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Dopamine Suppresses Osteoclast Differentiation via cAMP/PKA/CREB Pathway

Lufei Wang[1,#], **Lichi Han**[2,#], **Peng Xue**[1], **Xiangxiang Hu**[1], **Sing-Wai Wong**[3], **Meng Deng**[4], **Henry C. Tseng**[5], **Bo-Wen Huang**[6], **Ching-Chang Ko**[6,*]

[1]Division of Oral and Craniofacial Health Sciences, University of North Carolina Adams School of Dentistry, Chapel Hill, NC, United States

[2]Department of Oral Medicine, Medical College, Dalian University, Dalian, China

[3]Division of Comprehensive Oral Health, University of North Carolina Adams School of Dentistry, Chapel Hill, NC, United States

[4]Division of Craniofacial and Surgical Care, University of North Carolina Adams School of Dentistry, Chapel Hill, NC, United States

[5]Duke Eye Center and Department of Ophthalmology, Duke University Medical Center, Durham, NC, United States

[6]Division of Orthodontics, The Ohio State University College of Dentistry, Columbus, OH, United States

Abstract

How the nervous system regulates bone remodeling is an exciting area of emerging research in bone biology. Accumulating evidence suggest that neurotransmitter-mediated inputs from neurons may act directly on osteoclasts. Dopamine is a neurotransmitter that can be released by hypothalamic neurons to regulate bone metabolism through the hypothalamic-pituitary-gonadal axis. Dopamine is also present in sympathetic nerves that penetrate skeletal structures throughout the body. It has been shown that dopamine suppresses osteoclast differentiation via a D2-like receptors (D2R)-dependent manner, but the intracellular secondary signaling pathway has not

[*]Corresponding author: Ching-Chang Ko, DDS, MS, PhD, Professor and Chair, Division of Orthodontics, The Ohio State University College of Dentistry, 305 West 12th Avenue, Columbus, OH 43210, United States. ko.367@osu.edu.

[#]contributed equally to this work

Credit Author Statement

Author contributions

Lufei Wang: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Lichi Han**: Conceptualization, Data curation, Formal analysis, Investigation, Writing - review & editing. **Peng Xue**: Conceptualization, Supervision, Writing - review & editing. **Xiangxiang Hu**: Methodology, Writing - review & editing. **Sing-Wai Wong**: Resources, Writing - review & editing. **Meng Deng**: Resources, Writing - review & editing. **Henry C. Tseng**: Conceptualization, Supervision, Writing - review & editing. **Bo-Wen Huang**: Conceptualization, Supervision, Writing - review & editing. **Ching-Chang Ko**: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

Declaration of interest

The authors have no conflicts of interest to disclose.

been elucidated. In this study, we found that cAMP-response element binding protein (CREB) activity responds to dopamine treatment during osteoclastogenesis. Considering the critical role of CREB in osteoclastogenesis, we hypothesize that CREB may be a critical target in dopamine's regulation of osteoclast differentiation. We confirmed that D2R is also present in RAW cells and activated by dopamine. Binding of dopamine to D2R inhibits the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling pathway which ultimately decreases CREB phosphorylation during osteoclastogenesis. This was also associated with diminished expression of osteoclast markers that are downstream of CREB. Pharmacological activation of adenylate cyclase (to increase cAMP production) and PKA reverses the effect of dopamine on CREB activity and osteoclastogenesis. Therefore, we have identified D2R/cAMP/PKA/CREB as a candidate pathway that mediates dopamine's inhibition of osteoclast differentiation. These findings will contribute to our understanding of how the nervous and skeletal systems interact to regulate bone remodeling. This will enable future work toward elucidating the role of the nervous system in bone development, repair, aging, and degenerative disease.

Keywords

dopamine; dopamine receptor; osteoclasts; cAMP-response element binding protein (CREB); cyclic adenosine monophosphate (cAMP); protein kinase A (PKA)

1. Introduction

Bone remodeling is a lifelong dynamic process in which old bone is resorbed by osteoclasts (OC) and subsequently replaced with new bone synthesized by osteoblasts [1]. An imbalance between bone resorption and formation often leads to metabolic bone diseases. For example, pathological bone loss due to increased resorption is associated with osteopenia, osteoporosis, or Paget's disease of bone [2]. How this delicate balance is regulated is not fully understood, but is likely orchestrated by intracellular molecular pathways in bone cells in response to hormones, growth factors, or mechanical stimuli [3, 4]. How the nervous system regulates bone remodeling is an exciting area of emerging research in bone biology, supported by accumulating evidence that inputs from the central and peripheral nervous system may regulate bone remodeling [5, 6]. A number of neurotransmitters and their receptors have also been demonstrated to regulate the activity of bone cells [7–9].

Dopamine (DA) is a member of the catecholamine family of neurotransmitters found in diverse neuronal subtypes in the central nervous system [10]. DA has two categories of receptors: D1-like receptors (D1R) family (Drd1 and Drd5) and D2-like receptors (D2R) family (Drd2, Drd3, Drd4) that differentially regulate intracellular level of second messenger cyclic adenosine monophosphate (cAMP) [11]. DA can be released by hypothalamus neurons and regulates bone metabolism through the hypothalamic-pituitary-gonadal axis [12]. Accumulating clinical evidence have suggested the close relationship of DA with bone health and diseases. For example, the high risk of osteoporosis observed in Parkinson's disease patients as well as the increased fracture risk associated with antipsychotic medications [13–15]. In these examples, low DA level from dopaminergic degeneration

or pharmacological DA receptor blockade leads to excessive pituitary prolactin secretion and hence a reduction of gonadal steroid hormones, which adversely impacts bone metabolism.

In the peripheral nervous system, DA is stored in sympathetic nerves and can directly act on bone cells to regulate bone metabolism. We previously discovered that DA enhances osteoblast differentiation and mineralization *in vitro* [16, 17]. which can enhance bone formation. For bone resorption, Hanami *et al* discovered that DA or D2R agonist but not D1R agonist inhibits OC differentiation *in vitro*, suggesting a D2R-signal-dependent regulatory effect on osteoclastogenesis [18]. The D2R-signal-dependent anti-osteoclastogenic effect has also been demonstrated in a peri-implant osteolysis model [19].

However, the intracellular pathway by which D2R signal regulates the expression of OC-specific genes has not been fully elucidated. In addition, effect of D1R blockade on *in vitro* OC differentiation has also been reported [20], which further demonstrates that the mechanism by which dopaminergic signal regulates osteoclastogenesis is very complex and remains to be elucidated. In the present study, we found that cAMP-response element binding protein (CREB) activity responds to DA treatment in osteoclastogenesis. Considering the critical role of CREB in osteoclastogenesis--CREB alone or in cooperation with other transcription factors may transactivate many OC-specific genes [21], we hypothesize that CREB may be a critical target in DA's regulation of OC differentiation. By testing the above hypothesis, we have identified an intracellular signaling pathway (D2R/cAMP/PKA/CREB) as a molecular mechanism by which DA mediates OC differentiation.

