Polycytosine RNA-Binding Protein 1 Regulates Osteoblast Function via a Ferroptosis Pathway in Type 2 Diabetic Osteoporosis

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Abstract

In recent years, type 2 diabetic osteoporosis has become a research hotspot for the complications of diabetes, but the specific mechanism of its occurrence and development remain unknown. Ferroptosis caused by iron overload is considered to be one of the important cause of type 2 diabetic osteoporosis. Polycytosine RNA-binding protein 1 (PCBP1), an iron ion chaperone, was considered as a protector of ferroptosis. The present study aimed to investigate the existence of ferroptosis and specific role of PCBP1 in the development of type 2 diabetes. Firstly, a Cell Counting Kit-8 assay was used to detect the changes of osteoblast viability under the influence of high glucose (HG) and/or ferroptosis inhibitor given at different concentrations and at different times. In addition, the morphological changes of mitochondria in osteoblasts under high glucose were examined via transmission electron microscopy, and the expression levels of PCBP1, ferritin and the ferroptosis-related protein glutathione peroxidase 4 (GPX4) under the action of high glucose were detected via western blotting. Furthermore, a lentivirus was used to silence and overexpress PCBP1. Western blotting was used to detect the expression of levels of the osteoblast functional proteins osteoprotegerin (OPG) and osteocalcin (OCN), while flow cytometry was used to detect the changes of reactive oxygen species (ROS) levels in each group. Under the action of high glucose, the viability of osteoblasts was significantly decreased and the number of mitochondria undergoing atrophy was significantly increased, PCBP1 and ferritin expression levels were increased and GPX4 expression was decreased. Western blotting results demonstrated that infection of the lentivirus overexpressing PCBP1, increased the expression levels of ferritin, GPX4, OPG and OCN, compared with the high glucose group. The flow cytometry results identified a reduction in ROS, and an opposite result was obtained after silencing PCBP1. In conclusion, it was suggested that PCBP1 may protect osteoblasts and reduce the harm caused by ferroptosis by promoting ferritin expression under a high glucose environment. Moreover, it was indicated that PCBP1 may be a potential therapeutic target for treating type 2 diabetic osteoporosis.

Introduction

In recent years, the incidence of diabetes, especially type 2 diabetes, has increased annually, and it seriously harms human health ¹. There are >100 complications of diabetes, including diabetic osteoporosis, diabetic nephropathy, diabetic foot, diabetic heart disease, diabetic vascular disease and diabetic peripheral neuropathy, etc ². Among them, type 2 diabetic osteoporosis is particularly common, especially in the elderly. As individuals age, they have a significantly higher risk of fractures, which seriously affects the patient's quality of life and exerts a heavy burden on society ³. There are multiple types of osteoporosis. Compared with type 1 diabetic osteoporosis and other types of osteoporosis, type 2 diabetic osteoporosis has a more complicated environment and pathogenesis, including high glucose (HG) toxicity, inflammatory response, AGEs(Advanced glycation end products) aggregation and iron ion metabolism disorders ⁴. However, the exact cause of type 2 diabetic osteoporosis has not been fully elucidated.

Iron is an important trace element that sustains the life of an organism. The metabolic process of iron has a complete control system in advanced animals, including humans, to ensure that the body has a steady state of iron. Moreover, iron homeostasis is of great significance for cells to maintain their physiological functions⁵. Ferritin is the main storage protein of intracellular iron, and it is widely expressed in animals, plants and microorganisms. Ferritin can protect cells from the toxic effects of excessive iron and can release iron ions to meet the functional requirements of cells when iron is deficient⁶, and thus, is essential for the maintenance of iron homeostasis.

Ferroptosis is a non-apoptotic programmed death pathway that is dependent on iron ions and reactive oxygen species (ROS). When iron ions are excessively accumulated in cells, the large amount of ROS produced by the Fenton reaction attacks biological macromolecules, resulting in an imbalance of iron homeostasis in the cell, ultimately leading to cell death⁷. There are numerous studies investigating ferroptosis; however, there are fewer articles regarding bone metabolism. For example, Lu *et al*⁸ reported that ferroptosis may occur in the pathogenesis of osteoporosis, as detected using a mouse model of osteoporosis and bioinformatics methods. Seibt TM et al⁹ found that glutathione peroxidase 4 (GXP4) is a key regular of ferroptosis. Our previous studies have revealed that iron overload caused by high glucose is an important factor causing osteoblast injury, and ROS levels are significantly increased during this period¹⁰. These findings suggest that ferroptosis is closely associated with the pathogenesis of diabetic osteoporosis, but the specific occurrence process and whether ferritin is involved in this process require further examination.

