Comparison of Simulated Microgravity and Hydrostatic Pressure for Chondrogenesis of hASC

Liliana F. Mellor; Andrew J. Steward; Rachel C. Nordberg; Michael A. Taylor; Elizabeth G. Loboa

BACKGROUND: Cartilage tissue engineering is a growing field due to the lack of regenerative capacity of native tissue. The use of bioreactors for cartilage tissue engineering is common, but the results are controversial. Some studies suggest that microgravity bioreactors are ideal for chondrogenesis, while others show that mimicking hydrostatic pressure is crucial for cartilage formation. A parallel study comparing the effects of loading and unloading on chondrogenesis has not been performed.

METHODS: The goal of this study was to evaluate chondrogenesis of human adipose-derived stem cells (hASC) under two different mechanical stimuli relative to static culture: microgravity and cyclic hydrostatic pressure (CHP). Pellets of hASC were cultured for 14 d under simulated microgravity using a rotating wall vessel bioreactor or under CHP (7.5 MPa, 1 Hz, 4 h · d^{-1}) using a hydrostatic pressure vessel.

RESULTS: We found that CHP increased mRNA expression of Aggrecan, Sox9, and Collagen II, caused a threefold increase in sulfated glycosaminoglycan production, and resulted in stronger vimentin staining intensity and organization relative to microgravity. In addition, Wnt-signaling patterns were altered in a manner that suggests that simulated microgravity decreases chondrogenic differentiation when compared to CHP.

DISCUSSION: Our goal was to compare chondrogenic differentiation of hASC using a microgravity bioreactor and a hydrostatic pressure vessel, two commonly used bioreactors in cartilage tissue engineering. Our results indicate that CHP promotes hASC chondrogenesis and that microgravity may inhibit hASC chondrogenesis. Our findings further suggest that cartilage formation and regeneration might be compromised in space due to the lack of mechanical loading.

KEYWORDS: chondrogenesis, mechanical stimulation, simulated microgravity, cyclic hydrostatic pressure.

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Our bodies have adapted to constant mechanical stimulation produced by gravity and daily activities here on Earth. Microgravity, such as that experienced by astronauts during spaceflight, prolonged bed rest, and/or paralysis have been shown to cause deterioration of load-bearing tissues such as bone, skeletal muscle, and articular cartilage as a result of diminished mechanical load. The musculoskeletal system requires appropriate mechanical loading for development, maintenance, and repair. Articular cartilage is the main load-bearing tissue within the synovial joint and maintenance of articular cartilage homeostasis is heavily dependent on mechanical loading patterns. There is evidence that moderate loading can help maintain cartilage homeostasis;[3] conversely, astronauts on long-term space missions are subject to long periods of mechanical unloading, which may be detrimental to cartilage health.[1] Understanding how chondrogenesis is impacted in unloading conditions is essential for maintenance of cartilage health on extended missions, to prepare for cartilage-related injuries, and for proper bone fracture healing in space. On Earth, patients on long-term bed rest have also been documented to experience loss of articular cartilage thickness due to extended periods of unloading.[19] Therefore, understanding the complex interplay between unloading and loading in cartilage development and homeostasis could also have important implications on Earth

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by providing optimized methods for in vitro tissue-engineered cartilage culture.

In order to understand the consequences of loading and unloading on cartilage formation, the effects of cyclic hydrostatic pressure (CHP)[18] and simulated microgravity (s-μG)[11,21,43] on chondrogenesis of human adipose-derived stem cells (hASC) have been studied separately. We and others have proposed and investigated cyclic hydrostatic pressure as a loading mechanism that favors cartilage formation and maintenance.[33] The hydrostatic pressure vessel mimics the mechanical load that a joint would experience during moderate exercise. It has been shown that intermittent loading of hydrostatic pressure within the physiological range of 7-10 MPa increases cartilage matrix synthesis and improves chondrogenesis. Cyclic hydrostatic pressure has also been shown to stimulate chondrogenic gene expression and matrix production of human bone-marrow derived mesenchymal stem cells (hMSC)[2,24,25] and hASC,[28–30] even in the absence of chondrogenic growth factors.[12,25,30]

At the opposite end of the loading spectrum, s-μG has also been studied within the context of chondrogenesis. Simulated microgravity bioreactors have been used as a tool to culture primary chondrocytes in order to prevent cell dedifferentiation.[22] It has also been used to culture and expand both hMSC and hASC while maintaining stemness.[44,45] However, conflicting results have been published regarding whether or not microgravity is favorable to hMSC or hASC chondrogenesis. Although it has been suggested that microgravity activates the p38 MAPK pathway to simulate chondrogenesis,[43] others have found that collagen II production is inhibited in microgravity.[21] Altogether, the current body of literature suggests that both loading and unloading conditions are favorable for chondrogenesis; however, a comparative study of the two environments has not been evaluated to truly determine the appropriate mechanical environment for optimal chondrogenic differentiation.

