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14. ABSTRACT This grant investigates the potential use of calcitriol regulation of mullerian inhibitory substance (MIS) expression as an incremental therapy for prostate cancer. We have established that calcitriol (1,25-dihydroxyvitamin D3) directly stimulates MIS expression by binding to the vitamin D receptor (VDR) and directing the hormone-receptor complex to bind to a vitamin D regulatory element (VDRE) in the MIS promoter. We have attempted to demonstrate that combination of calcitriol and MIS achieve increased potency to inhibit prostate cancer cell growth compared to either drug alone. However, this has not yet been definitively accomplished although new data are included in this revised report. We further demonstrated the interaction of calcitriol-VDR with other transcription factors, SF-1, SOX-9 and GATA-4, to act together to synergistically increase MIS expression in prostate cancer cells. We believe that some of calcitriol's action to inhibit prostate cancer cell growth are due to stimulation of MIS and this work plans to substantiate this hypothesis and lay the ground work to translate this information to clinical trials in men with prostate cancer. The major focus of the work completed was to document the VDRE in the MIS promoter. This has now been completed and the manuscript published. Preliminary data showing further effects of MIS with and without calcitriol are now included in this report and the findings are confusing.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	12
Reportable Outcomes.....	13
Conclusion.....	13
References.....	14
Appendices.....	16

INTRODUCTION

The initial report that I submitted was not found acceptable because our efforts to complete Tasks 3 and 4 in the SOW were not covered. I apologize for this. The explanation is that the data thus far obtained for these Tasks is not definitive and difficult to interpret. I therefore did not include the material in the original report. In this revised report, all of our efforts on Tasks 3 and 4 are detailed.

Regarding Tasks 1 and 2. These have been carefully completed and are the subject of our published paper in ENDOCRINOLOGY. The paper is included in the Appendix. I did refer to this material in the Body of the previous report but I will make reference to that material more obvious in this revised report.

We proposed that MIS regulation is an important element contributing to the ability of 1,25-dihydroxyvitamin D₃ (calcitriol) to inhibit the growth and progression of prostate cancer cells. We have shown that calcitriol acts by several pathways to inhibit the growth of prostate cancer (PCa) cells [1-6]. In recent studies we showed that calcitriol, in addition to multiple other pathways, also stimulates the expression of MIS in a classical human prostate cancer cell line, LNCaP [5]. We further showed that the up-regulation of MIS expression is mediated directly by calcitriol binding to the vitamin D receptor (VDR) and the hormone-receptor complex subsequently interacts with a vitamin D regulatory element (VDRE) in the MIS gene promoter. We have now completed the studies defining the regulatory element in the MIS promoter. **The manuscript describing the VDRE in the MIS promoter has now been published and is**

included in the Appendix. New data to study the efficacy of calcitriol combined with MIS to inhibit PCa cell growth are now included in this revised report.

BODY

Task 1 and 2. To Study the Regulation of MIS by Calcitriol in PCa and Determine the Molecular Mechanism by Which Calcitriol Induces MIS in PCa.

These data are detailed in the published paper that is provided in the Appendix.

This paper completed Tasks 1 and 2 and was published in a front-line journal, ENDOCRINOLOGY. The details will not be repeated in the body of the report but are provided completely in the manuscript. However I will describe the results in the Body of the report.

In brief, Calcitriol (1,25-dihydroxyvitamin D₃) inhibits the growth of a variety of cancer cells including human prostate cancer. Müllerian inhibiting substance (MIS) also exhibits anti-proliferative and pro-apoptotic 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 anti-proliferative activity. **As detailed in the Published paper that is supplied in the Appendix,** we identified a 15 bp sequence GGGTGAgcaGGGACA in the MIS promoter that was highly similar to DR3-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. Co-expression of steroidogenic factor 1 (SF-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 (RXR) bound to the MIS VDRE and the binding was increased by calcitriol. Chromatin immunoprecipitation assays showed that VDR and RXR were present on the MIS promoter in prostate cancer cells. In conclusion, we have 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 anti-proliferative actions of calcitriol in some cancers, especially prostate cancer.

The work supported by this grant as Task 1 and Task 2 is detailed in a manuscript provided in the Appendix. The paper has now been published in the journal ENDOCRINOLOGY.

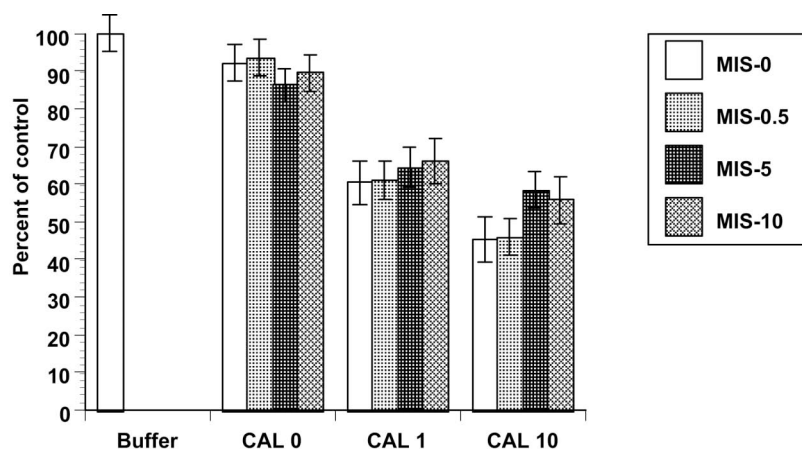
ADDITIONAL DATA ON TASKS 3 AND 4.

These data were not included in the original Final Report because I did not feel they were definitive. However, since that report was found unacceptable because our efforts to perform these tasks were not included, I have added the data from these inconclusive experiments to this Revised Final Report. Many of these experiments depend on using MIS as a reagent. This material is not available commercially. We have relied on receiving the MIS from another research lab which has been forthcoming with only limited amounts of this material that they have to work hard to purify and produce for their own purposes. Since our initial experiments were negative, it has been hard to ask for and obtain additional amounts of this valuable commodity. However Task 3 and Task 4 from our overly-ambitious SOW were initiated and some experiments were completed. These additional data are included in this Revised Final Report and will be discussed below. It is possible that some of the variability and difficulty in these experiments derives from using MIS of variable activity.

Task 3. Studies to Assess the Role of MIS on the Antiproliferative Activity of Calcitriol

Two experiments were completed studying Calcitriol alone, MIS alone and the combination on LNCaP PCa cell growth to determine whether MIS added to calcitriol would be a useful therapeutic combination with increased activity to inhibit PCa growth. In the first experiment (Figure 1), Calcitriol (CAL) at 1 and 10 nM inhibited LNCaP prostate cancer cell growth as expected. However MIS at 0.5, 5 and 10 $\mu\text{g/ml}$ did not inhibit PCa growth when given alone or increase the growth inhibiting activity of calcitriol when given in combination.

