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► **To cite this version:**

Sophie Lamothe, Valérie Bernard, Sophie Christin-Maitre. Gonad differentiation toward ovary. *Annales d'Endocrinologie = Annals of Endocrinology*, 2020, 81, pp.83 - 88. <10.1016/j.ando.2020.04.004>. <hal-03490495>

HAL Id: hal-03490495

<https://hal.science/hal-03490495v1>

Submitted on 3 Jun 2022

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GONAD DIFFERENTIATION TOWARD OVARY

La différenciation gonadique vers l'ovaire

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Résumé La différenciation gonadique dépend d'un ensemble de signaux cellulaires et hormonaux qui interagissent entre eux dans un ordre défini, avec des fenêtres d'action très précises, pour contribuer à la mise en place de l'appareil génital et à l'établissement d'un phénotype masculin ou féminin. La recherche a initialement progressé sur les étapes de la différenciation de la gonade en testicule, notamment suite à l'identification en 1990 du facteur SRY sur le chromosome Y. Les mécanismes impliqués dans la détermination de la gonade en ovaire ont été plus longs à identifier. Grâce à des patients porteurs de développement sexuel différent (DSD) ou de modèles animaux d'inactivation de gènes, les cascades impliquées dans l'activation et le maintien du développement ovarien ont beaucoup progressé ces dernières années.

Mots clefs : différenciation gonadique, ovaire, gonade, développement ovarien, phénotype, facteur SRY

Abstract Gonad differentiation depends on a set of cellular and hormonal signals interacting in a specific order, with very precise windows of action, to contribute to the establishment of the genital tract and a male or female phenotype. Research initially focused on the stages of gonad differentiation toward testis, in particular following the identification in 1990 of the SRY factor on chromosome Y. The mechanisms involved in gonad differentiation toward ovary took longer to identify. Thanks to patients with different sexual development (DSD) and animal knock-out models, description of the cascades involved in the activation and maintenance of ovarian development has progressed considerably in recent years.

Keywords : gonad differentiation, ovary, gonad, ovarian development, phenotype, SRY factor

Introduction

Gonad determination is a concept that emerged, scientifically, mainly since the late 1950s. The decisive role of the Y chromosome in the transformation of the gonad into the testis represented a major advance in the understanding of gonad determination. In 1990, the SRY factor (sex-determining region of the Y chromosome) was identified. The mechanisms involved in the transformation of the gonad into an ovary took longer to identify. In recent years, knowledge of the factors necessary for differentiation of the gonad toward ovary has progressed markedly. Thanks to patients with different sexual development (DSD) and animal models of gene knock-out, the cascades involved in the activation and maintenance of ovarian development are improving.

After a historical review of the theories of sex determination, this chapter will address the different stages and the main factors involved in the formation of the undifferentiated gonad and its transformation toward the ovary, and the factors involved in the maintenance of the ovary in the adult gonad.

I. HISTORY OF GONAD DETERMINATION

Theories of sexual determination have existed since ancient times. According to Anaxagoras (500-428 BC), if the sperm comes from the right testicle it will produce a boy, and if it comes from the left testicle, a girl. Others, such as Empedocles (496-435 BC), believed that sperm that enter a warm uterus induce males and females when entering in a cold uterus become females, these temperature differences being attributed to colder or warmer menstrual flow. A century later, Aristotle described new theories of sexual determination, according to which the male is the bearer of the divine principle and transmits human nature. The woman, devoid of seed, contributes only raw material to generation. "The very first genetic deviation is the birth of a female instead of a male" (1). Aristotle admitted that the female is necessary to conserve sexual difference; but women are nonetheless a failure of human nature (2). New explanations later refer to mechanisms of the animal world: depending on the species, the bi-potential gonad engages in male or female differentiation according to environmental factors such as temperature or behavior, and not according to genetic heritage. This is called environmental sex determination. In lizards, for example, an egg incubation temperature of eggs below 30°C induces male sex and a higher temperature female sex. The opposite is true for turtles (3). In fish, different types of social factor can determine sex. For example, in clownfish, sex is determined by the social rank of the individual. These fish live in groups in anemones. A female in a monogamous couple dominates the colony alongside an adult male, with infertile males and juveniles around this couple. The aggressive behavior of the dominants toward infertile males prevents their sexual maturation and the aggressive behavior of the female prevents the adult male from

becoming a female. If the female dies, the male of the couple becomes the dominant female while one of the infertile males becomes the fertile male (4).