In the past decade, hASC have received increasing attention for musculoskeletal research due to their relative ease of harvest, proliferative capacity, and multipotent differentiation potential.[13,26,27] We and others have demonstrated that hASC are highly responsive to the loading conditions in which they are cultured.[30,45] However, it is currently unclear which mechanical environments best promote hASC chondrogenesis in vitro. The objective of this study was to evaluate hASC chondrogenesis simultaneously in both CHP and s-μG environments in order to better understand the effects of these two mechanical stimuli on chondrogenic differentiation (**Fig. 1**). Since our bodies are adapted to constant mechanical loading here on Earth, we hypothesized that the physiological loading simulated by CHP would result in greater chondrogenic differentiation of hASC when compared to s-μG. Understanding the implications of loading and unloading on hASC will be essential to the development of mechanically competent tissue-engineered cartilage constructs and to understanding the effects of microgravity on cartilage development during long-term space missions.

METHODS

Equipment

A rotating wall vessel (RWV) bioreactor, designed by NASA scientists at the Johnson Space Center,[32] was used to simulate microgravity (s-μG). The bioreactor consists of a cylindrical vessel with a flat silicone rubber gas transfer membrane for oxygenation.[4,16] The RWV allows for solid body rotation around a horizontal axis, resulting in randomization of the gravitational vector, low shear stress, three-dimensional spatial freedom and oxygenation by diffusion of dissolved gases from the reactor chamber. During rotation, the cells are exposed to a constant rotation, producing vector-averaged forces comparable with that of near-Earth free fall orbit.[16]

CHP was applied in a custom bioreactor filled with paraffin oil (STE Oil) and maintained at 37°C as described previously.[12,30] The sealed bags exposed to CHP were placed into the pressure vessel while the normal gravity (1 G) control pellets were maintained in sealed bags in a 37°C incubator. The pressure vessel (Parr Instruments, Moline, IL) was connected to a hydraulic cylinder (Miller Fluid Power, Des Plaines, IL) that was pumped using a computer controlled MTS 858 Mini Bionix II load frame. CHP was applied at an amplitude of 7.5 MPa at a frequency of 1 Hz for 4 $h \cdot d^{-1}$ for 2 wk.

Procedure

Excess adipose tissue was obtained from five premenopausal donors (24 to 36 yr old women, 3 Caucasian, 1 Native American, and 1 of unknown ethnicity) in accordance with an approved IRB protocol at UNC Chapel Hill (IRB 04-1622) and as described previously by our lab.[7] Human ASC were isolated from the tissue using a method described previously by our lab and others.[5,7]

At second passage, 100,000 cells of each cell line were seeded in a single T-75 flask in complete growth medium (CGM) comprised of alpha-modified minimal essential medium (with L-glutamine) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Premium Select, Atlanta Biologicals, Lawrenceville, GA), 200 $mmol \cdot L^{-1}$ L-glutamine, and 100 I.U. penicillin/100 $\mu g \cdot ml^{-1}$ streptomycin (Mediatech, Herndon, VA). The cells were allowed to proliferate at 37°C in 5% carbon dioxide until reaching 80% confluency and were then passaged. The amassed cells were then characterized for osteogenic and adipogenic potential, ensuring the cells differentiated representative of an average of the five cell lines.[7]

After the third passage, hASC were resuspended at a density of 250,000 cells per pellet. Cells were centrifuged at 300×g for 5 min to allow a pellet to form at the bottom of a 15-ml falcon tube and subsequently maintained in CGM. For the hydrostatic pressure vessel, pellets ($N = 8$) were sealed into sterile bags with 10 mL of CGM. Half-medium exchanges were performed every 2 d and media samples were collected for biochemical analyses. For the RWV bioreactor, pellets ($N = 8$) were placed inside the RWV bioreactor with 10 ml of CGM and rotated constantly at 11 rpm inside of a 37°C/5% CO_2 incubator. The pellets were constantly suspended in the middle of the vessel and not on the

