CHAPTER 8

MICROGRAVITY AND BONE CELL MECHANOSENSITIVITY

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ABSTRACT

Stress derived from bone loading which affects bone cells is likely the strain-induced flow of interstitial fluid along the surface of osteocytes and lining cells. The response of bone cells in culture to fluid flow includes prostaglandin (PG) synthesis and expression of prostaglandin G/H synthase inducible cyclooxygenase (COX-2). Cultured bone cells also rapidly produce nitric oxide (NO) in response to fluid flow as a result of activation of endothelial nitric oxide synthase (ecNOS), the enzyme which also mediates the adaptive response of bone tissue to mechanical loading. Earlier studies have shown that the disruption of the actin-cytoskeleton abolishes the response to stress, suggesting that the cytoskeleton is involved in cellular mechanotransduction.

Microgravity, or near weightlessness, is associated with the loss of bone in astronauts, and has catabolic effects on mineral metabolism in bone organ cultures. This might be explained as resulting from an exceptional form of disuse under near weightlessness conditions. We found earlier that the transduction of mechanical signals in bone cells also involves the cytoskeleton and is related to PGE\textsubscript{2} production. Therefore it is possible that the mechanosensitivity of bone cells is altered under near weightlessness conditions, and that this abnormal mechanosensation contributes to disturbed bone metabolism observed in astronauts.

In the experiment “FLOW”, we tested the hypothesis of altered cell bone mechanosensitivity using an in vitro model developed for space flight experiments. The specific aim of FLOW was to test whether the production of early signaling molecules that are involved in the mechanical load-induced osteogenic response (NO and PGE\textsubscript{2}) by bone cells is changed under microgravity conditions compared to 1xg conditions. FLOW was one of the Biological experiment entries to the Dutch Soyuz Mission “DELTA” (Dutch Expedition for Life Science, Technology and Atmospheric Research). FLOW was flown by the Soyuz craft, which was launched on April 19, 2004, on its way to the International Space Station (ISS).
Osteocytes, osteoblasts, and periosteal fibroblasts were isolated from chicken skull and incubated in plunger boxes, developed by Centre for Concepts in Mechatronics (Nuenen, The Netherlands), using plunger activation events for single pulse fluid shear stress stimulations. Cultures in-flight were subjected to microgravity and simulated 1xg level by centrifugation. Ground controls were subjected to identical culture environment and fluid shear stress stimulations. Due to unforeseen hardware complications, results from in-flight cultures are considered lost. Ground control experiments showed an accumulative increase of NO in medium for osteocytes (as well as for osteoblasts and periosteal fibroblasts). Data from the online-NO sensor showed that the NO produced in medium by osteocytes increased sharply after pulse shear stress stimulations. COX-2 mRNA expression revealed high levels in osteoblasts compared to the other cell types tested. In conclusion, preparations for the FLOW experiment and preliminary ground results indicate that the FLOW setup is viable for a future flight opportunity.
INTRODUCTION

It has been well documented that bone tissue is sensitive to its mechanical environment. Subnormal mechanical stress as a result of bedrest or immobilization results in decreased bone mass and disuse osteoporosis (1). Spaceflight produces a unique condition of skeletal unloading as a result of the near absence of gravity. Studies of animals and humans subjected to spaceflight agree that near weightlessness negatively affects the mass and mechanical properties of bone (for a review, see (2)). Although the exact mechanism whereby bone loss as a result of spaceflight occurs is still unknown, recent in vivo studies suggest that bone cells are directly sensitive to near weightlessness. Using organ cultures of living bone rudiments from embryonic mice, Van Loon et al. (1995) showed that 4 days of spaceflight inhibited matrix mineralization, while stimulating osteoclastic resorption of mineralized matrix (3). Monolayer cultures of the human osteoblastic cell line MG-63 responded to 9 days of near weightlessness with reduced expression of osteocalcin, alkaline phosphatase, and collagen Iα1 mRNA (4). Reduced prostaglandin production was found in cultures of MC3T3-E1 osteoblastic cells exposed to 4 days of near weightlessness, probably due to inhibition of serum-induced growth activation (5). In addition near weightlessness induced prostaglandin E2 (PGE2) and interleukin-6 production in rat bone marrow stroma cultures, an observation that may be related to alterations in bone resorption (6). These results suggest that mineral metabolism and bone cell differentiation are modulated by near weightlessness, and that bone cells are directly responsive to microgravity conditions.

