Title	Estrogen increases expression of the human prostacyclin receptor within the vasculature þÿ t h r o u g h a n E R ± - d e p e n d e n t m e c h a n i s m
Authors(s)	Turner, Elizebeth C.; Kinsella, B. Therese
Publication date	2010-02-26
Publication information	Journal of Molecular Biology, 396 (3): 473-486
Publisher	Elsevier
Link to online version	http://dx.doi.org/10.1016/j.jmb.2010.01.010
Item record/more information	http://hdl.handle.net/10197/3162
Publisher's statement	þÿ T h i s i s t h e a u t h o r s v e r s i o n o f a w o r k t h a t w a s a c c e p t e d f o Molecular Biology. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in Journal of Molecular Biology, 396 (3): 473-486 DOI 10.1016/j.jmb.2010.01.010.
Publisher's version (DOI)	10.1016/j.jmb.2010.01.010

Turner EC & Kinsella BT. *J Mol Biol*. (2010), **396**(3):473-86.

Estrogen increases expression of the human prostacyclin receptor within the vasculature through an ERα-dependent mechanism.

Running Title: Prostacyclin Receptor Gene Regulation by Estrogen

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Abstract

Prostacyclin and the prostacyclin receptor (IP) are implicated in mediating many of the atheroprotective effects of estrogen in both humans and in animal models but through unknown mechanisms. Hence, herein the influence of estrogen on IP gene expression in endothelial EA.hy926, human erythroleukemia 92.1.7 and primary human (h) aortic smooth muscle (1^{o} hAoSM) cells was investigated. Estrogen increased hIP mRNA levels, promoter (PrmIP)-directed reporter gene expression and cicaprost-dependent cAMP generation in all cell types, effects that were abrogated by actinomycinD and the general estrogen receptor (ER)-α/ERβ antagonist ICI 182,780. Furthermore, the ERα-selective agonist 4,4',4"-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), but not the ERβ-agonist 2,3-bis(4-Hydroxyphenly)-propionitrile, significantly increased hIP mRNA and PrmIP-directed gene expression. Deletional and mutational analysis of PrmIP uncovered an evolutionary conserved estrogen-response element (ERE) while electrophoretic mobility shift, antibody-supershift and chromatin immunoprecipitations assays confirmed the direct binding of ERα, but not ERβ, to PrmIP both *in vitro* and *in vivo.* Moreover, immunofluorescence microscopy corroborated that estrogen and PPT increased hIP expression in 1^{o} hAoSMCs. In conclusion, the hIP gene is directly regulated by estrogen that largely occurs through an ERα-dependent transcriptional mechanism and thereby provides critical insights into the role of prostacyclin/hIP in mediating the atheroprotective effects of estrogen within the human vasculature.

Key Words: Prostacyclin Receptor, gene expression, transcription, Estrogen-response element (ERE), promoter.

Introduction

The prostanoid prostacyclin plays a central role in haemostasis, acting as a potent inhibitor of platelet aggregation and as an endothelium-derived vasodilator [1; 2]. The prostacyclin receptor (IP) is abundantly expressed throughout the vasculature, including in platelets/megakaryocytes, macrophages, vascular endothelial and smooth muscle cells, various other tissues including the heart, kidney, lung, thymus, spleen and in sensory neurons of the dorsal root ganglion [2; 3]. The IP is primarily coupled to Gs/adenylyl cyclase activation, mediating prostacyclin inhibition of platelet aggregation and vascular tone [2; 3].

The cardioprotective effects of prostacyclin within the myocardium and vasculature are well documented [4; 5]. Alterations in the levels of prostacyclin, its synthase or receptor, the IP, are associated with a range of vascular dysfunctions including stroke and myocardial infarction [6; 7]. Multiple and frequent single nucleotide polymorphisms occur within the coding sequence of the human IP that correlate with predisposition to cardiovascular (CV) disease including enhanced intimal hyperplasia and platelet activation in deep vein thrombosis [8]. As a major product of cyclooxygenase (COX)-2, prostacyclin acts as a potent pro-inflammatory mediator and is abundantly produced during myocardial ischemia and hypoxia, offering cardioprotection [9; 10]. Recognition of the importance of prostacyclin for haemostasis and CV integrity has been critically highlighted by various clinical trials that established that certain COXIBs, the sub-class of non-steroidal anti-inflammatory drugs that selectively inhibit COX2, depress prostacyclin generation predisposing patients to increased risk of thrombotic stroke and myocardial infarction [11; 12]. While $IP^{-/-}$ null mice display normal vascular function, they exhibit enhanced thrombotic tendency in response to vascular injury in addition to reduced acute inflammatory responses [3].

The protective role of estrogens in the heart and vasculature have also been established and gender-specific differences in the incidence of CV disease occur both clinically and in animal studies [5]. For example, hormone/estrogen replacement therapy can prevent the primary onset of coronary artery disease in post-menopausal women [5], although such effects are not without controversy [13; 14]. The effects of estrogen are largely mediated through its binding to one of two estrogen receptor (ER)α and β subtypes, members of the nuclear receptor superfamily [15; 16]. ERα and ERβ display distinct patterns of expression and biological function largely acting as transcription factors to modulate expression of target genes by either direct binding to the estrogen-responsive element (ERE) with the consensus 5'-GGTCAnnnTGACC-3' palindromic sequence [17] or by indirect interaction with other transcription factors, such as Sp1 and Ap1 [15]. Ligand-activated ERs may, in turn, recruit co-activators such as CBP/p300, SRC-1, TIF2 or co-repressors including N-CoR and SMRT [18; 19].

In addition to such classic genomic regulation by estrogen and analogues, more rapid non-genomic effects also occur and it is thought that some of these CV protective actions may be mediated by direct effects on the vessel wall [5; 16; 20]. Consistent with this, there is accumulating evidence that many of the cardio-protective effects of estrogen are mediated due to its increased synthesis & release of the endothelial-derived vasodilators nitric oxide and prostacyclin [21]. For example, estrogen induces the synthesis and expression of COX1, COX2 and prostacyclin synthase, resulting in up to 6-fold increases in systemic prostacyclin levels. Moreover, in the female low density lipoprotein receptor null mice ($LDLR^{-/-}$), estrogen stimulated both COX2 expression and prostacyclin formation resulting in a substantial atheroprotection [22]. In the same study, further disruption of the IP gene abrogated the atheroprotective effects of estrogen and accelerated atherogenesis in the double $LDLR^{-/-}/IP^{-/-}$ null mouse [22]. However, despite this, the actual molecular basis of the role of the IP in mediating such estrogen-induced atheroprotection remains to be established. Moreover, it is currently unknown whether estrogen may directly, or indeed indirectly, affect IP expression levels possibly accounting for such effects [22] and hence, critically, remains to be investigated.

The overall aim and rationale of the current study is to address this deficit by characterizing the hIP gene, focusing primarily on delineating the mechanism determining its aforementioned role in mediating the response to estrogen within the vasculature. Herein, we have uncovered a consensus *cis*-acting ERE in the hIP promoter critical for the transcriptional regulation of hIP expression by estrogen in a host of cells of vascular origin, including in the human endothelial EA.hy926 and megakaryocytic human erythroleukemia (HEL) 92.1.7 cell lines [23] and cultured primary human aortic smooth muscle cells (1^{o} hAoSMCs). The data outlined provide compelling evidence that the hIP gene is a *direct* target of estrogen that occurs through an ERα-dependent mechanism and, accordingly, not only provides a molecular genetic basis for understanding the modes of regulation of hIP expression in health and disease but also for the combined protective roles of estrogen and prostacyclin within the CV system.

Results

Estrogen-regulation of hIP Expression in EA.hy926 and HEL 92.1.7 cells.

The incidence of CV disease is less pronounced in women than men and this difference narrows post-menopause, consistent with the widely acknowledged atheroprotective actions of estrogens in pre-menopausal females [20; 24; 25]. However the underlying mechanisms of cardio-protection are largely unknown. In recent animal studies [22; 26], the cardioprotective effects of estrogen in $LDLR^{-/-}$ mice were shown to be mediated in part by COX2-derived prostacyclin release. Furthermore, the *anti*-atherogenic effects of estrogen were abrogated in $IP^{-/-}$ null mice and atherogenesis accelerated in double $LDLR^{-/-}/IP^{-/-}$ null mice [22]. The rationale of the current study was to establish whether estrogen may directly or indirectly regulate the human prostacyclin receptor (hIP) expression, providing a possible molecular basis accounting for some or all of these effects [22; 25].

Initially, RT-PCR analysis was used to examine possible regulation of hIP mRNA expression by 17β-estradiol (E_2) in the human endothelial EA.hy926 and megakaryocytic HEL 92.1.7 cell lines, where the E_2-responsive COX2 and -non-responsive GAP3'DH transcripts served as controls. Quantitative real-time RT-PCR analysis established that E_2-treatment increased hIP mRNA in EA.hy926 (2.5-fold, $P < 0.0001$; **Figure 1A**) and HEL (1.3-fold, $P = 0.008$; **Figure 1B**) cells, while pre-incubation with the transcriptional inhibitor actinomycin D (ActD), but not with the translational inhibitor cycloheximide (CHX), completely abrogated the E_2-induction of hIP mRNA expression (**Figure 1A & 1B**, respectively). Moreover, E_2 also resulted in significant increases in COX2 mRNA in both cell types but did not affect GAP3'DH mRNA expression (**Supplemental Figure 1**).

Previous studies have defined the human IP promoter (here-on-in referred to as PrmIP) as nucleotides -2427 to -744, relative to the translational start codon (+1) [23]. Genetic firefly luciferase reporter assays were used herein to examine PrmIP-directed gene expression. Stimulation with E_2 resulted in concentration-dependent increases of PrmIP-directed luciferase expression in EA.hy926 and HEL cells (10 nM; 2-fold, $P < 0.0001$ and 1.7-fold, $P = 0.0006$, respectively; **Figure 1C & 1D**). In addition to its classic ERα- and/or ERβ-dependent genomic regulation, E_2 can mediate more rapid non-genomic effects such as through activation of GPR30, a member of the G protein coupled receptor superfamily [27], and activation of the mitogen activated protein kinase (MAPK) and phosphatidyl inositol 3' kinase (PI3'K)-dependent signalling cascades [27; 28]. Hence, to establish whether those pathways might contribute to the E_2-induction of PrmIP-directed gene expression, the effect of the p42/p44 extracellular signal-regulated kinase (ERK) and the PI3'K inhibitors PD98059 and Wortmanin, respectively, was examined. While E_2 led to a time-dependent increase in PrmIP-

directed gene expression in both EA.hy926 and HEL cells, neither PD98059 nor Wortmanin significantly affected that expression in either cell type (**Figure 1E & 1F**). Furthermore, treatment with either PD98059 and Wortmannin alone had no effect whatsoever on basal PrmIP-directed luciferase expression in either cell type (**Figure 1E & 1F**). Hence, these data establish that hIP gene expression is indeed up-regulated by E_2 and that this occurs at the transcriptional level through a possible ERα/ERβ-dependent mechanism.

