Systematic Review and Meta-Analysis

Medicine

OPEN

Asperosaponin VI induces osteogenic differentiation of human umbilical cord mesenchymal stem cells via the estrogen signaling pathway

Junting Niu, MD[a], Yiqing Wang, MD[a], Yao Meng, MD[a], Wanli Qi, PhD[b,*], Jianping Wen, PhD[a]

Abstract

Background: Asperosaponin VI (ASA VI) is an active ingredient found in the traditional Chinese herb Radix Dipsaci, which is used to treat fractures. ASA VI combined with osteogenic medium can induce osteogenic differentiation of rat-derived stem cells. However, whether ASA VI alone can induce osteoblast differentiation of human mesenchymal stem cells (MSCs) remains unclear.

Methods: ASA VI human-derived binding proteins were searched in the PharmMapper database, osteogenesis-related signaling pathways were obtained through a literature search, and proteins contained in these signaling pathways were queried in the Kyoto Encyclopedia of Genes and Genomes database. SystemsDock was used to perform online molecular docking of target proteins to evaluate their binding abilities, and validation experiments were performed.

Results: A total of 620 ASA VI target proteins and 12 osteogenesis-related signaling pathways were queried, and 17 intersecting targets were screened. Molecular docking results showed that these targets had high binding affinity for ASA VI. We selected estrogen receptor 2 and its estrogen signaling pathway for experimental validation. The results showed that ASA VI can induce the osteogenic differentiation of MSCs through the estrogen signaling pathway.

Conclusion: ASA VI can independently induce osteogenic differentiation of human umbilical cord MSCs, and the estrogen signaling pathway plays an important role in this process. Thus, ASA VI may have potential as an anti-osteoporosis drug.

Abbreviations: ALP = alkaline phosphatase, ASA VI = Asperosaponin VI, ESR2 = estrogen receptor 2, FBS = fetal bovine serum, H-DMEM = high-sugar Dulbecco Modified Eagle Medium, hUC-MSCs = human umbilical cord mesenchymal stem cells, MMP2 = matrix metalloproteinases-2, mRNA = messenger ribonucleic acid, MSCs = mesenchymal stem cells, OD = optical density, OP = osteoporosis, OPG = osteoclastogenesis inhibitory factor, OPN = osteopontin, PCR = polymerase chain reaction, RUNX2 = runt-related transcription factor 2, TGF-β = transforming growth factor-β, UC = umbilical cord.

Keywords: asperosaponin VI (ASA VI), estrogen signaling pathway, osteogenic differentiation, osteoporosis (OP)

1. Introduction

Osteoporosis (OP) is a systemic metabolic bone disease associated with increased fracture risks and is a major public health concern.[1] Currently used drugs for OP include bisphosphonates, parathyroid hormone, calcitonin, etc. Among them, bisphosphonates are the first-line treatment in most developing countries. However, the side effects of these drugs include liver, kidney, and digestive tract damage, as well as dizziness, fever, etc, which limit their use.[2] OP has been characterized as a bone density-reducing disease, and causes dynamic imbalances in osteoclast- and osteoblast-mediated bone resorption and production.[3] These osteoblasts are derived from multipotent mesenchymal stem cells (MSCs). Promoting the differentiation of MSCs into osteoblasts has been suggested as a therapeutic strategy for treating OP.[4]

Because of the lack of effective clinical drugs, various natural medicinal compounds derived from plants have been evaluated for treating OP.[5] Radix Dipsaci is a traditional Chinese herbal medicine commonly used to treat fractures.[6] Asperosaponin VI (ASA VI) is the major active ingredient in Radix Dipsaci.[7] Studies have shown that ASA VI can not only induce osteogenic differentiation

This work was supported by the Provincial Special Fund for Industrial Innovation of Jilin Province [grant number 2017C059-1].

The authors have no conflicts of interest to disclose.

The datasets generated during and/or analyzed during the current study are publicly available.

Supplemental Digital Content is available for this article.

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How to cite this article: Niu J, Wang Y, Meng Y, Qi W, Wen J. Asperosaponin VI induces osteogenic differentiation of human umbilical cord mesenchymal stem cells via the estrogen signaling pathway. Medicine 2022;101:50(e32344).

Received: 27 October 2022 / Received in final form: 30 November 2022 / Accepted: 30 November 2022

http://dx.doi.org/10.1097/MD.0000000000032344

of rat adipose[8] and bone marrow MSCs[9] in the presence of osteogenic medium, but also inhibit inflammation and osteoclast formation to prevent bone loss.[10] However, osteogenic media alone can also induce osteogenic differentiation; thus, whether ASA VI can induce the osteogenic differentiation of human stem cells in the absence of osteogenic medium remains unclear.

We focused on osteogenic differentiation-related signaling pathways and applied the network pharmacology method to identify potential ASA VI target proteins in these signaling pathways, followed by determination of possible ASA VI-mediated osteogenesis-associated signaling pathways.[11] The induction of the osteogenic differentiation of human umbilical cord MSCs (hUC-MSCs) by ASA VI alone was evaluated, and the related signaling pathway was validated (Fig. 1).

2. Material and Methods

2.1. Predicting ASA VI targets in osteogenic differentiation signaling pathways

We identified relevant osteogenic signaling pathways through a comprehensive literature review, and evaluated key proteins from each of these signaling pathways for relevance using the Kyoto Encyclopedia of Genes and Genomes database.[12] The ASA VI 2D structure file was queried and downloaded from the PubChem database[13] and used for docking and molecular evaluations. We identified likely human protein partners of ASA VI using the PharmMapper database,[14] set to a confidence score of >0.4. The UniProt database[15] was used to convert these protein names into standard gene names, and intersections between likely ASA VI-binding proteins and their key osteogenesis signaling pathway proteins were obtained to identify the proteins most likely to be involved in ASA VI-induced osteogenic differentiation using a Venn diagram.