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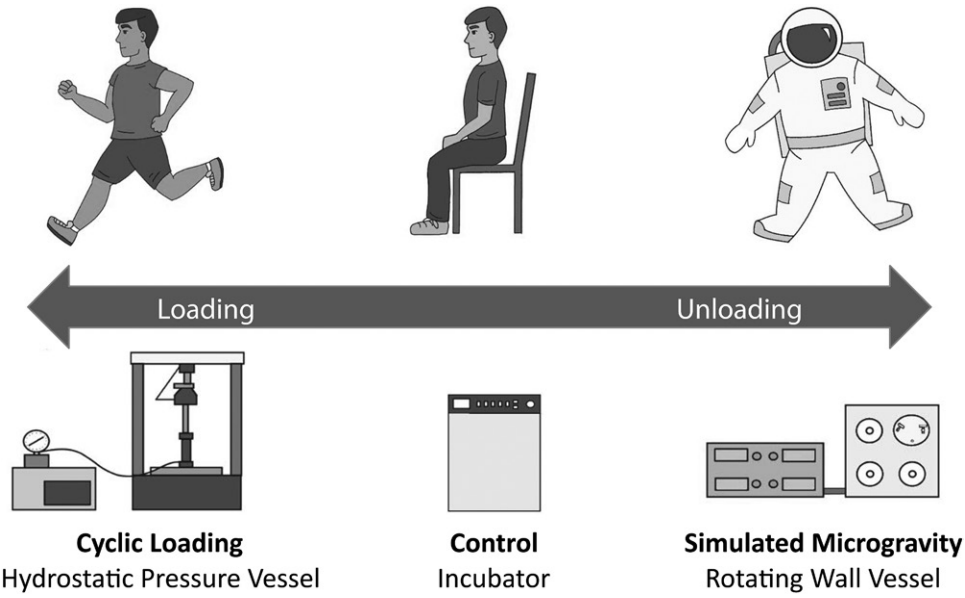


Fig. 1. The spectrum of mechanical loading conditions applied to articular cartilage in the synovial joint. At one end of the spectrum, articular cartilage is subjected to cyclic hydrostatic pressure when an individual walks or runs. Alternatively, astronauts on long-term space missions experience mechanical unloading within their joints. To simulate these conditions experimentally, loading can be simulated using a hydrostatic pressure vessel and unloading can be simulated in a rotating wall vessel bioreactor.

vessel wall. We checked daily that no air bubbles were formed in the media, which causes turbulence inside the vessel. Pellets from all three groups (s-μG, 1 G, and CHP) were collected and analyzed after 14 d.

Pellets ($N = 3$) were digested with papain (125 $\mu g \cdot mL^{-1}$) in 0.1 M sodium acetate, 5 $mmol \cdot L^{-1}$ L-cysteine-HCl, and 0.05 M EDTA (pH 6.0, all Sigma-Aldrich, St. Louis, MO) at 60°C for 18 h. DNA content was assessed using a Hoechst DNA binding assay. Aliquots of the papain digest and DNA standards (type 1 calf thymus, Sigma-Aldrich) were mixed with 0.2 $\mu g \cdot mL^{-1}$ Hoechst 33,258 dye and the fluorescence was assessed (Ex/Em: 352/461). Sulfated glycosaminoglycan (sGAG) content was quantified using a modified dimethylmethylene blue (DMMB) dye-binding assay with a chondroitin sulfate standard, as described previously.[35] Briefly, aliquots of the papain digest were mixed with a dye solution consisting of 80 μM DMMB (Sigma-Aldrich), 1% ethanol (Acros Organics, Geel, Belgium; VWR, Radnor, PA), 40 $mmol \cdot L^{-1}$ guanidine-HCl (Calbiochem, EMD Millipore, Billerica, MA; VWR), 315 μM formic acid, and 25 μM sodium hydroxide (Sigma-Aldrich) at a pH of 3.5 for 30 min and then centrifuged. The supernatant was removed and the remaining pellet resuspended in a dissociation buffer of 10% isopropanol (Alfa Aesar, Haver Hill, MA; VWR) and 4 M guanidine-HCl (Calbiochem, VWR). The resultant solution was measured colorimetrically at 656 nm. Media samples were also analyzed for sGAG content using the modified DMMB assay, and subsequently added to that accumulated within pellets to yield the total sGAG produced. All assays were performed in triplicate.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was used to determine relative mRNA expression changes of chondrogenic-specific genes in response to both mechanical stimuli. Total RNA was extracted from hASC pellets ($N = 3$) after 14 d of culture in CHP, s-μG, or static culture. Total RNA was extracted from each pellet by homogenizing each pellet with an RNase free disposable pellet pestle (Fisher Scientific, Waltham, MA) in 1 mL of TRIZOL reagent (Life Technologies, Carlsbad, CA), followed by a chloroform (Ricca Chemical Company, Arlington, TX) extraction. The extracted solution was incubated with an equal volume of isopropanol (Sigma-Aldrich) and 1 μL glycoblue (Life Technologies) at room temperature for 10 min. The solution was then centrifuged and the precipitate was washed once with 75% ethanol (VWR). The solution was centrifuged again and the supernatant was removed from the sample and resuspended in DEPC treated water (Mediatech, Manassas, VA). Total RNA yield and purity were analyzed using a NanoDrop 2000 Spectrophotometer (Sigma-Aldrich) and adjusted to a standard concentration prior to cDNA synthesis. To quantify mRNA expression, 120 ng of total RNA was reverse transcribed into cDNA using a high capacity first strand cDNA synthesis kit (OriGene, Rockville, MD) as per manufacturer's instructions. qRT-PCR was performed using 1 μL cDNA, 10 μL SYBR Green (Applied Biosystems, Foster City, CA), 7 μL DEPC treated water (Mediatech), and 1 μL forward and reverse primer (Life Technologies) for genes indicative of chondrogenesis and housekeeping control. The primers used in this study include: Collagen II 5′ TGG CCC TCA AGG ATT TCA AG 3′, 3′ ACC ATC ATC ACC AGG CTT TC 5′; Aggrecan 5′ CAG GCA GAT CAC TTG AGG TTA G 3′, 3′ CTC CCT AGT AGC TGG GAT TAC A 5′, Sox9 5′ TGA CCT ATC CAA GCG CAT TAC 3′, 3′ GCT TGC TCT GAA GAG GGT TTA 5′; and β-actin 5′ CAC TCT TCC AGC CTT CCT TC 3′, 3′ GTA CAG GTC TTT GCG GAT GT 5′. Real time qRT-PCR was performed using an ABI 7000 Sequence Detection system (Applied Biosystems). Samples were assayed in triplicate in one run (40 cycles) per gene. qRT-PCR data were analyzed using the $\Delta\Delta C_T$ method as described previously[20] with β-actin as the endogenous control. Relative quantification values are presented as fold changes in gene expression relative to the control group.

