

Positional cloning of the PIS mutation in goats and its impact on understanding mammalian sex-differentiation

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Abstract – In goats, the PIS (polled intersex syndrome) mutation is responsible for both the absence of horns in males and females and sex-reversal affecting exclusively XX individuals. The mode of inheritance is dominant for the polled trait and recessive for sex-reversal. In XX PIS^{-/-} mutants, the expression of testis-specific genes is observed very precociously during gonad development. Nevertheless, a delay of 4–5 days is observed in comparison with normal testis differentiation in XY males. By positional cloning, we demonstrate that the PIS mutation is an 11.7-kb regulatory-deletion affecting the expression of two genes, *PISRT1* and *FOXL2* which could act synergistically to promote ovarian differentiation. The transcriptional extinction of these two genes leads, very early, to testis-formation in XX homozygous PIS^{-/-} mutants. According to their expression profiles and bibliographic data, we propose that *FOXL2* may be an ovary-differentiating gene, and the non-coding RNA *PISRT1*, an anti-testis factor repressing *SOX9*, a key regulator of testis differentiation. Under this hypothesis, *SRY*, the testis-determining factor would inhibit these two genes in the gonads of XY males, to ensure testis differentiation.

PIS mutation / goat / gonad differentiation / XX sex-reversal / ovary development

1. HISTORY OF THE MUTATION

Spontaneously occurring hornless mutants exist in various ruminant species. They are particularly appreciated in farming since they make it unnecessary to surgically withdraw the horns, an operation currently performed on cattle and goats. In the early 1920's, pedigree analysis in goats indicated that

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hornlessness is monogenic, autosomal and dominant. This situation made it particularly easy to produce large herds of hornless goats, and the mutation, called *Polled* (P), has been extensively used in crosses in many countries (Eastern Europe, Switzerland, France, South America, Israel...). One surprising issue of hornless goat herds is the existence of abnormal sex-ratios, with an excess of male animals, amongst which many are in fact sterile. Some of these males appear as sex-reversed XX individuals, since the genetic sex is discordant from the phenotypic sex. A key linkage between hornlessness and intersexuality was published as early as 1944 [1], indicating unambiguously that whereas hornlessness is inherited in a dominant fashion, intersexuality is recessive. Other XX male sex-reversals have been identified in many mammalian orders, but only in goats is the phenotype so clear-cut, to the point where genetic females can be confounded with normal males. The existence of a visible marker (absence of horns) makes goats a very interesting model for identifying key sex-determining genes in mammals. Beside its sex-reversing effect on XX individuals, the *Polled* mutation (renamed PIS for polled intersex syndrome) is also responsible for infertility in PIS^{-/-} XY individuals, resulting apparently from a mechanical obstruction of the epididymis. Also, heterozygous PIS^{+/-} individuals appear mildly hyperfertile.

2. LINKAGE ANALYSIS OF THE PIS MUTATION

With the development of genomic tools and especially microsatellite markers, linkage analysis of the PIS mutation has become possible. Taking advantage of the dominance of the hornless trait, families were collected in French herds, and genome scanning located the mutation to goat chromosome 1 on band 1q43 [14, 15]. Its genetic localization was then refined by the targeted isolation and genotyping of new microsatellite markers using comparative mapping and selected chromosomal fractions. The PIS interval was thus reduced to a ~1 cM region of CHI1q43. The human homologous region was precisely identified as a HSA3q23 DNA segment encompassing the *COPB2* gene formerly "*COP*" [16].

In order to clone the PIS mutation, a chromosome walk inside the region of interest has been realized, leading to the isolation of 91 BAC from a constructed goat BAC library [11]. This led to the construction of six BAC contig elements of CHI1q43 (ICC1 to ICC6) covering altogether 4.5 Mb, one of which (ICC3, 1500 kb) encompasses the PIS locus (Fig. 1). Thirty polymorphic microsatellites were isolated from this 4.5 Mb region. Genotyping showed that ICC3 encompasses a ~400 kb interval where no recombinants are

