ORIGINAL ARTICLE

Oestrogen receptor α regulates the odonto/osteogenic differentiation of stem cells from apical papilla via ERK and JNK MAPK pathways

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Abstract

Objectives: Oestrogen receptor (ER) is a common nucleus receptor that is essential for the regulation of cell growth, proliferation and differentiation. This study was to examine whether ER α can affect the proliferation and odonto/osteogenic differentiation of stem cells from apical papilla (SCAPs).

Materials and Methods: Stem cells from apical papillas were isolated, purified and then transfected with ER α lentiviruses. The proliferation capacity was investigated by cell counting kit-8 (CCK-8) assay and flow cytometry. The odonto/osteogenic differentiation ability was analysed by alkaline phosphatase (ALP) activity, alizarin red staining, western blot assay (WB) and real-time RT-PCR. MAPK pathway and its downstream transcriptional factors were explored by WB assay.

Results: As indicated by CCK-8 assay and flow cytometry, ER α had no significant effect on the proliferation of SCAPs. When ER α was overexpressed, the ALP activity and the formation of calcified nodules were significantly enhanced in SCAPs. Moreover, the odonto/osteogenic markers (DMP1/DMP1, DSPP/DSP, RUNX2/RUNX2, OCN/OCN) in SCAPs were significantly up-regulated at both mRNA and protein levels. On the contrary, the odonto/osteogenic differentiation ability of SCAPs was remarkably inhibited after suppression of ER α . Mechanistically, the protein levels of phosphorylated ERK and JNK significantly increased after ER α overexpression. Moreover, some downstream transcriptional factors of MAPK pathway were simultaneously activated by ER α overexpression.

Conclusions: Together, the data accumulated here indicated that ER α can enhance the odonto/osteogenic differentiation of SCAPs via ERK and JNK MAPK pathways.

1 | INTRODUCTION

Regenerative medicine is recently concentrating on guiding differentiation of stem cells into lineage-specific, functional cells which can create and repair damaged tissues.¹ Stem cell therapies are attractive for treating a large amount of diseases, including oral diseases.²

Stem cells from apical papilla (SCAPs) are a population of stem/progenitor cells residing in the apical papilla of developing human permanent teeth.³ They can differentiate into neurocyte, adipocyte and osteoblast lineages in vitro under proper conditions, and has been reported to have effects on neuronal repair.⁴ SCAPs have been shown to form more uniform dentin-like tissue and possess much higher proliferation rate, dentinogenic capacity than other dental stem cells, being potentially a preferable cell source for tooth

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regeneration.^{5,6} SCAPs can give rise to primary odontoblasts, which are responsible for the formation of primary root dentin during the odontogenesis.⁵⁻⁷ Apical infection caused by dental caries, trauma and periodontal diseases may block the tooth development.⁸ Some documents have found that SCAPs may still exist and work at the site of the root development process during infections.⁹ In vivo studies, SCAPs have been proved to form a dentin/pulp complex when transplanted with HA/TCP particles in immunodeficient mice.⁵ It is reported that SCAPs could also be used in combination with periodontal ligament stem cells to form a bioroot.¹⁰

A wide array of biochemical (growth factors, hormones and microRNAs) and biophysical (electrical, magnetic and ultraviolet ray) elements are currently being investigated in vitro for their capacity to aid stem cell regeneration.¹¹ It is well established that oestrogen plays an important role in affecting growth, differentiation and function in multiple systems and organs in the organism.¹² The predominant biological effects of oestrogen are generally exerted by activation of oestrogen receptor (ER).¹³ ER is a member of the receptor superfamily, and it is an oestrogen-dependent transcription factor which accounts for the regulation of bone metabolism.¹⁴ Some studies have found that the expression of ER is up-regulated during the osteogenic differentiation of rat calvaria osteoblasts.¹⁵ ER is mainly divided into 2 types (ie ER α and ER β) and ER β exhibits a more limited expression pattern.¹⁶ ER α has the ability to decrease bone turnover and increase trabecular bone volume in male mice.¹⁷ Oestrogen and ER axis is multiply reported to have effects on the proliferation, apoptosis and differentiation of mesenchymal stem cells (MSCs).¹⁸ Some studies have proved that ER can activate related signalling pathways (Wnt/ β -catenin, mTOR and PI3K/Akt/STAT3) to regulate the proliferation-associated genes of MSCs.^{19,20} Researches suggest that oestrogen is involved in the differentiation of bone marrow MSCs¹³ and dental MSCs-mediated morphogenesis of tooth.²¹

