Low Fluid Shear Stress Regulates Osteogenic Differentiation of Human Mesenchymal Stem Cells through Notch1-Dll4 Signaling

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***Abstract*— Osteoporosis is a common bone and metabolic disease that is characterized by bone density loss and microstructural degeneration. Human bone marrow-derived mesenchymal stem cells have great potential for bone tissue engineering and cell-based therapy due to their excellent multipotency, especially osteogenic differentiation. Although low fluid shear force plays an important role in bone osteogenic differentiation, the cellular and molecular mechanisms underlying this effect remain poorly understood due to a lack of effective tools to detect gene expression at the single-cell level. Here, we presented a double-stranded nucleic acid biosensor to examine the regulatory role of Notch signaling during osteogenic differentiation. The effects of orbital shear stress on hMSC proliferation, morphology change, osteogenic differentiation and Notch1-Dll4 signaling were examined. Osteogenic differentiation was studied by characterizing alkaline phosphatase (ALP) activity. We further investigated how orbital shear modulates Notch1-Dll4 signaling during osteogenic differentiation. Our results showed Notch1-Dll4 signaling is involved in orbital shear-regulated osteogenic differentiation. Inhibition of Notch signaling will mediate the effects of shear stress on human osteogenic differentiation.**

Keywords: osteogenic differentiation, mesenchymal stem cells, Notch signaling, shear stress

I. Introduction

Osteoporosis is a systemic metabolic bone disease with bone mass loss and microstructural degeneration. In recent years, the cost for treating osteoporosis is increasing due to the increased aged population and space travel, causing challenges to public health care. In space, the reasons for developing osteoporosis is mainly related to low (micro- to zero-) gravity conditions, with possible contributions of cosmic ray radiation.[1] For example, bone density loss occurs in the weightless environment of space due to the lack of gravity force. Thus, the bone no longer needs to support the body against gravity. Astronauts lose about 1% - 2% of their bone mineral density every month during space travel. Osteoporosis is one of the major consequences of long-duration spaceflights in astronauts, seriously undermining their heath.[2] The current treatment of osteoporosis is to stimulate osteogenesis or inhibit bone resorption through drug-based agents, i.e., bisphosphonates.[3] However, drug-based agents are limited due to their side effects and lack of capability of regaining the lost bone density. Thus, there is an urgent need for alternative therapeutic approaches for osteoporosis, especially therapies that are able to counteract bone mass bone, which is crucial for prolonged Space missions.

Human bone marrow-derived mesenchymal stem cells are ideal candidates for cell-based therapies for bone tissue engineering and regenerative medicine due to their multipotency. Under mechanical or chemical stimulation, hMSCs can be induced to differentiate into different lineages, including osteoblasts (bone), neuroblasts (neural tissue), adipoblasts (fat), myoblasts (muscle), and chondroblasts (cartilage)[4]. Osteogenic differentiation is a dynamic process and involves several significant signaling pathways, including YAP/TAZ signaling, Notch signaling, and RhoA signaling [5, 6]. Although it has been shown that low fluid shear force, including that encountered in microgravity models, regulates *in vitro* osteogenic differentiation,[7-10] the fundamental mechanisms that are underlying this effect remains poorly characterized due to a lack of effective tools to detect gene expression profiles at the single-cell level.

Here, we present a double-stranded locked nucleic acid biosensor to investigate the effects of low orbital fluid shear stress on hMSCs proliferation and osteogenic differentiation. The phenotypic behaviors, including cell morphology, proliferation, and differentiation, were compared and characterized. We further tracked Notch 1 ligand Delta-like 4 (Dll4) gene expression by incorporating this biosensor with hMSCs imaging during osteogenic differentiation. Finally, we examined how Notch1-Dll4 signaling regulates osteogenic differentiation of hMSCs that is under orbital shear stress. Pharmacological administration is applied to disrupt Notch1-Dll4 signaling to investigate the molecular mechanisms that govern osteogenic differentiation. Our results provide convincing evidence that orbital shear stress induces osteogenesis through Notch1-Dll4 signaling.

