Original Article

DOI: 10.5582/ddt.2015.01008

Tauroursodeoxycholic acid attenuates inorganic phosphate-induced osteoblastic differentiation and mineralization in NIH3T3 fibroblasts by inhibiting the ER stress response PERK-eIF2α-ATF4 pathway

Fang Liu[1,2], Yazhou Cui[1], Pinglan Ge[1,2], Jing Luan[1], Xiaoyan Zhou[1], Jinxiang Han[1,*]

[1] *Key Laboratory for Biotech Drugs of the Ministry of Health, Shandong Medical Biotechnological Center, Shandong Academy of Medical Sciences, Ji'nan, Shandong, China;*
[2] *School of Medicine and Life Sciences, University of Jinan-Shandong Academy of Medical Science, Ji'nan, Shandong, China.*

Summary **Ectopic ossification occurs in a wide range of common and genetic diseases, but its molecular mechanisms and effective therapeutic targets remain to be clarified. The aim of the study is to investigate whether endoplasmic reticulum (ER) stress is involved in ectopic ossification and ER stress inhibitor tauroursodeoxycholic acid (TUDCA) has potential to treat the pathological conditions. In this study, inorganic phosphate (Pi)-induced NIH3T3 fibroblasts induced osteogenesis and mineralization was used as an *in vitro* model for ectopic ossification. Various concentrations of TUDCA (0.1, 1, 5, 10 μM) were added during osteogenic induction. Osteoblast differentiation and minerlization were determined by RT-qPCR, alkaline phosphatase (ALP) activity assay, Alizarin Red-S (AR-S) staining, and calcium deposition. ER stress signalling components were detected by Western-blot analysis. We found ER stress was activated by inorganic phosphate in NIH3T3 cells. During osteogenic induction, TUDCA inhibited NIH3T3 cells ALP activity and mineral nodule formation. In addition, TUDCA caused decreased expression of osteoblastic markers *Runx2*, *Col1a1*, *ALP*, *OCN*. Mechanistically, TUDCA inhibited the ER stress response PERK-eIF2α-ATF4 pathway during osteogenesis. In conclusion, TUDCA could inhibit fibroblasts mineralization *via* supressing the ER stress response PERK-eIF2α-ATF4 pathway, and has potential pharmacologic and therapeutic applications for treating ectopic ossification associated diseases.**

Keywords: Endoplasmic reticulum stress, ectopic ossification, tauroursodeoxycholic acid, eIF2α, AFT4

1. Introduction

Ectopic ossification is a complex process characterized by the formation of ectopic bone or the deposition of calcium phosphate complexes within muscles, connective tissue, or nerves in aberrant locations (*1-4*). The ossification processes may be caused by heredity, surgical intervention or trauma (*2,3*). Common diseases for ectopic ossification include diffuse idiopathic skeletal hyperostosis, ankylosing spondylitis, osteoarthritis, and other heritable disorders of vascular and skin mineralization (*2,4*). The molecular mechanism of ectopic ossification has always been a concern, but the exact pathogenic mechanism remains elusive. At present, in the surgical setting, the two most common preventative modalities are radiotherapy and indomethacin, a non-steroidal anti-inflammatory drug (NSAID), but both treatment modalities carry the risk of particular side effects (*2*), therefore, effective treatment is extremely urgent.

The endoplasmic reticulum (ER) is a cellular compartment that plays a critical role in protein synthesis, folding, and transportation including important proteins in osteoblast differentiation, such as osteocalcin and

**Address correspondence to:*
Dr. Jinxiang Han, Key Laboratory for Rare Disease Research of Shandong Province, Key Laboratory for Biotech Drugs of the Ministry of Health, Shandong Medical Biotechnological Center, Shandong Academy of Medical Sciences, Ji'nan, Shandong 250062, China.
E-mail: jxhan9888@aliyun.com

