Article

Discovery Medicine 2024; 36(183): 788–798
https://doi.org/10.24976/Discov.Med.202436183.74

Tauroursodeoxycholic Acid Mitigates Oxidative Stress and Promotes Differentiation in High Salt-Stimulated Osteoblasts via NOX1 Mediated by PGC-1α

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Published: 20 April 2024

Background: High-salt diet (HSD) is a pivotal risk factor for osteoporosis (OP). Accumulating evidence has supported that tauroursodeoxycholic acid (TUDCA), a naturally produced hydrophilic bile acid, exerts positive effects on the treatment of OP. This study is committed to shedding light on the impacts of TUDCA on high salt-treated osteoblasts and probing into its underlying mechanisms of action.
Methods: Cell counting kit-8 (CCK-8) assay was used to determine the viability of osteoblasts. Alkaline phosphatase (ALP) staining and Alizarin red S (ARS) staining were used to measure osteoblast differentiation. Reverse transcription-quantitative PCR (RT-qPCR) and western blot were used to examine the expression of osteogenic markers. Western blot was also used to analyze the expression of superoxide dismutase-2 (SOD2), peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α), and NADPH oxidase 1 (NOX1). The production of reactive oxygen species (ROS) was evaluated via dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay. Following PGC-1α knockdown in TUDCA-pretreated osteoblasts exposed to NaCl, the aforementioned functional experiments were implemented again.
Results: MC3T3-E1 cell viability was not significantly impacted by increasing concentrations of TUDCA. However, in NaCl-exposed MC3T3-E1 cells, the viability loss, oxidative stress, and decline of differentiation were all dose-dependently obstructed by TUDCA treatment. Moreover, NaCl exposure reduced PGC-1α expression and increased NOX1 expression, which was then reversed by TUDCA. PGC-1α deletion partially abolished the effects of TUDCA on PGC-1α and NOX1, differentiation, and oxidative stress in NaCl-treated osteoblasts.
Conclusions: TUDCA might protect against high salt-induced OP via modulation of NOX1 mediated by PGC-1α.

Keywords: high salt; osteoporosis; TUDCA; PGC-1α; NOX1; oxidative stress

Introduction

Osteoporosis (OP) is a frequently-occurring clinical bone disease, characterized as a bone mass reduction, skeletal microstructure degeneration, and decline in bone mineral density, leading to a high propensity for fractures [1,2]. Accumulating clinical researches have confirmed that diet, lifestyle, and estrogen level are crucial risk factors for OP, the incidence of which also displays an upward trend with aging in the elderly [3,4]. As estimated, over one-third of people aged 50 years and older suffered from OP between 2001 and 2015 in China [5]. High-salt diet (HSD) has been regarded as a vital contributor to vasculature, heart, kidney, skin, brain, and bone diseases [6]. Meanwhile, high salt intake is deemed as one of the major causes of OP [7]. Currently, the drugs applied to treat OP clinically are primarily focused on inhibiting bone resorption. However, the bone formation and resorption imbalance may also result in bone loss [8]. Hence, developing novel drugs to accelerate bone formation is conducive to treating HSD-related OP.

Tauroursodeoxycholic acid (TUDCA), a naturally produced hydrophilic bile acid formed from the association of ursodeoxycholic acid (UDCA) with taurine, is abundant in the bile of mammals that has been widely applied to the therapy for hepatic diseases [9]. Emerging evidence has illustrated that TUDCA also elicits protective activities in multiple human diseases, including diabetes, obesity, and neurodegenerative diseases, dependent on its cytoprotective, anti-inflammatory, and antioxidative effects [10,11]. Notably, TUDCA has been reported to potentiate osteoblast proliferation, differentiation, and mineralization *in vitro* [12]. Further, TUDCA may suppress renal injury and inflammation induced by chronic high salt [13]. Nonetheless, whether TUDCA functions in high salt-induced OP remains to be defined.

Peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α) present in mitochondria-rich tissues is a transcription factor responsible for mitochondrial biogenesis [14]. Further investigation has revealed that PGC-1α promotes the degradation of NADPH oxidase

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1 (NOX1), and up-regulation of NOX1 has been reported to induce the release of reactive oxygen species (ROS) and mitochondrial damage [15,16]. PGC-1α has been supported to facilitate bone formation and hamper bone resorption [17]. Moreover, inhibition of NOX1 may obstruct the process of OP [18]. Additionally, PGC-1α expression has been noted to be activated by TUDCA [19]. Hence, the relationship among TUDCA, PGC-1α, and NOX1 in OP also needs to be specified.

The present work aims to ascertain the influences of TUDCA on OP and explore the latent action mechanism.

Materials and Methods

Cell Culture and Treatment

Alpha-minimum essential medium (α-MEM; Servicebio, Wuhan, China), along with 10% fetal bovine serum (FBS; Servicebio, Wuhan, China) was applied for the incubation of murine osteoblast-like cell line MC3T3-E1 from BeNa Culture Collection (BNCC360180; BNCC, Beijing, China) at 37 °C with 5% CO_2. MC3T3-E1 cells were validated using the short tandem repeat (STR) method and were confirmed to be free of mycoplasma infection. Cells were pretreated by TUDCA (0, 0.1, 0.15, and 0.2 mM; Aladdin, Shanghai, China) for 2 hours prior to exposure to NaCl (0, 25, 50, and 100 mM) for 48 hours.

The differentiation of MC3T3-E1 cells maintained in α-MEM decorated by 10% FBS was induced following the addition of osteogenic medium (OM) comprised of 10 nM dexamethasone, 50 µg/mL ascorbic acid, and 5 mM β-glycerophosphate for 14 days.

Plasmid Transfection

The specific small interfering RNAs (siRNAs) targeting PGC-1α (siRNA-PGC-1α-1/2) and the negative control (siRNA-NC) synthesized by Qiagen (Hilden, Germany) were delivered into MC3T3-E1 cells by siLentFect™ reagent (BioRad Laboratories, Hercules, CA, USA) in line with the manufacturer's protocol. Cells were collected for follow-up experiments at 48 hours post-transfection. The sequences of siRNAs were as follows: siRNA-PGC-1α-1, 5′- GAAGACACCTTCCTCTCCTCCTTCT-3′; siRNA-PGC-1α-2, 5′- AGACACCTTCCTCTCCTCCTTCTTT-3′.

