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Genes essential for early events in gonadal development

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Abstract. The acquisition of a sexually dimorphic phenotype is a critical event in mammalian development. The basic underlying principle of sexual development is that genetic sex—determined at fertilization by the presence or absence of the Y chromosome—directs the embryonic gonads to differentiate into either testes or ovaries. Thereafter, hormones produced by the testes direct the developmental program that leads to male sexual differentiation. In the absence of testicular hormones, the female pathway of sexual differentiation occurs. Recent studies have defined key roles in gonadal development for two transcription factors: Wilms' tumor suppressor 1 (WT1) and steroidogenic factor 1 (SF-1). After presenting a brief overview of gonadal development and sexual differentiation, this paper reviews the studies that led to the isolation and characterization of WT1 and SF-1, and then discusses how interactions between these two genes may mediate their key roles in a common developmental pathway.

Key words. SF-1; WT1; DAX-1; MIS; sex determination.

Introduction

Prior to sexual differentiation, the ovaries and testes cannot be distinguished and therefore are called bipotential or indifferent gonads. These bipotential gonads arise from the urogenital ridge, a region adjacent to the mesonephros that ultimately contributes cell lineages to the adrenal cortex, gonads and kidney. The testes and ovaries have functional counterparts that serve corresponding functions in reproduction. These counterparts include the Leydig and theca cells, which comprise the steroidogenic compartment, the Sertoli and granulosa cells, which support germ cell maturation, the germ cells (spermatocytes and oocytes), and the peritubular myoid and stroma cells—which form the connective tissue of the gonads.

After sexual determination, the testes and ovaries can be distinguished histologically, largely because the testes organize into two distinct compartments: the testicular cords and the interstitial region. The testicular cords—precursors of the seminiferous tubules—contain the fetal Sertoli cells and the primordial germ cells, which migrate into the gonad from a position outside of the urogenital ridge. The interstitial region, surrounding the testicular cords, contains the steroidogenic Leydig cells and the peritubular myoid cells. In contrast, the ovaries have an amorphous, 'ground-glass' appearance and exhibit little structural differentiation until late in gestation.

The internal genitalia derive from the genitourinary tract, which again initially is identical in male and female embryos. At the indifferent stage, male and female embryos have two identical sets of paired ducts: the Müllerian (paramesonephric) ducts and the Wolffian (mesonephric) ducts. If the Y chromosome activates the male developmental pathway, testes develop and ultimately effect male sexual differentiation by causing the Müllerian ducts to degenerate and the Wolffian ducts to develop into the seminal vesicles, epididymis and vas deferens. In the absence of testicular

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hormones, the Wolffian ducts regress and the Müllerian ducts form the oviducts, Fallopian tubes, uterus and upper vagina. As predicted by the classic studies of Jost [1, 2], the critical mediator of Müllerian duct regression in males is a glycoprotein hormone, Müllerian-inhibiting substance (MIS, also known as anti-Müllerian hormone or AMH), which is produced by Sertoli cells within the testicular cords. Testicular androgens, synthesized by Leydig cells in the interstitial region, cause male differentiation of the Wolffian ducts and external genitalia.

The external genitalia, like the internal genitalia, also derive from structures that initially are found in both sexes, including the genital tubercle, urethral folds, the urethral groove and the genital swellings. Again, androgens are critical for virilization, although full virilization of the external genitalia requires the conversion of testosterone to dihydrotestosterone by 5α-reductase [3].

As summarized in the chapter by Koopman in this volume, we now know that a single gene, designated *SRY* for *sex-determining region-Y chromosome*, mediates male sexual determination (reviewed in [4]). However, the mechanisms by which *SRY* activates the male pathway remain undefined. The structural homology of SRY to transcriptional regulators of the high-mobility-group family led to the hypothesis that SRY activates downstream genes, which in turn mediate the conversion of the bipotential gonad into a testis. Direct target genes of SRY, however, have not been isolated, and the role of SRY as a regulator of gene transcription thus remains to be proven. In an effort to extend our understanding of sexual differentiation, other groups have tried to identify additional genes that play important roles in early stages of gonadal development and sexual differentiation.