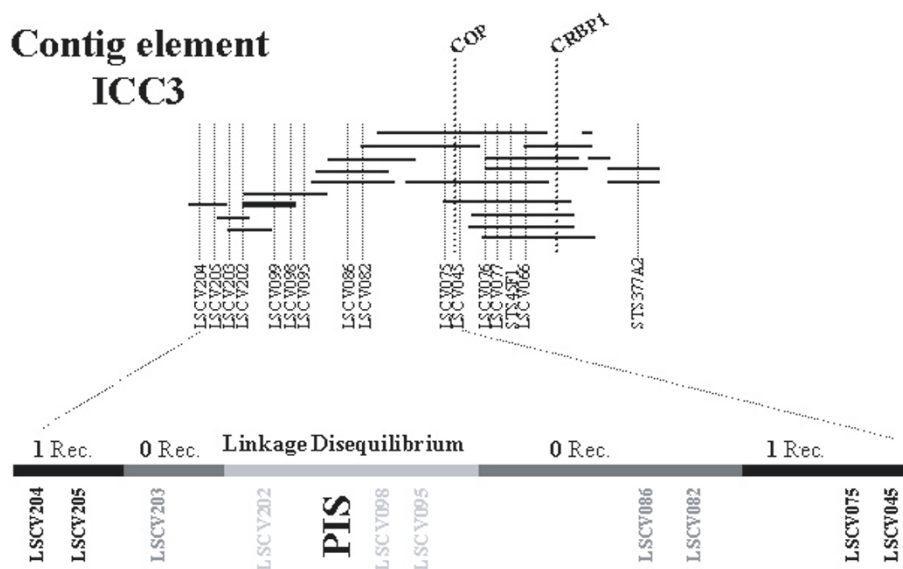


Figure 1. Details of the sub-contig ICC3 enclosing the PIS mutation. The lines in the upper part represent the BAC (the thick one: BAC 376H9). The lower part represents different genomic regions surrounding the mutation, with 1 recombinant (black) to 0 recombinants, and in the middle the region presenting a linkage disequilibrium (light grey). The position of two genes (*COP2* and *RBPI*) with those of 16 genetic markers (LSCV or STS) are indicated by dotted lines.

observed inside the resource families. Furthermore, a strong linkage disequilibrium among unrelated animals was detected with the two central markers of this region, suggesting a probable location for PIS in a ~100 kb DNA region contained in one BAC (376H9). High resolution comparative mapping with human data shows that this DNA segment is the homologue of the human region associated with Blepharophimosis Ptosis Epicanthus inversus Syndrome, BPES [12]. This finding suggests that a homologous gene(s) could be responsible for the pathologies observed in humans and goats.

3. POSITIONAL CLONING OF THE PIS MUTATION

Four microsatellites were isolated from the goat BAC 376H9, making it possible to characterize haplotypes associated with the PIS mutation. In order to characterize this BAC transcriptionally, it was subcloned in a specific exon-trapping vector. The subclones were transfected into COS-7 cells, exogenous mRNA was prepared and reverse-transcribed 12 h after transfection.

Sequencing made it possible to obtain 20 putative exons, one of which displayed a specifically gonadal pattern of expression. This element belongs to a 1.5 kb transcript without an open reading frame that we called *PISRT1* for PIS-regulated transcript 1. In order to physically position the coding and non-coding markers inside 376H9, a systematic sequencing approach was used. The BAC was first subcloned in large (~10 kb) plasmid inserts, which were then sequenced by a primer-walking approach. The sequences were then assembled on ~70 kb of the BAC. By haplotype analysis, the mutation was located between the two markers LSCV210 and LSCV211. The number of recombinant haplotypes between these two markers suggested that they are physically separated by ~25 kilobases. A targeted effort was performed towards this region, that could not be cloned in large plasmids due to its richness in repetitive sequences. Each progress in the sequence was monitored by PCR on genomic DNA from normal and *PIS*^{-/-} individuals. This screen enabled us to define a deleted element in all affected individuals. Sequencing through the deletion made it possible to define an 11.7 kb DNA fragment that is missing in mutant goats (Fig. 2). This element is composed for 80% of two copies of a partial ruminant LINE (2.2 and 2.6 kilobases), oriented head to head, 25 kilobases apart from *PISRT1*. There is no evidence of any coding sequence in the deleted fragment, suggesting that it probably affects the expression of this chromosome region by a long-range effect on chromatin structure [8].

