

The *SOX* gene family: function and regulation in testis determination and male fertility maintenance

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Abstract The *Sox* (*Sry*-type HMG box) genes encode a group of proteins characterized by the existence of an SRY (sex-determining region on Y chromosome) box, a 79 amino acid motif that encodes an HMG (high mobility group) domain which can bind and bend DNA, which is the only part in SRY that is conserved between species. The *Sox* gene family functions in many aspects in embryogenesis, including testis development, CNS neurogenesis, oligodendrocyte development, chondrogenesis, neural crest cell development and other respects. The *Sox* gene family was originally identified through homology with *Sry*. The *Sry* gene is the mammalian testis-determining gene. It functions to open the testis determination pathway directly and close the ovary pathway indirectly. *Sry* and *Sox9* are the most important two genes expressed during testis determination. Besides, researchers have found that *Sox8* and *Sox9* have functions in the male fertility maintenance after birth. In this review, information was evaluated from mouse or from human if not mentioned otherwise.

Keywords *SOX* · *SRY* · *SOX8* · *SOX9* ·
Testis determination · Male fertility maintenance

Abbreviations

Dax1 DSS(dosage-sensitive sex reversal)-CAH
(congenital adrenal hypoplasia) critical
region on the X chromosome protein
Fgf9 Fibroblast growth factor 9

Wnt4 Wingless-related MMTV integration site 4
Wt1 Wilms' tumor suppressor gene 1
AMH/MIS Anti-Müllerian ducts hormone/Müllerian
inhibiting substance
Sf1 The steroidogenic factor 1

Introduction

Sex development

The mammalian gonad (testis in male and ovary in female) begins development as an undifferentiated, bipotential anlage known as the genital ridge. This contains four cell lineages that comprise the gonad: the germ cells, connective tissue cells, steroid-producing cells (Leydig cells in the testis and theca cells in the ovary), and supporting cells (Sertoli cells in the testis and granulosa cells in the ovary) [1]. The genital ridges (gonadal primordia) are a pair of thickened rows of coelomic epithelial cells on either side of the midline in the trunk of an embryo that are the precursors of the gonads. They develop at about 7 weeks embryonic age in human, programmed to follow the default pathway to develop as an ovary, but if the Y chromosome is present, it will develop as a testis due to the action of the testis-determining gene on the Y chromosome [2].

TDF/Tdy & SRY/Sry

In 1959, in the study of two human disorders of sex development, researchers were sure that the Y chromosome carries a gene that determines maleness, this gene has been named *TDF* (testis-determining factor) in humans and *Tdy*

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(testis-determining Y chromosome) in mice [3]. During the development of the testis, cell-autonomous activity of *Tdy/TDF* triggers the Sertoli cell differentiation, and subsequent steps in testis differentiation may be a consequence of Sertoli cell activity [4].

It took about 30 years for researchers to determine the exact *Tdy* and *TDF*. In 1990, the *SRY* gene in human and *Sry* gene in mice had been confirmed as the testis-determining gene on the Y chromosome, respectively [5–9]. *Sry* is short for a sex determining region on the Y chromosome, which is specific for mammals, with it, the genital ridge will develop into testis and later steps into male differentiation, and to the contrary, the genital ridge will enter the female pathway.

Male determination

Because the genital ridge follows the default pathway to develop as an ovary, the central event in mammalian sex determination is the differentiation of testis from the genital ridge, rather than developing ovary. All other differences between the sexes in eutherian mammals are later effects due to hormones or factors produced by differentiated gonads, and for this reason, sex determination is equivalent to testis determination [9].

Male determination is initiated by activation of a Y-chromosome-located gene *Sry* within somatic cells of the genital ridge [5, 6, 9], and this pathway is regulated by many other factors so as to form a regulated framework at last.

SOX gene family

Those that encode proteins with more than 60 % similarity to the SRY HMG box region have been termed as *Sox* (*Sry*-type HMG box) genes [10]. This gene family is highly conserved across evolution (except for *Sry*), they were originally identified through homology as they contain an HMG box closely related to that of SRY [11–13].

