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Research article

ARMCX3 regulates ROS signaling, affects neural differentiation and inflammatory microenvironment in dental pulp stem cells

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ABSTRACT

Background: The neural differentiation of dental pulp stem cells (DPSCs) exhibits great potential in the treatment of dental pulp repair and neurodegenerative diseases. However, the precise molecular mechanisms underlying this process remain unclear. This study was designed to reveal the roles and regulatory mechanisms of the armadillo repeat-containing X-linked 3 (ARMCX3) in neural differentiation and inflammatory microenvironment in human DPSCs (hDPSCs).

Methods: We treated hDPSCs with porphyromonas gingivalis lipopolysaccharide (Pg-LPS) to simulate the inflammatory microenvironment. Then the lentiviral vectors were introduced to construct stable cell lines with ARMCX3 knockdown or overexpression. The expression of neural-specific markers, ARMCX3 and inflammation factors were estimated by immunofluorescence (IF), quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) assays. Additionally, we used IF assays and specific kits to investigate the regulatory role of ARMCX3 on reactive oxygen species (ROS) signaling. Moreover, a ROS inhibitor was utilized to verify whether ROS inhibition reversed the effects of ARMCX3 in Pg-LPS-treated hDPSCs.

Results: This work illustrated that Pg-LPS treatment significantly enhanced ARMCX3 expression and inflammatory response, and inhibited neural differentiation in hDPSCs. ARMCX3 knockdown effectively accelerated neural differentiation and controlled inflammatory cytokines at a lower level in hDPSCs in the presence of Pg-LPS. Additionally, knockdown of ARMCX3 notably reduced ROS production and ROS inhibition effectively eliminated the roles of ARMCX3 overexpression in hDPSCs. Besides, all results were proved to be statistically significant.

Conclusion: This investigation proved that ARMCX3 affected neural differentiation and inflammation microenvironment in hDPSCs at least partly by mediating ROS signal. These findings provided a new perspective on the mechanism of neural differentiation of hDPSCs and help to better explore the therapeutic schedule of pulpitis and neurodegenerative diseases.

1. Introduction

In recent years, stem cells have shown great potential in treating nerve injuries and defects [1,2], because stem cells could replenish nerve tissue by generating neuron-like cells [3]. Dental pulp stem cells (DPSCs), originate from the neural crest, exhibit obvious higher expression of neuronal marker compared with other stem cells [4,5]. DPSCs could differentiate into neurons and glial cells, and exert

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neuroprotective abilities [6,7]. Besides, cells regulate their microenvironment by producing a variety of factors, and paracrine interaction also play a modulatory effect on neighboring cells [8]. It is well known that the tumor microenvironment, as an ecological system of tumor cells, is crucial for the development of diverse cancers, including gastric cancer [9], cholangiocarcinoma [10] and pancreatic cancer [11], and affects the effectiveness of therapeutic interventions [10]. Inflammatory response is a key factor in the microenvironment [12,13]. DPSCs have the ability of self-renewal, and also regulate dental pulp repair and regeneration by affecting immune and inflammatory responses [14–16], and the inflammatory microenvironment has been proved to be vital for the differentiation of DPSCs [17]. For example, Hong et al. demonstrated that OCT4A up-regulates the self-renewal ability of hDPSCs by targeting FTX in the inflammatory microenvironment [18]. Chen et al. revealed that SNHG7 accelerates osteogenic differentiation of hDPSCs in inflammatory environments through hsa-miR-6512e3p expression [19].

Previous studies have pointed out that inflammation and pulp regeneration are mutually antagonistic processes [20] and inflammation plays a crucial role in the self-repair process of the dental pulp [17]. The production of excessive inflammatory factors destroys the pulp structure and causes tissue necrosis. However, moderate inflammatory response is beneficial to the development of DPSCs Specifically, on the on hand, high levels of inflammatory stimulation prevented osteogenic differentiation of hDPSCs [21,22]. The unresolved inflammation is one of the reasons why damaged pulp cannot be fully repaired [16]. Besides, several genes such as Resolvin E1 promotes differentiation of hDPSCs by resolving inflammation [16]. On the other hand, moderate inflammatory level initiates the proliferation, migration and differentiation of DPSCs, ultimately accelerates tissue regeneration [16,17,20]. For instance, Liu et al. discovered that low dose of pro-inflammatory factor TNF-α [17] enhances the differentiation ability of hDPSCs [23]. Therefore, maintaining a low level of inflammatory activation is essential for pulp repair.

The members belonging to Armadillo repeat-containing X-linked (Armcx) family contain multiple Armadillo domains [24]. Increasing evidence showed that Armcx family are closely related to the malignant development of various tumors [25,26]. For example, it is reported that overexpressed ARMCX1 suppresses gastric cancer metastasis by mediating PAR-1/Rho GTPase signal [27]. ARMCX3, localized on the X chromosome, has been proved to be tightly associated with mitochondrial dynamics [25]. Additionally, recent studies illustrated that ARMCX3 is highly expressed in the central nervous system and exerts a vital role in the regulation of neuronal mitochondrial transport, including calcium ions (Ca^{2+}) [24]. Moreover, bioinformatic analysis demonstrated the increased expression of ARMCX3 in inflammatory periodontal tissue [28]. However, the specific roles of ARMCX3 in neural differentiation of DPSCs and modulation of their inflammatory environment are uncovered.

Lipopolysaccharide (LPS), as a major component of the cell wall of Gram-negative bacteria, is produced at a high level in pulpitis tissues [29] and is often used to construct inflammation models *in vitro* [30]. In the preliminary experiment, we employed porphyromonas gingivalis (Pg) LPS (Pg-LPS) to activate the inflammatory response of human DPSCs (hDPSCs) and discovered that Pg-LPS treatment notably suppressed neural differentiation and increased the expression of ARMCX3 in hDPSCs. Therefore, we hypothesized that ARMCX3 might affect the differentiation of hDPSCs in the inflammatory microenvironment.

In this study, we revealed that Pg-LPS effectively upregulated ARMCX3 expression in hDPSCs. The knockdown of ARMCX3 reduced the level of inflammatory response, and promoted neural differentiation of hDPSCs. This work was likely to provide a promising target for the repair of pulp injury and the treatment of neurodegenerative diseases.

2. Materials and methods

2.1. Neural differentiation of hPDSCs

The hDPSCs (CP-H231, China) were cultured in a specific complete medium (CM-H231, Pricella, China). The cells were grown in a cell incubator (HH.CP-T, JTONE, China) containing 5 % CO_2 at 37 °C for 48 h.

For the induction of neural differentiation, passages 3–5 of hDPSCs were seeded into 6-well plates (2×10^4 cells/well). Then the Neurobasal medium (21103049, Gibco, USA) with 40 ng/mL Recombinant Human bFGF Protein (M9406, AbMole, USA), 20 ng/mL Recombinant Human EGF Protein (M9415, AbMole, USA), 1 × B-27 (17504044, Gibco, USA) and 1 % Penicillin-Streptomycin solution (60162ES76, YESEN, China) was added to each well. The cells were induced in a cell incubator (HH.CP-T, JTONE, China) containing 5 % CO_2 at 37 °C for 7 days and the medium was changed every 3 days. The steps were displayed in Supplementary Fig. S1.

