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Distinct roles for AF-1 and -2 of ER-alpha in regulation of MMP-13 promoter activity

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Running Title: AF-1 and AF-2 domains of ER-α modulate MMP-13 activity

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Abstract

Previous studies have indicated that ER-α can influence the activity of the MMP-13 promoter. ER-α activity is mediated by two separate transcriptional activation domains (AF-1 and AF-2). The present study focused on analyzing the roles of these domains on the activation of the MMP-13 promoter. Transfection of synoviocytes with an ER-α construct lacking the C-terminus AF-2 domain led to significant elevation in MMP-13 promoter activity compared to wild type ER-α. Progressive deletions in the N-terminal AF-1 domain led to significant losses in MMP-13 promoter activity. MMP-13 promoter mutagenesis indicated that an AP-1 regulatory site was essential for ER-α mutant activity. Thus, both AF-1 and AF-2 domains of ER-α are required for regulation of MMP-13 promoter activity. As ER variants and ER related proteins have been implicated in bone and joint disorders, these findings provide understanding of the possible role of ER variants in the development of such conditions.
INTRODUCTION

Subsets of women are prone to developing osteoarthritis (OA) following menopause when estrogen levels in the body decline (1, 2). It is well documented that the enzyme matrix metalloproteinase 13 (MMP-13) effectively degrades collagen which is the principal constituent of cartilage, and thus may play a major role in the pathogenesis of diseases such as osteoarthritis (reviewed in 3, 4). Transgenic mice over-expressing MMP-13 show evidence of degradation of articular cartilage and exhibit joint pathology similar to those found in OA (5). Furthermore, studies have shown that ovariectomy of rats leads to elevations in the expression of the rodent MMP-13 in osteoblastic cells (6).

The expression of MMP-13 is low in the cartilage of normal skeletally mature adults and this likely enables healthy connective tissue remodeling without damaging the tissues (7). However, in osteoarthritis-like conditions the level of MMP-13 expression increases considerably (including in synovium), resulting in aberrant destruction of cartilage tissue (7, 8, 9, 10). Based on its importance in normal and disease states, better understanding of the potential mechanisms by which MMP-13 expression is regulated by ER-α (+/- estrogen) may provide valuable insights into the onset and progression of OA, particularly in a subset of post-menopausal females.

The detection of estrogen receptors (ER) in several tissues of the joint indicates that these tissues are likely responsive to the hormone estrogen. Several studies have established the presence of both MMP-13 and ER in joint tissues (11, 12, 13, 14, 15) and parallel in vitro studies have indicated that both alpha and beta isoforms of ER (ER-α and ER-β) can elevate the activity of the MMP-13 promoter and this modulation is decreased depending upon whether or not the ER-α or ER-β is complexed with estrogenic ligands (16, 17). It is well established from previous studies that both the alpha and beta isoforms of ER function as ligand-inducible transcription factors and regulate the expression of estrogen responsive genes (reviewed in 18, 19). Transcriptional activation or repression of genes by ER occurs through the estrogen
response element (ERE) or through ER tethering to other transcription factors that act through the AP-1 or other transcriptional regulatory sites often present in the promoter (20, 21). Recent studies have shown that ER-α can regulate the expression of a member of the matrix metalloproteinase family of genes – MMP-13, primarily through the AP-1 transcriptional regulatory site (16).

ER-α is a modular protein that is comprised of six functional domains denoted A-F, each with specific functions, such as ligand binding, dimerization and DNA binding and transactivation. The activating potential of ER-α is mediated by two separate transcriptional activation domains (AF-1 and AF-2) that allow it to stimulate the transcription of specific genes (22). The relative activities of the AF-1 and AF-2 domains of ER-α vary depending upon the cellular environment and the promoter of the specific regulated gene. Furthermore, since the AF-1 and AF-2 domains are located at the amino and carboxyl terminal ends of the protein, respectively, synergism between the two AF sites via coactivators may also play a role in exerting their influence on downstream promoters (23).

Further to this discussion, naturally occurring variants of ER that lack the ligand binding exon, apparently through alternative splicing, have been reported in breast cancer tissue (24) and in ligaments (11). In addition, a family of estrogen receptor related proteins (ERR), also lacking ligand binding potential have been described in bone and other tissues (25). Recent studies have indicated that expression of some of these ERR can be modulated in joint tissues during inflammatory joint disease development (26). Therefore, not only is it of interest to understand the basic biology of ER variants, there may also be clinical relevance to their presence.

The presence or absence of estrogen has an influence not only on the MMP-13 activity in synoviocytes (16), but also on other MMPs such as MMP-2, MMP-7 and MMP-9 which are expressed in tissues such as the cornea (27). With the aim of increasing our understanding of the role of ER in regulation of MMP-13, in the present study we have analyzed the roles of the
activation functions and ligand binding domains of ER-α on the activation of the MMP-13 promoter in the presence and absence of estrogen in a rabbit synoviocyte cell line.

**Methods and Materials**

**Cell culture** --- The rabbit synoviocyte cell line HIG-82 was obtained from the American Type Culture Collection (Rockville, Md). Cells were maintained at 37°C under 5% CO₂ in Ham’s F-12 Nutrient Media (Life technologies) supplemented with 10% Fetal calf serum (InVitrogen Canada Inc.) and 1% Penicillin/Streptomycin. Cells were subcultured 1:4 with 0.25% trypsin. This cell line was previously shown to be negative for endogenous ER-α and ER-β expression (16,17).

**Construction of rabbit MMP-13 Luciferase reporter vector** -- The promoterless pGL2 Basic luciferase reporter vector (Promega, Madison, WI) was used to construct a plasmid containing the proximal 370bp of the rabbit MMP-13 promoter. The MMP-13 promoter construct p370-Luc was generated by PCR using a 5′-sense primer containing the appropriate MMP-13 promoter sequence with a Kpn-I site introduced at the 5′-end and the same 3′-antisense primer 5′-CCAACAGTACCGGAATGCCAAGC-3′ (nucleotide 49 to 71) that corresponds to a pGL2 Basic sequence downstream of the multiple cloning site. The 5′-primer used for p370-Luc construct contained MMP-13 sequence (underlined) was

-370 to –356 (5′-CGGGTACCATGGGCGTACACATA-3′).

