

PCBP2 as an intrinsic aging factor regulates the senescence of hBMSCs through the ROS-FGF2 signaling axis

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Abstract

Background

It has been reported that loss of PCBP2 led to increased reactive oxygen species (ROS) production and accelerated cell aging. Knockdown of PCBP2 in HCT116 cells leads to significant down-regulation of fibroblast growth factor 2 (FGF2). Here, we tried to elucidate the intrinsic factors and potential mechanisms of BMSCs aging from the interactions among PCBP2, ROS and FGF2.

Methods

Unlabeled quantitative proteomics were performed to show differentially expressed proteins in the replicative senescent human-derived bone marrow stromal cells (RS-hBMSCs). ROS and FGF2 were detected in the loss-and-gain cell function experiments of PCBP2. The function recovery experiments were performed to verify whether PCBP2 regulates cell function through ROS/FGF2-dependent ways.

Results

PCBP2 expression was significantly lower in P10-hBMSCs. Knocking down the expression of PCBP2 inhibited the proliferation while accentuated the apoptosis and cell arrest of RS-hBMSCs. PCBP2 silence could increase the production of ROS. On the contrary, overexpression of PCBP2 increased the viability of both P3-hBMSCs and P10-hBMSCs significantly. Meanwhile, over-expression of PCBP2 led to significantly reduced expression of FGF2. Overexpression of FGF2 significantly offset the effect of PCBP2 overexpression in P10-hBMSCs, leading to decreased cell proliferation, increased apoptosis and reduced G0/G1 phase ratio of the cells.

Conclusion

This study initially elucidates that PCBP2 as an intrinsic aging factor regulates the replicative senescence of hBMSCs through the ROS-FGF2 signaling axis.

eLife assessment

In this **valuable** study, the authors aimed to identify and characterize intrinsic factors that govern the aging process of bone marrow mesenchymal stromal cells (BMSCs), which are believed to be related to osteoporosis. The authors conclude that PCBP2 is an intrinsic aging factor, the decrease of its expression during aging results in cell proliferation activity decrease and cell senescence. The study provides **convincing** evidence in support of its conclusions.

Introduction

With the aging of the population, the incidence of osteoporosis is increasing globally. It is believed that the decrease in the number and "adaptability" of bone marrow mesenchymal stromal cells (BMSCs) is one of the key factors leading to osteoporosis[1]. With aging, the proliferation and function of BMSCs are impaired due to intrinsic and environmental factors[2], however, the underlying mechanism remains largely unknown.

The poly(rC)-binding protein 2 (PCBP2) is a RNA-binding protein and regulates gene expression at multiple levels including mRNA metabolism and translation. It has been reported that knock-out of PCBP2 led to decreased expression of p73 in a variety of cell lines, which in turn led to increased reactive oxygen species (ROS) production and accelerated cell aging[3]. However, whether PCBP2 influences cell aging through a ROS-dependent way is still unknown. ROS is a by-product of aerobic metabolism, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\cdot$), and has a wide range of biological targets and reactivity[4]. The disorder of ROS affects many cell functions, such as cell proliferation, cell apoptosis, autophagy and cellular senescence[4, 5]. In our pilot study, we found that the expressions of PCBP2 was significantly lower in P10-hBMSCs than in P3-hBMSCs. Therefore, we speculate that PCBP2 may be one of the intrinsic factors of aging of the BMSCs, and participates in the regulation of the cellular function of the cells by acting on the production of ROS.

In GSE95024 downloaded from the GENE EXPRESSION OMNIBUS (GEO) database, we analyzed by bioinformatics methods and found that after knockdown of PCBP2 in HCT116 cells, the expression of fibroblast growth factor 2 (FGF2) was significantly down-regulated in the cells. FGF2 has been reported to have functions in cell proliferation, senescence and G2/M arrest[6–8]. FGF2 was also crucial to the stemness maintenance of BMSCs with fibronectin and bone morphogenetic protein 4 [9]. FGF2-induced inhibition of RhoA/ROCK signaling played a key role in BMSCs differentiation into endothelial cells[10]. FGF2 isoforms were also found to be able to inhibit the mineralization of BMSCs [11]. Meanwhile, it has been reported that reactive oxygen species could mediate FGF2 release[12]. IL-1 β promoted FGF-2 expression in chondrocytes through the ROS/AMPK/p38/NF- κ B signaling pathway[13]. FGF-2 could be released by plasma-produced ROS[14].

As mentioned above, our understanding of the interactions among PCBP2, ROS and FGF2 in the aging process of BMSCs seems to be insufficient. The in-depth exploration of the mechanism of aging of the BMSCs is expected to provide additional information for the pathogenesis and clinical intervention of osteoporosis. We hypothesize that PCBP2 is an intrinsic aging factor of BMSCs and regulates the senescence of hBMSCs through a ROS and FGF2 dependent pathway.

Materials and Methods

Hayflick model of cellular aging

The human BMSCs (hBMSCs) were obtained from healthy male individuals who underwent traumatic femoral or tibia shaft fracture treatment by intramedullary nailing. Cell extraction and passage were performed as previously described[15]. hBMSCs were cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose containing 10% fetal bovine serum. It is well accepted that the expansion of BMSCs in culture will accelerate senescence, and the differentiation potential will decrease from the 6th generation. In the 10th generation, the average number of cumulative population doublings drops from 7.7 to 1.2[16]. Thus, after passage of the hBMSCs cells, the third-generation cells were labeled as P3 (*in vitro* non-senescence model, P3-hBMSCs) and the tenth-generation cells were labeled as P10 (*in vitro* replication senescence model, P10-hBMSCs). The cells were incubated in an incubator at 37 °C with 5% CO₂, and the culture medium was changed every two days.

GSE95024 analysis

The GSE95024 data was downloaded from the GEO database, including four groups of HCT116 cells with silenced PCBP2 and four groups of negative controls. R3.5.2 software was used to screen out differentially expressed genes, and logFC>=2 and p<0.01 was regarded as significance. The STRING (<https://string-db.org/cgi/input.pl>) tool was used to predict the interaction among differentially expressed genes in protein levels.

Unlabeled quantitative proteomics

The hBMSCs cells were lysed with RIPA, and the protein content of the cells was quantified using BCA Protein Assay kits (Abcam, USA). Dithiothreitol (1 M), 200 µL UA buffer, 40 µL trypsin buffer, 25 µL 25 mM NH₄HCO₃ and 50 µL 0.1% trifluoroacetic acid (TFA) were added in sequence and then centrifuged to obtain the peptides, which were quantified using the BCA kits. After desalting with a RP-C18 solid phase extraction column, the peptides were washed with 90% acetonitrile-water containing 0.1% TFA. After elution with 90% acetonitrile-water containing 0.1% TFA, the sample was reconstituted with 0.1% formic acid in water and finally analyzed using Liquid Chromatography/Mass Spectrometry (Thermo Electron Corporation, LCQ Deca XP MAX10).

Transfection

Cells were inoculated into 24-well plates, and when the cell confluence reached 30–50%, they were transfected with small interfering RNA (siRNA) to knockdown PCBP2 (sequence: 5' - GGCCTATACCATCAAGGA-3'), or transfected with plasmids over-expressing PCBP2 or FGF2. In accordance with the transfection procedure provided by Gene Pharma (Shanghai Gene Pharmaceutical Technology Co., Ltd.), 48 hours after transfection, the cells were used for subsequent experiments.

Real-time quantitative PCR (QRT-PCR)

The extraction of total RNA and reverse transcription were performed according to our standard protocol, as described previously [17, 18]. Reverse transcription was performed using Superscript II (ThermoFisher Scientific) and Fast SYBR Green (ThermoFisher Scientific) was used

for qPCR. The sequences of the primers used were as in [table 1](#).

Western blotting (WB) experiment

The steps for protein extraction and WB were the same as our previously published procedure [17]. According to the WB protocol, the primary antibodies, all of which were human anti-rabbit antibodies PCBP2 (Abcam, USA), FGF2 (Abcam, USA), FRS2 (Abcam, USA), and α -tubulin (Cell Signaling Technology, CST, USA) were incubated with nitrocellulose (NC) membrane overnight at a dilution of 1:1500, and then incubated with secondary HRP-conjugated antibody (1:1000, rabbit anti-mouse antibody, Abcam, USA) for 1 hour. Finally, the proteins were detected using Pierce SuperSignal West Pico Chemiluminescence Detection Kits (Thermo Fisher, MA, USA), and image and protein density were photographed and calculated by Image J system, respectively.