2. Materials and methods

2.1. Cell culture and OC differentiation

RAW 264.7 (ATCC® TIB-71) cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS and penicillin/streptomycin at 37°C, 5% CO_2. RAW cells were seeded at a density of 4×10^4 cells/cm^2 and received receptor activator of nuclear factor kappa-B ligand (RANKL) (10 ng/mL, R&D systems) stimulation for 3d ~ 4d to become mature OC, as indicated in specific experiments. Then the cells were fixed with 4% paraformaldehyde, permeabilized with Triton X-100, and stained with tartrate-resistant acid phosphatase (TRAP) staining solution [22]. Images were captured by Nikon Eclipse Ti-U inverted microscope (Nikon) and TRAP positive, multinucleated (≥3 nuclei) cells were counted as OC. For bone resorption pit visualization, RAW cells were seeded on bone slices (BioVendor). After 3~5 days RANKL treatment, cells were removed from bone slices by sonicating 10 minutes and then the bone slices were stained with 1% toluidine blue for 4 minutes [23]. Images were captured by Nikon SMZ18 stereo microscope (Nikon).

Primary OC differentiation were performed as previously described [22]. Femurs were harvested from 8-week-old C57BL/6J male mice and bone marrow cells were flushed out into α-MEM supplemented with 10% FBS and penicillin/streptomycin. Non-adherent cells were removed overnight and re-plated at a density of 1.5×10^5 cells/cm^2 with 30 ng/mL M-CSF (R&D Systems) for growing OC precursor cells. After 3 days, OC precursor cells were treated with 30ng/mL M-CSF and 10 ng/mL RANKL (R&D Systems) for 4 days to differentiate to OC. All animal procedures were approved by Institutional Animal Care

and Use Committee at the University of North Carolina at Chapel Hill and the Ohio State University, and were conducted in accordance with the NIH guidelines.

2.2. Chemicals

The concentrations of DA hydrochloride (Acros Organics) were indicated in specific experiments. D1R agonist SKF38393 (Sigma-Aldrich) and D2R agonist quinpirole (Sigma-Aldrich) was used at a concentration of 100 nM and 100 nM, respectively. D1R antagonist SCH23390 (10nM, Sigma-Aldrich), D2R antagonist haloperidol (10nM, Sigma-Aldrich), protein kinase A (PKA) inhibitor H89 (10 μM, Sigma-Aldrich), extracellular signal-related kinase 1/2 (ERK1/2) inhibitor SCH772984 (300 nM, Selleckchem), Akt 1/2/3 inhibitor MK-2206 (5 μM, Selleckchem), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitor KN-93 (10 μM, Selleckchem) were applied on the cells for 30 minutes before the DA treatment. Adenylate cyclase (AC) agonist Forskolin (10 μM, Sigma-Aldrich) and cAMP analog 8-Br-cAMP (50 μM, Selleckchem) that activates PKA was used together with DA treatment.

2.3. Cell viability assay

Cell viability in response to various concentrations of DA was detected by the RealTime-Glo™ MT cell viability assay (Promega) according to the manufacturer protocol. The luminescence (relative light unit, RLU) was read by the Cytation 5 imaging reader (Bio-Tek).

2.4. RT-qPCR

Cells were harvested by RNAzol (Molecular Research Center) for total mRNA extraction and cDNA reverse-transcription was performed by using iScript™ cDNA Synthesis Kit (Bio-Rad). RT-qPCR was performed on StepOnePlus Real-time PCR system (Applied Biosystems) using the iTaq™ Universal SYBR Green Supermix reagent (Bio-Rad). The primers are listed in Supplementary Table 1. The relative expression of target genes was normalized to housekeeping gene β-2-microglobulin (*B2m*) based on the comparative CT (ΔΔCT) method.

2.5. Western blot

Cells were lysed by RIP A buffer plus phosphatase & protease inhibitor cocktail. The total protein was separated by using XCell SureLock Mini-Cell (Invitrogen) sodium dodecyl sulfate-polyacrylamide gel electrophoresis system and transferred onto a nitrocellulose membrane by using Trans-Blot Cell system (Bio-Rad). Target protein was immunodetected using primary antibodies phospho-CREB (Serl33) (Cell Signaling, #9198), CREB (Cell Signaling, #9197), PKA-Cα (Cell Signaling, #4782), β-Actin (Santa Cruz, #sc-47778), and corresponding peroxidase-conjugated secondary antibodies (Merck Millipore). Protein was visualized by enhanced chemiluminescence solution (Thermo Fisher). Band images were captured by ImageQuant LAS 4000 camera system (GE Healthcare) and quantified by NIH ImageJ software.

2.6. CREB luciferase reporter assay

CREB transcriptional activity was determined by measuring the luciferase reporter activity in cells transfected with p-CREB-Luc reporter vector (Signosis, #LR-2008), which contains CREB cis-acting enhancer element upstream of a minimal TA promoter. 1×10^5 cells seeded in 24-well plate were transfected with 5μg plasmid using Lipofectamine 2000 (Invitrogen), according to the manufacturer protocol. 24 hours post transfection, the cells were washed off before being treated with indicated agents, and lysed for luciferase activity detection using Nano-Glo luciferase assay system (Promega). The luminescence (RLU) was read by the Cytation 5 imaging reader (Bio-Tek).

2.7. cAMP immunoassay detection

Intracellular cAMP level was detected by a fluorometric competitive ELISA kit (Abeam, #abl38880) according to manufacturer's instructions. RAW cells were treated with 100 μM DA in the presence of 10 ng/mL RANKL and lysed at indicated time points. HRP-labeled cAMP and free cAMP within the cell lysates were loaded into anti-cAMP antibody-coated plates. The activity of HRP-cAMP conjugate was measured by the Cytation 5 imaging reader (Bio-Tek). Free cAMP concentrations in the samples were calculated using a calibration curve.

2.8. Statistical analyses

Experiments were performed in triplicate and repeated three times. Data was presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). The comparisons among multiple groups were assessed using one-way ANOVA followed by Bonferroni post-hoc test. $P < 0.05$ was set for significance.