PCR products were purified and precipitated by the QIA quick-spin PCR purification kit (Qiagen) and digested with KpnI/HindIII. The final product was then ligated into KpnI/HindIII digested pGL2 Basic and transformed into E.coli (DH-5α). Mutations were introduced into desired constructs using the Quick Change Site-Directed Mutagenesis kit (Stratagene). The mutagenic oligonucleotide primers were designed and synthesized for each mutant. The AP-1 site TGACTCA was changed by mutating TGA to ACT at the first three bases. The PEA-3 site consensus sequence AGGAAGC was altered to AACCAAGC and the Runx domain binding
sequence (RD site) AACCACA was changed to AGACACA. All reporter plasmids were purified on EndoFree Plasmid Maxi Kits (Qiagen, Mississauga, ON) according to the manufacturer’s instructions and subjected to sequencing analysis to verify the orientation and accuracy of each construct.

**Additional Control and ER-α Expression Plasmids** -- The internal control plasmid pRLSV40 was obtained from Promega (Promega, Madison, WI). The pRLSV40 plasmid constitutively expresses the Renilla form of luciferase under the strong SV40 promoter and thereby acts as an internal control in the dual luciferase assay. The expression vectors for ER-α such as WT-ERα, E41, A87, M109, ΔAB and ABCD were those used previously and were all subcloned into the same vector, pCMV5 (28).

**Subcloning of ΔAB, ABCD into the pSG5 vector** - The ΔAB and ABCD constructs were subcloned from pCMV5 (28) to the pSG5 vector (Stratagene, USA). The sequence and orientation of the sub-cloned fragments was confirmed by sequencing.

**Transient Transfection and Luciferase assay** -- The rabbit synoviocyte cell line, HIG-82, was transfected using the FuGene6 Transfection reagent (Roche Molecular, Indianapolis, IN) as directed by the manufacturer. HIG-82 cells were seeded on 12-well plates on the 24 hours before transfection at a density of $1 \times 10^5$ cells/well. The cells were washed with PBS prior to transfection and then transfected with a constant amount (1µg) of reporter plasmids (shown to be optimal in preliminary studies; data not shown), pRLSV40 (50 ng) and WT-ERα or ABCD or ΔAB (0.25 µg - 1 µg) using the Fugene 6 reagent. Transfection was performed in Ham’s F-12 Nutrient Medium in the absence of serum for a period of 24 hours. Immediately, after transfection, (10^{-12} to 10^{-8} M) 17β-estradiol (E_2) (Sigma, Oakville, ON) was added to the medium of appropriate wells in specific experiments. Twenty four hours after transfection and (+/-) ligand treatment, the medium was removed from the cells and the cells were washed once with phosphate buffered saline (PBS). The cells were then lysed directly in the wells with 1X Passive
Lysis Buffer (Promega Corp.). Luciferase activity was determined on cell lysates using a Turner TD-20 illuminometer and the Dual Luciferase™ Reporter Assay kit (Promega Madison, WI). All transfection experiments were repeated three or more times with results very similar to those reported.

**Western Blotting:** Western blotting was conducted on extracts of cells transfected with either WTER-α (Full Length) or ABCD or ΔAB, sub-cloned into the pSG5 vector to assess ER protein being expressed in transfected cells. Protein extracts of non transfected HIG-82 cells were used as negative controls for ER expression. Cells were transfected as described above, and were collected 24 hours later. The cell pellet was washed with PBS buffer (1X) and then protein was extracted using a mammalian cell lysis kit (Sigma-Aldrich Inc., USA). Aliquots of protein lysates were separated using 10% SDS-PAGE gels at 130 Volts for 70 mins. The separated proteins were then transferred to nitrocellulose membranes by standard methods and incubated with optimal dilutions of rabbit polyclonal antibodies for either the N-terminal of ER-α (H-184 obtained from Santa Cruz Biotechnology Inc. with epitope corresponding to amino acids 2-185 mapping at the N-terminus of estrogen receptor α) or the C-terminal of ER-α (HC-20 obtained from Santa Cruz Biotechnology Inc with epitope mapping at the C-terminus of ERα of human origin). The blots were washed 3X with PBS buffer containing Tween20 (0.025%) and then incubated with the secondary antibody (anti-rabbit HRP obtained from Amersham Inc, USA). The Amersham ECL™ western blotting reagents (GE Healthcare, UK) were used to detect the bands.

**Real Time PCR analysis:** Real Time PCR was performed to determine the influence of WT, ABCD or ΔAB constructs of ER-α on endogenous MMP-13 mRNA levels in HIG-82 cells following transfection. HIG-82 cells were cultured on 6-well plates on the day before transfection at a density of 1×10^5 cells/well. The following day, cells were transfected with WT-ERα, ABCD or ΔAB at concentrations ranging from 0.25 to 1.0 µg. After 24 hours of transfection, cells were
washed with PBS, total RNA was isolated using the TRIspin method (29) and then the SYBR®
Green reagent was subsequently used for fluorometric quantification of total RNA. One
microgram of total RNA from each sample was reverse transcribed using the Omniscript RT kit
(Qiagen Inc., Mississauga, ON) using random primers and Omniscript reverse transcriptase,
and diluted to 500 µL/µg RNA. All samples in an experiment were subjected to RT at the same
time to avoid variability.

Real-time PCR was performed on the RT preparations with rabbit specific PCR-primers
for amplifying the MMP-13 (Forward primer: 5′-TGGTCTTTCTTGCTACGCTTT-3′ and Reverse
primer 5′-ACTCTGCGGTGTAAGGTGT-3′ and probe: 5′-
TTGCTGCCCATGAGTTGGCCATTCCTTT-3’) and the housekeeping GAPDH gene (Forward
primer 5′-GCAGGATGCGTTGCTGACAATC-3′ and reverse primer 5′-
AGTATGATTCACCACACGGAAGT-3′). The reaction mixture for quantification of GAPDH
contained 7.5 µL RT (described above), 0.75 µL each of forward and reverse primer (10 µM),
12.5 µL Bio Rad iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), and 3.5 µL molecular
biology grade water, for a total reaction volume of 25 µL. For MMP-13 assessment, the reaction
mixture contained 0.75 µL each of forward and reverse primer (10 µM) along with 2.5µL of
probe Amplification and detection were performed using an iCycler Thermal Cycler (Bio-Rad,
Hercules, CA). The PCR program consisted of 3 minutes at 95°C for Taq activation, followed by
40 cycles of PCR amplification (30 seconds at 95°C, 30 seconds at 65°C, and 30 seconds at
72°C) and 1 minute at 95°C. At the completion of each run, melting curves for the amplicons
were measured by lowering the temperature slowly from 95 to 15°C while monitoring
fluorescence. The specificity of the PCR amplification was checked by examining the melting
curve (dF/dT vs temperature) for non-specific peaks. GAPDH levels were used as a reference
for normalization and relative quantification was analyzed using iCycler iQ Optical System
Software Version 3.0a (Bio-Rad, Hercules, CA).
**Statistical Analysis:** Statistical analysis of the data was performed using ANOVA, STDEVA, and STEYX in the Excel 5.0 software.

