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Rapamycin's osteogenic effects on bone marrow mesenchymal stem cells through triggering autophagy

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Abstract
Background: Although autophagy is crucial for the self-renewal and differentiation of stem cells, it is yet unknown how it affects bone marrow mesenchymal stem cells (BMSCs). This work used rapamycin (RAPA), a traditional autophagy agonist with osteo-regulatory effects, to examine the relationship between autophagy and osteogenic differentiation.
Methods: After osteo induction (0, 7, 14, and 21 d), real-time quantitative polymerase chain reaction, western blotting, and immune-fluorescence were used to examine the autophagy of rat BMSCs (RT-qPCR). Alizarin red staining, alkaline phosphatase tests, and RT-qPCR/Western blotting quantification of bone sialoprotein, type 1 collagen, alkaline phosphatase, osteopenia, and Runt-related transcription factor 2 mRNA and protein levels were also used to assess osteogenic differentiation.
Results: During osteogenic differentiation, the BMSCs' baseline autophagy level steadily reduced due to changes in BECN1, the ratio of lapidated (LC3-II) to unelucidated (LC3-I) microtubule-associated protein 1 light chain 3, and the expression of the specific autophagic target p62. On the other hand, it grew when RAPA concentration increased. Additionally, whereas 5 nM RAPA inhibited osteogenesis on days 14 and 21, 2 nM RAPA increased BMSC osteogenic differentiation on days 7 and 14. The autophagy inhibitor 3-methyladenine may reduce RAPA's ability to increase osteogenesis in BMSCs.
Conclusions: During osteogenic differentiation, the baseline autophagy level of the BMSC reduced with time. Yet, a suitable RAPA concentration encouraged BMSC osteogenic differentiation through activation of autophagy.

Keywords: Rapamycin, osteogenic differentiation, autophagy, 3-methyladenine, bone marrow mesenchymal stem cell

Introduction

One of the most difficult clinical therapeutic issues is the therapy of bone abnormalities brought on by osteoporosis, infection, and trauma [1-3]. Finding effective bone restoration techniques is thus still difficult. In order to replace crucial differentiation functions lost or altered in various disease states (such as bone defects brought on by diseases like osteoporosis), some disciplines, such as regenerative medicine and tissue engineering, have become more and more dependent on cell and tissue cultures [4, 5]. In the fields of tissue engineering and regenerative medicine, BMSCs are often used. Due to their multidirectional differentiation potential and capacity to differentiate into tissues including bone, cartilage, and fat under specific circumstances, they play essential roles in bone regeneration treatment. It's interesting to note that recent research has shown that BMSC autophagy control provides a potential molecular mechanism by which to influence their characteristics, playing a crucial role in their bone regeneration and therapeutic potential in numerous biological processes [8, 9]. In order to maintain their baseline metabolism and organelle balance, autolysosomes merge with lysosomes to generate autolysosomes, which break down their contents for the synthesis of nutrients or energy. This process is known as autophagy [10].

Growing data indicates that precise regulation of protein turnover and organelle quantity is necessary for the self-renewal and differentiation processes of mesenchymal stem cells (MSCs). It's interesting to note that recent research has shown that BMSC autophagy control provides a potential molecular mechanism by which to influence their characteristics, playing a crucial role in their bone regeneration and therapeutic potential in numerous biological processes [8, 9]. In order to maintain their baseline metabolism and organelle balance, autolysosomes merge with lysosomes to generate autolysosomes, which break down their contents for the synthesis of nutrients or energy. This process is known as autophagy [10]. Growing data indicates that precise regulation of protein turnover and organelle quantity is necessary for the self-renewal and differentiation processes of mesenchymal stem cells

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(MSCsTey saw a brief increase in autophagy after differentiation began and a continued drop in autophagy after differentiation. According to another study, autophagy activation was also seen in BMSCs in the early stage of lipogenic differentiation, peaking on day 3 and then rapidly declining on day 7 [14].

