# Low Androgen Induced Penile Maldevelopment Involves Altered Gene Expression of Biomarkers of Smooth Muscle Differentiation and a Key Enzyme Regulating Cavernous Smooth Muscle Cell Tone

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**Purpose:** We determined the effects of low androgens in the neonatal period on biomarkers of smooth muscle cell differentiation, Myh11 and Acta2, and on Pde5A expression in the penis.

**Materials and Methods:** One-day-old pups were treated daily with the gonadotropin-releasing hormone antagonist antide with or without dihydrotestosterone for 1 to 6 days. Tissues were collected at age day 7 and at adulthood at age 120 days. Penes were examined by quantitative reverse transcriptase-polymerase chain reaction, Western blot and immunohistochemistry. Testes were assayed for the intratesticular testosterone and steroidogenic enzymes *Cyp17α1* and *StAR*.

**Results:** Gonadotropin-releasing hormone antagonist exposure suppressed the neonatal testicular testosterone surge 70% to 80%. Quantitative reverse transcriptase-polymerase chain reaction revealed 80% to 90% reductions in *Cyp17α1* and *StAR* protein, and 40% to 60% reductions in *Myh11* and *ACTA2* as a result of gonadotropin-releasing hormone antagonist compared to controls. Dihydrotestosterone co-administration mitigated these decreases. Western blot confirmed the Myh11 decrease at the protein level. Immunohistochemistry of Acta2 confirmed cavernous smooth muscle cell loss at the tissue level. Also, gonadotropin-releasing hormone antagonist exposure decreased *Pde5a* expression and dihydrotestosterone co-administration mitigated the decrease. Comparison of data between 2 parts of the penis body (corpora cavernosa and corpus spongiosum) showed that antagonist induced decreases in *Myh11*, *Acta2* and *Pde5a* expression occurred only in the corpora cavernosa, implying that the latter is the target site of low androgen action.

**Conclusions:** As evidenced by gonadotropin-releasing hormone antagonist induced suppression of the neonatal testosterone surge and reduced steroidogenesis, low androgens in the neonatal period altered gene expression of biomarkers of smooth muscle cell differentiation. This led to loss of cavernous smooth muscle cells and consequently to penile maldevelopment.

**Key Words:** penis; growth and development; androgens; Pde5a protein, rat; animals, newborn

**Abbreviations and Acronyms**

Acta2 = α-smooth muscle actin
CC = corpus cavernosum
CS = corpus spongiosum
CSM = cavernous smooth muscle
DES = diethylstilbestrol
DHT = dihydrotestosterone
GnRH-A = gonadotropin-releasing hormone antagonist
Myh11 = myosin heavy chain 11
Pde5a = phosphodiesterase-5A
Q-RT-PCR = quantitative reverse transcriptase-polymerase chain reaction

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DEVELOPMENT of the penis from the genital tubercle depends on androgens, especially DHT.[1] Androgen receptors are localized in the rat,[2,3] mouse[4] and human[5] genital tubercle/phallus prenatally. A reduction in the

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prenatal testicular testosterone surge[3] or androgen receptor absence/mutation[6,7] results in permanent penile malformation, including hypospadias, a smaller phallus and/or female-like genitalia. Conversely, a decrease in the neonatal testosterone surge does not cause hypospadias because urethral folds are already fused at birth but it causes other penile malformations, including a smaller penis, and loss of CSM cells and cavernous spaces.[8] The latter 2 structures are essential for erection in a vascular penis such as that in humans.

Abnormalities in CSM cells and cavernous spaces lead to corporeal veno-occlusive dysfunction, which is responsible for 70% of reported cases of erectile dysfunction.[9,10] The number of CSM cells are decreased in impotent men,[11] diabetic rats and aged rats with erectile dysfunction,[12,13] and castrated rabbits and mice.[14,15] A reduction in testosterone correlated with decreased CSM cell content and *Pde5a* expression in rats[16] and with impaired CSM cell relaxation in patients with erectile dysfunction.[17] Disruption in androgen action during the masculinization programming window of days 15.5 to 18.5 of gestation[3] or in the neonatal period[8] resulted in male reproductive tract abnormalities, including a smaller penis. Together these findings imply an important role for androgens in normal development of the penis and the maintenance of normal CSM cell structure and function.