Cell Counting Kit-8 (CCK-8) experiment

The cells were inoculated into 96-well plates with 2000 cells/well and cultured for 24 hours. Then, 10 μ L of CCK-8 reagent (WST-8/CCK8, ab228554, Abcam, USA) and 90 μ L of serum-free medium were added to each well, and incubated for 2 hours in the 37°C, 5% CO₂ and humid incubator. The absorbance was measured at 450 nm, and the experiment was repeated three times.

Detection of reactive oxygen species

The cells were seeded on a black 96-well microplate with a transparent bottom at 2.5×10^4 cells per well, and cultured overnight. The medium was then removed and 100 μ L of 1X buffer was added to each well. Next, the 1X buffer was removed and 100 μ L of diluted DCFDA solution added (Section 6.3) to stain the cells. The cells were incubated with diluted DCFDA solution in the dark for 45 minutes. The DCFDA solution was then removed, 100 μ L of 1X buffer or 1X PBS added to each well, and fluorescence was measured immediately. In the presence of compound, medium or buffer, the fluorescence at Ex/Em = 485/535 nm was measured using automatic microplate reader (Bio-tek) end point mode.

Flow cytometry

For apoptosis detection, after culturing the cells in six-well plates for 24 hours, the cells were collected and washed with incubation buffer, suspended with 100 μ L of labeling solution, and incubated at room temperature for 10–15 minutes. Next, the fluorescent (SA-FLOUS) solution was added and was incubated at 4 °C in the dark for 20 minutes. Finally, the FITC fluorescence was detected at 515 nm and PI fluorescence was detected at 560 nm. Finally, samples were analyzed on FACSCalibur (BD Biosciences, San Jose, CA, USA). Data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

For cell cycle progression, after culturing the cells in six-well plate for 24 hours, the cells were collected and fixed with 75% ethanol at 4 °C for 4 hours. Next, 400 μ L of CCAA solution (PI staining solution, Engreen, New Zealand) and 100 μ L RNase A (100 μ g/mL) was added and incubated at 4 °C in the dark for 30 minutes. Finally, samples were analyzed on FACSCalibur (BD Biosciences, San Jose, CA, USA). The generated histogram was used to calculate the ratio of cells in the G0/G1, S or G2/M phases.

ROS inhibitor

5×10^6 hBMSCs were inoculated into 6-well plates, after the corresponding number of cell passages, P3-hBMSCs and P10-hBMSCs were stimulated with 2mM ROS inhibitor (NAC, KFS289, Beijing Baiolaibo Technology Co. LTD)

Gene name	Primer sequences
PCBP2	Forward: 5'-ATFGTCATTTTAGCTGGATC-3'
	Reverse: 5'-GATAGATCGTGAAATGCT-3'
FGF2	Forward: 5'-AGAAGAGCGACCCTCACATCA-3'
	Reverse: 5'-CGGTLAGCACACACTCCTTTG-3'
β-actin	Forward: 5'-GCTCTAGGCGGACTGTAC-3'
	Reverse: 5'-CCATGCCAATGTTGTCTCTT-3'

Three separate experiments were performed for each sample, and the $2^{-\Delta\Delta Ct}$ value was used to calculate the relative expression.

Table 1.

The primers used in qRT-PCR

Statistical analysis

Data were presented as the mean $\pm$ standard deviation (SD) for three repetitions per group. Student's t-test was used to analyze the differences between two groups, and one-way (ANOVA) was used to analyze the differences between multiple groups. P-value <0.05 was considered as statistically significance.

Results

Identification of differentially expressed proteins (DEPs) in replicative senescent hBMSCs

In our pilot study, unlabeled quantitative proteomics was used to analyze the proteins which were differentially expressed between P3-hBMSCs and P10-hBMSCs (**Supplementary Figure 1** [↗](#)). The sequencing results showed that 50 proteins were differentially expressed, of which 25 were significantly up-regulated and 25 were significantly down-regulated in the replication senescent cells (**Figure 1A** [↗](#)). PCBP2 was among the 25 significantly down-regulated proteins (**Figure 1A** [↗](#), red arrow).

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed, and a variety of GO functions and KEGG signaling pathways seemed to be associated with these 50 DEPs. In GO cellular component annotations, the DEPs were significantly enriched in vacuolar lumen pathways, mitochondrial ribosome pathways, and organellar ribosome pathways. With respect to molecular function, the cadherin binding pathway, lamin binding pathway, and cell adhesion molecule binding pathway were significantly enriched in these DEPs. GO Biological Process terms included the endomembrane system organization pathway, protein-containing complex disassembly pathway, and vesicle budding from membrane pathway (**Figure 1B** [↗](#)). Using KEGG pathway analysis, DEPs were significantly enriched in endocytosis pathways, terpenoid backbone biosynthesis pathways, and the ferroptosis pathway (**Figure 1C** [↗](#)). These 50 DEPs in senescent hBMSCs seemed to affect a wide range of biological functions and signaling pathways, which might be associated with the underlying causes of cellular aging.

Low expression of PCBP2 accentuated the characteristics of cell senescence in hBMSCs

As shown in **Figure 1A** [↗](#), the expression of PCBP2, a gene downstream of the ferroptosis pathway, was significantly down-regulated in P10-hBMSCs cells. The QRT-PCR (**Figure 2A** [↗](#)) and WB (**Figure 2B** [↗](#)) assays confirmed that the expression of PCBP2 was significantly lower in P10-hBMSCs than in P3-hBMSCs. A siRNA that knocked-down PCBP2 and a plasmid that over-expressed PCBP2 were constructed, and QRT-PCR and WB were used to evaluate the efficiency of knockdown and overexpression of PCBP2 in the cells (**Supplementary Figure 2** [↗](#)). CCK-8 results showed that after over-expressing PCBP2, the viability of both P3-hBMSCs and P10-hBMSCs increased significantly (**Figure 2C** [↗](#)). On the contrary, after silencing PCBP2, the viability of both P3-hBMSCs and P10-hBMSCs weakened significantly (**Figure 2D** [↗](#)).

Using flow cytometry, we found that, compared with P3-hBMSCs, P10-hBMSCs tended to remain in the G0/G1 phase of the cell cycle (**Figure 2E** [↗](#) and **F** [↗](#)). In P3-hBMSCs with over-expression of PCBP2, the cell cycle had no significant change. However, in P10-hBMSCs with over-expression of PCBP2, the number of cells in the G0/G1 phase decreased, while the number of cells in the mitotic phase increased significantly (**Figure 2E** [↗](#)). Meanwhile, in PCBP2 knocked-down P3-hBMSCs, the cell cycle was significantly blocked, with the number of cells in G0/G1 significantly increased while the numbers of cells in S phase and G2/M phase significantly decreased (**Figure 2F** [↗](#)). In PCBP2 knocked-down P10-hBMSCs, the cell cycle was further arrested, with the number of G0/G1

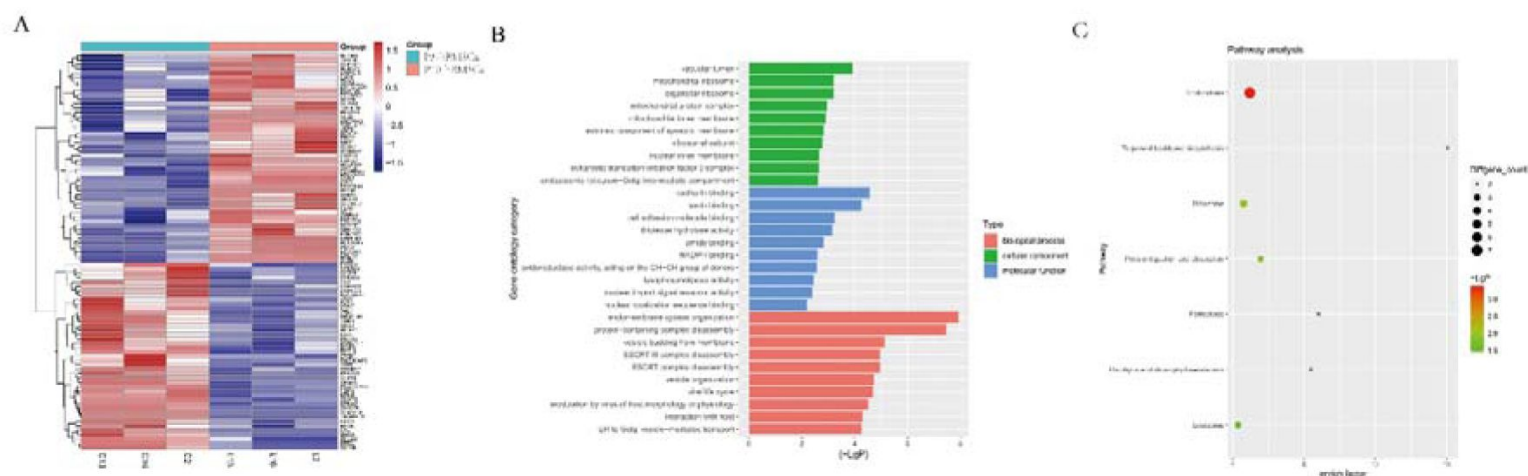


Fig. 1.

