Molecular Mechanisms of Sexual Development

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Key Words

 β -Catenin · Gonadal development · Sex determination · SOX9 · SRY

Abstract

Gonadal cellular organization is very similar in all vertebrates, though different processes can trigger bipotential gonads to develop into either testes or ovaries. While mammals and birds, apart from some exceptions, show genetic sex determination (GSD), other animals, like turtles and crocodiles, express temperature-dependent sex determination. In some groups of animals, GSD can also be overridden by hormone or temperature influences, indicating how fragile this system can be. This review aims to explain the fundamental molecular mechanisms involved in mammalian GSD, mainly referring to mouse as a major model. Conceivably, other mammals might show a molecular mechanism different from the commonly investigated murine species.

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Mammalian Sex Determination in 125 Words

Sex in mammalian embryos is genetically determined at fertilization. When eggs are fertilized by sperm carrying an X or Y chromosome, a female and male phenotype develops, respectively. The male pathway is activated by

a genetic factor, called testis-determining factor (TDF), which was identified more than 20 years ago as SRY, a gene carried by the Y chromosome. First, SRY triggers a cascade of molecular events, the SOX9 pathway, inducing the undifferentiated urogenital ridge, a gonadal primordium characterizing all embryos, to differentiate into testis. Successively, some hormonal products from specific testis cells, i.e. anti-Müllerian hormone (AMH) by Sertoli cells and testosterone by Leydig cells, head male phenotype development of the embryo. In the absence of SRY, a specific female molecular cascade, the β -catenin pathway, directs development of ovaries and female phenotype differentiation.

First Steps in Sex Determination

In both male and female murine embryos, the first sign related to sex development occurs at about 10.0 days post coitum (dpc) when a thickening of the epithelium, which gonadal somatic cells will derive from, appears along the coelomic surface of the mesonephros [Ross and Capel, 2005]. It is exactly when the XX and XY gonadal primordia show gonadal-specific gene expression, which is not sexually dimorphic until the activation of the male-specific pathway at 10.5 dpc. Knockout (KO) experiments in mice have determined that some of these genes are responsible for the early proliferation of gonads in both sex-

es before participation of sex-specific genes, as their disruption causes gonad regression in both XX and XY embryos. These genes are: *Emx2*, *Lhx9*, *Cbx2* (*M33*), *SF1* (*Nr5a1*), *Pod1*, and insulin receptors (*Insr*, *Igf1r*, *Insr*) [Luo et al., 1994; Miyamoto et al., 1997; Katoh-Fukui et al., 1998; Birk et al., 2000; Nef et al., 2003; Cui et al., 2004]. KO mice for these genes also show abnormal development of kidney and/or adrenal glands. Namely, the mesonephros, originating the gonad, derives from an intermediate mesoderm structure, the urogenital ridge, which includes pronephros, i.e. adrenal primordium, and metanephros, i.e. kidney primordium. This anatomical structure explains why mutations in gonad development often involve adrenal or kidney anomalies. Another gene, *Wtl*, is also related to early gonadal development in a more sophisticated way. In fact, *Wtl* gives rise to several isoforms, originating by alternative splicing or by different translation start codons. Two among such isoforms are denoted by a very interesting behavior. They are called KTS+ and KTS− as their difference is due to in the presence or absence of a triplet of lysine-threonine-serine (KTS) amino acid residues [Wagner et al., 2003]. *Wtl* KTS− KO mice show gonad regression of both XX and XY genotypes before 12.5 dpc [Kreidberg et al., 1993], while in *Wtl* KTS+ KO mice only XY embryos are affected as they develop a female phenotype [Hammes et al., 2001] as a consequence of absence of *Sry* activation or loss of stability. Thus, the action of *Wtl* can be related to 2 different steps in sex determination. The KTS− variant is involved in the development of the early gonad, ahead of sex-specific pathway activation, while the KTS+ variant is implicated in the male-specific pathway only. At the end of this step, at 10.5 dpc, several cells migrate from the mesonephros to the undifferentiated testis (a key step, together with cell proliferation), and successively they will be stimulated to differentiate into testis- or into ovary-specific cell lines. At that stage, a gonad can be influenced by the *Sry* gene, when present, and develop the typical architecture of vertebrate testis. If *Sry* is absent, another pathway can expand and direct ovary assembly. Thus, from 10.5 dpc only 1 way is possible: the undifferentiated ridge can develop into a testis or into an ovary once and for all.

SRY: The Godfather of Sex Determination

At 10.5 dpc a new actor, a gene discovered 20 years ago [Sinclair et al., 1990], shows up: *Sry* (sex-determining region of Y chromosome). *Sry* has been identified as the testis-determining factor (TDF), the key gene responsible

for testis development in XY embryos. Its implication was clearly proved observing testis development in XX mice transgenic for *Sry* [Koopman et al., 1991]. Furthermore, 15% of XY human embryos developing a female phenotype as a result of testis development failure show mutation in this gene [Berta et al., 1990].