II. Material and Methods

A. Cell Culture and Reagents

Human mesenchymal stem cells (hMSCs) were acquired from Lonza and maintained in mesenchymal stem cell basal medium (MSCBM) with GA-1000, L-glutamine, and

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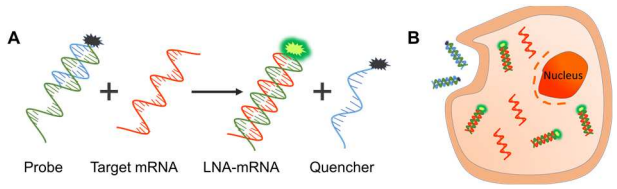

Figure 1. Single cell gene expression analysis of hMSCs. (A) Schematic illustration of double-stranded probe's binding scheme for mRNA detection. (B) Schematic of mRNA detection in hMSC using dsLNA/DNA probe.

mesenchymal cell growth supplements. Cells were grown at 37 °C and 5% CO_2 on a tissue culture dish in a humidified incubator with medium change every three days. Cells were passaged using 0.25% EDTA-Trypsin (Invitrogen), and passage 3-7 were used in the experiments. Nocth inhibitor DAPT (γ-secretase) was purchased from Sigma Aldrich. hMSCs were treated with DAPT (20 μM).

B. Stimulation of orbital shear stress

Cells were exposed to 20 rpm low orbital shear stress using a low-speed orbital shaker (Corning LSE, 6780-FP, orbit, 1.9cm, speed range, 3-60 rpm, length, 25.5 cm, width, 32cm). The orbital shear were applied to hMSCs after osteogenic induction for 6 hours per day for a total of 5 days. The orbital shake was placed inside the incubator. The orbital shear stress was calculated using the following equation:

$$\tau_{max} = a \times \sqrt{\rho \cdot \eta \cdot \omega^3}$$

Where τ_{max} is mear-maximal shear stress, a is the orbital radius of rotation, ρ is the density of cell culture medium, η is the dynamic viscosity of the medium, ω is the angular velocity and $\omega = 2\pi f$. f is the frequency of rotation (revolution per second).

C. Osteogenic Differentiation of hMSCS

For osteogenic differentiation, hMSCs were cultured on 24 well-plates with the initial concentration of 1x 10^6 cells/mL with a volume of 500 μL. Once the cells reach 50% confluency, basal medium were aspirated and osteogenic induction medium were added. Cells were images after 3 d and 5 d of osteogenic induction.

D. LNA Probe Preparation

The LNA probe complex was prepared according to our previous study [11-15]. Briefly, LNA probes have 20 base pair nucleotides designed for binding the Dll4 mRNA target. For mRNA detection, a fluorophore (6-FAM) was attached to the 5' end of the LNA probe. The binding affinity and specificity of LNA probe has characterized using mFold server and BLAST. The specificity of all sequeces are analyzed and evaluated by NCBI Basic Local Alignment Search Tool (BLAST) database. LNA probes and corresponding target DNA sequences are synthesized by Integrated DNA Technologies Inc (IDT). After four days of osteogenic induction, the prepared LNA probes (100 μM) were transfected into hMSCs using Lipofectamine 2000 following manufacturing instructions.

E. Alkaline Phosphatase Activity (ALP) Staining

Alkaline phosphate is an one of the reliable markers for early osteogenic differentiation since it is produced by osteogenic cells such as osteoblasts.[16] To quantify hMSCs osteogenic differentiation, cells were stained for alkaline phosphatase (ALP) using the alkaline phosphatase kit (Sigma-Aldrich). To label the nucleus and actin assist in determining the cells, Hoechst 33342 (1:2000)) and phalloidin (1 : 30) staining was performed. Then the cells were fixed by 4% cold- Paraformaldehyde (PFA) for 2 minutes which enable the maintenance of the ALP activation. A ratio of 1:1:2, Fast Red Violet solution, Naphthol AS-BI phosphate solution and water were prepared for the ALP staining. As the mixture transfer to the well plates, the staining process lasts for 15 minutes under room temperature, also protect from the light.

F. Imaging and Statistical Analysis

Images were captured using ZOE inverted fluorescence microscope. All fluorescence images were taken with the same setting for comparison. Data collection and imaging analysis were performed using NIH ImageJ software. All experiments were repeated at least three times or by different individuals. For measuring fluorescent intensity, at least 100 cells were quantified for each group. Results were analyzed using independent, two-tailed Student *t*-test in Excel (Microsoft). $P < 0.05$ was considered statistically significant.

