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Research Article

The mTORC1 Pathway during Osteogenesis in Rat Bone Marrow Mesenchymal Cell Under Uniaxial Stretch: Implications for Distraction Osteogenesis

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Abstract

Background: To investigate the mechanistic target of rapamycin complex 1 (mTORC1) pathway during osteogenesis of rat bone marrow mesenchymal cells (BMMSCs) under dynamic stretching by a uniaxial stretch apparatus.

Materials and Methods: Rat BMMSCs were adopted to stretch by the custom made uniaxial stretch apparatus. The displacement of silica gel film was 10% for 4 hours. To investigate the role of mTORC1 during the process of osteogenesis, rapamycin and MHY1485 were administrated to regulate the mTORC1 signal during stretching. 0, 2, 4, 8 and 16 hours after the stretch beginning, the mTORC1 signal were evaluated by western blotting and RT-PCR. The osteogenic effect, such as ALP, Runx2, CON and Col1 were also detected.

Results: mTORC1 signal was detected during the osteogenic process of rat BMMSCs under uniaxial stretch. The osteogenic effect was obviously inhibited when the endogenous mTORC1 signal was down-regulated by rapamycin. However, when the endogenous mTORC1 signal was increased by MHY1485, the osteogenic effect was enhanced.

Conclusion: The results of this study suggest that the mTORC1 signal may play an important role during the process of rat BMMSCs osteogenesis under uniaxial stretch by promoting osteogenic differentiation.

Keywords: *Mechanistic Target of Rapamycin Complex; Rat; Bone Mesenchymal Stem Cell; Uniaxial Stretch*

Introduction

Distraction Osteogenesis (DO), regarded as an endogenous tissue engineering, has become a promising technique for treatment of limb lengthening, correction of deformity, and reconstruction of large bone defect. DO is a continuous and active process of callus regeneration under appropriate mechanical stimulation, which is different from the fracture healing or embryonic bone development. Osteogenesis during osteodistraction is characterized by a series of cellular processes and complex molecular events. During past decades, though the histomorphological features and many biomechanics of DO have been extensively exploited, the exact process has not yet been thoroughly elucidated with respect to cellular and molecular events.

The mechanistic target of rapamycin (mTOR) is an evolutionarily conserved Ser/Thr kinase, which belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and nucleates at least two distinct multi-protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [1]. mTORC1 contains deptor, PRAS40, raptor, mLST8, mTOR and TTI1-TEL2 and is the sensitive target of rapamycin. In a wide variety of cell types, it is regarded as integrating both intracellular and extracellular signals, including growth factors, nutrients,

energy levels, and cellular stress to modulate cell proliferation and differentiation [2]. mTORC2 mainly contains rictor and is resistant to rapamycin. The two best characterized targets of mTORC1 are S6 kinase1(S6K1) and 4EBP1 [3]. S6K1 can phosphorylate a series of substrates including estrogen receptor alpha (ERα) and S6 ribosomal protein (S6) to control gene transcription, protein synthesis, and other biological processes [4-6]. The hyperphosphorylation of 4EBP1 dramatically reduces its affinity for eIF4E, thereby allowing eIF4E to assemble with eIF4G, facilitating the recruitment of other translation initiation factors to form the eIF4F complex and initiate cap-dependent translation [7]. Recent experimental evidences indicated that the mTOR may contribute to the maintenance of bone homeostasis and mesenchymal stromal cells lineage differentiation [8]. mTORC1 is ubiquitously expressed in all types of cells to regulate growth and metabolism. Though, the important pathway is a central controller of organ growth and development, the mTORC1 pathway's role during DO was scarcely reported.

Hence, we adopted rat bone marrow mesenchymal cells (BMMSCs) to investigate the mTORC1 signal pathway under mechanical stimulation using a uniaxial stretch apparatus to achieve new information about mTORC1 signal pathway during DO.