Reverse Transcription-Quantitative PCR (RT-qPCR)

Following the purification of total RNA from cells adopting SevenFast® Total RNA Extraction Kit (Seven Biotech, Beijing, China), the synthesis of cDNA was performed with the aid of All-in-one First Strand cDNA Synthesis Kit (Seven Biotech, Beijing, China) using the following thermocycling conditions: 70 °C for 5 minutes, 42 °C for 1 hour and 70 °C for 15 minutes. Relative gene expression was calculated following PCR amplification employing 2× SYBR Green qPCR MasterMix (Seven Biotech, Beijing, China) using the $2^{-\Delta\Delta Cq}$ method and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) acted as the loading control. The following thermocycling conditions were used for qPCR: Initial denaturation at 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The oligonucleotide primers that were used for PCR are as follows: Alkaline phosphatase (*ALP*) Forward: 5′-CCACTCTGGACTTGGTGGTC-3′, Reverse: 5′-GGACCTGAGCGTTGGTGTTA-3′; osteocalcin (*OCN*) Forward: 5′-AGACAAGTCCCACACAGCAG-3′, Reverse: 5′-TTAAGCTCACACTGCTCCCG-3′; Osteopontin (*OPN*) Forward: 5′-CCTTGCTTGGGTTTGCAGTC-3′, Reverse: 5′- ACAGGGATGACATCGAGGGA-3′; Runt-related transcription factor 2 (*RUNX2*) Forward: 5′-GACTGTGGTTACCGTCATGGC-3′, Reverse: 5′-ACTTGGTTTTTCATAACAGCGGA-3′; *GAPDH* Forward: 5′-GGCAAATTCAACGGCACAGT-3′, Reverse: 5′-TAGGGCCTCTCTTGCTCAGT-3′.

Western Blot

Total protein was acquired following the lysis of cells in Radioimmunoprecipitation Assay (RIPA) lysis buffer (Seven Biotech, Beijing, China). Polyvinylidene fluoride (PVDF) membranes were used to shift the protein samples resolved by 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following, the membranes soaked in 5% bovine serum albumin (BSA) were successively immunoblotted with primary antibodies overnight at 4 °C and goat anti-rabbit secondary antibody (ab205718; 1/2000; Abcam, Cambridge, UK) for 1 hour. The blots were visualized by the Super Enhanced electrochemiluminescence (ECL) Prime (Seven Biotech, Beijing, China), and ImageLab4.0 software (Bio-Rad, Hercules, CA, USA) was applied to analyze the gray values. ALP (ab229126; 1/1000; Abcam), OCN (ab93876; 1/1000; Abcam), OPN (cab283656; 1/1000; Abcam), RUNX2 (ab236639; 1/1000; Abcam), superoxide dismutase-2 (SOD2; ab68155; 1/1000; Abcam), PGC-1α (ab191838; 1/1000; Abcam), NOX1 (ab131088; 1/1000; Abcam), GAPDH (ab9485; 1/2500; Abcam) and β-actin (ab8227; 1/1000; Abcam) primary antibodies were utilized here.

Cell Counting Kit-8 (CCK-8)

In short words, MC3T3-E1 cells seeded in a 96-well plate were treated by TUDCA (0, 0.1, 0.15, and 0.2 mM) alone for 48 hours or NaCl (0, 25, 50, and 100 mM) for 48 hours or pretreated by TUDCA (0.1, 0.15 and 0.2 mM) for 2 hours prior to exposure to 100 mM NaCl for 48 hours. A total of 10 µL cell counting kit-8 (CCK-8) solution (Seven Biotech, Beijing, China) was pipetted to each well for 2 hours in conformity to the product manual, followed by the measurement of the absorbance at 450 nm with a microplate reader (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China).

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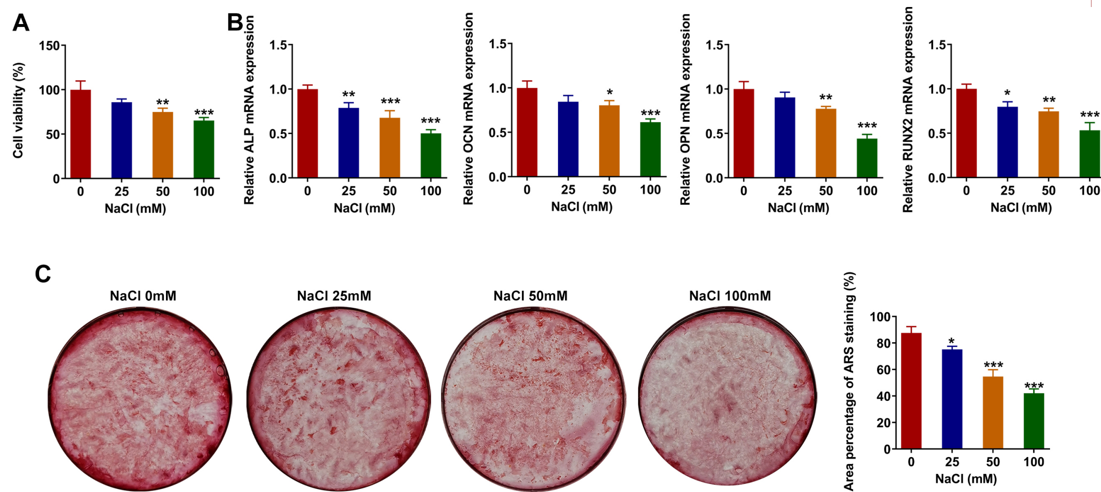

Fig. 1. High salt dose-dependently diminishes the viability and osteogenic differentiation of MC3T3-E1 cells. (A) CCK-8 assay appraised MC3T3-E1 cell viability. (B) RT-qPCR tested the expression of osteogenic markers. (C) ARS staining estimated osteogenic differentiation. Magnification, ×1. Data are representative of three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NaCl (0 mM). CCK-8, cell counting kit-8; RT-qPCR, reverse transcription-quantitative PCR; ARS, Alizarin Red S; ALP, Alkaline phosphatase; OCN, osteocalcin; OPN, Osteopontin; RUNX2, Runt-related transcription factor 2.