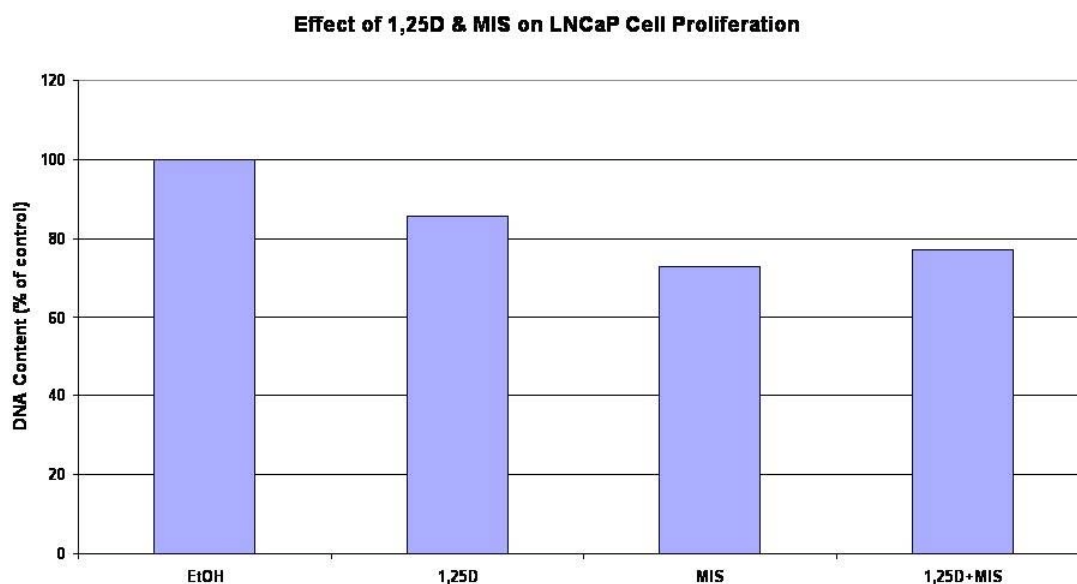
Figure 1. LNCap cell growth inhibition after 6 days of therapy with dose-response treatment of cells with calcitriol, MIS and combinations.



In a second experiment using a different batch of MIS (Figure 2), MIS did exhibit some growth inhibition activity when given by itself and but did not enhance the calcitriol effect when given in combination. This amount of activity was disappointingly slight. Calcitriol (1,25D) at 10 nM inhibited LNCaP cell growth to 85.6% of control, MIS at 5 $\mu\text{g/ml}$ to 73% of control but the combination was 77% of control. In other words, MIS showed modest growth inhibition but the

combination was no better than either drug alone. And the findings differed from the first experiment where MIS showed no anti-proliferative activity.

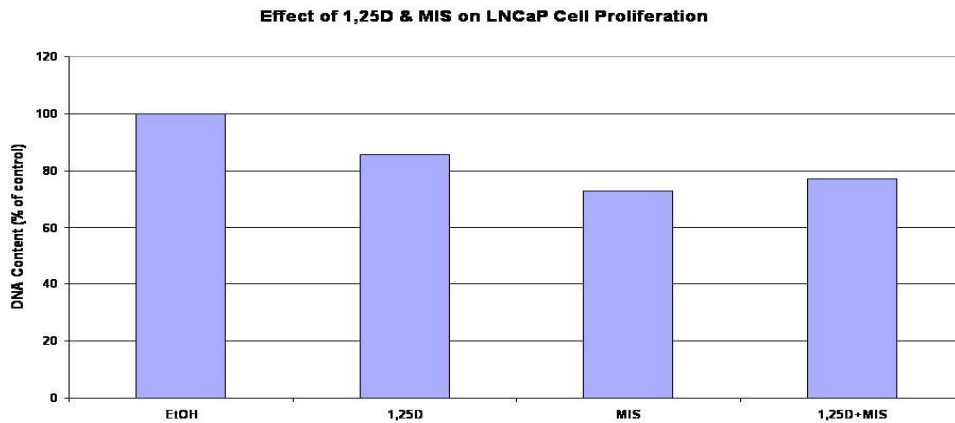
Figure 2.



Both of these experiments were 6-day growth experiments and growth was quantified by measuring the accumulated DNA.

We next performed two additional experiments over a shorter 3-day time course and assessed LNCaP cell growth by [^3H]-thymidine accumulation (Figure 3). These experiments were done twice in triplicate. Calcitriol (1,25D) at 10 nM inhibited growth to 87% of control, MIS at 5 ug/ml inhibited not at all (to 96% of control) and the combination inhibited to 74% of control, slightly more than calcitriol alone.

Figure 3.



In conclusion, the data from multiple growth experiments were variable and inconsistent. It is possible that the MIS in the first experiments was inactive but our collaborator checked it for us and found it to be active. We therefore have no explanation for the inconsistent data. We must conclude that the combination of calcitriol and MIS does not appear to have a significant advantage over calcitriol alone.

Task 4. Studies to Investigate the Role of MIS on the Downstream Actions of Calcitriol

A. Immuno-neutralization.

Our first approach was to immuno-neutralize MIS using commercial anti-MIS antibodies to determine whether removing MIS from the system would affect the ability of calcitriol to exhibit antiproliferative activity. These experiments failed when the antibody or its vehicle proved toxic to the cells.

B. siRNA

Our second approach was to knock-down MIS using siRNA to silence the MIS gene and delete its contribution to calcitriol's action and thereby determine whether lack of MIS would affect calcitriol's anti-proliferative activity. These experiments failed when the siRNA obtained from Santa Cruz Biotechnology Company was unsuccessful in its task to knock down the levels of MIS mRNA. In these experiments calcitriol remained as active in the control as in the siRNA treated groups.

