



Folate ameliorates homocysteine-induced osteoblast dysfunction by reducing endoplasmic reticulum stress-activated PERK/ATF-4/CHOP pathway in MC3T3-E1 cells

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Abstract

Introduction Homocysteine (Hcy) is considered a newly identified risk factor for osteoporosis. Nevertheless, the underlying mechanism of folate (FA), a key factor in the metabolism of Hcy, in protection against osteoblast dysfunction remains unclear. The purpose of this study was to investigate the mechanism by which FA attenuates Hcy-induced osteoblast damage.

Materials and methods The Hcy-induced MC3T3-E1 cells were treated with different concentrations of FA. Cell morphology, cell density, cell proliferation ability, alkaline phosphatase (ALP) activity and mineralization capacity were observed and determined; the gene expression of B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (BAX) and ERS-associated factors, including glucose-regulated protein 78 (GRP-78), activating transcription factor 4 (ATF-4) and growth arrest and DNA damage inducible gene 153 (CHOP/GADD153), were assessed by RT-PCR; and protein levels of GRP-78 and ATF-4 were analyzed by western blotting.

Results Hcy suppressed the proliferation, differentiation and mineralization ability of MC3T3-E1 cells in a concentration-dependent manner and activated the ERS signaling pathway. After intervention with different concentrations of FA, the cell viability and density, ALP activity, number of mineralized nodules, calcium content and Bcl-2 gene expression were all significantly increased, whereas the gene expression of GRP-78, CHOP/GADD153, ATF-4 and Bax was markedly down-regulated, and protein levels of GRP-78 and ATF-4 were also markedly decreased.

Conclusion The adverse effects of Hcy on osteoblast differentiation are dose dependent. FA not only protects against osteoblasts apoptosis but also has a direct osteogenic effect on Hcy-induced osteoblasts, which could be partially mediated by inhibition of the PERK-activated ERS pathway.

Keywords Folate · Homocysteine · Endoplasmic reticulum stress · Osteoblasts

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Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid formed as a key intermediate in the methionine cycle. Hcy levels vary considerably among individuals due to genetic background, medication and diet. Elevated plasma concentrations of Hcy, known as hyperhomocysteinemia (HHcy), have been proven to be closely related to several chronic diseases, including atherosclerotic cardiovascular disease [1], Alzheimer's disease [2], senile macular degeneration [3] and cancer [4]. Moreover, several studies have previously shown that Hcy promotes osteoclastic bone resorption, inhibits osteoblastic bone formation and induces osteoporosis via endoplasmic reticulum stress (ERS), mitochondrial injury and other pathways [5, 6]. In addition, Hcy also affects the

bone reconstruction process and increases the risk of fracture by upregulating intracellular reactive oxygen species and activating matrix metalloproteinases [7].

Folate (FA) is a key cofactor in the metabolism of the Hcy methylation pathway. Consequently, impairment of the methylation process due to FA deficiency results in the accumulation of Hcy. A preliminary study found that the plasma Hcy concentration of ovariectomized rats was significantly increased, while bone mineral density and alkaline phosphatase (ALP) activity were markedly decreased. However, FA administration markedly reduces levels of Hcy, increases bone ALP activity and enhances bone mineral density in an osteoporosis model of ovariectomized rats [8], but the specific mechanism has not been well described.

Based on existing data, we hypothesized that FA plays a bone-protective role by alleviating Hcy-induced ERS and improving the proliferation and differentiation of osteoblasts. Consequently, we assessed the effect of FA on the proliferation and differentiation of osteoblasts induced by Hcy, as well as the expression of apoptosis-related genes B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (BAX) in osteoblasts. Then, we investigated the gene and protein expression of key factors of the ERS pathway, including glucose-regulated protein 78 (GRP-78), activating transcription factor 4 (ATF-4) and growth arrest and DNA damage inducible gene 153 (CHOP/GADD153). The present study explored the possible bone protection mechanism of FA at the cellular and molecular levels, and provides a theoretical basis for the prevention and treatment of osteoporosis.

Materials and methods

Cell culture and treatment

MC3T3-E1 cells, which are preosteoblast cells with osteoblast differentiation ability, were used for the *in vitro* experiments and obtained from the Chinese Academy of Medical Sciences. The cells were cultured in low-glucose DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 0.5% penicillin and streptomycin (Sigma, USA) in a humidified incubator with 5% CO₂ at 37 °C. To induce differentiation, cells were seeded into 6-well culture plates and allowed to reach confluence. The media was then replaced with serum-free and low-glucose DMEM containing different concentrations of Hcy (0, 10⁻⁵, 10⁻⁴, 10⁻³ and 10⁻² mol/L, Sigma, USA) and cultured to specific times and assessed for the effect of Hcy on osteogenic differentiation. Based on the experimental results, the optimal inhibitory Hcy concentration of 10⁻³ mol/L was selected for subsequent experiments. Twenty-four hours after culture in serum-free low-glucose DMEM containing 10⁻³ mol/L Hcy, MC3T3-E1 cells were treated with various concentrations of FA (0,

10⁻⁷, 10⁻⁶, 10⁻⁵ and 10⁻⁴ mol/L, Yuanye Biology, Shanghai) to induce osteoblast differentiation (Fig. 1). All experimental manipulations were approved by the Ethics Committee of The First Hospital of Lanzhou University, Gansu, China.

Determination of cell viability

Cell viability was determined using the LIVE/DEAD™ Viability Kit (Thermo Fisher Scientific). Live cells are labeled with Calcein AM, which is hydrolyzed into a bright green fluorescent product after entering the cell. When the membrane integrity of dying or dead cells is compromised, ethidium homodimer-1 can enter the cell and bind to nucleic acids, exhibiting bright red fluorescence. Cells were cultured in 96-well plates at a density of 1×10⁴ cells/well for 48 h and then incubated with LIVE/DEAD reagents for 10 min at room temperature. Stained cells were observed under a fluorescence microscope (Olympus BX51 Microscope), and twenty representative images were acquired to compute cell density and the live cell percentage. Sampling areas were manually determined after visually assessing the entire sample region.

