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REVIEW

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# How does Hedgehog signaling participate in the cross-interaction of hormones and testis development?

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**Key words:** Hedgehog signaling, Hormone, Testis, Leydig cell, Gonadal dysgenesis

**Abstract:** Hedgehog (HH) signaling has been researched for decades and Hedgehog has 3 homologs: Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh). Dhh is the one involved in male gonad and germ cell development. The distribution of molecules in Hedgehog signaling in testis indicated that Hedgehog signaling executes important functions during testis development. The patients with Dhh signaling deficiency develop dysgenesis of gonads and hormone production which demands further exploration of gonad HH signaling. Some results proved the indispensable roles of HH signaling in gonad and germ cell development and the interaction with hormones. This review evaluates HH functions in the testis and how HH affects and is affected by hormones and provides novel insights about HH signaling to the readers.

## Introduction

The hedgehog (HH) gene was first discovered in *Drosophila* which encodes a protein participating in cell-cell communication during segmental patterning [1,2]. Hedgehog proteins were secreted as a morphogen to conduct cells' proliferation and development through short- and long-range signaling [3,4]. In vertebrate animals, there are 3 types of HH protein: Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh), they play different roles in different parts of the body during development.

The gradient of Shh directs subsequent morphogenesis of limb buds [5–7] and neural development [8], Shh takes part in the development of the cerebellum [9], bladder [10], neural tube [11] and the differentiation of neural cells, neural stem cells, oligodendrocyte precursor cells and astrocytes [12–16]. Some neurodegenerative diseases are related to Shh [17–19]. In general, the Shh takes part in the development of the nervous system. Ihh was expressed by prehypertrophic and hypertrophic chondrocytes which participate in regulating the endochondral ossification [20,21], the activation of Ihh signaling could drive resting zone chondrocytes to convert into osteoblasts eventually [22,23]. Ihh plays roles in the formation and specialization of craniofacial, bone, and cartilage [24–26]. Ihh takes charge of bone and cartilage formation. Dhh plays an important role in gonads especially testis development and hormone secretion [27,28], and even the evolution of scrotal testis [29]. But their roles are not always strictly separated: Shh could be involved in the formation and patterning of craniofacial structures [30]; Ihh may participate in the proliferation of colonic tumor cells [31]; Dhh functions in perineurium development [32] and participates in nerve injury and neuropathies [33,34], also the differentiation of chondrocytes [35]. The coordination of Ihh and Shh can mediate epithelial-stromal cross-talk with the function of primary cilia during decidualization in mice [36]. The relationship of the three HH components and molecular mechanisms in HH signaling had been reviewed before [37].

In addition to the typical functions that were reviewed previously, some research indicates that the HH signaling and the role Dhh plays in testis development have a close relationship with hormones and their production [38]. As the producer of testosterone in male vertebrates, the normal development and function of Leydig cells contribute a lot to male gonad development [39]. However, the null mutation of Dhh suppressed the production of testosterone by Leydig cells and spermatogenesis [40,41], which could be the defects of steroidogenic enzyme expression levels in Leydig cells [42]. Some *in vitro* research even showed that the HH signal pathway could regulate the steroidogenesis enzymes through a combination with their promoters [43]. More and

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more research indicated that there is a deep relationship between HH signaling, hormone production, and testis development.

During the past years of research on HH signaling and gonads, researchers have found the indispensable relations between them. Since cholesterol is needed during the processing of mature HH, some researchers discovered a close relationship between HH signaling and androgens [44]. Others found the important functions of HH in steroidogenic glands [45,46], gonadal development, and some clinical cases [27,47]. However, this previous research didn't realize that HH signaling has other molecular relationships in gonads. That is HH has relations with different kinds of hormones, especially a deep interaction with androgens. The interactions of HH signaling with hormones and the signaling functions in gonad development are not separated but related. In this review, we will take several parts to focus on HH functions in the testis and discuss why HH signaling is so important for testis development and hormones, dig into the deeper interaction of HH signaling and hormones, and propose our theory about HH signaling in testis. We searched for different types of HHs in different species and summarized the available information in Table 1.

**TABLE 1**

**The first report of Hedghog's function in various organs**

| Species | Protein name | Protein description on Uniprot | First source | Published year |
|---|---|---|---|---|
| *Cynops pyrrhogaster* | Sonic hedgehog protein | Activated in Animal Cap Explants | Takabatake et al. [48] | 1996 |
| *Danio rerio* | Desert hedgehog protein | Involves in patterning events | Avaron et al. [49] | 2006 |
| *Danio rerio* | Sonic hedgehog protein | Involves in patterning of brain and eyes | Roelink et al. [50] | 1994 |
| *Rattus norvegicus* | Sonic hedgehog protein | Involves in patterning events like anterior-posterior axis of the developing limb bud and induces ventral cell fate in the neural tube and somites | Roelink et al. [50] | 1994 |
| *Danio rerio* | Indian hedgehog B protein | Induces somite patterning and muscle pioneer differentiation | Currie and Ingham [51] | 1996 |
| *Drosophila melanogaster* | Protein hedgehog | Establishes the anterior-posterior axis and patterns imaginal disks | Nüsslein-Volhard and Wieschaus [52] | 1980 |
| *Gallus gallus* | Sonic hedgehog protein | Involves in patterning events and induces ventral cell fate in the neural tube and somites | Riddle et al. [53] | 1993 |
| *Gallus gallus* | Indian hedgehog protein | Take part in cartilage differentiation | Andrea et al. [54] | 1996 |
| *Homo sapiens* | Desert hedgehog protein | Functions in neural and endothelium integrity, required for testis development and spermatogenesis | Drummond I.A. submitted to EMBL/GenBank/DDBJ databases (https://www.uniprot.org/citations/CI-6IAIPE6O37A4J, accessed on 10 October 2024) | 1996 |
| *Homo sapiens* | Indian hedgehog protein | Functions in endothelium cell integrity maintenance and growth and differentiation of the endochondral skeleton | Chang et al. [55] | 1994 |
| *Homo sapiens* | Sonic hedgehog protein | Involves in patterning events and induces ventral cell fate in the neural tube and somites | Chang et al. [55] | 1994 |
| *Mus musculus* | Indian hedgehog protein | Take part in cartilage differentiation | Echelard et al. [56] | 1993 |
| *Mus musculus* | Desert hedgehog protein | Functions in neural and endothelium integrity, required for testis development and spermatogenesis | Echelard et al. [56] | 1993 |

