Bone Research(2013) 1: 98-104.
www.boneresearch.org

ORIGINAL ARTICLE

# Dynamic Fluid Flow Mechanical Stimulation Modulates Bone Marrow Mesenchymal Stem Cells

Minyi Hu, RobbinYeh, Michelle Lien, Morgan Teeratananon, KunalAgarwal, Yi-Xian Qin*

Department of Biomedical Engineering, Stony Brook University, Stony Brook, NY 11794-5281, USA

**Osteoblasts are derived from mesenchymal stem cells (MSCs), which initiate and regulate bone formation. New strategies for osteoporosis treatments have aimed to control the fate of MSCs. While functional disuse decreases MSC growth and osteogenic potentials, mechanical signals enhance MSC quantity and bias their differentiation toward osteoblastogenesis. Through a non-invasive dynamic hydraulic stimulation (DHS), we have found that DHS can mitigate trabecular bone loss in a functional disuse model via rat hindlimb suspension (HLS). To further elucidate the downstream cellular effect of DHS and its potential mechanism underlying the bone quality enhancement, a longitudinal *in vivo* study was designed to evaluate the MSC populations in response to DHS over 3, 7, 14, and 21 days. Five-month old female Sprague Dawley rats were divided into three groups for each time point: age-matched control, HLS, and HLS+DHS. DHS was delivered to the right mid-tibiae with a daily "10 min on-5 min off-10 min on" loading regime for five days/week. At each sacrifice time point, bone marrow MSCs of the stimulated and control tibiae were isolated through specific cell surface markers and quantified by flow cytometry analysis. A strong time-dependent manner of bone marrow MSC induction was observed in response to DHS, which peaked on day 14. After 21 days, this effect of DHS was diminished. This study indicates that the MSC pool is positively influenced by the mechanical signals driven by DHS. Coinciding with our previous findings of mitigation of disuse bone loss, DHS induced changes in MSC number may bias the differentiation of the MSC population towards osteoblastogenesis, thereby promoting bone formation under disuse conditions. This study provides insights into the mechanism of time-sensitive MSC induction in response to mechanical loading, and for the optimal design of osteoporosis treatments.**

***Keywords*:** bone adaptation; mechanical loading; noninvasive stimulation; osteoporosis; osteopenia

***Bone Research*** (2013) **1**: 98-104. doi: 10.4248/BR201301007

## Introduction

Bone loss due to functional disuse osteopenia, classified as secondary osteoporosis (1), leads to osteoporosis-related fractures and high medical costs (2, 3). While millions of people are affected by these conditions, patients subjected to prolonged immobility or bed-rest (e.g., due to spinal cord injury, and nonunion), as well as astronauts who participate in long-duration spaceflight missions, are the common targets of disuse osteoporosis (1). Subsequent health complications associated with the physiological and/or pathophysiological changes in the skeleton, such as increased risks of falls and fractures, often require long-term recovery. Unfortunately, a mechanistic approach to facilitate the development of clinical treatments for osteoporosis is still lacking.

Mesenchymal stem cells (MSCs) are pluripotent cells defined by their capacity for self-renewal and potential differentiation into the cells that form different types of tissues, such as bone (4). MSCs can differentiate into osteoblasts, and bone formation begins with MSC proliferation and condensation (5, 6). In addition to

*Correspondence: Yi-Xian Qin
E-mail:Yi-Xian.Qin@stonybrook.edu
Tel: 631-632-1481
Fax: 631-632-8577
Received 28 December 2012; Accepted 24 January 2013

targeting the resident bone cell population, strategies of new treatments for osteoporosis have been aimed at regulating the fate of MSCs (7-10). Pharmacological agents, (*e.g.* statins) have been shown to increase bone marrow osteogenesis (11). A large amount of published works have pointed out that external mechanical signals are able to regulate the osteogenesis of MSCs. The ability of different types of macro-level mechanical loading, e.g. fluid flow, tension, compression, hydrostatic pressure, *etc*, to alter the differentiation patterns of MSCs toward osteogenesis and chondrogenesis, has been studied (6, 12). Moreover, more complex 3D systems have been used by tissue engineers to explore the role of mechanical forces on MSC adhesion and differentiation, as well as promoting the growth of both bone and cartilage (13-19). *In vivo* studies using mechanical stimulation, such as whole body vibration, have also demonstrated their effects on MSC proliferation and differentiation toward osteogenesis (20-22).

