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AMH induces mesonephric cell migration in XX gonads

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Abstract

Migration of mesonephric cells into XY gonads is a critical early event in testis cord formation. Based on the fact that anti-Müllerian hormone (AMH) can induce testis cord formation in XX gonads, we investigated whether AMH plays a role in the induction of cell migration. Addition of recombinant AMH induced mesonephric migration into XX gonads in culture. AMH-treated XX gonads displayed increased vascular development and altered morphology of the coelomic epithelium, both features of normal testis differentiation. AMH did not induce markers of Sertoli or Leydig cell differentiation. We examined early testis development in *Amh*-deficient mice, but found no abnormalities, suggesting that any function AMH may have in vivo is redundant. Other transforming growth factor (TGF- β) family proteins, bone morphogenetic proteins (BMP2 and BMP4) show similar inductive effects on XX gonads in culture. Although neither BMP2 nor BMP4 is expressed in embryonic XY gonads, our findings suggest that a TGF- β signalling pathway endogenous to the XY gonad may be involved in regulation of mesonephric cell migration. The factors involved in this process remain to be identified.

Keywords

AMH; Testis; Mesonephros; Migration

1. Introduction

The transforming growth factor β (TGF- β) superfamily of secreted signalling molecules is involved in a wide range of developmental processes, including cell proliferation, differentiation, migration, and death. Members of the gene family include the TGF- β s, inhibins/activins, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), and anti-Müllerian hormone (AMH, also called Müllerian inhibiting substance). These secreted factors dimerize and bind to a pair of serine/threonine kinase receptors, termed type I and type II, on target cells (reviewed by ten Dijke et al., 1996). Upon ligand binding, the two receptors form a complex and type I receptors are phosphorylated and

activated by the type II receptor kinases. The type I receptors transduce the signals by phosphorylating intracellular proteins of the Smad family to regulate transcription of downstream genes (Attisano and Wrana, 2000). While TGF- β family members function in a wide range of tissues throughout development, many are also specifically involved in different aspects of reproductive development (Josso and di Clemente, 1999; Ingman and Robertson, 2002). However, little is known about the functions of the TGF- β family members during the initial stages of gonad organogenesis.

AMH has been extensively characterized at both the biochemical and functional level. AMH is secreted by pre-Sertoli cells of the embryonic XY gonad (Blanchard and Josso, 1974; Donahoe et al., 1977; Tran et al., 1987; Munsterberg and Lovell-Badge, 1991) and induces the regression of the Müllerian duct, the anlagen of the female reproductive tract. Phenotypic analysis of mice lacking a functional *Amh* gene product confirmed the requirement of AMH for Müllerian regression and also uncovered a function for AMH in regulation of Leydig cell proliferation in the adult testis (Behringer et al., 1994). No role for AMH during early testis development has been identified, yet several lines of evidence support the idea that AMH could function during this period. In some vertebrates, such as birds and alligators, expression of AMH is observed at the earliest stages of testis differentiation and precedes expression of *SOX9* (Oréal et al., 1998; Western et al., 1999). This is in contrast to the mouse, in which *Sox9* is one of the earliest markers of Sertoli cell differentiation and is required for normal initiation of *Amh* transcription (De Santa Barbara et al., 1998; Arango et al., 1999). The observation that *AMH* expression is independent of *SOX9* expression and occurs very early during gonad differentiation in some lower vertebrates raises the possibility that AMH could function at an early step in the sex determination cascade in these organisms.

Additional evidence comes from studies of freemartinism. Freemartins are bovine female embryos united to a male twin by chorionic vascular anastomoses. Ovaries from freemartins lose their germ cells and in some cases develop seminiferous tubules (Jost et al., 1972). Serum concentrations of AMH are similar in freemartins and their male twins, suggesting that AMH may mediate the disruption of ovarian development (Vigier et al., 1984). This idea is supported by the finding that recombinant AMH can induce a freemartin effect in fetal rat ovaries in culture, resulting in depletion of germ cells and formation of testis cord-like structures (Vigier et al., 1988). We have further investigated the effects of AMH on the development of XX gonads in culture to better understand how AMH induces this freemartin effect and to identify potential roles of AMH during normal testis development.