2.2. Molecular docking verification

The 3D structure of ASA VI was obtained from the PubChem compound database, and crystal structures of the ASA VI target proteins were acquired from the RCSB Protein Data Bank. Two proteins (MATK and SOCS3) which lacked comprehensive crystal structure data were omitted. Molecular docking evaluations between ASA VI and the 15 protein targets were performed using SystemsDock (http://systemsdock.unit.oist.jp).[16] Using the Cytoscape tool, we constructed The ASA VI target protein and the signaling pathway data were Imported into the Cytoscape tool, used the cytohubba plug-in to calculate the degree value, and constructed the ASA VI-target protein-signaling pathway network diagram..[17]

2.3. Chemicals and reagents

α-Minimum Essential Medium, high-sugar Dulbecco Modified Eagle Medium (H-DMEM), and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY). ASA VI was purchased from PuYi (Nanjing, Jiangsu, China) and fulvestrant (estrogen receptor antagonist) was purchased from MedChemExpress (Monmouth Junction, NJ). Anti-CD44-PE, anti-CD31-PE, anti-CD73-PE, and anti-CD45-PE were purchased from SinoBiological (Beijing, China), and the reverse transcription kit was purchased from Axygen (San Francisco, CA). The alkaline phosphatase (ALP) assay kit was purchased from Jiancheng (Nanjing, Jiangsu, China).

2.4. Isolation and culture of hUC-MSCs

UC samples were obtained from puerpera (after obtaining informed consent from the puerpera and approval from the Ethics Committee of the Second Affiliated Hospital of Jilin University). Additionally, hUC-MSCs were isolated[18] and cultured in α-Minimum Essential Medium (supplemented with 10% FBS), and maintained in a 5% CO_2 incubator at 37°C.[19] When the primary cultured cells reached 90% confluence at the bottom of the cell culture flask, they were trypsinized with 0.25% trypsin, and culture medium was used to stop the digestion reaction. A pipette was used to gently pipette the cells and place them in to a centrifuge tube, and they were then centrifuged at 1000 r/min for 5 minutes. The supernatant was discarded, and the cells were placed into new medium, mixed, and passaged. Cultures were carried out in T75 flasks (labeled as P1). Cell growth was observed, and the medium was changed every 3 days. When the cell coverage reached 90%, the cells were digested and passaged in T25 culture flasks at a 1:3 dilution.

2.5. Surface antigen identification of hUC-MSCs

P3 cells in good condition were digested, and suspended with phosphate buffered saline at the density of 1×10^5 cells/mL. A total of 50 μL cells were incubated for 30 minutes on ice in the dark with 1 μL of the following antibodies: PE-labeled anti-CD44 (Cat:12211-MM02-P), anti-CD73 (Cat:10904-MM07-P), anti-CD31 (Cat: 10148-MM13-P), and anti-CD45 monoclonal antibodies (Cat: 10086-MM05-P) were detected by flow cytometry.

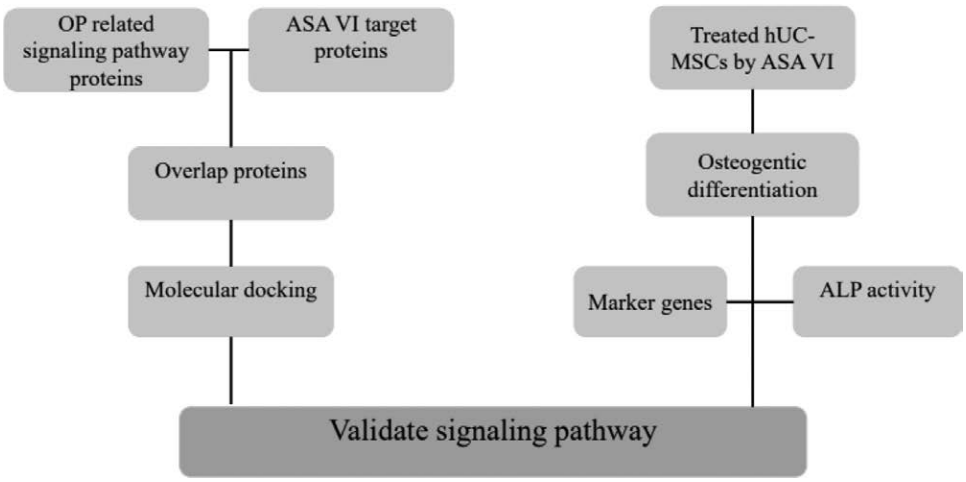

Figure 1. Network pharmacology analysis and experimental framework for evaluating Asperosaponin VI (ASA VI)-mediated treatment of osteoporosis.

Table 1

Gene primer sequences for qRT-PCR detection.

Gene	Forward primers	Reverse primers
β-actin	5′-CGTGGACATCCGCAAAGAC-3′	5′-TGGAAGGTGGACAGCGAGGC-3′
OPN	5′-TGATGCTACAGACGAGGAC-3′	5′-AATCACATCGGAATGCTC-3′
TGF-β	5′-ACTACTACGCCAAGGAGGTCA-3′	5′-GAGCAACACGGGTTCAGGT-3′
RUNX2	5′-CAGATGGGACTGTGGTTA-3′	5′-TGTGAAGACGGTTATGGT-3′
OPG	5′-CCCTTGCCCTGACCACTA-3′	5′-CATTTGAGAAGAACCCATC-3′
ESR2	5′-ATCTGTATGCGGAACCTC-3′	5′-CATCCCTCTTTGAACCTG-3′
MMP2	5′-ATCGAGACCATGCGGAAGC-3′	5′-GGAAGGCACGAGCAAAGG-3′

ESR2 = estrogen receptor 2, MMP2 = matrix metalloproteinases-2, OPG = osteoclastogenesis inhibitory factor, OPN = osteopontin, qRT-PCR = quantitative reverse transcription-polymerase chain reaction, RUNX2 = runt-related transcription factor 2, TGF-β = transforming growth factor-β.

