·Basic Research·

Effect of dihydrotestosterone on the expression of mucin 1 and the activity of Wnt signaling in mouse corneal epithelial cells

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Abstract

• **AIM: To explore the effects of the androgen dihydrotestosterone on the expression of mucin 1 (MUC1) and the activity of Wnt signaling in mouse corneal epithelial cells.**

• **METHODS: Primary mouse corneal epithelial cells were isolated from the corneas of BALB/c mice. Quantitative real-time polymerase chain reaction, immunofluorescence and Western blot analysis were used to quantify the differential expression of selected genes. The androgen receptor was silenced by transfecting cells with androgen receptor shRNAs. TOP-Flash and FOP-flash reporter plasmids were used to measure β-catenin-driven transcription.**

• **RESULTS: Dihydrotestosterone treatment increased MUC1 expression and activated the Wnt signaling pathway and led to the translocation of β-catenin and upregulation of the Wnt downstream target gene TATA box binding protein and urokinase plasminogen activator. These effects were prevented by downregulating the androgen receptor.**

• **CONCLUSION: Androgens may protect against dry eye by regulating the expression of MUC1 which is stimulated by the activation of Wnt signaling *via* the androgen receptor. An understanding of the mechanisms associated with androgen-mediated protection against dry eye is an important step in developing new therapies for this disease.**

• **KEYWORDS:** dihydrotestosterone; dry eye; β-catenin; mucins; androgen receptor

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INTRODUCTION

Dry eye disease, is a common multifactorial ocular surface disease that occurs due to insufficient tears to lubricate and nourish the eye or when tears evaporate too quickly [1]. The signs and symptoms of dry eyes include ocular discomfort, feeling of dryness and eye fatigue, corneal hyperemia and keratoconjunctival epithelial disorders. Dry eye is more frequent in women (11.9%) than in men (9.0%), and is associated with aging [2]. The number of dry eye patients is increasing yearly in association with an aging population, decreased androgen levels and increased exposure to video display terminals[3]. Advanced dry eye may damage the ocular surface and eventually impair vision.

Mucins are an important component of the tear film (TF) and are associated with the etiology of dry eye [4]. Mucin 1 (MUC1) is one of the most studied membrane-tethered mucins produced by the ocular surface epithelium that protects the ocular surface [5], stabilizes the TF and maintains the homeostasis of the ocular surface [6-7]. Mucin deficiency may reduce the stability of the TF, increase ocular surface tension and tear evaporation, which leads to the development and progression of dry eye[8].

The Wnt signaling pathway is an important modulator of cell function in a variety of cell types [9]. The activation of the Wnt signaling pathway is essential to corneal epithelial health and repairation [10]. Accumulation and nuclear translocation of the junctional protein β-catenin triggers the activation of the Wnt signaling pathway [11], leading to the transcription of β-catenin/Tcf enhancer factor[12], resulting in the upregulation of downstream target genes such as TATA box binding protein (TBP) [13] and urokinase plasminogen activator (UPA)[14].

Eyes are one of the many target organs of sex hormones[15-18]. The androgen receptor is expressed in human [16], rabbit[15,18], and mouse [17] lacrimal glands, cornea and other ocular tissues. Sex hormones play a key role in the development and progression of dry eyes [19]. Dry eye disease is more prevalent with androgen deficiency associated with Sjögren's syndrome, aging, menopause, complete androgen-insensitivity syndrome and anti-androgen use [16]. Androgen inhibits the

progression of dry eye [20], however, the mechanism remains unclear.

Women who use postmenopausal hormone replacement therapy, particularly estrogen alone, are at increased risk for dry eye [21]. Treatment with a combination of estrogen and androgen methyltestosterone reduced the symptoms of dry eye in postmenopausal women [20]. Androgen treatments may be associated with side effects of increased hirsutism [22]. It is therefore important to elucidate the mechanism underlying the androgen treatment for dry eyes. Dihydrotestosterone (DHT) is a potent androgen with a high affinity for the androgen receptor. The purpose of the present study was to explore the effects of DHT, on the expression of mucins and the activity of Wnt signaling in mouse corneal epithelial cells.

