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REVIEW

Etiology of Hypospadias: A Comparative Review of Genetic Factors and Developmental Processes Between Human and Animal Models

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Abstract: Hypospadias is a congenital anomaly of the penis with an occurrence of approximately 1 in 200 boys, but the etiology of the majority of hypospadias has remained unknown. Numerous genes have been reported as having variants in hypospadias patients, and many studies on genetic deletion of key genes in mouse genital development have also been published. Until now, no comparative analysis in the genes related literature has been reported. The basic knowledge of penile development and hypospadias is mainly obtained from animal model studies. Understanding of the differences and similarities between human and animal models is crucial for studies of hypospadias. In this review, mutations and polymorphisms of hypospadias-related genes have been compared between humans and mice, and differential genotype–phenotype relationships of certain genes between humans and mice have been discussed using the data available in PubMed and MGI online databases, and our analysis only revealed mutations in seven out of 43 human hypospadias related genes which have been reported to show similar phenotypes in mutant mice. The differences and similarities in the processes of penile development and hypospadias malformation among human and commonly used animal models suggest that the guinea pig may be a good model to study the mechanism of human penile development and etiology of hypospadias.

Keywords: hypospadias, genetic factors, external genital development, animal models

Introduction and Review Objectives

Hypospadias is one of the most common congenital penile malformations, with an occurrence of approximately 1 in 200 male newborns.[1] Patients with hypospadias confront serious psychological problems and physical difficulties with urination and sexual functions. Generally, hypospadias represents a disruption of normal penile development between 8 and 14 weeks of gestation, resulting in the abnormal urethral opening on the ventral surface of the penis.[2] Based on the severity, hypospadias can be divided into three degrees: mildest anterior forms, with the urethral opening on the glandular or subcoronal position of penis, more severe middle hypospadias, in which the meatus opens on the midshaft of the penis, and the most severe posterior forms containing penoscrotal, scrotal, and perineal openings.[3]

Unfortunately, the etiology of hypospadias in the majority patients has remained unknown. Genetic and environmental factors are the main susceptibilities. Although androgen is known to be essential for the sexual differentiation and penile development, only a small percentage of severe hypospadias can be explained by the genetic syndromes or defects involving the androgen receptor (*AR*) gene.[4] Interactions between genes and

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environmental factors, including placental insufficiency, exogenous endocrine-disrupting chemicals, such as vinclozolin[5] and di-n-butyl phthalate,[6] acting on polymorphic genes or inducing epigenetic changes, may greatly contribute to hypospadias development. The mechanism of external genital development, and genetic and environmental factors underlying hypospadias and animal models had been intensively reviewed by different research groups.[7–11]

Our knowledge about penile development and hypospadias is mainly based on the studies in animal models. Mice[12] and rats[13] are the most commonly used laboratory animals for hypospadias modeling, in addition, other animals such as guinea pigs,[14] rabbits,[15] and dogs[16] have also been used in hypospadias research. Unfortunately, differences exist between human and animal models in the mechanisms of normal penile development and hypospadias malformation. Without considering the differences, results derived from animal models of hypospadias are less reliable. Therefore, understanding the differences and similarities between human and animal models is crucial to the mechanism study of hypospadias. This paper reviews the etiology of hypospadias, including the influences of genetic, epigenetic factors, as well as animal models and their differences and similarities compared with humans.

Literature Search and Data Synthesis

We conducted a literature search using PubMed for all publications before September 1, 2020 for hypospadias related genes with key words "hypospadias and gene", "hypospadias and polymorphism", "hypospadias and mouse", and "mouse external genitalia and genes", we also use "hypospadias" and several domestic animals to find out the other animal models, such as "rabbit", "dog", and "horse", of hypospadias. We included systematic reviews of all literature and revealed reported hypospadias related genes identified from humans and mice. Phenotypic data of mouse mutants were acquired through both the literature searching using PubMed and checking the Mouse Genome Informatics online database resource (http://www.informatics.jax.org/).

