Involvement of a matrix metalloproteinase in MIS-induced cell death during urogenital development

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SUMMARY

Programmed cell death of the Müllerian duct eliminates the primitive female reproductive tract during normal male sexual differentiation. Müllerian inhibiting substance (MIS or AMH) triggers regression by propagating a BMP-like signaling pathway in the Müllerian mesenchyme that culminates in apoptosis of the Müllerian duct epithelium. Presently, the paracrine signal(s) used in this developmental event are undefined. We have identified a member of the matrix metalloproteinase gene family, Mmp2, as one of the first candidate target genes downstream of the MIS cascade to function as a paracrine death factor in Müllerian duct regression. Consistent with a role in regression, Mmp2 expression was significantly elevated in male but not female Müllerian duct mesenchyme. Furthermore, this sexually dimorphic expression of Mmp2 was extinguished in mice lacking the MIS ligand, suggesting strongly that Mmp2 expression is regulated by MIS signaling. Using rat organ genital ridge organ cultures, we found that inhibition of MMP2 activity prevented MIS-induced regression, whereas activation of MMP2 promoted ligand-independent Müllerian duct regression. Finally, MMP2 antisense experiments resulted in partial blockage of Müllerian duct regression. Based on our findings, we propose that similar to other developmental programs where selective elimination or remodeling of tissues occurs, localized induction of extracellular proteinases is critical for normal male urogenital development.

Key words: Müllerian duct, MIS, MMP2, Apoptosis, Genital ridge, Urogenital development, Mouse, Rat

INTRODUCTION

Apoptosis or programmed cell death is a major mechanism underlying morphogenetic events that sculpt the developing embryo. Prominent examples of cell death during vertebrate development include refinement of the immune system, specification of neuronal projections, formation of digits, and tail resorption during amphibian metamorphosis (see Jacobson et al., 1997). Müllerian duct regression is another example of a vertebrate developmental program involving cell death that results in the elimination of the primitive female reproductive tract in males. As such, this apoptotic event is an essential aspect of normal male sexual differentiation. The extracellular factor responsible for initiating the Müllerian duct regression program is the TGFβ-like hormone, Müllerian inhibiting substance (MIS or AMH) (see Nef and Parada, 2000; Roberts et al., 1999b). Members of the TGFβ gene 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 (reviewed by Itoh et al., 2000). The MIS type II receptor (MISIIIR) is expressed exclusively in the mesenchyme surrounding the Müllerian duct (Allard et al., 2000; Baarends et al., 1994; Roberts et al., 1999a), and at least for Müllerian duct regression, MIS employs a BMP-like signaling pathway and is able to signal through the type I receptor, ALK2 (Clarke et al., 2001; Gouedard et al., 2000; Visser et al., 2001). The specificity of the MIS signaling cascade is established by the restricted expression patterns of both the MIS ligand and the MIS type II receptor. Under normal circumstances, the mammalian embryonic ovary does not express MIS during sexual differentiation; consequently, the Müllerian duct fails to regress in females, and develops into the fallopian tubes, uterus and upper vagina. Conversely, the corresponding male reproductive tract, the Wolffian duct, is maintained in the male embryo because of testicular testosterone production, and becomes the epididymis, vas deferens and seminal vesicle (Nef and Parada, 2000). Addition of bioactive MIS to female genital ridges in culture results in Müllerian duct regression, suggesting that following MIS stimulation, females possess the full program to undergo duct regression in the urogenital ridge; thereby providing a unique opportunity to explore this morphogenetic process at an experimental level.

