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Prostacyclin Receptors: Transcriptional Regulation and Novel Signalling Mechanisms

Helen M. Reid and B. Therese Kinsella

UCD School of Biomolecular and Biomedical Sciences, UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland.

†Corresponding author: Tel: 353-1-7166727; Fax 353-1-7166456; Email: Therese.Kinsella@UCD.IE

Running Title: Regulation of the Prostacyclin Receptor

Key Words: Prostacyclin receptor, estrogen receptor, sterol response element binding protein (SREBP), PDZK1(PDZ-domain-containing protein 1), IKEPP (intestinal and kidney enriched PDZ protein), C/EBP, CCAAT/enhancer binding protein; Rab11, gene, cardiovascular.

Abbreviations: AoSMC, human aortic smooth muscle cell; CAD, coronary artery disease; C/EBP, CCAAT/enhancer binding protein; ChIP, chromatin immunoprecipitation; COX: cyclooxygenase; CVD, cardiovascular disease; EC, endothelial cell; ER, estrogen receptor; ERE, estrogen response element; GIP, GPCR interacting protein; GPCR, G protein-coupled receptor; HEL, human erythroleukemia; HUVEC, human umbilical vein endothelial cell; IKEPP, intestinal and kidney enriched PDZ protein; IP, prostacyclin receptor; NHERF, Na+/H+ exchange regulatory factor; PDZ, Postsynaptic density-95, Discs large, Zonula occludens-1; PDZD, PDZ domain; PDZK1, PDZ-domain-containing protein 1; PKA, cAMP-dependent protein kinase A; PMA, phorbol 12-myristate 13-acetate; pGL3B, pGL3Basic; pRL-TK, pRL-thymidine kinase; PRR, PMA-responsive region; PTGIR, prostacyclin receptor gene; PTGS2, COX2 gene; RBD, Rab11 binding domain; RCT, reverse-cholesterol transport; RLU, relative luciferase unit; SEM, standard error of the mean; SNP, single-nucleotide polymorphism; SRE, sterol response element; SREBP, sterol response element binding protein; TAF, TATA-box binding protein associated factor; TBP, TATA-box binding protein; TFII D, transcription factor D for RNA polymerase II; TI, transcription initiation; SDM, site-directed mutagenesis; TX, thromboxane; URR, upstream repressor region; Y2H, yeast-two-hybrid.

Abstract:
The prostanoid Prostacyclin plays diverse physiologic roles within the vasculature and other systems, and is widely implicated in several cardiovascular, pulmonary and renal diseases. Despite this, knowledge of the factors regulating expression of the I prostanoid receptor (the IP) remained largely unknown. This review details recent advances in understanding the key transcriptional regulators determining expression of the PTGIR gene in the human vasculature and the identification of novel interacting partners of the IP that impact on its function therein. Included in this are the trans-acting factors that regulate expression of the PTGIR under basal- and regulated-conditions, particularly those determining its up-regulation in response to cellular differentiation, estrogen and low serum-cholesterol. Moreover, the functional implications of the interactions between the IP with PDZK1, a multi PDZ-domain containing protein essential for reverse-cholesterol transport and endothelialization, and the IP with IKEPP, the intestinal and kidney enriched PDZ protein, for the role of the prostacyclin-IP axis within the vasculature are reviewed.
Highlights:

- Transcriptional regulation of the PTGIR, the I Prostanoid receptor (IP) gene
- Basal regulation by Sp1, PU.1 & Oct-1 and by C/EPBδ via the “core promoter” & URR
- Switch of C/EPBδ to PU.1 “URR binding” & enhanced “core binding” upregulate the IP
- Estrogen increases IP expression via an estrogen receptor(ER)α-dependent mechanism
- Low serum-cholesterol increases IP expression via an SREBP1-dependent mechanism
- Functional interaction of the IP with PDZK1 and with IKEPP within the vasculature

Graphical Abstract:
I. Introduction:
The prostanoid prostacyclin, also referred to as prostaglandin (PG)I₂, plays an essential role in haemostasis and in the dynamic regulation of vascular tone acting as a potent inhibitor of platelet aggregation and as a dilator of vascular smooth muscle. It also exerts potent pro-inflammatory and anti-proliferative effects and promotes vascular repair following endothelial injury. Hence, prostacyclin plays a key protective role in the vasculature and imbalances in the levels of prostacyclin, or of its specific synthase or its receptor, are implicated in a diverse range of cardiovascular diseases (CVDs) including thrombosis, stroke, myocardial infarction, atherosclerosis, systemic and pulmonary hypertension. In addition to its role in the vasculature, prostacyclin is centrally involved in other systems including in the kidney where it regulates renal blood flow and glomerular filtration rates, and in the lung where it acts as a bronchodilator and is widely indicated in the treatment of pulmonary arterial hypertension.

Consistent with its role in the vasculature, prostacyclin is mainly produced within the endothelium and vascular smooth muscle where it is synthesised from arachidonic acid predominantly by the sequential actions of cyclooxygenase (COX)1 or COX2, to generate the intermediates PGG₂/PGH₂ and subsequently by prostacyclin synthase to yield prostacyclin. The actions of prostacyclin are mainly mediated through its specific cell surface prostacyclin receptor or, more properly according the IUPHAR nomenclature, the I prostanoid receptor or, in short, the IP, a member of the G protein coupled receptor (GPCR) superfamily. The IP is predominantly coupled to Gs-activation of adenyl cyclase, increasing cellular cAMP and, in turn, activation of cAMP-dependent protein kinase (PK) A to mediate many of the inhibitory actions of prostacyclin in platelets and in various types of smooth muscle (SM), including vascular (V) SM. In addition to the IP, prostacyclin can act as a weak agonist of PPARδ, a member of the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors, particularly at elevated concentrations.