Materials and Methods

Cell culture

All animal experiments were performed in accordance with the Animal Care and Use Guidelines of the South Medical University. Four-week-old Sprague Dawley rats were purchased from the Experimental Animal Center of South Medical University. BMMSCs from tibia and femur of rats were flushed out by Dulbecco modified Eagle medium (DMEM, Gibco, New York, NY) containing 10% (v/v) fetal bovine serum (FBS, Hyclone, Logan, UT). Discard the adipose layer by centrifugation at 400×g for 10 minutes. Cell pellets were suspended in 5 mL chilled Hanks, balanced salt solution, and laid over 8 mL percoll solution (1.073 g/mL). Mononuclear cell layer was removed from the interface after centrifugation at 800×g for 30 minutes and cultured in DMEM containing 10% FBS, 1% penicillin-streptomycin (hyclone, Logan, UT, USA) at 37°C culture conditions in the presence of 5% CO_2 under aseptic conditions. Non-adherent cells were removed by replacing the medium after 3 days. When the culture reached 80% to 90% confluences, cells were subcultured.

Cell stretch protocol

Cells at passage 3 (2×10^4 cells/cm^2) were seeded in the custom-made silica gel quadrangular ring (length: 40 mm × width: 30 mm) on the medical silica gel film (Dow Corning, Midland, MI; thickness: 0.2 mm × length: 130 mm × width: 40 mm). The ring, which was used to control the seeding area, was taken away 24 hours later when the cell grown adherently. When confluence reached approximately 70%, cells were serum starved for 24 h for synchronization. Then the medium was changed and mechanical stretch was applied 12 hours later. The displacement of silica gel film was 10% for 4 hours.

The cells were randomly divided into three groups. Group A was the inhibiting group, which was added rapamycin (Abcam, Cambridge, UK; 5 μM) 12 h before stretch applied. Group B was the control group, which was added dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) 12 h before stretch applied. Group C was the activating group, which was added MHY1485 (Selleck Chemicals, Shanghai, China; 2 μM) 12 h before stretch applied.

Detection of mRNA expression by quantitative Real-Time reverse transcription polymerase chain reaction

Cells were harvested and total RNA was isolated 0, 2, 4, 8, and 16 hours after the stretch beginning. The cDNA was synthesized using a PrimeScript™ RT reagent kit (TaKaRa, Japan) according to the manufacturer's instructions. SYBR Green quantitative real time reverse transcription PCR analyses were carried out on an ABI 7300 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Specific primers for each gene were showed in table 1. All these primer sequences were confirmed to be specific for the target gene alone using BLAST (National Institutes of Health, Bethesda, Md.). The PCR program was initiated by 10s at 95ºC before 40 thermal cycles, each of 5s at 95ºC and 31s at 60ºC. All these experiments were performed in triplicate. The threshold cycle value of samples was normalized by expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous house-keeping gene.

Primers	Sequence
Runx2	Forward GACTGTGGTTACCGTCATGGC
	Reverse ACTTGGTTTTTCATAACAGCGGA
OCN	Forward GACAAGTCCCACACAGCAACT
	Reverse ATTGGGGACCCTTAGGCCAT
Col1	Forward GCTCCTCTTAGGGGCCACT
	Reverse ATTGGGGACCCTTAGGCCAT
Raptor	Forward GACTCCAGTTCGAGACAGCC
	Reverse TGAATAGGAGCTCACACGCC
S6K1	Forward AGCAGAGCGGAATATTCTGGAG
	Reverse GGTAGAGTTTTCCACCGGTCT
4EBP1	Forward CCGGGAGGAACCAGAATCAT
	Reverse CATCGCTGGTAGGGCTAGTG
GAPDH	Forward AGGTCGGTGTGAACGGATTTG
	Reverse GGGGTCGTTGATGGCAACA

Table 1: *The specific primers for each gene.*

Detection of protein expression by western blotting

The total protein were collected from the cells 0, 2, 4, 8 and 16 hours after the stretch beginning using commercially available kit (Sigma, Saint Louis, MO) and the protein concentration was determined by the BCA assay (Pierce, Thermo Fisher Scientific Inc, IL, USA). Protein samples were denatured by heating to 95ºC for 5 minutes. 20 ug of total protein of each sample was separated on 4 - 20% gradient SDS-polyacrylamide gel and was transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) using electrophoresis. After blocking the non-special binding with 5% non-fat milk, the membranes were incubated overnight at 4ºC with primary antibodies: p-4BPE1 (1:1000, Cell Signaling, USA), p-S6K1 (1:1000, Cell Signaling, USA), Runx2 (1:1500, Abcam, UK), Col1 (1:2000, Abcam, UK), GAPDH (1:2000, Abcam, UK). Then the membranes were incubated with HRP-conjugated secondary antibody. Bound peroxidases were detected by chemiluminescence detection (Bio-Rad, Hercrles, CA, USA).

