The Desert Hedgehog Signalling Pathway in Human Gonadal Development and Differences of Sex Development

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Abstract

While the Hedgehog signalling pathway is implicated in numerous developmental processes and maladies, variants in the *Desert Hedgehog* (*DHH*) ligand underlie a condition characterised by 46,XY gonadal dysgenesis with or without peripheral neuropathy. We discuss here the role and regulation of *DHH* and its signalling pathway in the developing gonads and examine the current understanding of how disruption to this pathway causes this difference of sex development (DSD) in humans.

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The Hedgehog Signalling Pathway in Humans

The highly conserved Hedgehog (HH) family of signalling molecules plays a central role in the development of numerous organs during embryogenesis. Disruption to the ligands or signalling pathway can cause a variety of genetic disorders at different stages [Sasai et al., 2019] as well as cancer [Doheny et al., 2020; Iriana et al., 2021].

The canonical HH signalling pathway has been the subject of numerous excellent reviews [Briscoe and Thérond, 2013; Lee et al., 2016] and is only briefly described here with a focus on human. The HH morphogens are growth factors produced and secreted by a subset of cells. Humans, like many higher vertebrates, have 3 *HH* genes; *Sonic* (*SHH*), *Indian* (*IHH*), and *Desert* (*DHH*) *hedgehog*. This review focuses on *DHH*, the only ligand known to be involved in sex differentiation and differences of sex development (DSDs). All HH ligands undergo auto-processing and cleavage with the covalent addition of cholesterol [Porter et al., 1996] and palmitoylation [Pepinsky et al., 1998] by the hedgehog acyltransferase (HHAT) [Chamoun et al., 2001], which increase stability in the extracellular matrix and facilitate long-range spreading [Lewis et al., 2001; Zeng et al., 2001; Callejo et al., 2006]. Secreted HH ligands diffuse in a gradient, binding to the Patched (PTCH) receptor on target cells [Marigo et al., 1996; Motoyama et al., 1998]. PTCH is a constitutive repressor, indirectly destabilising the G-protein coupled receptor, Smoothened (SMO), the main pathway activator [Ayers and Thérond, 2010]. Upon HH binding, PTCH is degraded in the endosome [Incardona et al., 2000] and repression of SMO is released, leading to downstream activation of the GLI family of transcription factors, GLI1, GLI2, and GLI3 [Bai et al., 2004].

Fig. 1. DHH signalling during fetal testicular development. **A** During testicular development, the germ cells (green) are encased by the supporting Sertoli cells (blue) in the seminiferous tubules. Peritubular myoid cells (PMCs, ochre) lay down the basal lamina (orange) that surrounds the tubules. In the interstitial space between the tubules, the steroidogenic Leydig cells (purple) differentiate. **B** Sertoli cells produce DHH (red dots), and the immature interstitial cells express the PTCH receptor complex (black) and other DHH signalling components (not shown). DHH from the Sertoli cells is thought to act in a paracrine fashion to induce the differen-

tiation of precursors into the PMCs (which form the basal lamina) and the Leydig cells. Additionally, some studies have suggested autocrine DHH signalling within Leydig cells to maintain their steroidogenic function. **C** DHH also mediates germ cell maturation and spermatogenesis. DHH signalling likely starts in spermatogonia and primary spermatocytes, which express the PTCH receptor complex as well as pathway activators. Elongating spermatids express SuFu (black cross), a DHH pathway inhibitor, which might indicate that DHH signalling is switched off at the spermatid stage.

The Desert Hedgehog Pathway during Testis Development

During embryogenesis the main roles of DHH are in the formation of the peripheral nerve sheath and the development of the testis [Briscoe and Therond, 2013]. Testis development from the bi-potential gonad is triggered by the expression of the Y chromosome linked *SRY* gene, which induces the differentiation of the Sertoli cells via *SOX9*. Following this, development of interstitial cell types such as the peritubular myoid (PTM) and fetal Leydig cells (FLCs) is initiated. The germ cells, which have migrated to the gonad early in its development, become encased in the seminiferous tubules along with the supportive Sertoli cells, surrounded by a basement membrane laid down by the PTM cells (Fig. 1a) [for a detailed review see Eggers et al., 2014].

