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The Mullerian Inhibitor and Mammalian Sexual Development [and Discussion]

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The Müllerian inhibitor and mammalian sexual development

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SUMMARY

The elegant embryological experiments of Jost demonstrated the existence of a foetal testicular factor that is required to cause the regression of the Müllerian duct system, the anlagen of the uterus, oviducts and upper portion of the vagina, during male sexual development. The Müllerian inhibitor currently known as Müllerian-inhibiting substance (MIS) or anti-Müllerian hormone (AMH), is a member of the transforming growth factor-β (TGF-β) family of growth and differentiation factors. The genetic manipulation of the mouse germline has lead to the generation of animal models for MIS function. Female transgenic mice that chronically express MIS during embryogenesis are born without a uterus or oviducts and their ovaries lose germ cells and degenerate, recapitulating the phenotype of the bovine freemartin. Some male transgenic mice from very high MIS-expressing lines are feminized, suggesting alterations in androgen biosynthesis. Male mice homozygous for a targeted mutation of the MIS gene develop as male pseudohermaphrodites with both male (testes and Wolffian duct-derived) and female (Müllerian duct-derived) reproductive organs. Most are infertile because the development of two reproductive systems physically blocks the exit of sperm from these males. In addition, Leydig cell hyperplasia is detected in a proportion of these males and in one case a Leydig cell tumour was found. Recently, a gene encoding a TGF-β family type II Ser/Thr kinase membrane-bound receptor has been isolated that is expressed in both male and female gonads and in the mesenchyme surrounding the Müllerian ducts during embryogenesis. These findings suggest that MIS-mediated Müllerian duct regression occurs indirectly through mesenchymal tissue. A targeted mutation of this receptor has been established in the mouse germline. Mice homozygous for this receptor mutation should be useful in understanding the MIS signalling pathway for Müllerian duct regression and gonadal function.

1. INTRODUCTION

The expression of the sex determination genes initiates genetic cascades that result in the differentiation of the male and female sexual phenotypes in mammals (see figure 1) (Sinclair *et al.* 1990; Gubbay *et al.* 1990; Koopman *et al.* 1991). Several of the genes that encode factors that regulate the differentiaiton of the sexual phenotypes have been isolated (Cate *et al.* 1986; Picard *et al.* 1986; Call *et al.* 1990; Gessler *et al.* 1990; Lala *et al.* 1992; Honda *et al.* 1993; Baarends *et al.* 1994; di Clemente *et al.* 1994). The identification of these genes and the determination of their functions during sexual differentiation are key to our understanding of these complex developmental pathways (Koopman *et al.* 1991; Kreidberg *et al.* 1993; Luo *et al.* 1994; Behringer *et al.* 1994; Shawlot & Behringer 1995).

MIS is a dimeric glycoprotein that is expressed specifically in the Sertoli cells of the foetal and adult testes and granulosa cells of the ovary after birth (for review, see Cate *et al.* 1990). This gonadal hormone is a member of the TGF-β family of growth and differentiation factors (Cate *et al.* 1986; Massagué 1990). Its primary role is to cause the regression of the Müllerian duct system during male foetal development (for reviews, see Donahoe *et al.* 1987; Josso *et al.* 1993; Behringer *et al.* 1994). The subsequent synthesis of testosterone causes the differentiation of the Wolffian duct system into the vas deferens, epididymides, and seminal vesicles of the male reproductive system. A variety of additional functions have been assigned to MIS, including roles in gametogenesis, testicular differentiation and descent, and lung development (Josso *et al.* 1993).

The bovine freemartin provides a naturally occurring model for the consequences of ectopic MIS exposure during female embryogenesis (Jost *et al.* 1972). When there is a male and a female twin, chorioallantoic anastomosis can lead to the exposure of the female foetus to the blood of her male twin. The female, known as a freemartin, has regressed Müllerian duct derivatives, stunted ovaries with decreased numbers of germ cells and seminiferous tubule-like structures containing Sertoli cells.

