Müllerian inhibiting substance regulates its receptor/SMAD signaling and causes mesenchymal transition of the coelomic epithelial cells early in Müllerian duct regression

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Examination of Müllerian inhibiting substance (MIS) signaling in the rat in vivo and in vitro revealed novel developmental stage- and tissue-specific events that contributed to a window of MIS responsiveness in Müllerian duct regression. The MIS type II receptor (MISRII)-expressing cells are initially present in the coelomic epithelium of both male and female urogenital ridges, and then migrate into the mesenchyme surrounding the male Müllerian duct under the influence of MIS. Expression of the genes encoding MIS type I receptors, Alk2 and Alk3, is also spatiotemporally controlled; Alk2 expression appears earlier and increases predominately in the coelomic epithelium, whereas Alk3 expression appears later and is restricted to the mesenchyme, suggesting sequential roles in Müllerian duct regression. MIS induces expression of Alk2, Alk3 and Smad8, but downregulates Smad5 in the urogenital ridge. Alk2-specific small interfering RNA (siRNA) blocks both the transition of MISRII expression from the coelomic epithelium to the mesenchyme and Müllerian duct regression in organ culture. Müllerian duct regression can also be inhibited or accelerated by siRNA targeting Smad8 and Smad5, respectively. Thus, the early action of MIS is to initiate an epithelial-to-mesenchymal transition of MISRII-expressing cells and to specify the components of the receptor/SMAD signaling pathway by differentially regulating their expression.

KEY WORDS: MIS, MIS type I/II receptor, SMAD, Epithelial-to-mesenchymal transition, RNA interference, Organ culture, Rat

INTRODUCTION

Müllerian inhibiting substance (MIS; also known as anti-Müllerian hormone, AMH) is essential for normal male sexual differentiation. In mammalian males, the fetal testes produce and secrete both MIS, which causes Müllerian (paramesonephric) ducts to regress, and testosterone, which promotes the differentiation of Wolffian (mesonephric) ducts. Müllerian ducts, in the absence of MIS, continue to develop and differentiate as the oviduct, uterus, cervix and upper part of the vagina, whereas Wolffian ducts, which give rise to the male internal reproductive tract structures, epidiymides, vas deferens and seminal vesicles, degenerate without testosterone stimulation. Defects in either the gene for MIS or its receptor can result in a form of male pseudohermaphroditism characterized by Wolffian duct expression predominating in the coelomic epithelium, whereas Alk3 expression appears later and is restricted to the mesenchyme, suggesting sequential roles in Müllerian duct regression. MIS induces expression of Alk2, Alk3 and Smad8, but downregulates Smad5 in the urogenital ridge. Alk2-specific small interfering RNA (siRNA) blocks both the transition of MISRII expression from the coelomic epithelium to the mesenchyme and Müllerian duct regression in organ culture. Müllerian duct regression can also be inhibited or accelerated by siRNA targeting Smad8 and Smad5, respectively. Thus, the early action of MIS is to initiate an epithelial-to-mesenchymal transition of MISRII-expressing cells and to specify the components of the receptor/SMAD signaling pathway by differentially regulating their expression.

The molecular mechanisms leading to Müllerian duct regression have yet to be clarified. MIS functions, like other members of the transforming growth factor (TGF) superfamily, by binding to its specific type II receptor (MISRII), which presumably must recruit and phosphorylate a type I receptor to initiate a downstream signaling cascade (for a review, see Teixeira et al., 2001; Josso and McMahon, 1998; Clarke et al., 2001). When the Müllerian duct is first developing, the coelomic epithelial cells are thought to invaginate and migrate in a cranial-to-caudal manner to form the Müllerian duct (Gruenwald, 1941). The Müllerian duct is subsequently eliminated in a cranial-to-caudal fashion as a result of MIS action (Piccon, 1969; Tsuji et al., 1992), which is attributed to the cranial-to-caudal expression of MISRII (Allard et al., 2000). Expression of MISRII was found in the mesenchyme but not in the Müllerian duct epithelial cells at the time of regression in the male (Baan et al., 1994; Clara et al., 1994; Teixeira et al., 1996), thus MIS is believed to function via a paracrine mechanism to cause apoptosis in the Müllerian duct (Tsui et al., 1992; Catlin et al., 1997; Roberts et al., 1999; Allard et al., 2000). By contrast, MISRII transcripts are present in a polarized pattern in the coelomic epithelium of female urogenital ridges during the corresponding period (Parr and McMahon, 1998; Clarke et al., 2001). The cause of this sexually dimorphic pattern of MISRII expression has heretofore been uncharacterized.

