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Polymorphisms in the promoter regions for human MMP-1 and MMP-13 lead to differential responses to the alpha and beta isoforms of estrogen receptor and their ligand in vitro

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*** Manuscript****SUMMARY**

Estrogen receptors (ER) are present in connective tissues and therefore it is possible that the loss of estrogen after menopause influences the integrity of these tissues, contributing to development of degenerative conditions such as osteoporosis and osteoarthritis in a subset of women. Aberrant expression of matrix metalloproteinases (e.g. MMP-1 & MMP-13) has been implicated in the progression of these diseases. The present study investigated potential molecular mechanisms involved in the regulation of expression of MMP-1 and MMP-13 promoter variants by ER- α and ER- β (+/- estrogen) in a transient transfection system. The results demonstrate that the activity of human MMP-1 and MMP-13 polymorphic variants are elevated in the presence of ER- α and ER- β , and the single nucleotide polymorphisms present in the promoters of MMP-1 and MMP-13 variants lead to differential activities in response to the ER isoforms. Furthermore, the influence of 17- β estradiol also varies depending upon whether the alpha or the beta isoform of ER is the modulator of these polymorphic variants. These findings support the conclusion that ER isoforms may be contributing to disease development and/or progression in genetically distinct subsets of women following menopause, and provide mechanistic insights into how such contributions are manifested.

Introduction

The incidence of osteoarthritis (OA) and osteoporosis (OP) is more prevalent in females than in males, particularly after menopause when estrogen levels in the body decline. Degradation of articular cartilage is a feature of OA and degradation of bone matrix a feature of OP. In both conditions aberrant expression of matrix metalloproteinases (MMPs) has been implicated (reviewed in 1; 2, 3). In addition, depletion of estrogen as a result of ovariectomy has been observed to lead to increased cartilage degradation in a rodent model (4, 5). Furthermore, in vivo animal studies on ovariectomized sheep (6) and monkeys (7) also suggest that estrogen replacement therapy may have a protective effect against OA as it reverses the damage to biomechanical properties of cartilage and/or decreases the severity of cartilage degradation. In vitro studies, which have investigated the effects of 17- β estradiol on IL-1 beta induced MMP expression, suggest that high concentration of estrogen can lead to increased IL-1 beta stimulated cartilage degradation (8). Destruction of cartilage has also been associated to production of catabolic factors such as NO by chondrocytes (9). It is well known that the major actions of the hormone estrogen are generally mediated through two intracellular receptors, ER-alpha and ER-beta (discussed in 10, 11).

Previous studies have shown that both ER- α and ER- β can elevate the activity of the promoter for the rabbit MMP-13, a member of the family of MMPs. Furthermore, those in vitro studies have also shown that in the presence of the estrogenic ligand 17- β estradiol, the enhancement of rabbit MMP-13 promoter activity by ER is depressed (10,11). Thus, it would appear that the receptors for estrogen have potential to exhibit a modulatory role on MMP-13 expression, and it is governed in part by the presence and absence of the hormone estrogen.

Studies in the progression of atherosclerosis lesions in the abdominal aorta led to the discovery of two polymorphic variants in the promoter of the MMP-13 gene (12). Sequence analysis of the MMP-13 promoter revealed an insertion of an adenine residue at position -291 leading to a stretch of 12A residues in one variant form. Alternately, in the other MMP-13 allele there is an absence of the adenine residue at -nt 291 leading to a stretch of only 11 adenine residues. Furthermore, an A to G transition at nt -77 changes the PEA-3 transcriptional regulatory site from AGGAAG to GGGAAG, thus leading to the formation of another polymorphic mutation. Increased development of atherosclerosis lesions in the young black males have been attributed, in part, to the polymorphisms observed in the MMP-13 promoter (12). Thus, far it is not known whether

these MMP-13 polymorphic promoter variants are influenced differently by the alpha and beta isoforms of ER in vitro.

Some sequence variations in the promoters of other members of the metalloproteinase family have also been detected. In particular, the 1G and 2 G variants are two well characterized polymorphic variations in the promoter for MMP-1 (14). The insertion of a guanine residue at position -1607 leads to the formation of an Ets transcription factor binding site which along with the AP-1 site at nt -1602 leads to increased transcription of the 2G variant (13,14). In contrast, the 1G variant lacks this insertion of a guanine residue in its promoter and consequently is reported to exhibit lower basal transcriptional levels (13, 14). It is reported that MMP-1 is a proteinase produced by multiple cells in the joint inclusive of synovial cells, and it is believed to influence the onset and progression of osteoarthritis by promoting degradation of collagen fibrils (2). Besides arthritis, MMPs are also postulated to play a role in the metastasis of the tumors. Previous studies have indicated that the hormone estrogen has an inhibitory effect on the activities of MMPs in breast cancer cells (15). Interestingly, ovariectomy in rats leads to a marked elevation in the expression of the rodent form of MMP-13 in bone (16).

Joint tissues are recognized as estrogen responsive tissues as several studies have identified estrogen receptors in several connective tissues (17, 18). Different tissues express different levels of ER- α and ER- β , and furthermore these isoforms are influenced differently by estrogen agonists and antagonists. Therefore, the aim of the present study was to understand the influence of ER- α and ER- β on several of the known polymorphic variants of the human MMP-13 and MMP-1 promoters, and to decipher the potential role of estrogen in such modulation. Polymorphisms found in the MMP promoters and their response to the changes in hormonal levels may provide a partial explanation for why only subsets of women develop osteoarthritis or osteoporosis with different clinical phenotypes following menopause.

Materials and Methods

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 sub-cultured 1:4 after trypsinization with 0.25% trypsin. This cell line lacks endogenous ER and has been used in previous studies (10,11).

Isolation and cloning of the human MMP-13 promoters -- The MMP-13 promoter construct (12 AA) was isolated from the total DNA obtained from human HL-60 cells by amplification with PCR using specific primers generated from published sequences (19). This was labeled as the primary PCR reaction. The sequences of the primers for the human MMP-13 gene used in this primary PCR reaction were:

Forward 5'-CTTCTGCAGGGGACCAAAGATG-3' (nt -1174 to -1153)

Reverse 5'-CTCAAGAAGAGGAAGGCAGCCA-3' (nt 60 to 39)

The secondary or nested PCR reaction used 1ul of the primary PCR reaction as a template. The sequences of the secondary primers were:

Forward 5'-CTGAGAATGGAAAGGGTGGAGGTC-3' (nt -939 to -916)

Reverse 5'-TCTTGAATGGTGATGCCTGGG-3' (nt 23 to 3)

The 939 bp PCR fragment obtained from the secondary reaction was cloned directly into the PCR 2.1-TOPO vector (Invitrogen Inc.). Following this, the 939 bp fragment was released from the PCR 2.1-TOPO vector by digestion with KpnI / XhoI, and subcloned into the KpnI/XhoI site of the promoterless pGL2 Basic Luciferase reporter vector. The resulting plasmids were transformed into One shot E. coli and purified on the QI-A spin Miniprep Kit (Qiagen, Mississauga, ON), according to the manufacturer's instructions and subjected to sequencing analysis to verify the orientation and accuracy of each construct. Two polymorphic MMP-13 constructs were identified, one which had a stretch of 12 adenine residues and one with a stretch of 11 adenine residues. The PEA-3 site began with an adenine residue – **AGGAAG** in these constructs and hence they were given the label of 12AA and 11AA. Two more polymorphic constructs 12AG and 11AG were generated with a guanine residue at the PEA-3 site (**GGGAAG**) replacing the adenine residue. This was done using the Quick Change Site-Directed Mutagenesis kit (Stratagene Inc).

Human MMP-1 promoter constructs: The human 1G and 2G MMP-1 promoter constructs were a generous gift from Dr. C.E Brinckerhoff (Dartmouth Medical School, NH, USA). Both the 1G and 2G constructs were subcloned into the pGL-3 luciferase expression vector. The deletion fragments -1458 bp, -572 bp, -143 bp and -73 bp were generated by RT-PCR using a 5' forward primer containing the MMP-1 sequence (underlined) along with a MluI site (**ACGCGT**).

-1458 5'CG**ACGCGT**CCATAAGGTGCTGAAGTG3' (nt-1458 to -1441)

-572 5'CG**ACGCGT**ATGGCCTGGCTCTGAGTAA-3' (nt -572 to -555)

-143 5'CG**ACGCGT**CTGCTAGGAGTCACCAT-3' (nt -143 to -126)

-72 5'CGACGCGTTGAGTCAGACACCTCTGG-3' (nt -72 to -55)

The reverse primer with the Hind III restriction enzyme site was common for all the RT-PCRs.

5' CCAAGCTTCAGTGCAAGGTAAGTG - 3' (nt -51 to -36)

The PCR products were purified and digested with MluI/HindIII. The final products were then ligated into MluI/HindIII digested pGL3 basic Vector.

The PEA-3 site consensus sequence AGGAAG at nt -1385 was altered to AGCGAG using the Quick Change Site-Directed Mutagenetic kit (Stratagene, La Jolla, CA USA) and the nt -1458 construct. The mutation was verified by sequencing. The orientation and integrity of all reporter constructs were assessed by DNA sequencing.