MATERIALS AND METHODS

Cell Culture and Reagents Primary mouse corneal epithelial cells (mCEC) were isolated from the corneas of BALB/c mice. Cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 μg/mL penicillin, and 100 μg/mL streptomycin (Sigma, St. Louis MO, USA) in humidified air with 5% CO_2 at 37℃. The mCEC from second to eighth passage were used. All experiments were carried out in accordance with the guidelines and the approval of Xi'an Jiaotong University Institutional Animal Use and Care Committee and in compliance with the Association for Research in Vision and Opthamology (ARVO) statement for the use of animals in ophthalmic and vision research. In our study, mCECs was incubated with DHT (0.3 mg/mL) for 24h. Primary antibodies against androgen receptor (AR) (ab74272), MUC1 (ab45167) and β-catenin (ab32572) were obtained from Abcam (Cambridge, CB, UK). Anti-TBP (No.12578) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-lamin A (sc-6214), anti-UPA (sc-14019), and anti-GAPDH (sc-20357) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Real–time Polymerase Chain Reaction The differential expression of selected genes was verified by quantitative real-time polymerase chain reaction (qRT-PCR). Briefly, total RNA was extracted using the Fastgen200 RNA isolation system (Fastgen, Shanghai, China) according to the manufacturer's instructions. The cDNA was synthesized using a real-time PCR kit (TaKaRa, Japan) with extracted RNA as a template. An iQ5 Multi-color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using a SYBR Green real-time PCR Master Mix (Takara, Dalian, China) was used for detection. Differential gene expression was calculated according to the ΔΔCt method with GAPDH as the normalization control [23]. PCR primer sequences were as follows: GAPDH (Forward: 5'-GCATCTTCTTGTGCAGTG CC-3', Reverse: 5'-TACGGCCAAATCCGTTCACA-3'); AR (Forward: 5'-GCTCACCAAGCTCCTGGATT-3', Reverse: 5'-TCAGGAAAGTCCACGCTCAC-3'); MUC1 (Forward: 5'-CCAAGCGTAGCCCCTATGAG -3', Reverse: 5'-GTGG GGTGACTTGCTCCTAC -3').

Preparation of Cell Extracts and Western Blot Analysis Protein lysates were prepared on ice in RIPA buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate] with freshly added 0.1 mg/mL phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate and 1 mg/mL aprotinin. Protein concentrations were determined using the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA, USA). Aliquots of cell extracts containing 20-50 μg of total protein were resolved by 12% SDS-PAGE and transferred to 0.45 mm nitrocellulose membranes (Osmonics, Westborough, MA, USA). The membranes were blocked for 1h at room temperature with Blotto A [5% nonfat milk powder in TBS-T: 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.05% Tween-20], and then incubated for 1h at room temperature in Blotto A containing a 1:1000 dilution of primary antibodies. After washing in TBS-T buffer for 5min at room temperature, membranes were incubated for 1h at room temperature in Blotto A containing a 1:10 000 dilution of peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Amersham, Arlington Heights, IL, USA). After washing in TBS-T, electrochemiluminescence was performed according to the manufacturer's recommendation (Thermo Fisher Scientific Inc., Waltham, MA, USA). All results were normalized to GAPDH protein expression.

Androgen Receptor Silence in Mouse Corneal Epithelial Cells To silence AR, short hairpin RNA (shRNA) against AR (SH-AR1 and SH-AR2) and the negative control shRNA (SH-control) were purchased from GenePharm (Shanghai, China). The mCEC were cultured in six-well culture plates to a confluence of 60% -70%. Cells were serum starved overnight and then transfected with AR shRNAs or the negative control shRNA according to the manufacturer's instructions using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA). After 24h or 48h (24h for PCR and 48h for Western blot), the medium was replaced with normal growth medium, and the efficiency of silence was determined by qRT-PCR and Western blot analysis.