Whereas the type II receptor is unique for MIS signaling, several type I receptors may mediate MIS signaling in different tissue contexts. Dominant-negative (Clarke et al., 2001) and antisense (Visser et al., 2001) Alk2 can reverse the function of MIS in p19 embryonic carcinoma cells and in the rat urogenital ridge in organ culture, respectively. ALK6 can have MIS ligand-dependent interaction with MISRII in Chinese hamster ovary (CHO) cells (Gouédard et al., 2000); however, Müllerian ducts regress normally in male Alk6 (Bmpr1b) knockout mice (Clarke et al., 2001). Conditional inactivation of Alk3 (Bmpr1a) prevents Müllerian duct regression in male mice (Jamin et al., 2002), creating a phenotype identical to that seen by inactivating the MIS ligand or its type II receptor, and thus providing strong evidence that ALK3 is an MIS type I receptor in the mouse. When transgenic mice carrying the conditional mutation of Alk3 were bred with transgenic mice overexpressing human MIS, the female progeny had no uterus (Jamin et al., 2003), suggesting possible redundancy among different MIS type I receptors in the presence of high levels of MIS.

ALK2, ALK3 and ALK6 also mediate the signaling of bone morphogenetic proteins (BMPs). These type I receptors phosphorylate receptor-regulated SMADs (R-SMADs) 1, 5 and 8 at the C-terminal SXS5 motifs to transduce BMP signals. The
phosphorylated SMADs translocate into the nucleus complexed with SMAD4 and transcriptionally regulate specific sets of targeted genes (for reviews, see Massagué, 2000; Attisano and Wrana, 2002). MIS has been shown to activate SMAD1 (Gouédard et al., 2000; Clarke et al., 2001; Visser et al., 2001) and SMAD5 (Visser et al., 2001) in vitro, implying that R-SMADs 1, 5 and 8 may mediate Müllerian duct regression (Kobayashi and Behringer, 2003).

The present study was undertaken to define when and where the MIS type I receptors are employed and to determine which SMADs transduce MIS signals in the urogenital ridge during Müllerian duct regression. We adapted RNA interference (RNAi) (Calegari et al., 2002; Sakai et al., 2003; Soutschek et al., 2004) to test functional activity of the components of the MIS signaling pathway in a urogenital ridge organ culture assay, which recapitulates the morphological events occurring in vivo during Müllerian duct regression (Donahoe et al., 1977). We show that ALK2-mediated MIS signaling induces migration of SMIRI-expressing cells from the coelomic epithelium into the Müllerian duct mesenchyme, and thus is responsible for the sexual dimorphism of MISRII expression. MIS also orchestrates the spatiotemporal expression of its type I receptors and R-SMADs, which is necessary for Müllerian duct regression.

**MATERIALS AND METHODS**

**Animals, organ culture and recombinant human MIS**

Urogenital ridges were dissected from the embryos of timed pregnant rats (Harlan) and studied at developmental stages from E14 to E15 to determine gene expression patterns and morphological changes in vivo. Male or female urogenital ridges from timed pregnant rats at E14.5 were also dissected and then cultured, either immediately or after special treatment, on MilliCell-CM membranes (Millipore) over CMRL1066 medium (Life Technologies) supplemented with 10% female (to avoid an effect of bovine MIS in male serum) fetal bovine serum, penicillin/streptomycin and 10 nM testosterone. Cultures were carried out with or without recombinant human MIS at a final concentration of 6 μg/ml (42.5 nM).

To obtain bioactive recombinant MIS, the human MIS cDNA was stably transfected into CHO cells. MIS was purified from the serum containing media by immunoaffinity chromatography as described previously in detail (Ragin et al., 1992), using a monoclonal antibody developed in this laboratory (Hudson et al., 1990).

