SIRT2 Mediated Microtubule Acetylation in Osteogenic Differentiation

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Objective: *To assess the role of microtubule acetylation in the transportation of amorphous calcium phosphate (ACP)-containing vesicles that mediate the osteogenic differentiation process of rat bone mesenchymal stem cells (BMSCs).*

Methods: *Rat BMSCs were cultured and transfected with sirtuin 2 (SIRT2) overexpression plasmids for an in vitro model. The microtubule acetylation-related protein levels were detected by western blots. The microtubule acetylation and the secretion rate of extracellular ACP-containing vesicles were observed with immunofluorescence and live cell fluorescence imaging. The secretion of ACP was observed by transmission electron microscopy. The mineralised nodule formation was stained with Alizarin Red S staining and observed by microscopy.*

Results: *Microtubule acetylation was increased during osteogenic differentiation of BMSCs, and microtubule transport efficiency was enhanced. Mechanically, microtubule acetylation is the key reason for the increased transportation rate of ACP-containing vesicles and enhanced osteogenic differentiation, as both were blocked after SIRT2-mediated microtubule acetylation inhibition.*

Conclusion: *Microtubule acetylation mainly promotes the transportation and secretion of ACP vesicles, and ultimately promotes the osteogenic differentiation process.*

Keywords: *biomineralization, intracellular transport, microtubule acetylation, osteogenic differentiation*

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During bone tissue healing, mineralisation is a highly prevalent and tightly regulated process. Abnormalities in this process can result in severe pathological conditions such as osteoporosis, osteoarthritis, osteogenesis imperfecta and Paget's disease.[1] Currently, bone healing is classified into two categories: intramembranous ossification and endochondral ossification. Unlike endochondral ossification, which involves stages such as fibrous callus formation, bony callus formation and bony callus remodelling and restructuring to promote bone tissue regeneration, intramembranous ossification involves direct ossification of the bone gap and reconstruction of the Haversian system, enabling the direct connection of the two ends of the fractured bone and thereby restoring the mechanical continuity of the skeleton.[2,3]

For most of the bone healing process, one of the most important events is the recruitment of osteoprogenitor cells to the injury site to then differentiate into osteoblasts; these newly formed osteoblasts then secrete calcium phosphate salts to facilitate bone regeneration.[4] More specifically, the earliest form of calcium phosphate salt is an amorphous precursor called amorphous calcium phosphate (ACP), which is conveyed to the cell membrane before being released into the extracellular matrix via exocytosis.[4-6] Once delivered into the collagen matrix outside the cell, ACP can further convert into octacalcium phosphate and

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subsequently undergo hydrolysis to form hydroxyapatite, the primary mineral component found in mature bone,[7] ultimately facilitating collagen fibre mineralisation. While mechanisms for mineralisation of collagen fibres have been established, numerous unanswered questions persist regarding the generation and transportation of ACP precursors to extracellular sites for mineralisation that require urgent clarification.

A substantial body of research has revealed the presence of mineral particles within intracellular vesicles of osteoblasts (also named matrix vesicles). These particles are synthesised within the cells and subsequently discharged into the extracellular environment via exocytosis.[8-10] The aforementioned studies suggest that after the intracellular synthesis of mineral precursor ACP, it is primarily localised to organelles such as mitochondria and autophagosomes, and is then transported to the extracellular matrix (ECM) utilising a microtubule-mediated transport system to trigger collagen mineralisation.[5,8] As a major constituent of the cellular cytoskeleton, microtubules participate in types of biological process, which exhibit a dynamic nature that enables constant switching between growth and shrinkage, facilitating exploration and sensing of intracellular space. The stringent modulation of their dynamics is vital for microtubule structure and function. Notably, it has been demonstrated that alterations in the structural stability of the microtubule system have a direct impact on bone formation.[11-13]