TOP/FOP –Flash Assays The mCEC were plated at a concentration of 5000 cells/well on white-bottomed 96-well plates. Cells were serum-starved overnight and co-transfected with TOP-Flash or FOP-Flash reporter plasmid and the pRL-TK as a control. Cells were subsequently treated with DHT (0.3 mg/mL) for 24h based on the concentration established previously [24]. The cells were then harvested and lysed in Promega luciferase assay lysis buffer. Using the luciferase assay kit (Promega), the luminescence from the TOP- or FOP-Flash was detected with a Microplate luminometer (Turner Biosystems Instrument, Sunnyvale, CA, USA). The TOP/FOP-Flash readings were normalized

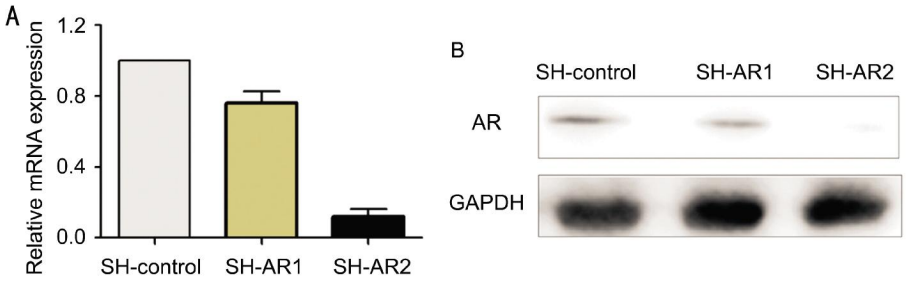

Figure 1 The efficiency of AR-specific shRNA in mCEC A: After transfection with shRNA plasmids for 24h, the mRNA expression of AR was detected by qRT-PCR; B: The AR protein expression was determined by Western blot after transfection with shRNA plasmids for 48h. GAPDH was used as a normalization control.

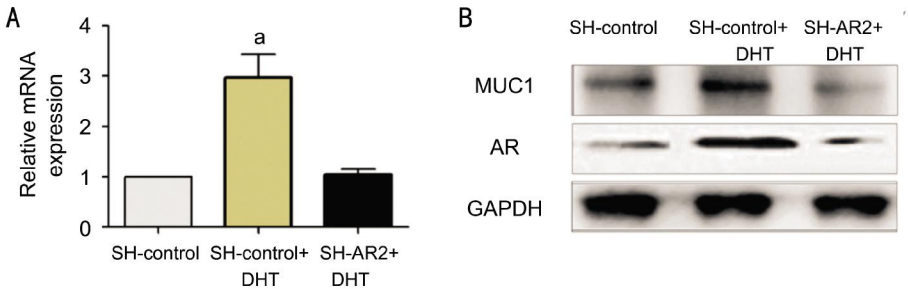

Figure 2 The effect of AR silience on MUC1 upregulated by DHT in mCEC A: Control and AR-specific shRNA-transfected mCEC were treated with DHT for 24h. The expression of MUC1 mRNA was measured by qRT-PCR. GAPDH was used as an internal control; B: The MUC1 and AR protein expression in mCEC was detected by Western blot after treatment with DHT. [a]P<0.05 *vs* SH-control group.

to the Renilla reniformis reading and the TOP/FOP ratio was used as a measure of β-catenin-driven transcription. Experiments were performed in triplicate.

Immunofluorescence Transfected mCEC cells were plated onto Lab-Tek CC2 glass chamber slides (Nalge Nunc International, Naperville, IL, USA). Cells were fixed in either ice-cold methanol or paraformaldehyde-lysine-phosphate buffer, with or without permeabilization in 0.2% Triton X-100. After treatment with 3% H_2O_2, the slides were incubated for 1h with monoclonal anti-MUC1 or anti-β-catenin. Normal mouse IgG was used as a negative control. The cells were further incubated with suitable secondary antibodies. Slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) with DAPI (4', 6'-diamidino-2-phenylindole dihydrochloride). The staining was examined under a Leica confocal or a Zeiss 100 M microscope.

