur marrow cells (STS-65)	CCU Ground control n=4	No	10% FCS $\alpha$ MEM with ascorbic acid, dexamethasone $\beta$ -glycerol- phosphate	Collected Day 4 & 5 At -20°C	mRNA levels for <i>PDGF<math>\beta</math></i> receptor, <i>EGFr</i> , <i>Shc</i> and <i>c-fos</i> were measured. <i>PDGF<math>\beta</math></i> receptor, <i>Shc</i> and <i>c-fos</i> were decreased by approximately 50% by day 5. <i>EGFr</i> was not changed. The authors suggested that there is a change in signal transduction in microgravity.	(87)

hFOB (STS-80)	CCM on Cytodex™ beds in hollow-fiber cartridges Ground control n=2	Yes	10% DMEM and Hams F12	Media collected on days 8, 12 in-flight stored at RT. Days 18 and 19 were collected postflight.	<i>IL-1α</i> and <i>IL-6</i> were decreased 8 hours after landing and returned to normal by 24 hours post landing. TGFβ messages were modestly reduced 8 hours post flight but were not significant. No change in growth, PGE <sub>2</sub> or glucose utilization was noted.	(86)
Primary chicken calvaria osteoblasts (STS-59)	Cellmax capillary culture units. Osteoblasts grown on Cytodex™ beads. N=2 or 4	Yes	αMEM10% FCS αMEM with ascorbic acid, β-glycerol- phosphate	Media samples removed during flight. Cartridges collected at landing and stored at -80°C.	No change in flight and ground control glucose utilization, however, there was a reduction in flight total RNA compared to ground. Reduced osteocalcin and collagen mRNA and protein expression suggesting that microgravity slows progression of differentiation.	(32)
ROS 17/2.8 (clinostat)	Clinostat experiment- cells grown in 25cm <sup>2</sup> falcon flasks	No	10% DMEM	Rotated on clinostat for 6, 12 and 24 hours. Fixed or frozen at time of collection	Clinorotation induced apoptosis of osteoblasts, suggested that cell death was associated with cytoskeleton disorganization.	(38)

**TABLE II: Effects of Microgravity on Cytoskeleton and Extracellular Matrix and ECM Mineralization**

Cell Type	Hardware, control, and Number of Replicates per data point	Flow	Media Type	Collection and storage	Change in growth or mRNA / Time in orbit	Ref.
ROS 17/2.8 (Biocosmos)	Plungerbox, parabolic flight	No	10% FCS-DMEM	2.5% gluteraldehyde room temperature	After 15 or 30 parabola, cells exhibited ruffling, cell area was significantly less and cell shape more irregular. PGE <sub>2</sub> content was significantly increased in parabolic flight samples. Inverse correlation between PGE <sub>2</sub> and cell area.	(72)
MC3T3-E1 (STS-56)	CMIX Ground control n=4	No	$\alpha$ MEM	Collected in ug days 1 and 4 Stored at 20°C	Cytoskeleton of flight osteoblasts had reduced number of stress fibers and seemed to be collapsed. Nuclei exhibited an elongation in flight samples. PGE <sub>2</sub> significantly increased in flight samples at 16h.	(28)
MG-63 (Foton-10)	Plunger box Ground and flight 1-g control n=2	No	10% FCS DMEM	Collected on Day 9 stored at +26°C. Fixed in 3.7% formaldehyde	Message expression for ALK, osteocalcin and Col I was significantly reduced. No change in Col I protein content. No major difference in morphology between flight and 1-G or Ground samples.	(66)
MC3T3-E1 (STS-76, 81 and 84)	Plungerbox Ground control & flight 1-g controls n=4	No	1% $\alpha$ MEM then activation with 10% $\alpha$ MEM	Collected in microgravity 3 hours and 24 hours Stored at -20°C	Significant changes in nucleus elongation and stellar cytoskeletal sturctures by 24 hours. Actin cytoskeleton has less surface area than 1-g and ground controls.	(75; 94)
ROS 17/2.8 (Bion-10)	Plungerbox	No	10% FCS-DMEM	Cells collected days 4 and 6.	Significant changes in cell shape, becoming rounder, covered with microvilli by day 4. By day 6, change more pronounced, with microgravity cells retracted and having long extensions with in-flight 1-g centrifuge controls resembling ground controls.	(82)
ROS 17/2.8 (parabolic flight)	Plungerbox	No	10% FCS-DMEM	Cells collected at 36 hours, fixed in 4% para-formaldehyde or 70% ethanol.	Global reductions seen in cell adhesion parameters during flight. Decreased cell area was associated with focal contact plaque re-organization.	(30)

