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Mammalian gonadal differentiation: the pig model

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In mammals, testicular differentiation is initiated by SRY (the sex-determining region of the Y chromosome) gene expression in Sertoli cell precursors, followed by upregulation of the SOX9 gene (SRY-related HMG box gene 9). Subsequently, differentiated testis produces two hormones that induce sexual differentiation of the internal and external genital tract. Knowledge of the molecular mechanisms involved in gonadal differentiation has increased greatly over the past decade. Several genes are involved in genital ridge formation in both sexes, and others act specifically in testicular or ovarian developmental pathways. As for other mammals, relatively few data are available on the first steps of ovarian differentiation in pigs. In this review, the expression profiles of most genes known to be involved in gonadal differentiation in pigs will be presented and compared with those observed in mice. The main feature of gonadal differentiation in the pig is fetal steroidogenesis, especially cytochrome P450 aromatase gene organization and expression. Another specific feature of gonadal differentiation in pigs is the appearance of numerous cases of XX sex-reversed animals. This intersex condition occurs as early as day 50 after coitus, during embryogenesis, and appears to be triggered genetically. It leads to a wide range of phenotypes, strikingly similar to those observed in humans. Identification of the genes involved in this pathology will improve our knowledge of mammalian gonadal differentiation and may allow the eradication of this genetic disease in pigs.

Introduction

In pigs, as in other mammals, sexual differentiation is achieved following two imbricated steps (Fig. 1). The first step leads to gonadal formation from a bipotential primordium localized at the anterior part of the mesonephros: the urogenital ridge. This developmental event, called sex determination, depends on XX or XY chromosomal status determined at the time of fertilization. The presence of a single gene on the Y chromosome, SRY, is sufficient to induce testicular differentiation (Sinclair *et al.*, 1990). In females, the absence of SRY testis-determining factor and the presence of two X chromosomes allows formation of the ovaries. The second step of sexual differentiation, known as secondary differentiation, leads to the development of internal and external genitalia. The internal ducts of males and females arise from the Wolffian and Müllerian anlagen, respectively. Female differentiation has been

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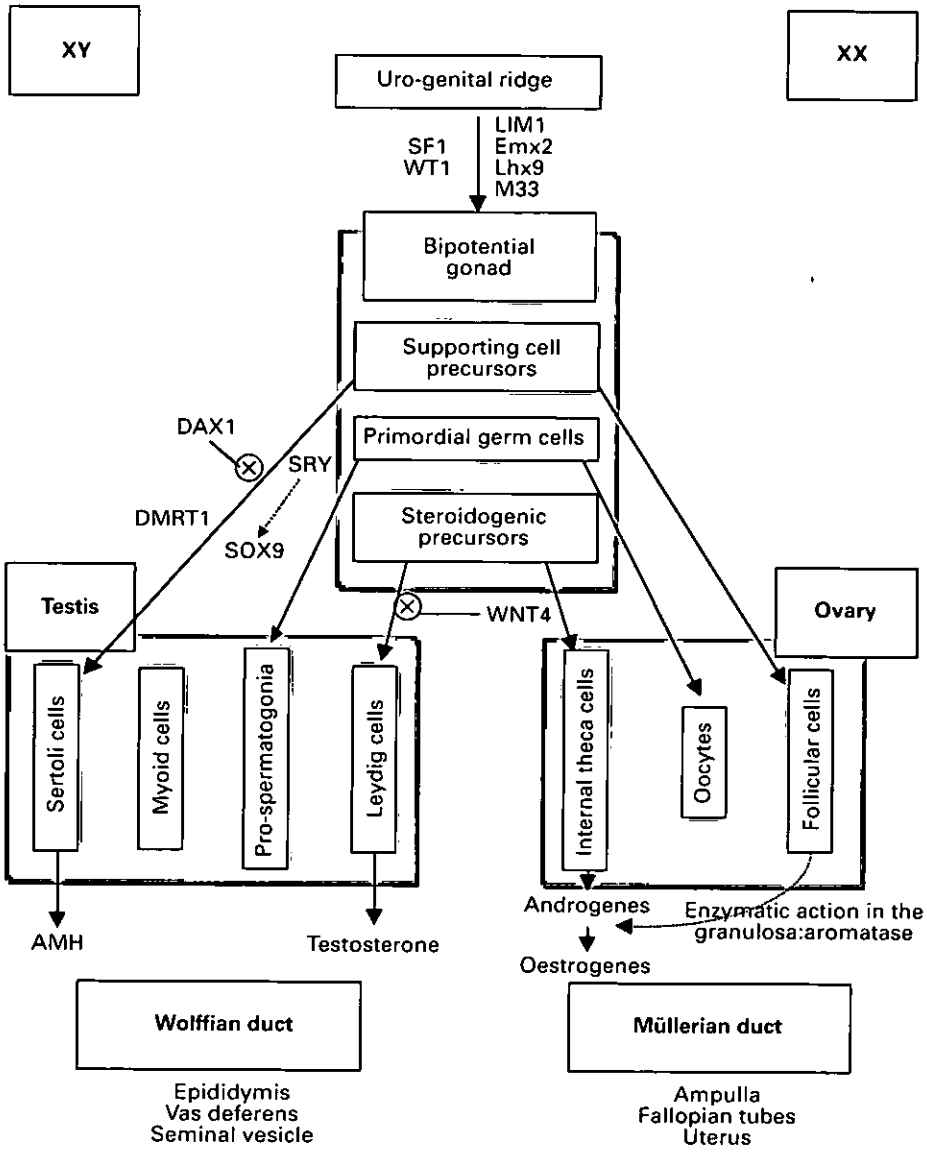