Our previous studies have detected the increased expression of ER α in the process of oestrogen-mediated differentiation of SCAPs.²² However, the exact effects of ER α on SCAPs and the underlying mechanisms have not been elucidated. In this work, we investigated the effects of ER α in SCAPs and demonstrated that ER α can promote the odonto/osteogenic differentiation of SCAPs by activating ERK and JNK MAPK pathways.

2 | MATERIALS AND METHODS

2.1 | Cell isolation and culture

Human third molars with immature roots were collected from donors (17-20 years old) at the Oral and Maxillofacial Surgery Department of Jiangsu Provincial Stomatological Hospital. The experimental procedures were approved by the Ethical Committee of Stomatological School of Nanjing Medical University and performed with the informed consent of the patients. The apical papilla was softly isolated from surfaces of roots and then minced and digested in a solution of 3 mg/mL type I collagenase (Gibco, Life Technologies, MA, USA) and 4 mg/mL trypsin (Gibco, Life Technologies) for 1 hour at 37°C. The

dissolved tissues were evenly plated into 6 cm culture dishes, then incubated in alpha minimum essential medium (α -MEM, Gibco, Life Technologies) supplemented with 10% foetal bovine serum (FBS, Gibco, Life Technologies), 100 g/mL streptomycin and 100 U/mL penicillin at 37°C in 5% CO₂. The media were changed after 24 hours thereafter every 2 days. After 7-10 days, the attached cells were collected and purified by the procedure of magnetic activated cell sorting as previously described.²³ Cells were passaged by 0.25% trypsin at a 1:3 split ratio after the cell density came up to 80%-85%. The experiments were carried out by using SCAPs within 3-5 passages.

2.2 | Flow cytometry

Enzyme digestion was used to collect SCAPs at the density of 1×10^4 cells/well. Then, cells were rinsed with 0.01 mol/L PBS twice and resuspended in 100 μ L wash buffer. After adding the primary antibodies (STRO-1, CD34, CD45, CD73, CD90, CD105) into each solution, the samples were incubated for 15 minutes at room temperature in the dark. Stained cells were washed twice with 0.01 mol/L PBS and analysed by FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). To investigate the effects of ER α on the proliferation of SCAPs, transfected cells were collected by using trypsin (Beyotime, Haimen, China) and fixed with 75% ice-cold ethanol at 4°C for 24 hours away from the light. Then, each sample was analysed by FACScan flow cytometer (BD Biosciences).

2.3 | Plasmid construction

The PLKO.1/ER α -shRNA plasmid, envelope plasmid (VSVG) and packaging plasmid (drpr) were mixed according to the ratio of 6.67: 2: 5 μ g, and then transfected 293T cells. After 48 hours, the viral supernatant was collected, centrifugated for 5 minutes at the speed of 2000 g, and at last stored under -70°C. The PMSCV-GFP/ER α plasmid, envelope plasmid (VSVG) and packaging plasmid (drpr) were mixed according to the ratio of 1.08 μ g: 0.27 μ g: 0.81 μ g, and then transfected 293T cells. After 72 hours, the viral supernatant was collected, centrifugated for 5 minutes at the speed of 2000 g, and at last stored under -70°C.

2.4 | Cell transfection

SCAPs cultured at 60%-70% confluence were transfected with the lentiviruses in 1 mL α -MEM supplemented with 10% FBS and 8 μ g/mL polybrene (Santa Cruz, Delaware Ave, Santa Cruz, CA, USA). 10 hours later, the infected cells were incubated in replaced conventional media. After 3 days, the antibiotic-resistant transfected cells were selected by applying puromycin in culture medium and passaged at the ratio of 1:3 for further use.

