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Expression of Estrogen Receptor Alpha and Beta is Decreased in Hypospadias

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Abstract

Purpose—Estrogenic endocrine disruptors acting via estrogen receptors α and β have been implicated in the etiology of hypospadias. However, the expression and distribution of estrogen receptors α and β in normal and hypospadiac human foreskins is unknown. We characterized the location and expression of estrogen receptors α and β in normal and hypospadiac foreskins.

Materials and Methods—We prospectively collected excess foreskin from 35 patients undergoing hypospadias repair and 15 patients undergoing elective circumcision. Hypospadias was classified as severe in 18 patients and mild in 17 based on the ectopic position of the meatus. mRNA expression levels in estrogen receptors α and β were quantified using reverse transcriptase polymerase chain reaction. Receptor location was characterized by immunohistochemical analysis. Additionally immunohistochemical analysis was performed in 4 archived human fetal penises.

Results—Mean $\pm$ SD ages were similar for the circumcision (9.5 ± 3 months) and hypospadias repair groups (9 ± 3 months, $p = 0.75$). mRNA expression levels in estrogen receptors α and β were significantly decreased in hypospadiac foreskin cases compared to controls ($p < 0.001$), while no statistically significant differences were seen between foreskins with severe and mild hypospadias. Estrogen receptor β immunostaining was strong in normal foreskin but weak in hypospadiac foreskin. Estrogen receptor β immunoreactivity was most intense in the stratum basale and stratum spinosum. Estrogen receptor α immunostaining was weak in normal and mild hypospadias foreskin, and undetectable in severe hypospadias. Fetal penises expressed strong estrogen receptor β immunopositivity in the urethral plate epithelium, corpus spongiosum, corpora cavernosa and penile skin, while estrogen receptor α immunostaining was not detected.

Conclusions—These data demonstrate a difference in estrogen receptor α and β expression and location in the foreskin of patients with hypospadias compared to controls. These findings are consistent with aberrant estrogenic effects having a role in the etiology of hypospadias.

Keywords

foreskin; hypospadias; penile diseases; penis; receptors; estrogen

Hypospadias is a common congenital abnormality characterized by an ectopic urethral meatus along the ventral surface of the penis proximal to the normal glanular location. Hypospadias is associated with penile curvature and incomplete development of the ventral

foreskin.^{1,2} Hypospadias affects approximately 1 in 200 to 300 live male births,¹ although recent epidemiological data suggest an increasing incidence.^{3–5} Although the etiology remains elusive, a possible explanation for the increasing incidence of hypospadias in industrialized countries is fetal exposure to estrogenic compounds or other environmental endocrine disruptors.^{4,6} Supporting this theory is the observation that male offspring of women exposed to diethylstilbestrol during pregnancy have a higher incidence of hypospadias,⁷ consumption of phytoestrogens (such as genistein) during gestation is associated with a higher incidence of hypospadias in boys,⁸ and in laboratory models neonatal exposure to estrogens and genistein causes hypospadias.^{9–11} Consistent with this theory is the discovery of estrogen receptors α and β , crucial for signal transduction of estrogenic substances, in the penile tissue of rat,¹² mouse¹³ and humans.^{14,15}

Human skin contains *ESR1* and *ESR2*, and is an important estrogen responsive organ.¹⁶ Although foreskin may have similar characteristics, to date the exact location and expression of estrogen receptors have not been defined in normal or hypospadiac human foreskin. Characterizing the cellular location and expression patterns of estrogen receptors in normal and hypospadiac human foreskin may expand our understanding of the causes of hypospadias beyond the murine model. In this study we used real-time polymerase chain reaction to qualify the mRNA expression of *ESR1* and *ESR2*, and immunohistochemical staining to determine receptor location. We hypothesize that the location and expression of *ESR1* and *ESR2* differ between normal and hypospadiac foreskin.

MATERIALS AND METHODS

This study was approved by the University of California, San Francisco human research committee. Any subject younger than 14 months undergoing initial hypospadias repair or elective circumcision at the University of California, San Francisco between 2005 and 2010 was eligible for the study. All subjects were prospectively enrolled, and informed consent was obtained preoperatively from parents/legal guardians. Excess preputial tissue was obtained from 35 male subjects with hypospadias undergoing surgical repair and 15 patients undergoing elective circumcision. The degree of hypospadias was determined by the position of the ectopic urethral meatus and was classified as mild (at or distal to mid shaft of penis) in 17 patients and severe (proximal to mid shaft of penis) in 18. Four human fetal penises were obtained from our archived tissue bank. Estimated gestational ages based on last menstrual period, head circumference and femur length were approximately 8 to 10, 14, 20 and 24 weeks.