3. Results

3.1. DA suppresses OC differentiation and CREB activity, which is dose-dependent

It has been reported that DA inhibits *in vitro* OC differentiation from human monocyte or murine bone marrow macrophages [18, 19]. Here we demonstrated that DA also suppresses OC differentiation from RAW 264.7 cells in concentration-dependent manner. RAW cells represent an OC precursor cell line that can be chemically induced by RANKL exposure to differentiate into mature OCs that can functionally resorb bone. The bone resorbing capacity of the RAW-derived OCs in our system was verified by the formation of resorption pit (Supplementary Fig. 1). In the following experiments, RAW cells were treated with various concentration of DA in the presence of RANKL for 4 days. Differentiation of RAW cells into OC was inhibited by DA treatment, and 100 μM DA showed the strongest inhibitory effects (Fig. 1A). TRAP staining showed decreasing number of OC with increasing DA concentration (Fig 1A, right panel). Expression of osteoclastic markers (*c-Fos, Nfatc1, Trap, Ctsk*) also generally exhibited an inverse relationship with DA concentration, demonstrating DA-mediated inhibition of osteoclastogenesis (Fig. 1B). We also investigated whether DA might be toxic to RAW cells through the MT cell viability assay. Within the concentrations used for treatment (10μM~100μM), DA did not show significant toxicity on RAW cells after

24, 72, and 96 hours of incubation (Supplementary Fig. 2). Therefore, the decrease in OC formation is not due to non-specific DA toxicity, but represents a direct inhibitory effect on the differentiation process.

We next investigated how DA modulates RAW differentiation into OCs by examining intracellular signaling pathways associated with both DA receptor activation and OC differentiation. At different stages of osteoclastogenesis (24h, 48h after RANKL treatment), a concentration-dependent inhibition of CREB phosphorylation by DA was observed (Fig. 2A and B). Consistently, DA also showed a concentration-dependent inhibition on the expression of osteoclastic genes (Fig. 2C). 100μM of DA treatment showed the strongest inhibitory effect. CREB actually plays an important role in the transactivation of osteoclastic genes during differentiation [21]. At the same time, CREB is a downstream target of dopaminergic signaling pathways [24]. Taken together, our data demonstrated that CREB activity is involved in the regulatory effect of DA on osteoclastogenesis, suggesting that CREB may be a critical node for connecting dopaminergic signal to OC differentiation.

3.2. D2R but not D1R signaling regulates CREB activity in osteoclastogenesis

DA receptors are seven-transmembrane G protein-coupled receptors that couple to different G proteins [25]. Upon binding to DA, Gαs-coupled D1R family activates AC and increases cAMP production. In contrast, Gαi/o-coupled D2R family suppresses AC activity and reduces cAMP production. It has been demonstrated that DA suppresses osteoclastogenesis via D2R signaling, and D2R agonist significantly inhibits OC differentiation from monocytes or macrophages [18, 19]. However, which intracellular signaling pathway and transcription factor is activated remains to be elucidated.

To determine the dopamine receptor subtype and signaling pathway involved, we first checked basal gene expression of DA receptors in RAW cells and RAW-derived OC by RT-qPCR. While all five subtypes of DA receptors were expressed, there were no differences in expression levels (Supplementary Fig. 3). We next investigated whether DA inhibited p-CREB through D1R or D2R. Subtype-specific agonists (SKF38393 for D1R and quinpirole for D2R) and antagonists (SCH23390 for D1R and haloperidol for D2R) were used. We found that D2R agonist reduced the p-CREB during osteoclastogenesis, which was similar to DA's effect. In contrast D1R agonist did not have any effect on the level of p-CREB (Fig. 3A and B). Consistently, DA's inhibitory effect on p-CREB was abolished when D2R was blocked with the D2R-specific antagonist, but the effect was not observed with D1R antagonist (Fig. 3A and B). These findings showed that DA inhibited p-CREB during osteoclastogenesis specifically through D2R.

We next checked their effects on CREB-mediated transcriptional activity by examining the expression of *c-Fos* and *Stat3*. These genes contain a cAMP response element (CRE) region in the promoter region so they can be transactivated by CREB [24]. Furthermore, we also assayed CREB transcriptional activity through a luciferase reporter assay containing CRE in the promoter. As anticipated, D2R agonist decreased the expression of *c-Fos* and *Stat3*, as well as p-CREB luciferase activity during osteoclastogenesis, comparable to DA's effects (Fig. 3C and D). Further, blocking D2R signaling successfully abolished DA's inhibitory effects while blocking D1R generally had no effect (Fig. 3C and D). We also

repeated these experiments utilizing primary mouse bone marrow mononuclear cells and observed similar effects on OC differentiation (Supplementary Fig. 4). Taken together, these results demonstrated that D2R mediated DA's ability to modulate CREB activity during osteoclastogenesis.

3.3. DA mediates CREB activity through cAMP/PKA pathway during osteoclastogenesis

A variety of protein kinases are capable of phosphorylating CREB in its kinase-inducible domain, which enables CREB a convergent target for multiple signaling cascades. Among DA-mediated signaling network, in addition to the canonical cAMP/PKA pathway, activation of Akt [26], PLCβ/Ca^{2+}/CaMKII [27], or cAMP/Epac/ERK pathway [28] have also been reported to activate CREB. Thus, we next investigated which of these signaling pathways might mediates anti-osteoclastogenic activity of DA. We combined DA with PKA inhibitor H89, or with the kinase inhibitor for other pathways, to determine their impact on CREB activity. DA combined with PKA inhibitor aggravated inhibition of both p-CREB level and CRE-luciferase activity during osteoclastogenesis (Fig. 4A–C). In contrast, DA combined with ERK, Akt, or CaMKII inhibitors showed little effect. We next checked the levels of cAMP, PKA, and p-CREB in response to DA during osteoclastogenesis. At various time points (10min, 30min, and 60min), level of cAMP, PKA, and p-CREB were coincidentally decreased in response to DA (Fig. 4D–F). Taken together, our data suggest that the cAMP/PKA signaling pathway mediates DA's inhibition of CREB activity during osteoclastogenesis.

3.4. Activation of cAMP/PKA/CREB pathway reverses DA-mediated inhibition of osteoclastogenesis

Given the critical role of cAMP/PKA/CREB pathway in the anti-osteoclastogenic effect of DA, we next investigated whether increasing cAMP/PKA/CREB activity reverses DA's inhibition of OC differentiation. To increase cAMP levels, we will utilize forskolin which is a known AC activator that increases cAMP production. Similarly, we will use 8-Br-cAMP, a cAMP analog, to activate PKA. When RAW cells were treated with forskolin or 8-Br-cAMP, a significantly increased phosphorylation of CREB and CREB transcriptional activity was observed, when compared to DA treatment (Fig. 5A–C). Further, DA-impaired OC differentiation was significantly improved by Forskolin or 8-Br-cAMP treatment by TRAP staining and quantitation of the number of mature OCs (Fig. 5D). Expression of most of the osteoclastic genes (*c-Fos, Nfatc1, Trap, Ctsk*) was also enhanced by Forskolin or 8-Br-cAMP as compared to DA inhibition (Fig. 5E). DA's inhibition of *c-Fos* expression was completely reversed by Forskolin or 8-Br-cAMP to normal control levels. Similar findings were also demonstrated using primary mouse OC precursor cells (Supplementary Fig. 5). Collectively, activating cAMP/PKA/CREB pathway reverses DA's inhibition of osteoclastogenesis, further demonstrating that DA regulates OC differentiation via the D2R/cAMP/PKA/CREB pathway.

