

Involvement of RBP4 in all-trans retinoic acid induced cleft palate

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Received June 29, 2016; Accepted April 24, 2017

DOI: 10.3892/mmr.2017.7327

Abstract. The current study was designed to elucidate the mechanism of retinol binding protein 4 (RBP4) in cleft palate induced by all-trans retinoic acid (atRA). To establish a cleft palate model in C57BL/6J mice, pregnant mice were administered atRA (100 mg/kg) by gavage at the tenth embryonic stage (E10.0). Control groups were given the equivalent volume of corn oil. Pregnant mice were dissected at E12.5, E13.5 and E14.5 to obtain the embryonic palates. The expression levels of RBP4 in the embryonic palatal mesenchyme (EPM) were determined by immunohistochemistry, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting. Human embryonic palatal mesenchymal cells were exposed to atRA to detect the variation in RBP4 induced by atRA *in vitro*. Small interfering RNA was used to suppress the expression of RBP4, and a plasmid overexpressing RBP4 was used to examine upregulated expression. The cell counting kit-8 assay was used to evaluate the effect of RBP4 on cell proliferation. The expression levels of p27 and cyclin D1 were determined by RT-qPCR and western blotting, while the expression levels of extracellular signal-related kinase (ERK) 1/2 and protein kinase B (AKT) were assessed by western blotting. At E14.5, RBP4 was strongly expressed in the EPM, while it was downregulated following atRA treatment, which induced cleft palate *in vivo*. *In vitro* experiments indicated that atRA suppressed the expression of RBP4 and altered the expression of p27 and cyclin D1 to cause growth inhibition. Knockdown of RBP4 resulted in decreased expression of cyclin D1 and increased p27, and suppressed proliferation. Overexpression of RBP4 reversed the inhibitory effect of atRA and promoted proliferation via the ERK1/2 and AKT signaling pathways. These results suggested that RBP4 was involved in cleft palate induced by atRA and it can be suppressed by atRA to cause growth inhibition in the embryonic palate.

Introduction

Retinol binding protein 4 (RBP4) is a member of the lipocalin family and is synthesized in the liver and adipose tissue as a retinol translocator (1,2). Abnormal metabolism of RBP4 can lead to disorders in the synthesis, transport and distribution of retinol, and affects embryonic growth and bone tissue differentiation (3-5). RBP4 has been demonstrated to be significantly decreased in children with non-syndromic cleft lip and/or palate (NSCLP) (6), suggesting that RBP4 levels are associated with the occurrence of NSCLP. Cleft face and palate have been observed in RBP knockout fetuses in animal models, along with obvious cranial and maxillofacial bone defects (7). These studies have indicated that RBP4 serves an important role in embryonic development, particularly in craniofacial growth. It is associated with cleft palate, in which the absence of RBP4 can lead to cleft palate.

Cleft palate resulting from multigenic and environmental factors is a common malformation (8,9). The formation of the secondary palate is a critical process in palatogenesis, including the growth, elevation and fusion of the palatal shelf. Failed elevation of the palatal processes is widely considered to be one of the primary causes of cleft palate and is attributed to growth inhibition of the embryonic palatal mesenchyme (EPM) (10). During the early phase of palatogenesis, large numbers of EPM cells proliferate for growth and elevation. When the proliferation of EPM cells is suppressed, the palatal processes become dysplastic and fail to elevate in an appropriate time. Palatal shelves cannot complete the contact and fusion, resulting in cleft palate.

As a derivative of vitamin A, all-trans retinoic acid (atRA) is involved in the differentiation, proliferation and apoptosis of palatogenesis (11). Previous studies have confirmed that atRA inhibits the proliferation and induces cell cycle arrest in EPM cells to cause palatal dysplasia and the development of cleft palate (12,13). Previous studies have demonstrated that atRA suppresses the expression of RBP4 in adipose tissues and liver (14). However, the effect of atRA on RBP4 in the embryonic palate requires further elucidation. The present study aimed to evaluate the function of RBP4 in the development of the atRA-induced cleft palate.

Materials and methods

Animals and atRA treatment. All animal experiments were approved by the Ethics Committee for Animal Experiments

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Key words: retinol binding protein 4, cleft palate, all-trans retinoic acid, embryonic palatal mesenchyme

of Sun Yat-sen University (IACUC: DB-15-0302). A total of 40 male and 40 female mice were used in the present study. The animals were housed at 22°C with a 12-h light/dark cycle, and given pellet food and tap water *ad libitum*. Specific pathogen-free C57BL/6J male mice aged 7–8 weeks (weight, 22–25 g) and female mice aged 4–5 weeks (weight, 14–18 g) were mated. Day 0 was defined as the presence of a vaginal plug. At embryonic day 10 (E10.0), 20 pregnant mice in the experimental group were administered atRA (100 mg/kg; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) in corn oil by gavage, and 20 pregnant mice in the control group received the same volume of corn oil. The mice were euthanized on E12.5, E13.5 and E14.5 by cervical dislocation to obtain embryonic palates.

Immunohistochemistry. A total of 58 embryonic palates were fixed in 4% paraformaldehyde for 48 h and dehydrated using an ethanol series. Next, they were embedded in paraffin in the coronal orientation. The paraffin blocks were sliced into 4 μ m sections. Every section was deparaffinized and hydrated using an ethanol series. The sections were retrieved by applying citric acid buffer (pH 6.0) for 30 min in a microwave for antigen retrieval, followed by incubation with anti-RBP4 (cat. no. ab109193; 1:200; Abcam, Cambridge, UK) for 18 h at 4°C. The sections were washed with phosphate-buffered saline (PBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated with secondary Ab-BIO (OriGene Technologies, Inc., Beijing, China) at 37°C for 1 h. Staining was performed with 3,3'-diaminobenzidine (DAB; OriGene Technologies, Inc.), and the presence of brown granules was considered to indicate positive staining. Subsequently, the sections were stained with hematoxylin for 3 sec. Images were obtained using a Zeiss Axioskop 40 (Leica Microsystems GmbH, Wetzlar, Germany).