Determination of Estrogen Receptor Specificity.

E_2-induced changes in transcription through ERα and/or ERβ regulate expression of distinct as well as overlapping sets of target genes [29]. Herein, immunoblot analysis confirmed endogenous expression of both ERα and ERβ in EA.hy926 and HEL cells and that expression is increased by E_2-stimulation (**Figure 2B & 2D**, respectively). The non-selective ERα/ERβ antagonist ICI 182,780 abrogated the E_2-induced increases in hIP mRNA (**Figure 2A & 2C**) and PrmIP-directed luciferase expression (**Figure 2E & 2F**). Moreover, the ERα-selective agonist 4,4',4"-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), but not the ERβ agonist 2,3-bis(4-Hydroxyphenly)-propionitrile (DPN), increased hIP mRNA in EA.hy926 and HEL cells (**Figure 2A & 2C;** P = 0.0003 and P = 0.0005, respectively). Furthermore PPT, but not DPN, also increased PrmIP-luciferase expression in both cell types (**Figure 2E & 2F;** $P < 0.0001$ and $P = 0.002$, respectively).

Ectopic expression of ERα also resulted in concentration-dependent increases in PrmIP-directed luciferase expression in EA.hy926 (**Figure 2G**) and, to a lesser extent, in HEL (**Figure 2H**) cells in response to E_2. This cellular difference may possibly be explained by the relatively high levels of endogenous ERα protein expression in HEL cells compared with EA.hy926 cells (**Figure 2B & 2D**). Over-expression of ERβ did not significantly affect PrmIP-directed luciferase expression in either cell line (**Figure 2G & 2H**). Western blot analysis confirmed that both ERα and ERβ were over-expressed to equivalent levels in the transfected EA.hy 926 and HEL cells.

As stated, the hIP is primarily coupled to Gs-mediated adenylyl cyclase activation leading to agonist-dependent increases in cAMP generation[2; 3]. Hence, herein, the effect of E_2 on cAMP generation in HEL and EA.hy926 cells in response to the selective IP agonist cicaprost was examined. E_2 significantly increased cicaprost-induced cAMP generation in both HEL (P = 0.0002, ANOVA; **Figure 2I & 2J**) and EA.hy926 (P = 0.0002, ANOVA) cells. The non-selective ERα/ERβ antagonist ICI 182,780 [30] abrogated the E_2-induced increases in cicaprost-dependent cAMP generation in both cells types while PPT, but not the ERβ agonist DPN, increased cicaprost-dependent cAMP generation in HEL (P = 0.0004, ANOVA; **Figure 2I & 2J**) and EA.hy 926 (P = 0.0002, ANOVA) cells. Taken together, these data indicate that ERα, but not ERβ, selectively

mediates E_2-inductions in PrmIP-directed gene expression in endothelial EA.hy926 and megakaryocytic HEL cells leading to functional increases in hIP expression in both cell types.

Identification of a Functional ERE within the PrmIP

Thereafter, 5' deletional- and genetic reporter-analyses localized the E_2-responsive region(s) within PrmIP. Initially, consistent with recently reported data in HEL cells[23], progressive 5' deletion of PrmIP identified the core basal promoter (-1022 to -895) and an upstream repressor region (-1761 to -1682) in EA.hy926 cells (**Figure 3**). E_2-treatment resulted in approximately 2-fold increases in luciferase expression-directed by the PrmIP-, PrmIP1- and PrmIP2- subfragments ($P < 0.0001$ in all cases; **Figure 3**) but did not affect luciferase expression by the smaller PrmIP3-, PrmIP4-, PrmIP5-, PrmIP6- or PrmIP7-subfragments. More specifically, deletion of nucleotides -1682 to -1575 resulted in a complete loss of E_2-induced expression in both EA.hy926 (**Figure 3**) and HEL (**Supplemental Figure 2**) cells, thereby localizing the E_2-responsive region.

Bioinformatic analyses of PrmIP predicted a putative ERE between -1682 to -1575 (5' nucleotide at -1654, containing a single nucleotide variation from the consensus ERE; **Figure 4**). Hence, the effect of E_2 on luciferase expression directed by PrmIP2 containing either that putative ERE or it's mutated ERE* equivalent (**Figure 4**) was examined. While E_2-stimulation yielded a 2-fold increase in PrmIP2-directed luciferase expression ($P < 0.0001$) in EA.hy926 cells, it had no effect on gene expression directed by the equivalent PrmIP2 subfragment containing the mutated ERE* (**Figure 4A**). Similarly, disruption of the ERE* completely inhibited the E_2-responsiveness of PrmIP2 in HEL cells (**Figure 4B**).

Electrophoretic mobility shift assays (EMSAs) and antibody shift assays further investigated the presence and specificity of nuclear/transcription factors capable of binding the putative ERE within PrmIP *in vitro*. Incubation of the biotinylated ERE probe, spanning nucleotides -1671 to -1637, with nuclear extract from HEL cells resulted in the formation of a major DNA-protein complex, designated C1, and a second more diffuse, slower migrating DNA-protein complex designated C2 (**Figure 5A**, lane 2). Both C1 and C2 complexes were efficiently competed by an excess of the corresponding non-labelled ERE probe (**Figure 5A**, lane 3). The specificity of nuclear factor binding to the ERE probe was verified whereby a competitor based on a consensus ERE sequence, but not on a randomised IP sequence, specifically competed both C1 and C2 DNA-protein complexes (**Figure 5A**, lanes 4 and 5). Moreover, supershift assays employing *anti*-ERα and *anti*-ERβ antibodies demonstrated specific and direct binding of ERα to the ERE probe of PrmIP in HEL cells as evidenced by the presence of an additional intense supershifted complex, whereas only a very weak ERβ-containing supershifted complex was evident (**Figure 5A**, lanes 6 & 7, respectively). As additional controls, it was determined that neither complex C1 nor C2 nor the

anti-ERα and *anti*-ERβ antibody- supershifted complex were generated by incubation of the ERE probe with the nuclear extract dialysis buffer (NEDB; **Supplemental Figure 3**).

To investigate whether endogenous ERα and/or ERβ actually binds to PrmIP *in vivo*, ChIP analysis was performed using *anti*-ERα and *anti*-ERβ specific antibodies. In vehicle-treated EA.hy926 and HEL cells, PCR amplification yielded products from input chromatin and chromatin recovered from the *anti*-ERα immunoprecipitates, but not from *anti*-ERβ or control normal rabbit IgG immunoprecipitates (**Figure 5B**; upper panels). Moreover, E_2-stimulation resulted in significant increases in amplicons generated in the *anti*-ERα derived immunoprecipitations in both EA.hy926 and HEL cells, while no amplicons were generated from *anti*-ERβ or control IgG immunoprecipitates in either cell line (**Figure 5B**; lower panels). Additionally, PCR analysis using primers for a non-specific region of PrmIP did not generate amplicons from ERα or ERβ immunoprecipitates in either cell type (**Figure 5C**). Collectively, these data confirm that ERα, and not ERβ, specifically binds to a functional ERE within PrmIP to mediate E_2-induced upregulation of hIP expression in both EA.hy926 and HEL cells.

E_2-regulation of IP Expression in Primary Human Aortic Smooth Muscle Cells

To determine whether the critical E_2-ERα-ERE-mediated regulation of PrmIP identified herein in human vascular endothelial EA.hy926 and megakaryocytic HEL cell lines may occur more widely within cells derived from the vasculature, the effect of E_2 on hIP expression was also investigated in primary human aortic smooth muscle cells (1^o hAoSMCs).

Semi-quantitative RT-PCR confirmed E_2 up-regulation of both hIP and COX2 mRNA expression in 1^o hAoSMCs (**Figure 6A**) while real-time RT-PCR revealed a 2.2-fold increase in E_2-induced hIP mRNA ($P < 0.0001$), an effect abolished by both ActD and ICI 182,780 (**Figure 6C**). Immunoblot analysis confirmed that ERα, and to a lesser extent, ERβ are expressed in 1^o hAoSMC and both are increased in response to E_2 (**Figure 6B**). Treatment with the selective ERα and ERβ agonists PPT and DPN, respectively, confirmed that ERα mediates E_2-upregulation of hIP mRNA in 1^o hAoSMCs ($P < 0.0001$; **Figure 6C**). Furthermore, E_2 led to a 2.2-fold ($P < 0.0001$) increase in PrmIP-directed luciferase expression in 1^o hAoSMCs (**Figure 6D**), an affect completely abrogated by ICI 182,780, while PPT, and not DPN, also significantly increased PrmIP-directed luciferase expression ($P = 0.0002$; **Figure 6D**). The antagonist ICI 182,780 also abrogated the E_2-induced increases in cicaprost-dependent cAMP generation, while PPT, but not DPN, significantly increased cicaprost-dependent cAMP generation in 1^o hAoSMCs ($P = 0.0002$ and $P = 0.0003$, respectively; **Figure 6E**). Moreover, ChIP analysis demonstrated that ERα, not ERβ, is capable of direct binding to PrmIP in 1^o hAoSMCs *in vivo,* and that binding is enhanced by E_2, consistent with findings in EA.hy926 and HEL cells (**Figure 6F & 6G**).

Thereinafter, indirect immunofluorescent staining of 1^{o} hAoSMCs with an affinity purified *anti*-IP antisera directed to it's intracellular $(IC)_2$ domain confirmed expression of endogenous hIP on the plasma membrane and intracellular membranes and established that exposure to E_2 for 24h resulted in substantial increases in hIP levels (**Figure 7A**). The antigenic IC_2 peptide completely blocked specific immunodetection of the hIP, thereby further validating specificity of the *anti*-IP antisera (**Figure 7A**). Expression of COX2 was also confirmed to be significantly increased by E_2 and showed distinct perinuclear staining (**Figure 7A**), consistent with previous reports [31]. Treatment with ICI 182,780 completely abrogated the E_2-induced increase in both hIP and COX2 expression (**Figure 7B**) while the ERα and ERβ selective-agonists PPT and DPN, respectively, demonstrated that the E_2-induced expression of both hIP and COX2 mainly occurs through an ERα-dependent mechanism (**Figure 7B**).