Genetic and Epigenetic Factors of Hypospadias

Genetic inheritance has long been inferred to be one of the causes of hypospadias.[17] Hypospadias was found to have a strong familial component and also to aggregate within more distant relatives. One large sample cohort study found 0.45% of boys had hypospadias; among these patients, 4.2% had at least one family member with a history of hypospadias, and the inheritance of hypospadias was transmitted equally through the paternal and maternal sides.[18] Hypospadias fathers were deemed to have high potential to have hypospadias sons, the incidence of hypospadias for those whose fathers were affected by hypospadias was 27% as reported.[19] And first degree relatives of hypospadias infants had more malformations other than hypospadias than controls.[20] In recent years, a lot of gene polymorphisms/mutations and some epigenetic marks have been revealed in hypospadias patients, with more and more studies suggesting multifactorial inheritance for the majority cases of hypospadias.

Current epidemiology and laboratory studies have suggested that epigenetic alterations may contribute to abnormal male sexual differentiation including hypospadias. Higher methylation level of *AR* gene in the foreskin, resulting in a decreased expression of the AR protein, might be involved in the pathogenesis of hypospadias.[21] Ohsako et al[22] reported a negative association of methylation level of the *SRD5A2* gene with the mRNA expression levels of CYP1 family genes in the preputial tissue of patients with hypospadias and suggested it is likely to indicate the involvement of chemical exposure and epigenetic change in the onset of hypospadias. Significant associations were observed between methylation levels of individual CpG sites and hypospadias, and DNA methylation patterns have been used in identifying and evaluating new candidate genes that may be involved in the etiology of hypospadias.[23] Nevertheless, data about the effect of methylation of genes on the development of hypospadias are limited, and the relation between other epigenetic factors involved in DNA or histone modifications with hypospadias is mostly unknown.

Mutations and Polymorphisms in Hypospadias Patients and Mice

Polymorphism in biology refers to the occurrence of two or more genetically phenotypes in a certain population. The polymorphisms of certain genes have been reported to cause hypospadias in humans, the comparison of key gene mutations and polymorphisms associated with hypospadias, and the relevant phenotypes between humans and mice are summarized in Table 1.

Table 1 Gene Variants and Relevant Phenotypes in Hypospadias Patients and Mice

Gene Name	Phenotype of EG (or Urogenital System) in Knockout Male Mice	Gene Variants in Hypospadias Patients	Clinical Hypospadias Type	Reference
AKR1C3 (HSD17B5)▲	Not available	c.643G>A	Penile	[51]
AR▲	Phenotypes vary from female-like EG, ambiguous genitalia to hypospadias, micropenis[24,25]	Methylation	Glandular	[21]
		c.2519G>A, c.2564G>A	Perineal	[27]
		c.2525T>C	Scrotal	
		c.1991C>T	Glandular	
		c.2577C>A	Penile	
		CAG repeat	Variable in different races	[28]
		GGN repeat	Penile	[30]
		c.1789G>A	Perineal	[46]
ATF3▲	No obvious phenotype[100]	rs11119982, rs3125289, rs1877474	Hypospadias	[101]
BNC2▲	Distal hypospadias in newborn mice[102]	c.916C>G, c.1735C>T, c.719A>G, c.847A>G, c.2768C>T, c.1240C>G, c.455A>G	Distal	[102]
BMP4▲	Hypoplasia of genital tubercle[77]	c.619C>G, c.668G>A, c.751C>T	Penoscrotal and penile	[80]
BMP7▲	Arrest in cloacal septation, and severe defects in genital urethra and mesenchyme[78]	rs6070007	Moderate	[74]
		rs6127980 rs6127978, rs6127985	Severe and mild Severe	
		c.907C>T	Glandular	[80]
CDH11▲	No obvious phenotype[114]	Loss of function mutation	Elsahy-Waters syndrome with hypospadias	[103]
CTNNB1▲	Hypoplasia or hypospadias[79]	347TCT→CCT, Ser45→Pro	Hypospadias	[81]
CYP11A1▲	Female EG[47]	c.666T>C	Mid-shaft	[48]
DGKK▲	Not available	rs1934179, rs7063116	Anterior or middle	[105]
		rs11091748, rs12171755	Anterior or middle	[104]
EMX2▲	Degeneration of the Wolffian duct and mesonephric tubules[97]	10q25.3-q26.12 microdeletion	Ranging from hypospadias to complete sex reversal	[98]
EPHRIN-B2Δ and EPHB2Δ	Hypospadias and anorectal malformations[84]		Not available	
ESR1▲	No obvious phenotype[33]	PvuII and XbaI C-A haplotype (rs2234693, rs9340799)	Mild	[34]

(*Continued*)

Table 1 (Continued).