The *Sox* family has at least 20 members, and has been divided into nine groups, among them, genes that function mostly in sex development are *Sry* in group A, *Sox8* and *Sox9* in group E [12, 14–16] (Table 1).

Functions of the SOX family

Functions of the SOX family in male development

The gonad is composed of cells derived from four lineages: the supporting cells, steroid-producing cells, connective tissue cells and germ cells. The *Sry* gene triggers the testis-determining pathway by inducing the embryonic somatic

cells that differentiate into Sertoli cells rather than granulosa cells, and the differentiation of Sertoli cells from the supporting cell lineage is thought to result in the differentiation of Leydig cells from the steroid-producing cell lineage, the induction of mitotic arrest in the germ cells and the proliferation and organization of connective tissue and blood vessels into the testicular pattern, this means that once Sertoli cells are determined, the male gonad testis is determined [1, 5, 6, 9, 17].

The procedure of male development can be divided into two stages: sex determination stage and testis differentiation stage.

Sex determination

This stage uses the differentiation of Sertoli cells as a symbol and *Sry* plays a significant role in this stage both in human and mouse.

A number of genes have been identified as potential regulators of *SRY* expression. These include several genes identified by mutation analysis in mice that are required for the early formation of bipotential genital ridges: *Emx2* [18], *Lhx9* [19], *Sfi* [20], *Gata4* [21] and *Wt1* [22, 23].

The *Sry* gene varies between species, it can act as a transcriptional factor to bind and bend genes “downstream” in the testis development pathway [5, 6, 17, 24–28], although HMG box is its functional domain, full-length SRY protein is essential for its DNA binding [29]. *Sry* is expressed in gonadal somatic cells and not in other lineages of the genital ridge, because Sertoli cells are the only somatic cells within the testis cords, so *Sry* is expressed in pre-Sertoli cells [1, 30, 31]. *Sry* acts as a switch to initiate the male pathway in a very short window of time at about 10.5–12.5 dpc (days post coitum, reaches a peak at 11.5 dpc) in the mouse XY gonad [1, 9, 24, 31, 32] and more exactly, the critical time window of *Sry* action required to induce testis formation is limited to approximately the first 6 h after the onset of endogenous *Sry* expression, that is, the period from 12 to 15 ts (approximately 11.0–11.25 dpc) (tail somites:ts) [33]. *SRY* expression commences in the gonadal ridge of 46, XY human embryos between 41 and 44 d.p.o. (days post-ovulation)/CS17-18 (Carnegie stage), the peak *SRY* expression is detected at 44 d.p.o./CS18, when sex cords are first visible, thereby defining testicular determination [34]. Delayed *Sry* expression does not induce early testis-specific cellular events that are required for Sertoli cell establishment and subsequent testis cord formation. On the contrary, this condition will tip the balance between FGF9 and WNT4 and switches genital ridge differentiation in the female pathway [33].

Sry's main and only function is to act as a molecular switch to activate the evolutionarily more conserved *Sox9*,

Table 1 Information of *Sox* family genes which are expressed in testis

Gene	Orthologue	Function	Article source
Group A	<i>Sry</i>	Human; Rodent; Marsupial	Testis determination [5–8]
Group D	<i>Sox5</i>	Human; Mouse	Highly expressed during spermatogenesis, detailed function remains unknown. [11]
	<i>Sox6/Sox LZ</i>	Human; Mouse	Highly expressed during spermatogenesis, encodes a leucine zipper-Containing protein, detailed function remains unknown. [30, 56]
Group E	<i>Sox8</i>	Human; Mouse	(1) Reinforcing <i>Sox9</i> function in testis differentiation of mice. (2) Substituting for <i>Sox9</i> where <i>Sox9</i> is either not expressed or expressed too late to be involved in sex determination or regulation of <i>Amh</i> expression; (3) To be a critical regulator of adult Sertoli cell function; (4) To be critical for the maintenance of adult male fertility. [2, 44, 57, 58]
	<i>Sox9</i>	Human; Mouse; Chicken	(1) <i>Sox9</i> is involved in Sertoli cell differentiation, the activation of <i>Mis</i> and <i>Sox8</i> , and the inactivation of <i>Sry</i> . (2) Conditional <i>Sox9</i> null mutants show normal embryonic testis development and are initially fertile, but become sterile from dysfunctional spermatogenesis at about 5 months. [2, 43]
Group F	<i>Sox17</i>	Mouse	(1) The mouse <i>Sox17</i> gene has two different mRNA isoforms which are termed as <i>Sox17</i> and <i>t-Sox17</i> . [59] (2) Form <i>Sox17</i> is expressed in spermatogonia, and the expression clearly declines from the early pachytene spermatocyte stage onward. Form <i>t-Sox17</i> expresses at the pachytene spermatocyte stage and was highly accumulated in round spermatids. (3) <i>Sox17</i> may function as a transcriptional activator in the premeiotic germ cells, and that a splicing switch into <i>t-Sox17</i> may lead to the loss of its function in the postmeiotic germ cells.

