Effects of angular frequency during clinorotation on mesenchymal stem cell morphology and migration

Carlos Luna¹, Alvin G Yew² and Adam H Hsieh¹,³

AIMS: To determine the short-term effects of simulated microgravity on mesenchymal stem cell behaviors—as a function of clinorotation speed—using time-lapse microscopy.

BACKGROUND: Ground-based microgravity simulation can reproduce the apparent effects of weightlessness in spaceflight using clinostats that continuously reorient the gravity vector on a specimen, creating a time-averaged nullification of gravity. In this work, we investigated the effects of clinorotation speed on the morphology, cytoarchitecture, and migration behavior of human mesenchymal stem cells (hMSCs).

METHODS: We compared cell responses at clinorotation speeds of 0, 30, 60, and 75 rpm over 8 h in a recently developed lab-on-chip-based clinostat system. Time-lapse light microscopy was used to visualize changes in cell morphology during and after cessation of clinorotation. Cytoarchitecture was assessed by actin and vinculin staining, and chemotaxis was examined using time-lapse light microscopy of cells in NGF (100 ng/ml) gradients.

RESULTS: Among clinorotated groups, cell area distributions indicated a greater inhibition of cell spreading with higher angular frequency ($P < 0.005$), though average cell area at 30 rpm after 8 h became statistically similar to control ($P = 0.794$). Cells at 75 rpm clinorotation remained viable and were able to re-spread after clinorotation. In chemotaxis chambers, clinorotation did not alter migration patterns in elongated cells, but most clinorotated cells exhibited cell retraction, which strongly compromised motility.

CONCLUSIONS: These results indicate that hMSCs respond to clinorotation by adopting more rounded, less-spread morphologies. The angular frequency-dependence suggests that a cell’s ability to sense the changing gravity vector is governed by the rate of perturbation. For migration studies, cells cultured in clinorotated chemotaxis chambers were generally less motile and exhibited retraction instead of migration.

npj Microgravity (2015) 1, 15007; doi:10.1038/npjmgrav.2015.7; published online 30 July 2015

INTRODUCTION

Intricate multi-scale interactions among cells, tissues, and organs fundamentally govern human health, which has evolved on Earth under a constant gravitational load of 1 g (9.8 m/s²). The biological mechanisms underlying the role of gravity in human health remain poorly understood, but their elucidation is necessary for enabling long-term manned space exploration. Numerous studies, supported by the National Aeronautics and Space Administration and other space agencies, have shown deleterious effects of space travel on the human body, such as accelerated bone loss,¹,² muscle tissue degeneration,³ and others.⁴ Importantly, these observations may have broader implications beyond spaceflight applications to provide a more detailed understanding of diseases on Earth. In particular, the musculoskeletal system has historically been a focal point in space biology research, because of the strikingly adverse changes that occur in astronauts.⁵ More recently, studies have begun to investigate mesenchymal stem cells for their roles in musculoskeletal lineage determination,⁶ bone repair,⁷ and tissue maintenance.⁸

Although the most relevant environment for performing microgravity research is in space, competition to use space-based facilities, like the International Space Station, is fierce and is further complicated by substantial time and resource investments.⁹ As a result, lower-cost and logistically simpler alternatives are attractive and include sounding rockets¹⁰ and parabolic flights,¹¹ or in-lab devices such as random positioning machines¹² and clinorotation devices (clinostats)¹³ to simulate microgravity. The selection of a specific technique usually depends on accessibility, cost, experimental design, and research question. Clinostats are among the most accessible methods to simulate microgravity, and they allow researchers to study living cells using standard laboratory tissue culture supplies.¹⁴

Clinorotation experiments have provided significant insight into the behavior of biological organisms in space. The technique was originally developed to study gravitropism in plant development, and has revealed that gravity serves a key role in statocyte function, which is believed to be directly involved in plant gravisensing and growth patterns.¹⁵ In animal and human cells, clinorotation has been used to recreate experiments that would otherwise be challenging to perform in space, such as high-resolution microscopy¹⁶ and assessment of stem cell differentiation.¹⁷ Cellular changes in microgravity have been associated with disruptions in the cytoskeleton and, consequently, changes in cell morphology and behavior.¹⁸,¹⁹