(Continued)

**Table 1 (continued)**

| Species | Protein name | Protein description on Uniprot | First source | Published year |
|---|---|---|---|---|
| *Mus musculus* | Sonic hedgehog protein | Involves in patterning events like anterior-posterior axis of the developing limb bud and induces ventral cell fate in the neural tube and somites | Echelard et al. [56] | 1993 |
| *Xenopus laevis* | Desert hedgehog protein | Involved in the patterning of anterodorsal ectoderm, nervous system and somites | Ekker et al. [57] | 1995 |
| *Xenopus laevis* | Indian hedgehog protein | Involved in the patterning of anterodorsal ectoderm, nervous system and somites | Ekker et al. [57] | 1995 |
| *Xenopus laevis* | | Guidance of axon, involved in the patterning of anterodorsal ectoderm, nervous system and somites | Ruiz i Altaba et al. [58] | 1995 |

## HH Signaling Influences Male Gonad and Germ Cell Development

*Tissue localization of HH signaling components*

To understand HH signaling, researchers did so many experiments and most of them concentrated on the downstream of HH. It will be easier to understand these issues by having a brief review of the main members downstream of HH in this signaling pathway. Ptc (Patched) takes the first position in the downstream of protein HH. Ptc suppresses the activation of Smoothened (Smo), the combination of HH and Ptc could initiate the degradation of Ptc and cancel the suppression of Smo by Ptc [59,60]. In drosophila, active Smoothened combines with kinesin Cos2 [61], and Cos2 regulates the activity of transcription factor Ci (*Cubitus interruptus*) [62]. In vertebrate cells, there are 3 formations of the transcription factor downstream of HH signaling homolog to Ci: Gli 1, 2, and 3 (glioma-associated oncogene family members 1, 2, and 3) [63], and Cos2's homologous protein are Kif7 and Kif27 [64,65]. Interestingly, Glis' regulation in vertebrates is not through Kif7 or Kif27. It is primary cilia that play the regulatory role of Cos2 in drosophila [66], Kif7 and Kif27 regulate the Smoothened, primary cilia, and Gli complex by organizing the cilium tip compartment instead [67,68].

Several members of HH signaling were detected after these years of research. HH protein has 3 homologous in vertebrate animals, but in testis, especially for mammalian animals, Dhh plays a more important role [47]. Expression of Dhh was initiated after the expression of sex-determining region Y (Sry) in precursors of Sertoli cells and will last in adulthood during the development of testis [69]. In some research, Dhh was detected in the cytoplasm of Sertoli cells, late condensing spermatids, and Leydig cells in Chinese tongue sole testis [70]. So Sertoli cells could be the main source of Dhh protein in the testis at first, germ cells, and Leydig cells could be the target of Dhh since HH was secreted to execute its function.

Ptc1 mRNA is specifically localized in the germ line in the testis [71]. However, further research showed that its protein localization indicated that Ptc1 was expressed in late spermatocytes, round spermatids, and Leydig cells, but not in other cells in the testis [72]. Ptc2 mRNA was detected in spermatogonia and spermatocytes [73], and Ptc3 protein was localized on the mid-piece of sperm [74]. As the receptor of HH protein, distributions of Ptc proteins and their mRNA give us a view on HH target cells primarily: Leydig cell and germ cell are HH protein target cell types in testis. Fortunately, the distribution of Smo protein made a supplementary support for this proposal. Smo protein was expressed in late spermatocytes, spermatids, and Leydig cells, but not in other cells [72].

Gli mRNAs were detected in Sertoli cells [75] and predominantly in spermatogonia and spermatocytes in adult testis [73]. Gli1 protein is expressed in Sertoli cells, spermatogonia, and spermatid [76]. Our proposed distributions of HH signaling members in testis are shown in Fig. 1.

This research suggests that Dhh is probably mainly produced by Sertoli cells and secreted to the testis microenvironment, downstream proteins of HH in the signaling pathway are expressed in the germ cells and Leydig cells, and it is Sertoli cells that make the decisions to direct these cells during testis development and function.

HH proteins are produced in the Sertoli cell's endoplasmic reticulum and secreted to the testis. HH combines with patched proteins which are located on the cell membrane of germ cells and Leydig cells to cancel Patched inhibition of Smoothen and Smoothen could function on primary cilia to recruit Gli and primary cilia activates Gli, Gli then transferred into the nucleus to promote or starts target genes transcription. The figure was drawn using Adube Illustrater 2024 software.