2. Examination of AMH function during early gonad development

In the mouse, the gonads arise as a thickening along the ventromedial side of the mesonephroi at approximately 10.5 days postcoitum (10.5 dpc). Initially identical in both XX and XY embryos, this bipotential primordium can develop as either a testis or an ovary. Testis development is initiated by expression of the Y-linked gene *Sry* between 10.5 and 12.5 dpc (Koopman et al., 1990, 1991; Lovell-Badge and Robertson, 1990). While the genetic targets of *SRY* are not known, a number of early cellular and morphological events downstream of *Sry* expression have been characterized. These early events include increased

cell proliferation (Schmahl et al., 2000), male-specific migration of cells from the mesonephros into the XY gonad (Buehr et al., 1993; Merchant-Larios et al., 1993; Martineau et al., 1997), and reorganization of Sertoli cells to surround germ cells and form testis cords. The migrating cells recruited from the mesonephros include peritubular cells that surround the testis cords and endothelial cells which are required for formation of an XY-specific vascular pattern (Martineau et al., 1997; Brennan et al., 2002). Blocking this migration prevents cord formation, demonstrating the importance of this process for testis morphogenesis (Buehr et al., 1993; Tilmann and Capel, 1999). We have investigated whether AMH may play a role in the regulation of this critical process.

Organ culture experiments using recombinant purified AMH protein were performed to determine whether AMH could induce migration of mesonephric cells into an XX gonad. The recombinant human AMH used in these experiments was purified from a CHO cell conditioned media transfected with the human *AMH* gene (Cate et al., 1986) using an immunoaffinity protocol described earlier (Ragin et al., 1992). Gonads from 11.5 or 12.5 dpc embryos were assembled with mesonephroi from ROSA 26 embryos, which constitutively express a *lacZ* reporter gene (Soriano, 1999). The samples were cultured for 40–48 h and stained for *lacZ* activity as previously described (Martineau et al., 1997). As expected, extensive migration of mesonephric cells into an 11.5 dpc XY gonad was observed, but no *lacZ*-positive cells were detected in XX gonads (Fig. 1A and B). However, when 11.5 dpc XX gonads were cultured in media containing 15 µg/ml recombinant human AMH protein, numerous cells migrated into the gonad (Fig. 1C). While this migration was consistently observed, the levels of AMH-induced migration were generally less than that seen in XY controls. Although migration is normally observed in XY (but not XX) gonads assembled at 12.5 dpc and cultured under the same experimental conditions, migration was not observed in 12.5 dpc XX gonads treated with AMH (not shown). This suggests that AMH has this inductive capacity only during a restricted developmental window.

Because many of the cells that normally migrate into the XY gonad contribute to the vasculature, we examined vascular formation in XX gonads cultured with AMH. Immunocytochemistry was performed on AMH-treated gonads and controls using antibodies against laminin, which labels the basal lamina beneath the coelomic epithelium, and PECAM-1, which labels germ cells and vascular endothelial cells. In XY controls, a large vessel, referred to as the coelomic vessel, can clearly be observed extending just beneath the coelomic epithelium of the gonad (Fig. 2A and B). This vessel is absent in XX gonads (Fig. 2C). In the XX gonad treated with 15 µg/ml AMH, a vessel is also observed along the anterior end of the gonad under the coelomic epithelium (Fig. 2E). While the vessels in the AMH-treated gonads did not always extend over the entire length of the gonad as is seen in XY controls, vessels of variable length were consistently observed beneath the coelomic epithelial layer in these samples. Interestingly, the AMH-treated XX gonads displayed another feature characteristic of XY development. The coelomic epithelium of these gonads was dramatically thickened as compared to XX controls. Epithelial cells were elongated, and multiple layers of cells were observed, similar to what is observed on the surface of XY gonads (Fig. 2B and F). It is not clear if this is due to increased proliferation of the cell layer, or if AMH alters the structural morphology of this region in some other way. It also

raises the possibility that AMH might not directly induce migration of vessels, but rather influence the characteristics of the coelomic surface in a way that allows the movement of vascular endothelial cells through this cell layer. These questions remain to be explored.