Polycytosine RNA-binding protein 1 (PCBP1) is a type of iron ion chaperone protein widely present in cells, which can bind iron ions and transfer iron ions to ferritin, thereby increasing the load of iron ions in ferritin¹¹. Among the PCBP family, PCBP1 is one of the most studied, and it has a wide range of functions, including regulating gene transcription, maintaining mRNA stability, promoting translation and acting as an iron ion partner¹²⁻¹⁴. Ryu *et al*¹⁵ reported that mice lacking PCBP1 exhibited small cell iron deficiency anemia and compensated by regulating erythropoietin. Protchenko O et al¹⁶ found that unchaperoned iron in PCBP1- delated mouse hepatocytes lead to production of ROS and indicated that PCBP1 may be a key factor in preventing ferroptosis. At present, to the best of our knowledge, no research has confirmed whether PCBP1 is involved in the occurrence and development of type 2 diabetic osteoporosis, and the relationship between changes in PCBP1 level and ferritin and ferroptosis in osteoblasts remains to be studied.

Therefore, the aim of the present study was to determine the existence of ferroptosis and the role of PCBP1 in the pathogenesis of type 2 diabetic osteoporosis. We hypothesized that the compensatory increase of PCBP1 in type 2 diabtic osteoporosis may lead to increased ferritin in osteoblasts, as well as reduce excessive accumulation of iron ions and slow the occurrence and development of ferroptosis. The present study first detected the expression levels of PCBP1 and ferritin in a high glucose environment, and then tested the relevant indicators, such as ferritin, after lentiviral silencing and overexpression of PCBP1. The current findings will help elucidate the pathogenesis of osteoporosis in type 2 diabetes and

could provide a theoretical basis for the treatment of metabolic diseases that target PCBP1 to regulate ferroptosis.

Materials And Methods

Cell culture and related reagents. The human osteoblast-like cell line, hFOB1.19, was purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences, and cultured in DMEM/F12 medium (glucose concentration 17.5 mmol/l; Hyclone; Cytiva) supplemented with 10% FBS (Hyclone; Cytiva) and 1% penicillin (Invitrogen; Thermo Fisher Scientific, Inc.), and cultured in a 34°C, 5% CO₂ incubator. The solution was changed every 2 days. When the cells were 80% confluent, they were subcultured with Trypsin-EDTA (Sigma, America).

PCBP1 lentiviral reagents were purchased from the Shanghai GeneChem Co., Ltd. PCBP1 (EPR11055, 1:500), ferritin (EPR3005Y, 1:500) and glutathione peroxidase 4 (GPX4, EPNCIR144, 1:500) antibodies were purchased from Abcam. Osteoprotegerin (OPG, sc-390518, 1:500) and osteocalcin (OCN, sc-390877, 1:500) antibodies were purchased from Santa Cruz Biotechnology, Inc. The human bone mineralization induction culture base was purchased from the Cyagen Biosciences, Inc. β -actin (bsm-33036M, 1:1000) and all secondary antibodies (Goat Anti-Rabbit IgG, bs-40295G-HRP, 1:5000. Goat Anti-Mouse IgG, bs-40296G-HRP, 1:5000) were purchased from BLOSS.

Cell viability analysis. Cell viability was detected using a Cell Counting Kit (CCK)-8 (Abcam) assay, according to the manufacturer's instructions. Cells from each group treated with different glucose concentrations (17.5, 26.25, 35, 43.75 and 52.5 mM, Sigma, America) and/or ferroptosis inhibitor (Ferrostatin-1, Fer-1, Sigma, America) were seeded in 96-well plates at a density of 6×10^3 cells/well, and cultured with 10% serum-containing medium. When the percentage of cell growth reached 70–90%, the serum concentration of the medium was reduced to 1% and the culture was continued to maintain the cells in the same division state as much as possible. After 24, 48 and 72 h, 10 μ l CCK-8 solution was added to each well, and the reaction was continued in the incubator for 4 h. The optical density (OD) value of cells in each well was observed using a microplate reader using a wavelength of 490 nm. Relative cell activity (%) = (OD value of wells in treatment group-OD value of blank group) / (OD value of control group-OD value of blank group).