Additional Control and Expression Plasmids -- The internal control plasmid for the dual luciferase assays, pRLSV40, which constitutively expresses the renilla form of luciferase under the strong SV40 promoter, was obtained from Promega (Madison, WI). The expression vectors for ER- α (pKCR2-ER α) and ER- β (pKCRE- ER- β) were a generous gift from Dr. Pierre Chambon (Strasbourg, France) and Dr. Koen Dechering (Organon, The Netherlands), respectively. Since the ER- α and ER- β constructs were obtained in different vectors, the inserts were cleaved from their original vectors and subcloned into the pSG5 vector. The orientation and integrity of the inserts were 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 and as described previously (20, 10). Previous analysis has revealed that this cell line is devoid of detectable ERs (10, 11). Briefly, HIG-82 cells were seeded on 12-well plates on the day before transfection at a density of 1×10^5 cells/well. The cells were washed with PBS prior to transfection and then transfected with a constant amount (1 μ g) of reporter plasmids (shown to be optimal in preliminary studies; data not shown), pRLSV40 (50 ng) and pSG5 vector alone (0.1-2 μ g), pSG5-ER β (0.5 μ g - 4 μ g) or pSG5-ER α (0.5 μ g) by means of the Fugene 6 Transfection Reagent. Transfection was performed in Ham's F-12 Nutrient Medium in the absence of serum. Immediately, after transfection, 17- β -estradiol (E2) (Sigma, Oakville, ON) was added to the medium in appropriate wells of 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 performed in triplicate and repeated three or more times with results very similar to those reported obtained.

Western Blot Analysis: The HIG-82 cells were either left un-transfected (Control) or were transiently transfected with ER- α (pSG5-ER α) or ER- β (pSG5-ER β) for 24 hr. At that time, the cells were washed and then lysed in Mammalian Cell Lysis Kit buffer (Sigma Chemical Co). The cellular extracts from control and transfected cells were separated on a 10% SDS-PAGE gel and then transferred to a nitrocellulose membrane. Western blotting was performed using a mouse monoclonal to ER β (ab1103) (obtained from Ab Cam. Inc.) or a rabbit polyclonal antibody for ER- α : ER- α (HC-20) (Santa Cruz Inc.).

Statistical Analysis: Statistical analysis of the data was performed using ANOVA, STDEVA, STEYX in the Excel 5.0 software.

Results

3.1. Influence of increasing concentrations of ER- β and ER- α on MMP-1 promoter variant activity.

The effect of increasing concentrations of ER- β and ER- α on MMP-1 promoter activity was examined in HIG-82 cells. Two well characterized MMP-1 promoter variants 1G and 2G were used in this study. HIG-82 cells were transiently co-transfected with increasing concentrations (0.5, 1 and 2 μg) of pSG5ER- β or pSG5ER- α DNA, simultaneously with either 1G or 2G constructs (1.0 μg) and the internal control plasmid, pRL-SV40 (0.05 μg). After 24 hours, the luciferase activity of the cell lysates was determined. In the absence of exogenous ER- α or ER- β , 1G and 2G activity were both detectable in the HIG-82 cells. Furthermore, the 2G variant consistently exhibited an ~ 2.5 fold higher activity than the 1G variant. The results presented in Figure 1A suggest that co-transfection with 0.5 μg of ER- β had a significant impact on activity levels of both the 1G and 2G MMP-1 promoter constructs. The activity of the 1G variant in the presence of ER- β was elevated approximately ~ 4 fold with respect to 1G activity in the absence of ER- β . The activity of the 2G variant in the presence of 0.5 μg of ER- β was elevated approximately ~ 3.7 fold when compared to the 2G activity in the absence of exogenously added ER- β . The activity of the 1G construct increased ~ 7.6 fold and ~ 13 fold when compared to 1G activity (- ER- β) in the presence of 1 and 2 μg of ER- β respectively. Similarly, the 2G variant also exhibited a ~ 4.6 fold and ~ 7.3 increase in activity in the presence of 1 and 2 μg of ER- β respectively. Although the activity of the 2G variant was always higher than that of the 1G variant in the absence or presence of ER- β , the proportional increase in activity for 2G was consistently less than the increase in activity for 1G at different concentrations of ER- β . In addition, the ratio of activity of 2G vs. 1G also decreased with increasing concentrations of ER- β .

The results presented in Figure 1B, show evidence of the effect of the alpha isoform of ER on the MMP-1 polymorphic constructs. The activity of the 1G variant increased 2.2 fold, 2.6 fold and 3.6 fold with concentrations of 0.5, 1.0 and 2 μg of pSG5ER- α DNA, respectively. The activity of the 2G variant showed a modest ~ 1.2 fold increase at 0.5 and 1 μg of ER- α DNA and detected activity increased approximately 2-fold with 2 μg of ER- β DNA when compared to 2G activity in the absence of ER- β . However, the 2G variant always exhibited higher activity as compared to the 1G variant in the absence or presence of the ER- α construct. These results indicate a strong influence of ER- α and ER- β on the activity of MMP-1 promoter variants in this in vitro model system.

Western blot analysis conducted on transfected and un-transfected cells indicated that un-transfected HIG-82 cells did not exhibit the presence of either ER- α or ER- β proteins (Figure 1C, Lane 1), and furthermore the cells transfected with pSG5-ER β or pSG5-ER α for 24 hrs exhibited the presence of these proteins at nearly identical levels (Figure 1C, Lanes 2 and 3). These results support the conclusion that the differences in activity levels detected above were not due to differences in the translation of the ER constructs.

3.2. Influence of 17- β estradiol on the modulating effects of ER isoforms on the activity of MMP-1 promoter variants.

Next, the effect of the estrogenic ligand, 17- β estradiol was investigated on the ER- α and ER- β mediated augmentation of 1G and 2G variant activities (see Figure 2A). HIG-82 cells were co-transfected with 1 μ g of pSG5ER- α or pSG5ER- β and the 1G variant along with 0.05 μ g of pRL-SV40 DNA. The activity of the 1G variant was found to be elevated approximately 2-fold in the presence of ER- α and ~3 fold in the presence of ER- β . When 10⁻⁸M 17- β estradiol was added to the transfection medium, the elevation in 1G activity levels induced by ER- β was found to decrease by approximately 75%. Interestingly, the elevation in activity with the 1G variant in the presence of ER- α remained uninhibited ($P > 0.05$, NS) in the presence of same concentration of 17- β estradiol (Figure 2A, lane 3). When the ligand, 17- β estradiol, was used at a concentration of 10⁻¹² M with ER- α or ER- β , no notable decreases in the activity of the 1G variant were again observed (data not shown). Figure 2B shows the response of the 2G construct with ER- α or ER- β (-/+ 17- β estradiol). The activity of the 2G variant was elevated modestly in the presence of ER- α and no decreases in activity levels were again observed in the presence of 10⁻¹² M 17- β estradiol (data not shown). However, the activity of the 2G variant decreased by ~30% when saturating amounts of 17- β estradiol (10⁻⁸M) were used (see Figure 2B lane 3). The activity of the 2G variant was elevated approximately 3-fold when ER- β alone was present, and this elevation was decreased ~70% in the presence of 10⁻⁸ M 17- β estradiol. At a concentration of 10⁻¹² M, the ligand once again did not exert any detectable impact on the activity of the 2G construct. These findings support the concept that effects of estrogen modulation on the ER isoforms may be different, and additionally these also influence the polymorphic variants differently.

3.3 Functional characterization of the promoter elements of the MMP-1 promoter involved in mediating the influences of ER- α & ER- β (+/-) ligands.

In order to characterize the promoter elements of the human MMP-1 promoter for their potential role in mediating the effects of the ER isoforms, a series of 5' deletion

constructs, ranging in length from 1458 bp to 73 bp of the MMP-1 promoter were generated (Fig.3 panel A). The 1458 bp MMP-1 construct lacks the important Ets and AP-1 binding sites present at position -1607 and -1602, respectively. Previous studies have shown these sites to be of importance in the activity of MMP-1 promoter (13). However, this construct still has several other transcriptional regulatory sites present such as the TATA box at -30 bp and AP-1 sites at -70, -181, -429 and -556 nt, and the PEA-3 sites at -88 and -1385 nt. which are proximal to the transcriptional start site. These constructs were used in co-transfection studies in HIG-82 cells along with ER- α or ER- β and the pRL-SV40, followed by luciferase assays to determine activity. The results presented in Figure 3B demonstrate that the activity of the -1458 construct was 75% lower than that of the intact 1G promoter in the presence of ER- α . In the presence of ER- β , a mean 70% decrease was observed in the activity of the -1458 construct when compared to the intact 1G construct (see Figure 3C). Interestingly, the mean percent decreases in activity observed for the -1458 construct with ER- α and ER- β were not significantly different. These results suggest that the AP-1 site present at position -1602 plays a primary role in elevating the activity of the MMP-1 promoter in response to ER- α and ER- β . Additionally, the ETS binding site present at -1607 bp in the 2G construct likely also contributes to the elevation in activity observed with the ER isoforms.

Next, the 572 bp fragment of the MMP-1 promoter was examined +/- ER- α or ER- β . In contrast to the significant decreases in activity seen for the 1458 bp fragment with ER- α and ER- β , this shorter fragment of MMP-1 demonstrated an 88% increase in the activity with ER- α and a 49% increase in activity in the presence of ER- β (Figure 3B & C Bar 4). When compared to the promoter of the -1458 bp fragment, this -572 bp fragment is missing the PEA-3 site at -1385 position. These results imply that the promoter activity is regulated positively and negatively by the various transcriptional regulatory sites of MMP-1 in this cell line, and suggest a negative regulatory role for the PEA-3 site at the -1385 location. To confirm this suggestion, the PEA-3 site at -1385 in the -1458 construct was mutated to yield a construct -1458M with this PEA-3 site inactivated. As shown in Figure 3D, in the presence of ER- α the activity of the mutated -1458 construct was actually significantly elevated compared to intact 1G. In contrast, in the presence of ER- β , the activity was partially restored compared to that with the 1G construct but the activity with the mutant was still less than that with the 1G variant.