2.2. Construction of stable cell lines

For overexpression of ARMCX3, full-length ARMCX3 cDNA was cloned into pCDH-CMV-MCS-EF1-Puro plasmid (V006738#, NovoPro, China) and the empty plasmid was used as negative control. To knock down ARMCX3, the fragments of short harpin (sh)-ARMCX3 (5′-CCACCTTGTTTATATGGTAAA-3′) were constructed into pLKO.1 puro plasmids (V010450#, NovoPro, China) and plasmids with scrambled sh-RNAs (sh-NC, 5′-UAAGGCUAUGAAGAGAUA-3′) were regarded as negative controls. Next, the Liposomal Transfection Reagent (40802ES01, YESEN, China) was employed to transfect the above plasmids and auxiliary plasmids (DR8.91 and VSVG) into HEK293T cells according to the manufacturer's instructions. After 48 h and 72 h, the supernatant was collected and added to hDPSCs in the logarithmic growth phase, followed by adding 2 μg/ml Polybrene (40804ES76, YESEN, China). After 24 h, the positive cells were screened using 25 μg/ml Ampicillin (ST007, Beyotime, China), which were regarded as stable cell lines.

2.3. Establishment of inflammation microenvironment and detection of inflammatory cytokines

For the induction of inflammation response, 1 μg/mL Pg-LPS (tlrl-pglps, Invivogen, USA) was employed to stimulate stable hDPSCs

for 24 h as reported in previous study [17]. The inflammatory cytokines including interleukin (IL)-6, IL-8 and tumor necrosis factor alpha (TNF-α) were quantified with corresponding ELISA kits (Jining Shiye, China) abiding by the manufacturer's protocol.

2.4. Quantitative real-time PCR (qRT-PCR)

TRIzol reagent (19201ES60, YESEN, China) was introduced to obtain total RNA from stable Pg-LPS-treated hDPSCs abiding by the instructions. Then qRT-PCR reaction was performed using One Step qRT-PCR Enzyme Mix (13197ES92, YESEN, China) as described in the manufacturer's guidelines. The transcription levels of all genes were referenced by β-actin. All information of primers were listed in Table 1.

2.5. Western blot assay

Total proteins collected by radioimmunoprecipitation (RIPA) lysis buffer (GD-Y2014, Shanghai Guduo Biotechnology Co., LTD, China) were isolated utilizing SDS-PAGE (WBK510-02, Shanghai Yongke Biotechnology Co., LTD, China), followed by shifting onto polyvinylidene fluoride (PVDF) membranes (WBF101, Shanghai Yongke Biotechnology Co., LTD, China). Then the membranes were treated with western blocking buffer (BP304, Shanghai Yongke Biotechnology Co., LTD, China) for 10 min and incubated with primary antibody (Table 2) overnight. Next, the membranes were incubated with second for 2 h antibody and protein bands were observed with enhanced chemiluminescence (ECL) kit (WBK101-01, Shanghai Yongke Biotechnology Co., LTD, China.

2.6. Immunofluorescence (IF)

The expression of neural-specific marker GFAP in hDPSCs was detected as described previously [31]. Simply, after 7 days of neural differentiation, the stable hDPSCs treated with Pg-LPS were immobilized with 4 % paraformaldehyde (P1110, Solarbio, China) for 30 min and permeated with 0.3 % Triton X-100 (T8200, Solarbio, China) for 10 min. The cells were then incubated with GFAP Rabbit mAb (A19058, Abclonal, China, 1:100) at 4 °C overnight. Next, the cells were treated with Goat anti-Rabbit IgG-AF488 Antibody (abs20025, Absin, China) for 2 h, followed by staining with 2 μg/ml DAPI dihydrochloride (C1002, Beyotime, China). Finally, the images were captured with confocal microscope (BZ-X, Keyence, Japan).

2.7. Reactive oxygen species (ROS) assay

The ROS levels in hDPSCs were measured with Reactive Oxygen Species Assay Kit (50101ES01, YESEN, China) abiding by the kit's protocol. The collected hDPSCs were incubated with 10 μM Dichloro-dihydro-fluorescein diacetate (DCFH-DA) for 15 min. Then the ROS content was observed by IF assay and the data were normalized with control group as the internal reference.

To block ROS signal, the hDPSCs were pre-treated with 5 mM Acetylcysteine (NAC, HY-B0215, MCE, USA) for 1 h.

2.8. Detection of oxidative stress indexes

The content of malondialdehyde (MDA), glutathione (GSH) and catalase (CAT) was estimated by Malondialdehyde (MDA) Content Assay Kit (BC0025, Solarbio, China), Reduced Glutathione (GSH) Content Assay Kit (BC1175, Solarbio, China) and Catalase (CAT) Activity Assay Kit (BC0205, Solarbio, China) according to the manufacturer's guidelines.

2.9. Measurement of total Ca^{2+} content

The stable hDPSCs treated with Pg-LPS were harvested and re-suspended in a new centrifuge tube. Then the 1 N Hcl (T8230, Solarbio, China) was added to the centrifuge tube and the Ca^{2+} level was detected with Calcium Assay kit (ab102505, Abcam, UK) abiding by the kit's instruction. Finally, a microplate reader (JC-MB36, Qingdao Juchuang Jiaheng Analytical Instrument Co., LTD, China) was employed to record the absorbance at 575 nm.

Table 1
Primers information.

Gene	Forward (5′-3′)	Reverse (5′-3′)
ARMCX3	GAGTGTGAATGCTGAAAATCAGCG	TCTCAGTCCAGCAAGCTGCACA
βIII-Tubulin	GGCCAAGGGTCACTACACG	GCAGTCGCAGTTTTCACACTC
GFAP	AACAACCTGGCTGCGTAT	ACTGCCTCGTATTGAGTGC
COX-2	TGAGTACCGCAAACGCTTCTC	TGGACGAGGTTTTTCCACCAG
iNOS	AGCAACTACTGCTGGTGGTG	TCTTCAGAGTCTGCCCATTG
β-actin	TAGCACAGCCTGGATAGCAACGTAC	CACCTTCTACAATGAGCT GCGTGTG

Table 2
Antibodies information.

Antibody	Item No.	Supplier	Country	Dilution
ARMCX3	25705-1-AP	Proteintech	USA	1:1000
β-actin	AC038	Abclonal	China	1:5000

2.10. Statistical analysis

The data were analyzed with Graphpad Prism 8.0 (GraphPad Software, USA) and presented as mean ± standard deviation (SD). Statistical differences were estimated with Student's t-test (two groups) or analysis of variance (ANOVA) (multiple groups). The $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ was considered as statistical significance.