Genes essential for early stages of gonadal development

From the above discussion, it is apparent that mutations in genes that are essential for development of the bipotential gonad—before sexual determination and differentiation take place—will impair testes formation in an XY background or ovary formation in an XX background. Recent studies have shown that two different transcription factors, WT1 and SF-1, play such pivotal roles in early gonadogenesis.

WT1: a critical mediator of urogenital development

The *WT1* gene initially was isolated through analyses of patients with Wilms' tumors, an embryonic kidney tumor arising from abnormal proliferation of the metanephric blastema (reviewed in [5]). Although Wilms' tumor generally presents sporadically, approximately 1% of Wilms' tumors occur in patients with similarly affected first-degree relatives. This finding led to the proposal that a tumor suppressor gene was mutated in these families in a manner akin to retinoblastoma. Efforts to map the gene(s) were largely guided by heterozygous deletions on human chromosome 11p13, which are associated with the WAGR syndrome [6]. Patients with this syndrome exhibit a variable phenotype that includes Wilms' tumors, aniridia, genitourinary abnormalities and mental retardation. Genitourinary abnormalities are observed only in a subset of patients and are relatively mild, involving cryptorchidism and hypospadias in males and horseshoe kidneys in males and females. The WAGR phenotype reflects deletions of several genes, including *WT1* [7–9] and the transcription factor *PAX6* [10], isolated mutations of which also are associated with aniridia in humans. Two other genes within the chromosomal region deleted in WAGR patients are expressed in the embryonic brain and therefore may contribute to the mental retardation phenotype [11, 12]. A small percentage of patients with familial Wilms' tumors inherit mutations or deletions of one WT1 allele, and then undergo somatic loss of the second allele due to gross chromosomal events. Other chromosomal regions associated with familial Wilms' tumors include 11p15 (the region associated with the Beckwith-Wiedemann syndrome) and chromosome 17. These other Wilms' tumor genes have not yet been identified, and remain an ongoing area of investigation.

WT1, by alternative splicing [13] and alternative translation start sites [14], generates at least eight different isoforms of a zinc-finger DNA-binding protein that is thought to regulate gene transcription by interacting with specific DNA recognition sequences upstream of target genes. Besides differing somewhat in their preferences for DNA binding [15], the different isoforms of WT1 also localize differentially within the nucleus. In vitro experiments demonstrated that the localization is controlled mainly by the presence or absence of the second alternative splice, which introduces three amino acids (lysine-threonine-serine, KTS) between zinc fingers 3 and 4. Whereas − KTS isoforms show a more diffuse nuclear staining, + KTS isoforms associate with spliceosomes, suggesting that they may participate in RNA processing [16, 17]. Studies to date have not yielded any evidence for tissue-related differences in the relative levels of the different WT1 isoforms in different tissues, and the molecular function of these WT1 isoforms remains unclear.

The first indication of an essential role for *WT1* in urogenital development came from analyses of its expression, which showed specific staining within the developing kidneys and gonads [18]. Subsequent analyses showed that point mutations in the *WT1* gene can lead

to mild abnormalities of the genital system, including hypospadias and cryptorchidism [19]. A clear role for *WT1* in development of the kidneys and gonads was established by analyses of *WT1* knockout mice [20]. In addition to renal agenesis, these *Wt1* knockout mice lacked gonads, and had impaired adrenal development (C. Moore et al. Development **126:** 1845–1857). As a result of their gonadal dysgenesis before the time that androgens and MIS are produced, the internal and external genitalia developed along the female program. These results, coupled with the structural similarity of WT1 with other transcription factors, suggest that WT1 regulates the expression of target genes that are essential for gonadogenesis in both males and females.