DEPs in replication senescent hBMSCs and their biological functions.

(A) A hub of 50 significantly DEPs induced by replication aging were shown as a heatmap. GO functions (B) and KEGG pathways (C) affected by these 50 DEPs. Notes: $P < 0.05$ and $\log_{2}FC \geq 2$ was regarded as indicating significance.

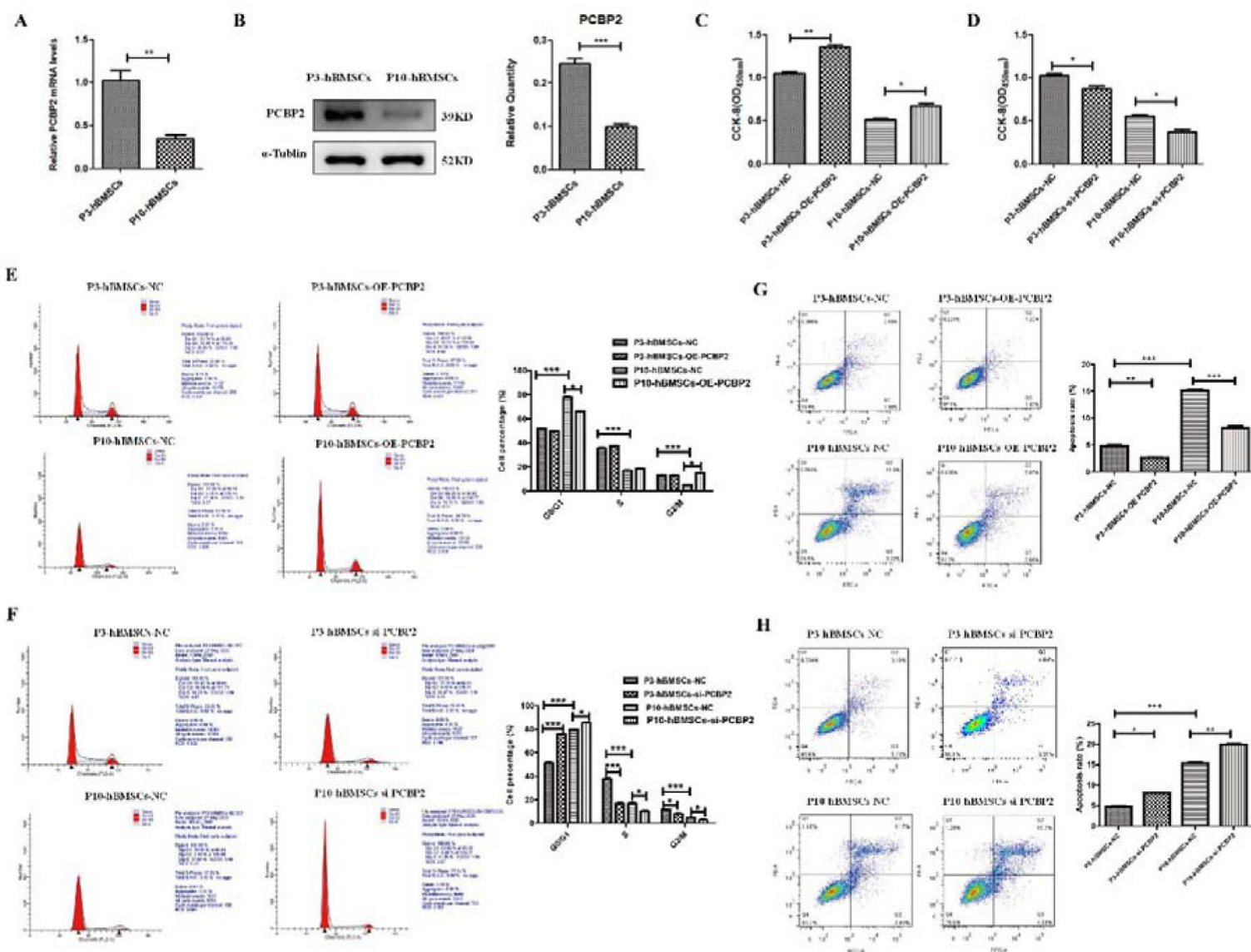


Fig. 2.

Low expression of PCBP2 accentuated the characteristics of cell senescence in hBMSCs

(A) RT-PCR and (B) WB detection for the expression of PCBP2 in P3-hBMSCs and P10-hBMSCs. CCK-8 assay for the effects of PCBP2 overexpression (C) and PCBP2 silencing (D) on the proliferation of P3-hBMSCs and P10-hBMSCs. Flow cytometry detection for the effects of PCBP2 overexpression (E) and PCBP2 silencing (F) on the cell cycle of P3-hBMSCs and P10-hBMSCs. The effects of PCBP2 overexpression (G) and PCBP2 silencing (H) on apoptosis of P3-hBMSCs and P10-hBMSCs were also detected by flow cytometry. Data were presented as mean $\pm$ SD (n = 3). *P < 0.05, **P < 0.01 and ***P < 0.001; β -actin and α -tubulin were used as the internal references for mRNA and protein detection.

phase cells significantly increased and the number of S phase and G2/M phase cells significantly decreased (**Figure 2F**). These results demonstrated that an appropriate amount of PCBP2 could maintain the normal cell cycle, and abnormally low expression of PCBP2 would lead to arrest of the cell cycle in normal cells.

Flow cytometry also demonstrated that the cell apoptosis was substantially higher in P10-hBMSCs than in P3-hBMSCs (**Figure 2G** and **H**). Over-expression of PCBP2 significantly reduced apoptosis in both P3-hBMSCs and P10-hBMSCs (**Figure 2G**), whereas silencing of PCBP2 significantly increased apoptosis in both P3-hBMSCs and P10-hBMSCs (**Figure 2H**). These results suggested that the decreased expression of PCBP2 induced by cell replicative senescence might promote the apoptosis of senescent cells.

Low expression of PCBP2 accentuated the cell senescence of hBMSCs in a ROS-dependent way

It is well known that ROS may have important effects on the cellular functions of different cells[19]. We used the functional gain-and-loss experiment to verify whether PCBP2 could influence the cellular functions of hBMSCs in a ROS-dependent pathway. The ROS detection results showed that over-expression of PCBP2 inhibited the production of ROS in both P3-hBMSCs and P10-hBMSCs (**Figure 3A**), while knockdown of PCBP2 increased the production of ROS in both P3-hBMSCs and P10-hBMSCs (**Figure 3B**). The increased ROS production caused by PCBP2 silence could be significantly rescued by adding 2mM of antioxidant NAC to PCBP2 silenced-P3-hBMSCs and P10-hBMSCs (**Figure 3C**). As shown in **Figure 2**, overexpression of PCBP2 promoted, while silencing PCBP2 inhibited the proliferation of P3-hBMSCs and P10-hBMSCs. Further CCK-8 results showed that the antioxidant NAC could significantly reverse the decrease in cell proliferation of P3-hBMSCs and P10-hBMSCs induced by silencing PCBP2 (**Figure 3D**). Moreover, the flow cytometry result showed that compared with P3-hBMSCs + si-PCBP2 group, the apoptosis of P3-hBMSCs + si-PCBP2+ NAC (2mM) group was substantially reduced, with similar results obtained in the P10-hBMSCs (**Figure 3E**). In addition, with the introduction of NAC (2mM) to PCBP2 silenced P3-hBMSCs and P10-hBMSCs, the proportion of cells in G0/G1 phase significantly reduced and the proportion of cells in S phase significantly increased. These results indicated that the low expression of PCBP2 induced by cell replicative senescence could inhibit cell proliferation, and induce cell apoptosis and cell arrest by increasing the production of ROS.