3. Results

3.1. ARMCX3 knockdown facilitates neural differentiation of hDPSCs

First, hDPSCs were treated with Pg-LPS for 24 h to activate intracellular inflammatory state. Fig. 1A showed that Pg-LPS treatment significantly up-regulated the expression of ARMCX3 in hDPSCs ($***p < 0.001$) (Fig. 1A). To verify the detailed functions of ARMCX3 in hDPSCs, we designed stable ARMCX3-knockdown cell line ($***p < 0.001$) (Fig. 1B and C). As expected, the increased level of ARMCX3 in Pg-LPS treated hDPSCs was notably down-regulated by ARMCX3 knockdown ($***p < 0.001$) (Fig. 1D and E). Subsequently, the neural differentiation specific medium was employed to induce neural differentiation of hDPSCs. After 7 days, differentiated hDPSCs exhibited a distinct neuro-like vertebral shape with longer synapses. Besides, Pg-LPS treatment obviously blocked the neural differentiation of DPSCs, while ARMCX3 knockdown effectively reversed the effects of Pg-LPS on DPSC (Fig. 1F). Additionally, IF assay verified that Pg-LPS notably down-regulated the level of neural-specific marker GFAP, whereas ARMCX3 deficiency dramatically increased that in hDPSCs (Fig. 1G). Meanwhile, we measured the expression of GFAP and neuron specific marker βIII-Tubulin in hDPSCs after induction. Fig. 1H illustrated Pg-LPS treatment reduced the level GFAP and βIII-Tubulin during neural-like differentiation of hDPSCs, which was notably counteracted by ARMCX3 knockdown ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) (Fig. 1H). These findings indicated that ARMCX3 knockdown facilitated neural differentiation of hDPSCs under Pg-LPS treatment.

3.2. ARMCX3 knockdown reduces inflammation response in hDPSCs

Next, we estimated the expression level of pro-inflammatory cytokines (IL-6, IL-8 and TNF-α) produced by Pg-LPS-treated hDPSCs. Fig. 2A indicated that Pg-LPS promoted the secretion of pro-inflammatory cytokines, while ARMCX3 deficiency effectively decreased that in hDPSCs ($**p < 0.01$, $***p < 0.001$) (Fig. 2A). Additionally, further assay pointed out that ARMCX3 knockdown notably down-regulated the production of inflammation factors including COX2 and iNOS in hDPSCs in the presence of Pg-LPS ($**p < 0.01$, $***p < 0.001$) (Fig. 2B). These data demonstrated that ARMCX3 knockdown could control Pg-LPS-induced inflammation at a lower level in hDPSCs.

3.3. ARMCX3 knockdown suppresses ROS signal in hDPSCs

Since the oxidative stress induced by ROS production is closely associated with inflammation response [32–34], we asked whether ARMCX3 deficiency inhibits cytokine expression by decreasing ROS generation. As exhibited in Fig. 3A, Pg-LPS treatment up-regulated the level of ROS, which was notably offset through ARMCX3 knockdown in hDPSCs ($***p < 0.001$) (Fig. 3A). Besides, we evaluated the antioxidant status in Pg-LPS-treated hDPSCs by measuring the levels of GSH, CAT and MDA. The data indicated that Pg-LPS significantly reduced the content of GSH and antioxidant enzymes CAT, and up-regulated the production of MDA. However, knockdown of ARMCX3 exhibited the opposite results in hDPSCs ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) (Fig. 3B and C). Moreover, previous studies proved that the occurrence of oxidative stress is usually accompanied by Ca^{2+} overload [35,36]. As expected, Pg-LPS treatment increased the level of Ca^{2+}, whereas ARMCX3 deficiency effectively reduced that in hDPSCs ($**p < 0.01$, $***p < 0.001$) (Fig. 3D). These observations illustrated that ARMCX3 downregulation exert a distinct role in inhibiting ROS signal in Pg-LPS-treated hDPSCs.

3.4. Inhibition of ROS blocks the effects of ARMCX3 on neural differentiation of hDPSCs

To verify whether the roles of ARMCX3 in the neural differentiation of hDPSCs is dependent on ROS signal, we constructed stable ARMCX3-overexpression hDPSCs ($***p < 0.001$) (Fig. 4A and B). Subsequently, the cells were treated with NAC, the ROS inhibitor, to blocked ROS generation. As exhibited in Fig. 4C, the overexpressed ARMCX3 up-regulated the level of ROS in Pg-LPS-treated hDPSCs, which was notably reversed by NAC treatment ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) (Fig. 4C). Neural induction experiment showed that ARMCX3 overexpression further suppressed neural differentiation of hDPSCs under Pg-LPS treatment, while NAC treatment effectively restored differentiation ability of hDPSCs (Fig. 4D). Additionally, ARMCX3 upregulation dramatically reduced the expression of neuro-associated markers GFAP and βIII-Tubulin in Pg-LPS-treated hDPSCs, whereas the effects were also counteracted remarkably by NAC treatment ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) (Fig. 4E and F). All these findings suggested that ROS production