Gene Name	Phenotype of EG (or Urogenital System) in Knockout Male Mice	Gene Variants in Hypospadias Patients	Clinical Hypospadias Type	Reference
		AGATA haplotype (SNPs 10–14: rs926779, rs3020364, rs6932902, rs3020371, rs3020375)	Hypospadias	[35]
ESR2▲	No obvious phenotype[33]	rs944050, rs3832949	Hypospadias	[37]
FGF8▲	No obvious phenotype[75]	c.590C>G, c.582–62G>A (rs3218238)	Hypospadias	[73]
FGFR2▲	Hypospadias[69]	c.550+27C>T, c.727+180T>G, c.382+52→G, c.2454C>T	Hypospadias	[73]
		c.830T>C (rs755793)	Mid-penile	
FGF10Δ	Hypospadias[68]	rs1482679	Severe	[74]
		rs16901816, rs2973644, rs2973646	Moderate*	
		rs6892212	Moderate* and severe*	
FKBP4Δ	Penile Hypospadias[31]	No variants found	Not available	[32]
GLI1▲	Not available	rs10783827, rs3825077*, rs3782126, rs2292657, rs4760259, rs2228226	Moderate and severe	[74]
GLI2▲	Widely opened urethra and preputial fusion defects[71]	rs4848125*, rs4143116, rs4848126*	Moderate	[74]
GLI3▲	No obvious phenotype[71]	rs6974655 rs9886211*	Mild Severe* and moderate#	[74]
		rs3801223	Moderate	
HAAO▲	Abnormal seminal vesicle morphology; external genital phenotype not available[115]	rs3816183	Anterior/middle and posterior	[106]
HOXA13▲	Hypospadias[86]	Mutation or polyalanine expansions	Hand–foot–genital syndrome with hypospadias not defined	[89,90]
HOXD13▲	Abnormal male accessory sex organs[87]	Polyalanine duplication at 5′-end	Distal penile, mid-shaft, and penoscrotal (HM)/ Distal (HZ)	[88]
HOXA4▲	No obvious phenotype[94]	rs6962314	Hypospadias	[95]
		c.385G>T, c.869C>G	Penoscrotal and penile	[80]
HOXB6▲	No obvious phenotype[93]	c.124C>A, c.367T>C	Scrotal and penile	[80]
HSD3B2▲	Not available	S213T	Scrotal	[49]
		S284R	Midshaft	
HSD17B3▲	Not available	rs2066479	Hypospadias	[50]
IRX6▲	Not available	rs6499755	Anterior/middle	[106]

(*Continued*)

Table 1 (Continued).

Gene Name	Phenotype of EG (or Urogenital System) in Knockout Male Mice	Gene Variants in Hypospadias Patients	Clinical Hypospadias Type	Reference
INSL3▲	Bilateral cryptorchidism without obvious penile phenotype[52]	C-19G, V18M, and R105H	Hypospadias with cryptorchidism	[53]
LAMA5△	Hypospadias[117]		Not available	
LSM1▲	Not available	rs775468919	Hypospadias	[107]
MAMLD1 (CXorf6) ▲	No obvious phenotype[54]	c.370G→T(E124X), c.589C→T (Q197X), c.1957C→T(R653X)	Penoscrotal	[56]
		c.1295T>C(V432A), 325delG	Penoscrotal and proximal	[55]
		CAG_{10}→CAG_{13}	Subcoronal	
MTHFR▲	Not available	C677T	Middle and posterior	[109]
MID1▲	No urogenital abnormalities[113]	c.712G>T, c.1679A>G, c.1230G>A (1230A, rs16986145)	Hypospadias	[108]
MYRF▲	Not available	c.2336+1G>A	Penoscrotal	[110]
NR5A1/ SF1▲	Abnormal gonad and adrenal gland development[58]	(CTGCAGCTG)×2 c.74A>G c.814A>C	Severe proximal Penoscrotal Ambiguous genitalia	[63,64,66]
		c.319C>T, c.103–3C>A, c.31G>T	Penoscrotal/scrotal	[65]
PGK2▲	No obvious phenotype	STR in the 3′ flanking region	Perineal	[111]
SHH△	Agenesis[72] or hypospadias[70]	rs9333613	Moderate	[74]
SMADIP1▲	Not available	935delG, deletion	Hypospadias and agenesis of the corpus callosum	[112]
SPRY1△ and SPRY2△	Absence of internal tubular urethra, and hypospadias-like phenotype[76]		Not available	
SRD5A2▲	No obvious phenotype[41]	V89L (rs523349), A49T (rs9282858)	Hypospadias	[43]
		c.168G>C, p.V89L rs7562326	Severe perineoscrotal Penoscrotal	[42,44]
		c.123G>A	Perineoscrotal	[45]
		c.16C>T, c.211C>T, c.59T>C, c.586G>C, c.607G>A, c.680G>A, c.737G>A, 218delT	Posterior	[46]
SRY▲	Sex reversal[59]	mosaic *SRY* mutation	Hypospadias and cryptorchidism	[67]
STARD3▲	Not available	rs1877031	Penoscrotal	[42]
STS▲	Not available	rs5934740, rs5934842, rs5934913, rs6639811, rs3923341, rs17268974, rs5934937	Penoscrotal	[42]
		rs17268974	Penile	