The importance of prostacyclin in protecting against CVD was highlighted by the finding that certain COX2-selective inhibitors/COXIBs, such as Vioxx, were associated with increased clinical incidence of thrombosis, myocardial infarction and/or stroke due to their selective impairment of prostacyclin synthesis without affecting levels of the pro-thrombotic thromboxane (TX)A₂, a prostanoid predominantly synthesised by COX1 in the anucleated platelet. Hence, though remaining somewhat controversial, the “imbalance theory” proposes that COXIBs may shift the balance between the anti-thrombotic prostacyclin and the pro-thrombotic TXA₂, thereby increasing the incidence of adverse thrombotic episodes in certain individuals. In line with this, IP null mice display enhanced tendency toward thrombosis, intima hyperplasia, atherosclerosis, and restenosis. Furthermore, endothelial progenitor cells (EPCs) from IP null mice fail to promote re-endothelialization and vessel repair in an experimental model of endothelial injury. Hence, the prostacyclin-IP axis plays a central protective role in promoting re-endothelialization, limiting neointima hyperplasia and vascular remodeling in response to the vessel wall injury that frequently accompanies atherosclerosis or certain vascular/surgical procedures, such as angioplasty or carotid endarterectomy for example. Somewhat consistent with this, several single-nucleotide polymorphisms have been identified within the IP gene that predispose individuals to CVD, including enhanced risk of deep vein thrombosis and intimal hyperplasia. However, despite this extensive appreciation of the importance of prostacyclin and its receptor, the IP, for vascular integrity, detailed knowledge of the factors regulating expression of the IP gene, the PTGIR, within the vasculature or indeed within any other system in which the prostacyclin-IP axis is physiologically important, remained poorly understood. This review details some of the recent...
advances that have led to the current understanding of the key transcriptional regulators determining expression of the human PTGIR within the vasculature, where studies were largely carried out in vascular model systems including in the megakaryoblastic platelet-progenitor human erythroleukemic (HEL) 92.1.7 and in the endothelial EA.hy 926 cell lines, in primary (1°) human umbilical vein endothelial cells (HUVECs) and/or in 1° human aortic smooth muscle cells (hAoSMCs).

2. Transcriptional Regulation of IP Expression

2.1. Identification of the Core Promoter Region of the Human PTGIR:

The structural organization of the human IP gene, the PTGIR, located on chromosome 19q13.3 is depicted in Figure 2A. In brief, it is composed of 3 exons separated by 2 introns, where exon (E) 2 and E3 are the main coding exons. E1, on the other hand, exclusively encodes 5’ untranslated region (UTR) where, within E1, the major transcriptional initiation (TI) site maps approximately to nucleotide -917 upstream of the translational initiation codon. The human IP promoter, here-on-in referred to as the PrmIP, lacks a conventional TATA box and initiator (Inr) element.

In order to characterize the human PrmIP, a 1.677 kb gene fragment surrounding the TI site was evaluated for promoter activity through a series of luciferase-based gene reporter assays initially in the platelet-progenitor HEL 92.1.7 cell line (Figure 2A & 2B). Using this gene-reporter approach combined with 5’deletional analysis, a series of successive 5’ sub-deletions denoted PrmIP1 – PrmIP7 were generated to map the main transcriptionally active regions within the human PrmIP (Figure 2B). Through this, an upstream repressor region (URR) was identified between PrmIP4 and PrmIP5. Furthermore, it was established that the region between PrmIP6 and PrmIP7 was the smallest sub-fragment with promoter activity and this region was found to correspond to the “core promoter” necessary for basal expression of the IP in HEL cells. Likewise in both the vascular endothelial (EA.hy 926 cell line & 1° HUVECs) and aortic smooth muscle (1° hAoSMCs) cell types, the core promoter mapped to PrmIP6. In addition, a URR was identified in the endothelial cell systems but was localised to the region between PrmIP1 and PrmIP2, different to the URR identified in HEL cells and 1° hAoSMCs, located between PrmIP4-PrmIP5. As elaborated upon below, while the trans-acting factors that bind and regulate the URR in HEL cells have been recently identified, the factor(s) that regulate the endothelial-specific URR remains to be investigated.

In all cell types examined, Sp1, PU.1 and Oct-1 were identified as the key trans-acting factors that regulate PTGIR expression through binding to their cognate cis-acting elements within the “core promoter/PrmIP6” (Figure 2C). As stated, the PrmIP lacks a conventional TATA box and initiator (Inr) element. The ubiquitously expressed transcription factor Sp1 can serve to attract key protein components of the basal transcriptional machinery to promoters lacking a conventional TATA box. The identification of direct interactions between Sp1, TATA-box binding protein (TBP) and TBP-associated factors (TAFs) led to the suggested role of Sp1 as an anchor for TAFs in TATA-less promoters whereby one or more Sp1 molecules bind to G/C-rich regions in such promoters to help establish a transcription pre-initiation complex. The PU.1 member of the Ets family is a product of the Spi-1 proto-oncogene first identified as the integration site of the Friend murine erythroleukemia virus. PU.1 acts as a master regulator of myeloid and B-cell development and is critical for hematopoietic development, particularly during early differentiation of myeloid, erythroid and B lineage cells. Like Sp1, PU.1 has also been shown to regulate certain TATA-less promoters, such as c-fes and M-CSF receptor. Given the fact that PU.1 binds to TBP in vitro, it is also thought to recruit TFII D (Transcription Factor D for RNA polymerase II), followed by other components of the basal transcriptional machinery. Ets family
members are often, if not always, found as subunits of multiprotein transcription complexes that are thought to be involved in the fine regulation of cellular promoter/enhancer sequences. Physical and functional interactions of PU.1 with partner proteins, in particular Sp1 family members, are critical to its role in gene expression and lineage determination. Octamer binding proteins belong to the POU (Pit-Oct-Unc) family of transcription factors that play critical roles in the regulation of gene expression in multiple cell types. Two POU domain proteins, the ubiquitously expressed Oct-1 and the lymphoid-specific Oct-2, have been shown to be involved in hematopoietic differentiation. Cooperative interactions of Oct-1 with either Sp1 or PU.1 are not without precedence and have been reported on other RNA polymerase II-regulated promoters. By way of example, Chen et al., described similar cooperative interactions between Oct-1 and Sp1 at the PU.1 promoter whereby successive disruption of an octamer motif and the Sp1 element lying in close proximity to each other did not lead to any further loss of activity relative to disruption of the octamer site alone.

Hence, in the case of the PTGIR, it is proposed that in the absence of a TATA box, Sp1 initiates the formation of the pre-initiation complex, thereby allowing recruitment of other specific factors, including PU.1 and Oct-1, and more general TBP factors and TAFs, along with RNA polymerase II, to form a multicomponent positive regulatory domain that is sufficient to confer basal expression of IP/PTGIR (Figure 2C). Noteworthy, bioinformatic analysis confirmed the absolute evolutionary conservation of the Sp1, PU.1 and Oct-1 binding elements within the proximal IP promoters from a range of species, including the horse, bovine, dog, rat and mouse. Coupled to this, the fact that Sp1, PU.1 and Oct-1 were found to be critical in regulating the PrmIP not only in the megakaryoblastic HEL 92.1.7 cells but also in the human endothelial and AoSM cells suggests that they provide a general, rather than a cell- or species-specific, model for transcriptional regulation of the IP. As elaborated upon below, additional control regions, such as the URR and other upstream cis-acting elements within the PrmIP are responsible for the tissue-specific and/or regulated expression of the IP.

