

Interaction of the Vitamin D Receptor with a Vitamin D Response Element in the Müllerian-Inhibiting Substance (MIS) Promoter: Regulation of MIS Expression by Calcitriol in Prostate Cancer Cells

Peter J. Malloy, Lihong Peng, Jining Wang, and David Feldman

Division of Endocrinology, Gerontology, and Metabolism, Department of Medicine, Stanford University School of Medicine, Stanford, California 94305

Calcitriol (1,25-dihydroxyvitamin D₃) inhibits the growth of a variety of cancer cells including human prostate cancer. Müllerian-inhibiting substance (MIS) also exhibits antiproliferative and proapoptotic actions on multiple cancer cells including human prostate cancer. In this study, we investigated whether calcitriol regulated MIS expression in prostate cancer, an action that might contribute to its antiproliferative activity. We identified a 15-bp sequence, GGGTGAgcaGGGACA, in the MIS promoter that was highly similar to direct repeat 3-type vitamin D response elements (VDREs). The human MIS promoter containing the putative VDRE was cloned into a luciferase reporter vector. In HeLa cells transfected with the vitamin D receptor (VDR), MIS promoter activity was stimulated by calcitriol. Coexpression of steroidogenic factor 1, a key regulator of MIS, increased basal MIS promoter activity that was further stimulated by calcitriol. Mutation or deletion of the VDRE reduced calcitriol-induced transactivation. In addition, the MIS VDRE conferred calcitriol responsiveness to a heterologous promoter. In gel shift assays, VDR and retinoid X receptor bound to the MIS VDRE and the binding was increased by calcitriol. Chromatin immunoprecipitation assays showed that VDR and retinoid X receptor were present on the MIS promoter in prostate cancer cells. In conclusion, we demonstrated that MIS is a target of calcitriol action. MIS is up-regulated by calcitriol via a functional VDRE that binds the VDR. Up-regulation of MIS by calcitriol may be an important component of the antiproliferative actions of calcitriol in some cancers. (*Endocrinology* 150: 1580–1587, 2009)

The classical actions of calcitriol include the regulation of calcium and phosphate metabolism, actions that determine the quality of bone mineralization. These classical calcitriol actions prevent rickets in children and osteomalacia in adults as well as play a role in the prevention of osteoporosis (1). The biological actions of calcitriol are mediated by the vitamin D receptor (VDR), a member of the steroid-thyroid-retinoid receptor superfamily of ligand activated transcription factors. Studies in VDR knockout mice (2, 3) and hereditary vitamin D-resistant rickets (HVDRR) in humans (4, 5) revealed multiple biological consequences of VDR signaling. More recently it has been recognized that calcitriol has a much wider range of actions that include prodifferentiation, antiproliferation, proapoptosis,

immunosuppression, and antiinflammation (1, 6). These actions have led to potential uses of calcitriol and less calcemic calcitriol analogs in the treatment of diseases such as osteoporosis, cancer, immunological diseases, diabetes, infection, and psoriasis among others (7).

Müllerian-inhibiting substance (MIS; also known as anti-Müllerian hormone) is a member of the TGF β superfamily that also includes activins, inhibins, and bone morphogenetic proteins (8). MIS is a glycoprotein that is secreted by Sertoli cells in testis and granulosa cells in the ovary. MIS binds to the MIS type II receptor (MISRII), a transmembrane serine threonine kinase, and recruits the type I membrane receptor activin A receptor, type II-like kinase 2 to initiate downstream signaling (9–11). In

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Abbreviations: BSCL, Berardinelli-Seip-type congenital lipodystrophy; ChIP, chromatin immunoprecipitation; HVDRR, hereditary vitamin D-resistant rickets; MIS, Müllerian-inhibiting substance; MISRII, Müllerian-inhibiting substance type II receptor; PCOS, polycystic ovarian syndrome; PMDS, persistent Müllerian duct syndrome; RXR, retinoid X receptor; SF-1, steroidogenic factor 1; VDR, vitamin D receptor, VDRE, vitamin D response element; WT, wild type.

developing male embryos, MIS initiates the regression of the Müllerian ducts that in a normal female embryo develop into the uterus, fallopian tubes, and upper vagina (12). Other roles for MIS have also been demonstrated: in Leydig cells in which MIS inhibits steroidogenesis (13, 14) and in the postnatal ovary in which MIS plays a role in follicle recruitment (15, 16).

Importantly, the growth of breast, cervical, endometrial, ovarian, and prostate cancer cells that express MISRII have been shown to be inhibited by MIS (17–24). In breast and prostate cancer cells, MIS up-regulates the immediate early gene 3 (*IER3/IEX-1S*) through an nuclear factor- κ B-dependent mechanism (20, 23, 24). In breast cancer cells, overexpression of *IER3* has been shown to inhibit cell growth (24). Furthermore, inhibition of prostate cancer cell growth by MIS was abolished by dominant-negative inhibitory- κ B α (*I κ B*), demonstrating that the growth-inhibitory action of MIS is mediated by nuclear factor- κ B (NF- κ B) in prostate (23). Recently we have shown that the MIS gene is up-regulated by calcitriol in prostate cancer cells (6).

In this report, we showed that the MIS promoter contains a functional vitamin D response element (VDRE) and its expression is regulated by calcitriol. Our findings demonstrate that MIS is a newly discovered direct target of the VDR that may have important implications in the anticancer activity of calcitriol.

Materials and Methods

Cell culture

HeLa cells were grown in DMEM containing 10% fetal bovine serum. COS-7 cells were grown in DMEM containing 10% bovine growth serum (Hyclone, Logan, UT). LNCaP and PC-3 prostate cancer cells were grown in RPMI 1640 containing 5% fetal bovine serum. Cells were incubated at 37 C in 5% CO₂. Cells were obtained from the American Type Culture Collection (Manassas, VA).

Promoter constructs

The human MIS promoter sequence between –657 and +23 (relative to the translational starting point at +1) was amplified by PCR using genomic DNA and oligonucleotide primers designed with *Mlu*I restriction sites. The amplified product was cloned into the *Mlu*I site in the promoter-less luciferase reporter vector, pGL3-basic (Promega, Madison, WI). The sequence was verified by sequencing. A single-point mutation in the putative VDRE sequence was constructed using the GenEditor site-directed mutagenesis kit (Promega). Deletion of the entire 15-bp VDRE sequence was performed by using a 40-base oligonucleotide primer that hybridized to 20 bases on either side of the 15-bp VDRE sequence using the GenEditor kit. All clones were verified by sequencing. The heterologous MIS VDRE reporter was constructed by insertion of four copies of the MIS VDRE into the *Mlu*I site in pGL3-promoter luciferase reporter vector (Promega).

