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Original Article

Preliminary Exploration of the Osteogenic Differentiation Mechanism of Bone Marrow Mesenchymal Stem Cells Regulated by SYVN1

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Abstract

Objectives: The osteogenic differentiation ability of bone marrow mesenchymal stem cells (BMSCs) is an important aspect of studying osteoporosis (OP). This study aims to explore the role of SYVN1 in regulating the osteogenic differentiation of BMSCs and to suggest its potential as a treatment for OP. **Methods**: BMSCs were differentiated using osteogenic induction. The expression of SYVN1 at different osteogenic induction time points was analyzed by Western blot (WB). The expression levels of osteogenic markers, including RUNX2, ALP, and OCN, were measured by RT-qPCR. EdU staining and colony formation assays were performed to evaluate the impact of SYVN1 on the proliferative ability of BMSCs. The effect of SYVN1 on osteogenic differentiation of BMSCs was assessed by alizarin red staining. The association of SYVN1 with the AMPK/mTOR pathway was confirmed through WB analysis. **Results**: The expression of SYVN1 decreased as BMSCs differentiation progressed. Overexpression of SYVN1 inhibited the osteogenic differentiation and proliferation of BMSCs, whereas silencing SYVN1 had the opposite effect. Furthermore, SYVN1 overexpression reduced the p-AMPK/AMPK ratio and increased the p-mTOR/mTOR ratio, effects that were reversed by the AMPK activator A-769662. **Conclusion**: SYVN1 overexpression inhibits the osteogenic differentiation and proliferation of BMSCs, potentially through modulation of the AMPK/mTOR pathway.

Keywords: AMPK/mTOR Pathway, BMSCs, Osteogenic Differentiation, SYVN1

Introduction

With the increasing aging of the population, osteoporosis (OP) has become a global health concern[1]. OP is a chronic metabolic disease characterized by low bone mineral density and microstructural deterioration. It primarily affects the elderly and postmenopausal women, leading to reduced bone strength and susceptibility to fragility fractures[2,3]. With advancements in molecular biology, gene therapy for bone metabolic diseases such as OP has emerged as a research hotspot. Bone homeostasis is a dynamic balance between bone formation and resorption, maintained by osteocytes, osteoblasts, and osteoclasts[4]. OP occurs when this balance is disrupted, favoring bone resorption over formation. Bone marrow mesenchymal stem cells (BMSCs), as multifunctional cells, can differentiate into osteoblasts, chondrocytes, and other cell types[5]. Studies have shown that BMSC differentiation is a key factor in determining bone tissue structure. As osteoblast precursors, BMSCs play a pivotal role in the progression of OP[6,7]. Therefore, exploring

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the mechanisms underlying the osteogenic differentiation of BMSCs may provide new insights for the early diagnosis and treatment of OP.

As an endoplasmic reticulum-related E3 ubiquitin ligase, Synoviolin 1 (SYVN1) is a key molecule mediating endoplasmic reticulum-associated degradation processes[8]. SYVN1 plays a role in the malignant progression of various tumors by regulating the ubiquitination and degradation of proteins such as p53[9], vimentin[10], and EEF2K[11]. Additionally, inhibition of SYVN1 protects mice from autoimmune encephalomyelitis induced by myelin oligodendrocyte glycoprotein[12]. Overexpression of SYVN1 has been shown to prevent spinal cord ischemia-reperfusion injury by regulating ferroptosis and serves as a prognostic marker for neurodegenerative diseases[13]. SYVN1 (also known as HRD1) has been found to counteract the inhibitory effect of octyl itaconate on osteoclast-related bone resorption, suggesting its involvement in osteoclast function. Furthermore, SYVN1 expression is significantly elevated in the synovium of patients with rheumatoid arthritis and is closely associated with disease progression[14]. Based on these findings, we aimed to investigate whether SYVN1 also affects the osteogenic differentiation of BMSCs.

In this study, SYVN1 expression was first examined during the osteogenic differentiation of BMSCs. Subsequently, the effects of SYVN1 on the osteogenic differentiation and proliferation of BMSCs were investigated to clarify its association with OP and explore its specific role in the progression of the disease.

Materials and Methods

Osteogenic Differentiation of BMSCs

BMSCs (ml096014, Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) were cultured in a cell culture incubator at 37°C with 5% CO_2. The culture medium consisted of DMEM supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). When the cell confluence reached 80%, osteogenic induction medium containing 5 μg/mL insulin, 0.1 μM dexamethasone, 0.2 mM vitamin C, and 10 mM β-glycerophosphate was added.