In order to identify potential mechanistic pathways that may contribute to mechanosensitivity of the hASC, a human Wnt signaling pathway PCR array (Qiagen, Hilden, Germany) was used to compare expression patterns of Wnt related genes in hASC incubated under cyclic hydrostatic pressure or simulated

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microgravity for 14 d. Using the RNA extracted in the previous section, cDNA was synthesized using an array-compatible RT2 First Strand kit (Qiagen). The mRNA from three separate experiments was pooled and quantified using a single set of arrays: one cyclic hydrostatic pressure plate and one simulated microgravity plate. If a gene did not amplify within either array, it was excluded from analysis.

On day 14, pellets ($N = 2$ per condition) were fixed in 10% zinc-buffered formalin (VWR) for 30 min and rinsed with PBS. Pellets were paraffin embedded and sectioned into 5-μm sections prior to staining. In order to examine cytoskeletal organization, sections were rehydrated, incubated in Antigen Retrieval Reagent-Universal (R&D Systems, Minneapolis, MN) for 10 min at 95°C and subsequently washed in water and PBS. Next, the sections were permeabilized in a 0.5% Triton-X solution for 45 min and washed in PBS. The sections were then incubated in a 1.5% BSA solution containing 1 $\mu g \cdot mL^{-1}$ antivimentin FITC (eBioscience, Inc., San Diego, CA) for 1.5 h, washed in PBS, mounted, and imaged using an EVOS FL Auto Cell Imaging System (Invitrogen) at 40× magnification.

Statistical Analysis

Statistical analysis was performed using Prism (version 6.07, GraphPad Software, La Jolla, CA). Biochemical results, both numerical and graphical, are expressed in the form of mean ± SD. Differences were determined using a one-way ANOVA with Bonferroni post hoc test. A level of $P < 0.05$ was considered significant.

RESULTS

RNA from hASC pellets cultured for 14 d under either s-μG or CHP was isolated and gene expression of chondrogenic genes compared to control pellets cultured in the incubator without mechanical load/unload stimulation (DOF = 2). Sox9 and collagen II expression was increased in CHP, compared to both control pellets ($P = 0.99$ and $P = 0.22$, respectively) and pellets cultured in s-μG ($P = 0.99$ and $P = 0.15$, respectively), although the difference was not statistically significant. Aggrecan gene expression was significantly increased in CHP conditions compared to both control ($P = 0.043$) and s-μG cultures ($P = 0.065$) (**Fig. 2A**). Total sGAG production was determined by summing the accumulation of sGAG in the pellet with that released into the media. Similar to the gene expression results, CHP significantly enhanced sGAG synthesis ($P = 0.0001$) while simulated microgravity had no effect ($P = 0.999$) (**Fig. 2B**).