Direct responses of bone cells to mechanical stimuli have been studied using several methods to apply mechanical stress in vivo (for a review, see (7)). Stretching or bending of the cell substratum has been widely used, but recent evidence indicates that fluid flow over the cell surface may better simulate the cellular effect of mechanical loading of bone in vivo (8-15). Strain (deformation) of the bone matrix as a result of mechanical stress in vivo causes flow of
interstitial fluid through the network of osteocyte lacunae and canaliculi (16, 17). Weinbaum et al. (1994) used Biot’s porous media theory to relate loads applied to a whole bone to the flow of canalicular interstitial fluid. Their calculations predict fluid shear stresses of 0.8 to 3 Pa as a result of peak physiological loading regimes (12). We have shown earlier that osteocytes are sensitive to fluid shear stress \textit{in vitro}, and release signaling molecules such as NO and PGE$_2$ in response to fluid shear stress (18).

FLOW was our entry to the Biological experiments carried by the Dutch Soyuz Mission “DELTA” (Dutch Expedition for Life Science, Technology and Atmospheric Research). Through DELTA, FLOW was flown by the Soyuz craft, which was launched on April 19, 2004, on its way to the International Space Station (ISS). The main scientific objective of the FLOW experiment was to test whether the production of early signaling molecules that are involved in the mechanical loading-induced osteogenic response (NO and PGE$_2$) by osteocytes is changed under microgravity conditions compared to 1xg conditions. Since we argue that especially the osteocyte, and not the osteoblast, is the mechanosensitive cell type within bone involved in mechanotransduction, the production of signaling molecules by osteocytes were compared to osteoblasts. Periosteal fibroblasts were used as negative controls. Chicken osteocytes, osteoblasts, and periosteal fibroblasts were incubated in plunger boxes developed by the Centre for Concepts in Mechatronics, using plunger activation events for single pulse fluid shear stress stimulations. Cultures in-flight were subjected to microgravity (µ-g) and simulated 1xg level by centrifugation. Ground controls were subjected to identical culture environment and fluid shear stress stimulations.
GROUND PREPARATIONS AND HARDWARE DEVELOPMENT

Dutch Soyuz Mission – FLOW hardware development

The experiment FLOW made use of plunger box units (PBU) (figure 1) developed by the Centre for Concepts in Mechatronics (CCM). One module contained two culture compartments, each holding a glass slide containing about 5x10^4 cells each (fig 1A). For each culture compartment fresh culture medium or RNA stabilization solution (RNALater, Ambion Inc., USA) were stored in three fluid reservoirs. The fluid was forced into the culture compartment by releasing a spring-loaded plunger by scorching a nylon thread by a heat wire at preset times. The fluid was led to the cultures via a system of internal channels and valves (as indicated by arrows in fig 1A). The spent medium was forced out of the culture compartment and found its way to the, now void, volume behind the just released plunger. These units were accommodated with a small NO probe and associated electronics for automated activation and digital data storage, during the experiment time-line for the Dutch Soyuz Mission (fig 1B).

The FLOW equipment for flight made use of 16 Type I experiment containers, which were installed in the KUBIK incubator facility, containing a centrifuge, built by COMAT Aerospace (Toulouse, France) for the European Space Agency (ESA) (fig 1C). A total of 6 containers in the flight centrifuge as 1xg controls, and 6 flight static (microgravity) containers were used. We designed the experiment to carry 4 units per cell type / g-level. This means that 12 cell cultures at 1xg and 12 cell cultures at μ-g were accommodated. An additional 2 containers per g-level were needed to command the experiment timeline and acquire and store the NO measurements.
Figure 1. Plungerbox. A. schematic representation of an automated tissue culture module (20x40x80 mm) made of a single block of polysulphone (PSU) as it was developed by the Centre for Concepts in Mechatronics (CCM; diagram courtesy of Dr. van Loon; HW, waste or sample holder; PL, plunger; FR, fluid reservoir). B. Plunger box unit with nitric oxide (NO) sensor (arrow). C. KUBIK incubator and centrifuge facility. Pictures courtesy of Centre for Concepts in Mechatronics (CCM).
Dutch Soyuz Mission – FLOW hardware fluid flow characterization

