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Research article

Effects of *all-trans* retinoic acid on goat dermal papilla cells cultured *in vitro*

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ABSTRACT

Background: All-trans retinoic acid (ATRA), a vitamin A-derived active metabolite, exerts important functions in hair biology. Previous studies indicated that excess ATRA hampered hair follicle morphogenesis and cyclic regeneration in adulthood, but other studies stated that ATRA promoted hair growth. Dermal papilla (DP), a cluster of specialized fibroblasts, plays pivotal roles in controlling development and regeneration of hair follicle. Several lines of evidence indicated that DP might be the target cells of ATRA in the hair follicle. To confirm this hypothesis, the present study was performed to explore the biological effects of ATRA on goat dermal papilla cells (DPCs) and clarify the roles of ATRA in hair biology.
Results: Our experimental results indicated that key signaling transducers of ATRA were dynamically expressed in distinct stages of goat cashmere growth cycle, and high-dose ATRA treatment (10^{-5} M) significantly impaired the viability of goat DPCs and lowered the ratio of proliferating cells. Otherwise, goat DPCs were stimulated to enter apoptosis and their cell cycle progression was severely blocked by ATRA. Moreover, the expression of fibroblast growth factor 7 (*Fgf7*), one of the potent hair growth stimulators secreted by DPCs, was transcriptionally repressed following ATRA treatment.
Conclusion: DPCs are the targets of ATRA in the hair follicle, and ATRA negatively regulates hair growth by the targeted suppression of cell viability and growth factor expression of goat DPCs. Through these observations, we offer a new mechanistic insight into the roles of ATRA in hair biology.
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1. Introduction

All-trans retinoic acid (ATRA), the active vitamin A metabolite, is well known for its prominent ability to modulate multiple cellular processes such as proliferation, differentiation, and apoptosis across many cell types [1,2].Recent studies demonstrated that ATRA mostly functions in a paracrine manner, and in its target cells, ATRA binds to nuclear retinoic acid (RA) receptors (RARα, β, and γ) or peroxisome proliferator-activated receptor-β/δ (PPARβ/δ); then, ATRA targets *cis*-acting elements of RA responsive genes, regulates transcriptional activity of genes, and induces diverse cellular responses [1,3,4].The intracellular level of ATRA and its partitioning between RARs and PPARβ/δ are adjusted by cellular RA-binding protein (CRABP1) and relative cellular level of CRABP2 and fatty acid–binding protein 5 (FABP5), respectively. CRABP1 directs ATRA to its catalyzing enzymes for degradation to reduce the intracellular level of ATRA, CRABP2 facilitates binding of ATRA to RARs, and FABP5 delivers ATRA to PPARβ/δ [5,6]. In some cell types with a high ratio of FABP5 to CRABP2, ATRA combines preferentially to PPARβ/δ and induces their proliferation. Conversely, ATRA mainly works with RARs and triggers cell growth inhibition, cell cycle arrest, or apoptosis [2,4,5].

Hair follicle is a mini-organ with a remarkable ability to periodically regenerate itself in the adulthood of mammals [7]. Typically, a hair follicle is composed of mesenchymal and epithelial compartments, and their intimate interaction is believed to play decisive roles in hair cyclical regrowth [7,8]. Dermal papilla (DP), the most important component of follicular mesenchyme-originated fibroblasts, participates in the process of hair cycling by secreting numerous growth factors to manipulate the proliferation and differentiation of follicular keratinocytes [9,10]. Thus, DP draws more attentions from researchers intended to uncover underlying mechanisms governing hair growth. Presently, successful cultivation of dermal papilla cells (DPCs) *in vitro* from various species provides useful models for the exploration of the above topic and sheds light on new mechanisms for elucidating hair diseases and others [11,12,13].

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The detrimental impact of excess ATRA in hair formation and growth has been noticed for decades, as evidenced by excess ATRA resulted in failure of hair morphogenesis and a premature phase transition of hair follicle from the anagen stage into the catagen stage, a phenomenon closely related to hair loss [14,15], whereas some reports pointed out that ATRA played positive roles in hair growth and was clinically applied for the treatment of hair loss [16,17,18]. Advances in comprehensive gene expression profiling of follicular cells discovered *Crabp1* and *Crabp2* as the feature genes of DP, thus indicating that DP might be the target cells of ATRA in the hair follicle and providing the possibility that DP mediates the biological output of ATRA in hair biology [13,19].Otherwise, unique expression patterns of *Rars* in DPCs or the intact hair follicle further supported the above hypothesis [20,21]. To solve this puzzle and clarify the effects of ATRA on DPCs, we isolated, cultivated goat DPCs, and treated them with different concentrations of ATRA. Our experimental results indicated that a higher dose (10^{-5} M) of ATRA inhibited cell growth of goat DPCs and decreased the expression of *Fgf7*, a potent growth factor secreted by DP to stimulate hair growth, thus demonstrating the inhibitory behavior of excess ATRA on hair growth and a new mechanism for explaining this puzzle.

2. Materials and methods

2.1. Chemicals and reagents

ATRA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich(Shanghai, China). ATRA was dissolved in pure dimethyl sulfoxide (DMSO) as the vehicle at 0.01 M and stored at -20°C for further preservation. MTT was dissolved in sterile phosphate-buffered solution (PBS) to a final concentration of 5 mg/ml. Both ATRA and MTT solutions should be carefully preserved in dark to maintain their biological activities. For immunocytochemistry identification, antibodies specific for smooth muscle α-actin (α-SMA) and CD133 were obtained from BOSTER Biological Technology Co. Ltd. (Wu Han, China).