SRY, a chromatin remodeling transcription factor, is characterized by the presence of a DNA-binding domain containing a high-mobility group 1 (HMG) box. The presence of an HMG box, highly related to the SRY HMG box, characterizes a gene family called SOX (SRY-related HMG box). This functional domain is able to bind the (A/T)ACAA(T/A) DNA sequence and consequently to bend DNA with an angle of 60–85° [Harley and Goodfellow, 1994]. This DNA bending property is essential to carry out its biological function. If this function is blocked, the undifferentiated XY gonad fails to develop into a testis [Pontiggia et al., 1994]. The HMG box is the only part of the SRY gene conserved in different mammalian species. It is also the only functional part of this gene, as most SRY mutations leading to failure of testis development reside in this domain. XX mice carrying human SRY under the control of mouse regulatory sequences [Lovell-Badge et al., 2002] or goat SRY with its own regulatory elements [Pannetier et al., 2006] develop into males, indicating that SRY from different species probably acts in a similar way. The importance of the HMG box in promoting the biological function of SRY is confirmed by the fact that replacing the *Sry* HMG box with *Sox3* or *Sox9* boxes causes sex reversal in transgenic XX mice [Bergstorm et al., 2000]. The SRY gene also possesses 2 nuclear localization signals (NLSs) placed at the N- and C-terminus of the HMG box domain, respectively. They are responsible for protein translocation into the nucleus, a key step in SRY function. Both NLSs are important, and they are not functionally redundant: a mutation involving just one of them is able to lead to testis development failure [Sim et al., 2005; Kaur and Jans, 2011]. These 2 NLSs recruit at least 2 proteins involved in SRY protein migration into the nucleus: calmodulin (N-NLS), whose action mode is still unknown, and importin-β (C-NLS), which facilitates transport through nuclear pore complexes [Harley et al., 1996; Forwood et al., 2001]. SRY also undergoes post-translational modifications; in particular, lysine acetylation and phosphorylation of 2 serines are the most relevant. Lysine (K136) acetylation promotes importin-β recruitment, whereas a 2-serine (S31, S33) modification increases the capacity to bind DNA and, consequently, transcription activity [Desclozeaux et al., 1998; Thevenet et al., 2004]. Finally, sev-

eral in vitro experiments show that SRY could interact with other proteins (KRABO, SIP1, WT1, PARP1). The significance as well as the real in vivo existence of these interactions are still unclear, though. SRY triggers testicular tissue differentiation from the undifferentiated gonad, and its expression is very peculiar in terms of time and space. In fact, *Sry* expression begins at 10.5 dpc in somatic cells of XY genital ridges, achieves its maximum level at 11.5 dpc, and comes to its end at 12.5 dpc [Gubbay et al., 1990]. Moreover, *Sry* shows a particular wave-like expression pattern in the genital ridge, starting at its central regions and then extending to both poles, the last regions where *Sry* expression can be observed [Bullejos and Koopman, 2001]. Noticeably, this expression profile is the same even during *Sry* expression termination. Consequently, a single cell is exposed to *Sry* mRNA for less than 48 h, i.e. the time span between 10.5 and 12.5 dpc. The *Sry* expression profile has to be precisely performed to maintain its proper function. In fact, any delay in its expression fails to induce a correct testis development, as clearly demonstrated by Hiramatsu et al. [2009] in transgenic mice carrying the *Sry* gene under the control of an inducible promoter (Hsp70.3). Even a 6-hour delay in *Sry* expression leads to a failure in testis development. The importance of respecting the critical window of *Sry* expression was also demonstrated observing the phenotypes of B6/Y^{DOM} mice. Y^{DOM} mice, carrying a specific Y-chromosome variant with a delayed *Sry* expression of about 10 h, are crossed into C57BL/6J (B6) genetic background. B6/Y^{DOM} mice show a variety of phenotypes that include both ovary and ovotestis [Eicher et al., 1992; Bullejos and Koopman, 2005].

