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Original Article

Phenytoin regulates osteogenic differentiation of human bone marrow stem cells by PI3K/Akt pathway

Zeliang Zhang [a], Wei Shang [b], Xicong Zhao [c], Lisong Lin [d, *]

[a] The First Affiliated Hospital of Fujian Medical University, No. 20 Chazhong Road, Fuzhou, 350001, China
[b] Department of Stomatology, The Affiliated Heping Hospital of Changzhi Medical College, Changzhi, Shanxi, 046000, China
[c] Department of Stomatology, The Affiliated Heping Hospital of Changzhi Medical College, Changzhi, Shanxi, 0460000, China
[d] Department of Oral and Maxillofacial Surgery, The First Affiliated Hospital of Fujian Medical University, Fujian Provincial Key Laboratory of Stomatology, No. 20 Chazhong Road, Fuzhou, 350001, China

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ABSTRACT

Background: We mainly studied the mechanism by which phenytoin promotes osteogenic differentiation of human jawbone marrow stem cells.
Methods: Bone marrow stem cells were extracted from jaw bone tissue debris obtained from 5 subjects undergoing implant restoration. Osteogenic and adipogenic experiments proved cells stemness, and the expression of ALP, RUNX2, and OSX were detected by qPCR and Western blot. High-throughput sequencing was used to extract differentially expressed genes, the network database predicted phenytoin drug targets, GO and KEGG enrichment combined with PPI network diagram to analyze the osteogenesis mechanism.
Results: Calcium nodules and lipid droplet formation were observed in osteogenic and adipogenic experiments. The concentration of phenytoin within 100 mg/L does not produce cytotoxicity. The results of PCR and WB indicated that 50 mg/L phenytoin significantly promoted the expression of ALP and RUNX2, and 25 mg/L phenytoin significantly promoted the expression of OSX. The results of network pharmacology suggest that phenytoin promotes bone formation by up-regulating FGFR2, S1PR1, TGFB3, VCAN core proteins and activating PI3K/Akt pathway.
Conclusions: Phenytoin activated the PI3K/Akt pathway to regulate the osteogenic differentiation of human jawbone marrow stem cells. https://data.mendeley.com/datasets/t3xstktt93/1.

1. Background

Maxillofacial bone defects caused by trauma, tumor, inflammation or congenital malformations are still difficult problems. The gold standard for clinical treatment of jaw defects is still vascularization bone tissue [1,2].Bone tissue engineering is the most promising method to replace autologous bone by combining cell biology and engineering principles to study biological substitutes that can repair bone defects and improve bone function. It mainly includes three factors: cells, scaffolds and biologically active cues. Cells and scaffold are the foundation and supporting parts, and biologically active cues is the key factor [3]. Due to the wide source and multiple differentiation potentials mesenchymal stem cells are regarded as the top cells. Biologically active cues play a significant role in inducing differentiation, usually belonging to protein-type polypeptide molecules. However, some problems remain to be solved in the process of clinical transformation, such as instability, immunogenicity and high costs. In recent years, with the development of bone tissue engineering, use small molecule drugs as biologically active cues to induce osteoblast differentiation has became a research hotspot. Organic compounds with a molecular weight of less than 1000 are defined as small molecule drugs. It has the advantages of not affecting genetic information, easy synthesis, low price, easy commercialization, stable performance, no immune rejection, etc., and have great prospects in tissue engineering and regenerative medicine [4].

* Corresponding author.
E-mail addresses: zzl0523@outlook.com (Z. Zhang), drshangwei@126.com (W. Shang), 306108609@qq.com (X. Zhao), dr_lls@163.com (L. Lin).
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List of abbreviations

PHT	phenytoin
BMSCs	bone marrow stem cells
ALP	Alkaline Phosphatase
GO enrichment	Gene Ontology enrichment
KEGG	Kyoto Encyclopedia of Genes and Genomes
PIP3	phosphatidylinositol-4,5-bisphosphate
Pten	phosphatase and tensin homologue
FOXOs	forkhead box protein
ENaC	Epithelial sodium channel

Phenytoin (PHT) has been used in the treatment of epilepsy for more than 80 years. Clinical observation showed that patients received treatment with low-dose phenytoin for a long time were prone to gingival hyperplasia caused by the proliferation of gingival fibrocytes and collagen [5]. And the thickness and density of jaw bone increased significantly, patients presented acromegaly-like face [6]. On the contrary, patients on long-term high-dose phenytoin therapy develop osteoporosis, which is more common in postmenopausal women [7]. Recently, there are increasing studies demonstrated that PHT has a wide range of biological activities and pharmacological effects. In 1958, Shapiro first reported on the use of phenytoin sodium in the treatment of gum wounds and found that PHT improved wound healing [8]. Now days, PHT has been widely used in the treatment of postoperative wounds, ulcerative surface, burn wound and refractory wound. PHT was co-cultured with bone marrow stem cells (BMSCs) and found to be equipped to induce osteogenic differentiation [9]. However, the mechanism of osteogenic induction by PHT has not been systematically discussed.

Ohta et al. injected different concentrations of PHT into mice, and found that the number of metaphyseal osteoblasts, the thickness, surface and volume of bone samples in the low-dose group treated with 1 mg/kg/day were increased, and the serum osteogenesis-related indicators increased significantly [10]. Frymoyer reported that fibular fracture rats received intraperitoneal injection of PHT 4 mg/kg significantly accelerated the rate of fracture healing compared with the control group [11]. Lau et al. research found that when the optimal stimulating dose of PHT was 5–10 mmol/L, thymine, cell number, specific activity of alkaline phosphatase and collagen synthesis is increased in bone-derived cells of the human hip. The levels of PHT in serum and the biochemical markers of bone formation were detected in 39 patients treated with PHT for a long time. The results indicated that these biochemical indicators were significantly connected with serum PHT levels, but had no concern with the dose or duration of PHT treatment [9]. Na et al. found that PHT can activate the MAPK pathway and participate in the migration and osteogenic differentiation of periodontal ligament cells [12]. Asgharian-Rezaee compared the osteogenic effect of PHT and dexamethasone, demonstrated that PHT can increase all osteogenic markers. The expression of ALP were significantly increased in the group of PHT, and the degree of mineralization was comparable to the group of dexamethasone [13]. D. IKEDO applied PHT to calcaneal cells of fetal rats and examined collagen synthesis, bone nodule formation, ALP activity, cell growth, osteocalcin and osteopontin mRNA expression. He found that 200 μmol/L of PHT increased the osteogenic indexes most significantly [14].O Nakade et al. is the first to confirm that TGF-pl was involved in the osteogenesis of PHT in human osteocytes [15]. The above studies on PHT-induced osteogenesis only tested osteogenic markers to verify the osteogenic effect of the drug, and no systematic research has been conducted on the specific mechanism of PHT. Osteogenesis is a complex dynamic process involving the participation of multiple molecules and the activation of multiple signaling pathways.

