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The Cell-Specific Nuclear Receptor Steroidogenic Factor 1 Plays Multiple Roles in Reproductive Function [and Discussion]

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The cell-specific nuclear receptor steroidogenic factor 1 plays multiple roles in reproductive function

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SUMMARY

The cytochrome P450 steroid hydroxylases exhibit tissue-specific and developmentally regulated gene expression. Recent studies showed that the orphan nuclear receptor steroidogenic factor 1 (SF-1) plays a key role in their gene regulation. In mouse embryos, SF-1 expression began at the inception of adrenal and gonadal development, suggesting that SF-1 plays a key role in the steroidogenic cell differentiation. SF-1 was also expressed in the developing pituitary gland and diencephalon, which raised the possibility that it also has additional roles in endocrine development. To examine the role of SF-1 in intact mice, we disrupted the gene encoding SF-1 by homologous recombination in embryonic stem cells; this approach ultimately permitted us to generate SF-1 knockout mice in which the gene encoding SF-1 was inactivated. These studies revealed essential roles of SF-1 in endocrine development that included adrenal and gonadal development, expression of several markers of pituitary gonadotropes, and formation of the ventromedial hypothalamic (VMH) nucleus. These results indicate that SF-1 acts at multiple levels of the reproductive axis to maintain reproductive competence.

1. INTRODUCTION

The cytochrome P450 steroid hydroxylases are expressed in a tissue-specific and developmentally regulated fashion (Miller 1988). Expression of these enzymes is generally limited to steroidogenic cells and, within these cells, is coordinately induced by trophic hormones. These coordinate profiles of expression raised the possibility that steroid hydroxylase expression is controlled by a shared transcriptional regulator. Studies analysing promoter activity of the 5′-flanking regions of these genes in cell lines derived from steroidogenic cells showed that multiple promoter elements were necessary for full levels of expression. Notably, each gene was regulated by elements that matched the AGGTCA 'half-site' motif for nuclear receptor family members (Rice *et al.* 1991; Morohashi *et al.* 1992). Recent studies described here indicate that these elements interact with a cell-selective nuclear receptor, steroidogenic factor 1 (SF-1), that is essential for adrenal and gonadal development. Evidence from cell culture models implicates SF-1 in the regulation of both key mediators of male sexual differentiation, whereas analyses of mice that are homozygous for a disrupted SF-1 allele indicate that SF-1 also plays key roles at the hypothalamic and pituitary levels of the reproductive axis.

2. RESULTS

(*a*) *Isolation and characterization of SF-1*

The similar sequences of the steroidogenic regulatory elements suggested that the same regulatory protein interacted with these sequences to coordinate the expression of the steroid hydroxylases (Rice *et al.* 1991; Morohashi *et al.* 1992). Because the protein that formed this complex was restricted to steroidogenic cell lines, we designated it steroidogenic factor 1 (SF-1). When we isolated an SF-1 cDNA (Lala *et al.* 1992), several important features became apparent. First, SF-1 belonged to the nuclear hormone receptor family, a diverse group of structurally related proteins that mediate transcriptional activation by various steroid hormones, Vitamin D, thyroid hormone and retinoids (reviewed by Evans 1988). The most striking match was to a cDNA isolated from embryonal carcinoma cells and designated embryonal long terminal repeat binding protein (ELP) because it inhibited transcription of retroviral LTRs (Tsukiyama *et al.* 1991). Isolation and characterization of genomic sequences encoding SF-1 ultimately revealed that both SF-1 and ELP arise from the same structural gene by alternative promoter usage and 3′ splicing (Ikeda *et al.* 1993). The gene encoding these two transcripts was designated *Ftz-F1* because it also resembles the FTZ-F1 *Drosophila* nuclear receptor, which encodes two developmentally regulated isoforms and which regulates the fushi tarazu (*ftz*) homeobox gene (Lavorgna *et al.* 1991, 1993).