Although clinorotation is a standard ground-based tool, previously reported studies on the effects of simulated microgravity in mesenchymal stem cells have yielded conflicting findings (Table 1). For instance, some researchers have shown that microgravity enhances proliferation,¹⁷ whereas others have...
The effects of angular frequency on hMSCs

C Luna et al

Table 1. Previously reported studies on mesenchymal stem cells under simulated microgravity

| Type of MSCs           | Experimental device          | Angular frequency | Duration | Results                                                                 | Ref |
|------------------------|-----------------------------|-------------------|----------|-------------------------------------------------------------------------|-----|
| Rat bone marrow        | Mitsubishi 3D clinostat     | 5 rpm             | 12 h     | Decrease in alkaline phosphatase (marker of osteoblastic differentiation) | 43  |
| Human adipose derived  | Rotatory bioreactor         | 11–25 rpm         | 21 days  | Microgravity promotes chondrogenesis via p38 MAPK pathway               | 21  |
| Rabbit bone marrow     | Rotatory bioreactor         | 20 rpm            | 14 days  | Microgravity promoted expression of collagen type II and Aggrecan        | 44  |
| Human bone marrow      | Rotatory bioreactor         | 16 rpm            | 7 days   | Decreased chondrogenic and osteogenic gene expression and increased adiogenic gene expression* | 22  |
| Rat bone marrow        | 2D clinostat               | 30 rpm            | 72 h     | Endothelial differentiation potential was improved under microgravity   | 45  |
| Human adipose derived  | 1D clinostat               | 15 rpm            | 1–12 days| Ultrasound stimulation enhances osteogenic differentiation in microgravity  | 46  |
| Human bone marrow      | Rotatory bioreactor         | b                 | 7 days   | Increased the expression of PPARγ2, receptor important for adipogenesis | 47  |
| Human bone marrow      | Rotatory bioreactor         | 9 rpm             | 7 days   | Microgravity affects integrin signaling and stress fibers, likely mediated by RhoA | 48  |
| Human bone marrow      | Rotatory bioreactor         | b                 | 7 days   | Microgravity disrupts integrin/MAPK signaling                           | 49  |
| Rat bone marrow        | 2D clinostat               | 30 rpm            | 3 days   | Microgravity enhances differentiation into neurons with more mature action potentials | 50  |
| Rat bone marrow        | 2D clinostat               | 30 rpm            | 24–96 h  | Microgravity inhibits proliferation and osteogenesis a                  | 20  |
| Human bone marrow      | 3D clinostat               | 5 rpm             | 2–4 weeks| Microgravity stimulates proliferation (13-fold) and cells can still differentiate after exposure to microgravitya | 17  |

Abbreviation: MSC, mesenchymal stem cell.

*Indicates research that did not indicate a fixed rotation speed, as rotation speed was varied to prevent sedimentation.

...demonstrated inhibition.20 Chondrogenic differentiation has also been found to be either promoted21 or suppressed.22 Similar inconsistencies have also been highlighted in a recent review.23 Some of these observed discrepancies could be due to differences in culture media formulation and cell source, but it is also possible that the choice of clinorotation parameters used for an experiment may substantially influence cell behavior.

A two-dimensional clinostat rotates a sample along the longitudinal axis to produce a time-averaged nullification of the gravity vector. Theoretically speaking, to simulate microgravity effectively, the period of rotation should be shorter than some time constant that governs the rate processes involved in the cellular grasivensing machinery that enable a cell to ‘perceive’ and respond to the changing trajectory of the gravity vector. For conventional clinostats, however, there are also practical considerations that limit the angular frequency of rotation.24 Specifically, rotation speed must be adjusted to balance sedimentation forces, centrifugal and Coriolis effects, and Stokes’ drag. Because of these constraints, the parameters of angular rotation depend on the particular design and implementation of each experimental system.