Gel mobility shift assay

Gel mobility shift assay was used to analyze VDR binding to the putative VDRE in the MIS promoter using complementary oligonucleotides for the MIS VDRE. COS-7 cell extracts overexpressing the VDR were incubated with 50 nM calcitriol before the addition of the [³²P]-labeled MIS VDRE probe followed by electrophoresis on nondenaturing gels and autoradiography as previously described (25).

Transactivation assays

The MIS promoter-luciferase plasmids were transfected into HeLa cells using HeLa Monster (Mirus Bio Corp., Madison, WI). Cells were

also cotransfected with pSG5-VDR and a control plasmid pRLnull to control for transfection efficiency. The transfected cells were treated with calcitriol for 24 h. Luciferase activity was determined using the dual-luciferase assay (Promega) and a Turner luminometer. In some experiments, an expression plasmid for steroidogenic factor 1 (SF-1) was cotransfected and the luciferase activity measured after calcitriol treatment.

Quantitative real-time PCR

LNCaP and PC-3 prostate cancer cells were treated with vehicle and graded concentrations of 1,25(OH)₂D₃ (in ethanol) for 6 h in medium containing 1% fetal calf serum. RNAs were isolated using RNeasy spin columns (QIAGEN, Valencia, CA). cDNA was prepared by reverse transcription using superscript III cDNA synthesis kit (Invitrogen, Carlsbad, CA). MIS (upper primer, 5'-CCTGGTGTAGCGGTGGAC and lower primer, 5'-GTCTCCAGGAAGGGGTCTG) and TBP (upper primer, 5'-TGCTGAGAAGAGTGTGCTGGAG and lower primer, 5'-TCTGATAGGCTGTGGGGTC) genes were then amplified from the cDNA using SYBR-green quantitative PCR kit (New England Biolabs, Ipswich, MA) and semiquantified using real-time PCR. Experiments were performed at least three times with duplicate determinations.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using the SimpleChIP enzymatic ChIP kit as described by the manufacturer (Cell Signaling Technology, Danvers, MA). In brief, LNCaP and PC-3 cells were treated with 100 nM calcitriol for 6 h and cross-linked by addition of 1% formaldehyde. Chromatin was prepared and digested with micrococcal nuclease for 12 min at 37 C. ChIPs were performed with rabbit anti-VDR polyclonal antibody (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit antiretinoid X receptor (RXR) polyclonal antibody Δ N197 (Santa Cruz Biotechnology), histone H3 antibody, and normal rabbit IgG. Antibodies were added to the chromatin digests and incubated with constant rotation overnight at 4 C. ChIP-grade protein G magnetic beads were added to capture the immune complexes. The beads were washed and the immunoprecipitates eluted with ChIP elution buffer. The cross-links were reversed by incubation at 65 C for 30 min. Proteinase K was added and incubated at 65 C for 2 h. The immunoprecipitated DNA fragments were then purified using spin columns. PCR was performed using primers MIS-2199867-U (5'-TGTTCTGGGGAGGGAAGAGCAGAC) and MIS-2200058-L (5'-ACGGCCAGGCAACCAACTAGACA) flanking the MIS VDRE and producing a 191-bp PCR product. Control primers 1664 bp upstream of the VDRE were MIS-2198069-U (5'-AGAAGGATCTGCATAGGCTGGGCA) and MIS-2198287-L (5'-TGAGGTCACTTGGCAAAGGCCTCA) and producing a 218-bp PCR product.

Statistical analyses

Transient transfections were performed in triplicate, and each experiment was repeated at least three times. The data were analyzed by the Student's *t* test and significant differences were designated as *P* < 0.05.

Results

The human MIS promoter is contained within a 789-bp sequence between the end of the SF3A2 gene and the start of the MIS coding sequence (26) (Fig. 1A). To determine whether putative VDREs were present in the MIS promoter, we used an *in silico*-based method to scan the promoter region of the MIS gene. We first analyzed the entire MIS promoter for VDREs using the Genetics Computer Group (Madison, WI) MAP program and a transcription factor database. The program identified a single VDRE-like sequence in the MIS promoter. As shown in Fig. 1B, the putative VDRE was located at –395 to –381 relative to the MIS translational start site. The putative VDRE sequence was