(*b*) *Ontogeny of SF-1 expression*

Based on the essential roles of steroid hormones in male sexual differentiation (Jost 1953), we used *in situ* hybridization to analyse the developmental profile of SF-1 in mouse embryos. As summarized in table 1, these analyses provided intriguing insights into the possible role of SF-1 in steroidogenic organ development (Ikeda *et al.* 1994). SF-1 transcripts were

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Table 1. *Profile of SF-1 expression during mouse development*

site	profile
adrenal gland	expressed from earliest stages of development of adrenal primordium (E10.5)
	as chromaffin cell precursors invade adrenal from neural crest at ~ E13, expression localizes to outer, cortical region
gonads	expressed in bipotential gonad of both sexes from earliest stages of gonadogenesis (~ E9)
	expression in testis persists after sexual differentiation (E12.5) in both Leydig and Sertoli cells
	expression in ovary decreases coincident with sexual differentiation (E12.5)
other sites	expressed in ventral diencephalon (precursor to endocrine hypothalamus)
	expressed in gonadotropes of anterior pituitary

present in the adrenal primordium from the inception of adrenal formation at approximately embryonic day 10.5 (E10.5). Later, as the sympathoadrenal precursors migrated into the adrenal gland from the neural crest to become the medulla, SF-1 expression was confined to the cortical region where steroidogenesis occurs. SF-1's expression at the earliest stages of adrenal differentiation suggested a key role in adrenal development. Similarly, SF-1 was expressed at very early stages of gonadogenesis (~ E9), with expression seen in both male and female embryos. At this stage of development, male and female gonads are histologically indistinguishable and are therefore termed indifferent or bipotential gonads. After ~ E12.5, as morphologic sexual differentiation is occurring, SF-1 expression persisted in the testes but was extinguished in ovaries. Moreover, SF-1 was expressed in both compartments of the foetal testes: the interstitial region, which contains foetal Leydig cells that produce androgens; and the testicular cords, which contain foetal Sertoli cells and primordial germ cells (PGCs). The expression of SF-1 by Sertoli cells and its sexually dimorphic pattern suggested that SF-1's role in the gonadal development extended beyond regulating the steroidogenic enzymes.

Finally, SF-1 was also expressed in discrete regions of the embryonic ventral diencephalon and anterior pituitary (Ingraham *et al.* 1994). Intriguingly, the ventral diencephalon is the analage of the endocrine hypothalamus, whereas the anterior pituitary includes the gonadotropes that produce gonadotropins, the predominant regulators of gonadal steroidogenesis. These findings suggested that SF-1 also functions at additional levels of the hypothalamic-pituitary-steroidogenic tissue axis.

(*c*) *The mouse Ftz-F1 gene is essential for adrenal and gonadal development*

To define the role of SF-1 in vivo, we used the technique of targeted gene disruption to 'knockout' the mouse *Ftz-F1* gene that encodes SF-1 (Luo *et al.* 1994). The strategy that we used abolished both ELP and SF-1 activity is shown in figure 1. In matings of heterozygous *Ftz-F1*- disrupted mice, -/- animals were born at the expected frequency of ~ 25%, indicating that neither ELP nor SF-1 is required for embryonic survival. As summarized in table 2, all knockout animals died shortly after birth and had depressed corticosterone levels, consistent with a pronounced impairment of corticosteroid biosynthesis. They also had female external genitalia irrespective of genetic sex, consistent with a failure to produce testicular androgens. These findings were all consistent with our