*HH signaling in the morphogenesis and development of testis*

Testis supplies the environment for germ cells' development. Besides germ cells, the correct function of HH signaling is needed for the formation and development of male gonads. A proper expression and secretion of HH protein is

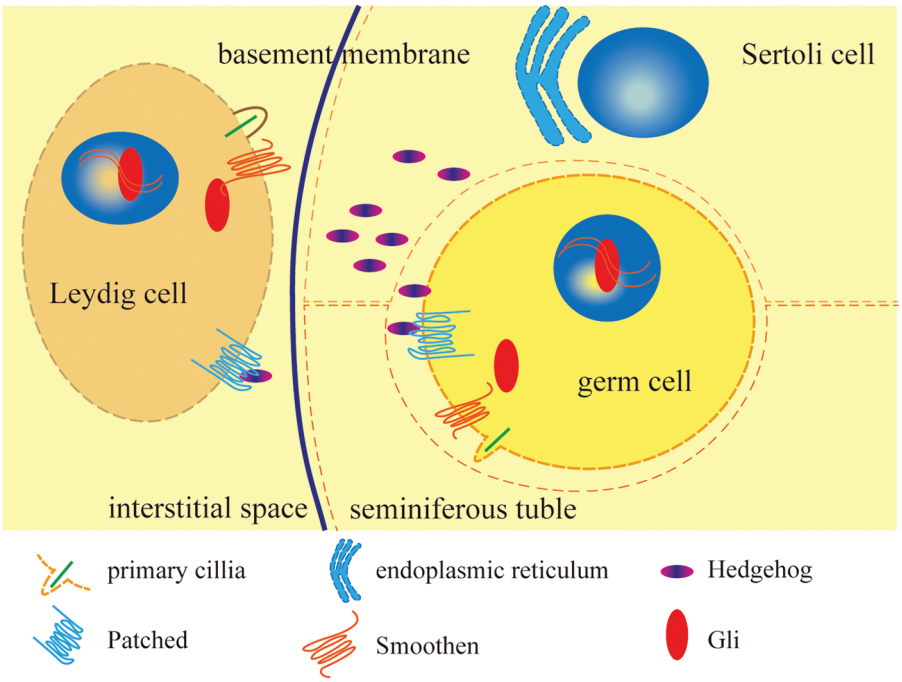


**FIGURE 1.** Distribution of HH signaling members and brief mechanism in testis.

required for the correct migration of germ cells in Drosophila embryos [77]. The *de novo* RNA-Seq analysis of *Onychostoma macrolepis* testis showed that the HH signaling pathway plays an important role in testis maintenance and spermatogenesis in general [78]. With the aging of Sertoli and Leydig cells, the HH signaling shows dysregulation through single-cell RNA sequencing in human testis [79]. HH signaling inhibitor Cyclopamine or Gli1/2 suppressor GANT61 treatment on mouse testis could abolish the appearance of fetal Leydig cells [80], and lead to defects in Wolffian duct morphogenesis of testis [81], and the Gli3 loss-of-function mutation leads to cryptorchidism and hypospadias in male mice [82]. The activation of HH signaling by SAG, an agonist of Smo, promotes laminin secretion and increases the proliferation of Leydig and germ cells, which contribute to the formation of the basal membrane and embedding of germ cells into Sertoli cells during the reconstruction progression of seminiferous tubule-like structures [83]. These animals treated with an inhibitor or activator of HH signaling offered us a conclusion that the activation of HH signaling is required for male gonad development, and the inhibition of HH signaling after animals' birth will defect testis morphogenesis and development.

What will it be if the change of HH signaling is not caused by drugs, but by the mutation of the individual fetus? Several reports could answer this question. The null mutation of Dhh in male mice could induce feminization, the polarity of Sertoli cells in their testes was lost, the basal lamina was irregular, and seminiferous cords were gapped during development [84]. In the masculinized Dhh-null adult males, the basement membrane was absent, but still exist abundant adult Leydig cells [40]. But in the Dhh null; Sf1+/− (steroidogenic factor 1) male mice, the gonad development was not as clear as in the normal mice and the adult Leydig cells failed to develop [85]. By contrast, consecutive activation of HH signaling induced the appearance of ectopic fetal Leydig cells in the ovary in female mice [86] and caused a reduction of testis and epididymis wet weight in male mice [87].

From these gene-edited or drug-treated animals, we can primarily know what happened about Dhh in testis: Dhh protein produced in testes to make sure the polarity of Sertoli cells and embed germ cells into Sertoli cells, direct laminin secretion to keep the structure of the basal membrane [88]. Testis can supply a proper environment for germ cell proliferation and differentiation in this way. Out of the seminiferous tubules, Dhh promises the development of adult and fetal Leydig cells with the help of full dose expression of Sf1. The proper expression of HH signaling is required for testis successful structural morphogenesis.

*Disturbance in HH signaling influences the survival and differentiation of germ cells*

Sperm production is highly correlated with mammalian reproductive security and HH signaling offers an inescapable contribution to germ cell survival according to this year's research. After the disruption of HH signaling, safe germ cell production would be problematic.

