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Hypospadias and anorectal malformations mediated by Eph/ephrin signaling

Selcuk Yucel^{*,†}, Christopher Dravis[†], Nilda Garcia^{**}, Mark Henkemeyer[†], and Linda A. Baker[†]

^{*}Akdeniz University School of Medicine, Department of Urology, Pediatric Urology Section

[‡]Division of Pediatric Urology, Department of Urology, University of Texas Southwestern at Dallas

[†]Center for Developmental Biology, University of Texas Southwestern at Dallas

^{**}Department of Surgery, Division of Pediatric Surgery, University of Texas Southwestern at Dallas

Abstract

Purpose—Despite extensive research, the molecular basis of hypospadias and anorectal malformations is poorly understood, likely due to a multifactorial basis. The incidence of hypospadias is increasing, thus making research in this area warranted and timely. This review presents recent molecular work broadening our understanding of these disorders.

Materials and Methods—A brief review of our recent work and the literature on the role of Eph/ephrin signaling in hypospadias and anorectal malformations is presented.

Results—Genetically engineered mice mutant for ephrin-B2 or EphB2;EphB3 manifest a variety of genitourinary and anorectal malformations. Approximately 40% of adult male heterozygous mice demonstrate perineal hypospadias. Although homozygous mice die soon after birth, 100% of homozygous males demonstrate high imperforate anus with urethral anomalies and 100% of homozygous females demonstrate persistent cloaca. Male mice compound homozygous for *EphB2*^{ki/ki}; *EphB3*^{Δ/Δ} also demonstrate hypospadias.

Conclusions—These mouse models provide compelling evidence of the role of B-class Eph/ephrin signaling in genitourinary/anorectal development and add to our mechanistic and molecular understanding of normal and abnormal embryonic development. As research on the B-class Ephs and ephrins continues, they will likely be shown to be molecular contributors to the multifactorial basis of hypospadias and anorectal malformations in humans as well.

Keywords

Hypospadias; Anorectal Malformation; Mice; Children; EphB; ephrin-B2

Hypospadias

Hypospadias is the second most common human birth defect, affecting 1 in 125 male births¹. Despite the high incidence, our current understanding of the etiology of hypospadias

Address Correspondence to: Linda A. Baker, MD, Associate Professor of Urology, Pediatric Urology, University of Texas Southwestern Medical Center, 5325 Harry Hines Boulevard, Dallas, TX 75235, Tel: 214-648-2278, Fax: 214-648-8786, Linda.baker@utsouthwestern.edu.

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is at best incomplete. It is well understood that the androgen signaling cascade, including testosterone (T) regulation, production and biosynthesis, the peripheral conversion of T by 5- α -reductase to dihydrotestosterone (DHT), and T/DHT-androgen receptor interactions^{2,3} mediate virilization of the genital tubercle, with phallic growth and urethral tubularization. Human intersex states best exemplify this signaling gone awry^{2,3}. It is also clear that altered androgen signaling, either on a genetic or hormonal level basis, does not completely account for all human cases of hypospadias and thus is collectively not solely responsible for hypospadias⁴. Additional molecules likely work independently or dependently with androgen signaling, either upstream, downstream or in concert. Recent investigations into several genetic syndromes have identified additional genes which when mutated or deleted cause hypospadias (Table 1), thereby making hypospadias a multifactorial birth defect.

Even with this information, most cases of hypospadias go clinically unexplained. This deficiency of knowledge is concerning given that there are several publications that suggest the incidence of hypospadias is increasing⁵. In addition, there is a significant controversial body of literature suggesting that prenatal exposure of male feti (animal or human) to “endocrine disruptors”, environmental compounds with antiandrogenic or estrogenic properties, may be mediating the increase in hypospadias and also cryptorchidism^{6,7}.