Tubulin post-translational modifications (PTMs) regulate microtubule properties and functions, attracting increased attention as the discovery of many more enzymes has enabled the functions of these rarely studied modifications to be determined.[10,14] While most known PTMs function on the outer surface of assembled microtubules, acetylation of a-tubulin's lysine 40 (K40) has been identified as a modification occurring on the inner surface, within the lumen of the microtubule.[15] Recent studies have pointed out that acetylation, as a type of post-translational modification in the microtubule system, plays an important role in regulating microtubule function.[16] More importantly, some studies illustrate that microtubule acetylation has various effects on biomineralisation depending on the tissue type. For example, Li et al[17] reported that promoting alpha-tubulin acetylation may contribute to interstitial mineral deposition for renal tubular epithelial; however, for the odontogenesis process, Zhan et al[18] did not find evidence of microtubule acetylation involvement in the differentiation of odontoblasts. In this study, the authors focused on biomineralisation related to osteogenic differentiation and paid closer attention to the level of microtubule acetylation within this process. The results showed that microtubule acetylation plays a role in the early stages of osteogenic differentiation in bone mesenchymal stem cells (BMSCs), whereas its significance diminishes in the later stages.

Therefore, this study constructed a BMSC osteogenic differentiation model and aimed to investigate the role of microtubule acetylation in biomineralisation and elucidate its internal mechanism in depth, and the results provide ideas for the development of new therapeutic strategies for regulating osteogenesis in the future.

Materials and methods

Chemicals

Foetal bovine serum and Dulbecco's modified Eagle medium were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Anti-α-tub (mouse mAb) was purchased from Abclonal (Woburn, MA, USA). Anti-α-tub (rabbit mAb) and -acet-α-tub antibodies were purchased from Proteintech (Chicago, IL, USA). Fluo-3 AM and Hoechst 33342 were purchased from Invitrogen (Thermo Fisher Scientific).

Cell culture

Rat BMSCs were purchased from Cyagen (Guangzhou, China), and then cultured in Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum. The culture medium was changed every 3 days. In vitro osteogenic differentiation was performed using the prepared osteoblast-inducing conditional media.

Plasmid construction and transfection

The SIRT2 overexpression plasmids were constructed from Vigenebio (Rockville, MD, USA) and prepared in growth medium and transfected utilising Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's protocol.

Cell fluorescence imaging for microtubule acetylation

After treatment of nontransfected or SIRT2 overexpressing plasmid-transfected BMSCs, the cells were incubated with antibodies against acet-α-tub (dilution 1:1000) and/or α-tub (dilution 1:200) overnight and the corresponding secondary antibody (dilution 1:100). Cellular fluorescence was observed using confocal microscopy (Leica,

Wetzlar, Germany) and analysed in Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

Western blot analysis

Cells were extracted and lysed, and the total proteins were collected. Then, the samples were heated at 100°C for 5 minutes for protein denaturation. 10 μg protein was separated by electrophoresis on a 10% sodium dodecyl sulphate (SDS) polyacrylamide gel. The separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked and incubated with a specific antibody and corresponding secondary antibody. The protein band was obtained using enhanced chemiluminescence (ECL) reagent (Meilunbio, Dalian, China) and analysed in ImageJ (National Institutes of Health, Bethesda, MD, USA).

RT-qPCR

Cells were extracted and lysed, and the total RNA were obtained. Then, cDNA was acquired from total RNA using Evo M-MLV RT Master Mix (Accurate Biology, Changsha, China). The target genes, SIRT2, were detected and analysed through real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) (LightCycler 96 Instrument, Roche, Basel, Switzerland). The mRNA expression levels of SIRT2 were normalized to those of GAPDH. The SIRT2 primer sequences were as follows: Forward Primer: CACGGCACCTTCTACACATCAC, Reverse Primer: CACGGCACCTTCTACACATCAC. GAPDH primer sequence: Forward Primer: GACATGCCGCCTGGAGAAAC, Reverse Primer: AGCCCAGGATGCCCTTTAGT.