The present laboratory hardware set-up as used for our regular research was scaled down and manufactured in compliance with space flight requirements. A characterization of the parallel-plate flow chamber was performed using analytical and numerical calculations assuming laminar viscous flow under isothermal conditions (see chapter 2 of this thesis). The parallel-plate flow chamber was downscaled from a width-to-height ratio of 80.0 to 33.3 (fig 2A). In order to validate this downscaled flow chamber, immortalized mouse calvarial osteoblast-like cells (MC3T3-E1) were used. These cells were cultured to near confluency in α-MEM with 10% fetal bovine serum (FBS). For fluid shear stress treatment, cells were plated at 2x10^5 cells/glass slide (5 cm^2), and incubated for 5 min in the presence of dynamic fluid shear stress with amplitude 0.7 Pa, average 0.7 Pa, and frequency of 5 Hz. Bone cell cultures showed similar mechanosensitivity (i.e., increased NO production) in response to dynamic wall shear stress averaging 0.7 Pa for a parallel-plate chamber width-to-height ratio of 80.0 reduced to 33.3 (fig 2B). Thus, our design conditions for downscaling the parallel-plate chamber for space flight requirements do not introduce artifacts for measuring the mechanosensitivity of bone cells. The NO produced was measured in the harvested media, as well as online with NO sensor probes incorporated in the PBU’s (see fig. 1B).

Fluid flow patterns in the actual geometry of the PBU’s developed by CCM for FLOW were simulated using Computational Fluid Dynamics software in the Technical University of Delft (flow chamber diagram, fig 2C). This work was done in collaboration with Prof. Frans Nieuwstadt and Dr. Mathieu Pourquie of the Hydrodynamics and Aerodynamics laboratory of the Technical University in Delft (The Netherlands). We found that the inclusion of an NO sensor in the PBU did not affect a homogenous fluid shear stress profile under laminar flows at the cell monolayer to induce a peak shear stress of 1.4 Pa, the stress needed to stimulate bone cells (fig 2D). The chamber height of the flight PBU’s were adjusted in order to achieve fluid shear stress pulse stimulations on the cells reaching a peak of 1.4 Pa.
NO sensor characterization and cell culture test on plunger boxes

An online NO sensor, manufactured by Innovative Instruments, Inc. (Tampa, FL, USA) has been characterized for its use in the FLOW experiment (fig 3A). The online NO sensor was found to exhibit a linear relation between its current signals to the increase of NO in solution. The temperature dependence of the sensor could be corrected by software; however, since the Dutch Soyuz Mission experiment involved a constant temperature environment, such a correction procedure was not necessary.
Figure 2. Parallel-plate flow chamber in vitro system. A. The parallel-plate flow chamber system was downscaled to have a width-to-height ratio of 33.3 from a previous ratio of 80.0 to fit into the space flight experiment requirements. B. Bone cells respond similarly to the same flow regime (PFSS, average shear stress = 0.7 Pa, 5Hz), for different flow chamber width-to-height ratios (33.3 and 80.0) up to nearly 2-fold NO production. T/C, PFF treatment over control ratio. C. Plunger box unit: culture chamber (single arrow), lid with on line nitric oxide sensor (two arrows). D. Homogenous velocity patterns in the plunger box unit implies a homogenous wall shear stress at 1.4 Pa (fluid velocity in arbitrary units).
Cell viability for the FLOW experiment was tested by using primary bone cells (chicken-derived osteocytes, osteoblasts, and periosteal fibroblasts), and back-up cell-lines (MLO-Y4 osteocyte-like cells, MC3T3-E1 osteoblast-like cells, and CCL-226 fibroblast-like cells) in a simulated experiment time-line for the actual space experiment, including ground preparations and transport duration and environment from Amsterdam to the hand-over at Baikonur, Kazakhstan. These tests showed that the cells survived prolonged culture at ambient temperatures for transport using a CO$_2$-independent medium (fig 3B and C). This viability test also indicated that the presence of the NO sensor was not harmful to the cell cultures.

