Prostacyclin receptor signalling and cell proliferation: Role in pulmonary hypertension

by

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I, Emilia Falcetti, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

I also confirm that the practical procedures resulting in the experimental data presented in the thesis was performed by me except for the following (also referenced within the body of the thesis):

- C1 and N1 antibody purification (performed by Ms Nuria Battle)
- C1 antibody western blotting (performed by Dr Sue Hall)
- Generation of HEK-293-IP cell line (performed by Professor Andy Tinker)
- Explantation and characterisation of PPH HPASM cell lines (performed by Dr Sue Hall)
- Explantation and characterisation of distal HPASM cell lines (performed by Dr John Wharton's lab)

For Mum, Dad and Sam

ABSTRACT

Prostacyclin and its stable analogues are used to treat pulmonary arterial hypertension (PAH), a disease associated with abnormal smooth muscle cell (SMC) proliferation. These analogues are thought to mediate their anti-proliferative effects through prostacyclin (IP) receptors linked to cyclic AMP (cAMP) generation, though other targets may be involved including peroxisome proliferator activated receptors (PPARs), transcription factors known to regulate cell growth. Thus the aim was to assess the role of the IP receptor and PPARγ in mediating the anti-proliferative effects of prostacyclin analogues in HEK-293 cells stably expressing the IP receptor (HEK-293-IP) and in SMCs derived from normal and hypertensive lungs.

In proliferation assays, the growth rate of HEK-293-IP cells was significantly decreased compared to cells expressing the empty vector. Furthermore, treprostinil and carbacyclin, and non receptor-dependent cAMP-elevating agents only inhibited proliferation in HEK-293-IP cells, suggesting the physical presence of the IP receptor is crucial in mediating the effects of both analogues and agents working downstream of the receptor. Protein kinase A and to a lesser extent, PPARγ appear to be involved since antagonists of these two pathways partially reversed the anti-proliferative effects of treprostinil. Using a dual luciferase reporter gene assay, I demonstrated that analogues could activate PPARγ *via* a novel IP receptor-dependent, cAMP-independent mechanism, likely to involve phosphorylation.

In separate studies, pulmonary SMC derived from young idiopathic PAH patients replicated at a faster rate compared to control cells. RT-PCR and immnuostaining showed that PAH cells expressed fewer IP receptors, although treprostinil still inhibited SMC proliferation, albeit through a mechanism largely involving PPARγ but not the IP receptor or cAMP. This contrasted to normal pulmonary SMC, where treprostinil inhibited proliferation *via* the IP receptor, cAMP and PPARγ. In conclusion, the IP receptor appears to play an important role in regulating cell growth and mediating the effects of prostacyclin analogues in normal but not in pulmonary hypertensive SMC.

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ABBREVIATIONS

5-HT$_7$	5-hydroxytryptamine-7 (serotonin receptor)
α2AR	α2-adrenergic receptor
AC	Adenylyl cyclase
AKAP	A-kinase anchor protein
AMPK	AMP activated kinase
ATP	Adenosine tri phosphate
BMP-II	Type II bone morphogenetic protein
BMPR-II	Type II bone morphogenetic protein receptor
cAMP	Cyclic adenosine monophosphate
CaMKII	Calmodulin dependent protein kinase II
CARBA	Carbacyclin
CICA	Cicaprost
CBP	CREB binding protein
cdk	Cyclin dependent kinase
cdki	Cyclin dependent kinase inhibitor
cGMP	Cyclic guanosine monophosphate
CHO	Chinese hamster ovary
CICA	Cicaprost
CO	Cardiac output
COX-2	Cyclo-oxygenase-2
CRE	Cyclic AMP response element
CREB	Cyclic AMP response element binding protein
DBD	DNA binding domain
DDA	2',5'-dideoxyadenosine
DP receptor	Prostaglandin D receptor
DTT	Dithiothreitol
E_m	Membrane potential
eNOS	Endothelial nitric oxide synthase
EP receptors	Prostaglandin E_2 receptors
ERK	extracellular-signal related kinase
ET-1	Endothelin-1
FBS	Foetal bovine serum
FPAH	Familial pulmonary arterial hypertension
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
GPCR	G-protein coupled receptor
G_s protein	Stimulatory G protein
H-89	N- [2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide

HEK-293 cells	Human embryonic kidney cells
HODE	Hydroxyoctadecadienoic acid
HPASMC	Human pulmonary arterial smooth muscle cell
IBMX	3-isobutyl-1-methylxanthine
ILO	Iloprost
IP receptor	Prostacyclin receptor
IP_3	Phosphatidyl inositol
IPAH	Idiopathic pulmonary hypertension
IPRA	IP receptor antagonist
K_{ATP}	ATP sensitive K^+ channel
LBD	Ligand binding domain
MAPK	Mitogen activated protein kinase
MEK	Mitogen activated protein kinase kinase
MaxiK	Large conductance Ca^{2+} activated K^+ channel
MHC	Myosin heavy chain
mIP receptor	Murine prostaglandin I_2 receptor
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
NO	Nitric oxide
NOS	Nitric oxide synthase
PAEC	Pulmonary arterial endothelial cells
PAH	Pulmonary arterial hypertension
PAP	Pulmonary arterial pressure
PASMC	Pulmonary artery smooth muscle cells
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PGC-1	PPARγ-co-activator 1
PGE_2	Prostaglandin E_2
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin, prostaglandin I_2
PGIS	Prostacyclin synthase
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
PPARs	Peroxisome proliferator-activated receptors
PPARα	Peroxisome proliferator-activated receptor α
PPARβ	Peroxisome proliferator-activated receptor β
PPARγ	Peroxisome proliferator-activated receptor γ
PPH	Primary pulmonary hypertension

PPRE	Peroxisome proliferator response element
PTx	Pertussis Toxin
PVR	Pulmonary vascular resistance
RB	Retinoblastoma
RO1138452	[(4,5-dihydro-1H-imidazol-2-yl) - [4- (4-isopropoxybenzyl) phenyl] amine]
ROSI	Rosiglitazone
RXR	Retinoid X receptor
SMC	Smooth muscle cells
SPH	Secondary pulmonary hypertension
TAE	Tris acetate EDTA
TGF-β	Transforming growth factor-β
TP	Thromboxane receptor
TREP	Treprostinil
TXA_2	Thromboxane A_2
TZD	Thiazolidinedione
VSMC	Vascular smooth muscle cells

Chapter 1

Introduction

1.1 Pulmonary Hypertension

The pulmonary circulation is a low resistance and low pressure system that allows blood into the lung microcirculation to promote gas exchange. Pulmonary hypertension is a serious chronic disorder of the pulmonary circulation. It is a progressive and often fatal haemodynamic abnormality characterised by a raised pulmonary-artery pressure (PAP). Although PAP in normal individuals varies with age, from early childhood to the fifth decade of life, its upper limit (mean PAP) is approximately 20 mm Hg (Mandegar *et al.*, 2004). In pulmonary hypertension however, PAP is raised significantly above this level and is clinically defined as a mean PAP of more than 25 mm Hg at rest rising to more than 30 mm Hg during exercise (Rubin, 1993).

PAP is a product of cardiac output (CO) and pulmonary vascular resistance (PVR). Elevated PVR has a harmful effect on the heart as the right ventricle is submitted to an increased work load necessary to overcome the downstream resistance. This eventually leads to right-ventricular hypertrophy and failure, a common cause of death in patients with pulmonary hypertension.

1.1.1 Classification of pulmonary hypertension

Pulmonary hypertension arises from a number of disease conditions. Associated or secondary hypertension (SPH) denotes the disease when it is in association with other cardiac or systemic disorders (Simonneau *et al.*, 2004). Idiopathic (sporadic) pulmonary arterial hypertension (IPAH) defines the disease in which pulmonary arteriopathy is the primary cause. IPAH was first discovered over 100 years ago in patients with right heart failure whose

necropsy showed no obvious reasons for pulmonary arteriosclerosis and was termed syphilitic pulmonary arteritis. In 1951 Dresdale and his colleagues coined the term 'primary pulmonary hypertension' (PPH) (Dresdale *et al.*, 1951). However, at the 3rd world conference on Pulmonary Hypertension in 2003, the term PPH was replaced with IPAH and was distinguished from familial PAH (FPAH) which is essentially the same disease, only supported by a genetic basis (Simonneau *et al.*, 2004).

Mutations in the type II bone morphogenetic protein receptor (BMPR-II) gene, a member of the transforming growth factor β (TGF-β) receptor superfamily, have been linked to cases of FPAH (Deng *et al.*, 2000) and the same genetic abnormality accounts for approximately 30% of cases of sporadic pulmonary hypertension. It displays autosomal dominant inheritance and genetic anticipation (Loyd *et al.*, 1995; Loyd *et al.*, 1984). TGF-β itself can strongly inhibits smooth muscle cell proliferation (McCaffrey *et al.*, 1997) as well as promoting cell differentiation (Adam *et al.*, 2000) although its effects are dependent on cell type and experimental setting. Similarly, type II bone morphogenetic protein (BMP-II) also inhibits vascular smooth muscle cell proliferation after balloon injury (Nakaoka *et al.*, 1997). However, smooth muscle cells isolated from the pulmonary arteries of patients with IPAH exhibited no anti-proliferative response to the BMPR-II ligands, BMP-2, BMP-4 and BMP-7, compared with cells from control patients or patients with SPH whose proliferation was inhibited by these agents (Morrell *et al.*, 2001). Genetic defects in BMPR-II do not explain all cases of FPAH or sporadic cases of IPAH, suggesting that other genes may be involved in the aetiology of the disease. Mutations in the gene that codes for activin receptor-like kinase (ALK 1), another TGF-β cell surface receptor,

appear to be responsible for the rare occurrence of pulmonary arterial hypertension in patients with hereditary hemorrhagic telangiectasia (Trembath *et al.,* 2001).

Serotonin has long been recognised as a potent naturally occurring vasoconstrictor and has been implicated in the pathogenesis of PAH. Outbreaks of PAH have been associated with use of fenfluramine-derivate anorexigens, appetite suppressant drugs which prevent serotonin uptake by platelets (reviewed in de Caestecker, 2006). A genetic link between anorexigenic drugs and PAH has been established following the observation that only a minority of patients who ingest these agents actually develop the pathology. A polymorphism in the promoter region of the human serotonin transporter (5-HTT) gene alters its transcriptional activity hence the level of its expression is predetermined based on its genotype. The polymorphism involves two alleles: the L and the S allele. The L allele has a 2- to 3-fold higher level of 5-HTT gene transcription compared to the S allele. It is believed that 60–70% of IPAH patients possess the L/L genotype, while L/L genotype is present in only 20–30% of the control population of Caucasian subjects, indicating that L/L genotype may confer genetic susceptibility to IPAH (Eddahibi *et al.,* 2001). This concept has recently been challenged as recent extensive analyses have failed to confirm a link between IPAH and the L/L genotype (Machado *et al.,* 2006; Willers *et al.,* 2006). Despite this, *in vitro* and *in vivo* experimental data support the hypothesis that increased 5-HTT expression, regardless of genotype, plays a role in the pathogenesis of PH (reviewed in de Caestecker, 2006)

Pulmonary arterial hypertension has also been divided into four distinct functional classes (I-IV) by the New York Heart Association (Table 1.1). This method of classification defines the severity of the disease, class I being the least severe and class IV being the most severe.

Class	Description
Class I	PAH without a resulting limitation of physical activity. Ordinary physical activity does not induce dyspnea or fatigue, chest pain, or near syncope.
Class II	PAH resulting in a slight limitation of physical activity. The patient is comfortable at rest, but ordinary physical activity causes undue dyspnea or fatigue, chest pain or near syncope.
Class III	PAH resulting in a marked limitation of physical activity. The patient is comfortable at rest, but less than ordinary activity causes undue dyspnea or fatigue, chest pain or near syncope.
Class IV	PAH resulting in an inability to carry out any physical activity without symptoms. The patient has signs of right heart failure. Dyspnea, fatigue or both may be present even at rest, and discomfort is increased by any physical activity

Table 1.1 New York Heart Association (NYHA) functional classification of pulmonary arterial hypertension (PAH). Classification modified from the NYHA classification of patients with cardiac disease. Adapted from the executive summary of the World Symposium on Primary Pulmonary Hypertension in Evian, France, in 1998.

Physiologically, PAH describes an intrinsic disease of the pulmonary vascular smooth muscle and endothelial cells due to abnormalities at cellular and molecular level (Rubin, 1997), such as mutations of cell surface receptors, dysfunctional ion channels and abnormal membrane transporters (reviewed in Mandegar *et al.,* 2004). The obliterative abnormality blocks the blood from flowing through small pulmonary vessels leading to chronic and sustained pulmonary hypertension. This occurs in both idiopathic and familial PAH as well as in disorders that produce obliterative pulmonary artery

disease as a complication of systemic processes, including collagen vascular disease or HIV infection.

1.1.2 Pathophysiology of IPAH

There are three main factors that combine to produce increased vascular resistance in obliterative pulmonary hypertension: vasoconstriction, vascular wall remodelling and thrombosis in situ (reviewed in Rubin, 1997). All three factors contribute to the narrowing of the lumen of small and medium sized arteries, a general histological finding in IPAH, in association with intimal and medial hypertrophy, concentric laminar intimal fibrosis, fibrinoid degeneration and formation of plexiform lesions (Wagenvoort, 1980; Bjornsson and Edwards, 1985; Pietra *et al.,* 1989). Figure 1.1 summarises the three factors and their causes.

1.1.2.1 In situ thrombosis

Endothelial cell dysfunction, and its interaction with growth factors and platelets, is thought to be partially responsible for thrombus formation as it promotes a pro-coagulant environment within the vascular bed. In fact patients with IPAH have been shown to have enhanced pro-coagulant activity due to increased levels of plasma fibrinopeptide-A and prolonged half-life of fibrinogen (Eisenberg *et al.*, 1990). Moreover, it has been reported that there is diminished fibrinolytic activity and increased levels of plasminogen activator inhibitor in 70% of patients with IPAH (Welsh *et al.*, 1996). In addition high levels of urinary metabolites of thromboxane A_2, a marker of platelet activation, have also been reported in these patients (Christman *et al.,* 1992). Platelet activation and aggregation may lead to

thrombus formation and be a significant factor in stimulating vasoconstriction and cellular proliferation by releasing vasoactive and mitogenic substances.

Platelets, fibroblasts, endothelial cells and thrombus formation are all involved in pulmonary vascular remodelling. It is becoming increasingly clear however, that vasoconstriction and even more significantly pulmonary artery smooth muscle cell (PASMC) proliferation lead to the vascular remodelling processes that characterise severe pulmonary hypertension.

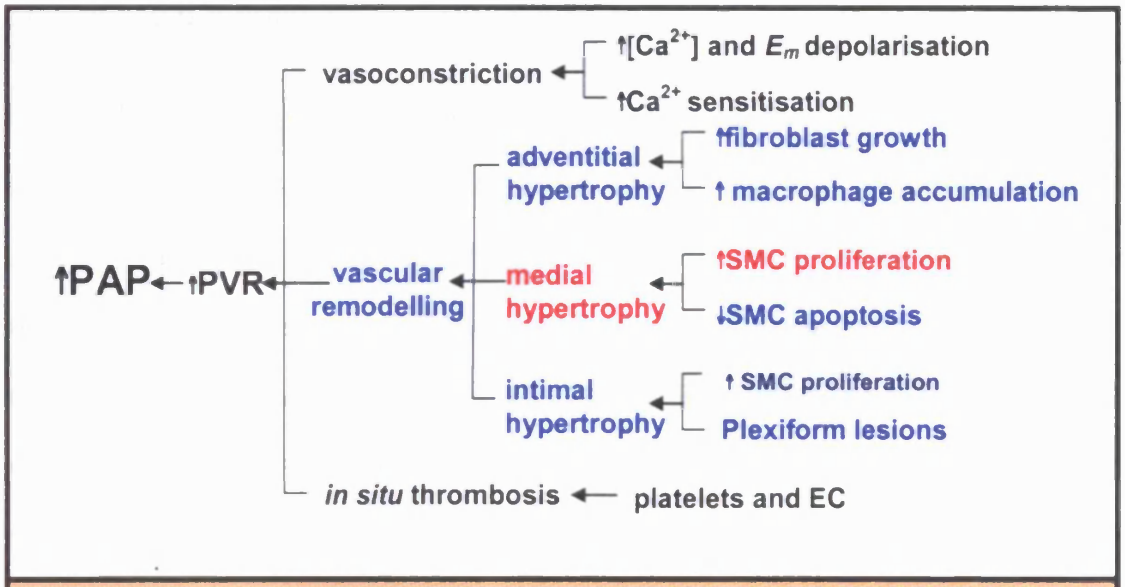

Figure 1.1 Diagram illustrating the three main factors contributing to the development of sustained elevation of pulmonary vascular resistance (PVR) and artery pressure (PAP) in pulmonary arterial hypertension (PAH). (*Em*) membrane potential, (SMC) pulmonary artery smooth muscle cells, (EC) pulmonary arterial endothelial cells. Adapted from Mandegar *et al*, 2004.

1.1.2.2 Pulmonary artery vasoconstriction

Smooth muscle constriction is elicited by a rise in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$). The contractile proteins, actin and myosin, contract PASMC in a Ca^{2+}-dependent manner. The degree of pulmonary vasoconstriction depends on both the level of $[Ca^{2+}]_{cyt}$ and the sensitivity of

the contractile apparatus to $[Ca^{2+}]_{cyt}$ (reviewed in Mandegar *et al.*, 2004). Control of $[Ca^{2+}]_{cyt}$ levels in PASMC is achieved principally, though not exclusively, in two distinct ways. The first is via trans-sarcolemmal influx of Ca^{2+} through Ca^{2+} permeable channels or exit of Ca^{2+} through membrane Ca^{2+}-Mg^{2+} ATPase pumps. The second is the mobilisation of Ca^{2+} from the sarcoplasmic reticulum (SR) through Ca^{2+} release channels or sequestration of Ca^{2+} back into the SR by Ca^{2+}-Mg^{2+} ATPase pumps (SERCA) (Berridge, 1993; Blaustein, 1988). There are two types of SR Ca^{2+} channels, which are named for their affinity for either the plant alkaloid ryanodine or the phospholipid metabolite inositol (1,4,5)-trisphosphate (IP_3).

In PASMC $[Ca^{2+}]_{cyt}$ can therefore be increased by Ca^{2+} release from the intracellular stores and through Ca^{2+} influx through Ca^{2+} channels. A number of Ca^{2+} channels are responsible for Ca^{2+} entry through the plasma membrane and the excitation-contraction coupling process is thus dependent on the function of these channels. These include voltage-dependent Ca^{2+} channels (VDCC) regulated by changes in membrane potential (E_m), receptor operated Ca^{2+} channels (ROC) activated by agonists binding to specific membrane receptors and store operated Ca^{2+} channels (SOC) activated by depletion of Ca^{2+} from intracellular stores (Nelson *et al.*, 1990; Thorneloe and Nelson, 2005). As well as passing Ca^{2+}, ROCs and SOCs are non-selective cation channels which when opened cause membrane depolarisation (Thorneloe and Nelson, 2005).

In addition, the activity of K^+ channels indirectly regulates the extent of Ca^{2+} influx through the VDCC by altering the E_m. For example, in smooth muscle, opening of potassium channels causes membrane hyperpolarisation thereby

reducing Ca^{2+} entry through VDCC and promoting relaxation. Down-regulated or dysfunctional K^+ channels can also lead to sustained membrane depolarisation and contribute to maintain elevated $[Ca^{2+}]_{cyt}$ levels found in pulmonary hypertensive cells, contributing to both vasoconstriction and cell proliferation (Remillard and Yuan, 2005). Indeed Yuan and colleagues (Yuan *et al.*, 1998) have shown that there was a lower voltage-gated K^+ (Kv) channel expression and decreased K^+ channel activity in PASMC from a small number of IPAH patients. More recently, enhanced expression of transient receptor potential (TRP) channel genes that encode channels responsible for non voltage-dependent Ca^{2+} entry were much higher in PASMC from IPAH patients than in those from normotensive or secondary pulmonary hypertension patients (Yu *et al.*, 2004). Sustained elevation of $[Ca^{2+}]_{cyt}$ induces a chronic state of vasoconstriction and is thus likely to contribute to smooth muscle cell hypertrophy and vascular remodelling.

1.1.2.3 Pulmonary arterial wall remodelling

In a normal state, the thickness and tissue mass of the pulmonary arterial walls are maintained at an optimum level by a balance between proliferation and apoptosis of PASMC, fibroblasts and pulmonary arterial endothelial cells (PAEC). In IPAH, however this balance is disrupted in favour of proliferation, leading to a thickening of the pulmonary arterial wall and a narrowing of the vessel lumen. The latter is eventually obliterated which leads to increased PVR. Vascular remodelling defines the structural changes that lead to hypertrophy and/or luminal occlusion. Loss of pulmonary vascular compliance and increased PVR due to vascular remodelling results in

pronounced pulmonary hypertension and is the principal pathological finding in IPAH (Rubin, 1997).

The molecular mechanisms underlying pulmonary artery remodelling are very complex but changes in Ca^{2+} homeostasis are likely to play a major role. Elevated $[Ca^{2+}]_{cyt}$ drives quiescent cells into the cell cycle where they undergo mitosis promoting cellular proliferation (Landsberg and Yuan, 2004). In addition it has been shown that resting $[Ca^{2+}]_{cyt}$ is significantly elevated in proliferating PASMC compared to growth-arrested cells, highlighting the fact that increased Ca^{2+} influx is required for cell proliferation as well as for smooth muscle contraction (Golovina *et al.*, 2001; Platoshyn *et al.*, 2000).

The balance of vasoconstrictor and mitogenic agents with vasodilator and anti-mitogenic agents is crucial in maintaining normal vascular function. In IPAH this balance is shifted more towards over production of vasoconstrictors such as endothelin (ET-1) and thromboxane (TXA_2) at the expense of vasodilators and anti-mitogenic factors such as prostacyclin (PGI_2) and nitric oxide (NO) (Christman *et al.*, 1992; Tuder *et al.*, 1999). Matrix metalloproteinases (MMPs) are also thought to have a role in the development of PAH. The gelatinases MMP-2 and MMP-9, which degrade collagen and elastin and regulate extracellular matrix deposition, have been shown to contribute to smooth muscle cell migration and proliferation (Frisdal *et al.*, 2001; Vieillard-Baron *et al.*, 2003). Activation of these metalloproteinases increases tenascin-C expression, a protein which promotes proliferation and suppresses apoptosis in PASMC (Cowan *et al.*, 1999). Furthermore MMP-2 has been found to be up-regulated in PASMC

from IPAH patients (Lepetit *et al.,* 2005) and is thought to regulate vascular tone and structure by cleaving vasoactive peptides (Martinez *et al.*, 2004).

1.1.2.4 Role of endothelium derived factors and phosphodiesterases in vascular wall remodelling

Endothelin

ET-1, produced by endothelial cells as well as smooth muscle cells (Kanse *et al.,* 1991), is an efficient survival and anti-apoptotic agent as well as being a potent potent vasoconstrictor peptide (Yanagisawa *et al.,* 1988). In addition it has mitogenic effects, promoting both inflammation and smooth muscle cell proliferation and has been found to play a role in vascular remodelling (Kirchengast and Munter, 1998; Davie *et al.*, 2002). Clinical and animal studies have implicated increased ET-1 production in the pathogenesis of primary and secondary pulmonary hypertension (Komai *et al.,* 1993; Noguchi *et al.,* 1997; Giaid, 1998). Indeed, endothelin antagonists appear effective in reversing the development of experimental pulmonary hypertension and are of proven benefit in the treatment of IPAH (Weinberger *et al.,* 2001; Attina *et al.*, 2005). The effects of ET-1 are mediated by the endothelin-A (ET_A) and endothelin-B (ET_B) receptors. In the cardiovascular system, ET_A receptors are found in smooth muscle cells and cardiac myocytes, whereas ET_B receptors are localized on both endothelial and smooth muscle cells (reviewed in Galie *et al.*, 2004). Human PASMC express both receptors and signalling through either type may prevent apoptosis (Davie *et al.*, 2002). The binding of ET-1 to ET_A and ET_B receptors in SMC activates phospholipase C (PLC), which leads to an increase of inositol triphosphate (IP_3) and intracellular Ca^{2+} leading to long-lasting

vasoconstriction. The increase of intracellular Ca^{2+} also stimulates protein kinase C, which mediates the mitogenic action of ET-1 (reviewed in Galie *et al.*, 2004). By contrast, activation of ET_B receptors in endothelial cells stimulates the release of NO and prostacyclin (Hirata *et al.*, 1993), prevents apoptosis (Shichiri *et al.*, 1997) and mediates the pulmonary clearance of circulating ET-1 (Dupuis *et al.*, 1996). It has been demonstrated that both ET_A and ET_B receptors are up-regulated in the vasculature of experimental models of both systemic (Wu *et al.*, 2000) and pulmonary hypertension (Li *et al.*, 1994; Soma *et al.*, 1999).

Thromboxane

Thromboxane A_2 (TXA_2) is produced primarily by platelets but also by the endothelium. It is a potent vasoconstrictor and smooth muscle mitogen. It is an arachidonic acid metabolite of the cyclo-oxygenase pathway which acts as an agonist for platelet aggregation and is likely to contribute to vascular remodelling and histopathologic changes associated with IPAH. In fact patients with IPAH have been found to have elevated levels of urinary 11-dehydro-TBX2, a urinary metabolite of TXA_2 (Christman *et al.*, 1992), as well as elevated synthesis of TXA_2 (Robbins *et al.*, 2001).