2.5 | Cell counting kit-8 assay

The proliferation ability of transfected SCAPs was detected by using the cell counting kit-8 (CCK-8, Dojindo, Tokyo, Japan) according to

the manufacturer's protocol. Cells were cultured in a 96-well plate at an initial density of 2×10^3 cells per well. Then, the complete medium was changed for the serum-free medium when cells adhered to the wall. At day 0, 1, 3, 5, 7 and 9, SCAPs were treated with CCK-8 regents at 37°C for 2 hours, and then the OD values were determined at 450 nm by a microplate reader (Bio-Tek, Vermont, USA).

2.6 | Alkaline phosphatase activity

Transfected SCAPs were plated at a density of 1×10^4 cells/well into 6-well plates (Corning, Life Sciences, NY, USA). Cells were cultured for 5 days and changed for fresh medium every other day. Then, they were rinsed with PBS for 3 times and incubated with 50 μ L 0.2% Triton X-100 for 2 hours at 37°C. The ALP activity was detected by using an ALP activity assay kit (Jiancheng, Nanjing, China) according to the manufacturer's instructions. The normalization of ALP activity to total protein was determined with a BCA kit (Beyotime, Shanghai, China).

2.7 | Alizarin red staining

The mineralization ability of transfected SCAPs in osteogenic induction group and uninduced group was investigated. Briefly, well-grown cells were plated into 6-well plates (Corning, Life Sciences). Then, cells were cultured in a mineralization-inducing medium containing α -MEM, 10% FBS, 100 U/mL penicillin, 100 g/mL streptomycin, 2 mmol/L L-glutamine (Sigma-Aldrich, Munich, Germany), 50 mg/L ascorbic acid (Sigma-Aldrich), 10 mmol/L β -glycerophosphate (Sigma-Aldrich) and 10 nmol/L dexamethasone (Sigma-Aldrich). Cells were fixed in ice-cold 70% ethyl alcohol for 30 minutes at room temperature after 2 weeks. Then cells were rinsed with deionized water and dyed with 100 μ mol L⁻¹ alizarin red (pH = 4.2, Sigma-Aldrich) for 2 minutes. To quantify the mineralization nodules, the alizarin red was dissolved with 10% cetylpyridinium chloride (CPC, Sigma-Aldrich) in 10 mmol/L sodium phosphate for 30 minutes at 25°C. The absorbances were respectively read at a wavelength of 560 nm using a microplate reader (Bio-Tek). The final calcium concentrations in each group were normalized with the total protein concentrations.

2.8 | Real-time reverse transcription polymerase chain reaction

Total RNA was extracted from SCAPs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The mRNA was subjected to reverse transcription by a PrimeScript RT Master Mix kit (TaKaRa, Dalian, China). Real-time RT-PCR was performed to analyse gene expression changes using SYBR Premix Ex Taq kit (TaKaRa Bio, Otsu, Japan) and ABI 7300 real-time PCR system. All primers were synthesized by the same manufacturer (Sangon Biotech, Shanghai, China). Table 1 showed the sense and antisense primers used for detection of the mRNA expression levels of *ER α* , *OCN*, *RUNX2*, *DSPP* and *DMP1*. Real-time RT-PCR reaction conditions

TABLE 1 Sense and antisense primers for real-time reverse transcription polymerase chain reaction

Genes	Primers	Sequences (5'-3')
<i>ERα</i>	Forward	ACTCAACAGCGTGTCTCCG
	Reverse	GGGCTCGTTCTCCAGGTAG
<i>DMP1</i>	Forward	TTTATAGGAAGTCTCGCATCT
	Reverse	TGGGACCATCTACGTTTT
<i>DSPP</i>	Forward	CGTTCAGGGAGTCCTAGCGGGAACG
	Reverse	GTGACTCTCCCTTCCATCTCTCTG
<i>RUNX2</i>	Forward	GAATGCCTCTGCTGTTATG
	Reverse	ACTCTTGCCCTCGTCCACT
<i>OCN</i>	Forward	AGCAAAGGTGCAGCCTTTGT
	Reverse	GCGCCTGGGTCTCTTCACT
<i>GAPDH</i>	Forward	GAAGGTGAAGGTCGGAGTC
	Reverse	GAGATGGTGATGGGATTTC

