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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 $5 \times 10^3$ 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 $2.5 \times 10^4$ 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.