Moreover, because differentiated MSCs include many autophagosomes, which are significantly diminished in later stages of differentiation, autophagy plays a crucial role in osteogenic differentiation [15]. To the best of our knowledge, no research has yet been done on the specific changes in BMSC basal autophagy levels during more precise differentiation phases, such as undifferentiated, early, middle, and late periods. Furthermore, it is uncertain if pharmaceutically modifying the degree of autophagy in this process might affect osteogenic differentiation. By binding to mTOR and activating the mTOR signaling pathway, the classic mammalian target of RAPA (mTOR) inhibitor rapamycin (RAPA) causes autophagy [16].

RAPA enhances autophagy and affects osteogenic differentiation in MSCs, according to studies [17–19], although the precise molecular processes by which it works are yet unknown. Moreover, the US Food and Drug Administration has authorized the use of RAPA for human treatment (such as post-transplant immunosuppression in humans), enabling its conversion for a particular]. To the best of our knowledge, no research has yet been done on the specific changes in BMSC basal autophagy levels during more precise differentiation phases, such as undifferentiated, early, middle, and late periods. Furthermore, it is uncertain if pharmaceutically modifying the degree of autophagy in this process might affect osteogenic differentiation. By binding to mTOR and activating the mTOR signaling pathway, the classic mammalian target of RAPA (mTOR) inhibitor rapamycin (RAPA) causes autophagy [16]. RAPA enhances autophagy and affects osteogenic differentiation in MSCs, according to studies [17–19], although the precise molecular processes by which it works are yet unknown. Moreover, the US Food and Drug Administration has authorised the use of RAPA for human treatment (such as post-transplant immunosuppression in humans), enabling its conversion for a particular.

Methods and materials
Cell culture
Male Sprague-Dawley rats (4 weeks old; Dr. Patnam Mahender Reddy Institute of Medical Sciences, Chevella, RR Dist, Telangana) sacrificed by cervical dislocation had their bone marrow removed. In a sterile setting, -modified Eagle's medium (-MEM; Gibco, USA) containing 10% foetal bovine serum (FBS; Cyagen Oricell, China) and 1% penicillin and streptomycin (Beyotime, China) was rinsed into the bone marrow cavities of the rats' femur and tibia. Ten, bone marrow cells were grown in an incubator at a constant temperature (5% CO_2, 37 °C). Every 2-3 days, the culture media was replaced. The following studies were performed using Te cells at phase three (P3).

BMSC identification
Initially, trypsin was used to break down 1 106 cells, and then 500 mL of PBS containing 5 mL of antibodies against CD34, CD45, CD90, and 105 was added (CD105; Abcam, USA). Cells were twice washed and resuspended in 100 L PBS following 40 min of incubation at 4 °C. The BD Accouri C6 fow cytometer (BD Biosciences, USA) 7965 was then used to examine the marker protein.

Osteogenic differentiation analysis
Alkaline phosphatase (ALP) activity
Osteoblast-inducing conditional media (MEM with 10% FBS, 1% penicillin-streptomycin, 10 mM - glycerophosphate, 10 nM dexamethasone, and 50 g/mL ascorbic acid) was used as the culture medium and BMSCs were seeded into 12-well plates at a density of 5 104 cells per well. RAPA (Abmole, USA) was added to osteoblast-inducing media at doses of 0, 2, or 5 nM for cell culture. All cultures were kept alive for 21 days, and different studies added 3-MA (Abmole, USA; 5 mM). Cells were stained using the BCIP/NBT ALP Staining Kit after 7, 14, and 21 days of cultivation (Beyotime, China). ALP Assay Kit (Beyotime, China) was also used to quantitatively assess the activity of alkaline phosphatase.