MTT assay

MC3T3-E1 cells (1×10⁴ cells/well) were seeded into 96-well plates and incubated overnight at 37 °C. After a 24-h incubation, the cells were treated with various concentrations of Hcy and/or FA for 48 h. Then, 100 μL MTT reagent (5 mg/mL) was added to each well and incubated for another 4 h. After the culture medium was removed, 150 μL of dimethyl sulfoxide (Sigma, USA) was added to each well, and the plates were shaken for 10 min. Optical densities (ODs) of cell supernatants were measured at 570 nm using an ultraviolet spectrophotometer (Shanghai Tianmei Instrument Company, Shanghai, China). Cell activity is expressed as the percentage of the value of normal cells.

Alkaline phosphatase (ALP) activity

MC3T3-E1 cells were plated into 6-well plates at a density of 5×10⁵ cells/well for 72 h. Then, the cells were washed three times with PBS, dissolved in 0.5 mL of Tris–HCl buffer (pH 7.6) containing 0.1% Triton X-100 and scraped into distilled water. ALP activity was measured using an ALP assay kit (Nanjing Jiancheng Biotechnology Co. Ltd., Nanjing, China) in accordance with the manufacturer's instructions. Absorbance was measured at 490 nm using a RT-6000 Enzyme Label Analyzer (Shenzhen Leidu Life Technology Co. Ltd., Shenzhen, China).

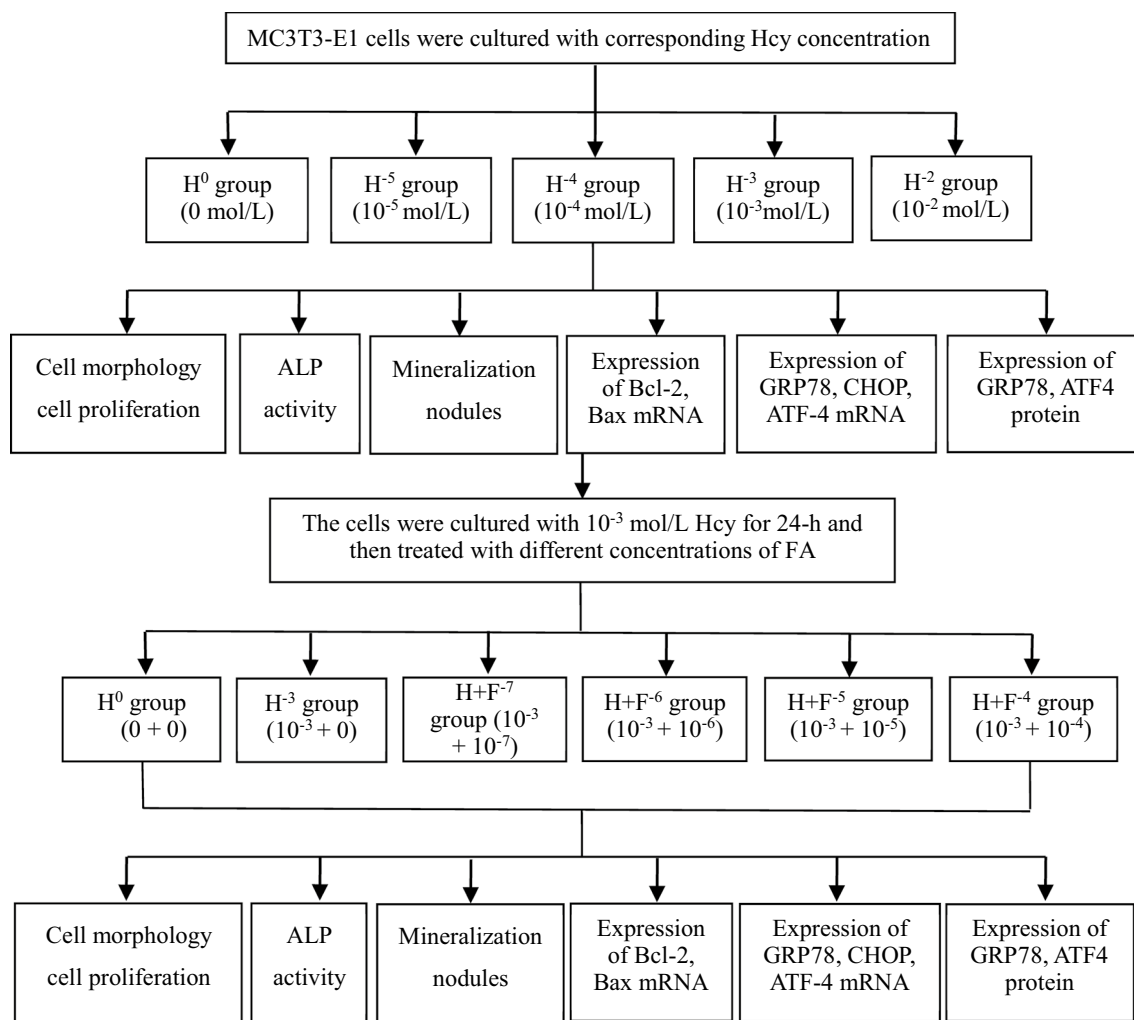


Fig. 1 Experimental flowchart

Mineralization assay

Alizarin red staining (ARS) was performed to determine the mineralization of MC3T3-E1 cells after 28 days of culture. Cells were washed twice with phosphate-buffered saline (PBS) and fixed in 95% ethanol for 10 min, and 1% alizarin red (Sigma, USA) was added and incubated for 30 min and then decolorized with 50% ethanol. A microscope (Olympus, Tokyo, Japan) was used to determine the number of mineralized nodules with a 10 mm coordinate network. To detect the calcium content in mineralized nodules, the absorbance was then measured at 570 nm [9]. The calcium content of the cell layer was normalized to the total DNA content.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cell monolayers using TRIzol reagent (TaKaRa, Tokyo, Japan). cDNA was synthesized

using the Prime Script™ RT reagent Kit according to the manufacturer's instructions (TaKaRa, Tokyo, Japan). Then, cDNA was used for RT-PCR assays conducted on an ABI 7300 FAST Real-Time PCR system (ABI, USA) using SYBR Premix Ex Taq™ (TaKaRa, Tokyo, Japan). A 40-cycle thermal program was used, consisting of pre-denaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s and annealing at 60 °C for 31 s. Each experiment was performed in triplicate. The relative mRNA expression was normalized to β -actin and calculated using the $2^{-\Delta\Delta CT}$ method. The sequences of RT-PCR primers are shown in Table 1.