Furthermore, a MMP-1 promoter fragment which is only -143 bp in length, was also assessed. A ~68% increase in the promoter activity as compared to intact 1G promoter activity was observed with this construct when ER- α was the modulator. Alternately, when ER- β was the modulator, the activity of this -143 bp MMP-1 construct decreased moderately (~5%) with respect to the intact 1G promoter construct. While overall both ER isoforms elevated the activity of MMP-1 promoter, subtle differences in the response of these transcriptional regulatory sites of the MMP-1 promoter to these ER isoforms were detected. These observations are further corroborated by the response of the -73 bp fragment of the MMP-1 promoter to the ER isoforms. The -70 bp construct containing only the AP-1 site was tested along with ER- α and a mean 24% decrease in the activity was observed compared to the intact 1G variant. When ER- β was the modulator, the -73 bp construct exhibited a mean 68% decrease in activity when compared to the 1G promoter (Figure 3 B & C, Bar 5).

3.4 Comparison of the effects of ER- α and ER- β on the human MMP-13 polymorphic promoter variants.

In this study, polymorphic promoter variants of a second matrix metalloproteinase, MMP-13, were also analyzed. Figure 4A illustrates the four polymorphic constructs of the human MMP-13 promoter used in this study. The variant 12AA contains a stretch of 12 A residues and the PEA-3 site has the consensus sequence AGGAAG. The 12 AG variant contains stretch of 12 A residues, but the PEA-3 site begins with a G residue. The 11AA and 11 AG constructs contain the stretch of 11 A residues and the PEA-3 site has either an A or a G residue, respectively. The HIG-82 cells were co-transfected with the individual human MMP-13 promoter variants, along with ER- α or ER- β and the internal control plasmid, pRL-SV40. After 24 hours, luciferase assays were used to analyze the cell lysates.

As shown in Figure 4B, no activity was observed for all four human MMP-13 constructs in the absence of either ER- α or ER- β . Both pSG5-ER α and pSG5-ER β induced expression of the human MMP-13 polymorphic variants. Interestingly, the induction of 11AA and 11AG MMP-13 promoter activity by the ER- β construct was consistently approximately 4.7 times higher than that observed for an equivalent amount of the ER- α construct (Figure 4B). The polymorphic variants 12 AA and 12 AG showed an elevation of approximately ~7.5 fold in activity in the presence of ER- β when compared to the influence of ER- α on these constructs. Furthermore, the activity of 12AA was 1.6 fold higher than that of 11AA suggesting that the presence of an extra adenine residue exerted a positive influence in elevating the activity of the 12AA

construct. Similarly, a 1.6 fold elevation in activity was observed when comparisons were made between the 11AG and 12AG constructs (Figure 4B). These results suggest that ER- β is a stronger modulator than ER- α of the human MMP-13 DNA constructs. The number (11 or 12) of adenine residues also appear to have an impact on the response of these constructs to ER-B with constructs with 12 adenine residues showing higher activity levels. Interestingly, this impact was observed only with ER- β and not with ER- α .

3.5 Polymorphisms in the human MMP-13 promoter lead to differential responses to ER isoforms (+/- 17- β estradiol).

In this aspect of the studies, polymorphic variants of the MMP-13 promoter were also analyzed for their response to the alpha and beta isoforms of ER in the presence and absence of the estrogenic ligand 17- β estradiol. The HIG-82 cells were co-transfected with the human MMP-13 polymorphic variants along with ER- α or ER- β and the internal control plasmid, pRL-SV40 in the presence and absence of 17- β estradiol. After 24 hours, luciferase assays were used to analyze the cell lysates.

The results presented in Figure 5A show the influence of ER- α on the human MMP-13 constructs. The activity exhibited by the 11AA MMP-13 promoter was used as the positive control and values obtained in the presence of ER- α were set to 100%. The activity of all of the human MMP-13 variants were high in the presence of ER- α . Interestingly, the activity of the human MMP-13 variants remained unaffected in the presence of estrogenic ligand, when ER- α was the modulator (Figure 5A).

Similar analyses with ER- β are shown in Figure 5B. The activities exhibited by the human MMP-13 promoter variants were again significantly increased by the addition of ER- β . Furthermore, all of the human MMP-13 constructs displayed an ~ 80% decrease in their activity in the presence of 17- β estradiol when ER- β was the modulator. Thus, some ER isoform-specific influences on the MMP-13 promoter activity may be operative in this system.

Discussion

The results of the present study suggest that genetic polymorphisms in the promoter of the human MMP-1 and MMP-13 genes can influence the response to the modulating influences of the ER isoforms. The activities of two well characterized polymorphic promoter variants of the MMP-1 gene, 1G and 2G were found to increase with increasing concentrations of ER- α and ER- β . Furthermore, the activity of the 2G variant remained consistently higher than that of 1G in the absence or presence of ER isoforms. Previous studies have indicated that the presence of the Ets binding site led to

increased transcription of the 2G variant in both normal fibroblasts and cancer cells (13,21,23). Additionally, the proportional increase in activity for 2G was less than the increase in activity for the 1G variant at different concentrations of ER- β (see Figure 1). Moreover, the ratio of activity of 2G vs. 1G also decreased with increasing concentrations of ER- β .

When the estrogenic ligand, 17- β estradiol was present at saturating levels along with ER- β , a suppression of 1G and 2G promoter activity was observed. Interestingly, repression of 1G activity when 17- β estradiol was present was insignificant ($p > 0.05$) when ER- α was the modulator. In contrast, the activity of the 2G variant was repressed by the ligand when ER- α was the modulator but not to the same extent as was observed when ER- β was the modulator. These results indicate that ER- β is likely a stronger modulator of MMP-1 variants than is ER- α . Several independent reports suggest that the two isoforms of the estrogen receptor have differential influences on different MMPs. Previous studies on rabbit MMP-13 regulation have also demonstrated ER- β to be a stronger modulator of this promoter than ER- α (11).

Abberant overexpression of MMP-1 protein has been correlated with the degradation of stromal layers leading to metastasis of primary tumor in cancer and also in progression of endometriosis (23, 24). One study has reported that MMP-1 and ER- β expression are elevated, but ER- α expression is down-regulated in the endometriotic lesions, suggesting that estrogenic effects on MMP-1 expression are modulated primarily through ER- β (25). Interestingly, bimodal effects of estrogen have been observed in a study on human retinal pigment epithelium. A very high level of 17- β estradiol (10^{-8} M) was found to suppress ER and MMP-2 expression whereas opposite effects were observed on both the proteins at a more physiological concentration of 10^{-10} M (26).

Several studies suggest that the synergistic interaction between the transcription factors binding to the closely spaced ETS and AP-1 transcription regulatory sites are essential for the basal activation of the 2G variant of MMP-1 (27, 28). In general, Ets binding proteins have been shown to bind to both c-fos and c-jun proteins, proteins which also bind to AP-1 sites (29), and furthermore it has been reported that Ets-1 and c-jun are the likely candidates to interact and influence the MMP-1 promoter activity (14). The present study also demonstrated that the Ets and AP-1 site at locus -1602 and -1607 to be one of the primary regulatory sites involved in the regulation of the MMP-1 activity in response to ER isoforms. Although, MMP-1 has several proximal promoter

elements, these two distal transcriptional regulatory elements appear to play an important role in the stimulation of MMP-1 promoter activity. In addition, a separate study which focused on the role of reactive oxygen species on the elevation of MMP-1 promoter activity also established that site-directed mutations of these Ets and AP-1 sites at nt -1607 and -1602 led to significant decreases in the activity of the MMP-1 promoter (30).

Deletion of the (-1607 bp) Ets and (-1602 bp) AP-1 sites in the -1458 construct led to a significant reduction in the activity of MMP-1 (see Figure 3), findings consistent with the results of Nelson et al. (30). The presence of multiple positive and negative cis-acting transcriptional regulatory elements have been reported in the promoter of the MMP-1 gene (13). Moreover the same study also reported the presence of several putative silencer elements located between -572 bp and -1772 bp in the MMP-1 promoter. Additionally, a number of silencer/antisilencer motifs have been described in the region between -1653 bp and -2672 bp of the MMP-1 gene and it is suggested that the anti-silencer motifs override regulation by the silencer motifs, similar to that seen in the regulation of the vimentin gene (13, 31). Sequence analysis of the -1458 bp construct used in the present study revealed the presence of one silencer element and no antisilencer elements. This may provide an explanation for the observed decrease in the transcriptional activity of this construct in the presence of ER- α and ER- β . It has also been suggested that more than one silencer element may be required for the repression of the MMP-1 gene (13), a possibility which may further explain the partial (~70%) depression observed in the activity of the -1458 bp construct compared to the intact 1G construct. Mutation of the PEA-3 site at the -1385 bp position led to increases in the activity of the -p1485M construct in the presence of ER- α and ER- β . These results support the conclusion that the intact PEA-3 site at the -1385 bp position has a negative regulatory role on the activity of the 1G construct. Furthermore, as the elevation in activity with -p1458M was less in the presence of ER-beta than in the presence of ER- α , there may be some ER isoform-specific influences on the impact of this negative regulatory site.