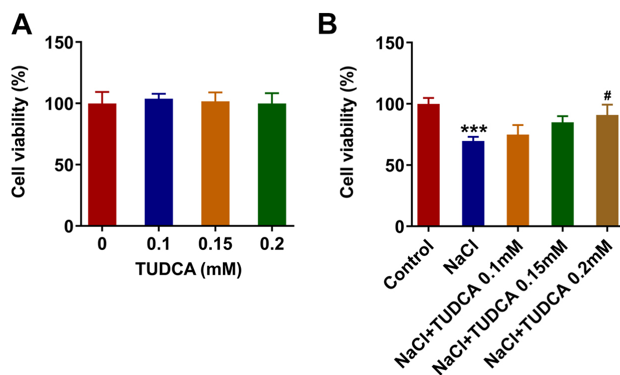

Fig. 2. TUDCA administration concentration-dependently elevates the viability of high salt-exposed MC3T3-E1 cells. (A) CCK-8 assay examined MC3T3-E1 cell viability when treated by increasing concentrations of TUDCA. (B) CCK-8 assay assessed the viability of TUDCA-pretreated MC3T3-E1 cells upon exposure to NaCl. Data are representative of three independent experiments. $^{***}p < 0.001$ vs. Control; $^{\#}p < 0.05$ vs. NaCl. TUDCA, tauroursodeoxycholic acid.

Alkaline Phosphatase (ALP) Staining

On the 14th day after osteoblastic induction, MC3T3-E1 cells subjected to transient transfection and indicated treatment were immobilized by cold propyl alcohol for 10 minutes prior to being probed with ALP staining solution (HASENSE, Jiangsu, China) for 4 hours. Afterwards, the cells were immersed in 2% cobalt nitrate for 3–5 minutes and 1% ammonium sulfide for 2 minutes. The images were captured under an inverted light microscope (Olympus, Tokyo, Japan).

Alizarin Red S (ARS) Staining

Prior to the cultivation with 2% Alizarin red S (ARS) solution (Nanjing SenBeiJia Biological Technology Co., Ltd., Nanjing, China) for 5 minutes, treated MC3T3-E1 cells were submerged in 4% paraformaldehyde at room temperature for 20 minutes and rinsed in distilled water, the images were captured under an inverted light microscope (Olympus, Tokyo, Japan). The area percentage of ARS staining was quantified by the ratio of the calcified area to the total area.

Estimation of Reactive Oxygen Species (ROS)

MC3T3-E1 cells were seeded in a 6-well plate at a density of 5×10^4 cells/well. After the indicated treatment, cells were maintained in a serum-starved medium decorated by 10 µM dichloro-dihydro-fluorescein diacetate (DCFH-DA; KeyGEN BioTECH, Nanjing, China) at 37 °C for 20 minutes. After washing phosphate-buffered saline (PBS), ROS production was calculated under a fluorescence microscope (Olympus, Tokyo, Japan) with an excitation wavelength of 485 nm and emission wavelength of 530 nm.

Statistical Analyses

GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analyses. All data from three parallel repeat experiments were denoted as the mean ± standard deviation. Statistical significances were measured using one-way analysis of variance (ANOVA) along with Tukey's post hoc test. The significance level was considered as $p < 0.05$.

