REVIEW

New insights into Mullerian inhibiting substance and its mechanism of action

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Introduction

One of the first factors to be secreted by fetal Sertoli cells during mammalian male sexual differentiation is the glycoprotein, Mullerian inhibiting substance (MIS), also known as anti-Mullerian hormone (AMH). MIS initiates the regression of the Mullerian ducts during a single, transient period during sexual development of the male; in the absence of MIS, the Mullerian ducts differentiate into the uterus, fallopian tubes and upper one-third of the vagina in the female (Josso *et al.* 1977, Lee & Donahoe 1993). In addition to this principal role for MIS, its continued postnatal expression supports hypotheses that MIS may have other roles such as regulation of gonadal function, testicular descent, lung development and suppression of tumor growth (Lee & Donahoe 1993, Teixeira & Donahoe 1996).

Regulation of MIS expression

The human gene, located on chromosome 19p13.2–13.3 is 4 kb long, encompassing five exons that yield a 2 kb mRNA (Cate *et al.* 1986). MIS mRNA expression is first detected in the mouse testis at 11.5 days post coitus (Munsterberg & Lovell-Badge 1991) and in the rat testis at 13 days post coitus (Josso *et al.* 1977), and it continues throughout fetal development. Messenger RNA expression decreases several days after birth and then decreases further peripubertally (Kuroda *et al.* 1990). MIS expression is first detectable in the female rodent several days after birth and persists throughout life (Munsterberg & Lovell-Badge 1991, Hirobe *et al.* 1992, Taketo *et al.* 1993). MIS protein expression appears to parallel mRNA expression in the rodent. Serum concentrations of MIS in males increase after birth to a peak in late infancy and then decline until puberty, when they remain stable; in females, MIS is first secreted by the ovaries in low concentrations postnatally and then increases at puberty (Lee *et al.* 1996).

The proximal 180 base pairs of the mouse MIS promoter appear to be sufficient for activation of MIS expression, and within this region there are several evolutionarily conserved motifs (Dresser *et al.* 1995, Giuli *et al.* 1997) (Fig. 1). Using deletion constructs of the –269 to +8 region of the human MIS promoter upstream of a luciferase reporter, we have been able to analyze the roles of the conserved elements. A region between –63 and –49 containing the sequence GTTTGT is protected by the HMG domain of the sex-determining region Y (SRY) protein in DNase footprinting experiments and the SRY HMG domain binds to this sequence in electrophoretic mobility shift assays (Haqq *et al.* 1993, 1994). As MIS expression in Sertoli cells follows approximately 1 day after the expression of SRY in rodents and shortly after SRY expression during the 7th week of human gestation, SRY is a candidate to regulate MIS expression through binding to this sequence. However, the functional significance of this element remains in question because, although SRY was able to induce expression of a luciferase reporter linked to the MIS promoter, no mutations of the MIS promoter, including mutations or deletions of this HMG element, were able to diminish this induction. It is possible that SRY requires the presence of an interacting factor to confer specificity for activation of this site.

Another conserved element in the human MIS promoter, the MARE-1 or M-1 site, is located between –93 and –69. This sequence conforms to the consensus sequence for the GATA family of transcription factors (Zon *et al.* 1991). GATA-1 is known to be expressed in adult Sertoli cells; however, in our laboratory, co-transfection of GATA-1 failed to activate an MIS-promoter-driven luciferase reporter construct. As novel GATA factors are discovered, they may be similarly tested for functional effects on the MIS promoter.