Western blotting

Western blotting was performed to assess the protein expression of GRP-78 and ATF-4. Briefly, protein concentration was measured using the BCA Protein Assay Kit (Solarbio). Equal protein concentrations were loaded, separated by SDS-PAGE (Solarbio), and transferred to PVDF membranes

Table 1 Primers sequences

| Gene | Forward primer | Reverse primer |
|---------------------|----------------------------|-----------------------------|
| <i>GRP-78</i> | 5'-CACAGACGGGTCATTCC-3' | 5'-CCTATGTCGCCTTCACT-3' |
| <i>CHOP/GADD153</i> | 5'-ATTGGGGGACCTATATCTC-3' | 5'-CTTGCTCTTCCTCCTCTTCC-3' |
| <i>ATF-4</i> | 5'-CCTATAAAGGCTTGCGGCCA-3' | 5'-CACTGCTGCTGGATTTTCGTG-3' |
| <i>Bcl-2</i> | 5'-CCAGATGGGAAGCAGAGCAA-3' | 5'-TGGGGGACAGGGGATAAGAG-3' |
| <i>Bax</i> | 5'-CACTAAAGTGCCCGAGCTGA-3' | 5'-GTGAGGACTCCAGCCACAAA-3' |
| <i>β-actin</i> | 5'-GTCCCTCACCTCCCAA AG-3' | 5'-GCTGCCTCAACACCTCAACCC-3' |

(Solarbio), which were blocked and incubated with a primary antibody at 4 °C overnight and then with secondary antibodies for 2 h at room temperature. Immunoreactivity was detected using an enhanced chemiluminescence system (Millipore). Quantification of western blots was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All data are shown as the mean ± standard deviation (SD) and were analyzed using one-way analysis of variance (ANOVA) with GraphPad Prism 7.0. *P* values less than 0.05 were considered statistically significant.

Results

FA improves the morphology of MC3T3-E1 cells induced by Hcy

Representative fluorescence images of MC3T3-E1 cells labeled with live/dead dye solution are shown in Fig. 2, where live cells are labeled in green and dead cells in red. After 48 h in culture, the cell population was uniformly distributed and established frequent cell-to-cell connections, and the typical morphology of MC3T3-E1 cells, such as polygonal, triangular and spindle-shaped cells, is shown in green. The effects of Hcy and FA on cell density and cell survival were further explored, and the results showed that after treatment of the cells with different concentrations of Hcy, a reduced density of cells and a lower percentage of live cells were observed and dead cells were appeared in Hcy-treated cells at concentrations of 10^{-3} and 10^{-2} mol/L (Fig. 2a, c, e). However, after adding FA to interfere with Hcy-induced MC3T3-E1 cells, the live cells gradually appeared denser and more fused, and dead cells gradually disappeared as the FA concentration increased (Fig. 2b). As shown in Fig. 2d, f, quantitative analysis also showed that the percentage of live cells increased from 21.8 to 93.2 and the density of cells increased from 6.5×10^4 to 10.5×10^4 cells cm^{-2} with the increasing FA concentration (0 – 10^{-4} mol/L).

FA stimulates cell proliferation in MC3T3-E1 cells induced by Hcy

The effects of Hcy on MC3T3-E1 cell proliferation were assessed using an MTT assay. We found that the cell proliferation ability of MC3T3-E1 cells exposed to different concentrations of Hcy (0 , 10^{-5} , 10^{-4} , 10^{-3} and 10^{-2} mol/L) was significantly decreased (Fig. 3a). Treatment of MC3T3-E1 cells with different concentrations of FA (0 , 10^{-7} , 10^{-6} , 10^{-5} and 10^{-4} mol/L) resulted in increased cell proliferation ability, suggesting a stimulatory effect on cell proliferation in response to FA. Meanwhile, the stimulatory effect of FA was dose dependent (Fig. 3b). The optimal stimulatory effect on cell proliferation was observed in response to 10^{-4} mol/L FA treatment.

FA promotes osteogenic differentiation in MC3T3-E1 cells induced by Hcy

Osteoblast differentiation is the most essential step for bone formation. To elucidate the effect of FA on the process of osteoblast differentiation, ALP activity was investigated after cells had been cultured in Hcy-containing medium for 3 days with or without FA. We found that the ALP activity decreased dramatically with increasing concentrations of Hcy (0 – 10^{-2} mol/L). However, MC3T3-E1 cells treated with FA exhibited a gradual increase in ALP activity (Fig. 4a, b).

