The Serine/Threonine Transmembrane Receptor ALK2 Mediates Müllerian Inhibiting Substance Signaling

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Müllerian inhibiting substance (MIS or anti-Müllerian hormone) is a member of the transforming growth factor-β family and plays a pivotal role in proper male sexual differentiation. Members of this family signal by the assembly of two related serine/threonine kinase receptors, referred to as type I or type II receptors, and downstream cytoplasmic Smad effector proteins. Although the MIS type II receptor (MISRII) has been identified, the identity of the type I receptor is unclear. Here we report that MIS activates a bone morphogenetic protein-like signaling pathway, which is solely dependent on the presence of the MISRII and bioactive MIS ligand. Among the multiple type I candidates tested, only ALK2 resulted in significant enhancement of the MIS signaling response. Furthermore, dominant-negative and antisense strategies showed that ALK2 is essential for MIS-induced signaling in two independent assays, the cellular *Tlx-2* reporter gene assay and the Müllerian duct regression organ culture assay. In contrast, ALK6, the other candidate MIS type I receptor, was not required. Expression analyses revealed that ALK2 is present in all MIS target tissues including the mesenchyme surrounding the epithelial Müllerian duct. Collectively, we conclude that MIS employs a bone morphogenetic protein-like signaling pathway and uses ALK2 as its type I receptor. The use of this ubiquitously expressed type I receptor underscores the role of the MIS ligand and the MIS type II receptor in establishing the specificity of the MIS signaling cascade. (Molecular Endocrinology 15: 936–945, 2001)

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INTRODUCTION

Müllerian inhibiting substance (MIS), also known as anti-Müllerian hormone (AMH), is a member of the large transforming growth factor-β (TGFβ) superfamily that includes TGFβs, activins, and the bone morphogenetic proteins (BMPs) (1). While many TGFβ factors exert diverse effects in multiple tissues, the principle function of MIS is to induce regression of the Müllerian ducts during vertebrate male sexual differentiation (2, 3). Biologically active MIS is secreted from embryonic Sertoli cells of the testis and through unknown mechanisms is delivered to the paramesonephric region in the genital ridge. There, it binds the MIS type II receptor that is expressed in the mesenchyme surrounding the epithelial Müllerian duct. MIS signaling results in cell death of the Müllerian duct, thereby eliminating the female reproductive tract in males (4–6). In addition to this well defined role during male sexual differentiation, more subtle but important roles for MIS in somatic cell proliferation and gonadal germ cell maturation have been revealed by genetic experiments in mice (7, 8). For example, loss of function studies show that male MIS null mice develop Leydig cell hyperplasia (8). Careful analyses of adult MIS $-/-$ ovaries also suggest that MIS-deficient ovaries exhibit enhanced primordial follicle recruitment eventually leading to premature ovarian failure (9). Finally, gain-of-function studies in mice in which MIS is overexpressed suggest a putative role for MIS in attenuating steroid production (7, 10). Presently, it is uncertain whether the same molecular machinery mediates all biological effects of MIS in both the embryo and adult. However, it is generally accepted that there is only one type II receptor for MIS in mediating Müllerian duct regression

based on data obtained from both mouse and human mutants (11, 12).

All members of the TGFβ family presumably mediate their biological effects by two related transmembrane serine/threonine kinase receptors, type II and type I. Extensive studies with some members of the TGFβ family have established that upon ligand binding to type II receptors, such as the MISRII, type I receptors are recruited to form a heteromeric receptor complex. The type I receptors are then transphosphorylated by the type II receptor in the GS box, which is located close to the transmembrane region in the cytoplasm. Activation of the type I receptor leads to phosphorylation of receptor-specific Smad proteins. Phosphorylated Smads associate with the common Smad4 and are then translocated to the nucleus where they participate in regulating gene expression (13, 14). Selective recruitment of Smad proteins by ligand-receptor complexes suggests that two major signaling pathways exist for members of the TGFβ superfamily. In the first pathway, Smad2 or Smad3 are recruited by both TGFβ and activin through their respective type I receptors, ALK5 (TβRI) and ALK4 (ActRIB). In the second, Smad1, Smad5, or Smad8 are recruited by BMPs through ALK2, ALK3, and ALK6 or so-called BMP type I receptors (15, 16). Thus far, almost all TGFβ-like factors examined appear to use these two distinct intracellular signaling pathways.

The precise delineation of the MIS signaling pathway has been hampered because bona fide MIS target genes and a partner type I receptor for MIS have not been identified. One previous report proposed that ALK6 could serve as the MIS type I receptor based on *in vitro* data (17). However, mice deficient in ALK6 survive into adulthood and appear to show normal Müllerian duct regression (18–19a). Unfortunately, mouse knockouts of other type I receptors are uninformative because of embryonic lethality; the early developmental delays observed in ALK2, ALK3, and ALK4 knockouts (12, 20–22) preclude examination of MIS-triggered events, such as Müllerian duct regression. To address the molecular nature of the MIS signaling pathway, we surveyed different cell types and potential reporters that might be useful in dissecting the components of the MIS signaling pathway. These studies suggested that the mouse embryonic carcinoma cell line P19 and the mouse *Tlx-2* homeobox gene promoter fused to a reporter gene would be useful in identifying type I receptor candidates. Here we report that nanomolar concentrations of bioactive MIS ligand induce a BMP-like signal through Smad1 and Smad5. Furthermore, our collective data obtained from cell studies, expression analyses, and antisense studies done in both cells and organ culture all strongly support the hypothesis that ALK2 is the type I receptor for MIS signaling in Müllerian duct regression and possibly in adult gonads.

RESULTS

A BMP Response Is Induced by Bioactive MIS Ligand and the MISRII

Attempts to identify a MIS type I receptor have been hampered by the lack of known MIS target genes. Moreover, MIS does not activate commonly used TGFβ-responsive reporter genes, such as 3TP-Lux or PAI-Luc, suggesting that MIS does not signal via the classic TGFβ pathway. To overcome these limitations, we investigated whether MIS induced a response in P19 mouse embryonic carcinoma cells, which have been shown to contain both BMP and TGFβ/activin signaling pathways. Two reporters were tested; the BMP responsive mouse *Tlx-2* promoter reporter (TLX2-Lux) and the activin responsive *Xenopus Mix.2* gene (A3-Lux) (23, 24). We observed significant induction (6-fold) of the *Tlx-2* promoter after transiently transfecting P19 cells with the MISRII and MIS stimulation; this MIS-induced response was completely dependent on transfection of the MISRII (Fig. 1A). By

