

**Transcriptional Regulation of the Human Prostacyclin Receptor Gene is Dependent on Sp1, PU.1 and Oct-1 in Megakaryocytes and Endothelial Cells.**

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## Summary

Prostacyclin plays a central role in haemostasis, inflammation and nociception. However, the factors regulating expression of the prostacyclin receptor (IP) gene in humans, or in other species, have not been identified. Herein it was sought to identify the key *trans*-acting factors and *cis*-acting elements regulating IP expression in the megakaryoblastic human erythroleukemia (HEL) 92.1.7 and the vascular endothelial EA.hy 926 cell lines. Using deletion and genetic reporter analyses, the essential core promoter, termed PrmIP, was localized to -1022 to -895 proximal to the transcription initiation site, while an upstream repressor region, localized to -1502 to -1271, was also identified. Bioinformatic analysis revealed evolutionary conserved Sp1, PU.1 and Oct-1 sites within the core PrmIP and disruption of those elements each led to substantial reductions in PrmIP-directed gene expression in both HEL and EA.hy 926 cells. Electrophoretic mobility shift assays (EMSAs) and supershift assays established that Sp1, PU.1 and Oct-1 can bind to elements within the core promoter *in vitro* while chromatin immunoprecipitation (ChIP) assays confirmed their specific binding to chromatin *in vivo*. Furthermore, combination mutations of the Sp1, PU.1 and Oct-1 elements revealed that they act independently to co-regulate basal transcription of the IP gene while ectopic expression of each of the *trans*-acting factors led to substantial increases in PrmIP-directed gene expression and IP mRNA expression in both HEL and EA.hy 926 cells. While EMSA and antibody supershift assays established that the Ets family member Fli1, but not Ets-1, is capable of binding to the PU.1 element within PrmIP *in vitro*, ChIP analysis established that neither Fli1 nor Ets-1 bind to that element *in vivo*. Collectively, these data provide critical insights into the transcriptional regulation of the IP gene in human megakaryocytic and endothelial cells, identifying Sp1, PU.1 and Oct-1 as the critical factors involved in its basal regulation in humans.

## Introduction

The prostanoid prostacyclin (prostaglandin (PG) I<sub>2</sub>) plays a central role in haemostasis acting as a potent inhibitor of platelet aggregation and as an endothelium-derived vasodilator<sup>1; 2</sup>. The actions of prostacyclin generally counteract those of thromboxane (TX) A<sub>2</sub>, a potent vasoconstrictor released principally by platelets, implying that factors regulating their relative levels and /or cellular responses are central to haemostasis and vessel tone<sup>2; 3; 4</sup>. Alterations in the relative levels of TXA<sub>2</sub> and prostacyclin or of their specific receptors contribute to a variety of vascular dysfunctions including thrombosis, stroke, systemic and pulmonary hypertensions, myocardial infarction and atherosclerosis<sup>5; 6; 7</sup>. Prostacyclin also imparts an important cardioprotective role<sup>8</sup>; it inhibits leukocyte-endothelial cell interaction<sup>9</sup> and promotes enhanced endothelial cell survival and/or proliferation supporting neovascularization and angiogenesis<sup>10</sup>. Prostacyclin and its analogues have been used clinically to improve pulmonary hypertension and various vaso-occlusive disorders such as that associated with Raynaud's syndrome and Buerger's disease<sup>1; 11; 12</sup>.

As a major product of cyclooxygenase (COX)-2 catalyzed metabolism of arachidonic acid, prostacyclin acts as a potent pro-inflammatory mediator and is abundantly produced during myocardial ischemia and hypoxia offering cytoprotection<sup>13; 14; 15</sup>. Moreover, the critical role of prostacyclin to vascular integrity has been further highlighted through clinical findings that certain COXIBs, the subclass of nonsteroidal anti-inflammatory drugs designed to selectively inhibit COX-2, depress prostacyclin generation predisposing patients to increased risk of thrombotic stroke and myocardial infarction<sup>16</sup>. Whilst mice deficient in prostacyclin receptors (IP<sup>-/-</sup>) display normal vascular function, they show enhanced thrombotic tendency in response to endothelium damage, for example<sup>14; 17</sup>. Significantly however, IP<sup>-/-</sup> null mice display altered pain perception and, in models of acute inflammation, exhibit substantially reduced inflammatory responses consistent with the central role of prostacyclin in nociception and as a potent pro-inflammatory agent<sup>14; 17; 18</sup>.

Prostacyclin mediates its actions through activation of its signature prostacyclin receptor, IP, a member of the G protein coupled receptor (GPCR) superfamily that is widely expressed within the vasculature including in platelets/megakaryocytes, macrophages, vascular endothelial and smooth muscle, in sensory neurons of the dorsal root ganglion and in various tissues such as in thymus, lung, heart and spleen<sup>2; 17; 18</sup>. While the IP is primarily coupled to activation of adenylyl cyclase (AC), mediating prostacyclin-inhibition of platelet aggregation and vascular tone, it may also regulate a number of other secondary effector systems, perhaps in a tissue- and /or species-specific manner<sup>2; 19</sup>. The cloned mouse (m) and human (h) IPs couple to both G<sub>s</sub> and to G<sub>q</sub>/phospholipase C $\beta$  activation and to intracellular calcium ([Ca<sup>2+</sup>]<sub>i</sub>) mobilization, while megakaryocytopoiesis of 1<sup>o</sup> h.stem cells is associated with reductions in prostacyclin-induced [Ca<sup>2+</sup>]<sub>i</sub> mobilization due to loss of G $\alpha$ <sub>16</sub> and increased G $\alpha$ <sub>s</sub> expression<sup>20</sup>. In addition to coupling to G<sub>s</sub>, the mIP, but not the hIP, couples to G<sub>i</sub> and to G<sub>q</sub> activation through a novel G protein switching mechanism, involving direct cAMP dependent protein kinase (PK) A- phosphorylation of the IP within its carboxyl terminal tail domain<sup>21; 22</sup>. A number of independent studies have established that the IP is subject to agonist-induced desensitization and down-regulation in human platelets and other cell types, providing an important mechanism of fine-tuning the cellular responses to prostacyclin *in vivo*<sup>23; 24; 25; 26; 27</sup>. While the hIP

undergoes agonist-induced protein kinase (PK)C phosphorylation, desensitizing its signalling<sup>27</sup>, its internalization is entirely independent of both PKC and of the classic GRK/ $\beta$ -arrestin-mediated mechanism<sup>26</sup> but, rather, largely occurs through a mechanism involving its direct interaction with Rab5a<sup>28</sup>.

Despite these studies and the wealth of information that has been accrued relating to the basic mechanisms of intracellular signalling by the IP<sup>2; 19; 21; 29</sup>, coupled to the breadth of knowledge highlighting the critical role of the prostacyclin in haemostasis, inflammation and pain<sup>9; 14; 16; 30</sup>, surprisingly to date the hIP gene remains largely uncharacterized and the factors regulating its expression have not been identified<sup>31</sup>. This study aims to address this scientific deficit by characterizing the hIP gene, focusing primarily on delineation of the mechanism determining its expression within the vasculature. Herein, we have identified a critical role for evolutionary conserved Sp1, PU.1 and Oct-1 *cis*-acting elements in the transcriptional co-regulation of the hIP in the megakaryocytic human erythroleukemia (HEL) 92.1.7<sup>32</sup> and in human endothelial EA.hy 926<sup>33</sup> cell lines. These studies represent the first detailed characterization of the human IP promoter and provide a strong molecular and genetic basis for understanding the modes of regulation of IP gene expression in health and disease.

## Results

### Functional Characterisation of the human Prostacyclin Receptor Promoter in human erythroleukemia cells

The central role of prostacyclin, signalling through its cognate prostacyclin receptor (IP), in haemostasis and vascular disease in addition to its role in inflammation, pain and analgesia is widely recognized<sup>1; 2; 34</sup>. However, the factors regulating expression of the IP gene in humans, or indeed in other species, have not been investigated in detail. Hence, the key aim of the current study was to identify the critical *cis*-acting elements and *trans*-acting factors regulating basal expression of the IP in the human erythroleukemia (HEL) 92.1.7 cell line and in human vascular endothelial cells. Through previous investigations, the structural organisation of the human IP gene was elucidated and the major transcription initiation (TI) site utilized in human lung tissue mapped to approximately nucleotide -875, relative to the translation start codon (+1)<sup>35</sup>. Consistent with those studies, herein the major TI was also investigated and was localized to a region between -875 to -774 in HEL 92.1.7 cells (data not shown). Hence, for subsequent studies herein, the human prostacyclin receptor promoter (PrmIP) is defined as nucleotides -2427 to -774, relative to the translational start codon (+1) and is located approximately 1.5 kb – 0.1 kb 5' and 3', respectively, of the major TI site at -875<sup>35</sup>.

Initially, genetic reporter assays employing firefly luciferase as specific reporter were used to characterise the PrmIP aiming to identify the major regulatory regions therein. Recombinant pGL3Basic (pGL3B) encoding PrmIP directed  $10.1 \pm 0.46$  relative luciferase units (RLU) in HEL cells while the empty pGL3B vector directed minimal activity ( $0.145 \pm 0.01$  RLU) and the pGL3Control vector, containing SV40 promoter and enhancer sequences, directed  $20.6 \pm 0.87$  RLU; **Figure 1: Panel A**). To identify the functionally important regulatory DNA sequences in its 5'-flanking region, a series of seven successive 5' deletions of PrmIP, each a common 3' site (-774), were generated (**Figure 1: Panel A**). Progressive 5' deletion to generate the PrmIP1 (-1761), PrmIP2 (-1682), PrmIP3 (-1575) and PrmIP4 (-1502) subfragments did not significantly affect PrmIP-directed luciferase expression in HEL cells (**Figure 1: Panel A**). Hence, from deletional analysis, it appears that sequences between -2427 and -1502 may not play a significant role in PrmIP-directed basal gene expression in HEL cells. However, 5' deletion of nucleotides -1502 to -1271, to generate PrmIP5 (-1271), yielded a 2-fold increase in luciferase expression (**Figure 1: Panel A**;  $p < 0.0001$ ) while further 5' deletion to generate PrmIP6 (-1271 to -1022) did not significantly affect the level of luciferase activity relative to that of PrmIP5. Conversely, further 5' deletion of nucleotides -1022 to -895 to generate PrmIP7 (-895 to -774) resulted in 14-fold decrease in luciferase activity in HEL cells (**Figure 1: Panel A**;  $p < 0.0001$ ) such that the level of luciferase expression directed by PrmIP7 ( $1.35 \pm 0.15$  RLU) was only marginally greater than that directed by the promoter-less pGL3B vector.

Collectively, these data suggest that the -1022 to -895 region, proximal to the TI, within the PrmIP represents the core promoter containing the positive regulatory elements required to direct efficient basal transcription in HEL cells while it also contains an upstream repressor sequence (URS) located between -1502 and -1271. Hence, the PrmIP6 (-1022 to -774) represents the smallest sub-fragment generated herein that contains the proximal core promoter. Consistent with that hypothesis, 3' deletion of nucleotides

spanning -1022 to -774 to generate 3'ΔPrmIP (**Figure 1; Panel B**) almost completely abolished PrmIP-directed transcriptional activity in HEL cells such that the level of luciferase activity was not substantially different from the empty pGL3B vector (compare  $0.303 \pm 0.05$  vs  $0.145 \pm 0.01$  RLU, respectively).

### Identification of Functional Sp1, PU.1 and Oct-1 Elements within the PrmIP.

Thereafter, it was sought to identify the key *cis*-acting elements located between -1022 and -895 within the PrmIP6 sub-fragment responsible for directing basal transcription from the core proximal promoter. Amongst the putative elements identified through bioinformatic analysis<sup>36</sup> were a consensus Sp1 (-967) element and closely positioned PU box<sup>37</sup> (-954, herein referred to as a PU.1 element) and octamer-binding (-949) elements (**Figure 2; panel A**). Furthermore, bioinformatic analysis revealed that the putative Sp1, PU.1 box and octamer sites are evolutionary conserved within the proximal IP promoters from a host of other species including those of horse, dog, bovine (**Figure 8B**), mouse and rat (data not shown).

Disruption of the Sp1 element (GGGCGG to GGATGG) through site-directed mutagenesis (SDM) resulted in a 2-fold reduction ( $p < 0.0001$ ) in luciferase expression compared to that directed by PrmIP6 (**Figure 2; Panel A**). Mutation of the PU.1 element (GAGGAA to GACGAA) also led to a 2-fold ( $p < 0.0001$ ) reduction in luciferase activity relative to that of PrmIP6. Similarly, disruption of the octamer element (AAATGA to AACTGA) at -949 led to a 1.3-fold ( $p = 0.0143$ ) reduction in PrmIP6-directed luciferase expression, albeit to a lesser extent than disruption of either the Sp1 ( $p = 0.0002$ ) or PU.1 ( $p = 0.0015$ ) elements alone.