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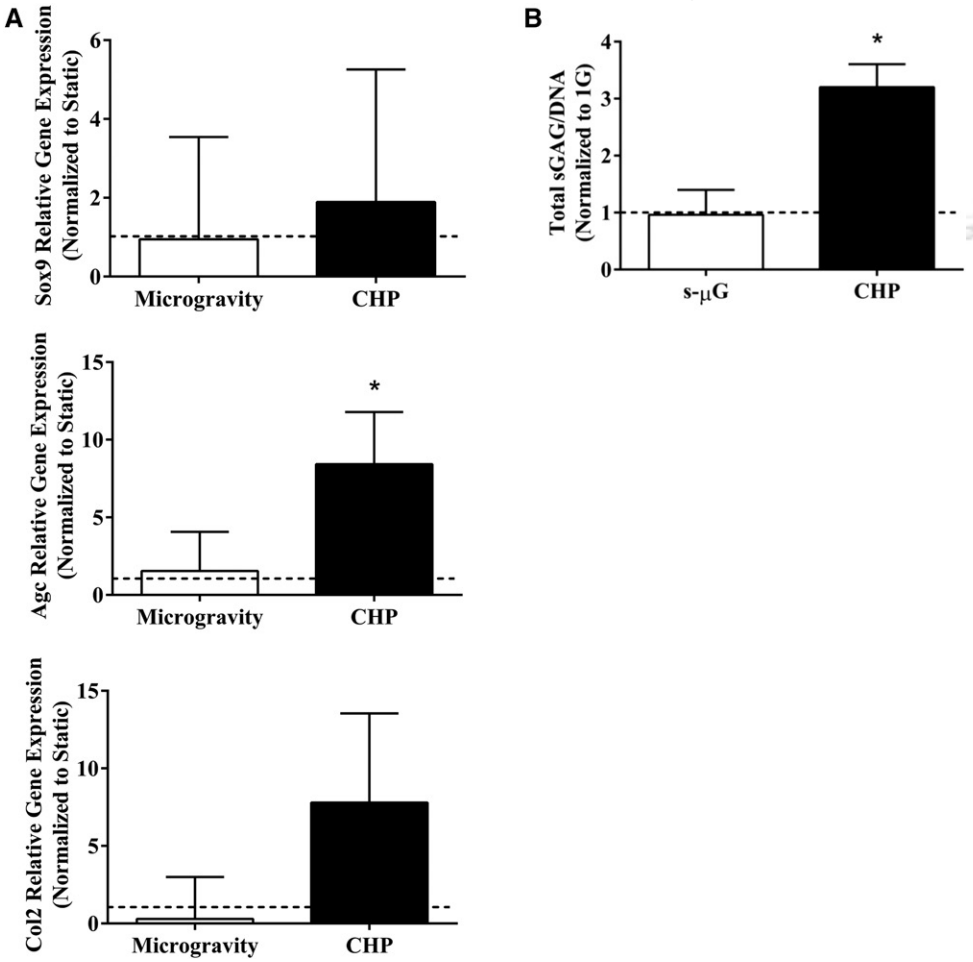


Fig. 2. Changes in chondrogenic mRNA expression and sulfated glycosaminoglycan synthesis under simulated microgravity and cyclic hydrostatic pressure. A) After 14 d of culture in either simulated microgravity (s-μG) or cyclic hydrostatic pressure (CHP), quantitative PCR was used to compare mRNA expression of chondrogenic genes. *$P < 0.05$. B) Total sGAG/DNA in microgravity and CHP conditions relative to culture in static conditions after 14 d of culture. *$P < 0.05$. Data is shown as mean ± SD.

A Wnt PCR array was used to compare gene expression of Wnt-related genes of pellets cultured in s-μG and CHP (**Fig. 3**). Several inhibitors of Wnt signaling were up-regulated in s-μG when compared to CHP pellets. Secreted frizzled-related protein 4 (SFRP4) had a 67.34 fold increase and SFRP1 had a 2.8 fold change; TLE1 and TLE/groucho, transcriptional corepressors of Wnt signaling, had a 4.42 and 2.8 fold change in s-μG compared to the CHP pellets, respectively. Only Axin2, a gene involved in the β-catenin destruction complex, was up-regulated in CHP by a 5.68 fold change. The other genes elevated in s-μG were Wnt signaling pathway activators. WNT3A, a Wnt ligand and β-catenin-dependent pathway activator, had a 30.98

