Sirtuin-1 (SIRT1) stimulates growth plate chondrogenesis by attenuating the PERK-eIF-2α-CHOP pathway in the unfolded protein response

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ABSTRACT

The NAD⁺-dependent deacetylase sirtuin-1 (SIRT1), has emerged as an important regulator of chondrogenesis and cartilage homeostasis, processes that are important for physiological skeletal growth and are dysregulated in osteoarthritis. However, the functional role and underlying mechanism by which SIRT1 regulates chondrogenesis remain unclear. Using cultured rat metatarsal bones and chondrocytes isolated from rat metatarsal rudiments, here we studied the effects of the SIRT1 inhibitor EX527 or of SIRT1 siRNA on chondrocyte proliferation, hypertrophy, and apoptosis. We show that EX527 or SIRT1 siRNA inhibits chondrocyte proliferation and hypertrophy and induces apoptosis. We also observed that SIRT1 inhibition mainly induces the PERK-eIF-2α-CHOP axis of the endoplasmic reticulum (ER) stress response in growth-plate chondrocytes. Of note, EX527 or SIRT1 siRNA-mediated inhibition of metatarsal growth and growth plate chondrogenesis were partly neutralized by phenylbutyric acid (PBA), a chemical chaperone that attenuates ER stress. Moreover, EX527-mediated impairment of chondrocyte function (i.e. of chondrocyte proliferation, hypertrophy, and apoptosis) was partly reversed in CHOP−/− cells. We also present evidence that SIRT1 physically interacts with and deacetylates PERK. Collectively, our findings indicate that SIRT1 deacetylates PERK and attenuates the PERK-eIF-2α-CHOP pathway and thereby promotes growth-plate chondrogenesis and longitudinal bone growth.

INTRODUCTION

Sirtuin-1 (SIRT1), a NAD⁺-dependent histone deacetylase, exerts a variety of biological function through the deacetylation of not only lysine residues in histone (1) but also non-histone proteins, such as p53 (2), forkhead family proteins (3) and p65/RelA subunit of nuclear factor-kB (NF-kB) (4). Through deacetylation, SIRT1 is involved in a variety of biological processes such as stress responses, DNA repair and inflammation (5,6), and plays a crucial part in regulating cell differentiation, proliferation, survival, and organism longevity (7,8).

Previous evidence indicates that SIRT1 exerts a regulatory role in cartilage homeostasis. In human chondrocytes derived from osteoarthritic (OA) patients, overexpressing or activating SIRT1 enhances the expression of cartilage anabolic genes such as col2a1 and aggrecan (9). It has also been shown that SIRT1 protects chondrocytes from radiation-induced senescence (10), and inhibits human chondrocytes apoptosis in vitro (11,12). Furthermore, inhibition of SIRT1 in human chondrocytes leads to OA-like gene expression changes (13) and cartilage-specific SIRT1 knockout mice show accelerated OA progression (14), suggesting that SIRT1 has a protective role in articular cartilage. With respect to SIRT1 effects on growth plate chondrocytes, previous studies indicate that SIRT1-null mice and mice with a SIRT1 point mutation exhibit significant reduction in body size (15,16), implicating that SIRT1 involved in growth plate chondrogenesis and, in turn, longitudinal bone growth.

In mammals, the long bone is mainly formed via endochondral ossification, which occurs at the epiphyseal growth plate where the cartilage template is first formed and then replaced by bone. The proliferation, hypertrophy, differentiation, and secretion into the extracellular matrix of chondrocytes in the growth plate lead to the formation of new cartilage, chondrogenesis (17). As new cartilage is continuously formed, the terminally differentiated chondrocytes undergo apoptosis, the metaphysis invades the growth plate with blood vessels and bone cell precursors that remodel the cartilage into bone tissue (18).
colleagues have shown that SIRT1 protects cardiomyocytes against ER stress induced apoptosis via regulating the PERK-eIF-2α pathway (23). However, whether and how SIRT1 modulates ER stress response in the growth plate chondrocytes has not been elucidated yet. Based on all these findings, we hypothesized that 1) SIRT1 facilitates longitudinal bone growth and growth plate chondrogenesis, and 2) such effects of SIRT1 are mediated by an unidentified pathway such as PERK-eIF-2α-CHOP.

We show that EX527 (a known specific SIRT1 inhibitor) inhibits metatarsal longitudinal growth and growth plate chondrogenesis, with such effects being partly neutralized by phenylbutyric acid (PBA, a chemical chaperone known to attenuate ER stress). In addition, we evaluate the effects of EX527 or SIRT1 siRNA on cultured chondrocyte proliferation, hypertrophy and apoptosis. Meanwhile, we observe that the inhibition of SIRT1 activity (by EX527 or SIRT1 siRNA) leads to the PERK-eIF-2α-CHOP axis of the ER stress response. Furthermore, we show SIRT1 regulates the PERK-eIF-2α-CHOP pathway partly through PERK deacetylation. Our results support the hypothesis that SIRT1 promotes growth plate chondrogenesis via maintaining ER homeostasis in PERK-eIF-2α-CHOP-dependent manner.