Mineralization of these cells was investigated using Alizarin red staining, and the total number of nodules per culture dish was quantified macroscopically. In regular culture medium without Hcy, an average of 100 nodules were counted per well. However, when different concentrations of Hcy were added to the medium, the number of mineralized nodules gradually decreased in a concentration-dependent manner as the Hcy concentrations increased (Fig. 4c, e). To determine whether the addition of FA modified the effects of Hcy, FA (10^{-7} – 10^{-4} mol/L) was added to the culture medium after 24 h of Hcy exposure, which showed that FA stimulated the number of nodules formed in a dose-dependent manner (Fig. 4d, f). In addition, calcium deposition in the mineralized nodules was also assessed using Alizarin red staining. Similarly, the calcium content was significantly inhibited by Hcy (Fig. 4g), whereas FA significantly

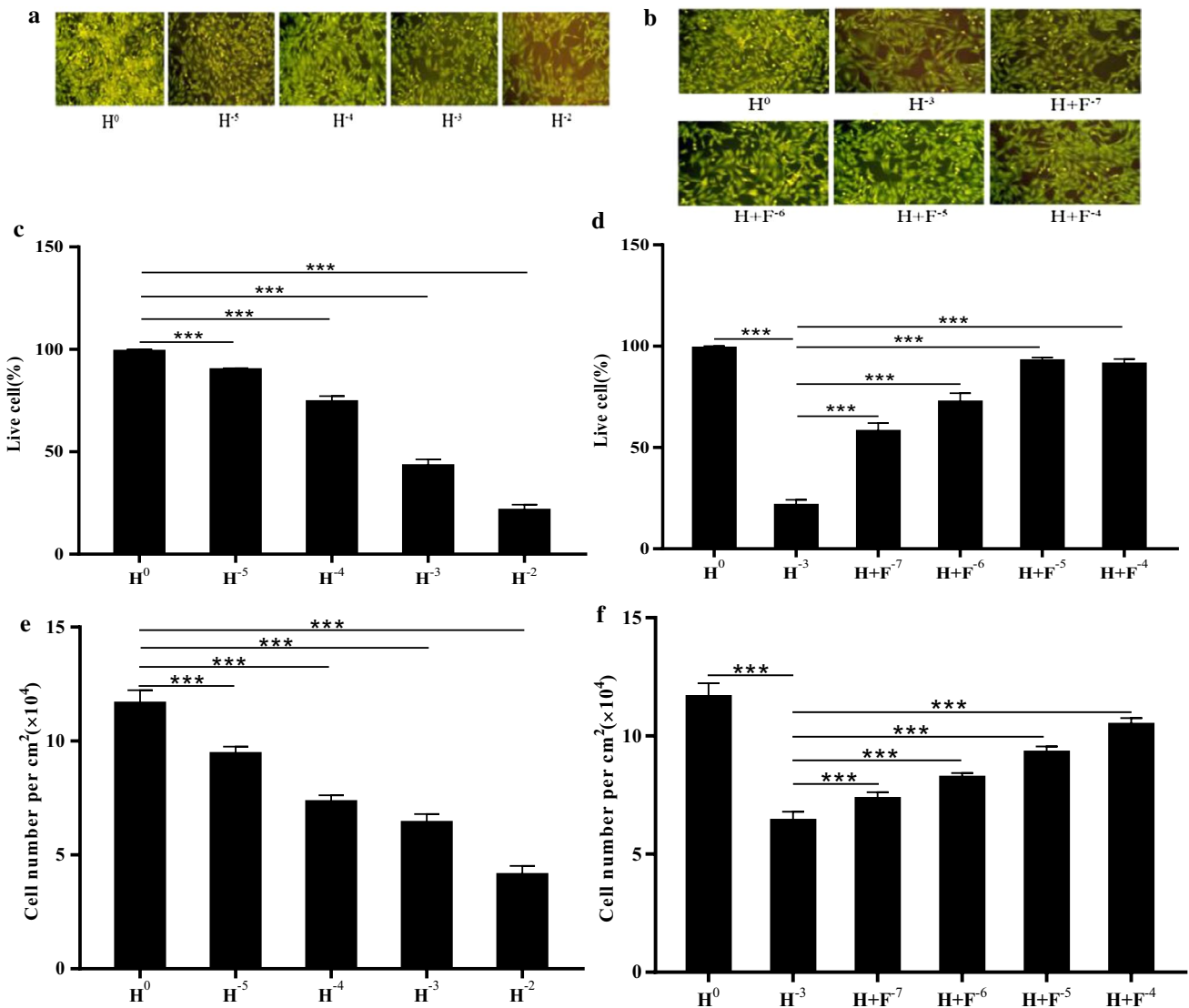


Fig. 2 Representative fluorescent images of MC3T3 cells with live cells stained as green and dead cells as red. **a** The morphology of MC3T3-E1 cells induced by Hcy. **b** The morphology of Hcy-induced

MC3T3-E1 cells treated with FA. **c, d** The percentage of live cells. **e, f** The number of cells per cm^2 was calculated ($\times 10^4/cm^2$). Data are represented as the mean \pm SD. *** $P < 0.001$

stimulated calcium deposition in all Hcy-containing media in a dose-dependent manner, with maximal stimulation being reached at a FA concentration of 10^{-4} mol/L (Fig. 4h). In summary, FA treatment attenuates the deleterious effects of Hcy on osteogenic differentiation.

FA reduces apoptosis induced by Hcy in MC3T3-E1 cells

We first examined gene expression levels of the apoptosis-related proteins Bcl-2 and Bax in cells with Hcy administration. The data showed that Bax expression was increased and Bcl-2 expression was decreased in cells exposed to Hcy (Fig. 5a), indicating that Hcy promoted apoptosis of

osteoblasts. Prolonged or severe ERS induces apoptosis. To examine whether FA ameliorates Hcy-induced apoptosis in osteoblasts, MC3T3-E1 cells were treated with different concentrations of FA after a 24-h incubation with 10^{-3} mol/L Hcy. The gene expression levels of Bcl-2 were increased while those of Bax were decreased as the FA concentration increased (Fig. 5b). Taken together, these results demonstrate that FA significantly ameliorates Hcy-induced apoptosis in MC3T3-E1 cells.