This hypothesis of sex determination sex by the environment persisted until the late 19th century, when new theories came to be proposed. The "*metagam*" theory was based on an external influence: the fertilized egg is asexual and sex is determined more or less late in the embryo. In "*progame*" theory, on the other hand, the ovum itself is sexually differentiated: there are male and female ova; the sperm plays no role in the determination of sex, but only gives the egg an incentive to develop; sex thus preexists fertilization. In "*syngamous*" theory, sex is determined at the very moment of fertilization; it results from the combination of the properties of the egg and the sperm which combine to form the fertilized egg (5).

The theory put forward by the Austrian embryologist Schenk (1840-1902) later made a big splash. Schenk had noticed a relationship between maternal nutritional status and the sex of the fetus. Pregnancy is accompanied by varying levels of glycosuria; if glycosuria is very high, miscarriage occurs and, when it is low, a girl is born; a perfectly healthy woman gives birth to a boy. According to Schenk, providing women who have only daughters with an appropriate diet, starting two or three months before conception and continuing for three months after, will obtain a boy (6). Even today, attempts to control sex determination through nutrition continue: according to certain theories, the diet to obtain a boy requires unstinting consumption of meat, salted, smoked or canned fish, salty aperitif crackers, and pastries, while having a girl requires eating vegetables, milk and dairy products (7).

Scientifically, Alfred Jost in 1947 showed that removing the testes in rabbit embryos induced a female phenotype at birth. This type of experiment gave rise to the hypothesis that the female phenotype was the consequence of non-differentiation of the gonad into testicle: differentiation "by default ". However, around 1961, genetic data highlighted the existence of sex chromosomes X and Y in mammals, as well as the major impact of the Y chromosome in testicular differentiation (8,9). In 1990, the SRY factor (Sex-determining Region on the Y chromosome) was identified. It is now known that there is an undifferentiated gonadal stage and then a positive determination stage from gonad to ovary.

II. FROM GENITAL CRESTS TO DIFFERENTIATED GONADE

II.a The undifferentiated or bi-potential gonad

The development of the gonad is the first step in organizing the reproductive system.

It begins very early, from the 3rd or 4th week of embryo development, with the establishment of an identical undifferentiated structure in XX and XY embryos.

At the start of their formation, the gonads are said to be "bi-potential" because they have the capacity to follow a path of male or of female differentiation. These undifferentiated gonads are composed of distinct cell populations: primordial germ cells (PGCs), from which originate gametes, and somatic cells. Each somatic line has a double potential: the support cell line, from which granulosa cells originate, and the steroid cell line, which evolve into theca cells (10).

The gonads and adrenals share a common developmental origin: the adreno-gonadal primordium (11). Initial gonads differentiate from the mesonephros (transient embryonic

kidneys). The PGCs then colonize the gonadal territory called the genital crest. The arrival of PGCs at the genital crests leads to proliferation of deep coelomic epithelial cells, which are able to surround the germ cells and thus form the primitive sexual cords. At this stage, the gonad still has a strictly identical morphology between the two sexes, with primitive sexual cords in both cortical and medulla regions (11).

Progression toward one sex or the other depends on the activation and repression of a large number of genes and a sufficient local concentration of specific proteins encoded by these genes: gonadal determination is a "dose-dependent" phenomenon. Several genes play a crucial role in the formation of genital crests, in particular, *WT1* (Wilm's Tumor gene 1) and *SF1* (Steroidogenic Factor 1). The proteins encoded by these genes are involved in the proliferation, differentiation and survival of somatic cells in the gonad.