Cell Transfection

The SYVN1 overexpression (OE-SYVN1) and knockdown (sh-SYVN1) plasmids were packaged with lentiviruses. Logarithmically growing BMSCs were digested and seeded into 6-well plates at a density of 100 cells/μL per well and cultured for 24 hours under standard conditions. Lentivirus solution (2 mL) and 1 μL of polybrene were added to each well and incubated for 48 hours. The medium was then replaced with fresh complete medium, and the cells were cultured further. After another 48 hours, puromycin was added to select stable transfected cell lines.

Table 1. Primer sequences used.

Primer	5'-3'
RUNX2-F	CGCCTCACAAACAACCACAG
RUNX2-R	TCACTGTGCTGAAGAGGCTG
OCN-F	CACTCCTCGCCCTATTGGC
OCN-R	CCCTCCTGCTTGGACACAAAG
ALP-F	TCAGGGCAATGAGGTCACAT
ALP-R	CCTCTGGTGGCATCTCGTTA
GAPDH-F	CCTGCCGGTGACTAACCCTG
GAPDH-R	TCCACCACTGACACGTTGGC

Alizarin Red Staining (ARS) Staining

After osteogenic differentiation, BMSCs were washed with PBS and fixed with a fixative. The cells were then stained with 0.1% alizarin red solution (TW-reagent, TW27103, Shanghai, China) for 30 minutes, and the mineralized matrix was observed under a microscope. After photographing, 1 mL of 10% cetylpyridinium chloride was added, and the absorbance at 562 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader.

BMSCs Flow Identification

After digestion and centrifugation, the BMSCs were resuspended in PBS at a concentration of 10^6 cells/mL. A 300 μL aliquot of the cell suspension was centrifuged at 4°C and incubated with the primary antibody for 20 minutes. Subsequently, the expression of cell surface markers on BMSCs was assessed by flow cytometry.

RT-qPCR

RNA was extracted from cells using the Trizol method, and the concentration and purity of the extracted RNA were determined using a Nanodrop spectrophotometer. cDNA was synthesized from RNA according to the instructions of the Takara reverse transcription kit (639505, Takara, Beijing, China). The target gene expression was then assessed using the amplification kit (CN830, Takara, Beijing, China). Relative gene expression was analyzed using the 2-ΔΔCT method, with GAPDH as the internal reference gene. The primers used are listed in Table 1.

Western Blot Assay

Total protein was extracted using RIPA lysis buffer, and the protein concentration was determined using the BCA protein assay kit. The protein was then separated by SDS-PAGE electrophoresis and transferred to a