**Figure 3.** Nitric oxide sensor calibration and cell cultures in the plunger box units (PBU). A. inNO 100 by Innovative Instruments, Inc. (Tampa, FL, USA) indicated linear increase in voltage in relation to sequential increase of nitric oxide concentration in solution. B. Osteocytes after 6 days of room temperature. C. Periosteal fibroblasts after 6 days at room temperature.
FLIGHT EXPERIMENT “FLOW” ON THE DUTCH SOYUZ MISSION
In this FLOW experiment, we tested the hypothesis of changed bone cell mechanosensitivity under near-weightlessness conditions. Gravity was applied using an onboard centrifuge. The response of the cells to flow stimulation was measured using online NO sensors. At the end of the experiment conditioned medium was tested for prostaglandin and NO production. Semi-quantitative polymerase chain reactions was performed to study COX-2 mRNA expression.

Bone cell cultures
Osteocytes were isolated from avian bone, using immunoseparation with a chicken osteocyte-specific antibody (gift from Dr. Nijweide, Leiden University, The Netherlands). Primary chicken osteocytes, osteoblasts and periosteal fibroblasts were harvested from calvariae as described by Ajubi et al. (20).

Primary cell cultures were prepared in Amsterdam, and transported at late access to Baikonur in Kazachstan. In this regard, a ground laboratory venue for handling primary cell cultures was set up for preparations in Baikonur. The following conditions were required for transport and storage conditions: temperature: ambient, 4-8ºC or lower; pressure: ambient (normal environmental conditions on earth or in flight); shock: minimum at post-launch, storage and after re-entry; humidity: ambient.
Experimental groups
The experiment consisted of six different groups (where $g = 9.8 \text{ m/s}^2$, acceleration due to gravity).

**Group 1**: $1 \times g$, osteocytes (OCY)
**Group 2**: $1 \times g$, osteoblasts (OB)
**Group 3**: $1 \times g$, fibroblasts (PF)
**Group 4**: $\mu$-g, osteocytes
**Group 5**: $\mu$-g, osteoblasts
**Group 6**: $\mu$-g, fibroblasts

Expected results
According to our working hypothesis the mechanosensitivity of bone cells under near weightlessness conditions is disturbed compared to unit gravity control conditions. If this hypothesis is correct and if there is a direct effect of near weightlessness on the cell, then the results as depicted in table 1 may be expected compared to control Group 1.

Table 1. Expected results

<table>
<thead>
<tr>
<th>Group</th>
<th>Medium components</th>
<th>mRNA (COX-2, ecNOS)</th>
<th>mRNA (COX-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (OCY)</td>
<td>$1 \times g$</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Group 2 (OB)</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Group 3 (PF)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 4 (OCY)</td>
<td>$\mu$-g</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 5 (OB)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 6 (PF)</td>
<td>0</td>
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<td>0</td>
</tr>
</tbody>
</table>

PGs, prostaglandins; NO, nitric oxide; COX-2, cyclooxygenase-2; ecNOS, endothelial cell nitric oxide synthase. ++, highest response; +, response; 0, no response.
**Experiment hardware**

We employed “standard” experiment container units based on “plunger boxes” developed by CCM. The only change was that these units had to be accommodated with the small NO probe and associated electronics for automated activation and digital data storage during experiment timeline, and some minor technical changes were implemented in order to achieve a peak shear stress stimulation of 1.4 Pa. Automated activation and digital data storage were dependent on a suitable power source provided by the KUBIK facility.

**Brief flight experiment protocol**

During the complete experiment time-line the medium was changed three times: the medium was refreshed two times, while during the last step RNA stabilization liquid was added. The experiment (phase B-C-D of experiment time-line, see fig 4) was conducted in the Soyuz using Type I Experiment containers, and had a microgravity group with an in-flight 1xg control group using a centrifuge and a ground control group. We used closed Type I containers with two extra type I containers used as electronics boxes for each group (in-flight microgravity group, 1xg-control group, ground control group). These two electronics boxes were needed for 1) experiment activation, and 2) online NO sensor signal processing. We required having power on all the experiment containers in the Kubik even before launch, since online NO detection was already interesting during the launch period (phase A of experiment time-line, see fig 4). However, during launch, centrifuge operation was not strictly necessary.
Figure 4. Schematic diagram of experiment protocol.