Cell culture and treatment with atRA. Human embryonic palatal mesenchymal (HEPM) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a 5% CO₂ atmosphere at 37°C. To study the effects of atRA, the cells were exposed to different concentrations of atRA (1 and 3 μ M). There were 3 duplicate cultures in each group, and experiments were performed in triplicate.

Gene silencing using small interfering RNA (siRNA). HEPM cells were cultured in 6-well plates (1 \times 10⁴ cells/well) in DMEM containing 10% FBS. When the HEPM cells had reached 70% confluency, they were transfected with a final concentration of 25 pmol RBP4 siRNA (Guangzhou RiboBio Co., Ltd., Guangzhou, China) using Lipofectamine RNAiMAX Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 72 h according to the manufacturer's instructions. An irrelevant siRNA (Guangzhou RiboBio Co., Ltd.) was used as the control. A total of 3 duplicate samples were used in each group, and experiments were performed in triplicate. These cells were collected for analysis of the associated mRNA and protein expression. Reverse transcription-quantitative polymerase chain reaction

(RT-qPCR) and western blotting were used to verify the knockdown efficiency of the target gene.

Overexpression of RBP4. RBP4 protein was overexpressed in HEPM cells to assess the function of RBP4 in the palatal mesenchyme according to a standard transient transfection protocol. The full-length RBP4 gene was cloned into pEZ-M98-GFP (iGeneBio Biotechnology Co., Ltd., Guangzhou, China) for the experiments. Cells transfected with pEZ-M98-GFP (without RBP4) were used as the control group. The HEPM cells were seeded into 6-well plates (1 \times 10⁵ cells/well) in DMEM containing 10% FBS. When the HEPM cells had reached 70% confluency, they were transfected with the above-mentioned constructs in Opti-MEM medium (Gibco; Thermo Fisher Scientific, Inc.), and Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transfecting nucleic acids (2500 ng/well) into cells according to standard procedures. Following transfection for 48 h, the medium was refreshed, and the cells were exposed to atRA (3 μ M) for 48 h. A total of 3 duplicate samples were used in each group, and experiments were performed in triplicate. The efficiency of the target gene overexpression was evaluated by western blotting.

RT-qPCR. Total RNA was extracted from mouse embryonic palates (60 in each group) or HEPM cells using a Qiagen RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the instructions provided by the manufacturer. A total of ~20 μ l cDNA was synthesized from 1 μ g of total RNA in a 20 μ l reaction mixture. The reaction mixture included total RNA, Anchored-oligo (dT)18 Primer, Random Hexamer Primer, PCR-grade H₂O, Transcriptor RT Reaction Buffer (5X), Protector RNase Inhibitor, Deoxynucleotide Mix and Transcriptor Reverse Transcriptase (all Roche Diagnostics GmbH, Mannheim, Germany). RT-qPCR was conducted using the Light Cycler 480 System (Roche Diagnostics GmbH) with SYBR Green I Master Mix (Roche Diagnostics GmbH) to quantitatively measure the mRNA levels. β -actin and GAPDH were used as reference genes. The following PCR primers (Generay Biotech Co., Ltd., Shanghai, China) were used in the experiments: RBP4 (Mus), forward 5'-GACACGGACTAC GACACC-3' and reverse 5'-CACGAGAAAACACAAAGG A-3'; β -actin (Mus), forward 5'-TCACCCACACTGTGCCCA TCTACGA-3' and reverse 5'-GGATGCCACAGGATTCCA TACCCA-3'; RBP4 (homo), forward 5'-GAGGACCCTGCC AAGTTCA-3' and reverse 5'-GGGAAAACACGAAGGAGT AGC-3'; P27 (homo), forward 5'-CAAACGTGCGAGTGT CTA-3' and reverse 5'-CAGTGCTTCTCCAAGTCC-3'; cyclin D1 (homo), forward 5'-TCCTACTACCGCCTCACA-3' and reverse 5'-ACCTCCTCCTCCTCCTCT-3'; GAPDH (homo), forward 5'-GGACCTGACCTGCCGTCTAG-3' and reverse 5'-GTAGCCCAGGATGCCCTTGA-3'.

Western blot analysis. 60 mouse embryonic palates in each group were treated with 1 U/ml dispase II (Roche Diagnostics, Indianapolis, IN, USA) for 15 min at 37°C to remove epithelia and obtain EPM at E12.5, E13.5 and E14.5. The palatal mesenchyme was flushed with cold PBS three times and lysed in cell lysis buffer (10% RIPA, 1% protease inhibitor, 1% phosphatase inhibitor; Sigma-Aldrich; Merck Millipore) on ice for 30 min. The protein was collected after the lysate