Alizarin red S staining

After 4 weeks of osteogenic differentiation using osteogenic induction medium, the BMSCs were fixed with 4% paraformaldehyde for 30 to 45 minutes, then treated with alizarin red S solutions (KeyGEN, Nanjing, China). The osteogenic differentiation status was observed through microscopy (Zeiss, Stereo Lumar V12, Oberkochen, Germany), and the alizarin red was further extracted with cetylpyridinium chloride and detected using a spectrophotometer at an absorbance value of 562 nm.

Alkaline phosphatase (ALP) enzyme assay

The ALP activity was detected using an Alkaline Phosphatase Assay Kit (Beyotime, Shanghai, China), and the experiments were carried out in accordance with the manufacturer's protocol.

Live cell fluorescence imaging for evaluating the transportation speed of Ca^{2+} containing vesicles

The cells were seeded in 24-well plates. After treatment with or without the SIRT2 overexpression plasmid, the cells were washed gently three times and stained with Fluo-3 AM (100 nM) (Thermo Fisher Scientific) at 37°C for 30 minutes. Cellular fluorescence was observed and captured in real time by confocal microscopy (Leica) and analysed using Image-Pro Plus 6.0 software.

Transmission electron microscopy (TEM)

Before TEM to observe the secretion of ACP, the cells were washed twice. The collected cells were fixed with 2.5% glutaraldehyde, dehydrated with graded ethanol solutions and acetone, and finally embedded. Ultrathin sections were prepared and observed via an H-7500 TEM instrument (Hitachi, Tokyo, Japan).

Statistical analysis

Data were presented as mean ± standard deviation (SD). Each experiment was repeated at least three times. A one-way analysis of variance was performed, combined with a Fisher least significant difference multiple comparison post-hoc test to measure statistical significance for three or more groups. For comparison between two groups, a Student *t* test was applied. $P < 0.05$ was considered statistically significant. SPSS 21.0 (IBM, Armonk, NY, USA) was used for statistical analysis.

Results

Microtubule acetylation during osteogenic differentiation of BMSCs

Some studies have confirmed that microtubule acetylation can effectively maintain the stability of microtubule structure, while reducing the microtubule acetylation level with overexpression deacetylase could have a significant inhibitory effect on cell morphology and function.[19] We therefore asked whether the microtubule acetylation level changed during the osteogenic differentiation process for BMSCs. The immunofluorescence experiment in this study showed that the acetylation level of the microtubule system in BMSCs was significantly increased during osteogenic differentiation