**Experiment phases (fig 4)**

0. Pre-launch period: samples were shipped to launch site and integrated into facility / launcher.

A. Pre-launch period: the experiment containers, now in the Kubik, were integrated into Soyuz and all experiment containers for FLOW (total 16: 8 on static and 8 on centrifuge positions) were powered via the Kubik. In this period we started to log the NO data from the cells. This phase needed to be as short as possible.

B. Post-launch period in which all containers were under microgravity until the crew was able to switch on the centrifuge. The centrifuge was turned on as soon as possible after launch. Estimated time to start the centrifuge was about 3 hours. Two hours after switch on the centrifuge it was temporarily switched off for about 15 minutes. The remaining part of this phase (i.e. 4 hours) was designated for the culture to overcome any possible disturbing effects of launch acceleration and vibration and start / stop of the centrifuge.

C. Experiment phase 1. This is the experiment phase in which the cells were exposed to near weightlessness or 1×g conditions. In this ‘early response period’ we measured on-line NO levels starting from baseline (i.e. fresh medium), and sampled this medium for on-ground...
prostaglandin levels. The replaced medium needed to be collected separately.

D. This is the extended experiment phase in which the cells were still exposed to near weightlessness or 1×g conditions. During this period we measured on-line NO levels starting from baseline (i.e. fresh medium), and sampled this medium for on-ground prostaglandin levels. At the end of this phase the medium was replaced by mRNA extraction fluid to “stabilize” the cellular mRNA. The replaced medium needed to be collected separately. The experiment containers were then left in place / stored until landing.

E. Post-experiment / storage phase.

Temperature requirements for the International Space Station (ISS)
Since the space experiment (phase B-C-D, fig 4) was anticipated to be finished in the Soyuz spacecraft, the ISS was the venue for sample storage. The optimum temperature range for storage was required to fall between 4ºC to 8ºC.

Materials returned to earth
All samples, spent media, digital NO sensor data were returned to earth. The preferred storage temperature was between 4ºC to 8ºC, hence early access was also required for retrieving all units as soon as possible after landing.

Requirements for the station crew
Step 1: Before launch: FLOW units were transferred to KUBIK incubator: to “centrifuge” or “static” condition as labelled on units (end of Phase A, fig 4)

Step 2: Before launch: KUBIK incubator should have been activated. It was required that activation of KUBIK relays an electronic signal to activate our automated experiment (Phase A-B-C-D) and use the online NO sensor after integration, to monitor NO production already before launch.
**NO determination**

The conditioned medium was assayed for NO. NO was measured as nitrite (NO$_2^-$) accumulation in the conditioned medium, using Griess reagent (1% sulfanilamide, 0.1% naphtylethelene-diamine-dihydrochloride, and 2.5 M H$_3$PO$_4$). The absorbance was measured at 540 nm. NO concentrations were determined using a standard curve derived from known concentrations of NaNO$_2$ in non-conditioned culture medium.

**RNA isolation and Reverse transcription**

Total RNA from the cells was isolated using Trizol® reagent with one modification, i.e. 5 µg of glycogen (Roche Diagnostics, Mannheim, Germany) was added to RNA and isopropanol solution prior to a centrifuge step to increase the RNA yield. Total RNA concentration was quantified spectrophotometrically. cDNA synthesis was performed using 0.5-1 µg total RNA in a 20 µl reaction mix consisting of 5 Units of Transcriptor Reverse Transcriptase according to the manufacturer’s instructions (Roche Diagnostics) with 0.08 A$_{260}$ units random primers (Roche Diagnostics), 1 mM of each dNTP (Invitrogen), and Transcriptor RT reaction buffer. cDNA was diluted 5 times and stored at −80ºC prior to real-time PCR.

