Testosterone regulates keratin 33B expression in rat penis growth through androgen receptor signaling

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Androgen therapy is the mainstay of treatment for the hypogonadotropic hypogonadal micropenis because it obviously enhances penis growth in pubescent microphallic patients. However, the molecular mechanisms of androgen treatment leading to penis growth are still largely unknown. To clarify this well-known phenomenon, we successfully generated a castrated male Sprague Dawley rat model at puberty followed by testosterone administration. Interestingly, compared with the control group, testosterone treatment stimulated a dose-dependent increase of penis weight, length, and width in castrated rats accompanied with a dramatic recovery of the pathological changes of the penis. Mechanistically, testosterone administration substantially increased the expression of androgen receptor (AR) protein. Increased AR protein in the penis could subsequently initiate transcription of its target genes, including keratin 33B (Krt33b). Importantly, we demonstrated that KRT33B is generally expressed in the rat penis and that most KRT33B expression is cytoplasmic. Furthermore, AR could directly modulate its expression by binding to a putative androgen response element sequence of the Krt33b promoter. Overall, this study reveals a novel mechanism facilitating penis growth after testosterone treatment in precastrated prepubescent animals, in which androgen enhances the expression of AR protein as well as its target genes, such as Krt33b.

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INTRODUCTION

Penis formation and growth are both androgens dependent, and androgen-dependent penile growth is believed to be responsible for 70%–75% of the adult penile length.1,2 It is supposed that androgens may affect penis formation and growth in different time windows, and this effect only occurs at 3 time points: in late gestation, the first 4 years after birth, and at puberty.3 Hence, androgen therapy with testosterone has been the mainstay of clinical treatment for the hypogonadotropic hypogonadal micropenis since the 1970s. Testosterone treatment usually successfully enhances penile growth in prepubescent microphallic patients, although most boys with a micropenis ultimately have a smaller than average penis in adulthood.4,5 Overall, a normal penis length/size depends critically on androgens, but how androgens mediate this process is still unclear.

Physically, testosterone is converted into 5α-dihydrotestosterone (DHT) by the enzyme delta4-3-ketosteroid-5α-reductase (5α-reductase), which binds to a specific androgen receptor (AR) protein.6-9 This steroid receptor complex translocates into the cell nucleus and initiates gene transcription, thus resulting in the expression of androgen target genes, which may play critical roles not only in the prostate, but also in penis growth.10,11

In this study, we clearly showed that testosterone treatment resulted in a dose-dependent stimulation of penis growth using a castrated rat model and found that it dramatically induced AR protein expression with an increased penis growth. To the best of our knowledge, we are the first to apply microarrays to screen the potential genes involved in penis growth at puberty, and KRT33B was identified as a novel AR target gene during penis growth after testosterone administration. Taken together, we demonstrated that androgens are critical for penile growth during puberty through upregulation of AR protein. In addition, we discovered KRT33B, a novel AR target gene, as a new candidate gene for rat penis growth. These findings may improve the understanding of mechanisms leading to penile development, thus providing potential molecular markers for optimal androgen treatment.

MATERIALS AND METHODS

Chemicals and reagents

Testosterone (25 mg ml−1) was purchased from Shanghai Tongyong Pharmaceutical Co. Ltd. (Shanghai, China). Rabbit polyclonal AR (N-20) and AR (N-20) X antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal KRT33B antibody (11048-1-AP) was obtained from Proteintech Group (Chicago, IL, USA). Primers for KRT33B and β-actin were designed by TaKaRa Biotec Technology (Dalian, China). Horseradish peroxidase-or tetramethylrhodamine isothiocyanate-conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The enhanced chemiluminescence (ECL) detection system

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was obtained from Amersham (Pittsburgh, PA, USA). The chromatin immunoprecipitation (ChIP) assay kit was purchased from Cell Signaling Technology.

Animal procedures
All animal procedures were approved by the Institutional Animal Care and Use Committee of the Medical School of Xi’an Jiaotong University. A total of 80 males Sprague Dawley (SD) rats aged 3 weeks old and weighing 40–50 g were obtained from the Laboratory Animal Center of Xi’an Jiaotong University and randomly assigned to eight groups (n = 10 for each group). All rats except the intact controls were castrated. Briefly, the animals were anesthetized with xylazine (10 mg kg⁻¹) and ketamine (90 mg kg⁻¹) by intraperitoneal (ip) injection and then subjected to a midline scrotal incision, and the testicles were removed. Four days after castration, all rats except the intact and castrated controls received various amounts of testosterone propionate (0.1, 0.5, 1, 5, 10, and 50 mg kg⁻¹, ip, daily) for 7 days, while intact and castrated controls received vehicle only. At the end of the experiment, all animals were sacrificed to obtain the penis samples for measurements and histological assessments. Considering the inhomogeneity of the penis, we separated the body regions for molecular biology analysis (Supplementary Figure S1). In addition, penile bones were dissolved prior to sectioning and homogenization.