Fig. 1. Simplified representation of mammalian sexual differentiation. The left part of the figure shows male development and the right part shows the female pathway. The different steps in this developmental process are boxed, from upper to lower, chromosomal sex, gonadal sex and internal phenotypic sex. The central part of the scheme represents the sex-specific fate of the different gonadal cell types in the bipotential gonad. Their differentiated counterparts are indicated in the final gonad (testis or ovary). Myoid cells of the testis have no precursors in the bipotential gonads because they migrate from the mesonephros. Principal genes implicated in gonadal differentiation are indicated. Crossed circles depict inhibitory regulations. LIM1 = Lhx1: LIM homeobox gene 1; Lhx9: LIM homeobox gene 9; Emx2: empty spiracles, *Drosophila* homologue 2; M33 = CBX2: chromobox homologue 2, *Drosophila* polycomb class; SF1: steroidogenic factor 1; WT1: Wilms' tumour gene 1; DAX1: DSS-AHC critical region on the X chromosome, gene 1; SRY: sex-determining region of Y chromosome; SOX9: SRY-related HMG box gene 9; DMRT1: doublesex- and Mab-3-related transcription factor 1; WNT4: wingless-type MMTV integration site family, member 4; AMH: anti-Müllerian hormone.

considered as the default pathway, which is 'overridden' in males by two testicular hormones: the anti-Müllerian hormone (AMH) and testosterone (Jost *et al.*, 1973). The production of AMH by Sertoli cells acts to suppress the Müllerian ducts, thereby preventing the formation of Fallopian tubes and uteri in males, whereas testosterone, secreted by Leydig cells, induces the formation of epididymis, vas deferens and seminal vesicles. The same process of a default pathway is involved in the formation of external genitalia (urethra, prostate gland, penis and scrotum), which differentiate from the urogenital sinus only in the presence of a male hormone: dihydrotestosterone.

This review will focus essentially on the first step of mammalian sexual differentiation, which is determined genetically. Firstly, the genes involved in this primary pathway will be described and their expression patterns in male and female pigs will be compared with those described in other species. In a second section, results concerning XX sex reversal, an abnormal gonadal differentiation observed frequently in pigs, will be presented.

Molecular basis of gonadal differentiation

The testis-determining factor: SRY

All the genes involved in gonadal differentiation have been discovered during the past decade. In 1990, the first, SRY, was isolated after 25 years of research. Indeed, Ferguson-Smith (1966) predicted that after uneven crossover between sex chromosomes during male meiosis, the testis-determining factor (TDF) could translocate from Y to X and cause sex reversal in humans. After numerous genetic studies on sex-reversed patients, a candidate gene was found in a 35 kb Y DNA fragment present in an XX male patient: SRY (Sinclair *et al.*, 1990). Thereafter, two pieces of experimental evidence were collected proving that SRY is TDF: (i) the open reading frame of SRY presents a mutation in XY female patients with gonadal dysgenesis (Cameron and Sinclair, 1997); and (ii) a 14.6 kb fragment containing the murine homologue (*Sry*) reverses the sex of XX mice when added by transgenesis (Koopman *et al.*, 1991). This intronless gene has been found in most mammalian species and represents the master gene in testicular differentiation. In pigs it has been located in the distal portion of the short arm of the Y chromosome (p12–p13), which is a conserved chromosomal position compared with that of humans (Yang *et al.*, 1993). SRY encodes a nuclear factor encompassing a conserved DNA binding domain of 79 amino acids (HMG box). Surprisingly, outside this domain, the SRY protein is poorly conserved among mammals, a fact that is particularly true for humans and mice (Whitfield *et al.*, 1993). Despite 10 years of research, the biological target of SRY and its molecular mechanism of action remain unknown.

The other genes

Sex determination appears to be a complex process involving two types of genes: some of the genes are implicated in the formation of the genital ridges in both sexes, whereas others are involved specifically in testis or ovary differentiation. To date, six genes encoding transcription factors have been classified in the first group: Wilms' tumour gene 1 (WT1), steroidogenic factor 1 (SF1), three homeobox genes (LIM1, Emx2 and Lhx9) and M33, a mouse homologue of the *Drosophila* polycomb genes. Five genes belong to the second group: the testis determining factor SRY, the SRY-related HMG box gene 9 (SOX9), the X-linked gene DAX1, DMRT1 and a gene of the Wnt/Wingless family WNT4.

Genes involved in early gonadogenesis. WT1 was first discovered in humans by genetic analysis of patients affected by renal failure (Wilms' tumour) associated with an XY sex

reversal phenotype (Gessler *et al.*, 1990; Pelletier *et al.*, 1991). The implication of WT1 in urogenital ridge formation was demonstrated clearly by knockout experiments in mice (Kreidberg *et al.*, 1993). Indeed, homozygous mutant embryos revealed failure in gonad and kidney development. The 10 exon WT1 gene contains two alternative spliced regions, one in exon 5 and a second in exon 9, giving rise to the addition or suppression of three amino acids: lysine-threonine-serine: KTS (Haber *et al.*, 1991). Therefore, WT1 encodes four distinct transcription factors, each containing four zinc finger domains. In addition to their DNA binding activity, WT1 proteins might also bind RNA and act as post-transcriptional regulators (Caricasole *et al.*, 1996; Bardeesy and Pelletier, 1998). WT1 transcripts are expressed very early in the mesonephros, then in the genital ridges and their expression persists in the gonad of both sexes throughout life (Pritchard-Jones *et al.*, 1990; Payen *et al.*, 1996). In addition to the involvement of WT1 in genital ridge formation, WT1 isoform without KTS can regulate the gene encoding AMH (Nachtigal *et al.*, 1998). The biological significance of isoform ratios (with or without KTS) has been brought to light by studying patients with Frasier syndrome (Barbaux *et al.*, 1997). In this syndrome, XY sex reversal is a consequence of intronic mutation preventing the production of the KTS-containing isoforms, thereby altering the isoform ratios in heterozygous affected patients. In conclusion, according to the expression pattern and the complexity of this gene, it is clear that WT1 is a key factor in gonadal formation and testis differentiation in mammals.

The nuclear hormone receptor SF1 homologous to the *Drosophila* fushi-tarazu factor 1 (Ftz-F1), also termed Ad4BP (adrenal 4 binding protein), was first isolated as a common regulator of cytochrome P450 steroid hydroxylases in the gonads and adrenal cortex (Lala *et al.*, 1992; Morohashi *et al.*, 1992). In the testis, expression of SF1 has been identified in Leydig cells as well as in Sertoli cells, where it contributes to maintaining high AMH expression (Shen *et al.*, 1994; Arango *et al.*, 1999). Another critical role in gonadal and adrenal development has been demonstrated by knockout experiments: mice lacking SF1 are devoid of gonads and adrenal glands (Luo *et al.*, 1994). Recently, a similar phenotype has been described in an XY woman patient harbouring a heterozygous 2 bp substitution in the DNA binding domain of SF1 (Achermann *et al.*, 1999). Hence, it is clear that SF1 is implicated at numerous steps in sexual differentiation as it regulates a large array of genes involved in gonadal formation and hormonal synthesis.