2.2. C/EBPδ & PU.1 Mediated Regulation of the PTGIR Expression:

The C/EBP family of transcription factors, composed of 6 members, C/EBPα through to C/EBPζ, are so called because they interact with the CCAAT (cytidine-cytidine-adenosineadenosine-thymidine) box motif found in many, but not all, eukaryotic promoter sequences. C/EBP members bind to DNA as homo- or hetero-dimers to regulate diverse processes including energy metabolism, innate and adaptive immunity, inflammation, hematopoiesis, adipogenesis, osteoclastogenesis, cell cycle, cellular proliferation and differentiation. In a study aimed at characterizing the functional URR identified in the megakaryoblastic HEL and hAoSMC lines (located between PrmIP4 - PrmIP5), C/EBPδ and PU.1 were identified as the key trans-acting factors that bind and repress PTGIR/IP gene expression under basal conditions. Consistent with this, it has long been established that PU.1 can physically interact with C/EBPδ (NF-IL6β) through an interaction dependent on the C-terminal 28 AA residues of PU.1 and that they can simultaneously bind to adjacent DNA binding sites to synergistically influence the basal transcription complex. Within the PrmIP, it is proposed that C/EBPδ binds to its cis-acting element and co-operates with PU.1 through its binding to the adjacent PU.1(b) element, also located within the URR and distinct from the PU.1(a) element previously identified within the core promoter, to repress the basal transcriptional apparatus. Interestingly, multi-sequence alignments confirmed that both the URR region and the PU.1(b) and C/EBP elements themselves are found within the PrmIP promoter regions from a range of other species, suggesting that this represents an evolutionary conserved mechanism of transcriptional regulation of the PTGIR. It is also noteworthy that the extent of overall identity between
the human PrmIP and the IP promoter sequences from all other non-primates is low but that part of the URR, encompassing the PU.1(b) and C/EBP elements, represents the most highly conserved promoter region across all species examined.

The pluripotent HEL92.1.7 cell line can undergo differentiation into the more platelet-like lineage in response to a host of physiologic and/or experimental cell differentiating agents including cytokines, growth factors or phorbol esters, such as phorbol myristic acid (PMA)\(^5\). During such cell differentiation, there is a concomitant increase in the expression of platelet proteins including the human IP and the T prostanoid receptor/TP. While Wilms tumour (WT) 1, early growth response (Egr) 1 and SpI were identified as the key factors responsible for the regulated expression of TP gene expression during such megakaryoblastic differentiation\(^5\), detailed characterization of the PrmIP identified two key PMA-responsive regions (PRRs) that regulate IP/PTGIR expression in response to PMA-induced differentiation of HEL cells\(^3\). More specifically, PRR1 maps to the URR within PrmIP4 while PRR2 is located within the "core promoter" region, contained within PrmIP6. Through a combination of approaches, it was established that while C/EBP\(\delta\) can bind to the URR/PRR1 region under basal conditions to repress IP/PTGIR expression in HEL cells, following PMA-induced differentiation C/EBP\(\delta\) repression is lifted and coincides with enhanced binding of PU.1 to its cis-acting elements within the UAR/PRR1 region\(^3\).

Furthermore, in response to PMA-induced differentiation, there is also enhanced binding of the key trans-acting factors required for basal expression of the PTGIR, namely SpI, PU.1 and Oct-1, to the PRR2 that maps to the "core region" of PrmIP/PrmIP6, further supporting or facilitating the PMA-induced increase in IP expression that occurs following PMA-induced differentiation of HEL cells\(^3\). Hence, collectively, the increased expression of the IP that occurs during PMA-induced differentiation of HEL cells involves the combined action of PMA-induced (a) alleviation of binding of repression by C/EBP\(\delta\) and enhanced binding of PU.1 to the URR/PRR1 region and (b) through enhanced binding of SpI, PU.1 and Oct-1 to the PRR2 that maps to the core promoter region of the PTGIR (Figure 3)\(^3\).

C/EBP\(\beta\) and C/EBP\(\delta\) are known to act synergistically to regulate transcription of various genes involved in immune and inflammatory responses\(^5\). Interestingly, COX2, primarily involved in prostanoid generation in response to inflammation, has been identified as a transcriptional target of both C/EBP\(\beta\) and C/EBP\(\delta\)\(^5\). During the initial stage of its induction, C/EBP\(\beta\), along with cAMP response binding protein (CREB), plays a major role in the transcriptional up-regulation of the COX2 gene (PTGS2). Thereafter, C/EBP\(\delta\) along with the transcriptional co-activator p300 and several other factors are subsequently recruited into a complex to sustain COX2 expression\(^5\). Therefore, both C/EBP\(\beta\) and C/EBP\(\delta\) are critical transcriptional regulators of genes involved in prostanoid biosynthesis and function, including the COX2/PTGS2 and IP/PTGIR genes. Mechanistically, in the case of the PTGIR, as the megakaryoblastic HEL cells undergo differentiation to the more platelet-phenotype there is a switch in transcription factor binding from predominantly C/EBP\(\delta\) binding to the URR to predominantly PU.1 binding to the PMA-induced PRR1 region to increase IP expression and signalling following cellular differentiation\(^3\).

**2.3. Transcriptional Regulation of PTGIR by Estrogen:**

In addition to that of prostacyclin, the protective role of estrogens in the myocardium and vasculature have also been categorically established being associated with gender-specific differences in the occurrence of coronary artery disease (CAD) and with lower incidence of stroke and other CV events in pre-menopausal women retarding atherogenesis and improving endothelial function\(^6\)\(^-\)\(^8\). On average, women develop vascular disease 10 years later than men and this has been attributed to the protective effects of the female sex hormones, particularly the estrogens, while in the estrogen-deplete post-
menopausal woman such gender differences disappear. The importance of estrogen is also strongly supported by observations in younger women with premature ovarian failure (POF) who display a higher risk of premature death, particularly due to CV disease\textsuperscript{60}. Furthermore, hormone/estrogen replacement therapy can prevent the primary onset of CAD in post-menopausal women\textsuperscript{61}, although such effects are not without controversy\textsuperscript{63, 64}.

The effects of estrogen are largely mediated through its binding to one of two estrogen receptor (ER)\textsubscript{α} and β isoforms, members of the nuclear receptor superfamily\textsuperscript{65}. ER\textsubscript{α} and ER\textsubscript{β} display distinct patterns of expression and function in various tissues but both act as ligand-dependent transcriptional regulators either by direct binding to the estrogen-responsive element (ERE) with the consensus palindromic repeat (5’-GGTCAnnnTGACC-3’), or indirectly by interacting with other transcription factors, such as Sp1 and Ap1, and by recruitment of its co-activators and/or co-repressors\textsuperscript{65, 66}.