Nitric oxide

NO is a potent endogenous vasodilator that directly relaxes vascular smooth muscle through stimulation of soluble guanylate cyclase and increased production of intracellular cyclic guanosine monophosphate (cGMP). It is produced from the terminal guanidine-nitrogen of L-arginine and oxygen in the endothelium by endothelial NO synthase (eNOS). It had been suggested

originally that the expression of eNOS is reduced in the lungs of patients with PAH (Giaid and Saleh, 1995) but this finding has not been confirmed in more recent investigations (Tuder *et al.*, 1999). However, there is evidence showing that whole body NO production, exhaled NO and urinary NO metabolites are all decreased in patients with PAH (Demoncheaux *et al.*, 2005; Girgis *et al.*, 2005). Current literature suggests that reduced bio-availability of NO in PAH is not principally the result of impaired eNOS expression but rather caused by complex pathways involving reduced L-arginine availability and increased NO degradation (Hoeper and Rubin, 2006).

Phosphodiesterases

There are 11 phosphodiesterase (PDE) isoform families responsible for the hydrolysis of cyclic nucleotides (Maurice *et al.,* 2003). cGMP, the intracellular second messenger of NO has gained substantial consideration as a possible target in the treatment of pulmonary vascular disease. PDE5, one of the PDEs responsible for the breakdown of cGMP, is abundantly expressed in PASMC (Murray *et al.,* 2002) and PDE5 inhibitors have been shown to have pulmonary vasodilatory effects in both experimental and human PAH (Zhao *et al.,* 2001; Aldashev *et al.*, 2005). In addition these inhibitors have antiproliferative effects on PASMC (Corbin *et al,* 2005; Wharton *et al.*, 2005). The inhibition of other PDE isozymes which breakdown cyclic AMP (cAMP), such as PDE1, PDE3 and PDE4, also suppresses proliferation and promotes apoptosis of PASMC, thus may have a role in pulmonary vascular remodelling and smooth muscle cell proliferation (Phillips *et al.*, 2005; Growcott *et al.*, 2006).

Prostacyclin

Prostacyclin (PGI_2), discovered by Sir John Vane and colleagues in 1976 (Moncada *et al.*, 1976), is the main prostanoid in the mammalian vasculature, and its production is greater in pulmonary than in systemic arteries (Shaul *et al.*, 1991). It is derived from arachidonic acid, which is converted by cyclo-oxygenase, primarily cyclo-oxygenase-2 (COX-2) into prostaglandin H (PGH_2) which in turn is eventually metabolised into PGI_2 by prostacyclin synthase (PGIS) (Fig. 1.2). PGH_2 is also the precursor of other prostanoids such as PGE_2, PGF_2, PGD_2 and TXA_2.

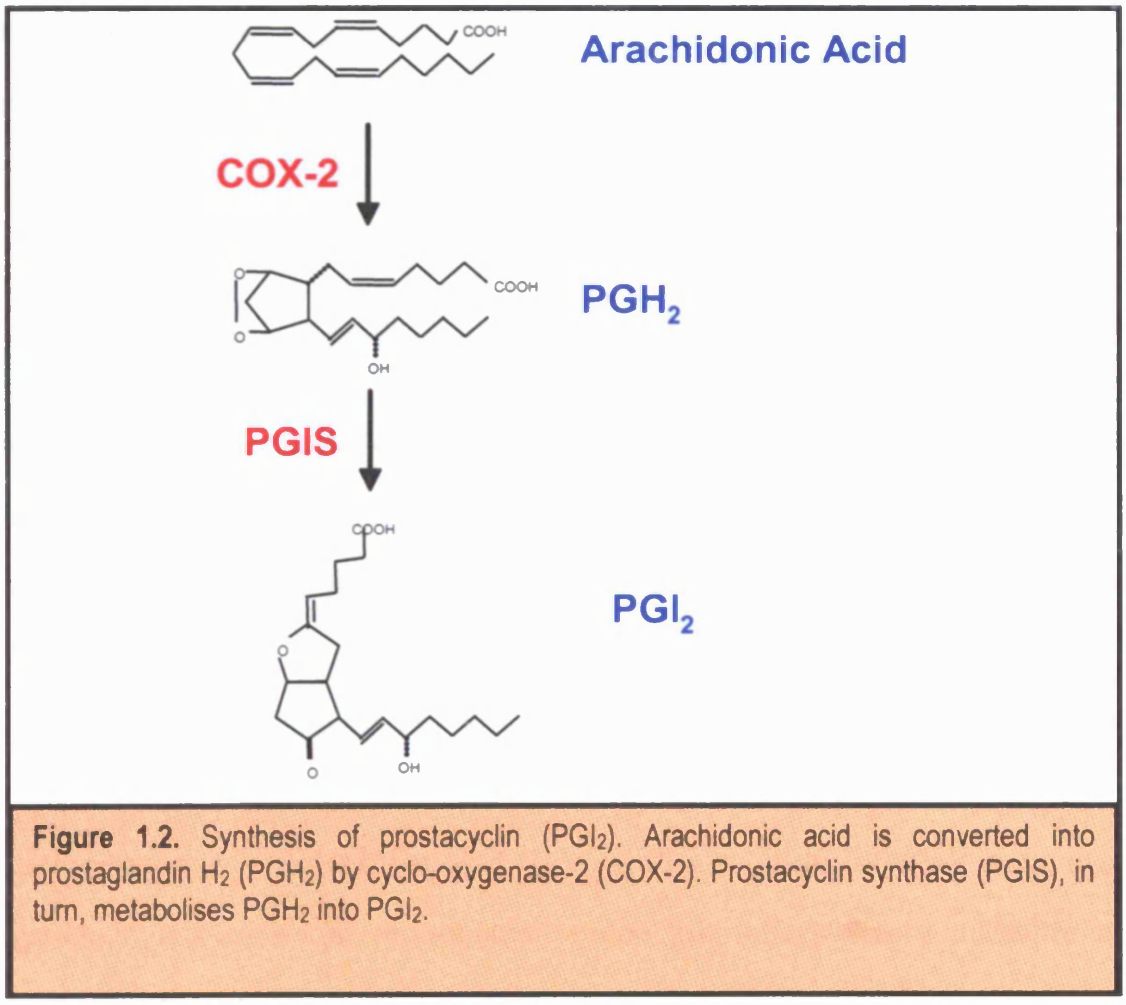

Figure 1.2. Synthesis of prostacyclin (PGI_2). Arachidonic acid is converted into prostaglandin H_2 (PGH_2) by cyclo-oxygenase-2 (COX-2). Prostacyclin synthase (PGIS), in turn, metabolises PGH_2 into PGI_2.

Several lines of evidence suggest that the major biological activities of PGI_2 are mediated by PGI_2 (IP) receptors coupled via the stimulatory G protein, G_s to adenylyl cyclase (Narumiya *et al.*, 1999). Prostacyclin, and its stable analogues induce inhibition of proliferation of vascular smooth muscle cells by stimulating the production of cyclic AMP (cAMP) (Wharton *et al.*, 2000; Clapp *et al.*, 2002) whereas relaxation appears to result from both cyclic AMP dependent and independent pathways (Turcato and Clapp, 1999; Orie *et al.*, 2006). In IPAH patients there is a decrease in prostacyclin production (Christman *et al.*, 1992) which may be the result of a lower expression of PGIS observed in the small arteries of such patients (Tuder *et al.,* 1999). Furthermore over-expression of PGIS in rats has been shown to inhibit proliferation and migration of smooth muscle cells in rat balloon-injured carotid arteries (Harada *et al.*, 1999). Prostacyclin and its stable analogues will be discussed in greater detail later.

1.1.3 Current treatment of IPAH

As yet lung transplantation is the only curative treatment for this disorder. Anticoagulants, such as heparin and warfarin, have been shown to be beneficial but their efficacy seems to be biased towards patients that display thrombotic lesions (Gaine and Rubin, 1998). They are given in combination with other therapies such as calcium-channel blockers (Ogata *et al.*, 1993). Long term therapy with calcium-channel blockers has achieved sustained haemodynamic improvement and survival in a minority of IPAH patients. It results in a substantial reduction in mean pulmonary artery pressure and pulmonary vascular resistance (Rich *et al.*, 1992). However, less than 10% of cases fall into this "responder" category (Runo and Loyd, 2003). In

addition some patients on calcium-channel blockers experience systemic hypotension, oxygen de-saturation or a decline in cardiac output and are unlikely to benefit from this vasodilatory therapy; in fact it may even cause further clinical deterioration (Gaine and Rubin, 1998). Another drawback to this therapy is that abrupt discontinuation results in rebound pulmonary hypertension which may be fatal (Gaine and Rubin, 1998). Apart from transplantation, anti-coagulation and calcium channel blockers, the development of most treatments for IPAH has focused on readdressing the balance between vasodilators and vasoconstrictors in the pulmonary vasculature.

1.1.3.1 PGI_2 and stable analogues

Prostacyclin in the form of epoprostenol (Flolan) has been the most widely studied drug in PAH. It improves haemodynamics and exercise tolerance and prolongs survival in IPAH patients who do not respond to vasodilator testing at cardiac catheterisation (Higenbottam *et al.*, 1993; Barst *et al.*, 1996; Barst *et al.*, 1994). Cohort analysis of patients with IPAH who were receiving continuous intravenous epoprostenol showed clinical benefit for patients in NYHA functional class III and IV (McLaughlin *et al.*, 2002; Sitbon *et al.*, 2002). The beneficial effects of prostacyclin may relate to their ability to inhibit smooth muscle cell proliferation (Wharton *et al.*, 2000; Clapp *et al.*, 2002), to decrease production of ET-1 (Prins *et al.*, 1994; Woods *et al.*, 2000) and increase its clearance in patients (Langleben *et al.*, 1999) and improve pressure-flow response to exercise (Castelain *et al.*, 2002). Epoprostenol has a short half-life in plasma (3 minutes) and is inactivated at low pH. Therefore its administration requires continuous intravenous infusion

with the use of a portable infusion pump connected to a permanent tunnelled catheter inserted into a subclavian vein (Barst *et al.,* 1996). Having an in-dwelling line may lead to complications such as exit-site infections and bleeding as well as sepsis. The dose of epoprostenol needs to be increased as the disease progresses to prevent symptom recurrence. It is not clear why this is so, but may be due to increased production of endogenous counter-mediators such as TXA_2 or to IP receptor desensitisation or down-regulation (Nilius *et al.,* 2000). Stable PGI_2 analogues with increased bioavailability have therefore been developed as an alternative to epoprostenol to try and circumvent some of these problems.

Schering's first compound Iloprost (Ventavis) has at least the same binding capacity to the IP receptor as PGI_2 but it is more stable and has a much more extended duration of action *in vivo* (Skuballa *et al.,* 1985). It has been approved for treating IPAH in Europe and is administered either intravenously or via an inhaler. Because of its relatively short duration of action compared to other analogues (plasma half-life of 15-30 mins), it must be inhaled as many as 6 to 12 times a day. The results of short-term studies and trials have been encouraging and this agent has the added advantage of relatively few systemic side effects (Olschewski *et al.,* 2002; Hoeper *et al.*, 2000). However the long term efficacy of inhaled iloprost and the effects of repeated inhalation remain to be established.

Beraprost sodium (Beraprost) is the only orally-available PGI_2 analogue; it has a half-life of 35–40 mins and is currently licensed for use in Japan. There have been two random clinical trials of Beraprost performed to date

(both in patients in NYHA class II and III). The first trial showed a significant improvement in the 6 minute walking distance test over 12 weeks in IPAH patients (but none in patients with PAH and associated conditions) although surprisingly there were no significant haemodynamic changes (Galie *et al.*, 2002). The second study, conducted over 12 months, showed that the significant improvement in walking distance seen at 3 and 6 months was lost at 9 months and thereafter (Barst *et al.,* 2003). There were no significant haemodynamic or survival benefits over placebo control patients at 12 months. While beraprost is not very efficacious over a prolonged period, it may prove beneficial as a component of combination therapy (Howard and Morrell, 2005).

Treprostinil (Treprostinil Sodium, Remodulin; UT-15) is a stable PGI_2 analogue, has a relatively long half life (~34-86 minutes) and can be administered as a continuous subcutaneous infusion. Treprostinil has been an approved therapy in the United States since 2002, and more recently in Europe. Patients undergoing treprostinil treatment frequently complain of severe pain at the site of infusion, a major hurdle for the widespread use of treprostinil. In November 2004 however, the FDA approved the intravenous use for those not able to tolerate subcutaneous infusion because of site or leg pain. Recent studies also show that there is a possibility of a successful switch from intravenous epoprostenol to intravenous treprostinil (Gomberg-Maitland *et al.*, 2005). In trials, treprostinil has given positive results in patients with class II-IV pulmonary arterial hypertension by improving indexes of dyspnea, signs and symptoms of pulmonary hypertension and haemodynamic measures (reviewed in McLaughlin *et al.*, 2002; Rubin, 2002).

In addition to having different half-lives, PGI_2 analogues have different affinities towards a wide range of prostanoid receptors including prostaglandin E_2 (EP) receptors (Kiriyama *et al.,* 1997; Clapp *et al.*, 1998; Abramovitz *et al.*, 2000). Figure 1.3 illustrates the chemical structure of PGI_2 and its analogues with their respective half-lives and their known receptor selectivities. The importance of receptor selectivity is yet to be fully established. There is little data upon which to judge whether or not the IP receptor is solely responsible for the beneficial clinical effects of PGI_2 or whether some effects may be mediated by the other receptors for which these compounds have affinity for.

Iloprost binds to the IP receptor with approximately the same affinity as it binds to EP_1 and EP_3 receptors (Abramovitz *et al.*, 2000). Cicaprost has a long *in vivo* half-life of over an hour and is described as the most selective IP receptor agonist exhibiting high binding affinity to the IP receptor (K_i ~10-20 nM). However, in species other than mouse, cicaprost has significant agonist activity at the EP_4 receptor (Narumiya *et al.,* 1999; Abramovitz *et al.,* 2000). In contrast, carbacyclin (Carbaprostacyclin, $cPGI_2$) has a relatively poor receptor selectivity having equal affinity for IP, EP_1 and EP_3 receptors, but does have the added feature of being membrane permeable. Treprostinil appears to be one of the most potent analogues in terms of inhibition of cell proliferation (Clapp *et al.*, 2002) but there is little data on receptor selectivity. Cicaprost and carbacyclin, although not approved therapies for PAH, have proved to be good tools in the laboratory due to their relative IP receptor selectivity and membrane-permeability respectively.

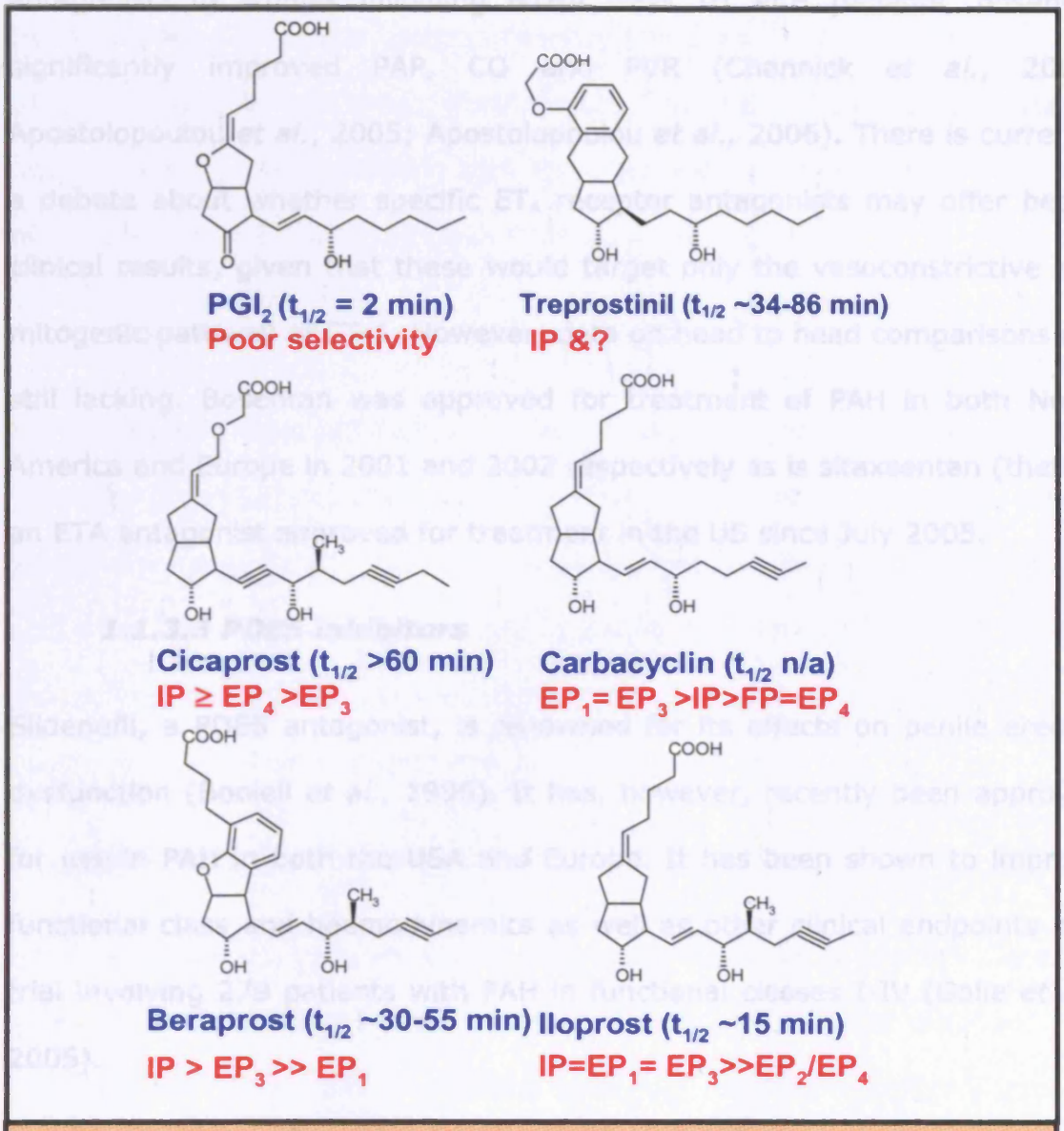

Figure 1.3. Structure of prostacyclin (PGI_2) and its stable analogues. Half-life ($t_{1/2}$) *in vivo* is shown in blue. Receptor selectivity is shown in red. (IP) PGI_2 receptor, (EP_{1-4}) prostaglandin E receptors, (FP) prostaglandin F receptor. (Kiriyama *et al*, 1997; Abramovitz *et al*. 2000).

1.1.3.2 Endothelin-receptor antagonists

Bosentan (Tracleer) is an orally active dual (ET_A and ET_B) endothelin receptor antagonist. In studies involving NYHA class III PAH patients, bosentan significantly improved PAP, CO and PVR (Channick *et al.*, 2001; Apostolopoulou *et al.*, 2005; Apostolopoulou *et al.*, 2006). There is currently a debate about whether specific ET_A receptor antagonists may offer better clinical results, given that these would target only the vasoconstrictive and mitogenic pathway of ET-1. However, data on head to head comparisons are still lacking. Bosentan was approved for treatment of PAH in both North America and Europe in 2001 and 2002 respectively as is sitaxsentan (thelin), an ETA antagonist approved for treatment in the US since July 2005.

1.1.3.3 PDE5 inhibitors

Sildenafil, a PDE5 antagonist, is renowned for its effects on penile erectile dysfunction (Boolell *et al.*, 1996). It has, however, recently been approved for use in PAH in both the USA and Europe. It has been shown to improve functional class and haemodynamics as well as other clinical endpoints in a trial involving 279 patients with PAH in functional classes I-IV (Galie *et al.*, 2005).

1.1.3.4 Potential future therapies for IPAH

Nitric Oxide

Since IPAH is associated with a defect in the production of NO, this agent has been proposed as a potential therapy (Giaid and Saleh, 1995). Short-term inhalation of NO has substantial pulmonary specific vasodilator effects

in humans (Giaid and Saleh, 1995) and there are isolated studies suggesting that treatment with L-arginine, the substrate for NOS, reduces PAP and increases exercise tolerance in patients with IPAH (Nagaya *et al.,* 2001). However long-term NO inhalation therapy has been shown to benefit only a small number of patients. Furthermore, its administration can cause haemodynamic deterioration and the generation of toxic by-products, making it unsuitable for home use (Hasuda *et al.,* 2000).

Vasoactive Intestinal Peptide

Vasoactive intestinal peptide is a member of the superfamily that secretes glucagon-growth hormone releasing factor. It too acts as a potent vasodilator and inhibits smooth muscle cell proliferation and platelet activation. In an encouraging study involving 8 patients with IPAH, inhaled vasoactive intestinal peptide produced a significant functional and haemodynamic improvement (Petkov *et al.,* 2003).

Selective Serotonin-reuptake inhibitors

Serotonin appears to have a role in the pathogenesis of various types of human and experimental forms of PAH. Therefore it has been proposed that specific treatments utilising selective serotonin reuptake inhibitors, such as fluoxetine, may provide protection against PAH (Eddahibi *et al.*, 2002; Marcos *et al.*, 2003). These agents are yet to be tested in PAH patients.

Combination therapy

The combined use of drugs with different mechanisms of action is the most likely emerging option for treatment of PAH. Most experts agree that a

combination of prostanoids, endothelin and PDE inhibitors will result in a better clinical outcome than each of the treatments alone. Some *in vitro* evidence comes from studies showing that combinations of PGI_2 analogues with PDE3 or PDE4 inhibitors results in a significant additive or synergistic elevation in cAMP and ensuing anti-mitogenic effects (Phillips *et al.,* 2005; Growcott *et al.,* 2006). There are only a few clinical studies and case series assessing combination therapy but the data presented are encouraging. Administering bosentan to patients receiving prostanoid treatment (intravenous or inhaled) improves exercise capacity and echocardiographic parameters of right heart function (Seyfarth *et al.,* 2005). In addition, a study by Hoeper and colleagues (Hoeper *et al.,* 2005), where PAH patients were treated with endothelin receptor antagonists, PDE5 inhibitors and prostanoids, demonstrated that such a combination approach significantly improved 1, 2 and 3 year survival rates compared to historical control groups treated with iloprost or beraprost alone.

Numerous other new compounds have been evaluated as potential treatment for PAH in experimental studies or small case series. Drugs used for other diseases such as statins, Rho-kinase inhibitors and imatinib mesylate, for example, have also been shown to be able to control the pathological vascular remodelling of PAH (reviewed in Ito *et al.*, 2007).

An open-label observational study on 16 PAH patients has shown that administration of simvastatin improves the 6-minute walk performance and haemodynamics without severe complications (Kao, 2005). A Rho-kinase inhibitor, Fasudil, when given intravenously to 9 patients with severe PH

exhibited a significant acute reduction in the pulmonary vascular resistance (Fukumoto *et al.*, 2005).

Platelet derived growth factor (PDGF) is involved in the proliferation of PASMC and PDGF receptors are upregulated in an animal model of PAH (Balasubramaniam *et al.*, 2003). Imatinib mesylate is a selective PDGF receptor antagonist shown to be able to reverse established severe PAH and prolong survival in two animal models of PAH (Schermuly *et al.*, 2005). Since this compound is tolerable and clinically used for the treatment of certain malignancies the result of this drug may be easily transferable to PAH therapies. Interestingly a case study reported that the oral administration of this drug improved exercise capacity, haemodynamics and functional class in a patient who was refractory to combination therapy (oral bosentan, inhaled iloprost and sildenafil) (Ghofrani *et al.*, 2005). Larger controlled trials are needed to identify the long-term benefits and anti-remodelling effects of these potential future treatments.

In summary, there have been many recent advances in our understanding of the pathobiology of pulmonary vascular disease and new medicines have been introduced into clinical practice. Surprisingly, however, the mechanism of action of prostacyclin and its analogues, to date the most effective treatment given to patients with IPAH, remains poorly understood.

1.2 The prostacyclin receptor

1.2.1 Expression and distribution

The IP receptor (Figure 1.4) is a membrane bound G-protein coupled-receptor (GPCR) belonging to the family of the prostanoid receptors

(Narumiya *et al.*, 1999). The human IP gene was first cloned from megakaryocyte and lung cDNA libraries and spans approximately 7kb, consisting of three exons and two introns. The gene maps to chromosome band 19q13.3 and encodes a protein of 386 amino acids folding into seven trans-membrane domains (reviewed in Smyth and Fitzgerald, 2002).