Most of what we know about the role of the DHH signalling pathway during testis development stems from animal models. This is summarised here. In mice, *Dhh* expression does not overlap with either *Shh* or *Ihh*, and *Dhh* is the main *Hh* involved in gonad development [Bitgood and McMahon, 1995; Shima et al., 2004]. *Dhh* expression in the mouse gonad starts from 11.5 dpc (days post coitum) and is maintained into adulthood [Bitgood et al., 1996; Yao et al., 2002; Szczepny et al., 2006]. In the early stages, *Dhh* expression is restricted to the Sertoli cells, likely inducing PTM and LC differentiation in a paracrine fashion [Bitgood et al., 1996; Clark et al., 2000; Pierucci-Alves et al., 2001; Yao et al., 2002] (Fig. 1b). PTM and LC precursors express the *Dhh* pathway receptors *Ptch1* and *Ptch2* from 11.5 dpc [Bitgood et al., 1996; Carpenter et al., 1998; Clark et al., 2000; Yao et al., 2002], and the *Gli* transcription factors (*Gli1-3*) are observed in the

fetal and adult interstitium from 14.5 dpc [Szczepny et al., 2006; Barsoum and Yao, 2011]. The role of Dhh appears to be conserved in the marsupial animal model, the tamar wallaby, where *DHH* is the only *HH* expressed during gonadal development [Chung et al., 2012], found in pre-Sertoli cells at the beginning of cord formation, when its receptors *PTCH1* and *PTCH2* are also expressed [O'Hara et al., 2011].

In mice, Dhh signalling initiates FLC differentiation and maturation through upregulation of the transcription factor *Sf1* (steroidogenic factor 1). *Sf1* in turn up-regulates the expression of the steroidogenic enzymes *Cyp11a1* (cholesterol side-chain cleavage; P450ssc) and *Hsd3b1* (hydroxyl-delta-5-steroiddehydrogenase, 3beta- and steroid delta-isomerase 1) [Yao et al., 2002; Park et al., 2007; Brokken et al., 2009]. Constitutively active Dhh signalling in *Sf1*-positive LC progenitor cells led to an increased number of FLCs in mouse fetal testis [Barsoum et al., 2013], while the treatment of fetal gonad explant cultures with an Hh signalling inhibitor severely disrupted FLC differentiation [Yao and Capel, 2002]. Dhh has also been implicated in adult LC (ALC) differentiation, increasing their proliferation and transition rate through their stages of development [reviewed in Ye et al., 2017]. Dhh may also maintain ALC steroidogenic function (Fig. 1b), as the loss of testosterone production in conditional *Wt1* (Wilms tumor protein) null mice could be partially rescued by treatment with SAG (a SMO agonist) [Chen et al., 2014]. Interestingly, Dhh protein expression has been described in mouse ALCs together with *Ptch1* and *Smo*, leading to the speculation that ALCs are capable of autocrine signalling [Morales et al., 2009], although further evidence is needed (Fig. 1b).

Dhh ablation in animal models confirms its importance during gonadal development. *Dhh* null XY mice display a range of testicular defects, depending on their genetic background. In some cases, XY nulls have abnormal FLC and ALC development with an interstitium containing primarily undifferentiated cells, leading to testosterone insufficiency [Clark et al., 2000; Yao et al., 2002]. Testis with disorganised and irregularly shaped Sertoli cells, abnormal peritubular tissue, and discontinued basal lamina that result in irregular and anastomotic tubules with germ cells located outside of the cords have also been described in mice lacking *Dhh* [Clark et al., 2000; Pierucci-Alves et al., 2001]. In some cases, *Dhh* null testes are significantly smaller, with seminiferous tubules only thinly lined with Sertoli cells [Bitgood et al., 1996]. Dhh mutant rats also show impaired LC development and insufficient androgen production [Kawai et al., 2011]. Wal-

laby ex vivo testicular cultures with inhibited Hh signalling have disorganised cords with some germ cells outside, likely due to a perturbation in the PTM cells and basal lamina, suggesting conservation of the mechanism [Chung et al., 2012].

Dhh also regulates germ cell maturation/spermatogenesis [reviewed in Walterhouse et al., 2003] (Fig. 1c). Adult XY *Dhh* null mice are infertile due to a lack of mature sperm, as germ cells do not progress towards the final stages of spermatogenesis [Bitgood et al., 1996]. Dhh signalling likely begins in spermatogonia and primary spermatocytes, which express the *Ptch* receptors, the Dhh activators *Smo* and *Fu*, and the *Gli* transcription factors [Persengiev et al., 1997; Carpenter et al., 1998; Fan et al., 2007; Morales et al., 2009]. At later germ cell maturation stages, Dhh signalling seems to be switched off in round and elongating spermatids, as indicated by both a shift of Gli1 protein expression from the nucleus to the cytoplasm [Kroft et al., 2001] and the expression of SuFu (Suppressor of Fused), a negative regulator of the Hh signalling pathway, in these cells [Szczepny et al., 2006].