Mechanical signals direct MSCs toward osteoblastogenesis and are anabolic to bone, while a reduction in mechanical forces on bone leads to adverse outcomes. *In vitro* simulated microgravity conditions, mimicking the reduced mechanical stress occurring in spaceflight and hind limb suspension (HLS), could alter the expression of differentiation-associated genes in osteoblasts and pre-osteoblasts (23-27). *Ex vivo* studies from HLS treatment in mice demonstrated a net consequence in suppressing osteogenic gene expression (27). A similar study in rats showed that 28 days of HLS negatively affected the growth potential of MSCs, but this adverse effect was removed upon recovery of normal gravity. Decreased osteogenic potential of MSCs from HLS resulted in an *in vitro* osteogenic induction condition. Low levels of osteoblastic gene markers were observed in the cells induced from the MSCs of the HLS rats, which eventually led to a decrease of bone mass. However, re-exposure to normal gravity or centrifugal force was able to reverse the observed changes (28).

Our group has recently developed non-invasive dynamic hydraulic stimulation (DHS) as a countermeasure for osteoporosis in rats (29). Demonstrated in a rat HLS model, DHS successfully mitigated trabecular bone loss under such disuse condition. The promising results suggest that DHS may potentially provide regulatory signals to osteoblast progenitors, which lead to phenotypic changes in bone structure. As this approach may provide a new alternative mechanical intervention for future clinical treatment for osteoporosis, it is important to elucidate the downstream cellular effects of DHS and its potential mechanisms to enhance bone quality. Therefore, the current *in vivo* longitudinal study was designed and aimed to evaluate the responses of MSC populations to DHS over 3, 7, 14, and 21days. It was hypothesized that mechanical signals derived from DHS may reveal a reduced bone marrow MSC growth under the disuse condition, occurring in a time-dependent manner.

## Materials and methods

*Experimental design*

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Stony Brook University. Ninety-three five-month old female Sprague-Dawley virgin rats (Charles River, MA) were used to investigate the effects of DHS on *in vivo* bone marrow MSC proliferation. Standard rodent chow and water ad libitum were provided for the animals, and they were housed in 18"x18"x24" (LxWxH) stainless steel HLS cages. The animal room had a 12:12 hours light: dark cycle and was temperature-controlled. Animals were randomly divided into 12 groups: 1) age-matched – day 3 ($n$=8), 2) HLS – day 3 ($n$=8), 3) HLS+DHS – day 3 ($n$=8), 4) age-matched – day 7 ($n$=8), 5) HLS – day 7 ($n$=8), 6) HLS+DHS – day 7 ($n$=8), 7) age-matched – day 14 ($n$=8), 8) HLS – day 14 ($n$=6), 9) HLS+DHS – day 14 ($n$=8), 10) age-matched – day 21 ($n$=8), 11) HLS – day 21 ($n$=8), 12) HLS+DHS – day 21 ($n$=7). The HLS procedure, similar to the setup from Lam's study (30), introduced functional disuse to the rat hindlimbs. Briefly, after cleaning with 70% alcohol, the animal's tail was lightly coated with tincture of benzoin. The dried and sticky tail was attached to a piece of surgical tape that formed a loop close to the end of the tail. Three strips of elastic adhesive bandage were used to secure the surgical tape that was placed on the tail. A tail harness apparatus and a swivel hook suspended from the top of the cage were linked to the loop formed by the surgical tape. To have the hind limbs about 2 cm above the cage bottom, the animals were suspended with an approximately 30° head-down tilt. The animal's forelimbs had full access to the entire cage. Throughout the entire study, animals' body weights and overall health were carefully monitored.