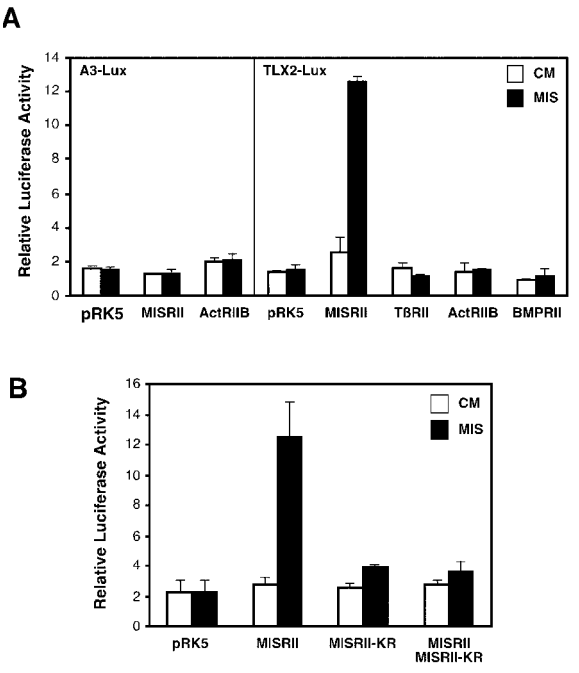

Fig. 1. MIS Induces MISRII-Dependent Transcription of the *Tlx2* Reporter Gene in P19 Cells

A, P19 cells were transiently transfected with an activin-responsive (A3-Lux) or BMP-responsive (TLX2-Lux) reporter gene together with the indicated type II receptors. Cells were incubated overnight in the presence of conditioned medium (CM, *open bars*) or bioactive MIS (*closed bars*). The relative luciferase activity was measured in cell lysates and normalized to β-galactosidase activity. Data are given as mean $\pm$ SD of triplicates and are representative of at least three independent experiments. B, P19 cells were transiently transfected with TLX2-Lux reporter and wild type-MISRII, kinase-inactive MISRII (MISRII-KR) or a combination of wild-type and mutant MISRII in a 1:1 ratio of expression plamids.

contrast, MIS did not induce the activin-responsive A3-Lux reporter (Fig. 1A), whereas activin did (data not shown). MIS induction of the *Tlx-2* promoter required the MISRII, whereas introduction of other type II receptors, such as BMPRII and ActRIIB or TβRII, failed to elicit MIS-dependent reporter activity (Fig. 1A). Transfection of a kinase-inactive MISRII (MISRII-KR), either alone or in the presence of the wild-type MISRII, completely inhibited MIS-induced *Tlx-2* activation (Fig. 1B). Thus, when coexpressed with wild-type MISRII, the MISRII-KR mutant served as a dominant-negative mutant receptor. Our findings are consistent with a previous report showing a 2-fold activation of another BMP-type promoter (Xvent-Luc) in P19 cells upon MISRII transfection and MIS stimulation (17).

Using the TLX2-Lux reporter assay in P19 cells, we then determined the effective concentration of bioactive MIS ligand needed to activate this reporter. A saturable and dose-dependent profile of MIS-induced Tlx-2 reporter activity was observed with significant activity observed at subnanomolar levels. The estimated ED_{50} (effective dose) for recombinant bioactive MIS (MIS-RARR) was determined to be approximately 18 nM MIS (Fig. 2); this value corresponds with the necessary concentration required for full Müllerian duct regression in organ explant culture assays of 15–20 nM (25). Taken together, these results demonstrate that MIS is capable of generating a BMP-like signal in P19 cells that does not require addition of type I receptors, but is solely dependent on the presence of the MISRII.

Dominant Negative Smad5, but not Smad2, Disrupts MIS Signaling

MIS-induced activation of a BMP-like signal suggests that MIS signaling is mediated through BMP receptor-specific Smads, such as Smad5, Smad1, or Smad8. Therefore, we investigated the effects of overexpressing representative Smads of either the BMP or TGFβ/activin signaling pathways. Increased reporter activity was observed after cotransfection of Smad5 or Smad1 and the MISRII (Fig. 3A and data not shown). In contrast, Smad2 failed to enhance MIS signaling above the baseline observed with transfection of only MISRII (Fig. 3A). To examine this issue further, we made use of a dominant-negative strategy to block endogenous Smad recruitment in P19 cells. Mutations of the carboxy-terminal phosphorylation sites of Smad proteins prevent dissociation from the activated receptor complex, thereby inhibiting activation (phosphorylation) of endogenous Smad proteins (26–28). We found that elevation of mutant Smad5 (Smad5–2SA), but not mutant Smad2 (Smad2–2SA), attenuated MIS-induced activation of TLX2-Lux reporter (Fig. 3B). Collectively,

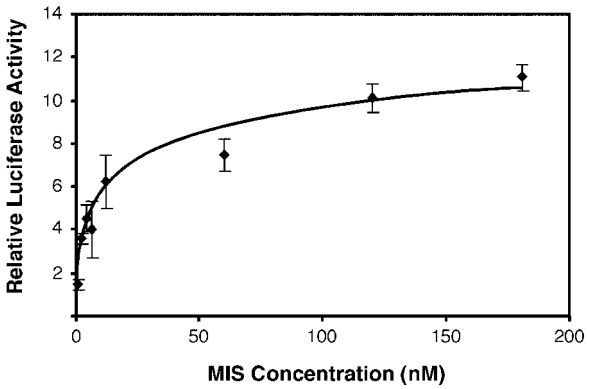

Fig. 2. Dose-Dependent Activation of TLX2-Lux by MIS

P19 cells were transiently transfected with TLX2-Lux reporter and MISRII. Cells were incubated overnight with increasing concentrations of bioactive MIS ligand (refer to *Materials and Methods* for quantitation). To control for potential activating factors present in conditioned medium, each assay point was normalized with control conditioned medium. Luciferase activity was determined in triplicate.

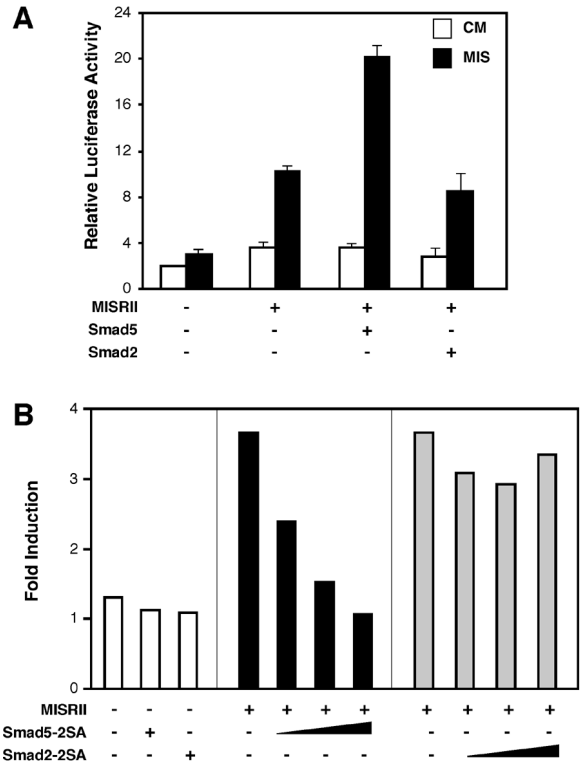

Fig. 3. Smad5 Is Involved in MIS Signaling

A, The TLX2-Lux reporter construct and MISRII expression vector were transfected into P19 cells with (+) or without (−) wild-type Smad5, wild-type Smad2. Transcriptional activity was measured after overnight incubation with conditioned medium (*open bars*) or bioactive MIS (*closed bars*), and normalized for transfection efficiency. In each transfection, equal amounts of DNA were transfected by adjusting with empty expression vector. Data are presented as mean ± SD of triplicates. B, MIS-induced activity of the TLX2-Lux reporter was measured with (+) or without (−) MISRII in increasing amounts of mutant Smad5–2SA, or mutant Smad2–2SA (10, 50, 100 ng/well). Data are expressed as fold induction of luciferase activity measured in the presence of bioactive MIS over activity measured in the presence of conditioned medium.

these data are consistent with the hypothesis that MIS signaling uses a BMP-like pathway.