**In situ hybridization**

Immediately after dissection at various times of gestation or after organ culture, urogenital ridges were fixed overnight at 4°C in 4% paraformaldehyde, embedded and sectioned at 7-10 μm. Deparaffinized and hydrated sections were microwaved in 0.01 M sodium citrate to unmask antigens by heating at 80-85°C for 10 minutes. Sections were blocked with 5% normal goat serum; incubated with rabbit anti-phosphoSMAD1/5/8 antibody (Cell Signaling) diluted at 1:100, with biotin-labeled goat anti-rabbit antibody (Vector) and with ABC reagent (Vector); developed with DAB reagent; and counterstained with 1% Methyl Green. For vimentin staining, sections were blocked using 5% normal donkey serum, then incubated with anti-vimentin antibody at a dilution of 1:100 (Santa Cruz Biotechnology) and FITC-conjugated secondary antibody. For immunofluorescence staining, urogenital ridges were fixed overnight at 4°C in 4% paraformaldehyde, embedded in paraffin wax and sectioned at 6 μm. Deparaffinized and hydrated sections were microwaved in 0.01 M sodium citrate to unmask antigens by heating at 80-85°C for 10 minutes. Sections were blocked with 5% normal goat serum; incubated with rabbit anti-phosphoSMAD1/5/8 antibody (Cell Signaling) diluted at 1:100, with biotin-labeled goat anti-rabbit antibody (Vector) and with ABC reagent (Vector); developed with DAB reagent; and counterstained with 1% Methyl Green.

**Whole-mount immunofluorescent microscopy**

Urogenital ridges were fixed in 4% paraformaldehyde overnight at 4°C, followed by washes with PBS, and permeabilized in 0.2% Triton X-100 in PBS for 15 minutes at room temperature. Samples were quenched in 0.1% sodium borohydride for 10 minutes at room temperature, blocked (1% BSA/5% normal goat serum in PBS) for 3 hours at room temperature, and incubated with rabbit anti-phosphoSMAD1/5/8 antibody (Cell Signaling) diluted at 1:100, with biotin-labeled goat anti-rabbit antibody (Vector) and with ABC reagent (Vector); developed with DAB reagent; and counterstained with 1% Methyl Green.

**siRNAs and RNAi in organ culture**

After testing multiple small interfering RNAs (siRNAs), the optimal targeting siRNA for each gene was selected as indicated in Table 1. The siRNAs were chemically synthesized, purified and duplexed by Qiagen-Xeragon, and resuspended to 20 nM following the manufacturer’s protocol. siRNA concentrations between 50 and 400 nM were tested for optimal silencing efficiency with less toxicity, and 200 nM was selected for further studies. Urogenital ridges were transfected with siRNA duplex in serum-free culture medium by using Oligofectamine reagent (Invitrogen). siRNAs were transfected into urogenital ridges and incubated with mammalian cell serum, then incubated with anti-vimentin antibody at a dilution of 1:100 (Santa Cruz Biotechnology) and Alexa fluor 568 secondary antibody (Invitrogen). For immunohistochemistry, urogenital ridges were fixed overnight at 4°C in 4% paraformaldehyde, embedded in paraffin wax and sectioned at 6 μm. Deparaffinized and hydrated sections were microwaved in 0.01 M sodium citrate to unmask antigens by heating at 80-85°C for 10 minutes. Sections were blocked with 5% normal goat serum; incubated with rabbit anti-phosphoSMAD1/5/8 antibody (Cell Signaling) diluted at 1:100, with biotin-labeled goat anti-rabbit antibody (Vector) and with ABC reagent (Vector); developed with DAB reagent; and counterstained with 1% Methyl Green.

**Table 1. The sequences for siRNA targeting**

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**RESULTS**

**Dynamic expression of MIS receptors and SMADs early in Müllerian duct regression**

In the rat embryo, the window of MIS responsiveness is from E14-E15. MIS has to be present during this period in order to achieve complete regression of the Müllerian ducts (Picon, 1969; Josso et al., 1976; Donahoe et al., 1977; Tsuji et al., 1992). Regression events, i.e. disruption of the basement membrane of the Müllerian duct and apoptosis of the Müllerian duct epithelial cells, occur after E15 in male rat urogenital ridges (Price et al., 1977; Trelstad et al., 1982; Allard et al., 2000). These facts prompted us to examine the expression profiles of the MIS receptors and SMADs in male rat urogenital ridges at E14-E15 in order to understand how they participate and cooperate in MIS signaling. At early E14 (E14.25), MISRII mRNA was expressed strongly in the cranial urogenital ridge (Fig. 1A), and then the expression was seen to extend craniocaudally along the urogenital ridge (Fig. 1B,C). Unexpectedly, MISRII expression was found predominantly in the coelomic epithelium, but not in the mesenchyme between the Müllerian and Wolffian ducts in male urogenital ridges (Fig. 1D). At this time, the pattern of MISRII expression was seen as different from that detected at E15.5 when MISRII expression appeared in a circumferential pattern around the male Müllerian duct epithelium (Clarke et al., 2001).