Interestingly, elevations in the activity of MMP-1 promoter activity compared to the 1G construct were observed when shorter constructs (-572 and -143 nt) of the promoter were used. The activity of these two constructs were elevated to somewhat similar levels ($p = 0.5$ when the activity of the -572 bp construct was compared to the activity of the -143 bp construct) when ER- α was the modulator. However, when ER- β

was the modulator, the activity of the -572 bp fragment was elevated more than that observed for the -143 bp construct. Thus, these findings in the present study suggest that ER- α and ER- β may use different regions of the MMP-1 promoter to mediate their influence. Furthermore, based on the studies discussed above, both of these short constructs lack the putative silencer elements which might contribute to their higher activity levels in the presence of ER- α and ER- β . In contrast to the elevated activity levels observed for the -572 bp promoter construct of MMP-1, the activity of the -73 bp fragment of the MMP-1 promoter demonstrated decreases in activity of ~24% and ~68% with ER- α or ER- β , respectively. Although the -73 bp construct lacks the putative negative regulatory element, the present findings are in agreement with the conclusions of others (32, 33), which indicated that the presence of only the proximal AP-1 element (at -73 bp) is not sufficient to confer maximal transcriptional activation of the MMP-1 promoter. Previous studies have reported that phorbol 12-myristate 13-acetate (PMA)-mediated transcriptional elevation of MMP-1 requires the proximal AP-1 site, as well as the distal transcriptional regulatory sites (33). Moreover, studies with the rabbit collagenase-1(MMP-1) promoter have indicated that mutation of the AP-1 site at the -77 bp position led to a reduction in the basal transcription of MMP-1 (34).

Previous studies have indicated that remodeling of connective tissues occurs in human atherosclerotic lesions and MMPs have been implicated in this process (35, 36, 12). Furthermore, genetic polymorphisms in the MMP-13 promoter have been linked to increased fibrous plaque observed in the abdominal aorta of young black males (12). The present study also evaluated the response of MMP-13 polymorphic mutants to the modulatory influences of the ER- α and ER- β . The presence of ER- α elevated the activity of MMP-1 promoter variants (12AA, 12AG, 11AA and 11AG) and addition of the estrogenic ligand, 17- β estradiol, at saturating concentrations (10^{-8} M) did not influence the, MMP-13 activity. However, when ER- β was the modulator, the human MMP-13 promoter variants did show an increase in the absence of estrogenic ligand and a decrease in the presence of 10^{-8} M 17- β estradiol. Similar to the observations with the human MMP-13 promoter variants, previous studies have demonstrated an elevation in the rabbit MMP-13 promoter with both ER- α and ER- β . In contrast to the human MMP-13 promoter variants, these elevations in rabbit MMP-13 promoter activity by both ER isoforms is subject to repression in the presence of 10^{-8} M estradiol (10,11). Interestingly, previous studies have reported a higher sequence homology between the rabbit and human MMP-13 promoter than between human and mouse MMP-13

promoters (37). However, the differences observed in the regulation of the rabbit and human MMP-13 genes by ER- α may be attributed in part, to cell-specific and species specific behavior of ER isoforms on the regulation of this gene.

It is well known that binding of steroid hormones to their specific intracellular receptors can modulate gene expression within hormone-responsive tissues. The results from the present studies suggest that MMP-1 and MMP-13 promoter variants are influenced differently by ER- α and ER- β depending upon the presence or absence of the hormone estrogen. These findings may have relevance to the functioning of knee components and bone integrity leading to osteoarthritis or osteoporosis in subsets of women following menopause. Genetic polymorphisms in the MMPs may also contribute to disease onset and progression in these subsets. Current association studies are investigating such relationships.

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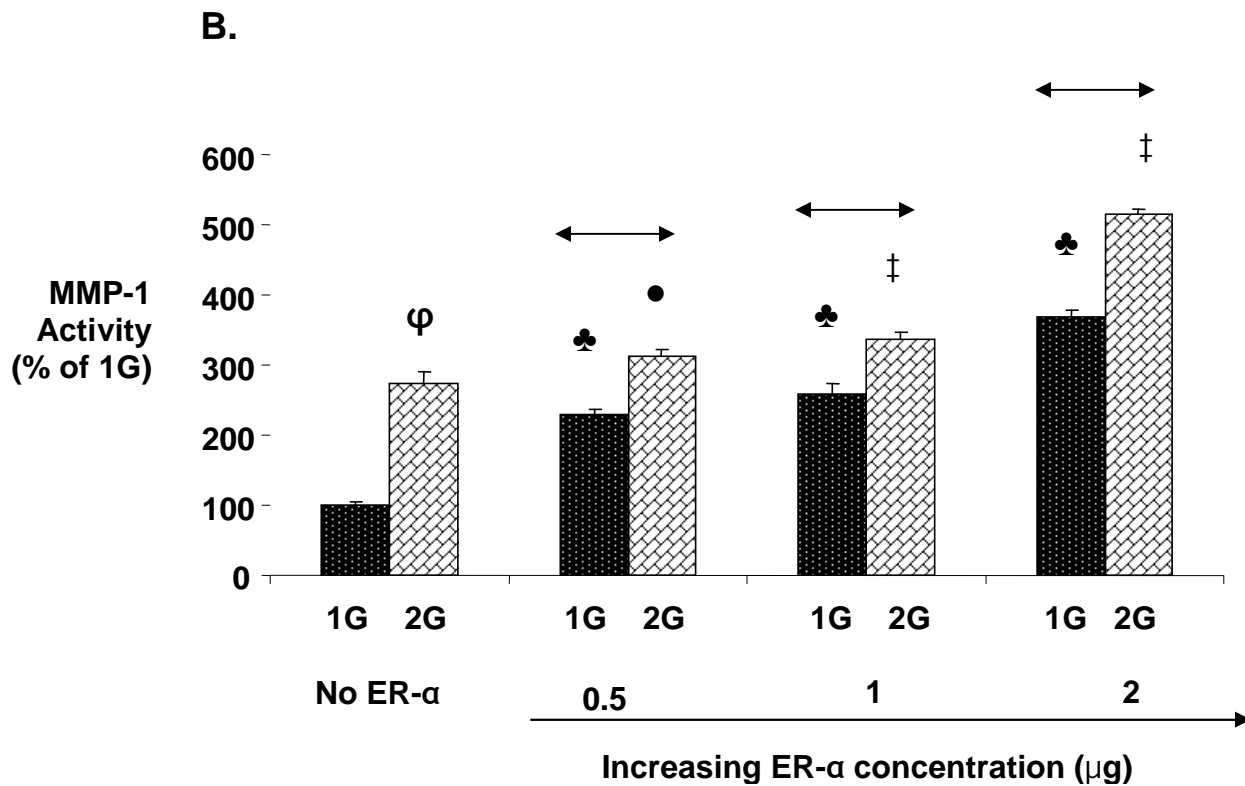
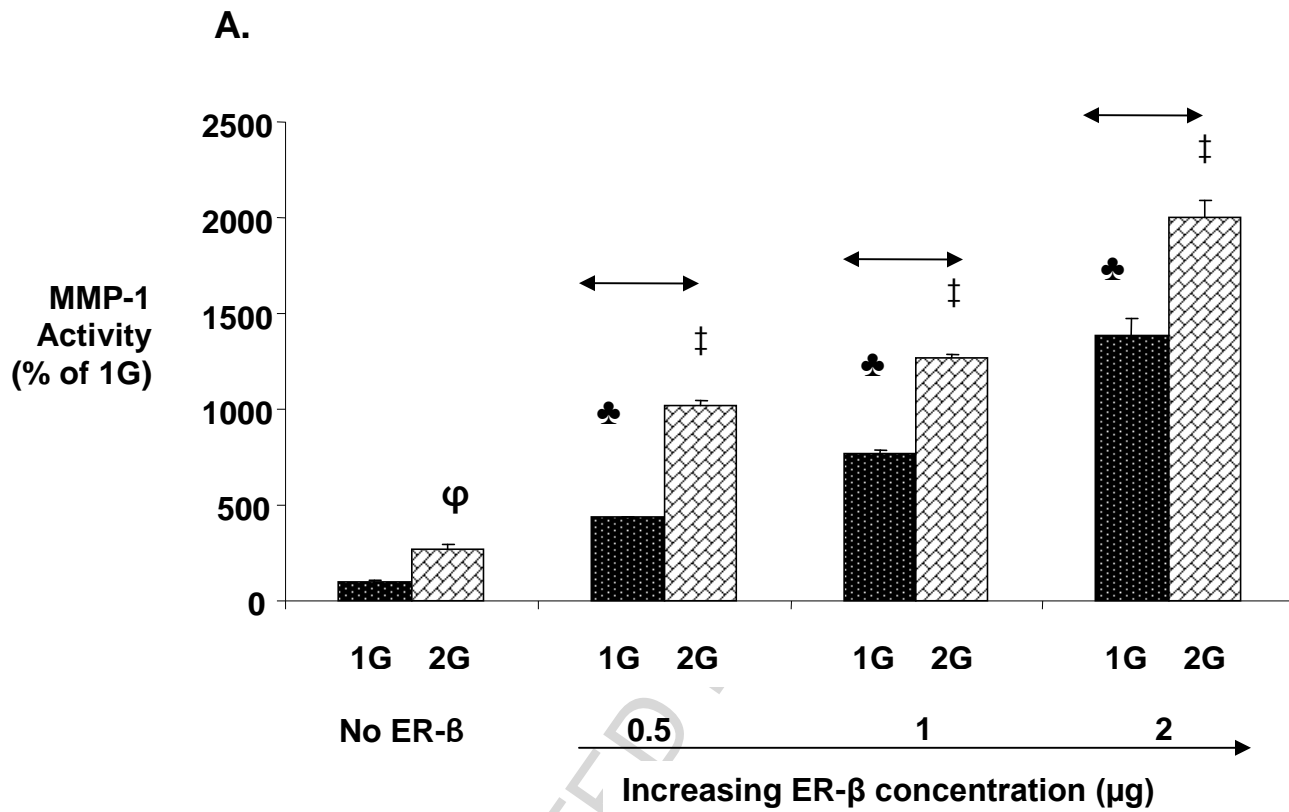
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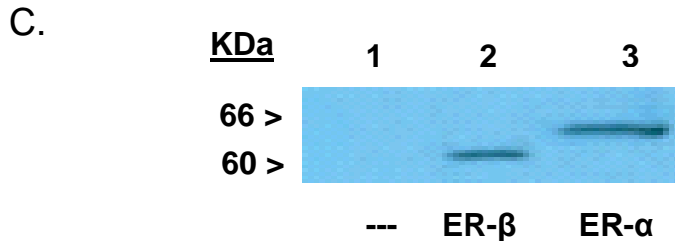