Desert Hedgehog in Human Testicular Development

In humans, *DHH*, composed of 3 exons, is located at 12q12.13 and encodes a protein of 396 amino acids [Tate et al., 2000]. Like the other HH proteins, DHH is likely produced as a 45 kDa precursor with the 25 kDa C-terminal driving autolytic cleavage assisted by the covalent addition of a cholesterol moiety to the 19 kDa N-terminal fragment, thought to be the active molecule [Mann and Beachy, 2004]. However, the extent of DHH processing in the human embryonic gonads is unknown. DHH can be cleaved in an in vitro system [Tajouri et al., 2018], yet DHH expressed in cultured cells such as HEK293T cells is often uncleaved [Pettigrew et al., 2014; Ayers et al., 2019]. Indeed, domain swapping experiments have demonstrated that the DHH C-terminal is less efficient in cleaving the SHH N-terminal than the SHH C-terminal [Pettigrew et al., 2014]. In culture, uncleaved DHH is not effectively secreted and accumulates intracellularly, unable to elicit paracrine pathway activation [Pettigrew et al., 2014], yet these cells are still able to induce HH signalling in a juxtacrine assay, including the activation of LCs derived from immature mouse testes (TM3 cells) [Pettigrew et al., 2014]. Based on this, it has been proposed that DHH is not processed or secreted in the Sertoli cells, instead eliciting a cellular response in the LCs in a juxtacrine fashion. Although based on the position and pro-

Fig. 2. DHH expression in human fetal testis. Buonocore et al. [2019] analysed DHH protein expression in human fetal testis. At 9 weeks post conception, they found cytoplasmic DHH primarily in interstitial Leydig cells, with some staining in Sertoli cells (AMH-positive). The source of DHH in Leydig cells is not known

and may have originated in Sertoli cells or in Leydig cells themselves. The latter would suggest a degree of autocrine DHH signalling in human Leydig cells. Figure reused with permission by authors from Buonocore et al. [2019].

posed effects of human *DHH* genetic variants, others have proposed that DHH auto-processing is necessary in the human gonads [Elzaia et al., 2020].

Recombinant human DHH shows an affinity to its PTCH receptor complex comparable to SHH and IHH [Pathi et al., 2001] and is therefore thought to activate the same pathway during development, and DHH likely plays a conserved role in the testis although some interesting differences may exist in humans. Single cell RNA seq data from human fetal gonads [Li et al., 2017] (<https://rgv.genouest.org/>) [Darde et al., 2015, 2019] show *DHH* expression in the Sertoli cells with a peak in expression at 12 gestational weeks. Pathway receptor/targets *PTCH1* and *PTCH2* become strongly expressed at 10 weeks of gestation in Leydig precursors, mature LCs, and primordial germ cells (PGCs). Similarly, the *GLI* genes can be observed in the same cell clusters with the strongest expression between 20 and 25 weeks of gestation. Of note, the *PTCH* receptor complex shows weak expression in Sertoli cells [Li et al., 2017], and the pathway activator *SMO* is expressed throughout the human gonad but absent from the Sertoli cells. This might suggest DHH produced by Sertoli cells can bind these cells via PTCH yet cannot activate signalling due to the absence of SMO. Just one study has looked at DHH protein in human fetal testis using immunohistochemistry and describes cytoplasmic expression of DHH predominantly in interstitial LCs but also in Sertoli cells at 9 weeks post conception [Buonocore et al., 2019] (Fig. 2). It is not clear whether this Leydig DHH originated in the secreting Sertoli cells or whether LCs are also a source of DHH, and DHH staining is still needed in earlier development phases (i.e., 5–7 weeks gestation) to truly understand its role in human testis differentiation. At later time points (19 gestational weeks), DHH protein expression is reportedly predominantly lo-

calised to Sertoli cells [Fowler et al., 2008], and maternal smoking during pregnancy appears to lead to a decrease in both *DHH* and *PTCH1* expression in male fetuses. Since maternal smoking is also associated with reduced testis size with decreased sperm count and quality in male offspring, a phenotype intriguingly similar to that of the *Dhh* knockout mice, it has been speculated that diminished *DHH* expression is responsible for this phenotype [Fowler et al., 2008].

Genetic Variants in DHH Cause 46,XY Gonadal Dysgenesis

It is well established that pathogenic variants in *DHH* are a rare cause of 46,XY DSD in humans, in particular causing 46,XY partial or complete gonadal dysgenesis with or without peripheral neuropathies (OMIM 607080, OMIM 233420). The *DHH* gene is highly conserved and shows 20 different *DHH* pathogenic variants that have been published in 24 cases (Fig. 3, Table 1) with variants falling in both the N- and C-terminal domains (Fig. 3) [Umehara et al., 1999, 2000; Sugie et al., 2002; Canto et al., 2004, 2005; Das et al., 2011; Werner et al., 2015; Eggers et al., 2016; Paris et al., 2017; Sato et al., 2017; Baldinotti et al., 2018; Rothacker et al., 2018; Tajouri et al., 2018; Ayers et al., 2019; Buonocore et al., 2019; Neocleous et al., 2019]. Given that DHH is a potent growth factor that signals in a concentration-dependent manner, it is not surprising that the vast majority of pathogenic cases reported are bi-allelic (homozygous or compound heterozygous), and the contribution of heterozygous variants in *DHH* to DSD is unclear. Two patients reported to carry a heterozygous variant (p.Leu363Cysfs*4) in *DHH* also had sex chromosome mosaicism (45,X/46,XY) [Canto et al., 2005] (Ta-