4. PIS IS A TRANSCRIPTIONAL REGULATORY REGION

Expression of *PISRT1* was studied first. This gene has a dimorphic expression pattern in the gonads of normal fetuses, with higher levels in the ovaries compared with testes during fetal life. Expressional studies were also performed on *FOXL2*, following its isolation in humans and its characterization as the gene responsible for BPES (OMIM 110100). This dominant syndrome leads to eyelid malformation in both sexes associated with female infertility [4]. In goats, *FOXL2* was localized at around 300 kb from the PIS deletion (Fig. 2).

The *PISRT1* and *FOXL2* transcription levels are found to be sexually dimorphic in the gonads from 36 days *post-coitum* (*dpc*), a stage corresponding to the beginning of testicular formation in males [9]. The expression levels of the two genes are higher in ovaries than in the testes of normal fetuses. Using *FOXL2*-specific antibodies, the protein has been investigated in the gonads of both sexes and has only been detected in the ovaries from 36 *dpc* to adulthood [3]. In XX *PIS*^{-/-} sex-reversed gonads, transcription levels were greatly reduced

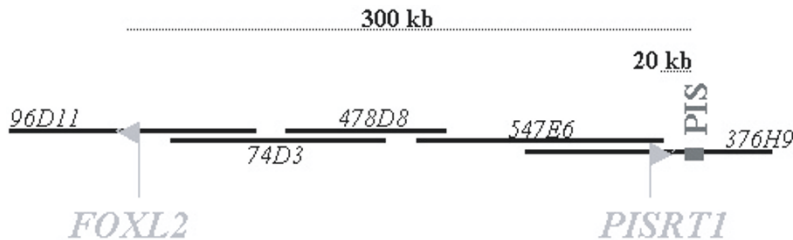


Figure 2. Schematic representation of the PIS region. The PIS mutation is represented on the right. It contains transcriptional regulatory elements of two genes (arrowheads), *PISRT1* and *FOXL2*, located at 20 and 300 kb from PIS, respectively. The lines represent the different BAC encompassing the region. The BAC addresses are given in italics.

as early as 36 *dpc* for *FOXL2* (Fig. 3) and only four days later, at 40 *dpc*, for *PISRT1*. When studied at later stages (56, 70 *dpc* and after birth), the transcription levels of these two genes remain practically undetectable in homozygous *PIS*^{-/-} sex-reversed gonads [8]. Moreover, *FOXL2* transcription levels were found to be similar between normal *PIS*^{+/+} and heterozygous *PIS*^{+/-} ovaries. This may explain why the heterozygous goats are fertile, as opposed to heterozygous *FOXL2*^{+/-} women affected by BPES type I [5].

FOXL2 and *PISRT1* are also expressed in goat developing eyelids (studied at 36 *dpc*), but their transcriptional levels are not affected by the PIS mutation, in accordance with the fact that there is no BPES phenotype in goats. In contrast, the transcriptional regulatory action of the PIS region on the two genes is also visible in the horn buds. Expression of *PISRT1* and *FOXL2* significantly increased in the horn buds of *PIS*^{-/-} and *PIS*^{+/-} 70 *dpc* fetuses, as compared with *PIS*^{+/+} controls [8].

In summary, the transcriptional effects of the PIS mutation are in complete agreement with the mode of inheritance of both traits. The polled trait is dominant and results from a gain of function mutation, precisely a gain of expression for the two genes. The XX sex-reversal trait is recessively inherited and results from a loss of function mutation (loss of expression). However, the role of each gene in the two phenotypes remains to be determined.

5. REPLACEMENT OF THE PIS-REGULATED GENES IN THE GONAD-DIFFERENTIATING CASCADE

Sex determination is governed by a series of genetic switches that influence cell fate and differentiation during critical periods of gonadal development.