Statistical Analysis Statistical analysis was performed using SPSS 18.0. The data were expressed as means ±standard deviation. Differences between means were tested for statistical significance using one-way ANOVA. Significance was identified at P<0.05. Each experiment was repeated at least three times.

RESULTS

Efficiency of shRNA in Mouse Corneal Epithelial Cells Two shRNA plasmids (SH-AR1 and SH-AR2) was constructed targeting AR gene and SH-Control as a negative control. The silencing efficiency of AR was examined by RT-PCR after transfecting with shRNA plasmids for 24h. The shRNA plasmids significantly reduced the AR mRNA level by 23.16% and 90.57% , respectively, compared with the SH-control group (P<0.05; Figure 1), which was confirmed by Western blot analysis. The AR protein expression was markedly suppressed after transfection with shRNA plasmids for 48h. In the following experiments, SH-AR2 plasmid was used to silence AR expression.

Effect of Androgen Receptor Silience on Mucin 1 Upregulated by Dihydrotestosterone DHT (0.03%) treatment significantly increased the MUC1 mRNA expression compared with the untreated control group (Figure 2A). However, the upregulation of the MUC1 by DHT treatment was inhibited by AR silience (Figure 2B), which indicates that the induction of MUC1 expression by DHT was attenuated by AR silencing.

Activation of the Wnt Signaling induced by Dihydrotestosterone TOP flash and FOP flash reporters are widely used to evaluate β-catenin-dependent signaling activity. Luciferase activity increased significantly with DHT treatment (Figure 3A). The translocation of β-catenin from cytoplasm to the nucleus is a key event during Wnt/β-catenin signaling activation. The β-catenin level remained unchanged in the total cellular protein fraction and increased in the nuclear fraction after DHT treatment for 24h (Figure 3B) indicate it translocated from the cytoplasm to the nucleus (Figure 3C). The UPA and TBP levels increased with DHT treatment (Figure 3D) and initiated theWnt/ β-catenin activity.

Effect of Androgen Receptor Silience on Dihydrotestosterone -induced Wnt Signaling activation mCEC was transfected with shRNA plasmid targeting AR before DHT treatment to determine the role of AR in the