FA reduces ERS induced by Hcy in osteoblast cells

In this study, the ERS response was assessed by detecting gene and protein expression levels of the ERS markers

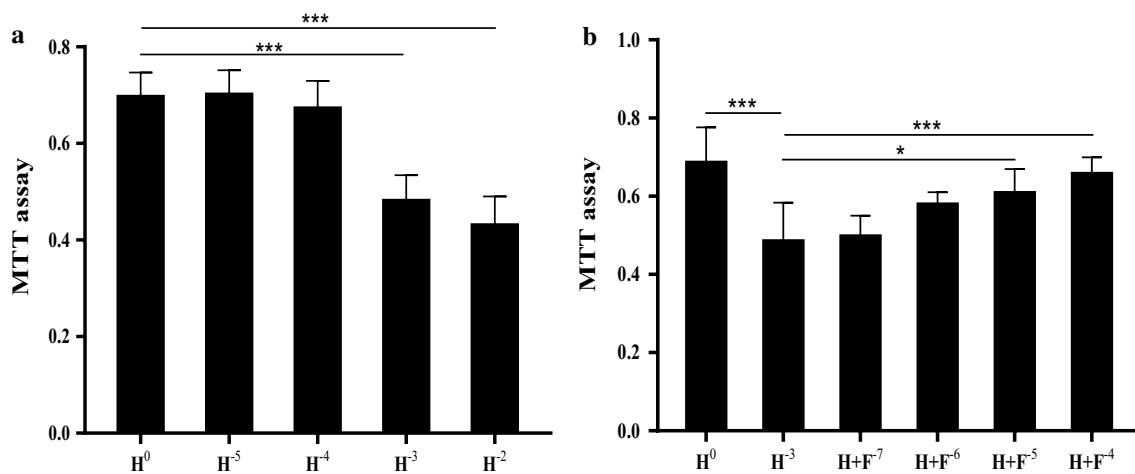


Fig. 3 Cell proliferation was evaluated using MTT assay. **a** The ability of cell proliferation in MC3T3-E1 cells with different concentrations of Hcy (0, 10^{-5} , 10^{-4} , 10^{-3} and 10^{-2} mol/L). **b** The ability of

cell proliferation in MC3T3-E1 cells after treated with different concentrations of FA (0, 10^{-7} , 10^{-6} , 10^{-5} and 10^{-4} mol/L). Data are represented as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

GRP78, CHOP and ATF-4. Gene expression of GRP-78, CHOP and ATF-4 as well as the protein expression of GRP-78 and ATF-4 were enhanced with increasing concentrations of Hcy (Fig. 6a–c), indicating that ERS is involved in Hcy-induced apoptosis in osteoblasts. To determine whether FA alters ERS in osteoblasts exposed to Hcy, MC3T3-E1 cells were treated with different concentrations of FA after a 24-h incubation with 10^{-3} mol/L Hcy. Quantitative analysis by western blot and PCR showed that expression levels of GRP78, CHOP and ATF-4 were significantly decreased in response to FA treatment (Fig. 6d–f). These findings suggest that FA treatment effectively rescues the Hcy-induced ERS response in MC3T3-E1 cells.

Discussion

The present study demonstrated that Hcy inhibits the proliferation, differentiation and mineralization of osteoblasts in a concentration-dependent manner and induces osteoblast apoptosis by activating the ERS signaling pathway. However, FA reverses these Hcy-mediated effects. After intervention with FA, osteoblast morphology, cell density, cell activity and cell proliferation ability were significantly improved, and the mineralization capacity of osteoblasts was enhanced, as demonstrated by the increase in ALP levels, mineralized nodule number and calcium content. In addition, FA protected against apoptosis of osteoblasts induced by Hcy by inhibiting the PERK pathway of ERS, further confirming our hypothesis.

Osteoporosis is a skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to increased bone fragility and fracture

susceptibility, which seriously affects patient quality of life. Imbalanced osteoblastic bone formation and osteoclastic bone resorption from various causes is the primary mechanism underlying osteoporosis and osteoporotic fracture [10]. Osteoblasts are major bone-forming cells that undergo proliferation, mineralization, renewal, and repair during the bone formation process, and each stage is affected and regulated by a variety of factors, including genetic backgrounds, hormone levels and cellular regulatory factors [11]. In recent years, HHcy has been identified as a new risk factor for osteoporotic fractures. A population-based prospective study found that increased plasma Hcy is associated with low bone mineral density and a high risk of fracture, while folic acid and vitamin B₁₂ supplementation reduce the risk of hip fracture [12]. In vivo, bone fragility at the femoral neck in Hcy-fed rats was increased by nearly 36%, the content of osteocalcin, a bone formation marker, decreased by 34%, and bone quality was consistently decreased in the presence of increased circulating Hcy levels [13]. Previous studies have shown that Hcy not only weakens the function of osteoblasts, promotes the activity of osteoclasts and increases bone resorption, but also results in a detrimental effect on bone by decreasing bone blood flow, increasing the levels of reactive oxygen species and activating the expression of matrix metalloproteinases that degrade extracellular bone matrix [6]. Furthermore, Hcy influences the formation of a stable bone matrix by inhibiting the collagen cross-linking enzyme lysyl oxidase in osteoblasts [14]. Similarly, our study found that Hcy not only reduces the density of MC3T3-E1 cells, hinders the proliferative capacity of osteoblasts but also inhibits the activity of ALP and decreases the number and calcium content of calcified nodules, suggesting a deleterious effect of Hcy on osteoblasts.