The *WT1* gene, located on chromosome 13, plays a major role in the development of the urogenital system, with expression in the mesenchyme of the renal vesicles, the glomerular epithelium of the kidney and the genital crests from the 6th week of development, and then in the fetal gonad. *WT1* binding to the promoter of the *Nr5a1* gene (nuclear receptor subfamily 5 group A member 1) enables expression and action during gonad development (12, 13). Studies in mice confirmed the role of *WT1* in urogenital development: mice mutated for *WT1* showed absence of development of the kidneys and gonads (12). In humans, mutations or deletions of the *WT1* gene are responsible for various syndromes, the prevalence of which is very low. They can induce full or partial 46, XY DSD and also impair the urogenital system. For example, Frasier syndrome combines 46, XY DSD and glomerular nephropathy. Denys-Drash syndrome combines 46, XY DSD, diffuse mesangial sclerosis and nephroblastoma. WAGR syndrome (Wilms's tumor, Aniridia and Genital anomalies with mental retardation) associates Wilms's renal tumor and total or partial aniridia, with DSD ranging from sexual ambiguity to testicular ectopy, and intellectual deficiency of varying severity.

The *SF1* protein is coded by the *Nr5a1* gene (nuclear receptor subfamily 5). It is expressed as early as the 6th week of development in the coelomic epithelium and the cells supporting the bi-potential gonad (14, 15). This transcription factor is involved in the development and proliferation of cells supporting the bi-potential gonad (16). The first loss of function mutation in *NR5A1* was described in humans in 1999 (17). It was identified in a 46,XY individual, phenotypically female, who developed postnatal adrenal insufficiency and bilateral cryptorchidism. Bilateral gonadectomy in adolescence identified two small dysgenic testes. In 2009, the involvement of *SF1* in premature ovarian failure or primary ovarian insufficiency (POI) was described for the first time (18). The *NR5A1* gene was sequenced in 4 families affected by both 46,XY dysgenesis and POI, and in 25 sporadic cases of POI without associated adrenal insufficiency. This sequencing identified mutations in several 46,XX members of the 4 families and in 2 of the 25 sporadic cases of POI. The spectrum of damage linked to *NR5A1* mutations is very wide. To summarize, *SF1* plays a role in testicular development and in maintaining the ovarian follicle pool.

II.b Sex determination

Sex determination enables the initial gonad or genital crest to initiate a developmental path leading to the formation of the ovary or the testis: i.e., gonadal sex. Differentiation is the process that guarantees the development of a genital tract and external genitalia according to gonadal sex, thereby constituting the phenotypic sex.

During ovarian determination, the primitive sex cords segment into clusters in which primordial gonocytes are found. These primitive sexual cords are connected to the mesonephrotic tubes by a network of cellular cords constituting the rete-ovari. The superficial epithelium of the female gonad continues to proliferate. It also gives birth to a second generation of cords, the cortical sex cords, that penetrate the underlying mesenchyme. The gonad determination process for the ovary is much longer than that for the testis. At the start of the 3rd month of pregnancy, the cortical area changes.

The gonocytes turn into oogonies and the cells of the sex cords organize around the germ cells, which results in the formation of primordial follicles (19). The mesenchymal elements give the ovarian stroma. In the center of the ovary, the spinal cords regress as well as the rete-ovari and the mesonephronic remains. All of these remnants are at the origin of Rosenmüller's organ (20).

II.c Factors determining the development of the ovary

II.c.1 Differentiation of ovarian somatic cells

In mammals, the formation of the testis or the ovary depends on a set of cellular and hormonal signals that interact in a specific order, with very precise windows of action, to contribute to the establishment of the genital apparatus and therefore a male or female phenotype.

Comprehensive research progressed in depicting the chronological stages of the differentiation of the gonad into testis (21). The SRY gene, discovered in 1990, located on the short arm of the Y chromosome, close to the pseudo-autosomal region, is considered the key gene for male determination (22,23). SRY protein is a transcription factor that modulates the expression of target genes essential for testicular differentiation, including *SrY-box* containing gene 9 (*SOX9*). Thus, the translocation of SRY on X chromosome induces, in 46,XX embryos carrying SRY, the development of testes, which can be normal (24). Conversely, a mutation of SRY in a 46,XY subject causes a defect in masculinization of the primitive gonads. This defect is often total, inducing streak gonads (25, 26). Thus, until recently, gonad differentiation toward ovary was considered to occur by default, due to the absence of the SRY gene and/or *SOX9*. It is now accepted that the formation of the ovary is the consequence of a brake on testicular development, but also of a positive determinism. There is indeed a cascade of events inducing gonad differentiation toward ovary (Figure 1).