To minimize the effects of these extrinsic stimuli, we recently developed a lab-on-chip clinorotation device (clinochip, Figure 1a) that confines adhered cells within a small region along the axis of rotation, limiting residual accelerations to levels below $10^{-4}$ g at different rotation speeds.25 Moreover, the clinochip is amenable to time-lapse microscopy, which enables us to characterize the kinetics of cell spreading, changes in morphology, and migration with minimal disruption to the simulated microgravity environment. In this study, we used this device to investigate how angular frequency affects human mesenchymal stem cell (hMSC) behavior. On the basis of the concept that there are specific rate processes that govern cellular grasivensing (Figure 1b), we hypothesize that hMSCs would exhibit angular frequency-dependent responses. The results of this work demonstrate that hMSCs can in fact ‘detect’ differences in rotation speed in a manner that causes heterogeneous population shifts toward more rounded morphologies and retraction of cell area. This could have significant implications on our basic understanding of stem cell regulation as well as on strategies that are being used in stem cell-based applications.

**MATERIALS AND METHODS**

**Materials**

hMSCs were obtained from a commercial source (PT-2501, Lonza, Walkersville, MD, USA), and confirmed to be mycoplasma-free. Clinochip fabrication required microscope slides (12-544-1, Fisher Scientific, Waltham, MA, USA) and 0.25-μm-thick polydimethylsiloxane sheets (HT-6240, Rogers Corporation, Rogers, CT, USA). Fluorescence visualization was performed for actin using 5% (w/v) Texas red phalloidin (Life Technologies, Gaithersburg, MD, USA) and for focal adhesions using 1 μg/ml fluorescein-conjugated anti-vinculin (MA1-34629, Life Technologies). Fibronectin (354008, BD Biosciences, San Jose, CA, USA) was used to enable cell adhesion, and nerve growth factor (NGF; 75-NGF, Life Technologies) was used for migration experiments. Calcein-AM and ethidium homodimer-1 (L-3224, Life Technologies) were used to perform cell viability assays.

**Experimental setup**

In general, we used a single experimental system for all of our experiments, as detailed previously.25 In brief, the system consists of a custom microscope-mounted gear system, driven by a computer-controlled stepper motor that rotates a small slide holder.25 This slide holder can accommodate various custom and commercially available lab-on-chip devices. A non-rotating slide holder enables having a static (standard gravity) control condition performed concurrently. The entire system is enclosed in an environmental chamber (Precision Plastics, Beltsville, MD, USA) and installed on an Olympus IX-81 epi-fluorescence microscope (Center Valley, PA, USA).

**Cell morphology platform**

A custom ‘clinochip’ device was used for visualizing cell spreading (that is, changes in cell morphology over time), and fabricated according to our previously described protocol.25 This clinochip consists of three layers
Cell migration was analyzed using commercially available Ibidi Chemotaxis cell attachment, the channel was then treated with 100 μM bronectin (P3) and P4 hMSCs were seeded into clinochip channels and incubated at 37 °C for 10 min to allow for cell attachment, followed by commencement of clinorotation. After termination of the clinorotation treatment, images were acquired at ×200 magnification for a total of 60 s, and a set of bright-field images of the clinochip were acquired at x200 magnification. At the end of the 8 h period, cells were fixed in 4% paraformaldehyde, and fluorescently stained for actin and vinculin. Captured images were analyzed using ImageJ (National Institutes of Health).

Cell viability and recovery after clinorotation
Cell viability was determined using calcein-AM (green) to indicate intracellular esterase activity for live cells and ethidium homodimer-1 (red) to indicate loss of plasma membrane integrity for dead cells. Calcein (final concentration 4 μM) and ethidium homodimer (final concentration 2 μM) were added to cell culture media that was used to plate hMSCs in bronectin-treated clinochips. After 10 min of attachment, clinochips were either rotated at 75 rpm or maintained at standard gravity conditions (non-rotated control) and also secrete NGF during tissue repair.28 In these migration experiments, NGF was prepared as a 100 ng/ml solution and injected into the top ports of the chamber. Following manufacturer’s instructions, the same volume was aspirated from the opposite port to create a chemical gradient.