The persistent Müllerian duct syndrome (PMDS) is a rare form of male pseudohermaphroditism with a variety of causes (Guerrier *et al.* 1989; Knebelmann *et al.* 1991; Carré-Eusebe *et al.* 1992; Imbeaud *et al.* 1994). In some cases, MIS levels are low or undetectable and correlate with structural lesions in the MIS

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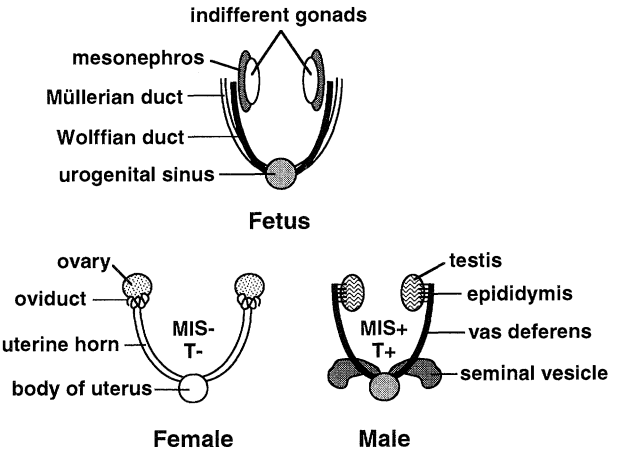

Figure 1. Mouse sexual development. The Müllerian ducts give rise to the uterus, oviducts, and the upper portion of the vagina. The Wolffian ducts give rise to the epididymides, vas deferens, and seminal vesicles. MIS produced by the Sertoli cells of the foetal testes causes the regression of the Müllerian ducts and testosterone (T) produced by Leydig cells induces the differentiation of the Wolffian duct system. The absence of both hormones during female foetal development permits the development of the Müllerian duct system while the Wolffian ducts passively regress.

gene. In other situations, however, MIS levels are within normal limits or are elevated, suggesting target-organ insensitivity. The recent cloning of a type II Ser/Thr kinase membrane–bound receptor that can bind MIS (Baarends *et al.* 1994; di Clemente *et al.* 1994) may help to explain these MIS-positive cases of PMDS and also help to define the MIS signalling pathway.

In vivo experimental approaches, primarily transgenic mice that chronically express MIS and MIS mutant mice, have been useful in the study of the function of MIS during embryogenesis and reproductive development (Behringer *et al.* 1990; Behringer *et al.* 1994; Lyet *et al.* 1995). Mice that ectopically express the receptor for MIS or receptor deficient mice should provide useful insights into the MIS signaling pathway during mammalian sexual and reproductive development.

2. MIS-OVEREXPRESSING TRANSGENIC MICE

The human MIS gene was chronically expressed in both male and female transgenic mice using the mouse methallothionein promoter (Behringer *et al.* 1990). Exposure of the female transgenic mice to MIS during embryogenesis resulted in the elimination of the Müllerian duct system and the degeneration of the ovaries. These results provided experimental confirmation *in vivo* for the central function of MIS during development (i.e. the regression of the Müllerian duct system). Transgenic males from lines that expressed very high levels of MIS were externally and internally feminized with undescended testes, suggesting abnormalities in androgen biosynthesis. In support of this idea, Lyet *et al.* (1995) have determined that these transgenic males have depressed levels of circulating testosterone. These gain of function studies provided insights into the potential *in vivo* functions of MIS. However, because the levels of MIS were quite high it was possible that abnormal MIS/receptor interactions were also occurring. Therefore, a loss of function approach was undertaken to complement these gain of function studies.

3. MIS MUTANT MICE

To understand the requirement of MIS during development, MIS mutant mice were generated by mutating the mouse MIS gene (Münsterberg & Lovell-Badge 1991) by homologous recombination in embryonic stem (ES) cells (Behringer *et al.* 1994). The gene-targeting vector deleted a portion of the first exon, and the entire first intron and second exon. Two independently derived ES clones successfully contributed to the germline of chimeric mice generated by blastocyst injection. Both MIS mutant mouse lines had the same phenotype. Female mutant mice were found to be normal and fertile, suggesting that MIS has a redundant role in ovarian function. Male mutants developed as male pseudohermaphrodites with both male (testes and Wolffian duct-derived) and female (Müllerian duct-derived) reproductive organs (see figure 2). The presence of a uterus in these males interferred with sperm transfer into females, rendering most (approximately 90%) infertile. Leydig cell hyperplasia was observed in about 30% of the mutant males greater than 10 weeks of age and in one case a Leydig cell tumour was found. These results confirmed that the primary role of MIS is the regression of the Müllerian ducts which is ultimately important for male fertility. In addition, these findings suggested a role for