Alizarin red staining (ARS)
The above-described cell culture conditions were used. In several tests, RAPA (0, 2, and 5 nM) and 3-MA (5 mM) were introduced. Cells were dyed with 1% alizarin red dye (Solarbio, China) for 15 minutes after being cultured for 14 and 21 days (Carl Zeiss, Germany). Next, using a microplate reader and 10% cetylpyridinium chloride (Boc Sciences, USA), the calcium nodules were quantitated (Molecular Devices, USA)

Cell viability
96-well plates with BMSCs planted into them at a density of 2 103 cells per well were then cultivated for 1, 3, and 5 days. The culture media contains varying amounts of RAPA (0, 2, 5, and 10 nM). Using the Cell Counting Kit (CCK)-8 solution, the effects of RAPA on cell proliferation were evaluated (Dojingdo, Japan). Briefly, each well received a dose of the CCK-8 solution. Using a spectrophotometer, the optical density of Te was determined at 450 nm.

RT-qPCR analysis
Using RT-qPCR, relative cytokine gene expression was measured. RAPA (Abmole, USA) was added to osteoblast-inducing media at concentrations of 0, 2, or 5 nM, and cells were grown under these conditions. 21 days were spent maintaining each culture, and 3-MA (5 mM) was introduced in several tests. BMSCs were used to isolate total RNA using the TRIZOL reagent (TaKaRa, Japan). UV spectroscopy was used to assess the total RNA's centration. The PrimeScriptTM RT Reagent kit with gDNA Eraser (Takara, Japan) was used to remove the genomic DNA at 42 °C for 2 minutes. Using the Prime Script RT kit and the manufacturer's instructions, RNA was reverse-transcribed into cDNA (Takara, Japan). Using a Roche 480 Light Cycler and the SYBR Green Kit (TaKaRa, Japan), the expression of the target genes was examined (Roche, Germany). The cycling conditions were: 60 °C for 30 s, 40 cycles of 95 °C for 5 s, and incubation at 95 °C for 30 s. Table 1 contains a list of the primers used.

Western blotting analysis
Western blotting was used to identify the relative expression of cytokine proteins. RAPA (Abmole, USA) was added to osteoblast-inducing media at concentrations of 0, 2, or 5 nM, and cells were grown under these conditions. 21 days

were spent maintaining each culture, and 3-MA (5 mM) was introduced in several tests. RIPA (Beyotime, China) was used to extract proteins from BMSCs while they were chilled. SDS-PAGE was used to load and separate proteins. The primary antibodies were directed against ALP (1:500; Abcam, USA), Runt related transcription factor 2 (1:500; HUABIO, China), bone sialoprotein (1:500; Proteintech, China), microtubule-associated protein 1 light chain (1:500; Wan leibio, China), Beclin 1 (1:500; SQSTM1/p62; Bioss, China), and GAPDH (1:5000; Abcam, USA).

Immunofluorescence staining: BMSCs were fixed in 4% paraformaldehyde, penetrated with 0.2% Triton X-100 (pH 7.4) for 10 min, and blocked with 5% bovine serum albumin after receiving RAPA treatment (0, 2, or 5 nM) for 0, 7, 14, and 21 d. The cells were then treated with a fluorescent secondary antibody (Abcam, USA) for 1.5 hours after being incubated with an anti-LC3 antibody (Pro-teintech, China) overnight at 4 °C. The nucleus was then stained with DAPI (Beyotime, China). Last but not least, pictures were taken using an inverted fluorescent microscope, the Axio Observer A1 (Carl Zeiss, Germany).

Table 1: Primer sequences for RT-qPCR

| Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Gapdh	ACG GCA AGT TCA ACG GCA CAG	GAA GAC GCC AGT AGA CTC CAC GAC
Alpl	CGT TTT CAC GTT TGG TGG CT	ACC GTC CAC CAC CTT GTA AC
Ibsp	ACA ACA CTG CGT ATG AAA CCT ATG AC	AGT AAT AAT CCT GAC CCT CGT AGC C
Runx2	CAG ATT ACA GAT CCC AGG CAGAC	AGG TGG CAG TGT CAT CAT CTGAA
Spp1	GAG CAG TCC AAG GAG TAT AAGC	AAC TCG TGG CTC TGA TGT TC
Col1a1	CGA GTA TGG AAG CGA AGG TT	CTT GAG GTT GCC AGT CTG TT
Lc3	GCG AGT TGG TCA AGA TCA TCC	CGT CTT CAT CCT TCT CCT GTTC
Becn1	AAT CTA AGG AGT TGC CGT TGT	GCC TCC AGT GTC TTC AAT CTT
Atg5	GAA GGC ACA CCC CTG AAA TG	CCT CAA CTG CAT CCT TGC AC