which in turn initiates the male differentiation program [3, 5, 6, 31, 35]. How can we obtain these results from the experiment?

Sry was identified as the only gene on the Y chromosome required to initiate male development because of two sets of experiments: (1) Mice or humans carrying deletions or point mutations in the HMG domain of the *Sry/SRY* gene show a autosomal XY female sex-reversed phenotype [5–7] and (2) *Sry* gene induced male sex reversal when it was transferred into XX mice. However, gene transfer of the human *SRY* gene was unable to induce the development of the male phenotype in XX female mouse embryos because the sequence and structure differences between human and mouse *SRY/Sry* [9, 17, 36]. More details about how *SRY* regulates *Sox9* and the specific functions of *Sox9* will be detailed in the following section.

Testis differentiation

This stage uses the differentiation of Sertoli cells as a start and the *Sox9* (SRY-box containing gene 9) functioning at this stage.

SOX9 promotes Sertoli cell differentiation from bipotential supporting cell precursors. At the same time, to ensure that Sertoli cells differentiate in sufficient numbers to induce normal testis development, the early testis produces prostaglandin D₂ (PGD₂, which recruits cells of the supporting cell lineage to a Sertoli cell fate), and the gene encoding prostaglandin D synthase (*Pgds*, the enzyme that produces PGD₂) is regulated by *Sox9* expression [37].

Haploinsufficiency of the *Sox9* gene in humans causes campomelic dysplasia (CD), an autosomal dominant disease of bone dysmorphology with approximately 75 % of XY patients also showing male-to-female sex reversal [30, 38], mutation analyses of patients with CD indicate that *Sox9* is involved in both skeletal development and sex determination [24]. Homozygous loss of *Sox9* in XY leads to ovary development and *Sox9*-overexpression in XX mice leads to testis development [2, 35, 39, 40].

Sox9 expresses in the supporting cell lineage, and *Sox9*-positive pre-Sertoli cells differentiate into Sertoli cells. *SOX9* is present in the cell cytoplasmic compartment in male and female gonads before gonads differentiate, but later its expression becomes restricted to the nuclei of

Sertoli cells while it remains cytosolic in a female embryo of the corresponding stage [30, 41].

Sox9 is expressed at high levels in Sertoli cells, and at lower levels in the germ cells, it is likely to be required for Sertoli cell differentiation, rather than proliferation [2]. High level expression of *Sox9* throughout the male other than the female genital ridge commences between 10.5 and 11.5 dpc: (1) At 10.5 dpc, before overt sexual differentiation, *Sox9* expression was limited to a faint, diffuse band on the lateral side of the genital ridge in both sexes. (2) At 11.5 dpc, *Sox9* expression differed strikingly between males and females, with a strong staining seen in male genital ridges [31]. (3) At 12.5–13.5 dpc, *Sox9* expression became localised to the sex cords in the testis, which at this stage consist of Sertoli and germ cells. (4) At 12.5 dpc both the Müllerian duct in female and the mesonephric duct in male express *Sox9*, by 13.5 dpc, SOX9 staining was retained only in the male and extended to the mesenchymal cells surrounding the Müllerian duct. (5) From 13.5 dpc until the duct has regressed, *Sox9* is expressed by the cells surrounding the Müllerian duct, a domain in which the AMH/MIS receptor is also expressed, this indicates that SOX9 is required for expression of the AMH/MIS [2, 35, 42].