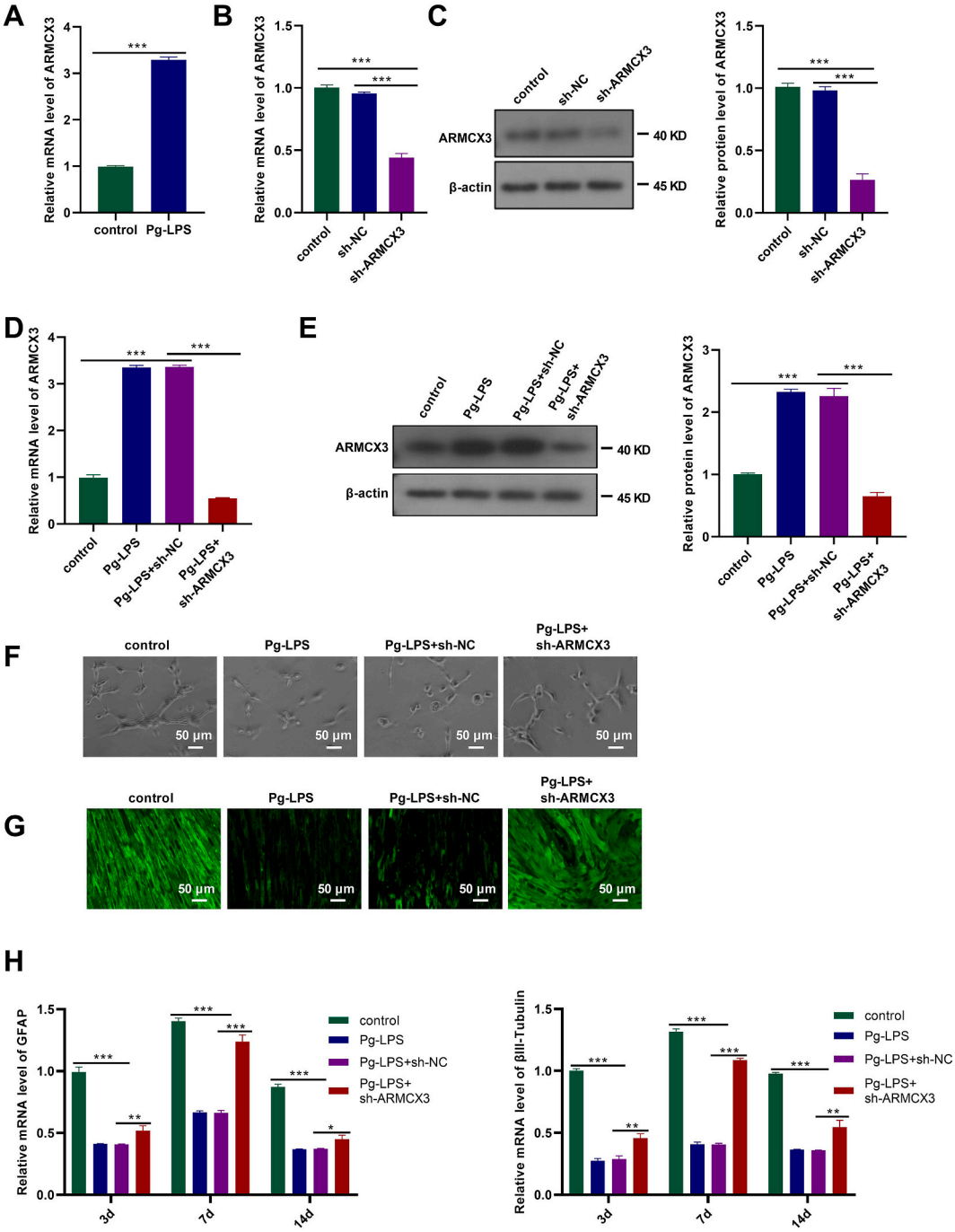

Fig. 1. ARMCX3 knockdown facilitates neural differentiation of hDPSCs. (A) The expression level of ARMCX3 in Pg-LPS-treated hDPSCs tested via qRT-PCR assay. (B–E) The expression level of ARMCX3 tested by qRT-PCR and Western blot assay (Figs. S2 and S3). (F) The detection of neural differentiation ability of Pg-LPS-treated hDPSCs. (G) The expression level of neural-specific marker GFAP (neural-specific marker) tested via IF assay. (H) The expression level of GFAP and βIII-Tubulin (neuron specific marker) tested via qRT-PCR assay. Scale bar = 50 μm $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

was closely involved in ARMCX3-mediated neural differentiation of hDPSCs.

3.5. Inhibition of ROS blocks the effects of ARMCX3 on inflammation microenvironment in hDPSCs

Furthermore, Fig. 5A proved that ARMCX3 overexpression further up-regulated the level of pro-inflammatory cytokines (IL-6, IL-8

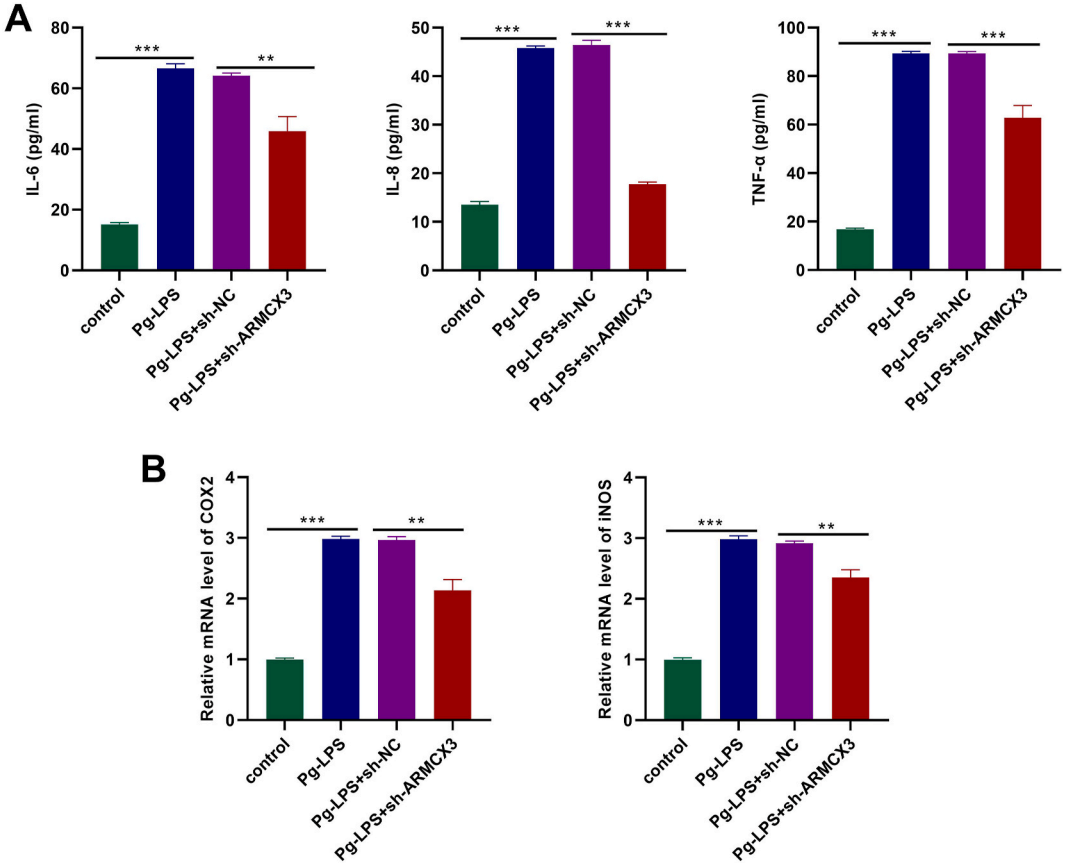

Fig. 2. ARMCX3 knockdown reduces inflammation response in hDPSCs. (A) The protein level of pro-inflammatory cytokines in Pg-LPS-treated hDPSCs determined by ELISA assay. (B) The mRNA level of COX2 and iNOS in Pg-LPS-treated hDPSCs tested via qRT-PCR assay. $^{**}p < 0.01$, $^{***}p < 0.001$.

and TNF-α) secreted through Pg-LPS-treated hDPSCs, which was effectively reversed by NAC treatment ($^{**}p < 0.01$, $^{***}p < 0.001$) (Fig. 5A). Meanwhile, NAC reduced the expression of COX2 and iNOS activated by ARMCX3 overexpression in hDPSCs under Pg-LPS treatment ($^{**}p < 0.01$, $^{***}p < 0.001$) (Fig. 5B). Taken together, these data verified that overexpressed ARMCX3 further triggered inflammation response in Pg-LPS-treated hDPSCs at least partly by ROS generation.

4. Discussion

Overall, this work firstly revealed the roles of ARMCX3 in hDPSCs. Specifically, ARMCX3 knockdown notably promoted neural differentiation and suppressed inflammation response in hDPSCs under Pg-LPS treatment. Besides, deletion of the ARMCX3 obviously reduced ROS production, while ARMCX3 overexpression exert a opposite role. Moreover, the inhibitor of ROS signal effectively reversed the effects of overexpressed ARMCX3 in neural differentiation and inflammation response in Pg-LPS-treated hDPSCs.

Dental pulp, the unmineralized oral tissue, is crucial to the sensation and defense reserve of teeth [37]. Promoting pulp repair can help restore the function of damaged teeth and avoid sequelae such as tooth loss [37,38]. However, there are still some knowledge gaps in the molecular mechanism of pulp inflammation, which needs to be further explored. Additionally, neurodegenerative diseases are regarded as a vital cause of disability and death [39]. Facial nerve is easily damaged and axon regeneration is difficult due to overstrain. Unsatisfactory treatment for nerve injury will seriously reduce patients' quality of life [40]. Hence, it is imperative to reveal the pathogenic mechanisms related to neurodegenerative diseases, which will have a great impact on human health in the future. Previous reports illustrated that pulp is innervated by a variety of nerves [41]. These nerve fibers can produce diverse signaling molecules to regulate tooth germ development. Additionally, dental pulp nerve regeneration plays a crucial role in pulp repair [42]. Emerging evidence revealed that dental pulp contains a variety of cells [43–45] including abundant stem cells [45]. Thereinto, DPSCs are isolated from the dental pulp tissue of the third molar [45], which exhibited rapid proliferation and strong self-renewal ability [46–48]. What's more, DPSCs have the potential for multidirectional differentiation, including odontoblast differentiation and neuronal differentiation [3,17]. Recent studies illustrated that DPSCs has neuroprotective properties and is a promising tool for treating neurodegenerative diseases as well as neurological disorders [3,49]. Therefore, exploring the molecular mechanism of neural differentiation of DPSCs is important not only for dental pulp injury, but also for various neurological diseases.