Results
BMSC identification

Rat BMSCs had the characteristic fibroblast and spindle form (Fig. 1A). According to an immunophenotype study, BMSCs positively expressed the hematopoietic markers CD34 and CD45 while negatively expressing the MSC markers CD90 and CD105, which is consistent with BSMC features (Fig. 1B).

BMSC osteogenic differentiation is associated with time-dependent autophagy modulation

The induced group seemed to have more ALP activity and calcium deposition than the control group, according to both ARS and ALP staining (Fig. 2A). Moreover, rising ALP activity was seen as induction time increased (Fig. 2B). Moreover, the expression of the osteogenesis-related proteins ALP and BSP significantly increased on days 0, 7, 14, and 21 following osteogenic stimulation, indicating effective BMSC osteoblast differentiation (Fig. 2C and D).

Several techniques were employed to evaluate the autophagy levels in BMSC during osteogenic differentiation in order to ascertain whether autophagy was involved. Several crucial autophagy-related proteins and genes underwent changes, which were seen. The ratio of LC3-II to LC3-I was greatest on day 0 and subsequently declined throughout the course of the next three differentiation phases. The time-dependent rise in autophagy cargo protein p62 levels points to a decrease in autophagic flux. Similar to this, we found reduced BECN1 levels (Fig. 2C and E). During osteogenic differentiation, Lc3, Becn1, and Atg5 expression were all consistently downregulated with time (Fig. 2F). Moreover, the LC3 immunofluorescence data supported the BMSCs' autophagy alterations (Fig. 2G). Our findings collectively showed that autophagy was modulated in a complicated, time-dependent manner during BMSC osteogenic development.

Cell viability with RAPA
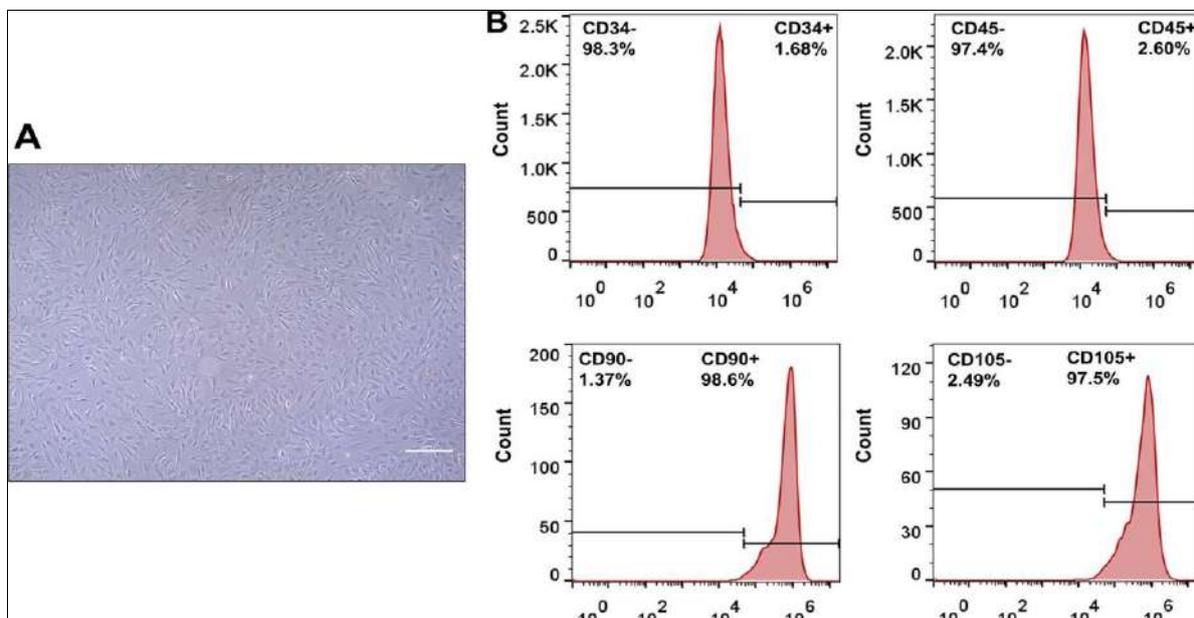
Using a CCK-8 test, the cytotoxicity of various RAPA doses was assessed (Fig. 3A). Comparative to the control group, 10 NM RAPA reduced cell growth starting on day 5. According to the ISO standard [20], cytotoxic potential is represented by cell viability below 70%. For the previous five days, however, the cell survival rates in the 0 nM, 2 nM, and 5 nM groups were greater than 70%. These findings imply that there was no detectable cytotoxicity in BMSCs at any of the three RAPA doses. Hence, in future tests, RAPA at 2 and 5 nM were utilized.