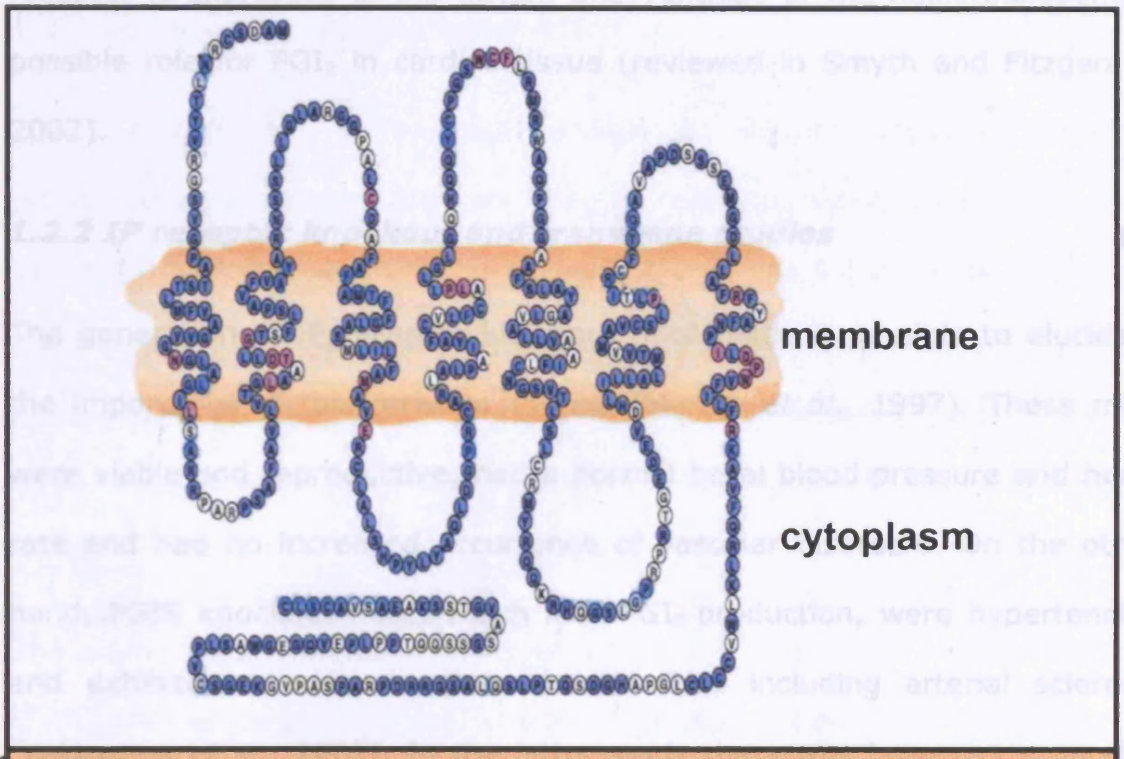

Figure 1.4. Diagram representing the IP receptor. This G protein coupled receptor (GPCR) consists of 386 amino acids forming seven trans-membrane domains. The C terminus is located in the cytoplasm and the N terminus is located extra-cellularly. Adapted from Cayman Chemicals.

The IP receptor is expressed in a number of tissues including kidney, liver, lung, heart and aorta (Boie *et al.*, 1994; Nakagawa *et al.*, 1994). In the vasculature it is mainly localised in platelets and vascular smooth muscle cells (Oliva and Nicosia, 1987). It appears to be confined to the arterial side of the circulation, even in the pulmonary vasculature (Oida *et al.*, 1995)

consistent with the notion that venous smooth muscle cells contain primarily EP type rather than IP receptors (Coleman *et al.,* 1994). Nevertheless, both isolated human pulmonary arteries and veins exhibit the same sensitivities to the relaxant effect of cicaprost (Walch *et al.,* 1999). IP receptors are present in fibroblasts too where they appear to mediate the anti-migratory effects of PGI_2 analogues (Kohyama *et al.,* 2002). In addition, the IP receptor is expressed in the atrium and ventricle of the heart indicating a possible role for PGI_2 in cardiac tissue (reviewed in Smyth and Fitzgerald, 2002).

1.2.2 IP receptor knockout and transgene studies

The generation of IP receptor knockout mice made it possible to elucidate the importance of this receptor *in vivo* (Murata *et al.*, 1997). These mice were viable and reproductive, had a normal basal blood pressure and heart rate and had no increased occurrence of vascular disorders. On the other hand, PGIS knockout mice, which lack PGI_2 production, were hypertensive and exhibited renal and aortic abnormalities including arterial sclerosis (Yokoyama *et al.,* 2002). In the latter study there was however, a parallel increase in the biosynthesis of other eicosanoids such as PGE_2 and TXA_2 probably as a result of a move to other synthetic pathways which could account for the different phenotype observed between PGIS and IP receptor knockout mice.

The ability of PGI_2 and its analogues to inhibit cell proliferation appears to rely on IP receptor activation. In VSMC from IP knockout mice, cicaprost had no anti-proliferative or cAMP elevating effects (Fujino *et al.*, 2002; Kothapalli *et al.,* 2003). Moreover, IP deficient mice lacked a hypotensive response to

cicaprost as well as being more susceptible to thrombus formation after endothelial damage was evoked (Murata *et al.*, 1997). In addition, these mice developed a greater degree of cardiac hypertrophy (Hara *et al.*, 2005), pulmonary hypertension and pulmonary vascular remodelling after chronic exposure to hypobaric hypoxia than did wild type mice (Hoshikawa *et al.*, 2001).

1.2.3 IP receptor signal transduction

The IP receptor is a "relaxant" prostanoid receptor together with the prostaglandin D receptor (DP) and the two prostaglandin E receptors EP_2 and EP_4 (Coleman *et al.*, 1994). Receptors activate intracellular signalling cascades by coupling to heterotrimeric G proteins. The IP receptor primarily activates adenylyl cyclase (AC) via the stimulatory G protein, G_S (Namba *et al.*, 1994), hence agonist binding to the receptor results in an increase in cAMP.

The main target for cAMP in mammalian cells is to activate protein kinase A (PKA, cAMP-dependent protein kinase). PKA is regulated by two catalytic (C) and two regulatory (R) subunits. Cyclic AMP binds to the R dimer and this leads to the dissociation of the R dimer from the C dimer. The dissociation of the C subunits activates them and results in the phosphorylation of the PKA phosphorylation consensus sequence X-Arg-Arg-X-Ser-X on target proteins (reviewed in Koyama *et al.*, 2001). In addition cAMP has also been shown to act in a PKA-independent manner by directly activating guanine nucleotide exchange factors (cAMP-GEFs) such as Epac-1 and -2 which promote the formation of GTP bound, active, Rap 1, a small Ras-like G protein involved in

cellular proliferation, and Rap 2 (Kawasaki *et al.*, 1998; de Rooij *et al.*, 2000).

Similar to other GPCRs, the IP receptor may couple to more than one G protein. In expression systems, as well as coupling to G_s, the IP receptor has also been shown to couple to G_q. This in turn activates PLC leading to IP_3 turnover and mobilisation of intracellular Ca^{2+} (reviewed in Smyth and Fitzgerald, 2002). Some have also reported coupling to the inhibitory G protein, G_i (Lawler *et al.*, 2001). There is controversy over whether the receptor couples dependently or independently of G_s/G_q and whether it couples to G_i at all (Wise and Jones, 2000).

Differential coupling of the IP receptor to multiple signalling pathways can be regulated by C-terminal modification. Isoprenylation and glycosylation of the C-terminal are both thought to be required for efficient coupling of the IP receptor to both G_s and G_q (Hayes *et al.*, 1999; Miggin *et al.*, 2002; Smyth *et al.*, 1998). In addition, the IP receptor possesses multiple cysteine residues in the C-terminal that are capable of being palmitoylated. Differing patterns of palmitoylation have been demonstrated to be required for coupling to G_s- versus G_q-mediated signal transduction pathways (Miggin *et al.*, 2003).

1.2.3.1 Mechanisms of IP receptor-mediated vasorelaxing effects of PGI_2 analogues

PGI_2 and its stable analogues promote vasorelaxation in a wide variety of vascular beds (Siegel *et al.*, 1989) including guinea-pig aorta (Clapp *et al.*, 1998; Turcato and Clapp, 1999), rat tail artery (Schubert *et al.*, 1997; Orie

et al., 2006) and human pulmonary artery and vein (Walch *et al.*, 1999). It is readily assumed that the IP receptor and subsequent elevation of cAMP mediates the major biological activities of PGI_2 and its analogues, since these agents readily increase cAMP in many different smooth muscle cell types (Vegesna and Diamond, 1986; Turcato and Clapp, 1999; Clapp *et al.*, 2002) and relaxation is commonly potentiated by agents preventing the breakdown of cAMP (Holzmann *et al.,* 1980; *Tanaka et al.,* 2004}. One of the final cellular events associated with PGI_2-induced vasorelaxation is membrane hyperpolarisation of smooth muscle cells resulting from activation of plasma membrane K^+ channels (Siegel *et al.*, 1989; Tanaka *et al.*, 2004). Depending on the vascular bed, the main channels involved are the ATP sensitive K^+ (K_{ATP}) channel, the large conductance Ca^{2+} activated K^+ (MaxiK) channel or the inward rectifier K^+ channel (Tanaka *et al.*, 2004; Orie *et al.*, 2006). The former two channels appear to be important in mediating part of the dilatory effects of iloprost in the pulmonary vasculature (Dumas *et al.,* 1997). Originally, K^+ channel activation was thought to be driven exclusively through a cAMP-dependent mechanism (Schubert *et al.,* 1997) although there is growing evidence for the involvement of cAMP independent mechanisms (Turcato and Clapp, 1999; Orie *et al.*, 2006) possibly through direct G_s protein coupling to the channel (Tanaka *et al.*, 2004)

1.2.3.2 Mechanisms of IP receptor-mediated anti-proliferative effects of PGI_2 analogues

Vascular smooth muscle cells are usually quiescent *in vivo* but in pulmonary vascular disease they undergo proliferation. PGI_2 and its stable analogues

inhibit smooth muscle cell proliferation and DNA synthesis by blocking the progress from the G_1 phase into the S phase of the cell cycle (Wharton *et al.*, 2000; Kothapalli *et al.,* 2003). Cyclic AMP-elevation appears to be the main signalling mechanism by which the receptor mediates inhibition of proliferation in a variety of cell types: 2',5'-dideoxyadenosine (DDA), an adenylyl cyclase inhibitor, attenuated both the acute stimulation of cAMP production as well as the inhibition of smooth muscle cell proliferation induced by iloprost, cicaprost and treprostinil (Wharton *et al.,* 2000; Clapp *et al.,* 2002; Phillips *et al.,* 2005). Different PGI_2 analogues inhibit proliferation with different potencies. Treprostinil appears to be the most potent analogue in human PASMC with an IC_{50} of 4.2nM (Clapp *et al.*, 2002). Specificity to the IP receptor does not appear to confer increased potency, in fact cicaprost, the most IP specific analogue to date, has a less potent anti-proliferative effect than either treprostinil or iloprost (Clapp *et al.,* 2002).

It has been shown very recently that this IP receptor dependent increase in cAMP activates PKA (Phillips *et al.,* 2005). What is acting directly downstream of PKA remains to be elucidated. It is well known that cAMP inhibits SMC proliferation *in vitro* (Assender *et al.*, 1992; Indolfi *et al.*, 1997) and reduces the formation of neointimal lesions after arterial injury *in vivo* (Indolfi *et al.,* 1997). It does so primarily by being able to inhibit both the extracellular-signal regulated kinase (ERK) and the phosphoinositide-3 kinase (PI3K) mitogenic pathways (reviewed in Bornfeldt and Krebs, 1999; Koyama *et al.,* 2001).

The ERK signalling pathway mediates mitogenic responses induced by a variety of growth factor receptors in many cell types including SMCs

(Servant *et al.*, 1996; Nelson *et al.*, 1998). Activation of this cascade leads to the activation of the small GTP-binding protein Ras and then sequential phosphorylation events lead to activation of the protein kinases, Raf, mitogen-activated protein (MAP) kinase kinase (also known as MAPKK or MEK) and ERK (also known as MAPK). cAMP, via PKA, inhibits activation of the ERK pathway in many cell types including arterial SMC (Graves *et al.*, 1993) however the exact mechanism is still unclear and may involve more than one target (reviewed in Bornfeldt and Krebs, 1999). Several studies suggest that the inhibition may be at the point of Raf given the ability of PKA to phosphorylate both Raf-1 and B-Raf isoforms (Wu *et al.*, 1993; Peraldi *et al.*, 1995). Again, the precise mechanism is uncertain but a reduction in the binding affinity between the PKA-phosphorylated Raf and Ras appears to be the most described mechanism (reviewed in Bornfeldt and Krebs, 1999).

PI3K comprises a family of agonist-stimulated lipid signalling enzymes that initiate signalling cascades by generating three distinct membrane lipids, the polyphosphoinositides PtdIns-3-P, PtdIns-3,4-P_2, and PtdIns-3,4,5-P_3 (reviewed in Toker, 2000). There are three classes of PI3Ks, each responsible for generating one of the three polyphosphoinositides. Class I PI3Ks are activated in many cell types in response to growth factor or hormone stimulation, such as platelet derived growth factor (PDGF) and insulin, and are crucial for cell proliferation and several other cellular processes (Toker, 2000). A number of protein kinases are activated downstream of PI3K, including protein kinase B (PKB, Akt), p21-activated kinase, protein kinase Cζ, and the mammalian target of rapamycin (mTOR) which is inhibited by elevation of cAMP levels (Toker, 2000; Scott and Lawrence, 1998). In addition to blocking the PI3K pathway, cAMP has been

shown to up-regulate levels of $p27^{kip1}$ in SMC (Fukumoto *et al.*, 1999; Kronemann *et al.*, 1999). Up-regulation of this cyclin dependent kinase inhibitor (CDKI) causes cell cycle arrest by inhibiting the activity of cyclin dependent kinases (CDK) 2 and 4 (Fukumoto *et al.*, 1999). Interestingly, Stewart and colleagues show that cicaprost also regulates $p27^{kip1}$ levels and that this is causally related to its antiproliferative effect (Stewart *et al.*, 2004).

G_1 phase progression is controlled by a sequential activation of cyclins and CDKs, specifically cyclin D1-cdk4/6 and cyclin E-cdk2. These regulate cell cycle progression by phosphorylating pocket proteins such as pRb, which releases sequestered E2F and in turn activates cyclin genes such as cyclin A (Takahashi *et al.*, 2000). This occurs possibly through interaction with the cell cycle-dependent element (CDE) and the cell cycle gene homology region (CHR) on the cyclin A promoter (Zwicker *et al.*, 1995; Liu *et al.*, 1998). The net result is progression into the S phase of the cell cycle. It has been shown that cicaprost blocks proliferation at the G_1 to S phase of the cell cycle, partly as a result of inhibiting activation of the cyclin A promoter and induction of cyclin A (Kothapalli *et al.*, 2003). In addition it has also been shown to block the activation of cyclin E-cdk2 (Kothapalli *et al.*, 2003).

Moreover, the cyclin A promoter contains a cAMP response element (CRE) (TGACGTCA), a site necessary for the efficient activation of this cyclin (Desdouets *et al.*, 1995). The CRE binding protein (CREB) family of transcription factors, which includes CREB, ATF1 and CRE modulator (CREM), can be activated by phosphorylation by several kinases in response to cAMP, calcium, stress and mitogenic stimuli. PKA is mainly responsible for

phosphorylating CREB at Ser133 (Mayr and Montminy, 2001). Phosphorylation of CREB promotes recruitment of the transcriptional co-activator CREB binding protein (CBP) and p300 (Chrivia *et al.,* 1993; Arias *et al.,* 1994), two highly related histone acetyltransferases (HATs). CBP/p300 then interacts with polymerase II complexes to mediate transcription. Transcription appears to peak 30 minutes after the increase in cAMP (Mayr and Montminy, 2001). Cicaprost has been shown to inhibit the binding of CREB and phospho-CREB to the CRE element in the cyclin A promoter (Kothapalli *et al.,* 2003). It is still unclear how cicaprost can mediate this inhibition of CREB binding given that it is well established that IP couples to G_s to increase cAMP levels. Kothapalli and colleagues suggest a possible role for G_i signalling as they have shown that pertussis toxin reverses the effects of cicaprost on cyclin E-cdk2 and CRE occupancy, an effect that prevents cicaprost from inhibiting cell proliferation.

It is possible that activation of CREB through IP receptor signalling may have effects other than those observed on cyclin A transcription. CREB has been shown to cooperate with BMP-stimulated Smad signalling (Ionescu *et al.*, 2004) and also appears to be involved with the peroxisome proliferator-activated receptors (PPARs), a family of transcription factors involved in cell metabolism, differentiation and growth which can be activated by some PGI_2 analogues (Forman *et al.,* 1997). CITED2, a CBP/p300- interacting protein, has been shown to be a PPARα co-regulator (Tien *et al.,* 2004) and PPARγ-co-activator 1 (PGC-1) contains a CRE motif, even though PPARγ itself does not possess one (Conkright *et al.*, 2003). It is thought that CREB regulates this gene through an indirect mechanism.

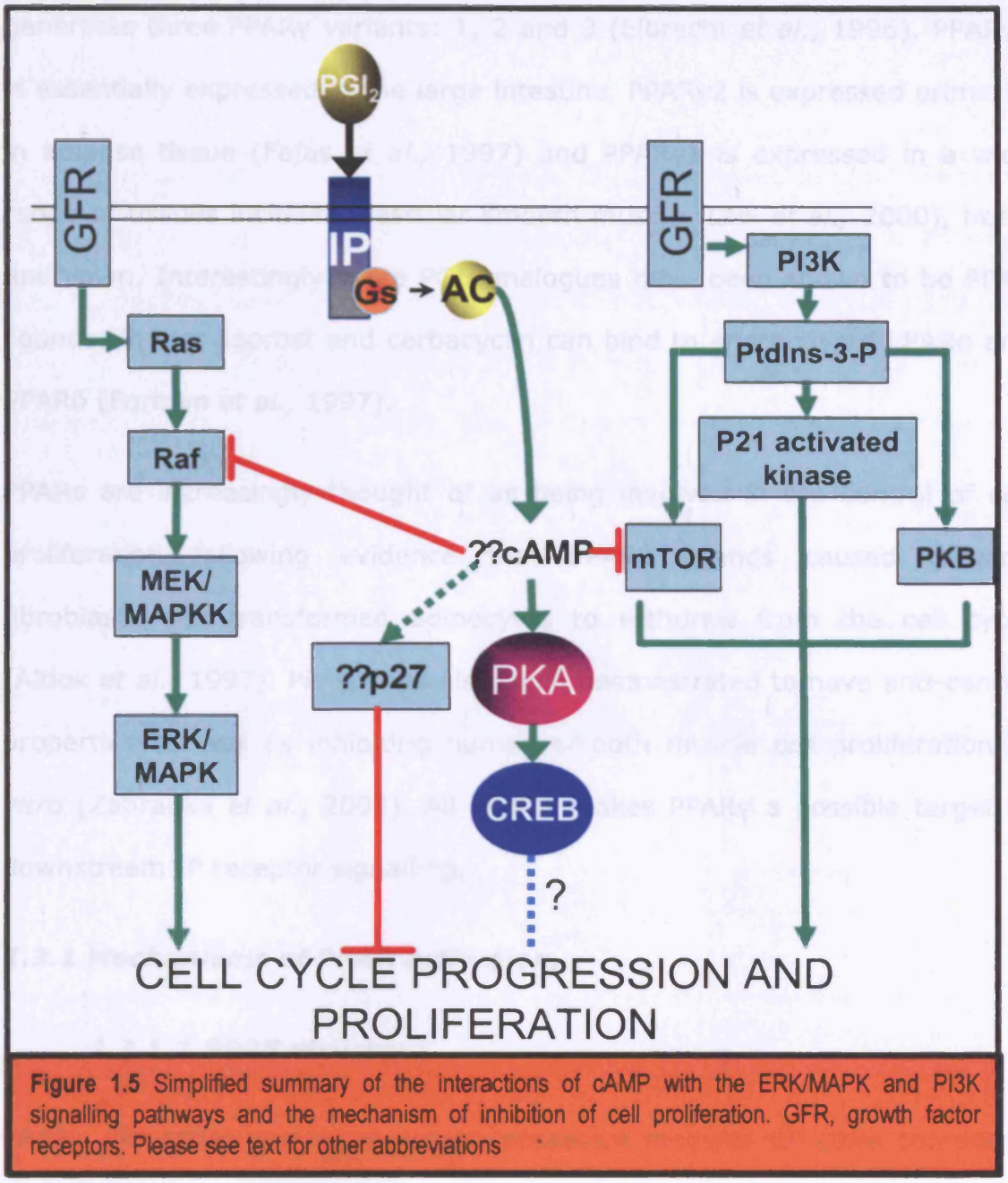

Figure 1.5 Simplified summary of the interactions of cAMP with the ERK/MAPK and PI3K signalling pathways and the mechanism of inhibition of cell proliferation. GFR, growth factor receptors. Please see text for other abbreviations

1.3 PPARs

Peroxisome proliferator-activated receptors (PPARs) are nuclear transcription factors which regulate target gene expression by binding to specific peroxisome proliferator response elements (PPREs) in the promoters of target genes. There are three isoforms of PPARs; α, δ and γ which are encoded by separate genes. Alternate promoter usage and splicing

generates three PPARγ variants: 1, 2 and 3 (Elbrecht *et al.*, 1996). PPARγ3 is essentially expressed in the large intestine, PPARγ2 is expressed primarily in adipose tissue (Fajas *et al.*, 1997) and PPARγ1 is expressed in a wide range of tissues including vascular smooth muscle (Law *et al.*, 2000), heart and colon. Interestingly some PGI_2 analogues have been shown to be PPAR ligands; in fact iloprost and carbacyclin can bind to and activate PPARα and PPARδ (Forman *et al.*, 1997).

PPARs are increasingly thought of as being involved in the control of cell proliferation following evidence that PPARγ ligands caused growing fibroblasts and transformed adipocytes to withdraw from the cell cycle (Altiok *et al.*, 1997). PPARγ has also been demonstrated to have anti-cancer properties as well as inhibiting human smooth muscle cell proliferation *in vitro* (Zahradka *et al.*, 2003). All of this makes PPARγ a possible target in downstream IP receptor signalling.

1.3.1 Mechanisms of PPAR activation.

1.3.1.1 PPAR structure

PPARs, like other nuclear receptors, possess a modular structure composed of functional domains. There are four main domains named A/B, C, D, and E/F (Figure 1.6) (reviewed in Desvergne and Wahli, 1999). The C domain, also known as the DNA binding domain (DBD), and the E/F domain, known as the ligand binding domain (LBD), are the most highly conserved regions across the receptor isoforms. The DBD consists of two zinc fingers that specifically bind PPREs. The LBD, located in the C-terminal half of the

receptor, has been shown to be composed of 13 α-helices and a small 4-stranded β-sheet.

The ligand-dependent activation domain, AF-2, is located in the C terminus of the LBD and is involved in the generation of the receptors' co-activator binding pocket (Nolte *et al.*, 1998). A ligand-independent activation function, AF-1, is found in close proximity to the N terminus of the receptor in the A/B domain (Werman *et al.*, 1997). The D domain is a hinge region that can modulate the DNA binding ability of the receptor and is involved in co-activator interaction.

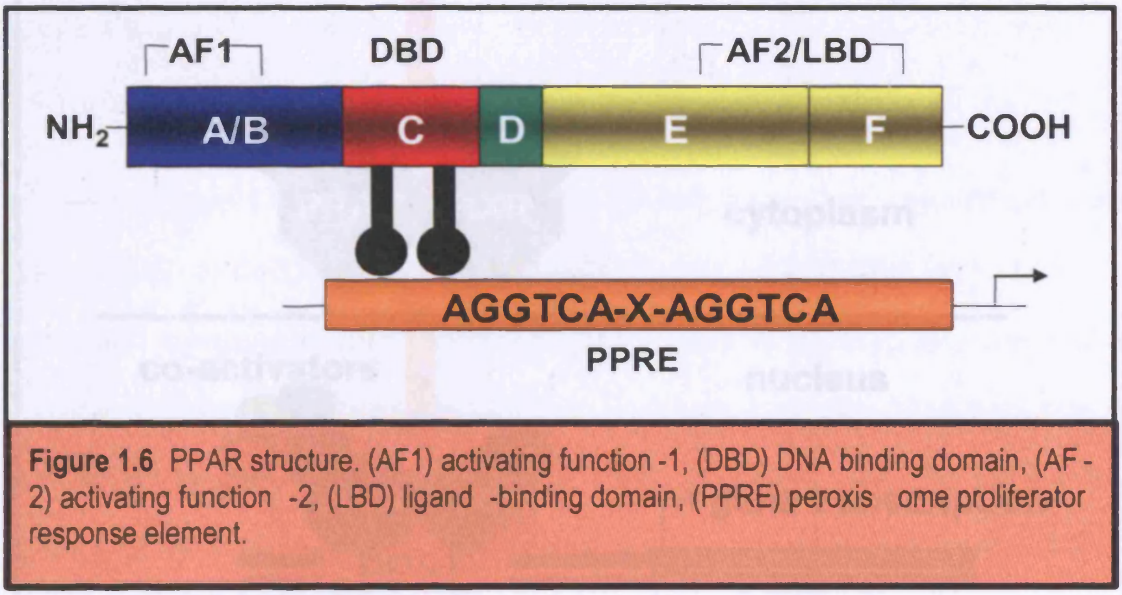

Figure 1.6 PPAR structure. (AF1) activating function -1, (DBD) DNA binding domain, (AF-2) activating function -2, (LBD) ligand -binding domain, (PPRE) peroxis ome proliferator response element.

1.3.1.2 Ligand binding

Classically PPARs are activated upon binding of a ligand to the LBD. Following this, PPARs heterodimerise with the retinoid X receptor (RXR). Like PPARs, RXR exists as three distinct isoforms: RXRα, β, and γ, all of which are activated by the endogenous agonist 9-*cis* retinoic acid (Mangelsdorf *et al.*,

1992), however the specific roles of these different isoforms of the PPAR:RXR complex have not yet been elucidated. The PPAR:RXR heterodimer binds to PPREs (Figure 1.7). PPREs are direct repeat elements consisting of two 6-base-pair consensus sequences (AGGTCA) separated by a single nucleotide spacer.