In chicken, single-cell transcriptome analysis of germ cells showed the participation of HH signaling during germ cell mitotic arrest [89]. The inhibition of HH signaling by cyclopamine, an inhibitor of Smo, increased the number of germ cells which suffered apoptotic and spermatogonia and inhibited the proliferation of primordial germ cells [83]. This is also discovered in rat testis *in vivo* and Medaka SG3 spermatogonial stem cell line *in vitro* [90–92]. Further research suggested that Dhh could rescue cell proliferation [91], and SAG, an agonist of Smo, could also rescue the number of germ cells in a reconstruction of the tubule-like structure system [83]. Treatment with cyclopamine on mice

testis *in vitro* culture system defected the germ cells' entrance of meiosis [93] and mitosis [94]. The deletion of the Ptc1 gene leading to germline anatomical defects and sterile in *Caenorhabditis elegans* [71] gives us more evidence to emphasize the importance of HH in germ cells' development.

In the Dhh-null masculinized mice, the spermatogonia were evident but the spermatocytes underwent cell death [41] and are absent, which suggests the process of spermatogenesis was inhibited without influencing germ stem cells. However, the knockdown of Gli1 in *Gallus gallus* decreased the quantity of primordial germ cells [95]. The reason for this difference could be the function of Gli, which regulates the cell cycle by binding to the regulator of the cell cycle (rgcc) directly [92].

The above evidence showed us that HH signaling is indispensable in germ cell development, but what be the outcome if more HH signaling in testes would happen? Some research showed the motility and number of sperm in the HH-continuous activated mice were reduced [87], and the overexpression of Gli1 promoted the differentiation of embryoid body cells into spermatogonial stem cells [95] while leading to the halt of spermatogenesis at the pachytene primary spermatocyte stage and apoptosis of germ cells [96]. It is obvious that overactivation of HH damages the cell cycle and physical function of germ cells. A proper activation of HH signaling was needed for germ stem cells to generate normal sperm and Gli1 plays a significant role in embryonic cells to differentiate into germ cells.

*HH signaling regulates Leydig cell differentiation*

As constitutions of the testis, adult and fetal Leydig cells make up the testis structurally and hold the function of androgen production and secretion [97,98]. Fetal Leydig cells develop during the tenth week of fetal life and experience 2 waves before the first year of life for humans, which occur in 3 waves in pigs. Fetal Leydig cells express hydroxysteroid dehydrogenase (3β-HSD) and Sf-1, which hold the function of steroid synthesis. While adult Leydig cells experience functional differentiation at the very beginning of fetal life (Luteinizing hormone/human chorionic gonadotropin-independent) and pubertal period (Luteinizing hormone/human chorionic gonadotropin-dependent) for humans, express several HSD (including 3β-HSD), possess the high capacity of testosterone synthesis [99,100]. During the differentiation of adult Leydig cells, the cells acquire the ability of androgen production, and the expression of relative enzymes is increased [101]. The expression of these enzymes could be regarded as a Leydig cell differentiation criterion. The damage to Leydig cell development and function could be one cause of the testis defect.

The fetal Leydig cell differentiation is initiated by HH signaling [102], and the null of HH is disrupted by upregulation failure of the Sf1 and P450 Side Chain Cleavage enzyme (P450 scc) [42]. Inhibition of HH signaling by cyclopamine also led to a significant loss of P450 scc, which suggests that the Leydig cell's function was damaged [103].

Adult Leydig cells provided a more complex view: Adult Leydig cells were evident in Dhh-null masculinized mice [40], but lost in feminized mice and TF male pseudohermaphroditism rat [104]. Furthermore, adult Leydig cells failed to develop in the Sf1+/−; Dhh null mice gonad but were normal in Sf1+/−; Dhh+/− male mice. This research indicates that HH signaling was indispensable for fetal and adult Leydig cell development, while Sf1 expression dose is decisive for adult Leydig cells.

Activation of HH signaling increased the proliferation and differentiation of Leydig cells [83,105], upregulated Sf1 expression, and induced ectopic fetal Leydig cells developed in the ovary [86]. The differentiation of progenitors of Sf1+/3βHSD- steroidogenic cells into Sf+ or Sf−/3βHSD- cells was also upregulated in Smo consecutive expressed mice [87]. By contrast, inhibition of HH signaling decreased Leydig cell number, especially the fetal: cyclopamine reduced the number of steroidogenic Leydig cells [35], abolished the appearance of fetal Leydig cells but not its maintenance, and this effect was not mediated by Gli1/2 [80]. Loss-of-function mutation of Gli3 decreased the transcription level of Sf1 and a number of differentiated fetal Leydig cells [82], which indicates the Leydig cell differentiation-induced function is executed by Gli3 and mediated by Sf1.

To sum up, HH secreted by Sertoli cells and combined with Leydig cell progenitors to induce the expression of P450scc, leads to fetal Leydig cell differentiation by Gli3's transcription activation, promise the generation of adult Leydig cells with enough dose of Sf1. The HH signaling functions in the testis development are shown in Fig. 2.

HH ligands secreted by Sertoli cells combine with the receptor on target cells: Leydig cells and germ cells to initiate the differentiation of fetal Leydig cell progenitors and make sure the production of androgens, guarantee the differentiation of adult Leydig cell progenitors with enough expression of steroidogenic factor 1 (Sf1) and the testosterone production in adult Leydig cells. Germ stem cells keep the normal differentiation and proliferation by signals from HH and enough androgens. HH also makes sure these cells produce enough laminin to keep the structure of the testis (figure not shown). After all, HH keeps the cell types and functions developing and the testis structure forming normally during the development of the testes. The figure was drawn using Adube Illustrater 2024 software.