ALP activity measurement

For ALP activity assay, BMMSCs were scrapped from the film and suspended in ddH2O before freezing and thawing for three times. ALP activity was determined at 405 nm using p-nitrophenyl phosphate (pNPP) (Sigma Aldrich) as the substrate. A 50 µl of sample was mixed with 50 µl of pNPP (1 mg/mL) in 1 M diethanolamine buffer containing 0.5 mM $MgCl_2$ (pH 9.8) and incubated at 37°C for 15 minutes on a bench shaker. The reaction was stopped by adding 200 mL of 2M NaOH per 200 µl of reaction mixture. Total protein content was determined by the BCA method. ALP activity was calculated as nmol p-nitrophenol per minute per mg protein.

Results

Cell stretch and osteogenic signal of bone marrow mesenchymal stem cells before and after stretch

Cells obtained in primary culture grew quickly in the first 2 to 3 days and reached 80% to 90% confluences within 5 to 7 days. The BMMSCs were identified by the positive for CD29 and CD90 and negative for CD34 and CD45.

The ALP activity in each group increased during the 8h after stretch and declined during the last 8 hours. Before stretch, there were no differences among the three groups ($p > 0.05$, respectively). The ALP activity in the group C presented the highest values at the other four time points after stretch beginning, and there were significantly differences among the three groups ($p < 0.05$, respectively).

The relative Runx2 mRNA level in the group A reduced gradually during the 8h after stretch beginning and increased during the last 8 hours. However, the relative Runx2 mRNA level in the group B increased gradually during the 8h after stretch beginning and decreased during the last 8 hours. The relative Runx2 mRNA level in the group C presented the highest values at the four time points after stretch beginning, and there were significantly differences among the three groups ($p < 0.05$, respectively). Before stretch, there were no significantly differences among the three groups ($p > 0.05$, respectively)

The relative OCN mRNA level in the group A increased 2h after stretch gradually and reduced in last 14 hours. However, the relative OCN mRNA level in the group B increased gradually during the 8h after stretch and decreased during the last 8 hours. The relative OCN mRNA level in the group C presented the highest values at the four time points after stretch, and there were significantly differences among the three groups in the last two time points ($p < 0.05$, respectively). Two hours after stretch beginning, there were no significantly differences among three groups ($p > 0.05$, respectively). Four hours after stretch beginning, there was not significantly difference between group A and group B ($p = 0.794$). Before stretch, there were not significantly differences among the three groups ($p > 0.05$, respectively).

The relative Col1 mRNA level in the group A reduced gradually during the 8h after stretch beginning and increased during the last 8 hours. However, the relative Col1 mRNA level in the group C increased gradually during the 16h after stretch beginning. The relative OCN mRNA level in the group C presented the highest values at the four time points after stretch beginning, and there were significantly differences among the three groups 4 and 8 hours after stretch beginning. Two hours after stretch beginning, there were not significantly differences among three groups ($p < 0.05$, respectively). 2 hours and 16 hours after stretch beginning, there was not significantly difference between group A and group B ($p = 0.333$, $p = 0.066$, respectively). Before stretch, there were not significantly differences among the three groups ($p > 0.05$, respectively).

mTORC1 signal of bone marrow mesenchymal stem cells before and after stretch

The relative Raptor mRNA level in the group A reduced gradually during the 8h after stretch and increased during the last 8 hours. However, the relative S6 mRNA level in the group C increased gradually during the 8h after stretch beginning, and decreased during the last 8 hours. The relative Raptor mRNA level in the group C presented the highest values at the four time points after stretch beginning, and there were significantly differences among the three groups 4 and 8 hours after stretch beginning ($p < 0.05$, respectively). Two hours after stretch beginning, there was not significantly difference between group A and group B ($p = 0.129$). 16 hours after stretch beginning, there were not significantly differences among the three groups ($p > 0.05$, respectively). Before stretch, there were no significantly differences among the three groups ($p > 0.05$, respectively).