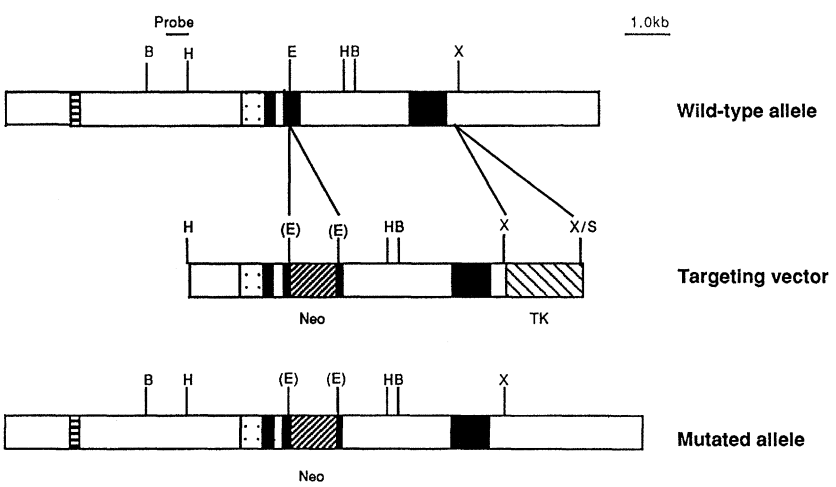

Figure 1. Strategy for disrupting the mouse *Ftz-F1* gene. A schematic summary of the strategy used to disrupt the mouse *FTZ-F1* gene. Solid areas indicate shared exons, horizontally lined areas denote SF-1-specific exons, and stippled areas denote ELP-specific exons. The Neo selectable marker and thymidine kinase (TK) gene were inserted into a plasmid containing the indicated *Ftz-F1* genomic sequences. The Neo gene disrupts the second zinc finger region required for DNA binding. Restriction endonuclease sites include B, BglII; X, XhoI; H, HindIII; E, Eco47 III. The position of the probe used for Southern blotting analyses is indicated.

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Table 2. *Features of* Ftz-F1-*disrupted mice*

found at expected frequency of 25% of live births, indicating that the *Ftz-F1* gene is not required for prenatal survival
look normal, but all have female external genitalia irrespective of genetic sex
fail to gain weight despite normal suckling
die shortly after birth unless treated with corticosteroids

anticipation that SF-1 was an essential regulator of adrenal and gonadal steroidogenesis. Surprisingly, the *Ftz-F1*-disrupted animals also lacked adrenal glands and gonads, demonstrating the essential role of SF-1 in adrenal and gonadal development. Developmental studies of *Ftz-F1*-disrupted embryos suggested that the earliest stages of gonadogenesis were relatively intact, but that the SF-1 deficiency ultimately was associated with the induction of apoptosis in the adrenal glands and gonads, with subsequent disintegration of the primary steroidogenic organs.

To see if the *Ftz-F1*-disruption affected the migration of the primordial germ cells (PGCs) to the gonads, we examined the patterns of alkaline phosphatase positive cells at different stages of development (Luo *et al.* 1994). At the earliest time points at which gonadal PGCs could be visualized with this technique, we saw no major differences in the numbers of alkaline phosphatase positive cells, suggesting that pgc migration was not impaired. Later, as the gonads regressed, the PGCs also disappeared, suggesting that the supporting structure of the gonad was required for their survival.

(*d*) *The Ftz-F1 gene also plays important roles at other levels of the hypothalamic-pituitary-gonadal axis*

As noted above, SF-1 was also expressed in the ventral diencephalon and the anterior pituitary, suggesting that it might act at additional levels of the hypothalamic-pituitary-steroidogenic organ axis. Consistent with this model, the *Ftz-F1*-disrupted mice did not express multiple proteins that comprise the normal complement of gonadotrope-specific markers, including the α-subunit of glycoprotein hormones, luteinizing hormone, follicle-stimulating hormone, and the gonadotropin-releasing hormone receptor (Ingraham *et al.* 1994). They also lacked the ventromedial hypothalamic nucleus (Ikeda *et al.* 1995), a region of the hypothalamus containing high concentrations of steroid hormone receptors that has been implicated in female reproductive behavior (Pfaff *et al.* 1995). These findings further illustrate the intimate link between SF-1 and the development of reproductive competence.

3. DISCUSSION

The central focus of our laboratory has been to define the mechanisms that regulate gene expression of the steroid hydroxylases, first in the adrenal cortex and more recently in gonads. These studies implicated SF-1 as a pivotal regulator of the steroid hydroxylases but more importantly, SF-1 has recently been shown to play additional essential roles in adrenal and gonadal development and to function at the hypothalamic and pituitary levels of the reproductive axis. The *Ftz-F1*-disrupted animals should provide an excellent model system in which to ascertain the target genes through which SF-1 exerts its actions in steroid organ development. For example, analyses of the 5′-flanking region of the gene encoding Müllerian Inhibiting Substance (MIS) identified a potential SF-1-responsive element, and showed a temporal correlation between SF-1 and MIS gene expression (Shen *et al.* 1994). If functional studies in intact mice ultimately support a role for this element in MIS gene expression, it will directly implicate SF-1 in both critical arms of male sexual development: androgen production by foetal Leydig cells and MIS production in foetal Sertoli cells.