*DHS protocol*

For the stimulated animals, daily DHS was applied in conjunction with HLS. The DHS setup was similar to the previous study (29). Briefly, DHS was delivered through a custom-made inflatable cuff placed around the right hind limb above the tibia. An oscillatory actuator-driven syringe, a force-controlled syringe and a pressure sensor, were connected to the stimulation cuff. The actuator-driven syringe was controlled by a programmable 100

MHz waveform/signal generator (Model 395, Wavetek). The hydraulic pressure was monitored by the pressure sensor throughout the entire treatment. With a stimulation frequency of 2 Hz, the pressure stimulation magnitudes were 30 mmHg static pressure + 30 mmHg (peak-to-peak) dynamic pressure. Daily stimulation of the "10 min on-5 min off-10 min on" loading regime was applied to each stimulated animal, while under anesthesia (isoflurane inhalation), for five days/week. The rats in groups 1-3 were stimulated for 2 days. The rats were euthanized in the end of the according time points.

*MSC quantification*

At the end of each time point, animals were sacrificed and the bone marrow was extracted from the right tibia using MEM culture media. Red blood cells in the bone marrow extracts were removed by incubation with Pharmlyse (BD Bioscience) at room temperature for 10 min. MSCs were isolated from the bone marrow extracts and quantified using flow cytometry analysis. Appropriate antibodies (BD Bioscience) for positive and negative MSC surface markers (CD29+/CD49e+/CD90.1+/CD45-/CD11b-) were applied with suggested concentrations; the cells were fixed at a final concentration of 1% formalin. Flow cytometry data were collected using FACS ARIA at Research Flow Cytometry Core Facility at Stony Brook University Medical Center.

*Statistical analyses*

The MSC numbers of each time point were normalized according to the total cell numbers within the bone marrow extracts, followed by normalization to age-matched values to obtain the MSC percentages. All the statistical analyses on the results were reported as mean±SD. GraphPad Prism 3.0 Software (GraphPad Software Inc., La Jolla, CA) was used to determine the differences between groups. One-way ANOVA with Tukey's post-hoc test were performed with normal equal variance. The level of significance was determined at $P<0.05$.

## Results

*Bone marrow MSC population – day 3*

Flow cytometry measurements indicated the MSC population, as represented by cells positive for CD29, CD49e, and CD90.1, and negative for CD45 and CD11b. Over three days, HLS started to lower the total MSC number in the bone marrow, although it was not statistically significant ($P>0.05$). At this stage, DHS has not shown any effects on the bone marrow MSC population under disuse condition (Figure 1).

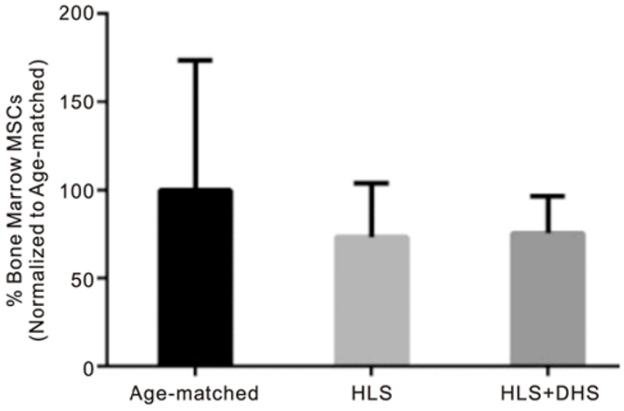


**Figure 1** Bone marrow MSC population – day 3. Graphs show mean±SD. HLSreduced the total MSC number in the bone marrow; although, it was not statistically significant ($P>0.05$). At this stage, DHS did not have any effect on the bone marrow MSC population under the disuse condition.

*Bone marrow MSC population – day 7*

By day 7, HLS reduced 67% of the total bone marrow MSCs compared to age-matched controls ($P<0.05$). On the other hand, DHS started to increase the MSC number within the bone marrow. Compared to the HLS group, this increase in bone marrow MSC number was up to 43% ($P>0.05$, Figure 2).