Previous studies have shown that MIS can activate or phosphorylate R-SMADs1, 5 and 8 in cell culture (Gouédard et al., 2000; Clarke et al., 2001; Visser et al., 2001). In this study, we examined the expression of phosphorylated SMAD1/SMAD5/SMAD8 (P-SMAD) in the urogenital ridge. Whole-mount immunofluorescence analysis showed no obvious P-SMAD expression in the urogenital ridge at early E14 (data not shown). After E14.5, expression of P-SMAD could be detected in male urogenital ridges. It appeared at low level at E14.5 and increased craniocaudally thereafter (Fig. 1M-O). Presence of P-SMAD in the male urogenital ridge after E14.5 implies that MIS is eliciting a functional response and MIS signaling may contribute to subsequent molecular events in the male urogenital ridge.

Sexually dimorphic pattern of Alk2 and Smad8 expression has previously been found in rat urogenital ridges at E15.5 (Clarke et al., 2001), and we examined their expression in male rat urogenital ridges at earlier stages. Little Alk2 expression was detected in the urogenital ridge before E14.5 (Fig. 1E). Thereafter, increased expression of Alk2 was seen in the anterior male urogenital ridge and extended craniocaudally (Fig. 1F,G). More Smad8 transcripts were also detected after E14.5 in male rat urogenital ridges (Fig. 1I,K). Cryosections showed that Alk2 and Smad8 mRNA was...
mainly localized in the coelomic epithelium (Fig. 1H,L). At this developmental stage, Alk3 expression was not detected in the coelomic epithelium but in the mesenchyme (data not shown).

At E15.5, Alk2 expression was increased in the fetal gonad but markedly reduced in the coelomic epithelium of the male urogenital ridge, and it began to disappear craniocaudally (Fig. 2A, arrow). Meanwhile, more Alk3 was detected in the mesenchyme, and its expression was much higher at E15.5 (Fig. 2B) than at E14.5 in the Müllerian duct mesenchyme in male urogenital ridges (data not shown). Concomitantly, prominent expression of P- smear was detected in the mesenchymal cells surrounding the Müllerian ducts of male urogenital ridges (Fig. 2D,F), but absent in the same area in the female (Fig. 2C,E), suggesting that functional MIS signaling continues in the peri-Müllerian duct mesenchyme. Smad5 also has a sexually dimorphic expression pattern at E15.5; its transcripts were expressed in the coelomic epithelium of female urogenital ridges (Fig. 2G), whereas male urogenital ridges expressed much less Smad5 in the coelomic epithelium adjacent to the Müllerian duct (Fig. 2H, arrow). Smad1 expression was weak and indistinguishable between male and female urogenital ridges from E14.5 to E15.5 (data not shown) (Clarke et al., 2001).

**MIS signaling induces a shift of MISRII expression from the coelomic epithelium to the mesenchyme**

To confirm that the expression of P- smear in male urogenital ridges at E14.5-E15.5 was the result of MIS action, we treated E14.5 female rat urogenital ridges in organ culture (Donahoe et al., 1977) with MIS at concentrations known to cause Müllerian duct regression (Ragin et al., 1992; Lorenzo et al., 2002). MIS treatment induced P- smear expression in two hours in female urogenital ridges (Fig. 3B). P- smear expression was first noted to increase along the outer region of the urogenital ridge lateral to the Müllerian duct (Fig. 3B,C, arrows), and later was also visualized medial to the Müllerian duct following treatment with MIS for 30 hours (Fig. 3D, arrowhead). This pattern is similar to that normally seen in male urogenital ridges (Fig. 3F), but not in untreated female urogenital ridges (Fig. 3E). These data suggest that the dynamic change of P- smear in male urogenital ridges at the corresponding developmental stage resulted from MIS activity.