Hypospadias is clearly a human disorder with phenotypic variability, ranging from the mild distal glandular hypospadias to the severe proximal perineal hypospadias (Figure 1). Fortunately, distal hypospadias is much more common, given that proximal hypospadias accounts for about 15% of cases. This information suggests that several mechanisms of maldevelopment might account for the phenotypic variability of hypospadias. In addition, proximal hypospadias cases can be associated with additional malformations, including penoscrotal transposition and anorectal malformations. Although not well recognized by urologists, the literature reports that up to 2.2–10.5% of patients with anorectal malformations also have hypospadias^{8–10}.

Anorectal malformations

Congenital anorectal malformations are common surgical problems affecting 1 in 1500 to 1 in 5000 live births, with an even gender distribution^{11,12,13,14}. In humans, they present as a spectrum of abnormalities ranging from an ectopic anus, to an imperforate anus with fistula to the distal genitourinary tract, to complex cloacal abnormalities. To date, three genetic mouse models of anorectal malformations exist, namely Sonic hedgehog (Shh)^{15–18}, Gli2 and Gli3^{18,19}, and ephrin-B2^{lacZ/lacZ} mutant mice.

Embryology

To fully understand hypospadias, anorectal malformations and how they might be linked, a review of normal embryology is warranted. In Figure 2, the process by which the cloaca (Latin – ‘sewer’) is septated is depicted, yielding separation of the urinary and fecal streams during normal embryonic development. Classic teaching describes the cloacal septation event occurring by the in growth of epithelium-covered mesenchymal folds, termed the cranial to caudal Tourneaux fold and the lateral to medial right and left Rathke folds. These folds, creating the urorectal septum, ultimately join the cloacal membrane, yielding the primitive urogenital sinus and the anorectal canal as two separate tubes. Although there has been a longstanding debate concerning the existence and nature of this septation event in human development^{20, 21}, it is true that by the early 7th week of human gestation the urogenital sinus and the anorectal canal are separate entities. Differentiation of the external genitalia (Figure 3) is not initiated until about the 8th week. This is simultaneous with when the testis or ovary differentiates depending on the activation of the SRY gene. In response to androgenic stimulation in the

male, the perineum and labioscrotal folds fuse in the midline, and the genital tubercle and urethral plate undergo elongation and masculinization through the 4th month of gestation, yielding an elongated tubularized urethra. In contrast, non-virilized female external genitalia consist of a small clitoris with a short urethra, surrounded by the open labioscrotal folds. The vagina forms between the urethra and the anorectum.

The molecular mechanisms and factors which drive urethral tubularization are not fully understood. For example, it is not known whether processes (epithelial cell adhesive events, epithelial–mesenchymal interactions or mesenchymal ingrowth) that guide cloacal septation (yielding the posterior urethra in males and entire urethra in females) might also mediate male anterior urethral tubularization/formation.

Endocrine disruptor theory and hypospadias

It is well established that humans continually ingest substances with known estrogenic activity, such as insecticides utilized in crop production, natural plant estrogens, by-products of plastic production and pharmaceuticals. In fact, the canned food industry uses some chemical substances with estrogenic activity to cover the inner surface of cans. All endocrine disruptors find their way into fresh or seawater to be accumulated in higher organisms at the top of food chain. Therefore, top predators such as large fish, birds, sea mammals and humans store the highest level of environmental contaminants in their body. For example, thinning of eggshells in birds was traced to the estrogenic activity of insecticides to which birds were exposed through their diet. Thus, humans and wildlife are constantly exposed to estrogenic compounds with the ability to cause reproduction problems, the so-called estrogenic endocrine disruptors^{22,23}.

Estrogenic contaminants are reported to disturb penile development in the American alligator²⁴. Also, potent estrogen estradiol-17 β disrupts penile development in mice²⁵. Diethylstilbestrol (DES) has been shown to cause urethral abnormalities in female neonatal mice²⁶. DES-related problems are well known in human literature. Sons of DES-exposed mothers have a higher ratio of hypospadias and other urethral anomalies and difficulty in passing urine²⁷. In mice, Kim et al. showed that treatment of pregnant mice with 17 α ethinyl estradiol and DES between 12 and 17 days of gestation causes hypospadias in almost 50% of pups²⁸. Another study confirmed the arrest in urethral tubularization in male mice under the effect of estrogenic compounds²⁹.