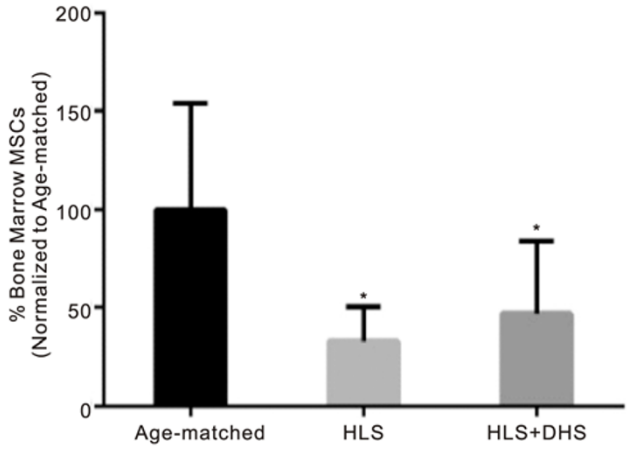


**Figure 2** Bone marrow MSC population – day 7. Graphs show mean±SD. HLS significantly reduced the total bone marrow MSCs compared to age-matched control ($P<0.05$). By this day, DHS started to elevate the MSC number within the bone marrow ($P>0.05$). *$P<0.05$ vs. age-matched.

*Bone marrow MSC population – day 14*

By day 14, the trend of the changes in bone marrow MSC populations was more apparent. While HLS greatly reduced 39% of bone marrow MSCs compared to age-matched control, the increase in the bone marrow MSC number in response to DHS reached up to 55% compared to the HLS group ($P>0.05$, Figure 3).

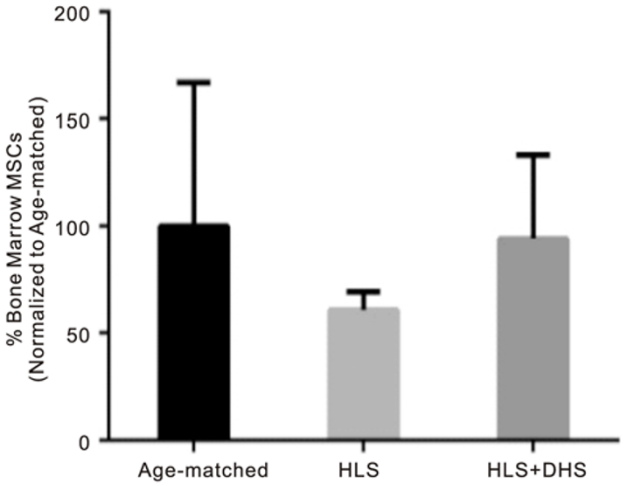


**Figure 3** Bone marrow MSC population – day 14. Graphs show mean±SD. HLS greatly reduced the bone marrow MSCs compared to age-matched control ($P$>0.05). The increase of MSC number of the DHS-treated group became more apparent compared to HLS ($P$>0.05).

*Bone marrow MSC population – day 21*

Interesting results were observed by day 21. HLS continued to reduce the bone marrow MSC number, 78% compared to age-matched controls ($P$<0.05). The elevated bone marrow MSC number by DHS was in conjunction with diminished HLS. Significant reduction of the MSCs in the bone marrow was again observed in the HLS+DHS group, 89% compared to the age-matched control ($P$<0.05, Figure 4). Overall, bone marrow MSC numbers under disuse showed a trend of being decreased at three weeks, but normalized significantly by week 2 after dynamic fluid flow loading (Figure 5).

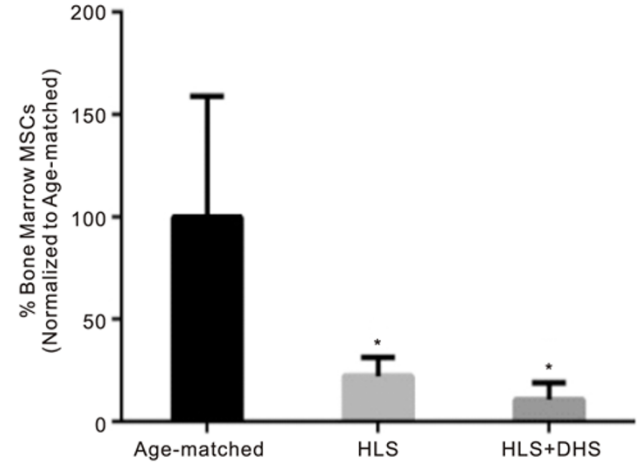


**Figure 4** Bone marrow MSC population – day 21. Graphs show mean±SD. HLS continued to reduce the bone marrow MSC number ($P$<0.05). The previous elevated bone marrow MSC number by DHS in conjunction with HLS was diminished. Significant reduction of the MSCs within the bone marrow was again observed in the DHS-treated group compared to age-matched control ($P$<0.05). *$P$<0.05 vs. age-matched.