The restricted expression of the MIS type II receptor to the Müllerian mesenchyme and not the epithelium implies that involution of the Müllerian duct is a consequence of an indirect or paracrine signal emanating from the Müllerian mesenchyme (Roberts et al., 1999a). This proposal predicts that MIS
signaling either activates a paracrine death factor leading to apoptosis of the epithelium, or alternatively, represses a survival signal required for growth of the epithelium. Paracrine signaling between the Müllerian epithelium and mesenchyme has also been inferred from mutant mouse strains lacking either Wnt4 or Wnt7a. Both of these Wnt signaling factors are expressed in, and are required for normal urogenital development. For example, Wnt4-null mice fail to develop Müllerian ducts, suggesting that Wnt4 signaling emanating from the mesenchyme is essential for formation of the Müllerian epithelium (Vainio et al., 1999). Likewise, persistence of Müllerian ducts observed in Wnt7a+/− male mice suggests that Wnt7a emanating from the epithelium is required for the maintenance of the MIS type II receptor in the mesenchyme (Parr and McMahon, 1998). These examples underscore the important role of Müllerian epithelial-mesenchymal interactions in reproductive tract remodeling and development.

While intracellular pathways leading to cell death are well defined (reviewed by Hengartner, 2000; Meier et al., 2000), it is less clear how extracellular cues, such as MIS, control spatial and temporal aspects of apoptosis. Therefore, the identification of downstream targets in the MIS signaling cascade represents the next step in unraveling the molecular details of this developmental apoptotic program. In searching for candidate genes in the MIS signaling cascade, we noted that previous studies reported a strong correlation between Müllerian duct regression and degradation of extracellular matrix. Indeed, during duct regression early reports noted the marked degradation of the basement membrane as judged by discontinuous fibronectin and laminin immunostaining, and the close or physical contact between the mesenchymal and epithelial cell layers (Ikawa et al., 1984; Paranko et al., 1984; Trelstad et al., 1982). More recently, apoptotic Müllerian epithelial cells were shown to localize with disruptions in the laminin basement membrane (Allard et al., 2000). These previous studies are consistent with the hypothesis that extracellular proteases might promote apoptosis in Müllerian epithelial cells.

In this study, we have identified a member of the matrix metalloproteinases gene family as the first candidate target gene in the Müllerian mesenchyme which is activated by MIS signaling. Initial expression and activity profiles of protease activity in embryonic genital ridges suggested that one member of this family, MMP2, may participate in Müllerian duct regression. Furthermore, a genetic mouse strain lacking MIS was used to confirm the link between MMP2 expression and the MIS signaling cascade. Finally, in vivo experiments using a rodent genital ridge organ culture model system showed a strong correlation between pharmacological inhibition or activation of metalloproteinases and the presence or absence of Müllerian duct regression, respectively. Taken together, our results suggest that MMP2, and possibly other members of the MMP family, are crucial for Müllerian duct programmed cell death during urogenital development.

**MATERIALS AND METHODS**

**Mice**
MIS-null mice on a C57/B6 background were a generous gift from Dr Richard Behringer (M.D. Anderson Cancer Center), Dr Zena Werb (UCSF) and Dr Douglas Hanahan (UCSF) kindly provided MMP2-null mice on FVB and C57/B6 backgrounds. Outbred Swiss Webster mice (Simonsen) were used for studies of wild-type embryos.

**Zymography**
Conditioned medium and lysates from genital ridges were electrophoresed on 7.5% SDS-PAGE gels containing 1 mg/ml gelatin (type A, 300 bloom; Sigma) in the absence of reducing agents. Gels were renatured in two washes of 2.5% Triton X-100 (Sigma) with shaking, for 30 minutes each wash. Gels were developed in 50 mM Tris-HCl pH 7.6, 15 mM CaCl2 for 24-48 hours at 37°C with gentle rocking, then stained with Coomassie brilliant blue and air dried in cellophane (Bio-Rad). To inhibit MMP activity in zymograms, 1, 10 Phenanthroline (Sigma) was dissolved in ethanol and added to developing buffer at a final concentration of 10 mM.