The majority of “anti-testicular” genes induce repression of SOX9 (21). The main players in female gonadal differentiation are the WNT4 / β catenin- pathway modulated by R-Spondin 1 (RSPO1) (27) and the Forkhead box L2 (FOXL2) transcription factor.

The WNT/ β -catenin pathway

When activated by WNTs (Wingless-type MMTV integration site), two transmembrane receptors form a heterodimer – which transmits a signal that causes β -catenin stabilization in the cell. β -catenin then acts as a transcriptional coregulator in the nucleus (28). Several WNT signaling pathways come into play when the undifferentiated gonad takes on a male or female destiny. Two main ligands have been identified, in particular *WNT4* (Wnt family member 4) and *RSPO1* (R-Spondin 1) (Figure 2).

The *WNT4* gene is located on chromosome 1 at 1p35. *WNT4* is expressed in the undifferentiated gonad of XX and XY embryos. Its expression decreases in the male gonad at the differentiation, while it persists in the developing ovary. In the 46,XX fetus, between 12 and 18 weeks of gestation, moderate *WNT4* expression is detected in the pre-granulosa and granulosa cells while expression is very low in the oocytes. At 19 weeks, *WNT4* expression increases intensively in oocytes (29). Mice with a XX karyotype, invalidated for *Wnt4* (*Wnt4* - / -), show partial sexual reversion, with a masculinization of the gonad. Support cells adopt characteristics of Sertoli cells and there is a decrease in the number of oocytes, suggesting that *Wnt4* is necessary to maintain the development of female germ cells (30). On the molecular level, *Wnt4* inhibits expression of Sox9 in mice (31). In humans, homozygous *WNT4* "loss-of-function" mutations are responsible for SERKAL syndrome (SEx Reversion, Kidneys, Adrenal and Lung dysgenesis). This syndrome is extremely rare, with prevalence <1/1,000,000 (32). It is lethal, and associates testicular development in 46,XX fetus, as well as renal, adrenal and pulmonary abnormalities (33). Heterozygous mutation of *WNT4* was first described in 2012 in a 46,XX individual. This 18-year-old patient consulted for primary amenorrhea. Her thelarche had occurred at the age of 12. The etiology of her amenorrhea was uterine agenesis. The patient also had renal agenesis (34). This phenotype is similar to that of Rokitansky-Kuster-Hauser syndrome but is accompanied by mild hyperandrogenism. This is a very rare disorder, and only a few cases have been reported (35, 36). Conversely, overexpression of *WNT4* by duplication of the gene was reported in a 46,XY individual (37). This patient presented sexual ambiguity with severe hypospadias, fibrous gonads, rudimentary uterus and vagina. *WNT4* thus appears to be a major player in ovarian development as well as the inhibition of testicular development. It acts by increasing the expression of *DAX1*, another anti-testicular actor (37). In all, *WNT4* has a brake role in the development of the gonad of the testis and decreases the synthesis of androgens by the gonad in women.