ROS detection. Cells in the logarithmic growth phase were collected and inoculated into 6-well plates at a density of 5×10^6 cells/ml, and were cultured in an incubator at 37.5°C for 72 h. We use 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma, America) to evaluate the ROS level. Briefly, DCFH-DA was diluted (1:1,000) with serum-free medium to a final concentration of 10 μ mol/l. After the cells were collected, they were suspended in diluted DCFH-DA, incubated in a cell incubator for 20 min, and inverted and mixed every 3–5 min. After washing the cells three times with serum-free cell culture solution, the ROS level in the cells in each group was measured using a flow cytometer at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Western blotting. After the cells were digested with Trypsin-EDTA, a lysis buffer (Abcam) containing protease and phosphatase inhibitor cocktail was added, placed on ice for lysis for 30 min and then centrifuged at 12,000 x g at 4°C for 30 min, following which the supernatant containing total protein was collected. After the total protein was extracted, the protein concentration was determined using the BCA method, and the protein sample (50 µg protein) was separated via 12% sodium lauryl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane (MerckMillipore, America) at 60 V for 2 h. The membrane was blocked with a blocking buffer containing 5% skim milk for 2 h and was then incubated overnight at 4°C with a primary antibodies (diluted at 1:100 to 1:1,000). Subsequently, the membrane was incubated with a HRP-conjugated secondary antibody (anti-mouse or anti-rabbit; IgG was diluted at 1:6,000 or 1:10,000) for 2 h at room temperature. The bands were visualized with an EC3 imaging system (Analytik Jena AG), and the ratio of the OD of each band to β -actin, as the internal reference protein, was measured using ImageJ 1.48V software (National Institutes of Health).

Lentiviral infection. The cells were seeded at a density of 4×10^4 cells/ml in a 96-well culture plate, with a volume of 90 µl per well, and were cultured in a 37.5°C incubator for 24 h. An appropriate amount of the virus stock solution was diluted with Enhanced Infection Solution to a titer of 1×10^8 TU/ml. This was added to the cells for virus infection, and then the culture plate was returned to the incubator for incubation. After the cells were infected for 3–4 days, the fluorescence expression was observed under a fluorescence microscope. At a later stage of infection, the cells were exchanged and passaged according to the growth of the cells, in order to obtain stable expressing cell lines.

Transmission electron microscope. After the cells were digested with Trypsin-EDTA, the suspended cells were collected via centrifugation and were washed three times with pre-chilled Phosphate Buffer Solution (PBS, Gibco, America). Then, the cells were fixed with 5% glutaraldehyde (Sigma, America) fixing solution for 1 h. After washing the cells again, they were routinely treated for the processes of dehydration, embedding, sectioning and staining. Morphological changes of mitochondria were observed under the transmission electron microscope.

Induction of mineralization. Cells were seeded at a density of 2×10^4 cells/cm² in a 6-well plate coated with 0.1% gelatin, and 2 ml medium was added to each well, which was then cultured in a 37.5°C, 5% CO₂ incubator. When the degree of cell fusion reached 60–70%, the medium in the 6-well plate was replaced with complete osteogenic induction differentiation medium (Cyagen Biosciences, Inc), and this was replaced with fresh osteogenic induction differentiation medium every 3 days. After 2–4 weeks of induction, cells were stained with Alizarin Red (Shanghai Beyotime Co., Ltd) and the cell morphology was observed under an inverted fluorescence microscope.

Statistical analysis. Each experiment was repeated ≥ 3 times, and the data are presented as mean $\pm$ SD. Data were analyzed using SPSS 22.0 software (IBM Corp.). An unpaired t-test was used for comparison between two groups, and the one-way ANOVA was used for comparison between multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Osteoblast activity is decreased under a high glucose environment, and it may be associated with ferroptosis. It was found that after the cells were treated for 24 h, groups with glucose concentration of 35 mM or higher showed an osteoblast promotion effect, but there was no significant difference between the four groups. After 48 h treatment, the cell viability of each group was slightly reduced compared with the control group, but there was no significant difference between all groups. After 72 h treatment, the viability of the three groups of cells with glucose concentration greater than 35.00mM was significantly reduced, but there was no statistical difference between the three groups (Fig. 1A). Compared with the control group, the high glucose group had lower osteoblast viability, and after the addition of the ferroptosis inhibitor, the osteoblast viability was increased compared with the high glucose group (Fig. 1B). The electron microscopy results identified that mitochondria in the high glucose group were fused and shrunken, and the staining intensity was deeper (Fig. 2). These results suggest that osteoblast activity was decreased in high glucose environments and may associated with ferroptosis.