**Organ cultures**
All organ culture experiments were performed on duplicate genital ridges in each experiment, and three independent experiments were performed (i.e., with three separate litters of embryos). Genital ridges from E14 or E15 (plug day=E0) Simonsen albino rats (Simonsen) were cultured on sections of MilliCell-CM membranes (Millipore) floating on 30 µl DMEM/F12 medium (Life Technologies) supplemented with penicillin/streptomycin, 10 µg/ml bovine insulin, 10 µg/ml human holo-transferrin (Sigma), and 10 nM testosterone (Sigma). 6His-tagged MIS was collected from the conditioned medium of stably transfected 293 cells as previously described (Roberts et al., 1999); the pro-hormone processing site mutant MIS-RARR was used to ensure efficient hormone processing by 293 cells (Nachtigal and Ingraham, 1996). 6His-tagged MIS was purified from conditioned medium using TALON metal affinity resin (Clontech), and stored at −80°C in the presence of 0.1% BSA. Purified MIS was added to organ cultures to 100 nM. This concentration is higher than that previously shown to cause Müllerian duct regression of 16 nM (Nachtigal and Ingraham, 1996), because MIS becomes unstable after purification on TALON resin and loses some bioactivity (L. M. R., unpublished observations). Organ culture medium was changed every 24 hours.

The following drugs were added to organ cultures as indicated in the text: 10 µM or 20 µM metalloproteinase inhibitor GM6001 and GM6001 negative control analog (Calbiochem; for detailed information on inhibitors and references, see www.calbiochem.com), 50 ng/ml ecotin (generous gift from Dr Charles Craik, UCSF), 200 µM phosphoramidon (Sigma), 200 µM caspase inhibitor Boc-D-FMK (caspase inhibitor III; Calbiochem), 50 µg/ml concanavalin A (Sigma), 100 µM leupeptin (Roche), 1 mg/ml aprotinin (Roche). For treatment of genital ridges with purified MMPs, 500 ng of recombinant active MMP2, MMP9, or stromelysin 1 was added directly to female genital ridges in the presence of 20 µM GM6001 or control analog for 24 hours. To allow better penetration of the tissue by enzymes, the surface of the Müllerian duct was punctured with a 27.5 gauge needle before application of MMPs. Control ridges were wounded in a similar manner; wounding did not significantly alter genital ridge morphology or cell death in organ cultures.

**TUNEL staining, in situ hybridization and immunostaining**
Genital ridges were fixed for TUNEL staining overnight at 4°C in 10% neutral buffered formalin, cryoprotected in 30% sucrose, embedded in OCT, and cut into 12 µm-thick sections. TUNEL staining was performed as previously described using the ApopTag FITC kit (Oncor) or In Situ Cell Death Detection Kit-AP (Roche) (Roberts et al., 1999a). Genital ridges were fixed for in situ hybridization and immunostaining overnight at 4°C in 4% paraformaldehyde, embedded and sectioned as above. In situ hybridization with digoxigenin-labeled RNA probes was performed as previously described (Roberts et al., 1999a). For immunostaining,
genital ridge sections were blocked for 30 minutes at room temperature in PBS with 10% normal goat serum and 0.2% Triton X-100 (Sigma). Sections were incubated overnight at 4°C with rabbit anti-laminin antibody (Sigma) diluted 1:500 in blocking buffer or rabbit anti-Pax2 antibody (BAbCo) diluted 1:150 in blocking buffer. Tissues were washed in PBS, and then incubated with goat anti-rabbit Cy3 secondary antibody (Cappel) at 1:200 in blocking buffer. Sections were washed in PBS, counterstained with 1 mg/ml Hoechst-33258 dye to visualize nuclei, and mounted with Fluoromount G (Southern Biotechnology Associates).

Morpholino antisense oligonucleotide treatment

Morpholino antisense oligonucleotides target the translational start site of messages to prevent translation of the targeted mRNA (Summerton and Weller, 1997). Special delivery morpholino oligonucleotide against rat MMP2 (5'-CTCCCCAGACCAATCGTG-CCTCCCAT-3') and control oligo containing four mismatched base pairs (5'-CTCgCCACgCAATCGTgGCTCgAT-3') were designed by GeneTools, LLC. Complete delivery solution containing 1.4 mM oligo was prepared according to the GeneTools protocol. Female genital ridges from early E14.5 embryos were submerged for 3 hours in delivery solution at 37°C, then placed on Millipore filters and cultured with MIS for 72 hours as described above. Data were analyzed by t-test (paired two sample for means).