ALK2 Is Involved in MIS Signaling

The results described above suggest that P19 cells contain an endogenous type I receptor capable of transducing MIS signaling when the MISRII is present. To test whether one of the previously identified type I receptors functions as a MIS type I receptor, multiple type I receptors (ALK1–6) were cotransfected with TLX2-Lux in P19 cells using lower concentrations of MISRII (1 ng/well) to observe additional activation. Under these conditions the strongest activation of the TLX2-Lux reporter was observed with a MISRII/ALK2 combination. Surprisingly, other BMP type I receptors known to signal through Smad5, such as ALK1, ALK3, and ALK6, failed to enhance the MIS response significantly, and ALK1 and ALK3 actually diminished MIS activation of the reporter (Fig. 4A). Western blot analysis revealed that each type I receptor was expressed at nearly equivalent levels (data not shown). That ALK2 participates in MIS signaling was further supported by using a dominant-negative or kinase-inactive ALK2 mutant receptor (ALK2-KR). Indeed, increasing amounts of ALK2-KR severely attenuated MIS-induced activation of TLX2-Lux (Fig. 4B). Although the dominant-negative ALK6-KR was previously reported to inhibit Xvent-reporter activity, in our hands we failed to observe inhibition of MIS-induced signaling with transfection of this mutant type I receptor (Fig. 4B and Ref. 17). Finally, increased activation of the MIS signaling by ALK2 was dependent on addition of biologically active MIS ligand and was not observed with either conditioned medium or inactive noncleavable MIS (MIS-RAGA, Fig. 4C).

ALK2 Is Expressed in All the MIS Target Tissues

Although our cell transfection studies suggested that ALK2 functions as a MIS type I receptor, supportive genetic data have been uninformative due to the early embryonic lethality observed in ALK2 null mice (21, 22). Therefore, we determined whether the expression profile of ALK2 coincides with the established expression patterns of MISRII. Based on previous studies regarding expression of the MISRII, we would expect ALK2 to be present in periductal mesenchymal cells surrounding the Müllerian duct as well as in both fetal and adult gonads. Indeed, we noted prominent expression of ALK2 in isolated urogenital ridges and gonads at several stages of embryonic development (Fig. 5, A and B). Interestingly, both ALK2 and MISRII levels diminished during the window of Müllerian duct regression, which commences at about E13 in male mice. Furthermore, similar to MISRII expression, ALK2 was easily detected in fetal gonads of both sexes, albeit at lower levels in fetal ovaries. By contrast, the other candidate MIS type I receptor, ALK6, is expressed at very low levels in both male and female urogenital ridges (Fig. 5A) and is barely detectable in

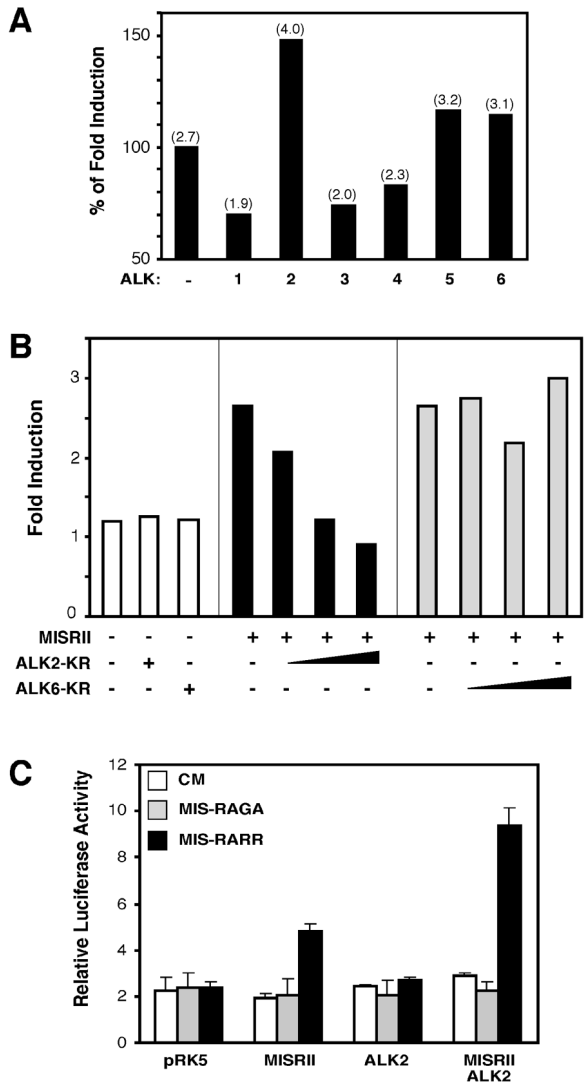

Fig. 4. ALK2 Transduces MIS Responses

A, P19 cells were transiently transfected with TLX2-Lux reporter and MISRII expression vector, either in the absence or presence of type I receptors (ALK1–6). Data are expressed as a percent of the maximum induction observed with transfection of MISRII alone, in the presence of MIS. The fold induction (+MIS over −MIS) is indicated *above* every *bar*. B, TLX2-Lux was transfected into P19 cells with MISRII, or MISRII together with increasing concentrations (10, 50, 100 ng/well) of kinase-inactive ALK2 (ALK2-KR) or kinase-inactive ALK6 (ALK6-KR). Data are expressed as fold induction as described in legend to Fig. 3. C, TLX2-Lux was transfected together with MISRII, ALK2 or MISRII, and ALK2 expression vectors. Cells were stimulated overnight with conditioned medium (CM; *open bars*), inactive (noncleavable) MIS (MIS-RAGA; *gray bars*) or bioactive MIS (MIS-RARR; *closed bars*). The relative luciferase activity was measured in cell lysates and normalized to β-galactosidase activity.

embryonic gonads of both sexes (Fig. 5B). *In situ* hybridization analysis of E13 mouse lower body sections showed more precisely that ALK2 is expressed in mesenchymal cells adjacent to the concentric Müllerian duct (Fig. 5C). This result agrees with the expres-