Figure 1: Effect of increasing ER- α or ER- β on the activity of MMP-1 promoter variants.

A. Rabbit synoviocytes (HIG-82) were transiently co-transfected with the human MMP-1 promoter constructs (1G or 2G), ER- β (pSG5-ER β), and the internal control plasmid, pRL-SV40. Increasing concentrations of pSG5-ER β ranging from (0.5 to 2 μ g) were used in this experiment. Cells transfected with 1G or 2G alone were used as controls. Luciferase activity was expressed as the ratio of 1G firefly luciferase activity and the Renilla luciferase reporter activity. Values represent the mean \pm S.E. for three determinations.

† $p < 0.001$ as compared with 1G activity in the absence of ER- β .

♣ $p < 0.0001$ as compared with 2G activity in the absence of ER- β .

⊕ $p < 0.001$ comparing the activity of 1G and 2G in the absence of exogenously added ER- β

↔ $p < 0.001$ comparing the activity of 1G and 2G in the presence of exogenously added ER- β

B. Rabbit synoviocytes (HIG-82) were transiently co-transfected with the human MMP-1 promoter constructs (1G or 2G), ER- α (pSG5-ER α), and the internal control plasmid, pRL-SV40. Increasing concentrations of pSG5-ER α ranging from (0.5 to 2 μ g) were used in this experiment. Cells transfected with 1G or 2G alone were used as controls. Luciferase activity was expressed as the ratio of 1G firefly luciferase activity and the Renilla luciferase reporter activity. Values represent the mean \pm S.E. for three determinations. † $p < 0.001$ as compared with 1G activity in the absence of ER- α .

† $p < 0.05$ as compared with 2G activity in the absence of ER- α .

• $p > 0.05$ Not Significant as compared with 2G activity in the absence of ER- α .

♣ $p < 0.005$ as compared with 1G activity in the absence of ER- α .

⊕ $p < 0.001$ comparing the activity of 1G and 2G in the absence of exogenously added ER- α

↔ $p < 0.005$ comparing the activity of 1G and 2G in the presence of exogenously added ER- α

C. Rabbit synoviocytes (HIG-82) were either left un-transfected or were transiently transfected with either ER- α (pSG5-ER α) or ER- β (pSG5-ER β). The protein extracts from transfected and un-transfected cells were separated on a 10% SDS-PAGE gel and then transferred onto a nitrocellulose membrane. Western blot analysis was conducted using the Mouse monoclonal to ER β (ab1103) (obtained from Ab Cam. Inc.) and rabbit polyclonal antibody for ER- α : ER- α (HC-20) (Santa Cruz Inc.). Lane 1 represents the un-transfected HIG-82 cells (Control); Lane 2. HIG-82 cells transfected with pSG5-ER β . Lane 3. HIG-82 cells transfected with pSG5-ER α .

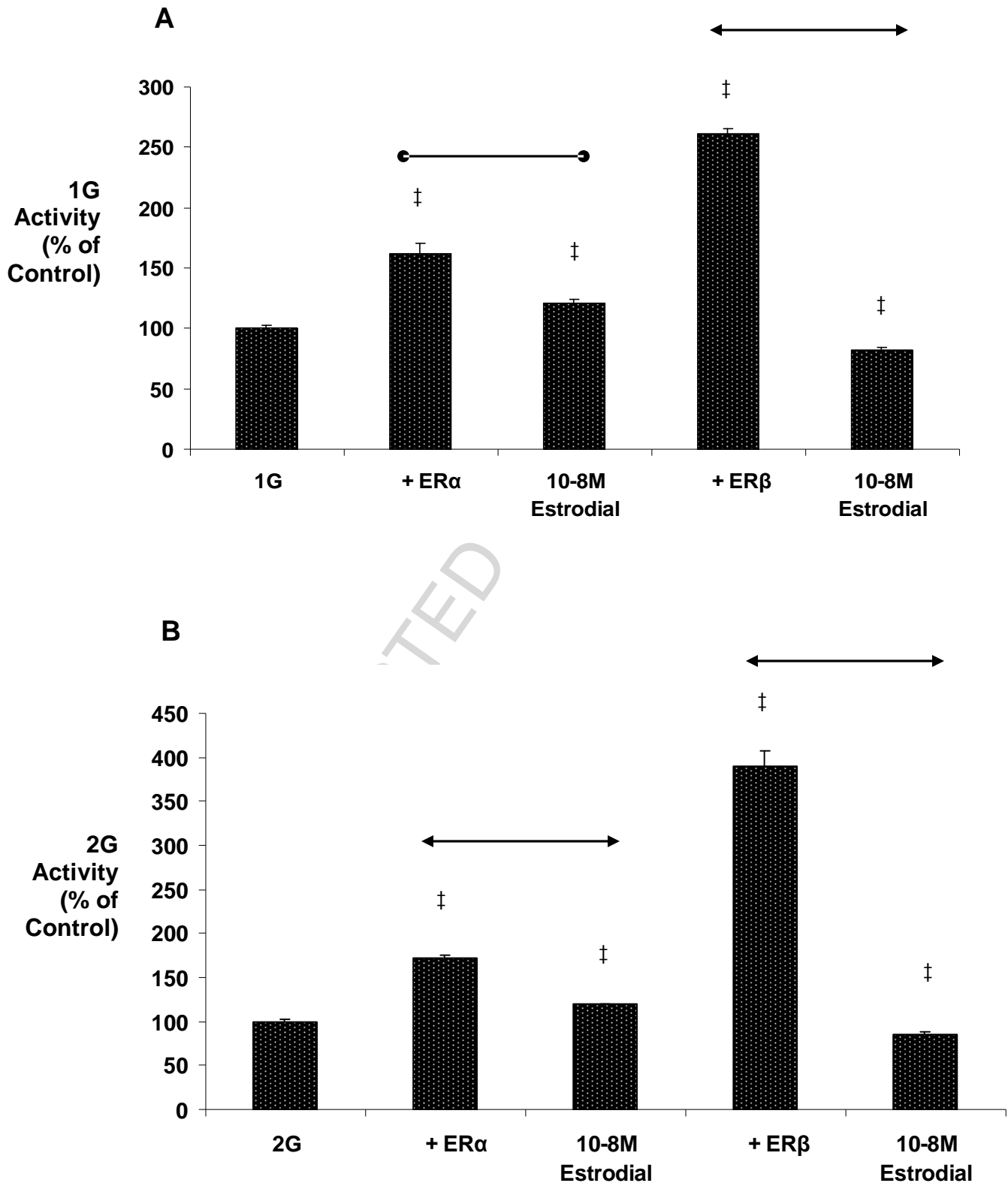
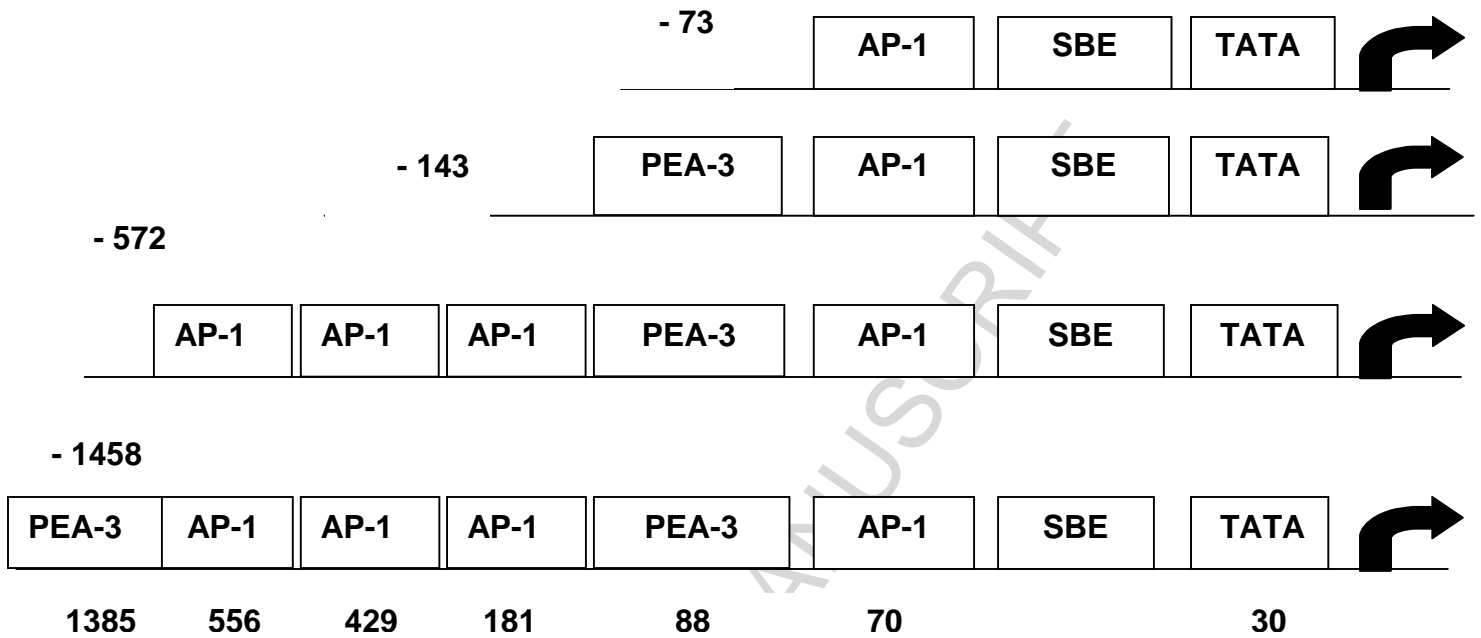