PCBP1 expression in osteoblasts is increased in a high glucose environment. The current study determined the expression level of PCBP1 in osteoblasts under a high glucose environment via western blotting (Fig. 3). It was found that PCBP1 expression was higher in osteoblasts under high glucose environment compared with the control group. This indicated that a high glucose environment can promote the expression level of PCBP1 in osteoblasts.

Lentiviral infection of PCBP1. To confirm the role of PCBP1 in diabetic osteoporosis, the expression level of PCBP1 in hFOB1.19 cells was altered. In total, > 80% of the cells expressed green fluorescent protein (Fig. 4A-C). Subsequently, the expression level of PCBP1 was detected via western blotting after PCBP1-encoded lentivirus infection (Fig. 4D), and the results confirmed that the cells were successfully transfected with PCBP1 lentivirus.

PCBP1 can reduce the occurrence of ferroptosis by increasing the expression level of intracellular ferritin in osteoblasts under a high glucose environment. Ferritin, the ferroptosis marker protein GPX4, the osteogenic marker proteins OPG and OCN (Fig. 5A), cellular ROS levels (Fig. 5B) and alizarin red-stained calcium nodules (Fig. 5C) were detected in osteoblasts under high glucose conditions alone or in combination with the infection of PCBP1 lentivirus via western blotting, flow cytometry and induction of mineralization, respectively.

The western blotting results demonstrated that, compared with the normal group, ferritin and PCBP1 expression levels were increased in the high glucose group, while the expression levels of the ferroptosis marker protein GPX4 and the functional indicator proteins OPG and OCN were decreased. Under the same conditions of high glucose concentration, the expression levels of ferritin, GPX4, OPG and OCN in the PCBP1 overexpression group were significantly increased compared with those in the high glucose alone group, while these expression levels were decreased in the knockdown group.

The flow cytometry results indicated that the ROS production was increased in the high glucose group compared with the normal group. Moreover, compared with the high glucose group, ROS production in the PCBP1 overexpression group was significantly reduced, while the knockdown group demonstrated the opposite result.

The osteogenic mineralization induction results identified that calcium nodule deposition was reduced in the high glucose group compared with the normal group. Compared with the high glucose group, calcium nodule deposition in the PCBP1 overexpression group was significantly increased, while an opposite result was found in the knockdown group. These results indicated that PCBP1 could reduce the toxic effect caused by ferroptosis on osteoblasts by promoting ferritin expression in a high glucose environment.

Discussion

The present study demonstrated that ferroptosis occurred in osteoblasts after high glucose treatment, and that PCBP1 and ferritin expression levels were higher compared with those in normal cells.

Ferroptosis is a recently discovered mechanism of cell death, and is an iron-dependent lipid peroxide aggregation process⁷. Ferroptosis has been a research hotspot in multiple fields in recent years. For example, epidemiology and animal studies have reported that iron affects cell proliferation^{17,18}. Moreover, abnormal iron metabolism and high intracellular iron content are characteristics of most cancer cells¹⁹. It has been suggested that oxidative stress may be the main cause of cancer, and that ferroptosis is involved in the pathogenesis of a variety of cancer types²⁰. In addition, ferroptosis can lead to numerous diseases, such as liver and kidney damage, iron overload cardiomyopathy and neurodegenerative diseases, amongst others^{21–23}. Studies have shown that ferroptosis may also be a potential mechanism of endothelial cell damage caused by particulate matter 2.5²⁴. Moreover, ferroptosis, as a novel death mechanism different from that of apoptosis and necrosis, has a wide range of effects on diseases and cell damage. For example, Zhang *et al*²⁵ revealed that upregulating GPX4 reduces the incidence of ferroptosis and helps decrease secondary brain injury after cerebral hemorrhage. Nguyen *et al*²⁶ summarized the basic information of ferroptosis discovery and further explained the role of ferroptosis in human cancer, while Zhu *et al*²⁷ compared the anticancer activity of artemisinin and its derivatives via a literature search and analysis, and reported that ferroptosis was a new mechanism for treating cancer. These studies indicate that ferroptosis is widely researched in the fields of tumors and nerve injury, but it is less frequently studied with regards to bone metabolism. The present findings suggested that ferroptosis may be involved in the pathogenesis of type 2 diabetic osteoporosis.