MC3T3-E1 (STS-76, 81 and 84)	Plungerbox Ground control & flight 1-g controls n=4	No	1% $\alpha$ MEM then activation with 10% $\alpha$ MEM	Collected in microgravity 3 hours and 24 hours Stored at -20°C	Elongated nuclei and stellar cytoskeleton present in microgravity samples, 1-g in-flight centrifugation reversed morphology changes to resemble ground controls.	(77)
Primary chicken calvaria osteoblasts (STS-59)	5-Cellco Cellmax capillary culture units. Osteoblasts grown on Cytodex™ beads.	Yes	$\alpha$ MEM10% FCS aMEM with ascorbic acid, $\beta$ -glycerol- phosphate	Media samples removed during flight. Cartridges collected at landing and stored at -80°C.	No change in flight and ground control glucose utilization, reduction in flight total RNA compared to ground. Morphology showed fewer osteoblasts and matrix development around beads and hollow fibers in microgravity.	(32)
hFOB (STS-80)	CCM on Cytodex™ beads in hollow- fiber cartridges Ground control n=2	Yes	10% DMEM /Hams F12 (growth)	Media collected on days 8, 12 in-flight stored at RT. Days 18 and 19 were postflight on the ground and stored in freezer.	No obvious change in cytoskeleton or morphology of cells growing on Cytodex™ beads.	(86)
ROS 17/2.8 (clinostat)	Clinostat experiment- cells grown in 25cm <sup>2</sup> falcon flasks	No	10% DMEM	Rotated on clinostat for 6, 12 and 24 hours. Fixed or frozen at time of collection	Cell viability decreased significantly over 24 hours of vector-averaged gravity. Clinorotation associated with disorganized actin cytoskeleton, condensation of F-actin and microspikes. Localization of integrin $\beta_1$ was perinuclear.	(38)



**Table III**

**Sequential responses of osteoblasts to gravity-induced signaling and gene expression**

<b>Seconds</b>	<b>Minutes</b>	<b>1-8 hours</b>	<b>&gt;18 hours</b>
<ul style="list-style-type: none"> <li>• G protein activation</li> <li>• Map Kinase Activation</li> <li>• PGE<sub>2</sub>, release</li> </ul>			→
			→
	<ul style="list-style-type: none"> <li>• <i>c-fos c-myc, egr-1</i> and <i>cox-2</i></li> <li>• Induction actin cytoskeleton signaling</li> <li>• Activation of nuclear transcription factors</li> </ul>		→
		<ul style="list-style-type: none"> <li>• Cytoskeletal changes</li> <li>• FAK formation</li> <li>• Autocrine growth factor synthesis</li> </ul>	<ul style="list-style-type: none"> <li>• Cell doubling</li> </ul>

## Figure Legends

**Fig. 1 Relationship of growth and differentiation gene expression.** Cell growth is regulated early in the growth period, with growth genes *c-fos*, *c-myc*, *cox-2* *egr-1* and transcription factors being actively made by the cell in the cell cycle during proliferation. Cell cycle is regulated partly by the cyclins, cyclin kinases (cdk), cyclin kinase inhibitors (cdki) and other cell cycle genes. During the very late proliferation stage and early portion of the differentiation stage, the collagen and mineralization genes are upregulated then alkaline phosphatase and matrix GLA proteins are upregulated in order for the extracellular matrix to mature. The final stages of mineralization occur during final differentiation where osteopontin and osteocalcin and collegians are upregulated. Adapted from (23).

**Fig. 2. Cell cycle regulation of immediate early genes and cyclins in the osteoblast.** Cell growth is defined by the cell cycle, which is divided into four phases, G1, S, G2 and M. When a cell is not growing, it is resting in the G1 or G0 phase. Growth activation is regulated at the border of the G1 phase just before the start of DNA synthesis (S-phase). The G1/S-phase transition in osteoblasts can be triggered either by a chemical signal like addition of sera or growth factors (95) or by mechanical stress(18; 21; 63; 64; 96-98). Once a cell enters into S-phase, it is committed to complete cell replication resulting in the production of 2 daughter cells at mitosis. Evidence from previous spaceflights suggest that the osteoblast is delayed in G1 presumably due to the lack of normal gravity force. Cells are first stimulated with growth factors (fetal calf sera) that stimulate synthesis of *c-fos*, *c-myc* and *cox-2*. Synthesis of PGE<sub>2</sub>, is induced prior to entry into S-phase. S-phase is associated with an increase of cyclin E and spike of histone-4 synthesis and DNA

synthesis. Once DNA replication is completed, cells enter G2 where proteins necessary for division are made before entry into mitosis.