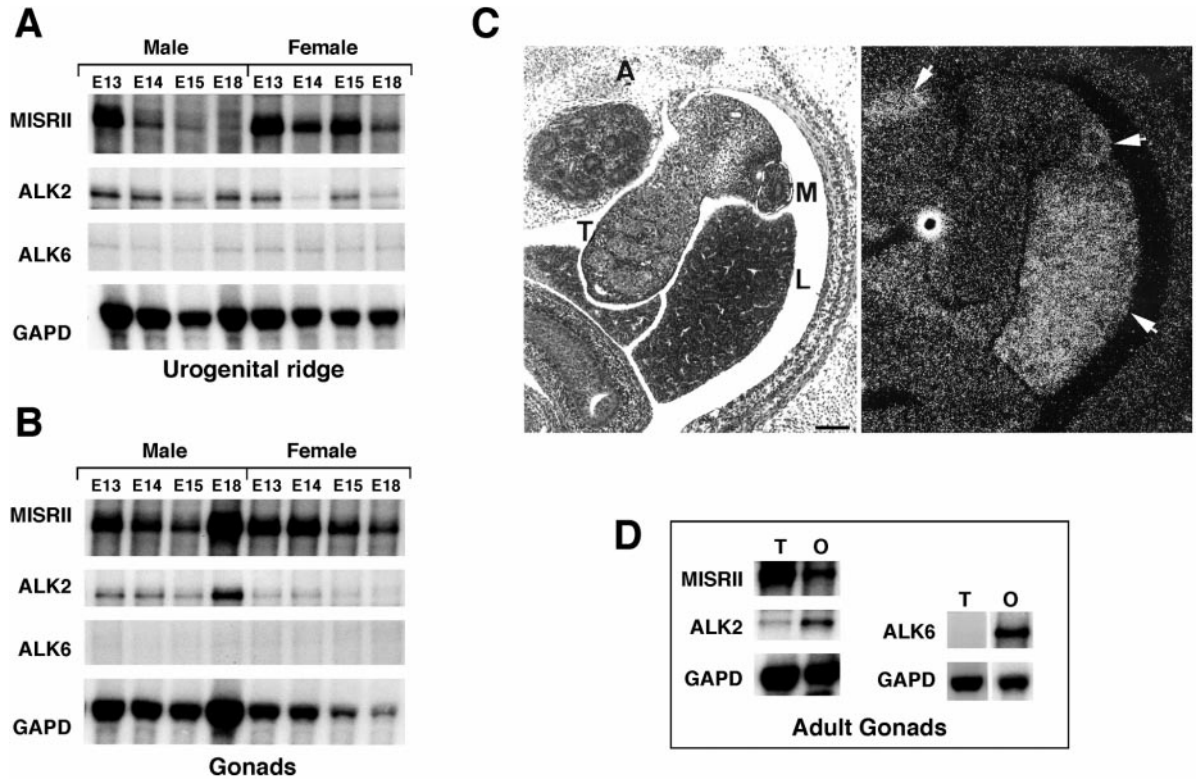

Fig. 5. ALK2 Is Expressed in All MIS Target Tissues

Levels of ALK2, ALK6, and MISRII mRNAs were measured in embryonic mouse urogenital ridges (A) and gonads (B) at various fetal ages by RNase protection assay. For staging of fetuses, refer to *Materials and Methods*. C, *In situ* hybridization analysis shows expression and localization of ALK2 mRNA in the urogenital ridge in an E13 male mouse. A transversal hematoxylin-eosin-stained section of the lower body is shown (*left panel*) along with a darkfield view (*right panel*). Sites of ALK2 expression are indicated by *white arrows*. Abbreviations for structures are as follows: A, adrenal; L, liver; T, testis, and M, the Müllerian duct as outlined by a *white broken circle* in *left panel*. The *scale bar* in the *left panel* equals 100 μm. D, Adult testis (T) and adult ovary (O) levels of ALK2, ALK6, and MISRII mRNAs were measured by RNase protection assay. GAPD mRNA measurements were included as a control for RNA loading.

sion pattern of the rat ALK2 (R1) in the genital ridge reported by Donahoe and co-workers (29). We also noted prominent ALK2 expression in the embryonic adrenal and liver (Fig. 5C). Given that the MISRII is also expressed in postnatal gonads (30–32), we determined the transcript levels of both ALK2 and ALK6 in these tissues. ALK6 mRNA expression was restricted to the ovary with virtually no signal observed in the testes; whereas ALK2 mRNA is expressed in both female and male gonads, albeit at a lower level in the testes (Fig. 5D). Taken together, our expression studies of ALK2 in both adult and embryonic reproductive tissues strongly support ALK2's role as a MIS type I receptor.

Antisense ALK2 Blocks MIS Signaling in Cells and in Organ Cultures

To determine whether endogenous ALK2 in P19 cells is capable of conferring MIS-induced activity in the presence of the MISRII, an antisense approach was undertaken to disrupt ALK2 protein expression. P19 cells were treated with morpholino antisense oligomers 24 h before cotransfection with TLX2-Lux and MISRII. The efficiency of morpholino oligomers resides in their prolonged stability and the efficient delivery system [ethoxylated polyethylenimine solution (EPEI)] in which almost 100% of the cells transfected take up these morpholino oligomers (Ref. 33 and Fig. 6A). Treatment of cells with an antisense ALK2 oligomer greatly diminished MIS-induced activation of TLX2-Lux to near basal levels (Fig. 6B). Incubation of cells with the delivery solution without antisense oligomers or with a control antisense ALK2 oligomer showed no inhibition of MIS-induced activity. The ALK2 control oligomer was identical to ALK2 except for four nucleotide mismatches. In contrast to the marked inhibition observed with the antisense ALK2 oligo, no inhibition of TLX2-Lux was observed using an ALK6 antisense oligomer (Fig. 6B). However, treatment of P19 cells with the ALK6 antisense oligomer decreased BMP4 signaling by approximately 50% (data not shown). Collectively, our findings suggest that MIS signaling is mediated through an endogenous ALK2 type I receptor present in P19 cells.