Ferritin is a natural reservoir of iron ions in the body, and serves an important role in iron storage and the control of intracellular iron distribution²⁸. Bu *et al*²⁹ reported that ferritin can reduce free iron in cells, thereby inhibiting lipid peroxidation, oxygen free radical damage and pro-inflammatory cascades that are induced when cells are stressed. Furthermore, Liu *et al*³⁰ examined the detoxification and utilization

mechanisms of high-concentration heavy metals in the body using blind shrimp from the deep-sea hydrothermal area, and these authors found that the heavy chain subunit of ferritin had iron oxidase activity, which could convert divalent iron into non-toxic trivalent iron and stored these iron. Santiago Gonzalez *et al*³¹ studied the iron metabolism of Schwann cells in myelinated nerve fibers, and identified that doublesex and mab-3 related transcription factor 1, ferritin and transferrin receptor 1 were key proteins for early iron uptake and storage in Schwann cells, as well as the normal myelination of the peripheral nervous system. Thus, based on the aforementioned findings, it was suggested that ferritin exerts a detoxification effect on iron overload and a protective effect on cells.

A previous study observed the upregulation of intracellular ferritin heavy peptide 1 and transferrin receptor 1 in mice fed with a high iron diet³². Furthermore, Li *et al*³³ have shown that ferroportin1 protein is localized in the cytoplasm of bone cells of rats and ferroportin1 may be involved in the pathological process of iron overload-induced bone lesion. Additionally, Zhou *et al*³⁴ revealed that at the same time as the bone iron content was decreased in iron responsive element binding protein 2-knockout mice, the gene transcription of ferritin was correspondingly reduced. Thomson *et al*³⁵ point out that when the concentration of iron ions in the cell is low, iron-regulatory proteins /iron-responsive elements (IRP/IRE) prevents the assembly of ribosomes, thereby inhibiting the translation of ferritin. When the concentration of iron ions increases, IRP/IRE decreases, thereby increasing the translation of ferritin to store excess iron. These studies indicate that changes in intracellular iron ions are closely associated with ferritin and ferritin is a important protector against iron overload, or ferroptosis in human body.

PCBP1, as an iron ion partner, can transfer iron ions to ferritin. Halon-Golabek *et al*³⁶ reported that, in the skeletal muscle of rats with amyotrophic lateral sclerosis, an increase in PCBP1 protein expression was accompanied by an increase in ferritin light and heavy chains. Moreover, Ryu *et al*³⁷ revealed the role of PCBP1 in iron flux via ferritin in developing erythroid cells, and showed that mice lacking PCBP1 could develop into mice with small cell anemia. Therefore, PCBP1 is important for the formation and function of ferritin. However, the effect of PCBP1 on ferritin in osteoblasts remains unknown.

Studies by Zarjou *et al*³⁸ have shown that ferritin regulates osteoblast activity and osteogenesis. However, to the best of our knowledge, no studies have yet elucidated the regulatory mechanism of ferritin in type 2 diabetic osteoporosis and its effect on type 2 diabetic osteoporosis. Our previous research has shown that osteoblasts from type 2 diabetic osteoporosis bone tissues will be overloaded with iron, causing the cells to produce an oxidative stress response, which ultimately affects the osteogenic function of the osteoblasts^{39,40}. However, the specific process and mechanism of iron overload are yet to be fully elucidated.

The present study demonstrated that the mitochondria demonstrated typical morphological changes⁷, and that the expression levels of PCBP1 and ferritin were significantly increased after high glucose treatment in osteoblasts. After lentiviral silencing and overexpression of PCBP1, ferritin was downregulated and upregulated, respectively. The current study identified a key role of PCBP1 in

regulating ferritin in type 2 diabetic osteoporosis. Ferritin is the main storage protein of intracellular iron and is an important component of intracellular iron homeostasis²⁸. Thus, it was suggested that the increase of ferritin in the high glucose environment was due to the regulation of PCBP1 and was to compensate for the intracellular iron overload.