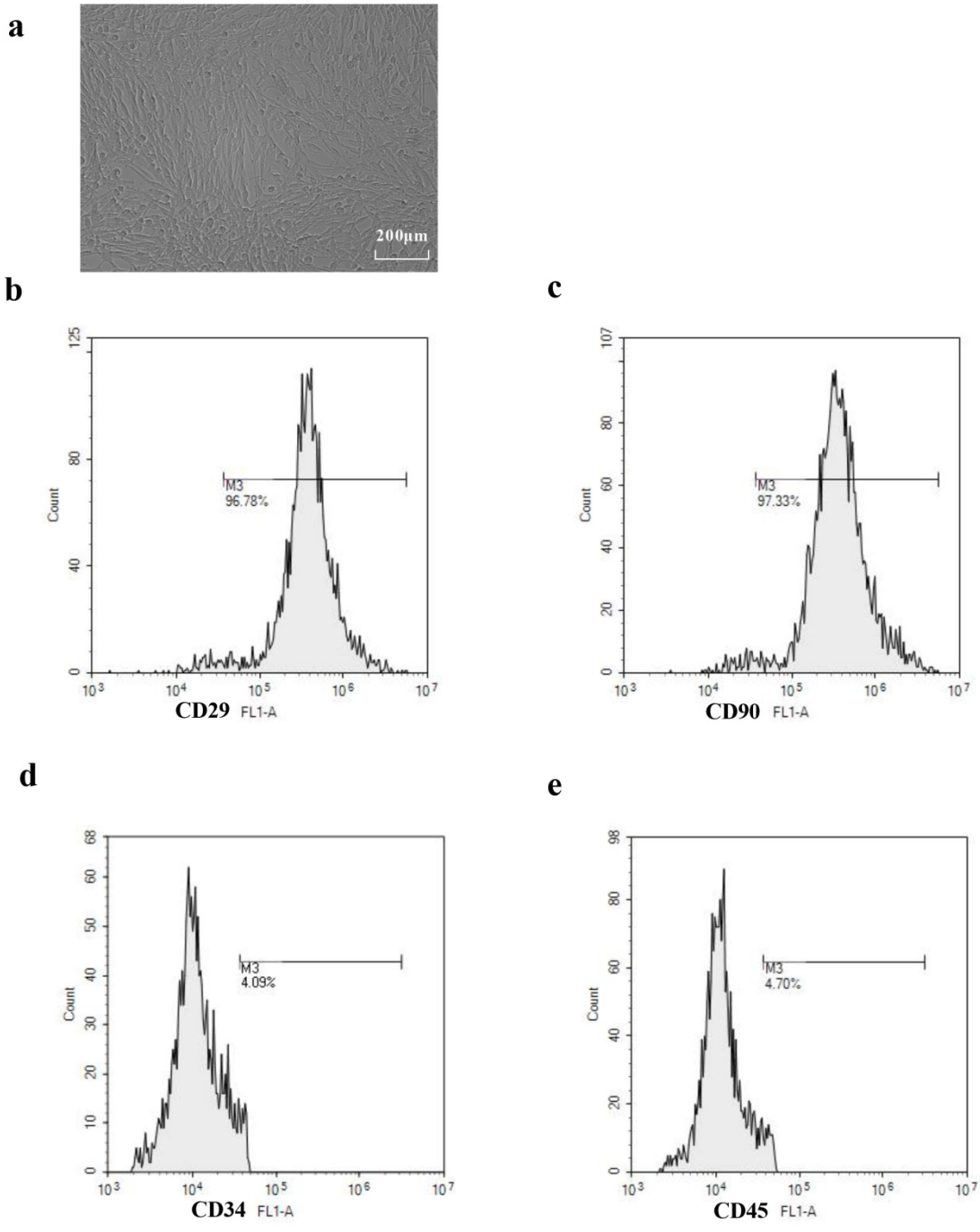

Figure 1. Identification of BMSCs. a. BMSCs appeared spindle shaped under a microscope. b-e. BMSC specific surface molecules (CD29,CD90, CD34, CD45) were detected by flow cytometry.

PVDF membrane. After blocking with 5% skim milk, the membrane was incubated with the primary antibody (anti-SYVN1: ab118483) at 4°C overnight. The membrane was then incubated with the secondary antibody for 1 hour. Protein bands were visualized using ECL chemiluminescent solution.

EdU Staining

BMSCs (2×10^5 cells/well) were seeded into 24-well plates. After adding preheated 2x EdU solution, the cells were incubated for 2 hours. Following PBS washing, cells were fixed with fixing solution at room temperature for 15