RESULTS
Effects of EX527 and PBA on metatarsal longitudinal growth
To determine whether SIRT1 regulates growth plate chondrogenesis, we cultured fetal rat metatarsals for 3 d in serum-free medium in the presence of EX527 (0-100μM), a specific SIRT1 inhibitor. During the 3 d of the culture period, 30μM (the lowest growth inhibiting concentration) EX527 significantly inhibited metatarsal longitudinal growth (figure 1A-1B). To further confirm the findings of SIRT1 on longitudinal growth, we obtained metatarsal bones from conditional knockout mice (SIRT1 cartilage specific cKO mice, generated using the Cre-loxP system). As expected, ablation of SIRT1 specific in chondrocytes inhibited metatarsal bones growth significantly after the 3 days of the culture (supplemental figure 1A). Immunohistochemical analysis of sections of 3 d metatarsal bones using the specific antibody against SIRT1 showed that the whole growth plate, including epiphyseal, proliferative, and hypertrophic chondrocyte, stains intensely for SIRT1, whereas 30μM EX527 caused a significant decrease of SIRT1 expression in growth plate (supplemental figure 1B). Western blot of lysates from cultured chondrocytes treated with EX527 confirmed that graded concentrations of EX527 caused a dose-dependent inhibition of SIRT1 expression, with a lowest inhibitory concentration of 30μM (supplemental figure 2A). We also analyzed the effect of 30μM EX527 on the expression of other sirtuin, SIRT2, in the metatarsal growth plate as well as in cultured chondrocyte. We found that there were no significant changes in the expression of SIRT2 in metatarsals (supplemental figure 1C) or chondrocytes (supplemental figure 2A) treated with 30μM EX527, indicating that 30μM EX527 could act specifically on SIRT1 without hitting other sirtuin.

Because we have previously demonstrated that the PERK-eIF-2α-CHOP axis of ER stress exerts an important role in growth plate chondrogenesis (22), we sought to determine whether EX527 inhibited metatarsal longitudinal growth via inducing the PERK-eIF-2α-CHOP axis of ER stress. As expected, TEM analysis showed that the ER cisternae were dilated in growth plate chondrocytes of EX527-treated metatarsals compared with control metatarsals (figure 1E). In order to confirm that inhibition of SIRT1 induced ER stress in chondrocytes, we then analyzed the expression of ER stress indicators in metatarsal growth plate by Western blot and RT-qPCR. Western blot of lysates from control and EX527-treated metatarsals confirmed EX527 significantly increased the phosphorylation of PERK and eIF-2α and the expression of CHOP compared to control metatarsals (figure 1C, supplemental figure 1D), and RT-qPCR also show a similar pattern of expression (figure 1D). To further confirm the effects of EX527 on PERK-eIF-2α-CHOP axis of ER stress, chondrocytes isolated from rat fetal metatarsal rudiments were cultured in the
presence of EX527 (0-100µM). As shown in supplemental figure 2A, EX527 induced this pathway in a dose-dependent manner, with higher concentrations (30 and 100µM) causing a statistically significant induction.

To determine whether the inhibitory effects of EX527 on metatarsal longitudinal growth are mediated by ER stress, metatarsals were cultured in the absence or presence of 30µM EX527, with or without 2mM phenylbutyric acid (PBA), a chemical chaperone known to attenuate ER stress. As expected, PBA effectively attenuated ER stress induced by EX527 in chondrocytes, as assessed by expression of p-PERK, p-eIf-2α, ATF4 and CHOP by Western blot (figure 1F, supplemental figure 1E) and RT-qPCR (figure 1G) respectively, and addition of PBA partially neutralized the inhibitory effect of EX527 on metatarsal longitudinal growth (figure 1H-I).

**Effects of EX527 and PBA on growth plate chondrogenesis**

Because the rate of longitudinal bone growth depends primarily on the rate of growth plate chondrogenesis, we evaluated the effects of EX527 on chondrocyte proliferation and hypertrophy. Treatment with EX527 significantly reduced the height of the epiphyseal and proliferative zones of the growth plate, where cell proliferation takes place (figure 2A, 2C). Consistent with these findings, we examined the *in situ* incorporation of BrdU into the metatarsal rudiments at the end of the culture period and observed that EX527 significantly inhibited the incorporation of BrdU into the growth plate epiphyseal and proliferative zones (figure 2D), whereas the addition of PBA partially neutralized the inhibitory effects of chondrocyte proliferation induced by EX527 (figure 2A, figure 2C-D). To assess chondrocyte hypertrophy, we examined the bone rudiments histologically. After 3 d in culture, 30 µM EX527 decreased the height of the growth plate hypertrophic zone, while co-treatment with PBA reversed growth inhibition in hypertrophic zone induced by EX527 (figure 2A, 2C). Inhibition of chondrocyte hypertrophy by EX527 was also confirmed by Coll10a1 immunohistochemistry (figure 2B). As shown in figure 2B and figure 2E, EX527 caused a marked decrease of coll10a1 expression, with such effect being partially abolished by the addition of PBA.

In light of the regulatory role of SIRT1 on apoptosis in other cell types, we evaluated the effects of EX527 on metatarsal growth plate apoptosis by *in situ* cell death. EX527 caused a significant increase in cell death compared with control (figure 2F), with such effect being neutralized by PBA (figure 2F).

**EX527 induced ER stress, inhibited proliferation, and hypertrophy, and increased apoptosis in cultured primary chondrocytes**

To further determine the interaction between SIRT1 and ER stress in chondrocyte, primary chondrocytes derived from rat fetal metatarsal rudiments were cultured in the absence or presence of 30µM EX527. As expected, EX527 reduced both SIRT1 protein (assessed by Western blot; figure 3A, supplemental figure 3B) and mRNA expression (assessed by RT-qPCR; figure 3B). Furthermore, chondrocytes treated with EX527 also exhibited ER stress, as evident by the induction of PERK and eIf-2α phosphorylation, and upregulation the expression of CHOP (figure 3A, supplemental figure 3B), similar to what we found in metatarsals study (figure 1C, supplemental figure 1C). We then restored SIRT1 expression in EX527-treated chondrocytes by overexpression plasmid of SIRT1, and the transfection efficiency was validated by fluorescence microscope (supplemental figure 3A) and reconfirmed by western blot (figure 3A) and RT-qPCR (figure 3B). After transient transfection, we verified that restoration of SIRT1 expression could ameliorate ER stress induced by EX527 in chondrocytes as detected by Western blot and RT-qPCR (figure 3A-B).

