Reduced Expression of Androgen Receptor and Myosin Heavy Chain mRNA in Cremaster Muscle of Boys with Nonsyndromic Cryptorchidism

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Purpose: To better define the developmental mechanisms of nonsyndromic cryptorchidism, we measured the expression of hormone receptor and muscle type specific mRNAs in target tissues of boys with and those without nonsyndromic cryptorchidism.

Materials and Methods: Prospectively collected cremaster muscle and/or hernia sac tissues from boys with congenital (79) or acquired (66) nonsyndromic cryptorchidism and hernia/hydrocele (controls, 84) were analyzed for hormone receptor (*RXFP2*, *AR*, *ESR1*, *ESR2*) and myosin heavy chain specific (*MYH1*, *MYH2*, *MYH7*) mRNA expression using real-time reverse transcriptase polymerase chain reaction. Log transformed mRNA, phenotype and feeding history data were statistically analyzed using Pearson's correlation, ANOVA and 2-sample t tests.

Results: *AR* mRNA expression was higher in cremaster muscle than in sac tissue, and significantly lower in congenital and acquired nonsyndromic cryptorchidism cases vs controls ($p < 0.01$). Type 1 (slow/cardiac) *MYH7* mRNA expression was also significantly reduced in both nonsyndromic cryptorchidism groups ($p \leq 0.002$), while a reduction in type 2 (fast) *MYH2* expression was more modest and significant only for the congenital cryptorchidism group ($p < 0.05$). Cremasteric *MYH7* and *AR* levels were strongly correlated ($r^2 = 0.751$, $p < 0.001$). *MYH7* and *ESR1* mRNA levels were higher and lower, respectively, in boys with nonsyndromic cryptorchidism who were fed soy formula. Expression of other genes was not measurable.

Conclusions: Our data suggest that boys with congenital and acquired nonsyndromic cryptorchidism differentially express *AR* and slow twitch specific *MYH7* mRNA in the cremaster muscle, and that *MYH7* expression is correlated with *AR* levels and soy formula use. These differences in gene expression may reflect aberrant hormonal signaling and/or innervation during development with the potential for secondary functional effects and failed testicular descent.

Key Words: cryptorchidism, androgens, estrogens, myosins

Abbreviations and Acronyms

AR = androgen receptor

E = embryonic day

ESR1 = ERα = estrogen receptor alpha

ESR2 = ERβ = estrogen receptor beta

INSL3 = insulin-like 3

MYH = myosin heavy chain

NSC = nonsyndromic cryptorchidism

RT-PCR = reverse transcriptase polymerase chain reaction

RXFP2 = relaxin/insulin-like family peptide receptor 2

Study received institutional review board approval.

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ISOLATED failure of testicular descent to scrotal position, or nonsyndromic cryptorchidism, occurs in 2% to 3% of boys, and is associated with long-term risks of subfertility and testicular malignancy.[1] NSC is associated with a constellation of reproductive tract and developmental abnormalities that may reflect damaging genetic and/or environmental influences. Epididymal defects, intrauterine growth retardation, subtle abnormalities of the hypothalamic-pituitary-gonadal axis and impaired spermatogonial development may

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occur in affected infants with NSC, and these varied phenotypes may have a common underlying etiology. Although the etiology of NSC in humans is unknown, it is hypothesized to be multifactorial and most likely due to altered signaling of INSL3/RXFP2 and/or AR pathways, which are essential for gubernacular development.[2] INSL3/RXFP2 signaling is essential for testicular descent while AR has a more general role in genital development, but specific mutations of these genes are uncommon in NSC.[3]