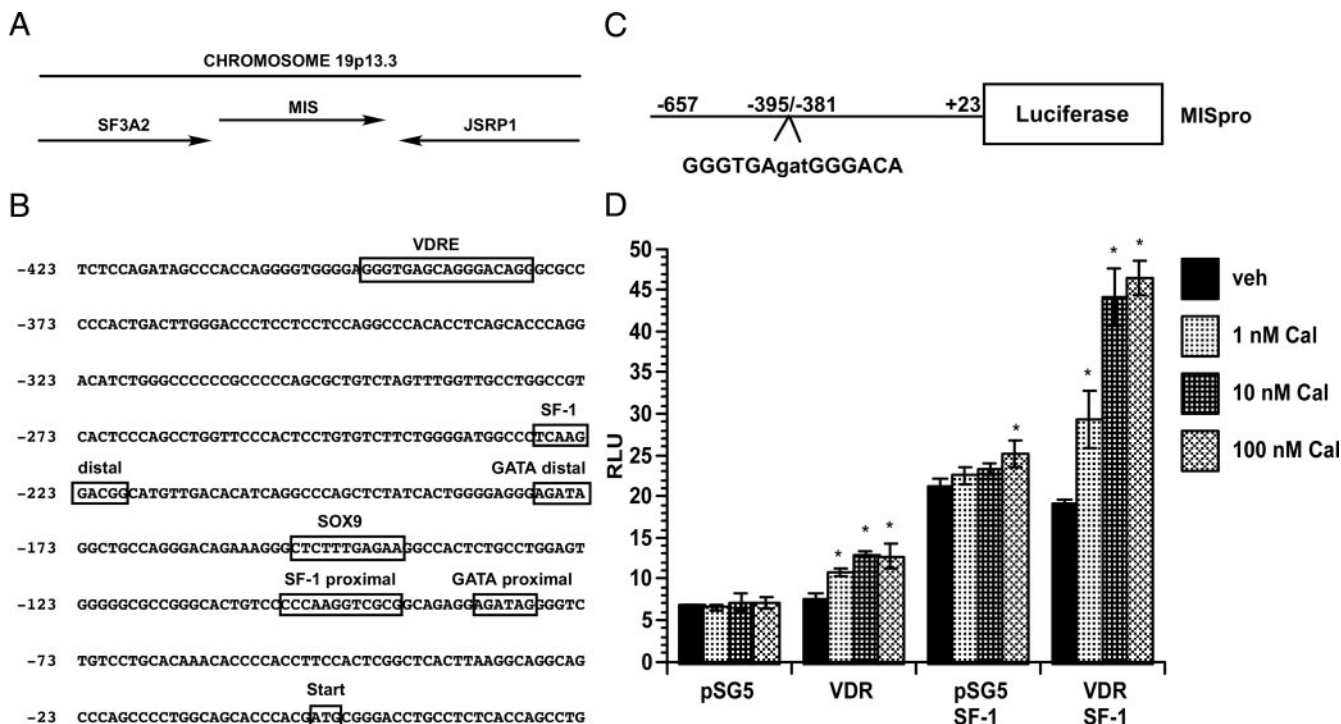


FIG. 1. The human MIS promoter containing a putative VDRE is activated by calcitriol, and SF-1 and VDR cooperate to increase MIS promoter activity in response to calcitriol. **A**, The human MIS gene on chromosome 19 is located between the SF3A2 gene and the JSRP1 gene. The MIS transcriptional start site is located only 748 bp downstream of the termination codon of the SF3A2 gene. Arrows indicate direction of transcription. **B**, Using *in silico* analysis, we identified a putative VDRE in the MIS promoter. The VDRE is located at nucleotides –381 to –396 relative to the ATG translation start site. The location of transcription factor binding sites for SF-1, Sry-type high-mobility-group box transcription factor (SOX)-9 and GATA-4 that regulate MIS promoter activity are also shown. **C**, A 680-bp fragment (–657 to +23) of the MIS promoter was cloned into the promoterless luciferase reporter vector pGL3-basic to generate the MIS promoter reporter construct (MISpro). **D**, Transactivation assays in HeLa cells transfected with the pSG5 vector without an insert or pSG5-VDR expression vector and the MIS promoter luciferase reporter construct or the combination of VDR and SF-1 expression vectors and the MIS promoter-luciferase construct. Cells were treated with vehicle or calcitriol (Cal) for 24 h. Luciferase activity was measured using the dual-luciferase assay. Shown is a representative experiment of at least three independent experiments. Values represent mean ± so of triplicate transfections. *, Calcitriol treatment significantly different from the vehicle-treated control.

located upstream of known transcription factor binding sites for SF-1, Sry-type high-mobility-group box transcription factor-9 (SOXa), and GATA-4 (Fig. 1B). The MIS VDRE exhibits a direct repeat 3 motif containing two hexameric sequences separated by a 3-bp spacer that is highly similar to several previously characterized VDREs. As shown in Table 1, the MIS VDRE is highly homologous to both the human and rat osteocalcin VDREs. The MIS VDRE and the human osteocalcin VDRE differ by only one nucleotide base in the 3-prime hexamer (Table 1).

Having identified a putative VDRE in the MIS promoter, we amplified a 680-bp DNA fragment from –657 to +23 containing the sequence and cloned it into the pGL3-basic luciferase reporter plasmid (Fig. 1C). We then transfected HeLa cells with the WT VDR cDNA expression vector and the MIS promoter reporter plasmid and examined transactivation after treatment

with calcitriol. As shown in Fig. 1D, in HeLa cells transiently transfected with the wild-type (WT) VDR, calcitriol treatment induced a dose-dependent increase in luciferase activity. At 100 nM calcitriol, there was an approximately 1.8-fold increase in luciferase activity *vs.* vehicle-treated cells. In cells transfected with the pSG5 vector control, calcitriol treatment failed to stimulate luciferase activity. These results demonstrated that the MIS promoter responds to calcitriol treatment.

Because SF-1 is a known major regulator of MIS promoter activity (27), we next determined the effects of coexpression of SF-1 and VDR on MIS promoter activity. As shown in Fig. 1D, in the absence of calcitriol, the basal MIS promoter activity was increased approximately 2.5-fold by transfection of SF-1 alone compared with transfection of VDR alone. A slight but significant increase in promoter activity was observed when the cells were treated

TABLE 1. Comparison of the human MIS VDRE and several previously described VDREs

| Gene | Location | Sequence |
|-------------------|----------------------|-------------------|
| Human MIS | –395/–381 | GGGTGA gca GGGACA |
| Human osteocalcin | –499/–485 | GGGTGA acg GGGGCA |
| Rat osteocalcin | –460/–446 | GGGTGA atg AGGACA |
| Human CYP24A1 | –169/–155 (proximal) | AGGTGA gcg AGGGCG |
| Human CYP24A1 | –291/–277 (distal) | AGTTCA ccg GGTGTG |
| Human IGFBP-3 | –3296/–3282 | GGTTCA ccg GGTGCA |

IGFBP, IGF binding protein.

with 100 nM calcitriol that was most likely due to activation of endogenous VDR. In the presence of both SF-1 and VDR, calcitriol stimulated a dose-dependent increase in MIS promoter activity. At 100 nM calcitriol, there was an approximately 2.4-fold increase in MIS promoter activity compared with vehicle control (Fig. 1D). The contribution of calcitriol and SF-1 gave a 7-fold increase in transactivation over basal and compared with the 2.5-fold increase by SF-1 alone. These results demonstrated that VDR and SF-1 cooperate to stimulate MIS promoter activity and that calcitriol is essential for maximum activity.

To confirm that the induction of the MIS promoter by calcitriol was mediated via the putative VDRE sequence, we constructed two mutations in the MIS VDRE (Fig. 2A). In one mutant, the 3-prime hexamer sequence GGGACA was mutated to GTGACA (MISpro mut1). In the second mutant, the entire 15-base GGGTGAgcaGGGACA VDRE sequence was deleted (MISpro Δ VDRE). As shown in Fig. 2B, in HeLa cells cotransfected with VDR and SF-1, calcitriol induced a dose-dependent increase in WT MIS promoter activity. On the other hand, the single-point mutation significantly reduced calcitriol-induced transactivation when compared with WT MIS promoter. Furthermore, deletion of the MIS VDRE sequence abolished transactivation by calcitriol. These results demonstrated that the VDR stimulated MIS promoter activity through the MIS VDRE sequence.