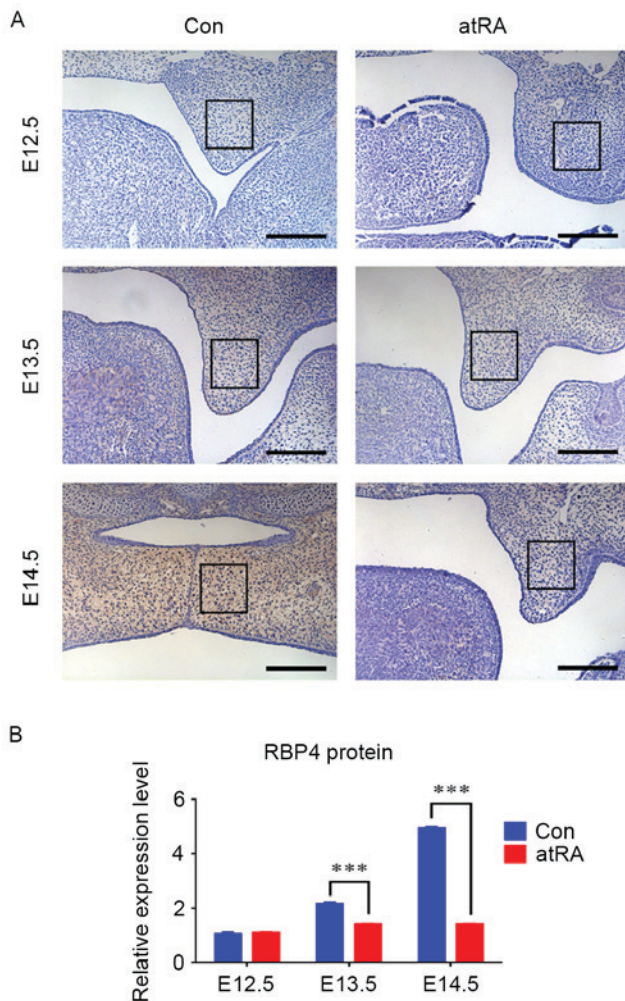


Figure 1. Immunohistochemistry assay demonstrating RBP4 expression in the EPM. RBP4 was significantly decreased at E13.5 and E14.5 in the EPM exposed to atRA when compared with the Con group. (A) Immunohistochemistry results (scale bar, 50 μ m), (B) quantification. *** P <0.001. RBP4, retinol binding protein 4; EPM, embryonic palatal mesenchyme; E, embryonic day; atRA, all-trans retinoic acid; Con, control.

solution was centrifuged at 4°C and 14,000 \times g for 30 min. The protein (30 μ g) was separated by 10% SDS-PAGE (CW BIO, Beijing, China) and transferred onto polyvinylidene difluoride membranes. The membrane was blocked in 5% bovine serum albumin (Beyotime Institute of Biotechnology, Shanghai, China) for 1 h and incubated overnight at 4°C with the following antibodies: Rabbit monoclonal anti-RBP4 (cat. no. ab109193; 1:1,000; Abcam), rabbit monoclonal anti-p27 (cat. no. 3688s; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) and rabbit monoclonal anti-cyclin D1 (cat. no. ab134175; 1:2,000; Abcam) antibodies. The membrane was incubated with horseradish peroxidase-coupled secondary antibodies for 1 h following three washes with Tris-buffered saline/Tween 20 (CW BIO). The membrane was detected using chemiluminescence with Immobilon western chemiluminescent horseradish-peroxidase substrate (EMD Millipore, Billerica, MA, USA).

Protein derived from cultured HEPM cells was also detected by western blotting. The following steps were repeated as described previously (15). In addition to the antibodies

mentioned above, the following antibodies were used: Rabbit monoclonal anti-extracellular signal-related kinase (ERK) 1/2 (cat. no. 4695s; 1:1,000; Cell Signaling Technology, Inc.), rabbit monoclonal anti-phosphorylated (p)-ERK1/2 (cat. no. 4370s; Thr202/Tyr204; 1:2,000; Cell Signaling Technology, Inc.), rabbit monoclonal anti-AKT (cat. no. 4691s; 1:1,000; Cell Signaling Technology, Inc.), rabbit monoclonal anti-p-AKT (cat. no. 4060s; Ser473; 1:1,000; Cell Signaling Technology, Inc.), rabbit monoclonal anti-proliferating cell nuclear antigen (PCNA) (cat. no. 13110s; 1:1,000; Cell Signaling Technology, Inc.).

Cell counting kit-8 (CCK8) assay. HEPM cells were cultured in a 96-well plate (5,000 cells/well) in DMEM at 37°C for 24 h. The medium was then replaced with fresh DMEM, and the cultures were exposed to atRA (3 μ M) or the transfection complex. Following 24, 48, 72 and 96 h, 10 μ l CCK8 (Dojindo Molecular Technologies, Inc., Tokyo, Japan) was added to each well for 2 h at 37°C. The absorbance was measured using a microplate reader at 450 nm.

Statistical analysis. All investigations, including individual experiments, were performed in triplicate. All data were analyzed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA) with the Student's t-test, or one way analysis of variance followed by the Bonferroni post hoc test to correct for multiple comparisons. The results are presented as the mean values \pm standard deviation, and P <0.05 was considered to indicate a statistically significant difference.

Results

RBP4 was downregulated in the EPM of cleft palate induced by atRA. At E12.5, the palate grew vertically along the side of the tongue, and the expression of RBP4 was negative without apparent differences in the EPM in either group (Fig. 1). At E13.5, the palatal shelves still grew in a vertical position alongside the tongue, and they appeared smaller in the atRA-treated group than that in the control group (Fig. 1A). There were more RBP4-positive cells in the EPM in the control group compared with the atRA group (Fig. 1; P <0.001). At E14.5, the bilateral palates shifted toward a horizontal position to contact one another in the control group. During this period, the medial edge epithelium was contacted to develop into the medial edge epithelial seam, and finally palate shelves fused. The expression of RBP4 was strongly positive in the EPM (Fig. 1A). By contrast, the palatal shelves appeared abnormally small and failed to undergo elevation and fusion, finally developing into cleft palate in the atRA-treated group at E14.5 (Fig. 1A). The incidence of cleft palate in the atRA-exposed groups was 96.3% (78 of 81 embryos). RBP4 was significantly downregulated in the EPM in atRA treatment compared with the control group (Fig. 1; P <0.001). The RT-qPCR results indicated that the mRNA levels of RBP4 were reduced at E13.5 (P <0.05) and downregulated at E14.5 in the atRA compared with the control group (Fig. 2A; P <0.01). Western blotting results identified that from E12.5 to E14.5, the protein levels of RBP4 were significantly reduced in the atRA-exposed vs. the control EPM, particularly at E14.5 (Fig. 2B; P <0.001).