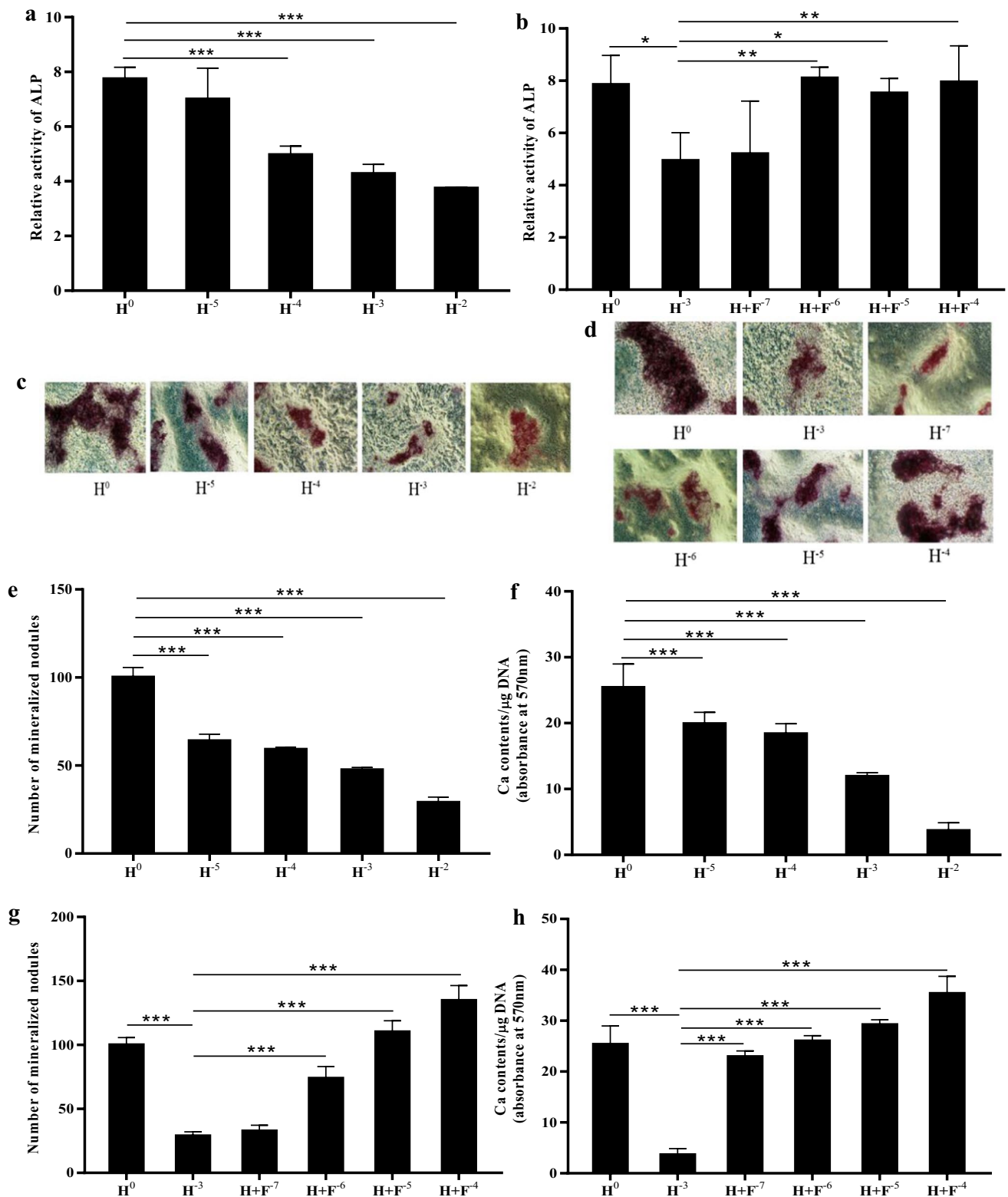


Fig. 4 The osteogenic differentiation in MC3T3-E1 cells. **a** Effect of Hcy on ALP activity. **b** Effect of FA on ALP activity in Hcy-induced MC3T3-E1 cells. **c** Effect of Hcy on the mineralization of MC3T3-E1 cells (100×). **d** Effect of FA on the mineralization of MC3T3-E1

cells induced by Hcy (100×). **e–h** Quantification of mineralized nodules number and calcium contents were performed in Hcy-induced cells treated with or without FA. Data are represented as mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

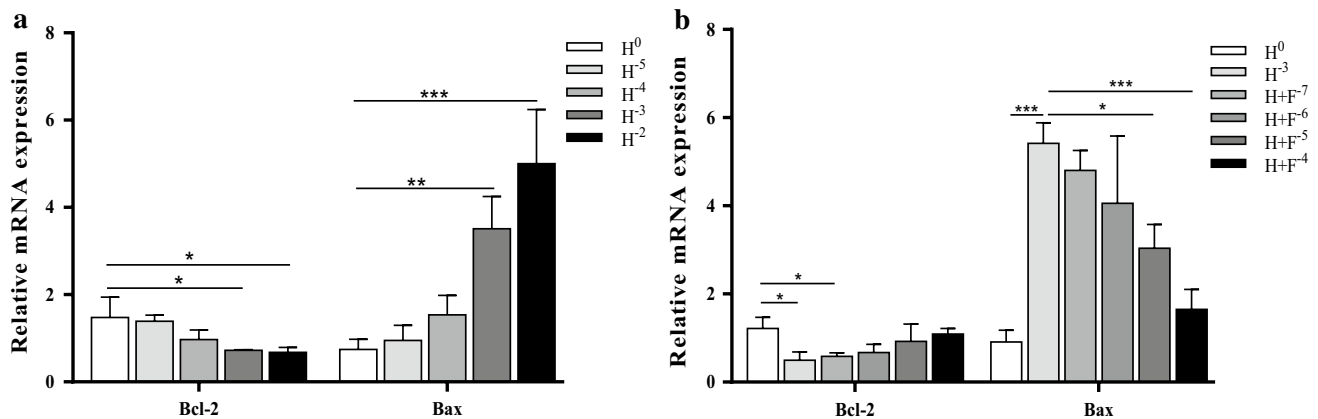


Fig. 5 FA protects MC3T3-E1 cells against apoptosis induced by Hcy. **a** The gene expression levels of Bcl-2 and Bax in cells interfered with Hcy for 24 h. **b** The expression levels of Bax and Bcl-2 with

different concentrations of FA after a 24-h incubation with Hcy at a concentration of 10^{-3} mol/L. Data are represented as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