RNA Extraction, and RT and Real-Time PCR

RNA extraction was performed using a protocol similar to those described previously.¹⁷ Briefly an RNeasy® Midi Kit was used to isolate RNA. The protocol was modified to eliminate DNA contamination by using proteinase K (1.3 mg, Roche Diagnostics GmbH, Mannheim, Germany) and RNase-free DNase I (20 μ l, Qiagen, Inc., Valencia, California). A NanoDrop® spectrophotometer was used to measure the quantity and purity of RNA.

RT-PCR was performed according to standard protocol. Briefly 2.5 μ g RNA were reverse transcribed in a reaction volume of 20 μ l and diluted with Tris-ethylenediaminetetraacetic acid buffer (10 mM Tris HCl, pH 8, 1 mM ethylenediaminetetraacetic acid). PCR primers were designed according to the specific target gene sequence published in PubMed and were synthesized by IDT®. The RT-PCR primers are outlined in the Appendix. SYBR® Green real-time quantitative PCR was performed using a 7300 fast sequence detection system (Applied Biosystems™) according to the manufacture instructions. Primer titration and dissociation experiments were performed so that no primer dimers or false amplifications would interfere with the result. Cycle threshold number was extracted for the reference

(GADPH) and target genes with auto baseline and manual thresholds. PCR was repeated 3 times for each sample. Expression levels of *ESR1* and *ESR2* are reported relative to GADPH using a 2 delta-delta cycle threshold.¹⁸ There was no difference in the amplification kinetics of GADPH and *ESR1/ESR2*.

Immunohistochemical Staining

Foreskin samples were fixed in formalin, paraffin embedded and sectioned at 5 μ m. Antigens were retrieved with antigen unmasking solution (Vector Laboratories, Inc., Burlingame, California). After blocking, slides were incubated overnight at 4°C with rabbit anti-ER β antibody (1:100 dilution, Upstate Biotechnology, Inc., Lake Placid, New York), and mouse anti-ER α antibody (1:30 dilution, cloneID5, Dakocytomation, Carpinteria, California). Staining of the tissue was performed with the Elite ABC kit (Vector Laboratories, Inc.), followed by hematoxylin counterstaining. Adult mouse uterus and prostate served as positive controls for ER α and ER β . Sections exposed to all steps except application of the primary antibodies were used as negative controls. Four human fetal penises from our tissue bank were also processed in a similar protocol. To confirm our results concerning the lack of detectable ER α in the human fetal tissue, we repeated the analysis using a different mouse monoclonal anti-ER α antibody (1:100 dilution, ab2746, abcam®). Stained serial sections were examined using a DM4000B brightfield microscope (Leica Microsystems, Inc., Buffalo Grove, Illinois). Digital images were acquired with a DFC500 camera (Leica Microsystems, Inc.). Once images were taken, the density of nuclear staining was graded on a subjective scale as none (0), weak (+), intermediate (++) or strong (+++). Each determination was based on the agreement of 2 observers who were blinded to the treatment group when inspecting the images.

Statistical Analysis

The difference in mRNA expression levels of *ESR1* and *ESR2* between controls, subjects with mild hypospadias and subjects with severe hypospadias was determined using ANOVA with Bonferroni correction. Statistical analysis was performed with SPSS® Statistics, version 17.

RESULTS

Subject Characteristics

Mean $\pm$ SD ages were similar for the circumcision (9.5 ± 3 months) and hypospadias repair groups (9 ± 3 months, $p = 0.75$). The meatal location in the 17 subjects with mild hypospadias was glanular in 2, subcoronal in 4, distal shaft in 5 and mid shaft in 6. The meatal location in the 18 patients with severe hypospadias was proximal penile shaft in 8, penoscrotal junction in 6 and perineal in 4.

mRNA Expression of *ESR1* and *ESR2* in Normal and Hypospadiac Foreskins

In normal human foreskin the mean $\pm$ SD expression level of *ESR1* mRNA (relative to GADPH) was $6.4\% \pm 3.3\%$, while that of *ESR2* was higher at $13.6\% \pm 6.9\%$ (fig. 1). Mean $\pm$ SD *ESR1* mRNA expression was significantly lower in mild ($1.9\% \pm 1.2\%$) and severe hypospadias foreskin ($1.0\% \pm 0.7\%$) compared to normal foreskin ($6.4\% \pm 3.3\%$, $p < 0.001$). There was no significant difference between mild and severe hypospadias in *ESR1* mRNA expression levels. Similarly mean $\pm$ SD *ESR2* mRNA expression was significantly lower in mild ($2.1\% \pm 1.9\%$) and severe hypospadias foreskin ($1.1\% \pm 0.9\%$) vs normal foreskin ($13.6\% \pm 6.9\%$, $p < 0.001$). There was no significant difference between mild and severe hypospadias in *ESR2* mRNA expression levels. Therefore, in normal foreskin *ESR1* and *ESR2* mRNA expression was higher than in hypospadiac foreskin.