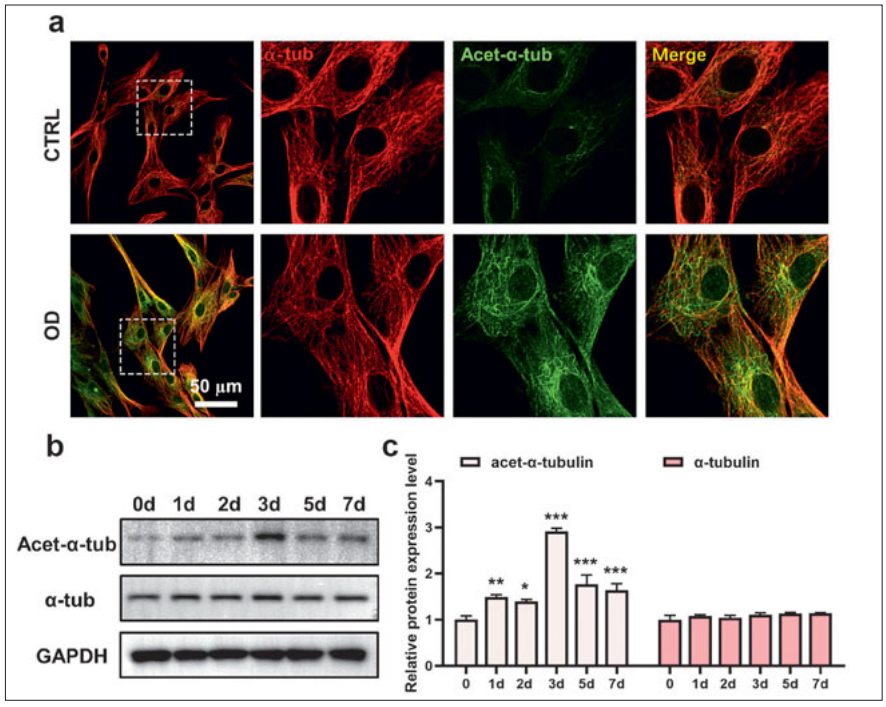

Fig 1a to c Acetylation of α-tubulin is involved in osteogenic differentiation of BMSCs. Representative fluorescence images of BMSCs immunostained for acet-α-tubulin (green) and α-tubulin (red) after osteogenic induction (OD) for 3 days **(a)**. Western blotting of acet-α-tubulin and α-tubulin expression in BMSCs treated with osteogenic induction for various durations. Changes in protein expression were quantified after normalisation to GAPDH **(b and c)**. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to the control group.

(Fig 1a). Similar results were obtained for the microtubule acetylation protein level: the protein levels of acetylated α-tubulin increased and peaked after 3 days of osteogenic differentiation and then declined until 7 days, but were still significantly higher than the base level (Fig 1b and c). The above data indicate that microtubule acetylation is likely to be an important biological phenomenon during osteogenic differentiation, but its exact role is still unknown.

Microtubule transportation efficiency of BMSCs was enhanced during osteogenic differentiation

Microtubules act as the main material transportation channel in cells and play a key role in ACP transportation and secretion. By using calcium ion marker Fluo-3 to label intracellular calcium ions, the transport rate of calcium ions was dynamically observed under a confocal microscope in living cells. The results showed that the transport rate of intracellular calcium during the osteogenic differentiation process for BMSCs was accelerated significantly (Fig 2a and b). Meanwhile, TEM data showed that the amount of extracellular ACP increased significantly after 7 days of osteogenic differentiation compared with the control group, indicating that ACP secretion occurred (Fig 2c). The above results suggest that microtubule transport efficiency is indeed significantly accelerated in the osteogenic differentiation process of BMSCs.

Microtubule acetylation is the key reason for the enhancement of transportation efficiency

Microtubule acetylation modification can improve microtubule work efficiency.[20,21] For example, acetylated microtubules can enhance microtubule transport efficiency and ultimately promote intracellular material secretion.[22,23] Thus, microtubule acetylation may be an important factor for regulating transport efficiency during the osteogenic differentiation process of BMSCs. To further clarify the crucial role of microtubule acetylation in osteogenic differentiation, the present authors constructed an overexpression plasmid for deacetylase SIRT2.[24] Existing studies have demonstrated that SIRT2 can interact with microtubule proteins through Co-Immunoprecipitation experiments.[25,26] Firstly, our data showed that the SIRT2 expression level was declined at 2 and 3 days and then increased to baseline at 7 days during BMSCs osteogenic differentiation, which indicated an opposite tendency compared to microtubule acetylation levels (Fig 3a). After overexpression of SIRT2, RT-qPCR detection showed that the gene expression of SIRT2 increased in BMSCs treated with osteogenic induction medium (Fig 3b). Importantly, immunofluorescence experiments showed the increase of acetylated α-tubulin protein expression level that occurred during BMSCs osteogenic differentiation was inhibited significantly after overexpression of SIRT2 (Fig 3c). Meanwhile, western blotting results also showed simi-