Sexual dimorphism of the external genitalia is determined by the presence and absence of androgen receptor signaling. The fetal testes produce testosterone which is the primary serum androgen. Within the developing genital tubercle, testosterone is converted into 5 α -DHT by the enzyme 5 α -reductase to create a more potent androgen. In the developing genital tubercle, 5 α -reductase type 2 isozyme has been shown to locate at the mesenchyme surrounding the developing urethra at the particular region where midline fusion occurs for urethral tubularization³⁰. In-utero exposure to anti-androgenic drugs inhibits the binding of testosterone and DHT to the androgen receptor to reduce the size of genital tubercle and anogenital distance. Likewise, 5 α -reductase inhibitors inhibit the distal development of the urethra resulting in hypospadias³¹.

The actions of androgens are elicited by a variety of downstream factors whose production, receptor binding and action must be intact. Unfortunately, today, we are still searching for these molecules that integrate the sex hormone balance with urethral tubularization.

B-class Eph/ephrin mutant mouse model

Our research group is investigating a novel genetically engineered murine model manifesting the unanticipated phenotype of hypospadias and genitourinary/anorectal malformations. The

Eph and ephrin gene families (Figure 4) are known for their roles in cell–cell signaling, cell sorting³², axonal guidance during neuronal development^{33,34}, delineation of embryonic cellular boundaries³⁵, vasculogenesis^{36,37} and epithelial–mesenchymal transitions³⁸. The Eph receptors, the largest subclass of receptor tyrosine kinases, and their membrane-bound ephrin ligands are two large highly conserved gene families expressed throughout invertebrates and vertebrates. Both the Ephs and ephrins are subdivided into A and B classes. For the most part, A-class Ephs bind to A-class ephrins and B-class Ephs bind to B-class ephrins, although there are a few exceptions to this rule. To understand their interactions, two cells are depicted in Figure 4, one expressing a B-class Eph molecule and the other expressing a B-class ephrin molecule. Upon activation of the EphB molecule by the ephrin-B molecule, a set of intracellular signals are activated within the EphB-expressing cell, which has been called the “forward” signal. Similarly, upon activation of the ephrin-B molecule by the EphB molecule, the “reverse” signal is activated within the ephrin-B-expressing cell. For ephrin-B2, it is clear that two ligands include EphB2 and EphB3. Thus, these molecules establish a system for cell–cell interactions and bidirectional signaling. Activation of these molecules alters cytoskeletal elements leading to alterations in cell morphology and in the nervous system, and cell–cell adhesive or repulsive events.

In order to better characterize the independent nature of “reverse” signaling, our group created a mutant mouse wherein the intracellular domain of the ephrin-B2 molecule was substituted by a lac Z cassette (ephrin-B2^{lacZ}). The adult male heterozygous mice (Figure 5) manifest hypospadias at approximately 30–40% penetrance, independent of genetic background. Figure 5A demonstrates a normal adult wild-type mouse with a normal penis and tubularized urethra (Figure 5C). In contrast, Figure 5B demonstrates the ephrin-B2^{lacZ/+} heterozygous hypospadiac adult mouse wherein the anogenital distance is decreased and the penis is hypospadiac, with an open urethral plate as indicated in Figure 5D. Given that ephrin-B2 binds to EphB2 and EphB3, adult mice functionally null for both EphB2 and EphB3 (*EphB2*^{ki/ki};*EphB3*^{Δ/Δ} compound homozygotes) also manifested perineal hypospadias and a reduced perineal distance (Figure 6), confirming the key role of this signaling pathway. In these hypospadiac mice, there is no sign of insufficient virilization, as judged by prostate and seminal vesicle size.