**Results**

3.1. **Protein levels of WT-ERα, ABCD DNA and ΔAB expressed in the transfected HIG-82 cells:**

The present study was focused on deciphering the possible roles played by the functional domains of ER-α in the modulation of MMP-13 promoter activity. A schematic representation of the different domains present in the WT-ERα, ABCD and ΔAB constructs is shown in Figure 1A. The WT-ERα contains the N-terminal AF-1 domain, central DNA binding domain and the C-terminal AF-2 / Ligand binding domain. As depicted in Figure 1A, the ABCD mutant of ER-α is deficient in the AF-2 and the ligand binding domains and the ΔAB is a mutant form of ER-α in which the AF-1 domain has been deleted.

As a first step, western blotting was performed to ensure that ΔAB, ABCD and WT-ERα were expressed in the HIG-82 cells following transfection. Prior to performing the western blotting, the ΔAB, ABCD and WT-ERα were first sub-cloned into the pSG5 vector from the pCMV5 vector as described in Material and Methods. The protein expression for WT-ERα and ΔAB was detected using the rabbit polyclonal antibody (HC-20) which recognizes the C-terminal portion of ER-α antibody (See Figure1B). As the ABCD construct is deficient in part of the C-terminus, hence it is not recognized by the HC-20 antibody. However, ABCD expression was detected when rabbit polyclonal antibody (H-184) to the N-terminus of the estrogen receptor α of human origin was used (see Figure 1B). The results from the western blot analysis indicate that ABCD, ΔAB and WT-ERα are all expressed at somewhat similar levels in the HIG-82 cells. Therefore, the increased or decreased levels of MMP-13 activity observed when ABCD or ΔAB is the modulator is likely not due to overtly unequal expression of ER-α mutants.

3.2. **Comparing the influence of increasing concentration of WT-ERα and ABCD DNA on endogenous and exogenous MMP-13 promoter activity**
Next, we examined the effect of WT-ERα and the ABCD mutant of ER-α (lacking the hormone binding and AF-2 domain) on endogenous MMP-13 mRNA levels in HIG-82 cells using real-time PCR. Non-transfected HIG-82 cells or cells transfected with increasing concentrations (0.25, 0.5, and 1 µg) of WT-ERα or ABCD plasmid DNA were analyzed 24 hours post-transfection for MMP-13 mRNA. The results presented in Figure 2A indicate that endogenous MMP-13 expression is influenced by the WT-ERα and ABCD mutant of the estrogen receptor alpha as endogenous MMP-13 mRNA levels were found to significantly increase following transfection with increasing concentrations of DNA. The results presented in Figure 2A suggest that transfection with 0.25 µg or 0.5 µg of WT-ERα did not have a significant impact on endogenous MMP-13 mRNA levels, although MMP-13 mRNA levels did increase at these DNA levels when ABCD was the modulator. However, mRNA levels for MMP-13 were increased by ~2.0 fold when the concentration of WT-ERα was increased to 1 µg. A 2-fold increase in the endogenous levels of MMP-13 was observed when 1 µg of ABCD was the modulator compared to when 1 µg of WT-ERα was the modulator. Thus, the ABCD construct lacking the AF-2 domain was a more effective modulator of endogenous MMP-13 mRNA levels than was the WT-ERα construct. The ΔAB ER construct, lacking the AF-1 domain did not influence endogenous MMP-13 mRNA levels at any of the concentrations tested (data not shown), findings consistent with results presented below.

The effect of increasing concentrations of the mutant truncated form of ER-α (ABCD) on MMP-13 promoter luciferase activity was examined in HIG-82 cells and again compared with the influence of increasing concentration of WT-ERα. The HIG-82 cells were transiently transfected with the WT-ERα or ABCD constructs at increasing concentrations ranging from 0 to 1µg, along with the p370-Luc MMP-13 promoter and the internal control plasmid, pRLSV40 (see Figure 2B). After 24 hours, the luciferase activities of the cell lysates were determined.
The results presented in Figure 2B demonstrate that increasing concentrations of ABCD and WT-ERα DNA led to corresponding elevations in MMP-13 promoter activity. MMP-13 activity was elevated to a greater extent under the influence of ABCD compared to WT-ERα. MMP-13 promoter activity was increased by ~30 fold by 1 µg of ABCD when present as the modulator, while only a ~10 fold increase was observed with the same concentration of WT-ERα (see Figure 2B). In general, MMP-13 promoter activity was elevated ~ 3 to 4 fold higher when the ABCD mutant of ER-α was the modulator as compared to the WT-ERα, across the concentration range assessed. HIG-82 cells transfected with only the p370-Luc construct (1.0 µg) and the internal control plasmid, pRLSV40 (0.05 µg) were used as a control and very low levels of MMP-13 activity were detected. These results indicate that when the AF-2/Ligand binding domains were deleted from ER-α constructs, a greater stimulation of MMP-13 promoter activity was observed than with equivalent doses of WT-ERα.

3.3. Comparing the influence of increasing concentration of WT-ERα and ΔAB DNA on MMP-13 promoter activity

Next, the effect of increasing concentrations of WT-ERα DNA was compared with the influence of ΔAB on MMP-13 promoter activity. HIG-82 cells were transiently transfected with either WT-ERα or ΔAB at increasing DNA concentrations ranging from 0 - 1 µg, concurrent with the p370-Luc construct (1.0 µg) and the internal control plasmid, pRLSV40 (0.05 µg). After 24 hours, the luciferase activity of the cell lysates were determined. In the absence of exogenous WT-ERα or ΔAB, minimal MMP-13 promoter activity was again detectable (see Figure 3).

The results presented in Figure 3 demonstrate that increasing concentrations of WT-ERα DNA again led to corresponding elevations in MMP-13 promoter activity. However, no significant stimulation in MMP-13 promoter activity was observed when ΔAB was the modulator, even when ΔAB levels were increased to 1 µg. In contrast, in the positive controls a 5-fold increase in MMP-13 promoter activity was observed when 0.25 µg of WT-ERα was used. Furthermore, increasing the amount of WT-ERα DNA, to 0.5 and 1 µg led to ~ 9 and ~ 11 fold
elevations in the activity of MMP-13 (See Figure 3). These results indicate that the presence of the AF-1 domain of ER-α is essential for up-regulating the activity of the MMP-13 promoter.