2.2. Skin sample collection

Experimental cashmere goats were obtained from the Shanbei Cashmere Goats Engineering Technology Research Center of Shaanxi Province, China. Three young and healthy female goats were randomly picked, and skin samples were harvested from the right mid-side of each selected goat at a distinct stage of cashmere growth cycle (anagen, catagen, and telogen stages). All goats used in the present study were intentionally labeled for consistent sampling. After collection, all skin samples were quickly washed thrice with sterile PBS and protected from RNA degradation using the RNAstore solution (CWBio, Beijing, China). All experimental procedures were approved by the Experimental Animal Manage Committee of Northwest A&F University.

2.3. Cell culture

Isolation, culture, and propagation of goat DPCs were performed as previously described [12,13]. Goat DPCs were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12;Thermo Fisher Scientific, MA,USA) containing 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel), 100 units/ml penicillin, and 100 μg/ml streptomycin purchased from Solarbio (Beijing, China). Cells were incubated at 37°C and 100% humidity in a 5% CO_2 incubator. The detection of goat DPCs aggregative behavior was carried out as previous report suggested [11]. Typically, the third to the fifth passage of goat DPCs was used in all experiments. Moreover, C2C12 cells were maintained in a way same as that of goat DPCs.

2.4. MTT assay

Cell viabilities were measured by the classical method – MTT assay. Concisely, goat DPCs were seeded into 96-well plates at a density of 5×10^3 cells/well, incubated for 24 h in conventional culture medium, and then, the DPCs were treated with ATRA (10^{-9}–10^{-5} M) or DMSO diluted at 1:1000 in culture medium as a control for 1, 2, or 3 d. Twenty microliters of the MTT solution (5 mg/ml) was added per well and incubated for 4 h at 37°C. After the complete removal of supernatant, 50 μl of DMSO was used to dissolve the formazan crystals. All samples were further incubated for 10 min at room temperature and quantified by measuring optical absorption at 570 nm using a microplate reader (BioTek, Vermont, USA). Six replicates were set for each experimental group.

2.5. Immunocytochemistry

Experimental procedures for the detection of specific α-SMA and CD133 protein expression in goat DPCs by an immunocytochemistry reaction were performed as according to those mentioned in previous studies [12,13]. Briefly, dilution of primary antibodies and their reactions to secondary antibodies were carried out according to the manufacturer's instructions. At the same time, the PBS solution without any primary antibody was set as the staining control to exclude any false-positive results in staining. Staining results were monitored using an inverted digital fluorescence microscope (Advanced Microscopy Group, USA).

2.6. Cell proliferation test

For EdU-based cell proliferation test, cell seeding and treatment with ATRA (10^{-9}, 10^{-7}, and 10^{-5} M) or DMSO as a vehicle control were performed in a manner same as that in the MTT assay described above. After 2 d of treatment, the cell proliferation ratio was assessed by Cell-Light™ EdU Apollo®488 *In Vitro* Imaging Kit (RiboBio, Guang Zhou, China) according to the manufacturer's instructions. Analysis of goat DPC proliferation status (ratio of EdU-positive cells to DAPI-positive cells) was performed using images of randomly picked five fields obtained under a digital fluorescence microscope (Advanced Microscopy Group, USA). All assays were performed thrice using triplicate wells.

2.7. Hoechst 33,258 staining

Cell apoptosis was observed by the Hoechst 33,258 nucleic acid staining method. Goat DPCs were seeded in 24-well plates at a density of 2.5×10^4 cells/well. Once attached after a 24-h incubation period, these cells were treated with ATRA (10^{-9}, 10^{-7}, and 10^{-5} M) and DMSO as mentioned above. After 2 d of treatment, the apoptosis status of goat DPCs were monitored using the Hoechst 33,258 staining kit (Beyotime, Beijing, China) with the manufacturer's instructions. Evaluation of apoptosis of the goat DPCs was carried out using images obtained under a fluorescence microscope (Advanced Microscopy Group, USA) from five randomly selected fields. The percentage of apoptotic cells was calculated as the number of apoptotic cells divided by that of all cells. Each treatment was performed thrice.

2.8. Cell cycle distribution analysis

We analyzed the cell cycle distribution of goat DPCs after treatment with ATRA(10^{-5} M) or DMSO for 48 h using Cell Cycle Analysis Kit (Beyotime, Beijing, China) and flow cytometry (Becton, Dickinson and Company, NJ,USA) as their direction suggested. All data were analyzed using FlowJo software V7.6.5 (Becton, Dickinson and Company, NJ, USA), and all experiments were performed in triplicates to assure reproducibility of the results.

2.9. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from tissues or cells were isolated using the ultraRNA pure kit (CWBio, Beijing, China) according to the manufacturer's instructions. Reverse transcription reactions were performed using the cDNA synthesis kit (Thermo Fisher Scientific, MA, USA). Quantitative real-time PCR analysis was performed in triplicates with Bio-Rad IQ5 Real-Time PCR system using RealStar Green Fast Mixture (Genstar, Beijing, China) according to the manufacturer's protocol. Relative levels of gene mRNA expression were determined by the $2^{-\Delta\Delta Ct}$ method. β-Actin was set as the internal control in all experiments. Information on genes of interest and their primers is detailed in Table 1.

2.10. Construction of the Fgf7 reporter plasmid and dual-luciferase assays

The putative *Fgf7* promoter region was defined as 2290 bp upstream to 61 bp downstream of the transcription start site (TSS) in the *Fgf7* mRNA transcript (*XM_005685797.3*) in the current experiment. A specific fragment was amplified from Cashmere goat genomic DNA using a forward primer 5'-CCGCTCGAGGCGGATGGTG ACTGTAG-3′ and a reverse primer 5'-CCCAAGCTTTTGCTGACCTCA TTGGA-3′; this fragment was then inserted into the pGL3-Basic vector, and the newly constructed plasmid was named as pGL3-Basic-FGF7. For dual-luciferase assays, C2C12 cells were seeded in 24-well plates at density of 8×10^4 cells/well overnight and transiently transfected with the same amount of pGL3-Basic or pGL-Basic-FGF7, with 20 ng of pRL-TK vector as the internal control using Lipofectamine® 3000 (Thermo Fisher Scientific, MA, USA) as per the manufacturer's suggestion of 24 h. Then, the cells were treated with 5×10^{-8} M ATRA or DMSO as the control for another 24 h after which reporter assays were performed using the Dual Luciferase Reporter Gene Assay Kit (Beyotime, Beijing, China) according to the manufacturer's instructions. No obvious toxicity was observed at the current dose tested in each well. The reporter activity was expressed as relative values compared with the vehicle control.