The relative S6K1 mRNA level in the group A reduced gradually during the 8h after stretch beginning and increased during the last 8 hours. However, the relative S6K1 mRNA level in the group C increased gradually during the 8h after stretch beginning and decreased during the last 8 hours. The relative S6K1 mRNA level in the group C presented the highest values at the four time points after stretch beginning, and there were significantly differences among the three groups at the last three time points after stretch ($p < 0.05$, respectively). Two hours after stretch beginning, there was not significantly difference between group A and group B ($p = 0.061$). Before stretch, there were not significantly differences among the three groups ($p > 0.05$, respectively).

The relative 4EBP1 mRNA level in the group A reduced gradually during the 8h after stretch beginning and increased during the last 8 hours. However, the relative 4EBP1 mRNA level in the group C increased gradually during the 12h after stretch beginning. The relative 4EBP1 mRNA level in the group C presented the highest values at the four time points after stretch, and there were significantly differences among the three groups 8 and 16 hours after stretch ($p < 0.05$, respectively). Two hours after stretch beginning, there was not significantly difference between group B and group C ($p = 0.054$). 16 hours after stretch beginning, there were not significantly differences among three groups ($p > 0.05$, respectively). Before stretch, there were not significantly difference among the three groups ($p > 0.05$, respectively).

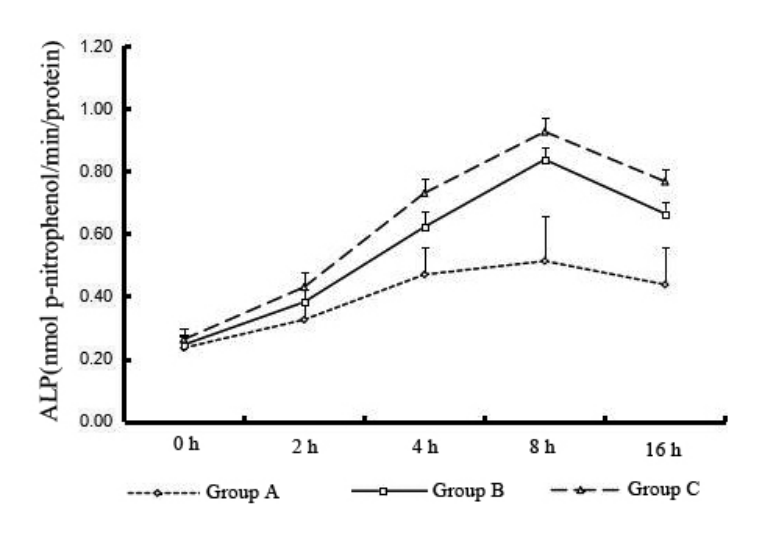

Figure 1: *The ALP activity in each group 0,2,4,8, and 16 hours after the stretch beginning.*