RESULTS

The embryonic Müllerian duct mesenchyme in males expresses MMP2

As a first step in determining whether MMPs participate in Müllerian duct regression we surveyed MMP expression in the genital ridge by zymography; this technique will detect any proteinase capable of degrading standard substrates, such as gelatin or casein. Gelatin zymography of mouse genital ridge extracts revealed one major MMP activity that corresponded in size to MMP2 (gelatinase A) and was zinc dependent (Fig. 1A and data not shown). Additional MMPs were not detectable by casein zymography (data not shown) suggesting that MMP2 is the only major proteinase expressed in the genital ridge. The precise localization of MMP2 in mouse genital ridges was examined by in situ hybridization during a developmental stage when apoptosis is first observed in the regressing male Müllerian ducts. Whole-mount in situ hybridization revealed prominent MMP2 expression along the length of the Müllerian duct in embryonic day (E) 13 male mouse genital ridges compared to the low level, dispersed MMP2 expression observed in female genital ridges (Fig. 1B). Closer examination revealed that MMP2 expression is highly enriched in the mesenchyme surrounding the Müllerian duct, only in male genital ridges. In both male and female genital ridges MMP2 expression can also be found in the coelomic epithelium sheathing the genital ridge (Fig. 1C). Consistent with our earlier analysis by zymography, we were unable to detect the closely related metalloproteinase MMP9 (gelatinase B) by in situ hybridization (data not shown). Taken together, these data suggest that MMP2 is the major proteinase expressed in a sexually dimorphic pattern in the developing genital ridge, raising the possibility that MMP2 is involved in Müllerian duct regression.

MMP2 expression is regulated during Müllerian duct regression

MMP2 expression was examined during different stages of Müllerian duct regression and compared with expression of the MIS type II receptor. Before regression at E12, dispersed, low levels of MMP2 expression were found in both male and female genital ridges (data not shown). During the initial stages of regression in male ridges (E13), as evidenced by the first TUNEL-positive, or dying cells (L. M. R., unpublished
The dynamic and sexually dimorphic MMP2 expression profiles during duct regression raise the possibility that the MIS signaling cascade regulates MMP2 transcription. We therefore examined MMP2 expression in male mice disrupted in the Amh locus encoding MIS. Male mice lacking either of the two major determinants of the MIS signaling cascade, the MIS ligand or the type II receptor, develop with female reproductive tracts (Behringer et al., 1994; Mishina et al., 1996). However, reproductive tract development is normal in Amh+/− heterozygous embryos (Behringer et al., 1994). MMP2 expression was examined in both the Amh+/+ or Amh−/− male genital ridges. As predicted, similar expression patterns were observed in both wild-type and Amh+/− ridges. By contrast, MMP2 expression was virtually undetectable in Amh−/− male genital ridges at both E13 and E14 (Fig. 2B). Indeed, this expression closely resembled the pattern of MMP2 expression observed in wild-type female ridges, with expression detected only in the coelomic epithelium (Fig. 2B and refer back to Fig. 1C). Expression of the type II MIS receptor was unchanged in the Amh−/− background (data not shown). The loss of MMP2 expression in Amh−/− Müllerian mesenchyme tissue strongly suggests that MMP2 is a candidate gene downstream of the MIS signaling cascade.

**Metalloproteinase inhibitors block Müllerian duct regression**

To test the potential role of MMP2 in Müllerian duct regression, we used the rat genital ridge organ culture system to chemically activate or inhibit protease activity. Genital ridge organ explants can be maintained for several days, during which full Müllerian duct regression takes place in male ridges or in female ridges after chronic exposure to recombinant MIS. We first used a broad range inhibitor of metalloproteinases, GM6001. At doses known to inhibit the sub-family of MMPs, we observed full repression of MIS-induced Müllerian duct regression in female genital ridges. By contrast, the negative control analog of GM6001 had no effect (Fig. 3A; Table 1). In addition, GM6001 effectively blocked normal duct regression in male genital ridges to levels almost equivalent to that observed for the caspase inhibitor, Boc-D-FMK, which prevents apoptosis (Fig. 3A; Table 1). TUNEL analysis confirmed that GM6001 blocked MIS-induced apoptosis in treated female genital ridges (Fig. 3B).