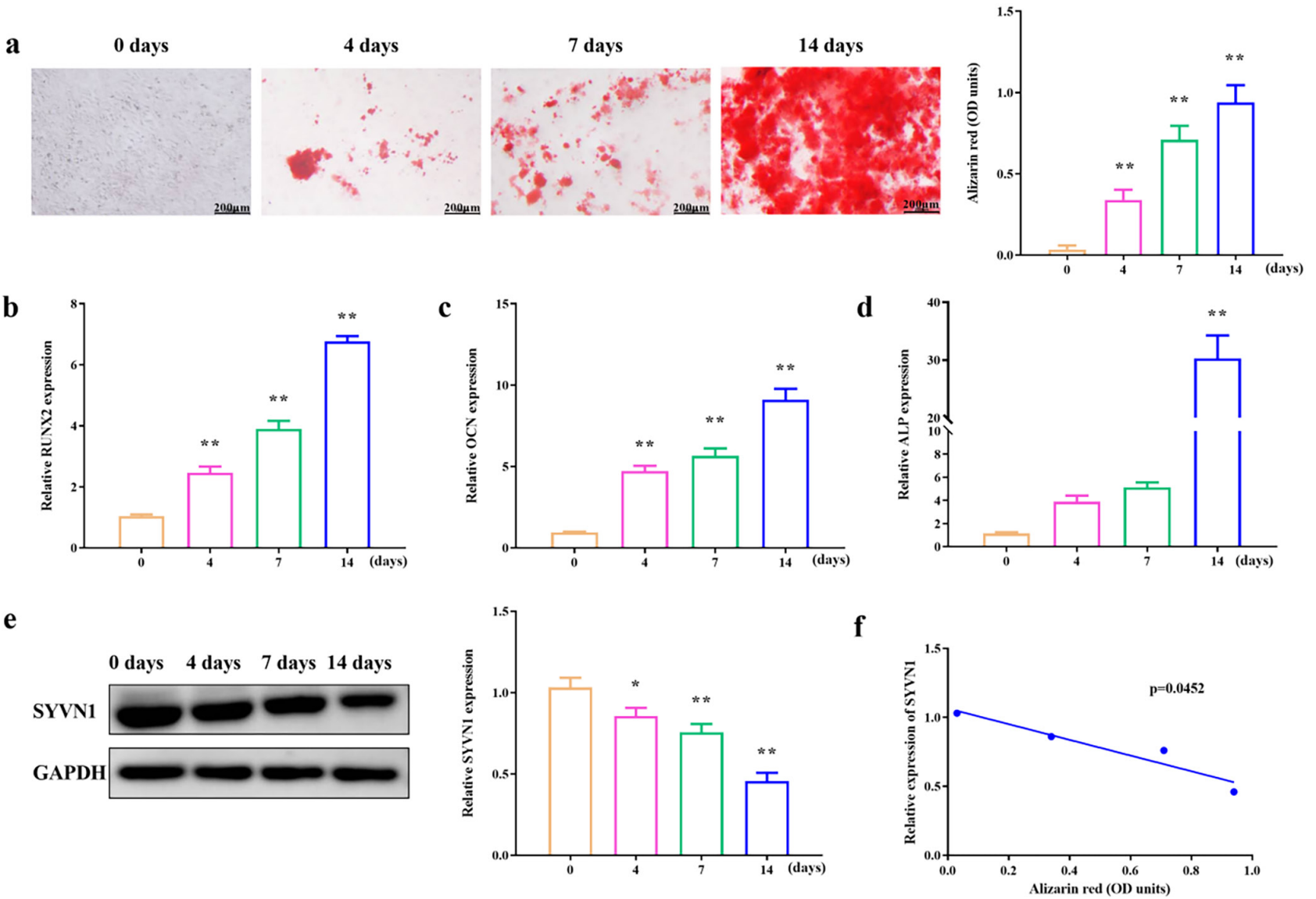

Figure 2. SYVN1 was underexpressed in osteogenic differentiation of BMSCs. a. ARS staining results during osteogenic differentiation of BMSCs. b-d. RUNX2, OCN and ALP in BMSCs osteoblast differentiation was measured by RT-qPCR. e. Western blot results showed SYVN1 expression in osteogenic differentiation of BMSCs. f. Correlation analysis between SYVN1 expression and ARS staining. *p<0.05; **p<0.01.