Network pharmacology uses nodes and connections to build network models and network diagrams to represent the interconnections of complex biological systems. By analyzing the composition and characteristics of the network, the multi-level and multi-angle biological network relationship between “drug-gene-target-disease” was revealed. To study the possible mechanism of pharmacological action and provide an important reference for discovering the pharmacological efficacy and mechanism of drugs.

In this study, we have a comprehensive analysis about the mechanisms of PHT regulated osteogenesis in BMCs using network pharmacology and molecular biology experiments.

2. Methods

2.1. Main reagents and materials

Phenytoin(MedChemExpress,USA), Osteogenic and Adipogenic differentiation media (Cyagen, USA)Cell Counting Kit-8(CCK-8, SEVENBiotech, China)ALP staining kit(Solarbio, China), TRIzol Reagent (Introgen, CA, USA),Phosphate-Buffered saline (PBS, SEVENBiotech, China), Penicillin-Streptomycin-Gentamicin Solution(100 ×) (SEVENBiotech, China), RIPAlysis buffer (Thermo Fisher Scientific), Inverted phase-contrast microscope (Olympus, Japan). Antibody ALP (Santa, USA sc-365765), Antibody RUNX2 (Santa, USA sc-390351), Antibody OSX (Santa, USA sc-393325), Antibody GAPDH (Santa, USA sc-47724).

2.2. Sample collection and cells culturing

Jaw bone tissue fragments were collected from 5 subjects aged 18–35 years old from the Department of Stomatology, Heping Hospital Affiliated to Changzhi Medical College.

During implant surgery, we collected the bone fragments and stored in PBS containing 1% Penicillin-Streptomycin-Gentamicin Solution, BMSCs was isolated under sterile conditions. Jaw bone tissue fragments were rinsed repeatedly with PBS, centrifuged at 1000r/min for 10min, and the supernatant was discarded. The cells were transferred to culture bottles and normal medium containing α-MEM containing 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin-Gentamicin Solution was added. Cells were cultured in an incubator at 37 °C and 5% CO2, and the medium was changed every 2 days until the cells reached confluence for cell passage. The experiments mainly used third to fifth passage cells. (The procedures followed in this study protocol were approved by the Ethics Committee of Changzhi Medical College and conformed to the ethical standards of the Declaration of Helsinki. All subjects voluntarily participated in the study and signed informed consent.)

2.3. Alizarin red and oil red O staining

Briefly, 24-well plates were seeded with 1×10^5 cells per well and then incubated in osteogenic and adipogenic differentiation media for 14 days with regular media changes. The cells were fixed with 4% paraformaldehyde and then stained with alizarin red or oil red O and photographed using a microscope.

2.4. Cell viability assay

Medium containing different concentrations of PHT were prepared, including A (PHT 12.5 mg/L), B (PHT 25 mg/L), C (PHT 50 mg/L) and D (PHT 100 mg/L). We set up 5 groups, group A, B, C, and D

were treated with corresponding medium containing drugs, and the group (−) was treated with general medium. Cells were seeded in 96-well plates at 3×10^3 cells/well, and Cell Counting Kit-8 assay was performed on the 1st, 3rd, and 5th day to detect the cell viability at each time point. The microplate reader accurately measured the absorbance value at 450 nm. The number of biological replicates for each group was n = 4. F-test was used to analyze the results, and the P-value <0.05 was considered to be significant.

The number of biological replicates for each experiment was n ≥ 3.

2.5. Alkaline phosphatase staining

Cells were cultured for 14 days, the medium was removed and rinsed with PBS. 300ul Alkaline Phosphatase (ALP) staining solution was added and incubated at 37 °C for 30mins, then rinsed three times with PBS. Images were captured using a microscope.

2.6. Real-time PCR analysis

Total RNA can be isolated from MSCs with TRIZOL reagent and reverse transcribed into cDNA with Revert Aid First Strand cDNA Synthesis Kit. Q-PCR was achieved using SYBR Green PCR Master Mix and a real-time system (QuantStudio 1, ThermoFisher Scientific, Massachusetts). GAPDH was used as the normalized gene and the expression of related mRNA was calculated basing on 2-△△Ct method. The number of biological replicates for each group was n = 4. F-test was used to analyze the results, and the P-value <0.05 was considered to be significant.

2.7. Western Blotting

BMSCs were treated with A (PHT 12.5 mg/L), B (PHT 25 mg/L), C (PHT 50 mg/L) and D (PHT 100 mg/L) of medium, and the control group (−) was treated with general medium, and the control group (+) was treated with osteogenic differentiation media. On the 14th day, cells were washed thoroughly in PBS and lysed to obtain proteins with RIPA lysis buffer. Further, electrophoresis experiments were performed on a 10% SDS-PAGE gel, the proteins were separated and electrotransferred to a 0.45 μm pore size polyvinylidene difluoride (PVDF) membranes. PVDF membranes were blocked with 5% BSA for 2 h at room temperature and incubated with primary antibodies ALP(dilution 1:100), RUNX2(dilution 1:100), OSX(dilution 1:100), and GAPDH(dilution 1:500), overnight at 4 °C. Finally, it was incubated with the second antibody labeled with horseradish peroxidase for 1 h to detect the binding of the primary antibody at indoor temperature. Protein bands then visualized using an ECL staining kit.