Expression of *Sox9* is strongly upregulated in the male gonad, while it is down-regulated in the female at the same time, coinciding a lot with the expression time window of *Sry* [31, 39, 42]. In later studies, *Sox9* was confirmed to be the only target of SRY in the sex developing pathway so far [31]. And because the relationship between SRY and SOX9 is dose dependent [24, 39], the expression amount of SRY per cell and the number of cells expressing the gene determining the extent of testis differentiation.

However, although *Sox9* is the only target of SRY that has been identified so far, overexpression of *Sox9* is required and sufficient to induce testis formation and it can substitute for *Sry*'s function [2, 43]. Moreover, high *Sry* expression persists in *Sox9* knock-out mice, indicating that *Sox9* activation leads to the downregulation of this gene [2, 31]. It is obvious that *Sry* may act only as a molecular switch to activate the evolutionarily more conserved *Sox9*, which in turn initiates the male differentiation process and has a dominantly fundamental role in testis determination in vertebrates [2, 3, 35].

Functions of the SOX family in male fertility maintenance

The fact that *Sox8* or *Sox9* mutants individuals which are initially fertile, later develop progressive seminiferous tubule failure and infertility [43] indicate that *Sox8* and *Sox9* function substantially in male fertility maintenance. There is indication of functional redundancy of both *Sox* genes in the maintenance of spermatogenesis [43].

Germ cells (in particular elongated spermatids) are dependent on Sertoli cells for the mechanical force to move them within the depth of the seminiferous epithelium. Each Sertoli cell is capable of forming 4 or 5 different and ever-changing microenvironments around germ cells at the same time [44].

In this background, researchers found that *Sox8*^{-/-} mice exhibited an age-dependent loss of this ability, which by 2 months resulted in signs of spermiation failure and inappropriate germ cell placement, by 5 months of age resulted in sterility and by 9 months of age, to a complete loss of the cycle of the seminiferous epithelium. As a product of adult Sertoli cells, elimination of SOX8 results in an age-dependent deregulation of spermatogenesis, which is characterized by sloughing of spermatocytes and round spermatids, spermiation failure and a progressive disorganization of the spermatogenic cycle, which resulted in the inappropriate placement and juxtaposition of germ cell types within the epithelium, even those sperm that did enter the epididymides displayed abnormal motility [44].

For *Sox9*, conditional null mutant mice showed normal embryonic and early postnatal development and were initially fertile, but became sterile after 5–6 months [43]. All above indicates that *Sox8* and *Sox9* are required for male fertility maintenance.

Although *Sox8* is not critically required for testis specification and development, it is obvious that *Sox8* is critical for the maintenance of adult male fertility beyond the first wave of spermatogenesis, and besides, the loss of *Sox8* resulted in progressive degeneration of the seminiferous epithelium through perturbed physical interactions between Sertoli cells and developing germ cells indicating that it is a regulator of Sertoli-germ cell adhesion [44].

How sex determination is regulated?

The sex determination is a pathway that represses ovary development and stimulates testis differentiation, and the gene *Sry* is a switch that opens a series of genes which pushes the genital ridge differentiating into the testis. And before sex determination, some genes are already existing, such as *Dax1*, *Sox9*, *Fgf9*, and *Wnt4*, they are initially expressed in similar patterns in XX and XY individuals prior to sex determination, waiting to function by establishing sexual dimorphism.

Fgf9 and *Wnt4* function antagonist to determine the fate of gonad and *Sry* breaks their balance

In the mouse, *Fgf9* and *Wnt4* are expressed in gonads of both sexes before sex determination. The fate of the gonad is controlled by an antagonism between *Fgf9* and *Wnt4*