Consistent with the observation in the metatarsal growth plate, EX527 significantly inhibited BrdU incorporation (figure 3C) as well as cyclin D1 and PCNA expression (figure 3D, supplemental figure 3C), with these effects being abolished by overexpression plasmid of SIRT1 (figure 3C-D). With respect to apoptosis, overexpression of SIRT1 neutralized the pro-apoptotic effect of EX527, as analyzed by TUNEL assay (figure 3E) and bcl-2/bax ratio of protein expression (figure 3F,
supplemental figure 3D). Lastly, to determine whether EX527 affects chondrocytes hypertrophy, we cultured chondrocytes with ITS for 7 d in the absence or presence of 30 μM EX527. Similarly, EX527 inhibited chondrocytes hypertrophy, as evident by reduced Col10a1 and MMP13 expression (figure 3G, supplemental figure 3E), whereas the addition of 10μM resveratrol, a SIRT1 activator, reversed the inhibition of Col10a1 and MMP13 expression induced by EX527 (figure 3G, supplemental figure 3E).

**Effects of PBA on SIRT1 siRNA-induced inhibition of chondrocytes function**

To further confirm whether inhibition of SIRT1 activity-mediated inhibition of chondrogenesis was caused by ER stress, chondrocytes were transfected with SIRT1 siRNA or control siRNA, and cultured in the absence or presence of 2mM PBA. Our finding indicated that PBA ameliorated ER stress induced by SIRT1 siRNA in chondrocytes as detected by Western blot and RT-qPCR (figure 4A-B, supplemental figure 4A), thus partially neutralizing the inhibitory effects on chondrocytes proliferation (figure 4C-D, supplemental figure 4B) and hypertrophy (figure 4G, supplemental figure 4D), and diminishing the pro-apoptotic effect of SIRT1 siRNA (figure 4E-F, supplemental figure 4C).

**Effects of CHOP siRNA on EX527-induced inhibition of chondrocytes function**

Because we observed that SIRT1 inhibition or knockdown (treatment with EX527 or transfection with SIRT1 siRNA) mainly induced the PERK-eIF2α-CHOP axis of the ER stress response in chondrocytes, we reasoned whether such inhibition of chondrocyte function was mediated by CHOP. To test this possibility, CHOP siRNA was transfected in chondrocytes, and its validation was measured by the reduction of CHOP protein expression by Western blot (figure 5A, supplemental figure 5A). The transfection of CHOP siRNA alone had minor effect on chondrocytes proliferation, hypertrophy and apoptosis given that basal level of CHOP in normal chondrocyte is low. However, CHOP siRNA abolished the suppression of EX527 on chondrocytes proliferation assessed by BrdU incorporation (figure 5B) as well as cyclin D1 and PCNA protein expression (figure 5C, supplemental figure 5B), and hypertrophy assessed by Col10a1 and MMP13 protein expression (figure 5G, supplemental figure 5D), meanwhile, neutralized the pro-apoptotic effect of EX527, as analyzed by TUNEL assay (figure 5D) and bcl-2/bax ratio of protein expression (figure 5E, supplemental figure 5C).

In addition, it has been reported that CHOP may form heterodimers with C/EBP-β and act as a transdominant negative inhibitor of C/EBP-β signaling (24), and C/EBP-β has also been indicated to regulate chondrocytes hypertrophy through interacting with RUNX2. Given this evidence, to explore the downstream mechanism that regulates chondrocytes hypertrophy, we performed RT-qPCR in EX527-treated chondrocytes with or without CHOP siRNA. Inhibition of SIRT1 decreased the mRNA levels of C/EBP-β and RUNX2, whereas treatment with CHOP siRNA partially abrogated such inhibitory effects (figure 5F), suggesting that CHOP may regulate chondrocytes hypertrophy through interaction with C/EBP-β and RUNX2.

**SIRT1 interacts with and deacetylates PERK**

It is known that SIRT1 could regulate protein activity through deacetylation on lysine residues. Therefore, to study the mechanism by which SIRT1 attenuates the PERK-eIF2α-CHOP pathway to maintain ER homeostasis, proteins acetylated on lysine residues were pulled down from primary chondrocytes lysates. In primary chondrocytes, only PERK was present in anti-acetyl lysine immunoprecipitates, whereas eIF2α and CHOP were not detected, indicating that among the members of the PERK-eIF2α-CHOP axis tested, PERK is the only lysine-acetylated protein (figure 6A). Moreover, reciprocal immunoprecipitation confirmed the acetylation of PERK on lysine residues (figure 6B). Of note, both depletion of SIRT1 with SIRT1 siRNA and inhibition of SIRT1 with EX527 significantly increased the acetylation level of PERK as shown in
To determine whether SIRT1 directly interacts with PERK in chondrocytes, co-immunoprecipitation assays were carried out. Immunoprecipitation of endogenous SIRT1 from primary chondrocytes lysates co-precipitated PERK (figure 6E), meanwhile, the reverse experiments, immunoprecipitating endogenous PERK and immunoblotting for SIRT1 (figure 6E), further confirming their physical interaction. Interestingly, thapsigargin (THG), which induces ER stress by inhibiting sarco/endoplasmic reticulum calcium ATPases, increased the level of PERK acetylation (figure 6F-G), and such level was similar when SIRT1 was inhibited by EX527 or SIRT1 siRNA (figure 6F-G), indicating that SIRT1 limits the acetylation level of PERK in order to maintain ER homeostasis in vitro. Furthermore, in parallel with acetylation, the level of phosphorylation of PERK was also increased in chondrocytes treated with THG, or treated with EX527 or SIRT1 siRNA (figure 6F-G), suggesting a dynamic interplay between these two post-translational modifications. Collectively, these results showed that PERK is acetylated on one or more lysine residues and that this acetylation is regulated, at least in part, by SIRT1 in order to maintain ER homeostasis in chondrocytes.