In addition to its classic genomic regulation, estrogen can mediate more rapid non-genomic effects such as through activation of GPR30, a member of the G protein coupled receptor superfamily\textsuperscript{67}. Moreover, it is now also accepted that many of its CV protective actions are actually mediated by direct effects on the blood vessel wall maintaining endothelium function and regulating plasma lipid\textsuperscript{68, 69}. For example, estrogen increases the synthesis & release of the endothelial-derived vasodilators and anti-platelet agents nitric oxide and prostacyclin and, in situations of vascular injury, impairs expression of a host of pro-inflammatory mediators including C-reactive protein\textsuperscript{61, 62}. More specifically, in the case of prostacyclin, estrogen increases the expression of its biosynthetic enzymes COX1, COX2 and prostacyclin synthase, resulting in up to 3-fold increases in systemic prostacyclin levels in rodents\textsuperscript{68}. Moreover, in the female atherosclerotic-prone low density lipoprotein receptor null mouse (LDLR\textsuperscript{-/-}), estrogen increased both COX2 expression and prostacyclin generation in the ovariectomized animal resulting in substantial atheroprotection\textsuperscript{69}. However, in the same study, further disruption of the IP (PTGIR) gene completely abrogated the atheroprotective effects of estrogen and accelerated atherogenesis in the double LDLR\textsuperscript{-/-}/IP\textsuperscript{-/-} null mouse\textsuperscript{69}, providing compelling evidence that many of the protective actions of estrogen within the CV system are indeed mediated by prostacyclin and its receptor, the IP. In fact, owing to those findings, through retrospective epidemiologic studies, that group provided evidence to suggest that NSAIDs and, in particular, selective COX2 inhibitors COXIBs that impair prostacyclin synthesis, may undermine the cardioprotective effects of estrogen replacement therapy in peri-menopausal women\textsuperscript{70}. However, despite such findings in both pre-clinical models and from observational studies in humans, the actual mechanistic basis of the role of the IP in mediating such estrogen-induced atheroprotection was unknown including, for example, whether estrogen may directly or indirectly regulate IP expression levels to account for such effects.

In the context of the latter, in studies investigating the key factors regulating expression of the human IP/PTGIR gene within the human vasculature\textsuperscript{29}, we discovered that the PrmIP contains a near perfect estrogen response element (ERE; 5’ -GGTCAaggTCACC-3’) which is also evolutionary conserved in other higher species orthologues. The frequency, proximity and conservation of the near classic ERE within the PrmIP led us to hypothesize that the PTGIR might indeed be directly regulated by estrogen which, in theory, might explain some of the combined cardioprotective and anti-thrombotic effects of estrogen and prostacyclin within the vasculature. In addressing this, we established that physiologic concentrations of estrogen (17β-estradiol) led to increased human IP mRNA and functional receptor expression and to increased PrmIP-directed gene expression in vascular endothelial (EA.hy926, 1° HUVECs) and megakaryoblastic (HEL 92.1.7) cells and in 1° hAoSMCs (Figure 4)\textsuperscript{30}. Moreover, this estrogen-regulation of the human IP occurred through a transcriptional mechanism involving direct
binding of ERα, but not ERβ, to the evolutionary conserved ERE located within an upstream steroid regulatory region of PrmIP30. Whether ERβ may regulate PTGIR gene expression in a cell type-specific manner or, indeed, whether it may act as a competitor of ERα-regulated expression of the IP, as occurs in the case of the BRCA2 gene71, requires further investigation.

Collectively, these data established that ERα serves as a trans-acting factor critical for regulation of the IP expression in response to estrogen and occurs through a direct “estrogen–ERα–ERE” mechanism. It is proposed that such regulation of IP expression may provide a mechanism, at least in part, to explain the combined effects of prostacyclin and the female hormone estrogen in protecting against CVD in younger estrogenic women72, 73.

2.4. Transcriptional Regulation of PTGIR by Cholesterol:
Elevated LDL-cholesterolemia is a well-recognized risk factor in CVD74, 75. In addition to estrogen, the clinical benefits of low serum LDL-cholesterol in the prevention of CAD are also widely recognized and can, in part, be accounted for by improvements in endothelial-dependent vasodilation by prostacyclin and nitric oxide76. For example, Statins yield pleiotropic beneficial effects not associated with their cholesterol-lowering properties77, 78, most notably improved cerebral blood flow associated with enhanced nitric oxide generation79, 80. Analysis of the effects of cholesterol and fatty acids on hepatic gene expression led to the discovery of a family of membrane-bound transcription factors named sterol response element binding proteins or SREBP and identified them as the master regulators of lipid homeostasis81. SREBP are members of the bHLH-LZ class of transcription factors and bind as dimers to a direct repeat “E-box” element referred to as a sterol response element (5'-ATCACCCCACTC')82. The first well-characterized functional sterol response element (SRE) was that of the human LDL receptor gene promoter83, which was then used for affinity purification and subsequent cloning of the SREBP trans-acting factor84. Family members SREBP1a and SREBP1c are known to activate genes involved in regulating general lipid metabolism, whereas SREBP2 regulates expression of genes involved in cholesterol homeostasis85. SREBP often cooperate with other DNA-binding proteins to achieve maximal transcriptional activation. For example, NF-Y and CREB cooperate with SREBP1 to regulate the HMG-CoA reductase gene, whereas SREBP1 cooperates with Sp1 to activate the LDL receptor gene86-88. Furthermore, SREBP1 and Sp1 have been identified as the two major transcription activators of the fatty acid synthase gene (FASN), one of the key enzymes in fatty acid synthesis89. Critically, regulation by these additional trans-acting factors permits modulation of SREBP’s transcriptional activity independently of its sterol-regulated proteolytic processing.