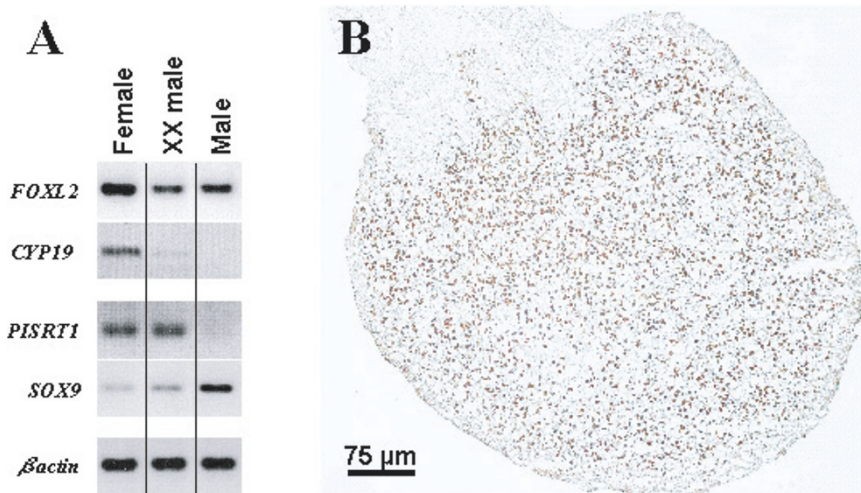


Figure 3. Expression profiles of sex-differentiating genes in the gonads of goat fetuses. (A) RT-PCR analysis of the PIS-regulated genes (*PISRT1* and *FOXL2*) with two other genes (*SOX9* and *CYP19* encoding the cytochrome P450 aromatase) in the genital ridges (gonad + mesonephros) of 36 dpc fetuses. At this stage, *FOXL2* expression is already affected in the XX sex-reversed gonads and the expression of *CYP19* is decreased. In contrast, *PISRT1* and *SOX9* expressions are the same as in the normal female. (B) Immuno-localization of the FOXL2 protein in a 45 dpc ovary. Despite the fact that some transcripts are detected in the male gonads (see in A), the protein FOXL2 has only been found in the ovaries and the eyelids.

In mammals, the identification of the testis-determining gene, *SRY*, has been a pivotal first step towards the unravelling of this genetic pathway [2]. It is now clear that numerous other genes, in addition to *SRY*, are necessary for normal testis development, such as *SOX9*, *WT1*, *SF1*, *DAX1*, etc. [6]. The analyses of these genes in humans with gonadal dysgenesis or sex-reversal, mouse models and using *in vitro* cell culture assays have revealed that sex determination results from a complex interplay between the genes in this network (Fig. 4). In contrast to the male pathway, few genes are known to be involved in ovary differentiation. Only two factors are known to be preferentially expressed in the early developing ovary of rodents, namely *Dax1* [13] and *Wnt4* [17]. These factors appear to act primarily as repressors of male development, rather than as active female-promoting signals.

Among the genes regulated by the PIS locus, *FOXL2*, encoding a fork-head transcription factor appears as an early determinant of ovarian differentiation. Its sexually dimorphic expression in embryonic ovaries of humans, ruminants, mice, chickens, and turtles, representatives of phylogenetically distant

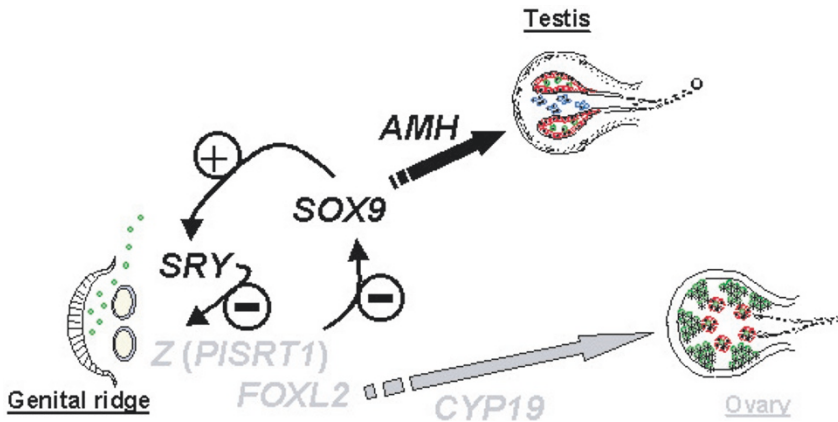


Figure 4. A schematic model of the sex-differentiation cascade with the PIS-regulated genes. In this model, *FOXL2* regulates *CYP19* gene expression and is determinant for the differentiation of the ovary. *PISRT1* acts as an anti-testis factor by inhibiting *SOX9* in the female pathway. Under this hypothesis, *PISRT1* must be inhibited in the male pathway; the sole candidate for triggering this inhibition remains *SRY*.