## Hormones Interact with HH Signaling in Testis Development

*Regulation of HH signaling changes steroidogenic enzyme expression*

Some research proves the combination of Glis and steroidogenic enzymes gene promoters straight. Cholesterol conversion to progesterone and estradiol could be triggered by HH signaling by inducing the Gli3-controlled P450 scc and Gli2-controlled 3β-HSD1 and aromatase by combining with their promoters in human trophoblasts [43], and the mutation of Gli3 also decreased Sf1 transcription level and testosterone production [82]. Gli2 interacts with RNA polymerase II and enhances the binding to the 11β-HSD2 gene's promoter in human trophoblast-like BeWo cells [106].

Besides the straight combination of Gli with promoters, the disturbance of HH signaling has a much deeper influence on hormone levels than we thought in mammalian

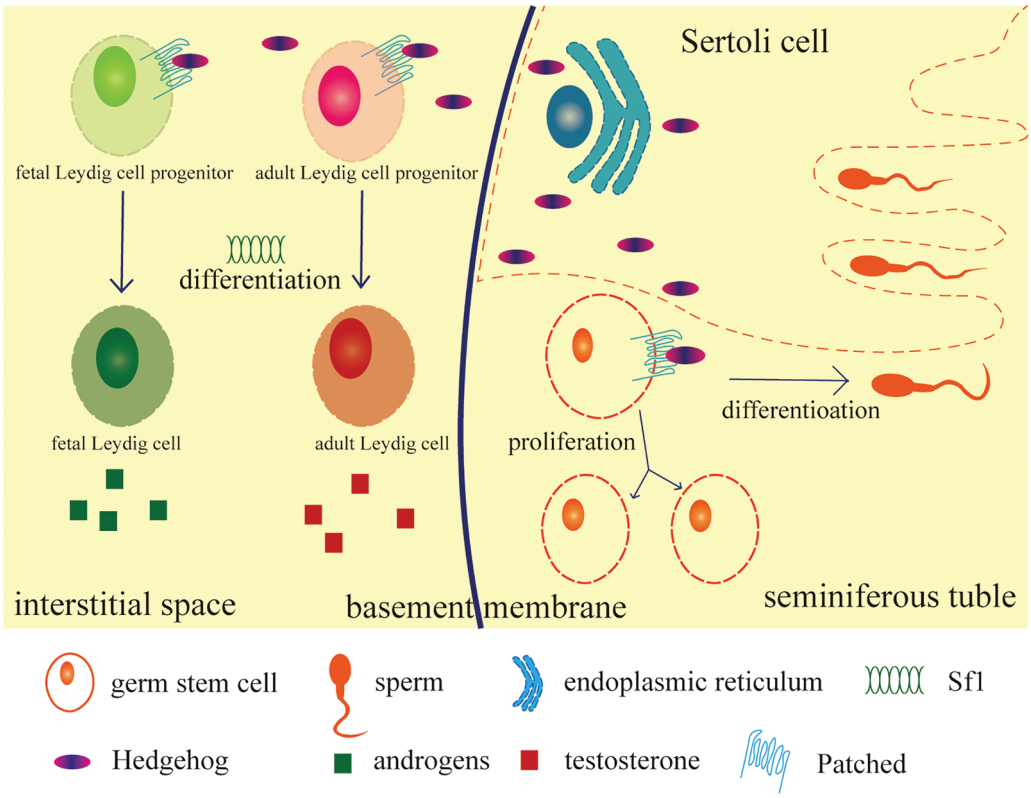


**FIGURE 2.** HH signaling function in testis.

animals and cells. Agonist of Smo treatment on prostate stromal cells upregulated the transcription of steroidogenesis enzymes, including Sf-1, SREBP (sterol regulatory element binding protein), CYP17A1 (a p450 cytochrome oxidase) RDH5 (retinol dehydrogenase 5) [107], and some other genes' expression involved in lipid metabolism and steroid biosynthesis [108]. SAG treatment can also partially rescue steroidogenic genes' expression in Wt1-deficient mice testes whose steroidogenic enzyme expression was decreased [109]. Cyclopamine treatment of *in vitro* cultured testis suppressed P450 scc and Hsd3β1 expression and secretion of testosterone [81]. The suppressed secretion of testosterone and impairment of P450 scc and other steroidogenic enzyme expressions would defect fetal Leydig cell differentiation and function, and that could be the reason why the researchers found adult but not fetal Leydig cells in HH-null masculinized mice which have been mentioned in the second part.

Deletion of Ptc resulted in significantly elevated ACTH (corticotrophin) release GH (growth hormone), and Prl (prolactin) expression in mice [110]. Activation of HH signaling by Shh treatment can increase GH and prolactin secretion in mice, even activate bone marrow stromal cells to steroidogenesis [111] and upregulate Sf1 expression and production of androgens in female mice which caused pseudohermaphroditism [86].

Loss-of-function mutation on Dhh in TF rats led to lower testosterone levels [104]. In Sf1+/−; Dhh+/− male mice, the Amh (anti-mullerian hormone), gonadal testosterone, and P450 scc were absent [85,94], Amh could also be reduced by cyclopamine treatment in a hanging drop gonad culture system [94].

These results indicate that HH signaling controls several enzyme's expressions which function in hormone production, then control several hormone levels and impact the health and development of individuals.

*Hormone treatment leads to changes in HH signaling members*
The influence between HH signaling and some hormones is bidirectional.