Based on our studies, SF-1 apparently acts at several positions within the hierarchy of gonadal development (see figure 2). Its early expression in the bipotential gonads of both male and female embryos and the absence of gonads in *Ftz-F1*-disrupted mice of both sexes suggest that SF-1 participates in critical early developmental events in the bipotential gonad. As SF-1 is expressed in both males and females with a timecourse that precedes the onset of Sry expression, it seems highly unlikely that Sry directly regulates SF-1 expression. It nonetheless remains possible that Sry indirectly regulates SF-1, perhaps by inhibiting a suppressor that inhibits SF-1 expression in the ovary. This model would predict that persistent ovarian expression of SF-1 during the period when SF-1 is normally not expressed may result in abnormal sexual differentiation, and transgenic experiments are currently underway to address this model.

Gonadal agenesis was also observed in mice with a knockout of the Wilm's tumor related gene WT1 (Kreidberg *et al.* 1993). Preliminary analyses in E11 *Ftz-F1*-disrupted embryos indicate that WT1 expression is maintained in the absence of SF-1; similarly, SF-1 expression is maintained in the WT1 null mice (Y. Ikeda, unpublished observation). These results suggest that, although they are both essential for gonadal development, WT-1 and SF-1 act independently in parallel pathways in gonadal development. Similar analyses, perhaps using more sensitive RNase protection or reverse transcription-polymerase chain reaction (RT-PCR) approaches, should provide novel insights into direct upstream and downstream actions of the expanding number of genes involved in gonadogenesis.

A final gene that may well interact, either directly or indirectly, with SF-1 is the recently-described *DAX-1* gene. This gene (described by Dr Camerino, this volume) was isolated by positional cloning from patients with congenital adrenal hypoplasia (Zanaria *et al.* 1994). These patients have an X-linked disorder characterized by adrenal hypoplasia and a subset of patients also exhibit hypogonadotrophic hypogonadism (Muscatelli *et al.* 1994), thus closely resembling the phenotype in the *Ftz-F1*-disrupted mice. Intriguingly, *DAX-1* encodes an orphan nuclear

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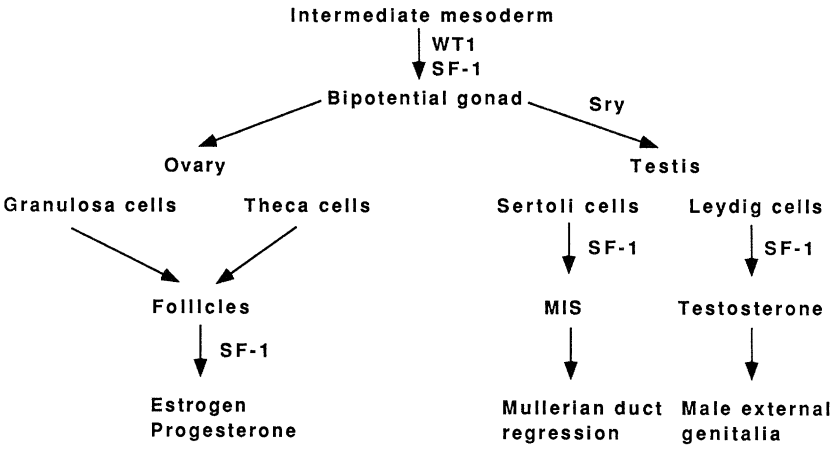

Figure 2. The roles of SF-1 in gonadal development and function. A model of gonadal development is shown, including sites at which SF-1 is believed to play important roles.

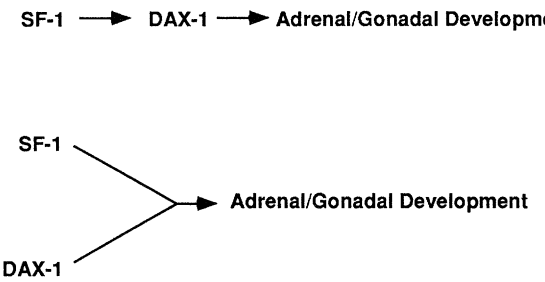

Figure 3. Possible interactions between SF-1 and DAX-1 in endocrine development. Two alternative models for potential interactions between SF-1 and DAX-1 are shown. (*a*) SF-1 directly regulates the expression of DAX-1, which then regulates genes essential for adrenal and gonadal development. (*b*) SF-1 and DAX-1 form a heterodimer that regulates genes essential for adrenal and gonadal development.