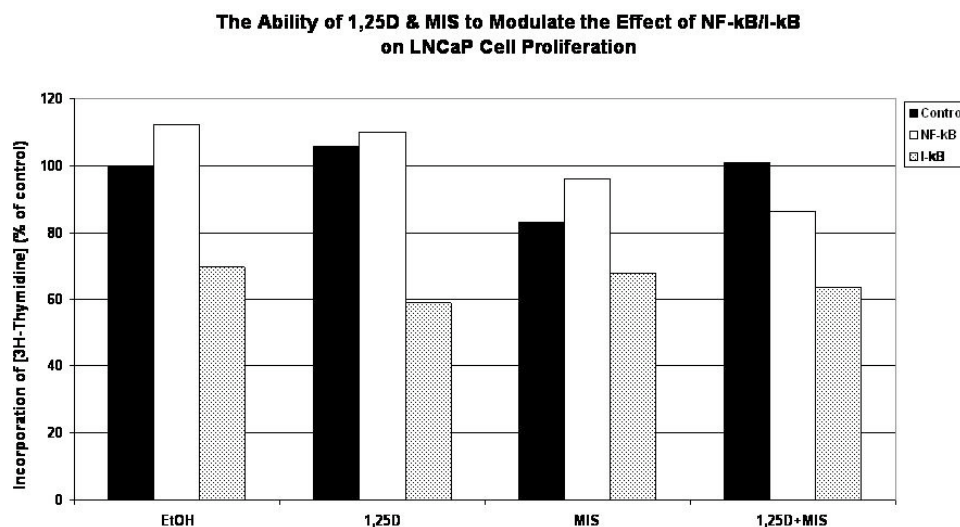
C. Studies of MIS Activity on NF- κ B and other immunological genes

LNCaP cells were treated with calcitriol (1,25D), MIS or the combination and a 98-gene array was used to assess the regulation of inflammatory target genes including NF- κ B components. The array was purchased from SAB Company and contains validated PCR primers to evaluate by PCR the expression levels of all of the genes of interest. This method provides an excellent way to assess the effects of MIS and calcitriol on the inflammatory pathway. The entire 98-gene array list is shown in the Appendix as well as the results of our experiments (Table 1). As can be seen, a number of genes exhibited major up-regulation by calcitriol and some by MIS. All genes stimulated by MIS were also stimulated by calcitriol and almost always to a greater extent. However, we did not find that any of the genes exhibited enhancement when both drugs were used. In fact, many of the genes that were stimulated by calcitriol were comparatively decreased by the combination and many genes stimulated by MIS were reduced by the combination. These findings left us unable to interpret the validity or the significance of the entire experiment.

D. Studies to Stimulate or block MIS Signaling to Determine How Much of Calcitriol's Activity is Abrogated

In these experiments we compared the ability of 10 nM calcitriol (1,25D), MIS 5 ug/ml and the combination in the presence or absence of NF-kB or IκB to affect [3H]-thymidine incorporation of LNCaP cells as a measure of LNCaP cell proliferation (Figure 4). NF-kB and IκB were transfected into LNCaP cells and the effect on cell growth was monitored by [3H]-thymidine incorporation.

Figure 4.



As can be seen, transfection of NF-kB into LNCaP cells caused a mild stimulation of growth that was not inhibited by calcitriol (1,25D). MIS had a minimal effect to inhibit this growth (96% of control) and the combination reduced the growth to 87% of control. When IκB was transfected into cells, there was a major slowing of growth to 70% in the control. Calcitriol reduced growth

further to 59% of control, MIS to 68% of control and the combination to 64% of control. The slowing of growth by I κ B indicates that NF- κ B blockade slows growth suggesting that LNCaP cell growth is being stimulated by NF- κ B. The calcitriol and MIS actions to further inhibit the growth were slight. Again these results do not strongly suggest that calcitriol plus MIS is a useful combination to treat prostate cancer

Interpretation of Data on Tasks 3 and 4

We completed Tasks 1 and 2 and the data are now published. The data obtained on Task 3 and 4 are variable and inconsistent. Multiple approaches were tried and the findings were not as useful for interpretation as hoped. Unfortunately, despite our serious efforts, we are unable to demonstrate that the combination of calcitriol and MIS improves the anti-proliferative activity of calcitriol. We could not consistently demonstrate that MIS exerted an anti-proliferative action in PCa cells. The findings suggest that combination therapy of calcitriol plus MIS is not a major improvement compared to calcitriol alone.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that MIS inhibits prostate cancer cell growth.
- Found that calcitriol stimulated MIS expression in prostate cancer cells.
- Characterized the mechanism for calcitriol to activate gene expression of MIS in prostate cancer.
- Identified the VDRE in the promoter of the MIS gene by which calcitriol directly interacts with the MIS gene to up-regulate MIS expression
- Demonstrated the collaboration of calcitriol and the transcription factor SF-1 to maximally stimulate MIS expression levels
- Found that the combination of calcitriol and MIS was no better than calcitriol alone in the inhibition of prostate cancer cell growth

REPORTABLE OUTCOMES

1. P.J. Malloy and D. Feldman. Mullerian Inhibitory Substance (MIS) is upregulated by 1,25-dihydroxyvitamin D₃ in LNCaP prostate cancer cells via a direct interaction of the vitamin D receptor with a vitamin D regulatory element in the MIS promoter. Endocrine Society 88th Annual Meeting, Boston MA June 24-27, 2006. poster 3-55.
2. H.D. Mason, L. Hanna, S. Rice, H. Brain, P.J. Malloy, D. Feldman, M Brincat, R. Galea, S.A. Whitehead, and L.J. Pellat, Role of Anti-Mullerian Hormone (AMH) in anovulatory polycystic ovarian syndrome. Endocrine Society 88th Annual Meeting, Boston MA June 24-27, 2006. Oral presentation OR38-3.
3. P. J. Malloy, L. Peng and D. Feldman, Müllerian Inhibiting Substance (MIS) is up-regulated by calcitriol in LNCaP prostate cancer cells via a direct interaction of the vitamin D receptor with a vitamin D response element in the MIS promoter. DOD Prostate Cancer Meeting, Atlanta GA Sept 2007. poster
4. A.V. Krishnan, J. Moreno, L. Nonn, P. Malloy, S. Swami , L. Peng, D. M. Peehl and D. Feldman, Novel pathways that contribute to the anti-proliferative and chemopreventive activities of calcitriol in prostate cancer. J Steroid Biochem. Mol. Biol. 103:694-702, 2007.
5. P. J. Malloy, L. Peng, J. Wang, and D. Feldman. 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. ENDOCRINOLOGY in press.

CONCLUSIONS

Calcitriol has many actions to inhibit prostate cancer. This work adds another important pathway to the repertoire by which calcitriol can have beneficial actions to slow prostate cancer growth and progression. Based upon our findings, we suggested that a study of the use of combination therapy administering calcitriol and MIS together may be warranted in the treatment of prostate cancer. Our work raised the possibility that direct stimulation of MIS expression by calcitriol via the VDRE documented in these studies may be an important new action of calcitriol. However, our attempts to demonstrate the enhanced activity of combination therapy were inconsistent and not conclusive but suggest that MIS plus calcitriol combination therapy is no better than calcitriol alone.

INDIVIDUALS FUNDED IN PART BY THIS GRANT

Aruna Krishnan, Ph.D
Srilatha Swami, Ph.D
Jining Wang, Ph.D.
Lihong Peng, Ph.D.

REFERENCES

See manuscript in Appendix for extensive list of citations.