Histological assessment
Tissues were washed twice with cold phosphate-buffered saline (PBS) and then fixed immediately in 10% polyformaldehyde. Forty-eight hours after fixation, dehydration, and paraffin embedding were conducted according to standard histological protocols. Sections (5-µm each) were evaluated according to standard protocols for staining with hematoxylin-eosin (HE) or Masson’s trichrome (showing fibrosis).

Microarrays
Since no commercial microarray for rat has been available, until now, we generated an alternate castrated mouse model to screen the potential genes involved in penis growth at puberty. Briefly, a total of 48 pubescent (age = 21 days) and 36 adult (age = 90 days) male Chinese Kun Ming mice were castrated and randomly divided into the control (saline) or testosterone (1 mg kg⁻¹, ip, daily) group, respectively. Seven days after treatment, the penis samples were collected for microarray analysis. The microarray experiments were performed by KangChen Bio-tech (Shanghai, China) using a custom-made Agilent 4 × 44 k microarray, which consisted of more than 41,000 mouse genes and transcripts. Genes with a two-fold change were considered significant.

Reverse transcription polymerase chain reaction
For reverse transcription polymerase chain reaction (RT-PCR), penis samples from each group were dissected and washed twice with cold PBS. After homogenization, the total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis and PCR were conducted according to standard PCR methods using SimpleChIP kit, according to the manufacturer’s instructions. Briefly, the MHCC-97L cells were plated in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum to achieve 50%–60% confluence before transfection. For each sample, 100 pmol of siRNA oligomer and 5 µl of lipofectamine 2000 Transfection Reagent were diluted in 250 µl of Opti-MEM I reduced serum medium without serum (Life Technologies, Grand Island, NY, USA), respectively. After mixing and incubation, 500 µl of oligomer-lipofectamine 2000 complexes was added to each well. The medium was changed after 4–6 h. The cells were incubated at 37°C for 48 h in a CO₂ incubator.

Western blotting analysis
Indirect immunofluorescence staining
Indirect immunofluorescence staining (IIF) staining was performed on cryostat sections of a normal pubescent rat penis. Briefly, cryostat sections were fixed in acetone at −20°C for 10 min. After rinsing in PBS, the samples were permeabilized for 1 min in PBS containing 0.1% Triton X-100. The samples were incubated with primary antibody overnight at 4°C, and then with secondary antibody for 30 min at 37°C in a water bath. Next, the sections were incubated with DAPI (0.1 µg ml⁻¹) in PBS for 5 min at room temperature and then covered with coverslips. At the end of each incubation interval, the sections were washed with PBS (pH 7.4) 3 times. The IIF results were documented with a photomicroscope.

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation assays were performed using a ChIP kit, according to the manufacturer’s instructions. Briefly, the cells were cross-linked, lysed, and sonicated using a Sonic Vibra Cell VC x 130PB (Sonics and Materials, Newtown, CT, USA). After sonication, the lysate was centrifuged and 1% volume of the supernatant was diluted in 900 µl of dilution buffer. The solution was precleared with 30 µl of ChIP-Grade Protein G Agarose Beads for 2 h at 4°C with rotation. The agarose beads were pelleted, and 2% volume of the supernatant was removed for input. The remaining supernatant was immunoprecipitated with the following antibodies: 10 µg of Histone H3, 10 µg of AR (N-20) X, and 1 µg of normal rabbit IgG, overnight at 4°C with rotation. Eluted protein-DNA complexes were digested with ribonuclease A and proteinase K. DNA fragments were purified by using spin columns. Purified DNA was analyzed by standard PCR methods using SimpleChIP Human RPL30 Extron 3 Primer and androgen response element (ARE) primer (from −2389 to −2232); forward, 5’-GGGACGTAGAGAGGAGAGAGAAGA-3’ and

Asian Journal of Andrology
reverse, 5'-CCACTATCAGTGAGTGCCAGTG-3'. PCR products were resolved by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

**Statistical analysis**
The quantitative data were presented as mean ± standard error of the mean. Statistical analyses were carried out by analysis of variance when more than two means had to be compared, or by the Student's t-test to compare paired data. In all cases, \( P < 0.05 \) was considered as statistically significant. Statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

**RESULTS**

Androgen modulates penis growth in pubescent rats
It is well-known that androgen plays an important role in the development of the male genital system; however, the molecular mechanisms are largely unknown. Initially, we generated an animal model to investigate whether androgen could affect penis growth during puberty. Indeed, our animal study showed a dose-dependent stimulation for the penis weight, length, and width by testosterone (0–1.0 mg kg\(^{-1}\)) treatment in castrated male SD rats \( (P < 0.05) \), while a higher dose of testosterone (5–50 mg kg\(^{-1}\)) did not further increase these parameters (Figure 1a and 1b).