collagen (*5*). Efficient functioning of the ER is essential for cellular function and survival. Once ER homeostasis is disrupted in a number of cellular stress conditions, such as UV, viral infection, nutritional deprivation and others (*6*), unfolded and misfolded proteins will accumulate in the ER lumen, resulting in ER stress, and the unfolded protein response (UPR) occurs (*7-12*). The ER stress response PERK-eIF2α-ATF4 pathway can be activated. Zhang *et al*. (*13*) reported PERK was required for prenatal and postnatal bone development. PERK was responsible for highly phosphorylated eIF2α, in Perk-/- mice, and the secretory pathway in osteoblasts cannot efficiently process and secrete procollagen, which caused severe skeletal dysplasias. Wei *et al*. (*14*) also found PERK was essential for neonatal skeletal development to regulate osteoblast proliferation and differentiation. Perk-/- mice were severely osteopenic, which was caused by a deficiency in the number of mature osteoblasts, impaired osteoblast differentiation, and reduced type I collagen secretion. Atsushi Saito *et al*. (*15*) demonstrated that ATF4 expression and function were influenced by the loss of PERK *in vivo* and *in vitro* and confirmed that ER stress during osteoblast differentiation activated PERK-eIF2α-ATF4 signaling followed by the promotion of gene expression essential for osteogenesis such as osteocalcin (*OCN*) and bone sialoprotein (*BSP*). Numerous reports have suggested ER stress occurs and is involved in osteoblast differentiation, however whether ER stress is associated with ectopic ossification remains uninvestigated.

TUDCA is a bile acid present in human bile at a low concentration and has a very good safety profile, and the normal concentration range in human plasma is 0.4-4 μM. It has been proved that TUDCA can enhance ER folding ability to prevent protein aggregation and thus protect cells against ER stress (*16*). TUDCA has been widely used in treatment of disease, such as, cholelithiasis, cholestatic liver disease, diabetes mellitus, obesity, and atherosclerosis, it works *via* preventing ER stress, as a classical ER stress inhibitor (*17-22*). Studies have demonstrated that TUDCA could markedly prevent Aortic valve (AV) calcification, and attenuate AV osteoblastic differentiation in both rabbit and mouse models of AV calcification *via* inhibition of ER stress and could suppress oxidized low density lipoprotein (oxLDL)-induced osteoblastic differentiation in cultured valvular interstitial cells (VICs) (*23*) and could alleviate advanced glycation end product-induced apoptosis and osteoblastic differentiation of stromal cells *via* alleviating ER stress (*22,24*).

In this study, we induced NIH3T3 cells mineralization with 3 mM inorganic phosphate ($NaPO_4$, Pi), treated with different amounts of TUDCA (0.1, 1, 5, 10 μM), examined the important indicators of osteoblastic differentiation and mineralization and ER stress response to investigate if ER stress is associated with ectopic ossification and can TUDCA inhibit fibroblasts ectopic ossification *via* supressing the ER stress response PERK-eIF2α-ATF4 pathway.

2. Materials and Methods

2.1. *Cell culture and treatment*

NIH3T3 cell line was acquired from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO_2 atmosphere at 37°C. The cells were cultured either in basic or osteogenic medium (OM) supplemented with 3 mM inorganic phosphate ($NaPO_4$, Pi) (Sangon Biotech, Shanghai, China) as described previously (*25*). Different concentrations (0.1, 1, 5, 10 μM) of TUDCA (Santa Cruz, USA) were added as indicated.

2.2. *Quantitative real-time polymerase chain reaction (RT-qPCR) analysis*

Total RNA was isolated from the cultures using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized with ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). RT-PCR was performed with THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) using LightCycler 480 thermocycler (Roche Applied Science, Mannheim, Germany). Relative mRNA levels of all genes were first normalized to the levels of 36B4 and then normalized to the average of negative control group levels. The primer sequences for the transcripts quantified by this method are shown (Table 1).

2.3. *ALP avtivity assay*

NIH3T3 cells were cultured with osteogenic medium (OM) for 9 days and then treated with TUDCA at given concentrations as described above in basic

Table 1. Quantitative real-time PCR was performed using each specific primer set

Target genes	Primer sequences
Runx2-fwd	AGTAGCCAGGTTCAACGATCTGA
Runx2-rev	GACTGTTATGGTCAAGGTGAAACTCTT
Col1a1-fwd	CACCCCAGCCGCAAAGAGT
Col1a1-rev	CGGGCAGAAAGCACAGCACT
ALP-fwd	TGGCTCTGCCTTTATTCCCTAGT
ALP-rev	AAATAAGGTGCTTTGGGAATCTGT
OCN-fwd	TGCTTGTGACGAGCTATCAG
OCN-rev	GAGGACAGGGAGGATCAAGT
36B4-fwd	AAGCGCGTCCTGGCATTGTCT
36B4-rev	CCGCAGGGGCAGCAGTGGT

fwd, forward; rev, reverse.

medium for 24 hours, ALP activity was performed by the standard protocols. Briefly, NIH3T3 cells were washed with phosphate-buffered saline (PBS) (pH 7.4) and lysed with non-denatured tissue/cell lysate, RIPA (Solarbio, China). ALP activity was assayed using the commercial kit (LabAssay ALP kit, Wako, Japan), and protein content was determined with BCA protein assay (Beyotime, Shanghai, China). ALP activity was normalized by the protein content.