- RSPO1

The *RSPO1* gene is located on chromosome 1 at 1p34.3. It is expressed early in ovarian development (38), and not expressed in testicular tissue. The R-Spondin 1 protein (*RSPO1*) plays a key role in gonad differentiation toward ovary, activating the WNT/ β -catenin pathway. It is part of a large family of growth factors. At gonadal differentiation, *RSPO1* is expressed in 46,XX but 46,XY gonads (39). It is also expressed in fibroblasts and regulates the proliferation and differentiation of keratinocytes (39). The phenotype of *Rspo1* *-/-* mutant mice is close to that of *Wnt4* *-/-* mice (27). In humans, the first "loss of function" homozygous mutations of *RSPO1* were described in 2006 in an inbred Italian family. Within the family, the 4 mutated brothers, with a karyotype 46,XX (*SRY*-negative), presented sexual ambiguity. Their external genitals were more of a male type but with hypospadias or greater ambiguities. Gonadal biopsies performed in some patients mutated for the *RSPO1* gene in some cases revealed testicular structures with Leydig cell hyperplasia and in other cases ovotestis with small areas of residual ovarian tissue (27). These patients also showed palmoplantar hyperkeratosis and a predisposition to squamous cell carcinoma of the skin (39). This pathology is extremely rare, with fewer than 10 cases described to date. However, their description has shown that R-Spondin 1 plays a key role in differentiating the gonad toward the ovary. Chronologically, it is one of the first stages of this differentiation. It is interesting to note that the two factors *WNT4* and *RSPO1* are also involved in the early stages of gonadal establishment, playing a role in the proliferation of coelomic epithelium cells. Chassot et al. demonstrated this phenomenon in 2012 in a *Wnt4* and/or *Rspo1* knock-out mouse model: whether mice were XX or XY, they exhibited decreased cell proliferation at the genital crests.

- **FOXL2**

The third known factor in positive ovarian determination is *FOXL2*, which is encoded by the *FOXL2* (*Forkhead box L2*) gene, located on chromosome 3 at 3q22.3. This transcription factor is expressed very early during ovarian development. An immunofluorescence study showed expression of *FOXL2* in gonad supporting cells, and especially pre-granulosa and granulosa cells (40). The role of *FOXL2* in ovarian determination was initially highlighted in goats, where loss of function of *Foxl2* had several impacts. Firstly, lack of feminization of the XX gonad with loss of aromatase expression and therefore absence of estrogen synthesis was observed. In addition, masculinization of the XX gonad occurs by overexpression of the male factor *SOX9*, resulting in differentiation of Sertoli cells and expression of anti-Müllerian hormone, causing Müller channels to regress (41). However, disabling the *Foxl2* gene in XX mice did not result in sexual reversion (42, 43). The ovaries of *Foxl2* *-/-* mice are small and severely disorganized, and primordial follicles do not form (44). Furthermore, overexpression of *Foxl2* in Sertoli XY cells was shown to induce disorganization of the seminiferous tubules and the development of an ovotestis-type gonad (45). Thus, there is a phenomenon of mutual exclusion within the somatic cells of the gonads between *FOXL2* for the ovary and *SOX9* for the testicle, inhibiting each other and resulting in two dichotomous states: male or female. In humans, heterozygous mutations of *FOXL2* were described in BPES

syndrome (Blepharophimosis-Ptosis-Epicanthus inversus), which leads to eyelid abnormalities, with diminished palpebral cleft and widening of the distance between the eyes called epicanthus. BPES can be associated with premature ovarian failure (BPES type 1) or be isolated (BPES type 2) (46-48). No abnormal sexual development has been described in individuals with *FOXL2* mutation to date.

II.c.2 Differentiation of ovarian germ cells

On arrival in the gonads, PGCs continue to actively proliferate. In the ovary, the germ cells cease mitotic multiplication to enter meiosis, and more precisely prophase I of meiosis. Meiosis in the human species stops before birth. Prophase I is divided into five stages (leptotene, zygotene, pachytene, diplotene, diacinesis) characterized by changes in the chromosomes. The first four stages take place during fetal life, then the germ cells become blocked at the diplotene stage. The last step (diacinesis) resumes at puberty. Meiosis is eventually completed when the egg fertilizes. After entering meiosis, ovogonies are referred to as oocytes I.

II.c.3 Genetic factors involved in prophase I of meiosis

One of the first factors necessary to engage germ cells in the process of meiosis is *DAZL* (Deleted in azoospermia-like). In mice, *Dazl* is expressed by male and female germ cells (post-migration). Inactivation of *Dazl* leads to infertility in both sexes, with absence of expression of the genes involved at the start of prophase I, including *Stra8* (*Stimulated by retinoic acid gene 8*), the gene that initiates prophase I (49). Early expression of *Dazl* upstream of *Stra8* allows the germ cells to be "competent" to enter meiosis. Once the cells have become "competent", several studies showed that entry in meiosis by female mouse germ cells is dependent on retinoic acid (RA), the active metabolite of vitamin A (50, 51). Induction of meiosis by RA has also been demonstrated in humans (52). Retinoic acid binds to its RAR receptor and the receptor-ligand couple activates one of its target genes, *Stra8* (53), which is required for pre-meiotic DNA synthesis, initiation of meiosis and progression of meiosis in germ cells.