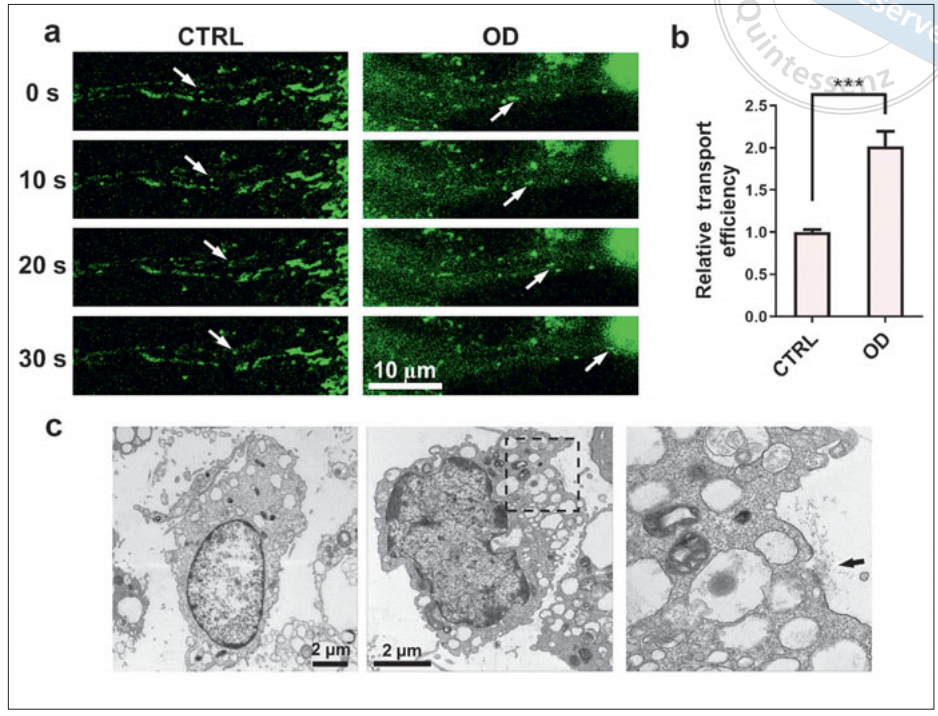

Fig 2a to c Microtubule-mediated intracellular transport increase during osteogenic differentiation of BMSCs. Live fluorescence images of calcium transportation (arrows) in BMSCs after osteogenic induction for 3 days. The translocation rate of calcium was quantified **(a and b)**. Representative TEM images showing releasement of ACP (arrows) after BMSCs were treated without or with osteogenesis induction medium for 7 days **(c)**. ***P < 0.001 compared to the control group.