(*Continued*)

Table 1 (Continued).

Gene Name	Phenotype of EG (or Urogenital System) in Knockout Male Mice	Gene Variants in Hypospadias Patients	Clinical Hypospadias Type	Reference
TGFBR2▲	Increased prostate gland adenocarcinoma and intraepithelial neoplasia incidence; penile phenotype not available[83]	rs6785358 (G allele)	Hypospadias	[82]
WT1▲	Deletion causes the failure of gonad and kidney development,[60] no gross abnormalities of EG were observed in conditional knockout males[61]	rs1799937, rs5030277, rs3858449, rs5030234 rs16574 rs12293750	Severe Severe and mild Mild	[74]

Notes: *The risk was restricted to non-Hispanic whites. #The risk was restricted to Hispanics. ▲ or △. The hypospadias related genes were first identified from human patients (▲) or mutant mice (△). "Not available" for mutant mouse phenotypes indicates either the phenotypes of EG in mutant male mice have not been recorded, or the mutant mouse strains are not available; for clinical hypospadias type indicates no relevant studies have been reported. If the severity of the human hypospadias was not defined, we use "hypospadias". Deletion regions involving multiple genes are not included.

Androgen masculinizes genital development through AR. Male mice with mutations in *Ar* gene develop feminized external genitalia (EG),[24] and disrupting AR in the androgen sensitive time window induces hypospadias.[25] In humans, mutations in *AR* underlie different forms of androgen insensitivity syndrome.[26] Polymorphism in *AR* gene contributes to susceptibility to severe hypospadias. Point mutation in exon 7 causes perineal or scrotal hypospadias.[27] Adamovic and Nordenskjold[28] have provided evidence that a higher number of the CAG repeat in the sequence of *AR* gene has a clear effect on the risk of hypospadias in Caucasians, while some other literature reported that there was no significant difference in CAG length between cases with hypospadias and controls in Japanese.[29] GGN repeat is another polymorphism of *AR* gene and was found to be associated with hypospadias in Iranians and Swedes, whereas the length of CAG repeat showed no significant difference between hypospadias cases and controls in the same group.[30] FK506-binding protein 4 (*FKBP4*) gene, also known as *FKBP52*, is an important AR activator, male mice with targeted ablation of *Fkbp4* develop hypospadias,[31] but no mutation was found in hypospadias patients.[32] The varieties of the populations and sample sizes may account for the inconsistency of the results. Clearly, the polymorphism of *AR* is complicated, and large sample sizes are necessary for the future studies in humans.

Estrogen receptor (ESR) has two major isoforms, ESR1 and ESR2. Deletion of either of them or double knockout caused no obvious external genital phenotype in mice.[33] But single nucleotide polymorphisms (SNPs) of *ESR1* and *ESR2* in humans were reported to have significant relationship with hypospadias.[34] Watanabe et al[35] have reported that the frequency of "AGATA" haplotype of *ESR1* is significantly higher in patients with hypospadias than in controls, which may enhance the ESR1 signaling and finally facilitate the development of hypospadias in the Japanese population. *ESR2* is expressed in fetal and adult testes.[36] The CA repeat in *ESR2* was found to be significantly prolonged in hypospadias patients compared with the controls, and the genetic variant of rs944050 in the heterozygous form in *ESR2* was more frequent in hypospadias patients than in controls.[37,38] Although deletion of *Esr1* and *Esr2* in mice has not shown detectable phenotypic change in EG, prenatal estrogen treatment in mice dramatically reduces penile size and induces a phenotype similar to human micropenis,[25,39] and there has been more evidence of estrogen's detrimental effects on penile masculinization; sequence variations in estrogen receptors may contribute to the development of hypospadias through increasing estrogen signaling. And, given genetic polymorphisms such as SNPs in hypospadias patients are variable, we think more data from hypospadias patients are necessary to draw clear conclusions.