2.4. *Mineralization analysis*

For mineralization analysis, NIH3T3 cells were cultured with osteogenic medium (OM) treated with TUDCA at given concentrations as described above for 14 days. Mineralization was determined using Alizarin Red-S (AR-S) staining kit (GENMED, USA) by the standard protocols. For quantitation of mineralization analysis, the stained cultures were incubated with 10% (w/v) cetylpyridium chloride at 37°C for 1 h, optical density of the supernatant was measured at 562 nm.

2.5. *Quantification of calcium deposition*

NIH3T3 cells were cultured with osteogenic medium (OM) treated with TUDCA at given concentrations as described above for 9 days. Calcium deposition in the plates was quantified by the o-cresolphthalein complexone method using a calcium colorimetric assay kit (Sigma, USA) in accordance with the manufacturer's instructions. Briefly, cells were washed with tris-buffered saline and decalcified using 0.6 N HCl for 24 hours at room temperature, and the calcium content of HCl supernatants was subsequently subjected to calcium colorimetric assay. After decalcification, each cell layer was washed with PBS and solubilized with 0.1 N NaOH containing 0.1% SDS. The protein concentration was measured with BCA protein assay (Beyotime, Shanghai, China). The calcium content of the cell layer was normalized to protein content.

2.6. *Western blot analysis*

Proteins were extracted from NIH3T3 cells under the designated conditions using Western Blot-IP lysis buffer (Beyotime, Shanghai, China). The lysates were incubated on ice for 45 min. After centrifugation at 14,000 × g for 15 min, the soluble proteins in the extracts were quantified. Protein-equivalent samples were subjected to SDS-PAGE for Western blotting. The following antibodies were used: anti-P-eIF2α (1:1,000, CST, USA), anti-T-eIF2α (1:1,000, CST, USA), anti-ATF4 (1:1,000, PTG, China), anti-Runx2 (1:1,000, Stan Cruz, USA), anti-β-actin (1:1,000, PTG, China). Immunoreactivity was determined using enhanced chemiluminescence (ECL) (Millipore Corporation, Billerica, MA, USA) chemiluminescence reaction.

2.7. *Statistics*

Measurements in each experiment were run in triplicate. For quantitative data, results are reported as the mean ± S.D. To determine the differences between groups, one-way analysis of variance (ANOVA) was carried out using SPSS software (version 17.0), with significance accepted at $p < 0.05$.

3. Results

3.1. *Effects of TUDCA on the expression of osteoblast-specific markers in Pi-induced NIH3T3 cells*

RT-qPCR was performed to measure the expression of osteoblast-specific markers including *Runx2*, *Col1a1*, *ALP* and *OCN*. As shown in Figure 1, compared with the osteogenic group, the mRNA expression of *Runx2*, *Col1a1*, *ALP* and *OCN* significantly decreased at 24 hours or 9 days with treatment of various concentrations of TUDCA (0.1, 1, 5, 10 μM) in a dose-dependent manner. However, the osteogenic group mRNA expression of *ALP* also decreased compared with the negative control group at 9 days.

3.2. *TUDCA inhibited ALP activity of NIH3T3 cells induced by inorganic phosphate*

ALP activity was assessed as an important indicator of osteoblastic lineage to study the effect of TUDCA on osteogenic differentiation. ALP activity was