4. Discussion

In our present study, we have identified a specific signaling pathway that mediates DA's inhibition of osteoclastogenesis (summarized in Fig. 6). We showed that D2R is the DA

receptor subtype that is activated by DA in RAW cells, and not D1R. Downstream from D2R, cAMP modulates PKA activity which ultimately act upon the transcriptional factor CREB. Phosphorylation of CREB is associated with the expression of downstream genes, specifically OC markers, that are transcriptionally controlled by CREB. Pharmacological activation of cAMP and PKA reverses the effect of DA on osteoclastogenesis. Therefore, we show for the first time that DA triggers the D2R/cAMP/PKA/CREB pathway to inhibit OC differentiation.

Peripheral DA is present throughout the body in sympathetic nerves and plays a critical role in the regulation of hormone secretion, blood pressure, immune response, circulation, as well as lung, kidney and gastrointestinal functions [29]. Bone tissues are highly innervated by sympathetic nerve fibers, where high level of DA is stored [30]. The presence of endogenous DA in bone marrow ($10^{-9} \sim 10^{-5}$ M) has also been reported [31, 32]. Moreover, mRNA and protein expression of five DA receptor subtypes in human or mouse OC precursors have been verified by recent studies, which is even higher in mature OCs [14, 18]. These evidences form the physiological foundation for peripheral DA to directly act on OC precursors and hence influences OC formation. In our experiments, the concentration of DA required to show effective inhibition of RAW cells-derived OC differentiation is higher than reported in mouse primary OCs [33]. This difference in DA sensitivity might be due to the relatively lower DA receptors expression in RAW cells (Supplementary Fig. 3). Despite this differential DA sensitivity, key results of our RAW cell experiments were confirmed using mouse primary OC precursor cells (Supplementary Fig. 4 and 5).

Previous reports suggested that D1R-signaling appears to have little impact on OC differentiation [18, 19], which is consistent with our finding that D1R agonist SKF38393 did not affect CREB activity or *c-Fos* expression (Fig. 3). However, D1R seems to have diverse effects on osteoclastogenesis under different conditions. D1R antagonist is reported to shows therapeutic potential on collagen-induced arthritis partly due to its anti-osteoclastogenic effect [20]. In contrast, selective Drd1 agonist A77636 inhibits osteoclastogenesis by downregulating NFATc1 through increased phosphorylation of eIF2α, and prevents osteolytic lesions in a bone model of tumor metastasis [34]. The diverse responses of OC differentiation to pharmacological D1R manipulation suggest that OC dopaminergic signaling is quite complex and requires further elucidation.

Recent reports have demonstrated D2R-dependent suppression of osteoclastogenesis [18, 19], but how D2R stimulation lead to downstream effectors that trigger osteoclastic genes expression has been unclear. A potential downstream signaling molecule is cAMP, which was described in a study by Hanami *et al* [18]. Our study expanded this study by identifying a complete pathway, D2R/cAMP/PKA/CREB axis, which acts as a molecular mechanism by which DA mediates osteoclastogenesis. Our data is also consistent with recent reports that pramipexole and quinpirole can also effectively inhibits OC differentiation from monocytes or macrophages [18, 19].

cAMP/PKA is thought to negatively regulate osteoclastogenesis by phosphorylating and deactivating Nfatc1, with crosstalk with Wnt signal or Ca^{2+}/CaMK [35, 36]. Moreover, Ramaswamy *et al* indicated that paternal allele deletion of *Gnas* (*Gnas*$^{+/p-}$)

showed decreased cAMP/PKA activity, decreased p-CREB, elevated Nfatc1, and enhanced osteoclastogenesis, which can all be reversed by pharmacological treatment that activates AC [37]. This is contrast to our finding that cAMP/PKA/CREB activity, Nfatc1, and osteoclastogenesis were uniformly decreased when responding to DA stimulation (Fig. 4 and 5). In view of the pro-osteoclastogenic effect of CREB--transactivating osteoclastogenic transcription factor *c-Fos* [38], the anti- or pro-osteoclastogenic findings for cAMP/PKA appears paradoxical and is in need of further investigation. Our data is consistent with Hanami *et al* study [18] which reported a reduction in cAMP level, along with decreased *c-Fos* and *Nfatc1* gene expression as well as decreased OC formation when D2R was activated. Moreover, as Fig. 5 showed, pharmacological activation of cAMP/PKA using Forskolin or 8-Br-cAMP exhibited pro-osteoclastogenic effects, implying that cAMP/PKA might mediate osteoclastogenesis preferentially by activating CREB rather than by deactivating Nfatc1, when responding to DA signal.

Clinical evidence regarding skeletal effects of DA, such as in Parkinson's disease patients or side effects of antipsychotic medications, mainly focus on hypothalamic-pituitary-gonadal axis as a potential mechanism [13, 15]. Our present work yields novel insight into how bone might be directly regulated by the nervous system, which in turn has possible clinical applications. First, dysregulated OC formation or activity can cause abnormal bone homeostasis. Excessive OC formation and activity are associated with osteoporosis, peri-implant osteolysis, and Paget's disease of bone. Our finding that D2R can inhibit osteoclastogenesis highlight the potential of using D2R agonists as novel drug therapies for these metabolic bone diseases. Secondly, the same D2R-CREB signaling pathway has been delineated for the regulation of synaptic function in neurons and suggest that cells in the nervous and skeletal systems utilize similar pathways to achieve different biological functions. This helps explain how abnormal bone loss might result as side-effects from psychiatric or neurological medications that modulate DA in the brain. Thirdly, because various resident cell types of the skeletal system (OCs, osteoblasts, chondrocytes, etc.) express receptors for neurotransmitters [39], our findings also support a hypothesis that the skeletal system is under tight regulation from the nervous system during growth, repair, remodeling, and aging. As such, this hypothesis predicts the existence of possible chemical mediators from bone, such as calcium released from bone matrix, that conversely act on neurons as part of a feedback system. Lastly, because DA and CREB also play critical roles in neuronal function, our data may help delineate a possible role for bone in maintaining normal function in the nervous system. Therefore, in addition to treating bone diseases, targeting bone dysfunction might constitute a novel approach to treating neurodegenerative diseases in the brain and eye.