Figure 1. Framework of how perceived gravity may bias cellular events under clinorotation. (a) The clinochip system consists of a stationary non-rotated control chip and a rotating clinochip, the speed of which can be controlled. (b) In the rotating cell-fixed frame of reference, the components of force caused by the gravity vector oscillate in a sinusoidal fashion during clinorotation. Lightly shaded blocks illustrate hypothetical time constants required for various intrinsic cellular events to occur. Forces experienced by cells are shaded in darker colors to help clarify the idea that slower clinorotation (upper graph) may fail to nullify gravity biases for some cell events, whereas faster clinorotation (lower graph) could provide greater probability of nullification for any given event.

First, a 100 μg/ml bronectin solution was injected into the viewing chamber. After 10 min of attachment, clinochips were marked with calcein-AM (green) and ethidium homodimer (red) to indicate loss of plasma membrane integrity for dead cells. Calcein and ethidium homodimer were added to cell culture media that was used to plate hMSCs in bronectin-treated clinochips. After 10 min of attachment, clinochips were either rotated at 75 rpm or maintained at standard gravity conditions (non-rotated control) and fluorescein micrographs were taken at 1, 4, and 8 h during clinorotation.

In addition to the live/dead assays, we acquired light micrographs of cell morphology in standard gravity following exposure to (a) 1 or 4 h of 30 rpm clinorotation, (b) 1 or 4 h of 60 rpm clinorotation, or (c) 1, 4, or 8 h of 75 rpm clinorotation. After termination of the clinorotation treatment, images were acquired in standard gravity every 30 min for 4 h, and then analyzed for cell area and shape factor using ImageJ (National Institutes of Health).

Statistical analyses
Because no prior publications have quantified cell areas during clinorotation, we relied on pilot data for descriptive statistics to make sample size calculations. Assuming a critical significance level of α = 0.05, statistical power of 0.9 (β = 0.1), and a detectable difference equal to the population s.d., we performed a power analysis to calculate an estimated sample size of n = 23, using the method of Sokal and Rohlf.29 To ensure adequate statistical power for cell area measurements, we chose to include at least n = 30 for each sample group (Table 2), acquired over several experimental replicates. All data are expressed as mean ± s.e.m. Variance did not markedly differ between groups (less than fourfold). Average cell areas among different clinorotation speeds were first log transformed to improve normality. Differences between clinorotation groups were then statistically analyzed using the Welch’s t-test for samples with unequal variance and unequal sample size, with Tukey post hoc tests for multiple pairwise comparisons.

Using Welch’s t-test for samples with unequal variance and sample size, we analyzed differences in cell velocity and directionality between 0 (n = 15) and 75 rpm (n = 6) only for cells that were actively migrating. Similarly, we used Welch’s t-test with Tukey post hoc test for multiple pairwise comparisons.
Effects of angular frequency on hMSCs
C Luna et al

RESULTS
Simulated microgravity had a profound effect on both the extent and kinetics of cell spreading (Figure 2). After the initial 10-min cell attachment and before rotation commenced, all of the groups possessed similar cell areas of ~500 μm². We found that hMSCs in the non-rotated 0 rpm condition (i.e., standard gravity) markedly increased spreading areas within 1 h after plating, reaching an average of 2000 μm². Their spreading area increased gradually with time, until they reached an average of 3000 μm² by 8 h. For clinorotated groups, cell spreading was impeded almost immediately. At 1 h, cells in the 30 rpm group were significantly smaller than control with an average area of 1500 μm², and the 75 rpm group had the lowest cell area of 775 μm². These results indicate that cell spreading is markedly affected by angular frequency and that these effects can be observed as soon as 1 h after clinorotation starts. Finer temporal resolutions and longer durations in future experiments may provide more detailed insight on the time constant of important cellular processes associated with cellular gravising.