Validation of BMSC autophagy

In order to measure autophagy in BMSCs, different RAPA concentrations (0 nM, 2 nM, and 5 nM) were utilized. Immunofluorescence allowed the observation of the autophagosomes (Fig. 3B). The BMSCs increasingly developed more LC3 puncta as the RAPA concentration rose. Secondly, we looked at how RAPA's activation of autophagy affected the expression of autophagy-related indicators in both proteins and mRNA (Fig. 3C). Becn1, Lc3, and Atg5 expression all increased in a dose-dependent manner. As anticipated, the ratio of LC3-II to LC3-I and the expression of the proteins BECN1 and p62 also followed the same pattern (Fig. 3D and E), demonstrating that autophagy increased with increasing RAPA concentration.

Autophagy levels in BMSCs with different RAPA concentrations

The variations in a number of autophagy-related proteins were then discovered on the seventh, fourteenth, and twenty-first days of osteogenic differentiation, respectively, to investigate whether RAPA could continuously trigger autophagy in BMSCs throughout osteogenic differentiation.

On days 7, 14, and 21, RAPA therapy elevated the LC3-II/LC3-I ratio and BECN1 levels in comparison to the control group, but dose-dependently decreased p62 levels (Fig. 4A–D). Immunofluorescence studies confirmed that LC3 production increased significantly and gradually as RAPA concentration increased (Fig. 4E)