A number of independent studies suggest that octamer sequence elements within certain promoters may be specifically regulated either by the ubiquitous Oct-1<sup>38</sup> or by the B lymphocyte- and/or neurone-specific Oct-2<sup>39;40</sup> or, in the certain cases, by both Oct-1 and Oct-2<sup>41;42</sup>. Hence, herein, the specificity of Oct-1 or Oct-2 binding to the octamer binding site within the PrmIP was investigated by examining the effect of heterologous over-expression of hemagglutinin (HA)-tagged forms of Oct-1 and Oct-2 on luciferase reporter expression. Western blot analysis confirmed that both Oct-1 and Oct-2 were over-expressed to equivalent levels in the transfected HEL cells (**Figure 2; Panel C**). However, ectopic expression of Oct-1, but not Oct-2, led to a significant increase in PrmIP6-directed luciferase activity ( $p < 0.0001$  and  $p = 0.1071$ , respectively; **Figure 2; Panel B**).

The Ets family of transcription factors are characterised by an evolutionary-conserved DNA-binding domain that binds to a purine-rich GGAA/T core sequence with additional flanking nucleotides often determining specificity<sup>43</sup>. Within PrmIP, a conserved putative PU.1 element<sup>37</sup> was identified (5'-GAGGAA-3') suggesting that the Ets family member PU.1 may be involved in hIP regulation. However, many family members, including PU.1, Fli1 and Ets-1, have been reported to play important roles in megakaryocytic and erythroid differentiation<sup>44</sup>. Therefore, the specificity of the PU box within PrmIP was also investigated by examining the effect of heterologous over-expression of PU.1, Fli-1 and Ets-1, on luciferase reporter expression. Immunoblot analysis confirmed that PU.1, Fli1 and Ets-1 were over-expressed to equivalent levels in the transfected HEL cells (**Figure 2; Panel E**). Ectopic expression of PU.1 led to a significant increase in PrmIP6-directed luciferase activity ( $p < 0.0001$ ). Similarly, over-expression of Fli-1

resulted in a significant increase in PrmIP6-directed luciferase activity ( $p = 0.043$ ), albeit to a lesser extent than ectopic expression of PU.1. Ets-1 heterologous over-expression did not significantly effect PrmIP-directed luciferase activity ( $p = 0.342$ , respectively; **Figure 2D&E**). Hence, collectively these data confirm that disruption of the Sp1, PU.1 and octamer elements each significantly impaired PrmIP6-directed transcription, and the putative octamer site at -949 is preferentially regulated by Oct-1, as opposed to Oct-2, and the putative Ets element is preferentially regulated by PU.1 and/or Fli1, but not Ets-1, in HEL cells.

### **Electromobility Shift Assays and Chromatin Immunoprecipitations to investigate Sp1, PU.1 and Oct-1 Binding.**

Electromobility shift assays (EMSAs) and antibody supershift assays were then employed to investigate the presence and specificity of nuclear/transcription factors capable of binding to the putative Sp1 element within PrmIP *in vitro*. Initially, the expression of Sp1 in HEL cells was confirmed by immunoblot analysis (**Figure 3: Panel B**). Incubation of the biotinylated Sp1 probe, spanning nucleotides -985 to -951, with nuclear extract resulted in the appearance of a major diffuse DNA-protein complex, designated C1 (**Figure 3A, lane 2**). Complex C1 was efficiently inhibited by an excess of corresponding non-labelled Sp1 probe (**Figure 3A, lanes 2 & 3**). The specificity of nuclear factor binding to the Sp1 probe was verified whereby a competitor based on a consensus Sp1 sequence, but not based on a randomized IP sequence, specifically competed the C1 complex (**Figure 3A, lane 4 & 5**). Moreover, pre-incubation of the nuclear extract with an *anti*-Sp1 antibody led to a decrease in the abundance of complex C1 and the appearance of a retarded supershift complex (**Figure 3A, lane 6**).

Furthermore, to investigate whether Sp1 actually binds to the IP promoter *in vivo*, chromatin immunoprecipitation (ChIP) assays were performed using chromatin extracted from HEL cells and antibodies directed to endogenous Sp1. PCR analysis using primers specific for the -1271 to -774 region of PrmIP generated amplicons from both the input chromatin and from the *anti*-Sp1, but not the normal rabbit IgG, immunoprecipitated chromatin (**Figure 3C**). Conversely, as additional controls, PCR analysis using primers for another region of the PrmIP, namely -1901 to -1555, only generated an amplicon from the input chromatin but not from the normal rabbit IgG or *anti*-Sp1 immunoprecipitated chromatin (**Figure 3D**). Hence, EMSA/supershift assays demonstrate that Sp1 specifically binds to the Sp1 probe *in vitro* while ChIP assays establish that Sp1 occupies elements within the -1271 to -774 region of the PrmIP chromatin *in vivo*. Collectively, these data show that we have identified a consensus Sp1 transcription factor binding site at -967 that is critical for efficient basal human PrmIP-directed gene expression in HEL cells.

Nuclear factor binding to the proposed PU.1 and Oct-1 elements was also investigated by EMSA and ChIP analysis. Owing to their close proximity, EMSA were performed using a 5'-biotinylated double-stranded PU.1/Oct-1 probe spanning nucleotides -963 to -929 of PrmIP containing both the putative PU.1 (-954) and Oct-1 (-949) elements. Incubation of the biotinylated PU.1/Oct-1 probe with nuclear extract from HEL cells resulted in the appearance of two main closely migrating DNA-protein complexes C1 and C2 and a third lesser abundant faster migrating complex designated C3 (**Figure 4A; lane 2, short and long exposures**). The specificity of nuclear factors binding was verified whereby complexes C1, C2 and C3 were

each efficiently competed by an excess of the corresponding non-labelled PU.1/Oct-1 probe whereas the non-specific competitor, based on a random IP gene sequence, failed to compete any of the C1-C3 complexes (**Figure 4A, lane 3 & 6, respectively**). Additionally, a consensus PU.1 sequence competed complex C2, but not C1 or C3 (**Figure 4; lane 4**). The consensus Oct-1 oligonucleotide mainly competed the DNA-protein complexes C1 and C3 but not C2 (**Figure 4A, lane 5**). The identity of the slower migrating C1 complex is unknown but may represent a tertiary PU.1/Oct-1 DNA complex. Immunoblot analysis also confirmed endogenous expression of Oct-1, PU.1, Fli-1 and Ets-1 in HEL cells (**Figure 4C-F**). Moreover, electromobility supershift assays employing *anti-Oct-1*, *anti-PU.1*, *anti-Fli-1* and *anti-ETS-1* antibodies demonstrated specific and direct binding of Oct-1 and PU.1 to the PU.1/Oct-1 probe of PrmIP in HEL cells as evidenced by the presence of supershifted complexes (**Figure 4B**). With respect to the other Ets family members, while no supershift occurred with antibodies directed to Ets-1, a weak supershifted immune complex was evident with the *anti-Fli1* sera, suggesting that Fli1 has the capability of binding to the PU.1 element within PrmIP, albeit to a lesser extent than PU.1.

To investigate whether endogenous Oct-1, PU.1, Fli1 and/or Ets-1 actually bind to the IP promoter *in vivo*, ChIP analysis was performed using the specific *anti-Oct-1*, *anti-PU.1*, *anti-Fli1* and *anti-Ets-1* antibodies and normal rabbit IgG as a background control. Immunoprecipitation of the cross-linked chromatin fragments obtained from HEL cells followed by semi-quantitative PCR using primers specific for the -1271 to -774 region of PrmIP surrounding the putative PU.1 site (-954) and Oct-1 (-949) elements generated amplicons from both the *anti-Oct-1* (**Figure 4G**) and *anti-PU.1* (**Figure 4H**), but not from the *anti-Fli1*, *anti-Ets-1* or control IgG immunoprecipitates (**Figure 4H**). Additionally, PCR analysis using primers for a non-specific region of PrmIP (-1901 to -1555) generated an amplicon from the input chromatin, no products were generated from the *anti-Oct-1*, *anti-PU.1*, *anti-Fli1* or *anti-Ets-1* immunoprecipitated chromatin (**Figure 4I**). Hence, these data confirm that both Oct-1 and PU.1, but not Fli1 or Ets-1, can specifically bind to the PrmIP promoter region of HEL cell chromatin located between -1271 to -774.

### **Examination of the Co-Regulation of the human IP promoter by Sp1, PU.1 and Oct-1 Transcription Factors in HEL cells**

Hence, studies herein have established that Sp1, PU.1 and Oct-1 specifically bind to the proximal PrmIP core region to regulate basal transcription of the hIP gene in HEL cells. In order to investigate whether Sp1, PU.1 and Oct-1 act independently or synergistically to regulate PrmIP-directed gene expression in HEL cells, the effect of mutating the *cis*-acting elements in combination was next examined (**Figure 5**). Initially, the double combinations, namely PrmIP6<sup>Sp1/Pu1\*</sup>, PrmIP6<sup>Sp1/Oct1\*</sup> and PrmIP6<sup>Pu1/Oct1\*</sup>, were generated and their ability to direct luciferase expression relative to that of the corresponding PrmIP6 subfragment containing wild type or singly mutated Sp1, PU.1 or Oct-1 elements was examined (**Figure 5**). While the PrmIP6<sup>Sp1/Pu1\*</sup> subfragment containing an intact Oct-1 element retained the ability to direct luciferase expression, the level of expression was some 3.2-fold less than that directed by the wild type PrmIP6 ( $p < 0.0001$ ). Moreover, luciferase expression directed by the PrmIP6<sup>Sp1/Pu1\*</sup> was 1.7-fold ( $p < 0.001$ ) and 1.6-fold ( $p < 0.0005$ ) less than that directed by the PrmIP6<sup>Sp1\*</sup> and PrmIP6<sup>Pu1\*</sup>, respectively. Hence, these data

suggest that both Sp1 and PU.1 elements can independently regulate PrmIP-directed gene expression in HEL cells and that disruption of both elements generates a promoter with a substantially impaired ability to initiate transcription through the Oct-1 element.

Consistent with this, the PrmIP6<sup>Sp1/Oct1\*</sup> and PrmIP6<sup>Pu1/Oct1\*</sup> subfragments containing intact Pu.1 and Sp1 elements, respectively, retained the ability to direct luciferase expression but with levels of expression reduced by some 2-fold ( $p < 0.0001$ ) and 2-fold ( $p < 0.0001$ ) relative to the wild type PrmIP6, respectively (**Figure 5**). Noteworthy, the level of luciferase expression directed by either the PrmIP6<sup>Sp1/Oct1\*</sup> and PrmIP6<sup>Pu1/Oct1\*</sup> subfragments was not significantly different than that directed by the PrmIP6<sup>Sp1\*</sup> ( $p = 0.6486$ ) or PrmIP6<sup>PU.1\*</sup> ( $p = 0.9185$ ), respectively. However, both PrmIP6<sup>Sp1/Oct1\*</sup> and PrmIP6<sup>Pu1/Oct1\*</sup> subfragments were significantly reduced relative to that directed by PrmIP6<sup>Oct1\*</sup> ( $p = 0.006$  and  $p = 0.011$ , respectively). These data suggest that both the Sp1 and PU.1 elements play a more significant role in PrmIP6-directed gene expression than that of Oct-1.

Disruption of all three *cis*-acting elements further impaired PrmIP-directed transcriptional activity such that PrmIP6<sup>Sp1/Pu1/Oct1\*</sup>-directed expression was reduced 6-fold ( $p < 0.0001$ ) relative to PrmIP6 (**Figure 5**). Moreover, the fact that transcriptional activity of PrmIP6<sup>Sp1/Pu1/Oct1\*</sup> was reduced relative to that of the double mutant PrmIP6<sup>Sp1/Pu1\*</sup> (2-fold;  $p = 0.0034$ ) further confirms a role for Oct-1. Collectively, these data reveal that the three Sp1, PU.1 and Oct-1 transcription factors act independently to co-regulate PrmIP-directed gene expression in the megakaryocytic HEL 92.1.7 cell line.

### Functional Characterisation of the Core PrmIP in human Endothelial EA.hy 926 cells

In order to examine whether the critical basal elements for PrmIP-directed expression identified herein in the megakaryocytic HEL 92.1.7 cell line may also be functionally important in vascular endothelial cells, it was also sought to characterise PrmIP-directed transcriptional activation in the EA.hy 926 cell line<sup>45</sup>. Human EA.hy 926 cells are a hybrid vascular endothelial cell line derived through fusion of primary human endothelial cells with the continuous human A549 cell line and are reported to represent an ideal model endothelial cell line<sup>46</sup>.