However, while a delayed expression of *Sry* seems to be very important in inducing critical effects in testis differentiation, no relevant events seem to derive from a premature expression of *Sry*. Inducing *Sry* expression before 10.5 dpc does not lead to either premature testis development or to activation of male-specific genes, which suggests that co-factors available from 10.5 dpc are needed for the action of SRY [Kidokoro et al., 2005]. As reported above, while the beginning of SRY expression is a critical parameter, its duration does not seem to be meaningful as several mammals show a persistent SRY expression beyond testis development. This expression profile was reported in goats [Pannetier et al., 2006], pigs [Parma et al., 1999], sheep [Payen et al., 1996], humans [Hanley et al., 2000], rabbits [Díaz-Hernández et al., 2008], and cattle [Ross et al., 2009]. The function of such persistent expression in these species is still unknown; though, a recent study performed in goats suggests its possible influence

on male fertility and testicular maintenance [Montazer-Torbati et al., 2010]. Exact temporal expression of SRY is not the only critical parameter; in fact, its quantity is similarly significant, as failure in testis differentiation occurs when a required threshold is not achieved. Although its precise mechanism of action is still unknown, several genes have been related to *Sry* regulation; e.g. its expression is decreased when factors such as GATA4, FOG2, CBX2, MAP3K4, insulin receptors, and WT1 (KTS+ variant) are inactivated in mice [Katoh-Fukui et al., 1998; Crispino et al., 2001; Hammes et al., 2001; Tevosian et al., 2002; Nef et al., 2003; Bogani et al., 2009; Manuylov et al., 2011].

Sry is expressed in the somatic cells of the XY genital ridge, inducing them to differentiate into Sertoli cells. Such cells are considered as the organizational center for the testis. In fact, they transmit signals to other precursor lineages to make them differentiate into other testis cell lines, and they draw cell migration from the mesonephros to set up male-specific vasculature [Coveney et al., 2008; Combes et al., 2009]. Another key step in testis differentiation consists in reaching a threshold number of Sertoli cells; chimera experiments (XX/XY) show that 30% XY cells are necessary for the gonad to develop into testis [cited by Brennan and Capel, 2004]. Moreover, it has been observed that shortly after *Sry* expression, all SRY-positive cells changed into Sertoli cells [Sekido et al., 2004], but not all Sertoli cells were SRY-positive [Palmer and Burgoyne, 1991], an observation suggesting the existence of paracrine signals supporting the action of SRY in the recruitment of Sertoli cells during testis formation, as described below.

To recap, SRY can act as TDF only under the following conditions: (a) SRY-scheduled time expression profiles must be precisely respected; (b) a threshold expression of SRY must be reached; (c) SRY migration in the nucleus must occur properly; (d) a discrete number of Sertoli cells must be differentiated. If these steps are fulfilled, SRY activates SOX9, and cells exposed directly to SRY or recruited by PGD2 pathway start to differentiate into Sertoli cells. At this point, only 1 chance is given to mammalian sex determination, which means that the gonad can develop exclusively into a testis.

In conclusion, SRY acts as a switch for male/female differentiating pathways and is crucial to activate SOX9, the autosomal gene responsible for heading Sertoli cell differentiation and subsequent formation of testis architecture.

Nevertheless, SRY is not conserved in all vertebrates; as a consequence, the molecular switch for male/female

pathways must be performed by other actors. In birds, homogametic sex is male (ZZ), while heterogametic sex is female (ZW). In the absence of *SRY*, the molecular switch seems to be played by *DMRT1*, a gene homologous to the *Drosophila* sex regulatory gene *doublesex* and to *Caenorhabditis* sex regulator *mab-3*. In fact, reduction of *DMRT1* protein expression in chicken leads to partial sex reversal of embryonic ZZ male gonads and to *SOX9* expression reduction, indicating that *DMRT1* acts through a dosage-sensitive mechanism [Smith et al., 2009]. In addition, in Medaka fish, where an XX/XY genetic mechanism underlies sex determination unlike some other fish, a *DMRT1*-like gene, *dmrt1bY*, triggers testis development [Matsuda et al., 2002]. Recently, Yoshimoto et al. [2010] isolated a W-linked gene, *DM-W*, as a paralogue of *Dmrt1* in *Xenopus laevis*, which shows a ZZ/ZW-type sex-determining system. The authors proposed that *Dmrt1* drives the expression of specific testis genes in ZZ gonads, while in ZW gonads *DM-W*, having no transactivation domain, acts as a *trans*-dominant negative factor and leads to repression of male-specific genes.

Intriguingly, deletion of the *DMRT1* locus in human chromosome 9p leads to XY gonadal dysgenesis [Raymond et al., 1999]. On the other hand, *Dmrt1* null mice show a mild phenotype characterized by post-natal testis defects [Raymond et al., 2000]. Thus, *DMRT1* involvement in mammalian sex determination is far from being clear.