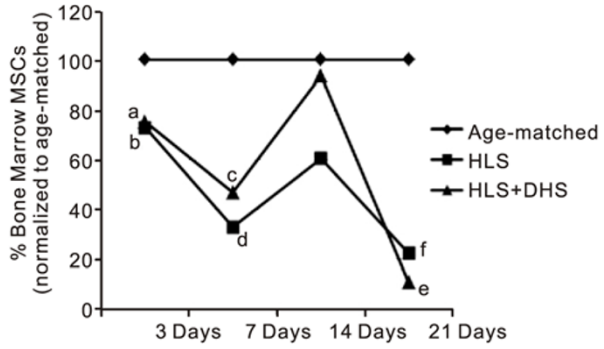


**Figure 5** Summary of bone marrow MSC numbers of age-matched, HLS and HLS+DHS groups over 3 days, 7 days, 14 days, and 21 days. Normalized to age-matched, MSC numbers of HLS and HLS+DHS groups had similar trends of reductions, which the MSC numbers were greatly reduced by day 7, peaked on day 14, and diminished by 21 days. Compared to HLS, the induction of the MSC number in response to DHS gradually increased between 7 to 14 days. [a]$P$<0.05 vs. age-matched – Day 3; [b]$P$<0.05 vs. age-matched – Day 3; [c]$P$<0.05 vs. age-matched – Day 7; [d]$P$<0.05 vs. age-matched – Day 7; [e]$P$<0.05 vs. age-matched – Day 21; [f]$P$<0.05 vs. age-matched – Day 21.

## Discussion

The present study focused on the mechanical contribution of DHS to the bone marrow cellular environment, and indicated that non-invasive DHS was able to promote the number of MSCs residing in the bone marrow. The external DHS serves as a direct coupling with oscillatory marrow fluid pressure, which may lead to influences in the interstitial fluid flow in bone and regulate skeletal mechanotransductive mechanisms. Longitudinal bone marrow MSC quantifications showed an interesting time-dependent manner of DHS and reduced MSC growth under disuse condition. The gradual changes of the MSC population size by DHS outlined the MSC activities and their contributions to the phenotypic changes in bone tissue.

To evaluate the changes in MSC population in response to DHS, flow cytometry analysis was employed to quantify the MSC cell counts of the bone marrow extracts. MSCs have been reported as expressing a series of surface markers, including CD90.1, CD29, and CD49e (31, 32). CD45 is often used as a negative marker of MSCs to be isolated from hematopoietic stem cells (HSCs) (31, 33, 34). CD11b is also often used as a negative marker of MSCs to isolate them from monocytes and macrophages (35). To specifically isolate the MSCs, antibodies for CD90.1, CD29, CD49e, CD11b and CD45 were utilized.

The data support the growing body of evidence of how MSC growth potential and osteogenic potential respond to the mechanical environment. Gravity is a

crucial factor for the differentiation and function of osteoblasts that are required for the maintenance of skeletal integrity. When exposed to normal gravity, MSCs are able to differentiate into osteoblasts followed by matrix mineralization under *in vitro* induction conditions (28, 36). Previous *in vivo* studies showed a decreased osteogenesis in young rats treated with HLS, signifying the important role of osteogenesis in maintaining bone mass under disuse condition (37, 38). Pan *et al* found a decreased *ex vivo* growth potential and osteogenesis of MSCs in the rat femurs subjected to HLS compared to the ones from the control rats (28). Once again, the effects of unloading on MSC osteogenesis indicated its effects on osteoprogenitors, which are closely correlated to the observed changes in osteoblastic bone formation. Bone formation is initiated with stem cell proliferation and condensation (5, 6). Changes in the mechanical environment, through low-magnitude mechanical loading, can potentiate the number of MSCs that reside in the bone marrow. Furthermore, these load-driven mechanical signals have been shown to bias the MSC differentiation toward osteoblastogenesis over adipogenesis, promoting bone formation (20).