Recombinant pGL3B plasmids encoding the full-length PrmIP and proximal core PrmIP6 promoters directed  $12.9 \pm 0.42$  RLU and  $24.1 \pm 0.52$  RLU, respectively, in EA.hy 926 cells while the empty pGL3B vector directed minimal luciferase activity ( $0.45 \pm 0.03$  RLU) and the pGL3Control vector directed  $33.2 \pm 1.4$  RLU (**Figure 6; Panel A**). Moreover, consistent with findings in HEL cells, 5' deletion of nucleotides -1022 to -895 to generate PrmIP7 resulted in a 17-fold loss ( $p < 0.0001$ ) in luciferase activity while 3' deletion of nucleotides -1022 to -774, to generate 3' $\Delta$ PrmIP, led to the near complete abolishment of reporter gene expression ( $p < 0.0001$ ; **Figure 6; Panel A**). These data establish that sequences -1022 to -895 within the core PrmIP region are essential for efficient basal transcription in both the megakaryocytic HEL and endothelial EA.hy 926 cell lines.

Herein, as stated, it has been established that Sp1, PU.1 and Oct-1 *cis*-acting elements located within PrmIP6 appear to be critical for the basal PrmIP-directed gene expression in HEL cells. Hence, to investigate whether these evolutionary conserved elements may play a role in basal transcription of the IP in

EA.hy 926 cells, Sp1, PU.1 and Oct-1 single and combination mutant-directed luciferase expression was also examined. Disruption of each of the Sp1, PU.1 and Oct-1 elements significantly affected reporter gene expression yielding 3-fold ( $p < 0.0001$ ), 3-fold ( $p < 0.0001$ ), 2.4-fold ( $p < 0.001$ ) reductions in PrmIP6-directed luciferase activity in EA.hy 926 cells, respectively (**Figure 6; Panel B**). Consistent with findings in HEL cells, mutation of the Oct-1 element alone had a lesser effect on PrmIP6-directed luciferase activity relative to disruption of either the Sp1 ( $p < 0.008$ ) or PU.1 ( $p < 0.001$ ) element disruption. Moreover, consistent with findings in HEL cells, ectopic expression of Oct-1, but not Oct-2, significantly increased PrmIP6-directed gene expression in EA.hy 936 cells (**Figure 6; Panels C & D**) and heterologous expression of Ets family members PU.1 and Fli1, but not Ets-1, led to an increase in PrmIP6-directed gene expression in endothelial cells (**Figure 6; Panels E & F**).

While the subfragment PrmIP6<sup>Sp1/PU.1\*</sup>, containing an intact Oct-1 element, retained the ability to direct luciferase expression, its level of expression was some 4.7-fold reduced relative to that directed by the wild type PrmIP6 ( $p < 0.0001$ ). Furthermore, luciferase expression directed by PrmIP6<sup>Sp1/PU.1\*</sup> relative to either singly mutated PrmIP6<sup>Sp1\*</sup> or PrmIP6<sup>PU.1\*</sup> subfragments was reduced by some 1.7-fold in both cases ( $p = 0.021$  and  $p = 0.0073$ , respectively).

Luciferase expression directed by the PrmIP6<sup>Sp1/Oct-1\*</sup> and PrmIP6<sup>PU.1/Oct-1\*</sup> subfragments containing intact PU.1 and Sp1 elements was reduced in each case by 4-fold ( $p < 0.0008$ ) and 3.2-fold ( $p < 0.0003$ ), respectively, relative to wild type PrmIP6 (**Figure 6B**). The level of luciferase expression directed by either the PrmIP6<sup>Sp1/Oct-1\*</sup> or PrmIP6<sup>PU.1/Oct-1\*</sup> subfragments was not significantly different than that directed by PrmIP6<sup>Sp1\*</sup> ( $p = 0.1167$ ) or PrmIP6<sup>PU.1\*</sup> ( $p = 0.2773$ ), respectively. However, expression by both PrmIP6<sup>Sp1/Oct-1\*</sup> and PrmIP6<sup>PU.1/Oct-1\*</sup> subfragments were significantly reduced relative to that of PrmIP6<sup>Oct-1\*</sup> ( $p = 0.0009$  and  $p = 0.0148$ , respectively). These data confirm that Sp1, PU.1 and Oct-1 independently regulate PrmIP and, consistent with the findings in HEL cells, provide further evidence that Sp1 and PU.1 impart a more significant role than that of Oct-1.

Furthermore, disruption of all three cis-acting elements almost completely abolished PrmIP6<sup>Sp1/PU.1/Oct-1\*</sup>-directed transcriptional activity yielding a 7-fold reduction in luciferase activity relative to the wild type PrmIP6 ( $p = 0.0001$ ). Moreover, transcriptional activity directed by PrmIP6<sup>Sp1/PU.1/Oct-1\*</sup> was reduced relative to that of PrmIP6<sup>Sp1/PU.1\*</sup> (1.5-fold;  $p = 0.0246$ ) further confirming a role for Oct-1 in endothelial cells.

To investigate whether endogenous Sp1, PU.1 and Oct-1 actually bind to the IP promoter in EA.hy 926 cells *in vivo*, ChIP analysis was performed using specific *anti*-Sp1, *anti*-PU.1 and *anti*-Oct-1 antibodies and normal rabbit IgG as a background control. Immunoprecipitation of the cross-linked chromatin fragments from EA.hy 926 cells followed by semi-quantitative PCR using specific primers for the -1271 to -744 region of PrmIP surrounding the putative Sp1 (-967), PU.1 (-954) and Oct-1 (-949) elements generated amplicons from each of the *anti*-Sp1, *anti*-PU.1 and *anti*-Oct-1 immunoprecipitates, but not from the IgG control (**Figure 6G; Upper panel**). In order to confirm the specificity of PU.1 binding within PrmIP6 in EA.hy 926 cells, ChIP analysis was also performed using antibodies directed to Fli-1 and Ets-1. While PCR amplicons were generated from the input chromatin, no products were generated from the *anti*-Fli-1, *anti*-

Ets-1 or the control IgG immunoprecipitates (**Figure 6G; Upper panel**). Additionally, PCR analysis using primers for a non-specific region of PrmIP (-1901 to -1555) generated an amplicon in the case of input chromatin only but not from the *anti-Sp1*, *anti-PU.1*, *anti-Fli1*, *anti-Ets-1* or *anti-Oct-1* immunoprecipitated chromatin (**Figure 6G; Lower panel**). These data confirm Sp1, Pu.1 and Oct-1, but not Fli-1 and Ets-1, can specifically bind to the PrmIP promoter region, between -1271 to -774, of chromatin in EA.hy 926 cells, consistent with the findings in HEL cells. Collectively, these data suggest that Sp1, PU.1 and Oct-1 co-regulate PrmIP-directed gene expression in EA.hy 926 cells but that while ectopic expression of Fli1 can mediate an increase in PrmIP-directed reporter gene expression, it does not bind to chromatin *in vivo*.

#### **Effect of Sp1, PU.1 and Oct-1 on PrmIP-directed Luciferase Activity and hIP mRNA expression.**

Thereafter, it was also sought to establish whether ectopic expression of Sp1, PU.1 or Oct-1 could induce changes in PrmIP-directed gene expression and in the levels of endogenous IP mRNA expression in either HEL 92.1.7 or endothelial EA.hy 926 cell lines. Initially, western blot analysis confirmed over-expression of Sp1, PU.1 and Oct-1 in transfected HEL and EA.hy 926 cells (**Figure 7: Panel A-C, insets**). Expression of Sp1 significantly increased PrmIP6-directed luciferase activity in HEL and EA.hy 926 cells ( $p = 0.0016$  and  $p = 0.0006$ , respectively). Similarly, over-expression of PU.1 and Oct-1 also led to significant increases in PrmIP6-directed expression in both HEL and EA.hy 926 cell lines ( $p = 0.0044$ ,  $p = 0.0001$ ,  $p < 0.0001$  and  $p = 0.0013$ , respectively; **Figure 7 A-C**). Moreover, RT-PCR analysis confirmed that heterologous over-expression of Sp1, PU.1 and Oct-1 each led to significant increases in hIP mRNA expression levels in both HEL and EA.hy 926 cells, without showing any effect on glyceraldehyde 3' phosphate dehydrogenase (GA3'PDH) mRNA expression levels (**Figure 7; Panels D & E**). Collectively, these data in human megakaryocytic HEL 92.1.7 and endothelial EA.hy 926 cells reveal that three Sp1, PU.1 and Oct-1 transcription factors independently co-regulate PrmIP-directed gene expression and identify them as the key *trans*-acting factors critical for its basal transcriptional regulation within the human vasculature.

## Discussion

The actions of prostacyclin generally counteract those of TXA<sub>2</sub> within the vasculature, where it serves as an important endothelium-derived *anti*-thrombotic and vasodilatory agent to regulate platelet aggregation and blood vessel tone<sup>2; 4</sup>. Alterations in the levels of prostacyclin or of its synthase or its receptor, the IP, have been associated to a range of vascular diseases including thrombosis, unstable angina, systemic and pulmonary hypertension<sup>1; 34</sup>. As a major product of COX-2, prostacyclin is also an essential pro-inflammatory mediator and, from studies initially generated in IP<sup>-/-</sup> null mice, is now widely regarded as a critical autocoid involved in the neurotransmission of pain<sup>34</sup>. However, despite the recognized role of prostacyclin in haemostasis, inflammation and nociception, the factors regulating expression of its target receptor, namely the IP, remains almost completely unexplored and unknown. The aim of the current study was to characterize the human IP promoter with the objective of identifying the essential *cis*-acting elements and *trans*-acting factors that determine its basal expression within the vasculature. To this end, HEL 92.1.7 and endothelial EA.hy 926 cells were employed as model platelet -progenitor megakaryocytic and vascular endothelial cells, respectively.

The human IP promoter (PrmIP) is defined herein as the 1.5 kb – 0.1 kb region 5' and 3' of the major transcription initiation (TI) site, identified at -875, and lacks a conventional TATA-box and initiator (Inr) element<sup>35</sup>. Successive 5' deletion of PrmIP (-2427 to -774, relative to the translational initiation codon at +1) localized the core promoter to a 248 bp region (-1022 to -774) surrounding the major TI at -875<sup>35</sup> while an upstream repressor sequence (URS) was also identified between -1502 and -1271 within PrmIP. Upstream repressor and activator sequences are gene-specific sequences that can fine-tune the rate of transcription initiation contributing to the regulation of gene expression<sup>47</sup>. While the identity of the *trans*-acting factor(s) that bind and modulate the URS uncovered herein within PrmIP is currently unknown, being the subject of on-going investigations, it is possible that they may play a role in regulating its expression in response to various stimuli and/or in a cell- or tissue- specific manner.

As stated, both 5' and 3'-gene deletions localized the core promoter to the PrmIP6 subfragment (-1022 to -774) and hence it contains the essential core elements required for basal transcription in both HEL and EA.hy 926 cell lines. In TATA-less promoters, assembly of the pre-initiation complex relies on the binding of multiple general transcription factors in proximity to the TI site<sup>48</sup>. Sp1 is a ubiquitously expressed transcription factor and can serve to attract key protein components of the basal transcriptional machinery to a promoter in the absence of a TATA-box<sup>49</sup>. The identification of direct interactions between Sp1, TATA-box binding protein (TBP) and TBP-associated factors (TAFs) led to the suggested role for 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<sup>50; 51; 52</sup>. Herein, bioinformatic analysis revealed the presence of an evolutionary conserved GC-rich box (5'-ggagGGCGgggc-3'), predicted to be a putative Sp1 binding element, localized some 90 bp 5' of the major TI within the PrmIP. Disruption of the Sp1 element significantly reduced PrmIP6-directed expression in both HEL and EA.hy 926 cell lines. Furthermore, EMSA and supershift assays confirmed the formation of a specific Sp1/DNA complex *in vitro* in HEL cells. ChIP analysis, using fragmented chromatin from HEL cells and

antibodies directed to endogenous Sp1, was consistent with the formation of a direct Sp1/DNA interaction within the basal regulatory region of PrmIP *in vivo*. Furthermore, ectopic over-expression of Sp1 led to a significant increase in PrmIP6-directed gene expression and hIP mRNA transcript levels. Collectively, these data demonstrate that Sp1 serves as a *trans*-acting factor critical in the basal regulation of human IP and, from studies in HEL cells, it occurs through a mechanism involving direct DNA/protein interactions. It is possible, or indeed likely, that in the absence of a TATA-element, Sp1 is acting as an initiator within the PrmIP during the formation of the basal pre-initiation complex.