were: 95°C for 30 s; followed by 40 cycles of 95°C for 5 s; 60°C for 31 s. mRNA levels were normalized to the amount of *GAPDH* (glyceralde-hyde-3-phosphate dehydrogenase). Relative gene expression values were calculated by the $2^{-\Delta\Delta C_t}$ method as previously reported.^{21,22} Each experiment was repeated independently at least 3 times.

2.9 | Western blot

To measure the expression of MAPK pathway-related proteins, transfected SCAPs at 48 hours were collected to obtain the cytoplasmic and nuclear protein with a Keygen Kit (Keygen Bio-Tech, Nanjing, China). To examine effects of *ER α* on the odonto/osteogenic differentiation of SCAPs, transfected cells were collected at day 0, 3 and 7 respectively. After washing thrice by PBS, SCAPs were lysed in RIPA buffer (Beyotime). The same amount of protein was subject to a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF, Millipore, Darmstadt, Germany) membranes. The transblotted membranes were blocked in 5% BSA for 2 hours at room temperature followed by an incubation with primary antibodies including *DMP1* (NBP1-45525, Novus, Littleton, USA), *RUNX2* (ab76956, Abcam, Cambridge, UK), *DSP* (sc-33586, Santa Cruz), *OCN* (ab93876, Abcam), *ERK* (#4695, Cell Signaling Technology), *p-ERK* (#4370, Cell Signaling Technology, Danvers, MA, USA), *JNK* (#9252, Cell Signaling Technology), *p-JNK* (#9255, Cell Signaling Technology), *P38* (#8690, Cell Signaling Technology), *p-P38* (#4511, Cell Signaling Technology), *c-Fos* (#2250, Cell Signaling Technology), *p-c-Fos* (#5348, Cell Signaling Technology), *c-Jun* (#9165, Cell Signaling Technology), *p-c-Jun* (#9261, Cell Signaling Technology), *Elk1* (#9182, Cell Signaling Technology), *p-Elk1* (#9186, Cell Signaling Technology), *H3* (#9728, Cell Signaling Technology) and β -ACTIN (AP0060, Bioworld, Nanjing, China) at 4°C overnight. After rinsed thrice in TBST for 30 minutes, the membranes were incubated with secondary antibodies for 1 hour at 4°C, and antibody-antigen reaction was detected using Western Blotting Imaging System.

FIGURE 1 Characterization of SCAPs and ER α expression in transfected SCAPs. A, Flow cytometry revealed that SCAPs were positive for STRO-1, CD73, CD90 and CD105 and negative against CD34 and CD45. B, The ER α expression at day 3 in Scramble group, sh-ER α group, GFP group and ER α group respectively. * $P < .05$ or ** $P < .01$. C, The ER α expression at day 3 in Scramble group, sh-ER α group, GFP group and ER α group respectively. * $P < .05$ or ** $P < .01$. D, Greyscale analyses of (C) by Image-Pro Plus 5.0 software

2.10 | Statistical analysis

Experiments were performed in triplicate and repeated more than 3 times independently. The results were presented as mean $\pm$ SD and analysed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Two-tailed Student's *t*-test was used for comparison of pairs. A *P* value of less than 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Characterization of SCAPs and cell transfection

There was a high expression of MSC markers (STRO-1, CD73, CD90 and CD105), while the hematopoietic makers (CD34 and CD45) were expressed at a low level in SCAPs, as indicated by flow cytometry (Figure 1A).

To detect the transfection efficiency, ER α expression in the transfected SCAPs using ER α overexpression or inhibition lentiviruses was measured by real-time RT-PCR and western blot assay at day 3. The results demonstrated a significant increase of ER α expression in ER α group as compared with that in GFP group ($P < .05$ or $P < .01$, Figure 1B-D), while the expression level of ER α was significantly decreased in sh-ER α group as compared with that in Scramble group ($P < .05$ or $P < .01$, Figure 1B-D).