**Discussion**

Recent evidence suggests that SIRT1, which is considered a longevity factor, may be implicated in the regulation of longitudinal bone growth. SIRT1-null mice are significantly smaller than WT littermates during embryogenesis and postnatal stages, and they do not survive longer than 1 month postnatally (25). In addition, SIRT1-heterozygous knockout mice show reduced growth and shortened long bones (26), and they also exhibit metabolic impairments and reduced bone mass (27,28). Furthermore, we have previously demonstrated a direct circadian regulation of Bmal1 in cartilage homeostasis mediated by SIRT1 (29). However, these findings provide only indirect evidence of SIRT1 regulating growth plate function.

In the present study, we demonstrated that inhibition of SIRT1 caused a significant suppression of metatarsal longitudinal growth, which resulted from induction of the PERK-eIF2α-CHOP axis of the endoplasmic reticulum (ER) stress response in growth plate chondrocytes, causing inhibition of the two main cellular events of growth plate chondrocyte proliferation and hypertrophy (30), and eventually leading to an inhibition of growth plate chondrogenesis. Meanwhile, the ubiquitous localization of SIRT1 in metatarsal growth plate further supports the finding of a uniformly suppressed chondrogenesis throughout the growth plate. All these findings indicate that SIRT1 expressed in growth plate chondrocytes facilitates chondrocytes proliferation and hypertrophy and prevents apoptosis.

Chondrocytes are known to have an increased ER burden caused by the synthesis and secretion of the large amount of ECM protein during development (19,31,32), they rely on the unfolded protein response (UPR) to maintain ER homeostasis. Previous studies in other cell types have shown that SIRT1 reduced ER stress and apoptosis of brown adipocyte by inhibiting Smad3/ATF4 signal (33), and SIRT1 protects cardiomyocytes against ER stress-induced apoptosis by attenuating PERK/eIF2α pathway (23). Of note, we observed that ER cisternae were enlarged and distended in growth plate chondrocytes of EX527-treated metatarsals, indicated that SIRT1 may facilitate maintaining ER homeostasis and protects chondrocytes against severe ER stress by attenuating PERK/eIF2α pathway (34). The discrepancies may reflect cell-type specificities including differentiation state.

Because we have recently demonstrated that PERK-eIF-2α-CHOP axis of ER stress exerts an important role in chondrogenesis (22), we hypothesized that SIRT1 facilitates maintaining ER homeostasis and protects chondrocytes against severe ER stress by attenuating PERK-eIF2-α-CHOP pathway. Indeed, we show that inhibition of SIRT1 in metatarsals and chondrocytes results in hyperactivation of PERK/eIF2α pathway.
Furthermore, we observe that reduction of ER stress with PBA partially restored EX527 or STRT1 siRNA-induced inhibitory effect on metatarsal longitudinal growth and chondrocyte function (i.e., proliferation, hypertrophy, and apoptosis). In addition, EX527-mediated impaired chondrocyte function was partly reversed in CHOP-/- cells, further supporting that SIRT1 mediates its protective effects, at least in part, by attenuation excessive ER stress via the PERK-eIF-2α-CHOP-dependent pathway.

The ability of cells to respond to ER stress is critical for cell survival, but chronic or irrecoverable levels of ER stress can lead to apoptosis (35). CHOP, a C/EBP homologous protein, also known as a pro-apoptotic factor, is understood to be a later event in the PERK-eIF-2α axis of UPR activation (36). CHOP can directly regulate death effectors, such as Bcl-2 and Bim, which subsequently render the cells more susceptible to apoptosis (37,38). It was reported that CHOP deficiency promotes cell survival in an ER stress–related model of type 2 diabetes (39). In a murine OA model, chondrocyte death and cartilage degeneration are decreased in CHOP-/- mice (40), indicating that CHOP plays an important role in ER stress-induced apoptosis. Consistent with these findings, downregulation of CHOP in chondrocytes could partly restore EX527-mediated inhibition of chondrocyte proliferation, and partly neutralize EX527-mediated proapoptotic effect. In addition to regulating apoptosis, CHOP can form heterodimers with C/EBP-β and act as a transdominant negative inhibitor of C/EBP-β signaling (24).

Several lines of evidence have implicated C/EBP-β as a key regulator in the transition of chondrocytes from proliferation to hypertrophy (41,42), and C/EBP-β interacts cooperatively with GADD45-β and RUNX2 to promote chondrocyte hypertrophy and growth plate matrix remodeling and turnover via upregulation of the expression of key markers of chondrocyte maturation including Col10a1 and MMP13(43,44).

Overall, our results support the hypothesis that SIRT1 inhibition, coupled with PERK-eIF-2α-CHOP axis of the ER stress, and downregulation of C/EBP-β and RUNX2 transcriptional activity, might result in impaired expression of Col10a1 and MMP13 for chondrocyte hypertrophy.

Although autophosphorylation of PERK has been intensively studied, the understanding of the molecular events regulating its autophosphorylation is poorly documented. Interaction of SIRT1 with eIF2α has been shown in HeLa cells and cardiomyocytes (23,45), however no evidence was reported on PERK acetylation/deacetylation. In our study, we found that as a lysine-acetylated protein, PERK physically interacts with SIRT1 and being deacetylated on its lysine residues. In chondrocytes, inhibition of SIRT1 promotes both hyperacetylation and phosphorylation of PERK, and then triggers the PERK-ATF4-CHOP axis of the ER stress, suggesting that SIRT1 may regulate PERK/eIF2α UPR pathway via deacetylation of PERK.

It is known that the activity of numerous targets of SIRT1 is regulated both by deacetylation and by phosphorylation, including PGC-1α, p53, FOXO and Beclin1 (6,46), however, the interplay between these two post-translational modifications is not fully understood. Our observations that PERK acetylation occurred earlier than phosphorylation when SIRT1 is inhibited (supplemental figure 6) further support the notion that SIRT1, by regulating the level of acetylation of PERK, may regulate its level of phosphorylation and thus its activity. Although additional studies are needed to clarify the relationship between acetylation and phosphorylation of PERK, our data provide a clue as to how SIRT1 attenuates PERK/eIF2α pathway in chondrocytes.