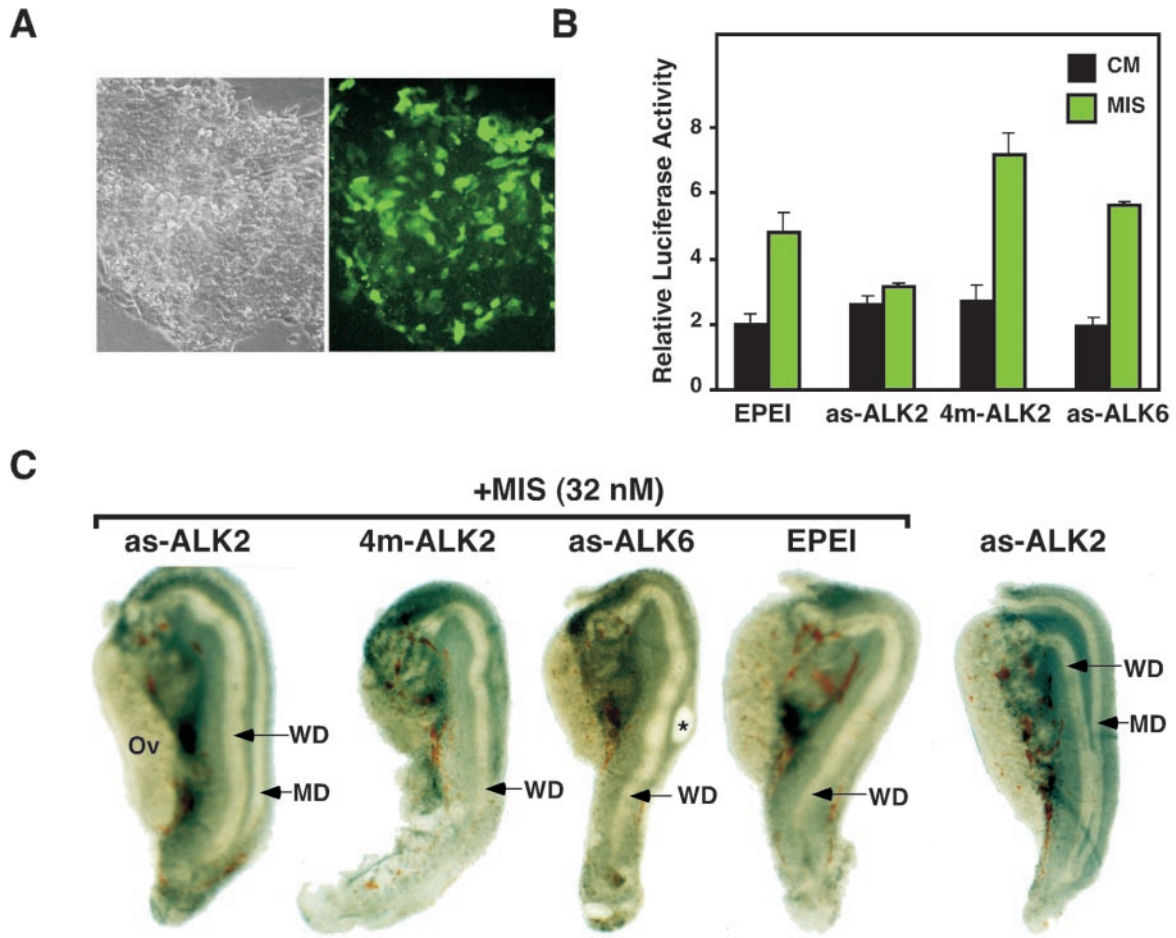

Fig. 6. Antisense ALK2 Oligomers Abrogate MIS Signaling in P19 Cells and in Urogenital Ridges

A, P19 cells were treated for 3 h with morpholino antisense oligomers using the special delivery system (EPEI). Cells were photographed with normal light (*left panel*) and with fluorescent light (*right panel*). Note that nearly all cells are fluorescent, indicating the efficient uptake of the fluorescent oligomer. B, Cells were transiently transfected with TLX2-Lux reporter and MISRII expression vector 24 h after treatment with antisense ALK2 oligomer (as-ALK2), mispaired ALK2 oligomer (4 m-ALK2), antisense ALK6 oligomer (as-ALK6), or delivery solution (EPEI). Luciferase activity was measured after treatment with conditioned medium (CM, *black bars*) or bioactive MIS (*green bars*), and normalized for transfection efficiency. C, Representative urogenital organ explants are shown after 3 days of culture in the presence of MIS after treatment with either an antisense ALK2 oligomer (as-ALK2), a control antisense ALK2 oligomer (4 m-ALK2), an antisense ALK6 oligomer (as-ALK6), or the delivery solution (EPEI); a persistent Müllerian duct (MD) was observed with antisense ALK2 oligomer and MIS treatment (*left panel*). Each condition was carried out in four independent experiments with an n = 4 or more. Also shown is a urogenital organ culture treated with ALK2 antisense and cultured for 3 days in absence of MIS (*right panel*). *Arrows* indicate the Müllerian duct (MD) and Wolffian duct (WD) in each panel and the ovary (Ov) is indicated in the *far left panel*. The *aterisk* indicates residual Müllerian duct.

To assess whether ALK2 mediates MIS signaling *in vivo*, a similar antisense approach was used in the Müllerian duct regression assay. In the absence of MIS, female urogenital ridges exhibit both the Müllerian (MD) and Wolffian (WD) ducts. In the presence of MIS, regression of the Müllerian duct occurs leaving only a visible Wolffian duct. Treatment of urogenital ridges with the antisense ALK2 oligomer partially or fully blocked MIS-induced regression (Fig. 6, *left panel*, as-ALK2). In contrast, treatment of the urogenital ridges with the control antisense ALK2 oligomer or the antisense ALK6 oligomer did not block MIS-induced regression (Fig. 6, m4-ALK2, as-ALK6). Furthermore, MIS-induced regression was unaffected by the special delivery reagent as judged by the normal loss of the Müllerian duct after incubation with MIS and the EPEI solution (Fig. 6, EPEI). In all cases, addition of morpholino oligomers alone had no effect on duct morphology (Fig. 6, right panel, no MIS, as-ALK2). These results strongly suggest that ALK2 mediates MIS-induced signaling in Müllerian duct regression.

DISCUSSION

The molecular signaling mechanisms of some TGFβ superfamily members have been studied in great de-

tail, and it is now established that at least two distinct signaling pathways exist. The first of these is a Smad2 and 3 signaling pathway shared by TGFβ and activin, and the second is a Smad1 and 5 signaling pathway shared by the BMP family (14). Here, we report that MIS activates a BMP-like pathway using Smad1 or Smad5 and requires bioactive MIS and the MIS type II receptor. Moreover, our cellular studies and antisense experiments indicate that the type I receptor that bridges the MISRII and Smad intracellular signaling is ALK2.

The best known function of MIS is induction of Müllerian duct regression in male fetuses (2, 3) where MIS induces apoptosis in the epithelial layer of the Müllerian duct by activating a signaling cascade in the mesenchymal layer (4). Although Müllerian ducts normally differentiate in female mice because MIS synthesis is restricted to embryonic testes, female Müllerian ducts are competent to respond to MIS *in vitro* and *in vivo* (8, 25). Indeed, several groups have established expression of MISRII in the mesenchyme surrounding the Müllerian duct of both sexes (4–6, 32). Here we report that the expression of ALK2 correlates well with the known expression pattern of the MISRII and is found in the urogenital ridge of male and female fetuses. *In situ* hybridization analysis revealed expression of ALK2 in the mesenchymal cells surrounding the Müllerian ducts. Interestingly, Smad5 is also expressed at a relatively high level in the mesenchymal cells surrounding the Müllerian duct (34). Most important, we believe that ALK2 plays an important role in Müllerian duct regression based on the blockage of duct regression after ALK2 antisense treatment in organ cultures. However, it is clear from our study and previous reports that ALK2 is not restricted to reproductive tissues. Despite the role of ALK2 in MIS signaling, the specificity and propagation of this signaling pathway are conferred by the MIS ligand and the MIS type II receptor and their subsequent association.

Our studies demonstrate that MIS signaling in P19 cells is mediated through an endogenous type I receptor. Interestingly, P19 cells express multiple endogenous BMP type I receptors, such as ALK2, ALK3, and ALK6, as well as endogenous activin type I receptor (ALK4) (35). In these cells, the identity of this endogenous BMP type I receptor involved in MIS signaling is likely to be ALK2. Consistent with this hypothesis, MIS signaling increased after cotransfection of ALK2 and was decreased in either the presence of a dominant-negative ALK2 or after treatment with antisense ALK2 oligomers. Although we found that other type I receptors were unable to significantly enhance MIS responsiveness in P19 cells, we noted that ALK2, ALK5, and ALK6 are all capable of forming a receptor complex with MISRII *in vitro* (data not shown). Interestingly, formation of these receptor complexes was ligand-independent, and addition of ligand showed no further increases in type I receptor recruitment by the MISRII. The lack of ligand-induced ALK2 recruitment could suggest that very few MISRII/ALK2 receptor complexes are needed for signal transduction and thus these low levels would fall below the limits of detection. Alternatively, the MIS ligand may induce a conformational change in the MISRII/ALK2 receptor complex rather than simply increasing their association. Whether all of these MISRII-type I receptor complexes are biologically relevant or merely reflect overexpression in a heterologous system remains uncertain. Nonetheless, our functional data suggest strongly that a MISRII/ALK2 receptor complex is capable of transducing the MIS signal.