SOX9: The Godfather's Yes-Man of Testis Differentiation

After over 20 years from its identification, the only ascertained direct target for *SRY* is *SOX9*. Unlike *SRY*, specific for mammals only, *SOX9* is conserved in many species [Morais da Silva et al., 1996] and much evidence demonstrated that it can direct testis development in mammals as well as in other vertebrates. Intriguingly, a *Drosophila* *Sox9* homologue, *Sox100B*, revealed to be essential for somatic testis differentiation [Nanda et al., 2009]. *Sox9* gain-of-function mutations switch male differentiation in XX gonads even in the absence of *Sry* inasmuch as in mice *Sox9* ectopic expression in XX gonads is sufficient to induce Sertoli cell differentiation and testis morphogenesis [Vidal et al., 2001]. A similar phenotype is described in transgenic mice carrying an insertion 1 Mb upstream of *Sox9* that performs a long-range effect on a gonad-specific enhancer, inducing over-expression of *Sox9* [Bishop et al., 2000]. In humans, only 1 case of

SOX9 duplication has been described in a 46,XX sex reversal patient [Huang et al., 1999], while a very recent description of a *SOX9* upstream duplication in a family showing 3 cases of 46,XX testicular disorder, with no other phenotypes, suggests the presence of sex-specific regulatory elements localized at the 5' end of the gene, able to activate *Sox9* gonad expression [Cox et al., 2011]. First evidence for the role of *SOX9* in testis development came from the observation of *SOX9* heterozygous mutations responsible for campomelic dysplasia (MIM 114290), a congenital skeletal disorder associated with various defects in testicular differentiation, up to complete male-to-female sex reversal phenotype in about 75% of XY patients [Foster et al., 1994]. However, heterozygous loss-of-function mutations do not cause sex reversal in mice, while homozygous embryos die at 11.5 dpc, when gonadal sex determination occurs. Experiments on conditional mutants demonstrated that *Sox9* homozygous specific inactivation in developing gonads interferes with Sertoli cell differentiation and testis cord formation, and gene expression analysis reveals down-regulation of male markers and up-regulation of ovarian factors as a sign of gonad sex reversal [Chaboissier et al., 2004; Barrionuevo et al., 2006]. More recent studies indicate that *Sox9* XY mutant gonads show an intermediate transcription profile between male and female gonads at 13.5 dpc. Moreover, follicles develop in mutant XY ovaries and some XY mutant females are partially fertile and produce viable offspring [Lavery et al., 2011].

SOX9 protein contains a highly conserved HMG box domain [Gubbay et al., 1990], including 2 NLSs [Sim et al., 2008] that interact with calmodulin (the N-terminal NLS) and with importin- β (the C-terminal NLS), as previously described for *SRY*, and a C-terminal transactivation domain [Südbek et al., 1996].

In mice, very low levels of *Sox9* expression were observed in the genital ridge of both sexes at 10.5 dpc, when *Sry* expression is at its very early activation. By 11.5 dpc, *Sox9* expression is absent in XX genital ridges, while it is up-regulated in XY genital ridges to support Sertoli cell lineage differentiation. Moreover, *Sox9* expression is maintained also after the *Sry* expression window is closed [Morais da Silva et al., 1996; Sekido et al., 2004]. This implies the existence of mechanisms for *Sox9* expression regulation and maintenance other than the mechanism *SRY* can provide. SF1 was supposed to have a role in initiating *Sox9* expression because early sex-independent expression of *Sox9* is lost in *SF1* (*Nr5a1*) mutant gonads [Sekido et al., 2004]. Recent results confirmed that SF1 is required for up-regulation of *Sox9*, acting synergically

with SRY on a *Sox9* testis-specific enhancer (TESCO, testis-specific enhancer of *Sox9* core element), and that in turn SOX9, together with SF1, maintains its own expression by an auto-regulatory loop [Sekido and Lovell-Badge, 2008]. On the other hand, in vitro co-transfection studies suggest that SOX9 is required for the male-specific expression of *SF1* [Shen and Ingraham, 2002]. Anyway, analysis of animal models highlighted other characters playing on this scenario. *Fgf9* mutant mice show that SOX9-positive loop is not sufficient to maintain its own expression. In this animal model, in fact, although *Sry* is correctly activated and *Sox9* levels are normal up to 11.5 dpc, *Sox9* expression is not maintained afterward, causing abortion of male differentiation pathway and change of somatic cells into a female program [Colvin et al., 2001; Schmahl et al., 2004]. In turn, *Fgf9* expression is down-regulated at 11.5 dpc in cultured gonads from *Sox9* mutant mice [Kim et al., 2006], and these findings associate the 2 genes in a feed-forward mechanism. FGF9 is a member of the fibroblast growth factor family; it is known to have mitogenic capacities in a number of cell types and is expressed in different proliferating regions [Colvin et al., 1999]. During sexual development *Fgf9* is expressed in both XX and XY gonads at 11.5 dpc; then, *Fgf9* expression becomes specific of XY gonads at 12.5 dpc. The most relevant cellular effect of *Fgf9* ablation is male-specific reduction of proliferation in the coelomic epithelium [Schmahl et al., 2004]. It may act as a paracrine factor to recruit nearby cells to Sertoli fate by its receptor FGFR2 [Kim et al., 2007; Bagheri-Fam et al., 2008], increasing the number of Sertoli precursors above the critical threshold needed for testis development. By 11.2 dpc, FGFR2 is localized in the cell membrane of most proliferating coelomic epithelial cells in both XX and XY gonads, while at 11.5 dpc FGFR2 is present in the nucleus of Sertoli precursor cells scattered in the interior of XY gonads. In *Fgf9* mutant mice, nuclear localization of FGFR2 is lost, indicating that it is dependent on FGF9 [Schmahl et al., 2004].