ER α and ER β IHC Staining in Normal and Hypospadiac Foreskins

In normal and mild hypospadias foreskin ER α immunoreactivity was weak and localized to the stratum stratum basale (fig. 2). In contrast, in severe hypospadias there was no detectable expression. In normal foreskin ER β was strongly expressed in the keratinocytes of the stratum basale and stratum spinosum. In contrast, in mild and severe hypospadias foreskin immunoreactivity was weak.

ER α and ER β IHC Staining in Normal Fetal Penis

ER α immunoreactivity was undetectable in all human fetal penile tissues examined (data not shown). In contrast, ER β was strongly expressed in human fetal penis, including the urethral plate epithelium, corpus spongiosum, erectile tissue, and penile epithelium and foreskin (fig. 3).

DISCUSSION

Skin is an important estrogen responsive organ and has the capacity to synthesize active steroids from inactive adrenal precursors.¹⁶ Hormone receptors have been described in normal human skin, and estrogen receptors in particular have been associated with skin tag malformations.^{19–21} As foreskin abnormalities are part of the constellation of anomalies that characterize hypospadias, a description of the cellular location and expression patterns of estrogen receptors in normal and hypospadiac human foreskin in infants may lend insight into the etiology of abnormal urethral development.

To our knowledge this is the first study evaluating *ESR1* and *ESR2* location and expression in normal and hypospadiac foreskin in infants of similar age. Our study reveals that normal foreskin in male infants contains *ESR1* and *ESR2* mRNA. Our immunolocalization results demonstrate that ER β is strongly expressed in foreskin, whereas ER α is weakly positive.

Our findings are in accordance with previous steroid receptor studies where ER β was the major receptor expressed in normal human skin.^{19,20} ER β immunoreactivity was strongly positive and mostly limited to the nuclear compartment of keratinocytes localized in the stratum basale and stratum spinosum, which is exactly what we observed in normal foreskin. In a related study using 12-week gestation human fetal penile tissue IHC staining also showed positivity for estrogen receptor in penile skin, although the exact location and expression and receptor subtypes were not further elucidated.¹⁵ Our human fetal data reveal that ER β is expressed in fetal foreskin, with the highest intensity noted in the stratum basale. In contrast to normal prepuce, our IHC results demonstrate that estrogen receptors are either weakly expressed or undetectable in the foreskin of boys with hypospadias. Taken together, these observations suggest that estrogen receptors, particularly ER β , have a role in the normal development of human foreskin.

Exposure to hormonally active substances during external genitalia development is associated with increased risk of congenital genitourinary malformations.^{4,6} We quantified mRNA expression levels of *ESR1* and *ESR2* in normal and hypospadiac human foreskins to determine if differences in ER subtypes exist that may further our understanding of genitourinary development. We found that foreskins of male infants with hypospadias have significantly decreased *ESR1* and *ESR2* expression levels compared to normal controls of similar ages. Although there was a trend toward lower estrogen receptor expression levels in severe grades compared to mild grades of hypospadias, the difference did not reach statistical significance. Our findings suggest that changes in estrogen receptor levels may be involved in normal and abnormal foreskin development. As a corollary, this finding may be akin to the dermatology literature where *ESR1* and *ESR2* levels have been shown to be significantly higher in skin tags compared to normal human skin,²¹ or the breast cancer

literature where *ESR1* and *ESR2* are differentially expressed in benign tumors, primary cancers and recurrent cancer.²²

In humans normal urethral development involves the midline fusion of the paired urethral folds with subsequent formation and remodeling of an epithelial seam.^{23,24} The abnormalities seen in hypospadias can be viewed as an abortive midline fusion of the urethral folds and corpus spongiosum. Endocrine disruptors interacting with estrogen receptors have been implicated in the increasing incidence of hypospadias.^{25–27} In a mouse model in utero estrogen exposure resulted in the failure of seam formation and the developmental arrest of tissue surrounding the urethra.⁹ In a related study using human fetal penile smooth muscle cells ER α and ER β were reported to be functionally active receptors.¹⁵ Furthermore, incubation of human fetal penile smooth muscle cells with increasing concentrations of estrogen (17 β -estradiol) resulted in a dose dependent decrease in cell proliferation. Taken together with our findings, these results suggest that estrogen and estrogen receptors may be involved in penile malformations.