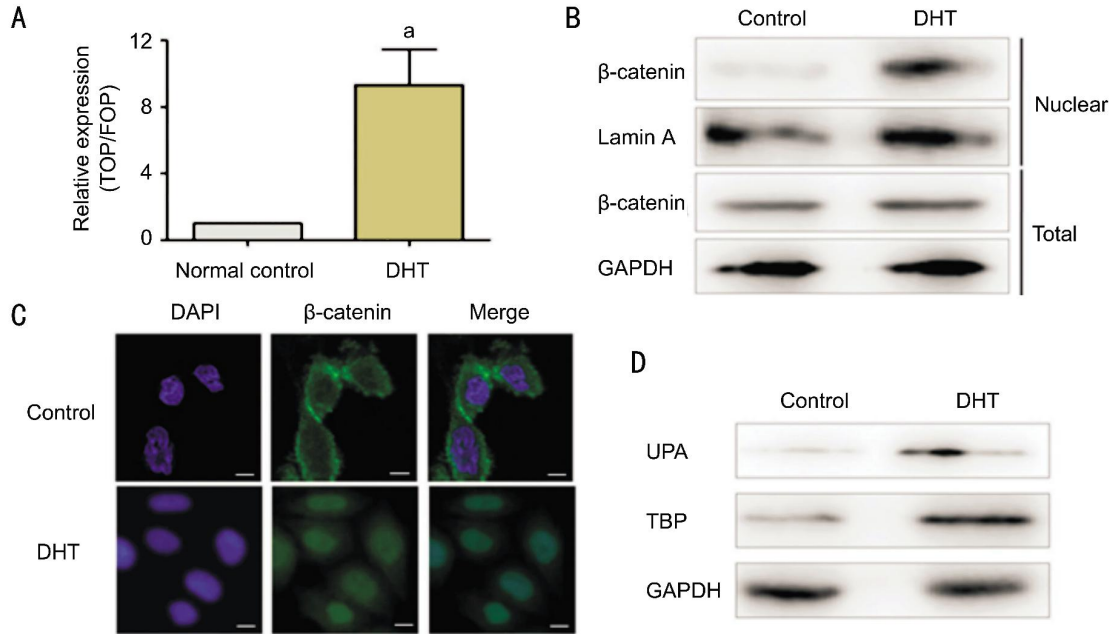

Figure 3 DHT induces activation of the Wnt Signaling in mCEC A: TOP/FOP flash assay was used to detect the activity of Wnt Signaling before and after DHT treatment. B: After DHT treatment for 24h, total and nuclear proteins were extracted and Western blot analysis was used to quantify the expression of β-catenin. GAPDH and Lamin A were used as internal controls for total and nuclear protein content. C: Representative images showing immunofluorescent staining of β-catenin (green) with propidium iodide (PI) (blue) counterstaining; bar: 15 μm. D: The protein expression of Wnt signaling target molecules, TBP and UPA, was estimated by Western blot after DHT treatment for 24h. $^{a}P<0.05$.

