Final Report: NCC 2-846
Title: Effects of Hypogravity on Osteoblast Differentiation.
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# I. INTRODUCTION

Weightbearing is essential for normal skeletal function. Without weightbearing, the rate of bone formation by osteoblasts decreases in the growing rat. Defective formation may account for the decrease in the maturation, strength and mass of bone that is caused by spaceflight. These skeletal defects may be mediated by a combination of physiologic changes triggered by spaceflight, including skeletal unloading, fluid shifts, and stress-induced endocrine factors. The fundamental question of whether the defects in osteoblast function due to weightlessness are mediated by localized skeletal unloading or by systemic physiologic adaptations such as fluid shifts has not been answered. Furthermore, bone-forming activity of osteoblasts during unloading may be affected by paracrine signals from vascular, monocytic, and neural cells that also reside in skeletal tissue. Therefore we proposed to examine whether exposure of cultured rat osteoblasts to spaceflight inhibits cellular differentiation and impairs mineralization when isolated from the influence of both systemic factors and other skeletal cells.

Growth of primary fetal rat osteoblasts on microcarriers enhances differentiation of osteoblasts and mineralization of the collagenous matrix. Using this culture system, we addressed the following questions: 1) Does spaceflight inhibit mineralization of the extracellular matrix? Osteoblast cultures were analyzed by light and electron microscopy to evaluate ultrastructural differentiation and the spatial organization of cellular and extracellular components of mineralizing cultures. 2) Does spaceflight delay the differentiation of osteoblasts? Differentiation was assessed both by histological analysis and by the steady-state expression of mRNA for genes that are markers of progressive osteoblast maturation, including alkaline phosphatase, ostepontin and osteocalcin. To assess metabolic activity and general health of the cultures, glucose and lactate concentrations in spent media were determined.

We were provided two flight opportunities with which to address the aims of this proposal. NIH.C1 (STS59) had a flight duration of 11 days and NIH.C3 (STS63) had a flight duration of 8

days. A detailed discussion of the protocols, results and conclusions follows.

## II. SUMMARY FLIGHT AND EXPERIMENTAL PROTOCOL

### A. NIH.C1 (STS59)

Fetal rat osteoblasts were prepared at KSC. Cells were purified by collagenase digestion, plated on microcarrier beads in Petri dishes, then loaded after 5 d into polypropylene fiber cartridges. Cells were maintained for 2 d on continuous flow CellCo Units, then transferred to the STL hardware and maintained on the unit for 4 d prior to launch. Basal controls were recovered at the time of launch, having been maintained on CellCo Units rather than STL hardware. There was a launch slip of 2 d. The flight duration was 11 d, having been extended in flight by one day. Therefore, the total time of cell growth in culture was 22 d. Previous studies showed that cells remained viable at least until 28 d in culture. Flight cartridges were recovered within 4 hrs after landing at Dryden while ground controls were recovered simultaneously at KSC. Two cartridges from each group were fixed for histological analyses, and two opened, the cells recovered, then frozen to -80C in freezing media.

NIH.C1 (STS59) time line:
(Launch= d0)

| Day: -11 | -6 | -4 | 0 | 11 | 11d + 4hr. |
|---|---|---|---|---|---|
| Isolate Cells | Cells in cartridges on CellCo units | Load on STL | *Launch* Recover Basal | *Landing* | Sample Recovery of Flight and Ground Controls |

At the time of sample recovery after flight, no visible signs of contamination were noted. However, spent media appeared acidic and there were obvious differences between samples in the amount of spent medium in the sump bags (see section IIA) due to possible problems with pump efficiency in this flight (See detailed discussion, Annual Report NIH.C1).

### B. NIH.C3 (STS63)

In contrast to NIH.C1, stock cultures of osteoblasts were plated onto tissue culture dishes in the laboratory of the principal investigator at Ames Research Center, Moffett Field, CA, then transported to KSC in order to circumvent problems encountered in the previous flight. At KSC, cells were plated onto beads, then loaded into cartridges and maintained in CellCo units as in NIH.C1. The

modified protocol was extensively tested in preliminary ground based experiments and shown to support osteoblast survival and differentiation.