3.4 Influence of progressive deletions in the AF-1 domain of ER-α on MMP-13 promoter activity.

The studies described above indicated a strong positive influence of the AF-1 domain of ER-α on the activity of the MMP-13 promoter. To further characterize the AF-1 domain for its role in mediating the effects of ER-α, a series of progressive AF-1 deletion constructs that led to deletion of regions corresponding to the N-terminal 40, 86, 108 or 180 amino acids, were utilized (28); see Fig.4 panel A). The complete N-terminal AF-1 domain of ER-α encompasses amino acids 1-180 (20). The deletion construct E41 is missing the bases corresponding to the first 40 amino acids of the AF-1 domain, A87 is missing the bases corresponding to the first 86 amino acid residues, M109 lacks the bases corresponding to the first 108 amino acid residues, and ΔAB is devoid of the entire AF-1 domain (bases corresponding to the 180 amino acids of the N-terminal region).

These constructs were evaluated using transient transfection assays in HIG-82 cells. The HIG-82 cells were transfected with p370-Luc (1 µg), one of the deletion constructs of AF-1 domain of ER-α (0.5ug) and the internal control plasmid, pRLSV40 (0.05ug) following the FuGene 6 transfection protocol. After 24 hours, the transfected cells were lysed and analyzed for MMP-13 promoter activity using luciferase assays.

The MMP-13 promoter activity observed with WT-ERα was set at 100% as presented in Figure 5B. When the E41 construct of ER-α was used, a mean 23% decrease in MMP-13 promoter activity was observed. Use of the A87 deletion construct led to a mean 55% decrease in MMP-13 promoter activity. Increasing the extent of the deletion in the AF-1 domain (M109 construct) led to a mean 63% decrease in MMP-13 promoter activity, and complete elimination of the AF-1 domain led to a mean 70% decrease in MMP-13 promoter activity. These results
indicate that progressive deletions across the AF-1 domain of ER-α lead to an increasing loss in the ability of ER to regulate MMP-13 promoter activity. However, even the complete removal of the AF-1 failed to completely eliminate ER influence on MMP-13 promoter activity, suggesting minor roles for other domains of ER-α.

3.5 Addition of the estrogenic ligand, 17-β Estradiol, leads to repression of MMP-13 promoter activity in the presence of WT-ERα and ΔAB, but no repression is observed when ABCD is the modulator.

The above results indicated that WT-ERα and ABCD are activators of MMP-13 transcriptional activity in the absence of any estrogenic ligand. Interestingly, ΔAB appears to be a very poor modulator of MMP-13 promoter activity under the same conditions. Subsequently, the influence of the estrogenic ligand, 17-β estradiol, was assessed for impact on WT-ERα, ABCD, or ΔAB mediated alterations in MMP-13 promoter activity. The HIG-82 cells were transfected with p370-Luc (1 ug), WT-ERα or ABCD or ΔAB (0.5ug) and the internal control plasmid, pRLSV40 (0.05ug) following the FuGene 6 transfection protocol. One set of transfected cells remained untreated, while other sets were exposed to increasing concentrations of 17-β estradiol (10^{-12}, 10^{-10} and 10^{-8} M).

As shown in Figure 5A, in the presence of WT-ERα alone, MMP-13 promoter activity was again elevated. However, an approximately ~60% decrease in MMP-13 promoter activity was observed when 10^{-12} M 17-β estradiol was added (see Figure 5A). Following further increases to 10^{-10} or 10^{-8} M estradiol, ~78 and 75% decreases in MMP-13 promoter activity were observed, respectively, with WT-ERα (see Figure 5A). These results suggest that increasing concentrations of 17-β estradiol led to a relative decrease in MMP-13 promoter activity in the presence of WT-ERα. Further, this experiment also indicated that 10^{-8} M 17-β estradiol is likely a saturating amount of hormone for the amount of receptor generated in the transfection experiments.
Consistent with the absence of the ligand binding domain, increasing concentrations of estradiol did not lead to decreases in MMP-13 promoter activity when ABCD was the modulator. MMP-13 promoter activity was again found to be elevated in the presence of ABCD when no estrogenic ligand was present. However, addition of increasing concentrations of 17-β estradiol did not lead to any significant decreases in MMP-13 promoter activity (See Figure 5B), as would be predicted because of the lack of a hormone binding domain.

While the ΔAB construct exhibited very modest influences on MMP-13 promoter activity (Figure 3), addition of 17-β estradiol did however exert a detectable effect on this residual activity, but only at the highest concentrations of estradiol tested (Figure 5C). Thus, at concentrations of 10^{-10} and 10^{-8} M 17-β estradiol, the ΔAB mediated MMP-13 promoter activity decreased by approximately 60 and 50%, respectively. Therefore, while the influence of the mutant ΔAB construct on MMP-13 promoter activity was considerably less than that of the WT-ERα, it was still modulated further by 17-β estradiol, consistent with the presence of the ligand binding domain in this N-terminally truncated ER.

3.6 Functional characterization of the MMP-13 promoter when modulated by WT-ERα, ABCD or ΔAB.

The results of the previous experiments demonstrated that MMP-13 promoter activity is modulated differently by WT-ERα, ABCD or ΔAB in the presence and absence of estrogen. The next step was to identify the specific elements in the MMP-13 promoter that play a role in ER-α mediated activity. The previously generated MMP-13 mutant promoter constructs (Lu et al. 2006) were used for these studies (See Figure 6A). In order to characterize the role of promoter elements of the rabbit MMP-13, DNA constructs with specific regulatory sites mutated (Runx: M370R2; PEA-3: M370P2; AP-1: M370A3 and AP-1+Runx+PEA-3 : M370A3R2P2) were transiently transfected into the HIG-82 cells along with either the WT, ABCD, or ΔAB mutants of ERα and the internal control plasmid, pRSV40L.

A. Modulation of MMP-13 promoter activity by WT-ERα
In the presence of WT-ERα, the MMP-13 promoter constructs exhibited varying degrees of activity proportional to the presence and role of each regulatory site. Maximum activity of the promoter was achieved when the MMP-13 promoter construct (p370-Luc) contained all the known transcription factor binding sites – the Runx, p53, PEA-3 and AP-1 sites. The activity level of cells transfected with wild type MMP-13 promoter (p370-Luc) was used as a control for this experiment and activity was set at 100%.

Site directed mutations in the Runx domain changed the normal Runx site of (AACCACA) to (AGACACA). As shown in Figure 6 B, a 22% decrease in luciferase activity was observed when only the Runx site was mutated (M370R2) compared to the activity levels of WT370 (Figure 6 B). The promoter activity of WT370 decreased by 60% when 17-β estradiol was present (Figure 6 B). In the presence of estradiol, the residual promoter activity of M370R2 decreased by 43%, compared to its activity in the absence of ligand (Figure 6 B).