1. J. Moreno, A.V. Krishnan, S. Swami, L. Nonn, D.M. Peehl, and D. Feldman, Regulation of prostaglandin metabolism by calcitriol attenuates growth stimulation in prostate cancer cells. *Cancer Res.* 65:7917-25, 2005.
2. J. Moreno, A. V. Krishnan and D. Feldman, Molecular mechanisms mediating the anti-proliferative effects of Vitamin D in prostate cancer. *J. Steroid Biochem. Mol. Biol.* 97:31-36, 2005.
3. L. Nonn, L. Peng, D. Feldman, D.M. Peehl, Inhibition of p38 by vitamin D reduces interleukin-6 production in normal prostate cells via MAP Kinase phosphatase 5: Implications for prostate cancer prevention by vitamin D. *Cancer Res.* 66:4516-4524, 2006.
4. J. Moreno, A.V. Krishnan, D.M. Peehl, D. Feldman, Mechanisms of vitamin D-mediated growth inhibition in prostate cancer cells: inhibition of the prostaglandin pathway. *Anticancer Res* 26: (4A) 2525-30, 2006.
5. A.V. Krishnan, J. Moreno, L. Nonn, P. Malloy, S. Swami , L. Peng, D. M. Peehl and D. Feldman, Novel pathways that contribute to the anti-proliferative and

chemopreventive activities of calcitriol in prostate cancer. J Steroid Biochem. Mol. Biol. 103:694-702, 2007.

6. A.V. Krishnan, J. Moreno, L. Nonn, S. Swami, D.M. Peehl and D. Feldman, Calcitriol as a chemopreventive and therapeutic agent in prostate cancer: Role of anti-inflammatory activity. J. Bone Miner. Res. 22: (Supple 2) v74-80, 2007.

Appendix

Table 1. Experimental Results of Calcitriol (1,25D), MIS and the combination on the expression levels of inflammatory target genes

Symbol	Well	Fold Up/Down Regulation	Fold Up/Down Regulation	Fold Up- or Down-Regulation
		1,25 D/control	MIS/control	D+MIS/control
AGT	A01	1.6	2.1	1.9
AKT1	A02	2.2	2.0	2.3
ATF1	A03	-2.8	-1.0	1.0
BCL10	A04	1.8	1.6	1.4
BCL3	A05	1.2	1.5	1.2
CFB	A06	1.4	1.5	1.2
BIRC2	A07	1.7	1.5	1.9
NOD1	A08	2.0	1.5	2.7
CASP1	A09	1.5	1.5	1.6
CASP8	A10	1.0	1.1	1.3
CCL2	A11	1.6	1.6	1.4
CD40	A12	1.9	1.2	2.6
CFLAR	B01	1.7	1.3	1.3
CHUK	B02	1.3	1.4	1.4
CSF2	B03	25	1.8	2.1
CSF3	B04	53	3.8	2.6
SLC44A2	B05	3.0	2.0	2.1
EDARADD	B06	53	3.8	3.1
EDG2	B07	53	3.8	2.6
EGR1	B08	1.9	1.1	2.2
ELK1	B09	1.0	1.0	1.3
F2R	B10	1.6	1.2	1.6
FADD	B11	2.4	1.5	2.3
FASLG	B12	53	3.8	2.6
FOS	C01	2.5	2.3	2.1
GJA1	C02	2.4	2.2	2.4
HMOX1	C03	1.3	1.6	1.8
HTR2B	C04	-1.0	1.3	1.3
ICAM1	C05	3.1	-1.5	1.0
IFNA1	C06	45	3.2	2.2
IFNB1	C07	1.0	1.3	1.4
IFNG	C08	53	3.8	2.6
IKBKB	C09	-1.1	1.2	1.2
IKBKE	C10	1.4	1.4	1.7
IKBKG	C11	1.3	-1.1	1.4
IL10	C12	14	1.0	1.3
IL1A	D01	1.9	1.0	3.1
IL1B	D02	2.1	1.1	-1.3
IL1R1	D03	2.6	1.7	2.1
IL6	D04	6.3	-1.7	1.6
IL8	D05	1.3	1.1	-1.1
IRAK1	D06	1.4	1.3	1.8

IRAK2	D07	1.6	1.3	1.6
JUN	D08	2.2	1.5	2.0
LTA	D09	2.5	1.3	1.4
LTBR	D10	-1.5	-1.0	1.3
MALT1	D11	-1.0	1.2	1.3
MAP3K1	D12	1.3	1.1	1.4
MYD88	E01	2.7	1.8	2.1
NLRP12	E02	4.0	1.0	2.3
NFKB1	E03	-1.3	1.2	1.2
NFKB2	E04	1.7	1.3	2.0
NFKBIA	E05	-1.2	1.6	1.4
PPM1A	E06	1.7	1.4	1.8
RAF1	E07	-1.3	1.1	1.1
REL	E08	12	15	17
RELA	E09	1.7	1.3	1.5
RELB	E10	1.1	1.1	1.9
TRIM13	E11	1.1	-1.0	1.0
RHOA	E12	-1.0	1.0	1.2
RIPK1	F01	-1.8	1.3	2.0
SLC20A1	F02	-1.1	1.3	1.5
STAT1	F03	1.1	1.2	1.5
TBK1	F04	-1.2	-1.1	1.1
TICAM2	F05	-1.2	1.1	1.0
TLR1	F06	5.6	1.0	1.4
TLR2	F07	2.0	-1.1	1.3
TLR3	F08	1.2	1.3	1.4
TLR4	F09	8.3	1.1	2.7
TLR6	F10	1.1	1.3	2.3
TLR7	F11	4.6	-3.0	-1.4
TLR8	F12	53	3.8	2.6
TLR9	G01	2.8	-5.0	-2.2
TMED4	G02	1.3	1.1	1.2
TNF	G03	-1.5	1.4	1.4
TNFAIP3	G04	1.2	-1.0	1.2
TNFRSF10A	G05	2.1	1.5	1.8
TNFRSF10B	G06	2.0	1.4	2.1
TNFRSF1A	G07	2.0	-1.2	1.6
CD27	G08	1.1	-1.0	1.4
TNFSF10	G09	1.0	1.4	1.5
TNFSF14	G10	1.2	1.8	4.2
TRADD	G11	-1.2	1.1	1.3
TICAM1	G12	1.4	1.2	1.7
B2M	H01	-1.2	-1.0	-1.0
HPRT1	H02	-1.3		-1.5
RPL13A	H03	1.3	-1.3	-1.1
GAPDH	H04	1.2	-1.1	1.4
ACTB	H05	1.1	1.3	1.2