In addition to WT1 and SF1, four other genes involved in urogenital ridge formation have been identified by knockout experiments in mice. Three of these genes belong to the homeobox gene family. Lhx9 invalidation results in failure in gonad formation without any additional major developmental defects (Birk *et al.*, 2000). Invalidation of the other two genes leads to a more marked change in the phenotype, with an absence of head structures for LIM1, and of kidneys and gonads for LIM1 and Emx2 (Shawlot and Behringer, 1995; Miyamoto *et al.*, 1997). The last gene in this group, M33, is one of the *Drosophila* polycomb genes. M33 null mice underwent a significant delay in gonadogenesis, leading to male-to-female sex reversal in XY fetuses (Katoh-Fukui *et al.*, 1998). The involvement of these genes in urogenital ridge formation has been demonstrated clearly in gene disruption experiments, but this strategy makes it impossible to reveal other putative functions later in sexual differentiation (such as WT1 and SF1). Conditional knockout will be needed to gain further information.

Sex differentiating genes. The Y specific gene SRY initiates a cascade of gene regulations resulting in maleness. One of the most important genes downstream from SRY is SOX9, which encodes a key transcription factor for testicular differentiation. SOX9 was first isolated by studying patients with a bone developmental defect, campomelic dysplasia, associated with 75% of XY individuals with a male-to-female sex reversal (Foster *et al.*, 1994; Wagner *et al.*,

1994). The structure of SOX9 is that of a typical transcription factor with a DNA binding domain (HMG box) and a transcriptional trans-activating domain (Südbeck *et al.*, 1996). From an evolutionary viewpoint, the structure and expression pattern of SOX9 appear to be highly conserved among mammals as well as in other vertebrates, such as birds and reptiles (Kent *et al.*, 1996; Morais da Silva *et al.*, 1996; Western *et al.*, 1999). A high expression of SOX9 is always correlated with testicular differentiation, independent of the presence of SRY. Abnormal upregulation of SOX9 in XX individuals is associated with female-to-male sex reversal in humans and mice (Huang *et al.*, 1999; Bishop *et al.*, 2000). It is now clear that SOX9 lies at a crucial step in testis formation and that progress in the understanding of this differentiation pathway could result from studies on its transcriptional regulation. However, defects in the expression of SOX9 leading to sex reversal indicate that there may be long range regulatory elements distributed as far as 1 Mb upstream from the coding region of the gene (Wunderle *et al.*, 1998; Pfeifer *et al.*, 1999). This fact has also been pointed out in XX mice with the description of the Odsex (ocular degeneration with sex reversal) mutation consisting of a 150 kb deletion localized at about 1 Mb upstream from *Sox9*. The authors conclude that the deletion encloses a female specific repressor binding site in this 5′ region (Bishop *et al.*, 2000), strengthening the suspicion that male specific genes such as SOX9 may be repressed in XX individuals (McElreavey *et al.*, 1993).

One putative repressor of male genes was discovered in 1994 by studying XY sex-reversed female patients harbouring a partial duplication (band p21) of their X chromosome (Bardoni *et al.*, 1994). In this dosage-sensitive sex reversal region (DSS), the DAX1 gene (DSS-AHC critical region on the X chromosome) appears to be the best candidate for triggering the absence of testicular differentiation by acting antagonistically to SRY (Swain *et al.*, 1998). This possibility was confirmed by the characterization of the *Dax-1* expression pattern in mice, which was expressed more highly in ovaries than in testes (Swain *et al.*, 1996). According to all these results, DAX1 was assumed to be a potential ovarian differentiating factor until the observation of *Dax-1*-null mice. Surprisingly, the disruption of *Dax-1* in female mice does not prevent ovarian differentiation or fertility (Yu *et al.*, 1998). In contrast, male spermatogenesis is affected in XY *Dax-1* –/Y mice.

A true ovarian determining gene was discovered by knockout experiments of *Wnt-4*, a signalling protein of the Wnt/Wingless family (Vainio *et al.*, 1999). Homozygous disruption of *Wnt-4* triggers the development of Leydig cells and steroidogenesis in female gonads. In contrast, sexual differentiation in *Wnt-4* –/– males appears to be normal at birth. The female dimorphic expression pattern of *Wnt-4* in mice also provides evidence for an ovary-differentiating role of this gene (Vainio *et al.*, 1999).

In conclusion, many factors involved in gonadal differentiation have been isolated and characterized over the past decade, but there may be other factors that are still unknown. For example, the genetic aetiology of many cases of sex reversal in humans and mammalian species remains to be elucidated and does not appear to be the result of mutations within known genes (Vaiman and Pailhoux, 2000). Monosomic deletions of chromosome 9p associated with male-to-female XY sex reversal in humans have indicated the possible role of DMRT1 in testis differentiation (Calvari *et al.*, 2000; Ottolenghi *et al.*, 2000). This gene, which is homologous to doublesex in *Drosophila* and mab-3 in *Caenorhabditis* (Raymond *et al.*, 1998), plays a crucial role in testis formation in reptiles and birds (Raymond *et al.*, 1999; Kettlewell *et al.*, 2000).