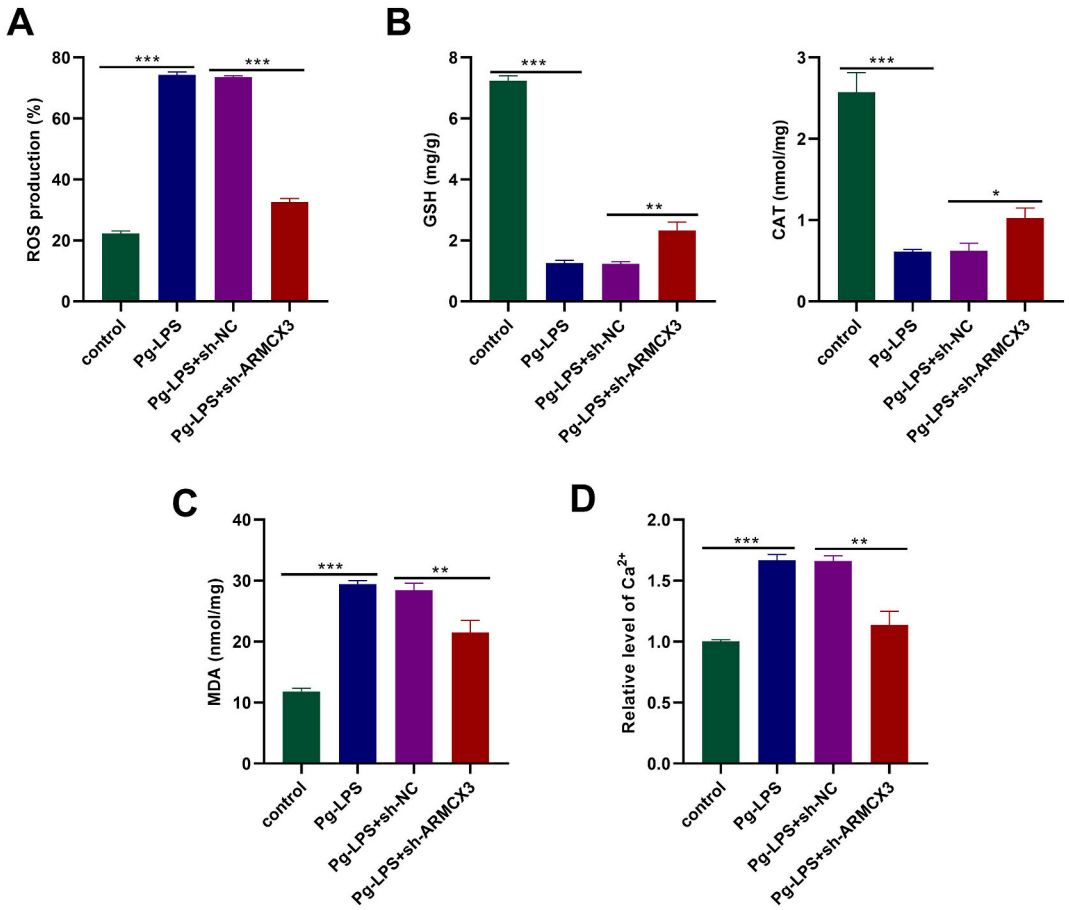

Fig. 3. ARMCX3 knockdown suppresses ROS signal in Pg-LPS-treated hDPSCs. (A) The production level of ROS in Pg-LPS-treated hDPSCs detected by IF assay. (B&C) The content of oxidative stress related indicators in Pg-LPS-treated hDPSCs detected by specific kits. (D) The level of Ca^{2+} in Pg-LPS-treated hDPSCs. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Inflammation is an indispensable stage in the process of pulp repair [50]. Accumulating evidence reported that excessive inflammation will cause irreparable injury to pulp tissue [51]. It is vital to control the inflammatory response at a lower level for regulating osteogenic differentiation of DPSCs and pulp repair [6–8]. For example, Chen et al. revealed that Resolvin E1 facilitates pulp repair by accelerating inflammation resolution [16]. Liang et al. found that circFKBP5 inhibits apoptosis as well as inflammation, thereby promoting osteogenic differentiation of DPSCs [52]. However, the detailed roles of inflammation in neural differentiation of DPSCs is still less studied. Interestingly, we constructed an *in vitro* inflammatory model by treating hDPSCs with 1 μg/ml Pg-LPS, and discovered that Pg-LPS treatment notably inhibited the neural differentiation of hDPSCs. The related molecular mechanism still needs to be further explored.

ARMCX3 is a recently reported member of the Armcx family [24,53], and there are few studies on its roles. Recent reporters revealed that ARMCX3 is involved in the processes of various diseases, such as obesity [54] and non-small cell lung cancer [25]. Bioinformatics analysis found that ARMCX3 expression was up-regulated in inflammatory periodontal tissues, suggesting that ARMCX3 might be associated with the process of pulp repair. Additionally, it has been reported that ARMCX3 exerts an obvious high expression in neurons and regulates the function of neuronal mitochondria [24,53]. Emerging evidence proved that ARMCX3 inhibited the proliferation of neurotropic progenitor cells by targeting Wnt signals, ultimately affecting the development of the chicken spinal cord [55]. More interesting, we discovered that Pg-LPS treatment notably increased the expression of ARMCX3 in hDPSCs. These findings aroused our interest in exploring the roles of ARMCX3 in inflammation microenvironment and neural differentiation in DPSCs.

GFAP is a specific protein of astrocytes, which exerts a series of functions in the central nervous system [56]. βIII-Tubulin is a marker of neurons, expressed in the early process of brain development [57]. Therefore, GFAP and βIII-Tubulin are commonly used to detect neural differentiation. This work proved that knockdown of ARMCX3 effectively up-regulated the expression level of GFAP and βIII-Tubulin in Pg-LPS-treated hDPSCs, while ARMCX3 overexpression played the opposite role. These results suggested that ARMCX3 knockdown promoted neural differentiation of hDPSCs in the presence of Pg-LPS. Besides, our findings also hinted that ARMCX3 might be involved in the development of neurons and even the central nervous system. In the future study, we will interfere with ARMCX3