The M2/MIS-RE1 site (Shen *et al.* 1994) at position –84 to –103 contains the sequence CCAAGGTCA at the 3' end, which conforms to the consensus steroidogenic factor 1 (SF1) binding site. SF1, an orphan receptor

Figure 1 MIS proximal promoter. In numbering the bases of the promoter region, bases are counted from the transcriptional start site.

of the steroid nuclear receptor superfamily, is expressed in the male and female mouse urogenital ridge before the expression of MIS and has an important role in normal sexual development (Ikeda *et al.* 1993). Null mutations of this gene lead to degeneration of the gonads, adrenals, and ventromedial hypothalamus (Luo *et al.* 1994, Ikeda *et al.* 1995). Mutations in the MIS-RE1 binding site lead to decreased expression of reporter genes in a postnatal Sertoli cell line and in transgenic mice (Giuli *et al.* 1997), whereas MIS expression in HeLa cells is induced by SF1 lacking the ligand-binding domain (Shen *et al.* 1994). In contrast, in our laboratory, when full-length SF1 and an MIS luciferase reporter were co-transfected into an embryonic male urogenital ridge-derived cell line, luciferase expression was decreased (Haqq *et al.* 1994). Thus, although it appears that SF1 may activate MIS expression, the precise nature of the interactions of SF1 with the MIS promoter remain unclear and may be developmentally dependent.

The human MIS gene does not contain a consensus TATA or initiator element (Cate *et al.* 1986, Guerrier *et al.* 1989). Experimental mutations in the human MIS promoter in our laboratory have identified the -6 to +10 region as containing a putative initiator site (N Morikawa, T Clarke, K Watanabe & P K Donahoe, unpublished observations). This region formed specific DNA-protein complexes with nuclear extracts from a 14.5 day post coitus rat urogenital ridge-derived cell line, and with purified transcription factor II (TFII)-I in electrophoretic mobility shift assays. Polyclonal antibodies were able to block the initiator TFII-I complexes, and excess molar amounts of initiators of other genes, such as the T-cell receptor-driven variable region B chain gene and terminal deoxynucleotidyl transferase, were able to compete with the complex. Thus human MIS may be initiator-driven via an initiator site that is distinct from the typical initiator consensus, PyPy(A+1)NT/APyPy.

MIS signaling

MIS is a homodimeric glycoprotein linked by disulfide bonds, with a molecular mass of 140 kDa (Cate *et al.*

1990). Along with the activins, inhibin, bone morphogenesis proteins, *Drosophila's* decapentaplegic, Vg-1 and a host of newly discovered growth and differentiation factors, it is a member of the transforming growth factor (TGF)- β family (Cate *et al.* 1990, Massague *et al.* 1994). The MIS molecule must be proteolytically cleaved between R427 and S428 to generate the bioactive carboxy (C)-terminal fragment (MacLaughlin *et al.* 1992, Pepinsky *et al.* 1988), which is a characteristic shared amongst many family members (Massague 1990). Furin and PC5, proprotein convertases expressed in the urogenital ridge, are candidate MIS processing enzymes that, by their temporal and spatial expression, may help confer tissue specificity to the actions of MIS actions (Nachtigal & Ingraham 1996). MIS is glycosylated at its amino (N) terminus, but the functional significance of this is unknown, as the N terminus does not appear to exhibit activity in an organ culture bioassay system, whereas holo-MIS and C-terminal MIS are active (Cate *et al.* 1990, MacLaughlin *et al.* 1992). Without the N terminus to confer stability, the half life of C-terminal MIS in serum is markedly reduced (R C Ragin & P K Donahoe, unpublished observations).

The actions of TGF- β family members are mediated by single transmembrane-spanning serine/threonine kinase receptors classified as types I and II. The type I and type II receptors share approximately 40% homology between their kinase domains. There is evidence that the type II receptors bind to ligand and then can recruit the type I receptors into a tetraheteromeric complex of two type II and two type I receptors (Fig. 2). Some of the type I receptors, such as Alk2 and Alk4 are shared by different type II receptors (Franzen *et al.* 1993). Of the multiple type I receptors that have been cloned, Alk2 is a candidate MIS type I receptor, as it is expressed around the Mullerian duct as it regresses (He *et al.* 1993).