To further demonstrate that the VDRE sequence specifically responds to calcitriol, four copies of the 15-bp VDRE sequence

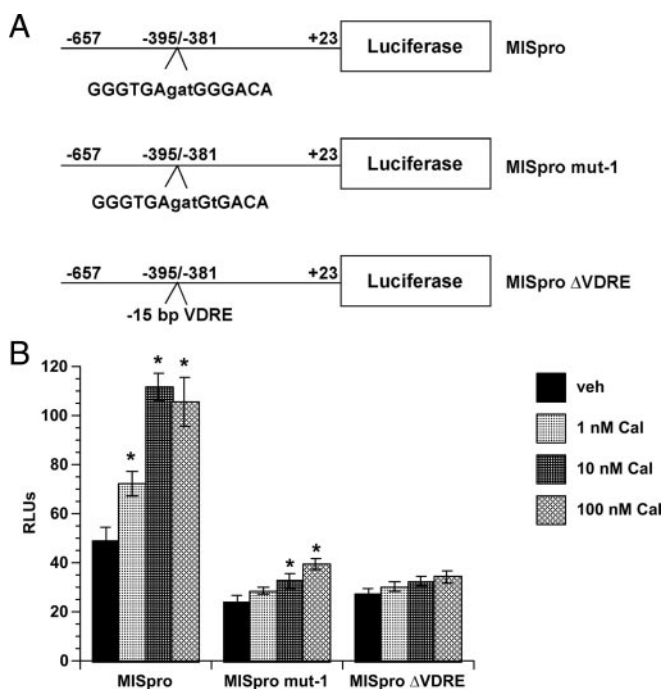


FIG. 2. Mutations in the MIS VDRE reduce calcitriol responsiveness. A, A single G-to-T point mutation in the 3-prime hexamer of the VDRE (MISpro mut-1) and a 15-bp deletion of the entire VDRE (MISpro Δ VDRE) were created in the MIS promoter-luciferase construct (MISpro). B, Transactivation assays in HeLa cells transfected with VDR and SF-1 expression vectors and the MIS promoter luciferase constructs. Cells were treated with vehicle (veh) or calcitriol (cal) for 24 h and luciferase activity measured. Shown is a representative experiment of at least three independent experiments. Values represent mean \pm SD of triplicate transfections. *, Calcitriol treatment significantly different from the vehicle-treated control. RLU, Relative luciferase units.

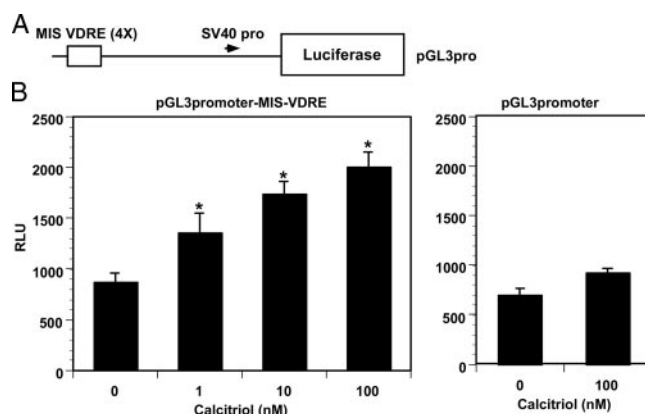


FIG. 3. The MIS VDRE confers calcitriol responsiveness to a heterologous promoter. A, Four copies of the 15-bp MIS VDRE were cloned upstream of the Simian virus 40 (SV40) promoter in the reporter vector pGL3promoter (pGL3pro). B, Transactivation assays in HeLa cells transfected with a VDR expression vector and the MIS VDRE (4X)-pGL3pro luciferase reporter construct. Cells were treated with vehicle or calcitriol for 24 h and luciferase activity measured. Shown is a representative experiment of at least three independent experiments. Values represent mean \pm SD of triplicate transfections. *, Calcitriol treatment significantly different from the vehicle-treated control. RLU, Relative luciferase units.

were cloned into pGL3-promoter, a heterologous promoter luciferase reporter vector containing a Simian virus 40 promoter (Fig. 3A). As shown in Fig. 3B, when HeLa cells transfected with the MIS VDRE-pGL3-promoter construct were treated with calcitriol, a dose-dependent increase in transactivation activity up to 2-fold was observed. HeLa cells transfected with the pGL3-promoter vector without an insert exhibited no calcitriol-induced increase in transactivation (Fig. 3C). These results demonstrated that the 15-bp MIS VDRE sequence conferred calcitriol responsiveness to a heterologous promoter.

We next determined whether the VDR binds directly to the MIS VDRE *in vitro* using gel shift assays (Fig. 4). For these assays, we used COS-7 cells that express endogenous RXR but only a small amount of VDR (28). As shown in Fig. 4, when extracts from COS-7 cells transfected with the pSG5 vector alone were incubated with the radiolabeled MIS VDRE probe, only a minor band shift was detected (Fig. 4, lane 2). When extracts of COS-7 cells that were transfected with the VDR WT cDNA expression vector in the absence of calcitriol, a band shift was detected with the MIS VDRE sequence (lane 3). Addition of calcitriol further increased the band intensity (Fig. 4, lane 4). Competition with unlabeled MIS VDRE WT sequence showed a decrease in band intensity (Fig. 4, lanes 7–9), whereas competition with a mutant MIS VDRE with mutations in the 5-prime and 3-prime hexamers (GtGTGAgcaGtGACA, MIS VDRE mut2) failed to reduce the complex (Fig. 4, lanes 10–12). Addition of VDR antibody 9A7 blocked the formation of the complex (Fig. 4, lane 14), whereas addition of RXR antibody decreased the band intensity and supershifted some of the complex (Fig. 4, lane 17) demonstrating that VDR and RXR were bound to the MIS VDRE sequence.