Antioxidant recovered the viability of senescent hBMSCs by suppressing FGF2 expression

In GSE95024, we found that after knockdown of PCBP2, 359 genes were significantly down-regulated and 332 genes were significantly up-regulated (**Supplementary Figure 3A**), 17 of which were significantly enriched in pathways related the role of proteoglycans in cancer (**Supplementary Figure 3B**), including seven down-regulated genes: TIMP3, PAK1, HPSE2, HSPB2, PRKACB, WNT8B, and PLAUR, and 10 up-regulated genes: CAV1, FRS2, CCND1, CAV2, AKT3, SHH, FGF2, PIK3R3, WNT7A, and THBS1. Analysis of a protein interaction network generated from the STRING database, in which these genes are involved, indicates that there is a significant correlation between FGF2 and the other genes (**Supplementary Figure 3C**). Therefore, we further explored the regulatory role of PCBP2 on FGF2. The QRT-PCR results showed that over-expression of PCBP2 led to significantly reduced expression of the mRNA levels of FGF2 in both P3-hBMSCs and P10-hBMSCs (**Figure 4A**). When PCBP2 was silenced, the mRNA levels of FGF2 significantly increased in both P3-hBMSCs and P10-hBMSCs (**Figure 4B**). The results of WB demonstrated that over-expression of PCBP2 led to significantly reduced protein expressions of FGF2 in both P3-hBMSCs and P10-hBMSCs (**Figure 4C**) and down regulation of PCBP2 led to a converse result (**Figure 4D**). This up-regulation of FGF2 from suppression of PCBP2 could be stopped by the antioxidant NAC (**Figure 4E**).

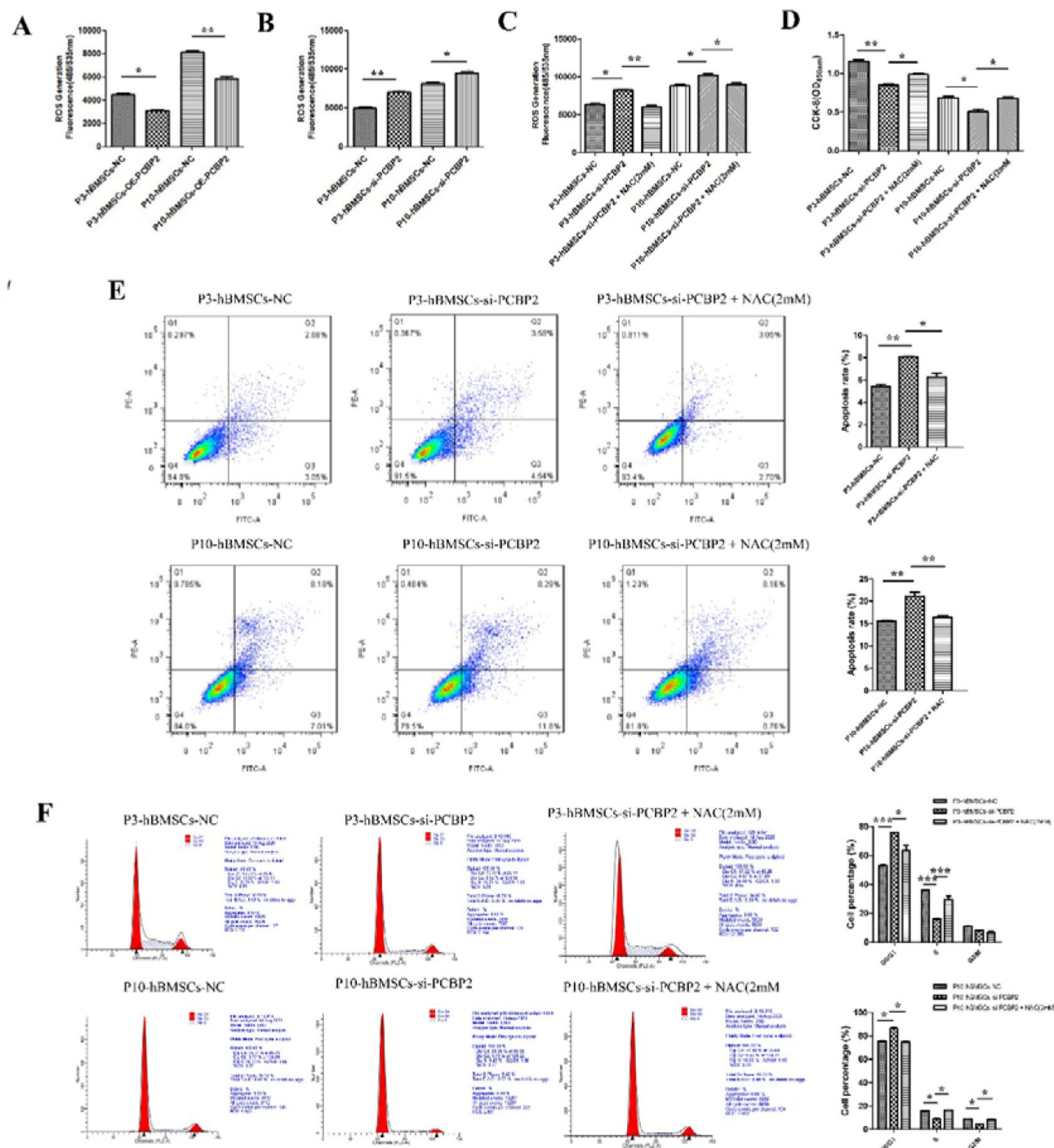


Fig. 3.

Low expression of PCBP2 inhibits cell proliferation, and promotes cell apoptosis and cell arrest in a ROS-dependent way.

(A) overexpression and (B) silencing of PCBP2 on ROS production in P3-hBMSCs and P10-hBMSCs. (C) The inhibitory effect of 2mM NAC on ROS production in P3-hBMSCs and P10-hBMSCs with silenced PCBP2. (D) CCK-8 test showed that the antioxidant NAC significantly recovered the suppressed cell proliferation in P3-hBMSCs with silenced PCBP2. Flow cytometry was used to detect the effects of 2mM NAC on the apoptosis (E) and cycle (F) of PCBP2 silenced P3-hBMSCs and P10-hBMSCs. Notes: Data were presented as mean $\pm$ SD (n = 3). *P < 0.05, **P < 0.01 and ***P < 0.001.