2.8. RNA-sequencing

The Agilent 2100 bioanalyzer performed quantification and qualification on the extracted RNA samples to accurately detect the integrity of the RNA. The mRNA of qualified samples was obtained for qPCR amplification and purification to obtain the final library. In the second step, initial quantification was performed using a Qubit2.0 Fluorometer, followed by detection of library insert size using an Agilent 2100 bioanalyzer. To ensure the quality of the library, qRT-PCR was used to accurately quantify the effective concentration of the library (the effective concentration of the library was higher than 2 nM). Afterward, clustering and sequencing were carried out. Clean reads were obtained through the following steps: raw data filtering, sequencing error rate checking and GC content distribution checking. DESeq2 R package (1.20.0) can be used for differential expression analysis, filtrating criteria are | log2 (FoldChange) | & gt; = 1 & padj = 0.05. Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis of differentially expressed genes were achieved through the ClusterProfiler R package.

2.9. Drug target prediction

The small molecule structures of phenytoin and phenytoin sodium were obtained from Pubchem database and downloaded as Structure2d_CId_175.sdf. The targets of phenytoin/phenytoin sodium were predicted from following databases:

Swiss Target Prediction http://swisstargetprediction.ch/
Drug bank(https://go.drugbank.com/)
TTD (http://db.idrblab.net/ttd/).
STITCH (http://stitch.embl.de/cgi/input.pl).
Pharm Mapper(http://lilab-ecust.cn/pharmmapper/index.html).
and CTD(https://ctdbase.org/).

2.10. Protein–protein interaction (PPI) networks

Intersecting drug targets obtained from online databases with sequencing results. The core targets in the union were inputed into the STRING (https://string-db.org/) database and Cytoscape 3.7.2 (Cytoscape Consortium, CA, USA) software to construct a visual PPI network. Using the CytoNCA plug-in to analyze the Centrality-Degree value, and the targets were further filtrated according to the Degree value.

2.11. Molecular docking

PHT small molecule ligands were downloaded from Pubchem and the structure was optimized using the software Chem3D.The saved files were converted to pdbqt format using AutoDockTool. UniProt can download the protein receptor of the PHT target, then the water molecule and small molecule ligand in the protein receptor were deleted using Pymol (https://pymol.org/2/). And use AutoDockTool to hydrogenate the saved protein receptor.Molecular docking was implemented using AutoDock Vina. We uploaded each processed receptor and its corresponding ligand separately. In order to allow the receptor and ligand to try docking at more positions, selecting the Number of docking parameters for docking as 15. By processing each receptor and its corresponding ligand, we obtained docking related results at 15 different positions. The lowest degree of connectivity was considered to be the best docking result for each receptor and its corresponding ligand. And Pymol was applied to display images of the receptor and the corresponding ligand.

3. Results

3.1. Cells culturing

BMCs were extracted from the jaw bone, which showed fusiform or polygonal protrusions under the microscope. After 21 days of adipogenic induction, adipoid cells and lipid droplets were formed. After 21 days of osteogenic induction, calcium nodules can be seen, which can be stained with alizarin red and appear as red lumps under the microscope. It is proved that the cultured cells have the characteristics of stem cells and have the ability of multidirectional differentiation (Fig. 1).

3.2. CCK-8

The results of CCK-8 cell proliferation assay showed that PHT at 12.5 mg/L to 50 mg/L promoted cells proliferation. There was no

significant difference between 100 mg/L and the group (−), indicated that the drug concentration higher than 100 mg/L may cause cytotoxicity (Fig. 2).

3.3. ALP staining

The result showed that the ALP staining of 25 mg/L,50 mg/L and group (+) was darker compared with group (−), and the 100 mg/L group had lighter color than the group (−). ALP expression was promoted by 25 mg/L, 50 mg/L and group (+) and inhibited by 100 mg/L group (Fig. 3).

3.4. Gene expression

Compared with the group (−), the expression of ALP and RUNX2 increased in the 50 mg/L group, and the OSX gene increased in the 25 mg/L group. The effect of drug induction was better than that of osteogenic induction solution, and the above results has statistically significance ($P < 0.001$). Western blot results also proved that the protein expression levels of ALP, RUN2, OSX was significantly higher in 50 mg/L group (Fig. 4).

3.5. Drug target prediction

By querying 6 databases, the protein names were converted into gene names, and finally the PHT drug target data were obtained. After taking the drug targets obtained in the above database and removed duplicate data, 814 phenytoin-related drug targets were predicted (Table 1).

3.6. Expression of differential genes-RNAseq

A total of 10629 genes were obtained from the intersection set of control and experimental results (Fig. 5A). Of these, 3602 genes had significant expression difference, including 1806 up-regulated genes and 1256 down-regulated genes (Fig. 5B). ClusterProfiler software performed GO functional enrichment analysis and KEGG pathway enrichment analysis of the differential gene set. For results of GO functional enrichment, a p-value≤ 0.05 was used as the threshold for significant enrichment. The results mainly involved DNA replication, DNA-dependent DNA replication, chromosome segregation, nuclear division and organelle fission. KEGG pathway enrichment mainly included the following pathways: protein digestion and absorption, DNA replication, cell cycle, receptor interaction, cytokine-cytokine and PI3K-Akt signaling pathway (Fig. 5C-D).