Besides the classical Wilms' tumor and the WAGR syndrome, *WT1* mutations are associated with two other clinical syndromes in human patients—Denys-Drash syndrome and Frasier syndrome. Denys-Drash syndrome is an autosomal dominant disorder characterized by gonadal and urogenital abnormalities in conjunction with diffuse mesangial sclerosis. The renal disease in Denys-Drash patients is quite severe, usually presenting in the first year of life and causing end-stage renal disease by age 3. The gonadal abnormalities of these patients vary, but generally are more severe than those associated with the WAGR syndrome, with streak gonads and sex reversal of external and internal genitalia at one extreme and varying degrees of pseudohermaphroditism in less severely affected XY males. Wilms' tumors are commonly seen in patients with Denys-Drash mutations. Denys-Drash syndrome almost always results from point mutations in the zinc-finger region that abrogate DNA binding; these mutated proteins are predicted to act in a dominant-negative fashion to inhibit function of the protein encoded by the wild-type allele [21–23]. In agreement with this notion it has been shown that WT1 is able to form homodimers at least in vitro [24, 25]. Extrapolating from the knockout mouse studies described above, it is probable that the degree of inhibition of *WT1* action correlates with the impairment of genitourinary development, with the most severe mutations leading to early gonadal dysgenesis and sex reversal of external and internal genitalia. It should, however, be noted that genetic modifiers also play important roles in the severity of the resulting phenotype. This is demonstrated in case studies of DDS patients where fathers were phenotypically normal despite carrying the same *WT1* mutation seen in affected offspring.

WT1 mutations also have been identified in patients with Frasier syndrome [26–28]. Unlike patients with Denys-Drash syndrome, these patients do not develop Wilms' tumors, but instead present with gonadal dysgenesis, male pseudo-hermaphroditism, and focal glomerular sclerosis. Their glomerulopathy is less severe than that associated with Denys-Drash mutations, with no evidence of renal insufficiency until after age 4 and preservation of some renal function until adolescence or young adulthood. The *WT1* mutations that cause Frasier syndrome cluster within intron 9 of the *WT1* gene, and apparently interfere selectively with the synthesis of splice variants of WT1 that insert the amino acids lysine-threonine-serine (+ KTS) between the third and fourth zinc fingers (fig. 1). Although the signifi-

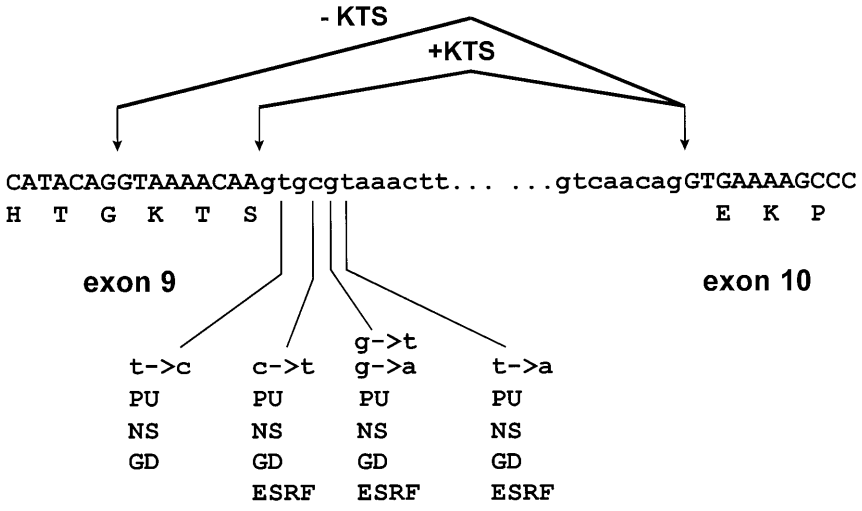

Figure 1. Mutations associated with the Frasier syndrome disrupt splicing at the second alternative splice donor site at the end of exon 9. PU, proteinuria; NS, nephrotic syndrome; GD, gonadal dysgenesis; ESRF, end-stage renal failure.

cance of the WT1 splice variants is not fully understood, this finding suggests that the + KTS isoform is essential for gonadal development but is not required to suppress the development of Wilms' tumors. This finding further suggests that the same *WT1* mutations that cause Frasier syndrome in boys may also cause some cases of focal glomerular sclerosis in girls, who escape diagnosis because they lack abnormalities of the external genitalia. If so, recognition of these mutations may considerably facilitate genetic counseling for family members.