We next investigated whether MIS directs Misr2 expression from the coelomic epithelium into the mesenchyme of the Müllerian duct. Female urogenital ridges were treated with MIS in organ culture, and the pattern of Misr2 expression was compared with that observed in untreated female counterparts. At E14.5, the coelomic epithelium adjacent to the Müllerian duct appeared thicker than that in other regions in both female (Fig. 4A, arrow) and male urogenital ridges (data not shown). The coelomic epithelium was separated from subjacent mesenchyme by a prominent basement membrane (Fig. 4A,G) (Ikawa et al., 1984), and was noted to have less vimentin expression (Fig. 4B, arrow). Before treatment commenced, Misr2 transcripts were localized to the coelomic epithelium lateral to the Müllerian duct (Fig. 4C). After treatment with MIS for 20 hours, Misr2 mRNA was observed in the mesenchyme adjacent to the Müllerian duct with reduced expression in the coelomic epithelium (Fig. 4H), in contrast to the untreated counterpart (Fig. 4E), in which Misr2 expression is indistinguishable from that at E14.5 (Fig. 4C). Prolonged treatment with MIS for 40 hours caused expression of Misr2 to diminish markedly in the coelomic epithelium and increase in the mesenchyme surrounding the Müllerian duct, notably, between the Müllerian and Wolffian ducts (Fig. 4K, arrowhead). In the untreated female urogenital ridges (without MIS for 40 hours), expression of Misr2 remained lateral to the Müllerian duct, predominantly in the coelomic epithelium (Fig. 4M). When MIS was removed from organ culture before Misr2 expression appeared around the Müllerian duct, the change of Misr2 expression did not proceed (data not shown). These data indicate that constitutive MIS signaling early in Müllerian duct regression contributes to the distinct male pattern of Misr2 expression.

**MIS induces migration of Misr2-expressing cells**

To determine whether a mechanism of epithelial-to-mesenchymal transition underlies the switch of Misr2 expression, we labeled the coelomic epithelium of female urogenital ridges at E14.5 with CM-DiI, which incorporates into cell membranes, with photostable fluorescence and no apparent adverse effects (Austin, 1995; Karl and Capel, 1998), and tracked the migration of fluorescent-labeled cells.
Exogenous MIS differentially regulates R-SMADs 1, 5 and 8 expression

To investigate whether the sexually dimorphic expression of Smad8 and Smad5 is dependent upon MIS, we treated E14.5 female urogenital ridges in organ culture with MIS and examined their expression. MIS treatment of female urogenital ridges resulted in increased expression of Smad8 (Fig. 5B,E). Smad8 expression was also induced by MIS added to female urogenital ridges after removal of the gonad (data not shown), indicating that this effect is not a result of other gonadal factors. In situ hybridization with probes targeting different regions in the Smad8 transcript (data not shown) confirmed that the regulated Smad8 was full length, not an isoform encoding C-terminus deleted Smad8 (Nishita et al., 1999). Treatment of E14.5 female urogenital ridges with MIS also resulted in decreased Smad5 expression in the coelomic epithelium adjacent to the Müllerian duct (Fig. 5H,K), similar to that seen in male urogenital ridges at the same developmental stage in vivo and in vitro (Fig. 2H, Fig. 5I,L). MIS had no noticeable effect on Smad1 expression in urogenital ridges (data not shown).

MIS spatiotemporally regulates Alk2 and Alk3 expression

To investigate whether MIS regulates Alk2 and Alk3 expression during Müllerian duct regression, we treated E14.5 female urogenital ridges in organ culture with MIS and examined expression over time. Treatment of E14.5 female urogenital ridges with MIS for 12 hours induced Alk2 expression (Fig. 5N,P) when compared with untreated ridges (Fig. 5M,O). Moreover, increased Alk2 expression was detected in the coelomic epithelium as early as 4-6 hours after treatment, and decreased after treatment for 24 hours (data not shown). Alk3 expression was increased only after culture for more than 24 hours with MIS. It was upregulated predominantly in the mesenchyme surrounding the Müllerian duct (Fig. 5R,T) when compared with untreated ridges (Fig. 5Q,S). Upregulation of both Alk2 and Alk3 both followed a cranial-to-caudal pattern.