**Real-time PCR**

Real-time PCR reactions were performed using the SYBRGreen reaction kit according to the manufacturer’s instructions (Roche Diagnostics) in a LightCycler (Roche Diagnostics).

cDNA (2 µl each) was diluted to a volume of 20 µl with PCR mix (Light Cycler DNA Master Fast start plus Kit, Roche Diagnostics) containing a final concentration of 0.2 pmol of primers. Relative housekeeping gene expression (18S; which expression was not subjected to time and/or treatment related variations) and relative target gene expression (COX-2) were determined.

Primers (Invitrogen) used for real-time PCR are listed in Table 2, and were designed using Clone manager suite software program version 6 (Scientific &
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Educational Software, NC, USA). The amplified PCR fragment showed extension over at least one exon-border except for 18S, which gene is encoded only by one exon. Values of relative target gene expression were normalized for relative 18S housekeeping gene expression.

<table>
<thead>
<tr>
<th>Table 2. Primers used for real time PCR</th>
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<tbody>
<tr>
<td>Target gene</td>
</tr>
<tr>
<td>18s forward</td>
</tr>
<tr>
<td>18s reverse</td>
</tr>
<tr>
<td>COX-2 forward</td>
</tr>
<tr>
<td>COX-2 reverse</td>
</tr>
</tbody>
</table>

Real Time PCR data analyses
With the Light Cycler software (version 2), the crossing points were assessed and plotted versus the serial dilution of known concentrations of the standards derived from each gene. PCR efficiency ($E$) was obtained by the formula: $E=10^{-1/slope}$ and the data were used if and only if the PCR efficiency was calculated between 1.85-2.0.

Statistics
Data were pooled from the results of at least four experiments for each cell culture group tested (Table 1). The differences between groups were analyzed with the non-parametric Wilcoxon signed-rank sum test of the S-Plus 2000 package (release 1). Differences were considered significant at a p-value < 0.05.

RESULTS AND DISCUSSION

Due to unforeseen hardware complications as a result of a lack of electronic power, the results from in-flight cultures are considered lost. However some...
data were measured from the ground control experiments. Ground control experiments showed an accumulative increase of nitric oxide in medium for osteocytes (as well as for osteoblasts and periostial fibroblasts, figure 5). Data from the online-nitric oxide sensor showed that the nitric oxide produced in medium by osteocytes increased sharply after the pulse shear stress stimulations (figure 6, shows data from one culture). The mRNA expression for COX-2 was higher in osteoblasts than in osteocytes and in periostal fibroblasts (fig 7). It was expected that osteocytes would show the highest COX-2 mRNA expression as a result of the pulse flow stimulations, since these cells are the most mechanosensitive bone cells. At this moment the question remains unanswered as to why osteocytes did not show a higher COX-2 mRNA expression than periostal fibroblasts and osteoblasts. These ground control results will determine the experiment design for the next opportunity for a flight experiment in the next Soyuz missions. Despite the setback from the flight experiment complications, the preparations for the FLOW experiment and preliminary ground results indicate that the FLOW setup is viable for a future flight opportunity.

**Figure 5.** Accumulative nitrite concentration in the medium. Nitrite, the stable metabolite of nitric oxide, increased for all cell types 2½ hrs (III) after the 1st (I) and 2nd (II) pulse shear stress stimulations (*, p < 0.05, increase greater than I and II, for all cell types). Values are mean ± SEM, n=4. OCY, osteocytes; OB, osteoblasts; PF, periostal fibroblasts.
FLOW has been considered as a potential candidate by the European Space Agency (ESA) for one of the next Soyuz missions in October, 2006. We were able to meet all necessary requirements in order to properly implement the FLOW experiment in the October, 2006 Soyuz mission.

Figure 6. Nitric oxide (NO) production by osteocytes, at the first and second pulse fluid shear stress stimulations by plunger activation (arrow) as monitored by the online-nitric oxide sensor.

Figure 7. COX-2 mRNA expression. Osteoblasts expressed higher mRNA expression for COX-2 compared to osteocytes and periosteal fibroblasts. Values are mean ± SEM, n=4. *Significantly higher than periosteal fibroblasts and osteocytes, p < 0.05. OCY, osteocytes; OB, osteoblasts; PF, periosteal fibroblasts.
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