Figure 2: Influence of ligand, 17- β estradiol on the modulating effects of ER- α and ER- β on MMP-1 promoter polymorphic variants.

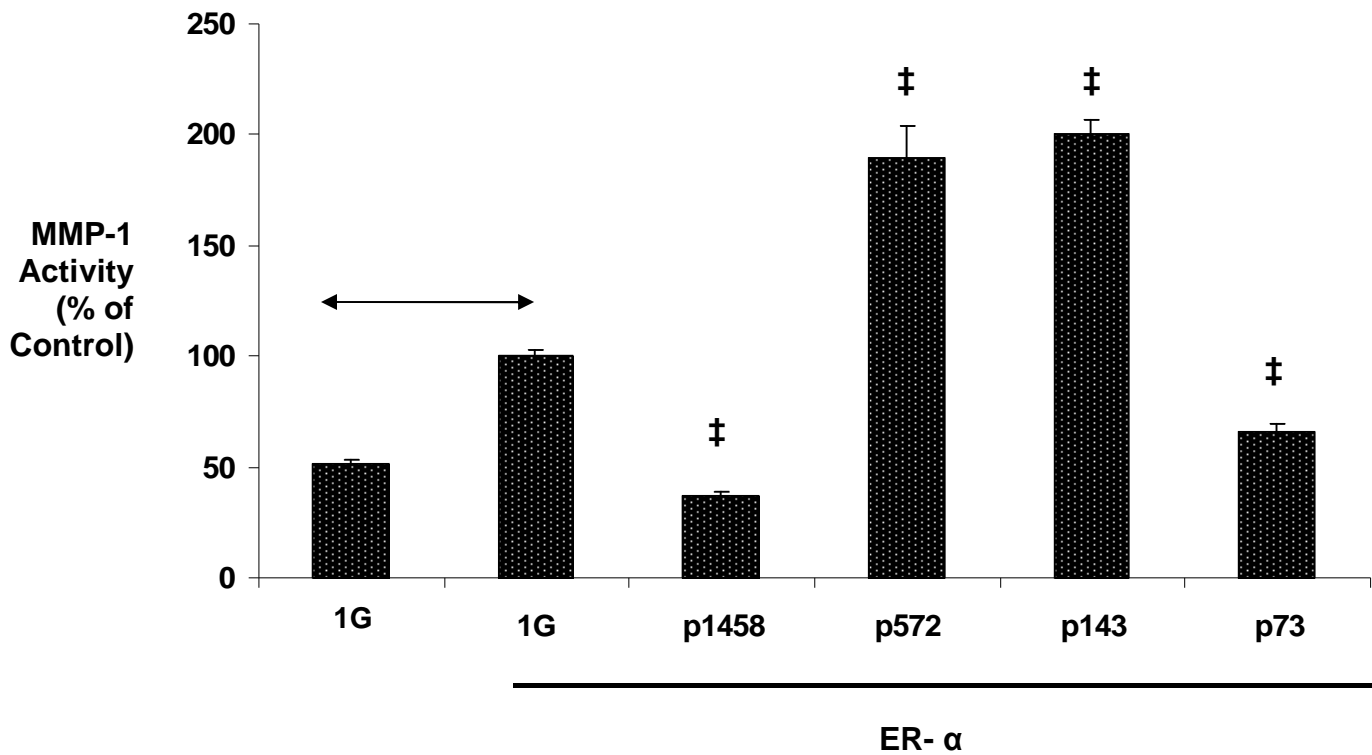
A & B. Rabbit synoviocytes (HIG-82) were transiently co-transfected with the human MMP-1 promoter constructs (1G or 2G), ER- α (pSG5ER- α) or ER- β (pSG5-ER β), and the internal control plasmid, pRL-SV40. One set of transfected cells was treated with 10^{-8} M 17- β estradiol. Cells transfected with 1G or 2G alone were used as controls in Figure 2A or 2B respectively. Luciferase activity was expressed as the ratio of 1G or 2G firefly luciferase activity and the Renilla luciferase reporter activity. Values represent the mean \pm S.E. for three determinations.

- P > 0.05 Not Significant: activity of 1G + ER- α compared to the activity of 1G + ER- α (10^{-8} M Estradiol)
- ↔ p < 0.05: activity of 1G + ER- β compared to the activity of 1G + ER- β (10^{-8} M Estradiol)
- ‡ p < 0.05: when compared to the activity of 1G and 2G without ER receptors or ligands.

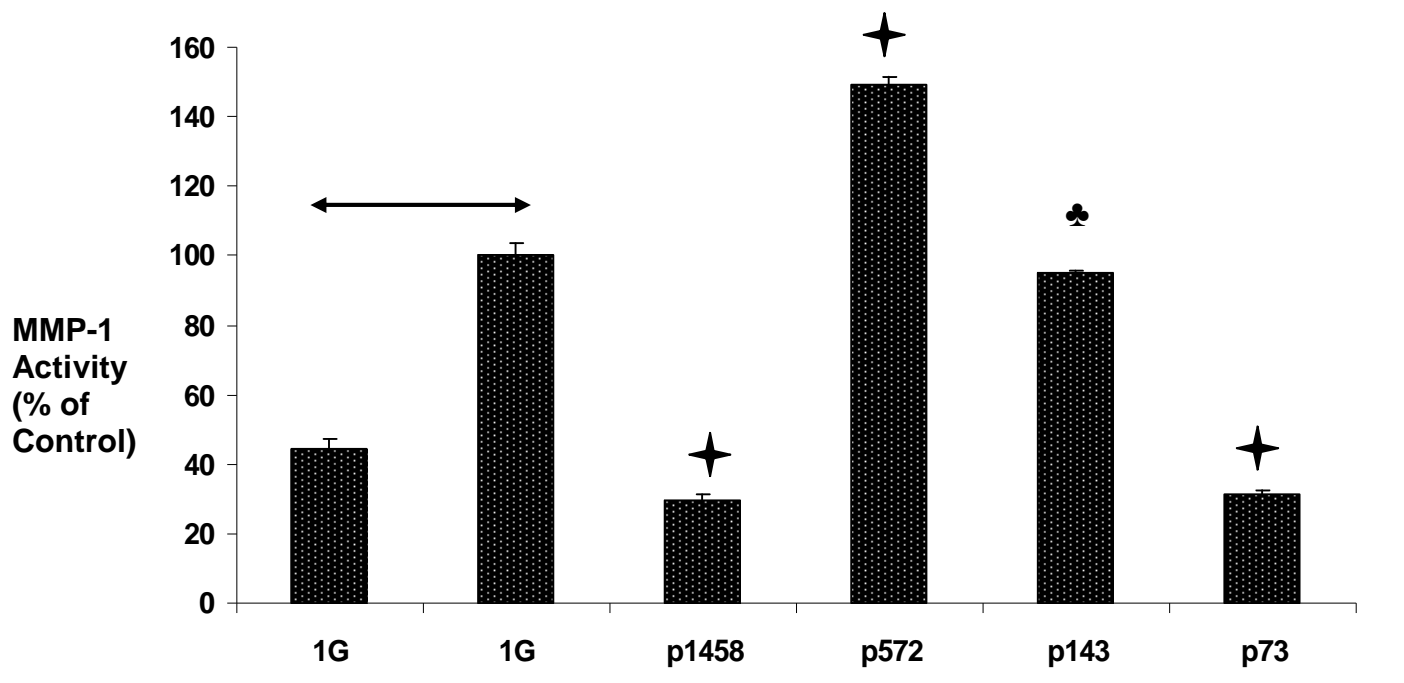
A.



B.



C.



D.