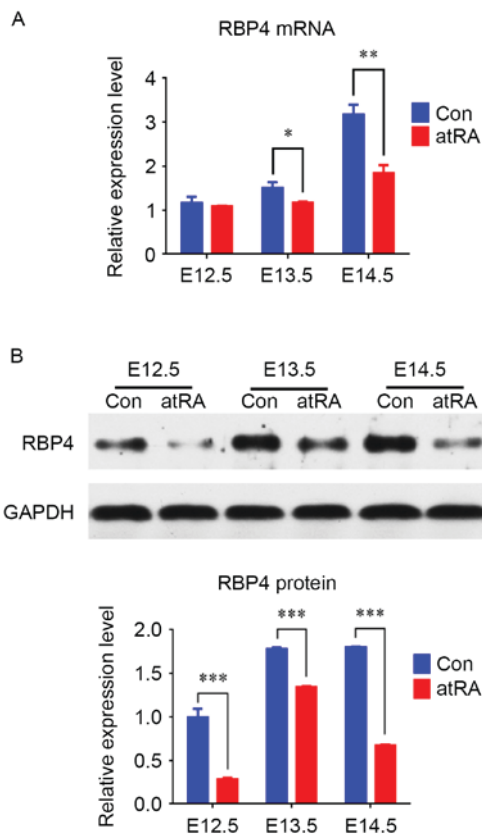


Figure 2. The expression of RBP4 was downregulated in the EPM treated with atRA. (A) When compared with the Con group, mRNA expression of RBP4 was reduced in the atRA group at E13.5 and E14.5. (B) Protein expression of RBP4 from E12.5 to E14.5 was lower in the atRA groups vs. Con. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. RBP4, retinol binding protein 4; EPM, embryonic palatal mesenchyme; atRA, all-trans retinoic acid; E, embryonic day; Con, control.

atRA induced RBP4 downregulation and regulated the expression of p27, cyclin D1, PCNA and the associated signaling pathway. To evaluate the effect of atRA on RBP4, HEPM cells were exposed to different concentrations of atRA (1 and 3 μM). The results indicated that the mRNA levels of RBP4 were decreased in HEPM cells treated with atRA (1 and 3 μM) (Fig. 3A; $P < 0.01$). The protein levels of RBP4 were also decreased in the atRA-treated groups compared with the controls (Fig. 4; $P < 0.001$). In contrast, elevated p27 mRNA levels (Fig. 3B; $P < 0.05$) and protein levels (Fig. 4; $P < 0.001$) were observed in HEPM cells exposed to atRA (1 and 3 μM). Cyclin D1 mRNA and protein levels were suppressed in the atRA-exposed groups compared with the control group (Figs. 3C and 4; $P < 0.001$). The protein levels of PCNA were reduced in the atRA-exposed groups compared with the controls (Fig. 4). Western blotting results identified that p-ERK1/2 was reduced in atRA-treated cells (Fig. 4; $P < 0.01$), and p-AKT was also reduced compared with the control group (Fig. 4; $P < 0.001$).

RBP4 suppressed the expression of p27 and stimulated the expression of cyclin D1 via the AKT and ERK1/2 signaling pathway. siRNA knockdown of RBP4 was performed to evaluate its involvement in cell proliferation (Figs. 5 and 6). The RT-qPCR and western blotting results demonstrated that

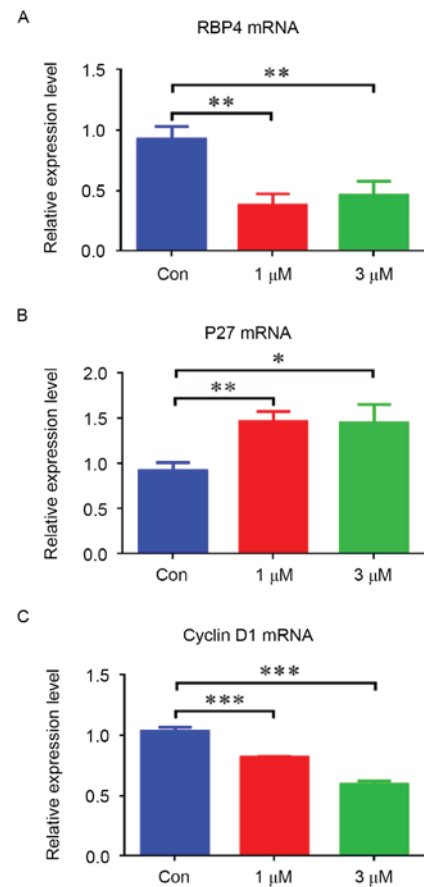


Figure 3. atRA suppressed RBP4 mRNA levels and altered the mRNA levels of p27 and cyclin D1 in human embryonic palatal mesenchymal cells. (A) mRNA expression of RBP4 was decreased in the atRA-treated group compared with that in the Con group. (B) mRNA expression of p27 was increased in the atRA-treated group compared with the Con group. (C) mRNA expression of cyclin D1 was reduced in the atRA-treated group compared with the Con group. There were 3 duplicate cultures in each group, and experiments were performed in triplicate. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. atRA, all-trans retinoic acid; RBP4, retinol binding protein 4; Con, control.