2.6. Experimental design

To evaluate the effects of ASA VI-mediated osteogenic differentiation on hUC-MSCs and the possible roles of the estrogen signaling pathway in this process, hUC-MSCs were divided into 3 treatment groups: the control group treated with H-DMEM (10% FBS); the ASA VI group treated with H-DMEM (10% FBS) with 10^{-5} M ASA VI; and the fulvestrant group treated with H-DMEM (10% FBS) with 10^{-5} M ASA VI and 10^{-7} M fulvestrant (an estrogen signaling pathway inhibitor).

2.7. Quantitative real-time polymerase chain reaction (PCR) analysis

First, hUC-MSCs (1×10^5 cells) were treated with ASA VI for 72 hours before total ribonucleic acid (RNA) was extracted using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA concentrations were measured using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA), and 2 μg of RNA was reverse-transcribed using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA). We then evaluated the transcription of osteopontin (OPN), osteoclastogenesis inhibitory factor (OPG), runt-related transcription factor 2 (RUNX2), transforming growth factor-β (TGF-β), estrogen receptor 2 (ESR2), and matrix metalloproteinases-2 (MMP2) using quantitative reverse transcription PCR (primers shown in Table 1). The reactions were performed using Bestar™ real-time PCR Master Mix (YEASEN, Shanghai, China), and messenger RNA (mRNA) expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method. β-actin was used as an internal control gene to normalize the obtained expression levels.

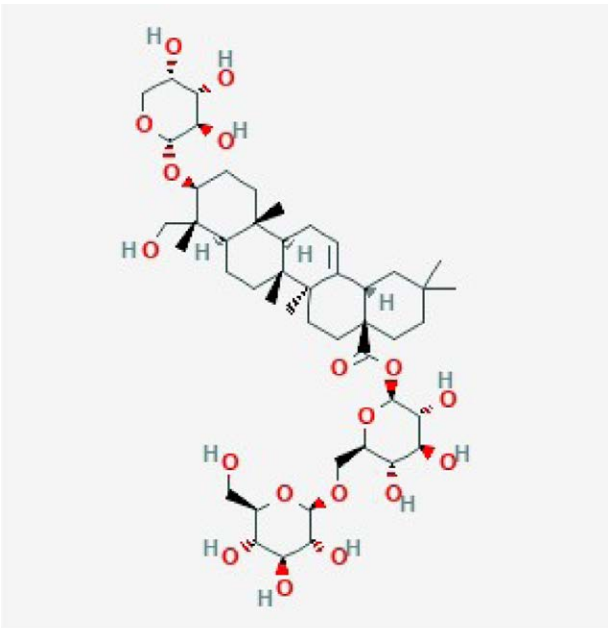

Figure 2. 2D structure of Asperosaponin VI (ASA VI).

2.8. Detection of ALP activity

P3 hUCMSC cells were seeded into 12-well plates at 5×10^4 cells/well, and randomly divided into 2 groups: control and ASA VI. The ASA VI group was used for ALP activity detection. Control cells were incubated in DMEM containing 10% FBS for 7 or 14 days. The absorbance at a wavelength of 562 nm was detected using a microplate reader according to the manufacturer's instructions. The ALP viability was calculated as follows: ALP viability = ([sample optical density [OD]-blank OD]/ [standard OD-blank OD]) × phenol standard concentration ÷ sample protein concentration.

2.9. Statistical analysis

Numerical data are expressed as the mean ± standard deviation of 3 independent experiments.[20] All statistical analyses were performed using GraphPad Prism7 (GraphPad, Inc., San Diego, CA). Student *t* tests were used to determine statistical correlations between the 2 groups, and significant differences

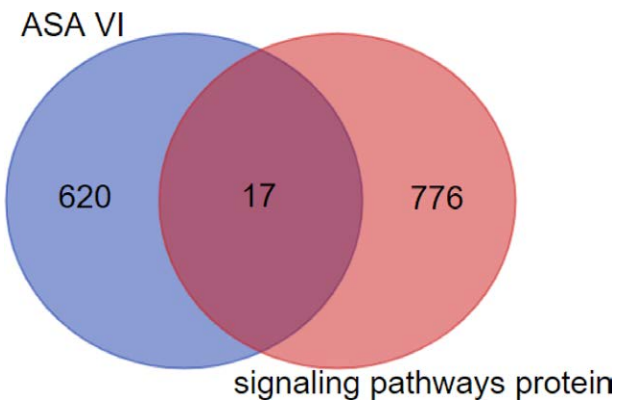

Figure 3. Venn diagram of the intersection of ASA VI target proteins and osteogenesis-related proteins. The blue circle represents ASA VI binding proteins, the pink circle represents signaling pathway proteins, and the overlapping region represents intersecting proteins (17 in total). ASA VI = Asperosaponin VI.

between groups were determined using 1-way analysis of variance. Statistical significance was set at $P < .05$.

3. Results

3.1. Proteins involved in osteogenic signaling pathways

We identified 776 proteins from 11 osteogenic-related signaling pathways for further analysis (Table S1, Supplementary Digital Content, http://links.lww.com/MD/I164).

3.2. Predicting ASA VI protein partners

The 2D structure file for ASA VI was downloaded from PubChem (Fig. 2). A total of 620 potential binding partners were identified in this analysis. The predicted ASA VI-binding proteins were intersected with osteoblast-related signaling pathway proteins to identify 17 osteogenic signaling proteins likely to respond to ASA VI-mediated induction (Fig. 3). Using the Cytoscape tool, we constructed a network which showed the relationships between ASA VI, the 17 target proteins, and 11 signaling pathways (Fig. 4).