In addition to the Sp1 element, a putative PU.1/Ets binding element was also identified within PrmIP6. The Ets family of transcription factors are known to play roles in a variety of cellular processes such as differentiation, apoptosis and development<sup>44</sup> and are characterised by an evolutionary-conserved DNA-binding domain that binds to a purine-rich GGAA/T core sequence in cooperation with other transcriptional factors and co-factors<sup>43</sup>. Fli1 is a member of the Ets family and was originally isolated from the proviral integration site of friend murine leukaemia virus-induced mouse erythroleukemia cells and it is preferentially expressed in the hematopoietic cell lineages and vascular endothelial cells<sup>43</sup>. Fli1 has been shown to be involved in the expression of various megakaryocytic genes such as GPVI<sup>53</sup>, GPIX and GPIIb/alpha<sup>54</sup>. The PU.1 member of the Ets family<sup>44</sup> is a product of the Spi-1 proto-oncogene first identified as the integration site of the friend murine erythroleukemia virus<sup>55</sup>. PU.1 acts as a master regulator of myeloid and B cell development<sup>56</sup> and is critical for hematopoietic development particularly during early differentiation of myeloid, erythroid, and B lineage cells<sup>57; 58</sup>. PU.1 has also been shown to regulate certain TATA-less promoters such as *c-fes*<sup>59</sup> and M-CSF receptor<sup>60</sup>. Given the fact that PU.1 binds to TBP *in vitro*, it is also thought to recruit TFIIID followed by other components of the basal transcriptional machinery<sup>61</sup>. Herein, significant reductions in PrmIP6-directed gene expression were observed upon disruption of the PU.1 element while heterologous over-expression of PU.1 and Fli1, but not Ets-1, yielded significant increases in PrmIP6-directed gene expression in both HEL and EA.hy 926 cell lines. EMSAs established that PU.1 was capable of binding to the PU.1 element in PrmIP6 in HEL cells whereby a specific consensus PU.1 competitor efficiently competed out DNA/protein complex 2, while antibody supershifts established that both PU.1 and, to a lesser extent, Fli1 but not Ets-1 generated retarded immune complexes. Superseding these findings, the specificity of PU.1 binding was also confirmed *in vivo* in HEL and EA.hy 926 cells whereby antibodies directed to endogenous PU.1, but not endogenous to Fli1 or Ets-1, led to immunoprecipitation of chromatin spanning the proximal region of the PrmIP. RT-PCR analysis further confirmed the role of PU.1 in hIP regulation whereby ectopic over-expression resulted in an increase in hIP gene mRNA transcripts in both HEL and EA.hy 926 cells. Collectively, these data indicate an important and specific role for the PU.1 member of the Ets family in regulating basal expression of the hIP in megakaryocytic and endothelial cell lines. While over-expression of Fli1 and antibody supershift assays suggested that it is capable of binding to the PU.1 element within the PrmIP, ChIP data established that it does not bind to either HEL or EA.hy 926 cell chromatin *in vivo*. It is notable that where Fli1 has been shown to regulate megakaryocyte-specific genes, such as in the case of the thrombopoietin, GPIX and GPIIb/alpha, their promoters each contain multiple binding sites for GATA-1, a well characterized zinc finger

transcription factor important for both erythroid and megakaryocytic differentiation<sup>54; 62</sup>. In those cases, physical interactions between Fli1 and GATA-1 mediate synergistic expression<sup>54; 62</sup>. Bioinformatic analysis of the PrmIP sequence from both humans and a host of other species suggests that it does not contain a GATA-1 binding element, possibly accounting for why Fli1 does not bind to the PU box *in vivo*, as assessed by ChIP analysis, even though it has the capacity to recognise it *in vitro*, as assessed by the antibody supershift assays.

Ets family members are often, if not always, found as subunits of multiprotein transcription complexes which are thought to be involved in the fine regulation of cellular promoter/enhancer sequences<sup>63</sup>. 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<sup>64</sup>. Within the PrmIP, the Sp1 and PU.1 elements lie in close proximity (within ~ 20 bp of each other) and therefore it is possible that they interact to co-regulate human IP expression. Consistent with this, simultaneous disruption of both the Sp1 and PU.1 elements generated a promoter PrmIP<sup>Sp1,PU.1\*</sup> with a substantially impaired ability to initiate transcription. Ets family and Sp1 transcription factors are known to co-transactivate several promoters, including the human T cell lymphotropic virus type 1 (HTLV-1) promoter and the immune coagulant fgl2/fibroleukin promoter<sup>65; 66</sup>. In the context of megakaryocytes, somewhat similar to the PrmIP, the TATA-less integrin  $\alpha$ IIb promoter is co-regulated by Sp1 and Ets binding to elements that lie proximal to its TI site and it is proposed that such regulation by Sp1 and Ets serves as a model for megakaryocyte-specific gene expression<sup>67</sup>. Whilst the finding that Sp1 and PU.1 are critical for the regulation of PrmIP within HEL cells is indeed consistent with the latter, we found a similar co-dependence within the endothelial EA.hy 926 cells suggesting that they also act outside of myeloid lineages to regulate IP expression. Moreover, it is also indeed worth noting that in the context of inflammation, PU.1 plays an essential role in the transcriptional upregulation of COX-2 in response to lipopolysaccharide in macrophages<sup>68</sup>. Hence, it is indeed plausible that PU.1 may have a role in the co-ordinate upregulation of prostacyclin synthesis, through COX-2, and signalling, through the IP, such as in response to bacterial sepsis.

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<sup>69</sup>. Two POU domain proteins, the ubiquitously expressed Oct-1 and the lymphoid-specific Oct-2, have been shown to be involved in haematopoietic differentiation<sup>70</sup>. Herein, disruption of the putative octamer element significantly reduced PrmIP6-directed reporter gene expression in HEL and EA.hy 926 cells. Moreover, ectopic expression of Oct-1 and Oct-2 showed preferential upregulation of PrmIP6-directed gene expression by Oct-1 in HEL cells, as opposed to Oct-2. Consistent with these findings, over-expression of Oct-1 also led to a significant increase in PrmIP6-directed gene expression in EA.hy 926 cells. Specific Oct-1 binding was also demonstrated by EMSAs whereby a specific Oct-1 oligonucleotide efficiently competed out two (C1 & C3) of the C1- C3 complexes generated using the over-lapping Pu.1/Oct-1 probe. The identity of the slower migrating C1 complex is unknown but it is possible that it represents a tertiary PU.1/Oct-1/DNA complex. In the presence of the *anti*-Oct-1 antibody, a supershifted complex was generated confirming direct Oct-1/DNA binding *in vitro*. However, superseding the EMSA and supershift assays, ChIP analysis using

specific antibody directed to endogenous Oct-1 confirmed that it specifically binds *in vivo* to the chromatin encoding the basal regulatory region of the human IP promoter in HEL cells. RT-PCR analysis further confirmed an important regulatory role for Oct-1 in both HEL and EA.hy 926 cells whereby ectopic over-expression of Oct-1 yielded an increase in hIP mRNA transcript levels. Hence, these data reveal an important role for the Oct-1 in PrmIP-directed transcriptional activity in megakaryocytic HEL and endothelial EA.hy 926 cells.

Consistent with this hypothesis, cooperative interactions between Oct-1 with either Sp1 or PU.2 have been reported on other RNA polymerase II –regulated promoters. Chen et al., (1996) 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 <sup>71</sup>. Moreover, Kistler et al., (1995) proposed a feed back mechanism at the PU.1 promoter whereby PU.1 itself binds to a putative Ets binding site cooperatively trans-activating transcription with Sp1 and Oct-1 <sup>72</sup>. A similar model has been proposed for transcription of the *fgl2* gene in endothelial cells whereby Sp1 initially binds, facilitating recruitment of further positive trans-regulators such as Ets-1 and Oct-1, forming a multi-protein complex that forms on a positive regulatory domain (PRD) proximal to the transcription initiation site <sup>73</sup>. Mutation of all three Sp1, PU.1 and Oct-1 *cis*-acting elements resulted in 6-fold and 7-fold ( $p = 0.1462$ ; ANOVA) reductions in PrmIP6-directed gene expression in HEL and EA.hy 926 cells, respectively. Collectively, these data suggest that cooperative binding of all three trans-acting factors Sp1, PU.1 and Oct1 is necessary to direct maximal basal expression from the PrmIP.

In conclusion, we have identified an URS (-1502 to -1271) and a proximal essential ‘core’ PrmIP region (-1022 to -895) within the human IP promoter. While the *trans*-acting factors regulating the URS are subject to ongoing investigations, a detailed characterisation of the proximal PrmIP core region revealed three functional Sp1, PU.1 and Oct-1 elements involved in co-regulating basal PrmIP activity in HEL and endothelial cells. Herein, we propose a model for basal transcription at the human IP promoter (**Figure 8; Panel A**). In the absence of a TATA-box, it is proposed that Sp1 initiates the formation of the pre-initiation complex, thereby allowing the recruitment of other specific, including PU.1 and Oct-1, and more general TBP, TAFs factors along with RNA polymerase II to form a multicomponent positive regulatory domain (PRD) that is sufficient to confer basal expression of the IP. Bioinformatic analysis confirmed the absolute evolutionary conservation of the Sp1, PU.1 and Oct-1 within the proximal IP promoters from a range of species including the horse, bovine, dog (**Figure 8; Panel B**), rat and mice (data not shown). 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 megakaryocytic HEL 92.1.7 cells, but also in the endothelial EA.hy 926 cell lines, suggests that they provide a general, rather than a cell- or species-specific, model for transcriptional regulation of the IP. It is likely that other upstream regulatory regions, such as the URS and other *cis*-acting elements, are responsible for the tissue-specific or altered expression of the IP such as in response to inflammation, pain or vascular disease. This study provides an important molecular and genetic platform that should provide a basis for

understanding how the many and diverse responses to prostacyclin are regulated at the level of transcription of the IP gene itself.

## Experimental Procedures

### Materials

pGL3Basic, pRL-Thymidine Kinase (pRL-TK) and Dual Luciferase® Reporter Assay System were obtained from Promega Corporation. DMRIE-C® was from Invitrogen Life Technologies. *Anti-Oct-1* (sc-232x), *anti-Sp1* (sc-59x), *anti-PU.1* (sc-22805x), *anti Fli1* (sc-236x), *anti-Ets.1* (sc-350x) and normal rabbit IgG (sc-2027) were obtained from Santa Cruz Biotechnology.

### Generation of Luciferase-based Genetic Reporter Plasmids

The human prostacyclin receptor (IP) promoter (PrmIP), defined as nucleotides -2427 to -774 located 5' of the translational ATG initiation codon, designated +1, was subcloned into the *KpnI* -*HindIII* sites of pGL3Basic to generate pGL3B:PrmIP. To identify sequence elements required for PrmIP activity, a series of 5'- and 3'-deletion subfragments were subcloned into pGL3Basic (pGL3b). Specifically, for all 5' deletions, PCR fragments were generated using the antisense primer Kin274 (5'-dCTCTCAAGCTTCTCTCCAGTCTTGCCCAGGCTC-3', complementary to nucleotides - 807 to -774 where the underlined sequence corresponds to the *HindIII* cloning site) in combination with specific sense primers designed to amplify progressively shorter regions of PrmIP. The list below identifies the recombinant plasmids encoding 5' deletion fragments of PrmIP generated in pGL3Basic, as well as the identity, sequence and corresponding nucleotides (Nu) of the specific sense primer used for each fragment. In each case, the - designation indicates nucleotides 5' of the translational start site (designated +1) and underlined sequences represent the *KpnI* cloning site.

- (1) pGL3B:PrmIP1 (primer Kin534; 5'-dGAGAGGTACCCAGCGGTGGTGGCTTGGCTGTG-3', Nu -1761 to -1739).
- (2) pGL3B:PrmIP2 (primer Kin535; 5'-dGAGAGGTACCGATCAAAATATGATTCCTGAAGG-3', Nu -1682 to -1662).
- (3) pGL3B:PrmIP3 (primer Kin536; 5'-dGAGAGGTACCGCCATGGAAGTCTCCCAAGC-3', Nu -1575 to -1552).
- (4) pGL3B:PrmIP4 (primer Kin537; 5'-dGAGAGGTACCCTCTGCAGGGTGAGCTGG-3', Nu -1502 to -1482).
- (5) pGL3B:PrmIP5 (primer Kin538; 5'-dGAGAGGTACCACCCTGAGACAGCCCAGG-3', Nu -1271 to -1249).
- (6) pGL3B:PrmIP6 (primer Kin539; 5'-dGAGAGGTACCGATGGATGTCCTGGATTG-3', Nu -1022 to -1002).
- (7) pGL3B:PrmIP7 (primer Kin540; 5'-dGAGAGGTACCGAGGAGGGTGGGAAGGAG-3', Nu -895 to -873).

For the 3' deletion, a PCR fragment was generated using pGL3B:PrmIP as template and primers Kin296 (5'-dGAGAGACGCGTAGCTACTCGGGAGGCTGAGGCAC -3'; Nu -2812 to -2778)

and Kin532 (5'-dGAGAAAGCTTCAATCCAGGACATCCATCTC -3'; Nu -1022 to -992) and was subcloned into the *MluI-HindIII* sites of pGL3Basic to generate pGL3B:3'ΔPrmIP. The identity and fidelity of all recombinant plasmids was verified by DNA sequence analysis.