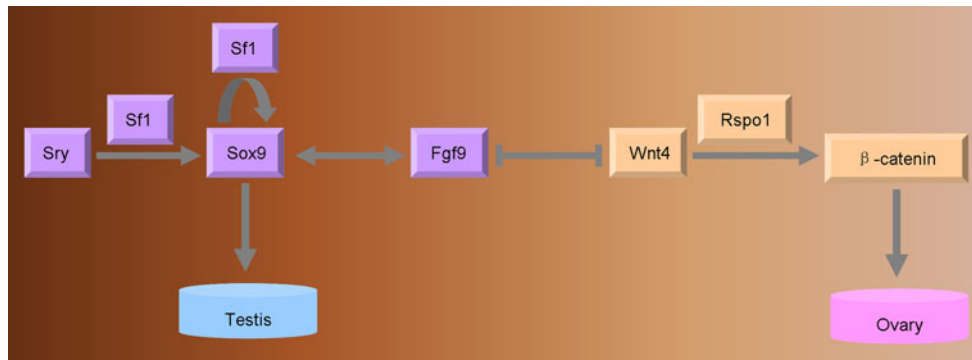


Fig. 1 The fate of the gonad is controlled by antagonism between *Fgf9* and *Wnt4* and *Sry* functions to destroy their balance. In this procedure, *Sry* gene has three functions: (1) breaking the balance between *Fgf9* and *Wnt4*; (2) inhibiting Wnt signaling at the level of β -catenin [51]; (3) initiating a positive feed-forward self-reinforcing loop between *Sox9* and *Fgf9* in XY gonads, to explain in detail is that *Sox9* gene is essential for *Fgf9* expression while *Fgf9* maintains *Sox9*

[33, 45]: loss of *Fgf9* leads to XY sex reversal [45, 46], up-regulation of FGF9 and repression of WNT4 leads to the testis pathway; loss of *Wnt4* in XX gonads is sufficient to up-regulate FGF9 and SOX9 despite the absence of *Sry*. Overexpression of FGF9 to WNT4 will upregulate the expression of *Sox9* and finally push the bipotential undifferentiated gonad developing into the male pathway. At the same time will *Wnt4* upregulate the expression of β -catenin (a subunit of the cadherin protein complex and has been implicated as an integral component in the Wnt signaling pathway) together with *Rspo1* (disruption of this gene can lead to complete female-to-male sex reversal in the absence of *Sry* [47]) to open the female pathway.

From the above, we can see that the main role of the *Sry* gene is to tip the balance between FGF9 and WNT4, and promote the development of testis. *Sry* normally initiates a positive feed-forward self-reinforcing loop between *Sox9* and *Fgf9* in XY gonads [48–50]. Besides, FGF9 is necessary for the down-regulation of WNT4 in differentiating XY gonads at or after bipotential stages [45]. However, in this pathway, *Sry* has another function, it inhibits Wnt signaling at the level of β -catenin in order to promote the male pathway in the pattern of blocking the female pathway [51] (Fig. 1).

Dax1 competes with *Sry* to destroy the male pathway

Dax1 is an orphan nuclear receptor localized at chromosome Xp21. In the mouse, *Dax1* is first expressed in the somatic component of the genital ridge at 10.5–11 dpc and peaks at around 12 dpc. In males, *Dax1* begins to be expressed and peaks at the same time as *Sry*, but the levels of DAX1 decreases dramatically as the testis cords begin to appear, whereas in females, *Dax1* continues to be expressed throughout the gonad after 12.5 dpc. This suggests that

expression, and at the same time, Sf1 and SRY cooperatively upregulate *Sox9* and then, together with Sf1, SOX9 also binds to the enhancer to help maintain its own expression after that of SRY has ceased [48–50]. *Sox9* gene regulate the expression of *Pgds* (prostaglandin D synthase), which produces PGD2 (prostaglandin D2) to recruit a supporting cell lineage to a Sertoli cell fate [37]

Dax1 could be involved in sex determination [52, 53]. Further researches showed that *Dax1* acts antagonistically towards *Sry*, it functions more as an ‘anti-testis gene’, rather than an ovary determinant, this is because *Dax1* competes with *Sry* and cooperates with SF1, which is essential for early gonadal development and AMH expression [48]. DAX1 may act as a co-repressor altering the properties of SF1 so that it no longer activates its target genes, it can also form heterodimers with SF1 to ensure *Sox9*’s repression in ovary development [52]. With the overexpression of *Dax1*, AMH is down-regulated, which results in the repression of ovary (Fig. 2).