NIH.C3 (STS63) time line:
(Launch= d0)

| Day: | -12 | -10 | -9 | -4 |
|---|---|---|---|---|
| | Isolate Cells | Cells on beads | Cells in cartridges | Load on STL |

| 0 | +9 | 9d+4hr |
|---|---|---|
| *Launch* Basal Cell Recovery | *Landing* | Sample Recovery |

Recovery of the samples after landing revealed obvious signs of bacterial contamination in 2 out of 4 flight cartridges and in 1 out of 4 ground control cartridges. Later, rigorous media sterility tests conducted in the principal investigator's laboratory of all of the spent media samples collected both before and after transfer of the cartridges to the flight hardware revealed that all of the cartridges were harboring contamination at the end of the flight period. Contamination was likely to have been introduced during or subsequent to transfer of the cartridges onto the STL flight hardware, since 1) none of the spent medium samples obtained from the cartridges at the time of handover to load on the STL hardware were contaminated and 2) the spent medium from some of the cartridges at the time of sample recovery was contaminated whereas the fresh medium supplied to the STL units was not.

Although we proceeded to analyze the samples from cultures that did not show obvious signs of contamination at the time of sample recovery in order to learn as much as possible from the experiment, all results obtained from this flight must be considered inconclusive since all of the cultures were harboring a suppressed bacterial contamination.

## II. RESULTS

### A. NIH.C1 (STS59)

#### 1. Metabolic Status

NIH.C1 Energy metabolism was assessed by measuring glucose and lactate concentrations in spent medium from flight and control cultures during 8 hours before flight and during the time the cartridges were on the STL unit (Table 1, 2). These values represent the average

rates of glucose consumption and lactate production during the period between media recoveries. Therefore, given the experimental protocol, the glucose and lactate values of conditioned medium recovered after landing include a period encompassing 4d prior to launch as well as the entire duration of flight (11d).

The amount of spent media recovered from the sump bags of control and flight cartridges varied substantially, indicating that pump efficiency was different in flight and control cartridges (see C1 annual report for detailed discussion).

*Table 1. Average Glucose Utilization and Lactate Production per Unit Volume; All samples included in data analysis.*

| | Control Cultures | Flight Cultures |
|---|---|---|
| Glucose Depletion (mg/l/hr) | | |
| Pre-flight (d-4.3 to d-4.0) | 21.6 ± 2.6 | 19.4 ± 3.0 |
| Flight (d-4.0 to d11) | 4.7 ± 0.6 | 2.7± 0.3* |
| Lactate Production (μmol/l/hr) | | |
| Pre-flight (d-4.3 to d-4.0) | 167 ± 7.4 | 151 ± 13.1 |
| Flight (d-4.0 to d11) | 53 ± 5 | 32 ± 1* |

Values are means ± S.D. measured on d7 or d22 and therefore represent the average rate of glucose depletion or lactate production. *indicates significant difference between flight and corresponding controls at P<.001 by Student-Newman-Keuls test. n=4 per group.

Once corrected by the total volume of medium pumped through each cartridge, the differences in glucose consumption and lactate production between flight and ground control samples were not significant (due to the variability in volume of medium pumped through the cartridges). However, when those samples in which the pumps may have malfunctioned were omitted from the statistical analysis, significant differences between flight and ground controls after recovery were evident (Table 2).

*Table 2. Rate of Glucose Utilization and Lactate Production: Outliers Omitted*

| | Control Cultures | Flight Cultures |
|---|---|---|
| Glucose Depletion (mg/hr) | | |
| Flight (d-4.0 to d11) | 0.354 ± 0.014 | 0.162 ± 0.019 * |
| Lactate Production (μmol/hr) | | |
| Flight (d-4.0 to d11) | 3.94 ± 0.23 | 1.99 ± 0.09 ξ |

Values are means ± S.D. for all samples (n=3 for each group). Values have been corrected for differences in the volume of medium pumped through individual cartridges (flight), as well as the amount of glucose and lactate present in the tubing and cartridge in pre-flight samples prior to transfer to the hardware. *no significant difference between flight and corresponding controls at P< .0001* or P, .0002ξ by Student-Newman-Keuls test.