II.d Formation of primordial follicles

Follicles form when the oocytes are blocked in prophase I of meiosis, from 14-15 weeks of gestation, in the human species. This process begins when the germ cells have colonized the gonads and divide to form clusters called cysts (or syncytia). These cysts are organized in ovigerous cords, where cytoplasmic bridges, surrounded by somatic ovarian cells, connect the oocytes to each other. The formation of primordial follicles results from the fragmentation of the ovigerous cords. These primordial follicles then consist of an oocyte surrounded by a few flattened somatic cells, the future granulosa cells. The different stages of follicle maturation are controlled by many factors and require a very precise dialogue between the oocyte and the somatic cells that surround it.

Among the important factors involved in the formation of primordial follicles are *FIGLA* factor, *SOHLH* factors, neurotrophins and *FOXL2*. In the ovaries of *Foxl2* *-/-* mice, the growing oocytes are (and remain) surrounded by a single layer of granulosa cells. This indicates that granulosa cell differentiation has been altered from the early stages of folliculogenesis. In addition, the oocytes remain clustered (up to 10 oocytes) and are surrounded by a layer of support cells. These structures persist for up to 8 weeks after birth as clusters of unfragmented oocytes and ovarian cords. These data show that *FOXL2* is necessary for the fragmentation of cysts and the formation of primordial follicles (43).

III. MAINTAINING THE OVARY

FOXL2, guardian of ovarian identity

Many active mechanisms maintain the differentiated state of the ovary. The “testicular” genes must be constantly suppressed. A major player in this process is *FOXL2*. In adult female mice, it is interesting to note that targeted *Foxl2* gene knock-out in follicular cells induces expression of the male gene *Sox9* in the cells; in such animals, follicular cells in the ovary then differentiate into Sertoli cells (44).

FOXL2 is expressed by granulosa cells, where it directly controls expression of aromatase, the key enzyme in the production of estradiol (54). It is by the joint action of *FOXL2* and estrogens, via their receptors (ER), that the major gene of male identity, *Sox9*, is kept silent. *FOXL2* works synergistically with the alpha estradiol receptor (ER α) and prevents the trans-differentiation of support cells into testicular cells in the adult ovary (55). Lack of expression of *FOXL2* or of both types of estrogen receptor (ER α and ER β) induces a lack of *Sox9* inhibition, inducing masculinization of somatic cells in the ovary (56). This illustrates an important role for *FOXL2*, estrogens and their two receptors ER α and ER β . These experiments showed that, in addition to its role in the morphogenesis of the female gonad, *FOXL2*, in adulthood, is a guardian of ovarian identity.

Another key factor in ovarian identity is the *Rspo1* gene. On the one hand, as described above, it plays a role in the inhibition of the *Sox9* gene by a molecular path distinct from that of *FOXL2*. On the other hand, in addition to its role in determining and differentiating PGCs in follicular cells (34), *Rspo1* is inhibited after birth, allowing maintenance of homeostasis in the adult ovary (57).

CONCLUSION

Different sexual developments (DSD) are rare pathologies, affecting between 1/4,500 and 1/5,000 births. Since the Chicago consensus conference, they have been classified as chromosomal, gonadal or phenotypic anomalies. There are 46,XX and 46,XY DSDs. Some etiologies are linked to an abnormality in the development of the gonad. Description of certain patients highlighted many factors involved in differentiating the undifferentiated

gonad toward the ovary. It is now recognized that there is a positive gonad differentiation, with the action of anti-testicular and pro-ovarian factors, such as *R-Spondin 1* and the *WNT4/β* catenin pathway. However, to date, many cases of DSD remain unresolved. New techniques in genetics, and particularly whole-genome study, should improve knowledge of ovarian determination. An interesting perspective is based on the fact that certain genes involved in gonad determination have been shown to be mutated in infertile patients, even in the absence of DSD. Therefore, research on gene differentiation in the ovarian gonad may in the near future elucidate certain so-called idiopathic etiologies of infertility.