We also tested additional protease inhibitors for their ability to block MIS-induced regression in female ridges. In surveying a number of other protease inhibitors, less robust effects were observed only with the general metalloproteinase inhibitor phosphoramidon and the potent serine protease inhibitor, ecotin (Table 1). Metalloproteinases have been shown to play an important role in cleaving inhibitory binding proteins from TGFβ-like ligands, to allow the ligands to signal through their receptors (reviewed by Smith, 1999). Therefore we tested whether inhibition of regression by GM6001 prevented propagation of MIS signal via its type I and type II receptors using the Tlx2 reporter system in P19 embryonic carcinoma cells (Visser et al., 2001). GM6001 had no effect on the ability of MIS to activate the Tlx2 reporter construct in P19 cells (Fig. 3C), implying that the obligate metalloproteinase activity in Müllerian duct regression lies downstream of MIS receptor(s) activation.

Current models of MMP2 activation propose that the
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membrane-type metalloproteinase 1 (MMP14/MT1-MMP) cleaves pro-MMP2 to its active mature form, as outlined in Fig. 4A (Sato et al., 1994). In this way, metalloproteinase inhibitors such as GM6001, and potentially the serine protease inhibitor ecotin, may block Müllerian duct regression indirectly by preventing the processing of pro-MMP2 to its active form. Consistent with this suggestion, we noted that the degree to which proteinase inhibitors prevented Müllerian duct regression correlated closely with the loss of mature MMP2 male genital ridges. For instance, ridges cultured with GM6001 showed almost no mature MMP2 as revealed by zymography, whereas some mature MMP2 was present with ecotin and phosphoramidon (Fig. 4B and data not shown). As expected, mature MMP2 was detected in male genital ridges treated with either the control GM6001 analog or the caspase inhibitor Boc-D-FMK, which would only effect the apoptotic machinery far downstream of the MIS signaling event (Fig. 4B).

MMP2 activation causes Müllerian duct regression

To test the hypothesis that MMP2 activation might trigger Müllerian duct regression we used the jack bean lectin concanavalin A (con A), to stimulate transcription of MMP14, as well as other MMPs (Atkinson et al., 1995; Overall and Sodek, 1990). An increase in MMP14 levels is predicted to activate latent MMP2, as outlined in Fig. 4A. Indeed, female genital ridges treated with con A showed a dramatic increase in the mature form of MMP2, which was almost completely abrogated by subsequent addition of the metalloproteinase inhibitor GM6001 (Fig. 4B). Remarkably, treatment of female genital ridges with con A resulted in complete Müllerian duct regression in the absence of MIS, while the neighboring Wolffian duct remained intact (Fig. 4C). Interestingly, the time course of con A-induced regression was rapid and was judged to be complete after 1 day of treatment compared with the 3 days normally required for MIS-induced regression. GM6001 prevented con A-induced regression (Fig. 4C). Because con A

Table 1. Effect of protease inhibitors on Müllerian duct regression

<table>
<thead>
<tr>
<th>Protease inhibitor</th>
<th>Target protease</th>
<th>% Regressed*</th>
<th>n†</th>
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<tbody>
<tr>
<td>Control analog</td>
<td>None</td>
<td>95</td>
<td>19</td>
</tr>
<tr>
<td>GM6001</td>
<td>Metalloproteinase</td>
<td>5</td>
<td>22</td>
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<tr>
<td>Phosphoramidon</td>
<td>Metalloproteinase</td>
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<td>8</td>
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<tr>
<td>Ecotin</td>
<td>Serine proteases</td>
<td>45</td>
<td>11</td>
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<tr>
<td>Aprotinin</td>
<td>Serine proteases</td>
<td>86</td>
<td>7</td>
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<tr>
<td>Leupeptin</td>
<td>Serine proteases</td>
<td>100</td>
<td>7</td>
</tr>
<tr>
<td>Boc-D-FMK</td>
<td>Caspases</td>
<td>0</td>
<td>9</td>
</tr>
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*Genital ridges with bo Müllerian duct.
†Total number of ridges tested. Experiments were repeated on 3-11 separate litters. Control analog and GM6001 were tested on male and female +MIS cultures; all other inhibitors were tested on males.
is capable of binding surface glycoproteins, we investigated whether con A could activate MIS receptors in the absence of MIS in our cellular Tlx-2 reporter system, as described above. Con A had no effect on MIS receptor signaling (Fig. 4D). The strong correlation between the ability of proteinase inhibitors or activators to inhibit or activate Müllerian duct regression, suggested that MIS signaling and MMP activation are closely linked.