In conclusion, we have demonstrated that SIRT1 facilitates longitudinal bone growth and growth plate chondrogenesis through regulation the PERK-eIF2α-CHOP axis of the ER stress (Schematic diagram in figure 6H). This study is the first to analyze the function of SIRT1 in growth plate, and extends our understanding of how SIRT1 interacts with key UPR pathways controlling growth plate chondrogenesis.

**EXPERIMENTAL PROCEDURES**

**Whole Metatarsal Culture**

The second, third, and fourth metatarsal bones were isolated from Sprague-Dawley...
rat embryos (20 days postcoitum) and cultured individually in 24-well plates. Each well contained 0.5 ml of minimum essential medium (HyClone) with 0.2% bovine serum albumin (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin (HyClone) and 50 μg/ml ascorbic acid (Sigma). Bone rudiments were cultured for 3 days in a humidified incubator with 5% CO₂ in air at 37 °C. The medium was changed on day 2. During the 3-day culture period, metatarsals were cultured in the absence or presence of EX527 (30 μM, Sigma, Cat.No.E7034), with or without PBA (2 mM, Sigma, Cat.No.SML0309).

Measurement of Longitudinal Growth- The length of each bone rudiment was measured under a dissecting microscope, using an eyepiece micrometer. To calculate the metatarsal growth rate, length measurements were performed at the beginning and at the end of the experiments. For each treatment group, 24 metatarsal bones isolated from 4 rat fetuses of the same litter were used. In order to confirm the effect of SIRT1 on metatarsal longitudinal growth, we performed 3 independent experiments from 3 pregnant mothers.

Quantitative Histological Analysis

At the end of the culture period, metatarsals were fixed in 4% phosphate-buffered paraformaldehyde overnight. After routine processing, three 5-μm-thick to 7-μm-thick longitudinal sections were obtained from each metatarsal bone and stained with toluidine blue. From each of the three sections, we measured the height of the epiphyseal zone, the proliferative zone, and the hypertrophic zone and calculated the average value. In the metatarsal growth plate, the epiphyseal zone is characterized by small and rounded cells, irregularly arranged in the cartilage matrix. The proliferative zone comprises cells with a flattened shape, arranged in columns parallel to the longitudinal axis of the bone. Eventually, chondrocytes, which are located farthest from the epiphyseal zone, stop replicating and enlarge to become hypertrophic chondrocytes (defined by a height of ≥9μm). All measurements were performed by a single observer blinded to the treatment regimen.

Immunohistochemistry

To detect SIRT1 and type X collagen (Col10a1) expression in the metatarsal bones, metatarsals were fixed in 4% phosphate-buffered paraformaldehyde overnight. After routine processing, 5-μm-thick to 7-μm-thick longitudinal sections were obtained. Sections were immunostained with SPLink Detection Kits (Zhongshan Biotechnologies, Beijing, People’s Republic of China; Cat.No.SP-9001). Briefly, sections were treated with 0.25% trypsin for 10 minutes at 37° before antigen retrieval after the pretreatment of 3% H₂O₂, and then were blocked using 5% goat serum for 15 minutes at room temperature (RT). Afterward, sections were incubated with designated primary antibodies overnight at 4°C. Antibodies included mouse anti-SIRT1 antibody at a dilution of 1:200 (Abcam, ab110304) and rabbit anti-Col10a1 antibody at a dilution of 1:200 (Abcam, ab58632). After three rinses with PBS, sections were incubated with respective secondary antibodies for 15 minutes at RT. Finally, sections were counterstained with hematoxylin.

BrdU incorporation

Cultured metatarsal bones were exposed to 5-bromo-2’-deoxyuridine (BrdU) (1:100, Invitrogen, 00-0103) for an additional 2 h before the end of culture period. The BrdU-labeled cells were visualized utilizing a BrdU labeling Kit (Invitrogen, Cat.No.93-3943) according to the manufacturer’s instructions. Primary chondrocytes were exposed to BrdU (1:1000, Roche) for 15 minutes before fixation. The BrdU-labeled cells were visualized utilizing a BrdU labeling and detection Kit (Roche, 11299964001) according to the manufacturer’s instructions. The percentage of BrdU-positive cells was calculated as the number of BrdU-labeled cells per grid divided by the total number of cells per grid. For each sample, the fraction of labeled cells in three distinct grid locations was calculated and averaged. All determinations were made by the same observer blinded to the treatment category.

TUNEL assay

Apoptotic cells in the metatarsal growth
plate and primary chondrocytes were identified by an In Situ Cell Death Detection Kit (Roche, 12156792910), according to the manufacturer’s instructions. Nuclei were counterstained with DAPI. TUNEL-positive cells were stained red fluorescence. The percentage of TUNEL positive cells was calculated as the number of TUNEL-labeled cells per grid divided by the total number of cells per grid. For each sample, the fraction of labeled cells in three distinct grid locations was calculated and averaged. All determinations were made by the same observer blinded to the treatment category.

Electronic microscopy (EM) analysis
Metatarsal bones were fixed in 2% glutaraldehyde, and embedded in epoxy resin (Epon). Ultrathin sections (80 nm) were stained with aqueous uranyl acetate and lead citrate and examined with a JEOL 2000EX transmission EM (JEOL).