The MIS type II receptor is expressed in the cells surrounding the fetal Mullerian duct, in fetal gonads during the period of sex determination and Mullerian duct regression, and in pubertal and adult rat gonads. The type II receptor mRNA temporal and tissue-specific patterns of

Figure 2 Model for MIS receptor signaling. A homodimer of MIS disulfide-linked at the carboxy-terminus binds to the extracellular cysteine-rich domain of the type II receptor. Downstream signaling occurs when the type I receptor is recruited and phosphorylated by the MIS-type II complex, which releases the negative inhibition exerted by FKBP12. (Reproduced with permission from Teixeira *et al.* 1996.)

expression have been shown to match that of MIS (Teixeira *et al.* 1996a,b).

All the type I receptors of the TGF- β family bind to FKBP12, a cytosolic protein known to bind the immunosuppressant macrolides, FK506 and rapamycin (Wang *et al.* 1994). Our current model postulates that binding of the MIS ligand to the receptor complex results in the phosphorylation of the type I receptor by the type II receptor, followed by the release of FKBP12, thus removing the inhibition of signal transduction by FKBP12 (Wang *et al.* 1996). Other members of the TGF- β type I receptor family have been shown to signal downstream through phosphorylation of members of the Smad family. These proteins, among others, are being investigated as substrates for the putative MIS type I receptor.

Physiological effects of MIS

The Mullerian ducts of male or female rat fetuses will regress fully in response to MIS until 15 days post coitus, but not after that (Donahoe *et al.* 1977, Josso *et al.* 1977). Evidence exists that MIS indirectly causes the dissolution of the Mullerian duct epithelium in the rat by its effects on the mesenchyme surrounding the Mullerian duct (Tsuiji *et al.* 1992). This irreversible dissolution in the human fetus occurs by 51 days post ovulation (Taguchi *et al.* 1984). Failure of the analogous events to occur in the male human fetus leads to the persistent Mullerian duct syndrome (PMDS), in which 46 XY males have normal external genitalia but have a cervix, uterus and fallopian tubes. Although many men with PMDS are infertile and have unilateral or bilateral undescended testes, with

Mullerian structures herniated into the inguinal canal, some are able to father children. Fifty percent of the cases of PMDS can be attributed to mutations in the MIS gene; in the remainder there is a mutation in the type II receptor, such as a deletion of exon 10 (Imbeaud *et al.* 1996). A patient with Mullerian duct retention has also been found to have a mutation in the splice donor site of intron two of the type II receptor, producing a receptor that failed to localize at the cell surface (Faure *et al.* 1996). Male MIS type II receptor-deficient mice display a phenotype of retained uterus and oviducts (Mishina *et al.* 1996), recapitulating that seen with MIS ligand knockout mice (Behringer *et al.* 1994) and in men with PMDS.

Although MIS does not appear to be essential for testicular differentiation in the male, it may affect subsequent testicular function. Focal Leydig cell hyperplasia and Leydig cell tumors were found in otherwise histologically normal testes of MIS-deficient male mice, while feminization and undescended testes were observed in some MIS overexpressing mice, presumably as a result of reduced Leydig cell androgen production (Behringer *et al.* 1990, 1994). A postnatal decline in MIS concentrations in the male occurs in parallel with the initiation of spermatogenesis and the intratesticular increase in testosterone (Cate & Wilson 1993, Al-Attar *et al.* 1997), and sperm isolated from adult MIS-deficient mice have normal morphological characteristics and functional abilities (Behringer *et al.* 1994), suggesting that MIS regulates the timing of spermatogenesis, but is not required for spermatogenesis.

Intra-abdominal descent of the testes during fetal development is guided by caudal enlargement of the gubernaculum and recession of the cranial suspensory ligament, events which may be under the direction of MIS (Hutson *et al.* 1997). Most patients with PMDS do not have testes correctly situated in the scrotum and have hypermobile testes, presumably because of a thin, elongated gubernaculum. This may indicate a role for MIS in the normal swelling of the gubernaculum (Hutson *et al.* 1994). In contrast to this, the testes of MIS-deficient and MIS-receptor-deficient mice undergo normal transabdominal descent (Behringer *et al.* 1994), suggesting either species differences or redundancy in MIS function. Thus any role that MIS may have in the descent of the testis remains unclear.