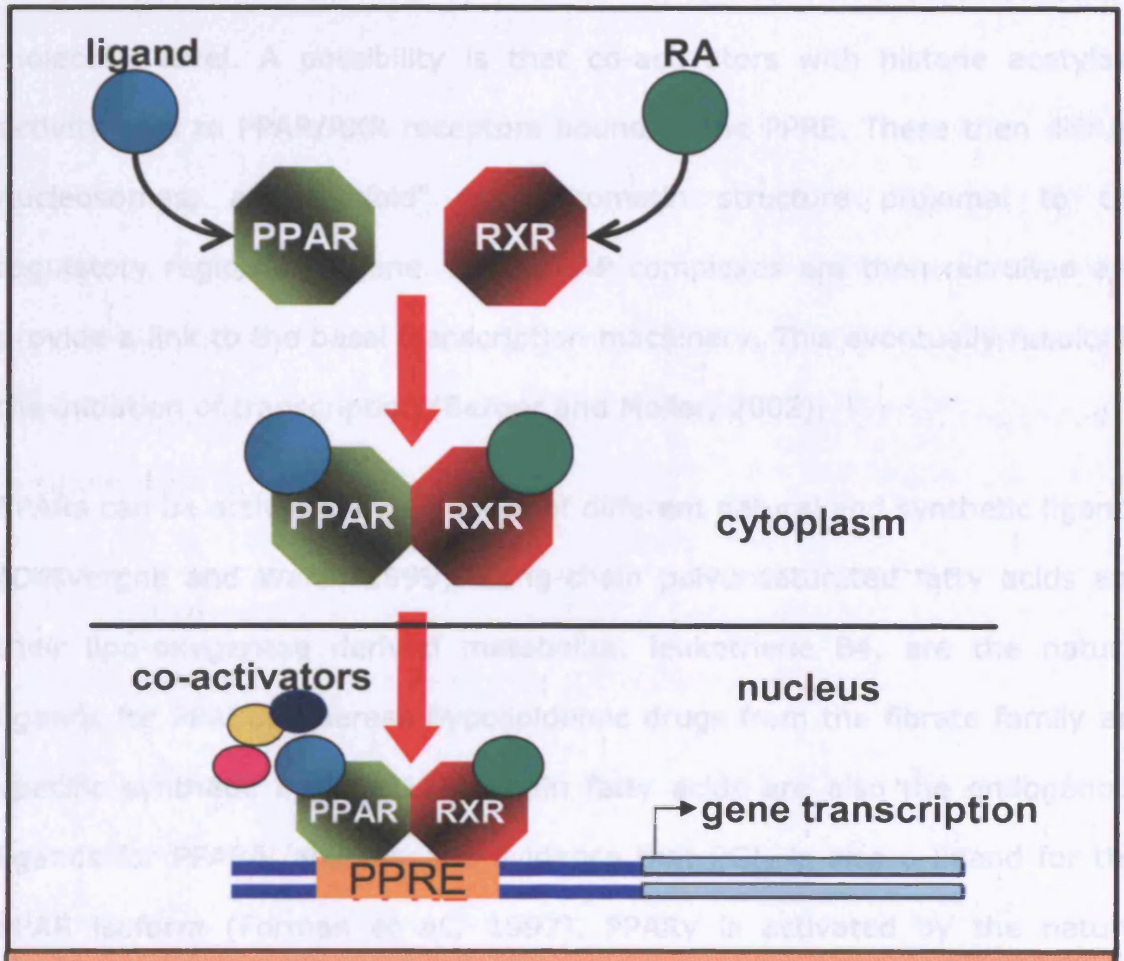

Figure 1.7 Mechanism of PPAR activation via ligand binding. Once activated the PPAR:RXR heterodimer translocates to the nucleus where it binds to the PPRE. Co-activators are also recruited leading to the regulation of gene transcription downstream. (RXR), retinoic acid receptor, (RA) retinoic acid, (PPRE) PPAR response element.

PPARs also recruit co-activators which are required for the successful increase in gene transcription; among these are also CBP/p300 and PGC-1. Co-activators such as CBP/p300 and steroid receptor co-activator (SRC)-1

possess histone acetylase activity able to remodel chromatin structure. Another set of co-activators, including members of the DRIP/TRAP complex such as PPAR binding protein (PBP)/TRAP220), form a bridge between the nuclear receptor and the transcription initiation machinery (reviewed in Berger and Moller, 2002). The exact role of a third group of co-activators, which includes PGC-1, RIP140, and ARA70, is not well understood at the molecular level. A possibility is that co-activators with histone acetylase activity bind to PPAR/RXR receptors bound to the PPRE. These then disrupt nucleosomes, and "unfold" the chromatin structure proximal to the regulatory region of a gene. DRIP/TRAP complexes are then recruited and provide a link to the basal transcription machinery. This eventually results in the initiation of transcription (Berger and Moller, 2002).

PPARs can be activated by a variety of different natural and synthetic ligands (Desvergne and Wahli, 1999). Long-chain polyunsaturated fatty acids and their lipo-oxygenase derived metabolite, leukotriene B4, are the natural ligands for PPARα, whereas hypolipidemic drugs from the fibrate family are specific synthetic ligands. Long-chain fatty acids are also the endogenous ligands for PPARδ, and there is evidence that PGI_2 is also a ligand for this PPAR isoform (Forman *et al.,* 1997). PPARγ is activated by the natural ligands 15-Deoxy-$\Delta^{12,14}$- prostaglandin J_2 (Forman *et al.*, 1995) and 9- and 13-hydroxyoctadecadienoic acid (Hampel *et al.,* 2006) as well as the insulin-sensitizing drugs from the thiazolidinedione (TZD) family, such as rosiglitazone and troglitazone, which are potent pharmacological ligands (Lehmann *et al.,* 1995; Murphy and Holder, 2000).

1.3.1.3 Phosphorylation

In addition to their direct ligand binding activation, PPARs are substrates for several kinases activated by a variety of endogenous or exogenous signals. PPAR phosphorylation regulates its trans-activating function, in a ligand-dependent or independent manner according to the isoform and/or cellular context (reviewed in Diradourian *et al.*, 2005).

Much of the literature on PPAR phosphorylation concentrates on PPARα. Treatment of hepatocytes with insulin has been shown to increase phosphorylation of this PPAR isoform. The extracellular signal-related kinase ERK-MAPK appears to be responsible for this phosphorylation (Shalev *et al.*, 1996). In addition, *in vitro* kinase assays have demonstrated that p38 MAPK phosphorylates serine residues located within the A/B domain of PPARα (Barger *et al.,* 2001) and calcium-dependent protein kinase (PKC) α and βII can phosphorylate the serine residues 179 and 230 and increase PPARα transcriptional activity in human liver cells (Blanquart *et al.*, 2004).

Of major interest to this project is the ability of PKA to phosphorylate PPARs. Lazennec and colleagues (Lazennec *et al.*, 2000) have shown that activated PKA results in the phosphorylation of all PPARs including PPARγ and this event was reversible using the PKA antagonist, H-89. Looking specifically at PPARα these investigators have shown that PKA-dependent phosphorylation enhances PPAR activity both in the absence and presence of exogenous ligands.

1.3.2 PPARγ

PPARγ plays a key role in the differentiation of adipocytes (Oberfield *et al.*, 1999). Interestingly there is evidence showing that carbacyclin too promotes terminal differentiation of preadipose cells from clonal lines and enhances differentiation of adipose precursor cells from murine or human adipose tissue (Negrel *et al.,* 1989; Vassaux *et al.*, 1992). The transcription factors C/EBPβ and C/EBPδ are critical for the differentiation of adipocytes and it has been demonstrated that the increase in cAMP levels caused by IP receptor activation is necessary and sufficient to up-regulate the rapid expression of these factors (Aubert *et al;* 2000).

Thiazolidinediones such as pioglitazone, troglitazone and rosiglitazone are all potent PPARγ ligands and are on the market as treatment for type II diabetes (reviewed in Meriden, 2004). Troglitazone however, was withdrawn due to hepatotoxic effects. They have been shown to decrease blood pressure in hypertensive rat models (Dubey *et al.*, 1993) as well as inhibit neointimal formation of balloon-injured vessels in rats (Law *et al.*, 1996). The latter may be a result of suppression of the mitogen activated protein kinase (MAPK) pathway (Goetze *et al.,* 1999) and inhibition of proliferation and migration of vascular smooth muscle cells by these ligands (Law *et al.*, 1996).

Thiazolidinediones have been reported to have both PPARγ dependent and independent effects. For example, troglitazone has been reported to decrease matrix metalloproteinase 9 (MMP9), the effect being mimicked by 15d-PGJ_2 suggesting involvement of PPARγ (Marx *et al.,* 1998). Interestingly, iloprost also has been shown to attenuate MMP-9 expression in

PASMC (Growcott *et al.*, 2006). However, iNOS mRNA expression can be induced by troglitazone and not 15d-PGJ_2 (Hattori *et al.*, 1999), highlighting potential PPARγ independent effects of this drug. The different effects of these two drugs may be explained possibly by their ability to recruit different PPARγ co-activators. The latter effects of troglitazone may be mediated in part through α-tocopherol (Law *et al.*, 2000). Because rosiglitazone lacks α-tocopherol, it is thought to be a more 'pure' ligand than the other thiazolidinediones and its vascular effects are likely to be mediated exclusively through PPARγ.

1.3.2.1 PPARγ and cell proliferation

There is a growing body of evidence for PPARγ mediating inhibition of cell proliferation. The PGJ_2 metabolite, 15-deoxy-$\Delta^{12,14}$-PGJ_2, a PPARγ ligand (Forman *et al.*, 1995; Kliewer *et al.*, 1995), as well as rosiglitazone, both strongly inhibit vascular smooth muscle cell proliferation (Sasaguri *et al.*, 1992; Ward *et al.*, 2004). Interestingly they induce G_1 arrest like PGI_2 and its stable analogues. This arrest is thought to be retinoblastoma (Rb)-dependent given that in the absence of Rb, activation of PPARγ results in cells accumulating in the G_2/M phase of the cell cycle, endo-reduplicating and undergoing apoptosis (Fajas *et al.*, 2003). 15d-PGJ_2 has also been shown to inhibit cyclin D1 expression and stimulate expression of the CDKI $p21^{Cip1}$ (Miwa *et al.*, 2000). $p21^{Cip1}$ is normally induced by several physiological stimuli that inhibit cell proliferation such as TGFβ and NO (Ishida *et al.*, 1997; Ishida *et al.*, 1999). In addition both troglitazone and rosiglitazone attenuated mitogen induced degradation of $p27^{Kip1}$, a CDKI which negatively regulates growth in a variety of cell types including

vascular smooth muscle cells, thus inhibiting cyclin-cdk activity and phosphorylation of Rb causing G_1 arrest (Sherr and Roberts, 1999).

Activation of PPARγ by both troglitazone and 15d-PGJ_2 can suppress thromboxane synthase gene transcription in macrophages (Ikeda *et al.*, 2000) as well as inhibiting thromboxane synthase mRNA expression and thromboxane-mediated cell growth in vascular smooth muscle cells (Sugawara *et al.*, 2002). It is thought that this effect of PPARγ is not achieved through binding to PPRE, but rather via a protein-protein interaction with the transcription activator protein Sp1 (Sugawara *et al.*, 2002). Also of interest is rosiglitazone's ability to increase prostanoid (PGD_2 and PGE_2) production in rat vascular smooth muscle cells at the level of both COX-2 expression and prostanoid formation (Bishop-Bailey and Warner, 2003). Moreover 15d-PGJ_2 activation of PPARγ induces endothelial cell apoptosis (Bishop-Bailey and Hla, 1999) and lack of PPARγ expression, via stable transfection of a dominant-negative PPARγ construct, inhibits apoptosis and facilitates cell growth and angiogenesis (Ameshima *et al.*, 2003).

Thiazolidinediones are also being tested as anticancer agents. There is however, controversy concerning the role of PPARγ in cancer. The anticancer evidence includes *in vitro* and *in vivo* studies suggesting inhibition of proliferation and differentiation (Sarraf *et al.*, 1998). In addition, enhanced PPARγ signalling has also been shown to restrain breast cancer (Suh *et al.*, 1999) and naturally occurring somatic mutations in the PPARγ gene have been reported in colorectal carcinomas (Sarraf *et al.*, 1999). Against this, there is evidence showing tumor promoting activity of PPARγ ligands in

mouse models of cancer (Lefebvre *et al.,* 1998; Saez *et al.*, 1998) as well as evidence suggesting that the anticancer effects of these agents are independent of PPARγ (Palakurthi *et al.*, 2001). This may highlight the importance of cell or tissue type in determining the eventual cellular effects of PPARγ activation.

Of major interest, is the apparent down-regulation of PPARγ in patients with IPAH (Ameshima *et al.,* 2003). Microarray gene expression screening has shown a decrease in PPARγ transcripts in random lung tissue samples from IPAH patients (Geraci *et al.*, 2001). PPARγ is normally abundantly expressed in human lung tissue especially in the endothelial cells. This expression was reduced or lacking in all angiogenic plexiform lesions of the pulmonary hypertensive lung as well as in the vascular lesions of a severe pulmonary hypertension rat model (Taraseviciene-Stewart *et al.*, 2001).

1.3.3 PPARδ

Unlike PPARγ, PPARδ is generally considered a stimulator of proliferation in a variety of cell types. The PPARδ agonist, GW501516 can stimulate proliferation of human breast and prostate cancer cell lines as well as increasing intestinal polyp growth (Stephen *et al.,* 2004). Moreover, this increase in cell proliferation was mainly observed in the absence of serum. GW501516 also prevented apoptosis stimulated by complete growth factor removal in colorectal cancer cell lines and this response was absent in cells lacking PPARδ (Stephen *et al.,* 2004). With regards to the vasculature, PPARδ has been shown to be up-regulated in vascular lesion formation and over-expression of this PPAR isoform in VSMC causes post-confluent cell proliferation by increasing both cyclin A and CDK2 as well as decreasing p57

(Zhang *et al.,* 2002). PPARδ activation appears to increase the expression of vascular endothelial growth factor (VEGF) in macrophages and VSMC (Bamba *et al.,* 2000; Yamakawa *et al.*, 2000) as well as enhancing COX-2 gene expression and increasing the proliferation of human hepatocellular carcinoma cells (Glinghammar *et al.,* 2003).

However there is also evidence supporting a pro-apoptotic role for PPARδ. Hatae and colleagues (Hatae *et al.,* 2001) even suggest that the apoptotic effects of prostacyclin are mediated by PPARδ. They showed that they could increase apoptosis in HEK-293 cells by over-expressing PGIS and that this effect was a result of endogenous PPARδ activation. In addition, Ali and colleagues (Ali *et al.,* 2006) suggest a role for PPARδ in mediating the antiproliferative effects of treprostinil in lung fibroblasts. However the authors of this study could only observe a small activation of PPARδ (in HEK-293 cells overexpressing this PPAR isoform) and this only occurred with high concentrations of treprostinil (30 μM or more). In addition they did not have an internal control for their PPARδ reporter gene assays. Furthermore, although treprostinil (100 μM) was marginally more potent in wild-type mice it was still able to inhibit proliferation in PPARδ knockout mice.

1.3.4 PPARα

PPARα is an important lipid sensor and regulator of the cellular energy-harvesting metabolism. It has been shown to play a key role in the regulation of cellular uptake, activation and β-oxidation of fatty acids (reviewed in Berger and Moller, 2002). PPARα has been shown to up-regulate the expression of the fatty acid transport protein (Martin *et al.,* 1997), a protein that transports fatty acids across the cell membrane. PPARα

is also thought to be involved in the transcription of long chain fatty acid acetyl-CoA synthase (Schoonjans *et al.,* 1996), ACO (Dreyer *et al.,* 1992), enoyl-CoA hydratase/dehydrogenase multifunctional enzyme (Marcus *et al.*, 1993) and keto-acyl-CoA thiolase (Zhang *et al.,* 1993), enzymes in the peroxisomal β-oxidation pathway.

PPARα has been a target for treatment of dyslipidemia and atherosclerosis. Fibrates, the main PPARα agonists, have substantial triglyceride lowering effects as well as modest HDL-raising efficacy (reviewed in Linton and Fazio, 2000). The mechanism by which PPARα lowers triglycerides is likely to include the suppression of hepatic apo-CIII gene expression while also stimulating LPL gene expression (Schoonjans *et al.,* 1996; Blanquart *et al.*, 2004). In addition, PPARα agonists also appear to have anti-inflammatory effects, particularly in VSMC (Devchand *et al.,* 1996; Chinetti *et al.,* 2001).

Novel data shows that PPARα may also have a role in controlling smooth muscle proliferation. Gizard and colleagues (Gizard *et al.*, 2005) have shown that the PPARα agonist, GW7647, inhibits proliferation of VSMC by controlling cell cycle progression into the S phase, similar to what has been demonstrated with PPARγ activation. PPARα appears to cause G_1 phase arrest by inducing the cell-cycle regulator and tumor suppressor gene p16. Although there is emerging data on the anti-proliferative effects of PPARδ (Ali *et al.,* 2006) and PPARα (Gizard *et al.,* 2005), the greatest body of evidence still concentrates PPARγ as the main regulator of VSMC proliferation.

1.4 Thesis aims

The main aim of the present study is to improve our understanding of the mechanism(s) by which PGI_2 analogues inhibit cell proliferation and to address the role of the IP receptor in mediating these effects.

In order to elucidate the importance of the IP receptor, a stable HEK-293 cell line expressing this receptor was used to examine the effects of i) PGI_2 analogues and ii) the antagonists of various transduction components in the signalling pathway, on cell proliferation and cAMP production.

Because there is evidence in the literature that PKA can phosphorylate and activate PPARγ (Lazennec *et al.*, 2000) and that some PGI_2 analogues can also directly bind to PPARα and PPARδ (Forman *et al.*, 1997), we hypothesised that PGI_2 analogues would also activate PPARγ and that this pathway would contribute to their anti-proliferative effects. Using a specific PPARγ reporter gene assay, we sought to determine whether PPARγ could be a downstream signalling component of the IP receptor pathway or act as an alternative intracellular target for PGI_2 analogues (Figure 1.8).

Finally, we sought to establish the role of the IP receptor in native human PASMC and compared the signalling mechanism observed in cells from 'control' patients to those derived from IPAH patients. Because of the faster rate of proliferation observed in IPAH versus control PASMC and in light of results obtained from the HEK-293-IP stable line we hypothesised that IPAH PASMC may lack or have down-regulated IP receptor expression. We sought to investigate whether this was true and whether PGI_2 analogues could still inhibit their proliferation.

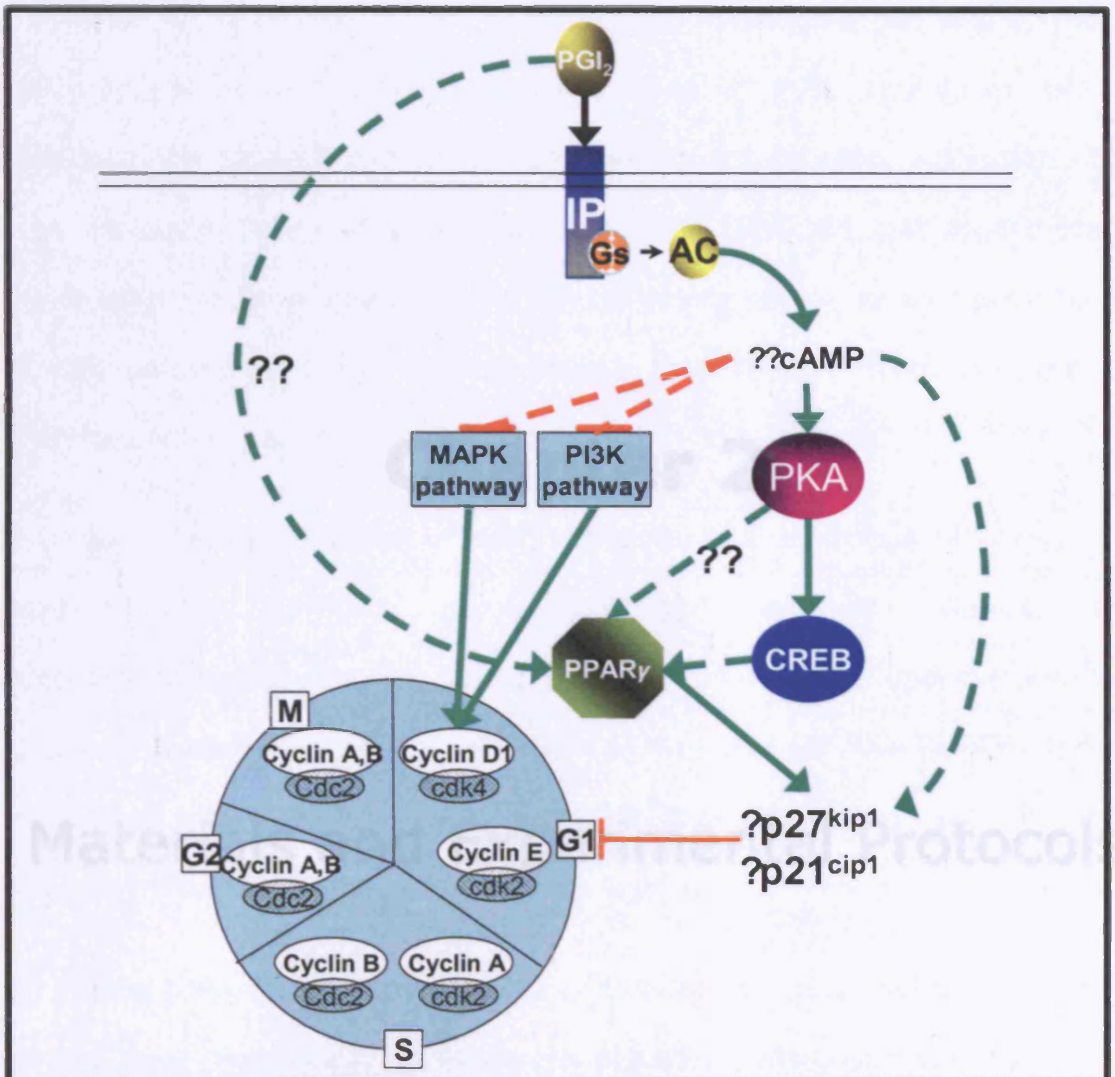

Figure 1.8 Potential pathways mediating the anti-proliferative effects of PGI_2 analogues. $PPAR_\gamma$ may be activated directly by cell permeable analogues or be a downstream target for PKA. (PGI_2) prostacyclin, (IP) prostacyclin receptor, (AC) adenylyl cyclase, (PKA) proein kinase A, (cdk) cyclin dependent kinase, (CREB) cAMP response element binding protein, (MAPK) mitogen activated protein kinase, (PI3K) phosphoinositide-3 kinase.

Chapter 2

Materials and Experimental Protocols

2.1 Introduction

The main focus of the present study was to investigate the role of the IP receptor in the anti-proliferative properties of PGI_2 analogues and to elucidate the signalling pathway downstream of receptor activation. This was assessed firstly in a newly established HEK-293 cell model stably expressing the IP receptor, or the control empty vector, as well as in native human pulmonary artery smooth muscle cells isolated from the lungs of IPAH or control patients.

Expression and localisation of the IP receptor in the various cell types was explored by RT-PCR, using especially designed primers, and immunofluorescent staining, using a novel IP-receptor specific antibody designed during this project. Functionality of the receptor was tested by measuring increases in intracellular cAMP upon stimulation with the PGI_2 analogue treprostinil with an ELISA-like assay.

To assess the proliferative response of cells to PGI_2 analogues a simple yet strong and reproducible proliferation assay was optimised using an automated cell counter. Various antagonists and other pharmacological tools were used in this assay to dissect potential signalling pathways, including the novel IP receptor antagonist RO1138452 developed by Roche (Palo Alto, California) (Bley *et al.,* 2006). To test the hypothesis of a potential role for PPARγ in the PGI_2 signalling pathway a dual reporter luciferase assay was optimised to measure PPARγ activation and to take into account the non-specific effects of our drugs of interest on full length promoters present in many commercially available luciferase plasmid constructs. The protocol was used extensively to attempt to dissect the

mechanism of activation of this nuclear receptor. The present chapter outlines more specifically all the assays, techniques and tools used in all the experiments contributing to this thesis as detailed above.

2.2 Cell culture

2.2.1 General Features

The cells used in this project were principally HEK-293 cells, an immortalised cell line of human origin derived from primary embryonic kidney cells transformed by exposure to sheared adenovirus DNA (adenovirus type 5)(Graham *et al.,* 1977). Their morphology is markedly epithelioid and they tend to continue dividing even after confluence is reached. A stable HEK-293 line expressing the IP receptor gene was established by Prof. Andrew Tinker at UCL and was used extensively in this project. These cells were generated by transfecting in a eukaryotic expression vector, pcDNA3.1/Zeo (Invitrogen, Paisley, UK), into which the human IP receptor gene was cloned. HEK-293 cells stably transfected with pcDNA3.1Zeo were generated as a control.