Steroidogenic factor 1 (SF-1): a critical mediator of endocrine development

SF-1 was described initially as an important regulator of the tissue-specific expression of the cytochrome P-450 steroid hydroxylases—enzymes that catalyze many key reactions in steroidogenesis [29, 30]. The subsequent isolation of a complementary DNA (cDNA) encoding *SF-1* showed that this critical regulator of the steroidogenic enzymes was itself a member of the nuclear hormone receptor family—proteins that mediate transcriptional activation by steroid hormones, thyroid hormone, vitamin D and retinoids [31]. Since its initial characterization, a number of groups have shown that *SF-1* regulates adrenal and gonadal expression of genes required for steroidogenesis, including the steroid hydroxylases, 3β-hydroxysteroid dehydrogenase, the adrenocorticotropin receptor and the steroidogenic acute regulatory protein (reviewed in [32]). Analyses in transfected Sertoli cells and transgenic mice further suggest that *SF-1* regulates the *MIS* gene [33–35]. In addition to the steroidogenic organs, *SF-1* transcripts also were detected in the anterior pituitary and hypothalamus. Collectively, these findings suggest that *SF-1* regulates the expression of both hormones that are critical for male sexual differentiation (androgens and MIS) and also raise the possibility that *SF-1* plays additional roles at other levels of the endocrine axis.

Analyses of *Sf-1* knockout mice dramatically confirmed essential roles of SF-1 at all three levels of the hypothalamic-pituitary-steroidogenic organ axis. Perhaps most strikingly, these *Sf-1* knockout mice lacked adrenal glands and gonads, undergoing loss of the primordial organs via programmed cell death at discrete stages of development when sexual differentiation normally takes place [36, 37]. These findings, which resemble closely the consequences of *Wt1* knockout on gonadal development, demonstrate unequivocally that SF-1 has essential roles in the early development of the adrenal and gonadal precursors. Consistent with the degeneration of testes before androgens and MIS are produced, *Sf-1* knockout mice also exhibit male-to-female sex reversal of the internal and external genitalia. They also have impaired expression of a number of markers of pituitary gonadotropes [38, 39], the pituitary cell type that regulates gonadal steroidogenesis. Finally, they lack the ventromedial hypothalamic nucleus [39, 40], a cell group in the medial hypothalamus linked to ingestive and reproductive behaviors [41].

The early embryonic expression of *SF-1* in the gonads and its established role as a transcription factor make it likely that *SF-1* is part of the hierarchical regulatory pathway that determines the expression of downstream genes required for gonadogenesis. Although mutations in *SF-1* have not yet been demonstrated in human patients, the human gene encoding SF-1 shares extensive homology with its mouse counterpart [42, 43] and is expressed in many of the same sites [44], suggesting that *SF-1* functions in humans as it does in mice. Inasmuch as the human *SF-1* gene resides on chromosome 9q33 [45], it is reasonable to speculate that patients will be found with abnormalities of gonadal development or sexual differentiation that map to this locus.

Genetic interactions in gonadal development

Sexual determination and differentiation require a complex set of events in the appropriate tissues at appropriate times of development. Defects in any of these steps can impair sexual differentiation. Although we know that SRY, encoded by the Y chromosome, is the primary mediator of male sex determination, considerable gaps remain in our understanding of just how SRY brings about these critical events in development. As summarized in figure 2, both X-linked genes (e.g. *DAX-1*) and autosomal genes (e.g. *SF-1* and *WT1*) also play critical roles in processes of sex determination and differentiation. An important goal for future studies is to define how these genes interact in a common developmental pathway to bring about these critical developmental events.