III. RESULTS AND DISCUSSION

A. LNA Biosensor

We first established an LNA bisonsor for detecting Dll4 mRNA gene expression in hMSC during osteogenic differentiation, **Figure 1A**. The prepared LNA biosensor probe

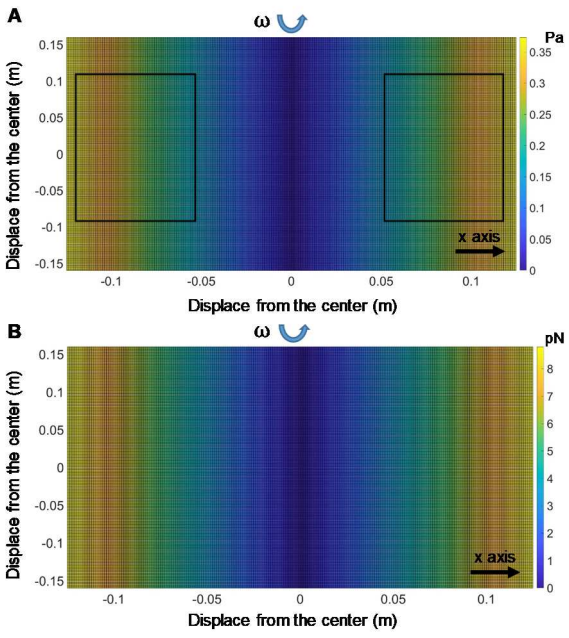

Figure 2. Simulated distribution of orbital shear stress and shear force. **(A)** Shear stress was calculated based on the displacement from the center of the shaker. The well plastes were placed in the area labeled as black rectangle. **(B)** Shear force was calculated based on the displacement from the center of the shaker.

was transfected into hMSCs to detect endogenous mRNA expression. In the presence of Dll4 mRNA, the LNA probe is thermodynamically displaced from the quencher to bind to the specific target sequences. The displacement reaction permits

the fluorophore to fluoresce, detecting the gene expression at the single-cell level, **Figure 1B**.

B. Simulaiton of Orbital Shear Stress and Analysis

The orbital shear stress was estimated using Strokes' second problem, which conceners a plate oscilating along one axis in the plane of the plate, with a liquid above it. Although orbital shaker does not produce uniform laminar shear stress on seeded cells, most of the cells were exposed near-maximum shear that is calculated as:

$$\tau_{max} = a \times \sqrt{\rho \cdot \eta \cdot (2\pi f)^3}$$

Where *a* is the orbital radius of rotation. The density of hMSCs culture medium is ~1.015 x 10^3 kg/m^3, the dynamic viscosity is 0.958 x 10^{-4} kg/m.s [17]. Since the cells in different wells were placed different locations on the orbital shaker, the shear stress is slightly different. Thus, we simulated the distribution of the shear stress over the shaker platform. Since the orbital shaker shakes along one axis (y), the shear stress along y axis are the same. At 20 RPM, the orbital shear stress were calculated, as shown in **Figure 2A**. The maximum shear stress is approximately 0.37 Pascal (3.73 dyne/cm^2), which is the edge of the shaker. At the center of the shaker, the shear stress is zero. The well-plates with the dimension of 120 mm x 85 mm were placed on the shaker, labelded in **Figure 2A**. Thus, the applied shear stress to different wells range from 1.5 dyne/cm^2 to 1.85 dyne/cm^2, which are similar to the values reported by others.[18-20] In our experiments, the wells were placed about 50 mm away from the center, the estimated shear stress is 1.5 dyne/cm^2. The applied shear force per each cell at different locations on the shaker platform were calculated, **Figure 2B**. The maximum force generated by orbital shaker is about 8 pN for the cells that were placed on the edge of the shaker. In our study, the shear forces for the cells that placed as illuatrated are in the range of 4-7 pN.