**Exogenous MMP2 causes apoptosis in the genital ridge**

If MMP2 activation is downstream of MIS-induced regression, it follows that MMP2 should trigger apoptosis within the Müllerian duct. Indeed, widespread cell death was observed in Müllerian ducts of female ridges cultured with activated MMP2, as indicated by condensed nuclei (Fig. 5A) and TUNEL staining (data not shown). Cell death was also noted in the Wolffian duct and the genital ridge mesenchyme (Fig. 5A), but was not present in control ridges. Staining for Pax2 protein, which marks both the Müllerian and Wolffian epithelia (Torres et al., 1995), revealed the complete destruction of the Müllerian duct in some MMP2-treated genital ridges (Fig. 5A). GM6001, but not its control analog, attenuated MMP2-induced apoptosis as illustrated by an intact Müllerian duct (Fig. 5A). These data demonstrate that active MMP2 promotes apoptosis in genital ridges. To test whether this pro-apoptotic activity is specific to MMP2, we tested the ability of two other MMPs, MMP3 (stromelysin 1) and MMP9 (gelatinase B), to cause death in organ culture. Activated MMP9 and the catalytic domain of MMP3, both caused death in genital ridges (data not shown), indicating that the ability of MMP2 to cause death in genital ridge is shared by other matrix metalloproteinases.

**Antisense oligonucleotides against MMP2 interfere with Müllerian duct regression**

The inhibition of Müllerian duct regression by GM6001 demonstrates that MMP activity is essential for Müllerian duct regression, but this inhibitor blocks a wide range of metalloproteinases. To test if MMP2 is required for Müllerian duct regression, we treated female genital ridges in organ culture with MIS and antisense MMP2 morpholino oligonucleotides. Previous studies have shown that morpholino oligonucleotides penetrate the genital ridge and work successfully to block MIS-induced regression when they are directed against the type I receptor, ALK2 (Visser et al., 2001). When genital ridges were treated with antisense MMP2 oligonucleotides we observed partial blockage of MIS-induced regression compared to ridges treated with the control MMP2 oligonucleotide containing four mismatches (Fig. 5B). Furthermore, Pax2 staining of these MIS-treated ridges revealed much higher numbers of Müllerian duct remnants in cultures treated with antisense MMP2 than in cultures treated with control oligonucleotides (Fig. 5B.C). These results provide strong evidence for the requirement of MMP2 in Müllerian duct regression.
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DISCUSSION

Epithelial-mesenchymal signaling underlies many morphogenetic events during development and in particular, plays a critical role in male reproductive tract development. We have identified a member of the matrix metalloproteinase gene family, MMP2, as the first candidate gene regulated by the MIS signaling cascade during Müllerian duct regression. Our data show that MMP2 expression is dependent on an intact MIS signaling system and is upregulated in the male Müllerian mesenchyme during duct regression. In addition, we find that inhibition of MMP2 activity blocks MIS-induced duct regression. Use of MMP2 antisense oligonucleotides in genital ridge organ cultures also sharply attenuated regression. Conversely, gain-of-function studies show that exogenous MMP2 or activation of MMP2 bypasses the requirement for MIS for Müllerian duct apoptosis and regression. Collectively, our study suggests that MIS signaling regulates MMP2 expression, and that MMP2 acts downstream of MIS to cause apoptosis in the epithelium of the duct.