Ongoing studies are examining potential interactions among these genes that may explain how they cooperate in gonadogenesis. Intriguingly, recent evidence supports direct functional interactions of *WT1* and *SF-1*, as well as interactions of *SF-1* with other genes implicated in gonadal and adrenal development. When analyzed in vitro with recombinantly expressed proteins or in mammalian two-hybrid assays, WT1 and SF-1 can form heterodimers [46]. The functional significance of this interaction is supported by the finding that cotransfection with WT1 markedly augments SF-1-dependent transcriptional activation of the *MIS* promoter. This effect was most pronounced with the − KTS isoform of WT1, a puzzling result in light of the apparent link between the + KTS form and gonadogenesis suggested

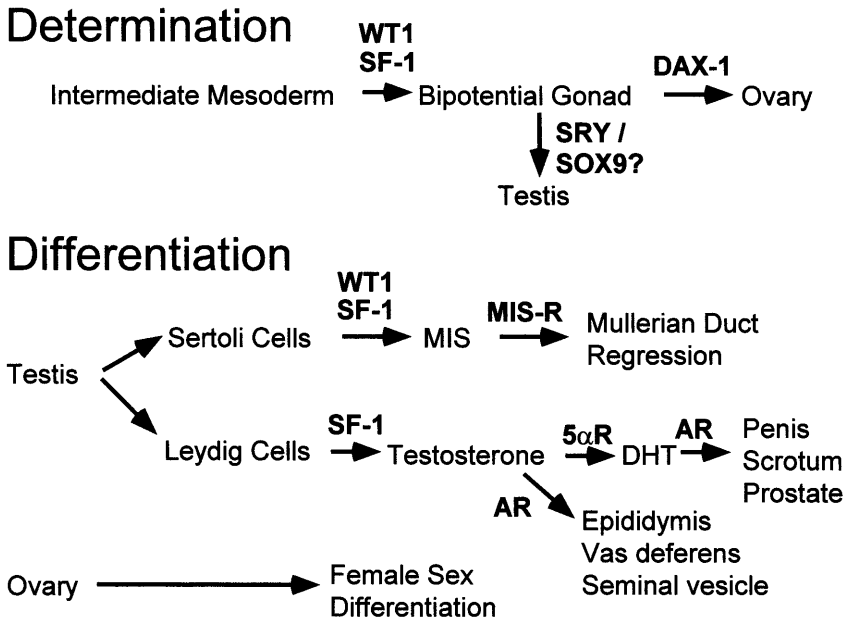

Figure 2. Summary of the molecular events in mammalian sex determination and differentiation. The positions of a number of genes believed to mediate key events in sex determination and differentiation are indicated, as discussed in the text. MIS, Müllerian inhibiting substance; MIS-R, Müllerian inhibiting substance receptor; 5αR, 5α-reductase; DHT, dihydrotestosterone; AR, androgen receptor.

by patients with Frasier syndrome. Alternatively, others have shown that cotransfection with WT1 increases the expression of reporter genes driven by the *SF-1* promoter, suggesting that WT1 may act in part to increase the levels of SF-1 (D. Lala and K. Parker, unpublished observation). In these studies, the + KTS isoform induced promoter activity most potently, providing a possible link with abnormal testes development in patients with Frasier syndrome. It is apparent that *WT1* is not absolutely essential for *SF-1* expression, as *Wt1* knockout mice still have detectable *Sf-1* transcripts in the degenerating gonads (K. Parker and J. Kreidberg, unpublished observation). Moreover, these two models are not necessarily antagonistic, and it remains plausible that *WT1* acts both to increase levels of *SF-1* transcripts and to facilitate its activation of downstream genes such as *MIS*. To further complicate the activation of the *MIS* gene, a recent study has demonstrated that, in addition to WT1, the SOX9 protein is also able to stimulate the *MIS* promoter in the presence of SF-1 at least in vitro [47]. Which of these factors are needed for *MIS* activation in vivo, or whether maybe all of them act together in a synergistic manner, will require a careful genetic analysis in the developing embryo.