**Fig. 3 Anabolic pathways for initiation of cell proliferation**

Tyrosine kinases such as FGF, ILGFs and EGF, and Serine/threonine kinase growth factors like TGF $\beta$  stimulate ras, raf and finally extracellular signal regulated kinase (ERK) are phosphorylated and translocated to the nucleus and activate nuclear transcription factors and induces growth related genes. The integrins can also stimulate ras and the MAPK pathway. Finally, studies have demonstrated that both PKA and PKC stimulate the MAPK pathway via raf. All pathways lead to activation of nuclear transcription factors and induction of immediate early genes.

**Fig. 4 Changes in MC3T3-E1 cytoskeleton and elongation of nuclei in microgravity.**

Structure of the F-actin cytoskeleton of 0-g and ground controls using a Zeiss Neofluar 100x oil objective using rhodamine phalloidin. Nuclei were stained with Hoechst 33258. Photos were from STS-56 samples.

**Fig. 5 Possible sites of regulation of G1-S transition**

- ① Stimulation of cell membrane by gravity causing release of arachidonic acid (AA) from membrane, perhaps through activation of c-PLA<sub>2</sub>. Mechanical stimulation also activates Ca<sup>++</sup> channels and G-proteins
- ② AA conversion into PGE<sub>2</sub> by cyclooxygenase and or PGI<sub>2</sub> by PGI<sub>2</sub> synthase
- ③ Export of the newly synthesized PGE<sub>2</sub>/ PGI<sub>2</sub> out of the osteoblast

- ④ Activation of seven-domain transmembrane receptors by PGE<sub>2</sub> and or by gravity interactions with the cytoskeleton
- ⑤ Activation of the seven-domain transmembrane EP2/4 receptors by PGE<sub>2</sub> or by gravity interaction with the cytoskeleton.

**Fig. 6 Osteoblast grown in microgravity or in 1-g centrifuge in flight.**

Osteoblasts were flown on STS-76, photos taken from samples 24 hours after addition of serum to quiescent cells in microgravity. Nuclei of the 0-g are partly elongated and phase contrast shows elongation of the whole cell and few cells in S/G2 stage. Also there were few mitotic figures in the 0-g sample. One-g flight samples have several cells that are in G2 (pink arrows) or just entering or undergoing mitosis (light blue arrows). Photos were taken with 20x objective.

**Fig. 7 Fibronectin network in osteoblasts grown in 1-g or 0-g environment.**

Osteoblasts were flown on STS-76, photos taken of samples 24 hours after addition of serum to quiescent cells in microgravity. Fibronectin was imaged with rabbit anti-fibronectin with a secondary G-anti rabbit rhodamine antibody. No significant difference was seen in the fibronectin matrix in microgravity.

**Fig. 8 Cytoskeleton and nucleus elongation with lack of mitogens**

Induction of either apoptosis or terminal differentiation in monocytes cultured in the 2% differentiation medium (DM) which causes mitogen deprivation. Proliferating C2C12 monocytes in growth medium (GM) were shifted to DM for 24, 48 or 72 hours. (A-D). Phase contrast photomicroscopy revealed morphological changes, with floating cells most

evident in DM 24 and 48 hour cultures. Green tags show apoptotic cells, Red shows muscle differentiation (MCH red) as well as cell shape details (E-H). Nucleus is displayed in (I-L), apoptotic cells seen in F,G and H also have condensed chromatin and cell shrinkage.

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**Fig. 9 Osteoblast grown in microgravity for 24 hours show differences in nuclear shape and actin cytoskeleton**

Osteoblasts were flown on STS-76, photos taken of samples 24 hours after addition of serum to quiescent cells in microgravity. Structure of the F-actin cytoskeleton of 0-g , 1-g flight and 1-g ground controls using a Zeiss Neofluar 100x oil objective using rhodamine phalloidin. Nuclei were stained with Hoechst 33258. Cells grown in microgravity had elongated nucle and elongated actin cytoskeleton. The microgravity grown cells did not have condensed DNA, however, there were few cells in G2 or mitosis . Growth of 1-g and ground controls showed normal actin cytoskeleton and cells in all phases of the cell cycle

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