Although numerous transcriptional targets of SREBP have been identified, little is known about their effects on expression of genes in extra-hepatic tissues or genes not directly associated with lipid homeostasis. The finding that endothelial prostacyclin levels may be increased by the cholesterol-responsive SREBP1 through its transcriptional up-regulation of COX2 was the first demonstration that the SREBP-mediated pathway(s) are present in vascular tissue90, 91. Reductions in LDL-cholesterol increase prostacyclin generation in endothelial cells by the selective transcriptional up-regulation of COX2, but not COX1 or prostacyclin synthase, and this occurs through binding of the cholesterol-responsive transcription factor SREBP to a SRE within the COX2 promoter90, 91. Through recent studies investigating the factors regulating the expression of the IP/PTGIR within the vasculature, it was discovered that IP expression may also be up-regulated in megakaryoblastic HEL and endothelial (EA.hy 926 and 16H7) cells cultured under low-serum cholesterol conditions (Figure 5)92. Through detailed mechanistic studies, it was established that this up-regulation of IP expression occurs
through a transcriptional mechanism involving binding of the cholesterol-responsive trans-acting factor SREBP1α, but not SREBP2, to a near-perfect evolutionary conserved SRE within the PrmIP (5’ CTCACCCAG 3’). Critically, it was established that the functional SRE is located immediately downstream/3’ of the Sp1, PU.1 and Oct-1 cis-acting elements within the “core promoter” region, and that binding of Sp1 to its cis-element participates in SREBP1-binding to the SRE and in regulating PTGIR expression in response to low serum cholesterol. These data are in keeping with previous studies showing SREBP1-dependent, but not SREBP2-dependent, transactivation of the LDLR and COX2 promoters and with the fact that SREBP1 is the predominant form expressed in endothelial cells.

Hence, discovery of the transcriptional regulation of the PTGIR by the cholesterol-responsive master regulator SREBP1 is significant and, mechanistically, may not only explain many of the protective roles of the prostacyclin-IP axis within the vasculature but may also explain, at least in part, some of the pleiotropic benefits of low-serum cholesterol and of cholesterol-lowering agents in improving/protecting vascular function. Furthermore, the fact that the human IP is also up-regulated by low-serum cholesterol in the platelet progenitor HEL cells suggests that low-serum cholesterol or cholesterol-lowering agents may also elevate IP expression in platelets, thereby conferring added anti-thrombotic benefits over-and-above the endothelial benefits in reducing overall risk of CAD. Collectively, these studies have led to significant advancements into elucidating the mechanisms of transcriptional regulation of the PTGIR within the human vasculature under both basal conditions and in response to cellular differentiation. Furthermore, they provide an important molecular and genetic platform for understanding the role of the prostacyclin-IP as a protective axiom within the vasculature and in mediating, at least in part, the cardio-protective effects of estrogen and of reduced serum-cholesterol.

3. Regulation of IP Signalling through Novel Protein:Protein Interactions:

As stated, the main physiologic actions of prostacyclin are mediated through the IP, a member of the GPCR superfamily that primarily couples to Gs/adenyllyl cyclase activation but also to other effectors in a cell- and/or species-specific manner. The IP is somewhat unusual among GPCRs in that it undergoes isoprenylation and palmitoylation within its carboxy-terminal tail (C-tail) domain, modifications critical for IP signalling and function. More specifically, the human IP undergoes farnesylation at Cys with its carboxy-terminal conserved -C^383SLC^386, or ‘CaaX’, motif and palmitoylation at Cys, and Cys (Figure 6). While neither lipid modification affect the ligand binding properties of the IP, they modulate its G protein coupling/intracellular signalling and, in the case of palmitoylation, may influence its ability to directly interact with Rab11a, to regulate agonist-induced trafficking of the IP following activation. Moreover, the IP is post-translationally modified by ubiquitination, which targets the mature and immature species to the lysosomal and 26S proteasomal/ERAD degradation pathways, respectively.

In addition to their classic interaction with heterotrimeric G-proteins, it is now recognized that GPCRs can interact with a wide range of functionally diverse proteins known collectively as ‘GPCR interacting proteins’ or ‘GIPs’, thereby regulating an array of other cellular events. Furthermore, due to its divergent sequence and capacity to contain functionally distinct binding motifs, the intracellular C-tail domain of the GPCR is the critical binding domain for such interactions between GPCRs with their specific GIP(s). To identify novel GIPs that interact with the IP, potentially shedding new insights into the role of prostacyclin within the vasculature including in cardioprotection, a yeast-two-hybrid approach was...
(Y2H)-based screen of a human kidney cDNA library was carried out where the carboxyl (C)-terminal tail domain of the IP (IP<sup>299</sup>–<sup>306</sup>) was used as the initial bait protein<sup>96</sup>, <sup>97</sup>, <sup>106</sup>, <sup>107</sup>. Through those studies, Rab11a was identified as a direct binding partner of the IP and this IP:Rab11a interaction was found to be dependent on a 14-residue Rab11 binding domain (RBD) located within its proximal C-tail domain of the IP, comprising Val<sup>299</sup>–Val<sup>307</sup>, adjacent to its palmitoylated residues at Cys<sup>308</sup>–Cys<sup>311</sup><sup>96</sup>, <sup>97</sup>. Moreover, it was established that the orientation of the RBD within the IP and its interaction with Rab11a may be regulated by agonist-dependent palmitoylation of the IP at Cys<sup>309</sup><sup>96</sup>, to dynamically regulate the trafficking and signalling of the IP<sup>97</sup>. In addition, as elaborated upon further below, it was discovered that the IP also interacts with members of the multi-PDZ domain proteins PDZK1 (NHERF3) and IKEPP (NHERF4) through interactions dependent on a PDZ-ligand(binding motif within the distal C-tail region of the IP<sup>106</sup>, <sup>107</sup>.

### 3.1. Role of PDZ Domain Proteins:

The frequently encountered postsynaptic density-95, disks large, zonula occludens-1 (PDZ) domain mediates protein:protein interactions by binding to the PDZ ligand located most typically, but not exclusively, at the extreme C termini of target proteins<sup>108</sup>–<sup>110</sup>. Through the formation of multiprotein complexes, PDZ interactions can participate in the coordination of key intra- and intercellular signaling systems, including intracellular routing or localization of proteins, cell polarity, as well as in the regulation of cell:cell interactions<sup>108</sup>–<sup>110</sup>. Structurally, the PDZ domain is composed of compact globular modules containing six anti-parallel β-strands (βA–βF) and two α-helices (αA and αB) with a highly conserved GLGF motif within its hydrophobic binding pocket that is responsible for the sequence-specific recognition of the PDZ ligand within the target protein(s). Depending on the nature of the three residues at their extreme C termini, the PDZ ligand of the target protein itself may belong to one of three classes, namely class I (Ser/Thr-Φ-COOH), class II (Φ-Φ-COOH), or class III (Asp/Glu-X-Φ-COOH), where Φ represents a hydrophobic amino acid and X can be any residue<sup>108</sup>–<sup>110</sup>.