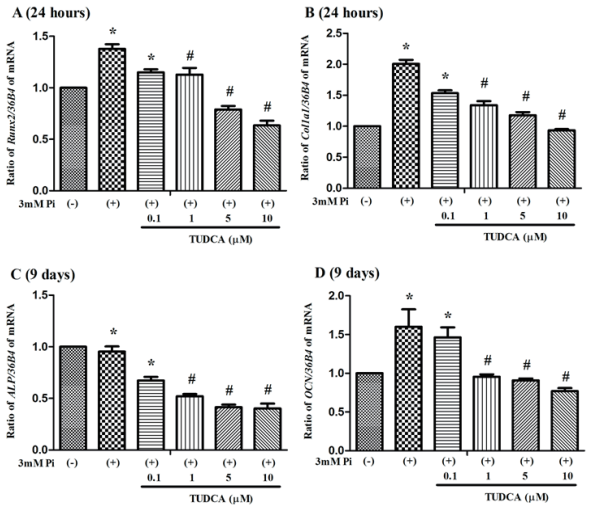

Figure 1. Effects of TUDCA on the expression of osteoblast-specific markers in Pi-induced NIH3T3 cells. Cells were cultured in osteogenic medium (OM) for 6 hours and then treated with TUDCA in basic medium for 24 hours or in osteogenic medium (OM) supplemented with TUDCA for 9 days. Total RNA was extracted and measured by RT-qPCR for *Runx2* **(A)**, *Col1a1* **(B)**, *ALP* **(C)** and *OCN* **(D)**. All mRNA expression levels were normalized to 36B4. (mean ± S.D., $n = 3$). * $p < 0.05$, *vs*. negative control group; # $p < 0.05$, *vs.* osteogenic induction group.

determined at 10 days (osteogenic induction for 9 days and treatment with TUDCA in basic medium for 24 hours). As shown in Figure 2, with osteoblast induction, ALP activity increased, when treated with various concentrations of TUDCA, ALP activity significantly decreased in a dose-dependent manner (21%, 34.8%, 35%, and 37.2% decreases with 0.1, 1, 5 and 10 μM of TUDCA, respectively). These data suggest that TUDCA can significantly inhibit ALP activity during osteogenic differentiation in NIH3T3 cells in a dose-dependent manner.

3.3. *TUDCA inhibited mineralization of NIH3T3 cells induced by inorganic phosphate*

To evaluate the effect of TUDCA on mineralization in the NIH3T3 cells matrix, alizarin red staining was performed at 14 days with osteogenic induction in the presence or absence of TUDCA at given concentrations. As is shown in Figure 3A, obvious matrix mineralization can be observed, but mineralization decreases with TUDCA treatment, the higher the concentration of TUDCA the more obvious inhibition. The quantitation of mineralization data (Figure 3B) suggests that TUDCA can significantly inhibit mineralization of NIH3T3 cells during osteogenic differentiation in a dose-dependent manner (3.5%, 8.3%, 10.4%, and 12.6% decreases with 0.1, 1, 5 and 10 μM of TUDCA, respectively).

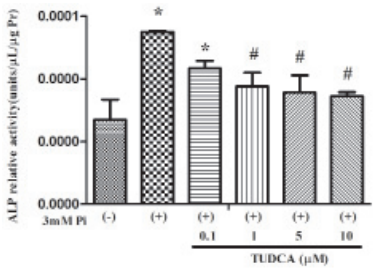

Figure 2. TUDCA inhibited ALP activity of NIH3T3 cells induced by inorganic phosphate. NIH3T3 cells were cultured in osteogenic medium for 9 days and then in basic medium with TUDCA (0.1, 1, 5, 10 μM) for 24 hours. ALP activity was assessed on day 10. (mean ± S.D., $n = 3$). $^{*}p < 0.05$, *vs*. negative control group; $^{\#}p < 0.05$, *vs*. osteogenic induction group.

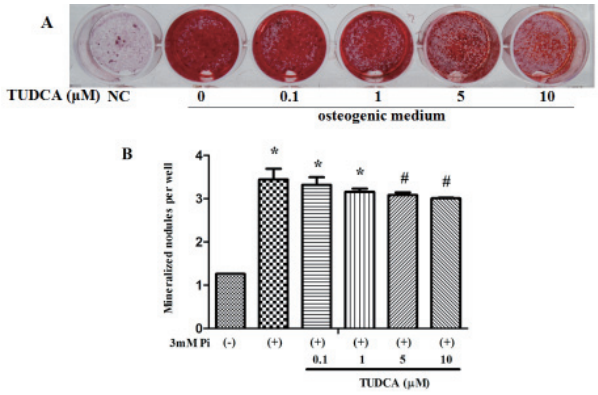

Figure 3. TUDCA inhibited mineralization of NIH3T3 cells induced by inorganic phosphate. Observations for mineralization **(A)** and quantitation of mineralization **(B)**. NIH3T3 cells were cultured in osteogenic medium (OM) with TUDCA (0.1, 1, 5, 10 μM) for 14 days and incubated with 10% (w/v) cetylpyridium chloride at 37°C for 1 h, optical density of the supernatant was measured at 562 nm. (mean ± S.D., $n = 3$). $^{*}p < 0.05$, *vs*. negative control group; $^{*}p < 0.05$, *vs*. negative control group; $^{\#}p < 0.05$, *vs*. osteogenic induction group. NC: negative control.