Collectively, data presented herein establish that E_2-upregulates expression of the hIP in several cell lineages derived from the human vasculature that occurs through direct binding of ERα to a functional ERE within PrmIP. Such regulation of the hIP expression adds to the growing appreciation of the importance of E_2-mediated regulation of the COX-derived prostacyclin metabolite and of its signalling & function within the vasculature.

Discussion

CV disease is the leading cause of morbidity and premature mortality, particularly in western societies, but coronary heart disease develops on average 10 years later in women than in men. This time delay has been partly attributed to the protective effects of female sex hormones, in particular the estrogens [16; 20]. Mechanistic studies carried out in *in vitro* cell/tissue preparations and in animal studies have demonstrated that both natural and synthetic estrogens exhibit *anti*-inflammatory and vasoprotective effects [24; 32; 33; 34; 35]. Moreover, 17β-estradiol (E_2) has been shown to lead to rapid endothelium-dependent and -independent dilation of coronary arteries in both women and men and to augment endothelium-dependent relaxation of coronary arteries *ex vivo* and to improve endothelial function [36]. The endothelium-derived prostacyclin not only plays a critical dynamic role in haemostasis and in the regulation of vascular tone but also, similar to E_2, acts as a critical cytoprotectant within the wider CV system [21; 37]. Expression of COX1, COX2 and prostacyclin synthase and, consequently, synthesis of prostacyclin are significantly elevated in response to E_2 [21] and compelling data generated in experimental animal models suggest that the *anti*-atherogenic effects of E_2 are mediated, at least in part, through the prostacyclin receptor/IP [22]. Herein, the aim of the current study was to carry out a detailed mechanistic study with the specific objective of seeking clarity *vis a vis* the possible regulation of IP expression by E_2 as suggested, but never actually demonstrated, from the numerous observations generated from such studies in both humans and animals.

Stimulation with E_2 resulted in the up-regulation of COX2 and hIP mRNA levels and increased PrmIP-derived gene expression and cicaprost-dependent cAMP generation in both model vascular endothelial (EA.hy926) and megakaryocytic (HEL 92.1.7) cells and 1^{o} hAoSMCs. The transcriptional inhibitor ActD and the non-selective ER antagonist ICI 182,780 completely abrogated any E_2-stimulatory effects on hIP expression in all cell lines. Moreover, the translational inhibitor CHX and the MAPK and PI3'K inhibitors PD98059 and Wortmannin did not abrogate E_2-stimulatory effects on hIP expression in both EA.hy 926 and HEL cells. Collectively, these data establish that E_2-regulation of the hIP occurs through a transcriptional mechanism and not through secondary events such as regulation of GPR30 and/or downstream activation of the MAPK or PI3'K signalling.

These findings identify the hIP, as well as confirming COX2, as a *bona fide* target of E_2 and may help, at least partly, to explain the observations in $IP^{-/-}$ null mice whereby the atheroprotective effects of E_2 are abrogated, highlighting a critical role for the hIP in mediating the cardioprotective effects of E_2 [22]. To our knowledge, data presented herein is the first demonstration of (h)IP regulation by estrogen within the vasculature.

The transcriptional effects of E_2 are largely mediated through two distinct nuclear receptors, ERα and ERβ, each encoded by unique genes but displaying distinct patterns of expression and function in various tissues [17]. ERα is the predominant subtype expressed in the breast, uterus, cervix, vagina and additional target organs whereas ERβ exhibits a more limited expression pattern and is primarily detected in the ovary, prostate, testis, spleen, lung, hypothalamus and thymus [38; 39]. To determine any possible ER subtype specificity in the E_2-mediated up-regulation of the hIP, the effect of the specific ERα and ERβ agonists was investigated. PPT, and not DPN, resulted in significant increases in hIP mRNA, PrmIP-directed gene expression and cicaprost-dependent cAMP generation in EA.hy926, HEL and 1^o hAoSM cells suggesting an ERα-dependent mechanism. Furthermore, immunofluorscence microscopy using selective *anti*-hIP antibodies corroborated these findings showing E_2-ERα-mediated increased expression of the hIP in 1^o hAoSMCs and provides evidence for similar modes of regulation of COX2 [22] and hIP in response to E_2 in the vasculature. Notably, while the ERβ agonist DPN did show modest inductions in immunoreactive expression of both COX2 and the hIP, ectopic expression of ERα, but not ERβ, significantly increased PrmIP-directed gene expression in all cell types understudy strongly suggesting an ERα-specific mechanism. Whether ERβ may regulate hIP gene expression in a cell-type specific manner or, indeed, whether it may act as a competitor of ERα-regulated expression of the hIP, as occurs in the case of the BRCA2 gene [40], requires further investigation.

Stimulation of target gene expression in response to E_2, or other ER agonists, largely occurs through one of two mechanisms [17; 38]. One such mechanism, exemplified by E_2-regulation of the progesterone receptor [41], occurs through 'direct binding' whereby the E_2-liganded ER binds directly to a specific ERE and interacts directly with co-activator proteins and components of the RNA polymerase transcription initiation complex resulting in enhanced transcription. The second mechanism is referred to as 'tethering' whereby ER interacts with other DNA-bound transcription factor(s), and not DNA, stabilising DNA/protein interactions and increasing transcription. An example of tethering is the interaction of ERα with Sp1, conferring E_2-responsiveness to the LDL receptor gene [42].

The consensus *cis*-acting ERE was originally identified by aligning homologous sequences in the 5' flanking regions of the estrogen-regulated vitellogenin A1, A2, B1, B2 and apo-VLDLII genes [43]. The minimal ERE sequence is a 13 bp palindromic inverted repeat (IR) with the consensus 5'-GGTCAnnnTGACC-3' [17; 44], which can act in an orientation- and distance-independent manner and, therefore, is defined as an enhancer element [44]. In the case of the ERα subtype, extension of the length of the ERE palindrome with an A/T rich sequence, of approximately 15 nucleotides, immediately flanking the 5' ERE is particularly important in

determining its binding affinity[17]. Herein, the site of action of E_2 was localised to -1682 to -1575 within the PrmIP and bioinformatic analysis revealed a near perfect ERE at -1654 with 12 bp of the 13 bp palindromic sequence identical to the consensus ERE and, notably, also containing a 5' flanking "A/T rich sequence", optimal for ERα binding [17]. Moreover, the ERE and flanking 5'A/T enriched-region were found to be highly conserved in a host of other species including within the dog, bovine and horse IP promoters (**Figure 4C**).

Hence, herein we investigated whether the evolutionary conserved ERE at -1654 mediates the E_2-induction of PrmIP-directed gene expression. Stimulation with E_2 led to significant increases in PrmIP2-directed luciferase expression in EA.hy926 and HEL cells, but did not have an effect on expression by those subfragments containing the mutated ERE*. Moreover, EMSA and antibody supershifts confirmed the specific binding of ERα, and to a much lesser extent ERβ, *in vitro*. Superseding these findings, ChIP analysis with fragmented chromatin from EA.hy926, HEL and 1^o hAoSM cells confirmed specific binding of ERα, and not ERβ, within the E_2-responsive region of PrmIP *in vivo*. Furthermore, E_2-stimulation led to a significant increase in specific ERα-DNA interaction in all cell types. Collectively, these data demonstrate that ERα serves as a *trans*-acting factor critical for regulation of the hIP in response to E_2 through a direct E_2-ERα-ERE mechanism.

In conclusion, we have identified an evolutionary conserved *cis*-acting ERE critical for the transcriptional regulation of the hIP in model and primary cells derived from the human vasculature and confirm that the hIP gene is regulated by E_2 through a direct ERα-ERE-dependent mechanism. These data provide an important molecular genetic platform for understanding the critical role of the hIP as a mediator of the atheroprotective effects of E_2 and its wider contribution to mechanisms of cardio-protection in humans. While it is appreciated that our studies are performed in cellular based systems, these molecular genetic studies are indeed likely to reflect the more physiological/clinical setting. Clinical trials involving E_2, such as Women in Health Initiative (WHI), have led to conflicting data regarding the clinical safety of HRT as a cardio-protectant, or not, post-menopause [14]. In light of the fact that the data herein pertaining to the hIP adds to a growing list of other proteins including COX1-, COX2- and prostacyclin synthase-associated with the important vasodilator prostacyclin and which are also upregulated by E_2, it is tempting to propose that data from the E_2 clinical trials might perhaps be re-evaluated or, indeed, any further trials involving E_2 may take a more detailed consideration of the prostanoid/prostacyclin-regulatory pathways/systems, such as within the vasculature.

Materials & Methods

Materials

pGL3Basic, pRL-Thymidine Kinase (pRL-TK), and Dual Luciferase® Reporter Assay System were obtained from Promega Corporation and pCRE-Luc from Strategene. DMRIE-C® was from Invitrogen Life Technologies and Effectene® from Qiagen. *Anti*-ERα (sc-7207x), *anti*-ERβ (sc-8974x), normal rabbit IgG (sc-2027) and goat *anti*-rabbit horseradish peroxidise (sc-2204) were obtained from Santa Cruz Biotechnology. *Anti*-HDJ-2 antibody was from Neomarkers. 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), 2,3-bis(4-Hydroxyphenly)-propionitrile (DPN) and ICI 182,780 were all obtained from TOCRIS. ActinomycinD (ActD), 17β-estradiol, cyclohexamide (CHX) and Wortmannin were from Sigma and PD98059 was obtained from Calbiochem.

Cell Culture

Human erythroleukemic (HEL) 92.1.7 cells [45], obtained from the American Type Culture Collection, were cultured in RPMI 1640, 10 % fetal bovine serum (FBS). Human endothelial EA.hy926 cells [46], obtained from the Tissue Culture Facility at UNC Lineberger Comprehensive Cancer Centre, Chapel Hill, NC, were cultured in DMEM, 10 % FBS. Primary human aortic smooth muscle cells (1° h.AoSMCs) were purchased from Cascade Biologics (C-007-5C) and routinely grown in either Smooth Muscle Cell Growth Medium 2 (Promocell GMBH, C-22062) supplemented with 0.5 ng/ml epidermal growth factor, 2 ng/ml basic fibroblast growth factor, 5 μg/ml insulin, 5% FBS or in M199, 10% FBS. All mammalian cells were grown at 37 ^{o}C in a humid environment with 5 % CO_2 and were confirmed to be free of mycoplasma contamination.