The mutation of steroid 5 alpha reductase (*SRD5A*), especially *SRD5A2*, is one of the main causes of pseudohermaphroditism in humans.[40] Interestingly, male *Srd5a2* knockout or *Srd5a1* and *Srd5a2* double knockout mice show fully formed EG.[41] As many different SNPs or mutations of human *SRD5A2* have been reported to correlate with severe hypospadias,[42–46] we believe different mechanism may exist in action of DHT in penile urethral development between humans and mice.

Steroidogenic genes also play important roles in normal genital development and sex differentiation. It has been known that deletion of cytochrome P450 family 11 subfamily A member 1 (*Cyp11a1*) induces to form female

EG in male mice,[47] but no study on external genital development in steroid sulfatase (*Sts*), hydroxy-delta -5-steroid dehydrogenase (*Hsd3b2*), hydroxysteroid 17-beta dehydrogenase 3 (*Hsd17b3*), *Hsd17b5*, or StAR related lipid transfer domain containing 3 (*Stard3*) gene knockout mice has been reported, although in humans, genetic variations in these genes have been found to be associated with different types of hypospadias.[42,48–51] Insulin-like hormone 3 (*INSL3*) gene, another Leydig-associated gene encoding a member of the insulin-like hormone superfamily produced mainly in gonadal tissues, was proved to be a cryptorchidism causal gene in mice,[52] mutations of this gene in humans were identified to be associated with cryptorchidism, and maybe hypospadias and micropenis as well.[53]

In addition, mastermind like domain containing 1 (*MAMLD1*), also known as chromosome X open reading frame 6 (*CXorf6*), is a causative gene for 46,XY disorders of sex development with hypospadias as a salient clinical phenotype and involved in the testosterone production, but mutant male mice exhibit normal genital and reproductive development.[54] Polymorphisms of this gene in humans are highly associated with hypospadias.[55,56] Ratan et al[57] indicated that Indian boys with isolated hypospadias had significantly higher incidence of *MAMLD1* polymorphism than a control group.

Genetic sex determination pathway genes control sex determination and gonad development, deletion of sex-determining region Y (*Sry*), the steroidogenic factor 1 (*Sf-1*) or Wilms tumor 1 (*Wt1*) in mice induces abnormal gonad development or male-to-female sex reversal.[58–61] It has been revealed that human mutations in any one of these genes cause impaired testis development,[62] and some of the genetic variations or duplications in these sex determination genes also induce severe hypospadias, but these hypospadias patients usually have other reproductive organ defects[63–67] (Table 1).

Most early external genital developmental genes also play important roles during sex differentiation. Deletion of any one of the fibroblast growth factor pathway genes *Fgf10*, *Fgfr2*, or hedgehog pathway genes *Shh*, *Gli2*, and *Gli3* in mice induces hypospadias.[68–72] And SNPs of these genes and some other genes in the same pathways, such as *GLI1*, have been found in hypospadias patients.[73,74] *Fgf8* knockout mice form normal EG,[75] however SNPs in *FGF8* in hypospadias patients have been reported,[73] which may suggest the different function of this gene in external genital development between humans and mice, but further investigation on larger sampling is required to clarify the causal role of *FGF8* in the development of hypospadias in humans. Sprouty (*SPRY*) genes are critical regulators of FGF signaling during embryonic patterning of the male GT, and the combined deletion of *Spry1* and *Spry2* in the male mouse embryo affected genital morphogenesis.[76] Until now, the variation of *SPRY1* or *SPRY2* in hypospadias patients has never been reported. In addition, deletion of bone morphogenetic protein pathway genes *Bmp4*, *Bmp7*, or Wnt/β-catenin signaling genes, such as *Ctnnb1*, can also induce external genital malformation including hypospadias in mice.[77–79] As expected, polymorphisms of all these genes, and transforming growth factor beta receptor 2 (*TGFBR2*) as well, have been detected in hypospadias patients,[74,80–82] although no obvious penile phenotype was observed in *Tgfbr2* knockout mice.[83] EPHRIN-B2 and its receptor EPHB2 play major roles in cell adhesion and tubular urethra formation, and male mice carrying the mutations develop severe hypospadias and incomplete midline fusion of the primitive cloaca.[84] How these early developmental genes interact with steroid hormone signaling and how they regulate sexual differentiation are interesting questions.