In addition, HE and Masson's trichrome staining were performed to investigate the pathological changes of the penis after castration and testosterone treatment. Compared with the intact control, more hyperplastic interstitial fibrotic tissue and slit-like blood vessels were extensively observed in the corpus cavernosum of the castrated control group. After testosterone administration, the number of hyperplastic interstitial fibrotic tissues and slit-like blood vessels gradually restored to normal levels (Figure 1c–1e).

![Figure 1: Effects of androgen on rat penis development. (a) Gross morphology of the rat penis by light microscopy with or without testosterone treatment. (b) Difference of the rat penis weight, length, and width after testosterone treatment. Values in each column that do not share the same letter indicate statistical significance; \( P < 0.05 \). (c) Histopathological assessment by H and E staining as indicated; *represents a blood vessel, ▲represents interstitial fibrosis. Scale bars = 100 μm. (d) Masson's trichrome staining of rat cavernous tissue. Smooth muscle and interstitial fibrotic tissues are stained in red and blue, respectively. Scale bars = 50 μm. (e) Quantitative changes in smooth muscle and interstitial fibrotic tissue contents were analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA); \( n = 4 \) for each group. *\( P < 0.05 \).]
Androgen increases AR protein expression in the penis

AR is known as a key factor in the androgen signaling pathway. To assess the effect of androgen on AR expression in the rat penis, western blotting and RT-PCR were performed. Our western blotting data showed that AR expression was dramatically reduced after castration and then gradually increased in a dose-dependent manner after testosterone treatment (Figure 2a, P < 0.05), while the RT-PCR results showed that Ar mRNA levels decreased after testosterone supplementation (Figure 2b). Furthermore, the IIF results clearly showed that androgen deprivation (castration) decreased AR (red color) expression in both the urethra and cavernous and that testosterone supplementation could restore its expression (Figure 2c).

Together, the results presented in Figure 2a–2c suggested that the androgen-manipulated rat penis growth at puberty was mediated by the androgen-AR signaling pathway.

Krt33b is identified as a candidate gene for androgen-regulated penis growth

To screen the growth-associated genes in the penis that are activated by testosterone, we analyzed the gene expression profiles of both pubescent and adult mice penises after treatment with or without testosterone using an oligonucleotide microarray. A total of 734 differentially expressed transcripts showed a two-fold induction in response to testosterone in the puberty group. Among these transcripts, 33 genes were more active in response to testosterone (>4-fold change) in the puberty group, but were less active (<4-fold change) in the adult group (Supplementary Table 1). Of these genes, 6 out of the 33 were keratin or keratin associated, which indicated that these genes may be crucial for penis growth during puberty. Therefore, we selected the most responsive gene (Krt33b) for further study.

Next, using fresh penis samples, we found substantially increased Krt33b mRNA levels after testosterone administration in the puberty group by RT-PCR. These results paralleled those obtained by microarray analysis (Figure 3).

Krt33b is a typical AR target gene that is expressed in the rat penis

To learn about the expression characteristics of Krt33b and AR, double-labeling IIF studies using anti-KRT33B and anti-AR antibodies were carried out. Our data showed that KRT33B (red color) and AR (green color) were co-expressed in the penis urethral epithelial cells and cavernous interstitial cells, in which most of the AR staining was nuclear but KRT33B staining was cytoplasmic (Figure 4). Meanwhile, IIF studies using anti-smooth muscle actin and anti-von Willebrand factor antibodies were performed to delineate the tissue architecture of the rat penis (Supplementary Figure S2).

Next, we extracted total proteins from penis samples from rats with various treatments and confirmed that KRT33B expression was regulated by androgen in a dose-independent manner (Figure 5a). Furthermore, treatment with the anti-androgen agent flutamide (50 mg kg\(^{-1}\), po, daily), which blocks the AR signaling pathway, resulted in a dramatic reduction of KRT33B expression (Figure 5b).