receptor that contains amino acids corresponding to the ligand-binding domain of nuclear receptors but lacks the zinc-finger DNA-binding domain typical of these transcriptional regulators. Two alternative models (see figure 3) could explain the strikingly similar phenotypes that accompany SF-1 and DAX-1 deficiency. First, SF-1 may act as an obligatory upstream regulator of DAX-1 gene expression, with DAX-1 then directly regulating genes that are essential for adrenal and gonadal development. Alternatively, it is possible that SF-1 and DAX-1 physically interact as a heterodimer, and that the heterodimeric species is the crucial component for adrenal and gonadal development. Further studies of these orphan nuclear receptors will undoubtedly provide new insights into the complex events in endocrine development and differentiation.

This work was supported by the Howard Hughes Medical Institute and the National Institutes of Health. We thank Dr Douglas Rice, Dr Andrea Mouw and Dr Deepak Lala for their key contributions to early studies of SF-1 and Dr Beverly Koller for invaluable assistance in preparing the *Ftz-F1*-disrupted mice. We also thank Dr Amanda Swain, Dr Robin Lovell-Badge, Dr Blanche Capel and Dr Giovanna Camerino for helpful discussions.

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Discussion

M. B. Renfree (*Department of Zoology, University of Melbourne, Parkville, Melbourne Vic. 3052, Australia*). How does SF-I regulate MIS expression?

K. L. Parker. Studies performed in collaboration with Dr Holly Ingraham (University of California, San Francisco), have identified a key promoter element that lies in the 5′-flanking region of the MIS gene. Mutation of this element markedly impairs promoter activity in tranfection experiments in primary Sertoli cells, and the element can clearly be shown in gel mobility shift experiment to bind SF-1. To date, co-transfection experiments have failed to demonstrate effects of SF-1 on promoter activity, raising the possibility that an endogenous ligand or coactivator is important for SF-1 mediated activation of MIS gene expression.

A. McLaren (*Wellcome/CRC Institute, Cambridge, U.K.*). Is SF-1 expressed in primordial germ cells? Also, in the SF-1 knock-out embryo, do the germ cells enter the genital ridges, and what becomes of them when the ridges degenerate?

K. L. Parker. Expression of SF-1 in the primordial germ has not been demonstrated, although the *in situ* studies lack the single-cell resolution that would be necessary to exclude expression by these cells. The primordial germ cells, as identified by alkaline phosphase staining, migrate into the genital ridges of both male and female SF-1 knockout embryos. Thereafter, as the genital ridges regress via apoptosis, there is a coincident loss of alkaline phosphatase positive cells, suggesting that they also die as the gonads degenerate.

N. Josso (*Unité de Recherches sur l'Enodcrinologie du Développement, Ecole Normale Supérieure, Département de Biologie, 1 rue Maurice Arnoux – 92120 Montrouge, France*). Dr Lovell-Badge mentioned that the onset AMH production by the testis occurs earlier than was previously thought, namely at 11.5 dpc in the mouse. Is there a difference in SF-1 expression 24 or 12 h earlier, say around 10 days?

K. L. Parker. The *in situ* hybridization approach used to measure SF-1 levels is only semi-quantitative, making it very difficult for us to exclude some differences in the levels of SF-1 expression in males and females at earlier stages of gonadogenesis. In addition, our analyses have only looked at mRNA levels, and therefore have not established the time that SF-1 protein first appears in the genital ridge. It is clear that both male and female embryos express readily detectable levels of SF-1 transcripts in the developing genital ridges at very early stages of gonadogenesis (approximately E9), and that SF-1 transcripts are present in both sexes at E10. Thereafter, coincident with critical events in sexual differentiation (E12.5-E13), we see that SF-1 mRNA levels increase considerably in the testis, but become very hard to detect in the ovary. This sexually dimorphic expression pattern is consistent with the model that SF-1 plays important roles in regulating the biosynthesis of both MIS and androgens, and thus contributes to the expression of the two key effector molecules for male sexual differentitation.

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