It is plausible that additional type I receptors or other plasma membrane proteins contribute to MIS signal transduction by their association with the MISRII/ALK2 receptor complex. For instance, ALK6 may indeed be part of the MIS signal transduction complex, as previously suggested, but at least in our system, ALK2 appears to be the essential signaling type I receptor. In contrast to a previous study, which suggested that MIS signals by recruiting ALK6 (17), we failed to observe any attenuation of MIS-induced signaling after overexpression of a dominant-negative ALK6-KR or treatment with antisense ALK6 oligomers. Potential reasons for this discrepancy may rest with the reporter used or the amounts of mutant type I receptor used in each study. In our hands, significant inhibition of the *Tlx-2* reporter was noted at a ratio of 1:10, MISRII: ALK2-KR. Collectively our data suggest that a role for ALK6 in the MIS signaling cascade will be less straightforward than previously proposed.

While a clearly defined role for MIS in adult reproduction remains to be established, the apparent modulation of gonadal cell function by MIS infers the presence of an active signaling pathway(s) in these tissues. In addition to a developmental role for MIS in Leydig cell proliferation, several groups also report that MIS dampens steroidogenesis in the ovary and testis by repressing transcription of key steroidogenic enzymes (7, 10, 36, 37). Whether steroidogenic enzymes are directly downstream of MIS signaling is still uncertain, given that responses in steroidogenic cell lines are not robust and require MIS treatment for a minimum of 2 days (Ref. 36 and our unpublished results). Nevertheless, gonadal expression of both MISRII and ALK2 in the embryo and adult suggests that, similar to the urogenital ridge, a competent MISRII/ALK2-receptor complex is able to function in these reproductive tissues. Likewise, the strong ALK6 expression in the adult ovary (Fig. 5D) raises the possibility that a MISRII/ALK6 complex could function in the adult female. Clearly, identification of MIS target genes may elucidate the full physiological relevance of MIS in gonadal function and may also reveal its potential interplay with additional TGFβ signaling pathways in reproductive function.

Our findings imply that MIS signaling activates a BMP-like pathway through ALK2 and Smad5. Presently, our studies are unable to exclude the possibility that novel type I receptors play a role in MIS signaling. The ability to conditionally ablate *ALK2* in mice in the

genital ridge would circumvent the embryonic lethality at E9.5 during the early stages of gastrulation (21, 22) and should provide definitive data as to its role in mediating MIS signaling during Müllerian duct regression. Obviously, an ideal promoter to execute this *in vivo* genetic strategy is the MISRII promoter. It will also be of interest to determine whether similar factors known to modulate ligand availability and ligand binding (*i.e.* follistatin, inhibin binding proteins) also function in the MIS signaling pathway. Continued investigation of this signaling cascade should provide new insights into the molecular mechanisms of developmental and adult reproductive physiology.

MATERIALS AND METHODS

Plasmids

The full-length rat MISRII cDNA was isolated from E14 genital ridges by RT-PCR using the following primers: sense, 5′-GACGAATTCCTTTAGTAGGATGCTG-3′; antisense, 5′-CC - G GTCGACGGACTTAGAGCCAGAGCC-3′. The sequence of this MISRII cDNA was verified by comparison to the published sequence (6) and subcloned into *Eco*RI/*Sal*I sites of pRK5 containing a carboxy-terminal Flag epitope tag. Type I receptor expression vectors were provided by Dr. J. Massagué (Sloan Kettering, New York, NY; human ALK1, mouse ALK3, and mouse ALK6), Dr. R. Derynck (University of California, San Francisco, CA; mouse ALK2 and rat ALK5), and Dr. L. Mathews (University of Michigan, Ann Arbor, MI; human ALK4) (38–40). Kinase inactive mutants, MISRII-K228R, ALK2-K235R, and ALK6-K230R, were generated by PCR-based mutagenesis strategy. TLX2-Lux and A3-Lux reporter genes and human Smad1 and human Smad2 expression vectors were provided by Dr. J. Wrana (Samuel Lunenfeld Research Institute, Toronto, Ontario, Canada) (23, 24, 26, 41). Mouse Smad5 expression vector was provided by Dr. X. Wang (Duke University, Durham, NC) (42), and human Smad2-SA and mouse Smad5–2SA constructs were provided by Dr. P. ten Dijke (The Netherlands Cancer Institute, Amsterdam, The Netherlands) (28, 43). The human BMPRII, mouse ActRIIB, and human TβRII were provided by Dr. M. Kawabata (Cancer Institute, Tokyo, Japan) (44), Dr. L. Attisano (University of Toronto, Ontario, Canada) (45), and Dr. R. Derynck (46), respectively.

Cell Culture, Transfections, and Luciferase Assays

P19 cells were cultured in α-MEM containing 7.5% calf serum and 2.5% FBS. For MIS-induced luciferase assays, P19 cells were seeded at 20% confluency in 12-well plates and transfected with TLX2-Lux or A3-Lux reporter plasmid (100 ng/well) and the indicated receptor expression vectors (25 ng/well) using Fugene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). Twenty-four hours after transfection, cells were cultured for 2 h in medium containing 0.2% serum followed by 16 h treatment with the MIS ligand (15 nM). Luciferase was measured using the luciferase assay system (PharMingen, San Diego, CA) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). In all transfections, β-galactosidase expression plasmid (pCMV5-βGal) served as internal control to normalize for transfection efficiency.

MIS Preparation and Enzyme-Linked Immunosorbent Assay (ELISA)

Recombinant bioactive (MIS-RARR) and inactive (MIS-RAGA) conditioned medium were obtained from stably transfected HEK-293S cells expressing these rat MIS cDNAs as described previously (25). Conditioned medium of wild-type HEK-293S cells served as the control. Collected media were concentrated approximately 30-fold using a Centriprep system (Millipore Corp., Bedford, MA). The integrity of recombinant MIS protein was determined by Western blot analysis using purified antirat MIS antibody (25), and the amount of MIS was measured by an in-house ELISA assay. The standard curve was generated using purified MIS at concentrations ranging from 0.025–1 μg/ml. Purified MIS or MIS-conditioned medium were serially diluted in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6) overnight at 4 C in a 96-well Immulon plate (Nunc, Rochester, NY), followed by incubation with blocking buffer (3% BSA, 0.02% NaN_3 in PBS) for 2 h at room temperature. Plates were incubated overnight at 4 C with a polyclonal MIS rabbit antibody (1:250 dilution) (25). The following day, wells were washed with PBST (0.1% Tween in PBS) and incubated with biotinylated conjugated donkey antirabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) (1:500 dilution) for 1 h at room temperature. After extensive washing with PBST, Streptavidin-peroxidase conjugate (Caltag Laboratories, Inc. Burlingame, CA) was added (1:500 dilution) for 30 min at room temperature. Wells were washed with PBST and incubated with BM blue (Roche Molecular Biochemicals) to visualize the complex. The reaction was stopped by adding 100 μl 1 M H_2SO_4, and the optical density was measured at 490 nm on a reader plate (BioTek, Winooski, VT). The molar concentrations of our recombinant HIS-tagged MIS were calculated based on an apparent molecular mass of 155 kDa as judged by SDS-PAGE in the absence of β-mercaptoethanol.