Exposure to estrogen receptor (ER) or its agonists downregulates HH genes predominantly via an ERα-dependent pathway in the prostate gland of male rats [112]. In contrast, agonists or antagonists of Erβ treatment, respectively, increased or blocked the proliferation in the NCI-H295A cell line (the human adrenal cell line) [113]. What's more, diethylstilbestrol (DES, estrogen compounds) and estrogen treatment increase expression of HH and HH signaling in mice experiment and rat SAH (subarachnoid haemorrhage) model [114,115]. Research about MLO-Y4 mouse osteocyte-like cells indicates that estrogen could influence the elongation of cilia to affect the activation of HH signaling [116].

There are also some other hormones that could regulate the HH signaling. FSH (follicle-stimulating hormone) is relevant to Dhh signaling [117], and leptin-induced Dhh signaling during human Leydig stem cell differentiation [105]. A high dosage of glucocorticoid treatment on NIH/3T3 cells (mouse fibroblast cell line) accumulated Smo in the primary cilia, conferring hypersensitivity to HH stimulation [118].

Androgens also suppress HH expression and its autocrine and paracrine activity in a dose-dependent manner [119], deprivation of androgen could rescue the Shh

and Dhh expression time-dependently in LNCaP (prostate cancer cell line) cells [120,121] and HH signaling was activated in androgen-independent prostate cancer cells [122]. The antagonist of AR (androgen receptor) flutamide treatment on rat fetalis *in utero* significantly decreased the mRNA level of Dhh and Ptc1 [81]. The mutation of Gli2 decreased the responsiveness of androgens in mice, but Gli3 could make some compensation in the process [123]. We summarized the hormones that influence or are influenced by HH signaling in Table 2. This evidence all showed a phenomenon that changed with different hormones, especially sex hormones, will influence the HH signaling members' transcription or protein function, and there seems to exist some relationship between the base of HH and hormone signaling that we want to explore further in the next section.

*HH signaling members interact with hormones*

As mentioned in this review, HH signaling has a deep relation with hormones, especially sex hormones. After years of

**TABLE 2**

**The hormones related to HH signaling**

| Hormones | Relationship with HH signaling | Published paper | Animals or cells |
|---|---|---|---|
| follicle-stimulating hormone (FSH) | Down regulate Dhh mRNA level | Mäkelä et al. [90] | Rats |
| Luteinizing hormone (LH) | Null of Dhh increased LH level | Clark et al. [40] | Mice |
| Anti-mullerian hormone (Amh) | Knock out of Dhh abolished Amh | Park et al. [85] | Mice |
| | Inhibitor of Smo treatment decreased Amh mRNA level | Szczepny [94] | Mice |
| Testosterone (T) | Knock out of Dh abolished T | Park et al. [85] | Mice |
| | Shh activated cells to produce T | Lubik et al. [107] | LNCaP |
| | Hh regulate the production of T positively | Levina et al. [108], Chen et al [109], Brokken et al. [81] | prostate stromal cells (PrSCs), mice |
| | | | Rats |
| Insulin-like 3 (INSL3) | Mis-activation of HH led to INSL3 mis-expression | Barsoum et al. [86] | Mice |
| | Gli3 lost of function lead to insufficient secretion of INSL3 | Kothandapani et al. [82] | Mice |
| | Suppression of HH signaling decreased secretion of INSL3 | Brokken et al. [81] | Rats |
| Insulin like growth factor 1 (IGF-1) | IGF-1 level decreased in Gli2 mutated individuals | Flemming et al. [124] | Human |
| Androgen and the receptor | Mis-expression of HH lead to mis-production of androgen | Kroft et al. [86] | Mice |
| | Gli3' lose-of function mutation leads to androgen secretion insufficient | Kothandapani et al. [82] | Mice |
| | Shh activates AR by combination with it | Miyagawa et al. [122] | LNCaP |
| | Androgen regulates HH protein level negatively | Chen et al. [119] | LNCaP |
| | Androgen regulate Gli activity positively | Li et al. [125] | LNCaP |
| | Suppression of HH signaling downregulates expression of AR target genes | | LNCaP |
| | Suppression of AR decrease Dhh and Ptc1 mRNA level | Lubik et al. [107], Sirab et al. [126], Brokken et al. [81] | Rats |
| | Suppression of AR increase HH signaling target genes expression | Sirab et al. [126] | LNCaP |
| Leptin | Leptin regulates Dhh signaling and influence T concentration | Arora et al. [105] | Human cells from patients |
| Progesterone | HH signaling regulates the expression of lipid metabolism-related enzymes and the production of progesterone | Tang et al. [43] | human choriocarcinoma cells (JEG-3 cells) |
| Estradiol | HH signaling regulates the expression of lipid metabolism-related enzymes and the production of estradiol | Tang et al. [43] | JEG-3 cells |
| Glucocorticoids | Glucocorticoids accumulate Smo protein on primary cilia and increase sensitivity of HH signaling | Wang et al. [118] | NIH/3T3 cells |

(Continued)

**Table 2 (continued)**

| Hormones | Relationship with HH signaling | Published paper | Animals or cells |
|---|---|---|---|
| Estrogen and the receptor | Shh interact with ERα and upregulate its expression | Sabol et al. [127] | breast cancer cell lines (MCF-7 and SkBr-3) |
| | Activation of ERα down regulates expression of HH signaling members | Katayama et al. [128] | Rats |
| | Estrogen suppresses the expression level of HH signaling member | Pu et al. [112] | Rats |
| | Activity of ERβ regulates Shh and Gli1 protein level | Medwid et al. [113] | NCI-H295A cells |
| Growth hormone (GH) | Shh treatment increase GH secretion | Vila et al. [129] | Human |
| | Gli2 mutated individual had low GH level | Flemming et al. [124] | Human |
| Prolactin | Shh increase the secretion of prolactin | Vila et al. [129] | Human |
| Gonadotropin | Gonadotropin level of Gli2-mutated individual was lower | Flemming et al. [124] | Human |
| Corticotropin-releasing hormone (CRH) | CRH upregulate HH signaling target genes, increase the secretion of ACTH with Shh | Vila et al. [130] | Human cells |

research, it was indicated that members of HH signaling could interact with AR and some other hormones to impact hormone target genes and HH signaling.