Table 1
Information of primers used in this study.

Gene	Primer sequence(5′-3′)	Product length (bp)
Crabp1	*F:GTGAACGCCATGCTGAGGAA* *R:GCACCGTGGTGGATGTCTTG*	*106*
Crabp2	*F:CTTTAGTGTTGTCCTCGGACGTTCG* *R:GCACGGTGGTGGAGGTTT*	*270*
Fabp5	*F:GGGATGGAAAGGAAAGC* *R:AAACCAGCAGTATGGAGATT*	*194*
Rarα	*F:ACCTGGTCTTCGCCTTCG* *R:CCTCCGTTTCCGCACAT*	*182*
Rarβ	*F:GGGCAAGTACACCACGAA* *R:TTTGGCAAACTCCACGAT*	*118*
Rarγ	*F:CTGCGGATCTGCACAAGGTA* *R:CCAGCGAAGGCAAAGACGA*	*131*
Igf-1	*F:TGCCAGTCACATCCTCCTCG* *R:ATAAAAGCCCCTGTCTCCAC*	*146*
Bmp2	*F:CCTTTATACGTGGACTTCAGTG* *R:GCCTTGGGAATCTTAGAGTTA*	*179*
Acta2	*F:CGGGACATCAAGGAGAAACTG* *R:TGAACGAGGGCTGGAACA*	*172*
β-catenin	*F:CTGCTGTTTTGTTCCGAATGTCT* *R:GGGCACCAATATCAAGTCCAA*	*141*
Fgf7	*F:ATGCGCAAATGGATACTGAC* *R:GGACTCATGTCATTGCAAGC*	*110*
β-actin	*F:CCTCTATGCCAACACAGTGC* *R:GTACTCCTGCTTGCTGATCC*	*211*

F: forward primer; R: reverse primer.

2.11. Transcription factor binding site prediction in the Fgf7 promoter region

To search for the possible RAR binding sites in the promoter region of the *Fgf7* gene, we used the online tool PROMO to predict potential target sites in the presumed DNA sequences [22]. The maximum matrix dissimilarity rate was set as 15% in the present study, and transcription factor binding sites (TFBS) defined in the TRANSFAC database were used to construct specific binding site weight matrices for TFBS prediction. PROMO online website: http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3.

2.12. Data analysis

All data herein were represented as mean ± standard deviation (sd). Statistical analysis was performed using GraphPad Prism 6.7 software. The two-tailed t-test was used to compare the mean values between two groups, and one-way analysis of variance (ANOVA) was applied for comparisons of the means among three or more groups. The criterion for statistical significance was $P < 0.05$, and statistical extreme significance was $P < 0.01$.

3. Results

3.1. Dynamic expression of key transducers in ATRA signaling pathway in cashmere growth cycle

Similar to other animals, hair regrowth in goat is under the precise control of molecular signals present within the goat [12,13]. To explore the biological roles of ATRA in hair cycling, we performed qRT-PCR to obtain the expression patterns of key transducers in ATRA signaling during cashmere growth cycle [8]. As shown in Fig. 1, we found that all key transducers showed a fluctuated expression pattern in all three stages. The expression level of *Crabp1* is significantly higher in the anagen stage than in the catagen stage ($P < 0.05$), and the *Fabp5* expression level is lower in the catagen stage than in the telogen stage ($P < 0.01$). Among the *Rars*, *Rarα* and *Rarβ* peaked in the catagen and telogen stages ($P < 0.01$), respectively. The other member of RARs RARγ showed an expression pattern similar to that of RARβ. These results indicated that ATRA signaling is closely related to cashmere regrowth.

3.2. Culture, identification, and aggregative behavior of goat DPCs in vitro

Microdissection of DP from surgically isolated hair follicle has been proven as a simple and feasible method to successfully culture DPCs *in vitro* [9]. To further unearth the possible roles of ATRA in hair cycling, we cultivated goat DPCs *in vitro* as previously reported and confirmed the identity of isolated cell population using primary antibodies that specifically target α-SMA and CD133, two well-known molecular markers of DPCs. As shown in Fig. 2a, goat DPCs outgrew from attached DP explant in 5 d, and their passaging cultures possessed a flattened and polygonal morphology resembling that in other species, and these cells tended to form a ball-like aggregate once reaching confluence. Results of immunofluorescence staining shown in Fig. 2b and c suggested that cultured goat DPCs exhibited positive and specific immunoreaction for both α-SMA and CD133. Our results verified the successful isolation and culture of goat DPCs.

3.3. ATRA decreased goat DPC viability and impaired their proliferation

To uncover the effect of ATRA on goat DPCs, we performed the MTT assay and EdU staining to monitor the status of cell viability and proliferation. As shown in Fig. 3a, ATRA severely impaired the activity of goat DPCs in a dose-dependent manner, and 48-h or 72-h