Another gene that interacts with *SF-1* in endocrine development is *DAX-1*. *DAX-1* encodes an atypical member of the nuclear receptor family that retains the conserved ligand binding domain but lacks the typical zinc-finger DNA binding motif [48], suggesting that DAX-1 regulates gene expression through protein-protein interactions. *DAX-1* was isolated initially by positional cloning of the gene responsible for X-linked adrenal hypoplasia congenita (AHC), a disorder in which patients present with ACTH-insensitive adrenal insufficiency due to impaired development of the adrenal cortex. If kept alive with corticosteroids, these AHC patients later may exhibit features of hypogonadotropic hypogonadism, reflecting a mixed phenotype of hypothalamic and pituitary gonadotropin deficiencies. The association of impaired adrenal development and hypogonadotropic hypogonadism resembles somewhat the phenotype in *Sf-1* knockout mice, suggesting that *DAX-1* and *SF-1* also may act in the same developmental pathway.

In support of this model, recent studies have shown that both genes are expressed in many of the same sites during embryogenesis, including the gonads, adrenal cortex, pituitary gonadotropes and the ventromedial hypothalamic nucleus (VMH) [49, 50]. Moreover, recent studies suggest several mechanisms by which SF-1 and DAX-1 may interact (fig. 3). One model (fig. 3B) proposes that DAX-1 can heterodimerize with SF-1, and that DAX-1 inhibits SF-1-mediated transcriptional activation because of this heterodimerization [51]. Related studies (fig. 3C) suggest that DAX-1 inhibits the

expression of SF-1-dependent target genes by recruiting the corepressor N-Cor to their promoters [52]. Alternatively, it has been proposed (fig. 3D) that DAX-1 interferes with SF-1 action by binding to hairpin loops in the 5′-flanking region of SF-1-responsive genes, presumably blocking access of the promoters to SF-1 [53]. Finally, there are reports that SF-1 can interact with promoter elements upstream of *DAX-1* to induce its expression (fig. 3E), thereby providing a cooperative link between these two genes [54, 55].

The functional consequences of these proposed interactions between DAX-1 and SF-1 appear to differ depending on the tissue. In the adrenal cortex, gonadotropes and the VMH, SF-1 and DAX-1 may cooperate to activate the expression of target genes required for tissue-specific functions. Consistent with this, the phenotypes of *Sf-1* knockout mice and *DAX-1* patients in these sites are generally concordant. In contrast, the actions of SF-1 and DAX-1 in gonadal cells appear to be antagonistic. SF-1 is required for testes