3.2 | ER α had no influence on the proliferation of SCAPs

CCK-8 assay and flow cytometry were performed to investigate whether ER α can exert an effect on the proliferation ability of

SCAPs. CCK-8 assay indicated that there was no significant difference ($P > .05$, Figure 2A) in cell growth between sh-ER α group and Scramble group, while ER α group also presented no distinct difference ($P > .05$, Figure 2A) as compared with GFP group. FCM analyses further confirmed that no significant difference ($P > .05$) was detected between sh-ER α group (23.16%, Figure 2B) and Scramble group (22.59%, Figure 2B), as indicated by the proliferation index ($PI = G_2M+S$). Meanwhile, there was no significant difference in proliferation index ($P > .05$) between ER α group (10.78%) and GFP group (9.15%, Figure 2B). Therefore, it was concluded that ER α was of no influence on the proliferation capacity of SCAPs.

3.3 | ER α enhanced ALP activity and mineralization capacity of SCAPs

After suppression of ER α in SCAPs, ALP activity was obviously down-regulated as compared with that in Scramble group ($P < .01$, Figure 3A) at day 5. Meanwhile, SCAPs generated more mineralization nodules in Scramble group as compared with that in sh-ER α group under osteogenic conditions (Figure 3B,C). CPC assay further demonstrated that the calcium concentration in Scramble group was significantly higher than that in sh-ER α group in osteogenic media ($P < .05$, Figure 3D). On the contrary, both ALP activity and mineralized nodules remarkably increased in ER α group as compared with those in GFP group ($P < .05$, Figure 3A-D) after overexpression of ER α in SCAPs.

3.4 | ER α promoted the odonto/osteogenic differentiation of SCAPs

After suppression of ER α in SCAPs, the mRNA levels of odonto/osteoblastic marker genes (*DMP1*, *RUNX2*, *DSPP* and *OCN*) were

FIGURE 2 Effects of ERα on the proliferation of SCAPs. A, CCK-8 assay for Scramble group, sh-ERα group, GFP group and ERα group at day 0, 1, 3, 5, 7 and 9 respectively. B, Cell cycle phases in Scramble group, sh-ERα group, GFP group and ERα group respectively

down-regulated at day 3 or 7 ($P < .05$, Figure 4A), while the protein levels of DMP1, RUNX2, DSP and OCN also obviously decreased at day 3 or 7 ($P < .05$ or $P < .01$, Figure 4C,D). On the contrary, the mRNA expression of DMP1, RUNX2, DSP and OCN was up-regulated at day 3 or 7 ($P < .05$ or $P < .01$, Figure 4B), while protein levels of DMP1, RUNX2, DSP and OCN also increased at day 3 or 7 ($P < .05$ or $P < .01$, Figure 4E,F). Collectively, the above findings indicated that ERα can regulate the odonto/osteogenic differentiation of SCAPs.

3.5 | ERα activated ERK and JNK MAPK pathways of SCAPs

Protein levels of p38, p-p38, ERK, p-ERK, JNK and p-JNK, as measured by western blot, are shown in Figure 5A. No change

in p38 and p-p38 expression was observed (Figure 5B). When ERα was repressed, the protein level of p-ERK was significantly down-regulated while the ERK expression was up-regulated. The expression level of JNK was down-regulated, while the p-JNK expression decreased more. The ratios of p-ERK/ERK and p-JNK/JNK had statistically significant differences between Scramble and sh-ERα groups ($P < .05$ or $P < .01$, Figure 5B). When ERα was overexpressed, the protein level of ERK was constant, while the p-ERK expression was significantly up-regulated. The expression level of JNK was down-regulated while p-JNK significantly increased. The ratios of p-ERK/ERK and p-JNK/JNK had statistically significant differences between GFP and ERα groups ($P < .01$, Figure 5B). It is suggested that ERα activated ERK and JNK MAPK pathways.