Primary chondrocyte culture
The cartilaginous portions of metatarsal bones isolated from Sprague-Dawley rat embryos (20 days postcoitum) were dissected, rinsed in PBS, then incubated in 0.2% collagenase (Sigma, C6885) for 2 hours. The cell suspension was aspirated repeatedly and filtered through a 70-μm cell strainer, rinsed first in PBS and then in serum-free DMEM, and counted. Chondrocytes were seeded at a density of 2×10^6 cell/ml in DMEM containing 10% FBS (Gibco). The culture medium was changed at 72h intervals. ITS-inducing culture was initiated at chondrocytes reaching 70–80% confluence by adding ITS (Sigma, I3146) (1:100) into the culture medium.

siRNA transfection
Chondrocytes were transfected with pools of siRNAs targeted for SIRT1 (Santa Cruz, sc-108043) or CHOP (Santa Cruz, sc-156118), a pool of siRNAs consisting of scrambled sequences was similarly transfected as control siRNA (Santa Cruz). siRNA was introduced to cells using Lipofectamine 2000 (Invitrogen, 11668027), according to the procedure recommended by the manufacturer. The transfected cells were cultured in DMEM containing 10% FBS for 48 hours after transfection.

SIRT1 plasmid transfection
Chondrocytes were transfected with the expression plasmid for SIRT1 or a control plasmid (Genechem Co, Shanghai, China) for 48h. The expression vector was introduced to cells using Lipofectamine LTX (Invitrogen, 15338100), according to the procedure recommended by the manufacturer.

Western blot
Whole-cell extracts were prepared by lysing cells with the RIPA buffer. Proteins were separated by 8–15% SDS-PAGE gel, separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membranes (Millipore) and were probed with the following primary antibodies: rabbit monoclonal antibodies against SIRT1 (Cell Signaling, 9475), PERK (Cell Signaling, 3192), Phospho-eIF2α (Cell Signaling, 3398), CHOP (Cell Signaling, 5554), rabbit polyclonal antibodies against Phospho-PERK (Santa Cruz, sc-32577), Bcl-2 (Santa Cruz, sc-492), Bax (Santa Cruz, sc-6236), Col10a1 (Abcam, ab58632), MMP13 (Abcam, ab39012), mouse monoclonal antibodies against eIF2α (Santa Cruz, sc-133132), Cyclin D1 (Santa Cruz, sc-450), PCNA (Santa Cruz, sc-25280), GAPDH (Santa Cruz, sc-365062). At last, the blots were visualized by an ECL detection system (Millipore) with a horseradish peroxidase-conjugated secondary antibody. A representative blot from three independent experiments is presented for each protein.

Immunoprecipitation
Cytoplasmic lysate (200μg) was incubated for 2 hours at 4°C with the corresponding antibodies coupled to 20μL of packed protein A+G Sepharose beads (Santa Cruz, sc-2002). Immune complexes were resolved by means of SDS-PAGE and immunoblotted with the indicated antibodies. To analyze the level of PERK acetylation, chondrocytes lysates were immunoprecipitated using anti-PERK antibody (Cell Signaling, 3192), and then immunoprecipitated proteins were run on SDS-PAGE and immunoblotted with
anti-PERK and anti-acetyl lysine antibody (Santa Cruz, sc-32268), respectively.

RNA extraction and Real time PCR
Total RNA from cultured chondrocytes was isolated by Trizol reagent (Invitrogen, 15596–026) according to the manufacturer’s instruction. The recovered RNA was further processed using RevertAid First Strand cDNA Synthesis kit (Thermo, K1621) to produce cDNA in accordance with the manufacturer’s instructions. The cDNA products were directly used for PCR or stored at −80°C for later analysis. Real-time quantitative PCR was performed in MJ Mini Real-Time PCR Detection System using SYBR Premix Ex Taq™ II (Takara, RR047A). Primers were as follows: rat GAPDH (forward 5’-TGA CGC TGG GGC TGG CAT TG-3’, reverse 5’-GCT CTT GCT GGG GCT GGT GG-3’); rat SIRT1 (forward 5’-TCG TGG AGA CAT TTT TAA TCA GG -3’, reverse 5’-GCT TCA TGA TGG CAA GTG G-3’), rat ATF4 (forward 5’-TCT GCT GCT TAT ATT ACT CTA ACC -3’, reverse 5’-GAG AAC CAC GAG GAA CAC GAG GAA CAC C-3’), rat CHOP (forward 5’-CTC TGA CTG GAA TCT GGA GAG TG-3’, reverse 5’-CTG AGT CAT TGC CTT TCT CCT TCG-3’), rat C/EBP-β (forward 5’-GAC AAG CAC AGC GAC GAG TA -3’, reverse 5’-GTG CTG CTT CTC CAG GTT-3’), rat RUNX2 (forward 5’-CCT TCC TCT CGA GAC CCT AA-3’, reverse 5’-ATG GCT GCT CCC TTC TGA AC-3’). Each experiment was performed in duplicate and experiments repeated 4 times independently. A dissociation curve analysis was conducted for each qPCR. Expression levels of the target gene were evaluated using a relative quantification approach (2^{ΔΔCt} method) against GAPDH levels.

Study approval
Animal care was approved by the Animal Experiment Administration Committee of the Medicine of Xi’an Jiaotong University in Shannxi, P.R.China.

Statistics
Statistical analysis was performed with the SPSS 17.0 software (SPSS Inc., Chicago, IL). All the experiments were repeated three or four times independently, and data were presented as mean ± SD. Statistical analysis was performed using two-tailed Student’s t-tests for two groups and one-way ANOVA for more than two groups. P values less than 0.05 were considered statistically significant.
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Conflict of interest

All authors state that they have no conflicts of interest.