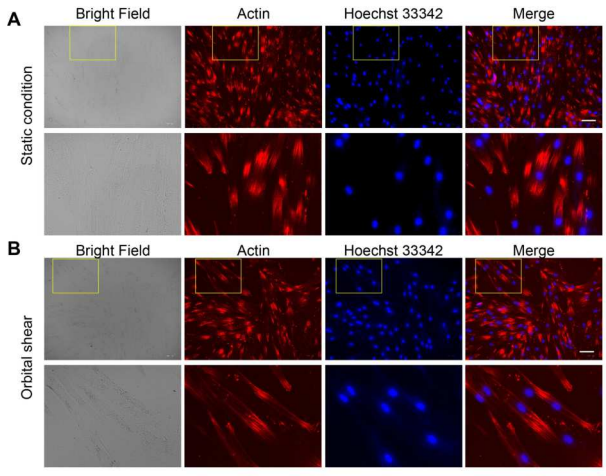

Figure 3. Effects of orbital shear stress on hMSCs morphology change. Representative images of hMSCs under static condition **(A)** and exposed to orbital shear **(B)**. The bottom panel showed the enlarged area of a yellow rectangle in the upper panel. hMSCs were exposed to orbital shear with 6 hours per day for 5 days. Samples were stained with F-actin (red; by phalloidin), and nuclei (blue; by Hoest 33342), respectively. Scale bar: 100 μm.

C. Effects of Orbital Shear Stress on hMSCs Morphology Change

To investigate the impacts of orbital shear stress on hMSCs morphology change, we quantified and compared the cell phenotypic behaviors, including cell area, cell length, cell aspect ratio, and cell perimeter of hMSCs. Cells subjected to orbital shear stress were compared to cells that were simply plated into tissue culture plates without shear (static condition). The control group provides a benchmark to account for any effects of exposing the cells to orbital shear stress. hMSCs cells were exposed to shear stress (~1.5 dyne/cm^2) for 5 days with 6 hours per day or static conditions. After five days of static or dynamic incubation, hMSCs were fixed, stained, and analyzed. **Figure 3** demonstrated the representative images of hMSCs under static conditions (**Figure 3A**) and hMSCs exposed to orbital shear stress (**Figure 3B**), respectively. Compared with hMSCs cultured under static conditions, the hMSCs exposed to orbital shear stress demonstrated a 55% increased of cell area, a 72% increase of cell length, a 16% increase of cell aspect ratio, and a 30% increase of cell perimeter, respectively, **Figure 4**. These results indicate that hMSCs are sensitive to low orbital shear stress with significant morphology changes. This finding is consistent with previously reported studies [18, 21].

D. Orbital Shear Stress Enhances hMSCs Osteogenic Differentiaiton

To investigate the effects of low fluid orbital shear stress on hMSCs osteogenic differentiation, hMSCs cells were placed on an orbital shaker after induction at the speed of 20 rpm with the estimated shear stress of 1.5 dyne/cm^2. A control group was placed in the static condition without applying shear. Osteogenic induction was initiated once the cells reached 50% confluency after cell seeding. After five days of osteogenic induction, ALP staining of hMSCs was conducted to analyze the early osteogenic differentiation. **Figure 5 (A-B)** show the representative bright field and fluorescent field images of hMSCs cultured with basal and induction medium under static conditions and orbital shear stress, respectively. ALP activity (early osteogenic differentiation marker) was quantified and compared by measuring the mean green fluorescent intensity of ALP stained hMSCs. The fluorescent intensity were normalized for better comparison. Under the static condition, ALP activity of hMSCs cultured in osteogenic induction medium increased 1.8 folds compared to hMSCs cultured in basal medium. Under orbital shear stress, the ALP activity was increased by 2.1 folds. Compared to static conditions, hMSCs exposed to orbital shear stress showed a 15% increase ((ALP intensity of hMSCs with shear – ALP intensity of hMSCs without shear)/ALP intensity of hMSCs without shear) of ALP activity after osteogenic induction, **Figure 5C**. We further quantify the differentiation percentage of hMSCs with and without orbital shear stress, calculated by number of ALP labeled cells per field/ total number of cells per field. With orbital shear stress, hMSCs differentiation percentage increased to 45.51%, compared to 38.02 % for hMSCs under static conditions. These results indicate that low shear stress