MMP2 is a regulatory target of MIS signaling

As a candidate target gene activated by the MIS signaling cascade, MMP2 is ideal as a paracrine factor because it is secreted by the Müllerian mesenchyme. Whether MMP2 is activated directly by downstream effectors of the MIS/ALK2 receptor complex, such as Smad1 or 5, is unclear at this time. It is worth noting that multiple Smad binding sites reside in both the proximal and distal MMP2 promoter (Harendza et al., 1995). However, our efforts to activate Mmp2 reporter constructs using an activated ALK2 receptor, which bypasses the requirement for the MIS ligand, consistently yielded only a modest induction of reporter activity (1.5- to 2-fold, data not shown). Although it is difficult to provide the precise link between MIS signaling and MMP2 transcription at this time, three lines of evidence lead us to conclude that MMP2 acts downstream of the MIS signaling event and upstream of epithelial apoptosis. First, MIS-induced signaling in our cellular assay was unchanged under conditions that either blocked or promoted MMP2 activity. Second, MMP2 expression is concomitant with the first TUNEL-positive cells observed in the dying epithelial duct. Third, whereas MMP2
inhibitors blocked both enzymatic activity and duct regression, caspase inhibitors failed to inhibit MMP2 activity, but did block regression; these data imply that caspase inhibitors prevent regression at much later stages of Müllerian duct apoptosis than those affected by MMP inhibitors.

That concanavalin A can specifically induce Müllerian duct regression while leaving other genital ridge structures intact, such as the Wolffian duct, is rather remarkable. This result infers indirectly that the elimination of the Wolffian duct in the female embryo utilizes a very different pathway from Müllerian duct involution. Alternatively, con A may trigger regression in the Müllerian duct only, because of high localized concentrations of MMP2 expression in the coelomic epithelium immediately adjacent to the Müllerian duct. Addition of con A to female genital ridges presumably activates latent MMP2, which predominates in the female genital ridge (Fig. 4B, female no treatment). By contrast, the absence of available pro-MMP2 in the vicinity of Wolffian duct may provide an explanation for the apparent selectivity of con A in Müllerian duct regression. Consistent with this hypothesis is the more global induction of cell death in female genital ridges after addition of activated MMP2 protein (see Fig. 5A), compared with the precise ablation of Müllerian duct structures after con A treatment. Clearly, the highly localized expression of MMP2 in the male Müllerian mesenchyme offer a plausible explanation for the selective apoptosis of the Müllerian duct in males.

MMP substrates in the embryonic genital ridge

One would like to know how MMP2 promotes duct regression. Does MMP2 cleave a substrate present on the epithelial cell surface or modify signaling molecule(s) secreted from the Müllerian mesenchyme (as schematized in Fig. 6)? Other developmental programs provide the best insight into how MMPs might mediate programmed cell death. Similar to Müllerian duct regression, elevated MMP activity has been implicated in both mammary gland involution (reviewed by Wiesen and Werb, 2000) and amphibian tadpole metamorphosis (Berry et al., 1998; Ishizuya-Oka et al., 2000; Su et al., 1997). Evidence from these developmental systems suggests that increased MMP activity leads to degradation of the extracellular matrix (ECM) and the loss of this structural support triggers apoptosis (Frisch and Francis, 1994; Meredith et al., 1993). Interestingly, tadpole intestinal epithelial death induced by the thyroid hormone, T3, can be reversed by addition of ECM (Su et al., 1997). During Müllerian duct regression, the localized degradation of the extracellular matrix, as evidenced by the disruption in laminin staining, may be sufficient to trigger epithelial apoptosis. Similarly, laminin degradation is also associated with hippocampal neuronal cell death induced by excitotoxicity (Chen and Strickland, 1997). In hippocampus, anti-laminin antibodies can bypass proteinases and sensitize neurons to cell death, raising the possibility that laminin could be the primary target of MMP2 during Müllerian duct regression. How the detachment of epithelial cells from the ECM causes apoptosis is still unclear, although death domain-containing receptors and caspase 8 have been implicated (Frisch, 1999; Rytoma et al., 1999). It will be of interest to examine the components of this pathway more fully in the genital ridge to determine their potential involvement in Müllerian duct regression.