RBP4 was effectively knocked down by siRNA both at the mRNA and protein levels; mRNA levels were repressed by 84.1% (0.17 ± 0.06 vs. 1.07 ± 0.12 ; $P < 0.001$; Fig. 5A), and protein levels were repressed by 56.7% (0.45 ± 0.02 vs. 1.04 ± 0.05 ; $P < 0.001$) (Fig. 6). Downregulation of RBP4 in HEPM cells transfected with RBP4 siRNA resulted in decreased expression of cyclin D1 at both mRNA (Fig. 5C; $P < 0.01$) and protein (Fig. 6) levels ($P < 0.001$), while increased p27 mRNA (Fig. 5B; $P < 0.05$) and protein (Fig. 6) levels were observed ($P < 0.001$). The protein levels of PCNA were reduced in RBP4-siRNA HEPM cells compared with the control group (Fig. 6; $P < 0.01$). The western blotting results demonstrated that p-ERK1/2 and p-AKT were reduced along with RBP4 (Fig. 6; $P < 0.001$). The western blotting results indicated that RBP4 was effectively upregulated in response to RBP4 overexpression in HEPM cells and the degree of overexpression was 51.2% (1.50 ± 0.02 vs. 0.99 ± 0.01 ; $P < 0.001$), cyclin D1 was also upregulated ($P < 0.001$), while p27 was downregulated (Fig. 7; $P < 0.001$). In the atRA-exposed group, RBP4 levels and cyclin D1 were decreased (Fig. 7; $P < 0.01$), however p27 was increased (Fig. 7; $P < 0.001$), as compared with the control

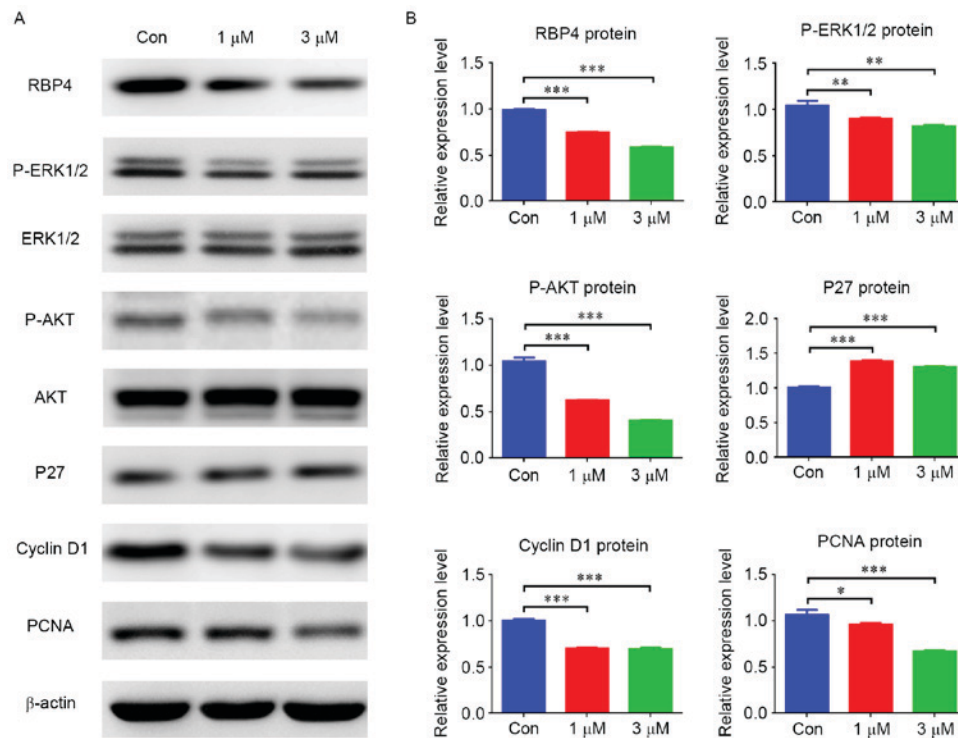


Figure 4. atRA inhibited RBP4 protein levels and altered the expression of associated regulators and signaling pathways in human embryonic palatal mesenchymal cells. (A) Protein levels of RBP4, cyclin D1, PCNA, p-ERK1/2 and p-AKT were all decreased in the atRA-treated group, however p27 was increased compared with the Con group. (B) Data for the protein levels are presented as the mean + standard deviation. There were 3 duplicate cultures in each group, and experiments were performed in triplicate. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. atRA, all-trans retinoic acid; RBP4, retinol binding protein 4; PCNA, proliferating cell nuclear antigen; p-, phosphorylated; ERK, extracellular signal-related kinase; AKT, protein kinase B; Con, control.

group. By contrast, RBP4 and cyclin D1 were not downregulated (Fig. 7; $P < 0.001$) and p27 levels did not increase (Fig. 7; $P < 0.001$) in the RBP4 overexpression plus atRA-treated group compared with the control. The western blot results additionally indicated that p-ERK1/2 and p-AKT were both upregulated in response to RBP4 overexpression in HEPM cells (Fig. 7; $P < 0.001$), and they were not downregulated in the RBP4 overexpression plus atRA-treated group ($P < 0.001$) compared with the control.

RBP4 promoted the proliferation of HEPM cells by CCK8 assay. When HEPM cells were exposed to atRA (3 μ M), cell proliferation levels were reduced from 24 to 96 h (Fig. 8A). siRNA knockdown of RBP4 in HEPM cells resulted in markedly reduced cell proliferation levels from 48 to 96 h (Fig. 8B). By contrast, RBP4 overexpression in HEPM cells resulted in enhanced cell proliferation levels from 48 to 96 h, with maximum proliferation observed at 96 h (Fig. 8C; $P < 0.01$). Compared with the control group, when the RBP4-overexpressing HEPM cells were exposed to atRA, cell proliferation levels were maintained, particularly at 72 h (Fig. 8C; $P < 0.001$).