Mutations in the PEA-3 site, which changed the sequence from AGGAAGC to AACAAGC (M370P2), resulted in a ~25% decrease in activity of the MMP-13 promoter when compared to control values (Figure 6 B). Addition of estradiol led to a ~52% decline in residual M370P2 activity as compared to its activity in the absence of any ligand. These results indicate that mutation in either the Runx or the PEA-3 site leads to similar decreases in MMP-13 promoter activity, and that estradiol was still an effective modulator of ER activity.

The AP-1 site was mutated in M370A3 and three bases were mutated to change the sequence from TGACTCA to ACTCTCA. A significant reduction in the activity of the MMP-13 promoter was observed (~87%) when the AP-1 domain was mutated as compared to the WT370 values (see Figure 6 B). Furthermore, under the influence of 17-β estradiol a further ~50% reduction in the residual activity of the M370A3 promoter was observed (see Figure 6 B).

Subsequently, the effect of mutating all three of the regulatory sites in the same construct was examined. Even when all three sites, i.e. AP-1, PEA-3 and Runx (M370A3P2R2) were mutated, MMP-13 promoter activity was depressed by ~86%, which is nearly identical to that
observed for the AP-1 mutant alone (~87%). These results indicate that AP-1 is likely the major element in regulating MMP-13 promoter activity in response to WT-ERα, and other domains such as Runx and PEA-3 play minor roles in modulating its activity.

**B. Modulation of MMP-13 promoter activity By ΔAB**

As shown in Figure 6 C, the activity levels of WT370 modulated by ΔAB in the absence of any ligand was set at 100%. A ~50% decrease in the WT370 activity level was observed when 17-β estradiol was present (see Figure 6 C). Next, the activity of the mutant M370R2, with a mutation in the Runx domain, or M370P2, with a mutation in the PEA-3 domain with ΔAB (+/-) 17-β estradiol was examined. The impact of these MMP-13 mutations was insignificant when ΔAB was the modulator (Figure 6C). Thus, mutations in the Runx and PEA-3 domains do not significantly impact MMP-13 promoter activity in the presence of the ΔAB mutant of ER-α. The activity levels of these mutants (M370R2 & M370P2) did decrease by ~50% in the presence of estradiol compared to their residual activity levels in the absence of ligand (Figure 6C, lanes 2B and lanes 3B).

A significant decrease (> ~80%) in promoter activity was observed when the AP-1 mutant (M370A3) was assessed along with ΔAB (+/-) estradiol (see Figure 6 C). Similar trends in the activity were observed for the mutant, M370A3P2R2, in which all the three regulatory sites are mutated (see Figure 6C). Therefore, the residual activity of ΔAB on the MMP-13 promoter was still mainly mediated via the AP-1 site.

**C. Modulation of MMP-13 promoter activity By ABCD**

As exhibited in Figure 6 D, promoter activity levels were the highest when all the transcriptional regulatory elements were intact as in the WT370 MMP-13 promoter construct, and when the ABCD mutant of ER-α was the modulator, and this was not altered in the presence of estrogen (See Figure 6 D). In contrast to findings with ΔAB and WT-ERα, the stimulatory activity of ABCD was not affected by mutations in the Runx (M370R2) or PEA-3 (M370P2) domains (Figure 6 D). Only when the mutations were in the AP-1 domain did the
MMP-13 construct show a ~80% decrease in activity in the presence of ABCD (Figure 6 D). Mutations in all three transcriptional regulatory motifs did not lead to further decreases in the activity of the MMP-13 promoter (see Figure 6 D), and the results were similar to those observed for M370A3 alone.

Furthermore, no decreases in the activity of either the WT370 or the mutant M370R2, M370P2 and M370A3 promoters was observed in the presence of the ligand, 17-β estradiol when ABCD was the modulator (See Figure 6D). These results again indicate that the ABCD construct is unable to respond to 17-β estradiol and that the AP-1 regulatory site of MMP-13 is the major regulatory site required for mediating the effects of ABCD (+/-) ligand.

Discussion

The results presented in this study in synoviocyte cells suggest that both AF-1 and AF-2 / ligand binding domains of ER-α play a role in modulating the activity of the MMP-13 promoter. Complete deletion of the AF-1 domain of ER-α (ΔAB construct) led to significant reductions in MMP-13 promoter activity when compared to the influence of WT-ERα on MMP-13 activity (see Figure 3B). Since the AF-2 / ligand binding domain is intact in the ΔAB construct, it is still able to mediate the effects of the ligand, 17-β estradiol, and addition of the hormone led to decreases in the residual MMP-13 promoter activity (see Figure 4C). Conversely, a constitutive elevation in the activity of the MMP-13 promoter was observed when the ABCD mutant ER with a deletion of the AF-2/ligand binding domain was used as the modulator. As expected, no decreases in the activity of the MMP-13 were observed when 17-β estradiol was present with ABCD as the modulator. Furthermore, similar protein expression of ABCD, ΔAB and WT-ERα was detected in western blot analysis; thereby ruling out the possibility that overexpression of ABCD can lead to higher activity of MMP-13. Our analysis of the MMP-13 promoter through site-directed mutagenesis studies have shown that regulation of MMP-13 promoter activity by WT, ABCD or ΔAB constructs of ER-α occurred primarily through the AP-1 transcriptional regulatory site. However, for full induction of MMP-13 promoter activity, other
regulatory sites such as Runx and PEA-3 also contributed in a minor fashion to the activation elicited by ER-α.

Previous studies have shown that both AF-1 and AF-2 regions of ER-α are required for full ER activity in most cellular systems (30, 23) Tora et. al.1989, McInerney et. al.1996). Moreover, it is also well recognized that the activation function of both the AF-1 and AF-2 domains of ER-α vary in a promoter and cell type specific manner (30, 31, 32). Previous reports have shown that the activity of the AF-2 domain is controlled by the hormone, estrogen (30). The ABCD mutant of ER-α used in this study lacks the AF-2/ligand binding domain. Therefore, decreases in MMP-13 activity in the presence of 17-β estradiol were not observed when ABCD was the modulator. The present observations are further corroborated by studies reported by An et al. (1999) (33) which suggested that the AF-2 domain is required for the estrogen mediated transcriptional repression of TNF-α since mutations in the AF-2 domain impair this repression.