Alk2 mediates the change of MISRII expression and is required for Müllerian duct regression

The functional importance of the MIS type I receptors and R-SMADs 1, 5 and 8 in Müllerian duct regression was investigated by RNAi in organ culture of male rat urogenital ridges. Multiple siRNAs designed to target Alk2, Alk3, Smad1, Smad3 and Smad8 were first studied in cultured MIS-responsive and MISRII-expressing R2C rat Leydig cells (data not shown) (Teixeira et al., 1999). The siRNAs that showed significant silencing of mRNA expression for each gene in cell culture were selected for subsequent use in organ culture (Table 1). Transfection of fluorescein-labeled siRNA into urogenital ridges could be visualized in the urogenital ridge, where it was seen to penetrate the coelomic epithelium, but not beyond (data not shown).

Male urogenital ridges were treated with control- or Alk2-siRNA, and expression of Misr2 and P-SMAD was examined. P-SMAD expression was markedly decreased in Alk2-siRNA treated male urogenital ridges (compare Fig. 6B with Fig. 6A, arrows). In the urogenital ridges treated with control-siRNA, Misr2 mRNA was detected in the mesenchyme around the Müllerian duct (Fig. 6C, arrowhead); however, in those treated with Alk2-siRNA, Misr2 expression was not evident in the area between the Müllerian and Wolffian ducts (Fig. 6D, arrowhead).

The selective expression of Wnt7a, which drives the expression of MISRII, in the Müllerian duct epithelium of urogenital ridges (Parr and McMahon, 1998) makes it a particularly useful marker
with which to study the Müllerian duct (data not shown), as it faithfully reflects Müllerian duct formation and regression. Detection of Wnt7a expression, which was able to locate remaining Müllerian duct epithelium in urogenital ridges, allowed us to monitor the effects of RNAi on Müllerian duct regression in organ culture and to examine the contribution of Alk2 as an MIS type I receptor in Müllerian duct regression.

Male urogenital ridges were treated with siRNAs and then cultured for additional 2 days. In situ hybridization showed that the Müllerian duct epithelium expressing Wnt7a was retained in the urogenital ridges treated with Alk2-siRNA (Fig. 6F,H, arrows), but not in control-siRNA treated urogenital ridges (Fig. 6E,G). Multiple siRNAs targeting different regions of Alk2 had similar effects (data not shown). These results suggest that Alk2 mediates essential MIS signaling in the transition of Misr2-expressing cells from the coelomic epithelium to the peri-Müllerian duct mesenchyme.

**SMAD8 but not SMAD5 mediates MIS signaling in Müllerian duct regression**

The role of SMAD1, SMAD5 or SMAD8 in MIS signaling and Müllerian duct regression was also investigated by RNAi. When male ridges were treated with control-siRNA for 12 hours, the entire Müllerian duct was still evident after culture for additional 36 hours (Fig. 7A). However, in Smad5-siRNA-treated urogenital ridges, regression was accelerated, as discontinuous Wnt7a expression was seen in the cranial area after culture for the same period (Fig. 7B, arrowheads). Moreover, when RNAi effect was examined in urogenital ridges after prolonged culture for additional 12 hours, Wnt7a expression still remained in the posterior region of control-siRNA urogenital ridges (Fig. 7C, arrow), but not in Smad5-siRNA-treated ridges (Fig. 7D), indicating that SMAD5 deficiency led to enhanced Müllerian duct regression. By contrast, treatment with Smad8-siRNA delayed Müllerian duct regression in male urogenital ridges, as Wnt7a expression was detected in the Smad8-siRNA (Fig. 7E, arrow), but not in control-siRNA treated urogenital ridges (Fig. 7F). Moreover, the effect of Smad8-siRNA on Müllerian duct regression was consistent with its specific gene silencing in cell culture, demonstrated by both RT-PCR and western (data not shown). Smad1-siRNA had no effect alone, and RNAi with both Smad1-siRNA and Smad8-siRNA simultaneously did not show a further inhibitory effect on Müllerian duct regression than that caused by Smad8-siRNA alone (data not shown).