FIGURE 3 Effects of ERα on ALP and mineralization capacity of SCAPs. A, ALP activity of SCAPs in Scramble, sh-ERα, GFP and ERα groups at day 5 respectively. * $P < .05$ or ** $P < .01$. B, The formation of mineralized nodules in SCAPs in 4 groups at day 14. C, Mineralized nodules under the inverted microscope in 4 groups. Scale bars = 100 μ m. D, Calcium contents in 4 groups by CPC assay. * $P < .05$

To further explore the role of MAPK pathway in ERα-mediated odonto/osteogenic differentiation of SCAPs, the downstream transcriptional factors of MAPK pathway were examined. The detected transcriptional factors expressed differently, as shown in Figure 5C. When ERα was repressed, the protein level of p-c-Jun was obviously decreased. The protein level of p-c-Fos was obviously down-regulated while the c-Fos expression had no change. p-Elk1 expression level was reduced, while the protein level of Elk1 was unaltered. When ERα was overexpressed, the expression of p-c-Jun increased. The protein level of p-c-Fos was significantly up-regulated, while the c-Fos expression was not altered. The protein level of p-Elk1 increased, while the Elk1 expression had no change. Our results demonstrated that ERα activated these transcriptional factors (c-Jun, c-Fos and Elk1).

4 | DISCUSSION

Various research studies have investigated the effects of oestrogen on the osteogenesis and osteogenic differentiation of MSCs.^{24,25} Oestrogen deficiency may result in the damage of periodontium and abnormal tooth development.^{26,27} It is recently reported that the concentration of ER plays a decisive role in the cellular responses to oestrogen.^{28,29} To date, little information is available concerning the effects of ERα on the committed differentiation of MSCs. The current study reveals for the first time that ERα can enhance the odonto/osteogenic differentiation capacity of SCAPs via ERK and JNK MAPK pathways.

In this study, ERα had no effect on the proliferation ability of SCAPs. Repression of ERα can decrease the number of mineralized nodules as well as the ALP activity of SCAPs. On the contrary, the formation of the mineralized nodules and the ALP activity of

SCAPs were up-regulated when ERα was overexpressed. ALP is an early marker during the osteoblast differentiation.²³ It is an enzyme protein secreted by osteoblasts, which can raise the local concentration of inorganic phosphate and accelerate the precipitation of calcium by hydrolysis of multiple phosphate.²¹ Among the odonto/osteogenic markers concerned, DSP and DSPP are specific biomarkers of tooth, which are highly expressed in mature odontoblasts and play an important role in the formation and mineralization of dentin, growth and development of tooth germ.^{30,31} DMP1 is a downstream factor of DSPP and regulated by it during the formation of dentin.³² RUNX2 is a vital nuclear transcription regulator of odonto/osteogenic differentiation.³⁰ It has been reported that RUNX2-knockout mice present the defective odontogenic ability while overexpression of RUNX2 can induce bone regeneration.^{33,34} OCN is a small γ -carboxyglutamate protein which is a marker of late phases of osteogenic differentiation.³⁵ Our results showed that the expression of odonto/osteogenic markers was decreased after repression of ERα at both mRNA and protein levels while the expression of these markers was up-regulated as ERα was overexpressed.

In terms of mechanisms, ERα has complex signalling pathways for different genes in different cell types in which the classic mechanism of ERα action is ligand-dependent.¹⁴ This model states that in the presence of oestrogen, the binding of ligand induces high affinity within ERα, which can promote its binding to specific DNA response elements-oestrogen receptor element (ERE) on the promoter region of target genes, thereby regulating the gene transcription level.¹⁸ Except the classic genomic way, ligand-independent activation (cross-talk with peptide growth factors) and ERE-independent genomic actions of ER (combining with specific transcriptional factors) are also well documented.¹⁸ Additionally, ER possesses a nongenomic effect which can induce biological effects in several minutes or even seconds via signal transduction pathways.^{36,37}