The mammalian gubernaculum comprises mesenchymal cells and an intrinsic (rodent) or extrinsic (human) cremaster muscle layer. The cell biology of hormone signaling in the gubernaculum remains incompletely defined. In vitro studies suggest that INSL3 and androgen stimulate gubernacular proliferation, and that their effects may be additive,[4,5] but the target cell(s) for hormonal signaling in the gubernaculum have not been specifically identified. Interestingly *Rxfp2* mRNA expression is diffuse throughout the fetal rat gubernaculum on embryonic day 14,[6] but immunostaining for INSL3 in the gubernaculum, which presumably localizes cell surface RXFP2, is limited to the muscle layer by E18 in mice,[7] suggesting that muscle may be the target of INSL3/RXFP2 signaling during a critical phase of testicular descent. AR and ERα proteins are also expressed in muscle, and AR has been found in the central mesenchyme of the rodent gubernaculum, possibly in fibroblasts or myofibroblasts, at E14.5 to E16.5 (mice) and E21.5 (rats).[8,9] Exposure to the antiandrogen flutamide during a critical window of gestation (E16 to E19)[10] causes cryptorchidism in rats, but is accompanied by minimal inhibition of fetal gubernacular size,[11,12] suggesting that the antiandrogen effect may not be associated with gubernacular growth per se. In addition, it was recently shown that flutamide decreases *Insl3* mRNA expression in fetal rats,[13] suggesting that a mechanism other than AR blockade may contribute to flutamide induced cryptorchidism. Evidence also exists for interactions between Insl3 and androgen signaling,[14] but how the complex interplay between Insl3 and androgen signaling determines testicular position has not been determined.

An emerging theme in deciphering mechanisms of testicular descent involves the role of muscle. Cryptorchidism occurs commonly in many syndromes associated with abnormal neuromuscular development,[15] supporting a role for muscle activity in the process of testicular descent. The cremaster muscle expresses *Rxfp2* and *Ar* in fetal mice and, thus, may represent a key target of critical hormone activities. Androgens stimulate myoblast differentiation and muscle hypertrophy, and regulate fiber type in skeletal muscle.[16] Accordingly we tested the hypothesis that altered expression of hormone receptor and muscle type specific mRNAs is present in target tissues of boys with NSC, potentially reflecting aberrant hormone dependent signaling during development.

MATERIALS AND METHODS

Patients

Clinical data and tissue samples were collected from boys recruited prospectively in institutional review board approved protocols with informed consent. We identified prepubertal participants attending a pediatric urology clinic scheduled for surgery to correct cryptorchidism (145 cases) or inguinal hernia/communicating hydrocele (84 controls). Exclusion criteria were reported gestational age younger than 36 weeks, other genital anomalies including hypospadias or micropenis; major abdominal wall defect or any defined syndrome such as cerebral palsy, the prune-belly syndrome or posterior urethral valves; or retractile testes in controls. Based on an existing electronic health record template used for more than a decade at our institution, parents were routinely asked for information regarding birth history and knowledge of first documentation of cryptorchid testicular position.

A surgeon coinvestigator provided documentation of intraoperative testicular position, ipsilateral hernia sac, epididymal anomaly and/or existence of an ipsilateral testicular appendage. The highest location of either testis was used in bilateral cases, and was defined as proximal for testes above the external inguinal ring (canalicular, abdominal) and distal for those at or below the external inguinal ring (superficial inguinal pouch, external ring, prescrotal, perineal). Participants with absent testes were excluded from analysis. Infant feeding type and breastfeeding duration data were obtained from a maternal and perinatal history questionnaire.

To best compare congenital and acquired forms of cryptorchidism, we adopted a conservative approach to limit misclassification of cases as acquired when cryptorchidism was present at birth but referral was delayed. We analyzed the cryptorchid age distribution and identified a major peak before age 2 years followed by a lower but sustained frequency during childhood. This distribution was similar to that reported by Hack et al,[17] although our postnatal peak was earlier and the secondary peak less pronounced, possibly because many boys were referred to us for evaluation before age 6 months. Based on our data and the recommendations of Bruijnen et al,[18] we categorized patients as having congenital NSC if surgery was performed at age 24 months or younger, or if families or pediatricians of older boys reported undescended testes at birth but referral was delayed. Boys older than 24 months and without a history of cryptorchidism were categorized as having acquired NSC.

Tissue Samples

We collected samples of the cremaster muscle and hernia sac (if present), tissues that are available from cases as well as controls. Although the gubernaculum is considered the primary signaling target during testicular descent, postnatally it is a fibrous remnant that is dramatically different in composition from its prenatal form, and is easily obtainable during orchiopexy but not hernia repair. A small portion of cremaster was removed during mobili-