Relationships among sex differentiating genes (Fig. 2)

Many studies have focussed on transcriptional regulation of the AMH gene. Indeed, AMH is the first hormone secreted by the differentiated testis. Two binding sites, one for SF1 and the

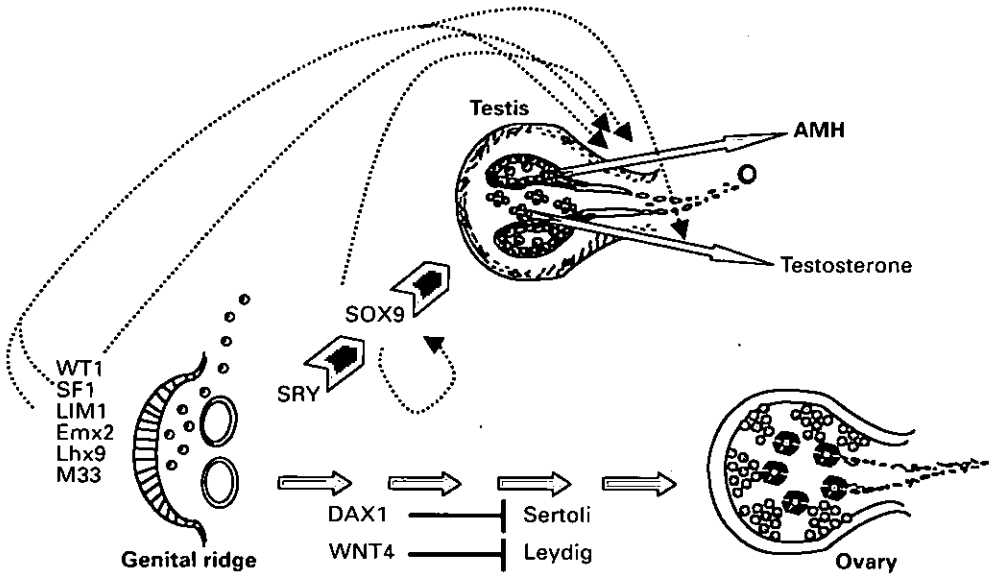

Fig. 2. Gene epistasis in gonadal formation. The upper part represents the male pathway initiated by SRY, with SOX9 as a key testicular factor. Dotted arrows indicate its role in AMH upregulation. AMH expression is also controlled positively by SF1 and WT1. The lower part shows the female pathway, including two male pathway inhibitors, DAX1 and WNT4. The six genes on the left are involved in genital ridge formation. LIM1 = Lhx1: LIM homeobox gene 1; Lhx9: LIM homeobox gene 9; Emx2: empty spiracles, *Drosophila* homologue 2; M33 = CBX2: chromobox homologue 2, *Drosophila* polycomb class; SF1: steroidogenic factor 1; WT1: Wilms' tumour gene 1; DAX1: DSS-AHC critical region on the X chromosome, gene 1; SRY: sex-determining region of Y chromosome; SOX9: SRY-related HMG box gene 9; WNT4: wingless-type MMTV integration site family, member 4; AMH: anti-Müllerian hormone.

other for SOX9, are highly conserved in the promoter region of the AMH gene among different mammalian species (Shen *et al.*, 1994). By targeted mutagenesis of these binding sites *in vivo*, it has been shown that, in mice, SOX9 triggers AMH expression and SF1 enhances it (Arango *et al.*, 1999). DAX1 and WT1 (-KTS) might also act as negative and positive modulators on AMH transcription through protein interaction with SF1 (Nachtigal *et al.*, 1998).

In conclusion, SOX9 is a key gene in mammalian sexual differentiation. Some questions remain to be answered. How is this gene upregulated in males and downregulated in females? What is the link between SRY and SOX9? Also, other than AMH, what male genes are regulated specifically by SOX9?

Gonadal differentiation: a cellular viewpoint

During the past 4 years, interesting results have been obtained using a co-culture system of gonads with mesonephri from a transgenic strain ubiquitously expressing β-galactosidase. Firstly, it has been shown that migration of mesonephric cells takes place into XY but not XX gonads from day 11.5 to day 16.5 after coitus in mice (Martineau *et al.*, 1997). The signal that triggers migration operates over long distances and behaves as a chemoattractant. By culturing XX gonads with XY gonads at their surfaces, as a sandwich, mesonephric cell

migration into the XX tissue took place. After this migration, the XX gonads organized cord structures and acquired male-specific patterns of gene expression (Tilmann and Capel, 1999). The migrating mesonephric cells give rise to three types of cell population in XY gonads: myoid, endothelial and some closely associated with endothelial cells. Finally, by using XX gonads transgenic for SRY, it has been shown that this migration event depends on the SRY gene and represents the earliest physiological sign of SRY action (Capel *et al.*, 1999).

The pig: a particular model of mammals?

To date, most of the gene expression patterns described have been observed in mice. In pigs, four studies present the time course of sex-determining genes during gonadal development (Daneau *et al.*, 1996; Parma *et al.*, 1997, 1999; Pilon *et al.*, 1998). The main results of these studies are summarized (Fig. 3) and compared with expression profiles in mice. No significant differences are noticeable for three genes: SOX9, DAX1 and AMH. There are three notable differences between these two species, two in males and one in females. In female mice, SF1 expression is stopped between germ cell meiosis (day 13.5 after coitus) and the beginning of folliculogenesis (day 18.5 after coitus) (Ikeda *et al.*, 1994). In pigs, SF1 is expressed continuously in gonads of both sexes from the time of formation of genital ridges (day 23 after coitus). Different levels of expression between sexes (high in males versus low in females) have been found using northern blot analysis at later stages (days 45–52 after coitus) (Pilon *et al.*, 1998). In mice, transcription of the SRY gene has been detected during a very short period of 2 days (days 10.5–12.5 after coitus) (Jeske *et al.*, 1995). Conversely, in pigs, SRY is expressed over a longer period of about 3 weeks. Expression persists > 2 weeks after the first sign of Sertoli cell differentiation (day 28 after coitus in pigs) (Parma *et al.*, 1999). The role of this persistence of SRY in domestic species is unknown but it could suggest the involvement of this gene in other developmental testicular processes, such as inhibition of male germ cell meiosis. The main difference is in the expression of aromatase. In pigs, P450 aromatase transcripts are detected very early in testicular differentiation, at the same time as AMH expression (Parma *et al.*, 1999). In mice, there is no expression of aromatase until birth in both sexes and in sheep and goats an expression peak is observed between gonadal differentiation and germ cell meiosis, but only in the female fetuses (Payen *et al.*, 1996; E. Pailhoux, personal observation). Of the species studied, the pig appears to be unique as far as P450 aromatase is concerned. There are three distinct functional genes encoding three isoforms (type I: ovary; type II: placenta; and type III: embryo), with presumed differences in substrate specificities, expression, activity and mode of regulation (Graddy *et al.*, 2000). The testicular isoform appears to be the same as that expressed in the adult ovary (Conley *et al.*, 1996). The testicular isoform also appears to be active, as oestrone sulphate was first detected in male pig fetuses at day 31 after coitus (Raeside *et al.*, 1993). This fetal production of oestrogens by the testis remains poorly understood. One putative cellular target could be the gonocytes, as proposed by Parma *et al.* (1999). In addition to aromatase, it seems that the entire fetal steroidogenic pathway is different in pigs compared with other mammalian species. We recently tested the expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) in gonads of male, female and intersex fetuses at days 50 and 70 after coitus and found similar high expression in all the samples tested. Also surprising was the expression profile of WNT4 (Fig. 5), which appears to be slightly higher in females, although a significant expression was also detected in males, in contrast to the case in mice. In conclusion, it appears that the specific steroidogenesis observed in pigs must be investigated further to gain a better understanding of the role these hormones play in the formation and maintenance of functional gonads and reproductive organs.