Name	Description	Role in Wnt signaling	Fold change	Wnt activity
SFRP4	Secreted frizzled-related protein 4	Wnt inhibitor	67.3373	↓
WNT3A	Wingless-type MMTV integration site family, member 3A	Wnt ligand: β-catenin-dependent pathway activator	30.9815	↑
WNT7B	Wingless-type MMTV integration site family, member 7B	Wnt ligand: β-catenin-dependent pathway activator	9.2749	↑
FZD9	Frizzled homolog 9 (Drosophila)	Receptor	6.0769	↑
WNT2B	Wingless-type MMTV integration site family, member 2B	Wnt ligand: β-catenin-dependent pathway activator	4.4795	↑
TLE1	Transducin-like enhancer of split 1	Transcriptional co-repressor	4.4178	↓
BCL9	B-cell CLL/lymphoma 9	Promotes β-catenin transcriptional activity	4.0652	↑
WNT1	Wingless-type MMTV integration site family, member 1	Wnt ligand: β-catenin-dependent pathway activator	3.7408	↑
TLE/groucho	Transducin-like enhancer of split/groucho	Transcriptional co-repressor	3.6133	↓
FZD7	Frizzled homolog 7 (Drosophila)	Receptor	3.5145	↑
CTNNB1	Catenin (cadherin associated protein), beta 1	Encodes β-catenin	3.0809	↑
FZD8	Frizzled homolog 8 (Drosophila)	Receptor	2.9554	↑
SFRP1	Secreted frizzled-related protein 1	Wnt inhibitor	2.7959	↓
WISP1	WNT1 inducible signaling pathway protein 1	Downstream target of Wnt1 signaling pathway	2.6635	↑
FRAT1	Frequently Rearranged In Advanced T-cell Lymphomas 1	Inhibits GSK-3-mediated phosphorylation of β-catenin	2.2553	↑
WNT10A	Wingless-type MMTV integration site family, member 10A	Wnt ligand: β-catenin-dependent pathway activator	2.1936	↑
WNT7A	Wingless-type MMTV integration site family, member 7A	Wnt ligand: β-catenin-dependent pathway activator	2.1043	↑
FZD1	Frizzled homolog 1 (Drosophila)	Receptor	2.0467	↑
TCF7L1	Transcription Factor 7-Like 1	Transcription factor	-2.6695	-
AXIN2	Axin 2	β-catenin destruction complex	-5.6818	↓
FZD3	Frizzled homolog 3 (Drosophila)	Receptor	-12.0919	↑

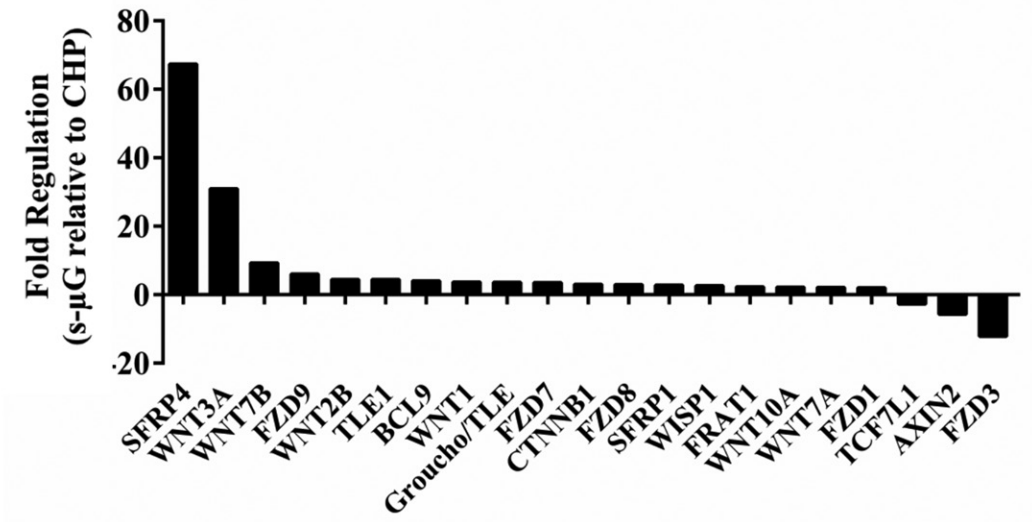


Fig. 3. Wnt signaling is dependent on mechanical stimulation. A Wnt PCR array was used to assess changes in Wnt-related genes between pellets cultured under simulated microgravity or cyclic hydrostatic pressure. Table (top) shows fold change (s-μG relative to CHP) for each gene that exhibited a fold change of 2% or higher, and briefly explains the role of each gene on Wnt signaling. The graph (bottom) represents fold change of simulated microgravity (s-μG) relative to cyclic hydrostatic pressure (CHP).

fold change when compared to CHP pellets. Among other Wnt ligands up-regulated in s-μG were WNT7B, WNT2B, WNT1, WNT 10A, and WNT7A. The receptors FZD9 and FZD8, and FZD7 and FZD1 were also up-regulated in s-μG.