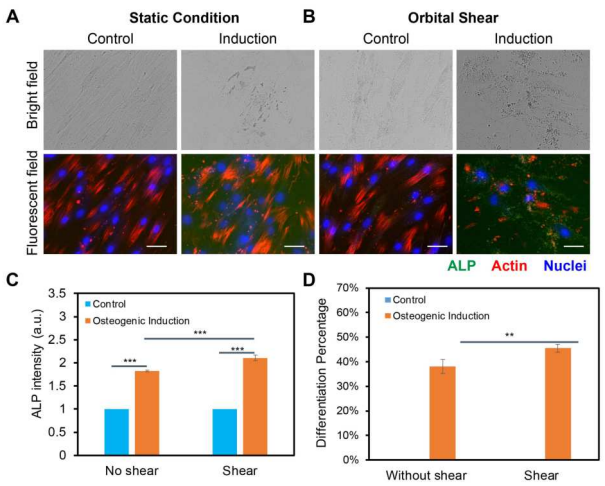

Figure 5. Orbital shear stress enhanced hMSCs osteogenic differentiation. Representative images of hMSCs cultured with basal culture medium and osteogenic induction medium under static condition (A), and exposed to orbital shear (B), respectively. hMSCs that were exposed to orbital shear stress for 5 days with 6 hours per day. Samples were stained with ALP (green; by ALP live stain), F-actin (red; by phalloidin), and nuclei (blue; by Hoest 33342), respectively. Scale bar: 100 μm. (C) Fluorescent intensity of ALP activity of hMSCs with and without orbital shear after 5 days of osteogenic induction compared to control group. (D) Osteogenic differentiation percentage with and without orbital shear stress. Data represent over 100 cells in each group and are expressed as mean± s.e.m. (n=6, ***, P<0.001, **, P<0.01)

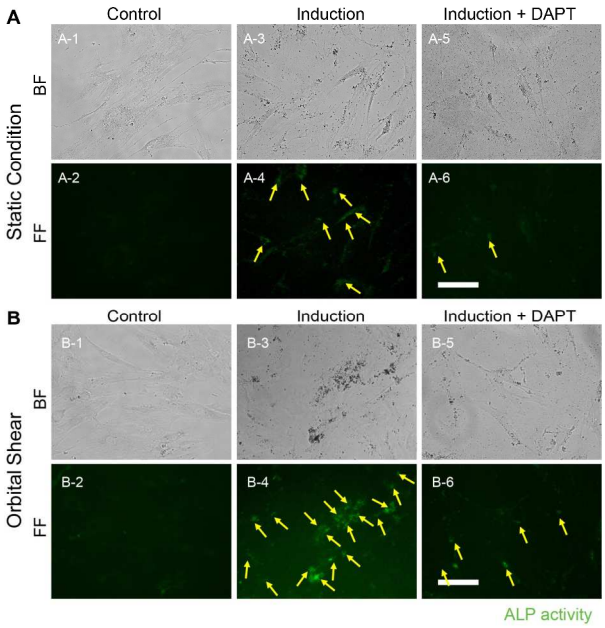

Figure 6. Involvement of Notch1-Dll4 signaling in regulating osteogenic differentiation with and without orbital shear. **(A)** Representative images of hMSCs in control, induction, and DAPT treatment groups without orbital shear (A) and orbital shear stress **(B)**. Images were taken after 5 days of induction. Green fluorescence signal indicating ALP activity. Yellow arrows indicate differentiated cells. Scale bar 100 μm.

has a significant influence on hMSCs osteogenic differentiation.

E. Notch1-Dll4 Signaling is Involved in Shear Stress Induced Osteogenic Differentiation

Previous studies have showed that Notch1-Dll4 signaling is involved during hMSCs osteogenic differentiation, disruption of Notch signaling mediated ALP activity, and osteogenic differentiation efficiency.[22] Here, in order to investigate whether orbital shear stress regulates osteogenic differentiation through Notch1-Dll4 signaling, we first perturbed Notch-Dll4 signaling by pharmaculeticual inhibition by using a γ-secretase inhibitor DAPT. hMSCs were treated with DAPT at a concentration of 20 μM during osteogenic differentiation with or without orbital shear stress to observe potential related effects on osteogenesis. A control group was designed without osteogenic induction. **Figure 6** show representative images of hMSCs under static and orbital shear stress that were cultured in basal medium, induction medium, and induction medium with the treatment of DAPT. As shown in **Figure 6**, DAPT treatment mediated osteogenic differentiation in both static condition and orbital shear condition. After Notch1-Dll4 signaling inhibition, ALP activity was further quantified and compared by measuring the mean fluorescent intensity to examine osteogenic differentiation efficiency. As shown in **Figure 8A**, after five days of osteogenic induction, ALP activity of hMSCs under static conditions was decreased by 28.8 % ((Fluorescent intensity with induction - Fluorescent intensity with DAPT)/ Fluorescent intensity with induction). Meanwhile, the ALP activity of hMSCs exposed to orbital shear stress was decreased by 18.2% with the treatment of DAPT. These results indicate that orbital shear stress rescued the effects of Notch1-Dll4 inhibition on hMSCs osteogenic differentiation.