3.4. *Effects of TUDCA on calcium deposition of NIH3T3 cells induced by inorganic phosphate*

To further evaluate the effect of TUDCA on calcification in NIH3T3 cells, quantification of calcium deposition was examined at 9 days under different conditions. In basic medium, NIH3T3 cells accumulated very little calcium mineral, in contrast, in the presence of 3 mM Pi, calcium deposition dramatically increased as shown in Figure 4 (calcified group versus uncalcified control: 41.5), but calcium deposition in NIH3T3 cells decresaed with TUDCA treatment (4.7%, 14%, 14.6%, and 32.6% decreases with 0.1, 1, 5 and 10 μM of TUDCA, respectively).

3.5. *ER stress is activated by inorganic phosphate in NIH3T3 cells*

To determine whether ER stress is activated by inorganic phosphate in NIH3T3 cells compared to other conditions, such as thapsigargin and tunicamycin (*6*), the expression of total and phosphorylated eIF2α in ER

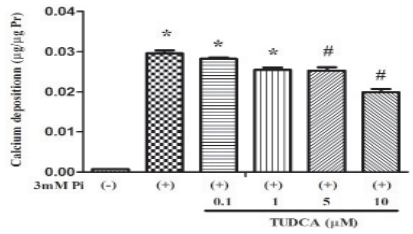

Figure 4. Effects of TUDCA on calcium deposition of NIH3T3 cells induced by inorganic phosphate. NIH3T3 cells were cultured in osteogenic medium (OM) with TUDCA (0.1, 1, 5, 10 μM) for 9 days. Cells were decalcified with 0.6 N HCl for 24 hours. The calcium content was determined by the o-cresolphthalein complexone method. After decalcification, the protein of the cell layer was extracted. The calcium content of the each cell layer was normalized to protein content. Data were mean ± S.D., $n = 3$. $^{*}p < 0.05$, *vs*. negative control group; $^{\#}p < 0.05$, *vs*. osteogenic induction group.

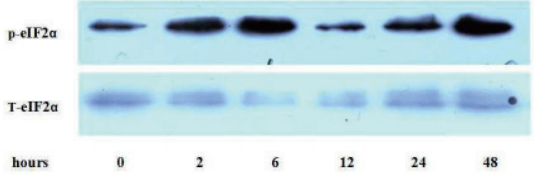

Figure 5. ER stress is activated by inorganic phosphate in NIH3T3 cells. NIH3T3 cells were cultured in osteogenic medium for 0, 2, 6, 12, 24 hours. The proteins were extracted for Western blot analysis of the expression of total and phosphorylated eIF2α (P-eIF2α and T-eIF2α).

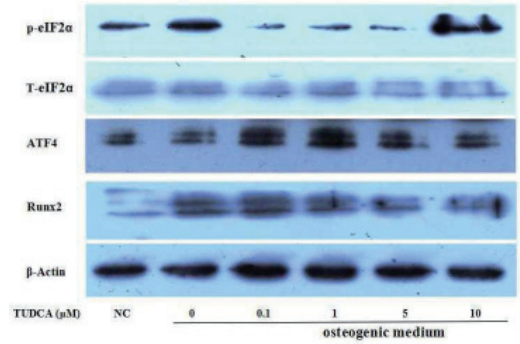

Figure 6. TUDCA inhibits the ER stress response PERK-eIF2α-ATF4 pathway in NIH3T3 cells induced by inorganic phosphate. Cells were cultured in osteogenic medium (OM) for 6 hours and then treated with TUDCA in basic medium for 24 hours. Proteins were extracted for Western blot analysis.

stress response mediated PERK-eIF2α-ATF4 signaling was evaluated by Western blot analysis. In Figure 5, inorganic phosphate ($NaPO_4$, Pi) was shown to induce phosphorylated eIF2α, especially at 6 hours and 48 hours, while no significant change occurred in the level of total eIF2α. Western blot analysis revealed Pi could stimulate ER Stress, like thapsigargin and tunicamycin.

3.6. *TUDCA inhibits the ER stress response PERK-eIF2α-ATF4 pathway in NIH3T3 cells induced by inorganic phosphate*

To further address the mechanism by which TUDCA inhibits Pi-induced NIH3T3 osteoblastic differentiation and mineralization, we examined the levels of total and phosphorylated eIF2α and ATF4 in the ER stress response PERK-eIF2α-ATF4 pathway in NIH3T3 cells induced by inorganic phosphate. As depicted in Figure 6, Western blot analysis demonstrated TUDCA significantly attenuated phosphorylation of eIF2α and ATF4, while no significant changes in the level of total eIF2α. Runx2 occurred, as a master regulator of osteoblast differentiation and bone formation, was examined, as depicted, TUDCA also attenuated the expression of Runx2. Together with other results, these results indicate that TUDCA can inhibit NIH3T3 fibroblast mineralization *via* suppressing the ER stress response PERK-eIF2α-ATF4 pathway.