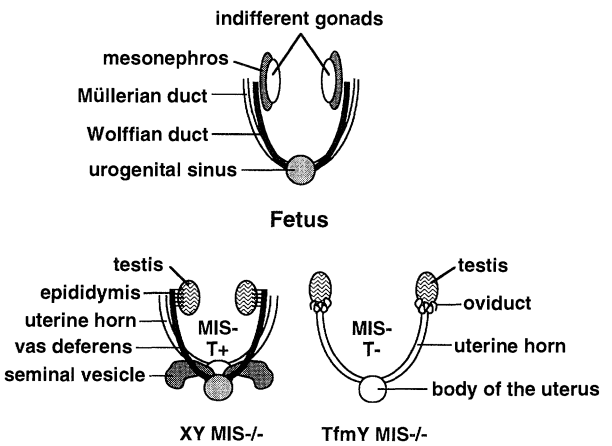

Figure 2. Male pseudohermaphroditism in MIS deficient and MIS deficient/androgen insensitive XY mice. XY individuals that lack MIS differentiate both the Müllerian and Wolffian duct systems and are overtly male in appearance. The presence of both types of reproductive organs severely hinders fertility. The physical association of the resulting oviductal tissue with the Wolffian duct derivatives blocks oviduct coiling. XY individuals that lack MIS and are also insensitive to androgens because of the *Tfm* mutation differentiate the Müllerian duct system but the insensitivity to androgens also results in the passive regression of the Wolffian duct system. While these mice have testes, they are female in appearance. Since the Wolffian duct system has been eliminated in these mice the oviductal tissue assumes its normal coiled morphology.

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MIS in the regulation of Leydig cell proliferation. The lack of abnormalities in testicular differentiation and descent, and in lung development, suggest that at least in mice, MIS is not essential for these processes.

The two central hormones of male sexual development, MIS and testosterone, were functionally eliminated by generating mice deficient for MIS that were also insensitive to androgens (see figure 2). This was accomplished by crossing mice with the MIS mutation to animals carrying the X-chromosome linked testicular feminization (*Tfm*) mutation which encodes a defective androgen-binding receptor (Charest *et al.* 1991; He *et al.* 1991). *Tfm*/Y MIS-deficient animals developed essentially as females. Externally they appeared female, internally they lacked Wolffian duct derivatives and had a uterus, oviducts and vagina, but in the place of ovaries they had small undescended testes. In MIS-deficient males the oviducts do not coil as in normal females because of an intimate physical association with the epididymides by connective tissue. In the *Tfm*/Y MIS–deficient 'males', the lack of Wolffian duct differentiation resulted in apparently normal oviductal coiling. Thus, the elimination of the Wolffian duct system is probably also important for normal female sexual development.

4. MIS-RECEPTOR MUTANT MICE

There are a number of patients with PMDS that have normal or elevated levels of MIS, suggesting target organ insensitivity. Likewise, a MIS-positive form of PMDS has been reported in dogs (Meyers-Wallen *et al.* 1989). One simple explanation for these findings could be a defective receptor for MIS.

Recently, a cDNA that encodes a receptor that can specifically, though weakly, bind the Müllerian inhibitor has been identified in rabbit (di Clemente *et al.* 1994). A homologous cDNA has also been isolated in rat, although ligand-binding information about this receptor has not been published (Baarends *et al.* 1994). These cDNAs encode a type II membrane-bound Ser/Thr kinase receptor of the TGF-β receptor family. The type II MIS-receptor is expressed in the somatic cells of the foetal and adult gonads and the mesenchymal cells adjacent to the Müllerian ducts of the foetus (Baarends *et al.* 1994; di Clemente *et al.* 1994). The structure of this receptor, its ability to specifically bind MIS, and its expression pattern suggest that MIS-mediated Müllerian duct regression occurs indirectly through mesenchyme tissue.

To determine whether signalling through this receptor is a requirement for Müllerian duct regression during male development and to uncover other essential functions of this receptor, we isolated the mouse type II MIS-receptor gene to generate receptor mutant mice by gene targeting in ES cells (Mishina *et al.* personal comm.). The mutation deletes the first six exons which encode the entire extracellular ligand-binding and transmembrane domain of the protein. It was predicted that this should functionally inactivate the type II MIS-receptor gene. Correctly targeted ES clones were obtained and two were found to be capable of contributing to the germline of chimeric mice generated by blastocyst injection (Mishina *et al.* personal comm.). The phenotype of mice homozygous for this targeted mutation should provide insights into the function of this receptor during Müllerian duct regression and gonadal development. In addition, the availability of the MIS-overexpressing transgenic mice, the MIS mutant mice, and now the MIS-receptor mutant mice provides the opportunity for intercrossing these animals to create compound mutants or mutants that overexpress MIS to characterize MIS-signalling pathways further. These unique genetic resources are beginning to shed light on the genetic pathway that contributes to the differentiation between the sexes.