Using both western blot analysis and immunohistochemistry previous observations have suggested that the HIG-82 cell line is devoid of detectable endogenous ER (16). Therefore, the influence of endogenous ER-α on the MMP-13 promoter activity can be ruled out. The results obtained from real time PCR experiments indicated that endogenous MMP-13 activity is stimulated by WT-ERα and the ABCD mutant, but not the ΔAB mutant, when they are introduced into the HIG-82 cell line (see Figure 1). The HIG-82 cell line which usually demonstrates a low constitutive expression of MMP-13 mRNA, showed a ~2-fold increase in the presence of WT-ER-α, and when the ABCD mutant of ER-α was the modulator, a ~4-fold increase in endogenous MMP-13 mRNA was observed.

In the present studies, MMP-13 promoter activity was found to be very low when ΔAB was the modulator as compared to the influence of the WT- ERα (see Figure 3B). The ΔAB is a deletion mutant of ER-α which is missing the entire AF-1 domain. The AF-1 domain is well characterized as the ligand independent transactivation domain of the ER-α and is speculated to synergize with the AF-2 domain to elicit the overall estrogen response on promoters
containing classical estrogen response elements (ERE) (30, 34, 20). Recently, it was shown that the cyclin G2 gene is down-regulated by ER-α when it is occupied by estrogen (35). Furthermore, a detailed mutational analysis of the ER-α revealed the necessity of the N-terminal region and the DNA binding domain to sustain the cyclin G2 repression. The present study also showed that progressive deletion of the AF-1 domain of ER-α led to corresponding decreases in the activity of the MMP-13 promoter. It appears that even the deletion of the equivalent of the first 41 amino acids from the N-terminal portion of the ER-α is sufficient to result in significant decreases in the stimulatory activity of ER-α on the MMP-13 promoter (see Figure 5B). These results are further substantiated by other studies which have shown that the first 21 amino acids of the AF-1 domain of ERα are required for cyclin G2 repression (35).

It has been speculated that in some cell types only one of the two AF domains may play a role in modulating the transcriptional activity due to the specific expression of distinct coactivators (20). Furthermore, Webb et al. (1999) have demonstrated that the more differentiated a cell is, the more likely that it will use the AF-1 domain of ER-α to mediate estrogen responses, and undifferentiated and dedifferentiated cells tend to depend more on the AF-2 domain of ER-α. The rabbit synoviocytes used in the present study can likely be classified as a differentiated cell line and the results presented in the present study show a definite lack of in stimulatory activity when the ΔAB construct, missing the AF-1 domain, is used as a modulator. Therefore, it is likely that the AF-1 domain of ER-α plays the major positive regulatory role in modulating the activity of MMP-13. However, since the absence of the AF-2/ligand binding domain leads to significant elevations in the stimulatory activity of the ABCD construct, a distinct negative regulatory role can also likely be assigned to the AF-2 domain.

The influence of ER on estrogen responsive genes can be mediated either through an estrogen response element (ERE), AP-1, or other sites in the promoter of target genes (36, 21). The rabbit MMP-13 promoter used in the present studies is well characterized and lacks the conventional ERE sequence, although it does have an AP-1 site. Other major
transcriptional regulatory sites present include the Runx domain, PEA-3 site and p53 site. Previous studies performed by our group have indicated that the AP-1 site of the rabbit MMP-13 promoter acts in conjunction with other regulatory sites such as the PEA-3 and Runx domains for mediating the effects of 17-beta estradiol in the presence of intact ER-α (16). These studies have been performed with MMP-13 promoter ranging from 670 bps to approximately 43 bps. The premise of tissue-specific expression of MMPs is becoming increasingly popular (37). The restricted expression of MMP-13 in cartilage and bone has been attributed to the Runx-2 site present in the promoter of MMP-13 (38). The protein binding to the Runx-2 site has primarily been found in the bone and cartilage cells. This site is considered essential for the optimal expression of MMP-13. Studies in both the human and rabbit genes have established the requirement of the AP-1 and Ets families of transcription factors that bind to their cognate sites just upstream of the TATA box (39, 40, 41). Furthermore, the activation of MMP-13 promoter by p38 through recruitment of AP-1 and the transcription factor Runx-2 in chondrocytes further emphasizes the tissue-specific mechanisms regulating MMP-13 gene expression (38).

The present study demonstrates that the AP-1 site of the MMP-13 promoter is also likely the primary locus for mediating the effects of WT-ERα, as well as the mutants ABCD and ΔAB in the presence and absence of the estrogenic ligand 17-β estradiol. Interestingly, mutations in the Runx and PEA-3 domains of the MMP-13 promoter did not appear to have a significant impact on the stimulatory activity of ABCD. Only mutations in the AP-1 domain, led to significant decreases in the activity of the MMP-13 promoter, when the ABCD construct of ER-α was the modulator (See Figure 6D). In contrast to these observations, mutations in the Runx and PEA-3 domains did lead to modest, but significant decreases in stimulation of MMP-13 promoter activity when ΔAB or WT-ERα were present as stimulators (See Figure 6 B & C).
Previous studies by Mengshol et al. (2001) have shown that the transcriptional response of MMP-13 to IL-1 in chondrocytes requires transcription factors that can bind to AP-1 and Runx-2. In a separate study, the AP-1 and Runx domain transcriptional factor binding sites on the rat MMP-13 promoter have been shown to be essential in both parathyroid hormone (PTH)-treated and differentiating osteoblastic cells in vitro (42, 43). Furthermore, it has been demonstrated that the Runx domain and AP-1 binding sites are organized in a specific helical pattern that facilitates the interaction of proteins that bind to these sites leading to the activation of the MMP-13 promoter activity (44). Thus, the AP-1 site in conjunction with other transcription factor binding sites appears to be critical for the transcriptional regulation of MMP-13 by some stimuli in certain cells, but the AP-1 site appears to be the dominant site for modulation of the MMP-13 promoter by ER-α in the HIG-82 cells.

It is well recognized that most of the effects of the hormone estrogen are mediated through either the alpha or the beta isoforms of ER. In addition to ER-α and ER-β, recently attention has turned to estrogen receptor related proteins (ERRs) which are classified as orphan receptors, as ligands which bind to these receptors have not been identified and they do not bind the naturally occurring endogenous estrogens (reviewed in 45). ERRs have been implicated in the development of breast cancer, as they are postulated to interfere with the estrogen signaling in the breast tissues (46). Although the presence of ERRs has been established in bone (47), cartilage (25) and in connective tissues such as ligaments (11), it is unknown if they play any role in the development of joint diseases such as osteoarthritis. As the present study has shown that ER constructs containing the AF-1 domain but lacking the ligand binding domain can effectively modulate MMP-13 promoter activity, ERs altered in or lacking ligand binding domains could potentially play a role in a variety of conditions to regulate expression of some genes.