The endoplasmic reticulum (ER) is primarily recognized as a protein-folding factory that is responsible for the biosynthesis, folding and processing of proteins. Under physiological conditions, abnormally folded or unfolded proteins can be degraded by the proteasome through ER-related degradation processes. However, in the case of pathology, the accumulation and aggregation of unfolded or misfolded proteins in the ER lumen causes ER dysfunction and triggers an adaptive signaling cascade called ERS [15]. GRP-78, an ER resident chaperone, regulates the activation of each of the three unfolded protein response pathways: inositol-requiring enzyme 1 α (IRE-1 α), activating transcription factor 6 (ATF-6) and PERK [16]. ATF-4, a dominant protein selectively translated in the PERK/eIF-2 α , is the primary regulator of transcription of key genes for adaptive response and has many biological functions, including participation in amino acid metabolism, bone metabolism and induction of apoptosis and autophagy [17–20]. ATF-4 cooperates with many regulators of osteogenic differentiation to regulate the expression of osteogenic-related genes, thereby regulating osteogenic differentiation [21]. However, under long-term sustained or excessive ERS conditions, the PERK/eIF-2 α pathway is activated, which stimulates increased biosynthesis of ATF-4 and further upregulates the expression of CHOP/GADD153, while high expression of CHOP induces osteoblast apoptosis by upregulating the expression of the pro-apoptotic protein Bax, downregulating the anti-apoptotic protein Bcl-2 and promoting the formation of apoptotic bodies and activation of caspases [22, 23]. Moreover, it has been shown that eIF-2 α activates a cytoprotective intracellular degradation system, autophagy, as an alternative pathway to degrade misfolded proteins accumulated in cells to reduce bone loss [24]. Of course, it has also been suggested that the effects of autophagy induced by ERS on cell survival are different and have dual roles, including both pro-survival

and pro-death, which may be dependent on the extent of ERS [25].

Studies have shown that high levels of Hcy upregulate the mRNA expression of GRP-78, ATF-4 and CHOP/GADD153 in MC3T3-E1 cells, suggesting that Hcy is an important factor mediating PERK activation-induced apoptosis in ER stress [26]. A previous study found that Hcy reduced osteoblasts viability in a dose- and time-dependent manner; at the same time, the expression of ERS-associated molecules, such as IRE-1 α , GRP-78 and ATF-4 was significantly increased in Hcy-treated osteoblasts, indicating that Hcy-mediated ERS activation promotes osteoblast damage [27]. In vivo, it has been shown that overexpression of CHOP, which appears as a result of ERS induced by Hcy, contributes to osteoblast apoptosis by upregulating the expression of Bax and downregulating Bcl-2 [28, 29]. Our results are consistent with previous reports showing that along with the increase in Hcy exposure intensity, an increase in the gene expression of GRP-78, ATF-4, CHOP/GADD153 and Bax and a decrease in Bcl-2 mRNA expression were observed, further confirming that Hcy induces osteoblast apoptosis by activating the ERS pathway. As a cell survival pathway, autophagy also plays a crucial role in maintaining bone homeostasis [30]. The relevance of Hcy to autophagy has been demonstrated in other cells, including hepatocytes, cardiomyocytes, and nerve cells [31–33], but whether Hcy regulates osteoblast differentiation by affecting autophagy is poorly understood. Studies have suggested that Hcy induces autophagy by activating oxidative stress, ERS and mitophagy. During the early stage, Hcy-induced autophagy has a protective effect on cell survival, whereas when the body is in a long-term stress state, the levels of autophagy-related proteins and calcium ions increase, resulting in mitochondrial autophagy and injury, ultimately leading to apoptosis [34]. This study did not assess the expression of