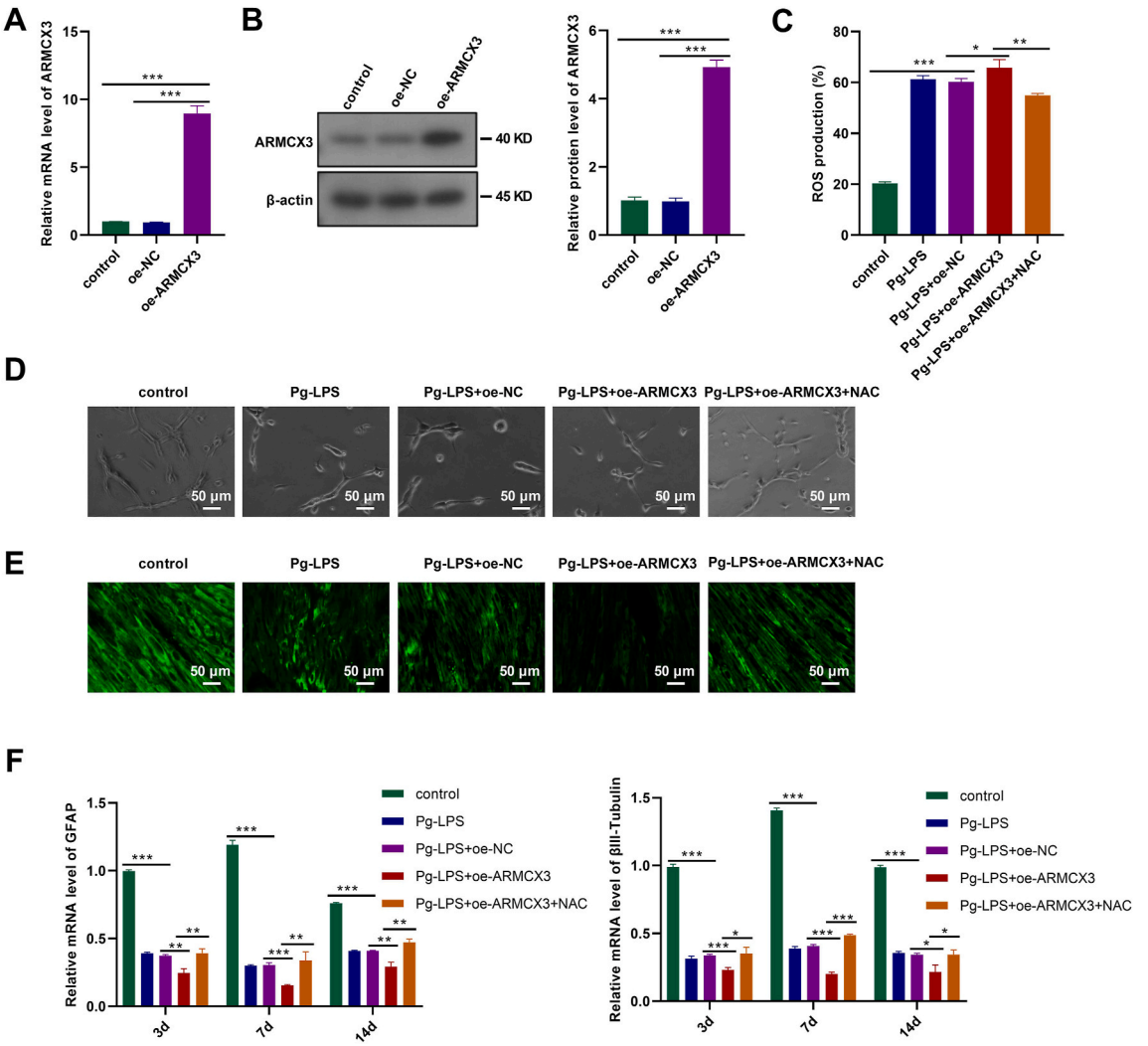

Fig. 4. Inhibition of ROS blocks the effects of ARMCX3 on neural differentiation of hDPSCs. (A&B) The expression level of ARMCX3 tested by qRT-PCR and Western blot assay (Fig. S4). (C) The production level of ROS detected by IF assay. (D) The detection of neural differentiation ability of hDPSCs. (E) The expression level of GFAP (neural-specific marker) tested via IF assay. (F) The expression level of GFAP and βIII-Tubulin (neuron specific marker) tested via qRT-PCR assay. Scale bar = 50 μm $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

expression in neurons to verify the roles of ARMCX3 in the development of the central nervous system. Moreover, ARMCX3 deficiency also suppressed inflammation response induced by Pg-LPS in hDPSCs, indicating that ARMCX3 participated in the regulation of the inflammatory microenvironment.

It is well known that excess ROS production leads to oxidative stress, closely linked to inflammatory responses in various diseases [34,58,59]. Besides, LPS can activate both inflammatory reaction and ROS generation remarkably [34], which is consistent with our present results. Moreover, ARMCX3 knockdown significantly reduced ROS level, up-regulated the expression of CAT (antioxidant enzyme) and GSH (non-enzymatic antioxidant) in hDPSCs. Meanwhile, the ARMCX3 down-regulation obviously decreased the level of Ca^{2+} triggered by ROS generation. These data implied that ARMCX3 might exerts its role in hDPSCs by ROS signal. To verify the hypothesis, we introduced NAC, a vital inhibitor of ROS signal, to treat hDPSCs that overexpress ARMCX3 in the presence of Pg-LPS. The results showed that after blocking ROS production, the inhibitory effects of ARMCX3 overexpression on neural differentiation also partially disappeared. Furthermore, ROS inhibition effectively abolished inflammation cytokines induced through ARMCX3 over-expression in Pg-LPS-treated hPDSC. This result verified that ARMCX3 affected inflammation microenvironment by mediating ROS signal. Collectively, our data established that ARMCX3 inhibited neural differentiation and enhanced inflammation response in hPDSCs via ROS generation.

Nevertheless, there are several limitations in this work. For example, it is unclear whether ARMCX3 regulates the differentiation in other directions of hPDSCs. We will induce hDPSCs differentiation in different directions in the absence of ARMCX3 to further investigate the effect of ARMCX3 on hDPSCs development. Besides, the molecular network through which ARMCX3 regulates neural