Human pulmonary artery smooth muscle (HPASM) cells were also used extensively in the project. Cells originated from the distal pulmonary arteries of the explanted lungs of 3 children suffering from IPAH who underwent lung or heart/lung transplantation and adult ('control') samples were obtained at lobectomy for bronchial carcinoma and lung transplantation from 3 patients with normal pulmonary arteries (Female, 54 years, lobectomy; Male, 51 years, lung transplant for emphysema; Male, 57 years, lung transplant for emphysema; courtesy of Dr. John Wharton,

Imperial College London; ethical approval, Brompton Harefield & NHLI REC 01-210).

2.2.2 Stable transfection of HEK 293 cells

A suspension of HEK-293 cells was transfected with pcDNA3.1/Zeo using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 2 µg of DNA was mixed with 250 µl of OptiMEM (Invitrogen) and 2.5 µl of Lipofectamine 2000 was mixed with another 250µl of OptiMEM. Both were incubated for 5 mins at room temperature prior to mixing the two together and incubating for a further 20 mins at room temperature. 400 µl of the DNA/Lipofectamine mixture was added to 2 ml of cells at a concentration of 1.3×10^6 per ml and mixed gently by pipetting followed by gentle shaking for 10 minutes. Transfected cells were then plated onto 6 well plates containing MEM (Invitrogen) + 10% FBS and no antibiotics. One 6-well plate would contain untransfected cells as a control. 48 hours post transfection, the media was replaced with media containing 400 µg/ml of zeocin to select for transfected cells. Following approximately one week of culture in the selective medium, individual zeocin resistant colonies were isolated using a sterile thin tipped cotton bud and transferred to 96 well plates for eventual expansion to T-75 flasks.

2.2.3 Isolation and characterisation of human peripheral pulmonary arterial smooth muscle cells (PASMC)

Three children suffering from IPAH had a bilateral lung or heart/lung transplant and the explanted lungs were taken with parental consent and local ethical permission (REC# 05/Q0508/45) and used for isolation of

pulmonary arterial smooth muscle cells. The children were 1) (IPAH 1; 1664) male aged 7 years 2) (IPAH 2; 1666) female aged 5 years and 3) (IPAH 3; 1672) male aged 5 years. All three had been receiving a continuous intravenous infusion of epoprostenol for 2 years combined with bosentan for more than 1 year. Lungs were obtained immediately after transplantation and bathed in ice cold PBS during dissection. Several peripheral pulmonary arteries were dissected away from the peripheral airway tree in one lower lobe and the adherent lung parenchyma and the adventitial layers were removed. 8-10 terminal branches were taken, each having an external diameter of approximately 250 μm. These arteries were collected into phosphate buffered saline (PBS) (Sigma) containing penicillin (300 μg/ml), streptomycin (300μg/ml) and gentamycin (180 μg/ml) where they remained for 45 mins at 4°C. They were then dissociated by incubation in a protease cocktail, containing elastase (Lorne Laboratories, Reading, UK) 0.125 mg/ml, collagenase (Sigma) 0.25mg/ml, trypsin inhibitor (Sigma) 0.06 mg/ml, bovine serum albumin 3.75 mg/ml and MEM vitamins 2.5μl/ml dissolved in DMEM/F12 buffered with HEPES and $NaHCO_3$ at 37°C, with continuous agitation for 30-45 mins. Dissociation was stopped by addition of growth medium (DMEM/F12 + 10% foetal bovine serum & penicillin and streptomycin) and cells were pelleted by centrifugation at 350g. They were re-suspended in growth medium and plated into uncoated 25 cm^2 flasks. In all the primary cultures the majority of cells settling and growing were fusiform and poorly spread with lamellipodia at both leading and trailing edges. Colonies of epithelioid cells were only seen in primary cultures. Cells were maintained in growth medium. At passage 1 and all later passages the cells grew rapidly and formed dense multilayered sheets of weakly oriented

fusiform cells that had a structure distinct from that of fibroblasts isolated from the adventitia of the proximal arteries of the same lung. Further characterisation of the PASM cells was carried out by immunohistochemical staining of confluent cell monolayers for smooth muscle-specific proteins, α-smooth muscle actin (clone 1A4, Sigma) and smooth muscle myosin heavy chain (MHC, clone SMemb, Yamasa Corp, Japan). In addition samples from each cell isolate were electrophoresed and blotted onto nitrocellulose then stained to show expression of α-smooth muscle actin, smooth muscle MHC (clone hSM-v, Sigma) and caldesmon (clone hHCD, Sigma). The isolation and characterisation of these PASM cells was carried out by Dr. Sue Hall, Institute of Child Health, UCL.

2.2.4 Cell maintenance and subculture

HEK-293 cells were grown in MEM containing Earle's salts and L-glutamine (Invitrogen, Paisley, UK) supplemented with 10% foetal bovine serum (FBS) (of European origin; Invitrogen) and 1% Penicillin-Streptomycin (from a 10,000 units/ml stock) (Invitrogen). IP receptor stable line cells were selected for by adding zeocin (400μg/ml) (Invitrogen) to the medium. DMEM/F12 1:1 with 10% FBS and penicillin/streptomycin media was used for smooth muscle cells. All cell types were kept at 37°C in a humidified incubator with 95% air and 5% CO_2. Cells grew as a monolayer on the surface of plastic tissue culture flasks (Sarstedt, Leicester, UK). All work with live cells was carried out under sterile conditions in a laminar flow tissue culture hood, using sterile and autoclaved equipment.

Cells were typically sub-cultured when they became 80-90% confluent. The medium was aspirated off, cells were washed with Ca^{2+} and Mg^{2+} free

phosphate buffered saline (PBS) (Invitrogen) warmed at 37 °C removed and then treated with enough 1X trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA·4Na; Invitrogen) to cover the flask for 1-2 mins. When all cells were dislodged, an excess of media containing FBS was added to neutralize trypsin. Cells were pelleted by centrifugation at 300 g for 5 mins at 25 °C and re-suspended in fresh medium at an approximate density of 1-2 × 10^6 cells/ml. Cells were then plated in new sterile culture flasks in a ratio ranging from 1:10-1:15 (or as required by protocol) by dilution in fresh medium.

HEK-293 cells were utilised for experimental procedures between passages 4 and 12. HPASM cells were utilised between passages 6 and 10.

2.2.5 Freezing down and reviving cell lines

If cells needed to be stored over a long period of time they were frozen down and cryo-preserved in liquid nitrogen. This was done by dislodging the cells from the flask with trypsin and pelletting them. They were then re-suspended in MEM containing 10% FBS, 1% Pen/Strep and 10% DMSO, aliquoted in 1 ml cryovials (5 aliquots were obtained from a confluent T-75 flask) and quickly chilled on ice. Subsequently they were kept at 4 °C, -20 °C and -80 °C for 20 mins at each temperature in order that the cells should freeze down gradually before being placed in a liquid nitrogen cell bank. To revive cells from their frozen state, they were de-frosted in a 37 °C water bath and immediately re-suspended in 5 ml of fresh medium and plated in a T-25 flask. After 24 hr cells were washed with PBS to remove all traces of DMSO and the media replaced. Cells were then cultured as normal.

2.3 Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

2.3.1 Total RNA isolation from mammalian cells

Total RNA isolation from cultured cells was achieved using an RNeasy Mini kit (Qiagen, Crawley, UK). Cells from a confluent T75 culture flask were washed with PBS then harvested by treatment with trypsin/EDTA for 2 mins, pelleted by centrifugation at 300g for 5 mins and the supernatant removed. Cells were disrupted and re-suspended in 600 µl of lysis buffer RLT containing 6µl β-mercaptoethanol. The sample was then homogenised thoroughly before adding 600 µl of 70% ethanol. 600 µl of the sample was then applied to an RNeasy mini column, centrifuged for 30 seconds at 16000 g and the flow-through discarded. This step was repeated with the remaining 600 µl of lysate. Following this, 700 µl of wash buffer RW1 was applied to the column, centrifuged for 30 s and again the flow-through was discarded. The column was washed twice with 500 µl of buffer RPE, once for 15 s and then for 2 mins. The column was finally transferred to a sterile 1.5ml Eppendorf and the RNA eluted with 25-50 µl of molecular biology grade water.

2.3.2 Measurement of DNA and RNA concentration

The concentration of purified DNA and RNA was measured by recording absorbance at 260 nm of a 1 ml solution of molecular biology grade water containing between 2 and 10 µl of RNA or DNA obtained from the final 25µl-50µl elution in the previous step (~0.05-0.2 µg) in a spectrophotometer (Cecil CE2041; Cecil Instruments, Cambridge, UK) using a quartz cuvette.

Before measuring the samples the spectrophotometer was zeroed using water alone. Using the relationship of 1 absorbance unit = 50 µg/ml of double stranded DNA or 40 µg/ml of RNA, and taking into account the dilution factor, the amount of DNA or RNA could be calculated. All RNA and DNA preparations were re-dissolved in molecular biology grade water or endotoxin free buffer TE (Qiagen) and stored at -20 °C when not in use.

2.3.3 Forward strand cDNA synthesis using SuperScript™II reverse transcriptase

DNA polymerase SuperScript™II reverse transcriptase (RT) (Invitrogen) was used to synthesise the complementary DNA strand from the total RNA isolated from cells. This is a point mutant of Moloney murine leukaemia virus RT which produces higher cDNA yields by eliminating RNase H activity. 5 µg of total RNA was used as a template, 250 ng of random primers and 1 µl of dNTP mix from a 10 mM stock were added and the volume was made up to 12 µl with molecular biology grade water. The mixture was heated to 65 °C for 5 mins and then quickly chilled on ice. 4 µl of 5X first-strand buffer (250 mM Tris-HCl pH8.3, 375 mM KCl, 15 mM $MgCl_2$) and 2 µl of 0.1 mM dithiothreitol (DTT) were then added and incubated at 42 °C for 2 mins prior to addition of 1 µl of SuperScript™II RT. The reaction was then incubated at 25 °C for 10 mins followed by 50 mins at 42 °C which allows the RT to work optimally. Finally the reaction was inactivated by heating at 70 °C for 15 mins.

2.3.4 Polymerase chain reaction

The polymerase chain reaction allows amplification of a target segment of DNA by up to a billion fold by design of apposite oligonucleotide primers complementary to the nucleotide sequence flanking both ends of the DNA sequence. Suitable primers were designed using OLIGO™ software that will work out an oligunucleotide sequence of approximately the right length, with an appropriate GC content and will also suggest an optimal annealing temperature. PCR requires unique DNA polymerase enzymes that are stable at very high temperatures (e.g. Taq polymerase, isolated from thermophile bacteria).

PCR can be summarised into three steps: 1) denaturation; the double stranded DNA template is separated at a temperature of 95 °C 2) annealing; an optimal temperature around 55 °C allows hybridisation of primers to the DNA template and finally 3) DNA synthesis; the DNA polymerase 'elongates' the primer using the dNTPs provided in the reaction mixture to synthesise the complementary strand of the template downstream of the primers. This cycle is usually repeated between 25 and 35 times resulting in exponential amplification of the required DNA sequence flanked by the oligonucleotide primers.

In this project Taq polymerase was used together with "Thermopol" buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton® X-100 pH 8.8) provided by the supplier or in some reactions the optimal concentration of Mg^{2+} was adjusted using a stock solution of $MgCl_2$. dNTPs were supplied as 100 mM stock solutions and were mixed in a 1:1:1:1 (dATP, dGTP, dCTP, dTTP) ratio to give a final concentration of 25

mM for each dNTP. Primers were synthesised commercially by Sigma-Genosys (Pampisford, UK) and dispatched as desiccated stocks. These were dissolved in molecular biology grade water to obtain a final concentration of 100 pmol/µl and the volume used was adjusted according to the concentration of oligonucleotide DNA used. Reactions were carried out in 0.2 ml eppendorf tubes placed in a Peltier thermal cycler (DNA Engine Dyad®, MJ Research, Waltham, USA) and run following an appropriate cycling protocol.

2.3.5 RT-PCR of human IP receptor in cultured cells

To determine the presence or absence of RNA message for the human IP receptor in the various cell lines used in these studies, we performed PCR, using IP receptor specific primers, on cDNA synthesised from the total RNA isolated from these cells. Negative controls were carried out both by performing the RT step in the absence of superscript II RT as well as performing the PCR step in the absence of cDNA. Primers were designed to span across two exons (so as to be able to exclude the amplification of any genomic DNA which may be a contaminant in the cDNA preparation) and amplified a 204 bp fragment of the IP receptor. Primers were also designed to amplify a 363 bp sequence of the human Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, a house keeping gene used as an internal control.

DNA sequences of primers:

Human IP receptor, accession id: NM_000960.3

Forward 5′ ATGTACCGCCAGCAGAAGCG 3′

Reverse 5′ GAAGCGGAAGGCAAGGAGCTC 3′

Human GAPDH, accession id: NM_002046

Forward 5′ AAGGTGAAGGTCGGAGTCAACG 3′

Reverse 5′ GGCAGAGATGATGACCCTTTTG 3′

PCR was carried out using the following cycling protocol with both sets of primers: 1.) 95 °C for 4 mins 2) 95°C for 30 s 3) 60 °C for 30 s 4) 72 °C for 30 s 5) 72°C for 10 mins 6) 4 °C. Steps 2, 3 and 4 were repeated 30 times. Products of the PCR reaction were loaded onto a 1.5% agarose gel following addition of DNA loading buffer (5:1 ratio) to facilitate the loading of samples into the wells. A 100 base-pair (bp) DNA ladder was also loaded onto the gel in order to assess the size of the PCR products. Gels were prepared by dissolving 1.5 g of agarose (Sigma Aldrich, Poole, UK) in 100 ml of 1X Tris acetate EDTA (TAE). The solution was heated at full power in a microwave and allowed to cool slightly before the addition of 10 µl (100 µg) of the fluorescent intercalating dye ethidium bromide needed to visualise DNA bands. The gel was allowed to set in a slab configuration with the insertion of a plastic comb to generate wells for DNA loading. Prior to running, the gel was placed in an electrophoresis tank filled with fresh 1X TAE buffer and allowed to equilibrate. An electrophoresis voltage of 100 V was applied across the electrodes of the tank. The movement of the tracker dyes, bromophenol blue and xylene cyanol contained in the DNA loading buffer was used to judge sample migration distance. Once samples had migrated an appropriate distance, gels were viewed under ultra-violet (UV) light

(312nm wavelength) using a dual intensity transilluminator (UVP Inc., Upland, California) equipped with a digital video camera set up to acquire, import and print photographs of the gel. The appropriate bands were excised from the gel and the DNA was extracted and purified using a Qiaex II gel extraction kit (Qiagen, Crawley, UK) before being sent to Cytomix (Cambridge, UK) for sequencing. Briefly gel sections were weighed and incubated for 10 mins with 3 volumes of QX1 buffer and 10 µl of QIAEX II at 50 °C. This allowed the agarose gel to dissolve while the nucleic acids were adsorbed onto the Qiaex II silica-gel particles. Samples were centrifuged for 30 s and the supernatant removed. The pellet was washed in QX1 buffer once and PE buffer twice before being left to air-dry completely. The pellet was then resuspended in 20 µl molecular biology grade water, incubated at room temperature for 5 mins then centrifuged. The supernatant containing purified DNA was quantified and sent for sequencing.

2.4 Determination of IP receptor protein expression and localization using antibodies.

2.4.1 Design of novel IP anti peptide antibody.

When the project was started there was no good commercially available specific IP receptor antibody. The Cayman antibody became available in the autumn of 2004. In 1998, Komhoff *et al* had used a polyclonal antibody generated against a synthetic peptide representing the N-terminal extracellular loop of the IP receptor to localize the receptor in the kidney (referred to from now on as the N1 antibody). In order to provide us with a tool for investigating IP receptor protein expression and localisation, we developed a novel polyclonal IP receptor anti-peptide antibody as well as

generating a stock of antibody raised against peptide sequence used by Komhoff and colleagues. Both the Cayman and the N1 antibodies were designed to recognise a peptide sequence within the extracellular N terminal tail of the IP receptor. The peptide sequence of the N1 antibody is RNLTYVRGSVGPAT, whereas the Cayman antibody targeted an overlapping sequence shifted to the N terminus (MADSCRNLTYVRGSVG). Because these antibodies were directed to the N-terminal, a C-terminal peptide sequence was used to generate a novel antibody (RRDPRAPSAVGKE, amino acids 329-343). Peptides corresponding to the amino acid sequences for the C1 and N1 antibodies were synthesized and conjugated to haemocyanin before injection into rabbits using standard protocols (Eurogentec Seraing, Belgium). To ensure that the antibodies were specifically reacting with the peptide antigen it was necessary to affinity purify the antibodies present in the terminal bleed. This was achieved by immunoaffinity purification against the peptide antigen coupled to a solid support. Immunoaffinity purification was carried out using the affigel gel-10 (Biorad, Hemel Hempstead, UK) by Ms Nuria Battle, a technician in the laboratory.

2.4.2 Immunofluorescent staining

Cells were cultured on 8 well Falcon™ slides (BD Biosciences, Oxford, UK) coated with a 0.01% (w/v) solution of poly-L-lysine (Sigma), washed briefly with PBS and fixed with 4% paraformaldehyde (Sigma) for 10 mins at 4 °C. Cells were washed again with PBS twice for 10 minutes at 4 °C prior to permeabilisation with 0.4% Triton X-100 (Sigma) for 20 mins at 4 °C. Cells were washed as before and incubated for 4 hr with an excess volume of blocking solution (2% (w/v) fraction V bovine serum albumin (Sigma), 5%

(v/v) goat serum (Sigma), 0.2% Triton X-100 (Sigma). During the blocking step anti-hIP antibodies (Cayman or personally designed) were prepared in PBS and centrifuged at 10,000 g for 5 mins at 4 °C to remove any aggregated material. To check for non-specific binding of the primary antibody, antibodies would be pre-incubated with the control peptide (4 µg/µl of antibody) for three hr to allow for the antibody to bind to the peptide. 100 µl of primary antibody (± control peptide) (1:100 dilution) was layered onto each well and incubated overnight in a moist chamber at 4 °C. Cells were washed with PBS four times for 15 mins at 4 °C and 100 µl of goat 488-conjugated anti-rabbit IgG, Alexa Fluor 488 (Invitrogen), together with a nuclear stain, TO-PRO-3 (Invitrogen), were applied (1:300 dilution) and incubated for 1 hr in a moist darkened chamber at room temperature. Cells were again washed in the dark, three times with PBS and once with distilled water to remove any residual salts. Slides were allowed to air-dry completely following the removal of the multi-chamber unit. Vectashield (Vector Laboratories Inc, Burlingame, USA), a non fluorescent anti-fade specialised mounting medium, was layered onto the slides before fixing cover slips in position with commercially available clear nail varnish. Slides were stored at −20 °C.

2.4.3 Visualisation of immunostained cells

Slides were viewed and analysed at X60 magnification (water immersion lens) using a Bio-Rad Radiance 2000 laser scanning confocal head mounted on a Nikon TE1000 (Hemel Hempstead, UK). Green fluorescence of FITC/Alexa 488 and far red fluorescence of TO-PRO-3 were measured at an excitation/emission wavelength of 492/520 nm or 644/657 nm,

respectively. Images of cells were taken at a focal plane taken from the middle of the cell of 0.1µm thickness. Images were studied using Bio-Rad LaserPix 4.0 software.

2.5 Measurement of cAMP levels

Cyclic AMP is thought to be the main second messenger mediator in the IP receptor signalling pathway. It is synthesized from ATP by membrane-bound adenylyl cyclase and is neutralized by hydrolysis to AMP by phosphodiesterases; therefore the concentration of cAMP in a cell is a function of the ratio of the rate of synthesis from ATP by adenylyl cyclase and its rate of breakdown to AMP by specific phosphodiesterases. Measuring cAMP was important in the present study to investigate receptor function and signalling.

2.5.1 cAMP sample preparation and purification

Cells were grown to 70-80% confluence in 6 well plates then starved in low serum (0.1%) medium for 48 hr. Media was then changed to one containing 10% FBS ± treatment and cells were incubated for 30 mins. In the case of treatment with antagonists, cells were pre-treated for 1 hr with the antagonists prior to addition of treprostinil for 30 mins.

Media was then aspirated from the plates and these were washed once with PBS. 250 µl of 0.1M HCl (VWR international, Lutterworth, UK) was then added to each well and incubated for 20 mins at room temperature. Cells were scraped with a rubber policeman and further dissociated by pipetting up and down until the suspension was homogenous and transferred to an

Eppendorf tube, centrifuged at 1000g for 10 mins and the supernatant then decanted into a clean test tube.

2.5.2 Protein assay

In order to correct for total protein concentration in experiments, a standard colorimetric assay based on the Bradford assay (Bradford, 1976) was used on the cell extracts. An eight point standard curve (0 to 2 mg/ml) of bovine serum albumin (BSA, Invitrogen) was loaded in triplicate on a clear 96 well plate. Cell extracts were diluted sufficiently to ensure that the protein concentration was in the range of the standard curve and loaded in triplicate into separate wells. 25 µl of reagent A (Biorad) followed by 200 µl of reagent B were then added to each well. The plate was gently shaken for 15 mins and the absorbance read at 690 nm in a GENios absorbance plate reader (TECAN, Reading, UK). Protein concentration of samples was calculated from the standard curve, by first subtracting the background reading from the blank, and then finally multiplying by the dilution factor of the sample.

2.5.3 cAMP enzyme linked immunoassay

To measure the cAMP levels in cell samples a competitive enzyme immunoassay was used (Cyclic AMP ACE EIA kit, Cayman Chemical, Ann Arbor, MI). This assay is based on competition between free cAMP and a cAMP-acetyl cholinesterase (AChE) conjugate (cAMP tracer) for a limited number of cAMP-specific rabbit antibody binding sites. Because the concentration of cAMP tracer is kept constant whereas the concentration of cAMP in the samples varies, the amount of cAMP tracer which binds to the

antibody is inversely proportional to the concentration of cAMP in the sample. This antibody-cAMP (either free or tracer) complex binds to the mouse monoclonal anti-rabbit IgG that has been previously attached to the well. Once the plate has been washed to remove any unbound reagents the amount of cAMP in a sample can be quantified by developing the plate using Ellman's reagent which contains the substrate to AChE. This enzymatic reaction emits a yellow colour that absorbs strongly at 412 nm and subsequently can be determined spectrophotometrically. A summary of the protocol is shown in Figure 2.1. AChE has several advantages over other enzymes commonly used for enzyme immunoassays: it does not self inactivate during turnover which allows multiple development of the assay if accidentally spilled. In addition, it is highly stable under assay conditions and it is not inhibited by common buffer salts and preservatives.

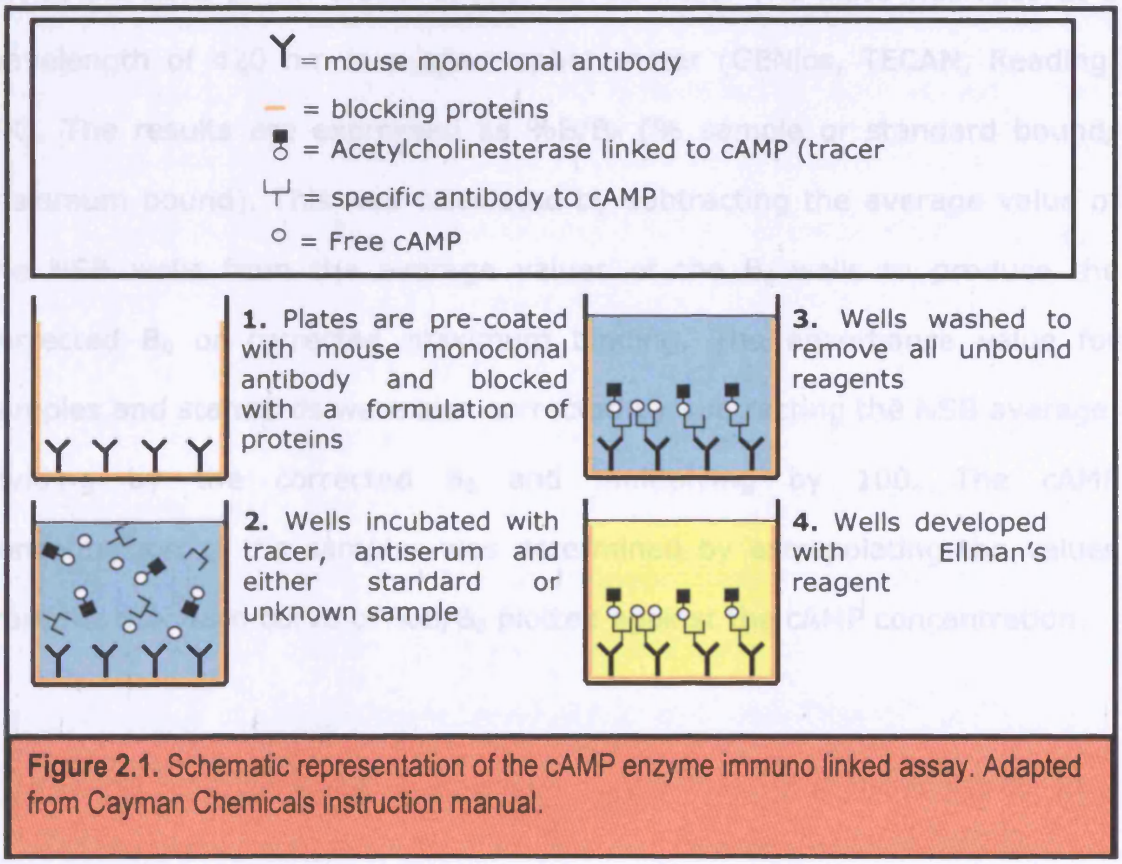

Figure 2.1. Schematic representation of the cAMP enzyme immuno linked assay. Adapted from Cayman Chemicals instruction manual.