5. Conclusion

In our study, we have identified D2R/cAMP/PKA/CREB as a candidate pathway that mediates DA's regulation of OC differentiation. These findings will promote our understanding of how the nervous and skeletal systems interact to regulate bone remodeling by characterizing the effect of the neurotransmitter DA on OCs. Finally, our findings support a clinical role of using DA receptors agonists/antagonists in the treatment of bone metabolic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Definition of Abbreviation

CREB	cAMP-response element binding protein
cAMP	cyclic adenosine monophosphate
PKA	protein kinase A
OC	osteoclasts
DA	Dopamine
D1R	D1-like receptors
D2R	D2-like receptors
RANKL	receptor activator of nuclear factor kappa-B ligand
TRAP	tartrate-resistant acid phosphatase
ERK1/2	extracellular signal-related kinase 1/2
CaMKII	Ca2+/calmodulin-dependent protein kinase II
AC	Adenylate cyclase
RLU	relative light unit
CRE	cAMP response element

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HIGHLIGHTS

- We identified a candidate pathway (D2R/cAMP/PKA/CREB) that mediates dopamine's regulation of osteoclast differentiation.
- Pharmacological activation of cAMP/PKA/CREB pathway reverses dopamine's inhibition of osteoclastogenesis.
- Dopamine and CREB may be promising mediators of neuronal regulation of bone remodeling.

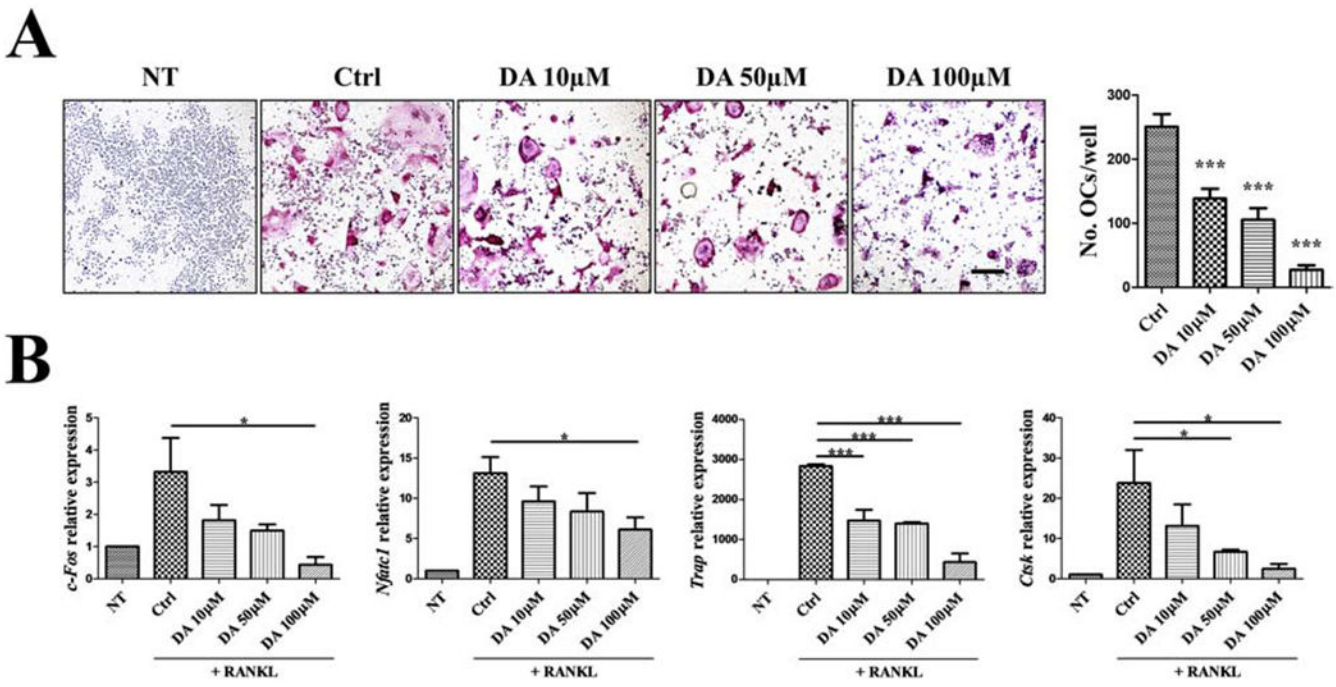

Fig. 1. DA inhibits OC differentiation from RAW cells.

RAW cells were treated with indicated concentration of DA in the presence of 10 ng/mL RANKL for 4 days. (A) TRAP staining and quantification of OC. TRAP positive, multinucleated OC in red were counted. n=4. Scale bar is 200μm. (B) Relative expression of osteoclastic genes (*c-Fos, Nfatc1, Trap, Ctsk*) on day 4 after DA+RANKL treatment were determined by RT-qPCR; normalized to *B2m*. n=3. *P < 0.05, **P < 0.01, ***P < 0.001 vs. Ctrl group. Data shown as mean ± SEM.