To investigate whether KRT33B was directly mediated by the androgen-AR signaling pathway, we selected HepG2 and MHCC-97L cells, which expressed both AR and KRT33B, for further study in vitro. Using siRNA transfection methodology, we found that the expression of KRT33B was obviously reduced by knocking down AR (P < 0.05), in both HepG2 and MHCC-97L cells (Figure 5c and 5d, respectively).

To clarify the mechanism of AR regulating Krt33b expression, a ChIP assay was carried out to identify the putative AREs in the KRT33B gene promoter. An approximately 2.4-kb sequence region upstream of the TATA box of the Krt33b gene is schematically shown in Figure 6a, and a homology screen for the semipalindromic AR consensus binding sequence GG\(^{+1}\),ACAnmTGGTCTC revealed the presence of one putative ARE. Interestingly, in our study, the ChIP assay clearly demonstrated that AR could directly bind onto the promoter AREs of the Krt33b gene in both HepG2 and MHCC-97L cell lines (Figure 6b and 6c, respectively).

Taken together, the results presented in Figures 4–6 implied that Krt33b gene expression could be regulated by androgen through AR directly targeting its ARE sequence.

DISCUSSION

Penis size among normal men varies considerably, and men’s anxiety regarding the “normality” of their penis size is widespread.\(^{1,2}\) Boys with a micropenis due to hypogonadotropic hypogonadism are frequently treated with testosterone to induce penis growth.\(^{3,4}\) A proportion show a poor growth response, and most boys with a micropenis ultimately have a smaller than average penis in adulthood.\(^{5}\) Recent animal studies have also identified that fetal or postnatal androgen treatment only advances the penis growth rate but does not increase its ultimate length.\(^{6}\) Hence, besides the action of androgen, there may be other factors that play different functions to coordinate the progression of penis growth.

To understand this process, an animal model of 21-day-old castrated male SD rats exposed with testosterone was generated. Testosterone administration (0–1.0 mg kg\(^{-1}\)) resulted in a dose-dependent stimulation of penis weight, length, and width increases. Consistently, hyperplastic interstitial fibrosis and aberrant slit-like blood vessels appeared after castration and were restored after testosterone supplementation. However, a higher dose of testosterone (5.0–50 mg kg\(^{-1}\)) did not increase these parameters further.

In addition, we also found that the AR protein levels in the rat penis increased in a dose-dependent manner after testosterone treatment (0–1.0 mg kg\(^{-1}\)), while Ar mRNA levels were decreased after testosterone administration. In fact, these inconsistent conclusions have also been reported by others.\(^{15–18}\) One study found that testosterone and DHT were not the major factors in the physiological downregulation of Ar in the rat corpora cavernosa.\(^{15}\) In addition, Takane et al. have reported that DHT accelerates the decline of penile Ar mRNA levels\(^{16}\) while Gonzalez-Cadavid et al. have demonstrated that androgen can upregulate the levels of Ar mRNA in rat penis smooth-muscle cells.\(^{17}\) Furthermore, Lin et al.\(^{18}\) have hypothesized that the in vivo AR downregulation in penile smooth muscle by androgen is mediated indirectly by a paracrine mechanism. Meanwhile, Zhou et al.\(^{19}\) have reported that the stabilization of AR protein requires its receptor to be occupied with androgens and that the AR NH\(_2\)-terminal domain has a specific role in stabilization by slowing the rate of ligand dissociation and AR degradation. Therefore, in our study, we assumed that androgen may modulate AR protein expression in the rat penis through posttranscriptional regulation.

Taken together, these results imply the importance of AR modulation in the setting of hypogonadotropic hypogonadism treatment. Furthermore, in clinical practice, high-dose testosterone should not be preferred, because compared with a lower dose; such doses of testosterone bring about more side-effects but no further stimulative effect on penis growth.

As described previously, AR exerts its transcriptional activity through activating its target genes, which may execute their biological function alone or together with other factors. Interestingly, using a gene expression array, we found that Krt33b mRNA levels in the pubescent rat penis were obviously upregulated after androgen treatment.
Mechanism of penis growth by androgen treatment
YM Ma et al

**Figure 2:** Ligand-dependent androgen receptor (AR) protein expression in the rat penis. Protein or mRNA levels of AR in the rat penis (n = 3–4 for each group) was determined using western blotting (a) or RT-PCR (b) with β-actin as an internal control, respectively; images are representative of three separate western blotting and PCR experiments. Quantitative analyses were carried out, and error bars represent mean ± standard error of the mean values in each column that do not share the same letter indicate statistical significance. P < 0.05. (c) Detection of AR protein expression in rat penis tissues by IIF. Cross cryostat sections of a penis probed with an anti-AR antibody (red) and DAPI (blue). Scale bars = 100 μm.