Osteogenic differentiation in BMSCs with different RAPA concentrations

After incubating cells in an osteogenic-inducing media with various RAPA doses for 7, 14, and 21 days, the effect of osteogenic differentiation was assessed. ALP activity and the quantity of mineralization nodules increased in the 2 nM group at 7 and 14 days compared to the 0 nM group, but differences between the two groups at day 21 were not significant (Fig. 5A– D). The osteogenic markers ALP, IBSP, RUNX2, SPP1, and COL1A1 consistently revealed the same trend in gene and protein expression levels between groups (Fig. 5E–K). BMSCs treated with 5 nM RAPA did not significantly differ from the 0 nM group on day 7 of osteogenic differentiation, in contrast to the 2 nM group. It did, however, prevent osteogenesis due to lower ALP activity and on days 14 and 21, it did, however, impede osteogenesis as evidenced by a decline in ALP activity and ARS staining and quantification (Fig. 5A–D). By using RT-qPCR and western blots on cells treated with 5 nM, the non-significant change on day 7 and osteogenic differentiation suppression on days 14 and 21 were further confirmed (Fig. 5E–K). The effects of various RAPA concentrations on BMSC osteogenic differentiation are confirmed by these findings, which may be connected to RAPA's encouragement of various autophagic levels during osteogenic differentiation.
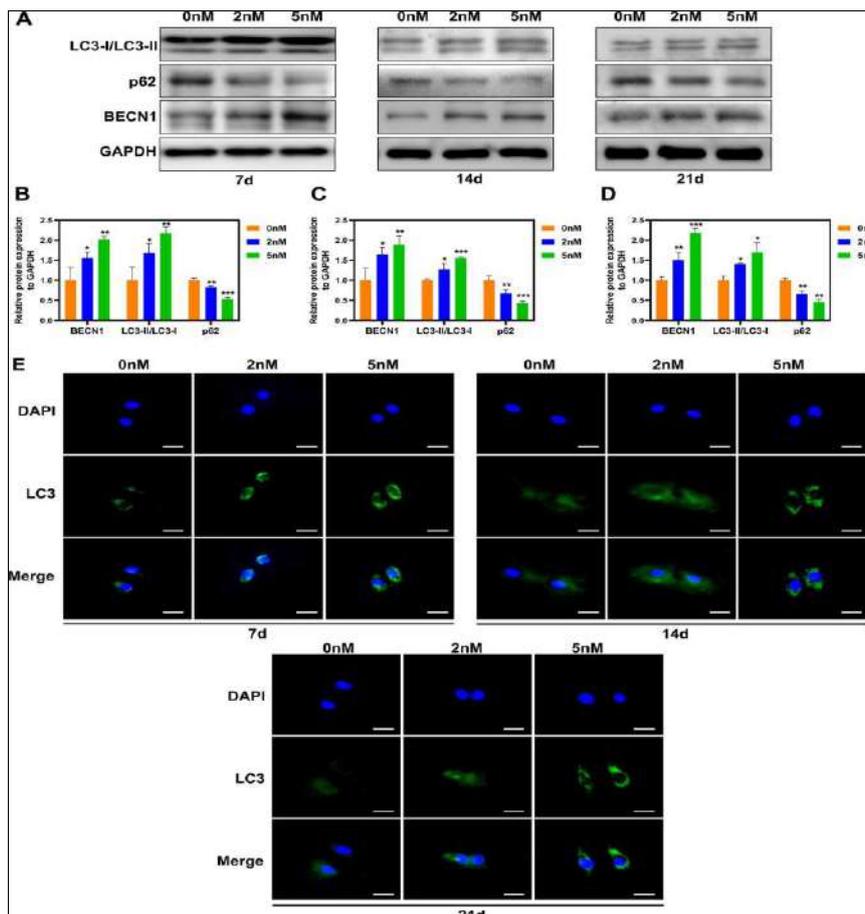
RAPA promotes BMSC osteogenic differentiation through its effect on autophagy We investigated whether RAPA stimulated BMSC autophagy by promoting osteogenic differentiation using the autophagy inhibitor 3-MA in light of the apparent activation of autophagy in BMSCs during osteogenic differentiation and encouragement of osteogenic differentiation [24]. We discovered that the 3-MA group had significantly lower levels of BECN1 protein and LC3-II/LC3-I ratio than the control group, although p62 expression was greater (Fig. 6A and B). In the 3-MA group, gene expression of Lc3, Becn1, and Atg5 reduced, demonstrating 3-MA's ability to prevent autophagy (Fig. 6C). Moreover, 3-MA reduced the protein expression of ALP, BSP, and RUNX2 on days 7 and 14 in order to block the osteogenesis differentiation that was induced by 2 nM RAPA (Fig. 6D–F). In the 2 nM RAPA with 3-MA group, ALP and ARS tests consistently showed lower ALP activity and mineralizing nodules (Fig. 6G and H). Moreover, 3-MA significantly reduced the gene expression level of osteogenesis that was induced by 2 nM RAPA (Fig. 6I and J). These results collectively revealed that 3-MA-induced autophagy inhibition hindered 2 nM RAPA-assisted BMSC osteogenic differentiation.

Conclusions

In conclusion, our study showed that BMSC basal autophagy decreases over time during osteogenic differentiation. Moreover, our findings suggested that sufficient RAPA concentrations may facilitate BMSC osteogenic differentiation by activating autophagy.

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