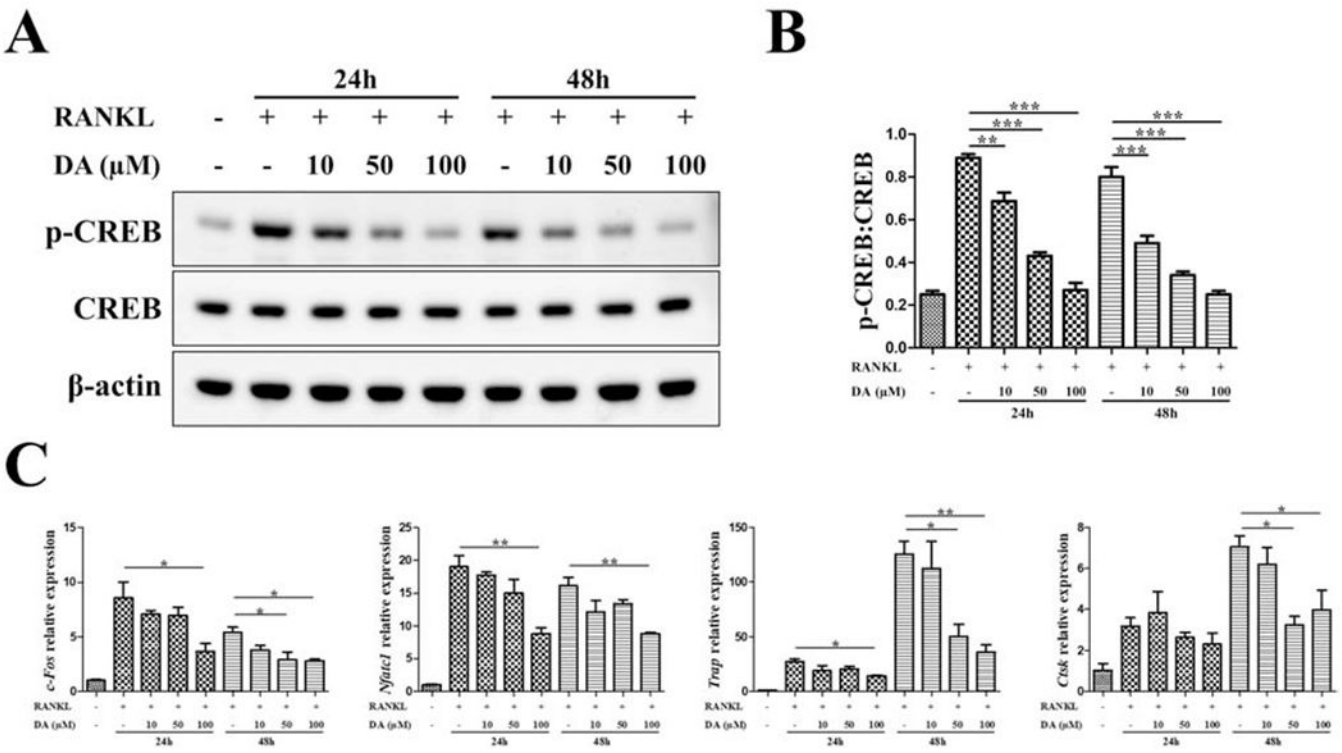

Fig. 2. CREB activity is inhibited by DA in osteoclastogenesis.

RAW cells were treated with indicated concentration of DA in the presence of 10 ng/mL RANKL. At different time points of osteoclastogenesis (24h, 48h), p-CREB and CREB level was detected by western blot (A) and quantitated as a ratio of p-CREB/CREB (B). (C) Relative expression of osteoclastic genes (*c-Fos, Nfatc1, Trap, Ctsk*) were determined by RT-qPCR; normalized to *B2m*. n=3 for all experiments; *P < 0.05, **P < 0.01, ***P < 0.001. Data shown as mean ± SEM.

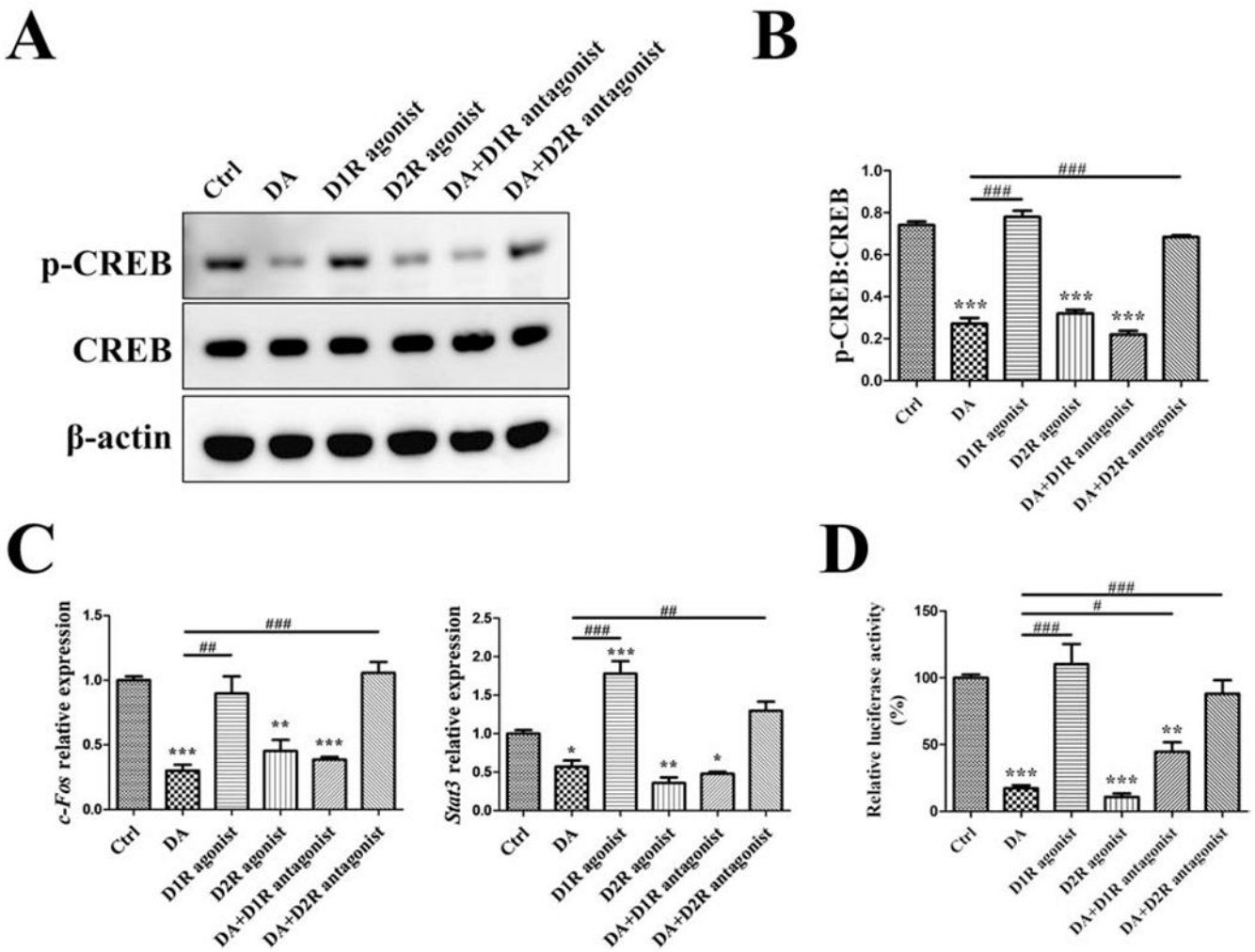

Fig. 3. D2R-dependent inhibition on CREB activity in osteoclastogenesis.

RAW cells were treated with vehicle (Ctrl), 100 μM DA, 100 nM D1R agonist SKF38393, 100 nM D2R agonist quinpirole, 100 μM DA plus 10nM D1R antagonist SCH23390 (after 30min SCH23390 pretreatment), or 100 μM DA plus l0nM D2R antagonist haloperidol (after 30min haloperidol pretreatment), in the presence of 10 ng/mL RANKL for 1 day. (A, B) Western blot detection of p-CREB/CREB. (C) RT-qPCR detection of *c-Fos* and *Stat3* genes; normalized to *B2m*. (D) For p-CREB luciferase activity assay, p-CREB-Luc reporter-transfected RAW cells were used to receive the treatment same as above. n=3 for all experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs. Ctrl group; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 vs. DA group. Data shown as mean ± SEM.