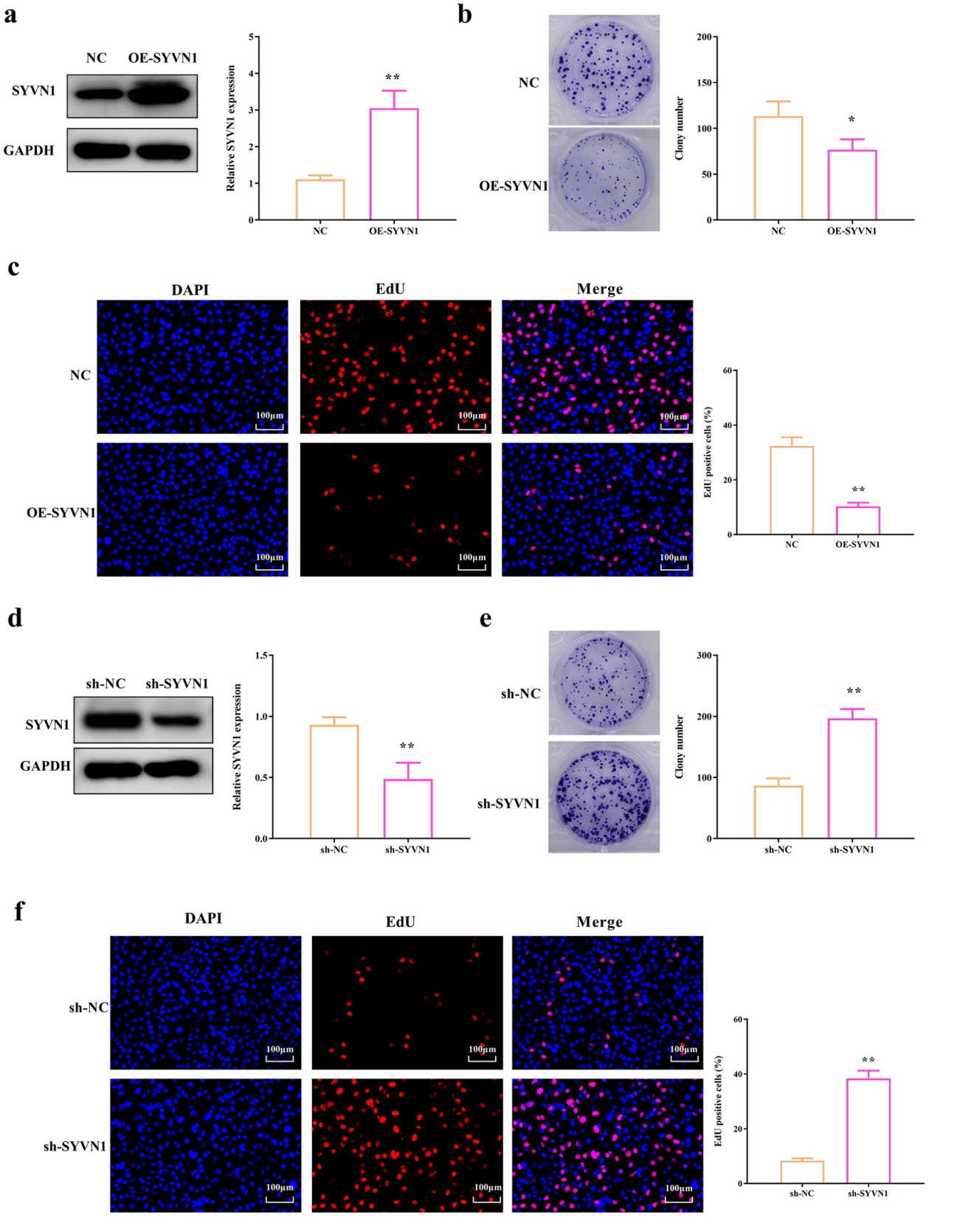

Figure 3. Effect of SYVN1 on proliferation of BMSCs. a. SYVN1 expression in BMSCs after transfection was investigated by Western blot. b. The results of plate cloning staining of BMSC cells transfected with SYVN1. c. EdU staining results of BMSC cells transfected with SYVN1. d. SYVN1 expression in BMSCs after transfection was investigated by Western blot. e. The results of plate cloning staining of BMSC cells transfected with si-SYVN1. f. EdU staining results of BMSC cells transfected with si-SYVN1. *p<0.05; **p<0.01.

minutes. Then, 1 mL of 0.3% Triton X-100 was added to each well and incubated at room temperature for 15 minutes. Next, 1 mL of Hoechst 3342 solution (1x) was added for nuclear staining. The EdU-positive cell rate was observed under an inverted fluorescence microscope.

Cell Cloning Formation Assay

Cells were digested with 0.25% trypsin and counted to prepare a single-cell suspension at a concentration of 500 cells/mL. The cell suspension was seeded into a six-well plate and cultured for 14 days. After fixation for 10 minutes, cells were washed with PBS and stained with 1 mL of Giemsa solution for 10 minutes. The number of cell clones was counted under a microscope.

Statistical analysis

Statistical analysis was conducted using SPSS software version 25.0. One-way analysis of variance (ANOVA) was employed for comparisons among multiple groups, while independent t-tests were used for comparisons between two groups. Data are presented as mean ± standard deviation. Statistical significance was set at a p-value of < 0.05.

Results

Identification of BMSCs

As shown in Figure 1a, the cell population exhibited a long spindle shape, which is consistent with the morphological characteristics of BMSCs. To further confirm that the cells isolated in this study were indeed BMSCs, flow cytometry was used to assess both positive and negative markers. The results showed that the expression of CD29 and CD90 was 96.78% and 97.33%, respectively (Figures 1b, c), while the expression of CD34 and CD45 was very low, at 4.09% and 4.70%, respectively (Figures 1d, e). In conclusion, these findings confirm that the cells isolated in this experiment are BMSCs, suitable for subsequent cell experiments.

SYVN1 Underexpression in Osteogenic Differentiation of BMSCs

BMSCs were subjected to osteogenic induction with different time gradients (0, 4, 7, and 14 days). The results revealed the formation of dark red calcium nodules around the cells in the osteoblast-induced group (Figure 2a). As the induction time increased, the depth of alizarin red staining also increased. RT-qPCR analysis of mRNA expression of osteogenic differentiation markers showed that, following osteogenic induction, the expression levels of RUNX2, OCN, and ALP were significantly elevated compared to baseline levels (Figures 2b, c, d), indicating successful osteogenic differentiation of BMSCs. Furthermore, SYVN1 gene expression was examined during osteogenic differentiation, and it was found that SYVN1 expression significantly decreased during the process (Figure 2e). Correlation analysis revealed that SYVN1 expression was negatively correlated with the degree of osteogenic differentiation of BMSCs (Figure 2f). These findings suggest that SYVN1 expression is associated with osteoporosis and osteogenic differentiation.

Effect of SYVN1 on BMSC Proliferation

First, SYVN1 was successfully overexpressed in BMSCs (Figure 3a). The proliferation ability of BMSCs was evaluated using the EdU assay and cell cloning formation assay. The EdU-positive cell rate decreased following overexpression of SYVN1 (Figure 3b), and the cell proliferation ability was weakened (Figure 3c). After successful silencing of SYVN1 (Figure 3d), the EdU-positive cell rate increased (Figure 3e), and the cell proliferation ability was enhanced (Figure 3f).