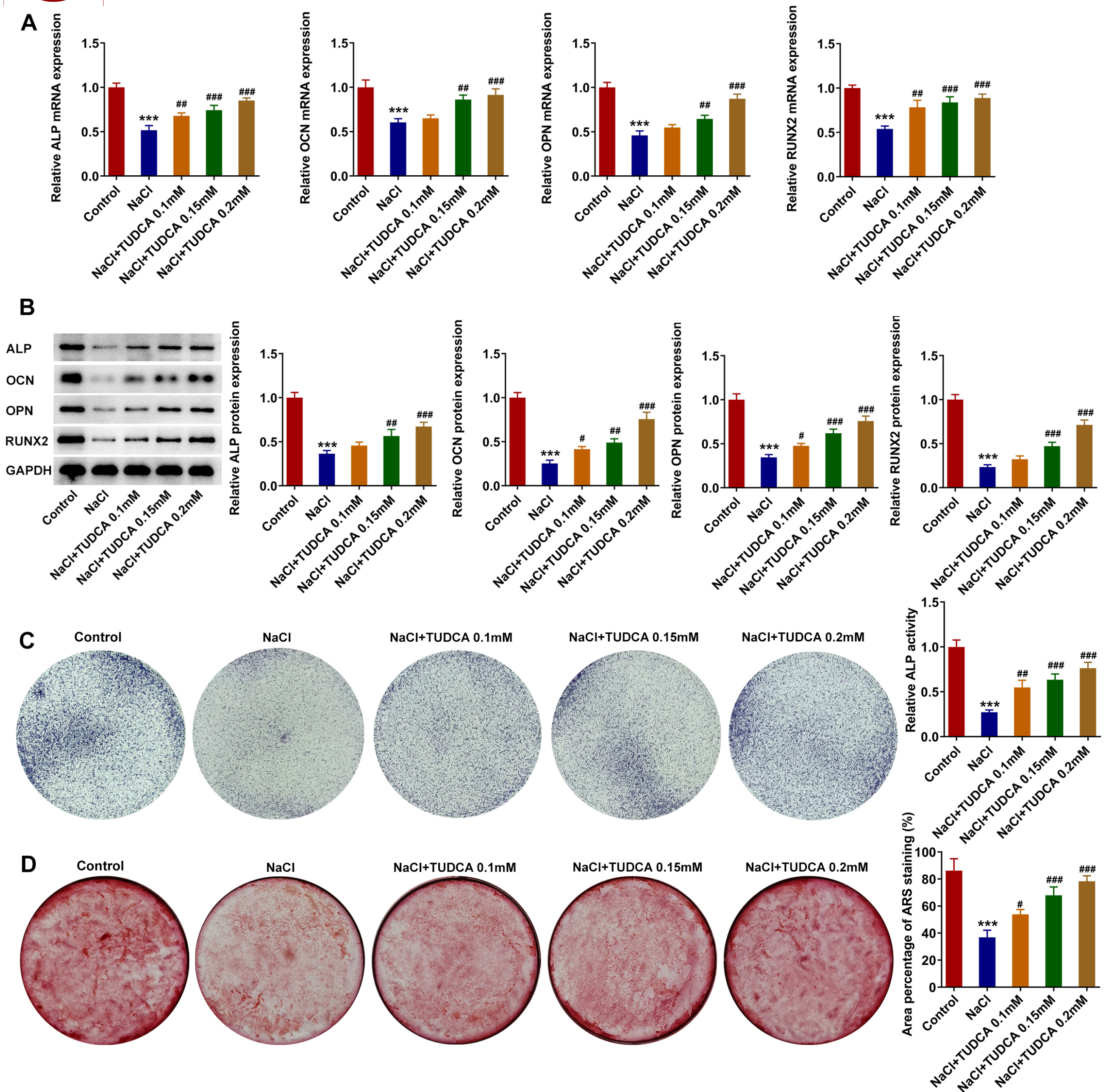

Fig. 3. TUDCA administration concentration-dependently potentiates the osteogenic differentiation of high salt-exposed MC3T3-E1 cells. (A) RT-qPCR tested the expression of osteogenic markers. (B) Representative western blot images and quantification of osteogenic markers. (C) ALP staining evaluated ALP activity. Magnification, ×1. (D) ARS staining estimated osteogenic differentiation. Magnification, ×1. Data are representative of three independent experiments. $***p < 0.001$ vs. Control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. NaCl.

Results

High Salt Dose-Dependently Diminishes the Viability and Osteogenic Differentiation of MC3T3-E1 Cells

To evaluate the effects of high salt on osteoblasts, MC3T3-E1 cells were exposed to varying concentrations of NaCl (0, 25, 50, and 100 mM) for 48 hours. NaCl treatment resulted in a notable decline in MC3T3-E1 cell viability in a dose-dependent manner (Fig. 1A). Further, to determine the impacts of high salt on osteogenic differentiation in MC3T3-E1 cells, the expression of osteogenic markers, including *ALP*, *OCN*, *OPN*, and *RUNX2* were analyzed. *ALP*, *OCN*, *OPN*, and *RUNX2* expression were all on a downward trend following challenges with increasing doses of NaCl (Fig. 1B). As depicted in Fig. 1C, the experimental results of ARS staining elucidated that NaCl concentration-dependently attenuated the ability of MC3T3-E1 cells to differentiate into osteoblasts. Conse-