We have previously shown that calcitriol induces MIS gene expression in LNCaP and PC-3 human prostate cancer cells and the human primary prostate cancer cell strain JBEpz (6). Treatment of LNCaP and PC-3 cells with graded concentrations of

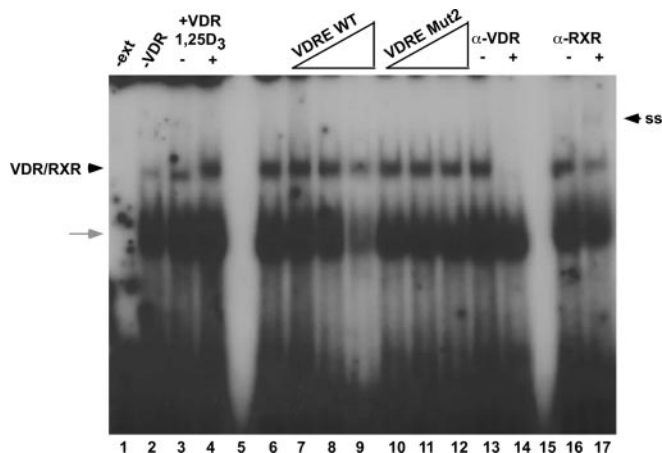


FIG. 4. The VDR binds to the MIS VDRE *in vitro*. A, [³²P]-labeled MIS VDRE was incubated with COS-7 extracts transfected with vector (pSG5) or pSG5-VDR. Lane 1, No extract; lane 2, pSG5 vector alone; lane 3, pSG5-VDR minus calcitriol; lane 4, pSG5-VDR plus 50 nM calcitriol. The remaining samples (lanes 5–17) all contained [³²P]-labeled MIS VDRE with pSG5-VDR plus 50 nM calcitriol. Lane 5, pSG5-VDR plus 50 nM calcitriol; lanes 6–9 contained increasing concentrations of unlabeled WT MIS VDRE; lanes 10–12 contained increasing concentrations of unlabeled MIS VDRE mut-2; lane 13, minus VDR antibody; lane 14 plus VDR antibody 9A7; lane 16, minus RXR antibody; lane 17, plus RXR antibody. The samples were electrophoresed on 5% polyacrylamide gels in 0.5 × Tris-borate buffer. Bands were visualized by autoradiography. Arrowhead indicates VDR-RXR complex, gray arrow indicates undetermined complex. ss, RXR antibody supershift complex.

calcitriol for 6 h resulted in a dose-dependent increase in MIS transcription (Fig. 5).

We then examined the presence of the VDR on the MIS promoter in intact prostate cancer cells using ChIP assays. LNCaP cells were treated with vehicle or 100 nM calcitriol for 6 h before ChIP assay. ChIP assays showed that in the absence of calcitriol both VDR and RXR were present on the MIS promoter (Fig. 6, upper panel, lanes 5 and 6). In the presence of calcitriol, there was no significant increase in VDR or RXR binding to the MIS promoter (Fig. 6, upper panel, lanes 10 and 11). Histone H3 was also present on the MIS promoter in both the absence and presence of calcitriol. No bands were detected with the IgG control antibody. As a negative control, we amplified a region about 1.6 kb upstream of the MIS translational start site (Fig. 6, lower panel). Only histone H3 was detected on the negative control sequence.

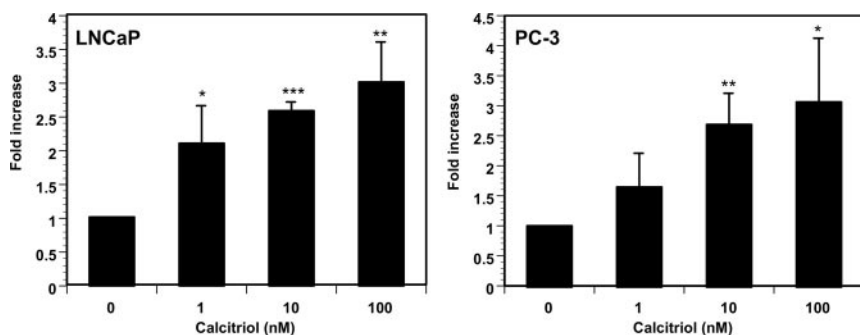


FIG. 5. The MIS gene is up-regulated by calcitriol in prostate cancer cells. LNCaP and PC-3 cells were treated with graded concentrations of calcitriol for 6 h. MIS gene expression was then analyzed by quantitative RT-PCR. Values represent mean ± SD of triplicate experiments. Asterisks indicate calcitriol treatment significantly different from the vehicle-treated control. *, *P* < 0.05; **, *P* < 0.005; ***, *P* < 0.001.

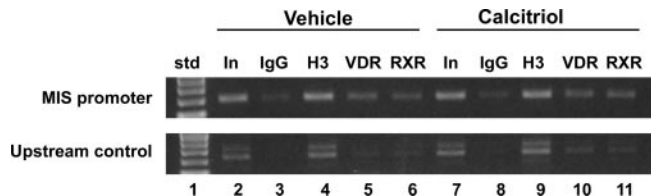


FIG. 6. The VDR is present on the MIS promoter in prostate cancer cells. LNCaP cells were treated with and without 100 nM calcitriol for 6 h at 37 C. The cells were then fixed with 1% formaldehyde for 10 min at ambient temperature. The samples were then analyzed by ChIP. Upper panel, PCR of MIS promoter containing VDRE; lower panel, control PCR of region about 1.6 kb upstream of the MIS promoter. ChIP assays were performed with antibodies to normal rabbit IgG, histone 3 (H3), VDR, and RXR. Std, 100 bp standard; In, input DNA extracted before ChIP assay.

These results demonstrated that VDR and RXR bind to the MIS promoter in intact prostate cancer cells.

Discussion

We have previously shown that calcitriol induces MIS gene expression in human prostate cancer cells (6). In the current study, we demonstrated that the MIS promoter contains a functional VDRE and that calcitriol directly up-regulates MIS gene expression via this response element. The MIS VDRE is highly similar to the human and rat osteocalcin VDREs, classical vitamin D target genes (Table 1). Coexpression of VDR and SF-1 increased basal MIS promoter activity that was further stimulated by calcitriol. Mutagenesis or deletion of the MIS VDRE significantly reduced or abolished responsiveness to calcitriol. In gel shift assays, VDR and RXR bind to the MIS VDRE, and the binding was increased by calcitriol. The 15-bp VDRE also conferred calcitriol responsiveness to a heterologous promoter. MIS gene expression was induced by calcitriol in both LNCaP and PC-3 cells. In intact prostate cancer cells, we showed that VDR and RXR were present on the MIS promoter using ChIP assays. These data demonstrate that the MIS promoter contains a functional VDRE that binds the VDR and is responsive to calcitriol.