Effect of SYVN1 on Osteogenic Differentiation of BMSCs

BMSCs overexpressing SYVN1 were induced to undergo osteogenic differentiation for 7 days. ARS staining was performed to visualize the calcium nodules formed during osteogenic differentiation and assess the osteogenic differentiation ability of BMSCs. Overexpression of SYVN1 impaired the osteogenic differentiation ability of BMSCs (Figure 4a). RT-qPCR analysis revealed that overexpression of SYVN1 hindered the expression of osteogenic markers RUNX2, OCN, and ALP in BMSCs (Figure 4b). Conversely, SYVN1 knockdown promoted osteogenic differentiation, showing an opposite effect (Figure 4c,d). Together, these results indicate that abnormal expression of SYVN1 negatively affects osteogenic differentiation of BMSCs.

SYVN1 Regulates the AMPK/mTOR Pathway in BMSCs

In the SYVN1 overexpression group, the ratio of p-mTOR/mTOR was increased, while the ratio of p-AMPK/AMPK was decreased (Figure 5a). In the SYVN1 silencing group, the p-mTOR/mTOR ratio was decreased, and the p-AMPK/AMPK ratio was increased (Figure 5b). Furthermore, the AMPK activator A-769662 reversed the effects of SYVN1 on p-mTOR/mTOR and p-AMPK/AMPK ratios (Figure 5c). Subsequent functional experiments confirmed that A-769662 reversed the inhibitory effect of SYVN1 on osteogenic differentiation (Figure 5d). Additionally, A-769662 disrupted the inhibitory effect of SYVN1 on BMSC cloning (Figure 5e), and reversed the SYVN1-induced reduction in the EdU-positive cell rate (Figure 5f). In conclusion, these findings demonstrate that SYVN1 regulates the AMPK/mTOR pathway during osteogenic differentiation of BMSCs.

Discussion

With the aging population and changing lifestyles, the incidence of osteoporosis continues to rise annually[15]. Osteoporotic fractures often lead to long-term mobility impairments and various complications in the elderly, significantly affecting patients' quality of life and overall