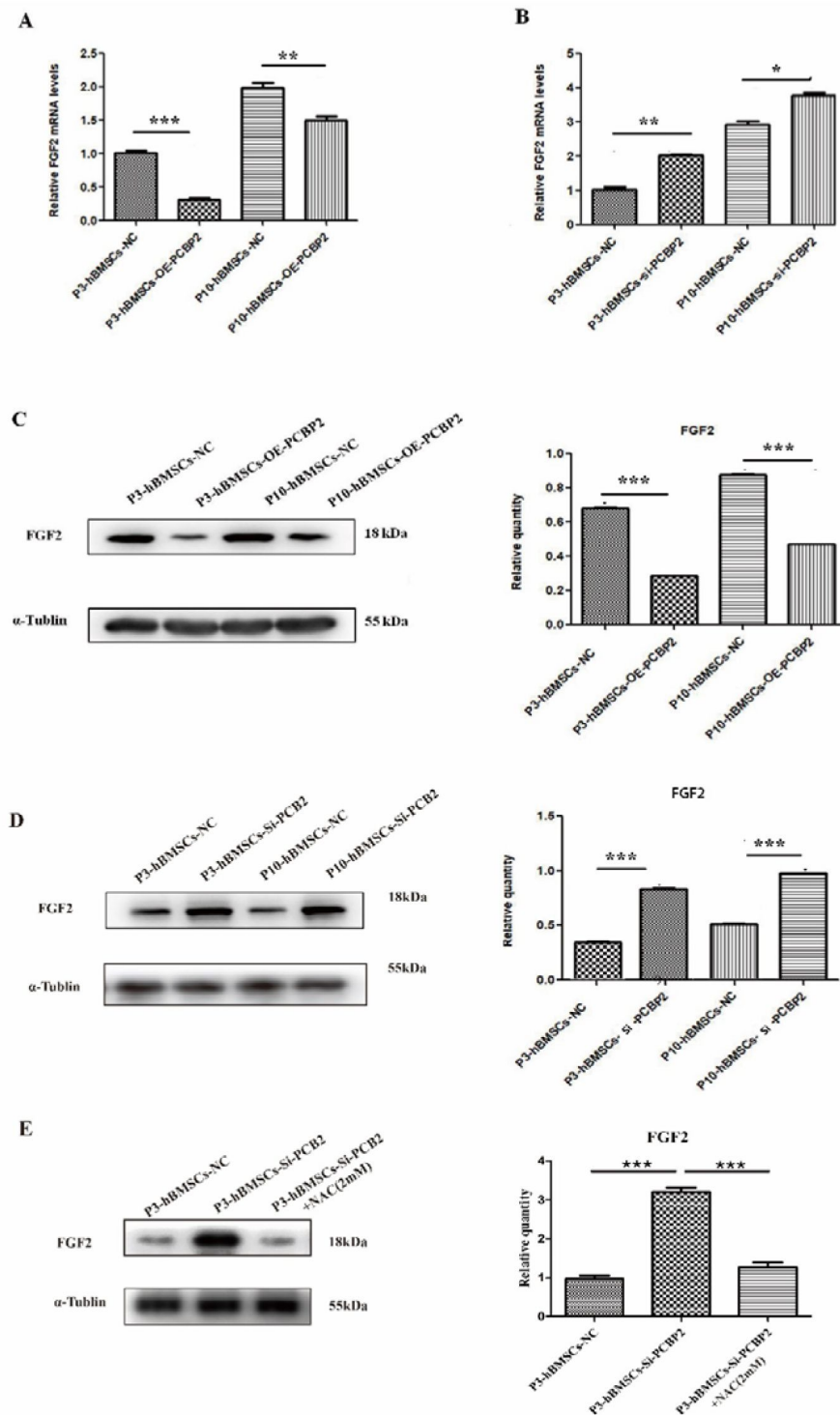


Fig. 4.

PCBP2 inhibits the expression of FGF2 in P3-hBMSCs and P10-hBMSCs.

The regulation of FGF2 mRNA levels by overexpression (**A**) and silencing (**B**) of PCBP2 was detected by RT-PCR, and were confirmed in protein levels via WB assay (**C**, **D**). (**E**) up-regulation of FGF2 protein level in P3-hBMSCs with silenced PCBP2 was stopped by 2mM NAC. Notes: Data were presented as mean $\pm$ SD (n = 3). *P < 0.05, **P < 0.01 and ***P < 0.001; β -actin and α -tubulin was used as the internal reference for mRNA and proteins detection.

Low expression of PCBP2 accentuated the senescent characteristics of hBMSCs through FGF2 overexpression

Then, the function recovery experiment was used to verify whether PCBP2 regulates cell function through FGF2. CCK-8 results showed that compared with the PCBP2 overexpression group, the cell proliferation of P3-hBMSCs (**Figure 5A**) and P10-hBMSCs (**Figure 5B**) decreased significantly in the OE-PCBP2+OE-FGF2 group. We further used flow cytometry to detect cell apoptosis in each group. In P3-hBMSCs, overexpression of PCBP2 could significantly inhibit cell apoptosis, and overexpression of FGF2 does not significantly reverse the inhibitory effect of PCBP2 on apoptosis (**Figure 5C**). However, overexpression of FGF2 significantly reversed the apoptotic effect of PCBP2 on P10-hBMSCs (**Figure 5D**). In addition, compared with the OE-NC P3-hBMSCs and OE-NC P10-hBMSCs, the G0/G1 phase cell ratio was significantly reduced (**Figure 5E**) and the S phase cell ratio was significantly increased in the OE-PCBP2 group (**Figure 5F**). Moreover, compared with P3-hBMSCs (**Figure 5E**) and P10-hBMSCs (**Figure 5F**) in the OE-PCBP2 group, overexpression of FGF2 significantly reversed the PCBP2-induced cell G0/G1 phase inhibition and S phase promotion. In addition to G0/G1 phase and S phase, overexpression of FGF2 can significantly reverse the effect of PCBP2 on the G2/M phase of P10-hBMSCs (**Figure 5F**). These results indicated that the introduction of PCBP2 promotes cell proliferation, and inhibits cell apoptosis and cell arrest by inhibiting the expression of FGF2.

Discussion

In this work, we identified an intrinsic factor of cell aging, PCBP2, via unlabeled quantitative proteomics and characterized its biological role in the cell replicative senescence of hBMSCs *in vitro*. Low expression of PCBP2 in the replicative senescent hBMSCs inhibited the proliferation, promoted the apoptosis and the cell cycle arrest through a ROS-FGF2 dependent pathway.

Generally, cell senescence is regarded as an irreversible cell cycle arrest, which is considered to be an evolutionary process established and maintained by the p16^{INK4A} and/or p53-p21 pathway[20]. However, with the suppression of p53 expression, senescent mouse embryonic fibroblasts rapidly re-entered the cell cycle[21], which inspired us with new understanding of cell senescence. The phenotype of cellular senescence is triggered by a variety of senescence stressors, which in turn affect multiple signaling pathways and gene expressions[20]. In order to validate the results of our pilot study we used QRT-PCR and WB analysis and confirmed that both the mRNA and protein expressions of PCBP2 were substantially lower in the senescent P10-hBMSCs than in the non-senescent P3-hBMSCs. Therefore, PCBP2 should be an intrinsic factor for cell aging and participate in the senescence of hBMSCs.

Cell cycle arrest, loss of cell proliferation and increase of cell apoptosis are the main characteristic of cell senescence[22]. In present study, we found that PCBP2 silencing significantly promoted the retention of hBMSCs in the G0/G1 phase and significantly reduced the proportion of cells in the S and G2/M phases. These results are consistent with previous reports that in MEFs, the loss of PCBP2 leads to the decrease of p73 expression, which in turn leads to the acceleration of cell senescence[3]. Meanwhile, we found that the apoptosis of replicative senescent hBMSCs increased after PCBP2 was silenced, while decreased after PCBP2 was over-expressed. After multiple proliferation and division of cells, the telomeres shorten, the cells reach the limit of normal cell division and turn into replicative senescence. This process is also known as Hayflick limit[23]. Over-expression of PCBP2 in the P10-hBMSCs promoted the proliferation of the replicative senescent cells, suggesting that PCBP2 might have the ability to extend the Hayflick limit or waken the cells from replicative senescence. Further studies are needed to verify these findings.