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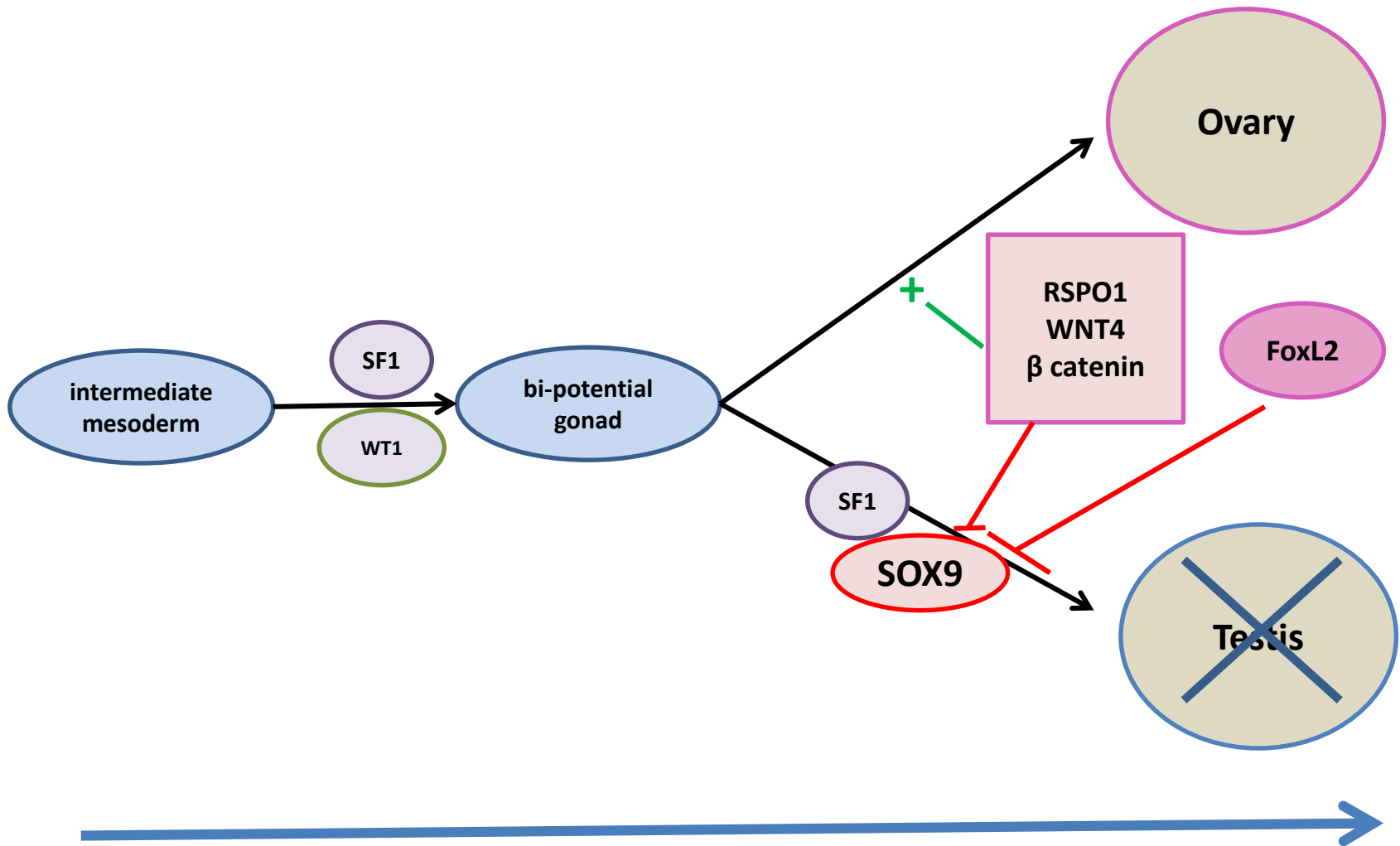