Fig. 3. Genetic variants in *DHH* reported in patients with DSD. A schematic figure showing the protein position of previously reported variants in *DHH*. Compound heterozygous variants are depicted by a dotted line joining the 2 variants and are shown above

the protein, homozygous variants are below. Yellow circles show missense variants, blue are in-frame deletions, and red are variants that cause frameshifts or premature stop codons. SP, signal peptide.

ble 1), and our group has described a number of DSD patients with heterozygous *DHH* variants although our functional studies have suggested that these are unlikely to be the major cause [Ayers et al., 2019]. Most patients with bi-allelic *DHH* variants are 46,XY female, with just 2 reportedly raised as males (Table 1). Complete gonadal dysgenesis has been described in 13 patients with streak gonads and/or Müllerian structures indicating no functional gonadal tissue (Table 1). Ten cases of confirmed or suspected partial gonadal dysgenesis have also been reported. One patient had histological findings suggestive of Leydig cell hypoplasia (Table 1). Five patients with *DHH* variants have reported gonadal cancer, and 7 had *DHH*-associated neuropathies (Table 1).

Several groups have used functional genomics to understand the underlying pathology of variants (Table 2).

Overexpression constructs of tagged *DHH* proteins have been used to assay cellular localisation and protein cleavage with varying results which appear to depend on the cells used. Our group found that *DHH* expressed in HEK293T cells was mostly full length, and expression levels/cleavage did not significantly differ for any variant tested with the exception of the frameshift variant p.Leu363CysFs*4 where no full length protein was observed (Table 2) [Ayers et al., 2019]. In these cells, transfected *DHH* was expressed in the cytoplasm and cell membrane. Cellular localisation of the 11 *DHH* variants tested did not differ to the wild type, although the p.Arg-164Pro variant had a slightly reduced staining [Ayers et al., 2019]. In contrast, another study found that *DHH* is cleaved when expressed in HeLa cells, and that a number of patient variants had reduced or absent self-cleavage

Table 1. Published cases of DHH-related DSD

Patient	Chromosomal change	cDNA (NM_021044.2)	Protein (NP_066382.1)	Zygosity	Karyotype	Gender	Published	Clinical notes	Cancer?	Neuropathy?
1	chr12:49488204A>G	c.2T>C	p.Met1Thr*	Homozygous	46,XY	Female	Umehara et al. [1999,2000]	Partial gonadal dysgenesis with premature female genitalia, a blind-ending vagina, and an immature uterus; testis on one side and streak gonad on the other	Not reported	Yes. Minifascicular formation with decreased density of myelinated fibres
2	chr12:49484991A>G	c.485T>C	p.Leu162Pro	Homozygous	46,XY	Female	Canto et al. [2004]	16-year-old with bilateral streak gonads, female external genitalia	Not reported	No
3	chr12:49483747delC	c.1086delG	p.Leu363Cysfs*4	Homozygous	46,XY	Female	Canto et al. [2004]	19-year-old with bilateral streak gonads, female external genitalia	Bilateral gonado-blastoma	No
4	chr12:49483747delC	c.1086delG	p.Leu363Cysfs*4	Homozygous	46,XY	Female	Canto et al. [2004]	26-year-old with bilateral streak gonads, female external genitalia	Bilateral dysgerminoma	No
5	chr12:49483747delC	c.1086delG	p.Leu363Cysfs*4	Heterozygous (XO/XY)	45,X/46,XY	Female	Canto et al. [2005]	19-year-old with right testis and left streak gonad, bilateral Fallopian tubes and epididymis	Right testis with gonado-blastoma	Not reported
6	chr12:49483747delC	c.1086delG	p.Leu363Cysfs*4	Heterozygous (XO/XY)	45,X/46,XY	Female	Canto et al. [2005]	19-year-old with right testis and left streak gonad, right epididymis and left Fallopian tube	No	Not reported
7	chr12:49488023_49488025delCTC	c.271_273delGAG	p.Glu91del [p.Glu90del in original publication, now updated]	Homozygous	46,XY	Female	Das et al. [2011]	26-year-old presented with primary amenorrhoea. She had bilateral streak gonads and female external genitalia	Not reported	Not reported
8	chr12:49488236_49488239dupGGCT	c.57_60dupAGCC	p.Gln21Serfs*71	Homozygous	46,XY	Female	Das et al. [2011]	17-year-old presented with primary amenorrhoea. Streak gonads visualised on ultrasound; hypoplastic uterus identified	Not reported	Not reported
9 (older sister of patient 10)	chr12:49485105C>T	c.371G>A	p.Arg124Gln	Homozygous	46,XY	Female	Werner et al. [2015]	Surgery for inguinal hernia during childhood - removed left gonad. Primary amenorrhoea at age 17. Hypogonadotropic hypogonadism noted. Vagina without upper cervix and no uterus, and no Müllerian remnants. Right gonad removed at age 30 - no seminiferous tubules or Leydig cells in histology	Invasive seminoma/dysgerminoma noted in right gonad at age 30	Yes. Numbness of extremities increased from age 20. Glove and stocking-like polyneuropathy