### 3.2. Interaction of the IP with PDZK1.

As previously stated, we recently identified the multi PDZ-domain protein PDZK1 (NHERF3) as a highly specific interactant of the IP (Figure 7)<sup>106</sup>. The intracellular scaffold or adapter protein PDZ domain-containing protein 1 (PDZK1) is a member of the Na<sup>+</sup>, H<sup>+</sup> exchanger regulatory family (NHERF) and is predominantly expressed in the brush border of the kidney and small intestine, in epithelial and endothelial cells, in macrophages and in the liver<sup>111</sup>–<sup>113</sup>. PDZK1 contains 4 PDZ domains, facilitating its binding to highly specific interacting partners<sup>114</sup>–<sup>116</sup>. Most notably, in the context of the CV system, through its interaction with the high density lipoprotein (HDL) scavenger receptor class B, type 1 (SR-B1), PDZK1 is essential for both reverse cholesterol transport (RCT) and for HDL-mediated vascular re-endothelialization<sup>112</sup>, <sup>117</sup>. Similar to prostacyclin and the IP, through its activation of SR-B1, HDL also plays an essential protective role within the CV system where it regulates re-endothelialization as well as RCT, maintaining endothelial integrity and protecting against atherosclerosis and restenosis<sup>118</sup>, <sup>119</sup>. By binding to its C-terminal PDZ ligand, PDZK1 plays an essential role in maintaining SR-B1 expression levels in the liver, thereby controlling HDL cholesterol levels, and is now also known to play a key role in HDL/SR-BI- regulation of EC migration, promoting re-endothelialization<sup>117</sup>, <sup>120</sup>, <sup>121</sup>. Hence, PDZK1<sup>−/−</sup> mice display both (i) marked hypercholesterolaemia due to a 95% decrease in SR-B1 expression in the liver and (ii) impaired re-endothelialization, leading to increased atherosclerosis &/or impaired vascular repair, and thus represent an important model of CV dysfunction<sup>117</sup>.
Since the discovery of an interaction of the IP with PDZK1, we have made significant advances in validating the interaction and in establishing that, similar to that of SR-B1, the interaction is likely to be functionally important with respect to the protective role of prostacyclin/IP within the vascular endothelium. In brief, key amongst those findings include: (i) PDZK1 directly interacts with the IP involving a Class I PDZ ligand at its C-terminus; (ii) while the interaction occurs constitutively, it is dynamically regulated in response to agonist (cicaprost)-activation of the IP (Figure 7A); (iii) while PDZK1 did not influence overall levels of the IP, it increases its functional expression at the plasma membrane enhancing agonist binding and cAMP generation; (iv) specifically, in the context of the role of prostacyclin in re-endothelialization, both cicaprost and HDL were confirmed to promote substantial EC migration and in vitro angiogenesis, and in an augmentative manner (Figure 7B), while (v) similar to that previously reported for the HDL/SR-B1-mediated EC responses, siRNA-disruption of PDZK1 abolished cicaprost-induced EC migration and in vitro angiogenesis, and without affecting VEGF-mediated responses. A number of studies have suggested that prostacyclin-induced endothelial migration and angiogenesis occurs through its regulation of PPARδ, rather than through the IP per se. However, in those studies, iloprost was used as the IP agonist, which, unlike the highly selective IP agonist cicaprost used in our own studies, is known to activate both the IP and PPARδ. Moreover, in our studies, the effects of cicaprost were blocked by the IP antagonist RO1138452, confirming that the cicaprost induced EC-migration and angiogenesis are mediated through the IP rather than PPARδ.

Collectively, given the role of prostacyclin throughout the CV system, identification of PDZK1 as a functionally important interactant of the IP sheds significant mechanistic insights into the protective roles of these key players, and potentially of HDL/SR-B1, in the vascular endothelium. Critically, we have discovered a novel link between the IP, PDZK1 and SR-B1, which potentially reveals new therapeutic approaches to vascular protection. However, in advance of being able to exploit such therapeutic potential, it is first necessary to understand key aspects of the IP:PDZK1 interaction, including pin-pointing the molecular interactions through detailed structural studies. Further to this, through detailed X-ray crystallography, the co-ordinates of the interaction between the IP with PDZD1 of PDZK1 have recently been solved to high resolution.

3.3. Interaction of the IP with IKEPP:

The IP was also found to interact with IKEPP (intestinal and kidney enriched PDZ protein/IKEPP/PDZK2) through binding of the class I type PDZ ligand at the C terminus of the IP with the domains PDZ domains (PDZD)1 and to a lesser extent PDZD2 of IKEPP (Figure 8). Compared to the other NHERF family members, IKEPP/NHERF4 has few known binding partners but among them include guanylyl cyclase C and a range of transporters in the renal and gastrointestinal systems to regulate anion and calcium secretion and carnitine transport.

The ability of the IP to bind both PDZK1 and IKEPP is not unusual as several other proteins can bind multiple NHERF family members. Moreover, while both IKEPP and PDZK1 possess 4 PDZ domains, their interaction with the IP differs with differential binding of the distinct PDZ domains. This too is not without precedence. For example, the organic cation transporters OCTN1 and OCTN2 display different binding preferences for PDZ domains within PDZK1 and IKEPP where both OCTN proteins bind to PDZD1, PDZD2 and PDZD4 of PDZK1 but bind to PDZD1 and PDZD3 of IKEPP. With respect to the IP, PDZK1 and IKEPP display clear structural and functional differences in their interaction and regulation of the IP. IKEPP interacts constitutively with the IP but upon agonist activation, this interaction is substantially increased coinciding with increased IP-induced
phosphorylation of IKEPP\textsuperscript{107}. While the identity of the IP-induced target sites of phosphorylation within IKEPP remain to be identified, at least 6 potential phosphorylation sites within IKEPP have been proposed\textsuperscript{130}.

IKEPP was originally identified in the kidney and gastro-intestinal system but, to date, there is no evidence of its expression in other tissue types\textsuperscript{123, 130, 131}. As stated, prostacyclin is widely implicated in the regulation of renal hemodynamics and the IP is expressed in renal tubules and in the cortex\textsuperscript{9, 10, 132}. Furthermore, prostacyclin is increasingly implicated in regulating renin release and in the development of renovascular hypertension-associated with obstructive vascular disease, including atherosclerosis\textsuperscript{4, 25}. A potential role for the interaction of the IP with IKEPP in renal hemodynamics is indicated by the colocalization of IKEPP and the IP to the epithelial lining of the distal and, to a lesser extent, the proximal renal tubules\textsuperscript{107}. In addition, consistent with the discovery of the expression of IKEPP in vascular endothelial cells, IKEPP co-localizes with the IP; is found therein in immune-complexes with the IP and, functionally, it plays a key role in IP-mediated endothelial cell migration and \textit{in vitro} angiogenesis\textsuperscript{107}. Collectively, these studies point to a novel, previously undiscovered role for the IP/IKEPP interaction within the vasculature and potentially in CVD that merits further investigation.