To understand the maldevelopment of the hypospadias in these adult mutant mice, the mutant mouse embryos were studied. In Figure 7, β-gal staining reveals that the ephrin-B2^{lacZ} protein is localized within the endodermal cells of the urethral plate. In these heterozygous mutant mice, urethral tubularization is incomplete, yielding perineal hypospadias. When compared to the wild-type animals as seen in the top panel, the bottom panel also clearly reveals that there is a defect of a persistent cloaca in these males. In the series of images in Figure 8, whole-mount visualization of these same embryos gives another view of the perineal defect at low and high power magnification. The tail has been amputated and is in the upper area of the images. The genital tubercle is then seen coming out towards the viewer in the lower portion of the images. Figure 8A shows a wild-type animal at embryonic day 16, demonstrating complete septation of both the urogenital sinus and the anorectal canal with closure of the perineum in the midline. In comparison, two *EphB2*^{u/x}; *EphB3*^{u/x} mutant littermates are seen in panels 8B and C. These two littermates with identical genotypes show phenotypic variability, with the mouse seen in panel B being less severely affected than the mouse in C, but it is clear that when they are both compared to the wild-type littermate, the perineum has not closed and there is an open cloaca. These data therefore suggest that ephrin-B2 and EphB2/EphB3 are key molecules involved in septation events in the perineum. This concept was further confirmed when we examined the ephrin-B2^{lacZ/lacZ} homozygous mice. As seen in Figure 9, the newborn wild-type male (left panel) demonstrates a normal anorectal canal, normal bladder and completely tubularized urethra. In contrast, on the right, the newborn male ephrin-B2^{lacZ/lacZ} homozygous littermate has a high imperforate anus. The arrow indicates the location of the rectourethral fistula entering at the base of the bladder neck. Female ephrin-B2^{lacZ/lacZ}

homozygous mice demonstrate persistent cloaca (Figure 10). This work has recently been published³⁹. Taken together, these murine data reveal the key role of B-subclass Eph/ephrin signaling in normal midline fusion events of the perineum, cloaca and external genitalia, and reveal gene dosage sensitivity, yielding hypospadias, anorectal malformations and cloaca.

To discriminate between EphB2-mediated forward (cell autonomous) and reverse (non-cell autonomous) signaling, a EphB2^{lac} mutation producing a kinase-inactive C-terminally truncated EphB2-β-gal fusion protein was bred to generate EphB2^{lac}; EphB3^{lac} compound heterozygotes. The EphB2^{lac}- encoded EphB2-β-gal fusion protein exhibits normal protein trafficking and ability to stimulate reverse signaling in adjacent ephrin-expressing cells.

Organ Culture System

In order to address whether EphB2 might be an androgen-regulated gene, an organ culture system was used wherein embryonic genital tubercles from the EphB2^{lac} Z⁺ mice could be grown. In these mice with normal genitalia, EphB2 expression is highlighted by the beta-galactosidase color reaction (blue). Embryonic day 12.5 (E12.5) genital tubercles from females and males were microdissected and grown in culture for 2 days with and without the presence of DHT. As seen in Figure 11, the female genital tubercle responded to DHT exposure with phallic enlargement, urethra closure distally, and increasing EphB2 expression in the urethral plate distal to the closure, all suggesting that EphB2 can be up-regulated by DHT exposure in the genital tubercle⁴⁰. Thus, EphB2 is a candidate androgen-regulated gene that might mediate the virilized genital phenotypes seen in female pseudohermaphrodites that are exposed to prenatal excess adrenal androgens.

Conclusions

The B-class Eph/ephrin cell–cell signaling pathway is crucial in mouse urethral and anorectal development and EphB2 is a candidate androgen-regulated gene. Many studies are currently underway further exploring the mouse model and the role these molecules may play in human hypospadias and urogenital/anorectal development.