Morpholino Antisense Oligo Treatment and Regression Assays

Special delivery morpholino antisense oligos (33, 47) were designed for ALK2 (as-ALK2: 5′-CTCCATCGACCATTG-TATAACC-3′) and ALK6 (as-ALK6: 5′-ATTTTCCAGAGC-TTCGTAAGAGCAT-3′) with guidance by Dr. Paul Marcos (Gene Tools LLC, Corvallis, OR). To create a control oligo, 4 mispairs were introduced into the antisense ALK2 oligo (4 m-ALK2: 5′-CTgCATgGACCATTGaATAtCC-3′). Antisense morpholino oligos were mixed with a delivery reagent, EPEI, according to the manufacturer's directions (Gene Tools) for 20 min incubation at room temperature. A complete delivery solution containing 1.4 μM of oligo was added to P19 cells for 3 h and medium was replaced with fresh medium. Transfection and ligand treatment were performed (as described above) 24 h after antisense oligo treatment. Female rat urogenital ridges were dissected from E14.5 embryos and incubated for 3 h with the delivery solution containing 1.4 μM of morpholino antisense oligos (as described above). Treated organs were cultured on MilliCell-CM biopore membranes (Millipore Corp.) floated in 0.2 ml medium as previously described (4, 25), in the presence or absence of MIS (32 nM) for 3 days.

RNAse Protection Assay and *in Situ* Hybridization

To obtain embryos for RNA protection assays and *in situ* hybridization studies, pregnant FVB mice were killed by cervical dislocation on embryonic (E) day 13, E14, E15, or E18; vaginal plug detection was considered to be E0. Fetal tissues were isolated and snap frozen in liquid nitrogen and stored at −80 C. Fetal sex was determined by PCR using placental genomic DNA, and total RNA was isolated as previously

described (48). An *Eco*RI-*Hin*dIII fragment containing 1–472 bp of the mouse ALK2 cDNA, and a *Hpa*I-*Apa*I fragment containing 79–551 bp of the mouse ALK6, both encoding the extracellular domain, were subcloned in pBKS and used to generate [^{32}P]-UTP-labeled antisense RNA probes. Mouse MISRII and control glyceraldehyde 3-phosphate dehydrogenase (GAPD) antisense RNA probes were generated (6) and used in RNase protection assays using 10 μg of total RNA as described previously (48). *In situ* hybridization analyses was performed as described previously (6) on tissue sections fixed overnight in Bouin's fixative, embedded in paraffin, and sectioned transversally at 8 μm. The same *Eco*RI-*Hin*dIII fragment of mouse ALK2 cDNA was used to generate sense and antisense [^{35}S]-UTP-labeled transcripts. Mice and rats were housed in accordance with NIH guidelines.

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Note Added in Proof

Similar findings showing the use of ALK2 in the MIS signaling pathway are reported in the following article (pp. 946–959) by Clarke *et al*. (19a).

REFERENCES

1. Massagué J 1998 TGF-β signal transduction. Annu Rev Biochem 67:753–791
2. Lee MM, Donahoe PK 1993 Müllerian inhibiting substance: a gonadal hormone with multiple functions. Endocr Rev 14:152–164
3. Josso N, Cate RL, Picard JY, Vigier B, di Clemente N, Wilson C, Imbeaud S, Pepinsky RB, Guerrier D, Boussin L, et al 1993 Anti-Müllerian hormone: the Jost factor. Recent Prog Horm Res 48:1–59
4. Roberts LM, Hirokawa Y, Nachtigal MW, Ingraham HA 1999 Paracrine-mediated apoptosis in reproductive tract development. Dev Biol 208:110–122
5. di Clemente N, Wilson C, Faure E, Boussin L, Carmillo P, Tizard R, Picard JY, Vigier B, Josso N, Cate R 1994 Cloning, expression, and alternative splicing of the receptor for anti-Müllerian hormone. Mol Endocrinol 8:1006–1020
6. Baarends WM, van Helmond MJ, Post M, van der Schoot PJ, Hoogerbrugge JW, de Winter JP, Uilenbroek JT, Karels B, Wilming LG, Meijers JH, Themmen APN, Grootegoed JA 1994 A novel member of the transmembrane serine/threonine kinase receptor family is specifically expressed in the gonads and in mesenchymal cells adjacent to the Müllerian duct. Development 120:189–197
7. Behringer RR, Cate RL, Froelick GJ, Palmiter RD, Brinster RL 1990 Abnormal sexual development in transgenic mice chronically expressing Müllerian inhibiting substance. Nature 345:167–170
8. Behringer RR, Finegold MJ, Cate RL 1994 Müllerian-inhibiting substance function during mammalian sexual development. Cell 79:415–425
9. Durlinger AL, Kramer P, Karels B, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen AP 1999 Control of primordial follicle recruitment by anti-Müllerian hormone in the mouse ovary. Endocrinology 140:5789–5796
10. Lyet L, Louis F, Forest MG, Josso N, Behringer RR, Vigier B 1995 Ontogeny of reproductive abnormalities induced by deregulation of anti-Müllerian hormone expression in transgenic mice. Biol Reprod 52:444–454
11. Imbeaud S, Faure E, Lamarre I, Mattéi MG, di Clemente N, Tizard R, Carré-Eusèbe D, Belville C, Tragethon L, Tonkin C, Nelson J, McAulife JB, Lababidi A, Josso N, Cate RL, Picard J-Y 1995 Insensitivity to anti-Müllerian hormone due to a mutation in the human anti-Müllerian hormone receptor. Nat Genet 11:382–388
12. Mishina Y, Suzuki A, Ueno N, Behringer RR 1995 Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. Genes Dev 9:3027–3037
13. Heldin CH, Miyazono K, ten Dijke P 1997 TGF-β signalling from cell membrane to nucleus through SMAD proteins. Nature 390:465–471
14. Wrana JL, Attisano L 2000 The Smad pathway. Cytokine Growth Factor Rev 11:5–13
15. Yamamoto N, Akiyama S, Katagiri T, Namiki M, Kurokawa T, Suda T 1997 Smad1 and Smad5 act downstream of intracellular signalings of BMP-2 that inhibits myogenic differentiation and induces osteoblast differentiation in C2C12 myoblasts. Biochem Biophys Res Commun 238:574–580
16. Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, Tamaki K, Hanai J, Heldin CH, Miyazono K, ten Dijke P 1997 TGF-β receptor-mediated signalling through Smad2, Smad3 and Smad4. EMBO J 16:5353–5362
17. Gouédard L, Chen YG, Thevenet L, Racine C, Borie S, Lamarre I, Josso N, Massague J, di Clemente N 2000 Engagement of bone morphogenetic protein type IB receptor and Smad1 signaling by anti-Müllerian hormone and its type II receptor. J Biol Chem 275:27973–27978
18. Baur ST, Mai JJ, Dymecki SM 2000 Combinatorial signaling through BMP receptor IB and GDF5: shaping of the distal mouse limb and the genetics of distal limb diversity. Development 127:605–619
19. Yi SE, Daluiski A, Pederson R, Rosen V, Lyons KM 2000 The type I BMP receptor BMPRIB is required for chondrogenesis in the mouse limb. Development 127: 621–630