**DISCUSSION**

**MIS-induced epithelial-to-mesenchymal transition underlies the change of MISRII expression**

During male sexual development, Müllerian ducts first form and then are eliminated as a consequence of MIS signaling. In the rat, MIS expression is first detected at E13 in fetal testes (Hirobe et al., 1992). However, a functional signaling pathway is not initiated until MISRII appears in the urogenital ridge. Our present work shows that functional MIS signaling, as documented by activation of R-SMADs1, 5 and 8, is not observed in the male urogenital ridge immediately until after the expression of MISRII. Interestingly, expression of MISRII and phosphorylated SMAD1/5/8 are localized in the coelomic epithelium at this stage. The downstream signaling events (e.g. upregulation of Alk2 and Smad8, and downregulation of Smad5) also appear initially in the coelomic epithelium. This explains why RNAi with the lipid-based transfection technique, which could only penetrate the surface coelomic epithelium, was effective in knocking down the components of MIS signaling in the urogenital ridge.
The epithelial cells of the Müllerian duct originate from the coelomic epithelium (Gruenwald, 1941), where expression of Misr2 is also first detected (Fig. 1A-D). MIS signaling leads to the appearance of Misr2 expression in the peri-Müllerian duct mesenchyme. By tracking the migration of DiI-labeled cells, we found that MIS induces the Misr2-expressing cells that originally reside in the coelomic epithelium to migrate into the mesenchymal compartment around the Müllerian duct, following disruption of basement membrane. MIS causes epithelial cells to become migratory, and thereby, initiates an epithelial-to-mesenchymal transition (for a review, see Thiery, 2002), driving Misr2 expression into the peri-Müllerian duct mesenchyme. Although we cannot completely rule out that non-Misr2-expressing cells migrate in response to an indirect effect of MIS and then begin to express Misr2 in the mesenchyme, our data strongly suggest that MIS directs Misr2-expressing cells from the coelomic epithelium into mesenchyme. The timeframe of the migration is in agreement with the period required for apoptosis to be observed in the Müllerian duct epithelium (Price et al., 1977; Roberts et al., 1999; Allard et al., 2000), implying that the Misr2-expressing cells as MIS effectors may have to arrive in the peri-Müllerian duct mesenchyme and/or become mesenchymal cells to exert significant paracrine effects on Müllerian ducts. The early epithelial-mesenchymal transformation is reminiscent of the subsequent transition of the epithelial duct cells to mesenchyme later during the regression phase (Trelstad et al., 1982; Austin, 1995; Allard et al., 2000), and illustrates that this cellular process is a key mechanism in Müllerian duct regression.

Before the Müllerian ducts develop, the Wolffian ducts occupy the lateral area of urogenital ridges beneath the coelomic epithelium where the Müllerian ducts are later destined to emerge (Gruenwald, 1941; Trelstad et al., 1982). The Müllerian duct forms between the Wolffian duct and the coelomic epithelium, and the Müllerian duct is initially separated from the coelomic epithelium only by a shared basement membrane and no intervening mesenchyme (Trelstad et al., 1982; Ikawa et al., 1984). The coelomic epithelium adjacent to the Müllerian duct expresses Misr2 and appears thicker than the epithelium covering other regions of the urogenital ridge (Fig. 4A-G). After peri-Müllerian duct mesenchyme forms under the influence of MIS, the coelomic epithelium adjacent to the male Müllerian duct becomes thinner and indistinguishable from that in lateral regions (Trelstad et al., 1982). MIS induces the Misr2-expressing epithelial cells to lose polarity and manifest a migratory phenotype, and thus facilitates the formation and patterning of the peri-Müllerian duct mesenchyme (Fig. 8). WNT signaling is associated with the epithelial and mesenchymal patterning of the female reproductive tract (Miller and Sassoon, 1998). β-Catenin, which transduces canonical WNT signaling, has been linked to the regulation of epithelial cell migration and epithelial-to-mesenchymal transition (Müller et al., 2002; Lu et al., 2003). Misr2-directed β-catenin knockout mice show defects in Müllerian mesenchymal development (Arango et al., 2005). MIS is able to activate the NF-κB pathway (Segev et al., 2001; Segev et al., 2002), which is also a stimulatory signal leading to epithelial-to-mesenchymal transition (Sosic et al., 2003; Huber et al., 2004). Translocation of β-catenin to the nucleus has also been correlated with MIS signaling (Allard et al., 2000). Therefore, MIS and WNT signaling pathways may function cooperatively in mediating epithelial-to-mesenchymal transition early in Müllerian duct regression.
Alk2 and Alk3 may act as sequential MIS type I receptors in Müllerian duct regression