Ferritin regulated by PCBP1 affects osteoblast function via ferroptosis. Park *et al*⁴¹ reported that the occurrence of ferroptosis was regulated by ferritin and transferrin receptors. Moreover, Mumbauer *et al*⁴² have shown that ferritin can protect cells from the harm caused by active oxygen accumulation and ferroptosis. Our previous research revealed that under high glucose conditions, ROS levels were significantly increased in osteoblasts, which could cause osteoblast function to decline. This study suggests that PCBP1 can reduce the occurrence of ferroptosis by promoting the formation of ferritin, which may become a potential target for the treatment of type 2 diabetic osteoporosis. Future studies will attempt to prepare targeted drugs carrying PCBP1, which can be targeted to the patient's osteoblasts via oral administration, intravenous drip or local injection, to inhibit the occurrence and development of osteoporosis, thereby improving or even reversing osteoporosis.

Abbreviations

PCBP1

Polycytosine RNA-binding protein 1

HG

high glucose

GPX4

glutathione peroxidase 4

OPG

osteoprotegerin

OCN

osteocalcin

ROS

reactive oxygen species

AGEs

Advanced glycation end products

DCFH-DA

2,7-Dichlorodihydrofluorescein diacetate

PVDF

polyvinylidene fluoride

PBS

Phosphate Buffer Solution

IRP/IRE

iron-regulatory proteins /iron-responsive elements

Declarations

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Maowei Yang conceptualized and designed the study. Hongdong Ma and Yueming Jiang performed the study and acquired the data. Hai Tian analyzed and interpreted the data. Yueming Jiang drafted the manuscript. Maowei Yang and Hongdong Ma are responsible for confirming the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Detection of osteoblast activity after the addition of different glucose concentrations and at different time points. (A) Osteoblasts significantly reduced their activity after 72 h under the action of ≥ 35 mM glucose concentrations. (B) Osteoblasts given HG combined with Fer-1 increased their activity compared with the high glucose group. Data are presented as the mean $\pm$ SD. * $P < 0.05$ vs. Control; # $P < 0.05$ vs. HG. HG, high glucose.

A

Ctrl

B

HG

Figure 2

Observation of mitochondrial changes in osteoblasts in a high glucose environment using an electron microscope. (A) In the control group, the mitochondria in the osteoblasts were normal and the ridge lines were clear. (B) In the high glucose group, the mitochondria in the osteoblasts were fused and shrunken, and the staining was deeper.

Figure 3

Detection of PCBP1 expression in osteoblasts under high glucose. Compared with the control group, PCBP1 expression was increased in the high glucose group. Data are presented as the mean $\pm$ SD.

*P<0.05 vs. Control

Figure 4

hFOB I. 19 cells were infected with PCBP1-encoded lentivirus, and the expression level of PCBP1 was detected via western blotting. (A) Expression level of green fluorescently labeled PCBP1 in hFOB I. 19 cells was detected using an inverted fluorescence microscope. (B) hFOB I. 19 cells were examined using a light microscope; (C) merging of A and B. (D) Western blotting results of the protein expression level of PCBP1 in hFOB I. 19 cells. Data are presented as mean $\pm$ SD. *P<0.05 vs. Control; #P<0.05 vs. empty vector virus control. PCBP1, polycytosine RNA-binding protein 1.

Figure 5

Regulation of PCBP1 on osteoblastic ferroptosis in a HG environment. (A) Western blotting results revealed that the expression levels of ferritin, GPX4, OPG and OCN after PCBP1 lentivirus transfection under a HG environment. (B) Flow cytometry results showed that the levels of ROS after PCBP1 lentivirus

transfection under a HG environment. (C) Alizarin red calcium nodule staining results identified the number of calcium nodules in osteoblasts after PCBP1 lentivirus transfection under HG environment. Data are presented as the mean $\pm$ SD. *P<0.05 vs. Empty vector virus control; #P<0.05 vs. Empty vector virus HG. PCBP1, polycytosine RNA-binding protein 1; HG, high glucose; GPX4, glutathione peroxidase 4; OPG, osteoprotegerin; OCN, osteocalcin.