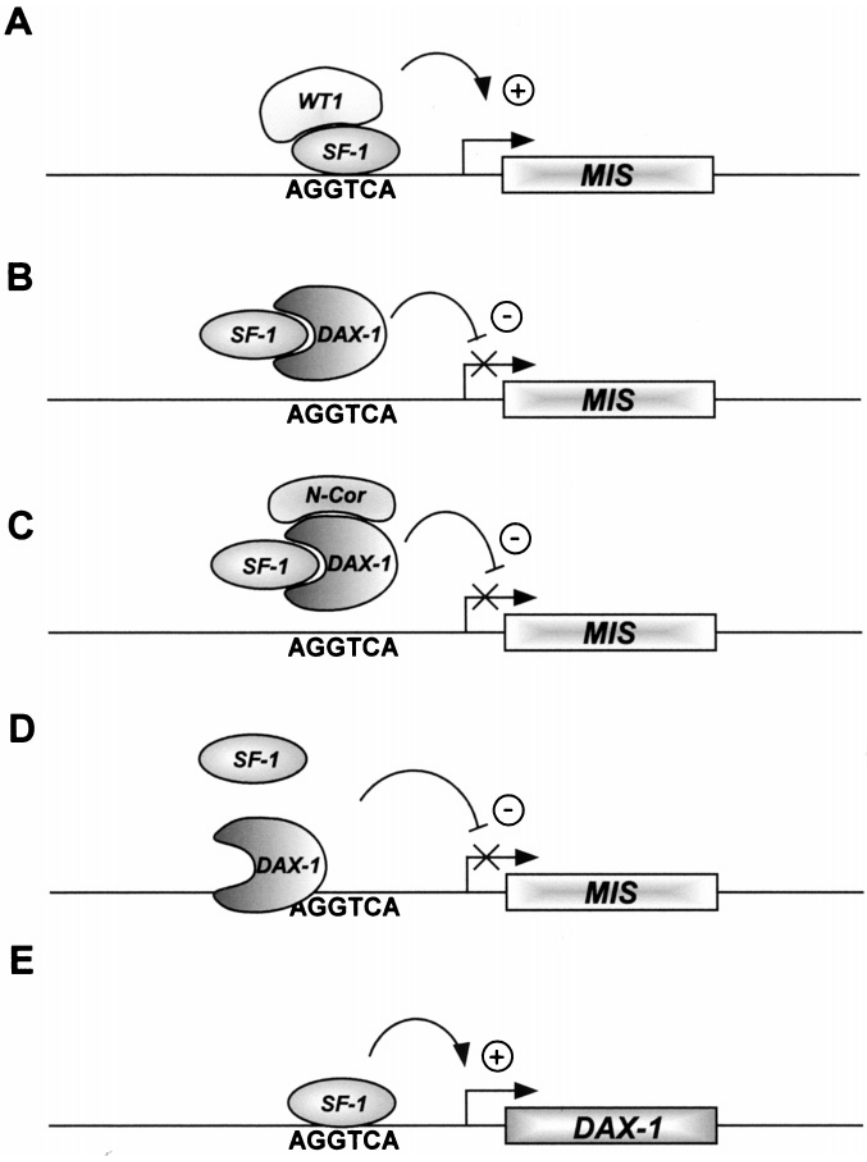

Figure 3. Potential interactions between SF-1 and DAX-1 in endocrine development. Models by which SF-1 and DAX-1 may interact in endocrine development are shown. (*A*) SF-1 induces transcription of the MIS gene by interactions with an AGGTCA promoter element. WT1 is likely to act as a stimulating cofactor in this activation process. (*B*) SF-1-DAX-1 heterodimerization inhibits activation of MIS. (*C*) DAX-1 recruits the corepressor N-Cor to SF-1-responsive promoters. (*D*) DAX-1 interacts with hairpin loops in SF-1-responsive promoters to interfere with SF-1-dependent transcriptional activation. (*E*) SF-1 activates the DAX-1 promoter.

development and male sexual differentiation, whereas its expression in the ovaries diminishes coincident with sexual differentiation [33, 34]. These findings suggest that SF-1 is essential for normal male sexual differentiation, but may impair ovarian development and female sexual differentiation. These findings lead to the proposal that a presumptive excess of DAX-1—in patients with dosage-sensitive sex reversal—would suppress SF-1 function and favor ovarian development, whereas the complete absence of DAX-1 would not impede SF-1 action and therefore would be compatible with normal testicular differentiation.

Summary

From the studies reviewed here, it is apparent that a number of the critical genes that mediate gonadal development and sexual differentiation have now been identified. Through a combination of studies in experimental model systems (e.g. knockout mouse and transgenic overexpression studies) and analyses of additional human patients with aberrant sex determination and/or differentiation, an improved understanding of these essential developmental pathways hopefully soon will emerge.

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