Genes in the PCR Array

PCR Array Catalog #:			PAHS-025		Open Gene Table in Web Browser
Position	UniGene	RefSeq	Symbol	Description	
A01	Hs.19383	NM_000029	AGT	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	
A02	Hs.525622	NM_005163	AKT1	V-akt murine thymoma viral oncogene homolog 1	
A03	Hs.648565	NM_005171	ATF1	Activating transcription factor 1	
A04	Hs.193516	NM_003921	BCL10	B-cell CLL/lymphoma 10	
A05	Hs.31210	NM_005178	BCL3	B-cell CLL/lymphoma 3	
A06	Hs.69771	NM_001710	CFB	Complement factor B	
A07	Hs.696238	NM_001166	BIRC2	Baculoviral IAP repeat-containing 2	
A08	Hs.405153	NM_006092	NOD1	Nucleotide-binding oligomerization domain containing 1	
A09	Hs.2490	NM_033292	CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	
A10	Hs.655983	NM_001228	CASP8	Caspase 8, apoptosis-related cysteine peptidase	
A11	Hs.303649	NM_002982	CCL2	Chemokine (C-C motif) ligand 2	
A12	Hs.472860	NM_001250	CD40	CD40 molecule, TNF receptor superfamily member 5	
B01	Hs.390736	NM_003879	CFLAR	CASP8 and FADD-like apoptosis regulator	
B02	Hs.198998	NM_001278	CHUK	Conserved helix-loop-helix ubiquitous kinase	
B03	Hs.1349	NM_000758	CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	
B04	Hs.2233	NM_000759	CSF3	Colony stimulating factor 3 (granulocyte)	
B05	Hs.631631	NM_020428	SLC44A2	Solute carrier family 44, member 2	
B06	Hs.352224	NM_080738	EDARADD	EDAR-associated death domain	
B07	Hs.126667	NM_057159	EDG2	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	
B08	Hs.326035	NM_001964	EGR1	Early growth response 1	
B09	Hs.701980	NM_005229	ELK1	ELK1, member of ETS oncogene family	
B10	Hs.482562	NM_001992	F2R	Coagulation factor II (thrombin) receptor	
B11	Hs.86131	NM_003824	FADD	Fas (TNFRSF6)-associated via death domain	
B12	Hs.2007	NM_000639	FASLG	Fas ligand (TNF superfamily, member 6)	
C01	Hs.25647	NM_005252	FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog	
C02	Hs.708288	NM_000165	GJA1	Gap junction protein, alpha 1, 43kDa	
C03	Hs.517581	NM_002133	HMOX1	Heme oxygenase (decycling) 1	
C04	Hs.421649	NM_000867	HTR2B	5-hydroxytryptamine	

				(serotonin) receptor 2B
C05	Hs.707983	NM_000201	ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor
C06	Hs.37026	NM_024013	IFNA1	Interferon, alpha 1
C07	Hs.93177	NM_002176	IFNB1	Interferon, beta 1, fibroblast
C08	Hs.856	NM_000619	IFNG	Interferon, gamma
C09	Hs.656458	NM_001556	IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
C10	Hs.321045	NM_014002	IKBKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon
C11	Hs.43505	NM_003639	IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma
C12	Hs.193717	NM_000572	IL10	Interleukin 10
D01	Hs.1722	NM_000575	IL1A	Interleukin 1, alpha
D02	Hs.126256	NM_000576	IL1B	Interleukin 1, beta
D03	Hs.701982	NM_000877	IL1R1	Interleukin 1 receptor, type I
D04	Hs.654458	NM_000600	IL6	Interleukin 6 (interferon, beta 2)
D05	Hs.624	NM_000584	IL8	Interleukin 8
D06	Hs.522819	NM_001569	IRAK1	Interleukin-1 receptor-associated kinase 1
D07	Hs.449207	NM_001570	IRAK2	Interleukin-1 receptor-associated kinase 2
D08	Hs.525704	NM_002228	JUN	Jun oncogene
D09	Hs.36	NM_000595	LTA	Lymphotoxin alpha (TNF superfamily, member 1)
D10	Hs.1116	NM_002342	LTBR	Lymphotoxin beta receptor (TNFR superfamily, member 3)
D11	Hs.601217	NM_173844	MALT1	Mucosa associated lymphoid tissue lymphoma translocation gene 1
D12	Hs.657756	NM_005921	MAP3K1	Mitogen-activated protein kinase kinase kinase 1
E01	Hs.82116	NM_002468	MYD88	Myeloid differentiation primary response gene (88)

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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

Running title: Calcitriol regulates MIS promoter activity

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Abbreviations: AMH, anti-mullerian hormone; BSCL, Berardinelli-Seip type congenital lipodystrophy; ChIP, chromatin immunoprecipitation; GMSA, gel mobility shift assay; HVDRR, hereditary vitamin D resistant rickets, LBD, ligand binding domain; MIS, Mullerian inhibiting substance; MISRII, Mullerian inhibiting substance type II receptor; PMDS, persistent Mullerian duct syndrome; RXR, retinoid X receptor; SF-1, steroidogenic factor 1; VDR, vitamin D receptor, VDRE, vitamin D response element.

ABSTRACT

Calcitriol (1,25-dihydroxyvitamin D₃) inhibits the growth of a variety of cancer cells including human prostate cancer. Müllerian inhibiting substance (MIS) also exhibits anti-proliferative and pro-apoptotic 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 anti-proliferative activity. We identified a 15 bp sequence GGGTGAgcaGGGACA in the MIS promoter that was highly similar to DR3-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. Co-expression of steroidogenic factor 1 (SF-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 (RXR) bound to the MIS VDRE and the binding was increased by calcitriol. Chromatin immunoprecipitation assays showed that VDR and RXR were present on the MIS promoter in prostate cancer cells. In conclusion, we have demonstrated that MIS is a target of calcitriol action. MIS is upregulated by calcitriol via a functional VDRE that binds the VDR. Upregulation of MIS by calcitriol may be an important component of the anti-proliferative actions of calcitriol in some cancers.

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) have revealed multiple biological consequences of VDR signaling. More recently, it has been recognized that calcitriol has a much wider range of actions that include pro-differentiation, anti-proliferation, pro-apoptosis, immuno-suppression and anti-inflammation (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, immunologic diseases, diabetes, infection and psoriasis among others (7).

Mullerian-inhibiting substance (MIS, also known as Anti-Mullerian hormone or AMH) is a member of the transforming growth factor- β (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 ALK2 in order to initiate downstream signaling (9-11). In 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 where MIS inhibits steroidogenesis (13, 14) and in the postnatal ovary where 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 NF- 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 IB- α demonstrating that the growth inhibitory action of MIS is mediated by NF- κ B in prostate (23). Recently, we have shown that the MIS gene is upregulated 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 anti-cancer 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 to +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 4 copies of the MIS VDRE into the *Mlu* I site in pGL3-promoter luciferase reporter vector (Promega).