For AR, the evidence is sufficient and convincing. The RNA sequencing analysis of androgen receptor axis inhibitors (ARPI) resistance patients emphasized the role of HH signaling primarily [131]. Other research in androgen-independent LNCaP (LNCaP-AI) cells showed Shh-activated AR (androgen receptor), and the impact could be caused by the binding of Shh-N-cholesterol through molecular dynamic simulation [122], this work primarily demonstrated the combination of HH protein and AR.

However, more research pointed to the interaction between Glis and AR. Knockdown of Smo attenuated AR signaling while overexpression of Gli1 partially reversed this inhibition in LNCaP cell culture [132]. Loss of AR elevated Shh-signaling activation in prostatic stromal cells [133]. It seems that Gli activity was needed for AR transcription activity under androgen-depleted conditions, this need will occupy Gli proteins and lead to a decrease in HH signaling activity.

The presence of dihydrotestosterone (DHT) or bicalutamide, an AR antagonist, affected the HH pathway target gene expression in different combinational treatments [126], androgen-activated AR significantly upregulated Glis transcriptional activity and the target genes expression by binding of AR to Gli2/3 [125,134], which means AR activity also impacted HH signaling target genes significantly. Counting on this research, we may find out a law: Glis combined with AR, activation of anyone will promote the other's activity but AR's promotion without androgen will occupy Glis and lead to a decrease of HH signaling. The three states in this hypothesis are shown in Fig. 3.

A: HH protein combines with and activates AR, and the complex translocates into the nucleus to start AR target gene expression; B: The combination of HH protein and patched led to the degeneration and permitted the activation of Gli proteins by Smoothen and primary cilia. Activated Gli combines with and activates AR, they are translocated into the nucleus to initiate AR target genes expression; C: Androgen activated AR, activated AR combines with and activates Gli, then the complex translocates into the nucleus to initiate Gli target genes expression. The figure was drawn using Adube Illustrater 2024 software.

The relationship among HH, Gli proteins, and hormones can explain what we have mentioned in the section 'Disturbance on HH signaling influences survival and differentiation of germ cells': primordial germ cell development was decreased in Gli1-knock down mice but is not affected in HH-null mice. The knockdown of Gli1 decreased the reaction of Gli proteins and hormones and led to a decrease of primordial germ cells [95], but the null of HH remained the reaction between Glis and hormones, which supports the hormones signaling and the production of primordial germ cells were not affected.

The proofs about ER or other hormones were not much but suggestable: The result of immunofluorescent and co-immunoprecipitation indicated the interaction of Shh and ERα protein in the MCF-7 cell line [127]. A computational study suggested the binding affinity of progesterone receptor and mature form of Shh and even more tendency for ERα to bind with the mature form of Shh than estradiol [135]. CRH increased Gli-dependent gene expression with Gli reporter plasmid, and the knockdown of Gli1 abolished the transcription stimulatory effect of CRH on POMC (proopiomelanocortin) [130], which suggested that Gli mediated CRH signaling.

## Clinical Cases of HH Signaling Mutated Gonad

The mutation of HH signaling caused some diseases in patients, and most diseases were related to hormone production. These cases were divided into Shh and Dhh mutation and they were connected to pituitary function and gonad development, respectively.

A female patient with Gli2 transcription activity lost mutation, her GH, insulin-like growth factor I (IGF-I), thyroxine (T4), and gonadotropin were quite lower than the