Another molecular signal seemingly involved in the recruitment of Sertoli cells during testis differentiation is prostaglandin D2 (PGD2). The exposure of cultured fetal mouse XX gonads to PGD2 resulted in *Sox9* expression, while *Sox9* expression was blocked in the presence of an inhibitor of PGD2 receptor [Wilhelm et al., 2005]. Moreover, it has been suggested that prostaglandin D2 synthase (PTGDS), an enzyme required to synthesize PGD2, is a direct target of SOX9 in Sertoli cells. SOX9 has been demonstrated to activate the *Ptgds* promoter in reporter assays, and SOX9 protein can bind *Ptgds* promoter in vivo [Wilhelm et al., 2007]. On the other hand, *Ptgds*^{-/-} XY

gonads exhibit reduced levels of *Sox9* transcription in vivo, but not complete sex reversal. Such findings suggest that PGD2-mediated signal is necessary to reinforce the recruitment of supporting cell precursors toward Sertoli cell fate [Moniot et al., 2009]. In Sertoli cells, SOX9 activates AMH transcription, a member of TGF- β superfamily, responsible for Müllerian duct regression [Knowler et al., 2003]. Concomitantly to the differentiation of Sertoli cells, a number of cellular events critical for testis morphogenesis occur in male gonads. Increased proliferation of interstitial cells, together with cell migration from the mesonephros, plays a crucial role in the development of testis cords, necessary for germ cell growth and maturation. Studies on cultured gonads demonstrated that, when blocking cellular migration from mesonephros at 11.5 dpc, testis cords do not develop properly [Buehr et al., 1993; Tilmann and Capel, 1999].

Migrating cells are mainly endothelial cells gathering within the gonad to give shape to a male-specific arterial network, branched from coelomic blood vessel. This vascular system is fundamental for testis growth and hormonal product distribution. Cell migration from the mesonephros should be regulated by PDGF (platelet-derived growth factor). Indeed, null mice for PDGF receptor (*Pdgfr*^{-/-}) show defects in cell migration and vascularization [Brennan et al., 2003].

Between 12.5 and 13.5 dpc, fetal Leydig cells, responsible for steroidogenesis, start to differentiate in the interstitial region of the XY gonad. At 11.5 dpc, *SF1* is expressed both in pre-Sertoli and pre-Leydig cell lines. Subsequently, *SF1* expression decreases in Sertoli cells and increases in differentiating Leydig cells, regulating genes involved in steroidogenesis [Hatano et al., 1994].

It was demonstrated that desert hedgehog gene (*DHH*) and PDGF receptor are male-specific factors required for early differentiation and expansion of the Leydig cell lineage.

Dhh and *Pdgfr* are expressed in Sertoli cells, while their corresponding receptor genes (*Ptch1* and *Pdgfr*, respectively) are expressed in the interstitium. Mutants in both *Dhh* and *Pdgfr* show defects in testis architecture and severe impairment of fetal Leydig cell differentiation [Bitgood et al., 1996; Yao et al., 2002; Brennan et al., 2003].

β -Catenin: The Pink Side of the Family

In 1947, Alfred Jost demonstrated by his renowned experiments on rabbits that male sex phenotypic development requires the presence of testes, while female sex

phenotypic development is able to proceed even in the absence of ovaries, which suggests the existence of an active genetic program for male phenotype differentiation against a default pathway for the female differentiation process [Jost, 1947].

Following TDF localization on the Y chromosome, Jost's hypothesis was translated from phenotype to gonad, and some authors postulated an active genetic mechanism for testis development and a passive mechanism for ovary differentiation. Several physiological and experimental evidences then contributed to erode the bases of this dogma in time. The existence of sex reversal syndromes, characterized by testis development and male phenotype in 46,XX subjects without SRY, suggests the existence of a genetically active program in XX gonads. This process, which may lead ovary differentiation and/or repress the testis developmental pathway, when disrupted could cause male genetic program activation in chromosomally female gonads.

A great number of genes that correctly coordinate processes as cell differentiation, migration, proliferation, and apoptosis are necessary to perform the development of such a complex organ as an ovary, with its morphologically and functionally specialized cell lineages organized into a precise conformation. Differential expression analyses in embryonic mice gonads during the critical sex determination period (10.5–13.5 dpc) demonstrated overexpression of 1,223 genes specifically in XX gonads, indicating the activation of a robust differentiation program also in female gonads [Nef et al., 2005]. Mutational analyses in humans and in animal models led to the identification of some genes involved in the early phases of ovary determination and differentiation.