### Site-directed Mutagenesis

Site-directed mutagenesis (SDM) was carried out using the Quik-Change™ method (Stratagene). The identities of the PrmIP elements subjected to SDM, with their starting positions in brackets, the nucleotides that were changed in underlined bold, templates used and names of the corresponding plasmids generated, as well as the identity, sequence and corresponding nucleotides of the specific primers used are listed below.

1. Sp1 (-967), from gGGCGg to gGATGg using template pGL3B:PrmIP6 to generate pGL3B:PrmIP6<sup>Sp1\*</sup>. Primers Kin595 (5'-dGGAGAACAGTGAGGGAGGATGGGGCAGAGAGAGG-3') and complementary Kin596.
2. PU.1 (-954), from aGGAAa to aCGAAa using template pGL3B:PrmIP6 to generate pGL3B:PrmIP6<sup>PU.1\*</sup>. Primers Kin574 (5'-dCGGGGCAGAGAGAGCGAAATGAAAAAGCTG-3') and complementary Kin 574.
3. Oct1 (-949), from aAATGa to aCTGa using template pGL3B:PrmIP6 to generate pGL3B:PrmIP6<sup>Oct1\*</sup>. Primers Kin576 (5'-dGCAGAGAGAGGACTGAAAAAGCTGGGGTG-3') and complementary Kin577.
4. Sp1/Oct1 (-967/-949), from aAATGa to aCTGa using pGL3B:PrmIP6<sup>Sp1\*</sup> as template to generate pGL3B:PrmIP6<sup>Sp1/Oct1\*</sup>. Primers Kin661 (5'-dGATGGGGCAGAGAGAGGACTGAAAAAGCTGGGGTGAGC-3') and complementary Kin662.
5. Sp1/PU.1 (-967/-954), from gGGCGg to gGATGg using template pGL3B:PrmIP6<sup>PU.1\*</sup> to generate pGL3B:PrmIP6<sup>Sp1/PU.1\*</sup>. Primers Kin572 (5'-dCAGTGAGGGAGGATGGGGCAGAGAGAG -3') and complementary Kin573.
6. PU.1/Oct1 (-954/-949), from aGGAAATGa to aCGAACTGa using pGL3B:PrmIP6 as template to generate pGL3B:PrmIP6<sup>PU.1/Oct1\*</sup>. Primers Kin590 (5'-dCGGGGCAGAGAGAGCGAACTGAAAAAGCTGG-3') and complementary Kin591.
7. Sp1/PU.1/Oct1 (-967/-954/-949), from aGGAAATGa to aCGAACTGa using template pGL3B:PrmIP6<sup>Sp1\*</sup> to generate pGL3B:PrmIP6<sup>Sp1/PU.1/Oct1\*</sup>. Primers Kin590 (5'-dCGGGGCAGAGAGAGACGAACTGAAAAAGCTGG-3') and complementary Kin591.

The fidelity of all plasmids was confirmed by DNA sequence analysis.

### Cell Culture

Human erythroleukemic (HEL) 92.1.7 cells, obtained from the American Type Culture Collection, were cultured in RPMI 1640, 10 % fetal bovine serum. Human endothelium cells EA.hy 926 cells <sup>45</sup> were obtained from the Tissue Culture Facility at UNC Lineberger Comprehensive Cancer Centre, Chapel Hill,

NC and were cultured in DMEM, 10 % fetal bovine serum. All mammalian cells were grown at 37 °C in a humid environment with 5 % CO<sub>2</sub> and were confirmed to be free of mycoplasma contamination.

### **Assay of Luciferase Activity**

HEL 92.1.7 were co-transfected with various pGL3Basic-recombinant plasmids, encoding firefly luciferase, along with pRL-TK, encoding renilla luciferase, using DMRIE-C® transfection reagent as per as previously described<sup>74</sup>. In the case of the EA.hy 926 cells, in brief, 24 hr 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) 5 µl DMRIE-C® reagent. Firefly and renilla luciferase activity was assayed 48 hr later using the Dual Luciferase Assay System®. To investigate the effect of over-expression of Sp1, PU.1, Oct-1 or Oct-2 on PrmIP6-directed luciferase expression, pCMVSPORT6-Sp1 (ImaGenes), pCMVSPORT6-PU.1 (Geneservice LTD), pSG5-Fli1 and pSG5-Ets-1 (Kind gift from Dr Dennis Watson, Medical University of South Carolina, USA), pcDNA3:HaOct-1 or pcDNA3:HaOct-2<sup>75</sup>, encoding hemagglutinin (HA)-epitope tagged Oct-1 and Oct-2, respectively, were transiently transfected into HEL cells along with recombinant pGL3B:PrmIP6. Briefly, pGL3B:PrmIP6 (1.5 µg per well) plus pRL-TK (200 ng per well) plasmids were transiently co-transfected along with either pCMVSPORT6-Sp1, pCMVSPORT6-PU.1, pSG5-Fli1, pSG5-Ets-1, pcDNA3:HaOct-1, pcDNA3:HaOct-2 or, as negative controls with pcDNA3 (0.5 µg per well) using DMRIE-C® as per the manufacturer's instructions. Firefly and renilla luciferase activity was assayed for 48 hr later 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).

### **Western Blot Analysis**

The expression of Sp1, PU.1 and Oct-1 proteins in HEL 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*-Sp1, *anti*-PU.1, *anti*-Fli1, *anti*-Ets-1 and *anti*-Oct-1 sera in 5 % non fat dried milk in 1 x TBS (0.01 M Tris-HCl, 0.1 M NaCl) for 2 hr at room temperature followed by washing and screening using goat *anti*-rabbit horseradish peroxidase (sc-2204) followed by chemiluminescence detection. HEL cells transiently co-transfected with pcDNA3, pcDNA3:HaOct-1 and pcDNA3:HaOct-2 were analysed by western blotting (60 µg whole cell protein per lane) using *anti*-HA 3F10 antibody; to confirm uniform protein loading, the blots were stripped and rescreened with *anti*-HDJ-2 antibody (Neomarkers) to detect endogenous HDJ-2 protein expression. HEL and EA.hy926 cells transiently co-transfected with pcDNA3, pcDNA3:HaOct-1, pCMVSPORT6-Sp1, pCMVSPORT6-PU.1, pSG5-Fli1 or pSG5-Ets-1 were analysed by western blotting (60 µg whole cell protein per lane) using *anti*-Oct-1, *anti*-Sp1, *anti*-PU.1, *anti*-Fli1 and *anti*-Ets-1 antibodies respectively.

### **Electrophoretic Mobility Shift and Supershift Assays**

Nuclear extract was prepared from HEL cells as previously described<sup>74</sup>. Oligonucleotides corresponding to the sense (5' end-labelled with biotin) and antisense strands of each probe (90 µM) were annealed by heating to 95 °C for 5 min followed by slow cooling to room temperature. Binding reactions were set up by incubating nuclear extract (2 µg total protein) with/without 300-fold molar excesses of non-labelled double-stranded competitors/non-competitors in 1 x Binding Buffer (20 mM Tris-HCl, pH 7.5., 80 mM NaCl, 1 mM EDTA, 100 ng of bovine serum albumin, 25 µg/ml poly(dI-dC), 10% glycerol, 1 mM DTT) for 45 min at room temperature. An appropriate concentration of biotin-labelled probe was then added and incubated for a further 45 min at room temperature after which protein-DNA complexes were then resolved by electrophoresis through 6% nondenaturing polyacrylamide gels. After electrophoresis, the DNA was transferred onto a Biotodyne B nylon membrane (Pall) and then visualized using the chemiluminescent-nucleic acid detection kit per the manufacturer's instructions (Pierce). The identities and sequences of the forward biotin-labelled oligonucleotide probes are listed below. Sequences of the corresponding non-labelled oligonucleotides are omitted.

1. Sp1 probe; Kin689(5'-[Btn]dGGAGAACAGTGAGGGAGGGCGGGGCAGAGAGAGG-3'; Nu - 984 to -950)
2. PU.1/Oct-1 probe; Kin693 (5'-[Btn]dGGCAGAGAGAGGAAATGAAAAAGCTGGGGTGAGC-3'; Nu -962 to -928)

The identities and sequences of the forward non-labelled competitor/non competitor oligonucleotides are listed below.

1. Sp1 probe; Kin768 (5'-dGGAGAACAGTGAGGGAGGGCGGGGCAGAGAGAGG-3'; Nu -984 to -950)
2. PU.1/Oct-1 probe; Kin770 (5'-dGGCAGAGAGAGGAAATGAAAAAGCTGGGGTGAGC-3'; Nu -962 to -928)
3. Sp1 consensus; Kin651 (5'-dATTTCGATCGGGCGGGGCGAGC-3')
4. PU.1 consensus; Kin826 (5'-dTAACCTCTGAAAGAGGAACTTGGT-3')
5. Oct-1 consensus; Kin773 (5'-dTGTCTGAATGCAAATCACTAGAA-3')
6. randomized non-specific probe; Kin335 (5'-dTGCGCCCGGCCTTCCATGCTCTTTGAC-3')

For electrophoretic mobility supershift assays, nuclear extract (2 µg total protein) was pre-incubated with 3 µg of *anti*-Sp1, *anti*-PU.1, *anti*-Fli1, *anti*-Ets-1 or *anti*-Oct-1 sera for 1 hr at room temperature prior to the addition of the relevant biotinylated Sp1 or PU.1/Oct-1 probes.

### ChIP analysis

Chromatin immunoprecipitation (ChIP) assays were performed essentially as described by Kock et al., 2007<sup>76</sup>. Briefly, HEL cells (1 x 10<sup>8</sup>) were grown in RPMI, 10 % FBS to 70 % confluency and collected by centrifugation at 2,000 g for 5 min at 4 °C, washed twice in ice-cold PBS and resuspended in 50 ml serum-free RPMI. EA.hy 926 cells (1 x 10<sup>8</sup>) were grown in DMEM, 10 % FBS to 70 % confluency and were

harvested by scraping and collected by centrifugation at 2,000 g for 5 min at 4 °C, washed twice in ice-cold PBS and resuspended in 50 ml serum-free DMEM. Formaldehyde (1 %)-cross linked chromatin was sonicated to generate fragments 500 bp to 1000 bp in length. Prior to immunoprecipitation (IP), chromatin was incubated with 60 µg normal rabbit IgG overnight at 4 °C on a rotisserie, after which 250 µl of salmon sperm DNA/protein A agarose beads (Millipore) were added and chromatin was precleared for 3 hr at 4 °C with rotation. Thereafter, *anti-Sp1*, *anti-PU.1*, *anti-Fli1*, *anti-Ets-1*, *anti-Oct1* (10 µg aliquots) or normal rabbit IgG (10 µg) were used for IP. All antibodies used for ChIP analysis were ChIP validated by the supplier (Santa Cruz) and have been widely used in the literature for such analyses<sup>77; 78; 79</sup>. Following elution of the immune complexes from the beads, cross-links were reversed by incubation at 65 °C overnight followed by protease digestion with proteinase K (Gibco-BRL #25530-031; 9 µl of 10 mg/ml) at 45 °C for 7 h. After precipitation, samples were resuspended in 50 µl dH<sub>2</sub>O. 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. The identities of the primers used for the ChIP PCR reactions, as well as their sequences and corresponding nucleotides within PrmIP are listed below.

1. Kin538, 5'-dGAGA GGTACC ACCCTGAGACAGCCCAGG-3', Nu -1271 to -1243
2. Kin274, 5'-dCTCTCAAGCTTCTCTCCAGTCTTGCCCAGGCTC -3', Nu -807 to -774
3. Kin676, 5'-dGAGAGGTACCCAGAGAGGGTCTCTG -3', Nu -1901 to -1886
4. Kin677, 5'-dCTCTAAGCTTGGAGACTTCCATGGC -3', Nu -1555 to -1540

### **Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total RNA was isolated from HEL 92.1.7 and EA.hy 926 cells using TRIzol reagent (Invitrogen Life Technologies). DNase 1-treated total RNA was converted to first strand (1<sup>o</sup>) cDNA with MMLV RT (Promega). PCR primers were designed to specifically amplify hIP mRNA sequences (5'-dGAAGGCACAGACGCACGGGA -3', Nu -57 to -37 of Exon 1; Kin264) and (5'-dGGCGAAGGCGAAGGCATCGC -3'; Nu 294 to 275 of Exon 2; Kin266) to generate a 348 bp amplicon 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<sup>o</sup> cDNA would be amplified, thereby eliminating genomic artefacts. The levels of hIP mRNA expression were determined by measurement of PCR product band intensities on densitometric scans; in each case, expression levels are represented as a ratio relative to GA3'PDH expression (i.e. hIP/GA3'PDH ± SEM, arbitrary units). Data are presented as mean changes in hIP mRNA expression in cells over-expressing either Sp1, PU.1 or Oct-1 relative to those levels in control (pcDNA3) transfected cells, set to a value of 1 (Relative Expression ± SEM, n = 3).