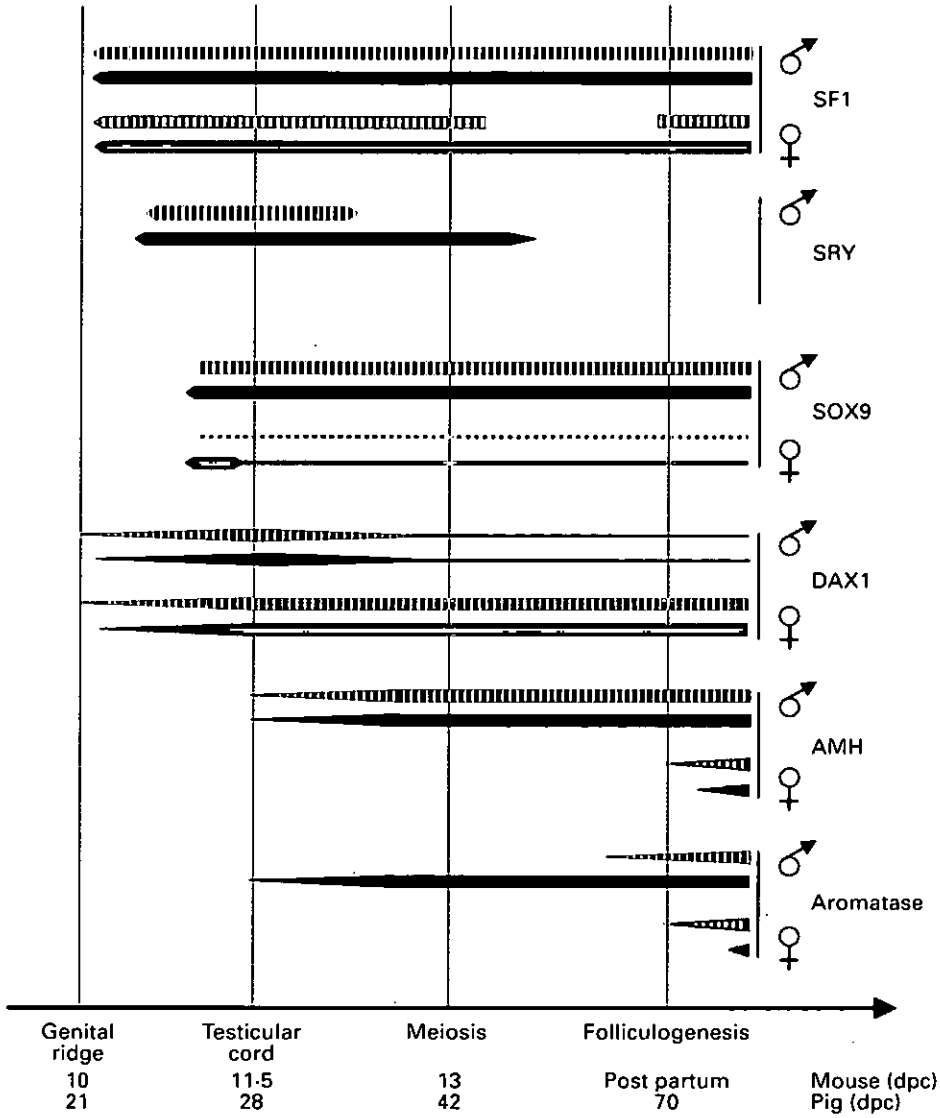

Fig. 3. Time course of gene expression during mouse and pig gonadal differentiation. The width of the lines is proportional to the relative expression. For each gene indicated on the right, the expression in gonads of male mice (striped black), male pigs (black), female mice (striped grey) and female pigs (grey) is shown during four main developmental processes, as indicated under the arrow symbolizing the time course of development. dpc: indicates the days after coitus at which these developmental processes occur. SF1: steroidogenic factor 1; SRY: sex-determining region of Y chromosome; SOX9: SRY-related HMG box gene 9; DAX1: DSS-AHC critical region on the X chromosome, gene 1; AMH: anti-Müllerian hormone.

Genes involved in ovary development

In the presence of two X chromosomes, the indifferent embryonic gonad develops into an ovary. Ovarian differentiation, germ cell meiosis and folliculogenesis begin during fetal life in pigs. Other key events, such as follicular growth and atresia, which occur throughout