Author contributions

Study design: S Wu, H Sun, X Kang, and W Yang. Data collection: S Wu, X Kang, X Jin and R Wang. Data analysis: S Wu, X Kang, D Feng and H Li. Data interpretation: S Wu, X Kang, W Yang and T Xie. Drafting manuscript: S Wu, H Sun, X Kang, and W Yang. Approving final version of manuscript: All authors. S Wu and H Sun take responsibility for the integrity of the data and the accuracy of the data analysis.
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Figure 1 Effects of EX527 and PBA on metatarsal longitudinal growth
A-B, fetal rat metatarsals (20 days post conception) were cultured for 3 days in serum-free MEM containing graded concentrations of EX527 (0–100μM, n=20–24/group). Bone length was measured at the beginning and at the end of the experiments using an eyepiece micrometer in a dissecting microscope. At the end of 3 days in culture, protein and total RNA were obtained from metatarsals and processed as described under “Materials and Methods”. C, the expression of SIRT1 and ER stress markers including PERK phosphorylation (p-PERK), eIF-2α phosphorylation (p-eIF-2α) and CHOP were detected by Western blot, a representative blot from four independent experiments was presented for each protein. D, relative expression of SIRT1, ATF4 and CHOP were examined by real-time PCR, results were presented as gene expression levels in all groups normalized to controls. E, transmission electron microscopy of growth plate chondrocytes displayed a normal structure of the rough ER in control metatarsals, but unusually dilated ER cisternae in EX527-treated metatarsals. Arrows indicate ER. Insets show a high magnification of selected areas. Scale bars =1μm. F, fetal rat metatarsals (20 days post conception) were cultured for 3 days in serum-free MEM containing EX527 (30μM, n=20/group) and/or PBA (2mM, n=20/group). The expression of p-PERK, p-eIF-2α and CHOP were detected by Western blot, a representative blot from four independent experiments was presented for each protein. G, relative expression of ATF4 and CHOP were examined by real-time PCR, results were presented as gene expression levels in all groups normalized to controls. H-I, bone length was measured at the beginning and at the end of the experiments using an eyepiece micrometer in a dissecting microscope. Data are expressed as means ± SD in each scatter plots. *P<0.05, **P<0.01.

Figure 2 Effects of EX527 and PBA on chondrocyte proliferation, hypertrophy and apoptosis in the metatarsal growth plate.
At the end of the experimental period, metatarsal bones were fixed and paraffin-embedded, and 5- to 7-μm-thick longitudinal sections were obtained. A, representative images were obtained from each treatment of metatarsal bones stained with toluidine blue. Insets show a high magnification of selected areas. Scale bars, 100μm. PZ, proliferative zone; HZ, hypertrophic zone. B, the expression of col10a1 was detected in cultured metatarsals by immunohistochemistry. Scale bars, 50μm. C, the heights of the epiphyseal, proliferative, and hypertrophic zones of the growth plate were quantitatively analyzed respectively (n=6/group). After 3 days in culture, BrdU was added to the culture medium and bone rudiments were incubated for an additional 2 h. D, representative images of the BrdU-positive cells. BrdU-positive cells were stained brown color (indicated by the arrow). Scale bars, 100μm. The number of BrdU-positive cells were analyzed separately for the epiphyseal zone and for the proliferative zone (n=6/group). E, relative expression of col10a1 was detected in growth plate chondrocytes of metatarsal bones by real-time PCR, results were presented as gene expression levels in all groups normalized to controls. F, representative images of the TUNEL-positive cells. TUNEL-positive cells were stained red fluorescence (indicated by the arrow). Quantification was showed on the right. Data are expressed as means ± SD in each scatter plots. *P<0.05, **P<0.01.

Figure 3 Effects of EX527 on proliferation, hypertrophy and apoptosis in cultured primary chondrocytes.
Chondrocytes were cultured for 48 h with or without EX527 (30μM). For the rescue experiment, chondrocytes were pre-treated with EX527 for 48 h and then transfected with either an empty vector (control plasmid) or a plasmid containing SIRT1 (SIRT1 plasmid) for additional 48 h. Both protein and mRNA expression were analyzed by Western blot (A) and real-time PCR (B), respectively. C, primary chondrocytes were labeled with BrdU and prepared for staining as described in Materials and Methods. A representative BrdU-positive cell is indicated by the arrow. Scale bars, 100μm. Quantification was showed on the right. D, the expression of CyclinD1 and PCNA were detected by Western blot in chondrocytes, a
representative blot from four independent experiments was presented for each protein. E, primary chondrocytes were labeled with TUNEL, a representative TUNEL-positive cell is indicated by the arrow. Scale bars, 100μm. Quantification was showed on the right. F, the expression of Bcl-2 and Bax were detected by Western blot, a representative blot from three independent experiments was presented for each protein. G, chondrocytes were cultured with ITS for 7 d, incubate in the absence or presence of 30μM EX527 and in combination with or without 10μM resveratrol. At the end of the culture period, chondrocytes were harvested, lysed, electrophoresed, and immunblotted for SIRT1, Col10a1 and MMP13, a representative blot from three independent experiments was presented for each protein. Data are expressed as means ± SD in each scatter plots. *P<0.05, **P<0.01.

Figure 4 Effects of SIRT1 siRNA and PBA on proliferation, hypertrophy and apoptosis in cultured primary chondrocytes.

Chondrocytes transfected with control siRNA or SIRT1 siRNA cultured in the absence or presence of 2mM PBA. A, at the end of the culture period, SIRT1, p-PERK, p-eIF-2α, and CHOP protein expression were determined by Western blot, a representative blot from four independent experiments was presented for each protein. B, ATF4 and CHOP mRNA expression was examined by real-time PCR, results were presented as gene expression levels in all groups normalized to controls. C, chondrocytes were labeled with BrdU and prepared for staining, a representative BrdU-positive cell is indicated by the arrow. Scale bars, 100μm. Quantification was showed on the right. D, the expression of CyclinD1 and PCNA were detected by Western blot, a representative blot from three independent experiments was presented for each protein. E, chondrocytes were labeled with TUNEL, a representative TUNEL-positive cell is indicated by the arrow. Scale bars, 100μm. Quantification was showed on the right. F, Bcl-2 and Bax protein levels were determined by Western blot, a representative blot from three independent experiments was presented for each protein. G, chondrocytes were treated with ITS for 7 d, cultured in the absence or presence of 30μM EX527 and in combination with or without 2mM PBA. At the end of the culture period, chondrocytes were harvested, lysed, electrophoresed, and immunblotted for Col10a1 and MMP13, a representative blot from four independent experiments was presented for each protein. Data are expressed as means ± SD in each scatter plots. *P<0.05, **P<0.01.