DAX1, a member of the nuclear hormone receptor superfamily, appeared to be an ideal candidate for an ovary-determining factor, being localized on chromosome X and identified in duplications in XY subjects characterized by male-to-female sex reversal [Bardoni et al., 1994; Zanaria et al., 1995]. Moreover, XY transgenic mice carrying extra copies of *Dax1* show complete sex reversal [Swain et al., 1998]. On the other hand, analyses of animal models carrying loss-of-function mutations demonstrated that this gene is not necessary for ovary development, while it is required for testis differentiation. Indeed, null mutations of *Dax1* in XX embryos do not affect ovarian development or fertility [Yu et al., 1998], but can cause complete male-to-female sex reversal in some XY genetic backgrounds [Meeks et al., 2003; Bouma et al., 2005].

Another candidate ovary gene was initially identified in goats as a factor causing polled intersex syndrome

(PIS). XX goats carrying homozygous *Polled* mutation showed female-to-male sex reversal in absence of SRY, associated with lack of horns. Mapping of the PIS locus in goats led to the identification of an 11.7-kb deletion that affects the transcription of *FOXL2* [Pailhoux et al., 2001]. In humans, mutation of *FOXL2* is responsible for blepharophimosis ptosis epicanthus inversus syndrome (BPES) associated with premature ovarian failure [Crisponi et al., 2001]. *FOXL2* is a basic helix-loop-helix transcription factor belonging to the forkhead/winged helix nuclear transcription factor family. In mice, *Foxl2* is specifically expressed in XX gonads by 12.5 dpc, mainly localized in committed pre-granulosa supporting cells [Schmidt et al., 2004], but null mutations do not seem to affect initial ovary development, while in *Foxl2* mutant ovaries defects in folliculogenesis become apparent in late development [Ottolenghi et al., 2005]. Moreover, *Foxl2* null mice show a reduced expression of the follistatin gene (*Fst*) in post-natal ovaries, while over-expression of *Foxl2* in mice results in an increase of *Fst* expression in XX gonads [Garcia-Ortiz et al., 2009]. *Fst*, a member of the TGF- β binding factor superfamily, plays an earlier role in ovarian development, as *Fst* null mice show partial XX sex reversal [Yao et al., 2004]. It was demonstrated by in vitro experiments that *Foxl2* up-regulates *Fst* expression cooperatively with *Bmp2*; moreover, *Bmp2* up-regulates *Fst* expression in ex vivo organ culture experiments [Kashimada et al., 2011]. However, recent results showed that conditional ablation of *Foxl2* in adult gonads determines an ovary-to-testis trans-differentiation of the somatic lineages. The mRNA expression profile of these mutant gonads shows the expression of testis-specific markers, including *Dax1*, *Dhh*, *Dmrt1*, and notably *Sox9* [Uhlenhaut et al., 2009]. This adulthood trans-differentiation seems to be driven by the activation of a *Sox9* testis-specific enhancer, TESCO, suggesting that *FOXL2* may repress in vivo *Sox9* expression through TESCO binding. Moreover, estrogen receptor 1 (ESR1) seems to synergize with *FOXL2* to repress TESCO regulatory element [Uhlenhaut et al., 2009]. Estrogens can induce male-to-female sex reversal in vertebrate species with genetic-based sex determination mechanisms as fish, reptiles and birds, but not in most eutherian mammals [Barske and Capel, 2008]. On the other hand, in goats *FOXL2* activates P450 aromatase gene transcription in early fetal ovary and the PIS phenotype is characterized by an earlier ovary phenotype than in *Foxl2* null mutant XX mice. These findings suggest that estrogen activity could be a predominant anti-testis or ovary-determining mechanism in goats.

WNT4 is one of the first best characterized markers for ovarian differentiation. WNT4 is a member of Wnt secreted signal molecules, which function as short-range ligands to locally activate receptor-mediated signaling pathway, and are involved in several embryonic developmental processes. The best known Wnt signaling pathway stabilizes β -catenin and activates LEF/TCF (lymphocyte enhancer factor/T-cell factor)-dependent transcription of downstream genes [Verheyen and Gottardi, 2010].

In mice, *Wnt4* is early expressed in the mesenchyme of mesonephros, but not in mesonephric tubules, and in the coelomic epithelium of gonadal anlagen during the bi-potential stage. When gonad sex differentiation starts at 11.5 dpc, expression decreases in male developing gonads, but persists in XX gonads and in the mesenchyme surrounding Müllerian ducts. In contrast to gonads, *Wnt4* is expressed in mesonephric mesenchyme of both sexes, and in mesenchyme cells underlying Müllerian ducts, while it is absent in Wolffian ducts [Vainio et al., 1999].