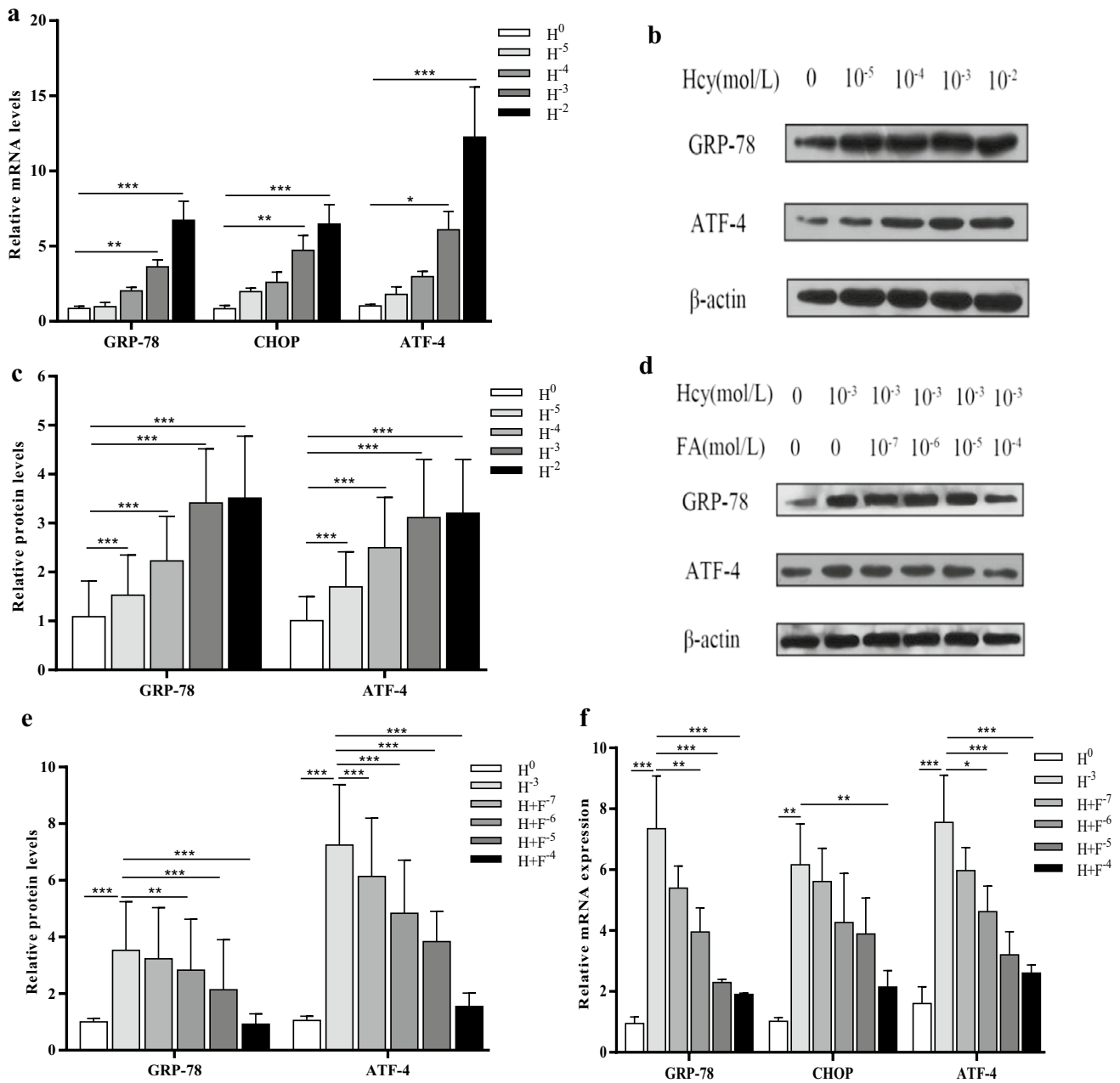


Fig. 6 The gene and protein expression levels of ERS related molecules. **a–c** Western blots and quantitative analysis were conducted to assess the expression levels of GRP78, CHOP and ATF-4 in MC3T3-E1 cells treated with Hcy. **d–f** The expression levels of GRP78,

CHOP and ATF-4 in Hcy-induced MC3T3-E1 cells after FA intervention. Data are represented as mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

autophagy-related proteins, and the role of autophagy in these processes is still unclear. Therefore, it is necessary to explore the mechanisms of autophagy in Hcy-induced osteoblast injury in the future.

FA is involved in multiple metabolic processes, such as carbon unit transfer, DNA methylation, methionine synthesis and purine synthesis in vivo and plays an important modulatory effect on cell division, homeostasis and gene

expression. Deficiency of FA can result in a variety of pathological mechanisms, such as hepatic steatosis, insulin resistance, endothelial cell dysfunction and osteoblast apoptosis. Among them, ERS has been proposed to explain observed pathological changes [35–38]. A previous study indicated that the mRNA and protein expression of the ERS-associated proteins Bip, PERK, EIF2 α and CHOP in Hcy-induced hepatocytes was upregulated both in vivo and

in vitro; moreover, expression of the apoptosis-associated proteins caspase-3 and caspase-12 was also increased. Most importantly, the ERS-associated apoptosis induced by Hcy was reversed after folate supplementation [35]. Another study demonstrated that FA deficiency may lead to ERS by interfering with the formation of disulfide bonds and activating the unfolded protein response, thereby activating the transcription of genes related to fat formation [36]. Hsu et al. found that folic acid deficiency is a risk factor for diabetes, which can induce ERS by upregulating CHOP expression and promoting apoptosis of insulin-producing pancreatic RINm5F islet β cells [37]. Clinical studies have reported that patients with insufficient folic acid intake have an increased risk of cardiovascular disease. The possible mechanism whereby this occurs is through to involve accumulation of Hcy causing folic acid deficiency, which triggers ERS and induces vascular endothelial cell dysfunction, promoting vascular smooth muscle cell proliferation, and further accelerating atherosclerosis formation [38].

To date, few studies have reported on the correlation between FA and bone metabolism. Clinical studies have found that the risk of osteoporosis and fracture in patients with folate deficiency is significantly increased, but the specific molecular mechanism remains unclear [39]. Adequate FA intake can prevent DNA damage, reduce oxidative stress and prevent apoptosis, which may be a potential mechanism for the protective effect of FA on bone cells [40]. Studies have shown that FA deficiency directly activates autophagy and induces cell death [41], but the effect of FA deficiency-induced autophagy on osteoblasts has not been reported. In addition, FA deficiency interferes with bone collagen connections and fiber formation by affecting Hcy metabolism, subsequently affecting bone mineralization capacity [42]. However, FA intervention reduces Hcy concentration in ovariectomized rats, increases levels of ALP in bone tissue homogenate, increases the bone mineral density of the lumbar spine and femur, improves the internal quality of the lumbar spine, and enhances the hardness and flexibility of the lumbar spine [8]. In our study, FA was used to interfere with Hcy-induced MC3T3-E1 cells to explore whether FA affected the proliferation, differentiation and calcification of osteoblasts through the ERS pathway. It was concluded that FA significantly attenuates the deleterious effects of Hcy on osteoblasts, as demonstrated by increasing cell viability and number, enhancing ALP activity and the mineralization ability of osteoblasts. Moreover, with increasing FA intervention concentration, the mRNA expression of GRP-78, ATF-4, CHOP/GADD153 and BAX in MC3T3-E1 cells was downregulated, while the mRNA expression of Bcl-2 gradually increased. Similar results were observed at the protein level, which further confirmed that FA has a beneficial effect on bone metabolism and partially attenuates the damage to

osteoblasts induced by Hcy through the PERK-mediated ERS pathway.

In conclusion, this study clearly demonstrates that the adverse effects of Hcy on osteoblast proliferation and differentiation are dose-dependent. FA not only significantly enhances cell viability and protects against osteoblasts apoptosis but also has a direct osteogenic effect on osteoblasts; furthermore, these actions could be partly mediated by inhibition of the PERK-mediated ERS pathway. However, whether FA achieves these bone-protective effects by inhibiting two other ERS pathways, IRE-1 α and ATF-6, remains unclear. Therefore, additional basic and clinical studies are still needed to further confirm the effects of FA on bone metabolism.

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Author Contributions SS, DZ and JJJ completed the project, analyzed the data, and drafted the manuscript; XLT and HXC designed the study, interpreted the data and contributed to critically revising the manuscript; HYZ, QMW and WNR participated in experimental operations; DHZ provided financial support and supervised data collection and manuscript writing. All authors read and approved the final manuscript.

Declarations

Conflict of interest All authors have no interests to disclose.

Ethical approval All authors have read the Journal's position on issues involved in ethical publication.

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