The cytoskeleton is known to influence how cells interact with their mechanical environment.[15,33] It has been previously shown that vimentin regulates chondrogenesis of adult human bone marrow-derived multipotent progenitor cells, and that knocking down vimentin compromises cartilage formation.[6] No changes were observed in actin intensity or organization in any of the mechanical environments evaluated (data not shown). However, vimentin staining intensity and organization was altered in hASC exposed to CHP relative to 1 G and s-μG, suggesting that changes in vimentin may regulate CHP mechanotransduction (**Fig. 4**).

DISCUSSION

Mechanical loading plays a critical role during stem cell differentiation as it activates specific mechanotransductive pathways that induce tissue-specific differentiation. In chondrogenesis, mechanical loading stimulates cartilage matrix synthesis and enhances the mechanical properties of the developing cartilage that will play an important role in mechanical load support and load transfer in the articular joint.[9,14] Hydrostatic pressure bioreactors can simulate the mechanical environment experienced in the joint cavity and several studies have demonstrated enhanced chondrogenesis of bone marrow derived mesenchymal and human adipose derived stem cells under this compressive stress.[33]

Conversely, previous investigators have also used microgravity bioreactors to induce chondrogenic differentiation

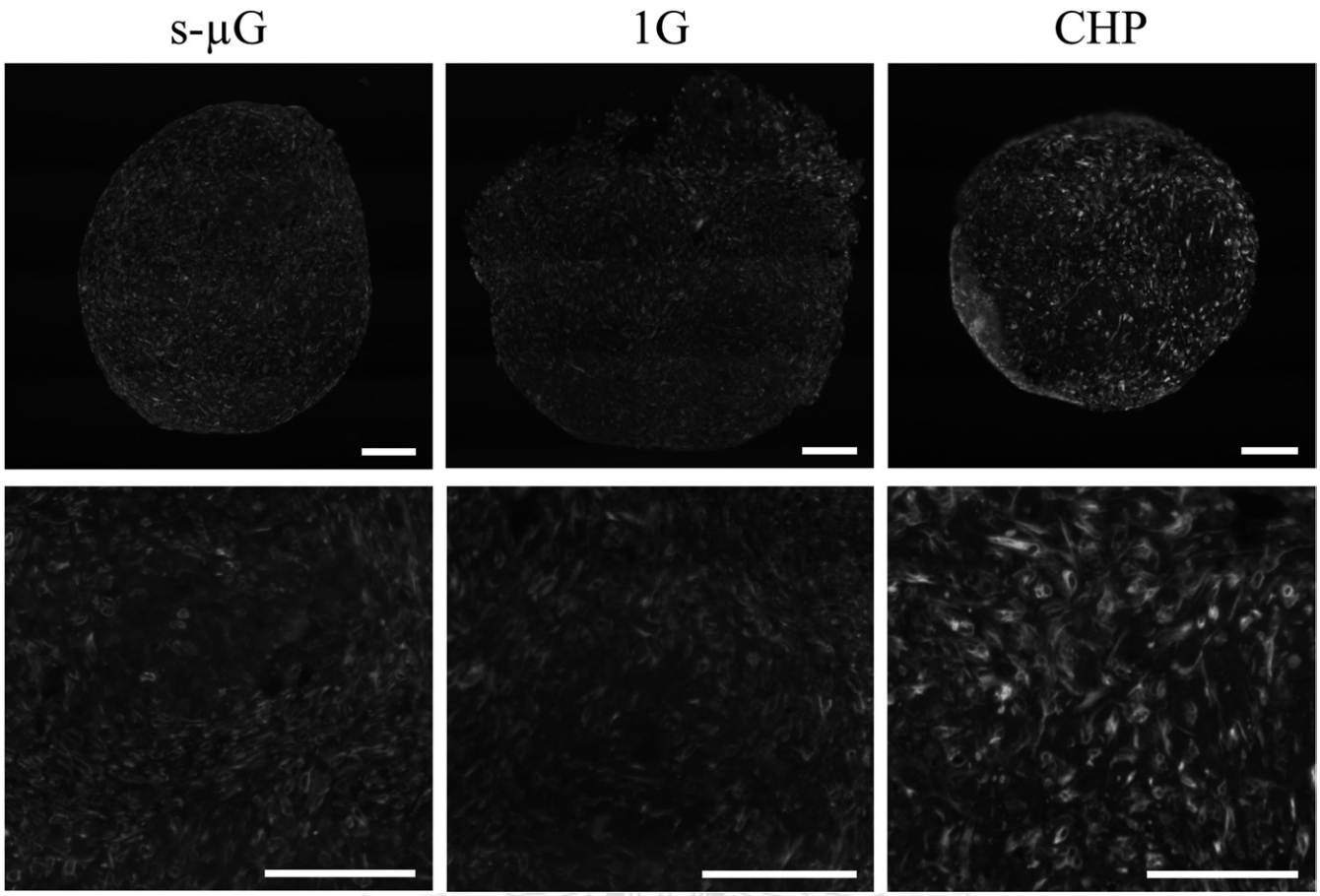


Fig. 4. Representative images demonstrating that CHP altered vimentin architecture relative to both microgravity and static conditions. Scale bars = 100 μm.