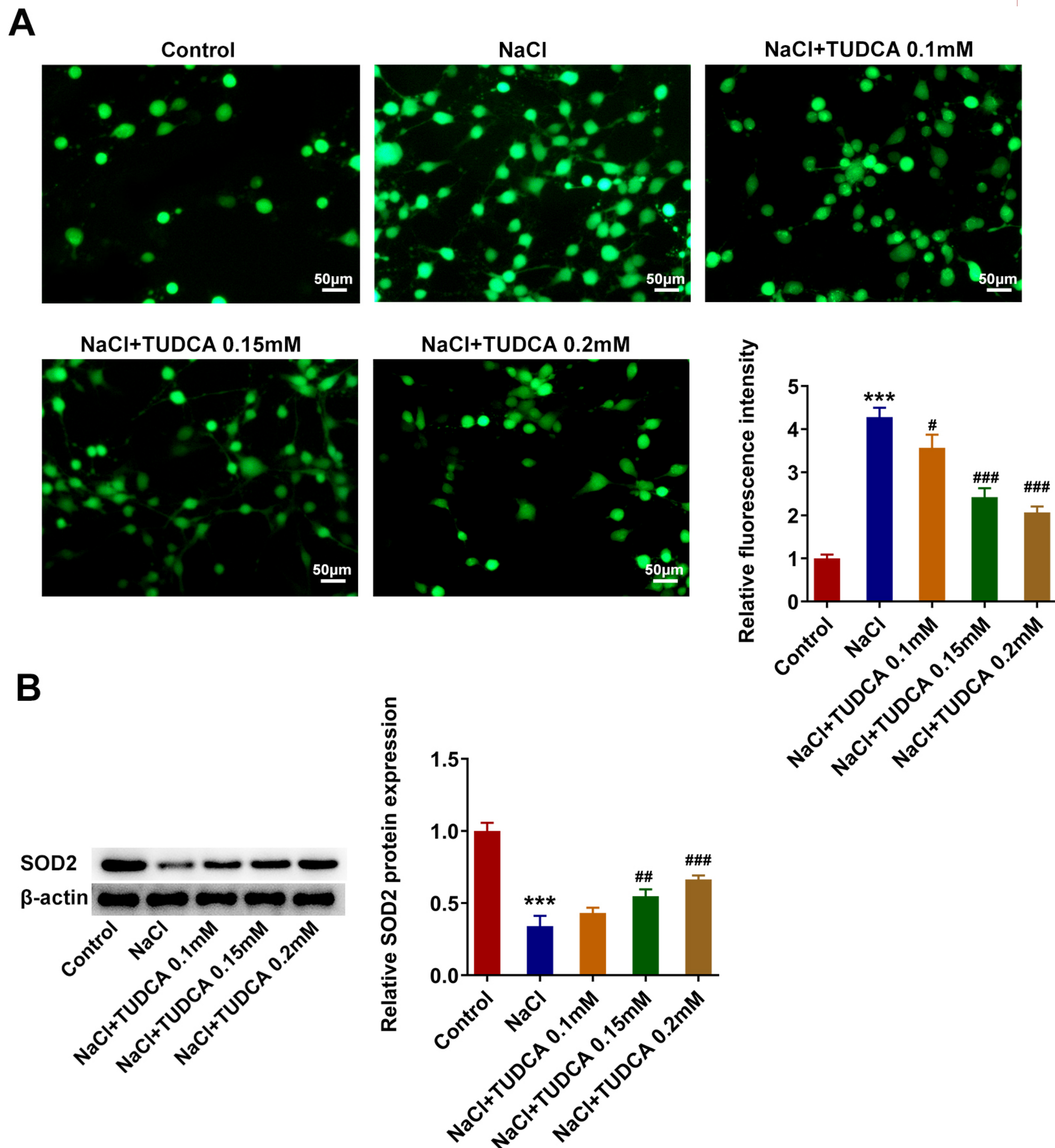

Fig. 4. TUDCA administration concentration-dependently ameliorates the oxidative stress in high salt-exposed MC3T3-E1 cells. (A) DCFH-DA staining evaluated ROS level. Magnification, ×200. (B) Representative western blot images of SOD2 expression and quantification. Data are representative of three independent experiments. $^{***}p < 0.001$ vs. Control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. NaCl. DCFH-DA, dichloro-dihydro-fluorescein diacetate; SOD2, superoxide dismutase-2; ROS, reactive oxygen species.

quently, 100 mM NaCl was selected for the appropriate induction dose for its prominent effect on MC3T3-E1 cell viability and osteogenic differentiation. Together, high salt significantly suppressed the viability and osteogenic differentiation in MC3T3-E1 cells.

TUDCA Administration Concentration-Dependently Elevates the Viability of High Salt-Exposed MC3T3-E1 Cells

To ascertain whether TUDCA played a protective role in high-salt-challenged MC3T3-E1 cells, CCK-8 assay was first applied to appraise MC3T3-E1 cell viability when treated by ascending doses of TUDCA (0, 0.1, 0.15 and 0.2 mM). No significant alternations in MC3T3-E1 cell viability were observed following administration of TUDCA (Fig. 2A). Meanwhile, the reduced viability of MC3T3-E1 cells exposed to NaCl was markedly improved by 0.2 mM TUDCA (Fig. 2B). In summary, TUDCA might protect against high salt-stimulated MC3T3-E1 cell viability loss.

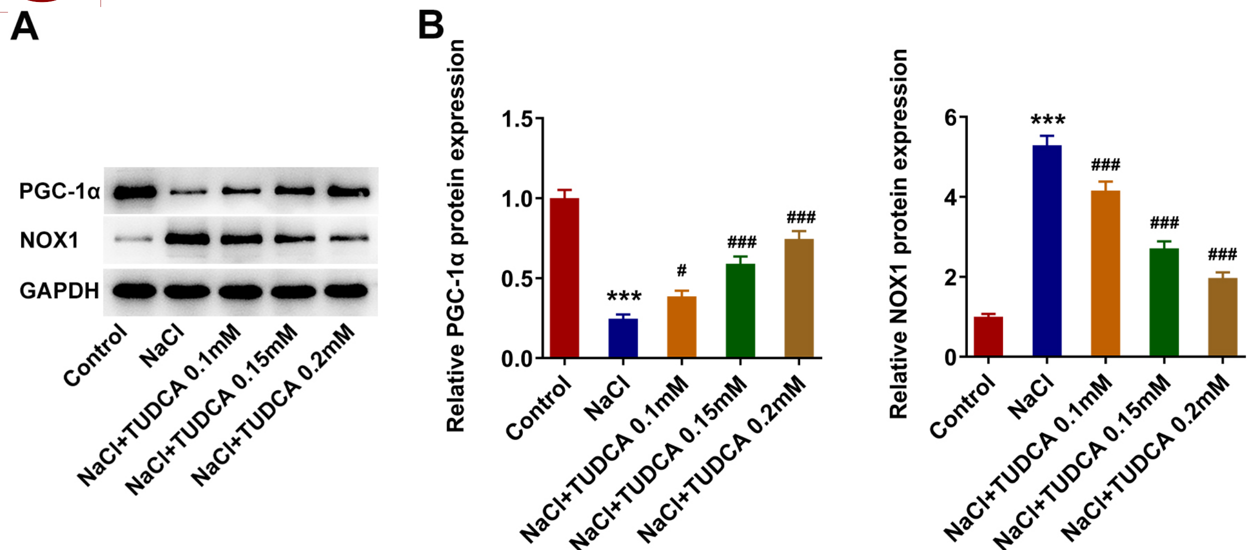

Fig. 5. TUDCA regulates PGC-1α and NOX1 in NaCl-treated MC3T3-E1 cells. (A) Representative western blot images of PGC-1α and NOX1 expression and (B) quantification. Data are representative of three independent experiments. $^{***}p < 0.001$ vs. Control; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. NaCl. PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator-1alpha; NOX1, NADPH oxidase 1.

TUDCA Administration Concentration-Dependently Potentiates the Osteogenic Differentiation of High Salt-Exposed MC3T3-E1 Cells

Osteogenic differentiation plays an important role in the pathogenesis of OP, and *ALP*, *OCN*, *OPN*, and *RUNX2* are essential osteogenic markers. As expected, the reduced *ALP*, *OCN*, *OPN*, and *RUNX2* expression in MC3T3-E1 cells challenged with NaCl were all increased again when treated with increasing concentrations of TUDCA (Fig. 3A,B). Also, as illustrated in ALP staining, ALP activity was eliminated by NaCl exposure in MC3T3-E1 cells and increased in TUDCA-treated MC3T3-E1 cells exposed to NaCl (Fig. 3C). ARS staining results indicated that NaCl exposure apparently suppressed the differentiation of MC3T3-E1 cells into osteoblasts, which was then reversed by TUDCA in a dose-dependent manner (Fig. 3D). To conclude, TUDCA might accelerate the osteogenic differentiation of high salt-treated MC3T3-E1 cells.

TUDCA Administration Concentration-Dependently Ameliorates the Oxidative Stress in High Salt-Exposed MC3T3-E1 Cells

Oxidative stress has been implicated as a causative factor in OP pathogenesis [20,21]. As illuminated in DCFH-DA, NaCl exposure distinctly raised ROS level in MC3T3-E1 cells, and TUDCA administration remarkably diminished ROS level in NaCl-treated MC3T3-E1 cells (Fig. 4A). Additionally, western blot analysis demonstrated that TUDCA significantly enhanced the expression of antioxidant SOD2 which was reduced in MC3T3-E1 cells exposed to NaCl (Fig. 4B). Overall, TUDCA might ease high salt-evoked oxidative stress in MC3T3-E1 cells.