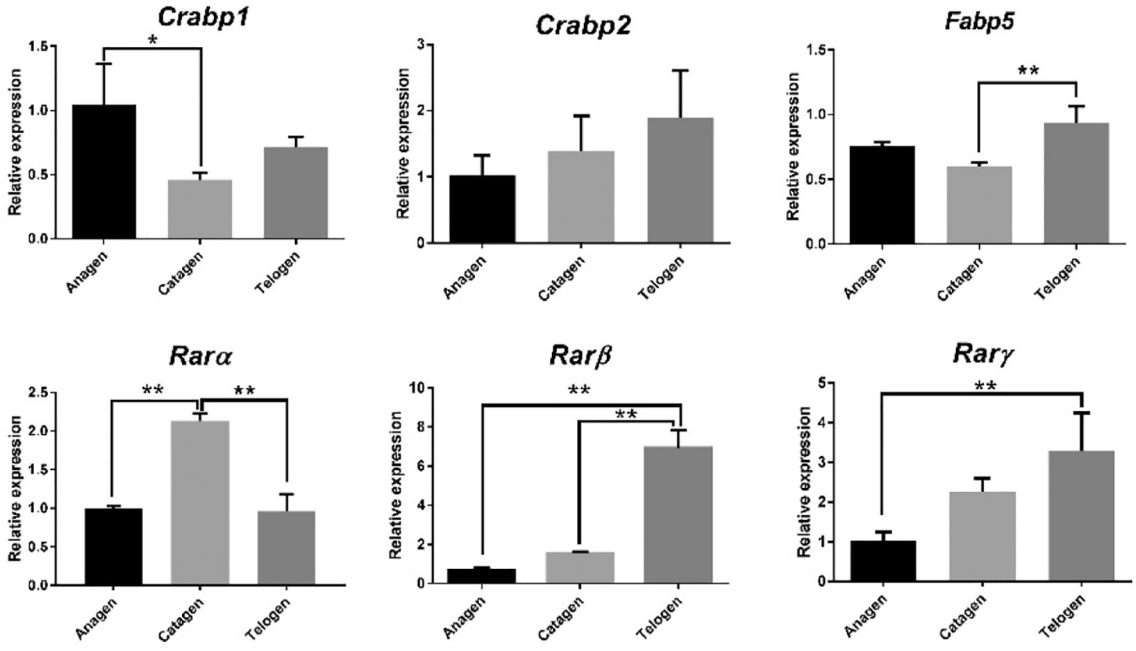

Fig. 1. Dynamic expression of key transducers in ATRA signaling in three stages of goat cashmere growth cycle. Total RNA from skin tissue samples in different cashmere growth stages (anagen, catagen, and telogen) was extracted and used for qRT-PCR analysis. Relative mRNA expression of genes involved was normalized with β-actin, and their expression levels in the anagen stage were set as the control, whose value is 1. $^{*}P < 0.05$, $^{**}P < 0.01$.

treatment with various concentrations (10^{-5}, 10^{-6}, and 10^{-7} M) of ATRA significantly depressed the viability of these cells ($P < 0.01$). At the same time, the EdU staining-based proliferation test result further confirmed the above-mentioned results, as shown in Fig. 3b by the obvious reduction of the numbers of EdU-positive cells by ATRA and in Fig. 3c by the ratio of proliferating goat DPCs at higher doses (10^{-5} and 10^{-7} M, $P < 0.01$). The present experiment confirmed the growth inhibitory role of ATRA on goat DPCs.

3.4. ATRA induced goat DPC apoptosis and blocked cell cycle progression

Except for the growth inhibitory role of ATRA, it was known to induce apoptosis and arrest cell cycle in many cell types [4]. To further clarify the effects of ATRA on goat DPCs, we treated the cells at different ATRA concentrations (10^{-5}, 10^{-7}, and 10^{-9} M) and employed Hoechst 33,258 staining to recognize apoptotic cells from normal ones. Results in Fig. 4a and b indicated that treatment with higher

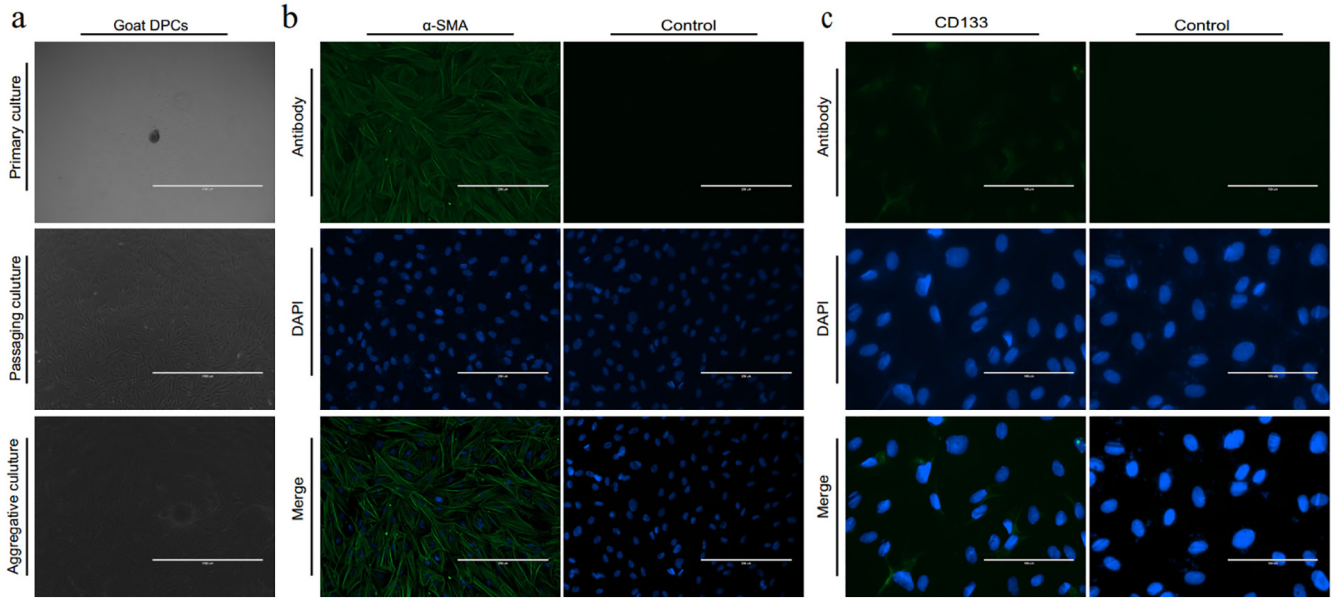

Fig. 2. Cultivation, passaging, and identification of goat DPCs. (a) Goat DPCs outgrew from the isolated DP explant in 5- to 7-d culture and were passaged after another 7 d of cultivation; passaged goat DPCs formed a ball-like structure resembled the *in vivo* morphology of DP, scale bar = 1000 μm. Protein expression and localization of α-SMA (b) and CD133 (c) in goat DPCs were monitored by immunofluorescence staining (red) and the nucleus was stained with DAPI (blue), scale bar = 200 μm and 100 μm, respectively.