Table 1 (continued)

Patient	Chromosomal change	cDNA (NM_021044.2)	Protein (NP_066382.1)	Zygosity	Karyotype	Gender	Published	Clinical notes	Cancer?	Neuropathy?
10 (younger sister of patient 9)	chr12:49485105C>T	c.371G>A	p.Arg124Gln	Homozygous	46,XY	Female	Werner et al. [2015]	Primary amenorrhoea at age 17. Normal female genitalia with blind-ending vagina and no Müllerian remnants. Gonadectomy - seminiferous tubules with Sertoli cells and some germ cells surrounded by fibrotic tissue. Some hyperplasia of Sertoli and Leydig cell-like cells - located in highly fibrous tissue with rare and atrophic seminiferous tubules	Seminoma/ dysgerminoma in situ with early invasion of the stroma in the right testis and a circumscribed gonado-blastoma	Yes. Glove and stocking-like polyneuropathy
11	chr12:49484985C>G	c.1058C>G	p.Ala353Gly	Homozygous	46,XY	Raised as female but transitioned to male	Eggers et al. [2016]	Ambiguous genitalia, blind ending vagina, palpable testes. Suspected partial gonadal dysgenesis	Not reported	Not reported
12	chr12:49484153G>A chr12:49484051G>A	c.680C>T c.782C>T	p.Ala227Val p.Pro261Leu	Compound heterozygous	46,XY	Female	Eggers et al. [2016]; Ayers et al. [2019]	Clitoral hypertrophy. Otherwise female outer appearance, short blind ending vagina. Both gonads palpable in labia majora. Histopathology: immature testicular tissue with reduced spermatogenesis and tubuli without lumen	Not reported	No sign at 20 years
13	chr12:49484087C>T chr12:49484968C>T	c.746G>A c.508G>A	p.Arg249His p.Gly170Ser	Compound heterozygous	46,XY	Male	Eggers et al. [2016]; Ayers et al. [2019]	Proximal hypospadias and chordee. Both gonads scrotal. Histology unknown	Not reported	Not reported
14	chr12:49484985C>G	c.491G>C	p.Arg164Pro	Homozygous	46,XY	Female	Eggers et al. [2016]; Rothacker et al. [2018]; Ayers et al. [2019]	Well-formed labia. Prominent clitoro-phallic structure. Normal vaginal opening with blind vagina. Both gonads in inguinal canal. Histopathology: bilateral dysgenetic testes; left para-gonadal biopsy showed Müllerian tissue resembling oviduct; right para-gonadal biopsy showed vaso-epididymal tissue	No	No sign at 14 years
15 (sister of patient 16)	chr12:49484957C>A	c.519G>T	p.Trp173Cys	Homozygous	46,XY	Female	Paris et al. [2017]	Presented with primary amenorrhoea at age 26. Female phenotype. Absence of gonads and uterus on ultrasound. No basal testosterone or response to hCG stimulation. Also reported psychomotor retardation, bilateral flat valgus feet, facial dysmorphism, increased volume of extremities, café au lait spots, and dental enamel anomalies	Not reported	Yes. Axonal sensory-motor neuropathy of the 4 limbs

Table 1 (continued)

Patient	Chromosomal change	cDNA (NM_021044.2)	Protein (NP_066382.1)	Zygosity	Karyotype	Gender	Published	Clinical notes	Cancer?	Neuropathy?
16 (sister of patient 15)	chr12:49484957C>A	c.519G>T	p.Trp173Cys	Homozygous	46,XY	Female	Paris et al. [2017]	Age 23. Female genitalia. Potential residual Müllerian structure on ultrasound. No reported dysmorphism	Not reported	No psychomotor retardation
17	chr12:49484984_49485744dup	c.304-572_492dup	p.?? 761-bp tandem duplication (c.304-572_492dup) with the 5' end located in intron 1 (chr12:49485744; GRCh37) and the 3' end in exon 2 (chr12:49484984; GRCh37)	Homozygous	46,XY	Female	Sato et al. [2017]	Amenorrhoea age 16. Undescended testis in left inguinal canal. Right gonad absent. No uterus. Blind ending vagina. Age 43 reported pigmentation of the nails and hyperkeratosis of soles. Pigmentation of all extremities observed by age 55. Also reported an XX sister with neuropathy carrying same mutation	Not reported	Yes. Sensory motor dysmyelinating polyneuropathy. Numerous nerve fascicles
18	chr12:49484984_49485744dup	c.304-572_492dup		Homozygous	46,XY	Female	Sugie et al. [2002]; Sato et al. [2017]	Normal female external genitalia with blind-ending vagina	Not reported	Progressive distal numbness and weakness of extremities from age 39. Sensory dominant neuropathy. Biopsy revealed minifascicle formation
19	chr12:49484922G>T	c.554C>A	p.Ser185*	Homozygous	46,XY	Female	Baldinotti et al. [2018]	Histology of left gonad described a dysgenetic-like testis structure with seminiferous tubules containing only Sertoli cells. Remnants of Müllerian structures present (immature Fallopian tubes but not uterus). Right gonad was dysgenetic with remnants of vas deferens. A 46,XX sister with peripheral minifascular neuropathy also reported	No	Yes. From 20 years, reported cramps, distal painful paresthesias/dysesthesias, and bilateral foot drop. At 52 years, equinovarus foot with indolent ulcers, and stepping and ataxic gait with severe peroneal atrophy and small hand muscle wasting, severe multimodal sensory loss with stocking-glove distribution, and loss of deep tendon reflexes. Nerve conduction studies were consistent with a severe mixed axonal and demyelinating motor sensory polyneuropathy
20	chr12:49484948G>C chr12:49483822delG	c.528C>G c.1011delC	p.Tyr176* p.Asn337Lysfs*24	Suspected compound heterozygous	46,XY	Female	Tajouri et al. [2018]	21-year-old reported with primary amenorrhoea. Hypergonadotrophic hypogonadism reported. Normal female external genitalia. Bilateral gonadectomy was performed	No	No