3.3. Molecular docking

We next performed molecular docking evaluations with each of the target proteins to further evaluate the likelihood of specific interactions between ASA VI and each of its predicted target proteins (Table 2). The results showed that all 15 target proteins had higher molecular docking scores than endogenous ligands, indicating good binding with ASA VI. Further research found that ESR2 and MMP2, with higher molecular docking scores, are jointly involved in the estrogen signaling pathway. ESR2 is an estrogen receptor, and its docking score with ASA VI is

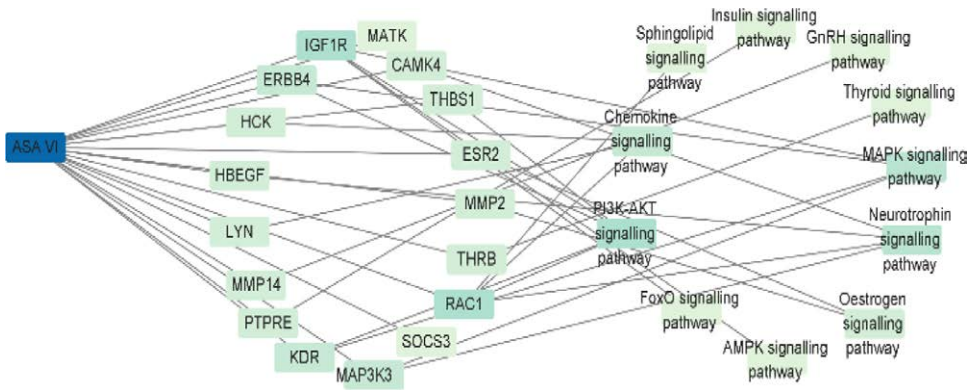

Figure 4. ASA VI-target proteins-signaling pathways network diagram. ASA VI = Asperosaponin VI.

Table 2

Docking scores determined using SystemsDock for the binding interactions between Asperosaponin VI (ASA VI), its endogenous ligands, and the predicted target proteins.

No.	Target protein name	Endogenous ligand docking score	ASA VI docking score	Signal pathways of the target protein
1	MAP3K3	3.88	8.342	MAPK signaling pathway Neurotrophin signaling pathway
2	ESR2	5.01	8.134	Oestrogen signaling pathway
3	IGF1R	4.09	8.092	MAPK signaling pathway AMPK signaling pathway FoxO signaling pathway PI3K-AKT signaling pathway
4	RAC1	4.1	8.006	Neurotrophin signaling pathway PI3K-AKT signaling pathway Chemokine signaling pathway Sphingolipid signaling pathway
5	PTPRE	5.01	7.997	Insulin signaling pathway
6	CAMK4	4.25	7.934	Neurotrophin signaling pathway
7	KDR	5.86	7.905	MAPK signaling pathway PI3K-AKT signaling pathway
8	MMP14	7.59	7.839	GnRH signaling pathway
9	LYN	3.62	7.835	Chemokine signaling pathway
10	HCK	4.5	6.464	Chemokine signaling pathway
11	ERBB4	4.64	5.06	MAPK signaling pathway PI3K-AKT signaling pathway
12	MMP2	4.83	4.881	Oestrogen signaling pathway
13	THBS1	5.25	4.639	PI3K-AKT signaling pathway
14	THRB	6.12	4.387	Thyroid signaling pathway
15	HBEGF	4.67	4.139	Neurotrophin signaling pathway

ASA VI = Asperosaponin VI, CAMK4 = calcium-dependent protein kinase IV, ERBB4 = epidermal growth factor receptor, ESR2 = estrogen receptor 2, GnRH = , HBEGF = proheparin-binding EGF-like growth factor, HCK = HCK proto-oncogene, IGF1R = insulin like growth factor 1 receptor, KDR = kinase insert domain receptor, LYN = tyrosine-protein kinase Lyn, MAP3K3 = mitogen-activated protein kinase 3, MMP2 = matrix metalloproteinases-2, MMP14 = matrix metalloproteinases-14, PTPRE = protein-tyrosine phosphatase epsilon, RAC1 = Ras-related C3 botulinum toxin substrate 1, THBS1 = thrombospondin 1, THRB = thyroid hormone receptor beta.

comparable to those of endogenous ligands (Fig. 5). Therefore, the estrogen signaling pathway was selected to study the effects of ASA VI on osteoblast differentiation in the subsequent cytological experiments.

3.4. Isolation of hUC-MSCs from the UC

hUC-MSCs were grown adherently and maintained in primary culture (Fig. 6), and arranged in parallel in the shape of long fusiforms. hUC-MSC surface markers were analyzed by flow cytometry. Among them, the positivity rates of CD31, CD45, CD44, and CD73 were 0.1%, 0.2%, 93.5%, and 40.0%, respectively (Fig. 7). The CD44 and CD73 surface markers of hUC-MSCs were highly expressed, while those of hematopoietic cells (CD31 and CD45) were lowly expressed. These results indicated that hUC-MSCs were successfully isolated from the UC.