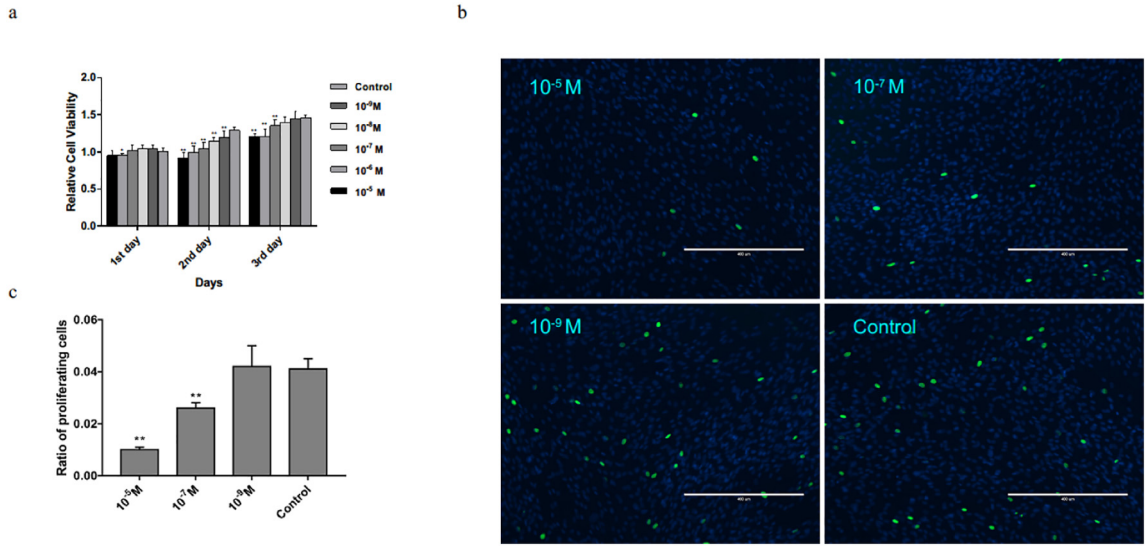

Fig. 3. Effects of ATRA on goat DPC viability and proliferation. (a) Goat DPCs were treated with different concentrations of ATRA or DMSO for 24 h, 48 h, and 72 h, and cell viability assays were performed by the MTT method. (b) Effect of ATRA on goat DPC proliferation for 48 h was also measured by EdU staining. (c) The graph represents the percentage of EdU-positive cells (yellow) in total cell population (yellow and blue). Cell nucleus was stained with Hoechst33342 (blue).$^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

doses (10^{-5} and 10^{-7} M) of ATRA significantly increased the quantity of apoptotic cells (condensed nucleus and dyed bright) and their percentage in all cells (stained blue, $^{**}P < 0.01$). Furthermore, by employing the fluorescence-activated cell sorting (FACS) method to analyze their cell cycle distribution, we found that ATRA potently abrogated the cell cycle progression of goat DPCs by detaining them in the G0/G1 phase (Fig. 4c and d, $P < 0.05$) and decreased their numbers in the G2 phase (Fig. 4c and d, $P < 0.05$). Briefly, all data herein proved the spurring effect of ATRA on the apoptosis of goat DPCs and its negative action on their cell cycle progression.