4. Discussion

NIH3T3 is a fibroblastic cell line with osteoblastic potential similar to C3H10T1/2 cells and MC3T3 cells, and can be induced into osteoblastic differentiation and mineralization *in vitro* (*26*), although it does not retain an ability to differentiate into osteoblast-like cells and is often used as a negative control during the study of osteoblastogenesis. As a mineralization inducer, inorganic phosphate ($NaPO_4$, Pi) has been widely used to induce osteoblast differentiation and mineralization (*25,27*), and Beck GR Jr *et al*. (*25*) has reported inorganic phosphate (Pi), as an inducer, can induce osteopontin expression in NIH3T3 cells in response to increased phosphate levels similar to the effect seen in MC3T3-E1 cells, namely, inorganic phosphate ($NaPO_4$, Pi) can induce osteoblastic differentiation and mineralization in NIH3T3 cells. Therefore, in this study, inorganic phosphate (Pi)-induced NIH3T3 fibroblasts induced osteogenesis and mineralization was used as an *in vitro* model for ectopic ossification.

Ectopic ossification occurs in a wide range of common and genetic diseases, but its molecular mechanisms and effective therapeutic targets remain to be clarified. In this study, we first explored the effect of TUDCA on the NIH3T3 fibroblast mineralization *in vitro* model for ectopic ossification. *Runx2* is a crucial transcription factor, is expressed in the earliest stage of osteogenic differentiation, and can trigger osteoblast differentiation as well as bone ECM proteins collagen type I (Col1a1) and osteocalcin (OCN) (*28,29*). ALP is an early marker of osteogenic differentiation. Col1a1 is the key for extracellular matrix (*30*). OCN is an osteoblast-specific marker for the late stage of osteoblast differentiation (*15*). Therefore, the expression of important osteoblast-specific genes, *Runx2*, *Col1a1*, *ALP* and *OCN* were assayed at 24 hours or 9 days. RT-qPCR results demonstrated that TUDCA significantly inhibited osteoblast-specific gene expression, and in addition, ALP activity, as an important indicator of osteoblastic differentiation also was assessed. Together with Alizarin Red-S (AR-S) staining and quantitation of mineralization and calcium deposition assays, all of these results indicated TUDCA could play an inhibition role in a dose-dependent manner in NIH3T3 cells during osteogenic differentiation. Therefore, we can conclude that TUDCA can inhibit ectopic ossification as a chemical chaperone.

A number of cellular stress conditions, UV, viral infection, and nutritional deprivation can trigger ER stress, and ER stress can also be induced by thapsigargin and tunicamycin (*6*). In this study, we also further demonstrated that ER stress could be activited by inorganic phosphate (Pi) in NIH3T3 fibroblasts, like thapsigargin and tunicamycin. Reports have suggested ER stress was induced during osteoblast differentiation and could activate ER stress response PERK-eIF2α-ATF4 pathways (*13-15*). In the PERK-eIF2α-ATF4 pathway, active PERK phosphorylates eIF2α, specifically promoting the translation of the activating transcription factor 4 (ATF4). ATF4 has been proven to be a critical transcription factor downstream of PERK signaling branches of ER stress, ATF4 can not regulate osteocalcin and bone sialoprotein transcription but also preserves mature osteoblast function including the synthesis of collagen, the most abundant extracellular protein found in bones (*15*). As a classical ER stress inhibitor, TUDCA has been proven to decrease the expression of phosphorylated eIF2α and ATF4 (*16-22*). In a recent study, Zhejun Cai *et al*. (*23*), found that TUDCA

protected against oxLDL-induced ER stress in VICs, significantly suppressed osteoblastic differentiation and protected against hypercholesterolemia-induced AV calcification in animal models. Therefore, in our study, we also investigated the effect of TUDCA on ER stress response mediated PERK-eIF2α-ATF4 pathway in an *in vitro* model for ectopic ossification, found TUDCA significantly suppressed the expression of phosphorylated eIF2α and ATF4, and the result is consistent with previous reports. Runx2, as a master regulator of osteoblast differentiation and bone formation, was examined, and TUDCA also attenuated the expression of Runx2. Taken together, we conclude ER stress is involved in ectopic ossification and TUDCA can inhibit mineralization *via* alleviating ER stress.