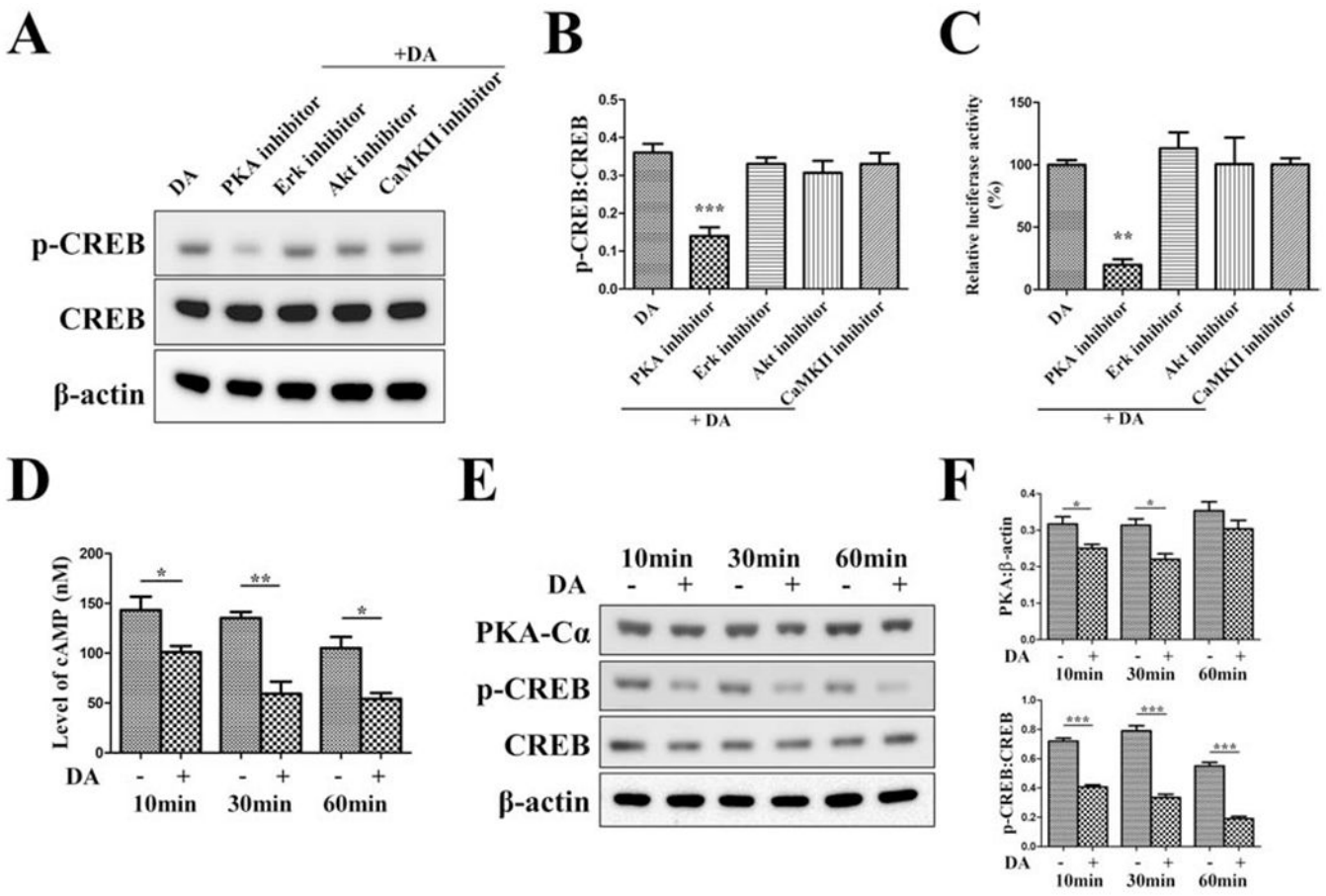

Fig. 4. DA mediates CREB activity through cAMP/PKA pathway during osteoclastogenesis.

For experiments in (A-C), RAW cells were pretreated with PKA inhibitor H89 (10 μM), ERK1/2 inhibitor SCH772984 (300 nM), Akt1/2/3 inhibitor MK-2206 (5 μM), or CaMKII inhibitor KN-93 (10 μM) for 30min and then stimulated with 100 μM DA plus indicated inhibitor in the presence of 10 ng/mL RANKL for 1 day. (A, B) Western blot detection of p-CREB/CREB. (C) For p-CREB luciferase activity assay, p-CREB-Luc reporter-transfected RAW cells were used to receive the treatment same as above. n=3 for all experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs. DA group. For experiments in (D-F), RAW cells were treated with 100 μM DA in the presence of 10 ng/mL RANKL and lysed at indicated time points. (D) intracellular cAMP concentrations were detected by a fluorometric competitive ELISA method. (E, F) Western blot detection of PKA and p-CREB/CREB. n=3 for all experiments. *P < 0.05, **P < 0.01, ***P < 0.001. Data shown as mean ± SEM.