5. CONCLUSIONS AND PERSPECTIVES

Current knowledge regarding MIS with respect to the regression of the Müllerian ducts and gonadal function can be summarized as follows. In males, MIS is expressed in Sertoli cells of the foetal testis. MIS leaves the foetal gonad and interacts with receptors on mesenchyme cells that surround the Müllerian duct epithelium. Signals transduced by the MIS receptor alter the phenotype of the mesenchyme cells to instruct, either actively or passively, the Müllerian duct epithelium to regress (see figure 3). In addition, MIS also acts in an autocrine–paracrine manner on Sertoli cells. Either directly or indirectly this results in the regulation of Leydig cell proliferation. In females, it is still not clear what role MIS plays in the gonad.

In summary, much has been learned about MIS, but a number of questions regarding its roles during development and reproduction remain to be answered. How is the MIS gene regulated? Is there a role for MIS in the ovary? How does MIS reach the mesenchymal cells surrounding the Müllerian ducts? How is the MIS receptor gene regulated? Does the type II MIS

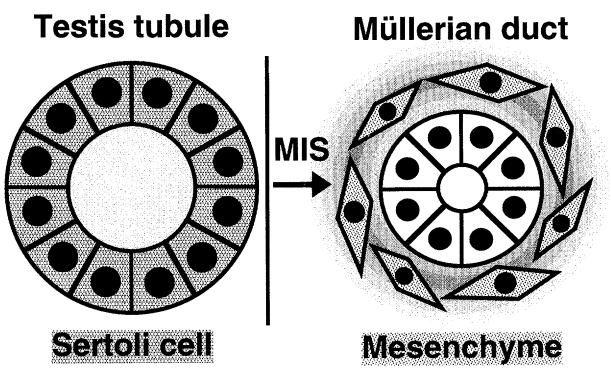

Figure 3. Current model for Müllerian duct regression. MIS produced by the foetal testes, by way of an unknown mechanism, reaches the region of the Müllerian ducts. The ligand then binds type II TGF-β family Ser/Thr kinase membrane bound receptors on mesenchyme cells that surround the Müllerian duct. Signals transduced by the interaction of MIS with this receptor alter the behaviour of the mesenchyme cells thereby instructing the Müllerian duct epithelium, either actively or passively, to regress. Receptors for MIS are also present on Sertoli cells, suggesting an autocrine function that ultimately regulates Leydig cell proliferation. The function of MIS in granulosa cells of the ovary after birth remains unknown.

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receptor interact with additional ligands besides MIS? What are the components of the MIS signal transduction pathway? Is there a type I or type III MIS receptor? What is the nature of the cell-cell interactions between the mesenchyme and the Müllerian duct epithelium that result in duct regression? The answers to these questions will not only define the mechanisms involved in this developmental pathway of sexual and reproductive development but may also provide fundamental information regarding more global biological mechanisms.

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Discussion

A. McLaren (*Wellcome/CRC Institute, Cambridge, U.K.*). Leydig cell hyperplasia has also been reported in the testes of aged male mice, and in the testes of XX/XY chimeric males at younger ages (McLaren 1975), which have presumably developed from gonads that could have been classified as ovotestes in the embryo.

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N. Josso (*Ecole Normale Supérieure, Département de Biologie, Montrouge, France*). There are several hypotheses that could explain the down regulation of testosterone production by AMH in Leydig cells, both prompted by what is known in granulosa cells (di Clemente *et al.* 1994). In ovaries, AMH is known to repress the transcription of aromatase and of the LH receptor. In the Leydig cell, AMH could conceivably affect other steroidogenic enzymes or the LH receptor. We favour the second hypothesis, in view of the Leydig cell hyperlasia observed in AMH knockout mice.

S. Imbeaud in our lab has detected a splice mutation in the donor splice site of the second intron of the human AMH receptor in a patient with the persistent Müllerian duct syndrome. This mutation, as well as others located in the intracellular part of the receptor, is autosomal recessive.

The localization of AMH receptors in the mesenchyme, but not the epithelium, of the Müllerian duct is in keeping with the work of Tsuji *et al.* (1992) indicating that AMH decreases the proliferation of mesenchymal but not epithelial cells in the 15-day-old Müllerian duct of the foetal rat.

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