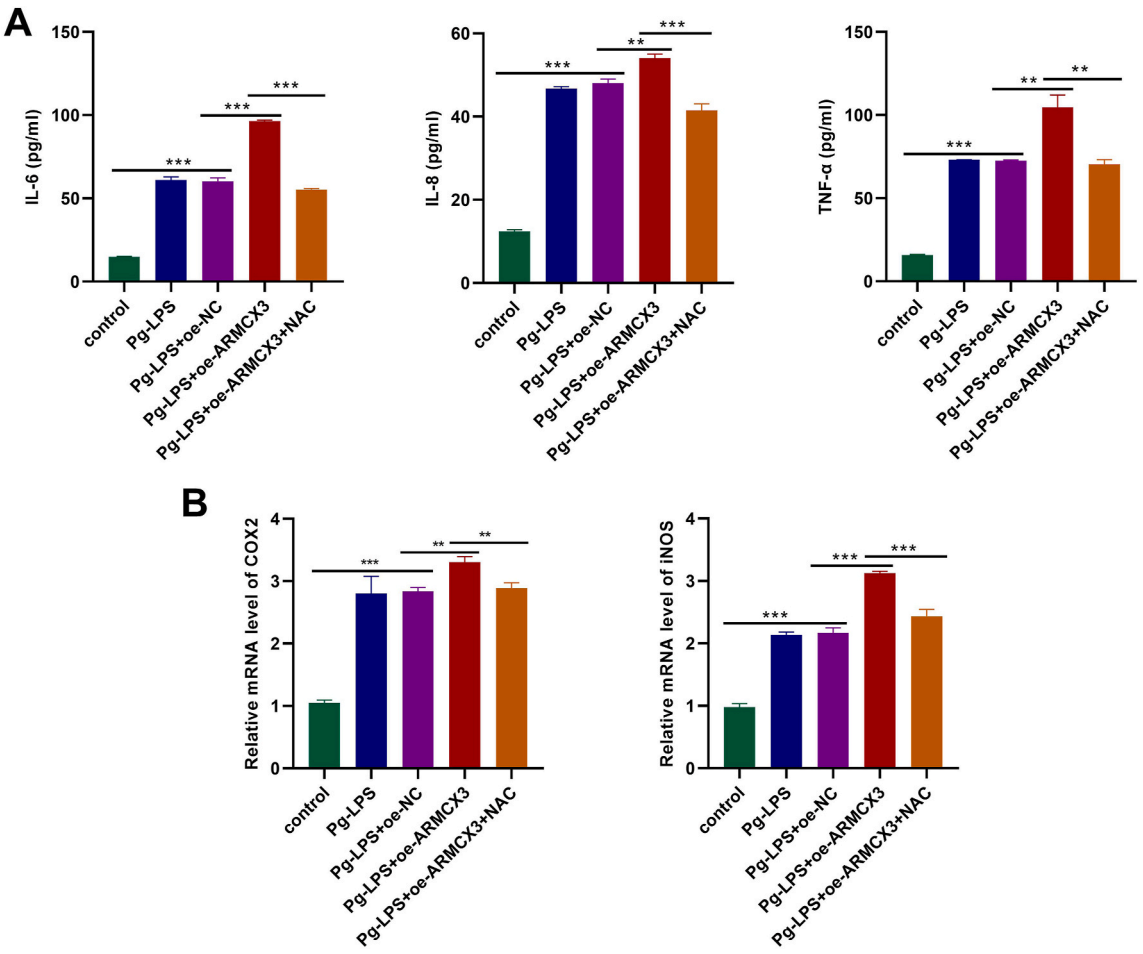

Fig. 5. Inhibition of ROS blocks the effects of ARMCX3 on inflammation microenvironment in hDPSCs. (A) The expression level of pro-inflammatory cytokines in Pg-LPS-treated hDPSCs determined by ELISA assay. (B) The mRNA level of COX2 and iNOS in Pg-LPS-treated hDPSCs tested via qRT-PCR assay. $^{**}p < 0.01$, $^{***}p < 0.001$.

differentiation of hDPSCs is unknown. More experiments will be conducted to uncover signaling pathways and downstream targets related to the role of ARMCX3 in neural differentiation of hDPSCs.

5. Conclusion

In summary, this study verified for the first time that ARMCX3 deficiency accelerated neural differentiation and suppressed the inflammatory microenvironment in hPDSC at least partly by mediating ROS signaling. These findings provided a promising therapeutic target for the treatment of pulpitis and diverse neurological diseases.

Data availability statement

Data included in article/supp. material/referenced in article.

Fundings

None.

CRediT authorship contribution statement

Quanying Zhou: Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Yi Lei:** Writing – review & editing, Project administration, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37079.

References

[1] S. Yi, et al., Application of stem cells in peripheral nerve regeneration, Burns Trauma 8 (2020) tkaa002.
[2] A.A. Al-Maswary, et al., Exploring the neurogenic differentiation of human dental pulp stem cells, PLoS One 17 (11) (2022) e0277134.
[3] P. Prateeksha, et al., KLF2 regulates neural differentiation of dental pulp-derived stem cells by modulating autophagy and mitophagy, Stem Cell Rev Rep 19 (8) (2023) 2886–2900.
[4] H. Zhou, et al., Metal-organic framework materials promote neural differentiation of dental pulp stem cells in spinal cord injury, J. Nanobiotechnol. 21 (1) (2023) 316.
[5] N. Kaukua, et al., Glial origin of mesenchymal stem cells in a tooth model system, Nature 513 (7519) (2014) 551–554.
[6] M. Song, et al., Human dental pulp stem cells are more effective than human bone marrow-derived mesenchymal stem cells in cerebral ischemic injury, Cell Transplant. 26 (6) (2017) 1001–1016.
[7] M. Irfan, S. Chung, C5L2 modulates BDNF production in human dental pulp stem cells via p38α pathway, Sci. Rep. 13 (1) (2023) 74.
[8] B. Liu, et al., Dental pulp stem cells induce anti-inflammatory phenotypic transformation of macrophages to enhance osteogenic potential via IL-6/GP130/STAT3 signaling, Ann. Transl. Med. 11 (2) (2023) 90.
[9] K. Rihawi, et al., Tumor-associated macrophages and inflammatory microenvironment in gastric cancer: novel translational implications, Int. J. Mol. Sci. 22 (8) (2021).
[10] R. Carloni, et al., Targeting tumor microenvironment for cholangiocarcinoma: opportunities for precision medicine, Transl Oncol 25 (2022) 101514.
[11] A. Di Federico, et al., Immunotherapy in pancreatic cancer: why do we keep failing? A focus on tumor immune microenvironment, predictive biomarkers and treatment outcomes, Cancers 14 (10) (2022).
[12] Y. Gu, et al., The proteomic characterization of the peritumor microenvironment in human hepatocellular carcinoma, Oncogene 41 (17) (2022) 2480–2491.
[13] Z. Tan, et al., The role of tumor inflammatory microenvironment in lung cancer, Front. Pharmacol. 12 (2021) 688625.
[14] M.P. De Miguel, et al., Immunosuppressive properties of mesenchymal stem cells: advances and applications, Curr. Mol. Med. 12 (5) (2012) 574–591.
[15] M.A. Cassatella, et al., Toll-like receptor-3-activated human mesenchymal stromal cells significantly prolong the survival and function of neutrophils, Stem Cell. 29 (6) (2011) 1001–1011.
[16] J. Chen, et al., Resolvin E1 accelerates pulp repair by regulating inflammation and stimulating dentin regeneration in dental pulp stem cells, Stem Cell Res. Ther. 12 (1) (2021) 75.
[17] W. Kang, et al., TAS2R supports odontoblastic differentiation of human dental pulp stem cells in the inflammatory microenvironment, Stem Cell Res. Ther. 13 (1) (2022) 374.
[18] H. Hong, et al., The pluripotent factor OCT4A enhances the self-renewal of human dental pulp stem cells by targeting lncRNA FTX in an LPS-induced inflammatory microenvironment, Stem Cell Res. Ther. 14 (1) (2023) 109.
[19] W. Chen, SNHG7 promotes the osteo/dentinogenic differentiation ability of human dental pulp stem cells by interacting with hsa-miR-6512-3p in an inflammatory microenvironment, Biochem. Biophys. Res. Commun. 581 (2021) 46–52.
[20] P.R. Cooper, et al., Inflammation-regeneration interplay in the dentine-pulp complex, J. Dent. 38 (9) (2010) 687–697.
[21] R. Ran, et al., Depletion of EREG enhances the osteo/dentinogenic differentiation ability of dental pulp stem cells via the p38 MAPK and Erk pathways in an inflammatory microenvironment, BMC Oral Health 21 (1) (2021) 314.
[22] H.M. Hu, et al., Artemisinin protects DPSC from hypoxia and TNF-α mediated osteogenesis impairments through CA9 and Wnt signaling pathway, Life Sci. 277 (2021) 119471.
[23] Y.K. Liu, Z.Y. Zhou, F. Liu, Transcriptome changes during TNF-α promoted osteogenic differentiation of dental pulp stem cells (DPSCs), Biochem. Biophys. Res. Commun. 476 (4) (2016) 426–430.
[24] G. López-Doménech, et al., The Eutherian Armcx genes regulate mitochondrial trafficking in neurons and interact with Miro and Trak2, Nat. Commun. 3 (2012) 814.
[25] J. Du, et al., Alex3 suppresses non-small cell lung cancer invasion via AKT/Slug/E-cadherin pathway, Tumour Biol 39 (7) (2017) 1010428317701441.
[26] S. Mirra, et al., ARMCX3 mediates susceptibility to hepatic tumorigenesis promoted by dietary lipotoxicity, Cancers 13 (5) (2021).
[27] L. Pang, et al., ALEX1, a novel tumor suppressor gene, inhibits gastric cancer metastasis via the PAR-1/Rho GTPase signaling pathway, J. Gastroenterol. 53 (1) (2018) 71–83.
[28] J. Liu, et al., Screening of crosstalk and pyroptosis-related genes linking periodontitis and osteoporosis based on bioinformatics and machine learning, Front. Immunol. 13 (2022) 955441.
[29] R. Arruda-Vasconcelos, et al., Investigation of microbial profile, levels of endotoxin and lipoteichoic acid in teeth with symptomatic irreversible pulpitis: a clinical study, Int. Endod. J. 54 (1) (2021) 46–60.
[30] F. Xu, et al., The potential application of concentrated growth factor in pulp regeneration: an in vitro and in vivo study, Stem Cell Res. Ther. 10 (1) (2019) 134.
[31] W. Zhou, et al., TIGAR promotes neural stem cell differentiation through acetyl-CoA-mediated histone acetylation, Cell Death Dis. 10 (3) (2019) 198.
[32] W. Mao, et al., RORα suppresses cancer-associated inflammation by repressing respiratory complex I-dependent ROS generation, Int. J. Mol. Sci. 22 (19) (2021).
[33] N. Das, et al., Intra-tumor ROS amplification by melatonin interferes in the apoptosis-autophagy-inflammation-EMT collusion in the breast tumor microenvironment, Heliyon 10 (1) (2024) e23870.
[34] O.J. Sul, S.W. Ra, Quercetin prevents LPS-induced oxidative stress and inflammation by modulating NOX2/ROS/NF-kB in lung epithelial cells, Molecules 26 (22) (2021).
[35] N. Zheng, et al., Oxidative stress-induced cardiomyocyte apoptosis is associated with dysregulated Akt/p53 signaling pathway, J. Recept. Signal Transduct. Res. 40 (6) (2020) 599–604.