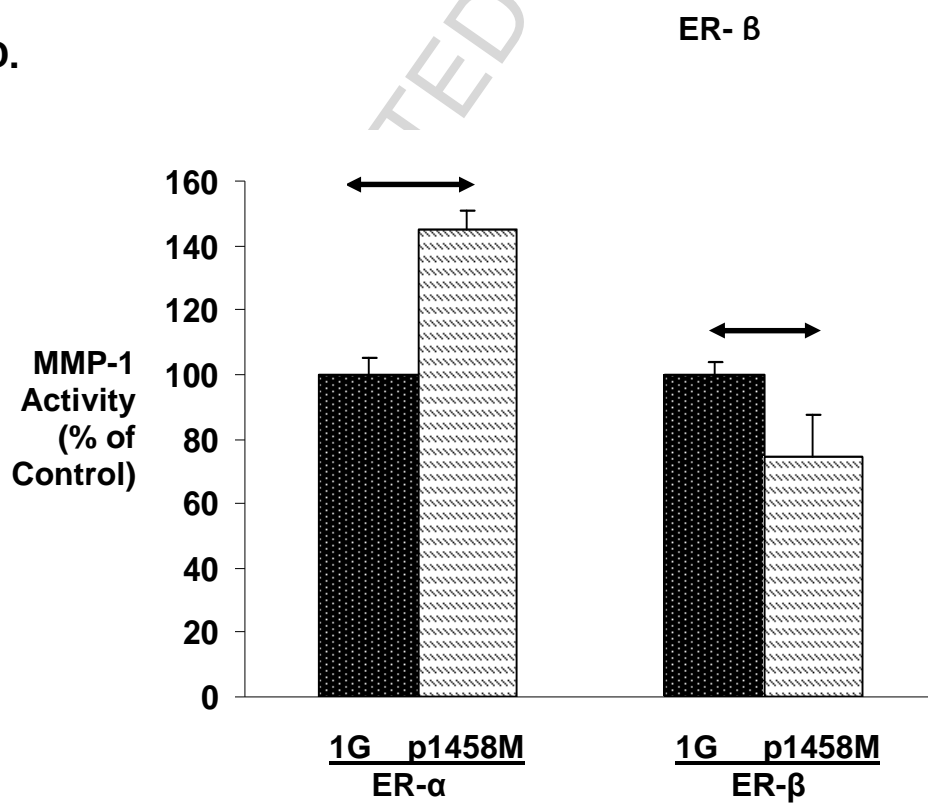


Figure 3: A) Schematic representation of the cloned DNA constructs containing promoter elements of human MMP-1.

All the constructs -1458, -572, -143 and -73 bps used in this study lack the distal Ets transcription factor binding site and the AP-1 binding sites present at distal locations at -1607 and -1602 bps. The -1458 bp construct contains the proximal promoter region stretching from transcriptional regulatory site, TATA box, at -30 nt to the PEA-3 site at -1385 nt. The construct -572 lacks the PEA-3 at position 1385 nt and the construct 143 lacks this PEA-3 plus three other AP-1 sites. The p73 MMP-1 promoter fragment contains only the AP-1 promoter element and the TATA box. All the promoter fragments were cloned into the vector, pGL2-Basic, which contains the promoterless luciferase gene. **B) Functional characterization of the promoter elements of human MMP-1 in response to ER- α .** Rabbit Synoviocytes, HIG-82 cells, were transiently co-transfected with 1G or deletion constructs of human MMP-1 promoter (-1458, -572 bp, -143 bp and -73 bp), along with 0.5 μ g of ER- α (pSG5-ER α) and 0.05 μ g of pRL-SV40. Luciferase activity was expressed as the ratio of firefly luciferase activity and the Renilla luciferase reporter activity. Values represent the mean \pm S.E. for three determinations and are expressed as percentage of 1G luciferase activity in the presence of ER- α .

‡ P < 0.005 when compared to luciferase activity of 1G in the presence of ER- α

↔ P < 0.0005 when compared to luciferase activity of 1G in the absence of ER- α

C) Functional characterization of the promoter elements of human MMP-1 in response to ER- β . HIG-82 cells were transiently co-transfected with 0.5 μ g pSG5-ER β along with either the human MMP-1 DNA (1G construct) or deletion constructs of human MMP-1 promoter such as (-1458, -572 bp, -143 bp and -73 bp) and the internal control plasmid, pRL-SV40. Luciferase activity was expressed as the ratio of firefly luciferase activity and the Renilla luciferase reporter activity. Values represent the mean \pm S.E. for three determinations and are expressed as percentage of 1G luciferase activity in the presence of ER- β .

♣ P > 0.05 Not Significant

★ P < 0.005 when compared to luciferase activity of 1G in the presence of ER- β

↔ P < 0.0005 when compared to luciferase activity of 1G in the absence of ER- β

D) Influence of PEA-3 site at -1385 nt position of 1G promoter on its activity in response to ER- α and ER- β . Rabbit synoviocytes, HIG-82 cells, were transiently co-transfected with 1G or mutated construct of human MMP-1 promoter (-p1458M), along with 0.5 μ g of ER- α (pSG5-ER α) or 0.5 μ g of ER- β (pSG5-ER β) and 0.05 μ g of pRL-SV40. Luciferase activity was expressed as the ratio of firefly luciferase activity and the Renilla luciferase reporter activity. Values represent the mean \pm S.E. for three determinations and are expressed as percentage of 1G luciferase activity in the presence of ER- α or ER- β .

↔ P < 0.05 when compared to luciferase activity of 1G in the presence of ER- α or ER- β

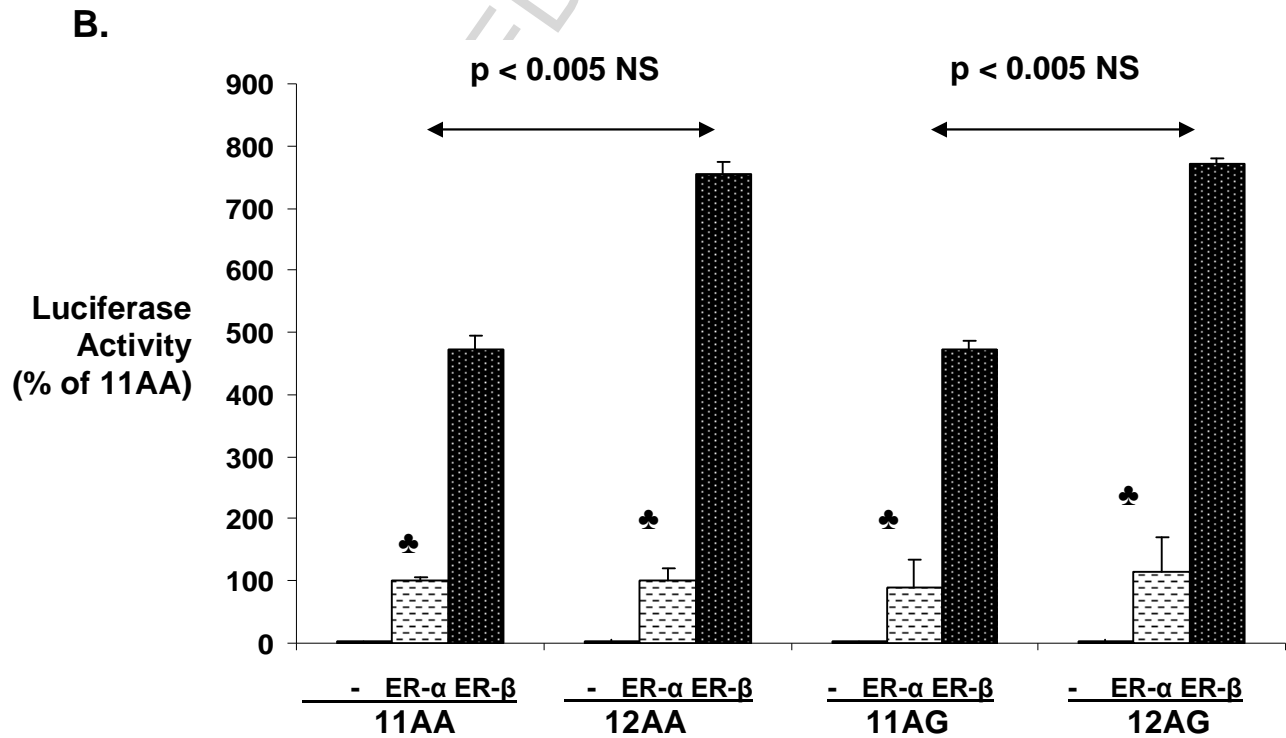
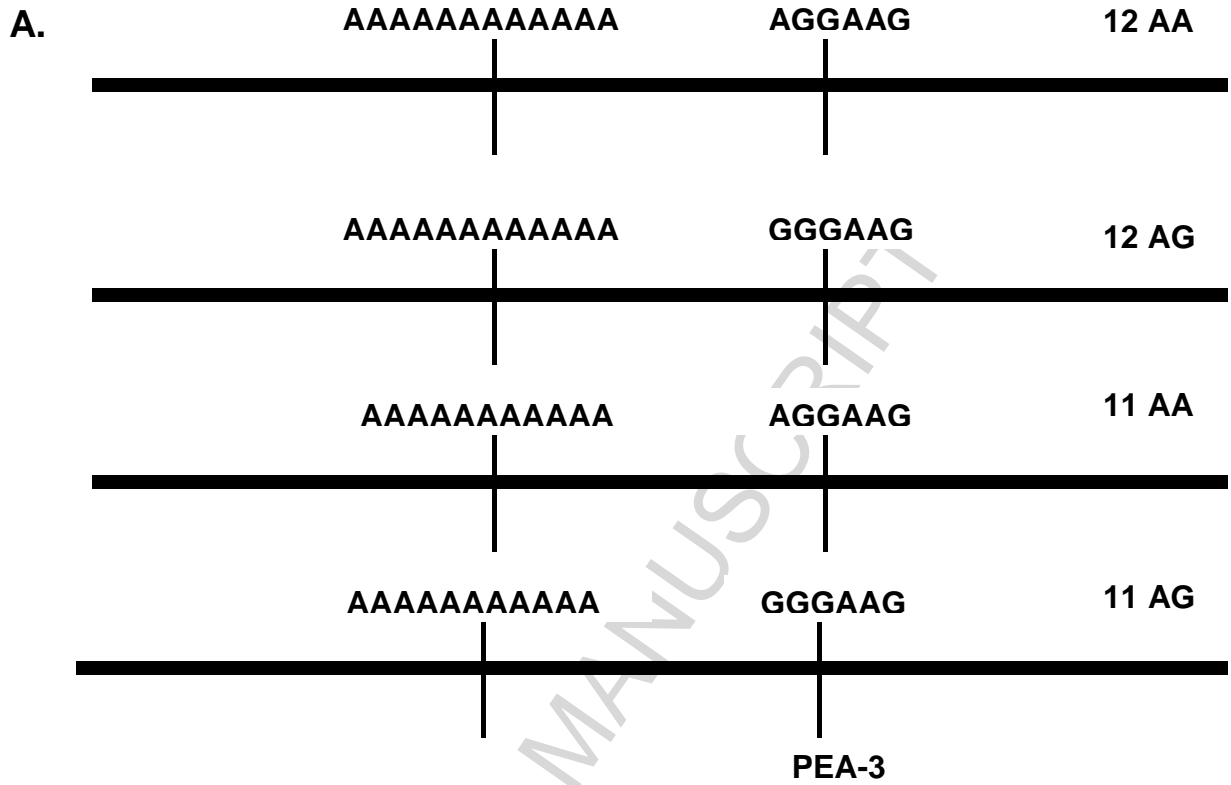