In addition, the chemical chaperone TUDCA has been proven to be protective in various diseases, such as, diabetes mellitus, obesity, and atherosclerosis, *via* prevention of ER stress. Previous reports show TUDCA enhances the adaptive capacity of ER, increases insulin sensitivity and reverses type 2 diabetes in obese mice by decreasing ER stress in the hypothalamus (*16*) and can alleviate advanced glycation end product-induced apoptosis and osteoblastic differentiation of stromal cells *via* alleviating ER stress (*22,24*). It was also discovered that TUDCA could decrease the lipid content of adipocytes and reduce body mass in obese humans by attenuation of ER stress (*31,32*) and prevent both the maturation of adipocytes from preadipocytes and weight gain in ob/ob mice by decreasing ER stress (*33,34*). Therefore, based on these facts, TUDCA might lead to a novel therapeutic approach in ectopic ossification associated diseases.

In conclusion, our results indicate that TUDCA can inhibit fibroblast's mineralization *via* suppressing the ER stress response PERK-eIF2α-ATF4 pathway. These findings provide novel insights into the mechanisms of ectopic ossification and TUDCA has potential pharmacologic and therapeutic applications for treating ectopic ossification associated diseases.

Acknowledgements

This study was supported by National Natural Science Foundation of China (81371909) and Key Projects in the National Science & Technology Pillar Program during the Twelfth Five-year Plan Period (2013BAI07B01).

References

1. Li Q, Jiang Q, Uitto J. Ectopic mineralization disorders of the extracellular matrix of connective tissue: Molecular genetics and pathomechanisms of aberrant calcification. Matrix Biol. 2014; 33:23-28.
2. Popovic M, Agarwal A, Zhang L, Yip C, Kreder HJ, Nousiainen MT, Jenkinson R, Tsao M, Lam H, Milakovic M, Wong E, Chow E. Radiotherapy for the prophylaxis of heterotopic ossification: A systematic review and meta-analysis of published data. Radiother Oncol. 2014; 113:10-17.
3. Edwards DS, Clasper JC. Heterotopic ossification: A systematic review. J R Army Med Corps. 2014. pii: jramc-2014-000277.
4. Li Q, Uitto J. Mineralization/anti-mineralization networks in the skin and vascular connective tissues. Am J Pathol. 2013; 183:10-18.
5. Kousteni S. Osterix finds its master. EMBO Rep. 2011; 12:382-383.
6. Hamamura K, Yokota H. Stress to endoplasmic reticulum of mouse osteoblasts induces apoptosis and transcriptional activation for bone remodeling. FEBS Lett. 2007; 581:1769-1774.
7. Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: Coordination of gene transcriptional and translational controls. Genes Dev. 1999; 13:1211-1233.
8. Ron D. Translational control in the endoplasmic reticulum stress response. J Clin Invest. 2002; 110:1383-1388.
9. Rutkowski DT, Kaufman RJ. A trip to the ER: Coping with stress. Trends Cell Biol. 2004; 14:20-28.
10. Boot-Handford RP, Briggs MD. The unfolded protein response and its relevance to connective tissue diseases. Cell Tissue Res. 2010; 339:197-211.
11. Marciniak SJ, Ron D. Endoplasmic reticulum stress signaling in disease. Physiol Rev. 2006; 86:1133-1149.
12. Minamino T, Komuro I, Kitakaze M. Endoplasmic reticulum stress as a therapeutic target in cardiovascular disease. Circ Res. 2010; 107:1071-1082.
13. Zhang P, McGrath B, Li S, Frank A, Zambito F, Reinert J, Gannon M, Ma K, McNaughton K, Cavener DR. The PERK eukaryotic initiation factor 2 alpha kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. Mol Cell Biol. 2002; 22:3864-3874.
14. Wei J, Sheng X, Feng D, McGrath B, Cavener DR. PERK is essential for neonatal skeletal development to regulate osteoblast proliferation and differentiation. J Cell Physiol. 2008; 217:693-707.
15. Saito A, Ochiai K, Kondo S, Tsumagari K, Murakami T, Cavener DR, Imaizumi K. Endoplasmic reticulum stress response mediated by the PERK-eIF2(alpha)-ATF4 pathway is involved in osteoblast differentiation induced by BMP2. J Biol Chem. 2011; 286:4809-4818.
16. Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, Görgün CZ, Hotamisligil GS. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science. 2006; 313:1137-1140.
17. Ceylan-Isik AF, Sreejayan N, Ren J. Endoplasmic reticulum chaperone tauroursodeoxycholic acid alleviates obesity-induced myocardial contractile dysfunction. J Mol Cell Cardiol. 2011; 50:107-116.
18. Galán M, Kassan M, Choi SK, Partyka M, Trebak M, Henrion D, Matrougui K. A novel role for epidermal growth factor receptor tyrosine kinase and its downstream endoplasmic reticulum stress in cardiac damage and microvascular dysfunction in type 1 diabetes mellitus. Hypertension. 2012; 60:71-80.
19. Galán M, Kassan M, Kadowitz PJ, Trebak M, Belmadani S, Matrougui K. Mechanism of endoplasmic reticulum stress-induced vascular endothelial dysfunction. Biochim Biophys Acta. 2014; 1843:1063-1075.