### **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  $\pm$  standard error of the mean (SEM). *P*-values  $\leq 0.05$  were considered to indicate statistically significant differences and \*, \*\*, \*\*\*, \*\*\*\* indicate *P*  $\leq$  0.05, 0.01, 0.001, 0.0001, respectively.

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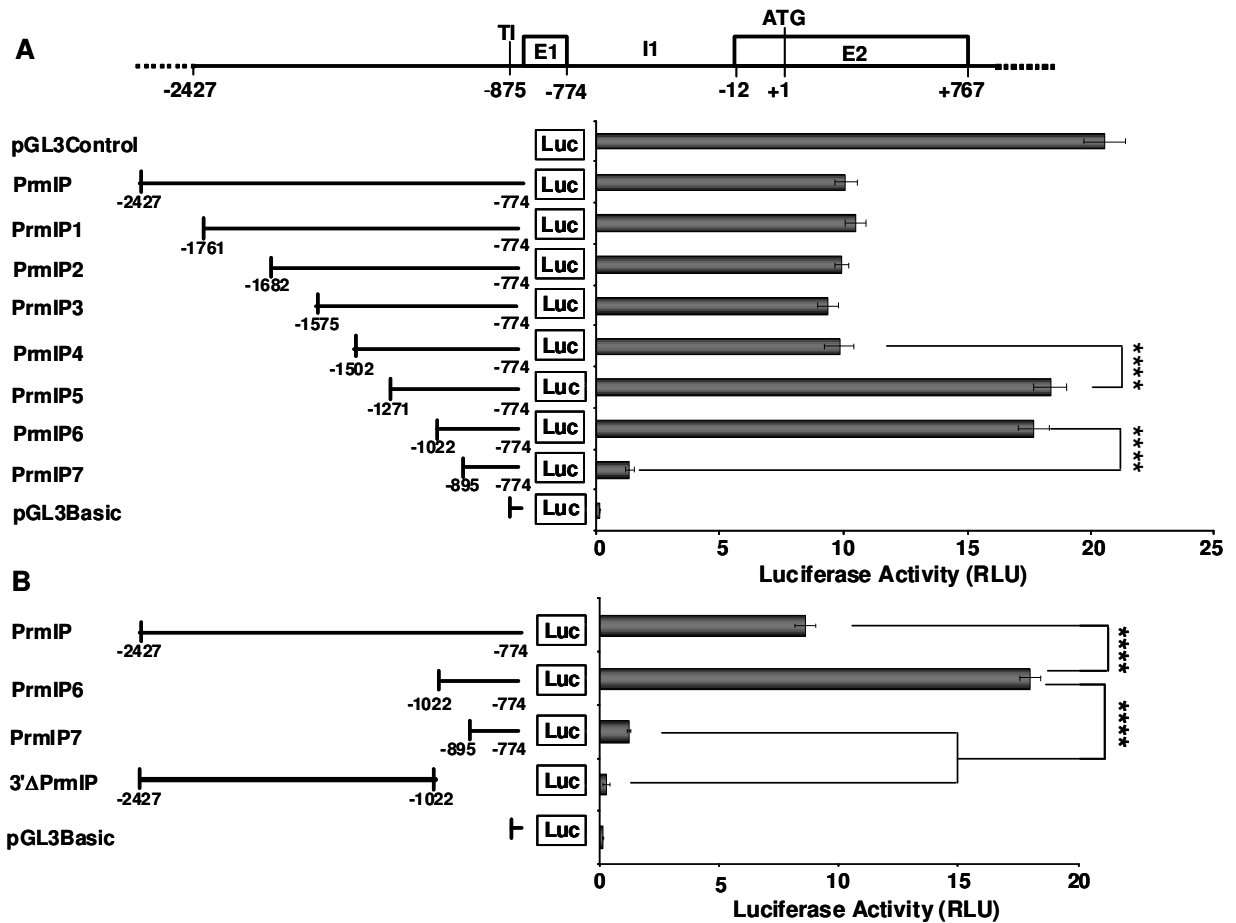
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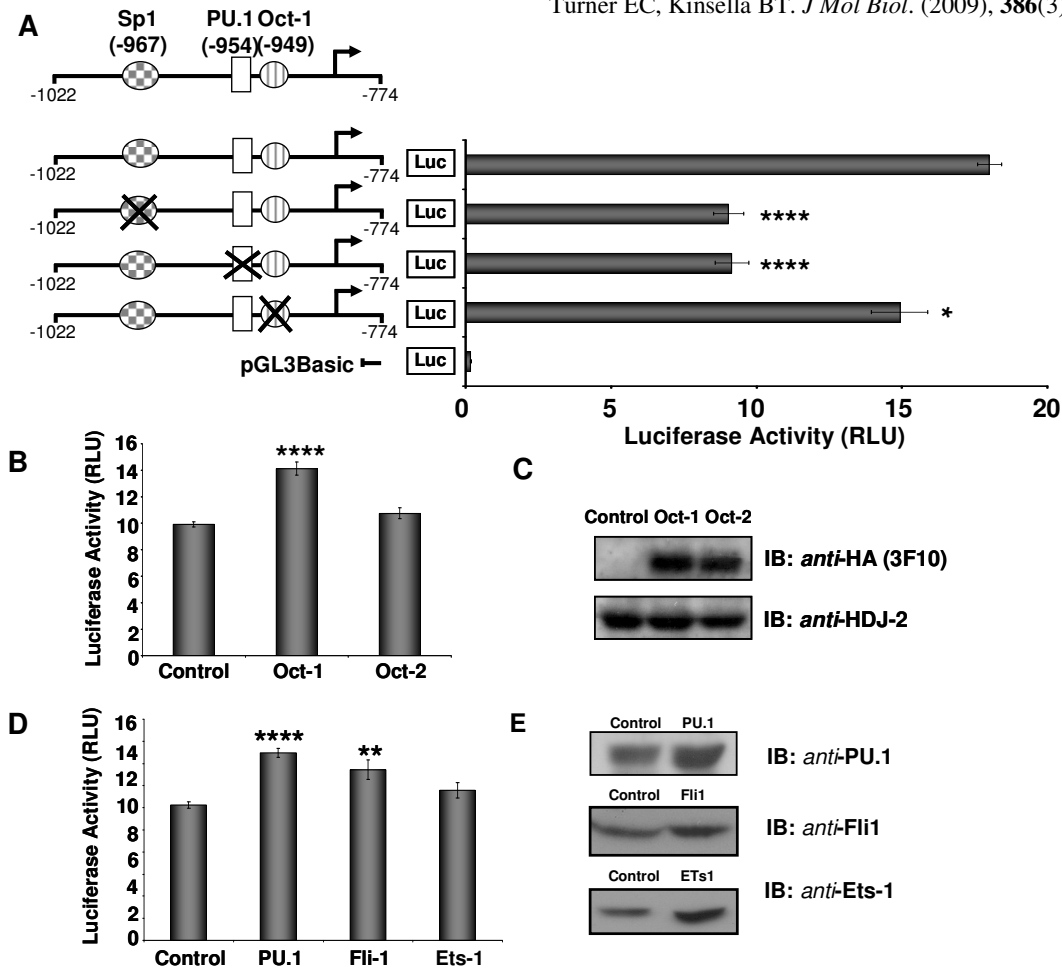
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## FIGURES

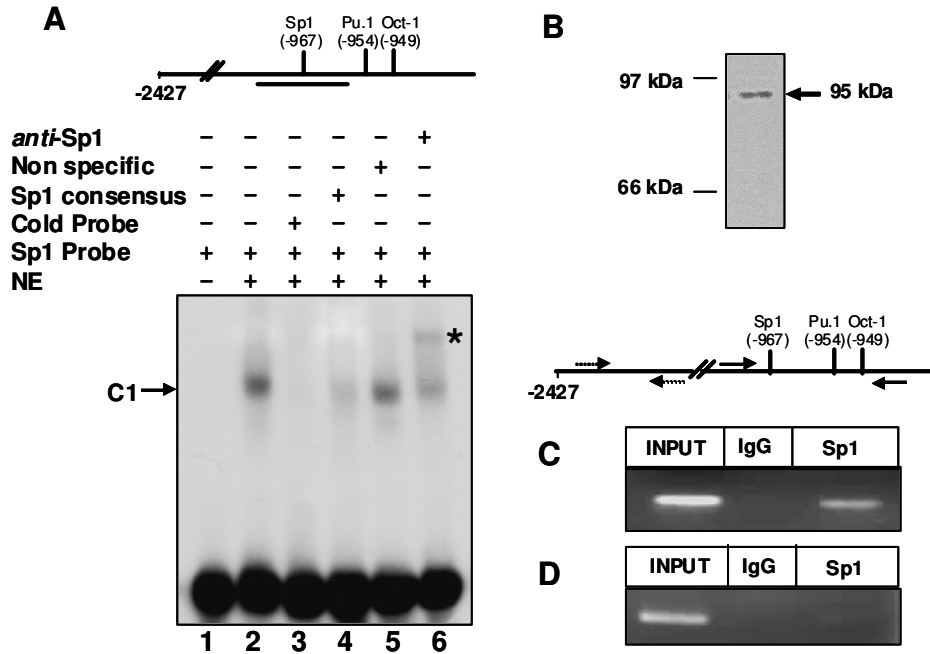


**Figure 1: Effect of 5' and 3' Deletions on PrmIP-directed Gene Expression in HEL 92.1.7 cells.**

A schematic of the human prostacyclin receptor (IP) genomic region spanning nucleotides -2427 to +767 encoding PrmIP, in addition to exon (E)1, intron (I)1 and E2 is shown. Nucleotide +1 corresponds to the translational start site (ATG) and nucleotides 5' or 3' thereof are given a - or + designation, respectively. Recombinant pGL3Basic encoding: **Panel A:** PrmIP (-2427 to -774), PrmIP1 (-1761 to -774), PrmIP2 (-1682 to -774), PrmIP3 (-1575 to -774), PrmIP4 (-1502 to -774), PrmIP5 (-1271 to -774), PrmIP6 (-1022 to -774) and PrmIP7 (-895 to -774) or **Panel B:** PrmIP (-2427 to -774), PrmIP6 (-1022 to -774), PrmIP7 (-895 to -774) and 3'ΔPrmIP (-2427 to -1022) or, as controls, pGL3Basic (panels A & B) or pGL3Control (Panel A) vectors were co-transfected with pRL-TK into HEL cells. Firefly and renilla luciferase activity was assayed 48 hr post-transfection; results are presented as mean firefly relative to renilla luciferase activity, expressed in arbitrary relative luciferase units (RLU  $\pm$  SEM; n = 6). The asterisks (\*) indicate that deletion of PrmIP sequences either significantly reduced or increased reporter gene expression in HEL cells where \*\*\*\* indicates  $p \leq 0.0001$ .