There are putative VDREs in the mouse (tGGTGAcctGGGcGg, -260/-246), rat (tGGTGAcctGGGggA, -254/-240), and bovine (GGGTGAgcaGcACg, -436/-422) MIS promoters (*lowercase letters* indicate differences between the human MIS VDRE GGGTGAgcaGGGACA), but we have not tested these elements for calcitriol responsiveness.

Although MIS is most known for its activity to initiate regression of Müllerian structures during male fetal development (11), many postnatal actions have been documented. MIS exhibits important actions on steroidogenesis (13, 14), follicle development (15, 16, 29), and ovarian and testicular function (30) and has been linked to polycystic ovarian syndrome (PCOS) (31–34). Whether calcitriol and VDR contribute to these activities by induction of MIS is currently unknown and warrants further investigation.

It has been suggested that the MIS locus is in the open chromatin state because a significant number of spliceosome-associated protein 62 (SAP 62) transcripts continue through the MIS gene (26). In our ChIP assays, we demonstrated that in the absence of calcitriol, VDR and RXR are present on the MIS promoter in prostate cancer cells. The presence of the VDR on the MIS promoter in the absence of calcitriol also suggests that the MIS locus is in the open state. MIS exhibits precise regulation despite its apparent open chromatin state, indicating that its expression is under stringent control. Because the VDR has been shown to interact with corepressors and silence the activity of some genes (35), it raises the possibility that the unliganded VDR silences MIS gene expression. Dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome (DAX-1), gene 1 has also been shown to inhibit MIS expression by interacting with SF-1 (36).

Our major interest to study MIS in relation to vitamin D stems from our investigation of various pathways to inhibit prostate cancer development or progression (6). We are especially focused on the potential of using calcitriol in combination therapy with other anticancer drugs (6). However, another reason for our interest in MIS was an observation in one of the cases of HVDRR that we previously reported (37, 38). This child, who has since died, had two rare genetic disorders in addition to HVDRR, generalized congenital lipodystrophy of the Berardinelli-Seip type (BSCL), and persistent Müllerian duct syndrome (PMDS). We found that the basis of his HVDRR was a mutation in the VDR ligand binding domain (H305Q) that altered the contact point for the 25-hydroxyl group in calcitriol (38). BSCL, a rare autosomal recessive disorder, was found to be caused by a splice site mutation in his BSCL2 gene (39). The BSCL2 gene product, seipin, is a transmembrane protein of unknown function localized in the endoplasmic reticulum (40). PMDS is usually caused by mutations in the MIS gene or the MIS receptor (MISR2) gene and is characterized by the presence of Müllerian derivatives in males (41). However, because the child already had two proven rare and unrelated autosomal mutations, we wondered whether this child could possibly harbor three unique rare mutations, which on a statistical basis would be extremely remote. Alternatively, we speculated that his PMDS might be caused by a downstream defect due to the mutated VDR. Our finding that MIS is regulated in part by calcitriol and the fact that VDR is expressed in Sertoli cells (42) makes this hypothesis feasible. The molecular basis for PMDS in the patient has not been discovered. PMDS has not been described in other cases of HVDRR (4, 5). However, the presence of retained Müllerian ducts may not cause symptoms in boys at an early age, and the presence of PMDS in other HVDRR boys may have been overlooked. In any case, the current study does prove that MIS is directly regulated by calcitriol and the loss of this action due to the mutation in the VDR may have caused PMDS in this child. We hope that any future cases of boys with HVDRR will be carefully checked for PMDS.

Our finding that the MIS gene is regulated by calcitriol suggests a role for the VDR in female reproduction. In females, MIS is expressed in granulosa cells of the ovary (43, 44). Although the exact biological functions of MIS are not fully understood, MIS may mediate follicle recruitment and selection, and inhibit aro-

matase activity (45, 46). Interestingly, in one VDR knockout mouse model, female mice had uterine hypoplasia due to impaired folliculogenesis (3). One explanation for these defects was that there was impaired estrogen synthesis in the knockout mice (47). Estrogen supplementation corrected the defects, whereas maintaining normal serum calcium through a rescue diet only partially increased aromatase activity, suggesting that VDR was required for full gonadal function (47). Also, in a 1 α -hydroxylase knockout mouse model, female mice exhibited impaired folliculogenesis, uterine hypoplasia, decreased ovarian size, and infertility (48). However, hypocalcemia in these mice leaves the precise direct role of calcitriol depletion unclear. Because calcitriol has been shown to stimulate aromatase activity in bone (49) and loss of VDR decreases aromatase in ovary (47), depletion of calcitriol in the knockout mice may have led to a loss of aromatase activity and subsequent decreased estrogen synthesis, contributing to uterine hypoplasia and infertility. Although these studies do not indicate whether MIS is an intermediate in the action of calcitriol on female reproduction, we speculate that regulation of MIS by calcitriol may play a role. In addition, PCOS has also been associated with high levels of MIS (31–34). The role of calcitriol and/or the VDR in the overproduction of MIS in PCOS also warrants investigation.

More recently, it has become clear that MIS has actions to inhibit cancer growth, and MIS is currently under intense investigation for use as an anticancer drug (18, 50, 51). Data indicate that MIS has antiproliferative activity against a variety of cancers including uterine, cervical, ovarian, breast, and prostate cancer, to name a few (17–24). In prostate cancer, MIS exerts dual actions to both inhibit androgen synthesis and promote tumor regression (13, 23, 52). Calcitriol also has multiple actions to prevent and inhibit prostate cancer growth (6, 7). These actions include cell cycle arrest, prodifferentiation, apoptosis, antiangiogenesis, inhibition of invasion and metastasis, and antiinflammatory activity (6, 7). The findings presented here demonstrate that up-regulating MIS is another anticancer action in the prostate. What proportion of the antiproliferative actions of calcitriol in prostate are due to stimulation of MIS is not yet clear. However, stimulation of endogenous MIS production by calcitriol would be additive to the anticancer activity of exogenous MIS used as cancer therapy. This leads us to speculate that combination therapy with MIS plus calcitriol should be more efficacious than MIS alone and should be considered in the investigation of MIS utility as an anticancer agent.

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Address all correspondence and requests for reprints to: Peter J. Malloy, Ph.D., S025 Division of Endocrinology, Gerontology, and Metabolism, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, California 94305-5103. E-mail: malloy@cmgm.stanford.edu.