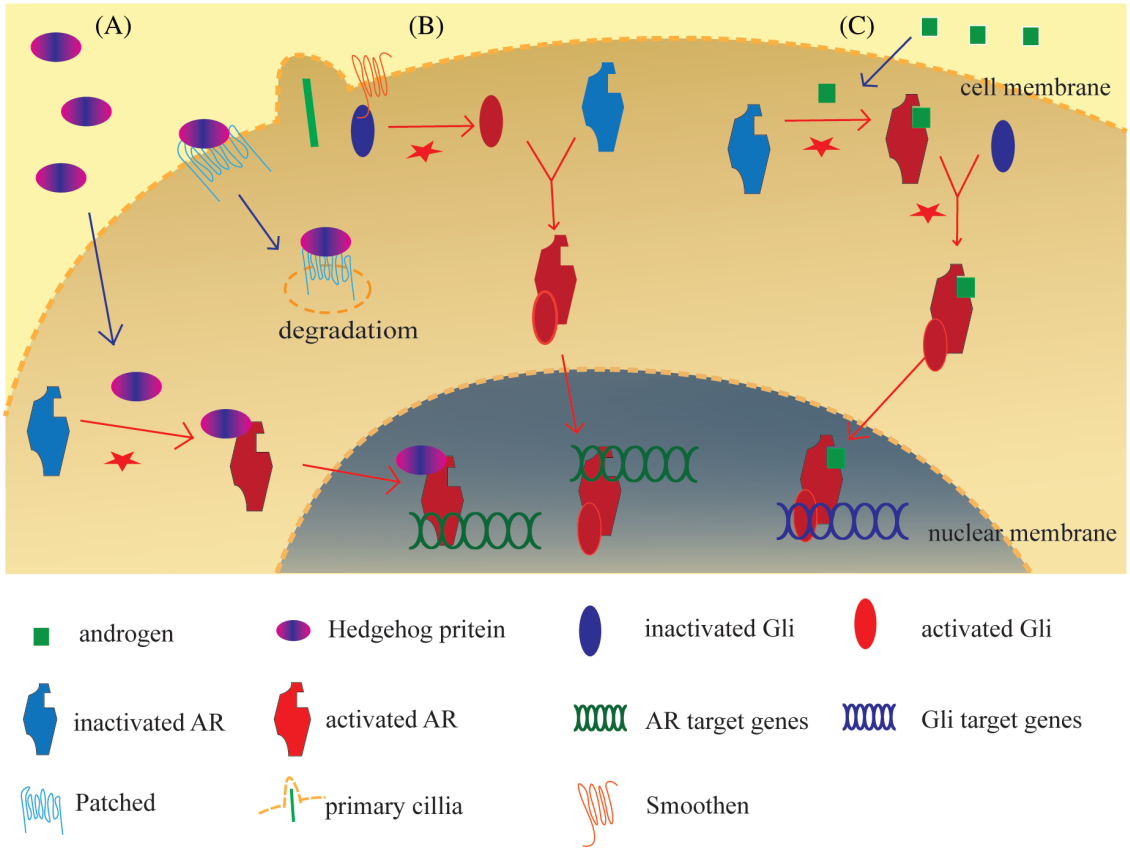


**FIGURE 3.** Three states of relation between HH signaling and androgen receptor (AR).

normal level. The GH replacement therapy helped to normalize her growth, but after the cessation, GH and IGF-I serum concentrations returned to subnormal values [124]. Research on some partial gonadal dysgenesis (PGD) and combined pituitary hormone deficiency (CPHD) patients revealed the mutation of Shh and HH interacting protein (HHIP) [136], which means HH signaling may participate in pituitary development and its hormone production.

SRY mutation causes gonadal dysgenesis in most cases [137], but in some of the other cases, mutation of Dhh may take charge. The mutations of Dhh were reported in some gonadal dysgenesis (GD) cases [138]. In some cases, the patients grew up as female but suffered from a defect of breast development, they were then found with 46, XY karyotype and homozygous mutation on the Dhh gene. The premature termination or loss of self-cleavage of Dhh protein or perturbation of the interaction of Dhh with its binding partners may contribute to GD [139], and in the other case, the patient got estrogen replacement therapy to rescue breast development [140]. The rescue of breast development was successful but had serious side effects: more obvious peripheral neuropathy, obesity, insulin resistance, fatty liver, and gastric ulcers.

One pure gonadal dysgenesis (PGD) patient with a homozygous missense mutation of Dhh was found the same heterozygous mutation in his father [141]. Two siblings with disorders of sex development (DSD) were found to compound heterozygous mutations of Dhh which were inherited from their parents [142]. The two reports suggested the risk that mutations of Dhh may be inherited and lead to the disease of offspring. Another PGD case was where a Dhh mutation altered a conserved residue among HH genes. It was found *in situ* seminoma and loss of Leydig cells in the peritubular, the patients with this mutation also suffered from polyneuropathy and change of peripheral structure [143]. HH homozygous mutation was also found in some individuals of PGD patients [144]. For these patients, the treatment after gonad development could not reverse the sex character but accumulated more hormone problems [124], and the hormone treatment couldn't save the defect during external genital development at fetal time in a mice experiment but the additional DHT rescued steroidogenic function of Leydig cells to some extent [82].

HH signaling is so important for a human's physical development, but gonadal dysgenesis patients with the mutation of HH signaling are not easy to find at their very young age before the gonad mature age. However, it is the premature stage for the patients to obtain medical aid before the gonad fails to develop since hormone therapy in adult patients has so many side effects and can't rescue the gonadal development. So the cure of gonadal dysgenesis patients should concentrate on the younger stage and still wait for further exploration.

## Conclusion and Perspectives

Since the discovery of HH signaling, more research concentrated on Shh and Ihh, and they also made some

progress in some diseases. The usage of Metformin could improve bronchopulmonary dysplasia through the regulation of Shh [145], the increase and over-expression of Shh could protect the development of embryonic spinal cord neocortical, neural stem cell number and expansion of the neocortex [146,147]. Some reports on Ihh uncovered the important role of micro RNAs in the function of this signaling by targeting Ihh or the signaling [148–150]. After a dozen years of research on Dhh, the signaling function of male gonads and hormones also gradually dis-enveloped. Dhh is produced in Sertoli cells in the testes and targets germ cell and Leydig cells to ensure germ cell and Leydig cell differentiation, androgen production, and integrity of testes structure during embryonic development.

HH signaling could be used to regulate cells and organoid states in experiments on testis [151]. The interaction of HH members and hormones reminds us of the possibility that hormone therapy for prostate cancers or other diseases may not be sufficient and HH signaling should be considered. The mutation of HH signaling members in gonadal dysgenesis provides a better view of this kind of disease and how to make better therapy plans to fight for the health of patients. However, research on HH signaling mostly concentrated on vertebrate animals and the difference with invertebrates was largely ignored. How this signaling evolved in the animal kingdom may support us with a better view of evolution.

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