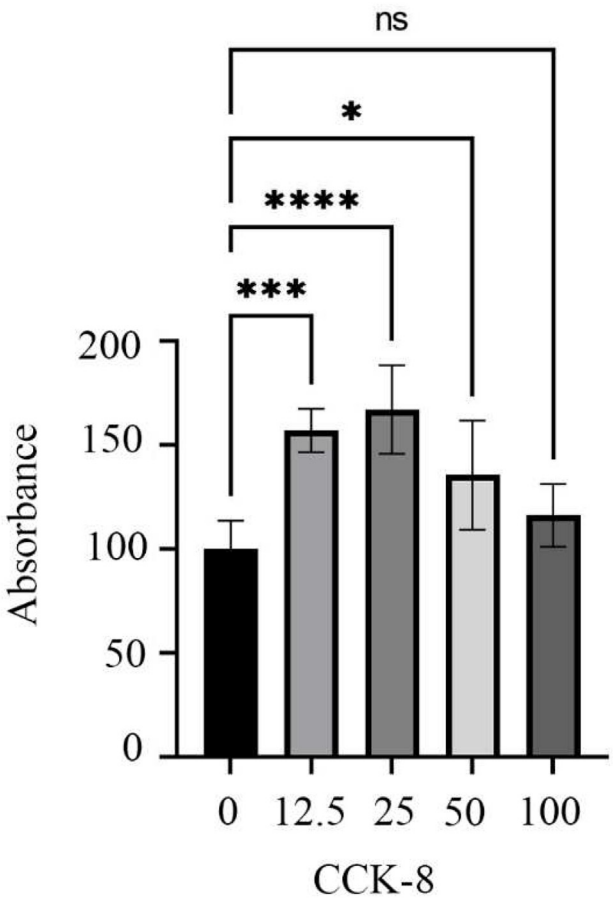

Fig. 2. Absorbance of CCK-8.

3.7. Drug targets regulate osteogenic genes and construction of PPI network

A total of 94 core genes were obtained by intersection of PHT drug targets and differential genes obtained by sequencing, including 61 up-regulated genes and 33 down-regulated genes (Fig. 6). By analyzing the biological functions of these genes, we can predict the biological role of PHT in the intervention of osteogenic differentiation of BMCs, which is mainly involved in the biological process of ossification. KEGG enrichment analysis suggested that the drug targets of phenytoin in regulating osteogenic differentiation were mainly involved: PI3K-AKt, chemical carcinogenesis, and AGE-RAGE signaling pathway (Fig. 7). PPI network could help us to further extract the core genes, and the top 50 genes were obtained by Degree value analysis. Among them, 8 genes were extracted: COL11A1, FGFR2, PTHLH, S1PR1, TGFB3, MMP14, IGF2, and VCAN (Fig. 8).

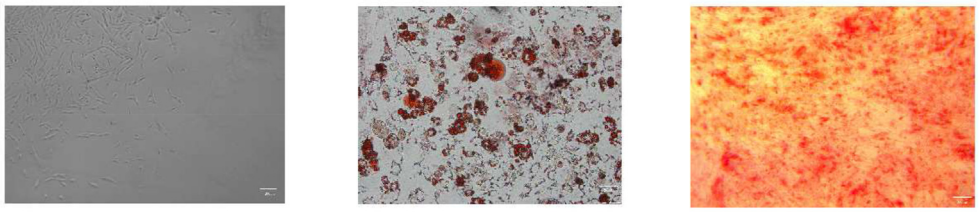

Fig. 1. A. Human bone marrow stem cells. (10X10) B. Oil Red O Staining. (20X10) C. Alizarin Red Staining. (4X10).

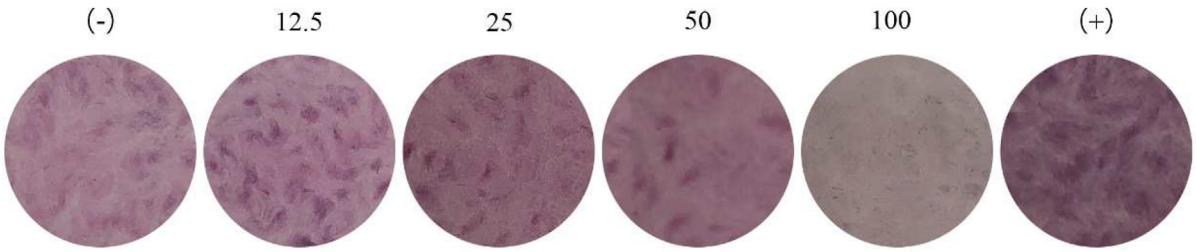

Fig. 3. The result showed that the ALP staining of 25 mg/L, 50 mg/L and group (+) was darker compared with group (−), and the 100 mg/L group had lighter color than the group (−).