To further investigate the relation of orbital shear stress and Notch1-Dll4 signaling, we examined Notch1 ligand, Dll4 mRNA expression of hMSCs under static condition, and orbital shear condition with basal culture medium, induction medium, and induction medium with DAPT treatment. **Figure 7** shows representative fluorescent images of hMSCs under different conditions. Dll4 mRNA expression of hMSCs was quantified and compared by measuring fluorescent intensity, **Figure 8B**. In both static and orbital shear conditions, a significant increase of Dll4 mRNA expression (static: 26.2 % increase; orbital shear: 72.1 % increase) was identified in hMSCs that were maintained in osteogenic induction medium compared to the cells maintained in basal medium. Further, with DAPT treatment, significant decrease of Dll4 mRNA of hMSCs were observed compared to osteogenic induction medium only, in both static and orbital shear conditions. Compared to static conditions, a significant increase (19.3%) of Dll4 mRNA was observed in hMSCs with the treatment of DAPT that were exposed to orbital shear.

Notch pathway is triggered by binding specific ligands to receptors. Here our results indicate that the expression of Notch ligand Dll4 was modulated during osteogenic differentiation. Orbital shear stress upregulates Dll4 mRNA expression of hMSCs under osteogenic induction, indicating the involvement of Notch1-Dll4 signaling in mechnoregulated osteogenic differentiation.

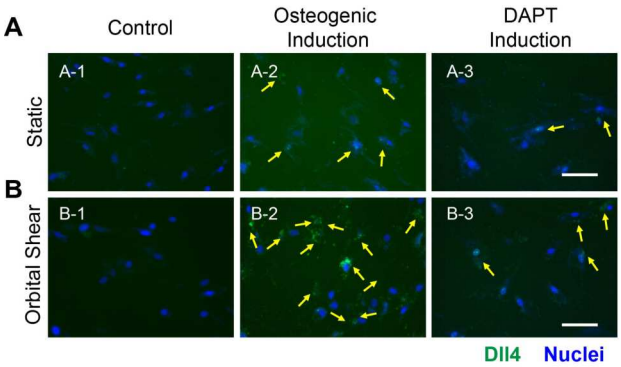

Figure 7. Notch1-Dll4 signaling in the regulation of hMSCs osteogenic differentiation. Representative fluorescence images of hMSCs in control, induction, and DAPT treatment groups under static condition **(A)** and orbital shear **(B)**. Green: Dll4 mRNA. Blue: nucleus, respectively. Scale bar: 100 μm.

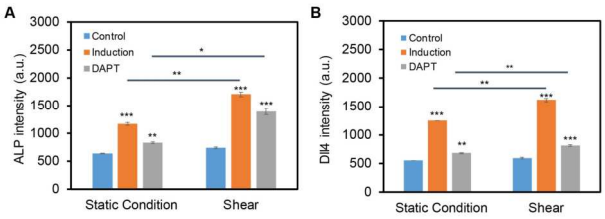

Figure 8. Notch1-Dll4 signaling regulates hMSCs osteogenic differentiation. **(A)** Comparison of ALP activity of hMSCs with and without orbital shear stress under different conditions. **(B)** Mean fluorescent intensity of Dll4 mRNA expression of hMSCs after 5 days of osteogenic induction under different conditions as indicated. Error bars, S.E.M., with n = 100 cells. p-Values were calculated using a two-sample t-test with respect to control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

IV. CONCLUSIONS

In this study, we demonstrated that low fluidic orbital shear stress enhanced hMSCs proliferation with the enhanced area, perimeter, and cell aspetic ratio. Osteogenic differentiation was promoted when hMSCs were exposed to orbital shear stress for five days of induction. We further elucidated the role of Notch1-Dll4 signaling in regulating osteogenic differentiation by exploiting LNA/DNA biosensor. Our results showed that orbital shear stress impaired Notch inhibition by inducing hMSCs osteogenic differentiation. In regards to the mechanism, we found that this effect may be mediated by inhibiting Notch1-Dll4 signaling by DAPT treatment.

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