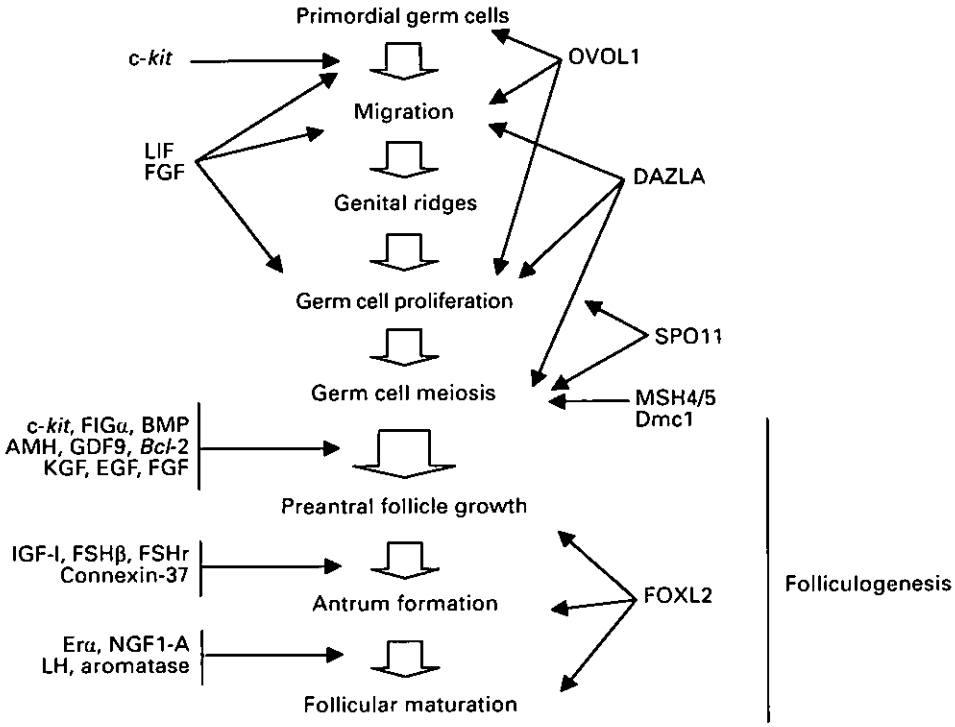

Fig. 4. Schematic representation of ovarian differentiation and function. Four main steps of germ cell life are represented (migration, proliferation, meiosis and maturation). A non-exhaustive list of factors involved in different steps is indicated on both sides of the diagram. OVOL1: OVO, *Drosophila* homologue-like 1; LIF: leukaemia inhibitory factor; FGF: fibroblast growth factor; DAZLA: deleted in azoospermia-like, autosomal; SPO11: *Saccharomyces cerevisiae* homologue of SPO11; MSH4/5: MutS *Escherichia coli* homologue 4/5; Dmc1: disrupted meiotic cDNA 1, yeast homologue; FOXL2: forkhead transcription factor L2; FIGα: factor in the germ line, alpha; BMP: bone morphogenetic protein; AMH: anti-Müllerian hormone; GDF9: growth/differentiation factor 9; *Bcl*-2: B-cell CII/lymphoma 2; KGF: keratinocyte growth factor; EGF: epidermal growth factor; IGF-I: insulin-like growth factor I; FSHβ: follicle-stimulating hormone, beta polypeptide; FSHr: FSH receptor; Erα: oestrogen receptor alpha; NGF1-A: nerve growth factor 1-A; and LH: luteinizing hormone.

prepubertal and reproductive life, are also initiated during fetal life. Four major phases can be distinguished: migration, proliferation and meiosis of the germ cells, and folliculogenesis. These steps are under the control of several transcription factors, growth and paracrine factors, and hormones (Fig. 4).

In pigs, urogenital ridge formation begins at day 21 after coitus (Pelliniemi, 1985). The migration and proliferation of germ cells depend on co-ordinated expression and interaction of different genes. Two factors: c-kit and c-kit ligand (stem cell factor; SCF) play a central role in the migration and colonization of germ cells to the developing genital ridge. SCF controls the early germ-line population and is an essential requirement for primordial germ cell survival (Godin *et al.*, 1991). Leukaemia inhibitory factor (LIF) is crucial for primary germ cell development (Donovan, 1994). It can stimulate primary germ cell proliferation in culture and may regulate primordial germ cell survival and proliferation *in vivo*. Apoptosis is responsible for primordial germ cell attrition in the developing fetal ovary. LIF might support primordial

germ cell survival by preventing primordial germ cell apoptosis (De Felici and Pesce, 1994). Another gene of interest during this step is the DAZLA gene. Indeed, the disruption of the DAZLA gene leads to a loss of germ cells and complete absence of male and female gamete production (Ruggiu *et al.*, 1997). DAZLA transcripts are localized in germ cells before meiosis and at the time meiosis begins. In the ovary, transcripts are not restricted to germ cells, but are found in somatic cells of primordial follicles (granulosa) as well.

Several genes have been shown to be involved in germ cell meiosis from day 42 after coitus in pigs. As oocytes transit from pachytene to diplotene before arresting, DNA repair proteins and other factors are required for proper chromosome alignment and recombination. Oogonia lacking MSH5 (homologue of bacterial MutS) or DMC1 (homologue of RecA) do not complete meiosis and are invariably lost from the ovary before diplotene. Null mice for Msh5 (Edelmann *et al.*, 1999) are viable but sterile. Meiosis in these mice is affected due to disruption of chromosome pairing in prophase I. The ovaries of Msh5 –/– females are normal in size at birth but degenerate progressively to become rudimentary, concomitant with the decrease in the number of oocytes from before day 3 post partum until adulthood. Null mutation of Dmc1 genes showed that homozygous mutant males and females are sterile, with arrest of gametogenesis in the first meiotic prophase (Habu *et al.*, 1996). In Dmc1 –/– female mice, normal differentiation of oogenesis was aborted in embryos and germ cells disappeared in the adult ovary.

Primordial follicles are formed perinatally in mammalian ovaries and the first appears at about day 70 after coitus in pigs. Folliculogenesis requires careful orchestration of developmental programmes in germ and somatic cells, as well as the interactions between them. In the initial stage of folliculogenesis, paracrine factors promote growth of the oocyte and adjacent somatic cells. FIGα (factor in the germ line alpha), a germline cell-specific factor (Soyal *et al.*, 2000), is crucial for the initial formation of primordial follicles. Mouse lines lacking FIGα have primordial follicles that are not formed at birth and a massive depletion of oocytes, resulting in shrunken ovaries and female sterility. In addition, many growth factors, fibroblast growth factor 8 (FGF8) and several members of the transforming growth factor β (TGFβ) family have been implicated in regulating early folliculogenesis. One factor, anti-Müllerian hormone (AMH), is expressed in granulosa cells surrounding oocytes and has been implicated in the recruitment of primordial follicles into the growth phase of folliculogenesis (Durlinger *et al.*, 1999). Other members of the TGFβ family, GDF9A (growth/differentiation factor 9) and BMP15 (bone morphogenetic protein 15), are first expressed in oocytes in primary follicles (McGrath *et al.*, 1995; Dube *et al.*, 1998). Female mice deficient in GDF9A are infertile because of an early block in folliculogenesis at the type 3b primary follicle stage. BMP15 is an oocyte-derived growth factor essential for female fertility. Natural mutation in this gene (Inverdale sheep carrying FecXI mutation) causes both increased ovulation rate and infertility phenotypes in a dosage-sensitive manner (Galloway *et al.*, 2000). Finally, the absence of gonadotrophins leads to atretic follicles that disappear from the ovary.