The assay was performed following manufacturer's instructions. Briefly, plates were set up to contain two blank (Blk) wells, two non-specific binding (NSB) wells, two maximum binding (B_0) wells and an eight point standard curve run in duplicate. Each sample was also assayed in duplicate. Samples for the standard curve were prepared by serially diluting a provided stock solution of 3000 pmol/ml cAMP, and adding these to the appropriate wells. Cell samples to be tested were diluted to a final protein concentration of 0.5mg/ml and added to the appropriate wells. Blank wells were left empty, all other wells were treated with 100 µl of EIA buffer followed by 50 µl of cAMP AChE tracer and 50µl of cAMP antiserum (apart from NSB wells where the latter was not added). The plate was covered and incubated for 18 hours at 4°C. The plate was then washed 5 times with the provided wash buffer and developed by adding 200µl of Ellman's reagent to each well and left in the dark on an orbital shaker for 90 mins. The plate was read at a wavelength of 420 nm in a spectrophotometer (GENios, TECAN, Reading, UK). The results are expressed as $\%B/B_0$ (% sample or standard bound/ maximum bound). This was calculated by subtracting the average value of the NSB wells from the average values of the B_0 wells to produce the corrected B_0 or corrected maximum binding. The absorbance value for samples and standards were also corrected by subtracting the NSB average, dividing by the corrected B_0 and multiplying by 100. The cAMP concentration of the samples was determined by extrapolating the values from the standard curve of $\%B/B_0$ plotted against the cAMP concentration.

2.6 Cell proliferation assays

To assess proliferation, cells were plated in 6 well plates at a density of 1 or 2 x 10^4/ml, in 2 ml of media containing 10% FBS. Cells were allowed to grow for 24 hr before starving them in a low serum medium (0.1%) or, in the case of HEK-293 WT, in media without serum. Cells were then kept in 'starving' conditions for 48 hr. Subsequently cells were incubated for 48 hr in media containing either 10% FBS (± treatment or solvent control) or 0-0.1% serum (control). The same batch of FBS was used for the majority of the experiments presented in this thesis to limit the variability in terms of growth factor composition and concentration. The same media and plating cell density were used for those studies in which proliferative characteristics of the cells were directly compared. For this purpose cells were incubated with media containing 10% FBS and were counted at 24 hr intervals (± treatment). Samples were prepared for counting by washing the cells with PBS, dislodging them with 500 µl of trypsin/EDTA and re-suspending thoroughly with MEM containing 10% FBS in a total volume of 2 ml. 500 µl of cell suspension was diluted at a ratio of 1:10 in cell-pack diluent (Malvern Instruments Ltd, Malvern, UK) to give a total volume of 5 ml in the sample beaker. Cells were counted using an automated cell counter, Sysmex F-520P (Malvern Instruments Ltd, Malvern, UK) which uses a direct current electronic resistance method of particle counting and sizing. The particle-counting detector block on the instrument has a transducer with an aperture of 100 µm and an internal and external electrode (Figure 2.2). The cell pack diluent is electrically conductive and when the transducer and electrodes are immersed in it allows a constant direct current to flow between the internal and external electrodes. When a cell passes through

the aperture during the counting process it decreases the flow of current, increasing the resistance between the electrodes. This resistance causes a voltage change between the electrodes proportional to the volume of the cell. These small voltage changes are detected and amplified by the instrument and electrical interference or artificial signals are eliminated by an electronic filter circuit.

The particle size distribution analyzer, PDA-500, processes particle detection signals from the F-520P and works out particle counts and information on particle volume. These data are transferred to a PC which analyses volume information for each particle and will calculate and display particle distribution in terms of volume or diameter equivalent to volume as well as total count per ml. Figure 2.3 shows an example particle distribution analysis and cell count using this set-up. Here, HEK cells expressing the IP receptor are counted with no FBS, with FBS, and with FBS + UT-15.

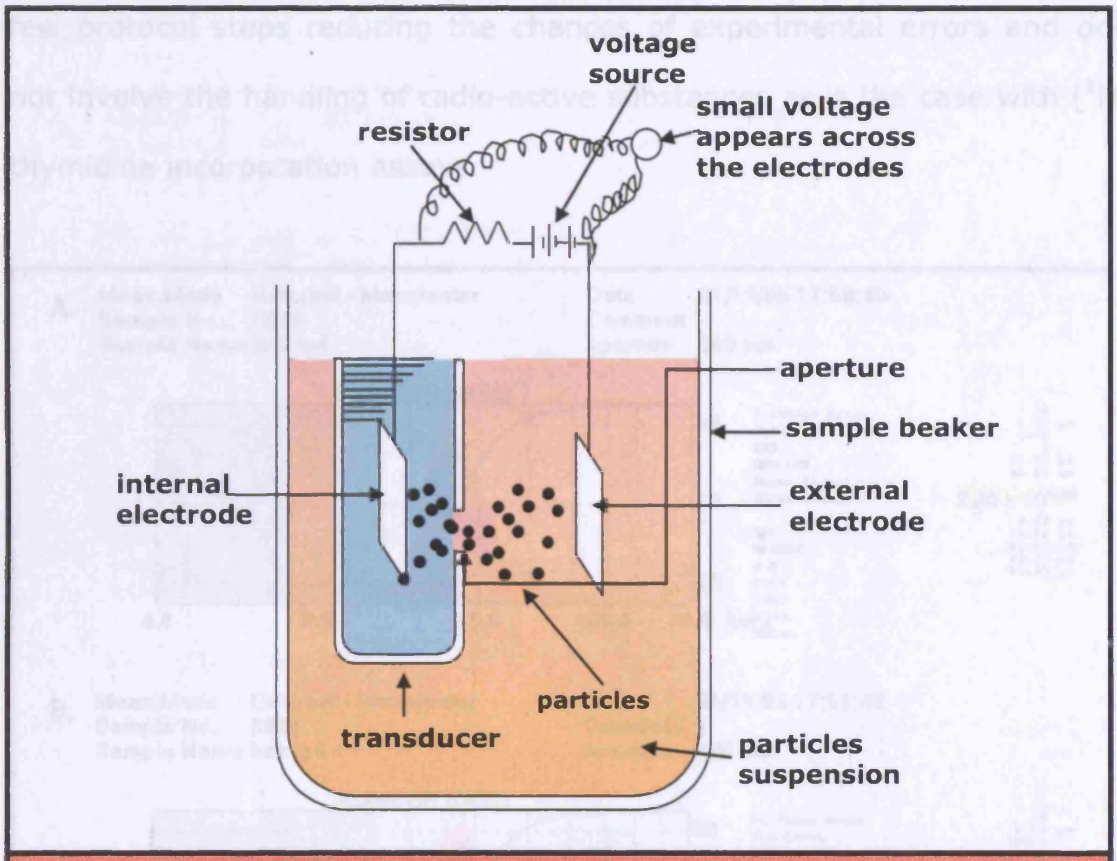

Figure 2.2. Sysmex F-520P particle-counting detector block. Adapted from the Sysmex F-520P Operator's manual

Particle distribution analysis allows the determination of dead or lysed cells as they are represented as a smaller peak ahead of the main peak on the distribution curve. PDA-500 allows you to reanalyse the curve to exclude counts from such peak (Figure 2.4). Checks were performed to ensure the accuracy of our cell counter by recounting samples using a haemocytometer and trypan blue (0.4 %; Invitrogen) exclusion, table 2.1 shows the direct comparison data for figure 2.3 obtained using the latter method.

This protocol was preferred to other methods used to measure cell proliferation, such as the tritium-labelled ([^{3}H]) thymidine incorporation and MTT yellow tetrazolium salt (MTT) assays due to its low cost and high throughput capabilities. It is simple, requires little cell manipulation and has

few protocol steps reducing the chances of experimental errors and does not involve the handling of radio-active substances as is the case with [^{3}H]-thymidine incorporation assays.

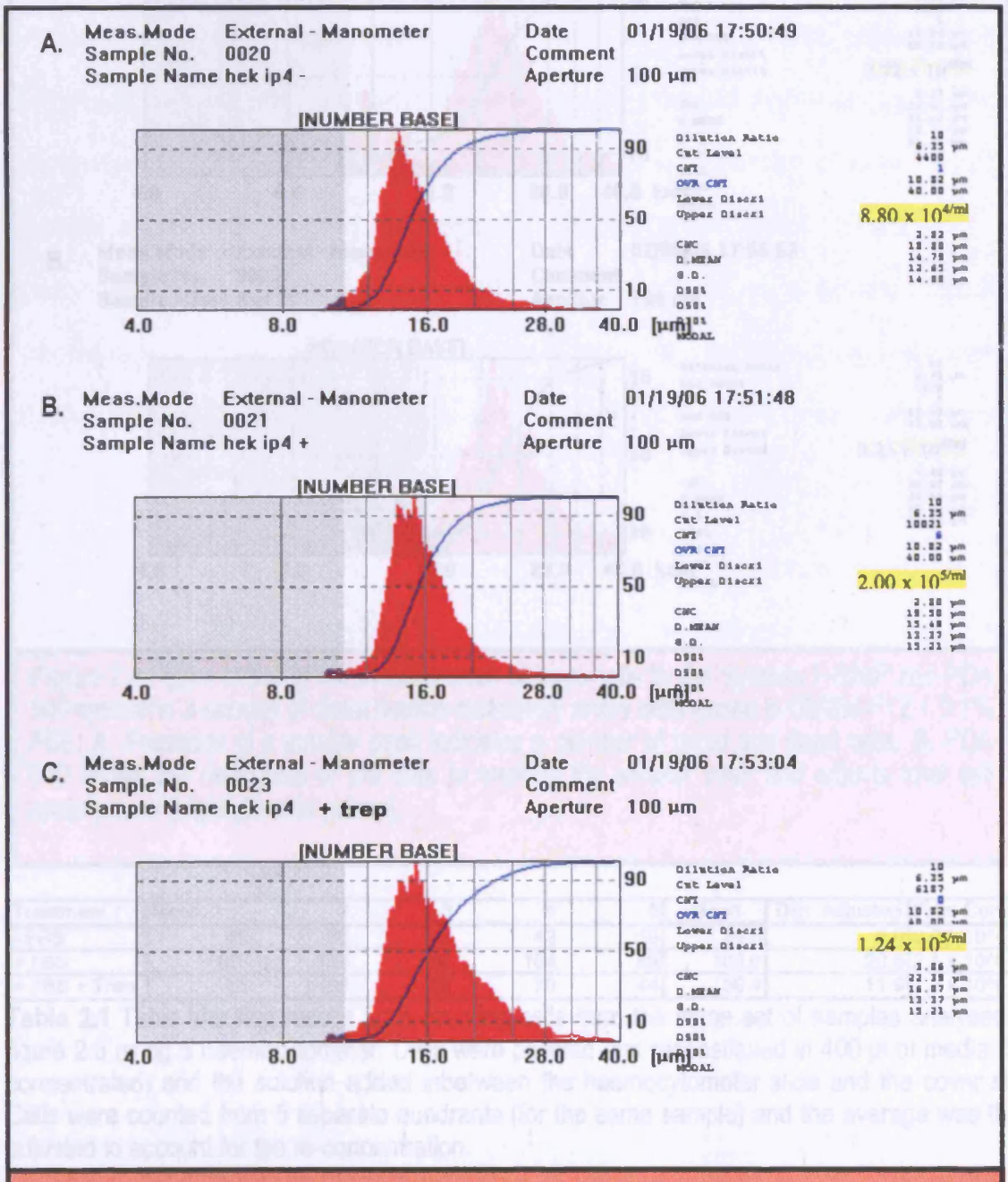

Figure 2.3 Figure showing the particle distribution analysis of three example cell counts as achieved with the Sysmex F-520P and PDA500 set-up. A. HEK-293-IP cells grown in MEM containing 0.1% FBS. B. HEK-293-IP cells grown in MEM containing 10% FBS and C. HEK-293 IPr cells grown in MEM containing 10% FBS + 100nM treprostinil (UT). The total cell count per ml is highlighted in yellow on the right.

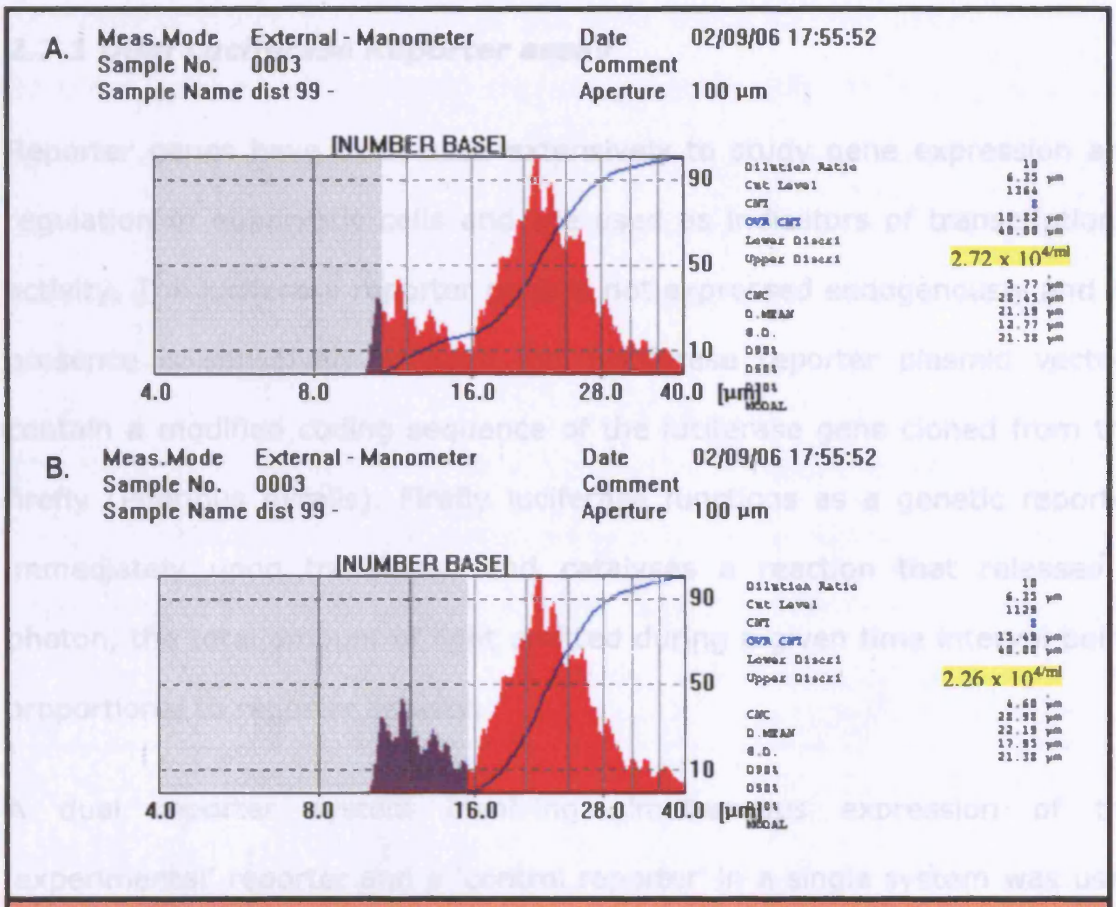

Figure 2.4 Figure showing the determination of dead cells by the Sysmex F-520P and PDA 500 system in a sample of distal human pulmonary artery cells grown in DMEM-F12 + 0.1% FBS. **A.** Presence of a smaller peak indicates a number of lysed and dead cells. **B.** PDA 500 allows the reanalysis of the data to exclude the smaller peak and adjusts total cell count per ml (highlighted in yellow).

Treatment	Meas. 1	2	3	4	5	Mean	Diln. Adjusted	Final Concn
- FBS	45	56	42	42	39	44.8	9.0	9.0 x 10^4
+ FBS	101	123	98	104	89	103.0	20.6	2.1 x 10^5
+ FBS + Trep	65	66	52	70	44	59.4	11.9	1.2 x 10^5

Table 2.1 Table showing results from counting cells from the same set of samples analysed in figure 2.3 using a haemocytometer. Cells were pelleted and reconstituted in 400 µl of media (5X concentrated) and the solution added inbetween the haemocytometer slide and the cover slip. Cells were counted from 5 separate quadrants (for the same sample) and the average was then adjusted to account for the re-concentration.

2.7 PPARγ activation reporter assays

2.7.1 Dual Luciferase Reporter assay

Reporter genes have been used extensively to study gene expression and regulation in eukaryotic cells and are used as indicators of transcriptional activity. The luciferase reporter gene is not expressed endogenously and its presence is sensitively assayed for. Luciferase reporter plasmid vectors contain a modified coding sequence of the luciferase gene cloned from the firefly (Photinus pyralis). Firefly luciferase functions as a genetic reporter immediately upon translation and catalyses a reaction that releases a photon, the total amount of light emitted during a given time interval being proportional to reporter activity.

A dual reporter system involving simultaneous expression of the 'experimental' reporter and a 'control reporter' in a single system was used to provide an internal control that serves as the baseline response. Normalising experimental reporter activity to the control reporter activity minimises experimental variability caused by differences in cell viability and

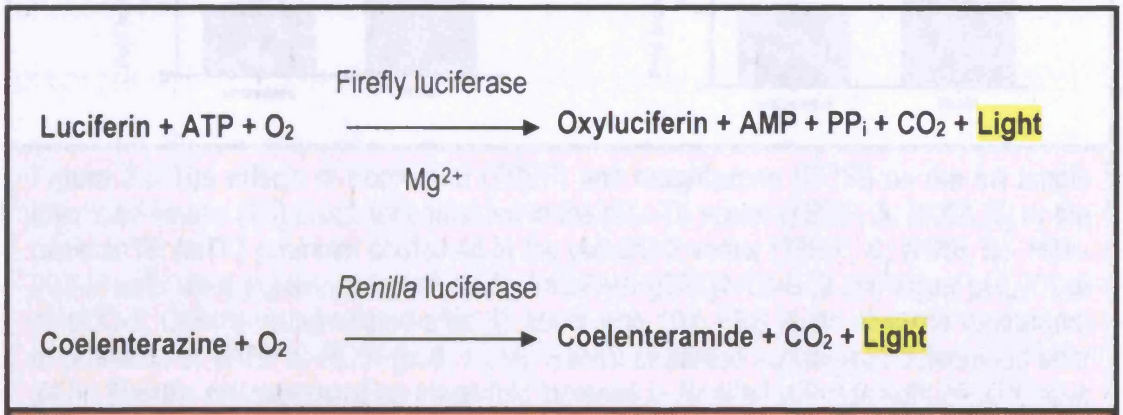

Figure 2.5 Bioluminescent reactions catalysed by firefly and *Renilla* luciferases.

transfection efficiency, non-specific effects on the promoter as well as differences in pipetting volumes, cell lysis efficiency and assay efficiency. Renilla luciferase activity (from the sea pansy Renilla reniformis) was used as our control reporter. Firefly and Renilla luciferases, because of their distinct evolutionary origins, have dissimilar enzyme structures and substrate requirements thus allowing to selectively discriminate between their respective bioluminescent reactions (Figure 2.5).

We found that treprostinil had a striking non-specific effect on the full length thymidine kinase promoter present in many commercially available vectors including the pTK-RL vector (Promega) commonly used in this type of study (Figure 2.6 A-B). Surprisingly, this was also observed with

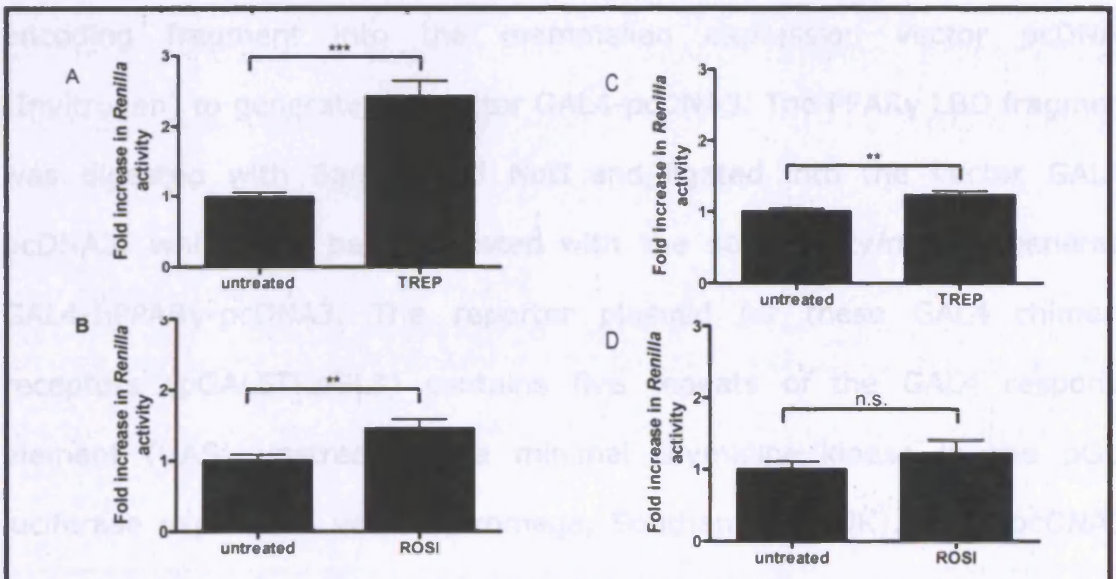

Figure 2.6 The effects of treprostinil (TREP) and rosiglitazone (ROSI) on the full length thymidine kinase (TK) promoter contained in the pRL-TK vector (TREP, **A**; ROSI, **B**) or the minimal TK (mTK) promoter contained in the pMLUC-2 vector (TREP, **C**; ROSI, **D**). HEK-293-IP cells were transiently transfected with LFABP(DR1)4-TK-GL3 and either pRL-TK or pMLUC-2. Cells were stimulated after 48 hours with 10% FBS in the absence (untreated) or presence of TREP or ROSI (both 1 μM). *Renilla* luciferase activity was determined after 24 hr. Results are expressed as mean fold increase in *Renilla* luciferase light units relative to untreated ± s.e.m. (n=12, 3 separate transfections). n.s. = non-significant, ** = $P<0.01$. *** = $P<0.001$

of study (Figure 2.6 A-B). Surprisingly, this was also observed with rosiglitazone albeit to a lesser extent. As such a minimal thymidine kinase promoter, a shortened version (120-140 bp) of the full length (716 bp) TK promoter with retained functionality, proved to be essential in plasmids used in our luciferase based reporter gene assays in order to minimise noise levels. Figure 2.6 (C-D) illustrates how using this latter type of promoter significantly reduced the occurrence of these non-specific effects to much lower, albeit still significantly different, levels.

2.7.2 Plasmids

A chimeric receptor containing the yeast GAL4 DNA binding domain fused to human PPARγ was created by insertion of a GAL4 DNA binding domain encoding fragment into the mammalian expression vector pcDNA3 (Invitrogen) to generate the vector GAL4-pcDNA3. The PPARγ LBD fragment was digested with BamHI and NotI and ligated into the vector GAL4-pcDNA3, which had been digested with the same enzymes to generate GAL4-hPPARγ-pcDNA3. The reporter plasmid for these GAL4 chimeric receptors (pGAL5TKpGL3) contains five repeats of the GAL4 response element (UAS) upstream of a minimal thymidine kinase in the pGL3 luciferase expression vector (Promega, Southampton, UK). GAL4-pcDNA3, GAL4-hPPARγ-pcDNA3 and pGAL5TKpGL3 were kindly provided by Professor Bart Staels (Pasteur Institute, Lille, France). The control vector, pMLuc2 (Merck Biosciences, Nottingham, UK), contains the minimal thymidine kinase promoter adjacent to the Renilla luciferase gene. Figure 2.7 summarises the vectors and their activities in the luciferase reporter gene assay.

2.7.3 Transient transfections of HEK-293 cells

Cells were co-transfected with pGAL5TKpGL3, pMLuc-2 and either GAL4-hPPARγ-pcDNA3 or GAL4-pcDNA3 using Lipofectamine 2000 (Invitrogen). Lipofectamine 2000 was chosen over other lipofectamines because of its high efficiency, its ability to transfect cells in suspension and its efficacy in the presence of serum. We found that transfection of cells in suspension was necessary in order to achieve homogenous transfection efficiency throughout the 96 well plates thus further minimising variability caused by transfection efficiency and pipetting.