Figure 2. Human mesenchymal stem cell (hMSC) spreading areas in simulated microgravity. (a) Time analysis of cell spreading of hMSCs in simulated microgravity at different rotation speeds (0, 30, 60, and 75 rpm). Note that increasing the rotation speed resulted in less-spreading area over time. NS Indicates statistically similar average areas (P > 0.05). (b) Representative micrographs of hMSCs spreading in a glass substrate coated with FN at different rotation speeds for 8 h (0, 30, 60, and 75 rpm). Note that cells at a speed of 30 rpm were more similar to control, whereas cells at 60 and 75 rpm have adopted a more rounded morphology.

Table 2. Number of cells measured for each condition in each experiment

| Experiment      | Condition | 0 rpm | 30 rpm | 60 rpm | 75 rpm |
|-----------------|-----------|-------|--------|--------|--------|
| Cell spreading  | NE = 6    | 30    | 30     | 30     | 30     |
|                 | 1 h       | 31    | 31     | 31     | 31     |
|                 | 2 h       | 30    | 49     | 57     | 30     |
|                 | 3 h       | 45    | 47     | 60     | 32     |
|                 | 4 h       | 44    | 48     | 60     | 45     |
|                 | 5 h       | 60    | 44     | 55     | 33     |
|                 | 6 h       | 37    | 40     | 58     | 36     |
|                 | 7 h       | 31    | 36     | 47     | 32     |
|                 | 8 h       | 31    | 37     | 50     | 36     |
| Cell morphology | NE = 3    | 20    | 20     | 20     | 20     |
|                 | 1 h       | 20    | 20     | 20     | 20     |
|                 | 4 h       | 19    | 20     | 20     | 21     |
|                 | 8 h       | 20    | 20     | 20     | 20     |
| Chemotaxis      | NE = 3    | Retract Null | 28 | dnp | 28 |
|                 | 1 h       | 15    | 28     | dnp    | 6      |
| Cell recovery   | NE = 2    | 0–5 h | 9      | 9      | 9      |
| Cell viability  | NE = 2    | 1 h   | dnp    | dnp    | 53     |
|                 | 4 h       | 33    | dnp    | dnp    | 53     |
|                 | 8 h       | 33    | dnp    | dnp    | 53     |

NE indicates the number of independent experimental replicates and dnp indicates did not perform.

pairwise comparisons to analyze differences in cell area between 30 and 75 rpm (at 0, 1, 2, 3, and 4 h) only for cells that were actively retracting (n = 28).
to cells that were considered rounded (Figure 3, black), values between 0.3 and 0.6 to semi-elongated cells (Figure 3, hatch) and values below 0.3 to elongated cells (Figure 3, gray). As expected from the previous area measurements, Figure 3 clearly shows that increasing clinorotation speed results in a larger percentage of rounded cells in its population. The number of elongated cells was significantly reduced with increasing clinorotation speed, and no elongated cells were found at 75 rpm.

Plotting cell circularity against cell area for each clinorotation speed at different time points, we clearly see a temporal increase in area associated with cell elongation in 0 rpm control cells (Supplementary Figure 3). This trend becomes disrupted with exposure to 30 and 60 rpm clinorotation, as cells tended to exhibit a broad range of circularity and area. However, only at 75 rpm the cell population maintains high circularity and smaller areas across time points (1, 4, and 8 h). Overall, our morphology analysis demonstrates that the cell population at 75 rpm possesses a more consistent morphology (average area, cell distribution, circularity versus area over time) over time.

Cell viability and recovery after clinorotation
To determine whether there was any loss of viability or if rounded cells were undergoing apoptosis, we exposed cells to 75 rpm and used a live/dead assay after 1, 4, and 8 h of clinorotation (Figure 4b). We found no difference in cell viability or changes in cell number when compared with control; in fact, the majority of cells were alive (>90%). Under light microscopy, we observed no evidence of apoptosis (i.e., membrane blebbing) for the cells that were not viable. This also indicates that our clinochip system can be used effectively without loss of cells due to lifting or cell death for at least 8 h.