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of stem cells for cartilage tissue engineering applications. Several studies have reported that chondrogenesis is enhanced in microgravity conditions when compared to static culture controls,[11,42,43] while yet other studies suggest that microgravity decreases cartilage formation when compared to similar static culture controls.[10,21]

The goal of this study, therefore, was to simultaneously compare the chondrogenic potential of hASC cultured in CHP, static culture, or s-μG to determine which of these mechanical loads is preferential for inducing cartilage formation. We found that hASC cultured in CHP exhibited significantly increased aggrecan expression when compared to static control cultures, while cells in s-μG had no significant change in gene expression relative to static controls. Similarly, total sGAG synthesis was significantly enhanced for hASC exposed to CHP when compared to static culture controls, while s-μG cultures had no change in sGAG production.

We further investigated the influence of CHP and s-μG on signaling pathways involved both in mechanotransduction and chondrogenesis. Wnt signaling has been previously identified as a major responder and transducer of mechanical stimuli in both bone[17,39] and cartilage.[38] We hypothesize that some of the results observed in our study could be explained by the modulation of Wnt signaling related genes. Moreover, the Wnt pathway has emerged over the past decade as a key regulator of chondrogenesis and osteogenesis as canonical Wnt signaling has been suggested to inhibit chondrogenesis and stimulate chondrocyte hypertrophy.[40]

We identified 21 genes that differed significantly between hASC stimulated by either cyclic hydrostatic pressure or simulated microgravity. The gene with the greatest fold upregulation in s-μG was SFRP4, which was previously found to be upregulated from early to late stage osteoarthritis.[23] This could suggest that hASC have a higher proclivity to forming an osteoarthritic phenotype in unloading conditions. In addition, Wnt3a was among one of the genes most highly upregulated in microgravity. Wnt3a has been shown to inhibit both chondrogenic differentiation[31] and osteogenic differentiation.[8] This could indicate that hASC cultured in simulated microgravity are inhibited from differentiating relative to hASC cultured in cyclic hydrostatic pressure.

MSC have a remarkable ability to sense and respond to their mechanical environment via "inside-out-in" signaling that involves mechanisms such as integrin binding, focal adhesion formation, cytoskeletal organization, and other downstream pathways. The cytoskeleton in particular is known to be a key regulator of MSC mechanotransduction as it acts to transfer mechanical signals throughout the cell.[15] The

mechanotransduction of CHP and s-μG is not well understood relative to deforming loads such as tension, uniaxial compression, and fluid flow. For example, actin stress fiber formation and related RhoA/ROCK signaling is often found to play a key role in mechanotransduction and differentiation resulting from deforming loads;[41] however, in agreement with previous studies, none of the nondeforming mechanical stimuli in the current study had an effect on actin intensity or organization.[35-37] Previous studies have demonstrated a crucial role for vimentin in the chondrogenic response of MSC to CHP.[34,35,37] In agreement with these previous studies, vimentin intensity and organization was altered in hASC exposed to CHP relative to both static and s-μG conditions. While CHP both enhanced chondrogenesis and altered vimentin organization, whether these changes in vimentin are driving chondrogenesis or are in response to a changing phenotype is still unclear.

Our study provides insight about the potential benefits of simulating physiological loading to stimulate chondrogenesis. However, additional in vivo studies will be valuable to determine if the changes observed in vitro at the gene expression level are similar at the tissue/organ level. Future studies could also implement the comparative approach used here to further investigate the molecular mechanisms of chondrogenesis, focusing on specific signaling pathways that can be triggered pharmacologically to induce chondrogenesis in a less labor-intensive manner.

In conclusion, through a parallel study of cultured hASC in CHP, s-μG, and static culture, we determined that the mechanical stimulation produced by CHP, which mimics the mechanical environment that articular cartilage experiences during daily activities here on Earth, enhanced cartilage formation when compared to cells cultured in microgravity bioreactors. Therefore, bioreactors that produce cyclic hydrostatic pressure should be used over microgravity bioreactors for cartilage tissue engineering purposes. Additionally, we hypothesize that fracture healing in space might be compromised as one of the first steps in bone healing includes production of new cartilage that will later undergo endochondral ossification as a component of normal secondary fracture healing.

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Liliana Mellor and Andrew Steward contributed equally to this work.

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