vertebrate groups, is consistent with a crucial role of *FOXL2* in the ovary-differentiation pathway [3, 4, 7, 10]. In addition, mutations in the *FOXL2* gene are responsible for BPES, a complex human disorder characterized primarily by malformations of the eyelids and other facial anomalies. Another key feature of BPES is gonadal dysgenesis or premature ovarian failure in a high proportion of female patients [4, 5]. In *PIS*^{-/-} goats, in which *FOXL2* expression is abolished, ovaries do not differentiate but testis-differentiation takes place inducing sex-reversal. The first molecular effect observed after the absence of *FOXL2* expression is a decreased transcription of the *CYP19* gene encoding the cytochrome P450 aromatase, responsible for the conversion of androgens into estrogens (Fig. 3). Moreover, the levels of aromatase transcripts were highly correlated with the expression profile of the *FOXL2* gene in goats and chickens and both proteins co-localize in the same gonadal area (data not shown). These data indicate that *FOXL2* may be considered as a possible regulator of aromatase gene expression in certain species of vertebrates. In adult mice, *FOXL2* transcription was detected in some oocytes, as well as in the granulosa cells and in cells of the *corpus luteum* [4, 7, 10]. In conclusion, *FOXL2* plays not only a primordial role in ovarian differentiation, but possibly also in follicle and oocyte maturation.

The second gene affected by the PIS mutation is a non-coding RNA, called *PISRT1*. Its expression is also suppressed in the ovaries of *PIS*^{-/-} animals but

later than *FOXL2*. Concomitantly with the *PISRT1* expression decrease, *SOX9* expression is up-regulated and the testis-differentiation commences, as attested by the presence of AMH (Anti-Müllerian Hormone) and testicular cord formation. Based on this observation and the fact that mutations of *FOXL2* alone are never associated to sex-reversed phenotypes but only with ovarian failure, we proposed that *PISRT1* could be an inhibitory factor of male genes and especially of the *SOX9* gene. On this assumption, the absence of *PISRT1* would be responsible for the masculinization of the $PIS^{-/-}$ female gonads by inducing *SOX9*. *PISRT1* expression has also been studied in mice, where, in contrast to goats, it is only expressed in adult ovaries [10]. This observation reinforces the idea that two distinct mechanisms of sex-determination could exist in mammals. The first, specific for the mouse, in which *Sry*, the trigger of a male cascade, directly activates *Sox9* which is then maintained to a male-specific threshold by an auto-regulatory loop. Rapidly *Sry* becomes unnecessary and its expression window is narrowed. In the second model, represented by ruminants and probably also humans, *SRY* inhibits anti-testis factors, such as *PISRT1*, via the PIS region (Fig. 4). This inhibition permits the activation of *SOX9* which must be maintained for a long time. This is consistent with the fact that in most mammals, *SRY* expression persists during a long period in order to initiate and maintain testis differentiation. Finally, the existence of a subtly-regulated balance between activator and repressor factors could participate in enhancing gene dosage sensibility of certain species, like humans, in sex-determination.

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REFERENCES

- [1] Asdell S.A., The genetic sex on intersexual goats and a probable linkage with the gene for hornlessness, *Science* 99 (1944) 124.
- [2] Berta P., Hawkins J.R., Sinclair A.H., Taylor A., Griffiths B.L., Goodfellow P.N., Fellous M., Genetic evidence equating *SRY* and the testis-determining factor, *Nature* 348 (1990) 448–450.