## 2. Morphology NIH.C1

Light and electron microscopic observations were performed on cell samples obtained from two flight cartridges and two ground control cartridges. By light microscopy the two groups appeared similar except there were more cells per section from the controls than from the flight samples. The cell morphology of both flight and ground controls appeared generally more fusiform (i.e. fibroblastic) rather than plump or cuboidal, which would be expected for well-differentiated osteoblasts. Cells which were attached to beads formed a cell/bead mass with additional cells scattered between the beads, whereas cells randomly scattered between beads, without attachment, never formed a discrete cell/bead mass.

Electron microscopy revealed that the majority of cells contained well-formed endoplasmic reticulum but small, poorly developed Golgi complexes. This correlated well with the absence of large accumulations of extracellular collagen. Cells from flight cultures were fewer in number and appeared even less differentiated than the control cultures. The small amount of extracellular collagen in both groups meant that mineralization in these cultures would be unlikely to occur and in fact was not seen.

All cells sampled from this experiment contained a large accumulation of lipid-like dense cytoplasmic inclusions. We have not previously observed accumulation of these inclusions in healthy, normal cell cultures, suggesting that there was some additional metabolic stress on these cultures which was not related to flight since the ground controls were also affected.

### 3. Northern Analysis NIH.C1

The cell yield was too low to purify sufficient amounts of mRNA for Northern analysis.

## B. NIH.C3 (STS 63)

### 1. Metabolic Status NIHC3.

Differences in volumes of spent medium recovered from sump bags were not evident in this flight (in contrast to NIH.C1), although contamination was evident (see Section IB)

*Table 2. Rate of Glucose Utilization and Lactate Production*

| | Control Cultures | Flight Cultures |
|---|---|---|
| Glucose Depletion (mg/hr) | | |
| Flight (d-4.0 to d8) | .428 | .281 |
| | .311 | .332 |
| | .332 | |
| Lactate Production (μmol/hr) | | |
| Flight (d-4.0 to d11) | 2.09 | 2.08 |
| | 1.59 | 2.07 |
| | 1.92 | |

Values shown are for each cartridge which did not show visible signs of contamination at the time of sample recovery, although they were later shown to harbor suppressed bacterial contamination. Values have been corrected for differences in the volume of medium pumped through individual cartridges (flight), as well as the amount of glucose and lactate present in the tubing and cartridge in pre-flight samples prior to transfer to the hardware.

### 2. Morphology NIH.C3.

In general, electron microscopy did not show any significant differences between those bone cells from flight and ground control groups. Two electron micrographs have been chosen and their description will indicate all findings for the many cells photographed from this flight project.

Photo A (Figure 1) is a single bone cell from the ground control collected following the recovery of cells after flight. The nucleus (N) shows very uniform chromatin staining, suggesting a very early stage in the differentiation process. This suggestion is supported by the cytoplasmic structure which shows very little organized endoplasmic reticulum (for collagen synthesis), and the Golgi structure (G) contains no visible secretory granules. Lipid inclusions and some mitochondria are often present in all bone cells regardless

of their differentiated state, although lipid inclusions were not as prevalent as in the C1 experiment. In the extracellular space, there is very little evidence of collagen fibril formation (F). Magnification =13,000X.

Photo B is a single bone cell recovered from the flight samples. Similar to the control samples, the nucleus is uniformly stained for chromatin, suggesting a limited degree of cellular differentiation. There is only a small amount of organized secretory granules suggesting a very low level of protein synthesis and secretion. As seen in the controls, lipid (L) inclusions and a scattering of mitochondria are also present in the cytoplasm. In the extracellular space, there is a very limited amount of collagen fibrils (F). Magnification=13,000X.

The bone cells appear to be actively dividing and increasing their cell mass in both the ground control and flight environment. In some areas of the cell mass, there was a greater amount of collagen formed than in the examples shown here (Figure 1). Nevertheless, there was never an amount of collagen formed which would indicate that mineralization might soon be initiated. We have seen in earlier studies, that collagen layers at least 10-15 microns in depth were necessary before any mineralization could be found. We never saw this degree of collagen formation in either the controls or flight samples retrieved from the cartridges. Thus the level of cell differentiation was never advanced enough so that the cells produced enough collagen for mineralization.