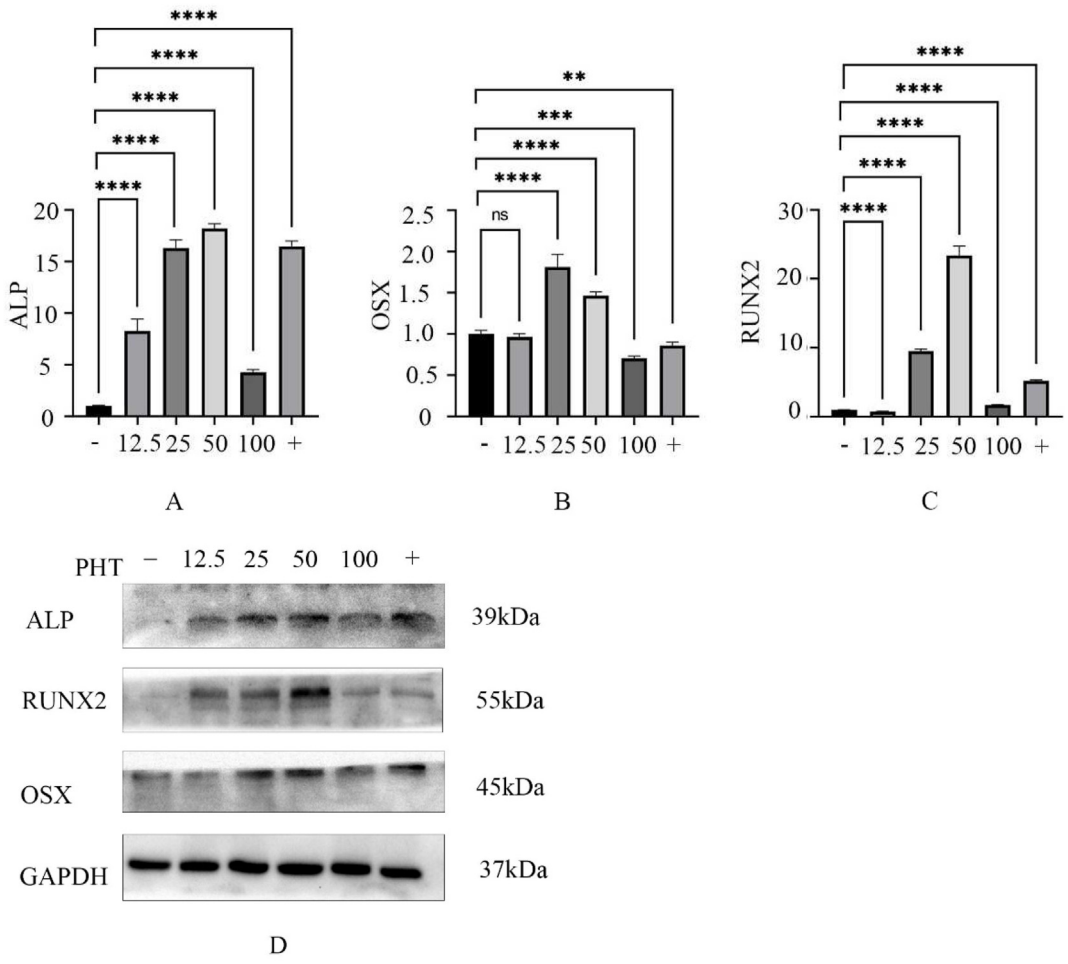

Fig. 4. A-C show the mRNA expression levels of ALP, OSX and RUNX2 genes at different drug concentrate. D. Western Blot experiment showed the protein expression levels of ALP, RUNX2 and OSX. (4X10).

3.8. Molecular docking

The Number of docking parameters selected for docking was 15, and the lowest degree of connectivity was used as the best docking result for each receptor and corresponding ligand, and statistics were performed. The matching values of FGFR2 S1PR1 TGFB3 VCAN were less than −7, indicating that them had strong binding ability to PHT drug target (Table 2) (Fig. 9).

4. Discussion

Our studies demonstrated that BMSCs had the potential of multi-directional differentiation, just like dental pulp stem cells and periodontal ligament stem cells. Appropriate concentration of PHT can accelerate the differentiation and proliferation of BMSCs. When the concentration of PHT was controlled within 100 mg/L, there was no toxic effect on the cells. The expression of osteogenic genes in the 50 mg/L group was significantly up-regulated, and the effect was better than that in the osteogenic induction solution group. RNA-sequencing combined with drug target prediction obtained 61 up-regulated genes and 33 down-regulated genes. PPI network and Cytoscape plugin helped us to further narrow the filtrating scope, and molecular docking finally identified 4 core targets of FGFR2, S1PR1, TGFB3 and VCAN. The core targets are mainly involved in fibrous tissue formation, cell adhesion, proliferation and migration.

Table 1
Phenytoin targets prediction.

Database	Targets
SwissTargetPrediction	120
ParmMapper	269
CTD	429
DrugBank	33
STITCH	10
TTD	1
Total	862

Previous studies have reported that these signaling pathways associated with osteogenesis include: BMPs/Smads pathway, Wnt/TGF-β pathway, Hedgehog pathway, MAPK pathway and PI3K/AKT pathway. Na et al. indicated that the MAPK pathway was involved in the PHT-regulated process of dental pulp stem cell migration to osteogenic differentiation [12]. Shang et al. demonstrated that PHT regulates the repair process of dental pulp stem cells by activating the BMP4/Samd pathway [16]. In our experiments, the results suggested that PI3K/AKT pathway was significantly upregulated. The correlation between PI3k-AKT signaling pathway and tumors, diabetes, and cardiovascular system diseases has been widely reported. In recent years, it has been found that this pathway also plays an important role in the process of osteogenesis [17,18]. Phosphatidylinositol 3-kinases (PI3Ks) are members of a unique family of lipid kinases that are regulated by growth factors such as insulin-like growth factor 1, fibroblast growth factor and epidermal growth factor. Upon activation by stimulation, bisphosphatidylinositol can be phosphorylated to triphosphatidylinositol. This response leads to the activation of many intracellular signaling pathways that regulate diverse functions such as cellular metabolism, survival, polarity, and vesicle trafficking [19].