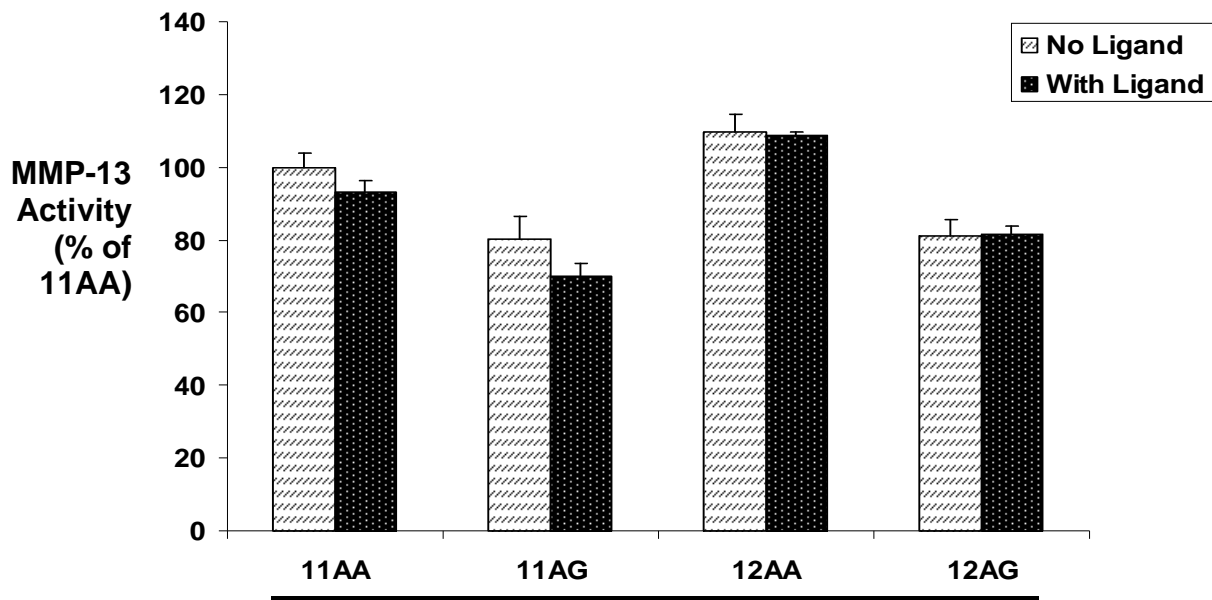
Figure 4: Comparison of the influence of ER- α and ER- β on the activity of polymorphic variants of the MMP-13 promoter.

A. Schematic representation of the polymorphic variations present in the promoter of human MMP-13 DNA. The construct 11AA has a stretch of 11 adenine residues. The PEA-3 site (AGGGAAG) in this construct starts with an A residue. The polymorphic variant 11AG has the same stretch of 11 adenine bases but the PEA-3 site starts with a Guanine residue (GGGGAAG). Both the constructs 12AA and 12AG have a stretch of 12 adenine residues but differ in the presence of adenine or guanine residues at the PEA-3 site as indicated in the figure above. All four constructs contain all the known key elements of rabbit MMP-13 promoter region such as Runx domain (RD), p53, PEA-3 and AP-1 binding sites. All the promoter fragments were cloned into the vector, pGL2-Basic, which contains the promoterless luciferase gene.

B. HIG-82 cells were transiently co-transfected with 0.5 μ g of pSG5-ER β or pSG5-ER α along with the human MMP-13 DNA constructs (11AA, 11AG, 12AA and 12AG), promoter (1 μ g) and the internal control plasmid, pRL-SV40 (0.05 μ g). Luciferase activity was expressed as the ratio of firefly luciferase activity and the Renilla luciferase reporter activity and values represent the mean \pm S.E. for three determinations. The activity of 11AA transfected with pSG5ER- α was set at 100%. The activity of the rest of the human MMP-13 constructs (\pm ER- α or ER- β) was expressed as percentage of the activity of 11AA (+ ER- α).

♣ P > 0.05 Not Significant

A



B

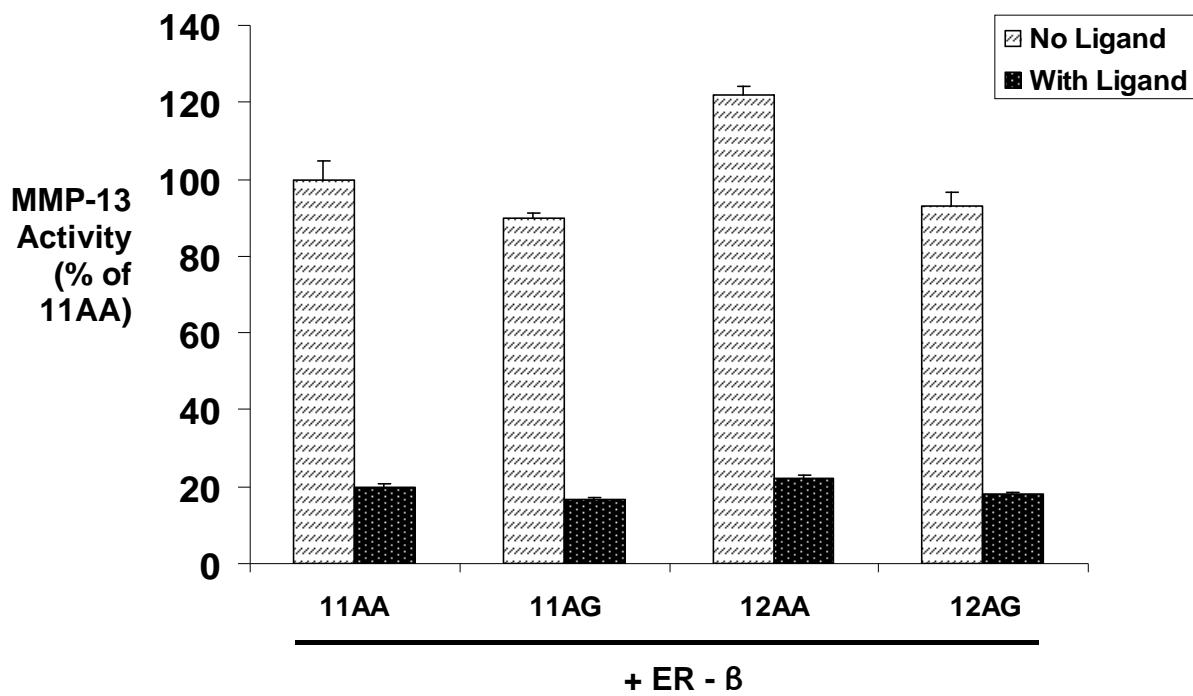


Figure 5: Polymorphisms in the MMP-13 promoter influence the response of the variants to the modulatory effects of ER isoforms. Rabbit Synoviocytes, HIG-82, were transiently co-transfected with either the polymorphic variants of the human MMP-13 promoter (12AA, 12AG, 11AA and 11AG along with 0.5 μ g of ER- α (Figure 5A) or ER- β (Figure 5B) and pRL-SV40. Cells were treated with 10^{-8} M $17\text{-}\beta$ Estradiol, for a period of 24 hours post transfection. Luciferase activity was expressed as the ratio of firefly luciferase activity and the renilla luciferase reporter activity. Values represent the mean \pm S.E. for three determinations and are expressed as percentage of 11AA luciferase activity without treatment with any ligand.