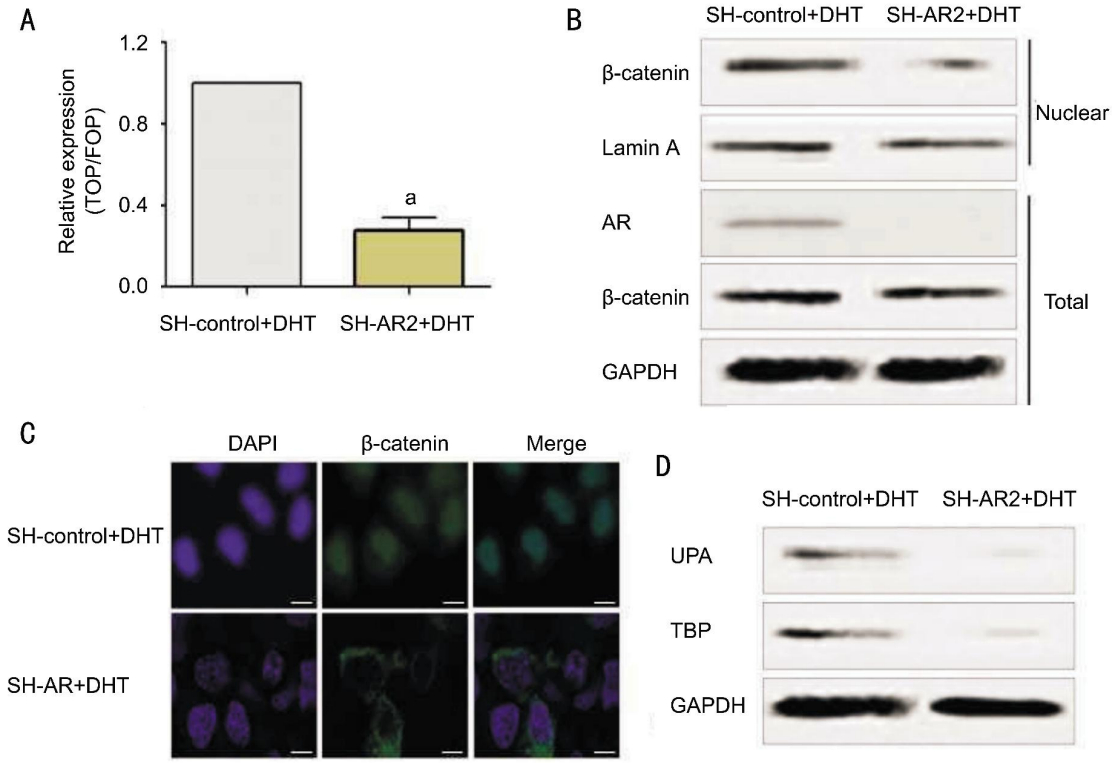

Figure 4 AR silence abolished Wnt signaling activation in mCEC A: The activity of Wnt signaling in SH-control and SH-AR-transfected mCEC in the presence of DHT was detected by TOP/FOP flash assay; B: SH-control and SH-AR-transfected mCEC were treated with DHT, and the β-catenin content in total cell and nuclear extracts was measured by Western blot; C: The expression and location of β-catenin were analyzed by immunofluorescence after SH-Control and SH-AR-transfected mCEC were treated with DHT; bar: 15 μm; D: Western blotting analysis was used to quantify the protein expression of the Wnt signaling target molecules, TBP and UPA after SH-Control and SH-AR-transfected mCEC were treated with DHT. $^{a}P<0.05$.

activation of Wnt signaling by DHT. AR silencing inhibited the activation of Wnt/β-catenin signaling by DHT (Figure 4A), inhibited the DHT-induced translocation of β-catenin into the nucleus (Figure 4B, 4C) and inhibited the DHT-induced

upregulation of the Wnt signaling target genes, UPA and TBP (Figure 4D). Taken together, these results indicated that DHT-induced Wnt Signaling activation was mediated by AR.

DISCUSSION

The major finding of this study is that the androgen DHT regulated the expression of MUC1 and induced Wnt signaling activation *via* AR. DHT treatment increased MUC1 expression, which is important in that the mucin layer plays a critical role in maintaining the stability of the tear film[5-8]. Three membrane-associated mucins (MUC1, MUC4, and MUC16) have been identified in the corneal epithelium[25]. The expression of MUC16 on the ocular surface glycocalyx provides a non-adhesive protective barrier for the epithelial surface [26]. It has been reported that the level of MUC1/A, a splice variant of MUC1, was lower in patients with dry eye disease compared with normal patients suggesting that the absence of MUC1/A contributed to dry eye disease [27]. However, this association is not always observed since the amount of MUC1 was higher in patients with dry eye associated with Sjögren's syndrome compared with patients without dry eye [28]. This discrepancy may be associated with increased MUC1, which was either compensatory or protective in the early stages of dry eye pathophysiology.

In this study, we found that MUC1 expression increased with DHT treatment and decreased when the AR for DHT was silenced. This strongly suggests that the AR is associated with the expression of MUC1. AR activation is closely related to the Wnt/β-catenin signaling pathway. DHT increased the activity of Wnt/β-catenin signaling, induced the translocation of β-catenin from the cytoplasm to the nucleus and increased the amount of two Wnt pathway downstream target genes, TBP and UPA. These effects were abolished when AR was silenced. We could see that catenin was still expressed at a very low level after AR silenced. This can be explained by the idea that there are other factors that influence Wnt signals in addition to the A-AR pathway. Wnt/β-catenin signaling plays a fundamental role in modulating a variety of physiological functions and cellular processes, such as the determination of cell fate [29], cell proliferation [30], migration [31], and polarity [32], and the maintenance of stem cells [33]. β-catenin is a key downstream effector of the canonical Wnt pathway[11]. β-Catenin has been shown to be a co-activator of the AR signaling in the presence of androgens. Co-localization of the AR and β-catenin has been found in human prostate cancer samples[34]. Additionally, the AR promotes β-catenin nuclear translocation in prostate cancer cells[35]. Our results most likely suggest the existence of a close relationship between AR signaling and the Wnt pathway in corneal epithelium.

In conclusion, the androgen DHT increases the expression of MUC1 and activates Wnt signaling that is attenuated by AR. This mechanism could be exploited as a therapy for dry eye.

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Conflicts of Interest: Qin L, None; **Pei C,** None; **Kang QY,** None; **Liu Z,** None; **Li L,** None.

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