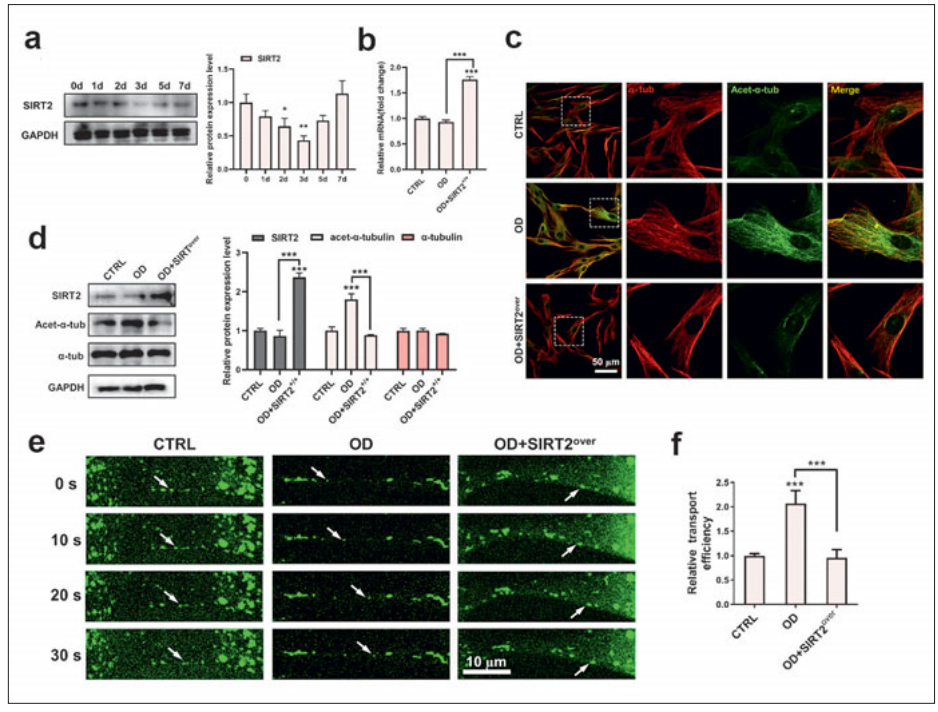

Fig 3a to f SIRT2 overexpression reduces the transportation efficiency via microtubule acetylation inhibition. Western blotting of SIRT2 expression in BMSCs treated with osteogenic induction for various durations. Changes in protein expression of SIRT2 were quantified after normalisation to GAPDH **(a)**. RT-qPCR detection of SIRT2 mRNA level in BMSCs after osteogenic induction for 3 days with or without SIRT2 overexpression. Changes in gene expression of SIRT2 were quantified after normalisation to GAPDH **(b)**. BMSCs were transfected with SIRT2 overexpression plasmid, then immunostained with antibodies against acet-α-tubulin (green) and α-tubulin (red), respectively **(c)**. Western blotting of SIRT2, acet-α-tubulin and α-tubulin expression in BMSCs after osteogenic induction for 3 days with or without SIRT2 overexpression. Changes in protein expression were quantified after normalization to GAPDH **(d)**. Live fluorescence images of calcium transportation (arrows) in BMSCs after osteogenic induction for 3 days with or without SIRT2 overexpression. The translocation rate of calcium was quantified **(e and f)**. *P < 0.05, **P < 0.01, ***P < 0.001 compared to the control group or the OD group as indicated.

lar results (Fig 3d). These data indicate that SIRT2 is an important enzyme that regulates microtubule acetylation, and its overexpression can indeed regulate the level of microtubule acetylation during osteogenic differentiation.

Secondly, after constructing a deacetylase SIRT2 overexpression plasmid to inhibit intracellular microtubule acetylation level, we evaluated the transport rate of calcium ion vesicles (such as ACP). The results showed that overexpressing SIRT2 can effectively inhib-