Discussion

atRA is essential for the development of embryos, by regulating morphogenesis, cell proliferation and differentiation (11,16). Embryos develop normally under low concentrations of atRA, while when exposed to overdoses of atRA, they develop defects in both animals and humans,

such as cleft palate (16,17). atRA has been widely used as a teratogen to establish animal models and cytotoxicity tests to determine the pathogenesis of cleft palate. Previous studies have confirmed that in pregnant mice exposed to atRA (100 mg/kg) at E10.0, the palatal shelves become dysplastic and fail to contact one another, resulting in cleft palate (17,18). In the mouse, palate shelves initially grow vertically alongside the tongue at E12.5 and then elevate to the horizontal position above the tongue, and contact with each other and fuse at E14.5 (19,13). Normal growth of EPM serves a key role in this process, a previous study indicated that atRA suppressed the process of growth and differentiation in the developing mesenchyme to cause an abnormally small palate and unfused palatal shelves (20). atRA has been demonstrated to inhibit the proliferation of EPM cells to cause cleft palate (13). The results additionally indicated that the palatal shelves were abnormally small and dysplastic in the atRA-exposed embryos, and unable to shift horizontally due to the inhibition effect of atRA.

RBP4 is a 21 kDa protein that is located in 10q23-24 in humans (21). RBP4 is primarily synthesized in the liver and transported to target tissues; it can also be detected in extrahepatic tissues such as adipose tissue, the kidneys and the lungs (22). RBP4, which is previously known as RBP, participates in retinol translocation, lipid metabolism, fetal growth, bone growth and inflammatory responses (23,24). A study reported that RBP knockout mouse have morphological abnormalities, including cleft palate and maxillofacial bone defects (7). This indicated that RBP4 serves an important role in normal physiological function and development,

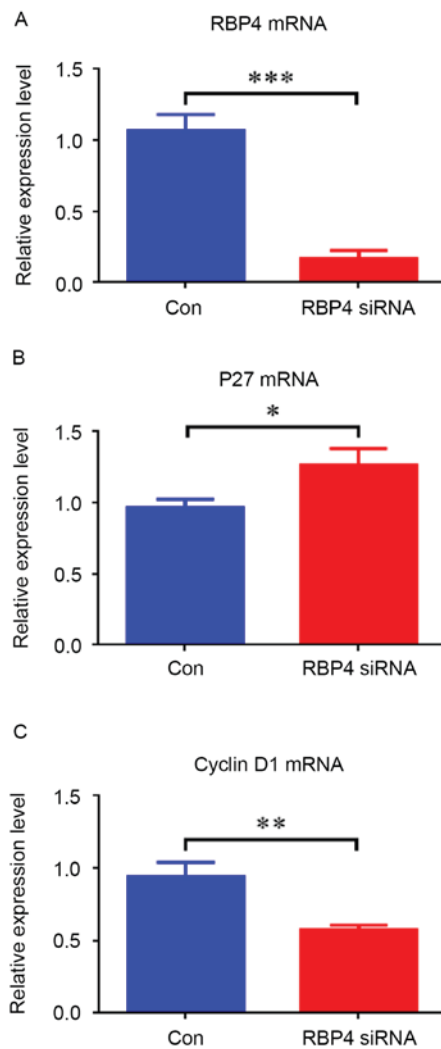


Figure 5. Knockdown of RBP4 induced upregulation of p27 and downregulation of cyclin D1 at the mRNA level. (A) When compared with the Con group, RBP4 mRNA levels were downregulated in HEPM cells treated with RBP4 siRNA. (B) P27 mRNA levels were upregulated in HEPM cells treated with RBP4 siRNA via Con. (C) Cyclin D1 mRNA levels were downregulated in HEPM cells treated with RBP4 siRNA compared with Con. A total of 3 duplicate samples were used in each group, and experiments were performed in triplicate. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. RBP4, retinol binding protein 4; HEPM, human embryonic palatal mesenchymal; siRNA, small interfering RNA; Con, control siRNA.

particularly in that of the palate and the maxillofacial region. It has been observed that the level of serum RBP4 is significantly lower in children with NSCLP than in those without (6). However, whether RBP4 can be detected in the EPM and the mechanism of RBP4 in cleft palate remains unclear. The present study observed that RBP4 was strongly expressed in the EPM at E13.5 and E14.5 in the normal embryonic palate, while it was downregulated in cleft palate induced by atRA. These observations indicated that RBP4 was involved in the development of the EPM and that the deficiency in RBP4 was associated with the occurrence of cleft palate.

In order to elucidate the mechanism of RBP4 in the cleft palate induced by atRA, HEPM cells were used in the *in vitro* experiments. The levels of RBP4 mRNA and protein were downregulated by atRA in HEPM cells. This observation

was consistent with the results of the *in vivo* animal experiments, demonstrating that atRA can significantly inhibit the expression of RBP4. Numerous studies have demonstrated that atRA can suppress the proliferation via the upregulation of p21/p27 and the downregulation of cyclin D1 in various cell lines (25-27). P27 structurally homologous to p21 is known as cyclin-dependent kinase inhibitor, which serves a negative role in cell proliferation and cell cycle progression (28,29). p27 expression can cause cell cycle arrest, while downregulation of p27 leads to cell cycle progression and cell proliferation (28,29). Cyclin D1 belongs to the highly conserved cyclin family, and function as regulator of cyclin-dependent protein kinases, promoting cell cycle progression (30). PCNA, a cofactor of DNA polymerase, is widely used as a proliferation associated marker (31). The results indicated that the expression of p27 was increased and cyclin D1 and PCNA were decreased in HEPM cells exposed to atRA, indicating that atRA inhibited the proliferation of HEPM cells. The CCK8 assay also proved that atRA had an inhibitory effect on cell proliferation. The western blotting results also indicated that p-AKT and p-ERK1/2 were both reduced in HEPM cells treated with atRA. It has been reported that the AKT and ERK1/2 signaling pathways are involved in proliferation and survival in different cells (32). Activation of AKT signaling promotes DNA synthesis and cell cycle progression, regulating cellular proliferation and survival (28). ERK1/2 has been demonstrated to be an essential signaling pathway to modulate cell proliferation and apoptosis (33). The results of the present study indicated that they may be involved in cell proliferation in HEPM cells.