### 3. Northern Analysis NIH.C3

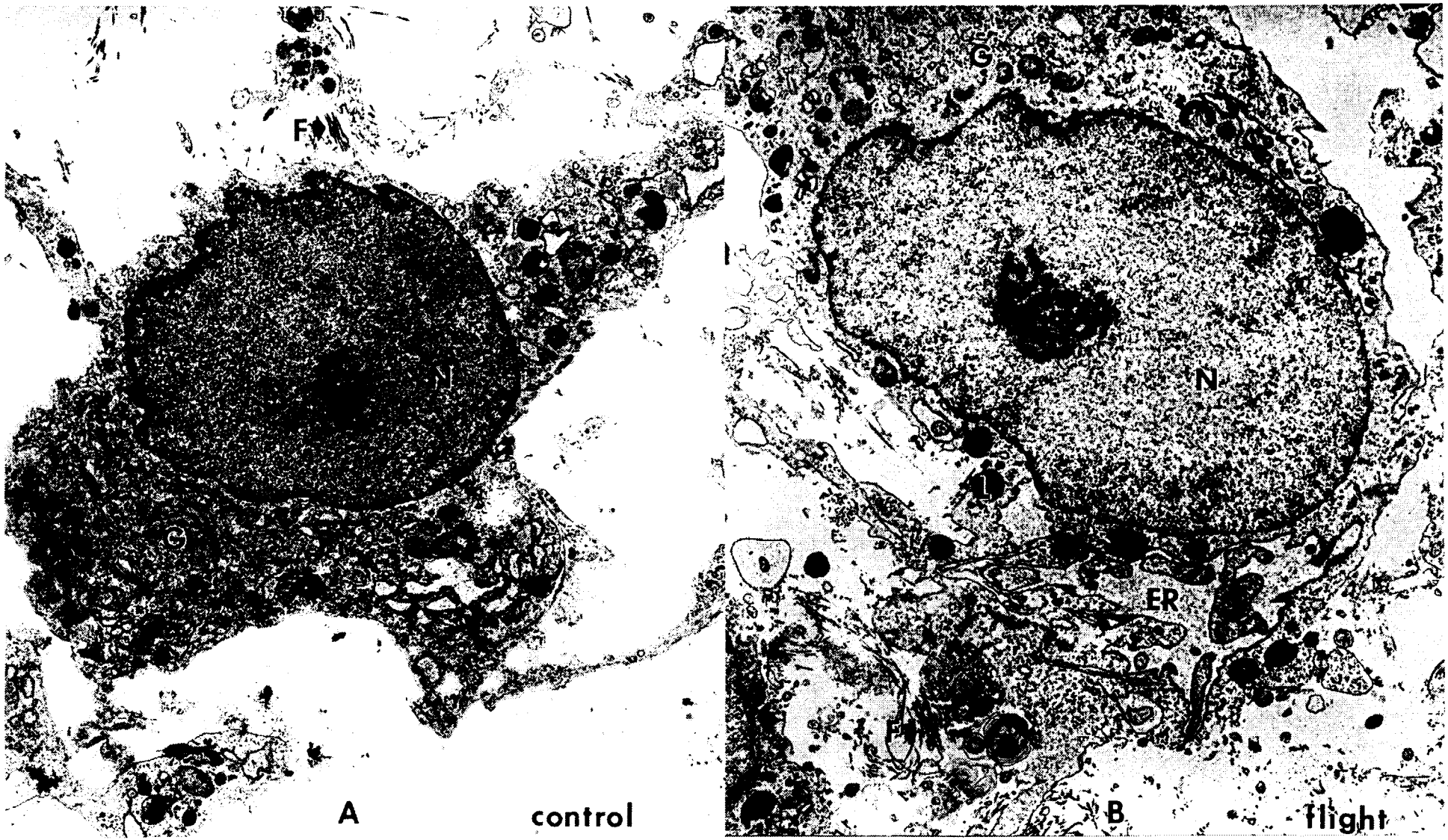
The steady-state expression of genes characteristic of the osteoblast phenotype was analyzed by Northern blotting. Alkaline phosphatase increases at early stages of osteoblast differentiation, and descreases once mineralized nodules form, whereas osteocalcin (OC) is expressed at late stages of differentiation and osteopontin (OP) is expressed at both early and late stages in cultured cells. Surprisingly, mRNA for alkaline phosphatase was not expressed at detectable levels in the cultures (data not shown) whereas both osteocalcin and osteopontin were expressed in flight and control cultures (Figure 2) . There were no consistent differences in mRNA expression evident between basal, ground control and flight samples.