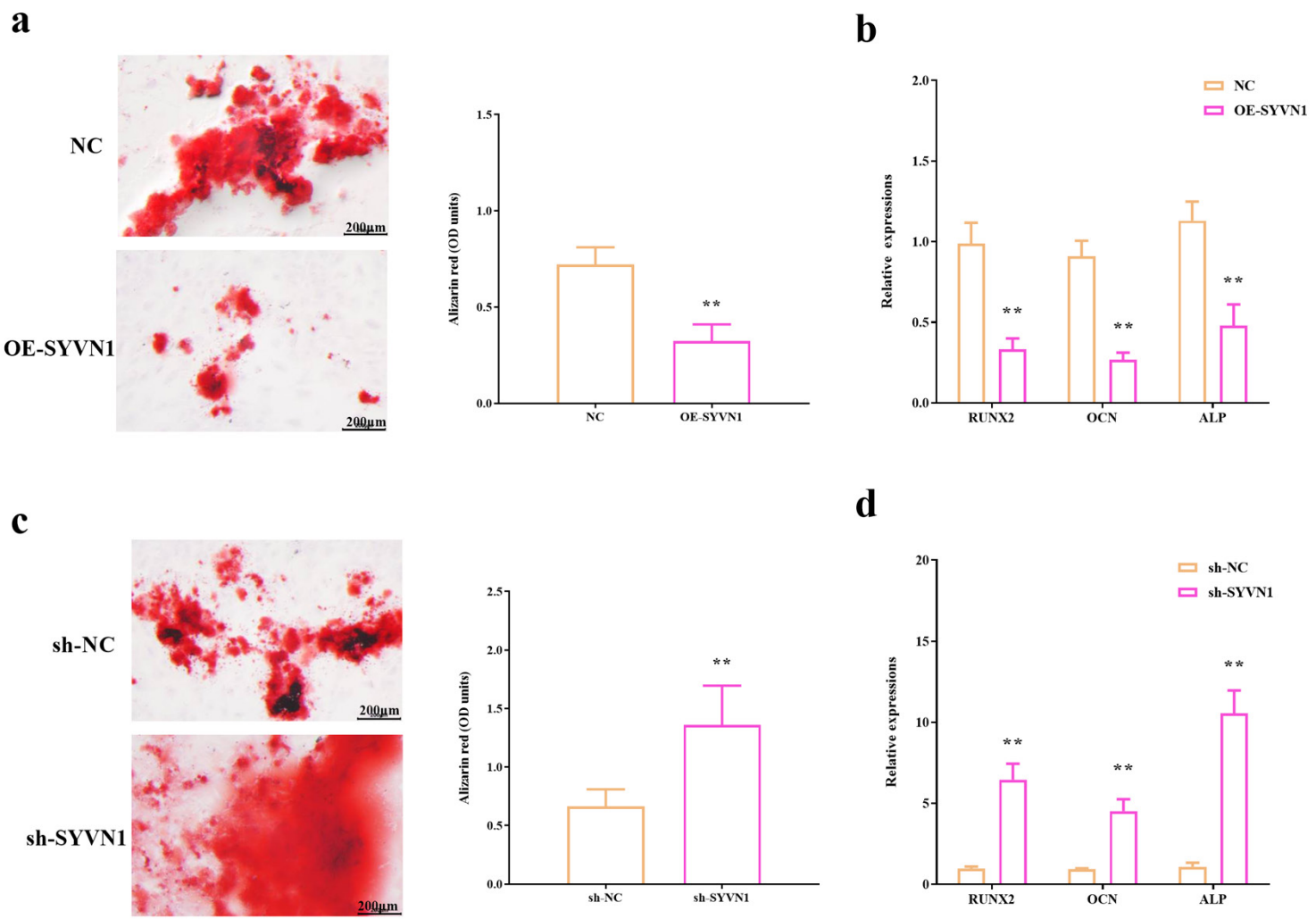

Figure 4. Effect of SYVN1 on osteogenic differentiation of BMSCs. a. The result of AR S staining of BMSC cells with SYVN1 overexpression. b. Osteogenic-related genes expression after overexpressing SYVN1 was verified by RT-qPCR. c. The result of AR S staining of BMSC cells with SYVN1 silencing. d. The expression of osteogenic-related genes after silencing SYVN1 was verified by RT-qPCR. **p<O.O1.