The freemartin effect occurs when a female fetus is exposed to the blood of a male twin *in utero*. In addition to MIS-directed Mullerian regression in the freemartin female, the ovarian germ cells regress, and the ovaries develop seminiferous tubules containing Sertoli cells. Female transgenic mice overexpressing MIS demonstrate a freemartin effect, with regression of the Mullerian ducts and masculinized ovaries with seminiferous tubules and few germ cells (Behringer 1990). The ability of MIS to redirect ovaries into a more testis-like gonad in females suggests at least an indirect role in male gonadal development. MIS may also have a physiological role in

the ovary, as MIS in rats is expressed after the first week of life in granulosa cells surrounding only preantral and small antral follicles (Hirobe *et al.* 1992, Ueno *et al.* 1989a,b, Baarends *et al.* 1995). MIS may have an indirect role in the inhibition of oocyte meiosis (Takahashi *et al.* 1986, Ueno *et al.* 1989a,b), by blocking aromatase and luteinizing hormone receptor synthesis in granulosa cells (diClemente *et al.* 1994), but a direct effect remains a possibility, as oocyte meiosis inhibition can be observed in isolated oocytes alone. Notably, MIS is not essential for female gonadal function and attainment of fertility, as MIS-deficient female mice are fertile (Behringer *et al.* 1994).

Respiratory distress syndrome, characterized by decreased surfactant production, is of greater morbidity and mortality in male neonates than in females (Miller & Futrakul 1968, Torday *et al.* 1981). The effects of MIS on surfactant accumulation and lung development may contribute to the sex bias of this disorder. MIS decreases the accumulation of disaturated phosphatidylcholine, a component of lung surfactant in fetal rats, and inhibits fetal lung branching morphogenesis in fetal rats by activation of apoptosis (Catlin *et al.* 1988, 1989, 1997). Proximity of the fetal gonad and lung probably allow this to occur through paracrine secretion of MIS activating the putative type II receptor localized in the embryonic lung (Caitlin *et al.* 1997).

Clinical applications for MIS

Serum measurement of MIS is a specific and sensitive marker for the presence of testicular tissue in boys with cryptorchidism. When measured either alone or in tandem with the measurement of human chorionic gonadotrophin stimulated testosterone, MIS concentrations may be used to guide the treatment of these patients. Similarly, serum MIS may be useful in the evaluation of the presence of any functioning testicular tissue in infants and children with ambiguous genitalia (Lee *et al.* 1997).

Purified MIS has demonstrated antiproliferative effects against vulvar carcinoma and ocular melanoma cell lines (Chin *et al.* 1991, MacLaughlin *et al.* 1992, Parry 1992). As ovarian epithelium-derived tumors arise from coelomic epithelium that normally invaginates during fetal development to form the Mullerian duct, this particular type of tumor is potentially responsive to the antiproliferative effects of MIS. Currently, preclinical evidence exists from our laboratory that human ovarian ascites cells from a large percentage of patients with stage III/IV ovarian epithelial cancer bind human recombinant MIS and are growth-inhibited by MIS. As a result, clinical trials are being planned to study the efficacy of MIS against epithelial ovarian tumors in humans (P K Donahoe, unpublished observations).

A variety of other types of ovarian tumors have been shown to secrete high levels of MIS. Although these

tumors, particularly granulosa cell tumors, are insensitive to any antiproliferative effect of MIS, increases in serum MIS may serve as a marker both of post-surgical residual tumor and of tumor recurrence (Gustafson *et al.* 1992, Rey *et al.* 1996). Serial measurements of MIS to allow early detection of recurrence may prove to be especially useful for patients with granulosa cell tumors, which have a poor prognosis in advanced stages (Powell & Otis 1997).

Summary

The primary function of MIS in mammals is to initiate regression of Mullerian structures in males as part of normal sexual development. As we learn more about its other roles, particularly its influence on the growth and differentiation of cell types within the gonad, a more thorough understanding of the receptors that MIS stimulates and the downstream signaling cascade with which it interacts will help in the development of diagnostic and therapeutic uses of MIS.

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