Gel Mobility Shift Assay (GMSA)

GMSA 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 over-expressing the VDR were incubated with 50 nM calcitriol prior to the addition of the [³²P]-labeled MIS VDRE probe followed by electrophoresis on non-denaturing gels and autoradiography as previously described (25).

Transactivation Assays

The MIS promoter-luciferase plasmids were transfected into HeLa cells using HeLa Monster (Mirus Bio Corporation, Madison, WI). Cells were also co-transfected with pSG5-VDR and a control plasmid pRLnull to control for transfection efficiency. The transfected cells were treated with calcitriol for 24 hr. Luciferase activity was determined using the dual luciferase assay (Promega) and a Turner luminometer. In some experiments, an expression plasmid for SF-1 was co-transfected and the luciferase activity measured following 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 hr 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'-TCTGAATAGGCTGTGGGGTC) genes were then amplified from the cDNA using SYBR-green qPCR kit (New England Biolabs, Ipswich, MA) and semi-quantified using real time PCR as previously described {Malloy, 2007 #2876}. Experiments were performed at least three times with duplicate determinations.

Chromatin immunoprecipitation (ChIP) Assay

ChIP assays were performed using the SimpleChIP enzymatic chromatin IP 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 hr 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), rabbit anti-RXR polyclonal antibody ΔN197 (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 hr. 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'-ACGGCCAGGCAACCAAACTAGACA) flanking the MIS VDRE and produce a 191 bp PCR product. Control primers 1664 bp upstream of the VDRE were MIS-2198069-U (5'-AGAAGGATCTGCATAGGCTGGGCA) and MIS-2198287-L (5'-TGAGGTCACCTTGGCAAAGGCCTCA) and produce 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 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 located upstream of known transcription factor binding sites for SF-1, SOX-9 and GATA-4 (Fig. 1B). The MIS VDRE exhibits a direct repeat 3 (DR-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 following treatment with calcitriol. As shown in Fig. 1D, in HeLa cells transiently transfected with the

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 versus 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.

Since SF-1 is a known major regulator of MIS promoter activity (27), we next determined the effects of co-expression 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 to transfection of VDR alone. A slight but significant increase in promoter activity was observed when the cells were treated 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 to vehicle control (Fig. 1D). The contribution of calcitriol and SF-1 gave a 7-fold increase in transactivation over basal and compared to 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 co-transfected 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 to 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 were cloned into pGL3-promoter, a heterologous promoter luciferase reporter vector containing an SV40 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 radio-labeled MIS VDRE probe, only a minor band shift was detected (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 (lane 4). Competition with unlabeled MIS VDRE WT sequence showed a decrease in band intensity (lanes 7-9) while 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 (lanes 10-12). Addition of VDR antibody 9A7 blocked the formation of the complex (lane 14) while addition of RXR antibody decreased the band intensity and supershifted some of the complex (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 calcitriol for six hours 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 hr prior to 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 both in the absence and presence of calcitriol. No bands were detected with the IgG control antibody. As a negative control, we amplified a region ~1.6 kbp upstream of the MIS translational start site (Fig. 6, lower panel). Only Histone H3 was detected on the negative control sequence. 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 upregulates 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). Co-expression 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 (tGGTGAcctGGGgCg, -260/-246), rat (tGGTGAcctGGGggA, -254/-240), and bovine (GGGTGAgcaaGcACg, -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 Mullerian structures during male fetal development (11), many post-natal actions have been documented. MIS exhibits important actions on steroidogenesis (13, 14), follicle development (15, 16, 29), ovarian and testicular function (30) and has been linked to polycystic ovarian disease (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 since a significant number of spliceosome associated protein 62 (SAP62) 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. Since 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. Dax-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 anti-cancer drugs (6). However, another reason for our interest in MIS was an observation in one of the cases of hereditary vitamin D resistant rickets (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 Mullerian 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 (MISRII) gene and is characterized by the presence of Mullerian derivatives in males (41). However, since 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 Mullerian 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 aromatase 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 while 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 1α -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. Since 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 lead 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 anti-cancer drug (18, 50, 51). Data indicate that MIS has anti-proliferative 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 while also promoting 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, pro-differentiation, apoptosis, anti-angiogenesis, inhibition of invasion and metastasis, and anti-inflammatory activity (6, 7). The findings presented here demonstrate that upregulating MIS is another anti-cancer action in the prostate. What proportion of the anti-proliferative 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 anti-cancer 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 anti-cancer agent.

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References

1. **Feldman D, Glorieux FH, Pike JW** 2005 Vitamin D, Second Edition. Elsevier Academic Press, San Diego
2. **Li YC, Pirro AE, Amling M, Delling 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 U S A* 94:9831-9835
3. **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
4. **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
5. **Malloy PJ, Pike JW, Feldman D** 2005 Hereditary 1,25-dihydroxyvitamin D resistant rickets. In: Feldman D, Glorieux F, Pike JW (eds) Vitamin D, Second Edition. Elsevier, San Diego, pp 1207-1238
6. **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
7. **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 Edition. Academic Press, San Diego, pp 317-382
8. **Massague J.** 1998 TGF-beta signal transduction. *Annu Rev Biochem* 67:753-791
9. **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
10. **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
11. **MacLaughlin DT, Donahoe PK.** 2004 Sex determination and differentiation. *N Engl J Med* 350:367-378
12. **Josso N, Clemente N.** 2003 Transduction pathway of anti-Mullerian hormone, a sex-specific member of the TGF-beta family. *Trends Endocrinol Metab* 14:91-97
13. **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 U S A* 98:3393-3397
14. **Fynn-Thompson E, Cheng H, Teixeira J.** 2003 Inhibition of steroidogenesis in Leydig cells by Mullerian-inhibiting substance. *Mol Cell Endocrinol* 211:99-104
15. **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
16. **Durlinger AL, Gruijters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JT, Grootegeed JA, Themmen AP.** 2002 Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. *Endocrinology* 143:1076-1084
17. **La Marca A, Volpe A.** 2007 The Anti-Mullerian hormone and ovarian cancer. *Hum Reprod Update* 13:265-273

18. **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 U S A* 103:17426-17431
19. **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 U S A* 102:111-116
20. **Hoshiya Y, Gupta V, Segev DL, Hoshiya M, Carey JL, Sasur LM, Tran TT, Ha TU, Maheswaran S.** 2003 Mullerian Inhibiting Substance induces NFkB signaling in breast and prostate cancer cells. *Mol Cell Endocrinol* 211:43-49
21. **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 U S A* 100:15601-15606
22. **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
23. **Segev DL, Hoshiya Y, Hoshiya M, Tran TT, Carey JL, Stephen AE, MacLaughlin DT, Donahoe PK, Maheswaran S.** 2002 Mullerian-inhibiting substance regulates NF-kappa B signaling in the prostate in vitro and in vivo. *Proc Natl Acad Sci U S A* 99:239-244
24. **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 NFkappa B-mediated pathway. *J Biol Chem* 275:28371-28379
25. **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
26. **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
27. **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
28. **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
29. **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*:18-21
30. **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
31. **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
32. **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
33. **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* 92:240-5