20. Lee YY, Hong SH, Lee YJ, Chung SS, Jung HS, Park SG, Park KS. Tauroursodeoxycholate (TUDCA), chemical chaperone, enhances function of islets by reducing ER stress. Biochem Biophys Res Commun. 2010; 397:735-739.
21. de Almeida SF, Picarote G, Fleming JV, Carmo-Fonseca M, Azevedo JE, de Sousa M. Chemical chaperones reduce endoplasmic reticulum stress and prevent mutant HFE aggregate formation. J Biol Chem. 2007; 282:7905-7912.
22. Chen Y, Liu CP, Xu KF, Mao XD, Lu YB, Fang L, Yang JW, Liu C. Effect of taurine-conjugated ursodeoxycholic acid on endoplasmic reticulum stress and apoptosis induced by advanced glycation end products in cultured mouse podocytes. Am J Nephrol. 2008; 28:1014-1022.
23. Cai Z, Li F, Gong W, Liu W, Duan Q, Chen C, Ni L, Xia Y, Cianflone K, Dong N, Wang DW. Endoplasmic reticulum stress participates in aortic valve calcification in hypercholesterolemic animals. Arterioscler Thromb Vasc Biol. 2013; 33:2345-2354.
24. Tanaka K, Yamaguchi T, Kaji H, Kanazawa I, Sugimoto T. Advanced glycation end products suppress osteoblastic differentiation of stromal cells by activating endoplasmic reticulum stress. Biochem Biophys Res Commun. 2013; 438:463-467.
25. Beck GR Jr, Zerler B, Moran E. Phosphate is a specific signal for induction of osteopontin gene expression. Proc Natl Acad Sci U S A. 2000; 97:8352-8357.
26. Shui C, Scutt AM. Mouse embryo-derived NIH3T3 fibroblasts adopt an osteoblast-like phenotype when treated with 1alpha, 25-dihydroxyvitamin D(3) and dexamethasone *in vitro*. J Cell Physiol. 2002; 193:164-72.
27. Beck GR Jr, Knecht N. Osteopontin regulation by inorganic phosphate is ERK1/2-, protein kinase C-, and proteasome-dependent. J Biol Chem. 2003; 278:41921-41929.
28. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. Cell. 1997; 89: 747-754.
29. Komori T. Regulation of bone development and maintenance by Runx2. Front Biosci. 2008; 13:898-903.
30. Couchourel D, Aubry I, Delalandre A, Lavigne M, Martel-Pelletier J, Pelletier JP, Lajeunesse D. Altered mineralization of human osteoarthritic osteoblasts is attributable to abnormal type I collagen production. Arthritis Rheum. 2009; 60:1438-1450.
31. Gregor MF, Yang L, Fabbrini E, Mohammed BS, Eagon JC, Hotamisligil GS, Klein S. Endoplasmic reticulum stress is reduced in tissues of obese subjects after weight loss. Diabetes. 2009; 58:693-700.
32. Gregor MF, Hotamisligil GS. Thematic review series: Adipocyte Biology. Adipocyte stress: The endoplasmic reticulum and metabolic disease. J Lipid Res. 2007; 48:1905-1914.
33. Basseri S, Lhoták S, Sharma AM, Austin RC. The chemical chaperone 4-phenylbutyrate inhibits adipogenesis by modulating the unfolded protein response. J Lipid Res. 2009; 50:2486-2501.
34. Ozcan L, Ergin AS, Lu A, Chung J, Sarkar S, Nie D, Myers MG Jr, Ozcan U. Endoplasmic reticulum stress plays a central role in development of leptin resistance. Cell Metab. 2009; 9:35-51.

(Received January 16, 2015; Revised February 11, 2015; Accepted February 22, 2015)