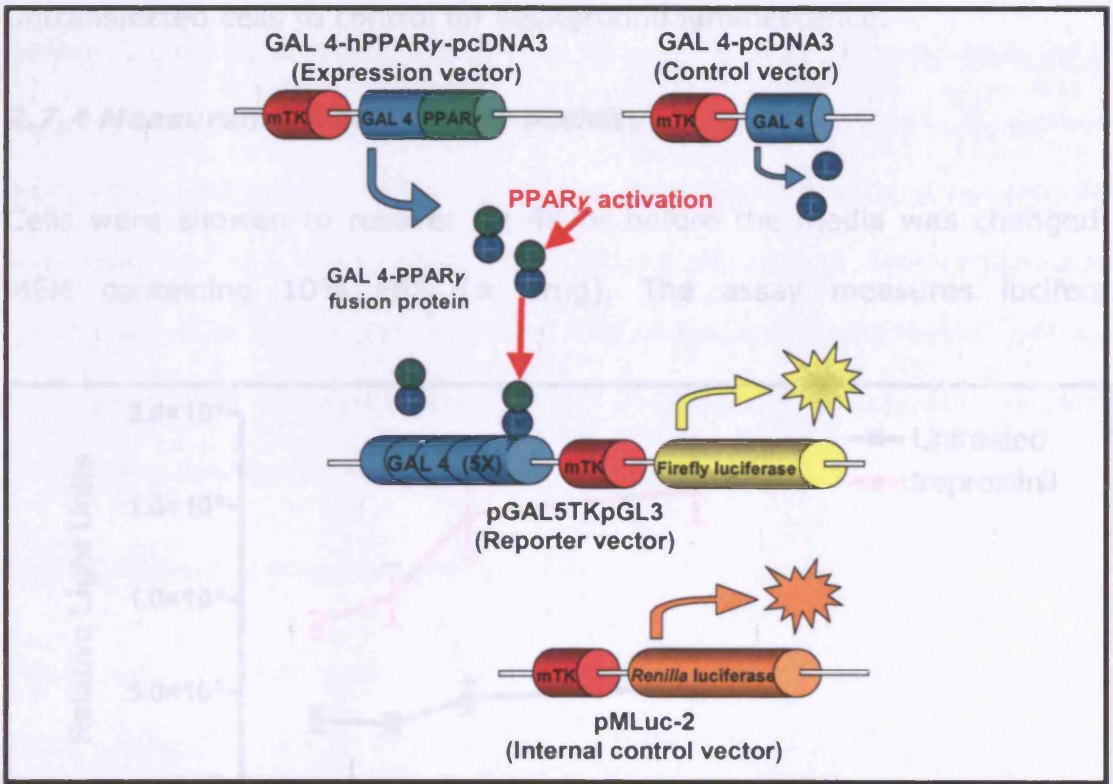

Figure 2.7 Schematics of vectors used in the dual luciferase reporter gene assay and their function. Cells were transfected with the reporter vector, pGAL5TKpGL3, the internal, control vector, pMLuc-2 and either the expression vector, GAL-4-hPPARγ-pcDNA3 or the control vector GAL-4-pcDNA3. The expression vector generates a GAL-4-PPARγ fusion protein that upon activation with a PPARγ ligand binds to one of the 5 copies of the GAL4 element on the reporter vector. This results in the production of firefly luciferase which can be detected and quantified. The internal control vector generates *Renilla* luciferase light which can be distinguished from firefly luciferase and is used to normalise the luciferase readings to control for transfection efficiency

Cells were kept in suspension at a density of 1.2-1.3 x 10^6 cells/ml. A total of 3.6 µg of plasmid DNA (3 µg pGAL5TKpGL3, 300 ng pMLuc-2 and 300 ng GAL4-hPPARγ-pcDNA3 or GAL4-pcDNA3) was dissolved into 400 µl of OptiMEM and 4 µl of Lipofectamine 2000 was dissolved in another 400 µl OptiMEM. Both were incubated at room temperature for 5 mins before mixing them together and incubating for a further 20 mins. Following this incubation, the lipofectamine mixture was added to 4 ml of the cell suspension and cells swirled for approximately 15 mins before plating out into 96 well plates. HEK-293 WT and IP-r cells were plated in 100 µl/well at a 1:20 and 1:10 dilution respectively. One column of wells was kept for untransfected cells to control for background luminescence.

2.7.4 Measurement of reporter activity

Cells were allowed to recover for 48 hr before the media was changed to MEM containing 10% FBS (± drug). The assay measures luciferase

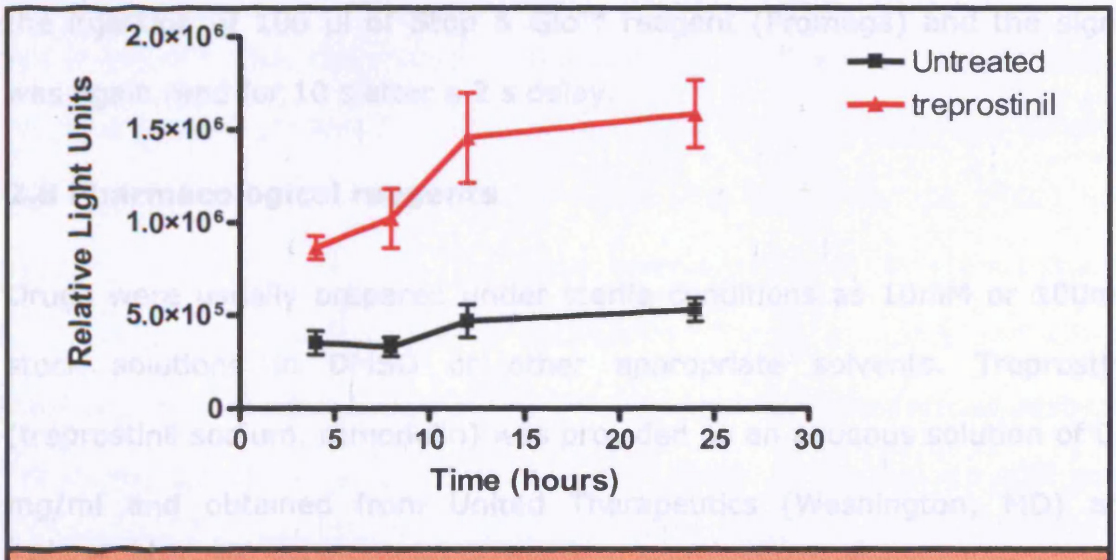

Figure 2.8 Time course of luciferase transcription. Cells were transfected with GAL4-hPPARγ-pcDNA3, pGAL5TKpGL3 and pMLuc-2 and allowed to settle for 48 hours. Cells were then either left untreated or treated with 1µM treprostinil. Luciferase measured at several time points. Results are expressed as mean luciferase light units normalised to Renilla luciferase ± s.e.m. (n=4).

expression cumulatively. Luciferase is a relatively stable compound and will not degrade readily once produced. Using this protocol allows transcriptional activation to be detected as early as 4 hr, with the signal peaking after about 24 hr (Figure 2.8).

Luciferase activity was therefore assayed at this time point, using either a Single or Dual Luciferase Assay kit (Promega) according to the manufacturer's instructions. Briefly cells were washed with PBS prior to 20 mins incubation with 20 µl/well of 1 x passive lysis buffer (100 mM potassium phosphate [pH 7.8], 0.2% Triton X-100, 0.5 mM DTT). The cells in lysis buffer were then frozen down and thawed out for maximal lysis before re-plating at 10µl/well into an opaque 96 well plate suitable for use in the Tropix TR717 microplate luminometer (Applied Biosystems, Warrington, UK). After a 2 s delay, the light signal produced by luciferase was read for 10 s following the injection of 100 µl luciferase reagent (Promega). The light signal from the Renilla luciferase was then initiated by the injection of 100 µl of Stop & Glo™ reagent (Promega) and the signal was again read for 10 s after a 2 s delay.

2.8 Pharmacological reagents

Drugs were usually prepared under sterile conditions as 10mM or 100mM stock solutions in DMSO or other appropriate solvents. Treprostinil (treprostinil sodium, remodulin) was provided as an aqueous solution of 0.6 mg/ml and obtained from United Therapeutics (Washington, MD) and cicaprost was provided in a buffer (9.9 mM Tris, 152 mM NaCl and 176 mM ethanol) at a concentration of 0.5 mg/ml and obtained from Schering AG (Berlin, Germany). The IP receptor antagonist RO1138452 was donated by

Roche (Palo Alto, CA) and GW7647 by GlaxoSmithKline (Research Triangle Park, NC). Carbacyclin (carbaprostacyclin) and H-89 were purchased from Biomol (Exeter, UK), Rp-cAMPS from Biolog Life Science Institute (Bremen, Germany), rosiglitazone from Alexis Corporation (Lausanne, Switzerland), 2'5'-dideoxyadenosine (DDA), pertussis toxin (PTx) and L165,041 from Merck Biosciences (Nottingham, UK), forskolin, 3-Isobutyl-1-methylxanthine (IBMX), quercetin, wortmannin, adenine 9-β-D-arabinofuranoside (Ara-A) and 5-Aminoimidazole-4-carbaxamide 1-β-D-ribofuranoside (AICAR) from Sigma-Aldrich (Poole, UK).

2.9 Statistical analysis

All data are presented as mean ± s.e.m. of n observations. IC_{50} values were determined using the sigmoidal dose-response curve fitting routine in GraphPad Prism 4 (Graphpad Software Inc. www.graphpad.com) using equation 2.1. For cell growth curves a variation of the sigmoidal dose response curve, the Boltzmann or Hill function was used to fit data. It allows for a bottom and top plateu and allows for a variable slope to better represent the lag phase of cell growth. For a single intervention between two groups an unpaired Student's t-test was performed. A t-test with Welch's correction was used if variances were significantly different between the two groups. For comparisons between 3 or more groups a one-way or two-way ANOVA test was used. Where appropriate a Bonferroni post-test correction for multiple comparisons was made. P values < 0.05 were considered to be statistically significant.

$$Y = \frac{A_1 + (A_2 - A_1)}{1 + 10^{(\mathrm{LogIC}_{50} - X)}}$$

Equation 2.1 Equation used to fit a sigmoidal dose response curve. A_1= initial Y value, A_2 = final Y value, IC_{50}, the concentration of an inhibitor that is required for 50% of the maximal inhibitory response.

$$Y = \frac{A_1 + (A_2 - A_1)}{1 + 10^{\wedge}\left(\frac{\mathrm{LogEC}_{50} - X}{\mathrm{slope}}\right)}$$

Equation 2.2 Boltzmann sigmoid equation used to fit proliferation curves. A_1 = initial Y value, A_2 = final Y value, EC_{50} = response halfway between A_1 and A_2. 'Slope' describes the steepness of the curve.

2.10 Further statistical considerations

In the proliferation studies presented in this thesis data was normalised and expressed as percentage growth relative to the untreated control, i.e. the difference in the growth response between cells starved in 0.1% FBS and growing in 10% FBS. This was necessary in order to minimise the noise of intra-experimental growth variability. The untreated control was given the arbitrary value of 100%. It was felt however that by attributing the value of 100% with no standard error would have been misleading as we would not be accounting for the variation in control cell growth as well as errors resulting from cell counting measurements. By not possessing error bars

the threshold for a treatment to be statistically significant would have been considerably lower. A solution was to extrapolate error bars for the control by dividing individual control values by the average for the control group. By doing this the average will always be 1 (or 100%) and all the individual normalised values could be used to determine the standard error of the mean. As shown in figure 2.9, analysing results with or without S.E.M error bars for the control made a difference to the resulting *P* values. This type of analysis was carried forward for all proliferation studies where the control treatment was normalised to 100% as well as luciferase assay experiments in chapter 4 where control bars were attributed a value of 1.

If data was pooled from more than one cell isolate for each cell type, for example when pooling two different HEK-293-Zeo or HEK-293-IP clones, or when pooling HPASM cells from 2 or 3 different IPAH or normal patients, a Student's t-test (2 different isolates) or ANOVA (3 different isolates) was performed to check that the distribution between the isolates was not significantly different. Data was thus only pooled if it followed that the distribution between isolates was not significantly different. In chapter 3 and 4, 2 isolates were always pooled to represent HEK-293-Zeo or HEK-293-IP cells. Figure legends in chapter 5 will state how many isolates were pooled for each experiment involving HPASM from normal or IPAH patients.

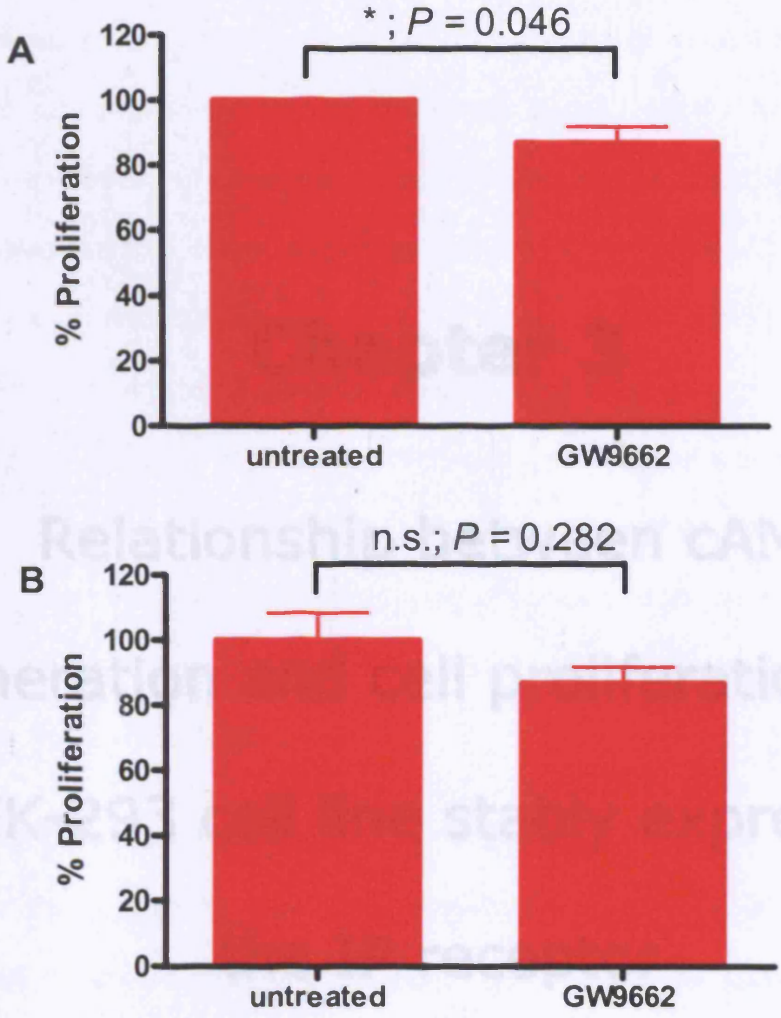

Figure 2.9 Figure demonstrating the difference in *P* values when analysing data with or without S.E.M error bars for the untreated control. In this example, growth arrested HEK-293-IP cells were stimulated with MEM + 10% FBS and either left untreated or treated with 1 μM GW9662. Cells were counted 48 hours following treatment. Data is expressed as % proliferation relative to the proliferative response mediated by 10% FBS alone and expressed as mean ± S.E.M. (n=9). A. Control expressed as 100% with no S.E.M bars. B. Individual untreated control values were divided by the average for the control group and the resulting normalised data was used to calculate S.E.M for the control. *P* values derived using student's t-test.

Chapter 3

Relationship between cAMP generation and cell proliferation in a HEK-293 cell line stably expressing the IP receptor

3.1 Introduction

The IP receptor is a cell surface G-protein coupled receptor consisting of seven transmembrane domains (Coleman *et al.,* 1994; Narumiya *et al.,* 1999). The human IP receptor gene (PTGIR) spans approximately 7.0 kb and is composed of three exons separated by two introns and has been assigned to human chromosome 19 (Ogawa *et al.,* 1995). It encodes a protein with a predicted molecular weight of 41 kDa, though the protein can be post-translationally modified, which can give rise to a protein of ~50-60 kDa. Modification can take the form of glycosylation, isoprenylation or palmitoylation and this can affect surface expression, G protein coupling and effector signalling (Smyth *et al.,* 1998; Miggin *et al.,* 2002; Miggin *et al.,* 2003; Hata and Breyer, 2004).

The IP receptor is the main target for PGI_2 and its stable analogues, although these agents can also bind with significant affinity to other prostanoid receptors including EP_1, EP_2, EP_3 and EP_4 (Kiriyama *et al.,* 1997; Hata and Breyer, 2004). In addition some analogues are known to be ligands for the nuclear receptors PPARα and PPARδ (Hertz *et al.,* 1996; Forman *et al.*, 1997) thus PGI_2 analogues have multiple targets which may mediate their effects. Upon activation, the IP receptor causes an elevation of intracellular cAMP *via* direct coupling to G_s and stimulation of adenylyl cyclase (Nakagawa *et al.*, 1994; Boie *et al.*, 1994) and subsequent activation of PKA. This mechanism is thought to account for the anti-proliferative effects of PGI_2 and its analogues in smooth muscle cells (Wharton *et al.,* 2000; Clapp *et al.,* 2002; Phillips *et al.,* 2005).

It has been established that increasing intracellular cAMP levels directly inhibits smooth muscle cell proliferation both *in vivo* and *in vitro* and that these growth inhibitory effects can be completely reversed by the inhibition of PKA (Indolfi *et al.*, 1997). In addition there is evidence showing that over-expression of AKAP75, a prototypic A-kinase anchor protein which anchors PKA to the membrane, inhibits proliferation of smooth muscle cells (Indolfi *et al.,* 2001). The authors of the study suggest that membrane targeting of PKA leads to suppression of SMC growth by amplifying and stimulating cAMP-PKA signalling, resulting in increased activation of cAMP-induced transcription and increased levels of the CDKI $p27^{kip1}$. Moreover, a very recent study has linked PKA to the differentiation effects of iloprost in VSMC (Fetalvero *et al.*, 2006). Both the PKA antagonist, myrPKI, and the ablation of the catalytic subunits of PKA via small interfering RNA (siRNA) inhibited the iloprost induced up-regulation of smooth muscle differentiation markers such as calponin, h-caldesmon and SM-α-actin (Fetalvero *et al.,* 2006). In addition it has recently been shown that a PKA antagonist can reverse the inhibitory effects of cicaprost on PDGF-induced mitogenesis in rat VSMC (Phillips *et al.,* 2005), the only study to date to have provided evidence for the much hypothesised role of PKA in PGI_2 analogue-mediated effects on cell growth.

In the present study, to better delineate the role of the IP receptor in modulating cell growth, a HEK-293 cell model was established in which the IP receptor was stably expressed (HEK-293-IP) and experimental data on these cells was compared to cells lacking the receptor (HEK-293-WT and HEK-293-Zeo). To validate this system and confirm that the HEK-293-IP cells did indeed express the IP receptor we performed RT-PCR to assess

mRNA levels and immunohistochemistry to assess protein expression in the three different HEK-293 cell lines.

Antibodies make invaluable tools when assessing the expression and localisation of a protein. No antibody against the IP receptor was commercially available until the autumn of 2004, when an antibody was marketed from Cayman Chemical (Ann Arbor, Michigan). In 1998 Komhoff and colleagues (Komhoff *et al.*, 1998) had developed an antibody targeting the N-terminal of the IP receptor and used it to describe the localisation of this receptor in the kidney. To my knowledge these were the only antibodies available during the course of my investigation although very recently a study exploring the localisation of the human IP receptor in the endometrium utilised a Santa Cruz (Santa Cruz, California) antibody targeted to the C-terminal of the IP receptor (Smith *et al.*, 2006).

In the present study we developed a novel anti-peptide antibody directed to the C-terminal of the IP receptor. This antibody was compared with the Cayman and the Komhoff antibodies and used to investigate receptor expression and localisation in cells used in our investigations. Generating a specific antibody or identifying the most reliable and specific antibody to the IP receptor will clearly be an extremely valuable tool for future studies.

The newly established HEK-293 cell line was used to characterise the IP receptor in isolation, in particular to establish the significance of this receptor in mediating the effects of PGI_2 analogues and the extent to which these effects can be attributed to the classical cAMP pathway. Using various pharmacological tools, experiments were designed to dissect the molecular

mechanisms involved in PGI_2 signalling, including the role of PKA, especially as they relate to the inhibition of cell proliferation.

RESULTS

3.2 RT-PCR confirms presence of mRNA in HEK-293-IP cells

Two clonal isolates of the IP receptor (HEK-293-IP) and control (HEK-293-Zeo) HEK-293 stable cell lines were generated using a standard transfection protocol (see chapter 2). Using specific primers to amplify a 204 bp sequence of the IP receptor, RT-PCR was performed on cDNA produced by 5μg of total RNA to confirm presence or absence of mRNA message in each cell type. A band of the appropriate size can be seen in lanes for both clonal isolates of HEK-293-IP cells (Figure 3.1 A) confirming presence of the IP receptor. To verify that the band visualised corresponded to the 204bp sequence of the IP receptor, the band was excised from the gel, the DNA purified and sent for sequencing (Cytomyx, Cambridge, UK). A sequence alignment of 162 base pairs of the product was performed using MegaBLAST (www.ncbi.com) which detected a 92.6% sequence identity to the human IP receptor (accession id: gi/39995095/ref/NM_000960.3). The expectation value (E-value) was $5e^{-26}$ ruling out the possibility that the sequence match was by chance related to another gene. No other sequences from any species showed any significant homology.

Very faint bands are present in both clonal isolates of HEK-293-Zeo cells (Figure 3.1 A) and HEK-293-WT (Figure 3.1 B) potentially suggesting weak expression of the IP receptor in these cells. The latter observation was

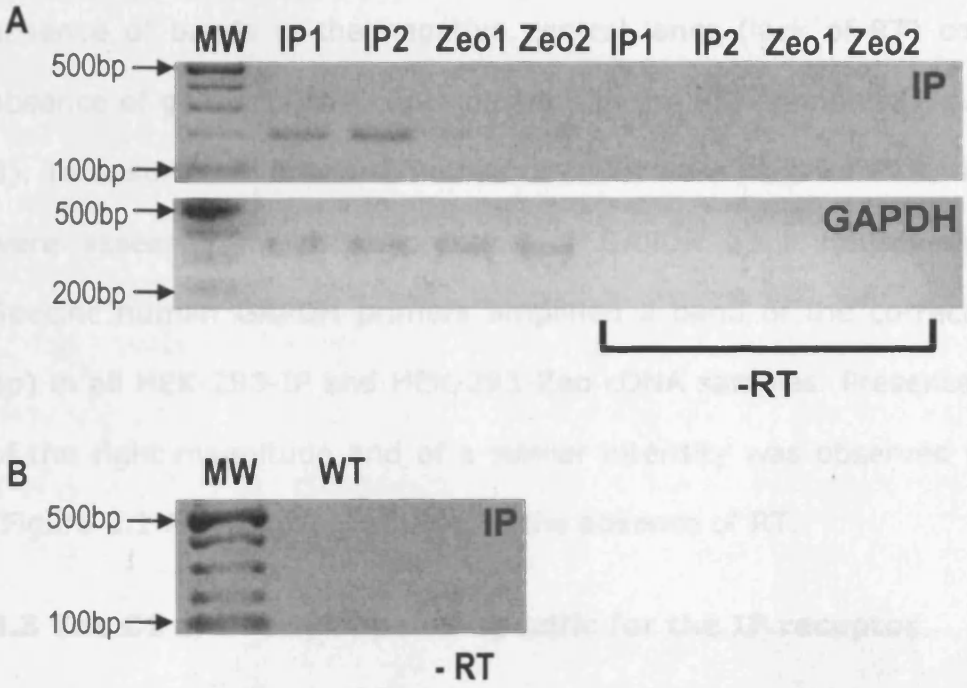

Figure 3.1 PCR was performed on reverse transcriptase cDNA products of 5μg of total RNA from two isolates of HEK-293-IP (**A**), HEK-293-Zeo (**A**) and HEK-293-WT (**B**) using IP receptor specific primers to amplify a 204 bp region of the receptor sequence and GAPDH primers to amplify a 363 bp band of this house-keeping gene as control. Reverse transcriptase controls (-RT) were negative in all cases. Identity of the IP receptor product was confirmed by DNA sequencing. This is a representative result which has been reproduced at least three times using different RNA preparations.

somewhat surprising given the described lack of prostanoid receptors in HEK-293 cells (Abramovitz *et al.,* 2000), however this low level of mRNA expression does not indicate presence of mature functional receptor protein as we show further on. Absence or, in the case of HEK-293-WT, near absence of bands in the negative control lanes (lack of RT) confirms the absence of genomic DNA contamination in the RNA samples (Figure 3.1 A & B). To ensure that similar amounts of cDNA were used for PCR, the samples were assessed for the expression of GAPDH as a housekeeping gene. Specific human GAPDH primers amplified a band of the correct size (363 bp) in all HEK-293-IP and HEK-293-Zeo cDNA samples. Presence of a band of the right magnitude and of a similar intensity was observed in all lanes (Figure 3.1 A) except, of course, in the absence of RT.

3.3 The C1 antibody appears specific for the IP receptor

To test whether the newly designed C1 antibody was specific for the IP receptor, both western blotting and fluorescence immunohistochemistry were performed on cells stably transfected with the IP receptor (HEK-293-IP) and cells lacking the receptor, namely HEK-293-Zeo.

Western immunoblotting with the C1 antibody developed here is currently being optimised by Dr. Sue Hall (Institute of Child Health, UCL) and encouragingly a band of ~60 kDa could be observed in HEK-293-IP protein samples which was near-absent in HEK-293-Zeo (Figure 3.2; figure provided by Dr. Hall). Dr. Hall also looked at the expression of the IP receptor in smooth muscle cells from "normal" (Normal 1 = 00/05 and Normal 3 = 99/09 in figure 3.2) and IPAH patients (IPAH1 = 1664, IPAH 2

= 1666 and IPAH 3 = 1672 in figure 3.2), these results are described in more detail later in the thesis (chapter 5).