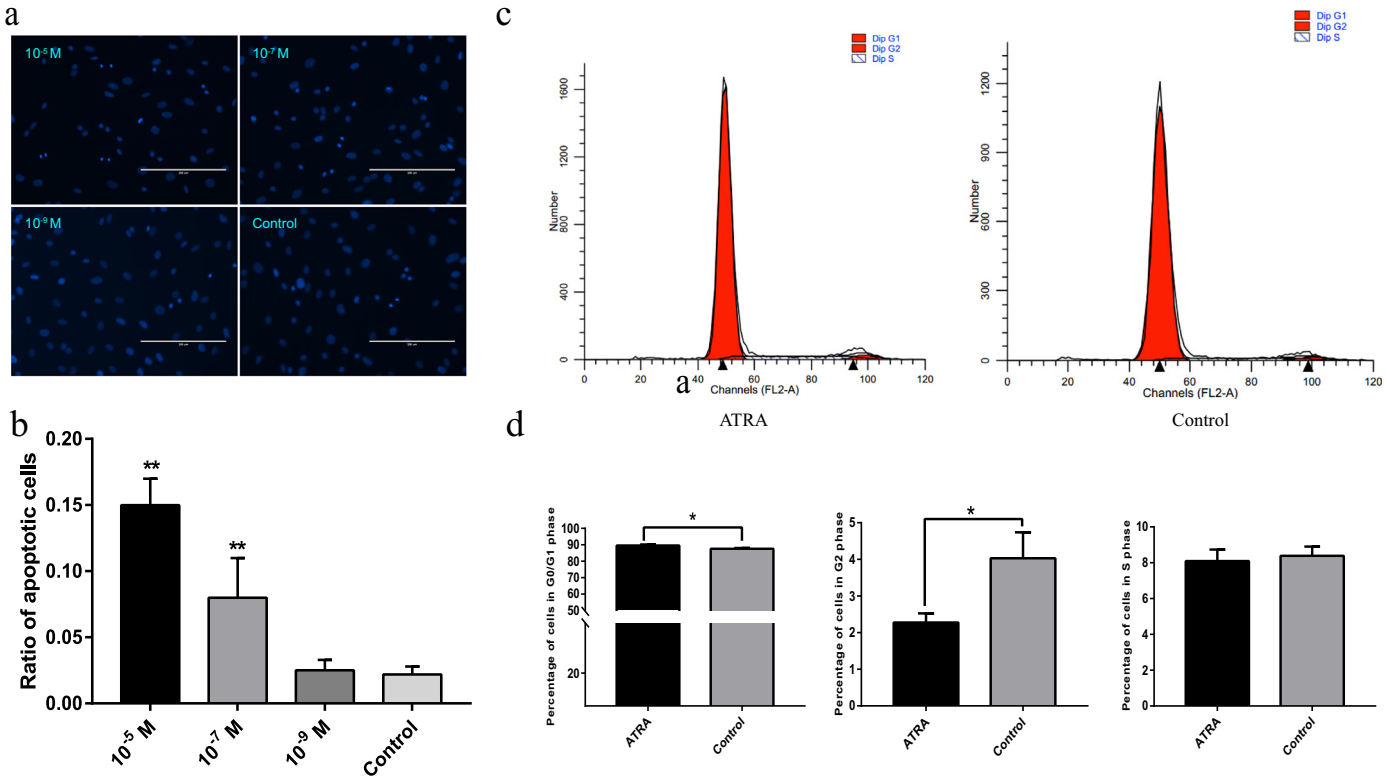

Fig. 4. ATRA stimulated goat DPCs to enter apoptosis and inhibited cell cycle progression. (a) Goat DPCs were treated with different concentrations of ATRA (10^{-5}, 10^{-7}, and 10^{-9} M) or DMSO as the control for 48 h, and Hoechst 33,258 staining was used to discriminate apoptotic cells (condensed nucleus and dyed bright) from normal ones (blue and large nucleus). (b) Percentage of apoptotic goat DPCs in Fig. 4a was counted with Image-Pro Plus 6.0. (c) Treatment of goat DPCs with 10^{-5} M ATRA or DMSO for 48 h, and cell cycle distribution was analyzed by FACS. (d) Percentage of goat DPCs in respective phases. ATRA arrested DPCs in the G0/G1 phase as shown by the significantly increased percentages of goat DPCs in the G0/G1 phase, $^{**}P < 0.01$,$^{*}P < 0.05$ compared with control group, scale bar = 200 μm.

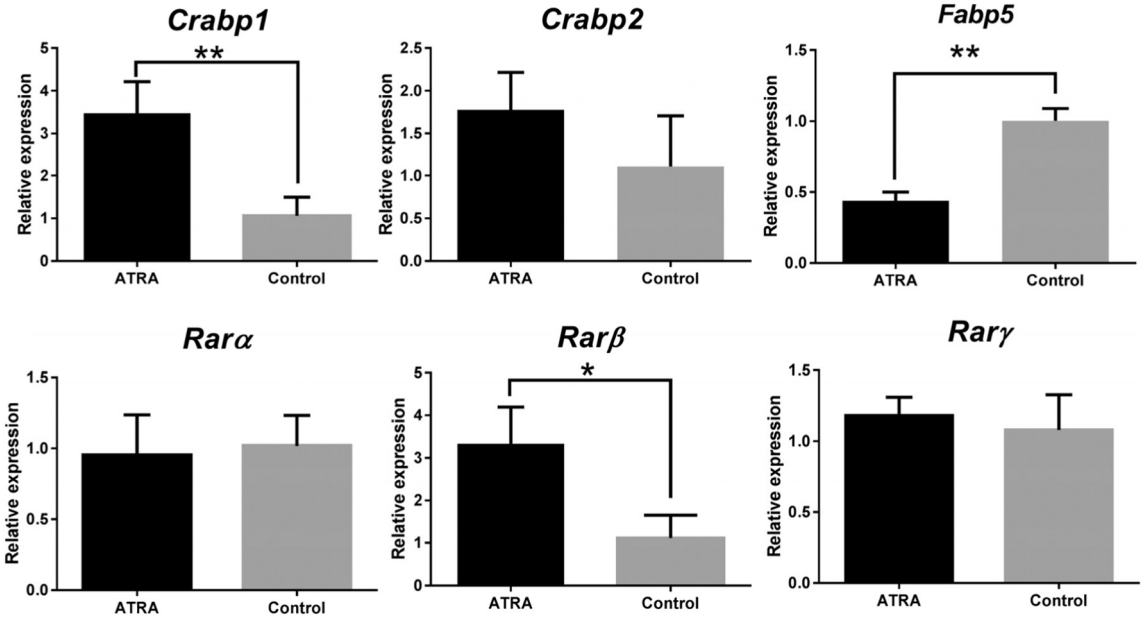

Fig. 5. Effects of ATRA on mRNA expression of key transducers in ATRA signaling in goat DPCs. Cells were treated with 10^{-5} M ATRA or DMSO as control for 48 h, and mRNA expression was defined using qRT-PCR. Relative expression of genes of interest was normalized with β-actin. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with the control group.

3.5. ATRA altered the expression of some key transducers of its signaling in goat DPCs

Previous studies identified the existence of *cis*-elements in promoter regions of key transducers of ATRA signaling, thus implying ATRA might reinforce its effects on target cells by transcriptionally activating the expression of these transducers [2]. To verify these studies and deeply elucidate the effects of ATRA on goat DPCs, we treated them with ATRA at 10^{-5} M for 48 h and the relative gene expression was determined by qRT-PCR. Our results in Fig. 5 indicate that only one member of *Rars*, *Rarβ*, positively responded to ATRA treatment ($^{*}P < 0.05$), whereas the expression levels of all three carriers showed dramatic changes after ATRA stimulation: *Crabp1* and *Crabp2* were upregulated, although *Crabp2* did not meet a significant criterion, but *Fabp5* was dramatically repressed by ATRA ($P < 0.01$).The differential responses of these key transducers to ATRA stimulation revealed their possible involvement in mediating the effects of ATRA on goat DPCs.