Wnt4 null XX mice show only a partial sex reversal phenotype: Müllerian ducts are absent and Wolffian ducts continue to develop, but no masculinization of external genitals was observed at birth [Vainio et al., 1999]. Mutant XX gonads develop a not-branched blood vessel resembling testis coelomic vessel and show the presence of steroidogenic cells derived from adrenal precursor [Jeays-Ward et al., 2003]. *Sox9* expression is initially up-regulated at 11.5 dpc, but rapidly down-regulated at 12.0 dpc and absent at 12.5 dpc [Kim et al., 2006]. Analysis of transgenic mice showed that gain-of-function mutation in XY gonads does not inhibit initial development of the coelomic vessel, but that it interferes with normal branching of testis vasculature [Jeays-Ward et al., 2003]. WNT4 antagonizes male vasculature development through the downstream effector FST [Yao et al., 2004]. In the ovary, WNT4 and FST repress inhibin beta-B (*Inhbb*), which in XY gonads is involved in the formation of the male coelomic vessel [Yao et al., 2006]. In humans, rare mutations in *WNT4* have been described in female patients with defects in Müllerian duct development and clinical signs of androgen excess [Biaison-Laubert et al., 2004]. Such evidences indicate that WNT4 is involved in Müllerian duct development and represses endothelial cell migration and coelomic vessel formation in XX gonads, but it does not function alone as a primary ovarian determining gene or as an opponent of the testis developmental pathway.

The double ablation of *Foxl2* and *Wnt4* genes in mouse XX gonads induces the development of ovotestes: testis-like tubules, expressing high levels of testis-specific genes as *Sox9* and *Amh* and containing differentiated spermatogonia, are present in the medullary region of the gonad, while in the cortical region, germ cells resemble late prophase oocytes, expressing meiotic markers [Ottolenghi et al., 2007]. Thus, sex reversal in these double mutant XX gonads is more evident than in single knockouts, indicating that FOXL2 and WNT4 could act through complementary pathways to repress the male fate in differentiating ovaries. Nevertheless, the incomplete phenotype suggests the existence of other ovarian factors needed to trigger ovary development and to oppose the male differentiation pathway in XX gonads.

In humans, the only gene found whose loss-of-function mutations cause testis development in XX patients, is *R-spondin 1*. In 2 independent cases, homozygous mutations of *RSPO1* were identified in XX subjects with complete female-to-male sex reversal, associated with skin defects [Parma et al., 2006].

In the absence of SRY, the gonads of these patients developed as functional testes, as shown by the absence of Müllerian derivatives and by internal and external genital masculinization. Moreover, *RSPO1* is required neither for male differentiation nor for fertility in XY subjects [Radi et al., 2005; Parma et al., 2006]. Expression analyses on embryonic murine gonads demonstrated expression of *Rspo1* in the urogenital ridge as early as 10.5 dpc in the coelomic epithelium. By 11.5 dpc sex-specific expression appears in the somatic cells of the XX gonads [Parma et al., 2006].

Studies on *Rspo1* null animal models clarified some aspects of *RSPO1* action in gonadal sex determination and ovary development. *Rspo1* KO XX gonads develop as ovotestes, containing seminiferous tubules together with nests of pachytene oocytes, and adult mice show masculinized external genitalia and persistence of both Müllerian and Wolffian ducts [Chassot et al., 2008; Tomizuka et al., 2008].

A differential expression analysis of genes involved in sex determination was performed in XX *Rspo1* mutant gonads versus wild-type gonads. Female markers, as *Bmp2* and *Foxl2*, do not show relevant differences between mutated and normal gonads, indicating a partial conservation of a female identity in the somatic lineage, even in the absence of *RSPO1*. On the contrary, male markers (*Fgf9*, *Pgds*, *Sox9*, *Amh*) exhibit an increased expression in *Rspo1* mutant mice, suggesting that in XX gonads *RSPO1* acts as a suppressor of the male differentiation pathway.

RSPO1 is a member of the protein family of R-spondins involved in the embryonic development of different organs [Nam et al., 2007]. They are secreted proteins that stabilize β -catenin through receptor interactions.

Analysis of mutant mice demonstrated that RSPO1 triggers female gonadal sex determination through β -catenin stabilization. Indeed, in *Rspo1* null mice XX gonads show a reduced regulation of β -catenin-specific targets (*Lef1* and *Axin*), and the mutant phenotype of XX *Rspo1*^{-/-} gonads is rescued by ectopic activation of β -catenin [Chassot et al., 2008]. The major role of the β -catenin pathway in sex determination of XX gonads was demonstrated by analysis of animal models. Ectopic expression of a dominant stable β -catenin allele in XY gonads is sufficient to disrupt male differentiation and induce ovary development [Maatouk et al., 2008]. On the other hand, specific inactivation of β -catenin in the putative precursors of granulosa cells causes defects in ovary differentiation, and mice show a phenotype similar to *Wnt4* and to *Rspo1* mutant mice [Tevosian and Manuylov, 2008; Liu et al., 2009].