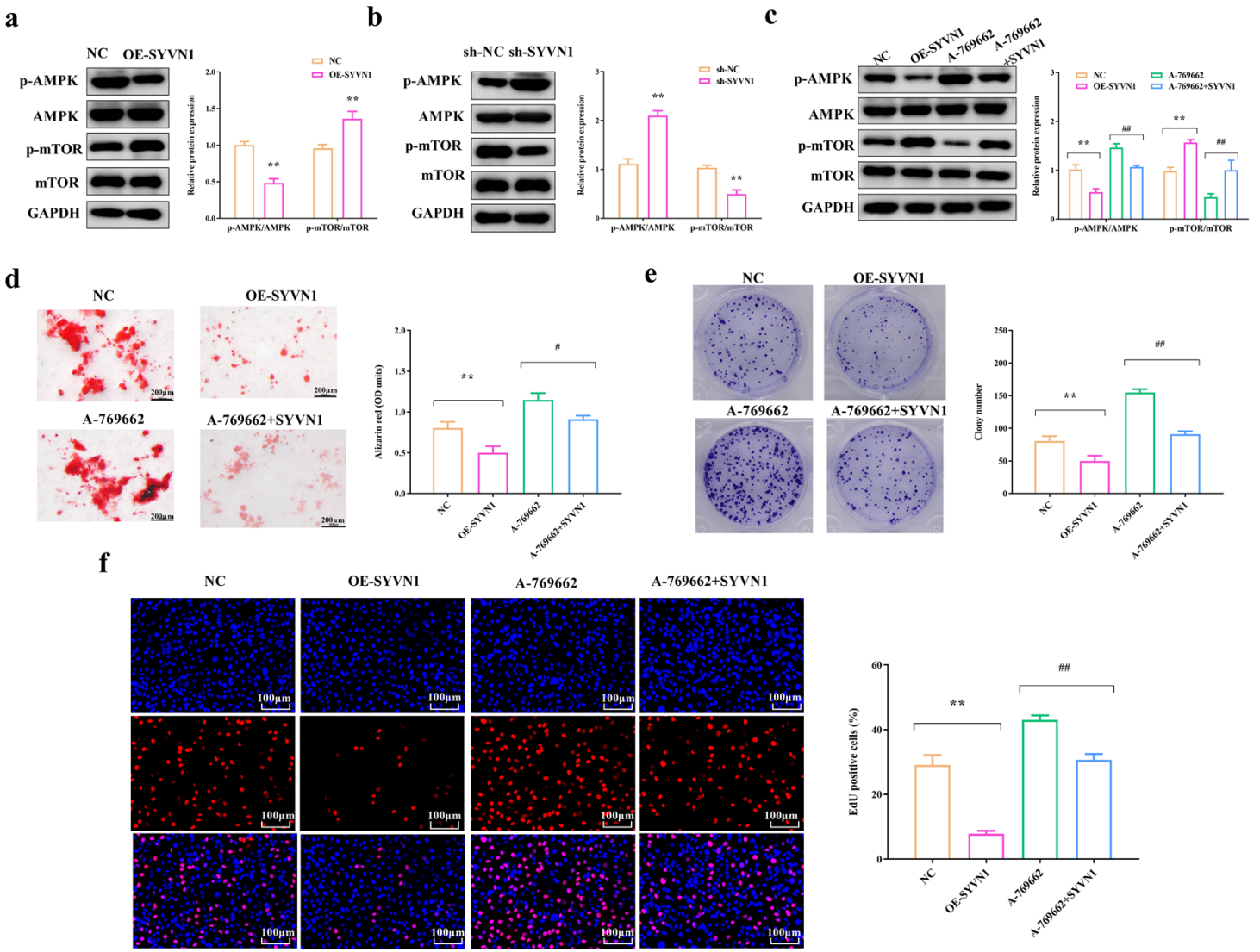

Figure 5. SYVN1 regulated AMPK/mTOR pathway in BMSCs. a, The investigation of SYVN1 overexpression on AMPK/mTOR pathway was determined by Western blot. b, The investigation of SYVN1 silencing on AMPK/mTOR pathway was determined by Western blot. c, Western blot verified the effects of AMPK activator A-769662 and SYVN1 overexpression on AMPK/mTOR pathway proteins. d, ARS staining. e, Cell cloning formation assay. f, EdU staining. #p<0.05, ##p<0.01: vs A-769662 group; **p<0.01: vs vector group.

health. Osteoporosis has increasingly become a global research focus, although its exact pathogenesis remains unclear[16]. In this study, the osteoblastic differentiation model of BMSC cells was utilized to investigate whether SYVN1 regulates osteoblastic differentiation through the AMPK/mTOR pathway.

SYVN1, an E3 ubiquitin ligase located on the ER membrane, plays a key role in the contractile homeostasis of vascular smooth muscle cells by negatively regulating endoplasmic reticulum stress[17]. In synovial fibroblasts, SYVN1 catalyzes IRE1 ubiquitination, promoting IRE1 degradation and inhibiting IRE1-mediated apoptosis, which contributes to the overgrowth of synovial fibroblasts[18]. In this study, we performed osteogenic induction differentiation of BMSCs and observed SYVN1 expression at different time points. SYVN1 expression was negatively correlated with the degree of osteogenic differentiation of BMSCs, suggesting that SYVN1 may play a role in the progression of OP by regulating osteogenic differentiation. We then performed stable overexpression and silencing of SYVN1 in BMSCs and conducted a series of cell function experiments. Overexpression of SYVN1 inhibited BMSC proliferation, clonal formation, and osteogenic differentiation, while SYVN1 knockdown had the opposite effect. Interestingly, SYVN1 has been shown to accelerate osteoclast differentiation by promoting the ubiquitination of Nrf2, indicating that SYVN1 may be involved in osteoclast activity[19]. Furthermore, SYVN1 may affect chondrocyte differentiation in Schmid type metaphyseal chondrodysplasia, though this remains to be confirmed[20]. In osteoarthritis, SYVN1 promotes cell proliferation, inhibits apoptosis, and suppresses autophagy[21], although its expression is upregulated in end-stage osteoarthritis tissues[22]. Therefore, the mechanism of SYVN1 in OP may differ from its role in osteoarthritis, warranting further investigation.

The AMPK/mTOR pathway plays a crucial role in bone homeostasis and metabolism[23]. It has been shown that this pathway promotes autophagy, thereby contributing to bone protection[24]. AMPK, a key energy sensor and metabolic regulator, is involved in cell autophagy, differentiation, apoptosis, and growth[25]. It not only indirectly regulates osteoblast and osteoclast function but also directly participates in osteoblast mineralization and osteoclast formation[26]. Under stress conditions, AMPK senses changes in the energy status and is activated via phosphorylation, which negatively regulates the downstream factor mTOR through feedback inhibition[27]. Our study demonstrated that overexpression of SYVN1 decreased the p-AMPK/AMPK ratio and increased the p-mTOR/mTOR ratio, while knocking down SYVN1 had the opposite effect, increasing the p-AMPK/AMPK ratio and decreasing the p-mTOR/mTOR ratio. This finding aligns with previous research showing that loss of SYVN1 leads to activation of the AKT and AMPK pathways[28]. Additionally, rescue experiments revealed that the AMPK activator A-769662 reversed the repressive effect of SYVN1 on BMSC proliferation and osteogenic differentiation. Based on these findings, we propose that SYVN1 affects the osteoporotic process by regulating the AMPK/mTOR pathway.

Conclusion

In conclusion, our results suggest that SYVN1 may influence osteogenic differentiation of BMSCs by regulating the AMPK/mTOR pathway, potentially serving as a key molecular mechanism underlying its involvement in OP regulation. However, further studies are needed to fully elucidate the role of SYVN1 in the pathological progression of OP, which could provide a novel target for therapeutic interventions.

Ethics approval

The study was approved by the Ethics Committee Board of The First Affiliated Hospital of Nanchang University (#CDYFY-IACUC-202407QR125).

Authors' contributions

ZP, ZW, YZ and XJ designed the study and drafted the manuscript. LY, XY, KS and NY were responsible for the collection and analysis of the experimental data. YH, NL,XZ,YW and JH revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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