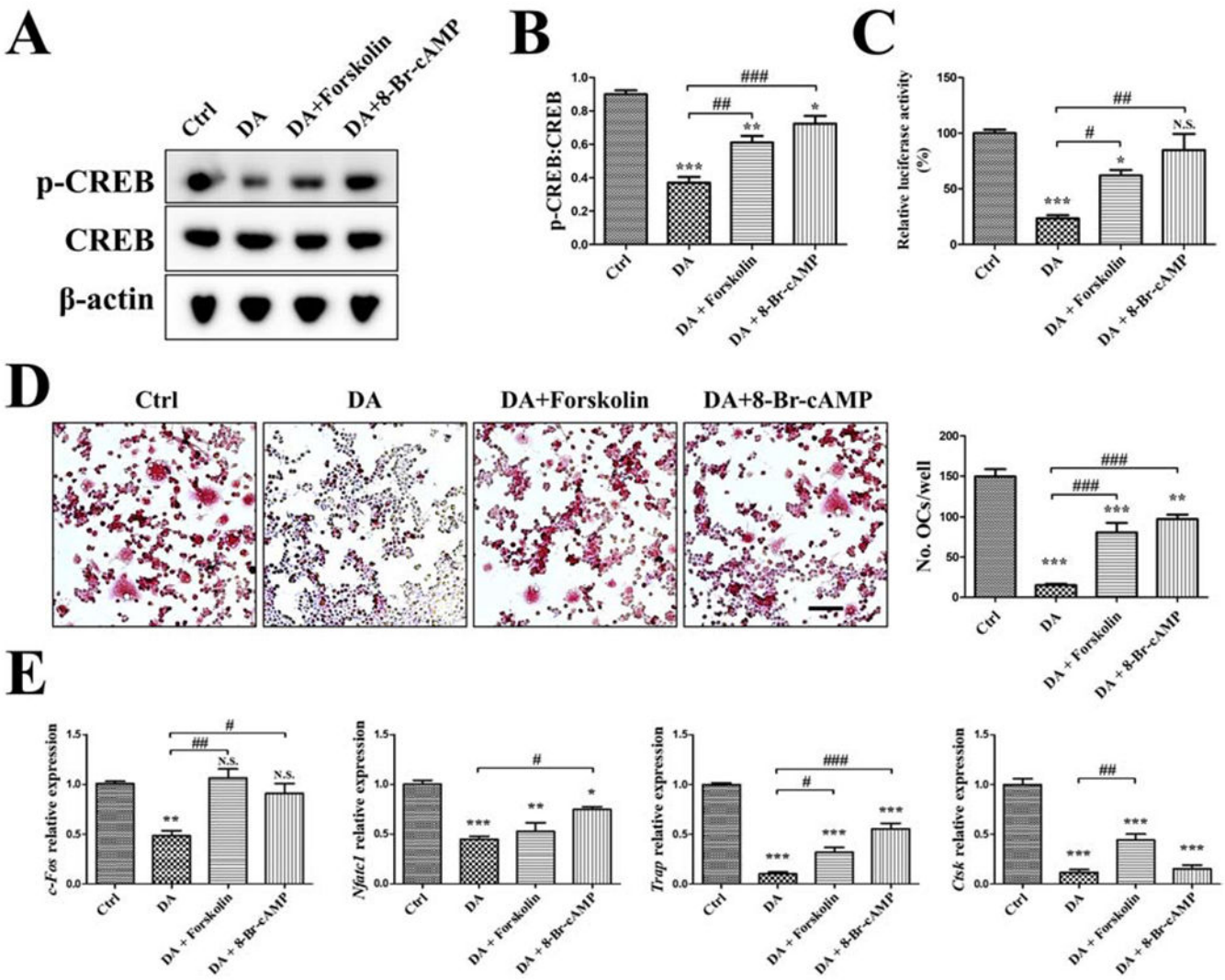

Fig. 5. Activation of AC/PKA partly abolishes DA's anti-osteoclastogenic effect.

RAW cells were treated with vehicle (Ctrl), 100 μM DA, 10 μM AC agonist Forskolin, or 50 μM PKA agonist 8-Br-cAMP as indicated, in the presence of 10 ng/mL RANKL for 3 days. (A, B) Western blot detection of p-CREB/CREB. (C) For p-CREB luciferase activity assay, p-CREB-Luc reporter-transfected RAW cells were used to receive the treatment same as above. (D) TRAP staining and quantification of OCs. TRAP positive, multinucleated OC in red were counted. Scale bar is 200μm. (E) RT-qPCR detection of osteoclastic genes (*c-Fos, Nfatc1, Trap, Ctsk);* normalized to *B2m*. n=3 for all experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs. Ctrl group; *P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 vs. DA group. Data shown as mean ± SEM.

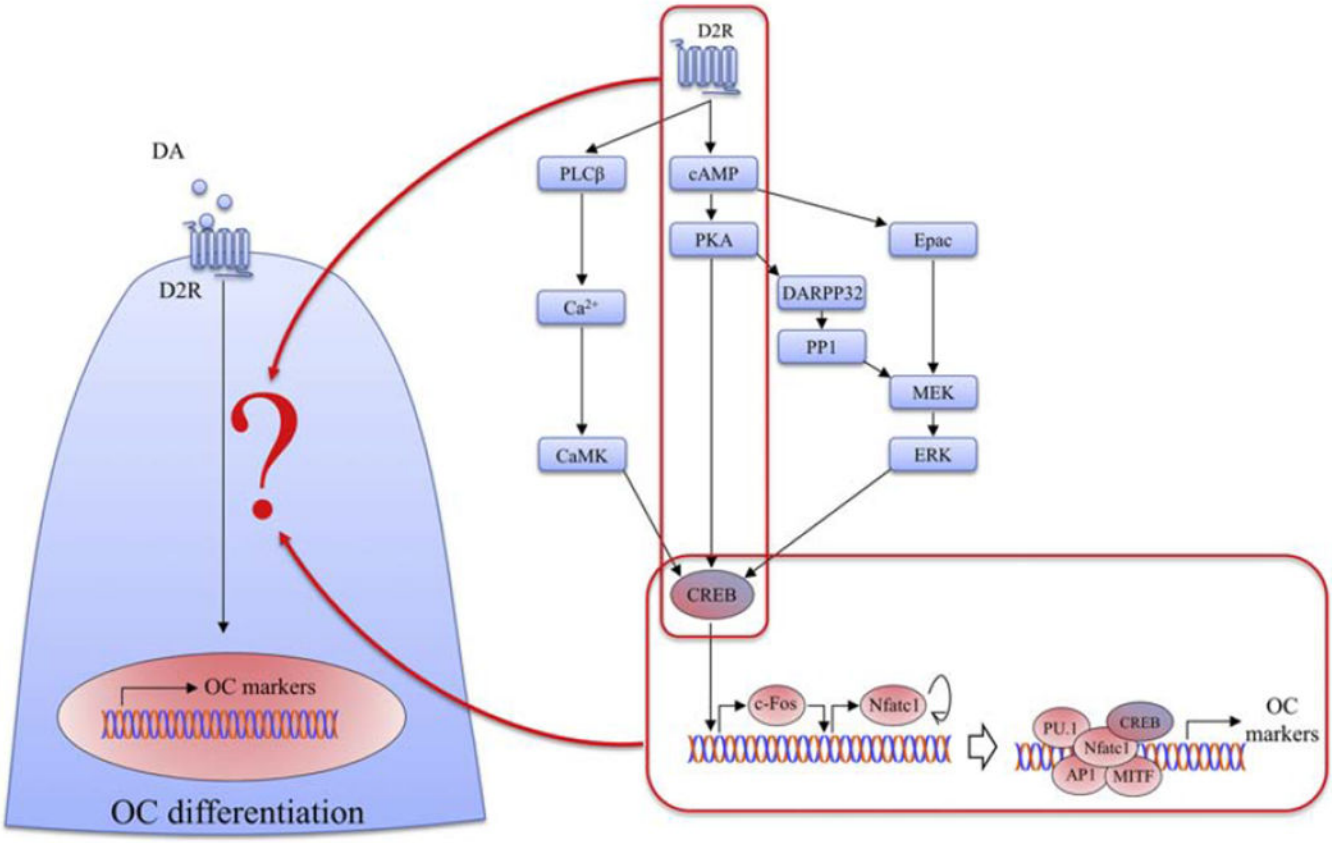

Fig. 6. Working model.

Our present study demonstrated DA regulates CREB activity via D2R/cAMP/PKA/CREB signaling pathway during osteoclastogenesis, which serves as a molecular mechanism by which DA modulates OC differentiation. DA, dopamine; D2R, D2-like receptors; OC, osteoclasts; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; CREB, cAMP-response element binding protein; PLCβ, phospholipase C-β; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; DARPP32, dopamine and cAMP regulated phosphoprotein 32kDa; PP1, protein phosphatase-1; Epac, exchange protein activated by cAMP; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-related kinase; Nfatc1, nuclear factor of activated T-cells, cytoplasmic 1; API, activator protein 1; MITF, melanocyte inducing transcription factor.