19a. Clarke TR, Hoshiya Y, Yi SE, Liu X, Lyons KM, Donahoe PK 2001 Müllerian inhibiting substance signaling uses a BMP-like pathway mediated by ALK2 and induces Smad6 expression. Mol Endocrinol 15:946–959

20. Gu Z, Nomura M, Simpson BB, Lei H, Feijen A, van den Eijnden-van Raaij J, Donahoe PK, Li E 1998 The type I activin receptor ActRIB is required for egg cylinder organization and gastrulation in the mouse. Genes Dev 12: 844–857
21. Gu Z, Reynolds EM, Song J, Lei H, Feijen A, Yu L, He W, MacLaughlin DT, van den Eijnden-van Raaij J, Donahoe PK, Li E 1999 The type I serine/threonine kinase receptor ActRIA (ALK2) is required for gastrulation of the mouse embryo. Development 126:2551–2561
22. Mishina Y, Crombie R, Bradley A, Behringer RR 1999 Multiple roles for activin-like kinase-2 signaling during mouse embryogenesis. Dev Biol 213:314–326
23. Tang SJ, Hoodless PA, Lu Z, Breitman ML, McInnes RR, Wrana JL, Buchwald M 1998 The Tlx-2 homeobox gene

is a downstream target of BMP signalling and is required for mouse mesoderm development. Development 125: 1877–1887
24. Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone Jr MA, Wrana JL, Falb D 1997 The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFβ signaling. Cell 89:1165–1173
25. Nachtigal MW, Ingraham HA 1996 Bioactivation of Müllerian inhibiting substance during gonadal development by a kex2/subtilisin-like endoprotease. Proc Natl Acad Sci USA 93:7711–7716
26. Macías-Silva M, Abdollah S, Hoodless PA, Pirone R, Attisano L, Wrana JL 1996 MADR2 is a substrate of the TGFβ receptor and its phosphorylation is required for nuclear accumulation and signaling. Cell 87:1215–1224
27. Abdollah S, Macías-Silva M, Tsukazaki T, Hayashi H, Attisano L, Wrana JL 1997 TβRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. J Biol Chem 272:27678–27685
28. Souchelnytskyi S, Tamaki K, Engström U, Wernstedt C, ten Dijke P, Heldin CH 1997 Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-β signaling. J Biol Chem 272:28107–28115
29. He WW, Gustafson ML, Hirobe S, Donahoe PK 1993 Developmental expression of four novel serine/threonine kinase receptors homologous to the activin/transforming growth factor-β type II receptor family. Dev Dyn 196: 133–142
30. Baarends WM, Uilenbroek JT, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen AP, Grootegoed JA 1995 Anti-Müllerian hormone and anti-Müllerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. Endocrinology 136:4951–4962
31. Baarends WM, Hoogerbrugge JW, Post M, Visser JA, De Rooij DG, Parvinen M, Themmen AP, Grootegoed JA 1995 Anti-Müllerian hormone and anti-Müllerian hormone type II receptor messenger ribonucleic acid expression during postnatal testis development and in the adult testis of the rat. Endocrinology 136:5614–5622
32. Teixeira J, He WW, Shah PC, Morikawa N, Lee MM, Catlin EA, Hudson PL, Wing J, Maclaughlin DT, Donahoe PK 1996 Developmental expression of a candidate Müllerian inhibiting substance type II receptor. Endocrinology 137:160–165
33. Summerton J 1999 Morpholino antisense oligomers: the case for an RNase H-independent structural type. Biochim Biophys Acta 1489:141–158
34. Meersseman G, Verschueren K, Nelles L, Blumenstock C, Kraft H, Wuytens G, Remacle J, Kozak CA, Tylzanowski P, Niehrs C, Huylebroeck D 1997 The C-terminal domain of Mad-like signal transducers is sufficient for biological activity in the Xenopus embryo and transcriptional activation. Mech Dev 61:127–140
35. Macías-Silva M, Hoodless PA, Tang SJ, Buchwald M, Wrana JL 1998 Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. J Biol Chem 273:25628–25636
36. Teixeira J, Fynn-Thompson E, Payne AH, Donahoe PK 1999 Müllerian-inhibiting substance regulates androgen synthesis at the transcriptional level. Endocrinology 140: 4732–4738
37. Racine C, Rey R, Forest MG, Louis F, Ferré A, Huhtaniemi I, Josso N, di Clemente N 1998 Receptors for anti-Müllerian hormone on Leydig cells are responsible for its effects on steroidogenesis and cell differentiation. Proc Natl Acad Sci USA 95:594–599
38. Franzén P, ten Dijke P, Ichijo H, Yamashita H, Schulz P, Heldin CH, Miyazono K 1993 Cloning of a TGF β type I receptor that forms a heteromeric complex with the TGF β type II receptor. Cell 75:681–692
39. ten Dijke P, Ichijo H, Franzén P, Schulz P, Saras J, Toyoshima H, Heldin CH, Miyazono K 1993 Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. Oncogene 8:2879–2887
40. ten Dijke P, Yamashita H, Ichijo H, Franzén P, Laiho M, Miyazono K, Heldin CH 1994 Characterization of type I receptors for transforming growth factor-β and activin. Science 264:101–104
41. Hoodless PA, Haerry T, Abdollah S, Stapleton M, O'Connor MB, Attisano L, Wrana JL 1996 MADR1, a MAD-related protein that functions in BMP2 signaling pathways. Cell 85:489–500
42. Yingling JM, Das P, Savage C, Zhang M, Padgett RW, Wang XF 1996 Mammalian dwarfins are phosphorylated in response to transforming growth factor β and are implicated in control of cell growth. Proc Natl Acad Sci USA 93:8940–8944
43. Tamaki K, Souchelnytskyi S, Itoh S, Nakao A, Sampath K, Heldin CH, ten Dijke P 1998 Intracellular signaling of osteogenic protein-1 through Smad5 activation. J Cell Physiol 177:355–363
44. Kawabata M, Chytil A, Moses HL 1995 Cloning of a novel type II serine/threonine kinase receptor through interaction with the type I transforming growth factor-β receptor. J Biol Chem 270:5625–5630
45. Attisano L, Wrana JL, Montalvo E, Massagué J 1996 Activation of signalling by the activin receptor complex. Mol Cell Biol 16:1066–1073
46. Feng XH, Filvaroff EH, Derynck R 1995 Transforming growth factor-β (TGF-beta)-induced down-regulation of cyclin A expression requires a functional TGF-β receptor complex. Characterization of chimeric and truncated type I and type II receptors. J Biol Chem 270: 24237–24245
47. Summerton J, Weller D 1997 Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucleic Acid Drug Dev 7:187–195
48. Visser JA, McLuskey A, Verhoef-Post M, Kramer P, Grootegoed JA, Themmen AP 1998 Effect of prenatal exposure to diethylstilbestrol on Müllerian duct development in fetal male mice. Endocrinology 139:4244–4251