We investigated whether clinorotation effects on cell morphology are reversible by performing time-lapse microscopy after cessation of clinorotation. Following cessation of clinorotation, all cells increased their areas and round cells became more elongated, similar to most of the cells in standard gravity controls (Figure 4a). Similar experiments were also repeated for 30 and 60 rpm after 1 and 4 h of clinorotation (Supplementary Figure 4).

Simulated microgravity inhibits cell migration by inducing cell rounding
For cell migration experiments, cells were seeded in Ibidi chemotaxis chips with a chemical gradient of 100 ng/ml NGF to promote cell migration. Under clinorotation, only a few cells migrated, whereas most cells did not (see Table 2 for cell numbers). Cells that were actively migrating at 75 rpm had a similar velocity ($P=0.391$) and directionality ($P=0.822$) when compared with control cells (Figure 5). Cells that were not migrating
under simulated microgravity exhibited morphological retraction (Figure 5). In other words, cell areas changed from fully spread morphologies to more rounded ones. This phenomenon is in agreement with our observations in cell-spreading experiments, even when the initial conditions were different (10 min for spreading assays versus 12 h for chemotaxis and migration assays).

We found that during clinorotation cells retracted their area, but did so independently of rotation speed \( (0\, h'\ P=0.539, \ 1\, h'\ P=0.288, \ 2\, h'\ P=0.963, \ 3\, h'\ P=0.848, \ 4\, h'\ P=0.689, \text{ between } 30 \text{ and } 75 \text{ rpm}) \). After 4 h of clinorotation during chemotaxis, cells at 30 and 75 rpm reached an average area of 2500 \( \mu m^2 \), which is a value similar to the spreading experiments for 30 rpm at 8 h \( (P=0.962 \text{, between } 30 \text{ and } 75 \text{ rpm}) \).
between 30 rpm, $P = 0.975$ between 75 rpm). However, we did not conduct experiments long enough to determine steady state–retracted cell areas.

Our results indicate that the cellular response to simulated microgravity is more dominant than chemotactic signals, suggesting the role of migration of hMSCs to tissue repair sites might be suppressed in microgravity. We base this conclusion on our observations of response to NGF at a concentration of 100 ng/ml. However, these results were obtained with only one concentration of NGF and it is possible that different concentrations or other chemotactic molecules can elicit a stronger chemotactic response. For example stromal-derived factor-1, which is known to be a strong homing signal for hMSCs. Nevertheless, because the response of adhered cells to clinorotation is retraction of cellular processes, we believe that the behavior will be similar, unless the chemotactic agent can also enhance cell adhesion.

**DISCUSSION**

Understanding how angular frequency, or rotation speed, of clinorotation affects cell behavior is a critical aspect of microgravity simulation experiments, so that the role of the changing gravity vector in cellular regulation can be appropriately considered. In this work we show that hMSCs can behave differently depending on clinorotation speed. In particular, although average cell area increased more slowly at 30 rpm compared with non-rotated controls, it continued along an upward trajectory at 8 h and seems to be converging with control values. For higher rotational speeds, cell area increases even more slowly, and appears to reach a plateau by 8 h at 75 rpm. This suggests that changes in cell morphology may approach a limit at an angular frequency that is close to 75 rpm, and almost certainly must exceed 30 rpm. Although these results are relevant to our clinochip configuration, results may vary for other clinostat devices, particularly for those that support cells in suspension.

Our results are consistent with other research showing that rotation speed affects animal cells, plants, and bacteria (*Escherichia coli*). For example, a study on osteoblastic ROS 17/2.8 cells reported that rotations at 10 and 40 rpm did not exhibit reproducible, detectable changes from stationary control cells. Only a speed of 50 rpm showed reproducible changes in actin cytoskeleton and cell surface integrin (β1) and apoptosis. The same effect has been observed with E. coli, which exhibited differential response as a function of clinorotation speeds from 10 to 50 rpm. Interestingly, the difference between 40 and 50 rpm is only 0.1674 of a cycle per second, indicating that cells can be highly sensitive to small changes in angular frequency.