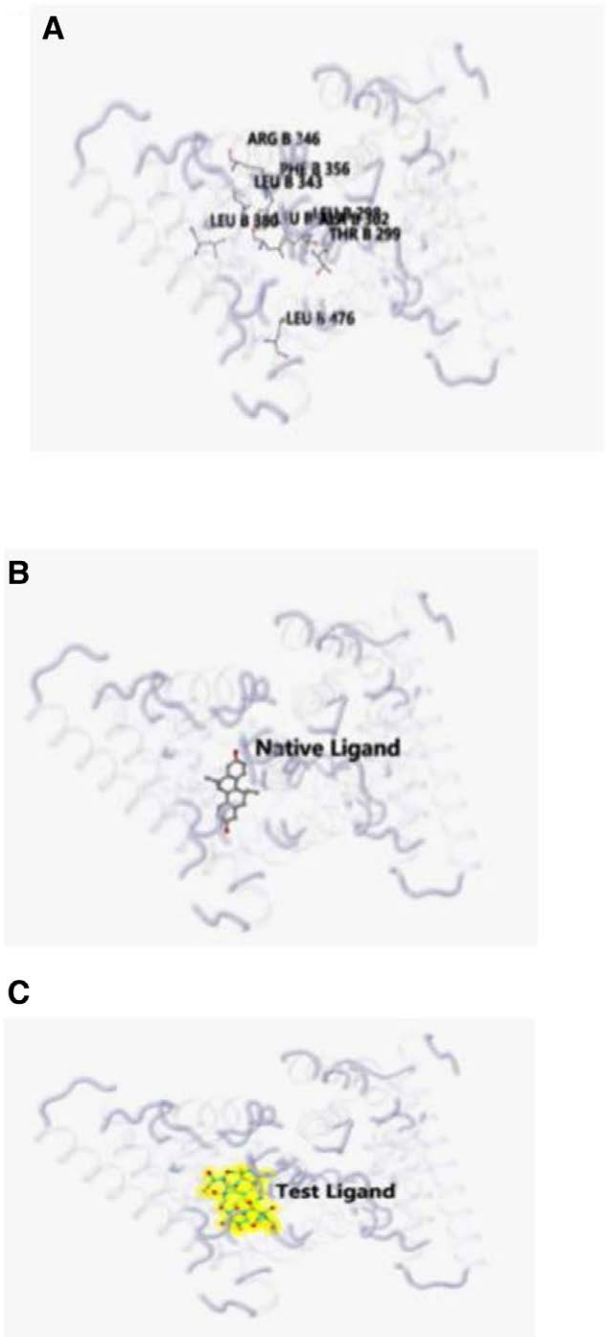

Figure 5. ASA VI combined with ESR2 in a location-specific simulation. (A) ESR2 binds to endogenous ligand sites. (B) Endogenous ligand binding site. (C) Combined position with ASA VI. The docking site of ASA VI with ESR2 is approximately the same as that of the endogenous ligand. ASA VI = Asperosaponin VI, ESR2 = estrogen receptor 2.

3.5. Osteogenic gene expression in ASA VI-treated hUC-MSCs

To explore whether ASA VI alone could induce the osteogenic differentiation of hUC-MSCs, we assessed the expression levels of osteogenic genes using quantitative reverse transcription-PCR. After treating hUC-MSCs with ASA VI for 3 or 5 days, osteogenic mRNA expression (OPN, OPG, RUNX2, and TGF-β) were significantly increased (Fig. 8). The results showed that ASA VI treatment enhanced the expression of these genes.

3.6. Effects of ASA VI on ALP activity in hUC-MSCs

ALP was used to explore the effects of ASA VI induction in the osteogenic differentiation of hUC-MSCs. ALP is an index of the early stages of bone formation.[21] After 7 days of ASA VI induction, it was observed that ASA VI promotes ALP activity ($P < .05$) compared to the control group, and at 14 days, ALP activity was significantly increased compared to the control group ($P < .05$) (Fig. 9).

3.7. Effects of the estrogen signaling pathway on gene expression during ASA VI-induced osteoblast differentiation

We used fulvestrant (an estrogen receptor antagonist) to explore the role of the estrogen signaling pathway in the osteogenic differentiation of ASA VI-induced hUC-MSCs. ESR2 and MMP2 are involved in the estrogen signaling pathway and were identified as critical nodes based on the bioinformatics and docking analyses in this study, highlighting the potential role of estrogen in ASA VI-mediated osteogenic differentiation. We found that

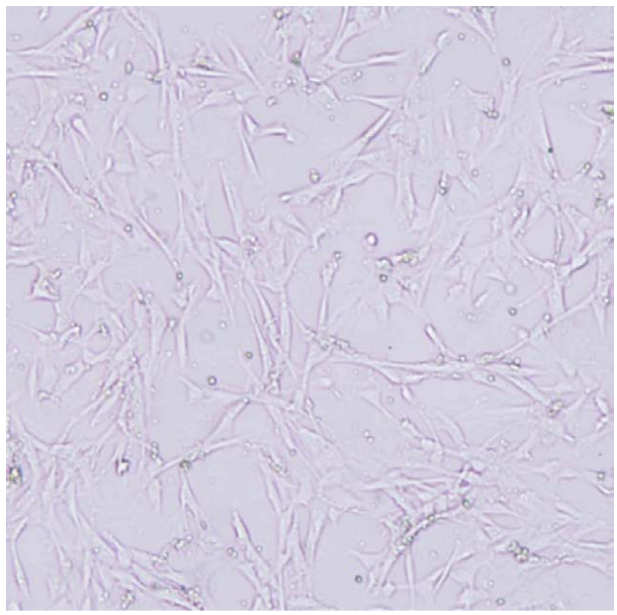

Figure 6. hUC-MSCs were spindle or fusiform in shape. hUC-MSCs = human umbilical cord mesenchymal stem cells.