In summary, the present study has characterized the requirements for ER-α modulation of the rabbit MMP-13 promoter in a transient transfection system. The results indicate that
specific domains of the ER-α, and especially the AF-1 domain, are critical for activity and that the ligand binding/AF-2 domain likely serves a modulating role. The findings also support the concept that naturally occurring ER-α proteins lacking ligand binding ability and thus modulation by estrogen, could also play a role in regulating gene expression in disease processes.

**Acknowledgements**

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References


A.

**Antibody ER-α (HC-20)**

Control

MW

**WT-ERα**

AF-1 DNA Ligand / AF-2

A/B C D E F

**ABCD**

A/B C D

Δ AB C D E F

B.

**Antibody ER-α (HC-20)**

Control WT-ERα Δ AB Control ABCD WT-ERα

MW

75

50

37
1A. **Diagrammatic representation of the structure** of WT-ER-α with all the functional domains (A-F), mutant ABCD without the ligand binding/ AF-2 domains and mutant ΔAB lacking the AF-1 domain.

1B. **Protein expression of WT-ER, ABCD and ΔAB in HIG-82 cells.**

HIG-82 cells were transiently transfected with either 0.5 µg of WT-ER or ABCD or ΔAB and 24 hours after transfection, cells were lysed and protein was extracted. Following separation using SDS-PAGE gel and transfer of the gel to a nitrocellulose membrane western blotting was performed. The panel on the left represents western blot performed using rabbit polyclonal antibody ER-α (HC-20) to detect WT-ERα and ΔAB and the panel on the right represents western blot performed using rabbit polyclonal antibody, ERα (H-184) to detect ABCD form of ER-α and the WT-ERα. HIG-82 cells were used as non-transfected negative controls in both cases (Control).
Increasing amounts of WT-ERα and ABCD DNA

Fold Increase in MMP-13 Levels

Control 0.25 µg 0.5 µg 1 µg

Fold Increase in MMP-13 Activity

Control 0.25 µg 0.5 µg 1 µg
Figure 2A. Effect of WT-ERα and ABCD on the endogenous levels of MMP-13 in the HIG-82 cells.

The mRNA levels for endogenous MMP-13 expressed prior to and after transfection with WT-ERα or ABCD in HIG-82 cell line as determined by real-time PCR. HIG-82 cells were either left un-transfected (No WT-ERα or ABCD) or were transiently co-transfected with (0.25 - 1 μg) of WT-ERα or ABCD. After 24 hours of transfection, total RNA was extracted from the cells and analyzed by Real-Time PCR.

- P > 0.05 when control without the transfected WT-ERα is compared to control without transfected ABCD

↔ P < 0.05 compare WT-ERα to ABCD

‡ P > 0.05 (Non-significant) when control without the transfected WT-ERα is compared to values obtained from cells transfected with increasing amounts of WT-ERα.

♥ P < 0.05 (Significant) when control without the transfected WT-ERα is compared to values obtained from cells transfected with increasing amounts of WT-ERα.

♣ P < 0.05 (Significant) as compared to control without the transfected ABCD

φ P > 0.05 (Not-Significant) as compared to control without the transfected ABCD

2B. Comparison of the effect of WT-ERα and ABCD mutant on the MMP-13 promoter activity in the absence of any ligand.

Increasing concentrations of ABCD and WT-ERα DNA leads to corresponding increase in the MMP-13 promoter activity. HIG-82 cells were transiently co-transfected with either WT-ERα or ABCD at varying DNA concentrations along with the rabbit MMP-13 DNA (p370-Luc 1 μg) and the internal control plasmid, pRL-SV40 (0.05 μg). Luciferase activity was expressed as the ratio of (p370-Luc) firefly luciferase activity and the (pRLSV40) Renilla luciferase reporter activity. Values represent the mean +/- S.E. for three determinations. Constitutive elevation in MMP-13 promoter activity is observed when ABCD mutant of ER-α is present as a modulator. MMP-13 promoter activity is elevated more when ABCD mutant of ER-α is the modulator as compared to the WT-ERα.

‡ P < 0.0005 as compared to control without the transfected WT-ERα

↔ P < 0.0005 compare WT-ERα to ABCD

♣ P < 0.0005 as compared to control without the transfected ABCD
Increasing amounts of WT-ERα and ΔAB DNA
Figure 3. Effect of increasing concentrations of WT-ERα and mutant of ER-α (ΔAB) DNA on the MMP-13 promoter activity in the absence of any ligand.

Increasing concentrations of WT-ERα DNA leads to corresponding increase in the MMP-13 promoter activity though no significant elevation in MMP-13 activity is observed when ΔAB is the modulator. HIG-82 cells were transiently co-transfected with either WT-ERα or ΔAB at varying DNA concentrations along with the rabbit MMP-13 DNA (p370-Luc 1 µg) and the internal control plasmid, pRL-SV40 (0.05 µg). Luciferase activity was expressed as the ratio of (p370-Luc) firefly luciferase activity and the (pRLSV40) Renilla luciferase reporter activity. Values represent the mean +/- S.E. for three determinations.

‡ P < 0.0005 as compared to control without the transfected WT-ERα

◆ P < 0.0005 compared WT-ERα to ΔAB
A.

<table>
<thead>
<tr>
<th></th>
<th>AF-1</th>
<th>DNA</th>
<th>Ligand / AF-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A / B</td>
<td>C</td>
<td>D</td>
<td>E</td>
</tr>
<tr>
<td>WT-ERα</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E41</td>
<td></td>
<td></td>
<td></td>
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<td>A87</td>
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<td></td>
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<tr>
<td>M109</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ AB</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

B.

WT-ERα: 120
E41: 60
A87: 20
M109: 20
Δ AB: 20

p = 0.0001
p = 5E-05
p = 0.004
p = 6.6E-05
Figure 4. Influence of AF-1 domain of ER-α on the MMP-13 promoter activity in the presence and absence of estrogen.

A. Diagrammatic representation of the WT- ERα and mutant constructs with progressive deletions in the A/B domain of ER-α used in the study.