The female pig gonad remains undifferentiated histologically until day 28 after coitus and final histological differentiation of the ovary is achieved by day 44 after coitus when the cortical cords have been organized and the medullary cords have degenerated (Pelliniemi and Lauteala, 1981). We analysed temporal expression of some of these genes involved in mammalian ovary development by RT–PCR. As examples, the results of three of these genes presenting typical expression profiles are summarized (Fig. 5). The ovarian-determining gene WNT4 was expressed as early as day 37 after coitus and throughout fetal life in both sexes, with a relatively higher expression in females. DMC1, which is essential for meiotic recombination, was first expressed during meiosis, between day 45 and day 52 after coitus, began to decrease at day 70 after coitus (last studied stage during fetal life) and was expressed

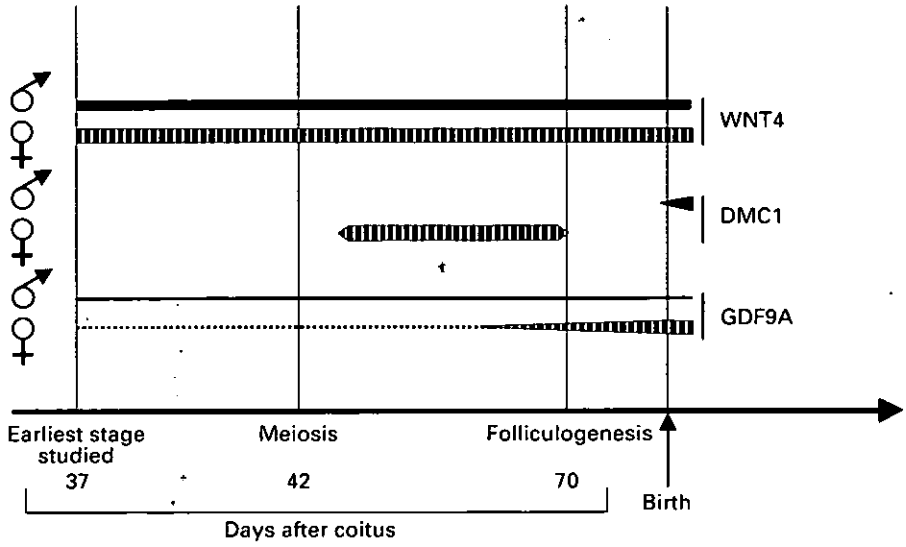

Fig. 5. Example of expression profiles of ovarian genes in pigs. These genes are involved in three different developmental steps: WNT4: ovarian differentiation; DMC1: germ cell meiosis; GDF-9A: folliculogenesis. The width of the lines is proportional to the expression. For each gene, the expression is shown for males (full line) and females (striped line). WNT4: wingless-type MMTV integration site family, member 4; DMC1: disrupted meiotic cDNA 1; GDF9A: growth/differentiation factor 9A.

in gonads of 5-week-old males. GDF9A, described as an oocyte-derived paracrine factor important for growth of follicles beyond the primary stage, increased at the beginning of folliculogenesis (day 65 after coitus).

These studies demonstrate that several genes, whether restricted in their expression to the ovary or expressed preferentially in the ovary, constitute critical determinants in ovarian development. Further investigations are also in progress, such as suppressive subtractive hybridization during the different stages of pig fetal ovarian development to isolate genes that are expressed differentially at key stages of ovarian development (before meiosis, during meiosis and during folliculogenesis).

Anomalous sexual development: sex reversal

In parallel with the classic scheme of sex differentiation in mammals, exceptions exist where XX individuals develop testes in the absence of the Y chromosome and, conversely, XY individuals without testes have been described. Such individuals have been observed in numerous mammalian species including humans, pigs, goats, horses, dogs, mice, marsupials and moles, and are called sex-reserved or intersexes (Pailhoux *et al.*, 1994a,b; Sanchez *et al.*, 1996; Whitworth *et al.*, 1996; Meyers-Wallen *et al.*, 1999; Bishop *et al.*, 2000; Buoen *et al.*, 2000; Sarafoglou and Ostrer, 2000). Some of these individuals result from mutations in the SRY gene (XY female) or from translocation of SRY to the X chromosome or autosomes (XX male). However, most sex-reversed individuals result from alterations of X-linked or autosomal genes remaining for the most part unknown. The genes known to be involved in sex determination, such as SRY, WT-1, DAX-1 and SOX9, have been isolated by genetic analysis of sex-reversed patients.

In pigs, the frequency of intersexuality ranges from 0.1 to 0.5% of females and may reach up to 20% in isolated herds. The animals described are mainly adults that were detected either by the breeder because of abnormal external genitalia or at slaughter through discovery of one or two abdominal testes. At slaughter, the incidence of intersexuality is > 0.2% of the total European pig population. In practical terms, instances of intersexuality represent a source of direct loss in the breeding herd resulting from sterility, genital infections reducing growth and viability and downgrading of carcasses for so-called boar taint. In addition, such animals may provoke losses due to their aggressive behaviour.

Intersex pigs are masculinized genetic females with a 38, XX chromosome constitution. We studied about 50 adult animals from an experimental breeding herd at INRA (Institut National de la Recherche Agronomique), where about 1% of females are intersexes, to elucidate the genetic aetiology of the pig sex reversal condition. PCR was used to test the DNA from blood and gonadal biopsies of intersex animals for the presence of cryptic Y chromosome-specific sequences, particularly the SRY gene. Intersex pigs result from three situations: the presence of XX and XY cells within the same animal (4%); the presence of a small fragment of the Y chromosome (containing the SRY gene) in XX individuals (2%); and the presence of an autosomal or X-linked mutation affecting genes in the sex-determining pathway or mimicking their effects (94%) (Pailhoux *et al.*, 1997).