[36] X. Yao, et al., Hsp90 protected chicken primary myocardial cells from heat-stress injury by inhibiting oxidative stress and calcium overload in mitochondria, Biochem. Pharmacol. 209 (2023) 115434.
[37] L. Zhu, et al., In vitro and in vivo evaluation of a nanoparticulate bioceramic paste for dental pulp repair, Acta Biomater. 10 (12) (2014) 5156–5168.
[38] M. Dalli, H. Çolak, M. Mustafa Hamidi, Minimal intervention concept: a new paradigm for operative dentistry, J Investig Clin Dent 3 (3) (2012) 167–175.
[39] Global, regional, and National burden of neurological disorders, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016, Lancet Neurol. 18 (5) (2019) 459–480.
[40] W. Xu, et al., VEGFA-modified DPSCs combined with LC-YE-PLGA NGCs promote facial nerve injury repair in rats, Heliyon 9 (4) (2023) e14626.
[41] K. Moe, et al., Development of the pioneer sympathetic innervation into the dental pulp of the mouse mandibular first molar, Arch. Oral Biol. 53 (9) (2008) 865–873.
[42] Q. Han, et al., Nell-1 promotes the neural-like differentiation of dental pulp cells, Biochem. Biophys. Res. Commun. 513 (2) (2019) 515–521.
[43] A. Al Madhoun, et al., Dental pulp stem cells derived from adult human third molar tooth: a brief review, Front. Cell Dev. Biol. 9 (2021) 717624.
[44] F. Diomede, et al., Decellularized dental pulp, extracellular vesicles, and 5-azacytidine: a new tool for endodontic regeneration, Biomedicines 10 (2) (2022).
[45] M. Tatullo, et al., Dental pulp stem cells: function, isolation and applications in regenerative medicine, J Tissue Eng Regen Med 9 (11) (2015) 1205–1216.
[46] Z. Xiao, et al., The potential therapy with dental tissue-derived mesenchymal stem cells in Parkinson's disease, Stem Cell Res. Ther. 12 (1) (2021) 5.
[47] V. Mattei, et al., Role of lipid rafts in neuronal differentiation of dental pulp-derived stem cells, Exp. Cell Res. 339 (2) (2015) 231–240.
[48] S. Aydin, F. Şahin, Stem cells derived from dental tissues, Adv. Exp. Med. Biol. 1144 (2019) 123–132.
[49] B.C. Heng, et al., EphrinB2 signalling modulates the neural differentiation of human dental pulp stem cells, Biomed Rep 9 (2) (2018) 161–168.
[50] D.K. Rechenberg, J.C. Galicia, O.A. Peters, Biological markers for pulpal inflammation: a systematic review, PLoS One 11 (11) (2016) e0167289.
[51] M. Irfan, et al., The role of complement C5a receptor in DPSC odontoblastic differentiation and in vivo reparative dentin formation, Int. J. Oral Sci. 14 (1) (2022) 7.
[52] C. Liang, et al., CircFKBP5 suppresses apoptosis and inflammation and promotes osteogenic differentiation, Int. Dent. J. 73 (3) (2023) 377–386.
[53] R. Serrat, et al., The non-canonical Wnt/PKC pathway regulates mitochondrial dynamics through degradation of the arm-like domain-containing protein Alex3, PLoS One 8 (7) (2013) e67773.
[54] A. Gavaldà-Navarro, et al., The armadillo-repeat containing X-linked protein 3, ARMCX3, is a negative regulator of the browning of adipose tissue associated with obesity, Int. J. Obes. 46 (9) (2022) 1652–1661.
[55] S. Mirra, et al., Function of Armcx3 and Armc10/SVH genes in the regulation of progenitor proliferation and neural differentiation in the chicken spinal cord, Front. Cell. Neurosci. 10 (2016) 47.
[56] E.M. Hol, M. Pekny, Glial fibrillary acidic protein (GFAP) and the astrocyte intermediate filament system in diseases of the central nervous system, Curr. Opin. Cell Biol. 32 (2015) 121–130.
[57] Y.Q. Jiang, M.M. Oblinger, Differential regulation of beta III and other tubulin genes during peripheral and central neuron development, J. Cell Sci. 103 (Pt 3) (1992) 643–651.
[58] R. Kang, et al., Deoxynivalenol induced apoptosis and inflammation of IPEC-J2 cells by promoting ROS production, Environ. Pollut. 251 (2019) 689–698.
[59] H.S. Park, S.R. Kim, Y.C. Lee, Impact of oxidative stress on lung diseases, Respirology 14 (1) (2009) 27–38.