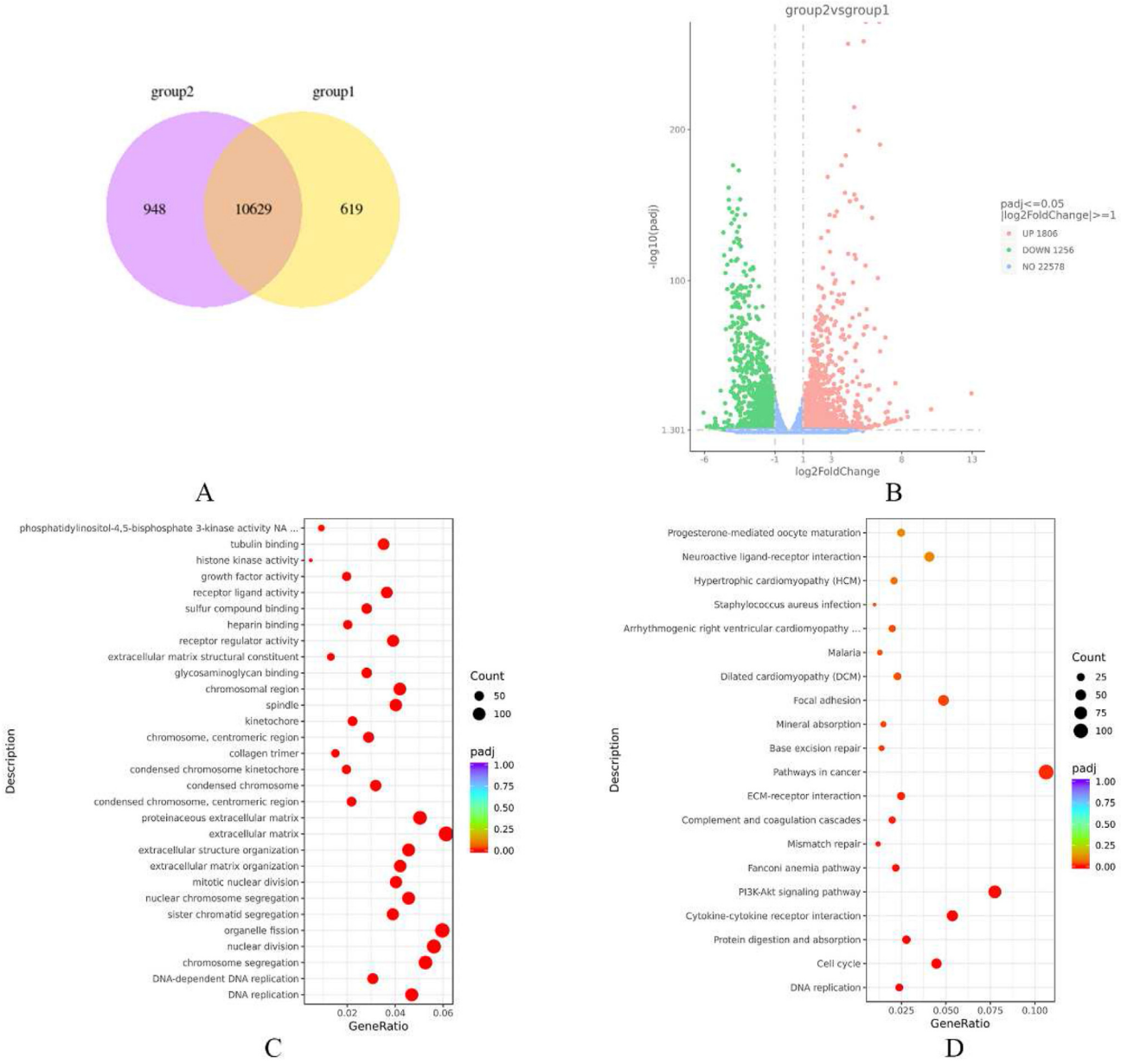

Fig. 5. A. Venn plot of differential genes. B. The volcano plot showing the expression pattern of DEGs in PHT and control group. C. GO functional terms enriched by the DEGs. D. The KEGG signaling pathway enriched by the DEGs.

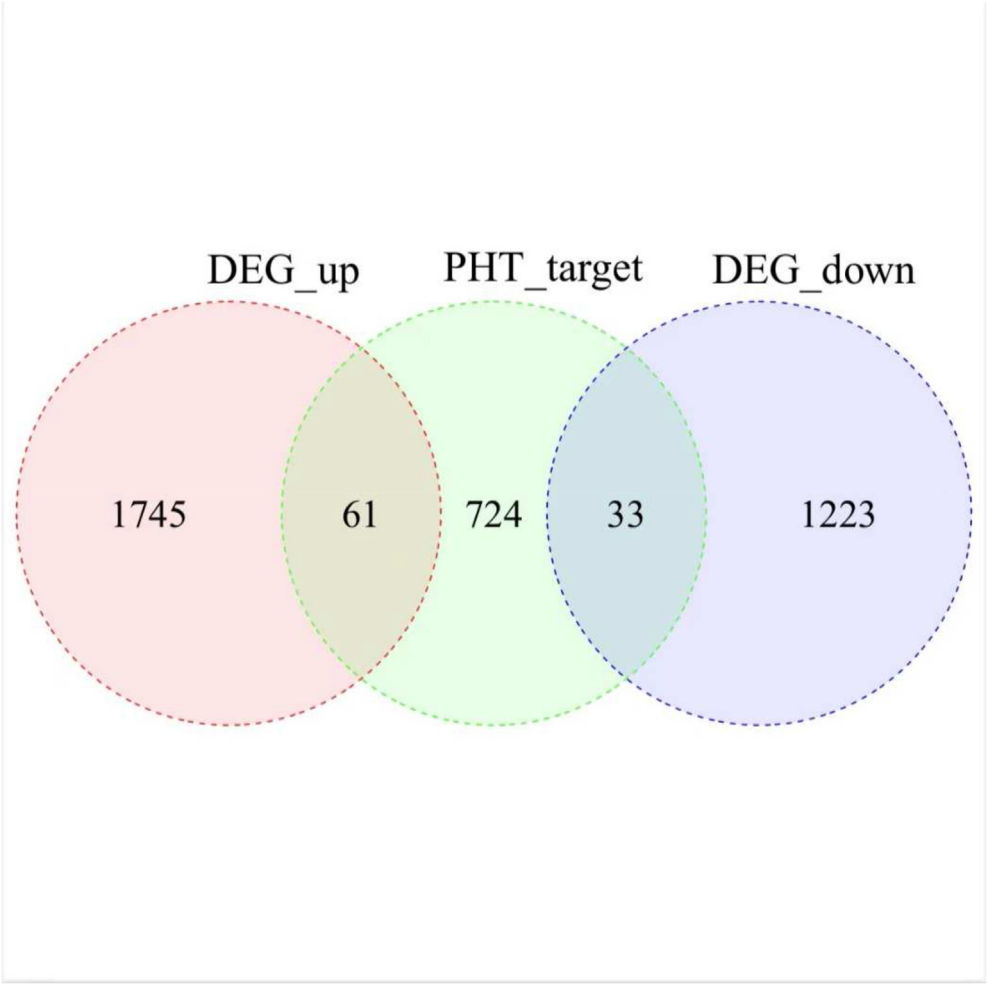

Fig. 6. Venn diagram showing the overlapping genes between DEGs and PHT target genes.