It is not known which cells are the targets of the AMH signal in these assays. The AMH type II receptor has been identified, and is specific for AMH signalling (Mishina et al., 1999). During embryogenesis, this receptor is expressed in mesenchymal cells surrounding the Müllerian duct and somatic cells of both the XX and XY gonad (Baarends et al., 1994). Thus, in our experiments, AMH might influence differentiation of gonadal somatic cells which subsequently secrete a migratory factor, or it could be signalling directly to mesonephric cells to stimulate their movement into the gonad. To investigate the first possibility, we examined markers of normal male development to determine if AMH induces differentiation of testicular somatic cells in XX gonads. Expression of *Sox9*, an early marker of Sertoli cell differentiation, was observed in XY gonads but not in XX gonads cultured under the same conditions. No *Sox9* expression was induced in XX gonads cultured with 15 µg/ml recombinant AMH (Fig. 3). Similarly, the p450 side chain cleavage (SCC) enzyme, an early enzyme in steroid biosynthesis and a marker of Leydig cell differentiation, was not expressed in AMH-treated XX gonads (Fig. 3B). These findings suggest that mesonephric migration induced by AMH is not a downstream effect of Sertoli or Leydig cell differentiation in the XX gonad. It also demonstrates that AMH cannot direct expression of *Sox9* or act as a switch for male development in higher vertebrates, at least not over the short time course of these assays. It is currently unclear whether AMH performs these functions in birds and reptiles, where it is expressed at much earlier stages.

As these findings implicated AMH as a potential inducer of mesonephric cell migration, we examined early stages of testis development in *Amh* null mutant embryos (Behringer et al., 1994). However, no abnormalities in early testis differentiation were discovered in these mutants. Migration occurred normally into 11.5 dpc *Amh* $-/-$ XY gonads when cultured with ROSA26 mesonephroi (Fig. 4A and B). Early morphological development of *Amh* $-/-$ XY gonads, including vascular development, was not impaired (Fig. 4C and D). Finally, expression of *Sox9* in *Amh* $-/-$ 12.5 dpc gonads demonstrated that Sertoli cell differentiation was not disrupted (Fig. 4E and F). These findings suggest that while AMH can induce features of testis development in XX gonads, it is not required for these processes in XY gonads. Therefore, if AMH does have a physiological role during normal testis morphogenesis, it is redundant with another factor or factors.

3. AMH and BMPs induce similar morphological changes in XX gonads

Genetic analyses have demonstrated that AMH is the only TGF- β family member to signal through the AMH type II receptor. However, that specificity is not found at the level of the type I receptor. Previous biochemical studies have identified two Bmp type I receptors, ActRIA (also called Alk2) and Bmpr1b (also called Alk6) as candidate type I receptors for AMH (Gouédard et al., 2000; Clarke et al., 2001; Visser et al., 2001). However, recent genetic analyses have demonstrated that Bmpr1a (Alk3) is required for normal Müllerian duct regression in vivo (Jamin et al., 2002). Bmpr1a also functions as a type I receptor for BMP2 and BMP4 (Koenig et al., 1994). Not only do pathways for BMPs and AMH

converge at the receptor level, but studies have demonstrated that AMH and BMP pathways utilize the same Smad effector proteins, specifically Smad1 and -5 (Gouédard et al., 2000; Clarke et al., 2001; Visser et al., 2001). Therefore, we investigated whether the effects induced by AMH are mimicked by other BMPs.

To examine this possibility, XX gonads were cultured in the presence of beads coated with BMP2 or BMP4 protein, as previously described (Furuta et al., 1997). Both BMP2 and BMP4 induced growth of vasculature into XX gonads, similar to the effects observed when AMH was added to cultures (Fig. 5). These findings may indicate that AMH is acting through a general BMP pathway endogenous to the gonad. However, this pathway must not involve BMP2 or BMP4, as we were unable to detect expression of either gene in embryonic XY gonads.

4. Future directions

These findings raise several questions regarding the roles of AMH and other TGF- β family members during gonad development. AMH can induce migration of mesonephric cells and development of a male-specific vascular pattern in cultures of XX gonads, but AMH is not required for these processes in XY gonads *in vivo*. Similar morphological effects are observed when recombinant BMP2 or BMP4 protein is added to cultures of XX gonads, yet neither of these molecules is expressed in XY gonads during normal development. There is much redundancy between signaling pathways for different BMPs (and AMH) at the intracellular level (Miyazono et al., 2001). It therefore seems possible that a different BMP is involved in the regulation of mesonephric migration during normal testis development. Future work in the lab will focus on determining whether TGF- β signalling pathways do regulate mesonephric cell migration during testis differentiation, and identifying which factors are involved in this process. We also intend to examine the mechanisms by which TGF- β factors are able to induce mesonephric migration. It is currently not clear whether these factors signal directly to cells of the mesonephros or if migration is downstream of other events. An analysis of whether BMP receptors are required in the gonad or the mesonephros should answer this. Hopefully, these studies will provide a better understanding of how the early stages of testis differentiation are regulated at the molecular level.