## IV. Summary and Conclusions

Results from STS-59 (NIH-C1) revealed that glucose utilization during spaceflight was significantly lower than ground control cultures, and the production of lactate concomitantly decreased. In addition, ultrastructural analysis by electron microscopy revealed that osteoblasts exposed to spaceflight possessed less well-organized, rough endoplasmic reticulum/Golgi apparatus than ground controls. This result indicates that spaceflight may inhibit the protein synthetic activity of osteoblasts, a fundamental requirement for new bone formation. Thus, spaceflight may regulate both energy metabolism and the activity of cultured osteoblasts.

Control and flight cell cultures on STS-63 (NIH-C3) acquired a bacterial contamination in the course of the experiment. Analysis of spent media samples revealed that the cultures acquired contamination at the time of transfer from ground-based Cellco units to the STL hardware. In general, light and electron microscopy did not show any significant differences in cell morphology between flight and ground control groups, and little evidence of collagen accumulation in either flight or ground control cultures. Northern analysis revealed that the cells expressed significant levels of mRNA for osteopontin as well as osteocalcin, which is a later marker of osteoblast differentiation. Thus the cells appeared to differentiate to a limited extent in the course of the experiment despite the contamination. Significant differences were not observed in the amounts of glucose consumed and lactate produced between flight and ground control samples at the end of the flight period. However, given the problem of contamination, data acquired from this flight are not informative and conclusions about the effects of spaceflight cannot be drawn.

Results from this project require confirmation in additional flight experiments. Future experiments should address the questions of whether microgravity alters energy metabolism as well as the differentiation and protein synthetic activity of cultured osteoblasts. To adequately answer these questions will require the use of both on-board centrifuge controls as well as methods to accurately quantify cell number in each sample (in order to correct glucose and lactate values).


F
N
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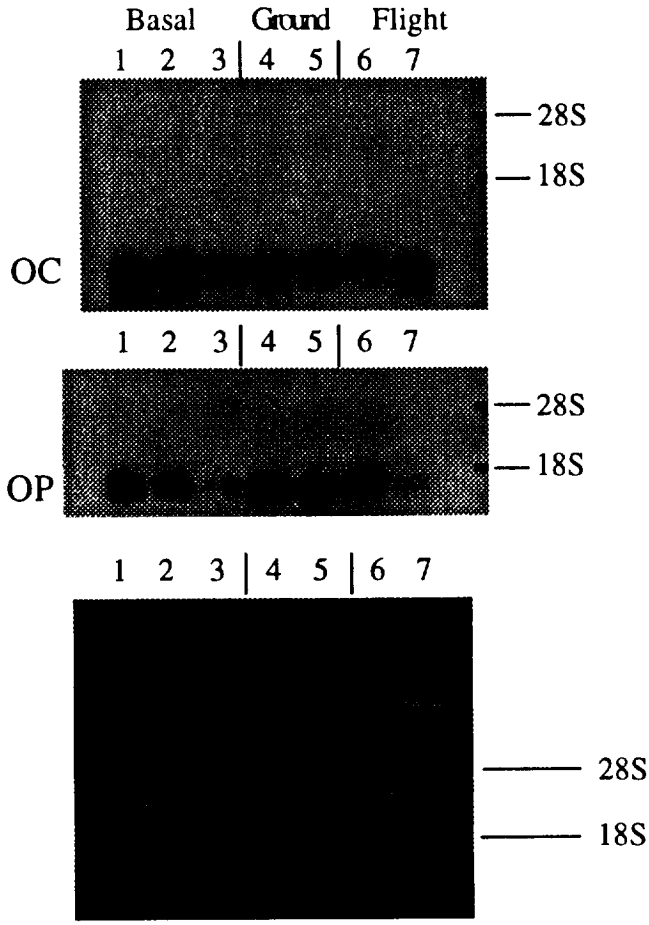
Basal
Ground
Flight
1 2 3 4 5 6 7
28S
18S
OC
1 2 3 4 5 6 7
28S
18S
OP
1 2 3 4 5 6 7
28S
18S

FIGURE 2