Table 1 (continued)

Patient	Chromosomal change	cDNA (NM_021044.2)	Protein (NP_066382.1)	Zygosity	Karyotype	Gender	Published	Clinical notes	Cancer?	Neuropathy?
21	chr12:49484948G>C chr12:49484199C>T	c.528C>G c.634G>A	p.Tyr176* p.Glu212Lys	Compound heterozygous	46,XY	Male	Tajouri et al. [2018]	At birth micropenis, penoscrotal hypospadias and undescended testis noted. Some response to human chorionic gonadotropin stimulation. Hypospadias repair and orchidopexy were performed	Not reported	No sign at 2 years 10 months of age
22	chr12:49484109A>G	c.724T>C	p.Phe242Leu	Homozygous	46,XY	Female	Buonocore et al. [2019]	Female external genitalia with complete gonadal dysgenesis and Müllerian structures	Not reported	Not reported
23	chr12:49484099C>G chr12:49484153G>A	c.734G>C c.680C>T	p.Arg245Pro p.Ala227Val	Compound heterozygous	46,XY	Female	Buonocore et al. [2019]	Partial virilisation and absent Müllerian structures	Not reported	Not reported
24	chr12:49484985C>G	c.491G>C	p.Arg164Pro	Homozygous	46,XY	Female	Neoleous et al. [2019]	19-year-old presenting with primary amenorrhoea, female external genitalia, streak gonads and no uterus	No sign at 19 years. Gonads removed	No sign at 19 years

[Elzaïat et al., 2020] (Table 2). In addition, HeLa cells transfected with DHH-GFP variants had 2 patterns of intracellular localisation, termed by the authors “granular” (cytoplasmic expression) and “ring-type” (homogenous expression surrounding the nucleus). A significantly lower proportion of the granular-type staining was described in some of the variants with impaired protein processing [Elzaïat et al., 2020] (Table 2). A cell-free in vitro cleavage assay has also shown that a fusion human DHH protein can undergo cleavage (with the addition of DTT or β -mercaptoethanol) [Tajouri et al., 2018] and 2 C-terminus patient variants led to reduced [p.(Glu212Lys)] or absent [p.(Asn337Lysfs*24)] cleavage efficiency in this system [Tajouri et al., 2018]. Interestingly, using HEK293T cells expressing wild type or variant DHH co-cultured with TM3 mouse LCs (known to be responsive to DHH [Pathi et al., 2001]), our group found reduced or absent pathway activity for 9 DHH variants (Table 2). This suggests that despite the lack of cleavage, variants in DHH affect its activity on LCs in this system.

Additional DHH Pathway Genes Contributing to Human Reproductive Disorders

Hedgehog Acetyltransferase

HHAT is a palmitoyl acyltransferase for HH proteins [Buglino and Resh, 2008], and its loss in mice affects Hh secretion and long-range diffusion, resulting in holoprosencephaly, acrania, and agnathia, likely due to reduced Shh signalling [Dennis et al., 2012], as well as disrupted testis cord formation and reduced LC differentiation [Callier et al., 2014]. In humans, *HHAT* is expressed in embryonic ovaries and testes at 9 weeks gestation, where it is higher in Sertoli cells compared to LCs [Callier et al., 2014]. Recessive variants in *HHAT* cause Nivelon-Nivelon-Mabille syndrome (NNMS; OMIM 600092), characterised by progressive microcephaly, vermis hypoplasia, and skeletal dysplasia. Just one 46,XY patient with NNMS has been described [Nivelon et al., 1992; Thauvin-Robinet et al., 2005; Callier et al., 2014], and this individual was born with normal external female genitalia. Histology confirmed testicular dysgenesis with dysplastic/immature seminiferous tubules and a dense interstitial compartment with reduced numbers of LCs. Persistent Müllerian and Wolffian duct structures were noted. The patient carried a homozygous *HHAT* p.Gly287Val missense variant inherited from unaffected carrier parents, which fell in the O-acyltransferase domain and abolished palmitoylation of HH proteins [Callier et al., 2014]. This sug-