A genetic approach was attempted using genome scanning of resource families to isolate genes involved in intersexuality in pigs. Analyses indicate that sex reversal in this species is controlled multigenically and five regions of the genome appear to be involved (E. Pailhoux, personal observation). Some of these regions correspond to regions described previously in humans, which also confer abnormal sex determination.

Phenotypically, all the animals studied ranged from true hermaphrodites (48%) to males with (50%) and without (2%) ambiguities. True hermaphrodites with both testicular and ovarian tissue in the same animal or in the same gonad have been observed and a few rare cases of pregnancy in these hermaphrodite sows have been obtained (Pailhoux *et al.*, 1997). Of the 50 animals observed, only one was a male without ambiguities. This phenotype, which was characterized by an external and internal male type genitalia, has not been described previously in pigs. The only visible defect was a unilateral cryptorchidism. From histological observations of gonadal biopsies, intersex gonads can be divided into different phenotypes. The most primitive type consists of unorganized tissue with neither ovarian nor testicular structures. The second type contains cords in interstitial stroma and thus resembles the sexually indifferent phase of embryonic gonadal differentiation. The third type occurs as a testis or an ovotestis and resembles the normal testis but with cords that are thinner and devoid of germ cells. The fourth type is similar but more rudimentary. The fifth occurs as an ovary or an ovotestis and consists of variable ovarian tissue (Pailhoux *et al.*, 1997). This broad variability of gonadal phenotypes is one of the main features of this pathology. The precise classification of the different cases of intersexuality in pigs reveals strong similarity with observations in human sex-reversed patients.

Pig fetuses were studied during gonadal development to determine the onset of the pathology (Fig. 6). The fetuses were obtained by crossing parents that had produced intersexes previously in their progeny. These molecular and histological investigations revealed that as early as day 50 after coitus, intersex gonads could differ from normal ovaries and present expression profiles of the male type for SOX9, AMH and P450 aromatase genes. Variability in gonadal phenotypes was observed at all the developmental stages tested, even within the same animal. The degeneration of germ cells observed in the testicular portion of all the intersex gonads was observed just after birth. The development of both non-functional ovaries and testis in intersex individuals with complete female chromosomes indicates that defective

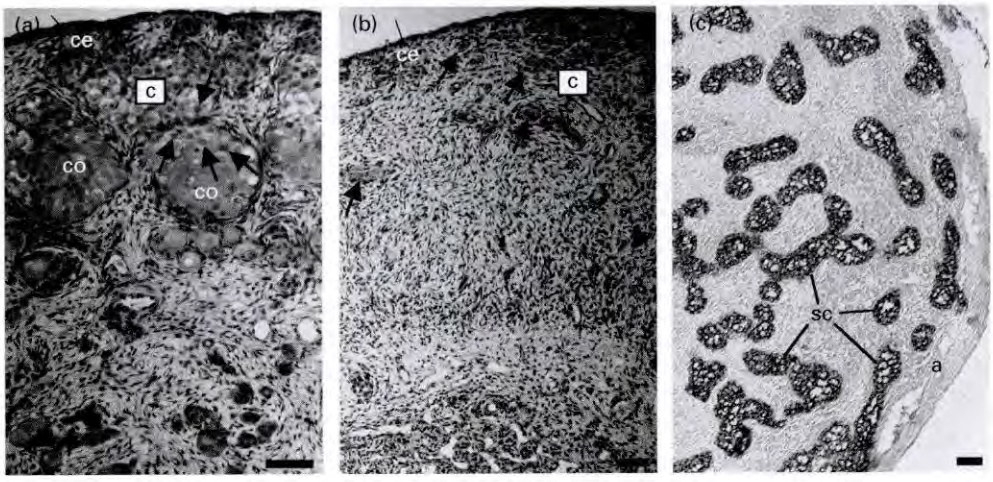

Fig. 6. Light micrograph of three gonadal sections from XX pigs. (a) Normal ovary at day 70 after coitus. (b) Ovary of an intersex pig at day 70 after coitus. Note the hypoplasia of the ovarian cortex associated with the reduction of germ cell number. (c) Immunohistological detection of anti-Müllerian hormone (AMH) in seminiferous tubules of a fetal XX sex-reversed gonad at day 50 after coitus. ce: coelomic epithelium; c: ovarian cortex; co: ovigerous cord; sc: seminiferous cord. The arrows show the germ cells. Scale bars represent 50 μm.

genes are involved in normal females, both in inhibition of male gonadal differentiation and maintenance of ovarian differentiation. Indeed, the ovarian pathway had been initiated in these gonads, but was altered and gave rise to severe ovarian dysfunction leading in some cases to complete transdifferentiation. Molecular identification of the genes involved in this pathology of XX sex reversal will greatly increase our understanding of sex determination and gonadal differentiation in mammals.

Conclusion

Our knowledge of the molecular mechanisms involved in mammalian gonadal differentiation has been improved greatly by the isolation and study of numerous genes. To date, the male pathway has been better characterized as a result of the discovery of two main genes: the testis-determining factor SRY and the Sertoli cell-specific factor SOX9. There is little information on the ovarian-determining pathway and very few factors have been identified. WNT4 is one factor that has been identified and appears to be an inhibitory testis factor. Likewise, two mutations (odsex in mice and polled intersex syndrome in goats) are indicative of the existence of this type of factor. Indeed, they reveal that XX sex reversal could be the result of suppression of such inhibitory effects. Similarly, investigations of pig intersexuality could give some insight into this type of gene. A better understanding of this pathology in pigs will also offer breeders the possibility of eradicating intersexuality and enhancing female fertility. Indeed, genes involved in ovarian sex reversal are likely to be critical factors in ovarian differentiation and function. Other interesting approaches for isolating ovarian-specific genes are being produced by the new transcriptome technologies. The systematic differential screening of transcripts involved in the crucial steps of ovarian differentiation (meiosis, folliculogenesis) along with delimitation of chromosomal segments implicated in reproductive quantitative trait loci will certainly improve the control of female fertility.

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