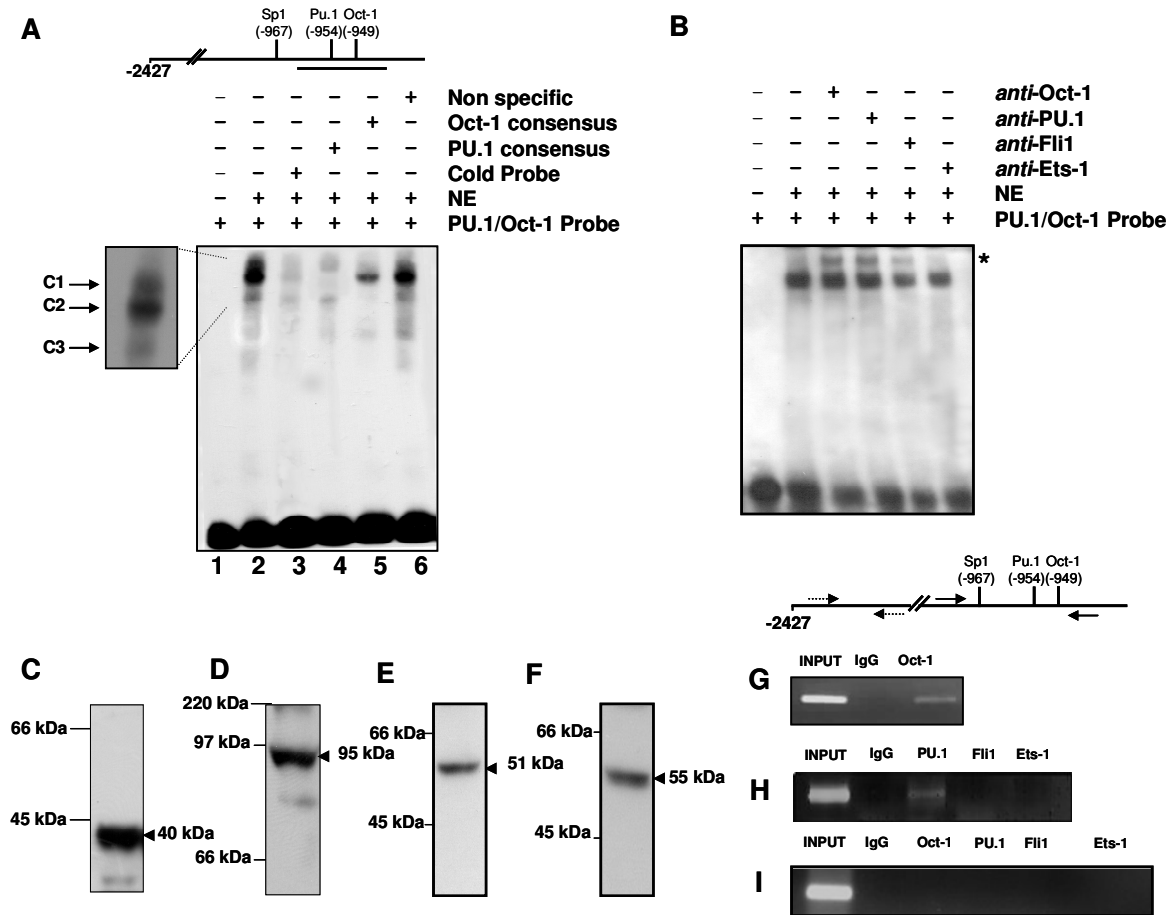


**Figure 2: Identification of Putative Sp1, PU.1 and Oct-1 Elements within the Core PrmIP.** **Panel A:** A schematic of PrmIP6 (-1022 to -774) in addition to the positions of putative Sp1, PU.1 and Oct-1 elements, where the 5' nucleotide is indicated in each case. Recombinant pGL3Basic encoding: PrmIP6, PrmIP6<sup>Sp1\*</sup>, PrmIP6<sup>PU.1\*</sup> and PrmIP6<sup>Oct1\*</sup> were co-transfected with pRL-TK into HEL cells. **Panels B & C:** HEL cells were transiently co-transfected with pGL3Basic:PrmIP6 in the presence of pRL-TK plus pcDNA3:HaOct-1 (Oct-1), pcDNA3:HaOct-2 (Oct-2) or pcDNA3 (Control). Cells were either assayed 48 hr post-transfection for mean luciferase activity (RLU  $\pm$  SEM, n = 5; **Panels A & B**) or by western blot analysis (60  $\mu$ g whole cell protein per lane; **Panel C**) using *anti-HA* 3F10 antibody (upper panel); to confirm uniform protein loading, the upper blot was stripped and rescreened with *anti-HDJ-2* antibody (lower panel). **Panels D & E:** HEL cells were transiently co-transfected with pGL3Basic:PrmIP6 in the presence of pRL-TK plus pCMVSPORT6-PU.1 (PU.1), pSG5-Fli1 (Fli1), pSG5-Ets-1 (Ets-1) or pcDNA3 (Control). Cells were either assayed 48 hr post-transfection for mean luciferase activity (RLU  $\pm$  SEM, n = 3; **Panel D**) or by western blot analysis (60  $\mu$ g whole cell protein per lane; **Panel E**) using *anti-PU.1*, *anti-Fli1* and *anti-Ets-1* antibodies. The asterisks (\*) in Panels A, B and D indicate significant changes in PrmIP6-directed luciferase expression in HEL cells, where \*, \*\* and \*\*\*\* indicate  $p \leq 0.05$ ,  $p \leq 0.01$  and  $p \leq 0.0001$ , respectively.



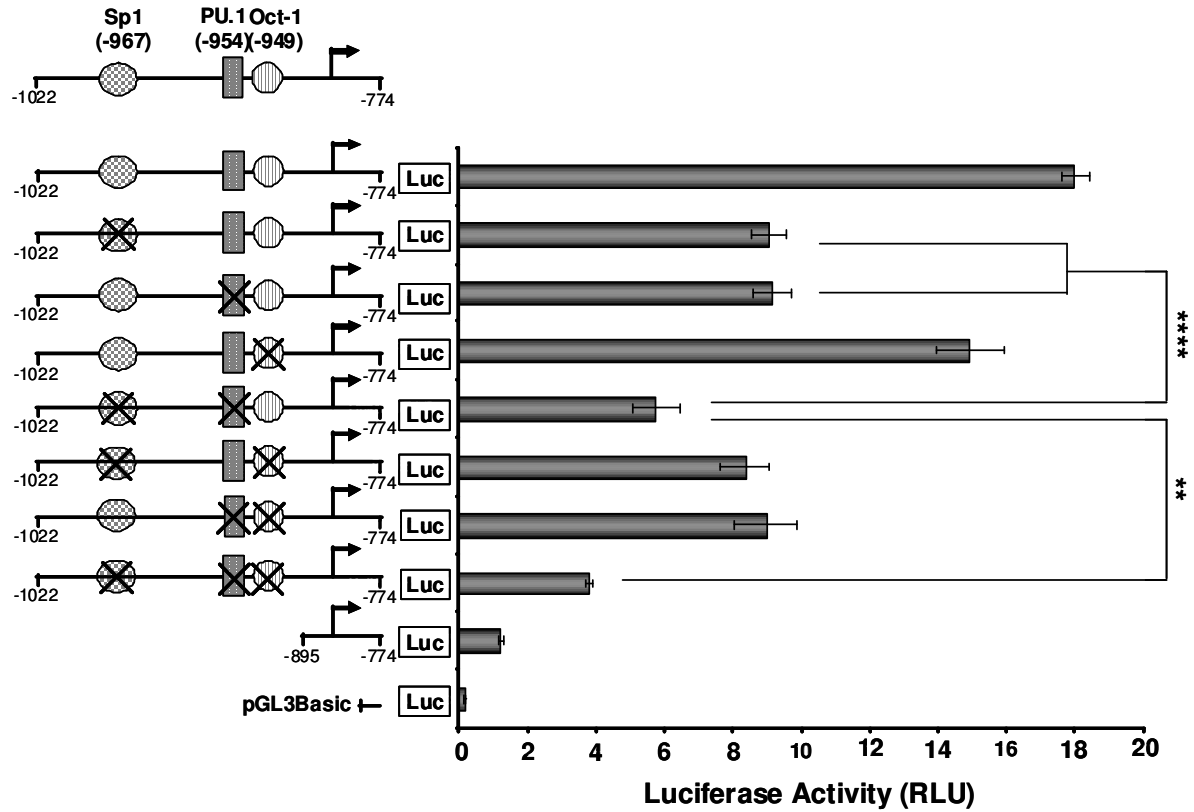
**Figure 3: EMSA and ChIP of Sp1 Binding to the Proximal PrmIP Region.**

**Panel A:** A schematic of PrmIP (-2427 to -774) in addition to the relative positions of putative Sp1, PU.1 and Oct-1 elements, where the 5' nucleotide of each element is indicated. EMSA and supershift assays were carried out using nuclear extract from HEL cells and a biotin-labelled double-stranded Sp1 probe (Sp1 probe) spanning -985 to -951 within PrmIP, as indicated by the horizontal bar. Nuclear extract was pre-incubated either with the vehicle (-), or with excesses of non-labelled Sp1 probe (+), a consensus Sp1 competitor, a randomized IP sequence competitor (non-specific competitor) or with *anti-Sp1* sera (*anti-Sp1*) prior to addition of the biotinylated Sp1 probe, as indicated. A major diffuse complex, designated C1, was observed. The star to the right indicates the supershifted transcription factor:DNA complex detected in the presence of the *anti-Sp1* serum. Images are representative of three independent experiments. DNA-protein complexes (C) are represented by the abbreviation C. **Panel B:** Western blot analysis of endogenous Sp1 expression in HEL cells (50  $\mu$ g whole cell protein per lane analysed), where the position of the molecular size markers (kDa) are indicated to the left of the panel. **Panel C & D:** Chromatin immunoprecipitation (ChIP) analysis: a schematic of the PrmIP genomic region and the forward and reverse primers used to amplify the proximal PrmIP region (-1271 to -774 as indicated by the solid arrows; used in Panel C) or, as controls, an upstream PrmIP region (-1901 to -1540 as indicated by the dashed arrows; used in Panel D) in immunoprecipitates of cross-linked chromatin fragments from HEL cells. PCR amplification was carried out using either template DNA extracted from the input chromatin (using approximately 1/20 of chromatin used in test ChIPs) or from normal rabbit IgG- or *anti-Sp1*- immunoprecipitated chromatin. Images shown are representative of three independent experiments.



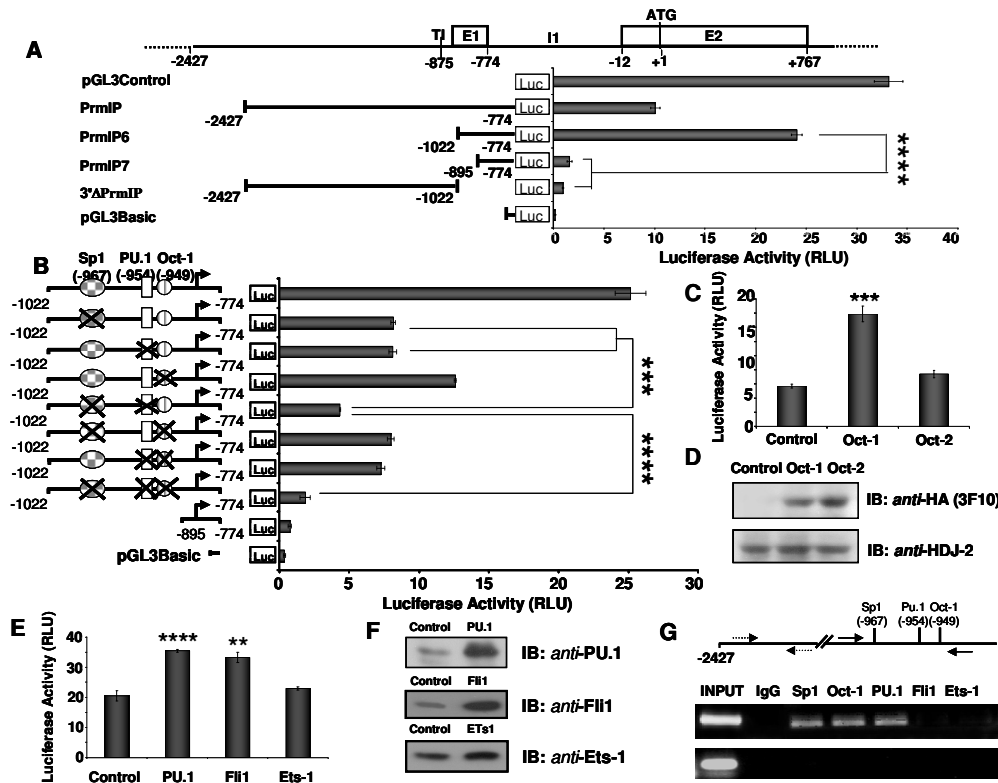
**Figure 4: Demonstration of PU.1 and Oct1 Binding to the Proximal PrmIP Region.**

EMSAs (**Panel A**) and supershift assays (**Panel B**) using nuclear extract from HEL cells and a biotin-labelled double-stranded PU.1/Oct1 probe spanning -943 to -929 of PrmIP, as indicated by the horizontal bar. **Panel A**: Nuclear extract was pre-incubated with either the vehicle (-) or with excesses (+) of non-labelled PU.1/Oct-1 probe (cold probe), a consensus PU.1 competitor, a consensus Oct-1 competitor, a randomized IP sequence competitor (non-specific competitor) prior to the addition of the biotinylated PU.1/Oct1 probe, as indicated. DNA-protein complexes (C) are represented by the abbreviation C. Three main complexes, designated C1, C2 and C3, were observed. The inset on the left shows C1-C3 complexes generated in the presence of nuclear extract only but represents a longer exposure of the same chromatogram. **Panel B**: Nuclear extract was pre-incubated with either vehicle (-), *anti*-PU.1 (+), *anti*-Fli-1 (+), *anti*-Ets-1 (+) or *anti*-Oct1 (+) sera prior to addition of the biotinylated PU.1/Oct1 probe, as indicated. The stars indicates the supershifted transcription factor:DNA complex detected in the presence of *anti*-PU.1 and *anti*-Oct-1 sera. Images are representative of three independent experiments. Western blot analysis of endogenous PU.1 (**Panel C**), Oct-1 (**Panel D**), Fli1 (**Panel E**) and Ets-1 (**Panel F**) expression in HEL cells (50  $\mu$ g whole cell protein per lane analysed), where the positions of the molecular markers (kDa) are indicated to the left of the panels. **Panel G, H & I**: ChIP analysis: a schematic of the PrmIP genomic region and the forward and reverse primers used to amplify the proximal PrmIP region (-1271 to -774 as indicated by the solid arrows; used in Panels G & H) or, as controls, an upstream PrmIP region (-1901 to -1540 as indicated by the dashed arrows; used in **Panel I**) in immunoprecipitates of cross-linked chromatin fragments from HEL cells using *anti*-Oct-1, *anti*-PU.1, *anti*-Fli1, *anti*-Ets-1 sera or normal rabbit IgG, as indicated. In each case, input represents PCR amplification of template DNA extracted from the input chromatin (using approximately 1/20 of that used in each of the test immunoprecipitates). The images shown are representative of three independent experiments.