Luciferase-based Genetic Reporter Plasmids

The plasmid pGL3B:PrmIP, encoding PrmIP (-2427 to -774, relative to the translation start codon at +1) from the human prostacyclin receptor (IP) in the pGL3Basic reporter vector, in addition to pGL3B:PrmIP1, pGL3B:PrmIP2, pGL3B:PrmIP3, pGL3B:PrmIP4, pGL3B:PrmIP5, pGL3B:PrmIP6 and pGL3B:PrmIP7 were previously described [23]. Site-directed mutagenesis of the ERE (-1654) from gGTCAagGTCAc to g**TG**C**T**ag**TG**C**T**c was carried by Quik-Change™ method (Stratagene) using pGL3B:PrmIP2 as template and primers Kin724 (5'-dCAAAATATGATTCCTGAAG**TG**C**T**AG**TG**C**T**CCAGAGCTTGGCCTGGGGC -3') and complementary Kin725 to generate pGL3B:PrmIP2^{ERE}*. The fidelity of all plasmids was confirmed by DNA sequence analysis.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from 1^{o} hAoSMCs, HEL 92.1.7 and EA.hy926 cells using TRIzol reagent (Invitrogen Life Technologies). DNase 1-treated total RNA was converted to first strand (1^{o}) cDNA with MMLV RT (Promega). PCR primers were designed to specifically amplify hIP mRNA sequences (5'-dGAAGGCACAGACGCACGGGA -3', Nu -57 to -37; Kin264) and (5'-dGGCGAAGGCGAAGGCATCGC -3'; Nu 294 to 275; Kin266) to generate a 348 bp amplicons and hCOX2 mRNA sequences (5'- dATCTCAGTCTTGAAGCCAATT-3', Nu 3119 to 3139; DT121) and (5'-dGAGCTAAATAGCAGTCCTGAG3', Nu 3339 to 3360; DT122) or, as an internal control, to amplify glyceraldehyde-3-phosphate dehydrogenase (GA3'PDH) mRNA (467 bp) (5'-dTGAAGGTCGGAGTCAACG-3'; Nu 527-545; Kin291) and (5'-dCATGTGGGCCATGAGGTC-3'; Nu 993-976; DT92). All primers were designed to span across an intron such that only PCR products from 1^{o} cDNA would be amplified, thereby eliminating genomic artifacts. The levels of hIP mRNA expression were determined by measurement of PCR product band intensities on densitometirc scans; in each case, expression levels are represented as a ratio relative to GA3'PDH expression (i.e. hIP/GA3'PDH ± SEM, arbitrary units). Real-time quantitative PCR was performed using the human prostaglandin I_2 receptor (hIP) gene expression assay (Hs00168765_m1) from Applied Biosystems as per the manufacturer's instructions using a 7900HT Fast Real-time PCR system (Applied Biosystems).

Assay of Luciferase Activity

HEL 92.1.7 and EA.hy926 cells were co-transfected with various pGL3Basic-recombinant plasmids, encoding firefly luciferase, along with pRL-TK, encoding renilla luciferase, using DMRIE-C® transfection reagent as previously described [23]. In the case of the 1^{o} hAoSMCs, in brief, 24h prior to transfection cells were plated in 6-well format to achieve 60-80 % confluency at time of transfection and were co-transfected with recombinant pGL3Basic (2 μg) and pRL-TK (200 ng) using 5 μl Effectene® reagent as per the manufacturer's instructions (Qiagen). Medium was supplemented 24h post-transfection with either 17β-estradiol (E_2; 10 nM), 4,4',4"-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT; 100 nM), 2,3-bis(4-Hydroxyphenly)-propionitrile (DPN; 100 nM) and/or ICI 182,780 (ICI; 100 nM) or, as a control, with vehicle [PBS, 0.1% EtOH] for 24 h. Alternatively, to examine the effect of MAPK and PI3'K inhibitors on E_2-induced PrmIP-directed gene expression the medium was supplemented with either PD98059 (PD; 10μM), wortmannin (Wort; 400 nM) or, as a control, with vehicle [Veh, 0.01% DMSO] for 30 mins prior to stimulation with 17β-estradiol (E_2; 10 nM) for 0 to 24 h. Firefly and renilla luciferase activity was assayed 16h later using the Dual Luciferase Assay System®. To investigate the effect of over-expression of

ERα and ERβ on PrmIP2-directed luciferase expression, pcDNA3.1-ERα and pcDNA3.1-ERβ (0-2.0 μg), or, as a negative control, pcDNA3.1, were transiently transfected into HEL, EA.hy926 or 1° hAoSMCs, as described above, along with recombinant pGL3B:PrmIP2. Firefly and renilla luciferase activity was assayed after 48h using the Dual Luciferase Assay System®. Relative firefly to renilla luciferase activities (arbitrary units) were calculated as a ratio and were expressed in relative luciferase units (RLU).

A reporter gene assay was performed to investigate changes in the intracellular levels of cAMP using the method described by Fitzgerald *et al.* [47] with minor modifications. In brief, luciferase reporter pCRE-Luc (1 μg; Stratagene) was co-transfected with 50 ng pRL-TK into HEL, EA.hy 926 and 1° AoSM cells. Cells were incubated 24h post-transfection with either vehicle (V; PBS, 0.01% EtOH), E_2 (10 nM), E_2 (10 nM) plus ICI 182,780 (100 nM), ICI 182,780 (100 nM), PPT (100 nM) or DPN (100 nM) for 24h. Cells were treated 48h post-transfection with IBMX (100 μM) at 37 °C for 30 min and then stimulated with either vehicle (V; DMSO) or 1 μM cicaprost at 37 °C for 3h. Firefly and renilla luciferase activity was assayed 52 hr post-transfection using the Dual Luciferase Assay System® and expressed as a ratio (relative luciferase units; RLU).

Western Blot Analysis

Both endogenous and ectopic expression of ERα and ERβ proteins in HEL, EA.hy926 and 1° hAoSM cells was confirmed by western blot analysis. Briefly, whole cell protein was resolved by SDS-PAGE (10 % acrylamide gels) and transferred to polyvinylidene difluoride (PVDF) membrane according to standard methodology. Membranes were screened using *anti*- ERα and *anti*- ERβ sera in 5 % non fat dried milk in 1 x TBS (0.01 M Tris-HCl, 0.1 M NaCl, pH 7.4) for 2h at room temperature followed by washing and screening using goat *anti*-rabbit horseradish peroxidise followed by chemiluminescence detection. To confirm uniform protein loading, the blots were stripped and rescreened with *anti*-HDJ-2 antibody (Neomarkers) to detect endogenous HDJ-2 protein expression. In all cases the relative levels of ERα/ERβ expression in vehicle- or E_2-incubated cells were normalized against HDJ-2 expression.

Electrophoretic Mobility Shift and Supershift Assays

Nuclear extract was prepared from HEL, EA.hy926 and 1° hAoSM cells essentially as previously described [48]. The identity and sequence of the forward biotin-labelled oligonucleotide probe is as follows: ERE probe; 5'-[Btn]d GATTCCTGAA**GGTCA**AGG**TCACC**AGAGCTTGGCCTG -3'; Nu -1671 to -1637 of PrmIP. The sequence of the corresponding non-labelled complementary oligonucleotide is inferred. The identities and sequences of the forward non-labelled competitor/non

competitors include (1). PrmIP ERE competitor; 5'-d GATTCCTGAA**GGTCA**AGG**TCACC**AGAGCTTGGCCTG -3'; Nu -1671 to -1637 of PrmIP. (2). Consensus ERE competitor; 5'-dGGATCTAGGTCACTGTGACCCCGGATC -3'. (3) Non-specific competitor; 5'-dTGCGCCCGGCCTTCCATGCTCTTTGAC-3'. For electrophoretic mobility supershift assays, nuclear extract (2 μg total protein) was pre-incubated with 3 μg of *anti*-ERα or *anti*-ERβ sera for 1h at room temperature prior to the addition of the biotinylated ERE probe. As additional controls, nuclear extract or nuclear extract dialysis buffer (NEDB; 20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.4 mM PMSF, 0.5 mM EDTA, 0.2 mM EGTA and 0.2 mM EGTA;[48]) was pre-incubated either with the vehicle (-) or with *anti*-ERα and *anti*-ERβ sera prior to incubation with the biotinylated ERE probe, as indicated.

ChIP analysis

Chromatin immunoprecipitation (ChIP) assays were performed as previously described [23]. PCR analysis was carried out using 2-3 μl of ChIP sample as template or, as a positive control, with an equivalent volume of a 1:20 dilution of the input chromatin DNA. Sequences of the primers used for the ChIP PCR reactions and corresponding nucleotides within PrmIP include.

1. 5′-dGAGAGGTACCCAGCGGTGGTGGCTTGGCTGTG-3′ , Nu -1761 to -1729
2. 5′-dCTCTAAGCTTGGAGACTTCCATGGC-3′ , Nu -1555 to -1540
3. 5′-dGAGAGACGCGTAGCTACTCGGGAGGCTGAGGCAC-3′, Nu -774 to -740
4. 5′-dGAGAGGTACCACCCTGAGACAGCCCAGG-3′, Nu -1271 to -1243

Immunofluorescence Microscopy

A polyclonal *anti*-IP antibody directed to intracellular $(IC)_2$ domain of the hIP (amino acid residues CLSHPYLYAQLDGPR; IP peptide) was raised in rabbits following conjugation to the carrier protein keyhole limpet haemacyanin according to standard procedures. Following affinity purification on SulfoLink-conjugated IP peptide resin (Pierce), indirect immunofluorescent detection of hIP and COX2 (*anti*-COX2; sc-1745) expression was determined in permeabilised (3.7 % paraformaldehyde, 0.2 % triton-X) or, as controls, in non-permeabilised cells. As additional controls, the *anti*-hIP antibody was pre-incubated with its cognate IP peptide (10 μg/ml) prior to exposure to cells. In parallel, nuclei was counter-stained with 4,6-diamidino-2-phenyl-indole (DAPI; 0.5 μg/ml, 1 min). All slides were imaged, at x 63 magnification, using Zeiss Imager.M1 AX10 microscope using AxioVision software (Version 4.4) for acquiring multichannel images with filters appropriate for AlexaFluor488 and DAPI fluorescence.