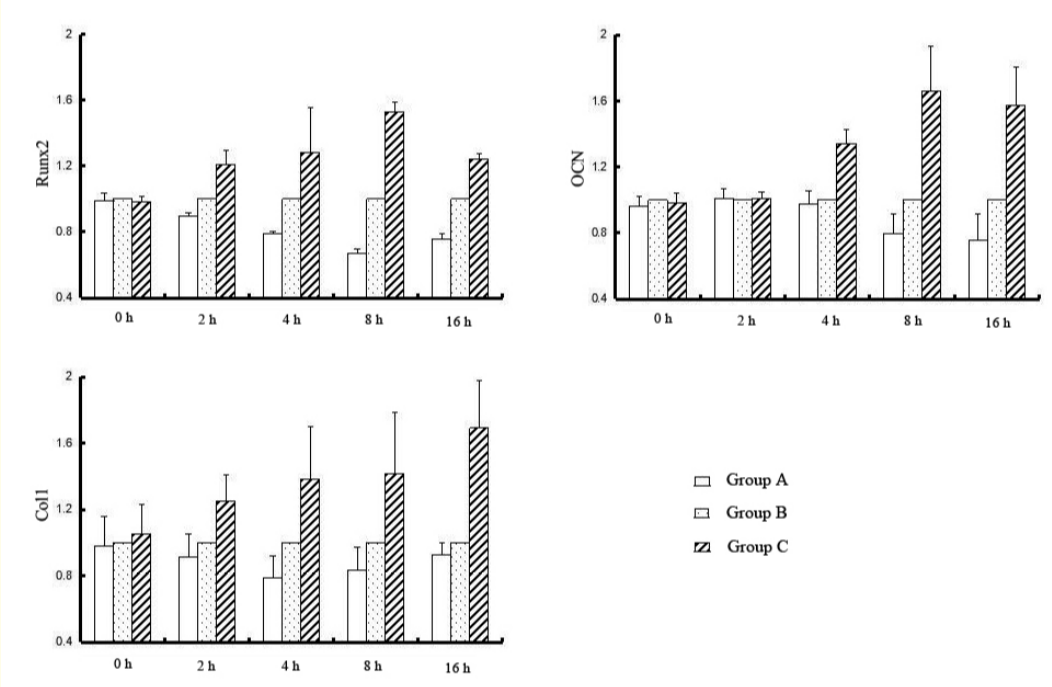

Figure 2: *The mRNA of osteogenic makers in each group 0,2,4,8, and 16 hours after the stretch beginning.*

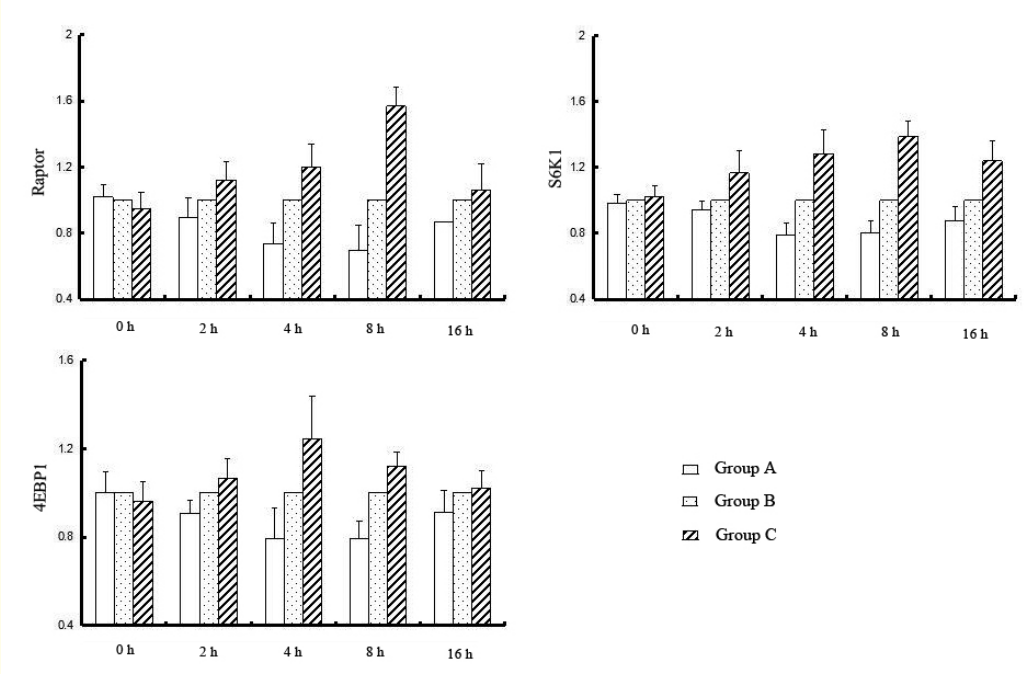

Figure 3: *The mRNA of mTORC1 signal in each group 0,2,4,8, and 16 hours after the stretch beginning.*

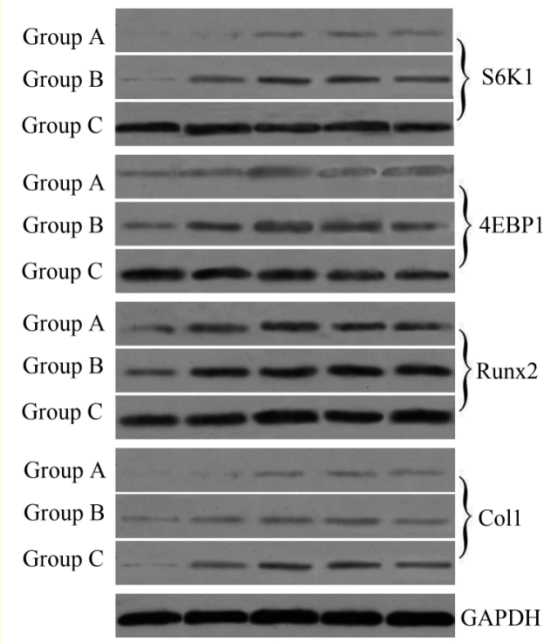

Figure 4: *The western blotting of osteogenic makers and mTORC1 signal in each group 0,2,4,8, and 16 hours after the stretch beginning.*

Discussion

In this preliminary cell stretch experiment, we adopted the stretching device which is characterized by uniaxial stretch stimulation and multiple units to elucidate the mTORC1 pathway expression during DO. The device could keep completely same frequency between each unit. Stretch stimulation is reciprocating cycle along the single axis which is similar to the mechanical stimulation during DO *in vivo*.