Table 2. Functional genomic findings for the 18 DHH variants reported

Variant	DHH protein, cleavage, and processing	Subcellular localisation	Activity
p.Met1Thr*	Missense mutation at the initiating codon which resulted in failure of translation of the <i>DHH</i> gene		
p.Glu91del [p.Glu90del in original publication]	Reduced cleavage in HeLa cells [Elzaïat et al., 2020]		Loss of activity in HEK/TM3 co-culture assay [Ayers et al., 2019]
p.Arg124Gln	Slightly reduced cleavage in HeLa cells [Elzaïat et al., 2020]		Partial loss of activity in HEK/TM3 co-culture assay [Ayers et al., 2019]
p.Leu162Pro	Abolished cleavage in HeLa cells [Elzaïat et al., 2020]	Altered subcellular localisation in HeLa cells [Elzaïat et al., 2020]	Loss of activity in HEK/TM3 co-culture assay [Ayers et al., 2019]
p.Arg164Pro	Abolished cleavage in HeLa cells [Elzaïat et al., 2020]	Slight reduction in expression levels [Ayers et al., 2019]	Loss of activity in HEK/TM3 co-culture assay [Ayers et al., 2019]
p.Gly170Ser	Reduced cleavage in HeLa cells [Elzaïat et al., 2020]		Partial loss of activity in HEK/TM3 co-culture assay [Ayers et al., 2019]
p.Trp173Cys	Abolished cleavage in HeLa cells [Elzaïat et al., 2020]	Altered subcellular localisation in HeLa cells [Elzaïat et al., 2020]	
p.Glu212Lys	Reduced cleavage efficiency in in vitro time course [Tajouri et al., 2018]		
p.Ala227Val	Abolished cleavage in HeLa cells [Elzaïat et al., 2020]	Altered subcellular localisation in HeLa cells [Elzaïat et al., 2020]	Loss of activity in HEK/TM3 co-culture assay [Ayers et al., 2019]
p.Arg249His	Reduced cleavage in HeLa cells [Elzaïat et al., 2020]		Partial loss of activity in HEK/TM3 co-culture assay [Ayers et al., 2019]
p.Pro261Leu	Abolished cleavage in HeLa cells [Elzaïat et al., 2020]	Altered subcellular localisation in HeLa cells [Elzaïat et al., 2020]	Loss of activity in HEK/TM3 co-culture assay [Ayers et al., 2019]
p.Asn337Lysfs*24	Abolished cleavage in in vitro assay [Tajouri et al., 2018]		
p.Arg353Gly	Abolished cleavage in HeLa cells [Elzaïat et al., 2020]		
p.Leu363Cysfs*4	Truncated protein detected in Western blot with less expression [Ayers et al., 2019]	Truncated form still expressed in HEK cells [Ayers et al., 2019]	Loss of activity in HEK/TM3 co-culture assay [Ayers et al., 2019]

gests that loss of HHAT disrupts DHH signalling in the human testis.

Cilia, Ciliopathies, and DSD

An interesting feature to vertebrate HH signalling is the importance of the primary cilia [Huangfu et al., 2003; Bangs and Anderson, 2017]. In the absence of HH, PTCH is present in the cilia and when HH binding inactivates PTCH, SMO accumulates in the cilia [Corbit et al., 2005; Rohatgi et al., 2007] where it controls the activation of the GLIs which are enriched at the cilia tip. Dhh signalling is thought to be regulated via the primary cilium in mouse LCs, as shown in TM3 culture cells [Nygaard et al., 2015], and cilia are also likely to play a role in human testis DHH signalling. PMCs are positive for primary cilia, and while mature LCs do not commonly possess primary cilia, less

differentiated LCs often do [Nygaard et al., 2015]. This would indicate that DHH signalling proceeds via the primary cilia in these cells. This idea is supported by findings in humans where defects in ciliary assembly or trafficking lead to numerous developmental disorders collectively referred to as ciliopathies. These include Bardet-Biedl (BBS), Joubert, and Meckel-Gruber syndromes [Reiter and Leroux, 2017]. Reproductive disorders are not uncommon in ciliopathies. For example, BBS patients often present with phenotypes such as LC or general testis hypoplasia [Green et al., 1989]. It has not yet been established whether these phenotypes are due to primary failure in testis differentiation or later from a disrupted adrenal-pituitary-gonadal axis [Soliman et al., 1996], and therefore, further work is required to understand the exact role of primary cilia in DHH signalling in the testis.