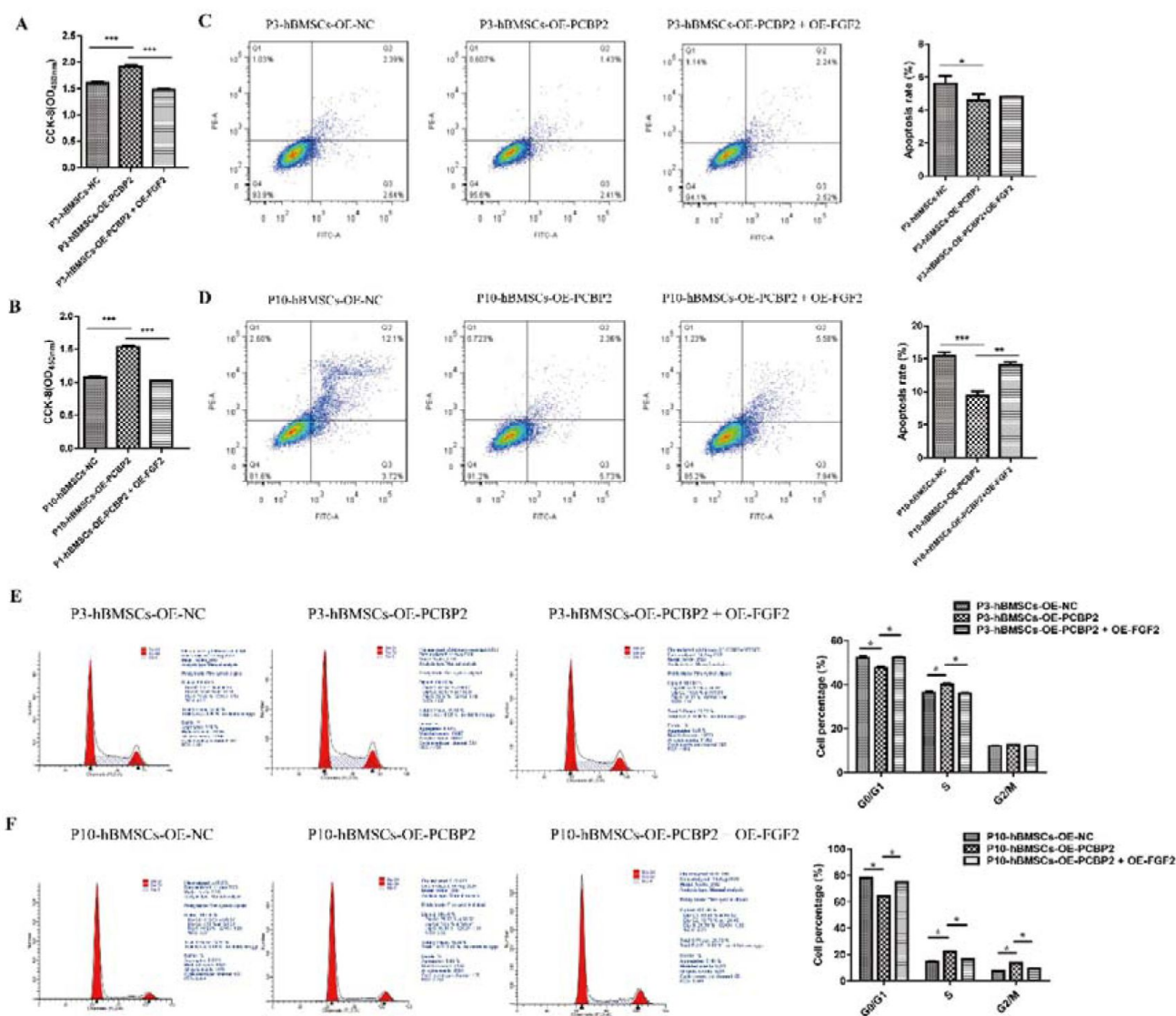


Fig. 5.

Overexpression of PCBP2 promotes cell proliferation, and inhibits cell apoptosis and cell arrest in a FGF2-dependent way.

CCK-8 detects the effect of overexpression of PCBP2 and FGF2 on the cell proliferation of P3-hBMSCs (**A**) and P10-hBMSCs (**B**). Flow cytometry was used to detect the apoptosis of P3-hBMSCs (**C**) and P10-hBMSCs (**D**) in NC, OE-PCBP2 and OE-PCBP2 + OE-FGF2 groups. In addition, in NC group, OE-PCBP2 group and OE-PCBP2+OE-PCBP2 group, the cell cycle of P3-hBMSCs (**E**) and P10-hBMSCs (**F**) were detected by flow cytometry. Notes: Data were presented as mean $\pm$ SD (n = 3). *P < 0.05, **P < 0.01 and ***P < 0.001.

Cell senescence can be induced by many factors, such as oxidative stress, cell culture and telomere shortening, and disturbances in mitochondrial homeostasis[24]. Abnormal mitochondrial homeostasis leads to an increase in the production of ROS[25]. Meanwhile, mitochondrial ROS aggravate cell senescence by enhancing DNA damage[26], which in turn forms the characteristic phenotype of senescent cells. Ren et al[3] reported that PCBP2 could inhibit the production of ROS by regulating the mRNA stability of p73 in MEFs. By interfering with the expression of PCBP2, we investigated the role of PCBP2 on the production of ROS in hBMSCs, and obtained similar results. It is report that PCBP2 is a versatile adaptor protein that binds iron and delivers it to ferritin for storage[27]. The main components of iron are stored in mitochondria, lysosomes, cytosol and nucleus, at concentrations of ~16μM, ~16μM, ~6μM and ~7μM, respectively[28]. Iron overload at the cellular level leads to an increase in ROS production, and result in oxidative stress[29]. However, the expression of PCBP2 was low in the replicative senescent hBMSCs, suggesting that the increase of ROS production in the senescent cells with low expression of PCBP2 might not depend on the transfer of iron. Future studies on the regulation mechanism of PCBP2 on ROS production are needed.

We found that down-regulating the expression of PCBP2 in hBMSCs could increase the ROS production in the cells. ROS are involved in regulating the activity of multiple signaling pathways, which are related to cell growth, cell apoptosis and the cell cycle [30]. Moderate level of ROS initiates the differentiation of stem cells, while high level of ROS leads to cell senescence and cell death of stem cells[31]. Through functional recovery experiments, we found that over-expression of PCBP2 promoted the proliferation and inhibited the apoptosis and cell cycle arrest of P10-hBMSCs in a ROS-dependent way. This result not only challenges the traditional theory that cell senescence is irreversible cell cycle arrest[32], but also indicates that the replicative senescent hBMSCs can be wakened in some extent by interventions on the production of ROS.

As mentioned in the introduction, FGF-2 can be released by ROS [12–14]. Our experiment also demonstrated that the increase of FGF2 induced by decreased PCBP2 expression can be reversed by oxidative stress inhibitors. Therefore, we concluded that PCBP2 inhibits ROS expression to a certain extent, and low ROS expression reduced FGF2 production, thus regulating the aging of hBMSCs cells. FGF2 promotes catabolism and subsequent anabolism by specifically binding to FGFR1[33], and is involved in regulating multiple downstream signal pathways, including PI3K/AKT, STAT1/p21 and RAS/MAPK kinase pathways[34]. Therefore, FGF2 plays an important role in regulating cell function. Through sequencing analysis, we found that when PCBP2 was knocked down, the expression of FGF2 was significantly increased, which was confirmed by QRT-PCR and WB. Combined with the functional recovery experiment, we found that overexpression of PCBP2 in the P10-hBMSCs could promote cell proliferation and make the cells re-enter into the S phase and G2/M phase, while inhibit the apoptosis of the cells by inhibiting FGF2. However, whether FGF2 mediates the cellular functions of hBMSCs through PI3K/AKT, STAT1/p21 and RAS/MAPK kinase pathways remains to be confirmed.

In conclusion, this study initially elucidates that PCBP2 as an intrinsic aging factor regulates the replicative senescence of hBMSCs through the ROS-FGF2 signaling axis. However, how does FGF2 interact with ROS in hBMSCs? In addition to FGF2 and ROS, does PCBP2 have other potential mechanisms for the aging of hBMSCs? These questions remain to be answered in future research.

Data Availability

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

Acknowledgements

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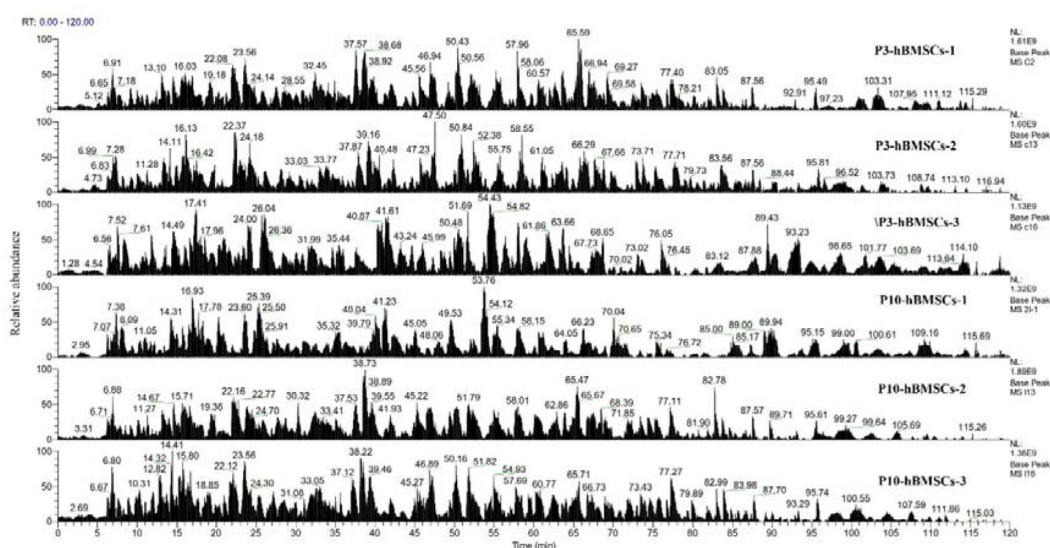
Acknowledgments

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Conflicts of interest

All authors declare no conflict of interest.