34. **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
35. **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
36. **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
37. **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 müllerian ducts in a Turkish boy born to consanguineous parents. *Am J Med Genet* 64:506-513
38. **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
39. **Magre J, Delepine M, Khallouf E, Gedde-Dahl T, Jr., 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, Navarro J, Nivelon-Chevalier A, Polak M, Robert JJ, Tric P, Tubiana-Rufi N, Vigouroux C, Weissenbach J, Savasta S, Maassen JA, Trygstad O, Bogalho P, Freitas P, Medina JL, Bonnicci F, Joffe BI, Loyson G, Panz VR, Raal FJ, O'Rahilly S, Stephenson T, Kahn CR, Lathrop M, Capeau J.** 2001 Identification of the gene altered in Berardinelli-Seip congenital lipodystrophy on chromosome 11q13. *Nat Genet* 28:365-370
40. **Ito D, Suzuki N.** 2007 Molecular pathogenesis of seipin/BSCL2-related motor neuron diseases. *Ann Neurol* 61:237-250
41. **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
42. **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 D $_3$. 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* 89:1364-70
46. **Nakhuda GS.** 2008 The role of mullerian inhibiting substance in female reproduction. *Curr Opin Obstet Gynecol* 20:257-64
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* 141:1317-24
48. **Panda DK, Miao D, Tremblay ML, Sirois J, Farookhi R, Hendy GN, Goltzman D.** 2001 Targeted ablation of the 25-hydroxyvitamin D 1alpha -hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. *Proc Natl Acad Sci U S A* 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* 86:393-7
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

TABLES

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

FIGURE LEGENDS

Figure 1. The human MIS promoter containing a putative VDRE is activated by calcitriol and steroidogenic factor 1 (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, SOX9 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 promoter-less 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 hr. Luciferase activity was measured using the dual luciferase assay. 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.

Figure 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 or calcitriol (cal) for 24 hr 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.

Figure 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 SV40 promoter in the reporter vector pGL3-promoter (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 hr 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.

Figure 4. The VDR binds to the MIS VDRE *in vitro*. A, [32 P]-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 6-17) all contained [32 P]-labeled MIS VDRE with pSG5-VDR plus 50 nM calcitriol. Lane 6, pSG5-VDR plus 50 nM calcitriol; lane 7-9 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 0.5X Tris-borate buffer. Bands were visualized by autoradiography. Arrowhead indicates VDR-RXR complex, grey arrow indicates undetermined complex, ss, indicates RXR antibody supershift complex.

Figure 5. The MIS gene is upregulated by calcitriol in prostate cancer cells. LNCaP and PC-3 cells were treated with graded concentrations of calcitriol for 6 hr. MIS gene expression was then analyzed by RT-qPCR. Values represent mean $\pm$ SD of triplicate experiments. Asterisks indicate calcitriol treatment significantly different from the vehicle-treated control * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.

Figure 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 hr at 37°C. The cells were then fixed with 1% formaldehyde for 10 min at ambient temperature. The samples were then analyzed by ChIP. A, PCR of MIS promoter containing VDRE; B, control PCR of region ~1.6kbp upstream of the MIS promoter. ChIP assays were performed with antibodies to normal rabbit IgG (IgG), histone 3 (H3), VDR and RXR. Std, 100 bp standard; In, input DNA extracted prior to ChIP assay.