3.6. ATRA transcriptionally depressed Fgf7 expression and the prediction of RAR binding sites in the Fgf7 promoter region

DP regulates hair growth by releasing growth factors to boost the proliferation and differentiation of follicular keratinocytes [7,9]. To testify whether ATRA impairs hair growth–promoting ability of goat DPCs or not, we quantified the expression of several growth factors and their upstream regulators closely related to that in goat DPCs after 10^{-5} M ATRA treatment. As indicated in Fig. 6a, we discovered that the expression of Fgf7, a powerful hair growth regulator, was negatively modulated by ATRA treatment ($P < 0.01$). Further, dual-luciferase analysis (Fig. 6b) proved that ATRA specifically inhibited the promoter activity of *Fgf7*, and discovery of multiple RAR binding sites in the promoter region by matrix-based prediction method (Fig. 6c) also supported the transcriptionally repressing role of ATRA on the *Fgf7* gene expression. All experiments present demonstrated ATRA impaired hair growth-promoting competence of DPCs by suppressing the release of growth factors.

4. Discussion

Although the essential roles of endogenous ATRA in hair follicle morphogenesis and cycling are undoubtable [23], the effects of exogenous ATRA on these processes is controversial. Using skin explants from embryonic chick and mouse, researchers found that excess vitamin A treatment caused metaplasia of hair follicle into glandular tissues [24,25]. Following studies expanded this knowledge by showing that excess RA can alter the signals originating from the dermis to instruct morphogenetic events of the hair follicle and promote phase transition in tissues [26,27]. Similarly, genetic manipulation-induced excessive endogenous RA accumulation in mouse embryo arrested the hair follicle development at the hair germ stage, and this abrogation was reversed by grafting the mutant skin on immunodeficient mice with a normal physiological level of RA [28]. These studies clearly suggested the detrimental effects of excess or exogenous RA on hair follicle morphogenesis. It has been reported that hair follicle cycling in adults shared nearly the same signals and mechanisms with its morphogenetic events [7,19,29]. Thus, it is reasonable to predict that excess RA might curb hair growth in adulthood, but studies hold opposite views about this. Kerstin Foitzik and his colleagues demonstrated that ATRA induced premature phase transition from anagen to catagen by elevating the expression of TGF-β2 in DP in organ-cultured human hair follicle, whereas another group showed that topical application of ATRA prolonged the anagen stage and shortened the telogen phase in a mouse model [17]. Moreover, ATRA was thought to be a hair growth stimulus and used for the treatment of hair loss in humans [16,18,30]. Presently, the dispute over this issue still exists.

DP is a population of functional fibroblasts formed by the embryonic dermis under the guidance from specific epidermal signals at the early stage of hair follicle development [11]. It plays pivotal roles in hair follicle biology by recruiting growth factors to instruct the proliferation and differentiation of follicular keratinocytes during hair formation [9]. The specific expression of *Crabp1* and *Crabp2* in intact DP *in vivo* [19,31] or *Rarβ* in cultured DPCs *in vitro* was observed by independent research groups [20,27]. These discoveries implied that DP or DPCs might be the target cells of ATRA in hair follicle and possibly mediate the effects of ATRA on hair follicle growth or