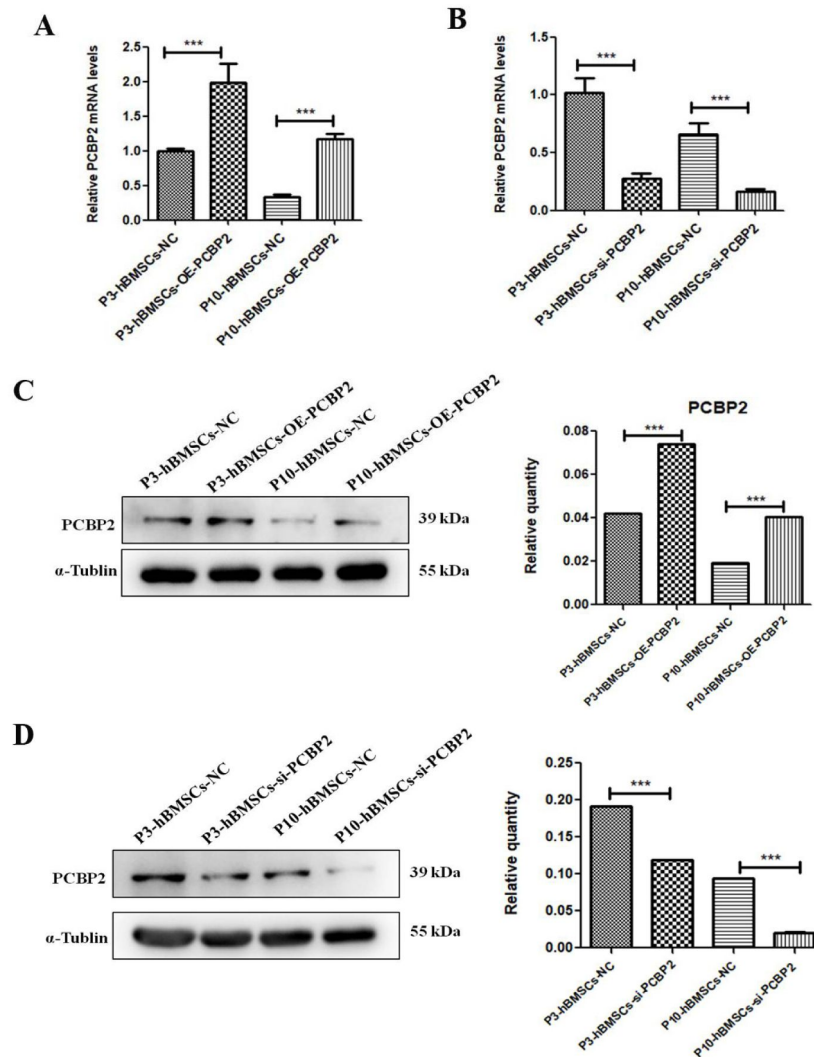
Supplementary figure legends



Supplementary Fig. 1.

Unlabeled quantitative proteomics for the DEPs between P3-hBMSCs and P10-hBMSCs.

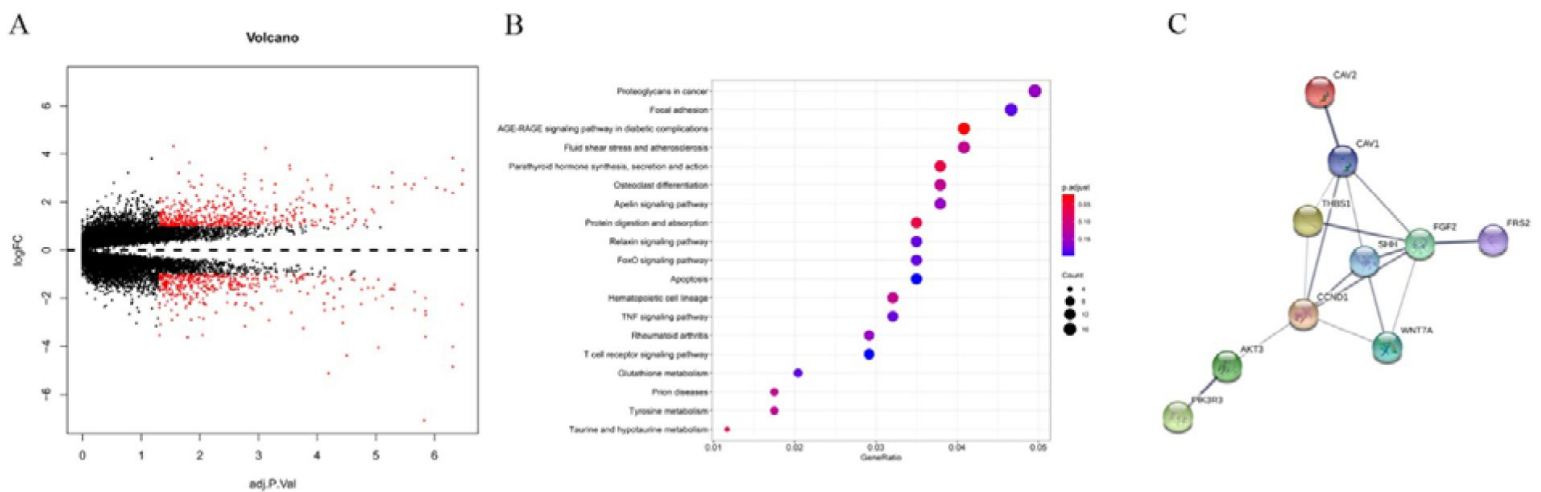
The enzymolysis products were separated by capillary high-performance liquid chromatography and then analyzed by Thermo Fisher Fusion Mass Spectrometer (Thermo Fisher). The content of each peptide was expressed by detecting the intensity of positive ions, and protein analysis was performed using Maxquant software (version number 1.6.0.1).



Supplementary Fig. 2.

Validation of transfection effect of PCBP2.

The mRNA expression of PCBP2 after transfection with plasmids that over-expressed PCBP2 (**A**) and siRNA that knocked-down PCBP2 (**B**). The effect of over-expressing PCBP2 (**C**) and knocking-down PCBP2 (**D**) as detected by WB. Notes: Data were presented as mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; β -actin and α -tubulin was used as the internal reference for mRNA and proteins detection.



Supplementary Figure 3.

Identification of the downstream genes of PCBP2.

Volcano gram of differentially expressed genes caused by PCBP2 silencing (**A**). Bubble diagram of KEGG pathway analysis of differentially expressed genes (**B**). The interaction of differentially expressed proteins was predicted by STRING website.