4. Summary/Conclusions:
Collectively, it is evident that significant advances have been made in identifying novel proteins and associated-pathways that influence the prostacyclin-IP axiom, providing new mechanistic insights into prostacyclin’s role in maintaining vascular integrity. Armed with this knowledge, it provides new pathways regulated by the cardio-protective prostacyclin that may be targeted potentially offering novel therapeutic approaches in treating many aspects of CVD or indeed in other conditions, such as pulmonary arterial hypertension (PAH) or renovascular hypertension, in which aberrant prostacyclin signalling and function is strongly implicated. At the genomic/transcriptional level, key factors including the female hormone estrogen and low serum-cholesterol already known to play critical roles within the CV system, in particular in cardio-protection and in re-endothelialization, also up-regulate expression of the IP. In the interests of “gender balance”, a remaining question worthy of investigation is what the impact of male hormones such as dihydrotestosterone on PTGIR/IP expression levels might be? Critically, the studies outlined herein provide a detailed molecular and genetic basis for understanding the main transcriptional factors determining expression of the IP within the vasculature and elsewhere. Furthermore, at the genomic level, several single nucleotide polymorphisms (SNPs) have recently been identified in the 5’ flanking region of the PTGIR\textsuperscript{31, 133} but it is unknown whether such SNPs may also contribute to population variations in IP expression levels and/or to predisposition to CVD/CAD. Through the many recent advancements outlined in this review, greater meaning and interpretation of such genome-wide association studies can also be garnered.

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Figure Legends

Figure 1. Structure of the Human Prostacyclin Receptor.
Three-dimensional representation of the human IP, depicting the seven transmembrane (TM) domains, TM1 to TM7, and the alpha-helical 8 domain (α-H8). The structural prediction was generated by online submission to the iterative TASSER (I-TASSER) algorithm, that builds three-dimensional protein structure models based on multiple threading consensus target-to-template alignments by LOMETS

Figure 2: Structural organization & Basal Transcriptional Regulation of the human Prostacyclin Receptor gene (PTGIR).
Panel A: The human prostacyclin receptor gene (PTGIR), located on chromosome 19q13.3, is composed of 3 exons separated by 2 introns (I1 and I2). Exon (E)1 encodes 52 bp of 5’ untranslated region (UTR) sequence and is located at nucleotides -772 to -824, relative to the translational initiation codon (at +1). E2 encodes 12 bp of 5’ UTR and the coding sequence for the translational initiation codon (ATG) through to amino acid 256 within TM6 of the IP (i.e., nucleotides +1 to +767) and E3 encodes aa 256 – 383 corresponding to TM6 through to the C-terminal residue (i.e., nucleotides +767 to +1149) in addition to the 3’ UTR. The transcription initiation codon (TI) maps approximately to nucleotide -917. Panel B & C: Recombinant pGL3B plasmids encoding PrmIP (−2449 to −772), its 5’ deletion derivatives PrmIP1−PrmIP7, mutated PrmIP6 variants or, as a control, pGL3B were co-transfected with pRL-TK into HEL 92.1.7 cells. At 48 hr post-transfection, Firefly and Renilla luciferase activities were assayed. Results are expressed as mean luciferase activity (RLU; ± S.E.M., n = 6). It was established that the region between PrmIP6-PrmIP7 (−1042 to −772), proximal to the T1 site contains the “core promotor” while an upstream repressor region (URR) was identified between PrmIP4-PrmIP5 (−1524 to −1293). Site directed mutagenesis (SDM) of the Sp1, PU.1 and Oct1 cis-elements within the core promotor reduced PrmIP-directed gene expression, while mutations of all 3 sites abolished PrmIP-directed luciferase activity showing that Sp1, PU.1 and Oct1 are the main trans-acting factors that bind and regulate the core PrmIP under basal conditions. Asterisks indicate that deletion or mutation of PrmIP sequences either significantly reduced or increased reporter gene expression in HEL cells, where * & **** indicates p ≤ 0.05 & p ≤ 0.00001, respectively. Panel D: The trans-acting factors Sp1, PU.1 and Oct-1 regulate basal transcription of the PTGIR gene by their interaction with the proximal core PrmIP where, in the absence of a TATA box, it is proposed that Sp1 initially binds to the proximal promoter acting as a preinitiation factor. Thereafter, other trans-acting factors, including PU.1 and Oct-1, in addition to TBP, TFIID and, finally, RNA polymerase II, are recruited to direct assembly of the transcriptional initiation complex, necessary for efficient basal PTGIR gene expression. Presented data was adapted from Turner & Kinsella, 2009.

Figure 3. PU.1-C/EBPδ cooperative binding and for PMA-mediated increases in PrmIP-directed gene expression.
Panel A. Chromatin immunoprecipitation (ChIP) analysis of C/EBPβ, C/EBPδ and PU.1 binding to PrmIP, using either input chromatin or chromatin extracted from anti-C/EBPβ, anti-C/EBPδ, anti-PU.1, normal rabbit IgG or no 1° antibody control immunoprecipitates from non-treated (0 hr) or PMA-treated (100 nM; 5 hr, 10 hr & 24 hr) HEL cells. PCR analysis was performed using primers to amplify the −1528 to −1327 region (indicated by solid arrows) or, as a negative control, primers to amplify the −1761 to −1577 region of PrmIP (indicated by dashed arrows). In the absence of PMA-induced HEL cell
differentiation, C/EBPδ and to a lesser extent PU.1 bind to mediate repression of PrmIP-directed gene expression. However, in response to PMA differentiation, there is a switch from predominant C/EBPδ binding to predominant PU.1 binding in this region, thereby converting the URR to a PRR1. **Panel B:** Hypothetical scheme for PMA-induced changes in transcriptional regulator binding in PrmIP. Under basal conditions, binding of C/EBPδ and PU.1 to its cis-acting element within the URR mediates repression of PrmIP expression. Following PMA treatment of HEL cells, (i) there is a complete loss of C/EBPδ binding, alleviating transcriptional repression in this region. (ii) This loss of repression coincides with increased binding of PU.1 to the URR, leading to enhanced transcriptional activation of PrmIP. (iii) Coincident with this, increased binding of Sp1, PU.1 and Oct-1 within the “core promoter” region further enhances the PMA-induced transcriptional activation of PrmIP. Presented data was adapted from Keating et al., 201031.