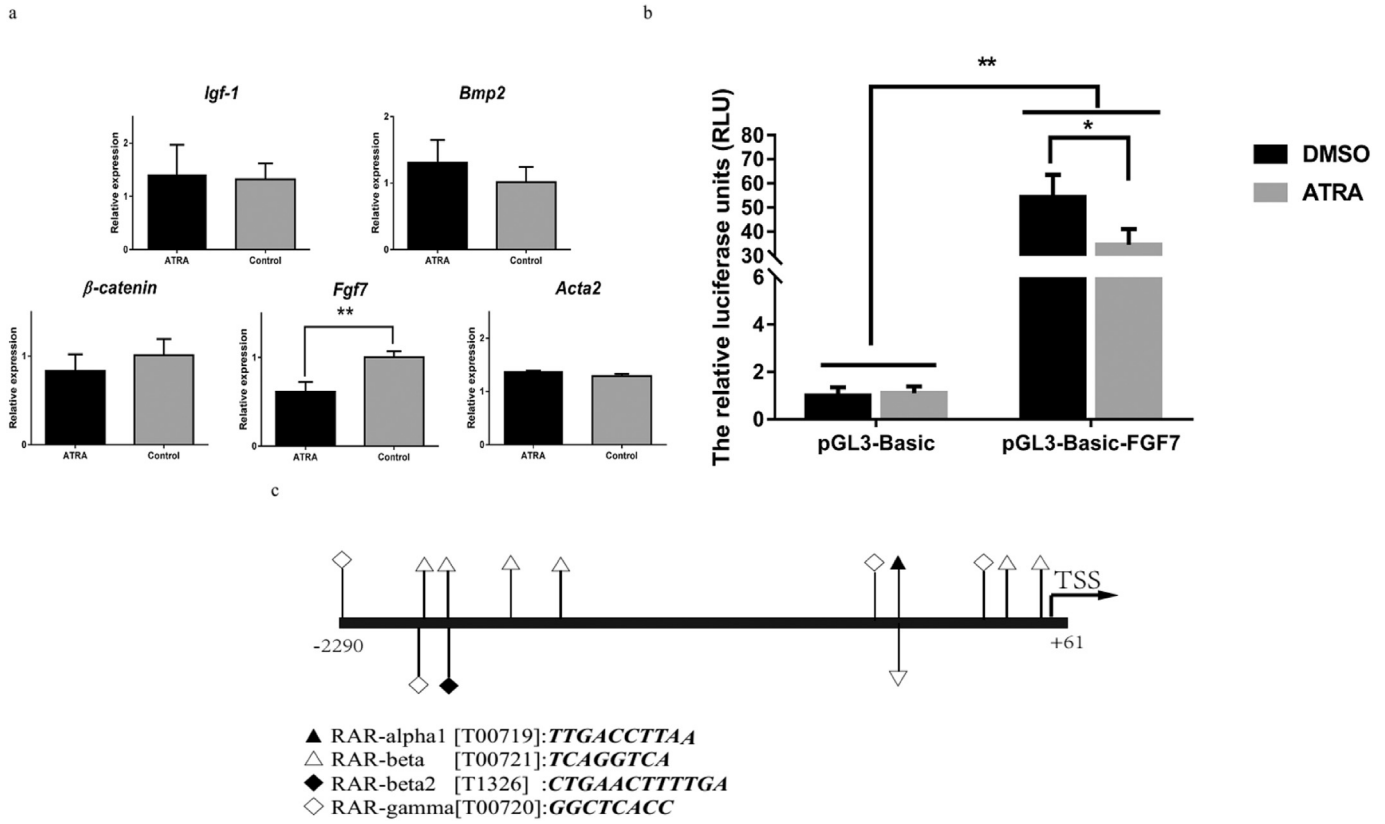

Fig. 6. ATRA transcriptionally depressed FGF7 expression and the prediction of RAR binding sites in the *Fgf7* promoter region. (a) Cells were treated with 10^{-5} M ATRA or DMSO as control for 48 h, and total RNA was harvested for the analysis of mRNA expression of genes involved in hair growth–promoting capacity of goat DPCs by qRT-PCR. Relative expression of genes was normalized with β-actin. $^{**}P < 0.01$ compared with control group. (b) After transfected with a plasmid, C2C12 cells were treated with ATRA (5×10^{-8} M) or DMSO as the control. Relative luciferase units (RLU) were determined by comparison with the native pGL3-Basic vector, whose value was set as 1 compared with the control group). (c) Prediction of RAR binding sites in the presumed promoter region of *Fgf7*, and maximum matrix dissimilarity rate was set as 15% in this study ($^{*}P < 0.05$, $^{**}P < 0.01$).

development. Based on this hypothesis, we designed and performed the present study. Our results indicated that a high dose of exogenous ATRA decreased cellular activity of goat DPCs and suppressed the expression of *Fgf7* at the transcriptional level. Otherwise, an increased expression level of *Rarβ* in ATRA-treated goat DPCs was also revealed. This study suggests that excess ATRA might adversely affect hair growth and this action is probably partially mediated by *Rarβ*.

Numerous studies have proven that the synthesis of ATRA occurs at sites of action and the precise levels of ATRA are tightly controlled by regulating several key steps in vitamin A metabolism [23]. In the hair follicle, diverse sites including DP, hair matrix, and outer root sheath are believed to be the locations of ATRA synthesis and action, thus implying the importance and complexity of ATRA functions in hair follicle development [31,32,33]. It is widely accepted that through alternate activation of two different nuclear receptors, RA imparts opposing effects on cell growth [34]. Binding to and activating RARs by CRABP2 delivery, RA inhibits cell growth; working with PPARβ/δ by FABP5 transportation, RA promotes cell proliferation [2]. In this study, we found that *Crabp2* and most *Rars* showed opposite expression levels compared with that of Fabp5 in the anagen stage (Fig. 1). Combing with differential expression results of these key transducers in the hair follicle in previous reports, this study suggests that *CRABP2*- and *FABP5*-mediated ATRA signaling pathways exert distinct influences on hair growth.

Cellular status of DP is closely related with hair growth as evidenced by the reduction in cell number in the telogen stage and its restoration in the anagen stage [10]. Otherwise, decrease in cell number inside DP often caused hair follicle miniaturization and eventually led to hair loss [35]. The present study showed that excess ATRA inhibited the activity of goat DPCs by lowering their viability and proliferation (Fig. 2), thereby driving them to enter apoptosis and blocking their cell cycle progression. This indicates that excess ATRA might negatively regulate hair growth *in vivo* by reducing DP cell number.

ATRA activates or depresses target gene expression by binding to the RA-response elements (RARE) in the promoter region of these genes with RARs and other proteins. Discovery of RAREs in the promoters of *Crabp2* and *Rars* indicates the existence of feedback loop to reinforce the effects of ATRA on target cells [1]. Increased expression of *Crabp2* and decreased expression of *Fabp5* (Fig. 5) further supported the growth inhibitory effect of excess ATRA on goat DPCs. Furthermore, ATRA-stimulated expression of *Rarβ* in goat DPCs affirmed the role of *Rarβ* in mediating RA-induced glandular metaplasia in the mouse skin [27] and indicated that *Rarβ* might be responsible for the effects of ATRA in goat DPCs.

FGF7 is a potent hair growth stimulator derived from DP and usually deemed as the target to treat hair loss [36,37]. The suppressed expression of FGF7 by ATRA in goat DPCs implied that excess ATRA damages the ability of DP to stimulate hair growth. Moreover, the discovery of multiple binding sites of RARβ in the presumed promoter of *FGF7* suggests that RARβ might exert a transcriptionally repressing role in the *Ffg7* gene expression. Taken together with previous results, we deemed that RARβ is the key RAR for the signaling transduction of ATRA in DP.

In summary, our results showed negative effects of excess ATRA in regulating cellular activity and growth factor expression of goat DPCs, thus shedding light on explaining the role of excess ATRA in inhibiting hair growth. Considering the structural complexity of the hair follicle, more endeavors are needed to clarify the effects of ATRA on other cell types of hair follicle, especially stem cells and hair matrix cells.

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Competing interests

The authors declare no competing interests.

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