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Fig. 1.

AMH induces migration of mesonephric cells into XX gonads. Gonads from 11.5 dpc CD-1 embryos were cultured with mesonephroi from 11.5 dpc ROSA26 mice for 48 h and stained for *lacZ* activity. (A) XX gonads cultured with a ROSA26 mesonephros did not contain any *lacZ*-positive blue cells. (B) In contrast, numerous cells migrated into an XY gonad. (C) Migration was also observed in XX gonads cultured with a ROSA26 mesonephros in the presence of 15 $\mu\text{g/ml}$ recombinant human AMH protein.

Fig. 2.

AMH induces vascular formation and alters epithelial morphology in XX gonads. 11.5 dpc gonads were cultured for 40 h in the presence or absence of 15 $\mu\text{g/ml}$ AMH.

Immunostaining was performed for PECAM-1 (Pharmingen), which labels germ cells (round, red) and vascular endothelial cells (elongated, red), and for laminin (H. Erickson, Duke University), which labels the basal lamina underlying the coelomic epithelium (green).

(A, and B) Cultured XY gonads display a characteristic vessel beneath the coelomic epithelium (small arrows), and a thickened coelomic epithelium containing multiple cell layers (large arrowheads). (C, and D) These features are absent in XX gonads. (E, and F) XX gonads cultured with AMH contain vessels extending underneath the epithelial layer (small arrows) and a epithelial morphology more similar to that observed in XY controls (large arrowheads). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

AMH does not induce expression of Sertoli or Leydig cell markers in XX gonads. Gonads and mesonephroi from 11.5 dpc embryos were cultured for 48 h in the absence or presence of 15 $\mu\text{g/ml}$ AMH and whole mount in situ hybridization for *Sox9* or *Scc* mRNA was performed as previously described (Tilman and Capel, 1999). (A) *Sox9* expression was observed in XY gonads (top), but was absent in XX gonads cultured under similar conditions (middle). XX gonads cultured in the presence of AMH (bottom) also did not express detectable levels of *Sox9*. (B) *Scc* expression was also observed in XY controls (top) but was not induced in AMH-treated XX gonads (bottom).

Fig. 4.

Normal early testis differentiation in *Amh*^{-/-} embryos. (A, and B) Gonads from wild-type (A) and *Amh*^{-/-} (B) 11.5 dpc XY embryos were cultured for 48 h with ROSA26 mesonephroi. Similar levels of mesonephric migration were observed in both samples. (C, and D) Immunostaining for laminin (green) and PECAM-1 (red) on 12.5 dpc wild-type (C) and *Amh*^{-/-} (D) XY gonads. *Amh* mutant gonads displayed normal morphology and coelomic vessel formation (arrows). (E, and F) Whole mount in situ hybridization for *Sox9* expression in 12.5 dpc wild-type (E) and *Amh*^{-/-} (F) XY gonads. Sertoli cell differentiation appears normal in *Amh* mutants, as demonstrated by *Sox9* expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

BMP2 and BMP4 induce vascular development and block germ cell meiosis in XX gonads. Beads were soaked with 10 mg/ml recombinant human BMP2 or BMP4 (Genetics Institute) or BSA. Ten to 15 beads were placed on the coelomic surface of 11.5 dpc XX gonads and samples were cultured for 48 h. Immunohistochemistry was performed with antibodies against PECAM-1 (red) and γ H2AX, a marker of meiotic cells (green, antibody from W. Bonner, NCI). XX gonads cultured with BSA-coated beads (A) displayed normal morphology. XX gonads cultured with BMP4-coated beads (B) or BMP2-coated beads (C, and D) displayed a large vessel underneath the coelomic epithelium (arrows). BMP2- and BMP4-treated gonads also contained fewer meiotic germ cells, a finding that is not discussed here. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)