TUDCA Regulates PGC-1α and NOX1 in NaCl-Treated MC3T3-E1 Cells

As reported, TUDCA can activate the expression of PGC-1α, which has been supported to widely participate in the development of OP. Intriguingly, the expression of PGC-1α and NOX1 were tested by western blot, and it was noticed that NaCl challenging declined PGC-1α expression whereas augmented NOX1 expression in MC3T3-E1 cells. In NaCl-treated MC3T3-E1 cells, TUDCA pretreatment dose-dependently elevated PGC-1α expression, whereas it cut down NOX1 expression (Fig. 5A,B), suggesting that TUDCA might modulate PGC-1α and NOX1 in NaCl-exposed MC3T3-E1 cells. 0.2 mM TUDCA was also utilized in the ensuing experiments due to its prominent effect.

PGC-1α Interference Abolishes the Effects of TUDCA on the Viability, Oxidative Stress, PGC-1α, and NOX1 in NaCl-Challenged MC3T3-E1 Cells

To substantiate that TUDCA exerted its protective activities in NaCl-treated MC3T3-E1 cells via modulation of NOX1 mediated by PGC-1α, PGC-1α was successfully knocked down following transfection of siRNA-PGC-1α-1/2 plasmids. Furthermore, siRNA-PGC-1α-1 was chosen for the follow-up experiments as the interference efficacy of siRNA-PGC-1α-1 was found to be more significant than siRNA-PGC-1α-2 (Fig. 6A). CCK-8 assay demonstrated that TUDCA overtly enhanced the diminished viability of MC3T3-E1 cells exposed to NaCl, which was then slightly suppressed again after deficiency of PGC-1α (Fig. 6B). In addition, the up-regulated ROS level in NaCl-exposed MC3T3-E1 cells was depleted by TUDCA administra-

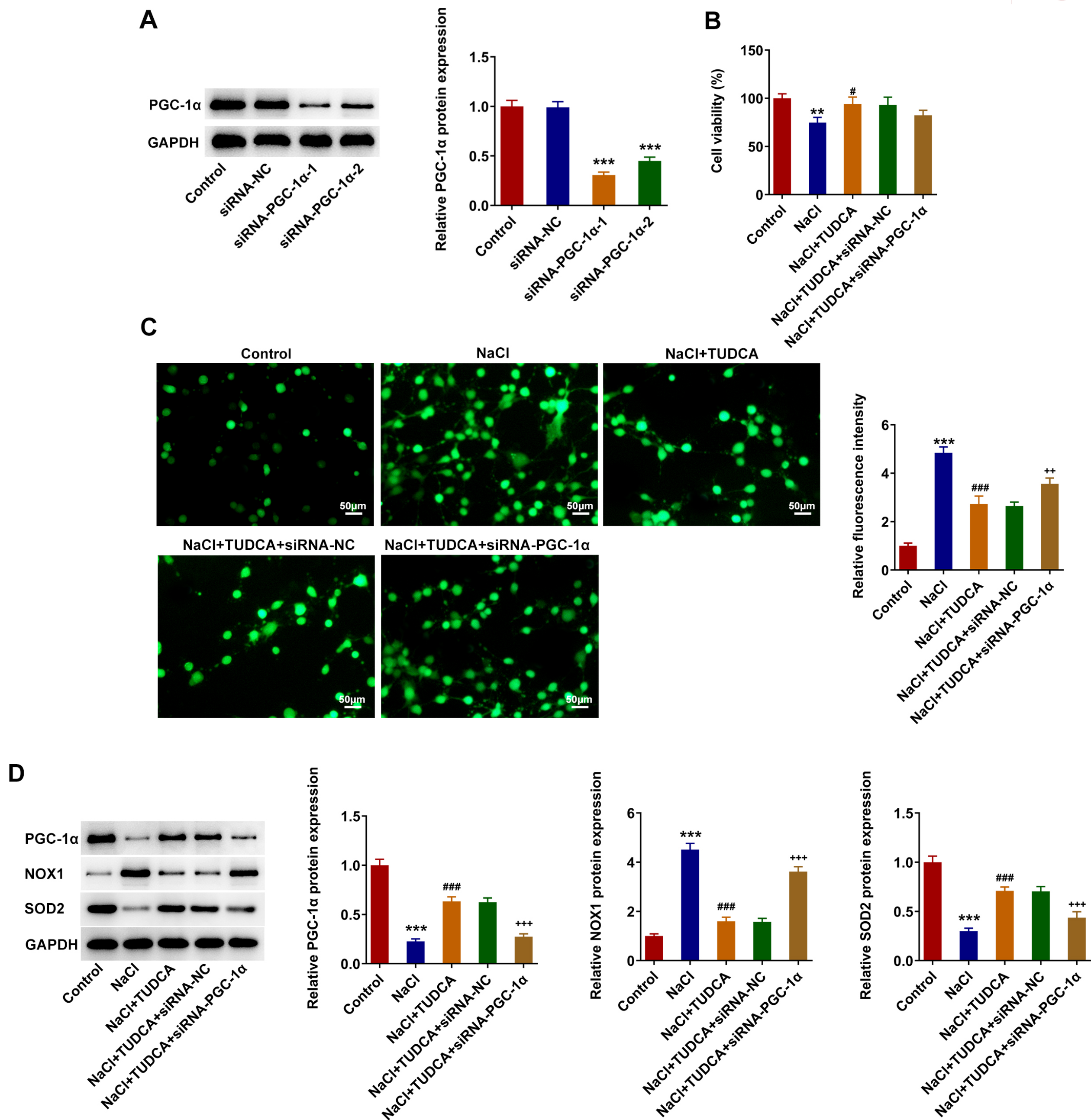

Fig. 6. PGC-1α interference abolishes the effects of TUDCA on the viability, oxidative stress, PGC-1α, and NOX1 in NaCl-challenged MC3T3-E1 cells. (A) Western blot examined the transfection efficacy of PGC-1α interference plasmids. $^{***}p < 0.001$ vs. siRNA-NC. (B) CCK-8 assay examined MC3T3-E1 cell viability. (C) DCFH-DA staining evaluated ROS level. Magnification, $\times$200. (D) Western blot examined SOD2, PGC-1α, and NOX1 expression. Data are representative of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. NaCl; $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. NaCl+TUDCA+siRNA-NC. SOD2, superoxide dismutase-2.