In addition, enzymes involved in lipid biosynthesis in the ciliary microenvironment that promote Hedgehog signalling may also be important contributors to DSD. *DHCR7* (7-dehydrocholesterol reductase) is an enzyme that synthesises cholesterol, and recent studies have shown that *DHCR7* localises near the ciliary base to prime the cilia for HH pathway activation [Findakly et al., 2021]. Children with variants in *DHCR7* develop Smith-Lemli-Opitz syndrome (SLOS; OMIM 270400), with elevated 7-DHC and low cholesterol levels and a broad phenotypic spectrum including underdeveloped external genitalia and poorly differentiated testicular tissue. These studies were not carried out in gonadal tissue/cell lines; however, given that HH molecules like DHH are cholesterol-modified and that cilia-based activation may require local cholesterol synthesis, it would be interesting to investigate whether variants in *DHCR7* lead to DSD via a decrease in gonadal DHH signalling.

Outstanding Questions

Despite the overwhelming evidence that DHH variants cause 46,XY gonadal dysgenesis, the exact role of DHH and its signalling pathway in the human gonad are still not well understood. Outstanding questions surround the expression, cleavage, and secretion of the DHH ligand in the human gonads. Further research into the relative importance of juxtacrine, paracrine, and even autocrine signalling is required and may reveal differential mechanisms for each gonadal cell type. Thus far, this research has been severely impeded by the difficulty in obtaining primary human embryonic gonadal tissue at a relevant time point for fetal gonadal development (i.e., weeks 5–9 of gestation). To add to this, an adequate human embryonic gonadal cell line is currently lacking. We and others have begun to address this issue using differentiation methods for pluripotent stem cells into several cell types of the embryonic gonad, that is, bipotential cells, early Sertoli cells, and immature LCs [Bucay et al., 2009; Buganim et al., 2012; Yang et al., 2015, 2017; Sepponen et al., 2017; Rodriguez Gutierrez et al., 2018; Chen et al., 2019; Knarston et al., 2020]. We have observed the induction of *DHH* expression in 3D organoid cultures, thus providing a valuable future tool to investigate the role of key testicular genes such as *DHH* during human fetal testis development [Knarston et al., 2020].

Interestingly, animal models suggest that the major role of Sertoli-secreted Dhh is to drive differentiation of the in-

terstitial LC and PTM cells, and mice nulls often have testes with reduced populations of these cells and disrupted testis cords. However, in humans, the majority of patients with bi-allelic DHH variants demonstrate complete gonadal dysgenesis, a more severe phenotype. This suggests that DHH may play an additional role in human testis, for instance in Sertoli cell differentiation or maintenance. Alternatively, human gonads may show a greater sensitivity to disruption of the testis cord basement membrane, observed in several animal models, perhaps resulting in complete degeneration/loss of the gonad. Interestingly, DHH has been suggested to play a role in vascular endothelial cells, in particular in maintaining the integrity of the blood-brain and the blood-nerve barrier by regulating endothelial tight junction expression [Chapouly et al., 2016; Caradu et al., 2018; Hollier et al., 2020]. DHH has also been shown to regulate the expression of proangiogenic factors in Schwann cells [Renault et al., 2013]. A similar role for DHH in the formation of the blood-testis barrier or testis vasculature is therefore feasible.

A DHH genetic diagnosis can guide clinical management and patient monitoring, in particular, in relation to gonadal malignancies and neuropathies. Indeed, 7 of the 24 patients previously reported with DHH-associated DSD also had neuropathy, and 5 patients had reported gonadal cancers (including seminoma in situ, dysgerminoma, and gonadoblastoma) [Canto et al., 2004, 2005; Werner et al., 2015]. However, functional studies are yet to reveal which DHH variants carry greater risk of these additional features or whether this is due to additional environmental or genetic factors. These genotype-phenotype correlations require more investigation and may be revealed only with additional reported cases and a greater understanding of the DHH pathway role and regulation in the human embryo.

It is also interesting that very few additional DHH pathway genes have been implicated in DSD thus far. This is likely explained by the fact that DHH, SHH, and IHH all converge on a central pathway, mutations in which lead to severe phenotypes or even embryonic lethality. In these cases, a fertility or sex-reversal phenotype may be overlooked. Currently, less than 50% of patients born with a DSD will receive a genetic indication [Eggers et al., 2016]. Future advances in sequencing technologies may identify variants in additional DHH signalling pathway components with gonadal-specific mosaicism as genetic risk factors or as oligogenetic contributors to DSD.

Conclusion

The HH signalling pathway is a well-studied regulator of embryonic development and when disrupted causes severe genetic defects. The HH morphogen DHH specifically regulates fetal gonadal development, and *DHH* variants cause 46,XY gonadal dysgenesis in humans, sometimes combined with peripheral neuropathy. A detailed understanding of how DHH signalling works in human is still lacking, and more research is required to understand the pathological mechanisms underlying DHH-related DSDs. This is required for better diagnosis, prognosis, and clinical management of these patients.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

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