B. Progressive deletion of AF-1 domain of ER-α leads to corresponding decrease in the activity of MMP-13 promoter. HIG-82 cells were transiently co-transfected with 0.5 µg WT-ERα or E41 or A87 or M109 or ΔAB DNA along with rabbit MMP-13 DNA (p370-Luc), containing the first 370 proximal bp. of the rabbit MMP-13 promoter fused to the pGL2 Basic reporter gene and the internal control plasmid, pRLSV40. Cells transfected with WT-ERα DNA were used as control. Luciferase activity was expressed as the ratio of firefly luciferase activity and the Renilla luciferase reporter activity. Values represent the mean +/- S.E. for three determinations and are expressed as percentage of p370-Luc Luciferase activity + WT-ERα DNA. (↔ p values < 0.05: significant difference observe when compared to the influence of WT-ERα on the activity of p370-Luc).
Figure 5: Effect of increasing concentrations of 17-β Estradiol on MMP-13 promoter activity when either WT-ERα or ABCD or ΔAB is the modulator. HIG-82 cells were transiently co-transfected with 0.5 µg WT-ERα or ABCD or ΔAB DNA along with rabbit MMP-13 DNA (p370-Luc), containing the first 370 proximal bp. of the rabbit MMP-13 promoter fused to the pGL2 Basic reporter gene and the internal control plasmid, pRL-SV40. Cells were treated with 17-β estradiol at concentrations ranging from (10^{-12} M to 10^{-8} M) for 24 hours. Cells transfected with WT-ERα or ABCD or ΔAB DNA without treatment with any ligand were used as control. Luciferase activity was expressed as the ratio of firefly luciferase activity and the Renilla luciferase reporter activity. Values represent the mean +/- S.E. for three determinations and are expressed as percentage of p370-Luc Luciferase activity + WT-ERα or ABCD or ΔAB DNA without treatment with 17-β Estradiol.

A) Increasing concentrations of estradiol leads to corresponding decreases in the MMP-13 promoter activity when WT-ERα is the modulator. The decrease in the MMP-13 promoter activity is observed even when very low concentration of (10^{-12} M) estradiol is used. † P Value < 0.05 when compared to MMP-13 promoter activity without the ligand.

B) Increasing concentrations of estradiol leads to corresponding decreases in the MMP-13 promoter activity when ABCD is the modulator. Lack of ligand binding / AF-2 domain in the ABCD mutant makes it indifferent to the presence of the ligand. ● P Value < 0.05 when compared to MMP-13 promoter activity without the ligand.

C) Increasing concentrations of estradiol leads to corresponding decreases in the MMP-13 promoter activity when ΔAB is the modulator. Low concentration of estradiol (10^{-12} M) has no influence on the MMP-13 promoter activity when ΔAB is the modulator. However, at higher concentrations of 10^{-10} and 10^{-8} M, a ~40 – 50 % decrease in the activity of MMP-13 promoter is observed. ‡ P Value < 0.05 when compared to MMP-13 promoter activity without the ligand.
A. Site directed mutant constructs of rabbit MMP-13 promoter

<table>
<thead>
<tr>
<th>Runx Domain</th>
<th>PEA-3</th>
<th>AP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT 370</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M370R2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M370P2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>M370A3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M370A3R2P2</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

B. WT-ERα

![Graph showing the effect of estradiol on WT370, M370R2, M370P2, M370A3, and M370A3R2P2 in the presence or absence of estradiol.]
C. 

\[ \Delta AB \]

\begin{tabular}{ccccccc}
Estradiol & - & + & - & + & - & + & - & + \\
WT370 & M370R2 & M370P2 & M370A3 & M370R2P2A3 & & & & \\
\end{tabular}

D. 

\[ \text{ABCD} \]

\begin{tabular}{ccccccc}
Estradiol & - & + & - & + & - & + & - & + \\
WT370 & M370R2 & M370P2 & M370A3 & M370R2P2A3 & & & & \\
\end{tabular}
Figure 6: Functional characterization of the MMP-13 promoter when modulated by either WT-ERα or ABCD or ΔAB mutant of ER-α.

A. Data showing the primary sites that were mutated by site directed mutagenesis in the different constructs.

B. HIG-82 cells were transiently co-transfected with wild-type and site-directed mutant constructs of rabbit MMP-13 promoter in pGL2-Basic vector (WT370, M370R2, M370P2, M370A3 & M370A3P2R2), WT-ERα or ABCD or ΔAB constructs of ER-α and internal control plasmid, pRLSV40. Cells were treated with 10^{-8} M 17β Estradiol after transfection for a period of 24 hours. Luciferase activity was expressed as the ratio of firefly luciferase activity and the Renilla luciferase reporter activity. Values represent the mean +/- S.E. for three determinations and are expressed as percentage of p370-Luc luciferase activity without treatment with 17β Estradiol. The signs (-) and (+) represent activity in the absence and presence of the ligand, 17β estradiol for WT370, M370R2, M370P2, M370A3 & M370A3P2R2 respectively.

- P < 0.05 when compared to WT370 activity modulated by WT-ERα
- ‡ P < 0.005 when compared to WT370 activity modulated by WT-ERα
- ⇪ P < 0.0005 Comparison of promoter activity of WT370 and mutants (+/- 17β estradiol)

C. HIG-82 cells were transiently co-transfected with wild-type and site-directed mutant constructs of rabbit MMP-13 promoter, ABCD – a mutant construct of ER-α and internal control plasmid, pRLSV40. Cells were treated with 10^{-8} M 17β Estradiol after transfection for a period of 24 hours. Luciferase activity was expressed as the ratio of firefly luciferase activity and the Renilla luciferase reporter activity. Values represent the mean +/- S.E. for three determinations and are expressed as percentage of p370-Luc luciferase activity without treatment with 17β Estradiol. The signs (-) and (+) represent activity in the absence and presence of the ligand, 17β estradiol for WT370, M370R2, M370P2, M370A3 & M370A3P2R2 respectively.

‡ P < 0.005
♣ P < 0.00005

⇣ P < 0.005 Comparison of promoter activity of WT370 and mutants (+/- 17β estradiol)

D. HIG-82 cells were transiently co-transfected with wild-type and site-directed mutant constructs of rabbit MMP-13 promoter, ABCD – a mutant construct of ER-α and internal control plasmid, pRLSV40. Cells were treated with 10^{-8} M 17β Estradiol after transfection for a period of 24 hours. Luciferase activity was expressed as the ratio of firefly luciferase activity and the Renilla luciferase reporter activity. Values represent the mean +/- S.E. for three determinations and are expressed as percentage of p370-Luc luciferase activity without treatment with 17β Estradiol. The signs (-) and (+) represent activity in the absence and presence of the ligand, 17β estradiol for WT370, M370R2, M370P2, M370A3 & M370A3P2R2 respectively.

♣ & ‡: P < 0.005 Comparison of promoter activity of WT370 and mutants (+/- 17β estradiol)