Hox genes regulate the vertebrate body plan.[85] Several Hox genes such as *Hoxa13* and *Hoxd13* are strongly expressed in developing EG, and deletion of them in mice causes hypospadias or male accessory sex organ malformation.[86,87] Mutation of *HOXA13* or polyalanine expansions in this gene in humans results in hand–foot–genital syndrome with hypospadias of variable severity in male patients, and mutation in human *HOXD13* can also lead to hypospadias in various forms in males.[88–90] *Hoxa4* gene was found to be expressed in mouse testis,[91] and *Hoxb6* may be expressed in male reproductive organs.[92] No obvious phenotypic change in reproductive organs has been observed in mutant mice deficient in either *Hoxa4* or *Hoxb6*,[93,94] but genetic variants of both genes have been found to be associated with human hypospadias.[80,95] Empty spiracles homeobox 2 (*Emx2*) gene is a transcriptional target of HOXA10 regulation in the reproductive tract,[96,97] mutation of this homeobox gene in humans induces hypospadias and concomitant other genital malformations.[98] More patient research is required to understand the roles of HOX genes and other homeobox genes in hypospadias malformation.

Activating transcription factor 3 (ATF3) is upregulated in penile tissue in hypospadias patients and estrogen-treated mice.[99] *Atf3* knockout mice are available, but no external genital phenotype has been reported.[100] We assume that

maybe there is no obvious external genital phenotype in *Atf3* knockout mice. However, in humans, the variants of *ATF3* have been identified as risk factors for hypospadias.[101]

Variants in human *BNC2*,[102] *CHD11*,[103] *DGKK*,[104,105] *HAAO* and *IRX6*,[106] *LSM1*,[107] *MID1*,[108] *MTHFR*,[109] *MYRF*,[110] *PGK2*,[111] *SMADIP1*,[112] and some other genes have also been reported to be associated with hypospadias. Although distal urethral defects were observed in *Bnc2* knockout newborn mice,[102] no urogenital abnormalities were present in *Mid1* knockout mice,[113] and also no external genital phenotype has been reported in *Cdh11*,[114] *Dgkk*, *Haao*,[115] *Lsm1*, *Myrf*, *Pkg2*,[116] or *Irx6* knockout mice. In addition, *Lama5* was found to be required for urethral and external genital development, and deletion of *Lama5* induced hypospadias in mice, suggesting it may be a potential hypospadias causal gene;[117] until now, no SNP or mutation of *LAMA5* has been reported in hypospadias patients (Table 1).

Only a Small Portion of the Human Hypospadias Related Gene Mutations Have Been Reported to Show Similar Phenotypes in Mutant Mice

In the 49 hypospadias related genes we listed in Table 1, the majority of them (41) were first identified from human patients. Mutations in 13 out of the 49 genes were shown to cause hypospadias in mice (Figure 1), thus far, only mutations in seven out of the 13 genes (*AR*, *BNC2*, *CTNNB1*, *FGFR2*, *FGF10*, *HOXA13*, and *SHH*) have been associated with an increased risk of hypospadias in humans, variations of the other six genes (*EPHRIN-B2*, *EPHB2*, *FKBP4*, *LAMA5*, *SPRY1*, and *SPRY2*) have not yet been identified in human hypospadias patients, we suggest more investigations of the association between genetic variance of these genes and hypospadias risk should be conducted in hypospadias patients. Among the 43 human hypospadias related genes we listed in Table 1 (at least one mutation or polymorphism of the gene has been reported to be associated with hypospadias in groups of patients) (Figure 1), only mutations in seven genes (*AR*, *BNC2*, *CTNNB1*, *FGFR2*, *FGF10*, *HOXA13*, and *SHH*) have been shown to cause hypospadias in both humans and mice, no typical hypospadias but other or even more severe urogenital anomalies were observed in the knockout male mice of 12 genes (*BMP4*, *BMP7*, *CYP11A1*, *EMX2*, *GLI2*, *HAAO*, *HOXD13*, *INSL3*, *NR5A1/SF1*, *SRY*, *TGFBR2*, and *WT1*), male mice deficient in 12 genes (*ATF3*, *CDH11*, *ESR1*, *ESR2*, *FGF8*, *GLI3*, *HOXA4*, *HOXB6*, *MAMLD1*, *MID1*, *PKG2*, and *SRD5A2*) displayed no obvious phenotypic external genital abnormalities, and data of the external genital development in mouse mutants of the remaining 12 human hypospadias related genes (*AKR1C3*, *DGKK*, *GLI1*, *HSD3B2*,