Figure 1. Factors involved in ovarian development over time before birth

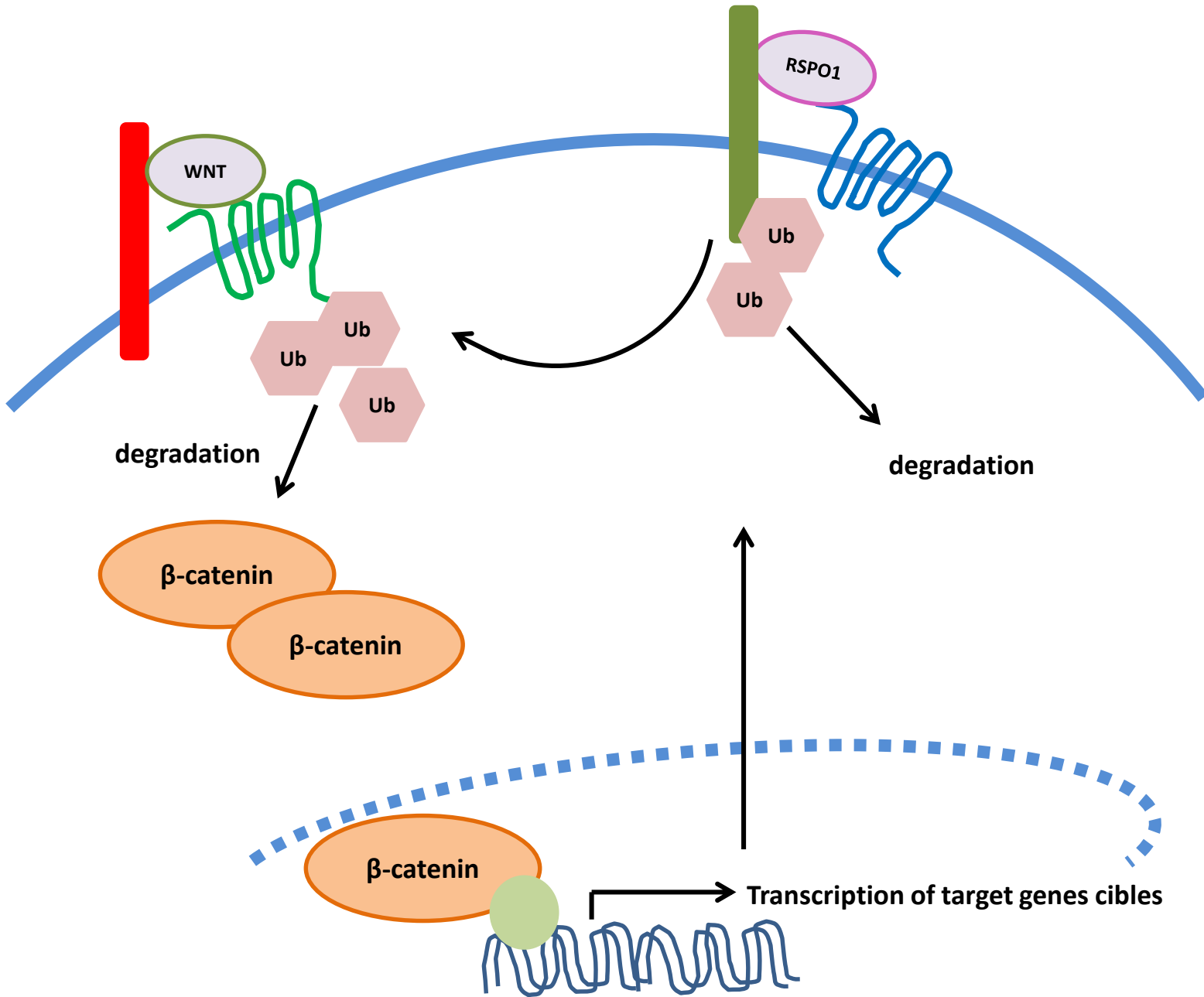


Figure 2. WNT/β-catenin and RSP01 signaling pathway