Statistical Analysis

Statistical analysis of differences were analysed using the two-tailed Students' unpaired *t*-test or, as specifically indicated in the text, using two-way analysis of variance (ANOVA). All values are expressed as mean ± standard error of the mean (SEM). *P*-values ≤ 0.05 were considered to indicate statistically significant differences and *, **, ***, **** indicate $P \leq$. 0.05, 0.01, 0.001, 0.0001 for two-tailed Students' unpaired *t*-test analysis and ### indicates $P \leq 0.001$ for ANOVA analysis, respectively.

Acknowledgements: The plasmids pcDNA3.1-ERα and pcDNA3.1-ERβ were kindly provided by Dr Leigh Murphy, University of Manitoba, Canada. This work was supported by The Health Research Board (RP2006/14) and Science Foundation Ireland (Grant No. SFI: 05/IN.1/B19).

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FIGURES

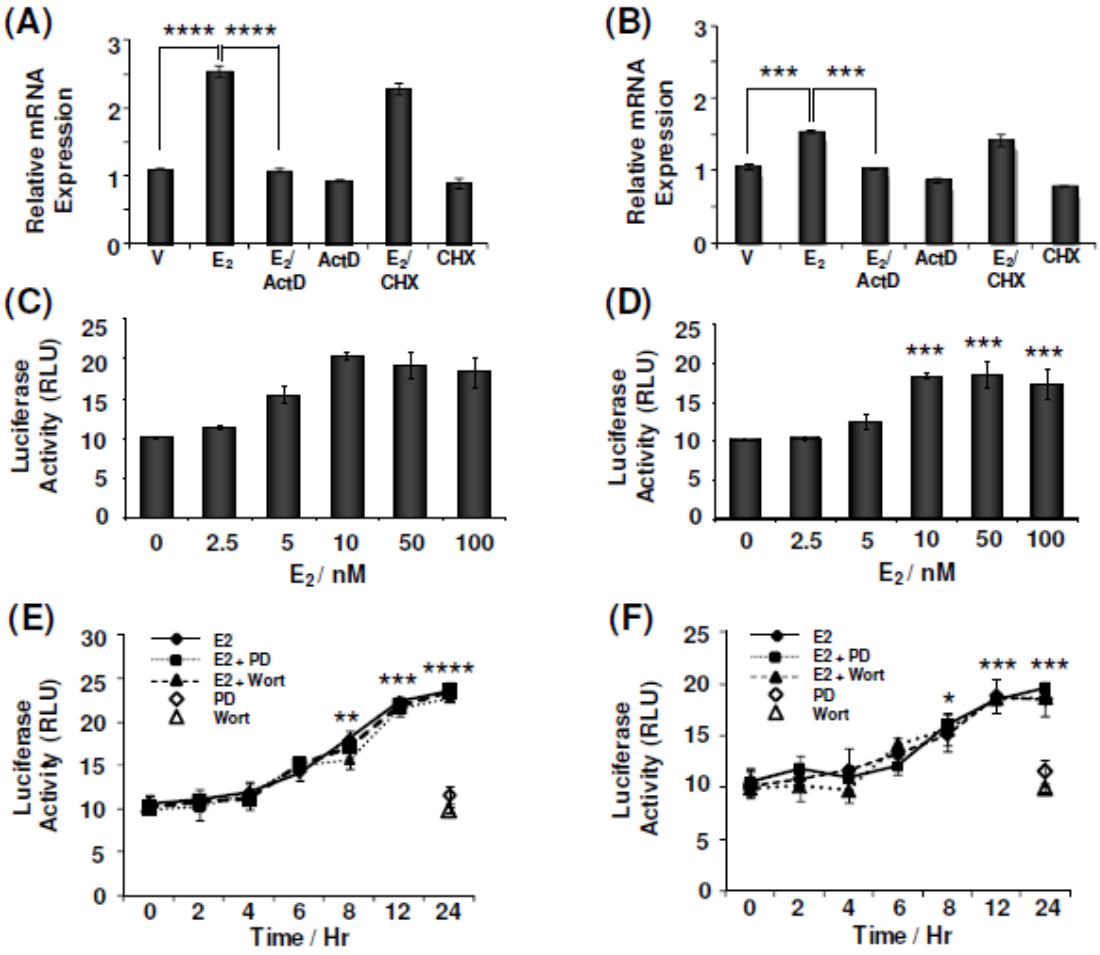


Figure 1: **Effect of E_2 on PrmIP-directed Gene Expression and Cicaprost-dependent cAMP Generation in EA.hy926 and HEL cells.**

Panels A & B: Quantitative real-time RT-PCR analysis of hIP relative to GA3'PDH mRNA expression in EA.hy926 (**Panel A**) and HEL (**Panel B**) cells pre-incubated for 24h with either vehicle (V), E_2 (10 nM) and/or ActD (10 µg/ml) and CHX (20 µg/ml). **Panel C & D**: Effect of E_2 (0 – 100 nM; 24h) on PrmIP-directed luciferase gene expression in EA.hy926 (**Panel C**) and HEL (**Panel D**) cells (RLU ± SEM; n = 6). **Panels E & F:** Effect of MAPK and PI3K inhibitors, PD98059 (PD; 10 µM; 0-24 h) and Wortmannin (Wort; 400 nM; 0-24 h) respectively, on E_2 (10 nM; 0-24 h)-induced PrmIP-directed luciferase gene expression in EA.hy926 (**Panel E**) and HEL (**Panel F**) cells (RLU ± SEM; n = 6). *P*-values ≤ 0.05 were considered to indicate statistically significant differences and *, **, ***, **** indicate $P \leq$. 0.05, 0.01, 0.001, 0.0001 for two-tailed Students' unpaired *t*-test analysis.

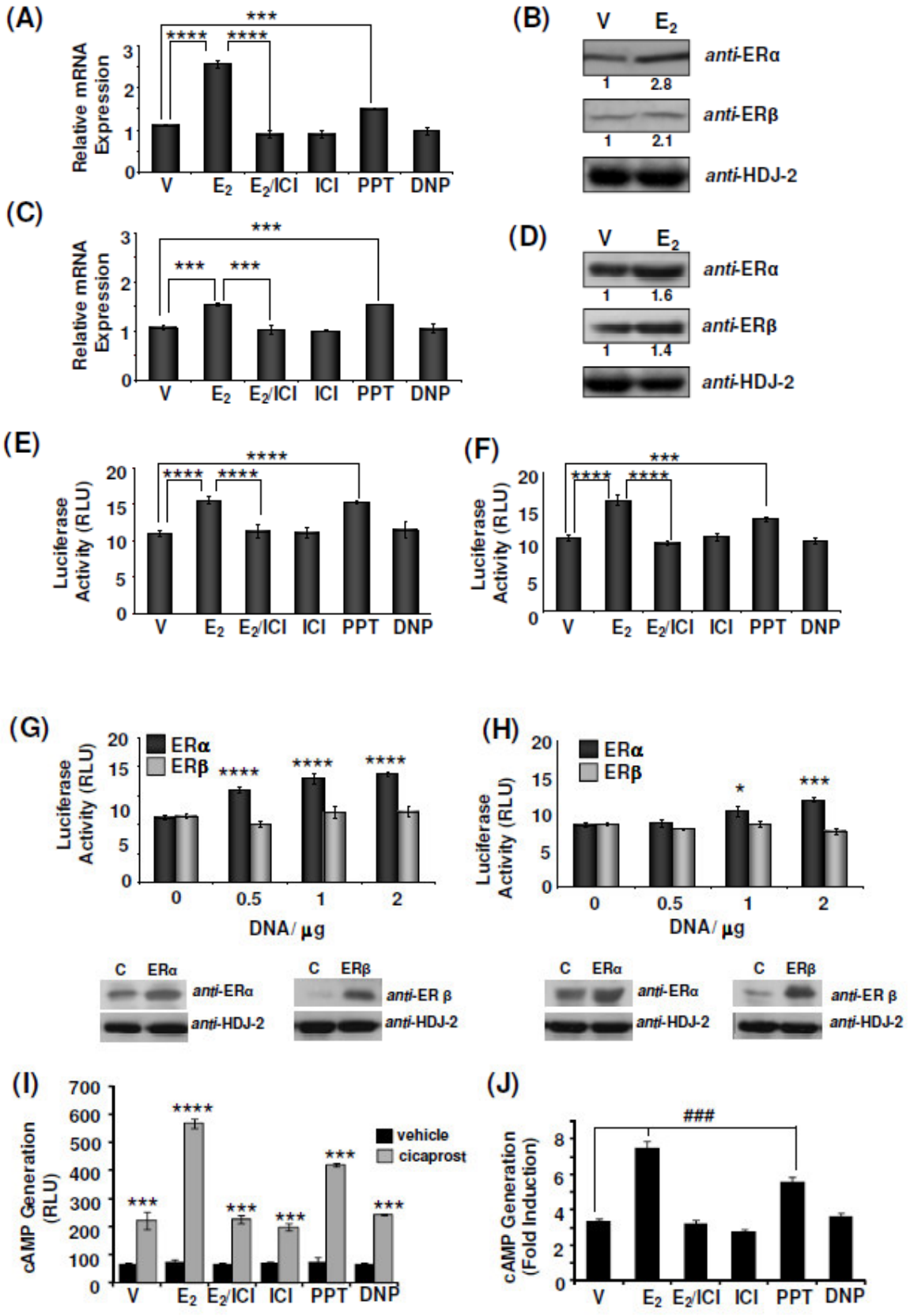

(A)
Relative mRNA Expression
V
E2
E2/ICI
ICI
PPT
DNP
(B)
anti-ERα
anti-ERβ
anti-HDJ-2
(C)
(D)
(E)
Luciferase Activity (RLU)
(F)
(G)
ERα
ERβ
DNA/ µg
(H)
C
(I)
cAMP Generation (RLU)
vehicle
cicaprost
(J)
cAMP Generation (Fold Induction)

Figure 2: Effect of ER Ligand Specificity on hIP Expression in EA.hy926 and HEL cells