Therefore, as a novel form of low-magnitude external mechanical loading on bone, DHS serves great potential to encounter bone marrow MSC reduction due to disuse (*e.g*. HLS). Combined with previous findings regarding DHS's mitigation effects on disuse trabecular bone loss, it is suggested that DHS may change the perfusion pressure associated with increased blood flow to the limbs (39), which mediates bone fluid flow (BFF) that, in turn, alters the mechanical environment of bone marrow MSCs and further leads to the expression of osteogenic factors (40). A limitation of the current study is that differentiation experiments on *ex vivo* MSCs were not included. However, our previous study has clearly shown the osteogenic adaptive response to DHS in a rat HLS model, indicating its osteogenic phenotypic outcome. This finding suggests that mechanical signals from DHS may have promoted the osteoblast differentiation from the MSC population in order to elicit the phenotypicosteogenic bone formation. This will also encourage our future investigations into the local cellular effects, osteoblasts and osteoclasts, in response to DHS.

One interesting finding in the present study, is the non-linear time-dependent fashion of DHS in encountering the reduced MSC growth under disuse condition. The gradual changes on the MSC quantity in response to DHS, suggest that the MSC growth activities seemed to take place within a certain time window (Figure 5). This, once again, supports our hypothesis that MSCs may respond to the DHS-derived mechanical signals, undergoing increase of growth and differentiation into osteoblasts over time. These step-wise and highly coordinated activities may eventually contribute to the phenotypic change in bone tissue. As observed in our previous work, bone growth was already accomplished by day 28. As an earlier event, it is reasonable to see enhanced MSC growth in response to the mechanical signals of DHS at earlier time points (7-14 days).

In summary, results of this study indicate that the MSC pool was positively influenced by the mechanical signals driven by DHS, which provides important information to elucidate the downstream cellular effects of mechanical signals and their contribution to enhance bone quality. DHS, as a novel and non-invasive intervention regulating skeletal adaptation, not only provides significant insights for future clinical applications, but also serves as an ideal tool to delineate the complex mechanotransductive mechanisms in the skeleton. This study may provide insights for the mechanism of time sensitive MSC growth in response to mechanical loading, and for the optimal design of osteoporosis treatments.

## Acknowledgments

This research was supported by the National Institute of Health (R01 AR52379, AR49286, and AR60621), the US Army Medical Research and Materiel Command, and the National Space Biomedical Research Institute through NASA Cooperative Agreement NCC 9-58. The authors are grateful to Dr. Clint Rubin for the stimulating discussions and to Alyssa Tuthill for technical assistance.

## References

1 Lau RY, Guo X. A review on current osteoporosis research: with special focus on disuse bone loss. J Osteoporos. 2011;2011:293808.

2 Turner AS. Animal models of osteoporosis--necessity and limitations. Eur Cell Mater. 2001;1:66-81.

3 Garnero P, Delmas PD. Contribution of bone mineral density and bone turnover markers to the estimation of risk of osteoporotic fracture in postmenopausal women. J Musculoskelet Neuronal Interact. 2004;4:50-63.

4 Lu X, Li S, Cheng J. Bone marrow mesenchymal stem cells: progress in bone/cartilage defect repair. Sheng Wu Yi Xue Gong Cheng Xue Za Zhi. 2002;19:135-139.

5 Karsenty G, Wagner EF. Reaching a genetic and molecular understanding of skeletal development. Dev Cell. 2002;2: 389-406.

6 Kelly DJ, Jacobs CR. The role of mechanical signals in regulating chondrogenesis and osteogenesis of mesenchymal stem cells. Birth Defects Res C Embryo Today. 2010;90:75-85.

7 Cheng SL, Shao JS, Charlton-Kachigian N, Loewy AP, Towler DA. MSX2 promotes osteogenesis and suppresses adipogenic differentiation of multipotent mesenchymal progenitors. J Biol Chem. 2003;278:45969-45977.