Figure 1

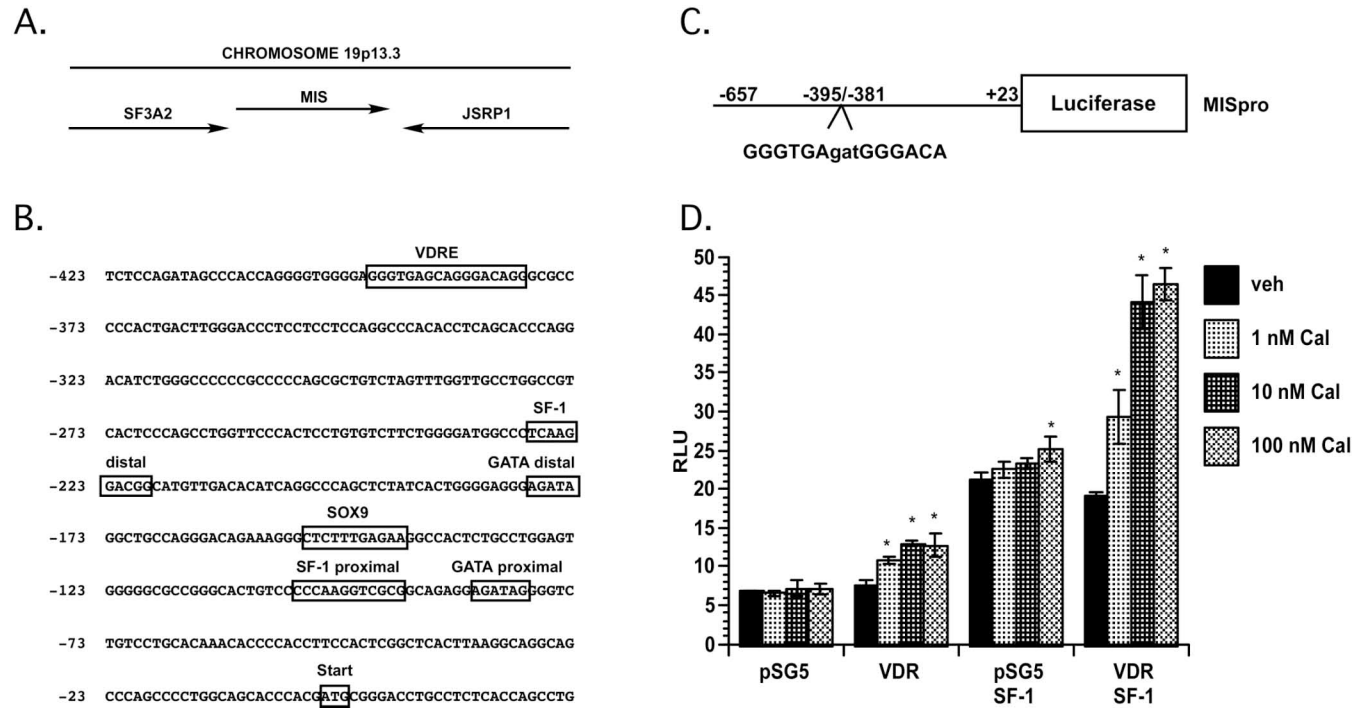


Figure 2

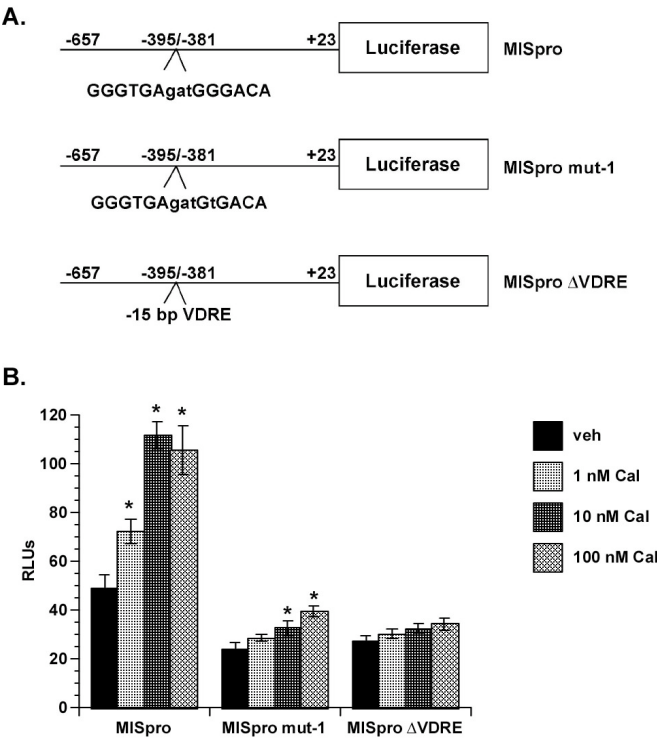


Figure 3

A.



B.

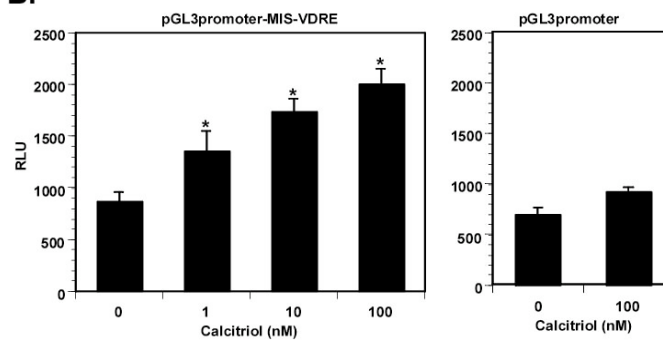


Figure 4

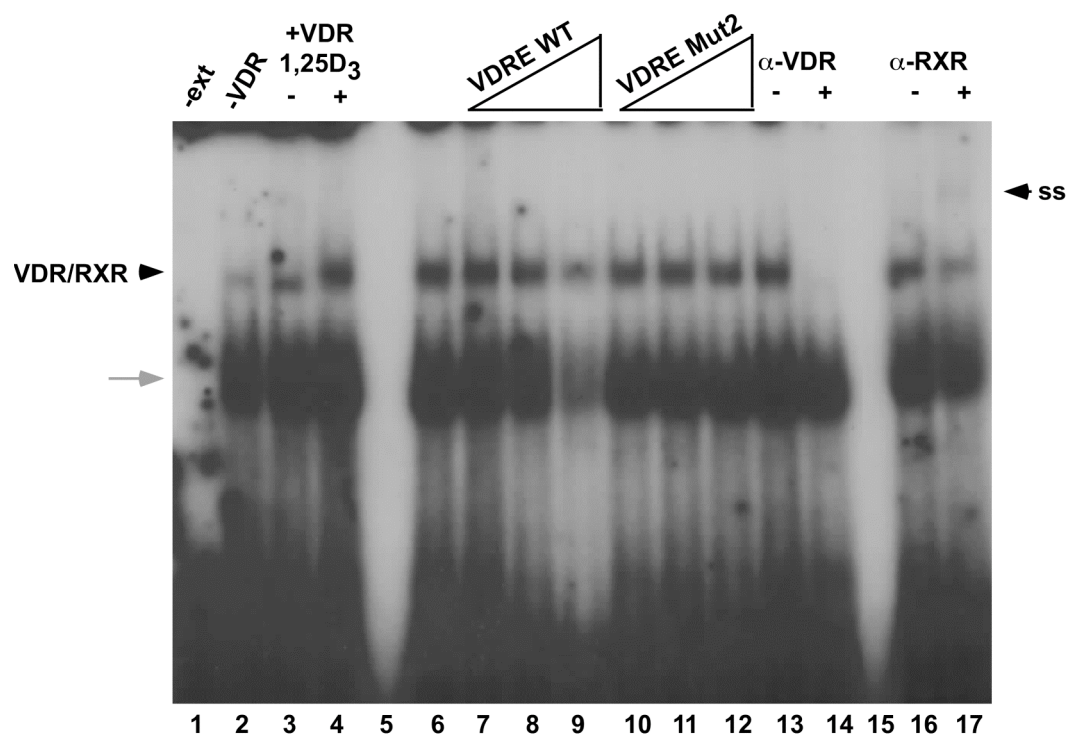


Figure 5

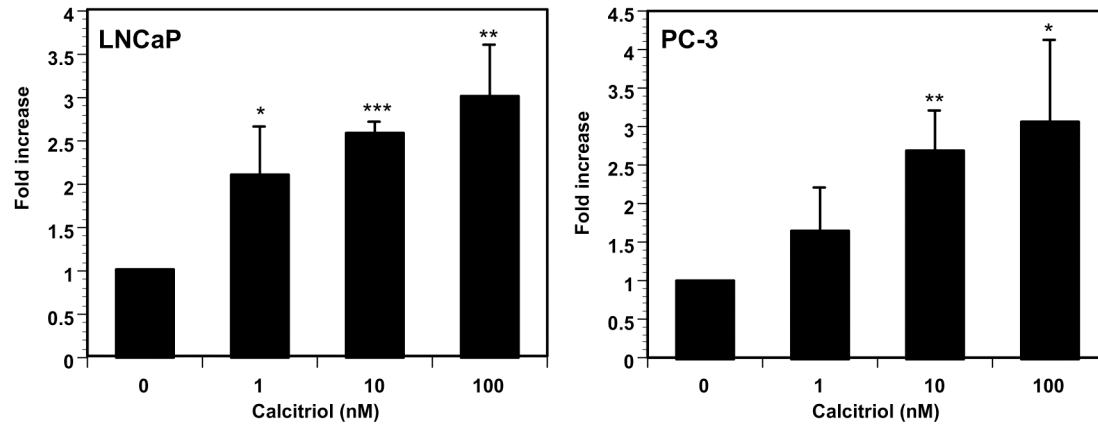


Figure 6