Panels A and C: Quantitative RT-PCR analysis of hIP relative to GA3'PDH mRNA expression in EA.hy926 (**Panel A**) or HEL (**Panel C**) cells pre-incubated for 24h with vehicle (V; PBS, 0.01% EtOH), E_2 (10 nM), E_2 (10 nM) plus ICI 182,780 (100 nM), ICI 182,780 (100 nM), PPT (100 nM) or DPN (100 nM). **Panels B & D**: Immunoblot analysis of ERα, ERβ and HDJ-2 expression in EA.hy926 (**Panel B**) and HEL (**Panel D**) cells pre-incubated for 24h with vehicle (V) or E_2 (10 nM). Relative levels of ERα/ERβ expression in vehicle- or E_2-incubated cells normalized against HDJ-2 expression are indicated below panels. **Panels E & F:** Effect of ER ligands on PrmIP-directed luciferase expression in EA.hy926 (**Panel E**) and HEL (**Panel F**) cells (RLU ± SEM; n = 6) where cells were pre-incubated for 24h with drugs or vehicle as in Panels A/C. **Panels G & H:** Effect of ectopic expression of ERα (0-2.0 μg) and ERβ (0-2.0 μg) on PrmIP-directed luciferase expression in EA.hy926 (**Panel G**) and HEL (**Panel H**) cells (RLU ± SEM; n = 6). Lower Panels: Western blot analysis of whole cell protein in **G & H** transfected with either the empty vector (C) or with vectors encoding ERα or ERβ and screened with the respective *anti*-ERα and *anti*-ERβ antisera and, as a protein loading control, *anti*-HDJ-2. **Panels I & J**: HEL cells were pre-incubated with vehicle (V; PBS, 0.01% EtOH), E_2 (10 nM), E_2 (10 nM) plus ICI 182,780 (100 nM), ICI 182,780 (100 nM), PPT (100 nM) or DPN (100 nM) for 24h and hIP-induced cAMP accumulation in response to vehicle (V; PBS, 0.01 % DMSO) or cicaprost (1 μM) determined (RLU ± SEM; n = 3), where data are represented as fold inductions in cAMP accumulation in Panel J. *P*-values ≤ 0.05 were considered to indicate statistically significant differences and *, **, ***, **** indicate $P \leq$. 0.05, 0.01, 0.001, 0.0001 for two-tailed Students' unpaired *t*-test analysis and ### indicates $P \leq 0.001$ for ANOVA analysis, respectively.

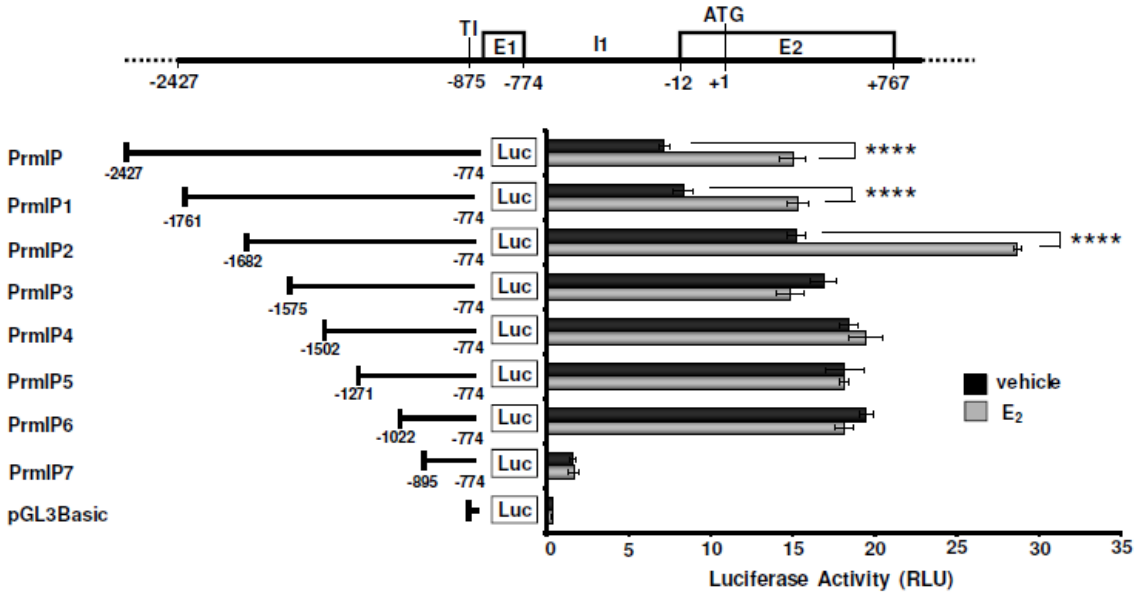


Figure 3: **Localisation of an E_2-Responsive Region within PrmIP by 5' Deletion Analysis.**
A schematic of the hIP genomic region, spanning nucleotides -2427 to +767, encoding PrmIP, exon (E)1, intron (I)1 and E2, where +1 corresponds to the translational start site. Effect of E_2 on PrmIP-, PrmIP1-, PrmIP2-, PrmIP3-, PrmIP4-, PrmIP5-, PrmIP6- and PrmIP7-directed luciferase gene expression in EA.hy926 cells pre-incubated for 24h with vehicle (V; PBS, 0.01 % EtOH) or E_2 (10 nM; RLU ± SEM; n = 6). *P*-values ≤ 0.05 were considered to indicate statistically significant differences and *, **, ***, **** indicate $P \leq$. 0.05, 0.01, 0.001, 0.0001 for two-tailed Students' unpaired *t*-test analysis.

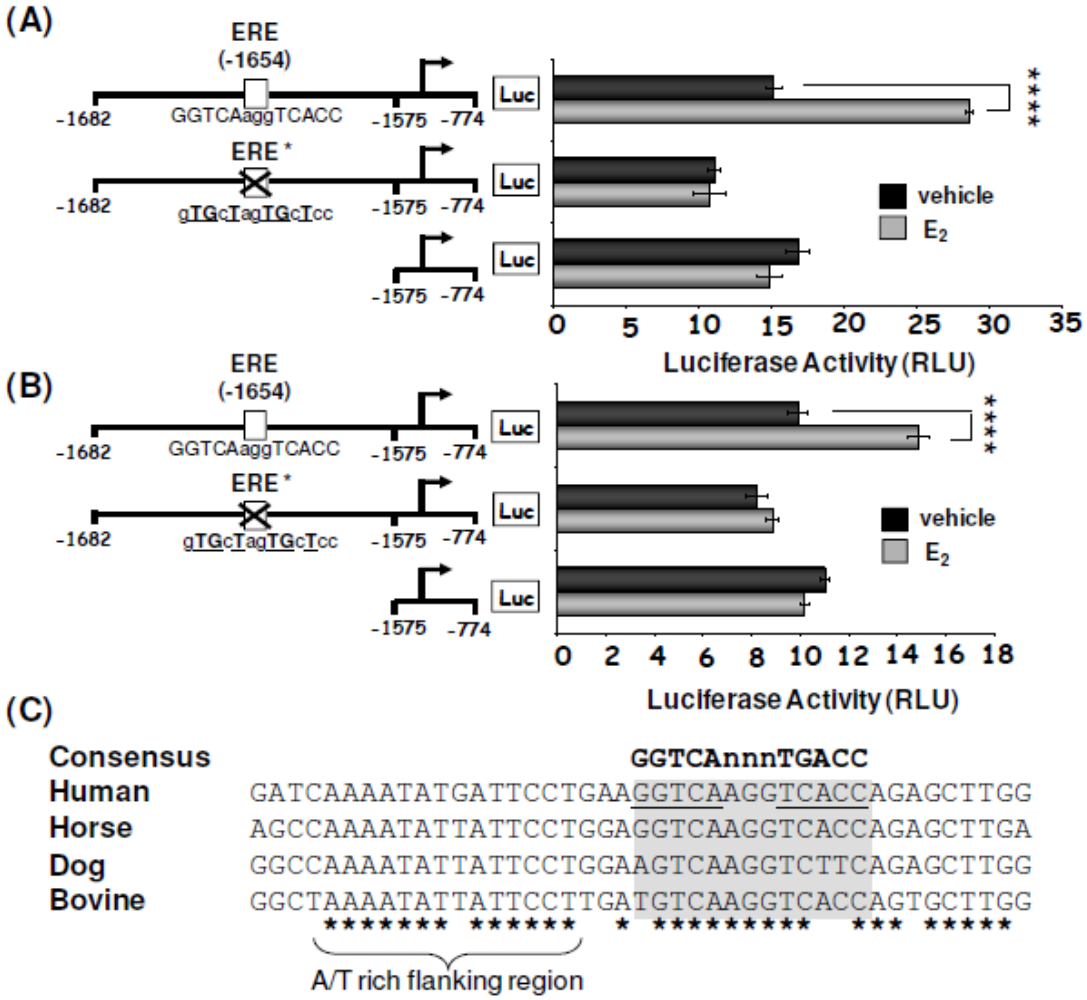


Figure 4: Identification of Putative ERE within PrmIP.

Panels A and B: A schematic of PrmIP2 in addition to the putative ERE, where the 5' nucleotide is indicated in brackets (-1654) and the actual sequence of the ERE and its mutated ERE* variant are given. Effect of E_2 on PrmIP2, PrmIP2^{ERE*} and PrmIP3-directed luciferase expression in EA.hy926 (**Panel A**) and HEL (**Panel B**) cells pre-incubated for 24h with either vehicle (V; PBS, 0.01% EtOH) or E_2 (10 nM; RLU ± SEM; n = 6). **Panel C:** Alignment of the putative estrogen-responsive region of human PrmIP with horse, dog, bovine IP promoter orthologue sequences. The consensus ERE is underlined in the human PrmIP sequence and highlighted by a grey box in the orthologues. *P*-values ≤ 0.05 were considered to indicate statistically significant differences and *, **, ***, **** indicate $P \leq$. 0.05, 0.01, 0.001, 0.0001 for two-tailed Students' unpaired *t*-test analysis.