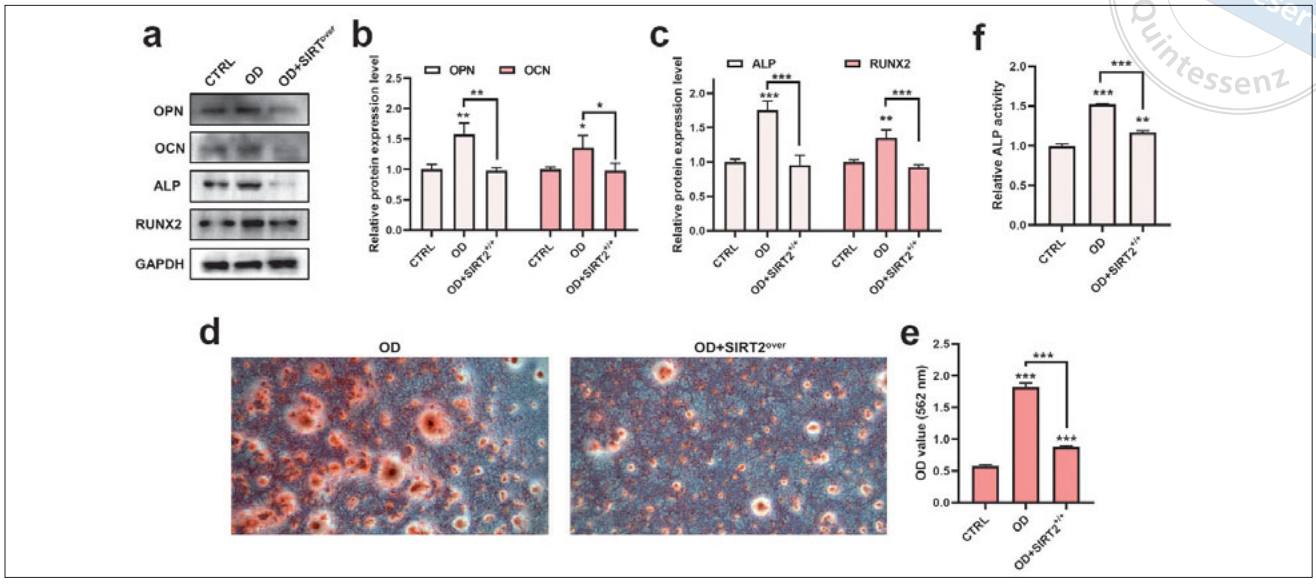

Fig 4a to f Reduced α-tubulin acetylation further hinders the osteogenic differentiation process. BMSCs were transfected with SIRT2 overexpression plasmid, then western blotting of matrix proteins and proteins related to osteogenic differentiation in BMSCs treated with osteogenic induction for 7 days. Changes in protein expression were quantified after normalisation to GAPDH **(a to c)**. Representative images of Alizarin red staining in BMSCs treated with osteogenic induction for 28 days (×50 magnification) **(d)**. Quantitative analyses of Alizarin red staining as treatment in **(d) (e)**. Quantitative analyses of ALP activities in BMSCs treated with osteogenic induction for 7 days **(f)**. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to the control group or the OD group as indicated.

it the transport rate of intracellular calcium ion vesicles (Fig 3e and f). Thus, we indicate that microtubule acetylation is one crucial factor for enhancing cellular transport efficiency during osteogenic differentiation.

Inhibition of microtubule acetylation blocks the osteogenic differentiation process

It is important to examine whether microtubule acetylation enhanced transportation efficiency will further affect the osteogenic differentiation effect and ultimately impact the biomineralisation process. The present authors detected the changes in the expression level of key osteogenic differentiation proteins in BMSCs under the condition of SIRT2 overexpression. The results showed that key osteogenic differentiation proteins and matrix proteins, such as ALP, Runt-related transcription factor 2 (RUNX2), osteocalcin (OCN) and osteopontin (OPN), increased during osteogenic differentiation, but they were all significantly inhibited under SIRT2 overexpression for reducing the intracellular microtubule acetylation level (Fig 4a to c). In addition, by using Alizarin red to detect the level of mineralised nodule formation, the results showed that osteogenic differentiation of BMSCs can achieve a good mineralisation induction effect that mainly manifests as a significant increase in the level of mineralised nodule formation. However, by overexpressing SIRT2 to reduce intracellular microtubule acetylation, the abovementioned mineralised nodule formation effect was significantly blocked (Fig 4d and e). We also detected ALP activity with or without overexpressing SIRT2 during osteogenic differentiation of BMSCs, and similar results were obtained to show that increased ALP levels were observed under osteogenic differentiation, but inhibited after overexpressing SIRT2 (Fig 4f). These data all suggest that the osteogenic differentiation process is indeed regulated by microtubule acetylation, where enhanced transportation efficiency is one main influential factor.