**Figure 4. Estrogen-mediated increases in PrmIP-directed gene expression.**
Panel A: Immunofluorescence microscopy of human IP and COX2 expression in 1° hAoSMCs preincubated for 24 hr with either vehicle (PBS, 0.01% EtOH) or 10 nM 17β-estradiol (E2) and immunolabelled with either anti-IP or anti-COX2 sera and Alexa Fluor 488-conjugated anti-rabbit IgG (green), followed by counterstaining with DAPI (blue). E2 mediates an increase in IP and COX2 expression. **Panel B:** Schematic of the estrogen-responsive element (ERE) located at -1783 to -1597 and upstream of the previously identified URR and core promoter regions, where ERα binds within PrmIP to regulate transcription of the PTGIR gene. Presented data was adapted from Turner & Kinsella, 201030.

**Figure 5. SREBP1-mediated increases in PrmIP-directed gene expression.**
Panel A: Immunofluorescence microscopy of 1° HUVECs cultured for 24 hr in high, normal or low serum-cholesterol and immunolabeled with anti-IP sera and Alexa Fluor 488-conjugated anti-rabbit IgG (green), followed by counterstaining with DAPI (blue). In low serum-cholesterol, IP expression is substantially increased. **Panel B:** In conditions of low-serum cholesterol, the SREBP1 precursor protein is transported to the Golgi apparatus where it is first cleaved by site-1 protease (S1P) (scissors) and then by S2P (scissors). The liberated, transcriptionally active basic helix-loop-helix (bHLH)-LZ domain of SREBP1 travels to the nucleus and directs the transcription of target genes through binding to cis-acting serum response elements (SREs) within their specific promoter regions. **Panel C:** In low serum-cholesterol, IP mRNA levels and PrmIP-directed gene expression are significantly increased through a transcriptional mechanism involving binding of Sp1 and SREBP1, but not SREBP2, to their adjacent consensus cis-elements within the core PrmIP promoter. Presented data was adapted from Turner & Kinsella, 201292.

**Figure 6. Structural organization of the human I prostanoid receptor.**
Two-dimensional serpentine representation of the human I prostanoid receptor (IP) composed of an amino-terminal extracellular domain (N-terminus), 7 transmembrane domains (TM)1-7, 3 extracellular loops (ECL)1-3, 3 intracellular loops (ICL)1-3 and an intracellular carboxyl-terminal domain (C-tail) is shown, where the TM s are in green. The conserved (Cys92-Cys170) and putative (Cys5-Cys165) disulfide bonds are indicated with dashed red lines. N-linked glycosylation sites are represented by hexagonal shapes on Asn7 and Asn78. The isoprenylation (Cys383) and palmitoylation (Cys308, Cys309 and Cys311) sites within the C-tail of the human IP are indicated by red or orange lines, respectively. Residues experimentally determined to be important for ligand recognition/binding or G protein coupling are in...
pink and black, respectively. Proline residues within the TM s important for receptor activation are in light blue. The endoplasmic reticulum (ER) export motif is purple\textsuperscript{101, 102}.

**Figure 7. Model of the interaction of PDZK1 with the IP and its role in endothelial cell migration & in vitro angiogenesis.** Panel A: In the absence of agonist, PDZK1 is constitutively associated in a complex with the IP, where PDZK1 is either not phosphorylated or basally hypo-phosphorylated. On agonist (cicaprost) stimulation: (i) the IP undergoes an agonist-induced conformational activation leading to dissociation of PDZK1; (ii) Released PDZK1 is then subject to enhanced IP-induced cAMP-dependent PKA phosphorylation at Ser\textsuperscript{505}, and (iii) this enhanced or nett hyper-phosphorylated PDZK1 triggers its re-association with the IP. The re-association of PDZK1 and IP is coincident with regulated (iv) de-phosphorylation of PDZK1 and its return to basal/hypo-phosphorylated levels. Consistent with this model, the phosho-defective PDZK1\textsuperscript{S505A} is found in a constitutive complex with the IP and undergoes agonist-induced dissociation but cannot undergo phosphorylation-induced re-association in response to receptor activation. In contrast, the phospho-mimetic PDZK1\textsuperscript{S505D} mimics the hyper-phosphorylated protein state (state iii), leading to sustained interaction with the IP. Panel B: Agonist activation of the IP, SR-B1 and VEGFR leads to enhanced endothelial cell migration and in vitro angiogenesis/tube formation. HDL/SR-B1-, but not VEGF/VEGFR-, mediated endothelial cell-migration is dependent on its interaction with PDZK1. Cicaprost activation of the IP promotes endothelial cell migration and tube formation in a PDZK1-dependent mechanism, where siRNA disruption of PDZK1 inhibits both cicaprost- and HDL-, but not VEGF-, induced endothelial cell responses. Presented data was adapted from Turner et al., 2011\textsuperscript{106}.

**Figure 8: Differential Modulation of the Interaction of IKEPP & PDZK1 with the IP.**
In the absence of agonist, both IKEPP and PDZK1 are constitutively associated in a complex with the IP where these interactions are largely dependent on PDZ domain 1 (PDZD1) and, to a lesser extent, on PDZD2 of IKEPP or, in the case of PDZK1, on PDZD3 and, to lesser extent, on PDZD1 and on PDZD4. Upon cicaprost stimulation (10 min; CICA), the association of IKEPP and PDZK1 with the IP is differentially modulated. Specifically, the association of IKEPP with the IP is enhanced in response to receptor activation, an effect that is abrogated by the selective IP antagonist RO1138452 and the combined activity of the PKA and PKC inhibitors, H-89 and Gö6983, respectively. On the other hand, in response to cicaprost (10 min) stimulation, there is a transient disassociation of PDZK1 from the IP complex and this occurs due to IP-induced PKA phosphorylation of Ser\textsuperscript{505} within the C-terminal regulatory domain of PDZK1. Cicaprost-stimulation promotes endothelial cell migration and in vitro angiogenesis, effects that are impaired by the IP antagonist RO113845 or siRNA-disruption of IKEPP or PDZK1 expression. Presented data was adapted from Reid et al., 2012\textsuperscript{107}. 

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Figure 3

(A) (B)

Figure 4

(A) Anti-hILP Anti-COX2

(B) Estrogen (E) & ER agonists

Increased gene expression

URR – Upstream Repressor Region
ERE – Estrogen Response Element

Figure 5

(A) Serum Cholesterol Levels

(B) Increased ProIP directed gene expression & hIP mRNA levels

(C) Estrogen (E2) & ERa agonists

Figure 6

SCRNL

ICL1

ICL2

ICL3

ECL1

ECL2

ECL3

N-Terminus

C-tail

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