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Figure 1.

Three cases of human hypospadias demonstrating phenotypic variability. The arrow indicates the location of the urethral meatus in each case. (A) Subcoronal hypospadias. (B) Penoscrotal hypospadias with mild scrotal clefting. (C) Perineal hypospadias with ventral chordee and completely bifid scrotum.

Figure 2.

Depiction of human embryonic development at weeks 5–7 of gestation. The allantois and hindgut drain into the cloaca. Classic embryology teaching suggests the cloaca is septated by the caudal and ventral growth of the Tourneux fold and the lateral to medial growth of the left and right Rathke folds. These folds coalesce, forming the urorectal septum which divides the primitive urogenital sinus from the anorectal canal. This process is controversial. Reproduced with permission⁴¹.

Figure 3.

(A) Male and female human external genitalia are identical at 6–7 weeks gestation as cloacal septation occurs. (B) Under the influence of androgens produced by the differentiating male testis, the genital tubercle elongates, the urethra tabularizes, the labioscrotal folds fuse, and the male perineum forms. Female external genitalia form in the absence of androgen action. Reproduced with permission⁴¹.

Figure 4.

The Eph and ephrin gene families are divided into A and B classes. Protein functional motifs are depicted by shapes; A-class ephrins are bound to the cell membrane by glycosylphosphatidylinositol (GPI) linkage while the B-class ephrins are transmembrane (TM) molecules. On the right, two cells are depicted, an Eph-expressing cell and an ephrin-expressing cell. Activation of the Eph receptor by an ephrin triggers the 'forward' signal in the Eph-expressing cell. Simultaneously, the ephrin is activated by the Eph, triggering the 'reverse' signal in the ephrin-expressing cell. Thus bidirectional signaling occurs and both gene families act as both receptors and ligands.

Figure 5.

(A) Adult male wild-type (+/+) mouse depicting normal anus (arrowhead) and penis (arrow). (B) Adult male ephrin-B2^{lacZ/+} heterozygous (+/-) mouse with normal anus (arrowhead), reduced anogenital distance and perineal hypospadias (arrow). (C) Cross section of adult +/+ penis with tubularized urethra (arrow). (D) Cross section of adult-B2^{lacZ/+} heterozygous (+/-) mouse penis with hypospadias (asterisk). Reproduced with permission³⁹.

Figure 6.

(A) Adult male wild-type (+/+) mouse depicting normal anus (arrowhead) and penis (arrow). (B) Adult male *EphB2^{ki/ki};EphB3^{Δ/Δ}* compound homozygote mouse with normal anus (arrowhead), reduced anogenital distance and perineal hypospadias (arrow). (C) Cross section of adult +/+ penis with tubularized urethra (asterisk). (D) Cross section of adult *EphB2^{ki/ki};EphB3^{Δ/Δ}* compound homozygous mouse penis with hypospadias (arrow). Reproduced with permission³⁹.

Figure 7.

Upper panel: consecutive cross sections of embryonic day 17 (E17) wild-type mouse penis demonstrating tubularized urethra (arrow) and normal septated anus (asterisk). From left to right, the sections progress from the distal penis inward into the perineum. Lower panel: consecutive cross sections of embryonic day 17 (E17) *ephrin-B2^{lacZ/+}* heterozygous mouse penis demonstrating incomplete proximal urethral tubularization and perineal closure (asterisk). In some more distal locations, urethral tubularization appeared more normal (arrow and arrowhead). The dark-blue staining indicates the high expression of *ephrin-B2^{lacZ}* in the tubularizing urethral epithelium, septating epithelium of the cloaca and urogenital sinus (not shown). From left to right, the sections progress from the distal penis inward into the perineum. Reproduced with permission³⁹.

Figure 8.