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References

- Feldman D, Pike JW, Glorieux FH 2005 Vitamin D. 2nd ed. San Diego: Elsevier Academic Press
- Li YC, Pirro AE, Amling M, Dellling G, Baron R, Bronson R, Demay MB 1997 Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia. *Proc Natl Acad Sci USA* 94:9831–9835
- Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T, Kato S 1997 Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nat Genet* 16:391–396
- Malloy PJ, Pike JW, Feldman D 1999 The vitamin D receptor and the syndrome of hereditary 1,25-dihydroxyvitamin D-resistant rickets. *Endocr Rev* 20:156–188
- Malloy PJ, Pike JW, Feldman D 2005 Hereditary 1,25-dihydroxyvitamin D resistant rickets. In: Feldman D, Pike JW, Glorieux F, eds. *Vitamin D*. 2nd ed. San Diego: Elsevier; 1207–1238
- Krishnan AV, Moreno J, Nonn L, Malloy P, Swami S, Peng L, Peehl DM, Feldman D 2007 Novel pathways that contribute to the anti-proliferative and chemopreventive activities of calcitriol in prostate cancer. *J Steroid Biochem Mol Biol* 103:694–702
- Feldman D, Malloy PJ, Krishnan AV, Balint E 2007 Vitamin D: biology, action, and clinical implications. In: Marcus R, Feldman D, Nelson DA, Rosen CJ, eds. *Osteoporosis*. 3rd ed. San Diego: Academic Press; 317–382
- Massague J 1998 TGF- β signal transduction. *Annu Rev Biochem* 67:753–791
- Clarke TR, Hoshiya Y, Yi SE, Liu X, Lyons KM, Donahoe PK 2001 Mullerian inhibiting substance signaling uses a bone morphogenetic protein (BMP)-like pathway mediated by ALK2 and induces SMAD6 expression. *Mol Endocrinol* 15:946–959
- Visser JA, Olaso R, Verhoef-Post M, Kramer P, Themmen AP, Ingraham HA 2001 The serine/threonine transmembrane receptor ALK2 mediates Mullerian inhibiting substance signaling. *Mol Endocrinol* 15:936–945
- MacLaughlin DT, Donahoe PK 2004 Sex determination and differentiation. *N Engl J Med* 350:367–378
- Josso N, Clemente N 2003 Transduction pathway of anti-Mullerian hormone, a sex-specific member of the TGF- β family. *Trends Endocrinol Metab* 14:91–97
- Trbovich AM, Sluss PM, Laurich VM, O'Neill FH, MacLaughlin DT, Donahoe PK, Teixeira J 2001 Mullerian inhibiting substance lowers testosterone in luteinizing hormone-stimulated rodents. *Proc Natl Acad Sci USA* 98:3393–3397
- Fynn-Thompson E, Cheng H, Teixeira J 2003 Inhibition of steroidogenesis in Leydig cells by Mullerian-inhibiting substance. *Mol Cell Endocrinol* 211:99–104
- McGee EA, Smith R, Spears N, Nachtigal MW, Ingraham H, Hsueh AJ 2001 Mullerian inhibitory substance induces growth of rat preantral ovarian follicles. *Biol Reprod* 64:293–298
- Durlinger AL, Grujters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JT, Grootegoed JA, Themmen AP 2002 Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. *Endocrinology* 143:1076–1084
- La Marca A, Volpe A 2007 The Anti-Mullerian hormone and ovarian cancer. *Hum Reprod Update* 13:265–273
- Pieretti-Vanmarcke R, Donahoe PK, Pearsall LA, Dinulescu DM, Connolly DC, Halpern EF, Seiden MV, MacLaughlin DT 2006 Mullerian inhibiting substance enhances subclinical doses of chemotherapeutic agents to inhibit human and mouse ovarian cancer. *Proc Natl Acad Sci USA* 103:17426–17431
- Renaud EJ, MacLaughlin DT, Oliva E, Rueda BR, Donahoe PK 2005 Endometrial cancer is a receptor-mediated target for Mullerian inhibiting substance. *Proc Natl Acad Sci USA* 102:111–116
- Hoshiya Y, Gupta V, Segev DL, Hoshiya M, Carey JL, Sasur LM, Tran TT, Ha TU, Maheswaran S 2003 Mullerian inhibiting substance induces NF κ B signaling in breast and prostate cancer cells. *Mol Cell Endocrinol* 211:43–49
- Barbie TU, Barbie DA, MacLaughlin DT, Maheswaran S, Donahoe PK 2003 Mullerian inhibiting substance inhibits cervical cancer cell growth via a pathway involving p130 and p107. *Proc Natl Acad Sci USA* 100:15601–15606
- Stephen AE, Pearsall LA, Christian BP, Donahoe PK, Vacanti JP, MacLaughlin DT 2002 Highly purified Mullerian inhibiting substance inhibits human ovarian cancer *in vivo*. *Clin Cancer Res* 8:2640–2646
- Segev DL, Hoshiya Y, Hoshiya M, Tran TT, Carey JL, Stephen AE, MacLaughlin DT, Donahoe PK, Maheswaran S 2002 Mullerian-inhibiting substance regulates NF- κ B signaling in the prostate *in vitro* and *in vivo*. *Proc Natl Acad Sci USA* 99:239–244
- Segev DL, Ha TU, Tran TT, Kenneally M, Harkin P, Jung M, MacLaughlin DT, Donahoe PK, Maheswaran S 2000 Mullerian inhibiting substance inhibits breast cancer cell growth through an NF κ B-mediated pathway. *J Biol Chem* 275:28371–28379
- Malloy PJ, Xu R, Peng L, Clark PA, Feldman D 2002 A novel mutation in helix 12 of the vitamin D receptor impairs coactivator interaction and causes hereditary 1,25-dihydroxyvitamin D-resistant rickets without alopecia. *Mol Endocrinol* 16:2538–2546
- Dresser DW, Hacker A, Lovell-Badge R, Guerrier D 1995 The genes for a spliceosome protein (SAP62) and the anti-Mullerian hormone (AMH) are contiguous. *Hum Mol Genet* 4:1613–1618
- Shen WH, Moore CC, Ikeda Y, Parker KL, Ingraham HA 1994 Nuclear receptor steroidogenic factor 1 regulates the Mullerian inhibiting substance gene: a link to the sex determination cascade. *Cell* 77:651–661
- Whitfield GK, Selznick SH, Haussler CA, Hsieh JC, Galligan MA, Jurutka PW, Thompson PD, Lee SM, Zerwekh JE, Haussler MR 1996 Vitamin D receptors from patients with resistance to 1,25-dihydroxyvitamin D₃: point mutations confer reduced transactivation in response to ligand and impaired interaction with the retinoid X receptor heterodimeric partner. *Mol Endocrinol* 10:1617–1631
- Themmen AP 2005 Anti-Mullerian hormone: its role in follicular growth initiation and survival and as an ovarian reserve marker. *J Natl Cancer Inst Monogr* 34:18–21 (Review)
- Josso N, Picard JY, Rey R, di Clemente N 2006 Testicular anti-Mullerian hormone: history, genetics, regulation and clinical applications. *Pediatr Endocrinol Rev* 3:347–358
- Cook CL, Siow Y, Brenner AG, Fallat ME 2002 Relationship between serum Mullerian-inhibiting substance and other reproductive hormones in untreated women with polycystic ovary syndrome and normal women. *Fertil Steril* 77:141–146
- Pigny P, Merlen E, Robert Y, Cortet-Rudelli C, Decanter C, Jonard S, Dewailly D 2003 Elevated serum level of anti-Mullerian hormone in patients with polycystic ovary syndrome: relationship to the ovarian follicle excess and to the follicular arrest. *J Clin Endocrinol Metab* 88:5957–5962
- Pellatt L, Hanna L, Brincat M, Galea R, Brain H, Whitehead S, Mason H 2007 Granulosa cell production of anti-Mullerian hormone is increased in polycystic ovaries. *J Clin Endocrinol Metab* 92:240–245
- Wang JG, Nakhuda GS, Guarnaccia MM, Sauer MV, Lobo RA 2007 Mullerian inhibiting substance and disrupted folliculogenesis in polycystic ovary syndrome. *Am J Obstet Gynecol* 196:77e1–77e5
- Hsieh JC, Sisk JM, Jurutka PW, Haussler CA, Slater SA, Haussler MR, Thompson CC 2003 Physical and functional interaction between the vitamin D receptor and hairless corepressor, two proteins required for hair cycling. *J Biol Chem* 278:38665–38674
- Nachtigal MW, Hirokawa Y, Enyeart-VanHouten DL, Flanagan JN, Hammer GD, Ingraham HA 1998 Wilms' tumor 1 and Dax-1 modulate the orphan nuclear receptor SF-1 in sex-specific gene expression. *Cell* 93:445–454
- Van Maldergem L, Bachy A, Feldman D, Bouillon R, Maassen J, Dreyer M, Rey R, Holm C, Gillerot Y 1996 Syndrome of lipoatrophic diabetes, vitamin D resistant rickets, and persistent Mullerian ducts in a Turkish boy born to consanguineous parents. *Am J Med Genet* 64:506–513
- Malloy PJ, Eccleshall TR, Gross C, Van Maldergem L, Bouillon R, Feldman D 1997 Hereditary vitamin D resistant rickets caused by a novel mutation in the vitamin D receptor that results in decreased affinity for hormone and cellular hyporesponsiveness. *J Clin Invest* 99:297–304
- Magre J, Delphine M, Khallouf E, Gedde-Dahl Jr T, Van Maldergem L, Sobel E, Papp J, Meier M, Megarbane A, Bachy A, Verloes A, d'Abronzio FH, Seemanova E, Assan R, Baudic N, Bourut C, Czernichow P, Huet F, Grigorescu F, de Kerdanet M, Lacombe D, Labrune P, Lanza M, Loret H, Matsuda F, *et al.* 2001 Identification of the gene altered in Berardinelli-Seip congenital lipodystrophy on chromosome 11q13. *Nat Genet* 28:365–370
- Ito D, Suzuki N 2007 Molecular pathogenesis of seipin/BSCL2-related motor neuron diseases. *Ann Neurol* 61:237–250
- Josso N, Belville C, di Clemente N, Picard JY 2005 AMH and AMH receptor defects in persistent Mullerian duct syndrome. *Hum Reprod Update* 11:351–356
- Stumpf WE, Sar M, Chen K, Morin J, DeLuca HF 1987 Sertoli cells in the testis and epithelium of the ductuli efferentes are targets for ³H 1,25 (OH)₂ vitamin D₃. An autoradiographic study. *Cell Tissue Res* 247:453–455