**Figure 3:** Differential gene expression of Krt33b in the mouse penis. (a) Relative gene expression of the Krt33b gene in the penis of mice at puberty and adulthood based on a gene expression microarray. (b) RT-PCR was carried out to confirm the changes of the Krt33b gene in the penis of mice at puberty and adulthood.

Furthermore, we provided evidence that the transcription of this gene was directly controlled by AR directly binding onto its gene promoter. Whether it is a truth or a coincidence, recent research has shown that pubic hair growth is highly relevant to penis growth. Sexual hairs such as pubic hair, a beard, and axillary hair arise from small vellus, unmedullated hairs during puberty under the influence of circulating androgens and represent particularly coarse, strongly pigmented, and medullated hairs in adults. According to their similar growth environment, human pubic hair and the penis seem to share a common growth mechanism.

During hair growth, numerous keratins organize into filaments to participate in the production of the hair shaft. The keratin multigene family includes the keratins, which are expressed in various types of soft keratins, and the hard keratins, which are involved in the formation of hard-keratinized structures such as...
The present study initially provided evidence that androgen-induced KRT33B is generally expressed in the rat penis and its gene transcription is directly controlled by AR. Therefore, we hypothesized that KRT33B may participate in the progression of penis growth during puberty. Thus, advanced biological techniques should be applied to investigate the potential functions of this gene in the future.

**AUTHOR CONTRIBUTIONS**

YMM and KJW participated in the design of the trial, conducted the data acquisition, interpreted and analyzed the data, and drafted and revised the manuscript. QD and QS designed the study and contributed the study materials. YG, PG, SX, and XYW conducted the data acquisition and analyzed the data. YGG and DLH designed the trial, interpreted the data, and drafted the manuscript. All authors read and approved the final manuscript.

**COMPETING INTERESTS**

The authors declare that they have no competing financial interests.

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Supplementary Information is linked to the online version of the paper on the Asian Journal of Andrology website.

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Figure 5: Effects of androgen/AR on Krt33b expression. The levels of KRT33B after testosterone (a) or flutamide (b) administration were determined using western blotting with β-actin as an internal control; the levels of KRT33B in HepG2 (c) and MHCC-97L (d) cells at 48 h after transient transfection of AR siRNA were determined using western blotting with β-actin as an internal control; western blots are representative of three independent experiments. *P < 0.05 (NC vs siAR in each group).

Figure 6: Androgen response elements (AREs) in the Krt33b promoter by a chromatin immunoprecipitation (ChIP) assay. (a) Framework of the human Krt33b gene. The predicted ARE sequence was located at the region from −2368 to −2354 upstream of the TATA box. ARE primers were designed according to this framework, −2389 and −2232 represent the initial sites of the forward and reverse primers, respectively. (b, c) ChIPs were performed using digested chromatin from HepG2 and MHCC-97L cells co-expressing ar and KRT33B. Purified DNAs were analyzed by standard PCR methods using SimpleChIP Human RPL30 Primers and ARE primers. PCR products were observed for each primer set in the input sample and various ChIP samples, but not in the normal IgG ChIP sample.

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as hairs, nails, claws, and beaks.24-26 The best-known function of keratins and keratin filaments is to provide a scaffold for epithelial cells and tissues to sustain mechanical stress, maintain their structural integrity, ensure mechanical resilience, protect against variations in hydrostatic pressure, and establish cell polarity.27 Previous studies have confirmed that some keratins can be regulated by androgens. Jave-Suarez et al.24 have shown that the expression of human hair keratin 7 is directly regulated by androgens. Similarly, Yoshida et al.25 also have reported that androgen regulates hair keratin 37 expression in the human occipital hair medulla. In the present study, we found that KRT33B was generally expressed in urethra epithelial cells and cavernous interstitial cells of the rat penis, and its expression was modulated by androgen. Meanwhile, one limitation of this study was that we did not separate the penile urethra from the corpus cavernosum for analyses.

**CONCLUSION**

The present study initially provided evidence that androgen-induced KRT33B is generally expressed in the rat penis and its gene transcription is directly controlled by AR. Therefore, we hypothesized that KRT33B may participate in the progression of penis growth during puberty. Thus, advanced biological techniques should be applied to investigate the potential functions of this gene in the future.
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YM Ma et al

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