Figure 5 CHOP siRNA partially reverses EX527-mediated inhibition of chondrocyte proliferation and hypertrophy, and neutralizes EX527-mediated proapoptotic effect.

Chondrocytes were transfected with CHOP siRNA for 72 h or cultured with or without 30μM EX527 and in combination with control or CHOP siRNA. A, the expression of CHOP was detected by Western blot, a representative blot from four independent experiments was presented for each protein. B, chondrocytes were labeled with BrdU and prepared for staining, a representative BrdU-positive cell is indicated by the arrow. Scale bars, 100μm. Quantification was showed on the right. C, CyclinD1 and PCNA protein expression were determined by Western blot, a representative blot from four independent experiments was presented for each protein. D, chondrocytes were labeled with TUNEL, a representative TUNEL-positive cell is indicated by the arrow. Scale bars, 100μm. Quantification was showed on the right. E, the expression of Bcl-2 and Bax were detected by Western blot, a representative blot from three independent experiments was presented for each protein. F, relative expression of C/EBP-β and Runx2 were examined by real-time PCR, results were presented as gene expression levels in all groups normalized to controls. G, chondrocytes were treated with ITS for 7 d, cultured in the absence or presence of EX527 and transfected with or without CHOP siRNA. At the end of the culture period, chondrocytes were harvested, lysed, electrophoresed, and immunblotted for Col10a1 and MMP13, a representative blot from four independent experiments was presented for each protein. Data are expressed as means ± SD in each scatter plots. *P<0.05, **P<0.01.

Figure 6 SIRT1 physically interacts with and deacetylates PERK

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A, immunoprecipitation of acetylated proteins from chondrocytes lysate followed by immunoblotting with the indicated antibodies. Input, supernatant before immunoprecipitation; IP, immunoprecipitate; IgG: negative control. B, PERK was immunoprecipitated from chondrocytes lysate and its level of acetylation was analyzed by immunoblotting with anti-acetyl-lysine antibody. C-D, PERK was immunoprecipitated from control or chondrocytes treated with EX527 (30μM) or SIRT1 siRNA and its level of acetylation was determined by immunoblotting with anti-acetyl-lysine antibody. The relative quantity of acetylated PERK were analyzed using ImageJ software and was shown on panel D. E, The physical interaction between endogenous SIRT1 and PERK was demonstrated by co-immunoprecipitation. SIRT1 was precipitated from chondrocytes lysate with anti-SIRT1 antibody and blotted with anti-PERK antibody, and vice versa. F-G, PERK was immunoprecipitated from control or chondrocytes treated with THG (80nM) or EX527 (30μM) or SIRT1 siRNA, and its levels of acetylation and phosphorylation were analyzed. The relative quantity of acetylated and phosphorylated PERK were analyzed using ImageJ software and was shown on panel G. A representative blot from three or four independent experiments was presented for each protein. Data are expressed as means ± SD in each scatter plots. *P<0.05, **P<0.01. H, proposed model of SIRT1 protection function in chondrocytes. SIRT1 protects chondrocytes against ER burden-induced injury and maintains ER homeostasis during the process of chondrogenesis.
Figure 1

A. Cumulative metatarsal growth (% control)

B. Increase length (μm)

C. Western blot analysis of SIRT1, p-PERK, PERK, p-eIF-2α, eIF-2α, CHOP, and GAPDH.

D. Relative SIRT1 mRNA expression

E. Representative images of control and EX527-treated samples.

F. Relative ATF4 mRNA expression

G. Relative CHOP mRNA expression

H. Cumulative metatarsal growth (% control) for EX527 and EX527+PBA groups.

I. Increase length (μm) for EX527 and EX527+PBA groups.

Figure 1
Figure 3
**Figure 4**

A. Western Blot analysis showing the expressions of SIRT1, p-PERK, PERK, p-eIF-2α, eIF-2α, CHOP, and GAPDH under different conditions of Control siRNA, SIRT1 siRNA, and SIRT1 siRNA + PBA.

B. Graphs showing the relative mRNA expression of ATF4 and CHOP under different conditions.

C. Images and graphs indicating the percentage of Brdu positive cells (%).

D. Western Blot analysis showing the expressions of CyclinD1, PCNA, and GAPDH under different conditions.

E. Images and graphs indicating the percentage of TUNEL positive cells (%).

F. Western Blot analysis showing the expressions of Bcl-2, Bax, and GAPDH under different conditions.

G. Western Blot analysis showing the expressions of Col10a1, MMP13, and their respective bands under different conditions of ITS, EX527, and PBA.
Figure 5
Figure 6

- A: Western blot showing protein bands for PERK, eIF-2α, and CHOP.
- B: IP: acetyl-lysine showing bands at 150kDa, 102kDa, 38kDa, 31kDa, and 24kDa.
- C: Control siRNA, SIRT1 siRNA, Vehicle, EX527.
- D: Graph showing ac-PERK/PERK levels.
- E: Input IP IgG showing bands at 150kDa, 102kDa, and 150kDa.
- F: THG, EX527, SIRT1 siRNA.
- G: Graph showing p-PERK/PERK levels.
- H: Schematic diagram illustrating the role of PERK, SIRT1, and CHOP in ER stress and cell fate.

Legend:
- PERK
- P-eIF-2α
- ATF4
- CHOP
- ER burden
- Proliferation
- Hypertrophy
- Apoptosis

** and * indicate statistical significance.
Sirtuin-1(SIRT1) stimulates growth plate chondrogenesis by attenuating the PERK-eIF-2α-CHOP pathway in the unfolded protein response
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