The *Wt1* gene is expressed very early during fetal development in both sex indifferent gonads in pre-Sertoli and Sertoli cells. *Wt1* is essential for the maintenance of Sertoli cells and seminiferous tubules in the developing testes. Besides, expression of *Sox9* in mutant Sertoli cells was turned off at embryonic day 14.5 after *Wt1* ablation, suggesting that WT1 regulates *Sox9*, either directly or indirectly, after *Sry* expression ceases [54]. Moreover, WT1 activated and up-regulated the human *SRY* gene and initiated a regulatory gene cascade in the male sex determination and differentiation pathway [22]. Mouse *Sry* can be a target for *Wt1*, at least in vitro [23].

Alternative splicing of exon IX inserts or removes three amino acids (\pm KTS) between zinc fingers III and IV that changes the DNA binding specificity of WT1. WT1 ($-$ KTS) has been proposed to be transcriptional activators of the *Dax1* [23]. Besides, *Dax1* is also an immediate downstream target of WT1 and only the WT1 ($-$ KTS) isoform. WT1 ($-$ KTS) can associate and synergize with SF1 to promote *Amh* gene expression, DAX1 can antagonize this synergy through a direct association with SF1 [53] (Fig. 2).

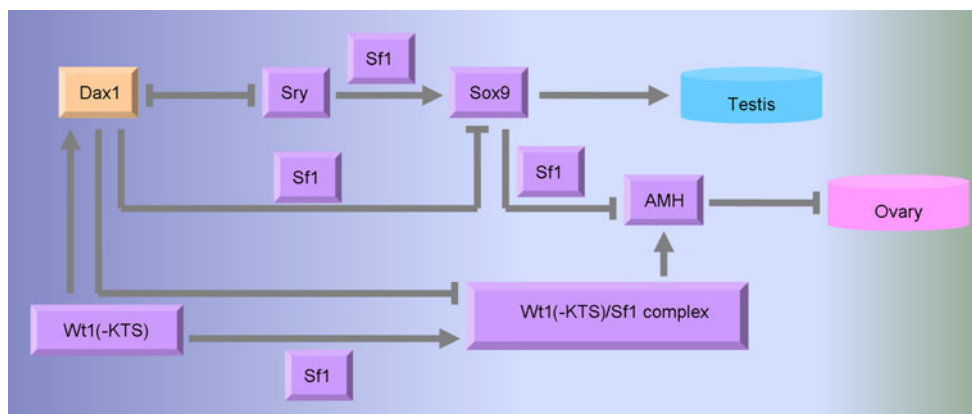


Fig. 2 *Dax1* acts antagonistically to wards *Sry* and the male pathway. By competing with *Sry* and cooperating with *Sf1*, which is essential for early gonadal development and AMH expression [48], *Dax1* achieves the ability to break the testis development. With the overexpression of *Dax1*, AMH is down-regulated, which results in the repression of ovary. Heterodimers of *Dax1* and *Sf1* ensure *Sox9*'s

repression in ovary development [52]. *Wt1* (–KTS) has been proposed to be transcriptional activators of the *Dax1* [23]. Besides, *Dax1* is also an immediate downstream target of *Wt1* and only the *Wt1* (–KTS) isoform. *Wt1* (–KTS) can associate and synergize with *Sf1* to promote AMH gene expression, *Dax1* can antagonize this synergy through a direct association with *Sf1* [53]