Specificity and versatility in the signaling responses of TGF-β family members are defined particularly by the type I receptors that a ligand can activate. For example, TGF-β activates ALK5 in Mink lung cells (Bassing et al., 1994), while ALK1 acts as a TGF-β type I receptor in vascular smooth muscle differentiation (Oh et al., 2000). Alk3 and ALK6 can serve as sequential type I receptors in BMP signaling, and control the production and fate of dorsal precursor cells from neural stem cells (Panchision et al., 2001). Alk2, as a functionally essential MIS type I receptor in the rat urogenital ridge (Visser et al., 2001), mediates the change of MISRII expression, and thus the migration and transition of the coelomic epithelial cells (Fig. 6). Alk2 has also been shown to regulate epithelial-to-mesenchymal transition during cardiac valve formation (Desgrozelle et al., 2005). Interestingly, constitutively active Alk3 can stimulate Ecadherin expression and antagonize the process of epithelial-to-mesenchymal transition (Zeisberg et al., 2003).

Analysis of Alk2 expression in the male urogenital ridge of the rat has previously shown that it is present in the Müllerian mesenchyme at E15, but not at E16 (He et al., 1993) when Müllerian duct regression is not yet complete. Alk3 is ubiquitously expressed in embryonic organs including the urogenital system by the time that MIS and MISRII are expressed (Dewulf et al., 1995). However, we noted that ALK3 expression favors the mesenchyme of the urogenital ridge instead of the coelomic epithelium where functional MIS signaling initially occurs (Fig. 2B). Alk2 expression is increased after E15.5, coincident with the appearance of Misr2 in the peri-Müllerian duct mesenchyme in male rat urogenital ridges (Fig. 2B), and this could also be recapitulated in female urogenital ridges upon MIS treatment (Fig. 5Q-T). Moreover, regulation of Alk3 expression appears to be a downstream event, as diminution of Alk2-mediated signaling with Alk2-siRNA also inhibits the upregulation of Alk3 in male urogenital ridges (data not shown). The spatiotemporal patterns of Alk2 and Alk3 expression imply that they may act sequentially as type I receptors for MIS signaling (Fig. 8); Alk2 functions early in MIS signaling and mediates the migration and transition of coelomic epithelial cells,
whereas ALK3 may participate in later MIS signaling in Müllerian duct regression, which has been well documented in the mouse (Jamin et al., 2002). The indispensable role of ALK2 in Müllerian duct regression was also documented in mouse urogenital ridges by performing RNAi at E12.5 (data not shown). Moreover, we also observed increased expression of Alk3 in the mouse at E14.5 (developmentally equivalent to ~E15.5 in the rat) in the Müllerian duct mesenchyme (data not shown), which appears later than ALk2 (Visser et al., 2001). Although incubation with siRNAs for duct mesenchyme (data not shown), which appears later than Alk2 (developmentally equivalent to ~E15.5 in the rat) in the Müllerian duct regression, suggesting that SMAD5 and SMAD8 can transduce specific signals in MIS pathways. In addition, targeting Smad5 expression with siRNA promoted Müllerian duct regression.

Increased SMAD8, similar to upregulated ALK2, can act to sustain and amplify the signaling cascade. Disruption of the feed-forward circuit by RNAi-mediated gene silencing of either SMAD8 or ALK2 affected the subsequent downstream signaling events, resulting in retained Müllerian ducts. However, our investigation did not reveal a clear role for SMAD8 in MIS-induced earlier epithelial-to-mesenchymal transition (data not shown). It is possible that this process is independent of SMAD signaling. Prolonged induction of Smad8 at E15–E16 over Alk2 expression was seen in the male peri-Müllerian mesenchyme (data not shown), suggesting that SMAD8 may play a role in later ALK3-mediated molecular events during Müllerian duct regression.

In conclusion, we identified the coelomic epithelium as the first target for MIS and found that MIS exerts a profound influence on the expression of its own signaling components early in Müllerian duct regression. These events elicit epithelial-to-mesenchymal transition and amplify the MIS signaling for subsequent regression of the Müllerian duct. Knowledge of the downstream MIS signaling events in the urogenital ridge will be important to the study of MIS at other target sites such as the coelomic epithelium of the ovary where oncogenic changes lead to ovarian cancer in mouse models (Orsulic et al., 2002; Connolly et al., 2003; Dinulescu et al., 2005) and presumably in humans.

References
Baanends, W. M., van Helmond, M. J., Post, M., van der Schoot, P. J.,
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