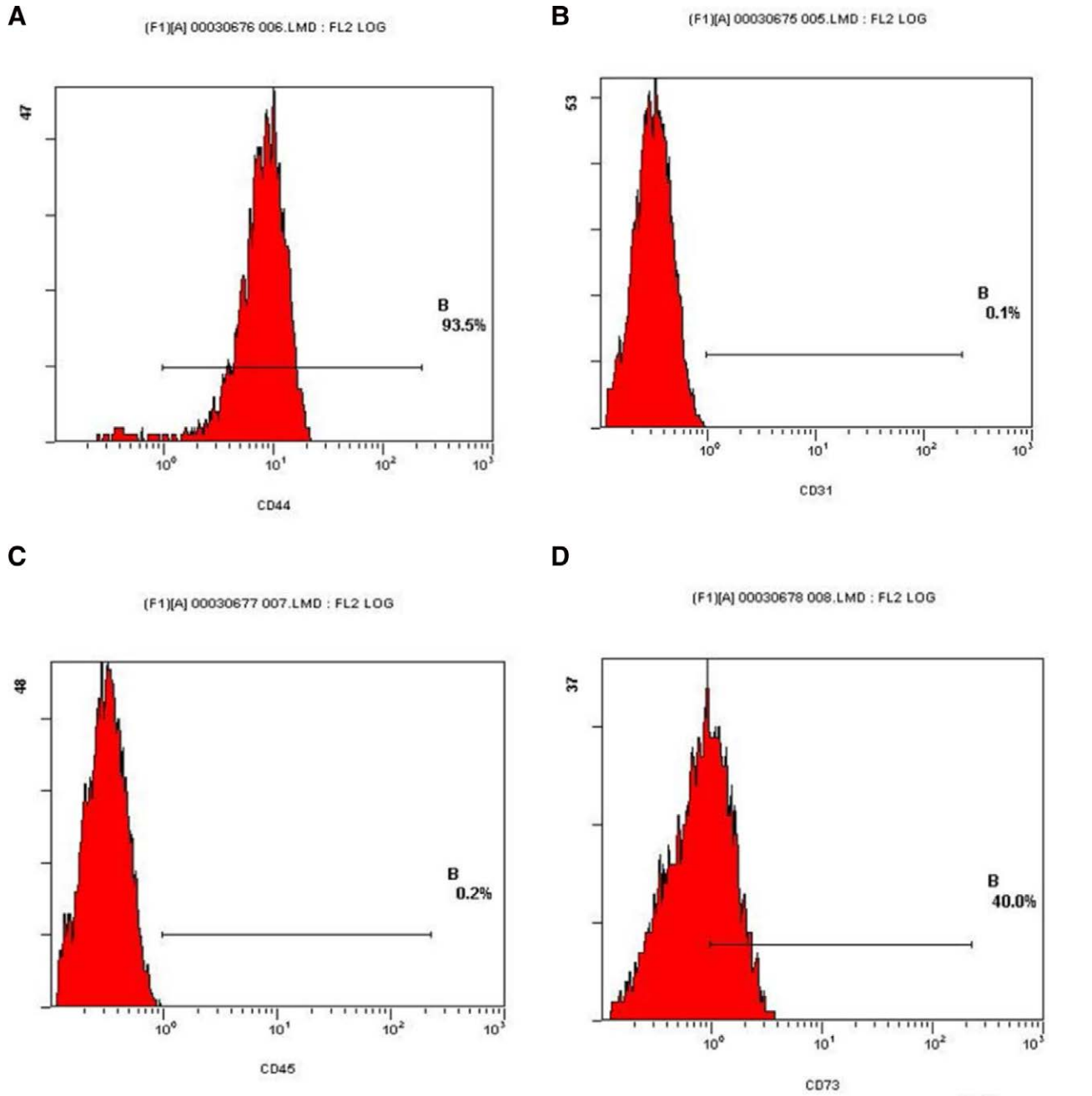

Figure 7. Flow cytometry detection of UC-MSC surface markers. (A) Anti-CD44 monoclonal antibody. (B) Anti-CD31 monoclonal antibody. (C) Anti-CD45 monoclonal antibody. (D) Anti-CD73 monoclonal antibody. UC-MSC = umbilical cord mesenchymal stem cells.

both ESR2 and MMP2 transcription was reduced in response to fulvestrant(Fig. 10), particularly when compared to the ASA VI group. The mRNA expression of the osteogenic genes OPN, OPG, RUNX2, and TGF-β were also reduced in response to fulvestrant(Fig. 10). These results indicate that the estrogen signaling pathway is involved in the ASA VI-induced osteogenesis of hUC-MSCs and that ESR2 and MMP2 are target proteins of ASA VI.

4. Discussion

OP includes senile OP and postmenopausal OP. Women suffering from postmenopausal OP account for as many as 30% of OP cases globally.[22] Bone mass loss caused by estrogen deficiency is a major cause of OP in menopausal women.[23] At present, estrogen replacement therapy has been used clinically to treat OP, but its use is limited due to significant side effects, including increased risks of cancer. However, the estrogen signaling pathway is significant in the treatment of OP.[24] Our network pharmacology results showed that 17 osteogenic differentiation-related signaling pathway proteins are target proteins of ASA VI. Molecular docking results showed that the crystal structures of 15 target proteins had high docking scores with endogenous ligands. Further analysis of the signaling pathways of these 15 target proteins found that ESR2 and MMP2 coexist in the estrogen signaling pathway. As an estrogen receptor, ESR2 not only has a higher content in bone tissue,[25] but also has a higher docking score with ASA VI than endogenous ligands, indicating that ASA VI can combine with ESR2 to exert estrogen-like effects. MMP2 is abundantly secreted by osteoblasts and participates in bone