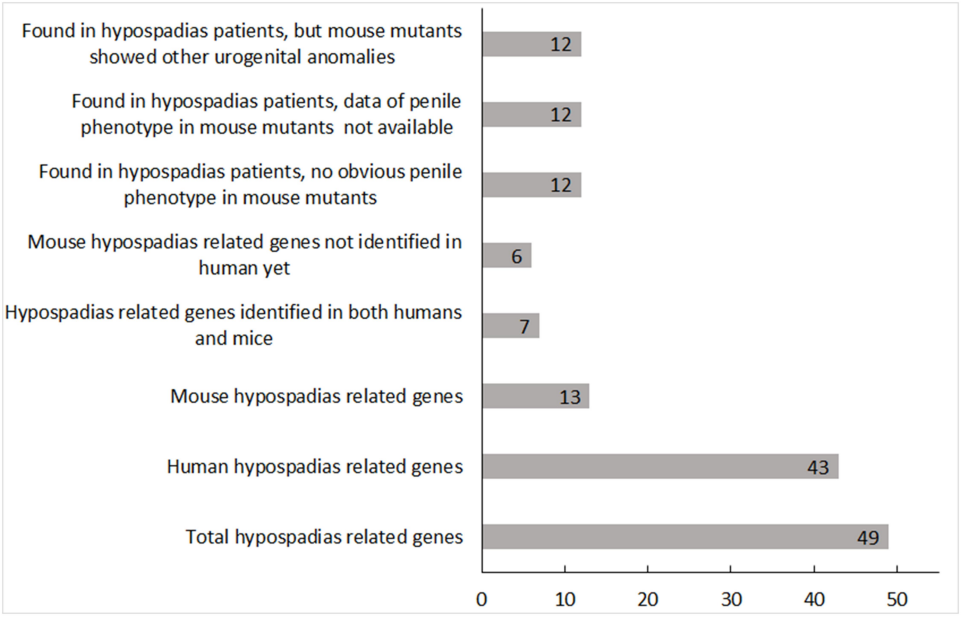

Figure 1 Genetic mutations found in hypospadias patients and mice. The figure shows the numbers of gene mutations found so far in hypospadias patients and mice. The x-axis indicates the number of genes, and the y-axis indicates the different groups. All the data were based on publications and online resources before September 1, 2020.

HSD17B3, *IRX6*, *LSM1*, *MTHFR*, *MYRF*, *SMADIPI*, *STARD3*, *and STS*) are still not available. There may be multiple reasons for the phenotypic differences caused by these gene mutations between mice and humans, the differences in the mechanisms of external genital development and hypospadias malformation between the two species are likely to be the main reasons.

Other Animal Models in Studies of the Etiology of Hypospadias

Mouse is the most commonly used animal model to investigate the etiology of hypospadias. However, due to the difference in the developmental mechanisms of penile urethra between mice and humans, mid-shaft or perineal hypospadias has only been observed in human hypospadias patients but never seen in mice.[118] Except for mice, rats have also been widely used in hypospadias related research, but the majority of the studies focused on the effect of environmental endocrine disrupting chemicals.[119,120] Hypospadias related genetic mutation in rats has never been reported. The detailed comparison of mice, rats, and humans was reviewed by Cunha et al,[118] and they suggested that mouse and rat models do not fully reflect the human condition. Rabbits are another useful model for hypospadias, and the fetal development of the rabbit phallus is relatively more analogous to that of the human.[15] A literature search suggests that the rabbit model is more commonly used for hypospadias repair studies, rather than for etiologic research of hypospadias.[121]

Hypospadias were also found in other domestic animals such as dogs,[122] cats,[123] sheep,[124] cattle,[125] and horses.[126] Moreover, polymorphisms were also found in *MAMLD1*, *SRD5A2*, and *AR* genes in hypospadias dogs.[127] Mice mutant for *Insl3* has no obvious penile phenotype, but the dogs with a heterozygous base change in *INSL3* gene showed similar hypospadias and cryptorchidism phenotypes to those of human C-19G, V18M, or R105H variants.[52,53,128] Dogs are the most widely used domestic animal model for hypospadias research, and the majority of the hypospadias studies using domestic animal models were case reports,[129,130] or focused on surgical repair.[131]