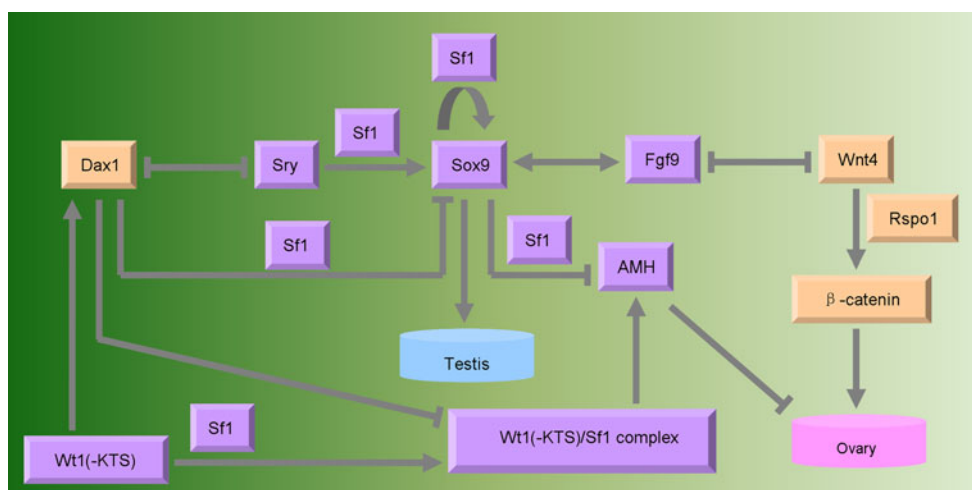


Fig. 3 AMH/MIS plays a significant role in repressing the Müllerian duct differentiation. In the male pathway, two processes occur. Firstly, the Wolffian ducts have to be maintained and stimulated to differentiate into the male tract and accessory organs. Secondly, the Müllerian duct system has to regress, due to the action of AMH/MIS

secreted by Sertoli cells [43, 55]. *Sox9* and *Sf1* are both involved in the expression of the AMH gene, in part as a result of their respective binding to the AMH promoter and in part because of their ability to interact with each other [48]

AMH/MIS plays a significant role in repressing the Müllerian duct differentiation

Shortly after testis development is triggered, Sertoli cells align into visible cord-like structures and begin to express AMH/MIS (anti-Müllerian ducts hormone/Müllerian inhibiting substance), this marks the start of the hormonal cascade required for male sexual differentiation.

Although the undifferentiated gonad is bipotential, the anlagen of the male and female reproductive tracts, the Wolffian and Müllerian ducts respectively, are unipotential, and besides, the survival and development of one versus the other depends on the type of gonad that differentiates. In the

female, the Wolffian duct system degenerates and the Müllerian ducts give rise to the oviducts, uterus and upper vagina, this does not depend on any factors produced by the ovary and is often considered part of the default pathway [41, 55]. In the male, therefore, two processes have to occur. At first is that the Wolffian ducts have to be maintained and stimulated to differentiate into the male tract and accessory organs, the vas deferens, seminal vesicles and epididymides, this is due to the influence of testosterone produced by Leydig cells in the testis [43]. Additionally, the Müllerian duct system becomes reduced due to the action of AMH/MIS secreted by Sertoli cells [43, 55]. *SOX9* and *SF1* are both involved in the expression of the *Amh* gene, in part as a result

of their respective binding to the AMH promoter and in part because of their ability to interact with each other [48] (Fig. 3).

Cytosolic expression of the AMH protein is only observed in Sertoli cells, not in the ovary [41], the primary role of AMH is to inhibit the differentiation of Müllerian ducts and it plays no critical role in testicular determination per se. It is involved in sex differentiation rather than sex determination.

Conclusions and perspectives

The *Sox* gene family in male development functions mainly in two aspects: testis development (which includes testis determination and testis differentiation) and male fertility maintenance.

Sry gene functions in testis determination. Its main role is to break the expression balance of *Fgf9* and *Wnt4* in the genital ridge and push the indifferentiated bipotential gonad development into testis. *Sox9* gene regulates testis differentiation in two ways. It is involved in the activation of AMH/MIS which functions in repressing the ovary pathway and is essential for Sertoli cell differentiation and seminiferous tubule formation. *Sox9* is sufficient to induce testis formation and it can substitute the *Sry* function.

Sox8 and *Sox9* mutant individuals show a phenotype of late-onset sterility in mice, which is fertility at newborn but gradually become completely sterile at about 5-months after birth.

Several factors are involved in the regulation of these processes, forming a well-organized framework, which can guarantee a normal sex development.

However, it still remains unknown whether this framework is the final one. Or, are there any other genes involved? What are the exact molecular mechanisms of *Sry* regulation? What are the precise functions of the non HMG-domain regions of SRY? What is the detailed relationship between *Sox8* and *Sox9* in the male development pathway? Many questions remain to be studied here.

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