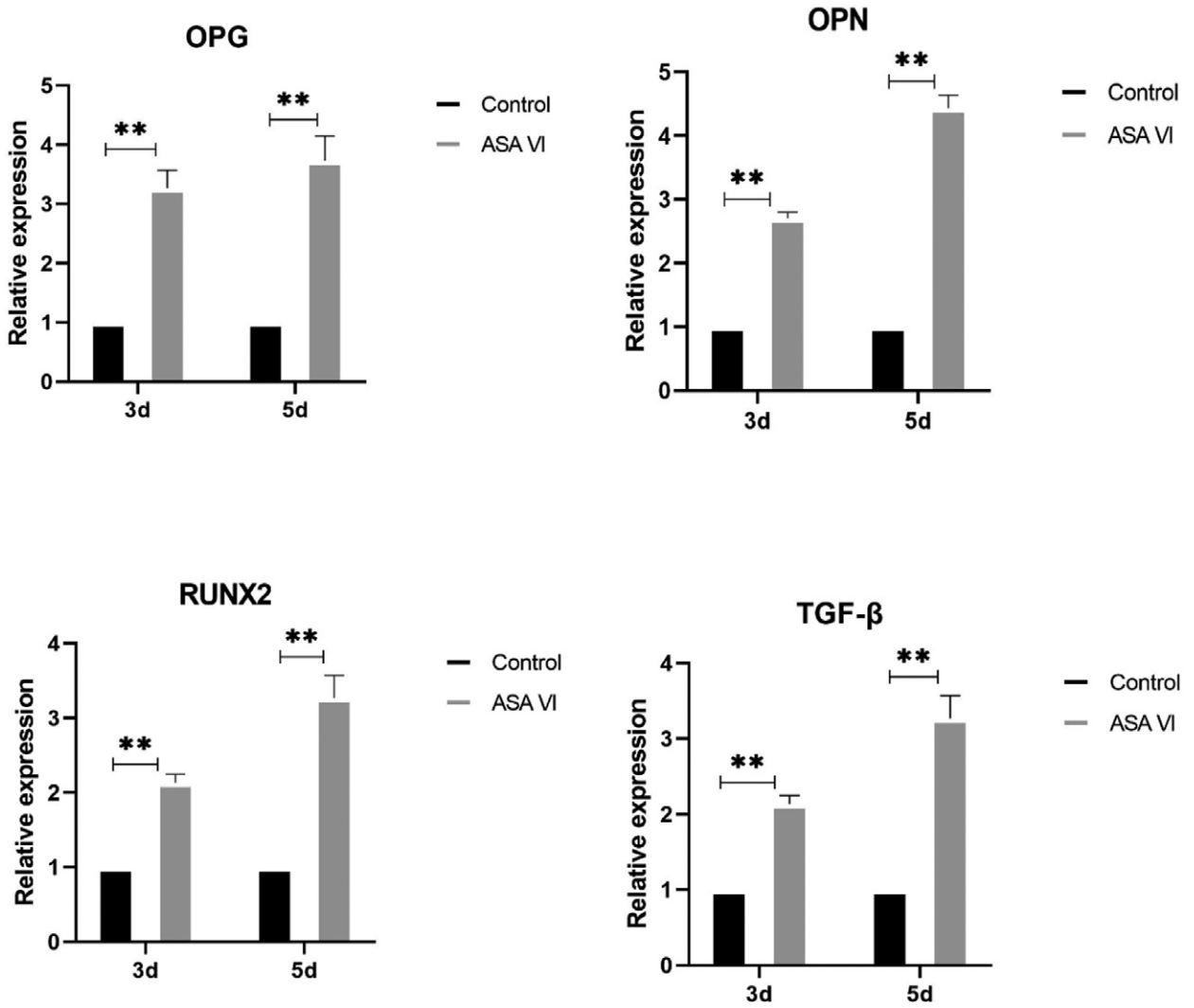

Figure 8. Asperosaponin VI (ASA VI) induces osteogenic marker transcription (** $P < .01$).

formation and bone turnover.[26] Therefore, ESR2 and MMP2 became the main target proteins of our research.

hUC-MSCs derived from placental tissue have multi-directional differentiation potential and can differentiate into osteoblasts, adipocytes, chondrocytes, etc.[27] Placental tissue is readily available and is often considered medical waste. The placenta is an important source of MSCs, and amniotic membrane, UC, chorionic membrane, and decidua MSCs have been isolated from the placenta. Studies have compared the osteogenic differentiation potential of the above MSCs, and the results show that amniotic membrane MSCs and UC-MSCs have higher osteogenic potential,[28] and we chose to use hUC-MSCs as they are easier to isolate and less immunogenic.

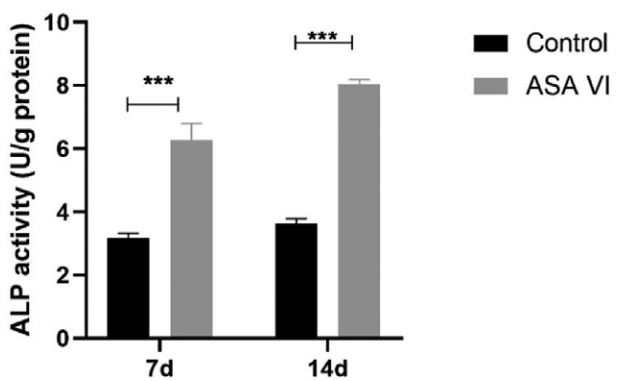

Figure 9. ALP activity, detected at a wavelength of 562 nm. ALP = alkaline phosphatase.

Here, we examined the osteogenic differentiation of human stem cells induced by ASA VI. Treatment of hUC-MSCs with ASA VI resulted in a significant increase in the expression of the osteogenic genes OPN, OPG, TGF-β, and RUNX2. OPN is an important component of the mineralized extracellular matrix of bones and teeth,[29] RUNX2 is involved in the regulation of osteogenic differentiation,[30] and OPG is a protective factor of bone tissue.[31] TGF-β is involved in bone remodeling, and can promote osteogenesis and regulate bone metabolism through various receptors and ligands.[32] A previous study showed that ASA VI combined with osteogenic induction medium (10 nM dexamethasone, 10 mM β-glycerophosphate, and 50 mM ascorbate phosphate) induces osteogenic differentiation in rat-derived stem cells.[8] Our results show that ASA VI alone can also induce osteogenic differentiation in hUC-MSCs.