Although a precise gravisensing mechanism has yet to be determined, it must be one that can detect subtle alterations to external forces in a short period of time. Our data indicate that it is linked to pathways that are involved in cell spreading and cytoskeletal organization, two phenomena that have been observed for different cell types. Potential candidates could be one or more molecules already known to play important roles in cellular mechanoregulation, such as the Rho family of GTPases, focal adhesions, or other cell surface receptors. Although mechanosensitive pathways seem the most direct apparatus for gravisensing, others have also suggested the importance of external environmental factors including fluid shear, fluid and nutrient exchange, oxygen content, buoyancy, and changes in the extracellular matrix. Thus, clinostats used to probe candidate gravisensing receptors should minimize external environmental variables that arise from clinostat operation.

In the context of a proposed framework for cellular gravisensing, the current results are consistent with our working hypothetical model (Figure 1b). Standard gravity would constitute a constant gravitational stimulus and be detectable to all cells. With intermediate timescales of gravitational nullification, the probability for a single cycle of rotational perturbation to evade gravisensing in each cell would assume a broad distribution. As any putative biochemical gravisensing reaction(s) would be stochastic, the probability for gravisensory reaction(s) in each cell would decrease with both lower angular frequency and exposure to longer duration perturbations. We did in fact observe such trends, as a result of the time lapse, single-cell measurement capabilities of the clinochip system.

For our clinochips, we do not expect that centripetal forces have a role with our observed angular frequency-dependence, as residual accelerations were calculated to be on the micro-scale regime. In addition, because the capillary number (indicative of viscous forces) is orders of magnitude smaller than surface tension, fluid shear forces are deemed negligible. There is also no enhanced nutrient transport that accompanies clinorotation in our system. Without these confounding factors, it seems reasonable to conclude that the cell morphology differences we observed were predominantly due to changes in the angular frequency of clinorotation.

We believe the rounding and retracted cell area of hMSCs in simulated microgravity inhibit migration in the presence of a chemical gradient. Interestingly, the kinetics of cell area changes during retraction were different from those during spreading, and were also angular frequency independent, suggesting that distinct gravisensing mechanisms may regulate different processes. Similarly, other cell processes, and even cell fate, could be affected. In the same way that microgravity may produce these cell behavioral alterations, other mechanical stimuli and substrate characteristics can also modulate cell function.

Stem cell morphology has a strong correlation with cellular phenotype and differentiation potential. It has been demonstrated in prior work that spread cells have a higher tendency to undergo osteogenesis and that cells with rounded morphologies are more susceptible to adipogenesis. Because of the short-term nature of this current study, it is not possible to establish whether hMSCs in our system would behave according to these trends. However, our data would suggest that cells at 30 rpm clinorotation would be more amenable to osteogenic differentiation, whereas cells at 75 rpm would tend toward adipogenic or chondrogenic lineages. This may help explain the conflicting reports on mesenchymal stem cells differentiation during clinorotation that we and others have observed. Future longer-term studies with the clinochip are required to explore hMSC differentiation potential. Specifically, as we have shown that the population distribution of cell morphologies can be controlled by the angular frequency of rotation, we may be able to derive cellular phenotypes desirable for stem cell renewal, repair, and tissue engineering.

**CONTRIBUTIONS**

CL, AY, AH conceived the project and analyzed the data, CL performed the experiments, AY developed the lab-on-chip clinostat, and all authors contributed to writing of the manuscript.

**COMPETING INTERESTS**

The authors declare no conflict of interest.

**FUNDING**

This work was supported by National Aeronautics and Space Administration Space Biology Program, Grant NNX13AM006G.

**REFERENCES**

1 Zwart SR, Morgan JL, Smith SM. Iron status and its relations with oxidative damage and bone loss during long-duration space flight on the International Space Station. *Am J Clin Nutr* 2013; 98: 217–223.

© 2015 Macmillan Publishers Limited