Discussion

The cytoskeleton is known to function in mechanical sensing and mechanical transduction. Interactions between cytoskeletal proteins, integrins and mechanical forces can affect the cell shape, proliferation and even differentiation.[27,28] Because of this, there has been increasing interest in the interplay between the cytoskeleton and stem cell differentiation. Interestingly, microtubules have been shown to play an indistinct role

in osteogenic differentiation, as it was observed that the morphology and structure of microtubules did not change significantly during the differentiation of mesenchymal stem cells.[28] However, during biomineralisation, osteoblasts must transport substantial amounts of mineral matrix out of the cell into the extracellular matrix. The microtubule system is a key component of the cytoskeleton involved in regulating this material transport. Thus, understanding how the biological function of microtubules evolves during biomineralisation is a compelling issue that warrants further investigation.

PTMs on microtubules are a key controller of microtubule properties and functions.[29] Among these, microtubule acetylation is recognised as the most stable modification, primarily regulated by the recruitment of the αTAT1 protein to the microtubule lumen, making it less susceptible to interference from other biological enzymes.[19,30,31] More intriguingly, a previous study by the present authors also reported that enhancing microtubule acetylation is beneficial for alleviating lysosomal autophagy blockades resulting from microtubule disruption, where the enhanced microtubule acetylation helps maintain microtubule structure, significantly promoting vesicle transport and fusion, and thereby sustaining normal metabolic functions.[32] Thus, in this study, exploring how microtubule acetylation mediates mineral transport could provide new insights into the biomineralisation process.

The present data demonstrate that microtubule acetylation plays a critical role in ACP-containing vesicle transportation during osteogenic differentiation, which is needed for biomineralisation.[33,34] However, other important factors also can influence ACP formation and biomineralisation, such as lysosome mediated biogenesis of matrix vesicles.[35] Some articles have indicated that matrix vesicles are vesicular structures rich in calcium and phosphate, contaiing organic materials such as acidic proteins. Lysosomes are thought to be one type of early matrix vesicle, capable of enriching calcium and phosphate and subsequently transporting ACP without crystallisation.[34,36] As we reported previously, lysosome transportation could also be mediated via microtubule acetylation.32 Given this, it is essential to investigate whether microtubule acetylation also participate in the lysosomal transportation and secretion, and then influence the biogenesis of matrix vesicles and ACP formation during biomineralisation. Exploring these issues could yield more valuable insights into the regulatory mechanisms of microtubule acetylation underlying biomineralisation.

Furthermore, osteogenesis is not limited to osteoblasts but also involves biological functions among various cell types within the bone microenvironment, including osteocytes, endothelial cells and immune cells. Given the wide range of cell types that participate in bone formation,[37] it is conceivable that modulation of microtubule acetylation during osteogenesis should have a widespread effect on a variety of cells. For instance, microtubule dynamics are known to influence the behaviour of osteoclasts during bone resorption and could impact the balance between bone formation and degradation.[12] As such, more research should be funded to investigate how the modulation of microtubule acetylation affects the biological process of other crucial cells within the bone microenvironment, which not only help to achieve a comprehensive understanding of the function of the microtubule system in bone formation, but also may pave the way for the development of novel therapeutic strategies that target the osteogenic microenvironment as a whole. Therefore, while the role of microtubule acetylation in osteogenic differentiation is becoming clearer, much remains to be explored regarding its function in other biological processes and cell types participating in bone formation. Future research should aim to expand understanding of the osteogenic microenvironment and the potential role of microtubule modifications across different cell types in bone formation.

Conclusion

In summary, microtubule acetylation is one of the key biological phenomena in the osteogenic differentiation of BMSCs, where increased microtubule acetylation could promote the transportation and secretion of ACP vesicles, and ultimately the osteogenic differentiation process.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Dr Xin Ru ZHOU contributed to the experiments and manuscript draft and editing; Drs Can ZHANG, Chen Rong XU, Xin Er TAN, Qian Qian HAN, Xi YANG and Tian Yu SUN contributed to the data collection or methodology; Dr Long Quan SHAO contributed to study supervision; Dr Jia LIU contributed to study conceptualisation, supervision and critical revision of the manuscript.

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