We examined the role of the estrogen signaling pathway in the ASA VI-induced osteogenic differentiation of hUC-MSCs. We used an estrogen receptor antagonist (fulvestrant) to inhibit binding of ASA VI to the estrogen receptor. The results showed that the expression levels of the bone marker genes, ESR2 and

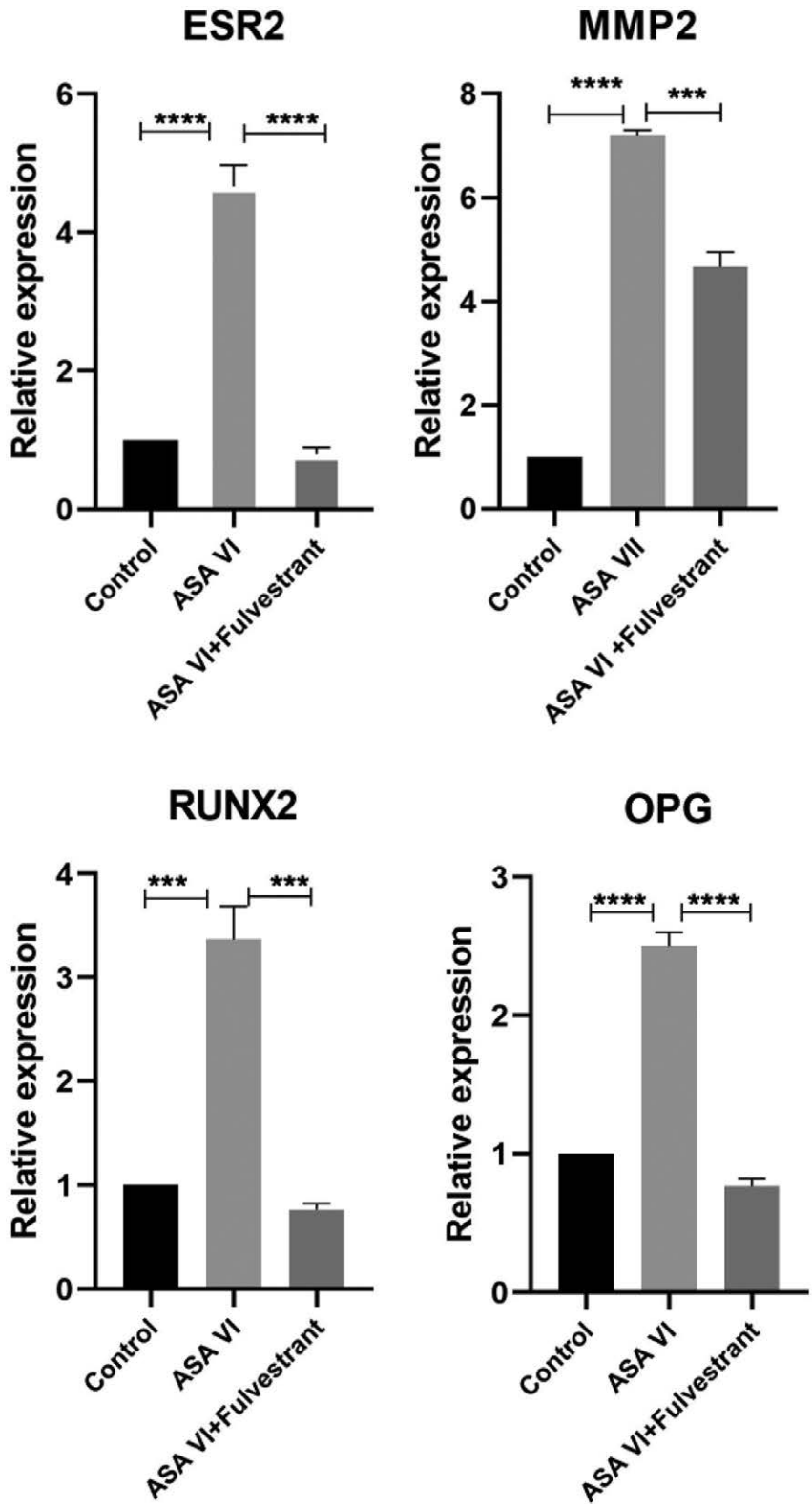

Figure 10. Effects of fulvestrant on the transcription of MMP2, ESR2, OPN, and OPG in Asperosaponin VI (ASA VI)-treated human umbilical cord mesenchymal stem cells (hUC-MSCs) (**$P < .01$). ESR2 = estrogen receptor 2, MMP2 = matrix metalloproteinases-2, OPN = osteopontin, OPG = osteoclastogenesis inhibitory factor.

MMP2, in the ASA VI group were significantly higher than those in the control group. However, the expression levels of these markers were significantly lower in the fulvestrant group, indicating that ASA VI can induce hUC-MSCs to successfully differentiate into osteoblasts and secrete large amounts of MMP2, but fulvestrant inhibited these effects. ASA VI played an estrogen-like role in the induction of hUC-MSC osteogenic differentiation, and participates in the estrogen signaling pathway.

Estrogen plays an important role in regulating bone mass in both sexes.[33] Estrogen replacement therapy was previously

used to treat patients with OP but is no longer used because of its side effects.[24] Some phytoestrogens may serve as alternatives to estrogen replacement therapies for treating OP,[34] such as isoflavones which are rich in phytoestrogens. Studies have shown that isoflavone preparations can significantly improve lumbar spine and femoral neck bone density in estrogen-deficient perimenopausal and postmenopausal women with OP.[35] We found that ASA VI activated the estrogen signaling pathway and exhibited a phytoestrogen-like effect. Therefore, ASA VI should be evaluated as a candidate for future treatments for OP.

Acknowledgments

We thank the laboratory members Ge Gao and Furong Jia for providing technical assistance. We thank Editage for language editing of the manuscript.

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Software: Junting Niu, Yiqing Wang, Yao Meng.
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Visualization: Junting Niu, Yao Meng.
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Writing – review & editing: Junting Niu, Jianping Wen.

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