Although the urethra and corpus spongiosum were not directly examined in our surgical cohort, it is possible that similar down-regulation of estrogen receptors occurs in the hypospadiac urethra and corpus spongiosum, as these penile tissues contain similar receptors to those found in foreskin. Indeed, we noted strong ER β immunostaining in the urothelium and corpus spongiosum, corpus cavernosum and penile skin in all of the human fetal specimens. Evrard and Balthazart reported that the corpus cavernosum, corpus spongiosum and urethral epithelium in humans expressed estrogen receptors α and β , with ER β exhibiting stronger immunostaining than ER α .²⁸ Similarly Crescioli et al reported estrogen receptors in the urethra, glans and stromal cells of the corpus cavernosum of the developing human fetal penis.¹⁵ Furthermore, in a cellular model of human fetal penile smooth muscle cells *ESR1* and *ESR2* were observed, with higher mean *ESR2* mRNA expression compared to *ESR1*.¹⁵ When viewed in this context, our findings raise the possibility that down-regulation of *ESR1* and *ESR2* may have a role in congenital penile malformations such as hypospadias.

Our results need to be interpreted with caution. Although our IHC and real-time PCR results correlated well with each other, we did not directly study normal or hypospadiac urethral tissue in patients undergoing surgery. It is possible that events occurring in the foreskin, whose epithelium is ectodermal in origin, may not be occurring in the urethra, whose epithelium is endodermal in origin. Furthermore, we did not study the events at the critical time of external genitalia development and urethral malformation. To help circumvent differences that may be attributed to postnatal hormonal changes, we evaluated male infants of similar age. Finally, we did not detect ER α in fetal penile tissue, although it was seen in the normal and mild hypospadias foreskins in the neonate but not in severe hypospadias. It may be that ER α is present in fetal penile tissue but our current methods/antibodies simply did not detect it. Alternatively it is possible that prenatal receptor expression levels may also change during development in humans, as noted in mouse¹³ and rat models.²⁹ Indeed, our laboratory continues to explore different modern IHC techniques and use of different antibodies in hopes of clarifying ER α expression in human fetal penile development.

CONCLUSIONS

In view of past observations that *ESR2* is ubiquitously expressed in human penile vasculature, corpus cavernosum, urothelium and now foreskin, as seen in this study, we propose that estrogen and estrogen receptors may have a significant role in normal and abnormal penile development. To our knowledge this is the first study of the expression and location of *ESR1* and *ESR2* in normal foreskin and preputial tissue in patients with

hypospadias. In normal foreskin there is strong ER β immunohistochemical staining but weak ER α staining. In hypospadiac foreskin ER β immunohistochemical staining is weak and ER α is weak (mild hypospadias) or undetectable (severe hypospadias). Similarly the mRNA expression levels of *ESR1* and *ESR2* are significantly lower in hypospadiac foreskin compared to normal foreskin. The differential expression of *ESR1* and *ESR2* in the foreskin of patients with hypospadias merits further investigation to elucidate the role of estrogen receptor subtypes in the etiology of hypospadias.

Acknowledgments

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Abbreviations and Acronyms

ER	estrogen receptor
<i>ESR1</i>	estrogen receptor alpha
<i>ESR2</i>	estrogen receptor beta
GADPH	glyceraldehyde-3-phosphate dehydrogenase
IHC	immunohistochemical
PCR	polymerase chain reaction
RT	reverse transcriptase

APPENDIX

Gene	Primer Sequences	Product Size (bp)
ESR1	F: AGATCTTCGACATGCTGCTGGCTA	137
	R: AGACTTCAGGGTGCTGGACAGAAA	
ESR2	F: TGGGCACCTTTCTCCTTTAGTGGT	85
	R: TCTTGCTTCACACCAGGGACTCTT	
GADPH	F: CATGTTCGTCATGGGTGTGAACCA	160
	R: AGTGATGGCATGGACTGTGGTCAT	

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Figure 1. Expression levels of *ESR1* and *ESR2* mRNA in human foreskin evaluated with real-time RT-PCR were significantly higher in normal vs hypospadiac foreskin ($p < 0.001$). No statistically significant differences were seen between severe and mild hypospadias foreskins.

Figure 2.

Expression of *ESR1* and *ESR2* in normal and hypospadiac foreskins. ER α immunostaining was weak in normal and mild hypospadias foreskins and limited to stratum basale. ER α immunostaining was undetectable in severe hypospadias. ER β immunostaining was strong in normal foreskin but weak in mild and severe hypospadias foreskin. ER β immunoreactivity was most intense in keratinocytes of stratum basale and stratum spinosum. H&E, reduced from $\times 100$.

Figure 3. ER β immunostaining. *A* and *B*, coronal sections of 8 to 10-week human fetal penis. Note strong ER β staining in urethral groove/plate, glans stroma and erectile tissue. *C* and *D*, transverse sections of 24-week human fetal penis at level of glans. Note strong ER β immunopositivity in foreskin epithelium, foreskin stroma, inner preputial lamina, urothelium and corpus spongiosum. H&E, reduced from $\times 200$. Insets reduced from $\times 25$.