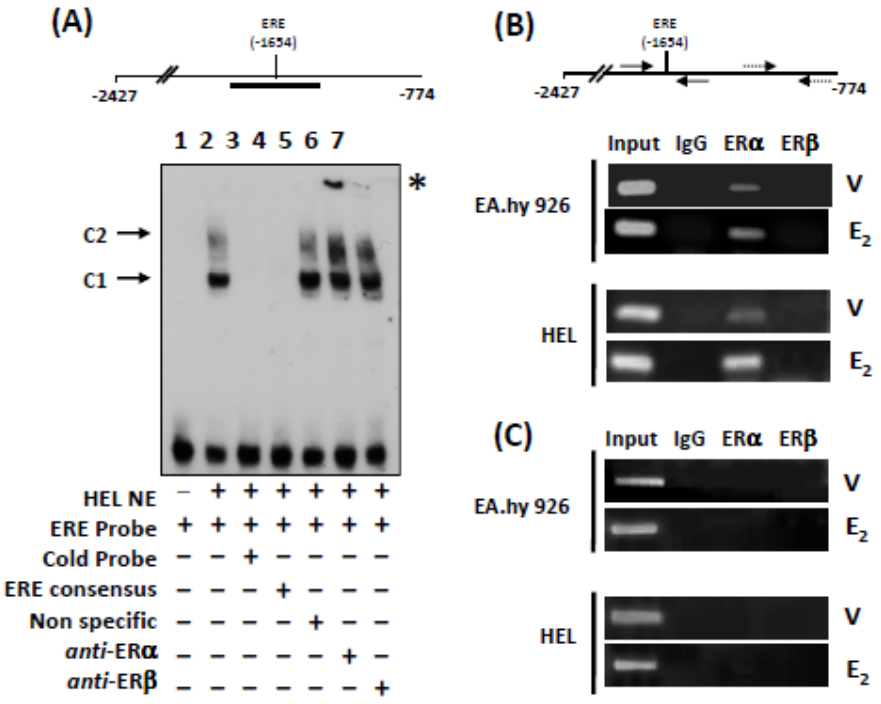


Figure 5: EMSA and ChIP of ERα Binding to PrmIP.

Panel A: A schematic of PrmIP (-2427 to -774) in addition to the putative ERE, where the 5' nucleotide (-1654) is indicated. EMSA and supershift assays were carried out using nuclear extract from HEL cells and a biotin-labelled ERE probe, as indicated by the horizontal bar. Nuclear extract was pre-incubated either with the vehicle (-) or with (+) non-labelled ERE probe, consensus ERE competitor, non-specific competitor or with *anti*-ERα and *anti*-ERβ sera prior to incubation with the biotinylated ERE probe, as indicated. Arrows to the left indicate DNA:protein complexes (C1 / C2) and the star to the right indicates supershifted transcription factor:DNA complexes. **Panels B & C:** ChIP analysis: a schematic showing the forward and reverse primers used to amplify either the estrogen-responsive (-1761 to -1746; solid arrows; **Panel B**) or, as controls, downstream (-1271 to -1005; dashed arrows; **Panel C**) sub-fragments of the PrmIP genomic region from immunoprecipitates of cross-linked chromatin from either EA.hy926 (**Panel B**) and HEL (**Panel C**) cells pre-incubated for 24h with vehicle (V; PBS, 0.01 % EtOH) or E_2 (10 nM). Images are representative of three independent experiments.

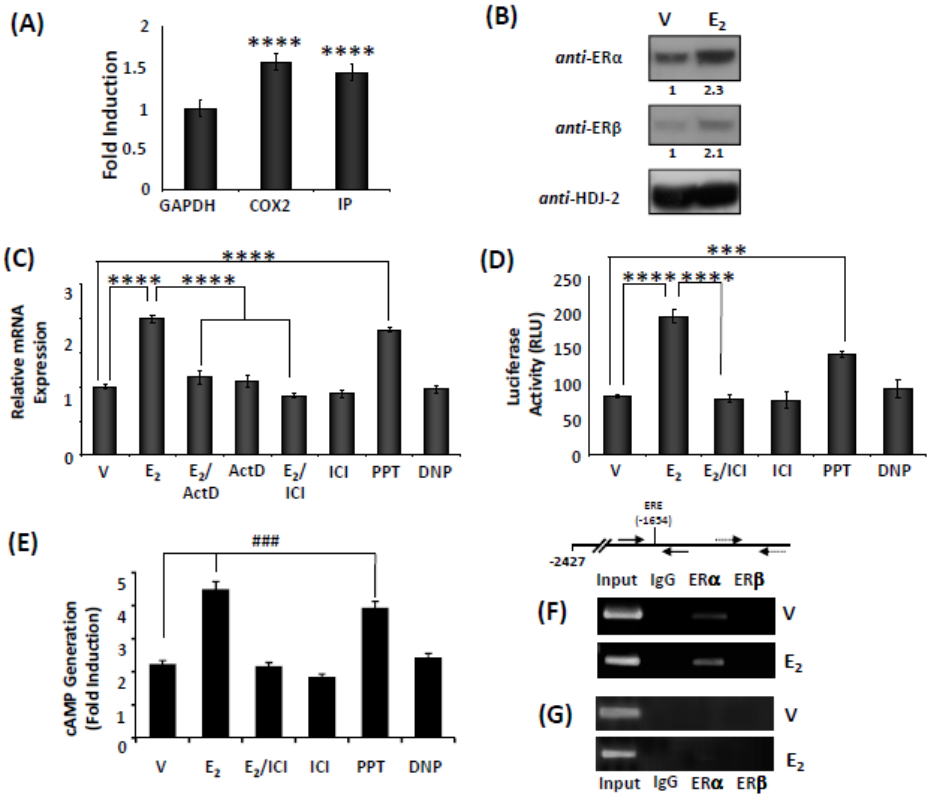


Figure 6:

Regulation of hIP/PrmIP Expression in 1° hAoSMCs.

Panel A: Quantitative real-time RT-PCR analysis of COX2 and hIP relative to GA3'PDH mRNA expression in 1° hAoSMCs pre-incubated for 24h with vehicle (V; PBS, 0.01 % EtOH) or E_2 (10 nM). **Panel B:** Immunoblot analysis of ERα and ERβ expression in 1° hAoSMCs pre-incubated for 24h with vehicle (V) or E_2 (10 nM). Relative levels of ERα/ERβ expression in vehicle- or E_2-incubated cells normalized against HDJ-2 expression are indicated. **Panels C**: Quantitative RT-PCR analysis of hIP relative to GA3'PDH mRNA expression in 1° hAoSMCs pre-incubated for 24h with vehicle (V), E_2 (10 nM), E_2 (10 nM) plus ActD (10 µg/ml), ActD (10 µg/ml), E_2 (10 nM) plus ICI 182,780 (100 nM), ICI 182,780 (100 nM), PPT (100 nM) or DPN (100 nM) for 24h (Relative Expression ± SEM, n = 3). **Panels D**: Effect of ER ligands on PrmIP-directed luciferase expression in 1° hAoSMCs pre-incubated for 24h with either vehicle (V), E_2 (10 nM), E_2 (10 nM) plus ICI 182,780 (100 nM), ICI 182,780 (100 nM), PPT (100 nM) or DPN (100 nM; RLU ± SEM; n = 6). **Panel E**: 1° hAoSMCs were pre-incubated with vehicle (V; PBS, 0.01% EtOH), E_2 (10 nM), E_2 (10 nM) plus ICI 182,780 (100 nM), ICI 182,780 (100 nM), PPT (100 nM) or DPN (100 nM) for 24h and hIP-induced cAMP accumulation in response to vehicle (V; PBS, 0.01 % DMSO)- or cicaprost (1 µM) determined. Data are represented as fold inductions in cAMP accumulation. Symbols ### represent $P \leq 0.001$ for ANOVA analysis. **Panel F & G**: ChIP analysis: a schematic showing the forward and reverse primers used to amplify either the estrogen-responsive (-1761 to -1746; solid arrows; **Panel F**) or, as controls, downstream (-1271 to -1005; dashed arrows; **Panel G**) sub-fragments of the PrmIP genomic region from immunoprecipitates of cross-linked chromatin from 1° hAoSMCs pre-incubated for 24h with vehicle (V; PBS, 0.01 % EtOH; upper panels) or E_2 (10 nM; lower panels). Images are representative of three independent experiments. *P*-values ≤ 0.05 were considered to indicate statistically significant differences and *, **, ***, **** indicate $P \leq .$ 0.05,

0.01, 0.001, 0.0001 for two-tailed Students' unpaired *t*-test analysis and ### indicates $P \leq 0.001$ for ANOVA analysis, respectively.