Previous reports have confirmed that RBP4 can enhance proliferation in vascular smooth muscle cells and rat aortic smooth muscle cells induced by hyperinsulinism (34,35). However, the effect of RBP4 on HEPM cells remains unclear. In the current study, the CCK8 assay demonstrated that RBP4 enhanced the proliferation of HEPM cells, and the growth inhibition induced by atRA was mitigated by RBP4 overexpression. RBP4 has been previously observed to promote cell proliferation by upregulating p-ERK1/2 in rat aortic smooth muscle cells (35). A previous study additionally confirmed that RBP4 can significantly increase the expression of p-AKT in vascular endothelial cells (36). In the present study, results suggested that RBP4 could upregulate the expression of p-ERK1/2 and p-AKT to activate the AKT and ERK1/2 signaling pathways in HEPM cells. However, the mechanism of how RBP4 affects p-ERK1/2 and p-AKT remains unclear, and further research is required. A previous study observed that activation of the ERK1/2 and AKT signaling pathways suppresses the expression of cyclin-dependent kinase inhibitors (p27 and p21) and increases the expression of cell cyclins (cyclin D1 and cyclin E) to promote cell cycle progression and cell proliferation (32). The results of the present study demonstrated that RBP4 could stimulate cyclin D1 and inhibit p27 to promote cell proliferation via the AKT and ERK1/2 signaling pathways. When RBP4 was suppressed, it can cause growth inhibition. The *in vivo* and *in vitro* studies indicated that depression of RBP4 in the palate could suppress the expression of p-ERK1/2 and p-AKT, and affect the expression of p27 and cyclin D1 to cause growth inhibition of EPM, leading to cleft palate. The results demonstrated that RBP4 served an

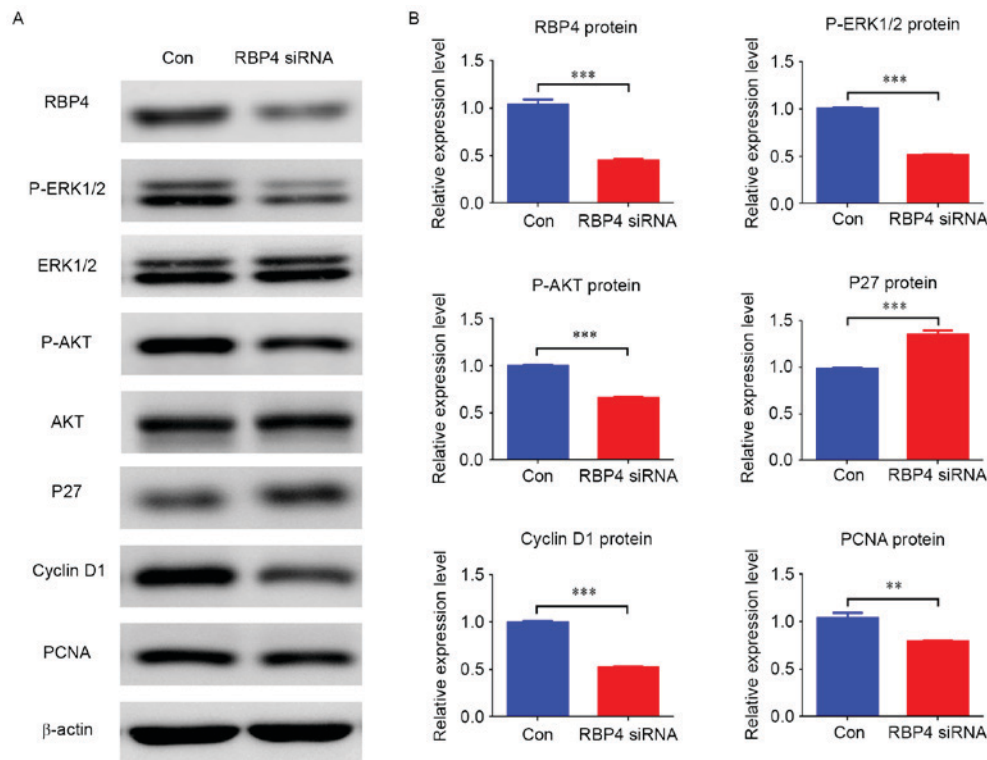


Figure 6. Knockdown of RBP4 altered the expression of related regulators and signaling pathways in HEPM cells. (A) Protein levels of RBP4, cyclin D1, PCNA, p-ERK1/2 and p-AKT were all downregulated in HEPM cells treated with RBP4 siRNA, however p27 was upregulated compared with the Con group. (B) Data for the protein levels are presented as the mean + standard deviation. A total of 3 duplicate samples were used in each group, and experiments were performed in triplicate. ** $P < 0.01$ and *** $P < 0.001$. RBP4, retinol binding protein 4; HEPM, human embryonic palatal mesenchymal; PCNA, proliferating cell nuclear antigen; p-, phosphorylated; ERK, extracellular signal-related kinase; AKT, protein kinase B; siRNA, small interfering RNA; Con, control siRNA.