FIGURE 4 Effects of ERα on the odonto/osteogenic differentiation of SCAPs. A, Real-time RT-PCR analysis of *DMP1*, *RUNX2*, *DSPP* and *OCN* gene levels in Scramble and sh-ERα groups at day 0, 3 and 7 respectively. * $P < .05$. B, Real-time RT-PCR analysis of *DMP1*, *RUNX2*, *DSPP* and *OCN* gene levels in GFP and ERα groups at day 0, 3 and 7 respectively. * $P < .05$ or ** $P < .01$. C, The protein levels of OCN, DSP, RUNX2 and DMP1 in Scramble and sh-ERα groups at day 0, 3 and 7 respectively. D, Grayscale analyses of (C). * $P < .05$ or ** $P < .01$. E, The protein levels of OCN, DSP, RUNX2 and DMP1 in GFP and ERα groups at day 0, 3 and 7 respectively. F, Grayscale analyses of (E). * $P < .05$ or ** $P < .01$.

Oestrogen membrane receptors have been reported to be involved in delivering mitogen-activated protein kinase (MAPK)-mediated phosphorylation, whereas the activator is likely to be trace of oestrogen in serum.¹⁴ MAPK is an important protein kinase in cells, which plays an important role in transmitting signals between the membrane and the nucleus and subsequently regulating cell proliferation, differentiation, survival, and apoptosis.³⁸ MAPKs mainly include 3 parallel signalling pathways, ie extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38.³⁹ Blockage of the ERK pathway can inhibit the RUNX2-mediated osteogenic differentiation,⁴⁰ while JNK pathway is closely related to cell differentiation and apoptosis.⁴¹ Our data demonstrated that the changes of ERα expression brought about the corresponding changes in the phosphorylation level of ERK and JNK, indicating an activation of ERK and JNK MAPK pathways by ERα overexpression.

To further explore the effect of MAPK pathway in the odonto/osteogenic differentiation of SCAPs, the expression of downstream transcription factors of MAPK pathway was then investigated. Our results revealed that c-Fos and c-Jun were phosphorylated after overexpression of ERα. It is reported that ERK and JNK pathways are likely to be responsible for the stimulation of c-Fos and c-Jun, which can then form AP-1 complex and thereby lead to activation of different target genes.⁴² The phosphorylation level of Elk-1 increased after the activation of JNK in this study, suggesting that the Elk-1 phosphorylation may correlate with the activation of JNK. Some studies have proved that ERα can directly interact with some nuclear transcription factors including AP-1, forming a ERα/AP-1 complex which can binds to the specific GC-rich promoter sequences and then activates the expression of several osteogenic genes.^{14,43} Moreover, ERα has many phosphorylation sites (Ser118), and phosphorylation state can control the

FIGURE 5 Effects of ERα on MAPK pathway and its downstream transcription factors of SCAPs. A, Protein levels of p38, p-p38, ERK, p-ERK, JNK and p-JNK in the cytoplasm of transfected SCAPs in Scramble, sh-ERα, GFP and ERα groups at 48 hours. β-ACTIN served as an internal control. B, Quantitative analysis for the ratios of p-p38/p38, p-ERK/ERK and p-JNK/JNK. **P* < .05 or ***P* < .01. C, Protein levels of c-Fos, p-c-Fos, c-Jun, p-c-Jun, Elk1 and p-Elk1 in the nuclei of transfected SCAPs in Scramble, sh-ERα, GFP and ERα groups at 48 hours. H3 served as an internal control

responses to transcription events.⁴⁴ Taken together, ERα carries out the functions in the differentiation process of SCAPs via the utilization of synergistic effects.

5 | CONCLUSIONS

This work identifies an important role of ERα in regulating the odonto/osteogenic differentiation of SCAPs by activating ERK and JNK MAPK pathways as well as their downstream transcriptional factors. These findings not only contribute to a more comprehensive understanding of the mechanism of stem cells differentiation but also provide potential therapeutic strategies for the tissue regeneration.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

AUTHOR CONTRIBUTIONS

Yanqiu Wang, Yadie Lu, Jinhua Yu: Study design. Yixiang Zhou, Yongchun Gu, Xiyao Pang, Jintao Wu, Zehan Li, Romila Gobin: Data collection. Yanqiu Wang, Yadie Lu: Manuscript preparation.

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