8 David V, Martin A, Lafage-Proust MH, Malaval L, Peyroche S, Jones DB, Vico L, Guignandon A. Mechanical loading down-regulates peroxisome proliferator-activated receptor gamma in bone marrow stromal cells and favors osteoblastogenesis at the expense of adipogenesis. Endocrinology. 2007;148:2553-2562.

9 Krishnan V, Bryant HU, Macdougald OA. Regulation of bone mass by Wnt signaling. J Clin Invest. 2006;116:1202-1209.

10 Menuki K, Mori T, Sakai A, Sakuma M, Okimoto N, Shimizu Y, Kunugita N, Nakamura T. Climbing exercise enhances osteoblast differentiation and inhibits adipogenic differentiation with high expression of PTH/PTHrP receptor in bone marrow cells. Bone. 2008;43:613-620.

11 Akune T, Ohba S, Kamekura S, Yamaguchi M, Chung UI, Kubota N, Terauchi Y, Harada Y, Azuma Y, Nakamura K, Kadowaki T, Kawaguchi H. PPARgamma insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. J Clin Invest. 2004;113:846-855.

12 Castillo AB, Jacobs CR. Mesenchymal stem cell mechanobiology. Curr Osteoporos Rep. 2010;8:98-104.

13 Holtorf HL, Jansen JA, Mikos AG. Flow perfusion culture induces the osteoblastic differentiation of marrow stroma cell-scaffold constructs in the absence of dexamethasone. J Biomed Mater Res A. 2005;72:326-334.

14 Datta N, Pham QP, Sharma U, Sikavitsas VI, Jansen JA, Mikos AG. *In vitro* generated extracellular matrix and fluid shear stress synergistically enhance 3D osteoblastic differentiation. Proc Natl Acad Sci U S A. 2006;103:2488-2493.

15 Bjerre L, Bunger CE, Kassem M, Mygind T. Flow perfusion culture of human mesenchymal stem cells on silicate-substituted tricalcium phosphate scaffolds. Biomaterials. 2008;29:2616-2627.

16 Duty AO, Oest ME, Guldberg RE. Cyclic mechanical compression increases mineralization of cell-seeded polymer scaffolds *in vivo*. J Biomech Eng. 2007;129:531-539.

17 Emans PJ, Pieper J, Hulsbosch MM, Koenders M, Kreijveld E, Surtel DA, van Blitterswijk CA, Bulstra SK, Kuijer R, Riesle J. Differential cell viability of chondrocytes and progenitor cells in tissue-engineered constructs following implantation into osteochondral defects. Tissue Eng. 2006;12:1699-1709.

18 LeBaron RG, Athanasiou KA. *Ex vivo* synthesis of articular cartilage. Biomaterials. 2000;21:2575-2587.

19 Bryant SJ, Chowdhury TT, Lee DA, Bader DL, Anseth KS. Crosslinking density influences chondrocyte metabolism in dynamically loaded photocrosslinked poly(ethylene glycol) hydrogels. Ann Biomed Eng. 2004;32:407-417.

20 Luu YK, Capilla E, Rosen CJ, Gilsanz V, Pessin JE, Judex S, Rubin CT. Mechanical stimulation of mesenchymal stem cell proliferation and differentiation promotes osteogenesis while preventing dietary-induced obesity. J Bone Miner Res. 2009;24:50-61.

21 Sen B, Xie Z, Case N, Styner M, Rubin CT, Rubin J. Mechanical signal influence on mesenchymal stem cell fate is enhanced by incorporation of refractory periods into the loading regimen. J Biomech. 2011;44:593-599.

22 Sen B, Xie Z, Case N, Ma M, Rubin C, Rubin J. Mechanical strain inhibits adipogenesis in mesenchymal stem cells by stimulating a durable beta-catenin signal. Endocrinology. 2008;149:6065-6075.

23 Patel MJ, Liu W, Sykes MC, Ward NE, Risin SA, Risin D, Jo H. Identification of mechanosensitive genes in osteoblasts by comparative microarray studies using the rotating wall vessel and the random positioning machine. J Cell Biochem. 2007;101:587-599.