References

- [1] Ganguly P, El-Jawhari JJ, Giannoudis PV, Burska AN, Ponchel F, Jones EA (2017) **Age-related Changes in Bone Marrow Mesenchymal Stromal Cells: A Potential Impact on Osteoporosis and Osteoarthritis Development** *Cell Transplant* **26**:1520–1529
- [2] Gibon E, Lu L, Goodman SB (2016) **Aging, inflammation, stem cells, and bone healing** *Stem Cell Res Ther* **7**
- [3] Ren C, Zhang J, Yan W, Zhang Y, Chen X (2016) **RNA-binding Protein PCBP2 Regulates p73 Expression and p73-dependent Antioxidant Defense** *J Biol Chem* **291**:9629–9637
- [4] Schieber M, Chandel NS (2014) **ROS function in redox signaling and oxidative stress** *Curr Biol* **24**:R453–462
- [5] Luo Z, Xu X, Sho T, Zhang J, Xu W, Yao J, et al. (2019) **ROS-induced autophagy regulates porcine trophectoderm cell apoptosis, proliferation, and differentiation** *Am J Physiol Cell Physiol* **316**:C198–C209
- [6] Cheng Y, Lin KH, Young TH, Cheng NC (2020) **The influence of fibroblast growth factor 2 on the senescence of human adipose-derived mesenchymal stem cells during long-term culture** *Stem Cells Transl Med* **9**:518–530
- [7] Zhou J, Liu Z, Zhang L, Hu X, Wang Z, Ni H, et al. (2020) **Activation of beta(2)-Adrenergic Receptor Promotes Growth and Angiogenesis in Breast Cancer by Down-regulating PPAR gamma** *Cancer Research and Treatment* **52**:830–847
- [8] Salotti J, Dias MH, Koga MM, Armelin HA (2013) **Fibroblast growth factor 2 causes G2/M cell cycle arrest in ras-driven tumor cells through a Src-dependent pathway** *PLoS One* **8**
- [9] Chen L, Carlton M, Chen X, Kaur N, Ryan H, Parker T, et al. (2021) **Effect of fibronectin, FGF-2, and BMP4 in the stemness maintenance of BMSCs and the metabolic and proteomic cues involved**
- [10] Li D, Cheng P, Jiang H, Cao T, Wang J, Gao Y, et al. (2018) **Vascularization converts the lineage fate of bone mesenchymal stem cells to endothelial cells in tissue-engineered bone grafts by modulating FGF2-RhoA/ROCK signaling**
- [11] Xiao L, Eslinger A, Hurley MJ, Jobe M, Bone MR, Tojot AS, et al. (2013) **Nuclear fibroblast growth factor 2 (FGF2) isoforms inhibit bone marrow stromal cell mineralization through FGF23/FGFR/MAPK in vitro** :35–45
- [12] Kalghatgi S, Friedman G, Fridman A, Clyne AM (2010) **Endothelial cell proliferation is enhanced by low dose non-thermal plasma through fibroblast growth factor-2 release** *Ann Biomed Eng* **38**:748–757
- [13] Chien SY, Huang CY, Tsai CH, Wang SW, Lin YM, Tang CH (2016) **Interleukin-1 β induces fibroblast growth factor 2 expression and subsequently promotes endothelial progenitor cell angiogenesis in chondrocytes** *Clin Sci (Lond)* **130**:667–681

- [14] Arjunan KP, Friedman G, Fridman A, Clyne AM (2012) **Non-thermal dielectric barrier discharge plasma induces angiogenesis through reactive oxygen species** *J R Soc Interface* **9**:147–157
- [15] Liu T, Zheng X, Wang C, Wang C, Jiang S, Li B, et al. (2021) **The m(6)A "reader" YTHDF1 promotes osteogenesis of bone marrow mesenchymal stem cells through translational control of ZNF839** *Cell Death Dis* **12**
- [16] Bonab MM, Alimoghaddam K, Talebian F, Ghaffari SH, Ghavamzadeh A, Nikbin B (2006) **Aging of mesenchymal stem cell in vitro** *BMC Cell Biol* **7**
- [17] Shi GX, Zheng XF, Zhu C, Li B, Wang YR, Jiang SD, et al. (2017) **Evidence of the Role of R-Spondin 1 and Its Receptor Lgr4 in the Transmission of Mechanical Stimuli to Biological Signals for Bone Formation** *Int J Mol Sci* **18**
- [18] Zheng XF, Zhao ED, He JY, Zhang YH, Jiang SD, Jiang LS (2016) **Inhibition of substance P signaling aggravates the bone loss in ovariectomy-induced osteoporosis** *Prog Biophys Mol Biol* **122**:112–121
- [19] Milkovic L, Cipak Gasparovic A, Cindric M, Mouthuy PA, Zarkovic N (2019) **Short Overview of ROS as Cell Function Regulators and Their Implications in Therapy Concepts** *Cells* **8**
- [20] Cheng LQ, Zhang ZQ, Chen HZ, Liu DP (2017) **Epigenetic regulation in cell senescence** *J Mol Med (Berl)* **95**:1257–1268
- [21] Dirac AM, Bernards R (2003) **Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53** *J Biol Chem* **278**:11731–11734
- [22] Dodig S, Cepelak I, Pavic I (2019) **Hallmarks of senescence and aging** *Biochem Med (Zagreb)* **29**
- [23] Liu J, Wang L, Wang Z, Liu JP (2019) **Roles of Telomere Biology in Cell Senescence, Replicative and Chronological Ageing** *Cells* **8**
- [24] Regulski MJ (2017) **Cellular Senescence: What, Why, and How** *Wounds* **29**:168–174
- [25] Victorelli S, Passos JF (2019) **Reactive Oxygen Species Detection in Senescent Cells** *Methods Mol Biol* **1896**:21–29
- [26] Passos JF, Nelson G, Wang C, Richter T, Simillion C, Proctor CJ, et al. (2010) **Feedback between p21 and reactive oxygen production is necessary for cell senescence** *Mol Syst Biol* **6**
- [27] Frey AG, Nandal A, Park JH, Smith PM, Yabe T, Ryu MS, et al. (2014) **Iron chaperones PCBP1 and PCBP2 mediate the metallation of the dinuclear iron enzyme deoxyhypusine hydroxylase** *Proc Natl Acad Sci U S A* **111**:8031–8036
- [28] Paul BT, Manz DH, Torti FM, Torti SV (2017) **Mitochondria and Iron: current questions** *Expert Rev Hematol* **10**:65–79
- [29] Andrews NC (1999) **Disorders of iron metabolism** *N Engl J Med* **341**:1986–1995
- [30] Zheng WL, Wang BJ, Wang L, Shan YP, Zou H, Song RL, et al. (2018) **ROS-Mediated Cell Cycle Arrest and Apoptosis Induced by Zearalenone in Mouse Sertoli Cells via ER Stress and the ATP/AMPK Pathway** *Toxins (Basel)* **10**

- [31] Bigarella CL, Liang R, Ghaffari S (2014) **Stem cells and the impact of ROS signaling** *Development* **141**:4206–4218
- 32. de Magalhaes JP, Passos JF (2018) **Stress, cell senescence and organismal ageing** *Mech Ageing Dev* **170**:2–9
- 33. Yan D, Chen D, Cool SM, van Wijnen AJ, Mikecz K, Murphy G, et al. (2011) **Fibroblast growth factor receptor 1 is principally responsible for fibroblast growth factor 2-induced catabolic activities in human articular chondrocytes** *Arthritis Res Ther* **13**
- [34] Teven CM, Farina EM, Rivas J, Reid RR (2014) **Fibroblast growth factor (FGF) signaling in development and skeletal diseases** *Genes Dis* **1**:199–213

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Editors

Reviewing Editor

Helen Lu

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Augusta University, Augusta, United States of America

Reviewer #1 (Public Review):

Summary:

This study aimed to elucidate the intrinsic factors and potential mechanisms of BMSCs aging from the interactions among PCBP2, ROS, and FGF2. This study represents the first study to reveal PCBP2 as an intrinsic aging factor to regulate the replicative senescence of hBMSCs through ROS-FGF2 signaling. This study provides convincing evidence to support the above conclusion.

Strengths:

This study utilized multiple in vitro approaches, such as proteomics, siRNA, and overexpression, to demonstrate that PCBP2 is an intrinsic factor of BMSC aging.

Weaknesses:

This study did not perform in vivo experiments.

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Reviewer #2 (Public Review):

Summary:

The authors were trying to identify and characterize the intrinsic factors that control the process of cell aging of bone marrow mesenchymal stromal cells (BMSCs), which is believed to be related to osteoporosis.

Strengths:

The method is reasonable. The concept and methods used in this work can be easily extended to other systems and cells to study their aging process. It is also interesting to further examine if PCBP2 functions as an intrinsic aging factor in these other cell types.

The results are solid, supporting the claims and conclusions. The authors successfully identified and characterized PCBP2 as one of the intrinsic aging factors for BMSC cells.

Weaknesses:

It is unclear if PCBP2 can also function as an intrinsic factor for BMSC cells in female individuals. More work may be needed to further dissect the mechanism of how PCBP2 impacts FGF2 expression. Could PCBP2 impact the FGF2 expression independent of ROS?

Additional context that would help readers interpret or understand the significance of the work:

In the current work, the authors studied the aging process of BMSC cells, which are related to osteoporosis. Aging processes also impact many other cell types and their function, such as in muscle, skin, and the brain.

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