tion and PGC-1α silencing increased ROS level again in TUDCA-pretreated MC3T3-E1 cells challenged with NaCl (Fig. 6C). On the contrary, the ascending SOD2 expression in NaCl-treated MC3T3-E1 cells imposed by TUDCA was decreased following PGC-1α absence. TUDCA pretreatment increased PGC-1α expression and declined NOX1 expression in NaCl-challenged MC3T3-E1 cells, which was then countervailed by PGC-1α inhibition (Fig. 6D). Collectively, TUDCA modulated PGC-1α-mediated NOX1 to enhance the viability and mitigate the oxidative stress in MC3T3-E1 cells treated by NaCl.

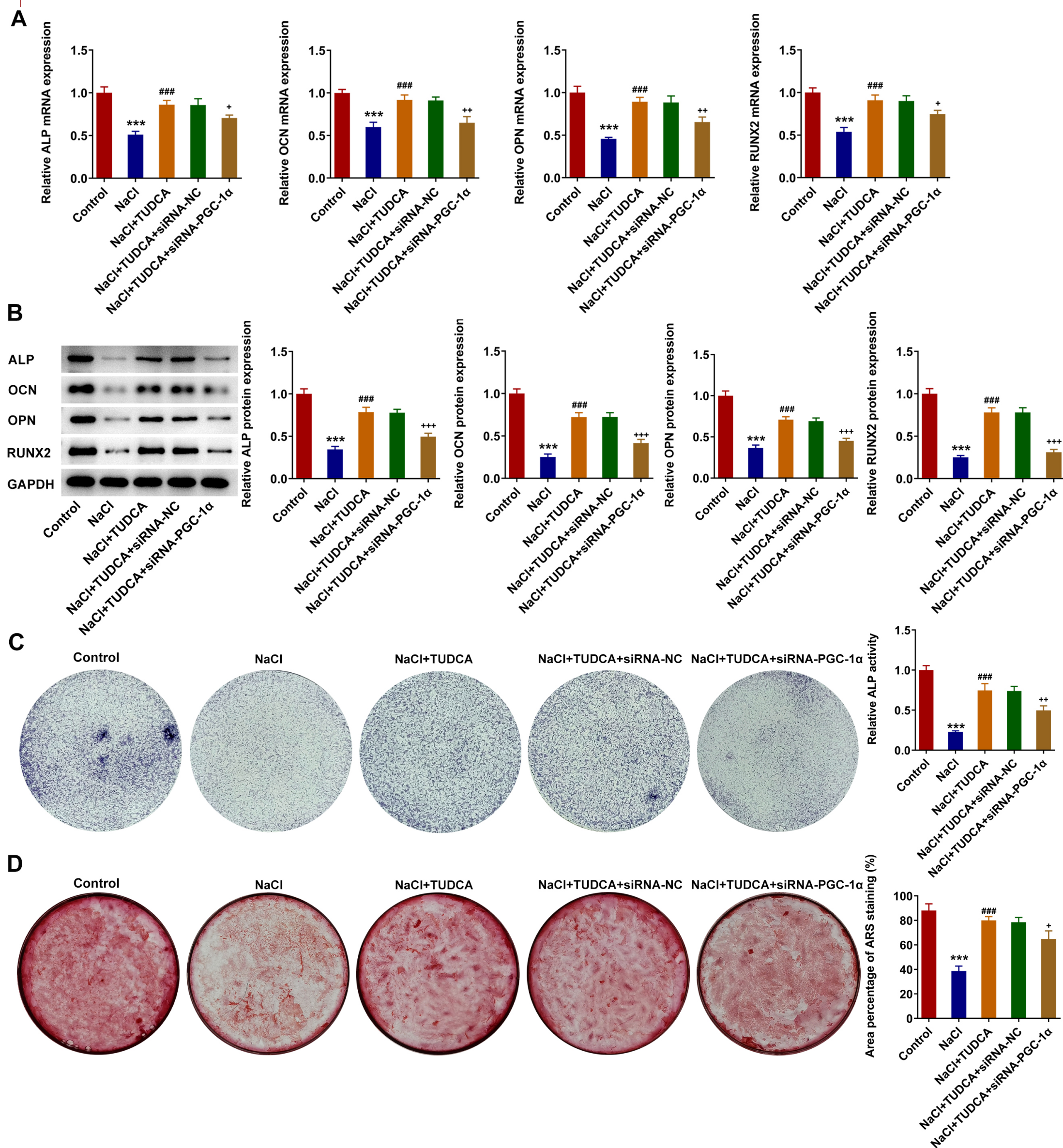

Fig. 7. TUDCA modulates PGC-1α-mediated NOX1 to facilitate the osteogenic differentiation of MC3T3-E1 cells treated by NaCl. (A) RT-qPCR tested the expression of osteogenic markers. (B) Western blot tested the expression of osteogenic markers. (C) ALP staining evaluated ALP activity. Magnification, $\times 1$. (D) ARS staining estimated osteogenic differentiation. Magnification, $\times 1$. Data are representative of three independent experiments. $^{***}p < 0.001$ vs. Control; $^{\#\#\#}p < 0.001$ vs. NaCl; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. NaCl+TUDCA+siRNA-NC.