Guinea Pig Is Another Optional Animal Model to Study Human Penile Development and Hypospadias

Appropriate animal models are important to study penile development and hypospadias. In humans, the male genital tubercle development involves an initial opening of the urethral plate to form a urethral groove from proximal to distal, and subsequent fusion of the proximal region to close the urethral and penile epithelia and extend the closed urethra distally (distal opening proximal closing zipper). Females show a similar urethral plate opening to form a urethral groove, but lack the following urethral closure seen in males (the single opening zipper), both males and females form urethral grooves before sex differentiation and tubular urethra formation.[132,133] In commonly used mice and rats, the penile tubular urethra forms by direct canalization of the urethral plate within the penis body[134–136] without forming an obvious urethral groove,[137] moreover, the penile urethral closing from proximal to distal accomplishes during embryonic development[14,132] in humans, but the process still continues after birth in mice and rats.[138]

Guinea pig has been used as an effective model to study sexual differentiation and the role of exogenous and endogenous steroid hormones in sexually dimorphic organ and behavioral development since decades before.[14,–139–141] Wang et al[14] elucidated the embryonic early development and sex differentiation of guinea pig EG and the pharmaceutical induction of male hypospadias and female penis in guinea pigs, revealing that fetal development of the guinea pig phallus is homologous to that of humans; the key steps in human external genital development, urethral groove formation (in both sexes), and subsequent "distal opening and proximal closing" (in males), which have never been shown in commonly used animal models such as mice and rats, were presented in guinea pigs; antiandrogen bicalutamide treatment in different time periods around the urethral tube closing stage successfully induced hypospadias with various severity in guinea pigs, the phenotypes were similar to those of human hypospadias (eg, penosrotal hypospadias); compared to mice and rats, guinea pigs have a relatively longer gestation (on average 68 days), the EG of newborn guinea pigs is well developed, and the antiandrogen-induced hypospadias phenotypes can be detected at birth, suggesting the key sexual differentiation process of the guinea pig EG occurs prenatally as well as that of humans; the findings suggest that the guinea pig is a good model to study the mechanisms of human penile urethral formation (eg, distal opening and proximal closing) and to evaluate the pathophysiological processes of hypospadias. In addition, Wang and Zheng[142] showed the differential expression pattern of *Shh* between the guinea pigs during urethral

groove formation and the mice at the comparable stage: *Shh* mRNA expression domain shifts out to the ventral surface of genital tubercle from proximal throughout to distal in guinea pigs, but is excluded from the ventral surface epithelium in midshaft and distal of mouse genital tubercle; suggested *Shh* expression in ventral surface epithelium of genital tubercle may play a causal role in urethral groove formation in guinea pigs, maybe in humans as well. Although the penile development in guinea pigs is similar to that in humans at early tubular urethral forming stage, there are some differences in the urethral closure process and penile development between guinea pigs and humans at later embryonic and postnatal stages, eg, guinea pigs, but not humans, form a baculum before birth, and develop penile spines after birth.[14] Nevertheless, the literature studying hypospadias based on a guinea pig model is still very limited.

Conclusion and Future Directions

In general, the knowledge about genetic factors underlying hypospadias is very limited, and the majority of variants discovered in hypospadias patients did not show similar phenotypes in mouse mutants. As we know, most genetic polymorphisms in hypospadias patients were identified by single gene sequencing, not considering whether the other gene(s) had sequence variants or not meanwhile. As technologies are available, and the genome sequencing is getting cheaper, whole genome sequencing of hypospadias patients should be performed in the future to reveal hypospadias causal genes in humans. The environmental factors may induce hypospadias through affecting developmental gene expression, which can be detected by transcriptome sequencing (RNA-seq), but the up or down expression levels of the affected genes may not be detected in hypospadias patients after birth; to understand the deep mechanisms of hypospadias, reliable animal models are required. We need to understand the similarities and differences in the processes of penile development and hypospadias malformation among humans, mice, and other animal models, and carefully select animal models based on specific research questions. For studies of mechanisms of urethral groove formation and the "distal opening and proximal closing" in tubular urethral formation, guinea pig may be one good choice.

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Disclosure

All authors declare they have no conflict of interest.

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