Rapamycin is a mTOR targeting inhibitor, which binds to FKBP12 to form the rapamycin/FKB12 complex and then prevents interaction of mTOR with target proteins in this signalling pathway. MHY1485 is a mTOR signal activator, which increases mTOR phosphorylation levels and phosphorylates downstream S6K1 and rpS6 proteins without affecting total mTOR content, total S6K1 and rpS6 levels. To investigate the role of mTORC1 pathway during osteogenesis under uniaxial stretch, we adopted those agents to regulate the mTORC1 pathway signal of the rat BMMSCs. In the group A, the rapamycin was added in the stretch unit to inhibit the mTOR signal, the mRNA level of Raptor, S6K1 and 4EBP1 were obviously down regulated during the 16 hours. The expression of pS6K1 and p4EBP1 in the group A were weaker than those in the group B and in the group C. The MHY1485 was administrated in the stretch unit to activate the mTOR signal in the group C, the mRNA level of Raptor, S6K1 and 4EBP1 were obviously upregulated when compared with the group B and A during the 16 hours. Those results proven that the signal of mTORC1 in the group A was down-regulated and which was also up-regulated in the group C.

Runx2 is a critical regulator of the differentiation of osteoblasts and chondrocytes which is essential for skeletal development. Osteocalcin and collagen I, which are essential bone proteins, are secretory glycoproteins produced almost exclusively by osteoblasts. ALP is a marker of osteoblast differentiation, which is a ubiquitous enzyme with an important role in osteoid formation and bone mineralization. These makers were adopted to evaluate the osteogenic effect during osteogenesis under uniaxial stretch when the mTORC1 signal was regulated. The results showed that cell osteogenic ability was down regulated when mTORC1 pathway was inhibited. While, the cell osteogenic ability was enhanced obviously after the mTORC1 pathway was activated. Those preliminary results supported that the mTORC1 pathway may play a crucial role during osteogenesis under stretch stimulus.

Recently, different studies of the role of mTORC signaling in osteoblast differentiation produced controversial results. Singha., *et al.* [9] found that rapamycin significantly inhibited proliferation in both MC3T3-E1 preosteoblastic cells and BMMSCs by reducing OCN, bonesialoprotein and osterix mRNA expression, ALP activity, Runx2 protein and mineralization capacity at the early stage of osteoblast differentiation, and Yeh., *et al.* [10] regarded this mechanism to that rapamycin could inhibit BMP-7 to activate the P70S6K, a downstream effector of mTOR in fetal rat calvarial cells. Huang., *et al.* [11] reported that activation of mTORC1 is required for preosteoblast proliferation and inactivation of mTORC1 is essential for their differentiation and maturation. However, Lee., *et al.* [12] regarded rapamycin as a potent stimulator of osteoblastic differentiation of human embryonic stem cells by modulating mTOR and BMP/Smad signaling. Furthermore, Chen., *et al.* [13] confirmed that decreasing mTORC1 signaling with deletion of mTOR or Raptor in mesenchyme resulted in death shortly after birth and skeletal discrepancy. And these controversies may be resulted from the differences in the cell types or cell differentiation-stages examined. From our preliminary results, the mTORC1 signal may play an important role during the process of rat BMMSCs osteogenesis under uniaxial stretch by promoting osteogenic differentiation.

Conclusion

To our knowledge, this the first direct report about the mTORC1 signal during DO. However, the exact mechanism about the mTORC1 during DO further exploration. And the mTORC1 activating agents might be useful in promoting osteogenesis during DO.

Acknowledgements

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Ethics Approval and Consent to Participate

This study was conducted with the approval of the Animal Ethics Committee of the first Peoples' hospital of Chenzhou, University of China South (N2015094562, approval date 2015-09-05).

Competing Interest

The authors declare that they have no competing interests.

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