Activated PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP3) to phosphatidylinositol-3,4,5-triphosphate (PIP3), recruits and activates PDK-1 and Akt to further activate downstream effector protein. During this process, the negative regulator of PI3K, phosphatase and tensin homologue (Pten), limits signal strength by reversing PIP3 to PIP2. Akt is an important signaling molecule of PI3K, phosphorylating downstream targets, including glycogen synthase kinase 3β, mammalian target of rapamycin, endothelial nitrous oxide synthase and double A lipid of disalicylic acid [20]. Akt affects bone formation and osteoblast survival by maintaining

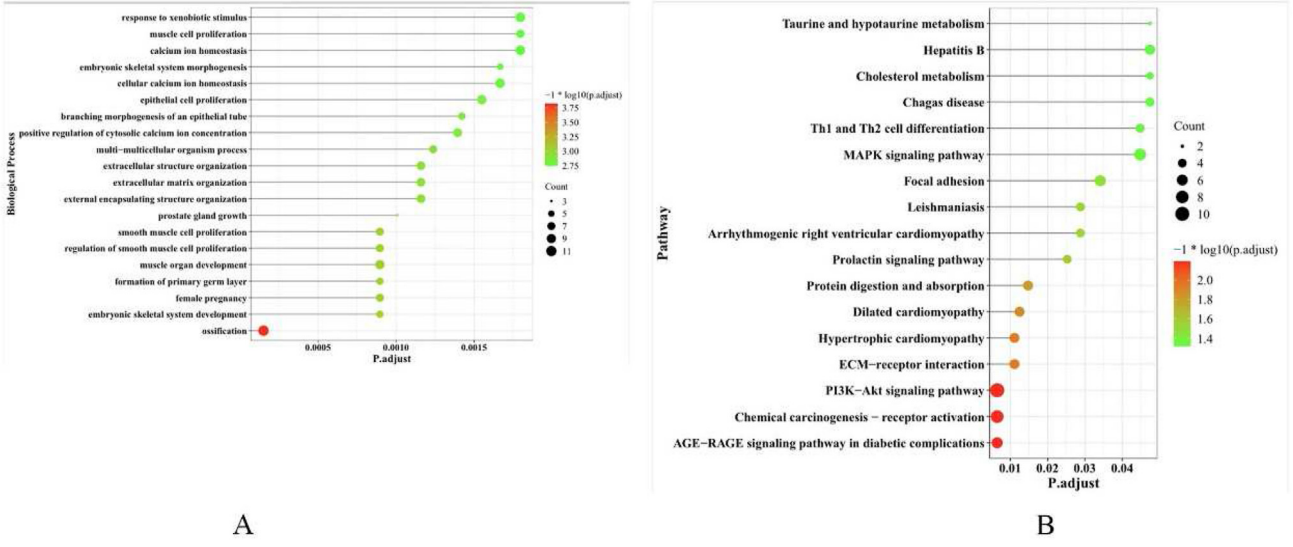

Fig. 7. PPI network.

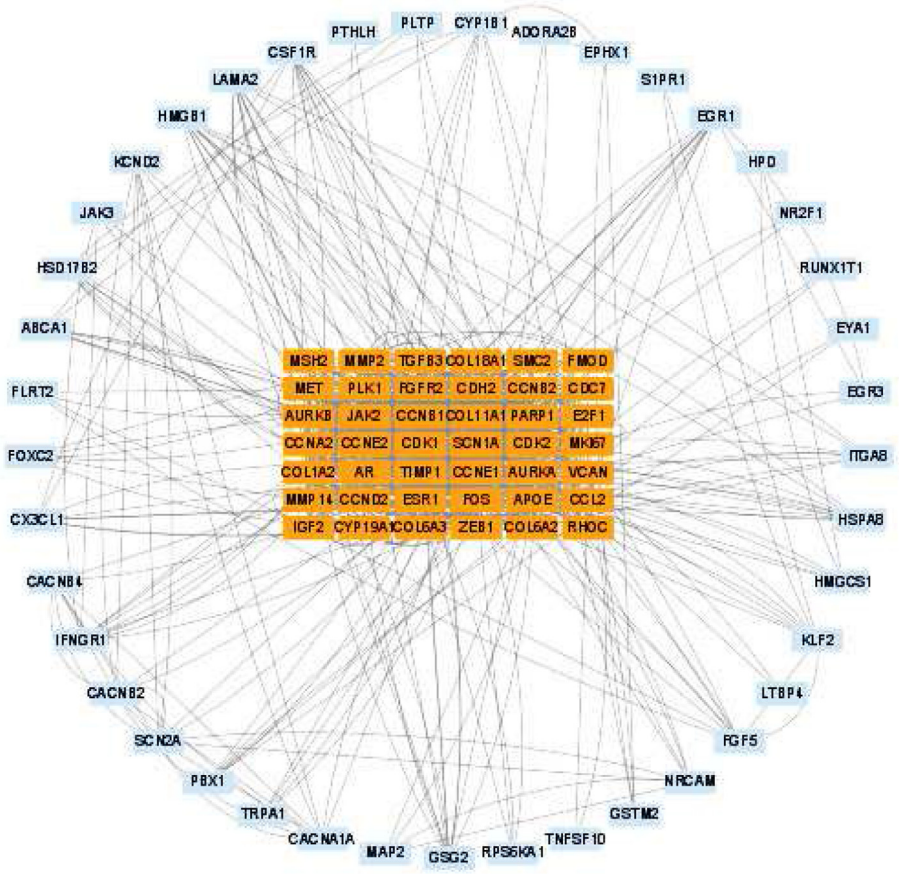

Fig. 8. A. The biological processes enriched by the upregulated PHT targets. B. The KEGG pathway enriched by the upregulated PHT targets.

Table 2
Results of molecular docking.

Protein	Ligand	affinity (kcal/mol)	center_x	center_y	center_z	size_x	size_y	size_z
COL11A1		−6.6	23.156	38.976	41.299	19	19	19
FGFR2		−7.4	53.834	13.169	15.203	19	19	19
PTHLH		−5.9	5.304	−5.365	−3.849	19	19	19
S1PR1	CID:1775	−8.1	4.371	13.952	−12.344	19	19	29
TGFB3		−7.3	18.59	74.281	52.101	19	19	19
MMP14		−6.8	35.104	21.952	9.337	19	19	19
IGF2		−6	3.895	−8.45	4.534	19	19	19
VCAN		−7.9	19.402	−7.644	104.657	19	19	19

forkhead box protein O (FOXOs) in the cytoplasm. Furthermore, Akt is closely related to bone morphogenic protein 2, which can regulate the differentiation of bone marrow mesenchymal stem cells into osteoblasts [21]. Studies have shown that Akt and its downstream targets in the PI3K signaling pathway are key points in regulating cartilage osteogenesis. Delayed osteogenesis and insufficient skeletal development were observed in Akt knockout mice.