- [3] Cocquet J., Pailhoux E., Jaubert F., Servel N., Xia X., Pannetier M., De Baere E., Messiaen L., Cotinot C., Fellous M., Veitia R.A., Evolution and expression of *FOXL2*, *J. Med. Genet.* 39 (2002) 916–921.
- [4] Crisponi L., Deiana M., Loi A., Chiappe F., Uda M., Amati P., Bisceglia L., Zelante L., Nagaraja R., Porcu S., Ristaldi M.S., Marzella R., Rocchi M., Nicolino M., Lienhardt-Roussie A., Nivelon A., Verloes A., Schlessinger D., Gasparini P., Bonneau D., Cao A., Pilia G., The putative forkhead transcription factor *FOXL2* is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome, *Nat. Genet.* 27 (2001) 159–166.
- [5] De Baere E., Dixon M.J., Small K.W., Jabs E.W., Leroy B.P., Devriendt K., Gillerot Y., Mortier G., Meire F., Van Maldergem L., Courtens W., Hjalgrim H., Huang S., Liebaers I., Van Regemorter N., Touraine P., Praphanphoj V., Verloes A., Udar N., Yellore V., Chalukya M., Yelchits S., De Paepe A., Kuttent F., Fellous M., Veitia R., Messiaen L., Spectrum of *FOXL2* gene mutations in blepharophimosis-ptosis-epicanthus inversus (BPES) families demonstrates a genotype-phenotype correlation, *Hum. Mol. Genet.* 10 (2001) 1591–1600.
- [6] Koopman P., The genetics and biology of vertebrate sex determination, *Cell* 105 (2001) 843–847.
- [7] Loffler K.A., Zarkower D., Koopman P., Etiology of ovarian failure in blepharophimosis ptosis epicanthus inversus syndrome: *FOXL2* is a conserved, early-acting gene in vertebrate ovarian development, *Endocrinology* 144 (2003) 3237–3243.
- [8] Pailhoux E., Vigier B., Chaffaux S., Servel N., Taourit S., Furet J.-P., Fellous M., Grosclaude F., Cribiu E.P., Cotinot C., Vaiman D., A 11.7-kb deletion triggers intersexuality and polledness in goats, *Nat. Genet.* 29 (2001) 453–458.
- [9] Pailhoux E., Vigier B., Vaiman D., Servel N., Chaffaux S., Cribiu E.P., Cotinot C., Ontogenesis of female-to-male sex-reversal in XX polled goats, *Dev. Dyn.* 224 (2002) 39–50.
- [10] Pannetier M., Servel N., Cocquet J., Besnard N., Cotinot C., Pailhoux E., Expression studies of the PIS-regulated genes suggest different mechanisms of sex determination within mammals, *Cytogenet. Genome Res.* 101 (2003) 199–205.
- [11] Schibler L., Vaiman D., Oustry A., Guinec N., Dangy-Caye A.-L., Billault A., Cribiu E.P., Construction and extensive characterization of a goat Bacterial Artificial Chromosome library with threefold genome coverage, *Mamm. Genome* 9 (1998) 119–124.
- [12] Schibler L., Cribiu E.P., Oustry-Vaiman A., Furet J.-P., Vaiman D., Fine mapping suggests that the goat *Polled Intersex Syndrome* and the human *Blepharophimosis Ptosis Epicanthus Syndrome* map to a 100-kb homologous region, *Genome Res.* 10 (2000) 311–318.
- [13] Swain A., Zanaria E., Hacker A., Lovell-Badge R., Camerino G., Mouse *Dax1* expression is consistent with a role in sex determination as well as in adrenal and hypothalamus function, *Nat. Genet.* 12 (1996) 404–409.

- [14] Vaiman D., Koutita O., Oustry A., Elsen J.-M., Manfredi E., Fellous M., Cribiu E.P., Genetic mapping of the autosomal region involved in XX sex-reversal and horn development in goats, *Mamm. Genome* 7 (1996) 133–137.
- [15] Vaiman D., Pailhoux E., Schibler L., Oustry A., Chaffaux S., Cotinot C., Fellous M., Cribiu E.P., Genetic mapping of the polled/intersex locus (PIS) in goats, *Theriogenology* 47 (1997) 103–109.
- [16] Vaiman D., Schibler L., Oustry-Vaiman A., Pailhoux E., Goldammer T., Stevanovic M., Furet J.-P., Schwerin M., Cotinot C., Fellous M., Cribiu E.P., High-resolution human/goat comparative map of the goat polled/intersex syndrome (PIS): the human homologue is contained in a human YAC from HSA3q23, *Genomics* 56 (1999) 31–39.
- [17] Vainio S., Heikkilä M., Kispert A., Chin N., McMahan A.P., Female development in mammals is regulated by Wnt-4 signalling, *Nature* 397 (1999) 405–409.