TUDCA Modulates PGC-1α-Mediated NOX1 to Facilitate the Osteogenic Differentiation of MC3T3-E1 Cells Treated by NaCl

To further demonstrate that TUDCA might function in NaCl-induced MC3T3-E1 cells by mediating PGC-1α and NOX1, the osteogenic differentiation of NaCl-treated MC3T3-E1 cells administrated with TUDCA and transfected with PGC-1α interference plasmids were detected. At the same time, TUDCA boosted ALP, OCN, OPN, and RUNX2 expression in NaCl-exposed MC3T3-E1 cells, while PGC-1α interference partially downregulated ALP, OCN, OPN, and RUNX2 expression in TUDCA-administrated MC3T3-E1 cells treated by NaCl (Fig. 7A,B). As expected, the improved ALP activity imposed by TUDCA in MC3T3-E1 cells challenged with NaCl declined again after PGC-1α was depleted (Fig. 7C). Besides, the results of ARS staining clarified that the aggravated osteogenic differentiation of NaCl-challenged MC3T3-E1 cells on account of TUDCA treatment was obstructed again after PGC-1α was silenced (Fig. 7D). All in all, PGC-1α depletion offsets the impacts of TUDCA on the osteogenic differentiation of NaCl-exposed MC3T3-E1 cells.

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Discussion

OP is a chronic bone disorder characterized by the imbalance between bone resorption by osteoclasts and bone formation by osteoblasts [22]. Osteoblasts derived from bone marrow mesenchymal stem cells (BMSCs) are capable of differentiating into mature osteoblasts. During the process of bone formation, the abnormal proliferation and apoptosis of osteoblasts under the stimulation of various factors are critical contributors to the decrease in bone mineral density, even bone-related diseases [23,24]. HSD has been increasingly recognized as an inducing factor for OP by destroying bone microstructure and suppressing osteogenic differentiation [7,25,26]. As reported, a high-salt environment and the consequently increasing osmolarity may enhance the release of inflammatory cytokines, profibrotic growth factors, and components of the renin-angiotensin-aldosterone system [27]. Also, sodium or chloride accumulation may contribute to lymphangiogenesis and autoimmune diseases via osmotic stimuli [28]. Here, ascending concentrations of NaCl were used to treat MC3T3-E1 cells, and the experimental results revealed that NaCl treatment notably diminished MC3T3-E1 cell viability in a dose-dependent manner. ALP, OCN, OPN, and RUNX2 are typical osteoblast differentiation markers [29]. ALP is a membrane-bound enzyme that stimulates osteoblast activity and bone mineralization. Osteoblasts secrete OCN and OPN that are also regarded as biomarkers of bone mineralization [30,31]. RUNX2 is an essential transcription factor for osteoblast differentiation [32]. Through investigation, it was observed that ALP, OCN, OPN, and RUNX2 expression were all on a downward trend in MC3T3-E1 cells upon exposure to varying doses of NaCl. As expected, the ability of MC3T3-E1 cells to differentiate into osteoblasts was concentration-dependently weakened by NaCl.

TUDCA has been well-documented to exhibit potential therapeutic benefits in various disease models [10]. Recent literature has underlined the potential protective role of TUDCA in OP through boosting osteoblast proliferation, differentiation, and mineralization *in vitro* [12]. Moreover, chronic high salt-triggered renal injury and inflammation can also be alleviated following the application of TUDCA [13]. Consistently, the administration of TUDCA alone exerted no apparent influence on MC3T3-E1 cell viability. Meanwhile, in NaCl-exposed MC3T3-E1 cells, TUDCA markedly improved cell viability in a dose-dependent manner. TUDCA reversed the suppressed differentiation of MC3T3-E1 cells into osteoblasts upon exposure to NaCl in a dose-dependent manner, which was also accompanied by the finding that the declined ALP, OCN, OPN, RUNX2 expression and ALP activity in NaCl-challenged MC3T3-E1 cells were all elevated again when treated by TUDCA.

Oxidative stress has been associated with the pathogenesis of OP, leading to osteoblast dysfunction, including an increase in osteoblast apoptosis and inhibition of osteogenic differentiation [20,21]. ROS, the excess production of which is widely accepted as a key player that induces oxidative stress, has been identified as a molecule that influences the differentiation of osteoclasts [33]. SOD2, which has been supported to exert antioxidant properties by eliminating superoxide radicals, also participates in OP [34]. The present work elucidated that NaCl exposure distinctly increased ROS levels while lessening SOD2 expression in MC3T3-E1 cells, and TUDCA administration remarkably diminished ROS levels and increased SOD2 expression in NaCl-treated MC3T3-E1 cells.

As reported, TUDCA treatment can activate PGC-1α expression [19], and PGC-1α can further decline NOX1 expression by promoting NOX1 degradation [15]. In agreement with these findings, the declined PGC-1α expression and augmented NOX1 expression in NaCl-challenged MC3T3-E1 cells were both reversed by TUDCA. PGC-1α is an important regulator of mitochondrial biosynthesis that has been supported to facilitate bone formation and hamper bone resorption [14,17]. Further studies in this study also proved that PGC-1α deletion partially abolished the impacts of TUDCA on PGC-1α and NOX1, viability, differentiation, and oxidative stress in NaCl-treated osteoblasts.

Conclusions

Collectively, TUDCA might protect against viability loss and oxidative stress and accelerate osteogenic differentiation via modulation of PGC-1α-mediated NOX1 in NaCl-exposed osteoblasts. This study uncovered the mechanisms underlying high salt-stimulated OP and implied that TUDCA might possess great therapeutic value for treating OP, even bone-related diseases. Nonetheless, *in vivo* animal models still become a requisite for verification. In addition, sodium and chloride are the key factors in maintaining normal osmotic pressure [27,28]. In further investigations, the impacts of osmotic pressure in NaCl-treated osteoblasts must be considered.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are availabe from the corresponding author on reasonable request.

Author Contributions

SML contributed to the design of the study and project administration. JJL and QY performed the experiments and analyzed the data. CLL performed the statistical analysis. SML and TW interpreted the data. SML, TW and CLL confirmed the authenticity of all raw data. SML and TW drafted and reviewed the manuscript and all authors were involved in revising it critically for important intellectual content. All authors read and gave final approval of the version to be published. All authors have participated suf-

ficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

The study was supported by the Startup Fund for scientific research, Fujian Medical University (2018QH1172) and Natural Science Foundation of Fujian Province (2019J01611).

Conflict of Interest

The authors declare no conflict of interest.

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