MMP2 may cause apoptosis by degrading a secreted survival factor, or by proteolytically activating a secreted death factor (see Fig. 6). There are numerous potential substrates in addition to extracellular matrix that localized MMP2 activity might affect in Müllerian duct regression. These include cleavage of proteins to small active peptides, cleavage that releases soluble factors from inhibitory binding proteins and finally, cleavage that converts a membrane-bound factor to a soluble form (reviewed by Vu and Werb, 2000). Given the importance of Wnt4 and Wnt7a in reproductive tract development, it is also possible that MMP2 activity targets components of these signaling pathways and may indirectly influence the modest increase in the subcellular relocalization of β-catenin to the nucleus during Müllerian duct regression (Allard et al., 2000).

MMP2 and other proteinases in Müllerian duct regression

While our expression studies and in vitro studies prompt us to focus on MMP2 as a downstream target of MIS signaling during Müllerian duct regression, we are unable to exclude a critical role for other proteinases during this developmental process. Indeed, our findings that a serine proteinase inhibitor can partially block MIS-induced regression would suggest that other classes of proteinases are likely to be involved. Our studies showing an attenuation of MIS-induced regression following treatment with MMP2 antisense oligonucleotides provides the strongest evidence that MMP2 participates in Müllerian duct regression. However, the inability to block MIS-induced regression completely with MMP2 antisense oligonucleotides,
which is possible using an ALK2 (MIS type I receptor) antisense strategy, indicates that additional proteases are involved. We have also noted normal urogenital development in Mmp2−/− mice (data not shown). These data imply that MMP2 may be insufficient for regression or that functional redundancy by this large gene family occurs during development. Recent genetic evidence supports the later possibility, at least in mice. Whereas Mmp2−/− mice appear to be normal in all respects, humans possessing homozygous inactivating mutations in Mmp2 suffer from arthritis and osteoporosis as a result of Vanishing Bone Syndrome (Itoh et al., 1997; Martignetti et al., 2001). The normal appearance of Mmp2-null mice would not have been predicted given the widespread expression of MMP2 throughout development. Interestingly, Mmp14-null mice do exhibit a similar phenotype to MMP2 human mutants; these mice display severe impairment of bone and cartilage development consistent with the fact that MMP14 activates the MMP2 enzyme (Zhou et al., 2000). The discovery of a dramatic phenotype in humans lacking MMP2 activity, but not in MMP2-null mice, suggests that in mice, other gene products compensate for the loss of MMP2. Given that humans and mice differ in their requirement for MMP2 function in bone homeostasis, a similar species difference may account for the observation that anti-MMP2 antisense oligonucleotides partially block regression in rat genital ridges, while Mullerian duct regression occurs normally in MMP2-null mice. In addition to activating MMP2, MMP14 shares some substrate specificity with MMP2 (Ohuchi et al., 1997), suggesting that MMP14 activity may compensate for loss of MMP2. While we have detected MMP14 expression throughout the genital ridge by in situ hybridization, preliminary experiments failed to reveal any obvious sexual dimorphism in MMP14 message levels, or regulation of MMP14 mRNA by MIS in the Mullerian duct (data not shown). It remains to be determined whether MIS regulates MMP14 post-transcriptionally, and if MMP14 can compensate for loss of MMP2 in Mullerian duct regression.

Future studies aimed at identifying additional target genes of the MIS signaling cascade should shed light on the potential role of other proteases in duct regression. Identifying relevant substrates of MMP2 in the genital ridge is also critical for understanding the molecular details of Mullerian duct regression, despite the fact that searching for proteolytic substrates continues to be technically challenging. Even so, such knowledge is likely to provide real insight into reproductive tract development, as well as other developmental programs where selective elimination or remodeling of tissues occurs.

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