The downstream of this signaling pathway is related to multiple osteogenic genes. High expression of Forkhead gene family members FOXO1, FOXO3 and FOXO4 was observed in chondrocytes and osteoblasts. RUNX2 regulates the expression of p85 and p110β subunits of the PI3K complex at multiple levels in the PI3K signaling pathway network.

As the downstream gene Osterix of Runx2, PI3K and BMP2 signaling pathway jointly up-regulate the expression of Osterix gene. FOXO1 has been proved to physically interact with ATF4 in osteoblasts to up-regulate protein synthesis under increased oxidative stress conditions [22]. Wang al et demonstrated that inhibition of p75NTR reduced alveolar bone mass and slowed bone development in mice. Up-regulation of P75NTR can promote the osteogenic differentiation of ectodermal mesenchymal stem cells by enhancing the expression of PI3K/Akt/β-catenin [23]. Skull formation is caused by endochondral ossification. Hallgrimsson et al. used the Pten $^{flox/flox}$ × col2a1 cre mouse model to study the impact of phenotypic variation on the shape of the skull, and found that the skull base of the mice in the treatment group was significantly shortened [24].

In addition to the above-mentioned proven signaling pathways, PHT may also affect osteogenesis in other ways. The healing process of bone injury is divided into intraperiosteal osteogenesis and

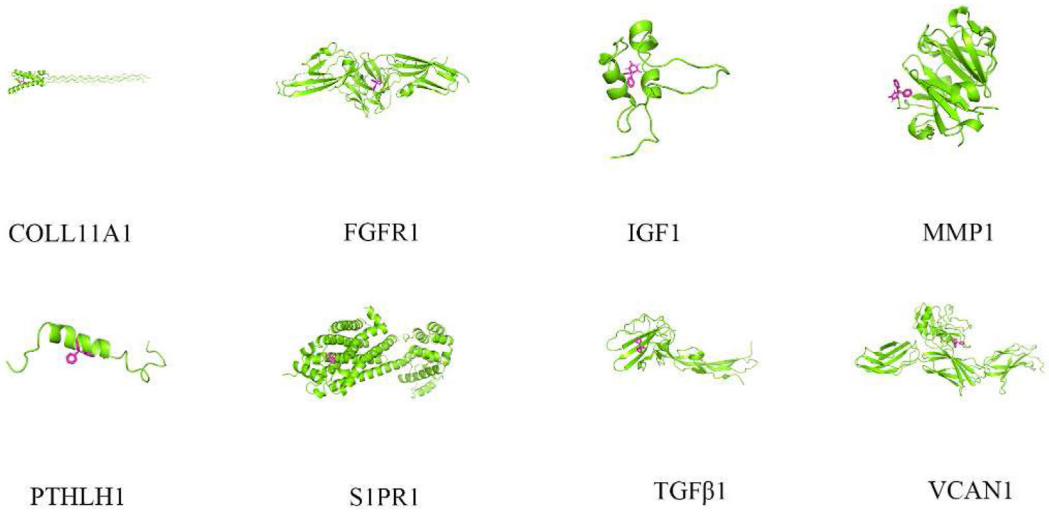

Fig. 9. The three-dimensional molecular docking diagram.

cartilage ossification. Mesenchymal stem cell differentiation at the site of injury and subsequent cartilage and bone formation are guided by a variety of microenvironmental signals. The overall process includes growth factors released from the bone matrix as well as changes in mechanical microenvironment and the oxygen tension, both pathways based on coordinated interactions between macrophages and osteoblasts [25,26].

In 1994, Epithelial sodium channel (ENaC) was first reported. It is a unique amiloride-sensitive voltage-independent sodium channel and belongs to the ENaC/DEG ion channel family. ENaC is a giant glycosylated molecule found in the apical membranes of epithelial tissues such as bladder, renal collecting duct, distal colon, sweat and salivary glands, lung, and taste buds, as well as in osteocytes such as osteoblasts and the middle of chondrocytes [27]. Daily sodium intake is essential for the human body, and abnormal sodium intake has also been proven to be closely related to bone metabolism diseases. The balance of sodium ions in the human body is regulated by ENaC. Chen et al. proved that 8-pCPTcGMP stimulates the proliferation, differentiation and expression of osteogenic genes of osteoblasts by regulating the expression of ENaC through the cGMP/PKGII signaling pathway [28]. Lu et al. suggested that changes in ENaC activity may be involved in dexamethasone-induced osteoblast differentiation and mineralization. However, the effect of dexamethasone on ENaC is not mediated through the glucocorticoid genome mechanism [29]. It has been confirmed that Na ion and ENaC affect bone formation. PHT has a stabilizing effect on excitable membranes of various tissues, can block Na + channel, reduce Na + influx, and thereby reduce its excitability. Whether PHT have a regulatory effect on ENaC remains to be further studied.

6. Conclusions

Small molecule drugs have many advantages as osteoinductive factors. PHT is a small molecule drug that has been used in clinical practice for many years. Previous studies have suggested that the mechanism of PHT inducing osteogenesis is related to the MAPK pathway, BMP4/samd pathway, the involvement of macrophages, and the regulation of Na ions. Here, we demonstrated that PI3K/Akt can also be activated by PHT to regulate osteogenesis. Further studies are needed to determine whether PHT has a regulatory effect on ENaC. Through experiments and literature review, we systematically describe the mechanism of PHT in inducing BMSCs to osteogenesis, hoping to provide some new ideas for bone defect repair in the future.

Ethics approval and consent to participate

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 [5]. Informed consent was obtained from all patients for being included in the study. The study was reviewed and approved by the Ethics Committee of Changzhi Medical College, with approval no: RT2022036.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Author contributions

Lisong Lin: Ideas, Supervision. **Zeliang Zhang:** Performing the experiments, and data/evidence collection, writing the initial draft. **Wei Shang**: Data analysis. **Xicong Zhao:** Performing the experiments.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2023.06.015.

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