Low power (upper panel) and high power (lower panel) view of the perineum of three E16 littermates with the amputated tail (t) and genital tubercle (g) viewed at top and bottom of photos, respectively. (A) *EphB2*^{+/+};*EphB3*^{Δ/Δ} mouse demonstrates closed perineum and tubularized urethra. (B and C) Two examples of *EphB2*^{+/+};*EphB3*^{Δ/Δ} mice with mild (B) and severe (C) delays in perineal closure and urethral tubularization, with cloaca (asterisk). Reproduced with permission³⁹.

Figure 9.

Left: sagittal section of E18 male wild-type mouse demonstrating normal spine, anorectum and male urethra. Right: sagittal section of E18 *ephrin-B2*^{lacZ/+} male with normal spine and high imperforate anus with rectourethral fistula at the bladder neck. Anal dimple is present.

Figure 10.

Left: sagittal section of E18 female wild-type mouse demonstrating normal spine, bladder (B), anorectum-hindgut (HG), vagina (V) and female urethra (asterisk). Right: sagittal section of E18 *ephrin-B2*^{lacZ/+} female with normal spine and high persistent cloaca (the arrow). Anal dimple is present. Reproduced with permission³⁹.

Figure 11.

E12.5 female EphB2^{lacZ/+} heterozygous genital tubercles were harvested and cultured in media with or without DHT for 2 days. The female genital tubercles responded to DHT exposure with phallic enlargement, urethral tubularization distally and increasing EphB2 expression in the glans and urethral plate distal to closure.

Table 1

Some genes associated with hypospadias when mutated or deleted

Human Genes known to cause Hypospadias	Human chromosome	OMIM Reference Number
<i>Intersex disorders</i>		
Androgen receptor (AR)	Xq11-q12	313700; 312300; 300068
Steroid 5-alpha-reductase-2 (SRD5A2)	2p23	607306
Luteinizing hormone (LH) receptor	2p21	152790
3 beta-hydroxysteroid dehydrogenase gene type II	1p13.1	201810
17-beta-hydroxysteroid dehydrogenase (HSD17B4)	5q2	601860
3-beta-hydroxysteroid dehydrogenase	1p13.1	109715
17-alpha hydroxylase deficiency	10q24.3	202119
Steroidogenic acute regulatory (StAR) protein	15q23-q24, 8p11.2	201710; 600617
Sex-determining region on Y chromosome (SRY)	Yp11.3	480000
SRY-BOX 9 (SOX9)	17q24.3-q25.1	608160
Receptor tyrosine kinase-like orphan receptor 2 (ROR2)	9q22	268310; 602337
Wilms' tumor suppressor gene-1 (WT-1)	11p13	607102; 194072; 194080; 136680
<i>Midline Malformation Syndromes</i>		
Midline 1 Ring Finger Gene (MID1) (FXY)	Xp22.3	300000
sal-like gene 1 (SALL1)	16q12.1	107480; 602218
Gli-Kruppel Family Member 3 (GLI3)	7p13	200990; 165240; 146510
<i>Limb-Genital Syndromes</i>		
Homeobox A13 (HOXA13)	7p15-p14.2	142959; 140000
Homeobox D13 (HOXD13)	2q31-q32	142989
Ephrin-B1 (EFNB1)	Xq12	304110
<i>Other Miscellaneous</i>		
Paired-like homeodomain transcription factor 2 (PITX2)	4q25-q26	180500; 601542
Protein O-Mannosyltransferase 1 (POMT1)	9q34.1	236670
SMADIP1 gene aka ZFX1B (Zinc Finger Homeobox 1B gene)	2q22	235730; 605802
Glypican-3 (GPC3)	Xq26	312870
X-linked lissencephaly (ARX)	Xp22.13	300004; 300382
Fraser 1 (FRAS1) gene	4q21	607830; 219000
S terol delta-7-reductase gene (DHCR7, 7-alpha dehydrocholesterol reductase)	11q12-q13	270400; 602858

OMIM: Online Mendelian Inheritance of Man (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>)