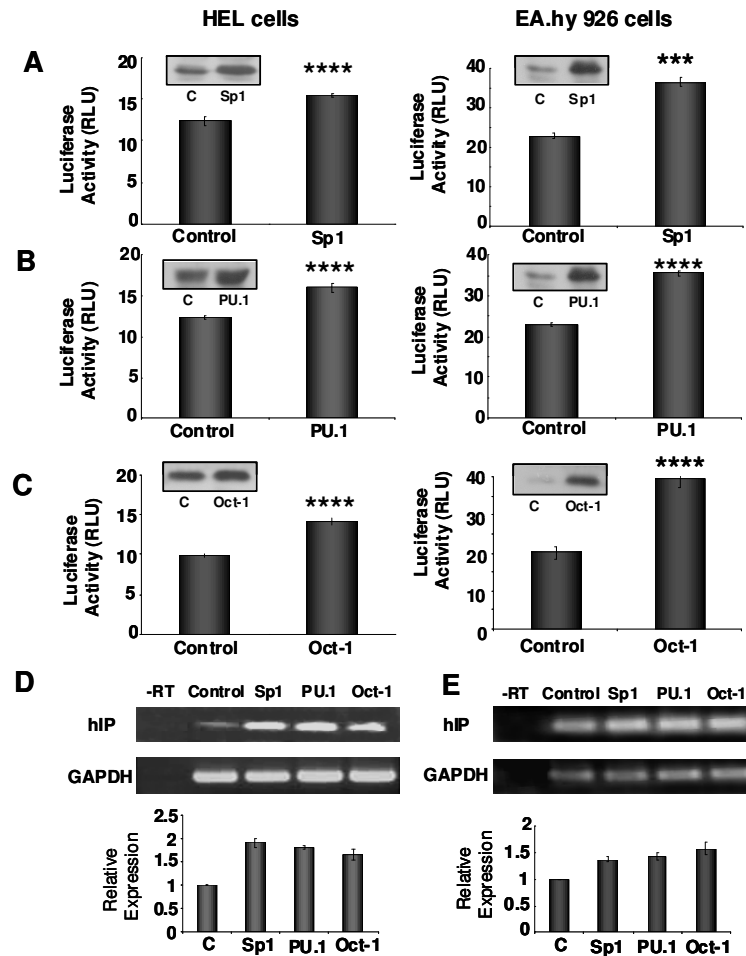
**Figure 5: Co-regulation of PrmIP activity by Sp1, Pu1 and Oct1.**

A schematic of the PrmIP6 genomic region, spanning -1022 to -774, in addition to the relative positions of putative Sp1, PU.1 and Oct-1 elements where the 5' nucleotide is indicated and the X symbol signifies mutated element. Recombinant pGL3Basic encoding: PrmIP6, PrmIP6<sup>Sp1\*</sup>, PrmIP6<sup>Pu1\*</sup>, PrmIP6<sup>Oct1\*</sup>, PrmIP6<sup>Sp1/Pu1\*</sup>, PrmIP6<sup>Sp1/Oct1\*</sup>, PrmIP6<sup>Pu1/Oct1\*</sup>, PrmIP6<sup>Sp1/Pu1/Oct1\*</sup> or PrmIP7 (-895 to -774) were co-transfected with pRL-TK into HEL cells. Mean luciferase activity was assayed 48 hr post-transfection (RLU  $\pm$  SEM, n = 6). The asterisks (\*) indicate that site-directed mutagenesis of PrmIP sequences significantly reduced luciferase expression in HEL cells where \*\* and \*\*\*\* indicate  $p \leq 0.01$  and  $p \leq 0.0001$ , respectively.



**Figure 6: PrmIP-directed Gene Expression in EA.hy 926 cells.**

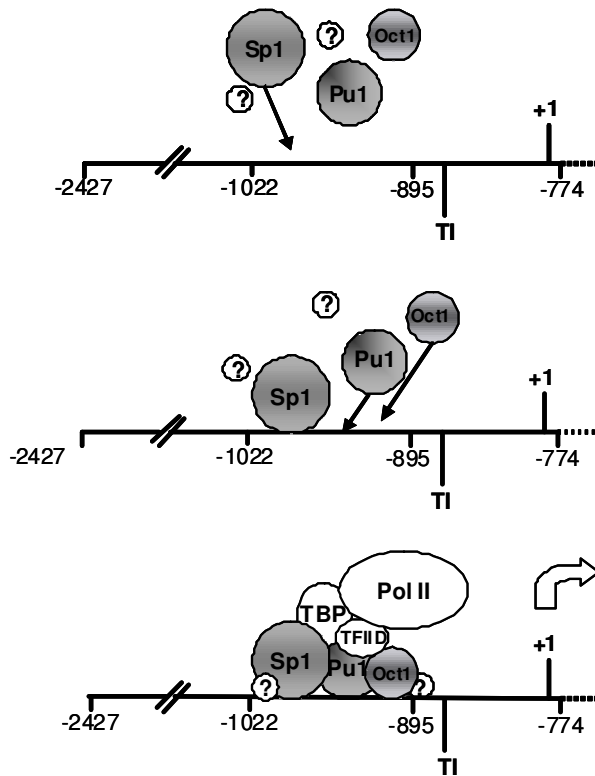
A schematic of PrmIP (-2427 to -774), in addition to exon (E)1, intron (I)1 and E2 is shown. **Panel A:** Recombinant pGL3Basic encoding: PrmIP, PrmIP6, PrmIP7, 3'ΔPrmIP or, as controls, pGL3Basic or pGL3Control vectors were co-transfected with pRL-TK into EA.hy 926 cells. **Panel B:** A schematic of the PrmIP6 (-1022 to -774), in addition to the positions of putative Sp1, PU.1 and Oct-1 elements, where the 5' nucleotide is indicated and the X symbol signifies mutated elements. Recombinant pGL3Basic encoding: PrmIP6, PrmIP6<sup>Sp1\*</sup>, PrmIP6<sup>Pu1\*</sup>, PrmIP6<sup>Oct1\*</sup>, PrmIP6<sup>Sp1/Pu1\*</sup>, PrmIP6<sup>Sp1/Oct1\*</sup>, PrmIP6<sup>Pu1/Oct1\*</sup>, PrmIP6<sup>Sp1/Pu1/Oct1\*</sup> and PrmIP7 (-895 to -774) or, as a control, pGL3Basic were co-transfected with pRL-TK into EA.hy 926 cells. Mean luciferase activity was assayed 48 hr post-transfection (RLU ± SEM, n = 5). The asterisks (\*) indicate that deletion mutagenesis of PrmIP sequences significantly reduced reporter gene expression in EA.hy 926 cells where \*\*\* and \*\*\*\* indicate  $p \leq 0.001$  and  $p \leq 0.0001$ , respectively. **Panels C & D:** EA.hy 926 cells were transiently co-transfected with pGL3Basic:PrmIP6 in the presence of pRL-TK plus pcDNA3:HaOct-1 (Oct-1), pcDNA3:HaOct-2 (Oct-2) or with pcDNA3 (Control). Cells were either assayed 48 hr post-transfection for mean luciferase activity (**Panel C**; RLU ± SEM, n = 5) or by western blot analysis (**Panel D**; 60 μg whole cell protein per lane) using anti-HA 3F10 antibody (upper panel); to confirm uniform protein loading, the upper blot was stripped and rescreened with anti-HDJ-2 antibody (lower panel). **Panels E & F:** EA.hy 926 cells were transiently co-transfected with pGL3Basic:PrmIP6 in the presence of pRL-TK plus pCMVSPORT6-PU.1 (PU.1), pSG5-Fli1 (Fli1), pSG5-Ets-1 (Ets-1) or pcDNA3 (Control). Cells were either assayed 48 hr post-transfection for mean luciferase activity (RLU ± SEM, n = 3; **Panel E**) or by western blot analysis (60 μg whole cell protein per lane; **Panel F**) using anti-PU.1, anti-Fli1 and anti-Ets-1 antibodies. The asterisks (\*) indicate that over-expression significantly increased PrmIP6-directed luciferase expression, where \*\*, \*\*\* and \*\*\*\* indicate  $p \leq 0.01$ ,  $p \leq 0.001$  and  $p \leq 0.0001$ , respectively. **Panel G:** ChIP analysis: a schematic of the PrmIP genomic region and the forward and reverse primers used to amplify the proximal PrmIP region (-1271 to -774 as indicated by the solid arrows; used in upper panel) or, as controls, an upstream PrmIP region (-1901 to -1540 as indicated by the dashed arrows; used in lower panel) in immunoprecipitates of cross-linked chromatin fragments from EA.hy 926 cells using anti-Sp1, anti-Oct-1, anti-PU.1, anti-Fli1, anti-Ets-1 sera or normal rabbit IgG, as indicated. In each case, input represents PCR amplification of template DNA extracted from the input chromatin (using approximately 1/20 of that used in each of the test immunoprecipitates). The images shown are representative of three independent experiments.



**Figure 7: Effect of Ectopic Expression of Sp1, PU.1 and Oct-1 on hIP Gene Expression.**

**Panels A-C:** HEL (left panels) and EA.hy 926 (right panels) cells were transiently co-transfected with pGL3B:PrmIP6 along with pRL-TK in the presence of either pCMVSPORT6-Sp1 (Sp1), pCMVSPORT6-PU.1 (PU.1), pcDNA3:HaOct-1 (Oct-1) or pcDNA3 (Control). Cells were harvested and either assayed for mean luciferase activity (**Panels A – C**; RLU  $\pm$  SEM,  $n = 3$ ) or by western blotting (60  $\mu$ g whole cell protein per lane) using *anti*-Sp1, *anti*-PU.1 and *anti*-Oct-1 antisera, respectively (inserted panels). The asterisks (\*) indicate that over-expression of either Sp1, PU.1 or Oct-1 significantly increased PrmIP6-directed luciferase expression relative to that in the control (pcDNA)-transfected cells, where \*\*\* and \*\*\*\* indicate  $p \leq 0.001$  and  $p \leq 0.0001$ , respectively. **Panels D & E:** Alternatively, cells were analysed by reverse transcriptase-PCR (RT-PCR) analyses to monitor hIP mRNA (upper panels) or GAPDH mRNA (lower panels) expression levels in either HEL (**Panel D**) or EA.hy 926 (**Panel E**) cells. The bar chart below Panels D and E represent mean changes in hIP mRNA expression in cells over-expressing either Sp1, PU.1 or Oct-1 relative to those levels in control (pcDNA3) transfected cells, set to a value of 1 (Relative Expression  $\pm$  SEM,  $n = 3$ ).

**A**



**B**

	<u>Sp1</u>	<u>Pu1 Oct1</u>
<b>human</b>	ACA----GTGAGGGGAGGGCGGGCCAGAGAGAGGAATGA	
<b>horse</b>	AAGCAGGGGGAGGGAGGGCGGGCCAGAGA--GGAAATGA	
<b>dog</b>	GATGAGAAGGAGGGAGGGCGGGGAGGGA--AGGAGGCTG	
<b>bovine</b>	AAGCAGGGGGAGGGAGGGCGGGCCAGAGAGAGGAATGA	
	*****	*****

**Figure 8: Model of the PrmIP-directed Basal Gene Expression in HEL 92.1.7 and EA.hy 926 cells.**

**Panel A:** Hypothetical scheme for the basal transcriptional regulation of PrmIP. Sp1, PU.1 and Oct-1 were identified as the *trans*-acting factors that regulate basal transcription of the hIP gene by their interaction with the proximal core PrmIP. In the absence of a TATA-box, it is proposed that Sp1 molecules initially bind to the proximal promoter acting as a pre-initiation factor. Following Sp1 binding, 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 human IP gene expression.

**Panel B:** Alignment of the proximal core region of human PrmIP with horse, dog, bovine IP promoter sequences. Grey highlights and asterisks indicate fully conserved bases. The consensus Sp1 and overlapping PU.1 and Oct-1 elements are underlined in the human PrmIP sequence.