We sought to understand the role of androgens, especially the neonatal testosterone surge, in CSM cell differentiation. To achieve this objective we investigated molecular changes in biomarkers of smooth muscle cell differentiation, Myh11 and Acta2, in rats treated neonatally with GnRH-A with or without DHT. These changes were studied in the whole penis (glans and body) at age 7 days to determine immediate effects soon after the end of treatment. They were also studied separately in 2 parts of the penis body, the CC and CS, in adulthood to determine whether effects were permanent and differentially expressed. (Notably, it was not possible to separate the CC and CS at age 7 days.) This separate determination was warranted because we previously reported that GnRH-A induced effects at the tissue level were limited to the CC.[8] In addition to smooth muscle cell biomarkers, we studied molecular changes in *Pde5a* expression because of its significance in penile erection.

## MATERIALS AND METHODS

### Animals

Sprague Dawley® rats were maintained at the Tuskegee University animal facility as previously described.[18] Rats were handled in accordance with the guidelines of the National Institutes of Health Guiding Principles for the Care and Use of Animal Research. All animal procedures were approved by the Tuskegee University institutional animal care and use committee.

### Treatment

At age 1 day 6 to 8 male pups from different litters were randomly assigned to each group. Each pup received daily subcutaneous injections of 50 μl normal saline (controls) and/or oil containing GnRH-A (15 mg/kg, Bachem, Torrance, California) with or without DHT (20 mg/kg, Sigma®) on postnatal days 1 to 6. Six-day treatment and these doses were based on our previous data[8] but the GnRH-A dose was increased from 10 to 15 mg/kg to ensure the maximal effect. Tissues were collected after $CO_2$ asphyxiation at age 7 days and adulthood at age 120 days. All samples were snap-frozen in liquid nitrogen and stored at −80C. The whole penis (glans and body) was homogenized at age 7 days. The CC and CS parts of the penis body were dissected under a stereo microscope and homogenized separately. The dorsal artery and vein were removed and discarded.

### Quantitative Reverse Transcriptase-Polymerase Chain Reaction

Q-RT-PCR was performed as described previously[18] using total RNA extracted from the whole penis and testis at age 7 days, and from the CC, CS and prostate in adulthood. Primers, including *Myh11*, *Acta2*, *Pde5a*, *StAR*, *Cyp17a1* and *Actb* as the housekeeping gene (catalogue No. PPR53316A-200, PPR59337B-200, PPR45092A-200, PPR45414A-200, PPR44710A-200 and PPR06570B-200, respectively) were obtained from Qiagen®.

### Western Blot

Western blot was done using 30 μg protein extracted from the adult CC, CS and prostate as described previously.[18] For primary antibodies rabbit polyclonal anti-Myh11 (sc-98705) and anti-Pde5a (sc-32884) were used with mouse monoclonal anti-ACTB (sc-47778, Santa Cruz Biotechnology, Santa Cruz, California) as the loading control. Horseradish peroxidase conjugated goat anti-rabbit IgG (sc-2004) for Myh11 and Pde5a, and goat anti-mouse IgG (sc-2061, Santa Cruz Biotechnology) for ACTB were used as secondary antibodies. Membranes were developed using Luminata™ Crescendo Western HRP Substrate.

### Immunohistochemistry and Histochemistry

Acta2 immunostaining was performed in formalin fixed adult penises as described previously[18] using anti-α-actin primary antibody (sc-130617, Santa Cruz Biotechnology). Avidin-biotin horseradish peroxidase complex and 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, California) were used as the detection system. For histochemical demonstration of fat we stained formalin fixed adult penis tissues in a solution of 1% osmium tetroxide and 2.5% potassium dichromate. Specimens were processed and embedded in paraffin wax followed by sectioning and staining with hematoxylin and eosin.

### Intratesticular and Plasma Testosterone

Testicular tissues and blood plasma were collected at necropsy and frozen at −20C until assayed as described previously.[18] Assay sensitivity was 0.2 ng/ml. All samples

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