43. Vigier B, Picard JY, Tran D, Legeai L, Josso N 1984 Production of anti-Mullerian hormone: another homology between Sertoli and granulosa cells. *Endocrinology* 114:1315–1320
44. Takahashi M, Hayashi M, Manganaro TF, Donahoe PK 1986 The ontogeny of Mullerian inhibiting substance in granulosa cells of the bovine ovarian follicle. *Biol Reprod* 35:447–453
45. Grossman MP, Nakajima ST, Fallat ME, Siow Y 2008 Mullerian-inhibiting substance inhibits cytochrome P450 aromatase activity in human granulosa lutein cell culture. *Fertil Steril* 89:1364–1370
46. Nakhuda GS 2008 The role of Mullerian inhibiting substance in female reproduction. *Curr Opin Obstet Gynecol* 20:257–264
47. Kinuta K, Tanaka H, Moriwake T, Aya K, Kato S, Seino Y 2000 Vitamin D is an important factor in estrogen biosynthesis of both female and male gonads. *Endocrinology* 141:1317–1324
48. Panda DK, Miao D, Tremblay ML, Sirosi J, Farookhi R, Hendy GN, Goltzman D 2001 Targeted ablation of the 25-hydroxyvitamin D 1 α -hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. *Proc Natl Acad Sci USA* 98:7498–7503
49. Yanase T, Suzuki S, Goto K, Nomura M, Okabe T, Takayanagi R, Nawata H 2003 Aromatase in bone: roles of vitamin D3 and androgens. *J Steroid Biochem Mol Biol* 86:393–397
50. Teixeira J, Maheswaran S, Donahoe PK 2001 Mullerian inhibiting substance: an instructive developmental hormone with diagnostic and possible therapeutic applications. *Endocr Rev* 22:657–674
51. Donahoe PK, Clarke T, Teixeira J, Maheswaran S, MacLaughlin DT 2003 Enhanced purification and production of Mullerian inhibiting substance for therapeutic applications. *Mol Cell Endocrinol* 211:37–42
52. Teixeira J, Fynn-Thompson E, Payne AH, Donahoe PK 1999 Mullerian-inhibiting substance regulates androgen synthesis at the transcriptional level. *Endocrinology* 140:4732–4738