β -Catenin is an intracellular bi-functional protein involved in cell-cell adhesion interactions; it also functions as a nuclear transcription factor. The major part of β -catenin is associated with cadherins within the adherent junctions, as a component of the cytoskeleton. However, a little dynamic pool of β -catenin shuttles between cytoplasm and nucleus to transduce the canonical Wnt signal [Kimelman and Xu, 2006]. The crucial point of Wnt signal transduction engages the inhibition of the constitutive degradation mechanisms. In the presence of Wnt signal, stable β -catenin translocates into the nucleus and regulates target gene expression by binding DNA through the nuclear transcription complex LEF/TCF. In cells not exposed to Wnt signal, the cytosolic pool of β -catenin is kept at low levels through degradation mediated by the ubiquitin pathway. Removing β -catenin from the LEF/TCF complex allows access to co-repressors inhibiting transcriptional function [Cavallo et al., 1998; Roose et al., 1998].

Unlike the XY gonad, where dramatic changes follow *Sry* expression, very few morphological changes occur in the female gonad until close to birth. In the ovary 2 regions can be distinguished: the cortex and the medulla, even if this distinction is not well appreciable at 12.5 dpc. Primordial germ cells, clustered in germ cell nests, localize in the cortex, while in the medullary region they undergo apoptosis. The first cellular event specific for the XX gonad is the entry of germ cells into meiosis, which in mice occurs between 13.5 and 14.5 dpc. It was demon-

strated that the entry of XX germ cells into meiosis is induced by retinoic acid. In the male gonad, entry into meiosis is prevented by up-regulation of CYP26B1, an enzyme that degrades retinoic acid [Bowles et al., 2006].

In fetal ovary, a coordinate action of secreted (RSPO1/WNT4) and intracellular (FOXL2) factors specifies and maintains the identity of granulosa cells, the somatic cell lineage derived from the same precursors as testis Sertoli cells.

Fights between Rival Gangs

In a bipotential gonad, a precarious equilibrium is established between factors needed for testis or ovary development, and the presence or absence of the Y chromosome leads the balance toward male or female mode. SRY activates *SOX9* transcription (maintained and reinforced through a feed-forward loop involving FGF9 and PGD2) that in turn activates Sertoli cell differentiation and, thus, testis development. Nevertheless, SRY must be active in a definite time slot. Otherwise RSPO1 and WNT4, through β -catenin stabilization, together with FOXL2 establish and maintain the female differentiating pathway of somatic cell progenitors by antagonizing *SOX9* expression. Molecular pathways of ovary and testis determination reciprocally cross at different levels. FGF9 and WNT4 act antagonistically: exogenous FGF9 exposure of gonadal systems induces *Sox9* expression and blocks *Wnt4* expression, suggesting that FGF9 acts both recruiting gonadal cells into a male differentiation program and blocking female signals. In turn, WNT4 is involved in male pathway repression. Indeed, *Fgf9* results de-repressed in *Wnt4* mutant XX gonads, where *Sox9* expression is activated in the absence of SRY [Kim et al., 2006].

The bipotential gonad hangs in the balance between 2 fates, both generated by extracellular signals and by an intracellular antagonism based on the relative equilibrium between β -catenin and *SOX9* amounts, a mechanism demonstrated to be involved in chondrocyte differentiation [Akiyama et al., 2002].

Similarly, in the bipotential gonad β -catenin and *SOX9* could be proposed as executive antagonistic molecules between female and male pathways, directing somatic precursors towards either granulosa or Sertoli cells. Anyway, such complex and multifactorial regulations, reciprocally positive and negative, suggest the existence of accessory 'remote controls' reinforcing these pathways.

A bipotential gonad is the only embryonic structure that can originate 2 different organs such as testis and

ovary. After an early phase of strength balance, when several factors contribute to raise the gonadal anlagen, the critical point for sex development, i.e. gonadal sex determination, coincides with the deployment of forces by 2 distinct and antagonistic molecular pathways, determined to prime gonadal differentiation toward a testis or an ovary. A crucial point for the genetic control of sex determination stands in the enhancement of one path-

way, coherent with the chromosomal sex, so that the opposite pathway cannot take control of the gonad.

SOX9 and β -catenin pathways seem to be the 2 major mechanisms involved in this sort of fight. Though, many other components are not answering the roll call, as several human patients affected by disorders concerning sexual development do not have a genetic diagnosis.

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