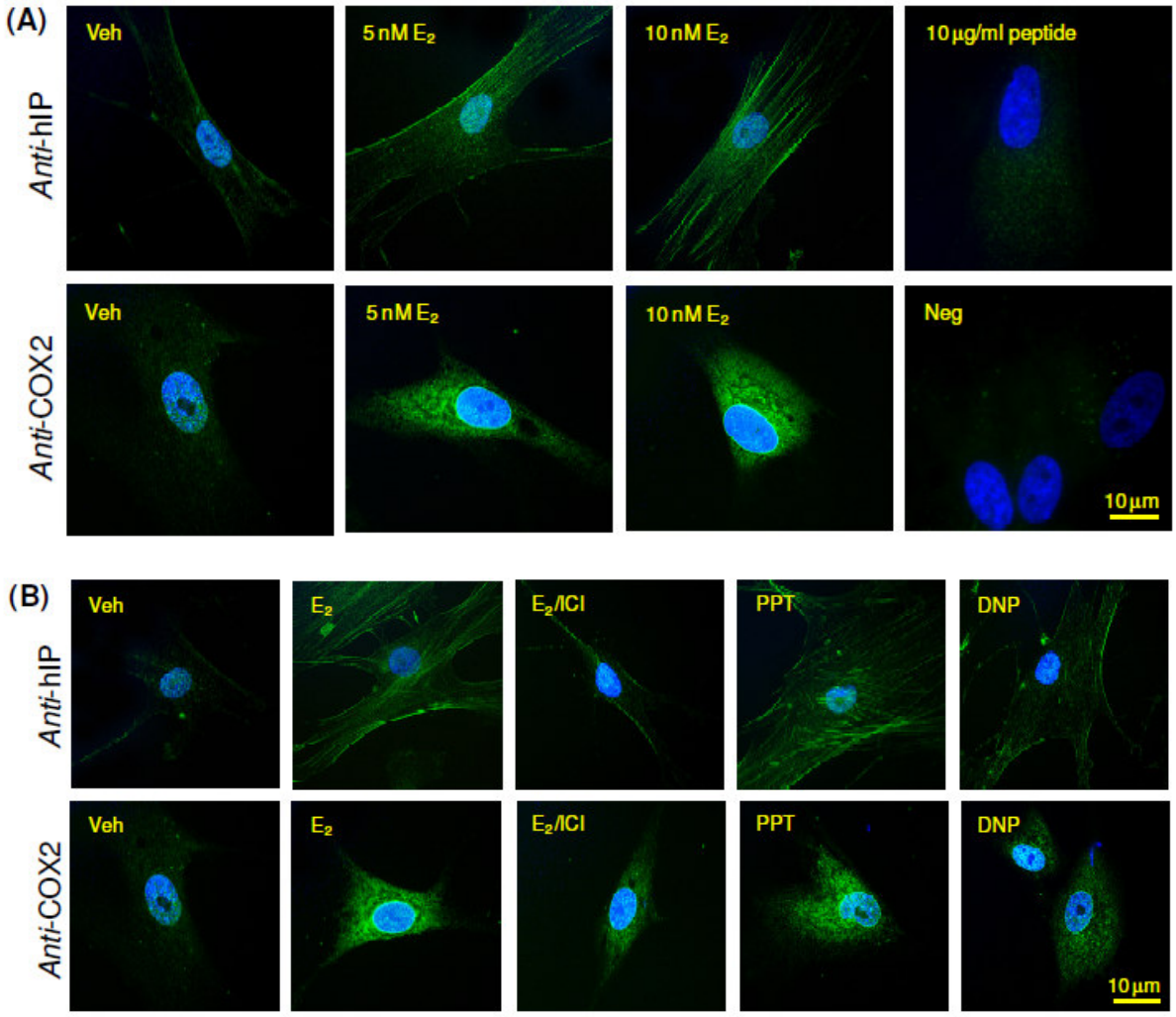
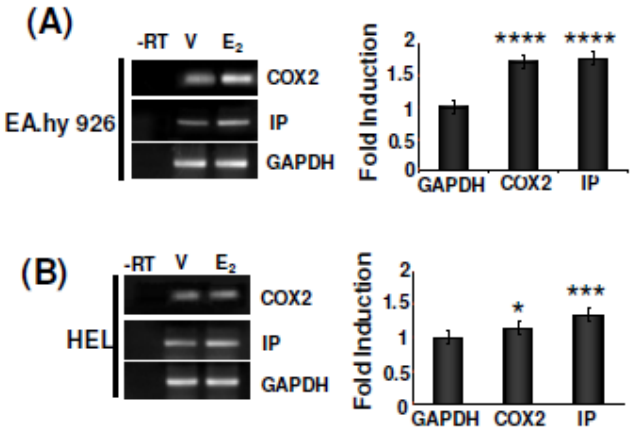


Figure 7: Immunofluorescence microscopy of human IP and COX2 expression in 1° hAoSMCs.

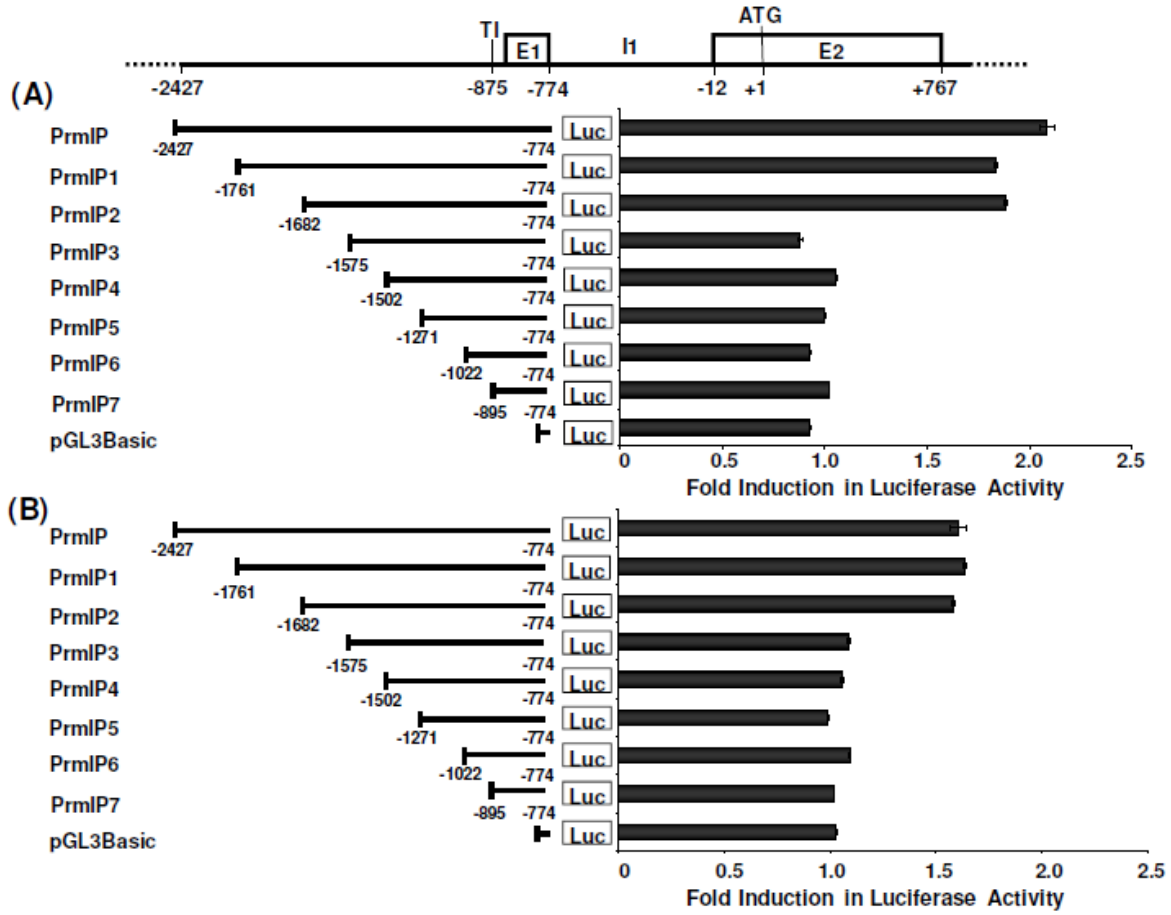
Panel A & B: Immunofluorescence microscopy of 1° hAoSMCs pre-incubated for 24h with either vehicle (V; PBS, 0.01 % EtOH), E_2 (5 nM or 10 nM; **Panel A**) or with vehicle (V), E_2 (10 nM), E_2 (10 nM) plus ICI 182,780 (100 nM), PPT (100 nM) or DPN (100 nM; **Panel B**) and immunolabelled with either *anti*-hIP (upper panels) or *anti*-COX2 (lower panels) sera and AlexaFluor488 conjugated *anti*-rabbit IgG (green), followed by counterstaining with DAPI (blue). Upper and lower right images in Panel A: Immunoscreening of 1° hAoSMCs with *anti*-hIP pre-blocked with the antigenic peptide (upper panel) or with the AlexaFluor488 conjugated *anti*-rabbit IgG alone (lower panel). Images are representative of three independent experiments.

Supplementary Figures:

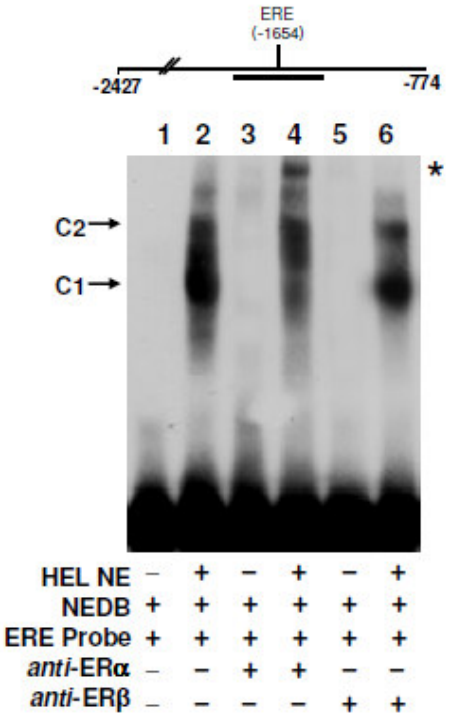


Supplementary Figure 1: **Effect of E_2 on hIP mRNA Expression in EA.hy926 and HEL cells.**

Panel A & B: RT-PCR analysis of COX2, hIP or GA3'PDH mRNA expression in EA.hy926 (**Panel A**) and HEL (**Panel B**) cells pre-incubated for 24h with vehicle (V; PBS, 0.01 % EtOH) or E_2 (10 nM), where the bar charts represent mean fold inductions ± SEM (n = 3). *P*-values ≤ 0.05 were considered to indicate statistically significant differences and *, **, ***, **** indicate $P \leq$. 0.05, 0.01, 0.001, 0.0001 for two-tailed Students' unpaired *t*-test analysis



Supplementary Figure 2: Localisation of an E_2-Responsive Region within PrmIP by 5' Deletion Analysis. A schematic of the hIP genomic region, spanning nucleotides -2427 to +767, encoding PrmIP, exon (E)1, intron (I)1 and E2, where +1 corresponds to the translational start site. Effect of E_2 on PrmIP-, PrmIP1-, PrmIP2-, PrmIP3-, PrmIP4-, PrmIP5-, PrmIP6- and PrmIP7–directed luciferase gene expression in EA.hy926 (**Panel A**) and HEL (**Panel B**) cells pre-incubated for 24h with vehicle (V; PBS, 0.01 % EtOH) or E_2 (10 nM; RLU ± SEM; n = 6). Results are expressed as mean fold-induction of luciferase expression in E_2- relative to vehicle-treated EA.hy926 (**Panel A**) and HEL (**Panel B**) cells.



Supplementary Figure 3: EMSA and Supershift Assay of ERα Binding to PrmIP.

Panel A: A schematic of PrmIP (-2427 to -774) in addition to the putative ERE, where the 5' nucleotide (-1654) is indicated. EMSA and supershift assays were carried out using nuclear extract from HEL cells or, as controls, with the nuclear extract dialysis buffer (NEDB; 20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.4 mM PMSF, 0.5 mM EDTA, 0.2 mM EGTA and 0.2 mM EGTA;[48]) and a biotin-labelled ERE probe, as indicated by the horizontal bar. For antibody supershift assays, nuclear extract or NEDB was pre-incubated either with the vehicle (-) or with *anti*-ERα and *anti*-ERβ sera prior to incubation with the biotinylated ERE probe, as indicated. Arrows to the left indicate DNA:protein complexes (C1 / C2) and the star to the right indicates supershifted transcription factor:DNA complexes. Images are representative of three independent experiments.