24 Pardo SJ, Patel MJ, Sykes MC, Platt MO, Boyd NL, Sorescu GP, Xu M, van Loon JJ, Wang MD, Jo H. Simulated microgravity using the Random Positioning Machine inhibits differentiation and alters gene expression profiles of 2T3 preosteoblasts. Am J Physiol Cell Physiol. 2005;288:C1211-C1221.

25 Carmeliet G, Nys G, Stockmans I, Bouillon R. Gene expression related to the differentiation of osteoblastic cells is altered by microgravity. Bone. 1998;22:139S-143S.

26 Zayzafoon M, Gathings WE, McDonald JM. Modeled microgravity inhibits osteogenic differentiation of human mesenchymal stem cells and increases adipogenesis. Endocrinology. 2004;145:2421-2432.

27 Visigalli D, Strangio A, Palmieri D, Manduca P. Hind limb unloading of mice modulates gene expression at the protein and mRNA level in mesenchymal bone cells. BMC Musculoskelet Disord. 2010;11:147.

28 Pan Z, Yang J, Guo C, Shi D, Shen D, Zheng Q, Chen R, Xu Y, Xi Y, Wang J. Effects of hindlimb unloading on *ex vivo* growth and osteogenic/adipogenic potentials of bone marrow-derived mesenchymal stem cells in rats. Stem Cells Dev. 2008;17:795-804.

29 Hu M, Cheng J, Qin YX. Dynamic hydraulic flow stimulation on mitigation of trabecular bone loss in a rat functional disuse model. Bone. 2012;51:819-825.

30 Lam H, Qin YX. The effects of frequency-dependent dynamic muscle stimulation on inhibition of trabecular bone loss in a disuse model. Bone. 2008;43:1093-1100.

31 Zangi L, Rivkin R, Kassis I, Levdansky L, Marx G, Gorodetsky R. High-yield isolation, expansion, and differentiation of rat bone marrow-derived mesenchymal stem cells with fibrin microbeads. Tissue Eng. 2006;12:2343-2354.

32 Harting M, Jimenez F, Pati S, Baumgartner J, Cox C, Jr. Immunophenotype characterization of rat mesenchymal stromal cells. Cytotherapy. 2008;10:243-253.

33 Wang C, Xu Y, Song WG, Chang WS. Isolation and culturation, phenotype detection of rat bone marrow mesenchymal stem cells. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2007;23:466-468.

34 Mafi P, Hindocha S, Mafi R,Griffin M, Khan WS. Adult

mesenchymal stem cells and cell surface characterization - a systematic review of the literature. Open Orthop J. 2011;5:253-260.

35 Marcus AJ, Coyne TM, Rauch J, Woodbury D, Black IB. Isolation, characterization, and differentiation of stem cells derived from the rat amniotic membrane. Differentiation. 2008;76:130-144.

36 Phinney DG, Kopen G, Righter W, Webster S, Tremain N, Prockop DJ. Donor variation in the growth properties and osteogenic potential of human marrow stromal cells. J Cell Biochem. 1999;75:424-436.

37 Barou O, Palle S, Vico L, Alexandre C, Lafage-Proust MH. Hindlimb unloading in rat decreases preosteoblast proliferation assessed *in vivo* with BrdU incorporation. Am J Physiol. 1998; 274:E108-E114.

38 Basso N, Bellows CG, Heersche JN. Effect of simulated weightlessness on osteoprogenitor cell number and proliferation in young and adult rats. Bone. 2005;36:173-183.

39 Raitakari M, Nuutila P, Ruotsalainen U, Teras M, Eronen E, Laine H, Raitakari OT, Iida H, Knuuti MJ, Yki-Jarvinen H. Relationship between limb and muscle blood flow in man. J Physiol. 1996;496: 543-549.

40 Kreke MR, Huckle WR, Goldstein AS. Fluid flow stimulates expression of osteopontin and bone sialoprotein by bone marrow stromal cells in a temporally dependent manner. Bone. 2005;36: 1047-1055.