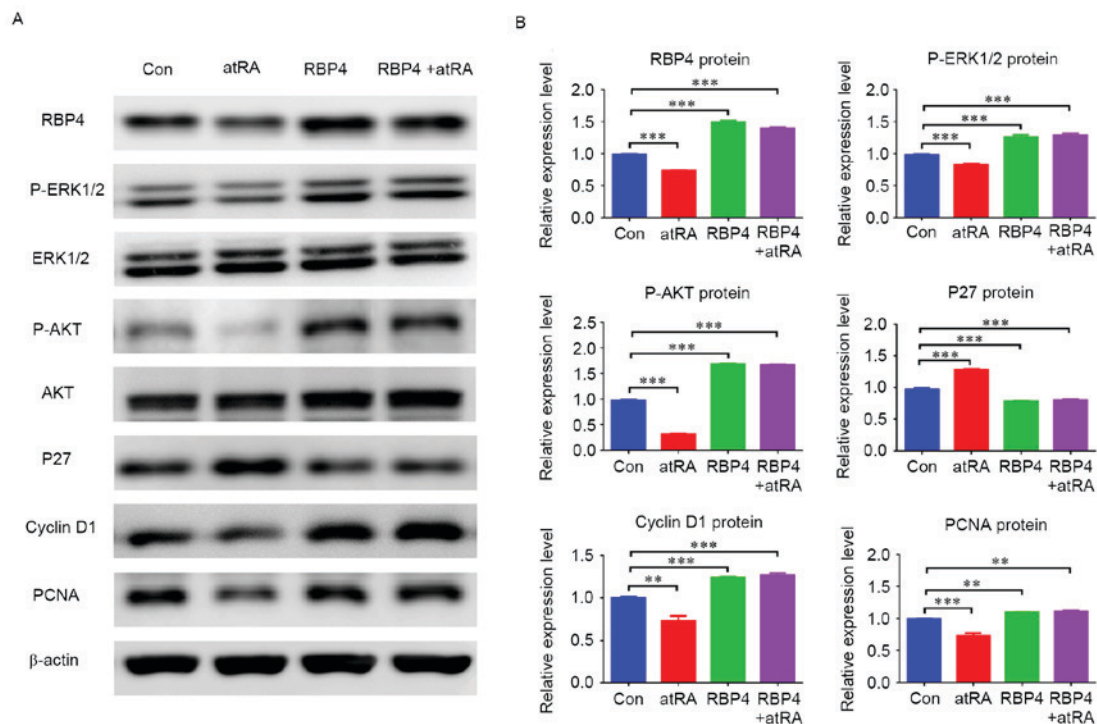


Figure 7. Overexpression of RBP4 altered the expression of related regulators and related signal pathways in HEPM cells. (A) The protein levels of RBP4, cyclin D1, PCNA, p-ERK1/2 and p-AKT were all upregulated in HEPM cells overexpressing RBP4, however p27 was downregulated compared with the Con group. The protein levels of RBP4, cyclin D1, PCNA, p-ERK1/2 and p-AKT remained upregulated in the RBP4 overexpressing group treated with atRA, and p27 was downregulated compared with the Con group. (B) The data for the protein levels are presented as the mean + standard deviation. A total of 3 duplicate samples were used in each group, and experiments were performed in triplicate. ** $P < 0.01$ and *** $P < 0.001$. RBP4, retinol binding protein 4; HEPM, human embryonic palatal mesenchymal; PCNA, proliferating cell nuclear antigen; p-, phosphorylated; ERK, extracellular signal-related kinase; AKT, protein kinase B; atRA, all-trans retinoic acid; Con, control.

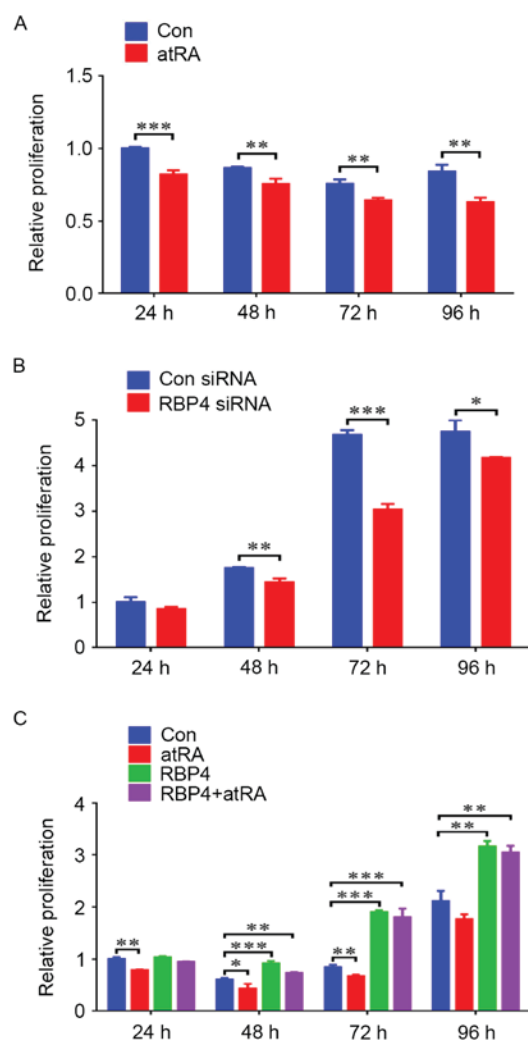


Figure 8. Effects of RBP4 on HEPM cells using the cell counting kit-8 assay. (A) The proliferation levels of HEPM cells treated with atRA (3 μ M) decreased from 24-96 h. (B) The proliferation levels of HEPM cells transfected with RBP4 siRNA decreased from 48-96 h. (C) The proliferation levels of HEPM cells overexpressing RBP4 increased from 24-96 h, and atRA had no effect on these cells. A total of 3 duplicate samples were used in each group, and experiments were performed in triplicate. * P <0.05, ** P <0.01 and *** P <0.001. RBP4, retinol binding protein 4; HEPM, human embryonic palatal mesenchymal; siRNA, small interfering RNA; atRA, all-trans retinoic acid; Con, control.

important role in cleft palate induced by atRA, indicating that it could be used as detection index or prophylaxis method for cleft palate.

In summary, the study confirmed that RBP4 is involved in cleft palate induced by atRA, and it is downregulated in the EPM treated with atRA. RBP4 can be suppressed by atRA to cause growth inhibition in the embryonic palate.

Acknowledgements

The current study was funded by grants from the National Natural Science Foundation of China (grant no. 81100739), the Project of Health and Family Planning Commission of Shenzhen Municipality (grant no. 201302202) and the Natural Science Foundation of Guangdong Province (grant no. S2011040004190).

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