C1 antibody staining can be visualised in HEK-293-IP (Figure 3.3 A) but not in HEK-293-Zeo (Figure 3.3 B) cells suggesting that this antibody is indeed specific for this receptor. Cells were fixed and permeabilised as previously described in the methods (see section 2.4.2) and then stained with the C1 antibody (1:100 dilution), by incubating cells overnight. Visualisation was carried out using a goat anti-rabbit FITC conjugated Alexa-Fluor 488 (green) secondary antibody (1:300). TO-PRO-3 (red) was used to visualise the nuclei. The staining is abundant in HEK-293-IP cells and appears to be localised at the cell membrane although it is also strongly expressed in the cytoplasm. To test whether the latter may have been due to increased receptor internalisation during cell growth, we investigated the effect of starving cells prior to incubation with the antibody. Staining cells that had been previously growth arrested for 48 hrs in MEM containing 0.1% FBS showed a similar pattern of cytoplasmic staining, although the concentration of staining at the cell margins appeared more intense, possibly indicating more receptor at or close to the membrane (Figure 3.3 C & D).

To control for possible non-specific staining by the fluorescent secondary antibody, cells were incubated in the absence of the primary antibody, C1. Under these conditions, no staining was observed in either HEK-293-IP or HEK-293-Zeo cells (Figure 3.4 A & B) confirming the specificity of the primary antibody. A further control was performed with the antigenic peptide, which should compete with the primary antibody, and thus prevent the signal from the secondary from being visualised. To perform this, cells

were pre-incubated for 3 hrs with the antigenic peptide prior to exposure with the primary antibody. Again no fluorescent signal was apparent (Figure 3.4 C), strengthening the validity of this antibody as specifically binding to the IP receptor.

3.3.1 Immuno-staining with the N1 and the Cayman antibodies

In order to compare the novel C1 antibody to the commercially available Cayman antibody and the N1 antibody previously utilised by Komhoff *et al,* (1998) we performed the same experiments as above using these two antibodies. Signal could be detected with both antibodies in the HEK-293 IP cells (Figure 3.5 A & C), though the pattern of staining differed from our antibody in that it was largely associated around or in the nucleus. Moreover, staining, albeit less, could be visualised in HEK-293-Zeo cells (Figure 3.5 B & D). Taken together, this suggests that these two antibodies probably lack specificity for the IP receptor.

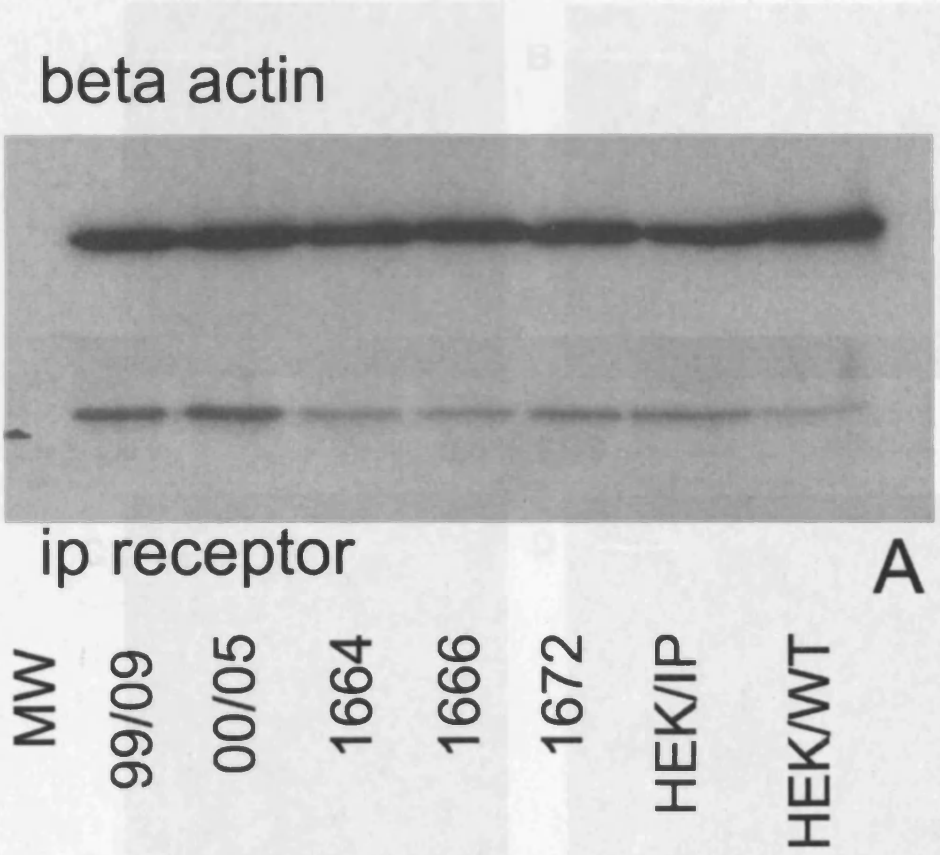

Figure 3.2 Lab work carried out by Dr. Sue Hall who also provided this figure. The C1 antibody was used to study expression of the IP receptor in HEK-293-Zeo, HEK-293-IP as well as normal (99/09, 00/05) and IPAH (1664, 1666 and 1672) vascular smooth muscle cells. The C1 antibody picks up a band at 60 kDa. Beta-actin was used as a positive control and all samples show the same expression of this protein.

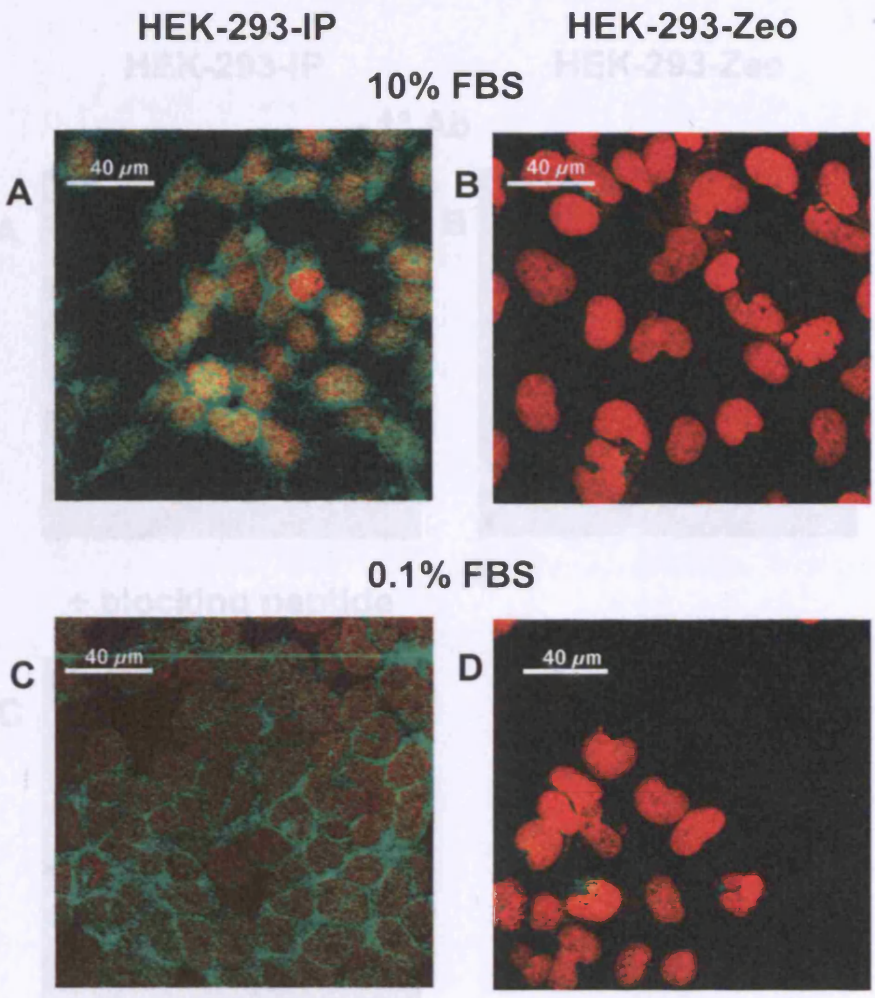

Figure 3.3 HEK-293 IP (**A, C**) and HEK-293 Zeo (**B, D**) cells stained with the C1 antibody. Growing (MEM + 10% FBS) (**A,B**) or starved (MEM + 0.1% FBS) (**C,D**) cells were fixed in 4% paraformaldehyde prior to staining with the C1 primary antibody (1:100 dilution). FITC conjugated Alexa fluor 488 (green) was used as a secondary antibody and TO-PRO-3 (red) was used to stain nuclei (both at 1:300 dilution). Cells were visualised using a confocal microscope with a X60 magnification water immersion lens. Images of 0.1 μm thickness were taken from the middle of the cells. Images are not derived from a Z-series but were taken as a single image at a level were nuclear staining was intense.

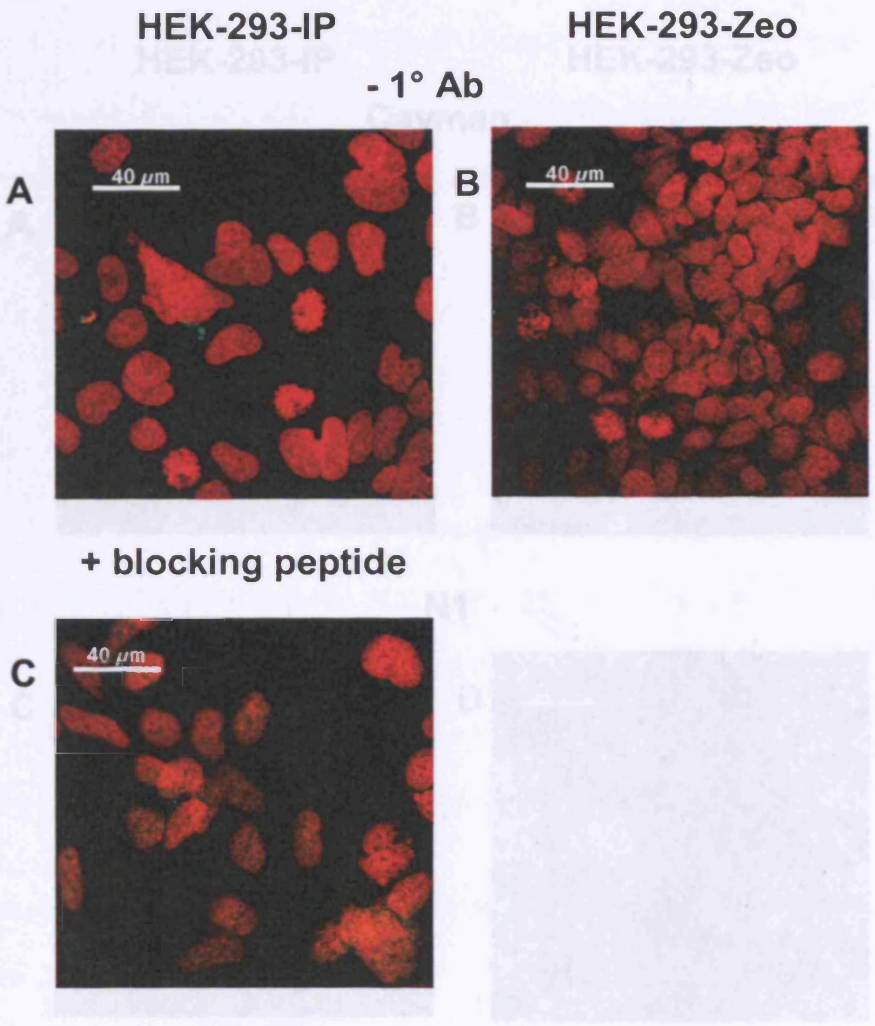

Figure 3.4 Immunohistochemistry controls. **A & B**. Growing HEK-293-IP and HEK-293-Zeo cells were fixed in 4% para-formaldehyde and immuno-stained with FITC conjugated Alexa fluor 488 (green) and TO-PRO-3 (Red) in the absence of primary antibody (both 1:300 dilution) **C**. Growing HEK-293-IP pre-incubated with C1 blocking peptide (4 μg/μl) prior to staining with primary antibody (1:100 dilution). Images are not derived from a Z-series but were taken as a single image at a level were nuclear staining was intense.

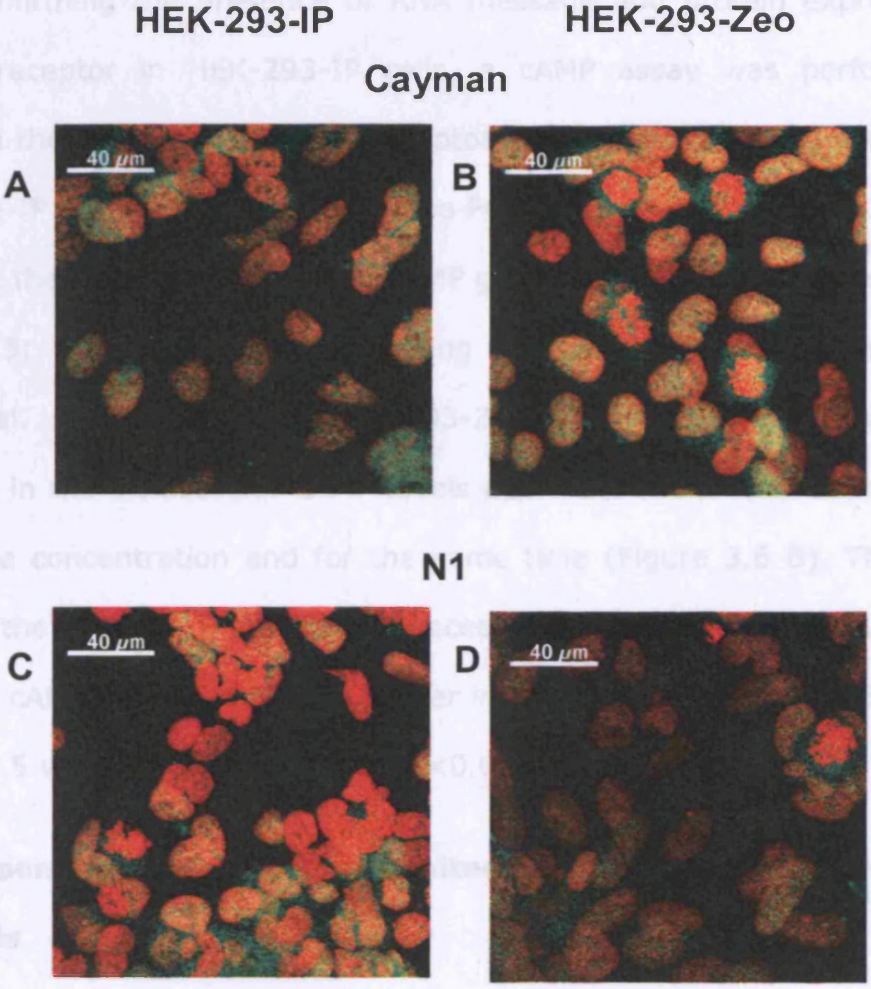

Figure 3.5 Growing HEK-293-IP and HEK-293-Zeo cells were fixed in 4% para-formaldehyde and immuno-stained with the Cayman IP receptor antibody (1:100) (**A,B**) or the N1 antibody (1:100) (**C,D**). FITC conjugated Alexa fluor 488 (green) was used as a secondary antibody and TO-PRO-3 (red) was used to stain nuclei (both at 1:300 dilution). Images are not derived from a Z-series but were taken as a single image at a level were nuclear staining was intense.

3.4 The IP receptor in HEK-293-IP cells is functional

After confirming the presence of RNA message and protein expression of the IP receptor in HEK-293-IP cells, a cAMP assay was performed to establish the functionality of the receptor in these cells. As expected, when HEK-293-IP cells were treated with the PGI_2 analogue treprostinil (1 μM) for 30 mins, the intracellular levels of cAMP greatly increased by ~40 fold (n=4, P=0.0013; Figure 3.6 A), confirming that the IP receptor is indeed functional. In contrast, the HEK-293-Zeo cells displayed no significant increase in the intracellular cAMP levels upon treatment with treprostinil at the same concentration and for the same time (Figure 3.6 B). This would suggest the absence of a functional receptor in these cells. Basal, untreated levels of cAMP were significantly higher in HEK-293-Zeo than in HEK-293-IP cells (39.5 vs 9.3 pmol/mg, n=3-6, P<0.05)

3.5 Presence of the IP receptor alters the proliferative rate of HEK-293 cells

One of the main phenotypic differences initially noted between cells with and without the IP receptor was a discrepancy in their proliferation rate. HEK-293-IP cells had a much reduced growth rate compared to either HEK-293-Zeo or HEK-293-WT cells (Figure 3.7). After 4 days of growth in media containing 10% FBS, cells expressing the IP receptor had grown approximately 50% less (HEK-293-WT, n=9 and HEK-293-Zeo and HEK-293-IP, n=18, P<0.001) than cells not expressing the receptor. Similar growth characteristics were observed for all clonal isolates of HEK-293-IP and HEK-293-Zeo tested (Figure 3.8).

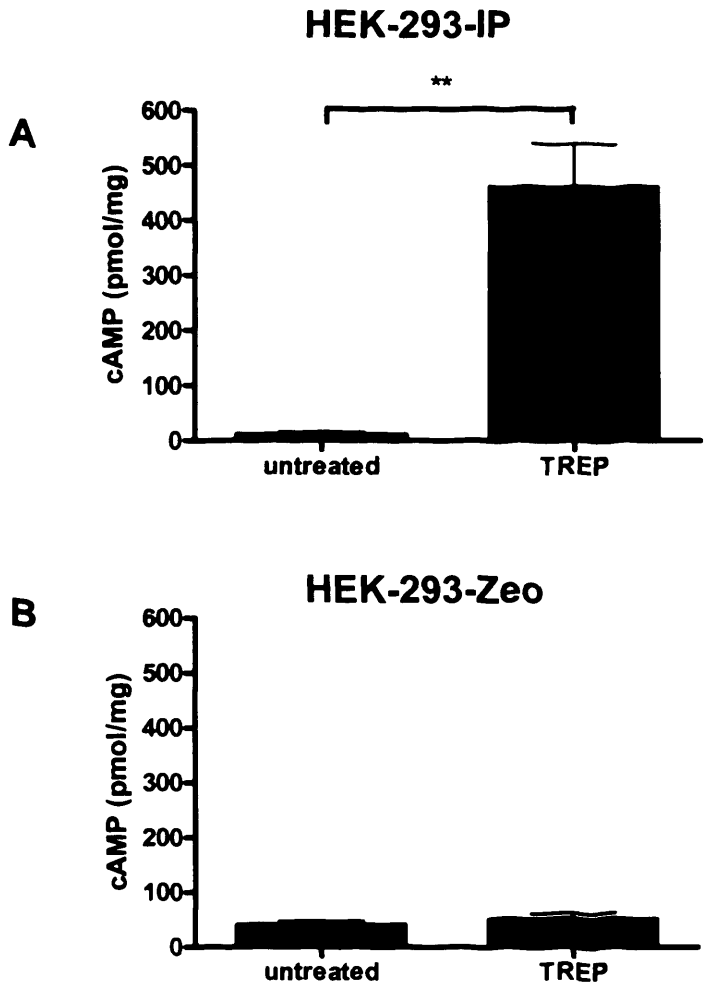

Figure 3.6 Cyclic AMP measurements in HEK-293-IP (**A**) or HEK-293-Zeo (**B**) cells. In both cases, cells were stimulated with 10%FBS ± either treprostinil (TREP; 100 nM). Cyclic AMP was extracted 30 mins after TREP stimulation and measured using an enzyme immunolinked assay. Results expressed as mean pmol of cyclic AMP per mg of total protein ± s.e.m (measurements in duplicate of 3-4 experiments). **=$p<0.01$.

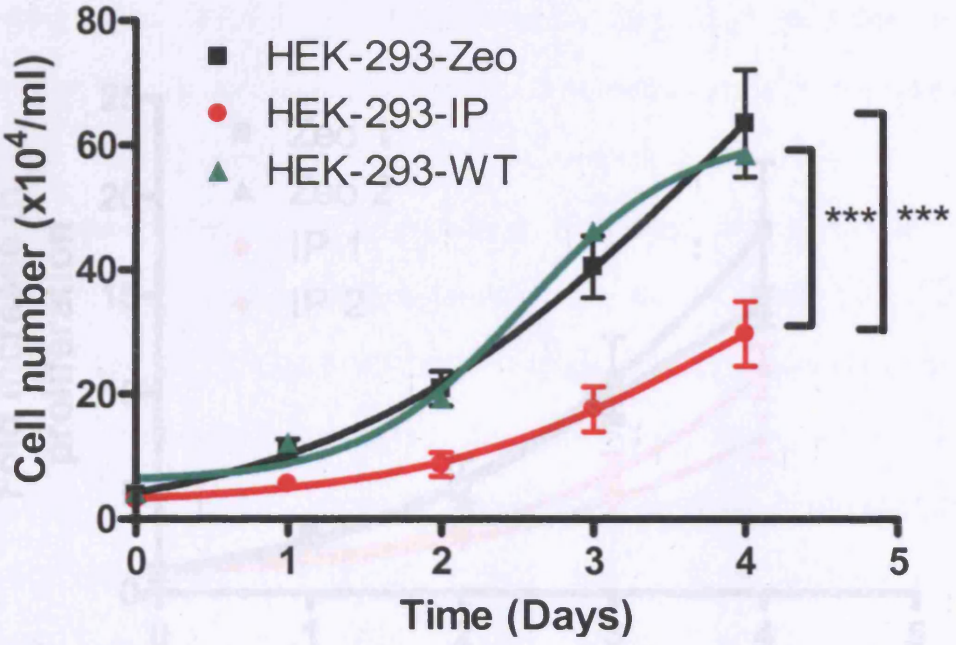

Figure 3.7 Proliferation curves of HEK-293-Zeo (■), HEK-293-IP (●) and HEK-293-WT (▲) cells. Growth arrested cells were stimulated with 10% FBS and counted at different time points as shown. Data shown as mean ± S.E.M. (n=9-18). *** = P<0.001.

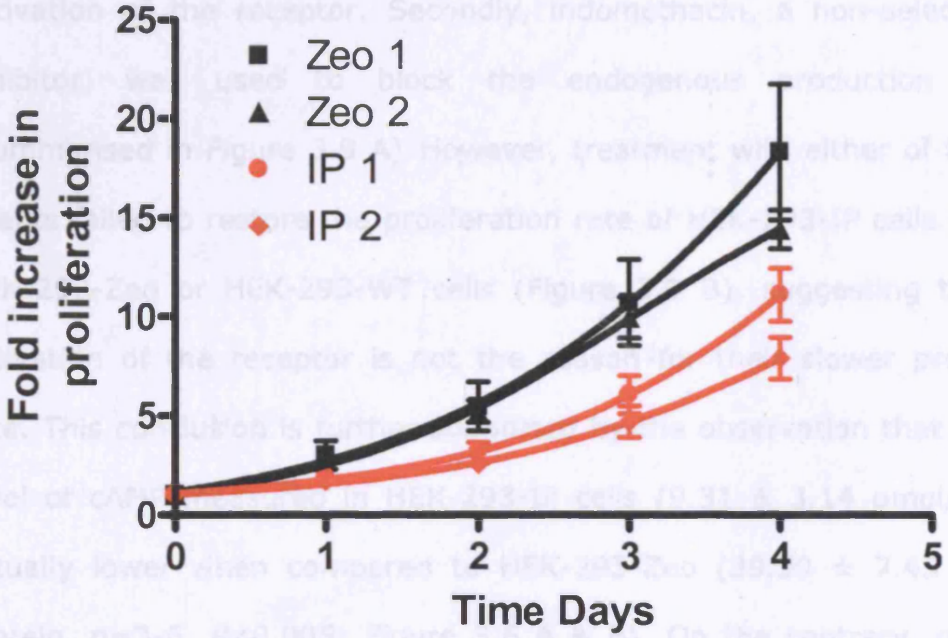

Figure 3.8 Proliferation curves of two clonal isolates of HEK-293-Zeo, 1 (■) and 2 (▲) and two clonal isolates of HEK-293-IP, 1 (●) and 2 (♦). Growth arrested cells were stimulated with 10% FBS and counted at different time points as shown. Data shown as mean ± S.E.M. (measurements in triplicate of 3 experiments).

One possible explanation for the lower proliferation rate found in the cells expressing the IP receptor was thought to be basal activation of the receptor by endogenously produced PGI_2. This theory was tested in two ways. Firstly, the novel IP receptor antagonist RO1138452 (subsequently referred to as IPRA from here onwards) was used to block any basal activation of the receptor. Secondly, indomethacin, a non-selective COX inhibitor, was used to block the endogenous production of PGI_2 (summarised in Figure 3.9 A). However, treatment with either of these two agents failed to restore the proliferation rate of HEK-293-IP cells to that of HEK-293-Zeo or HEK-293-WT cells (Figure 3.9 B), suggesting that basal activation of the receptor is not the reason for their slower proliferation rate. This conclusion is further supported by the observation that the basal level of cAMP measured in HEK-293-IP cells (9.31 ± 3.14 pmol/mg) was actually lower when compared to HEK-293-Zeo (39.50 ± 7.43 pmol/mg protein, n=3-6, $P<0.005$; Figure 3.6 A & B). On the contrary, one would have expected cAMP levels to be higher in the IP receptor expressing cells if indeed there was increased basal activation of the receptor due to endogenously produced PGI_2 production or enhanced receptor coupling to G_s.

3.6 Effect of G_i/G_o inhibition.

The unexpected lower basal level of intracellular cAMP in HEK-293-IP cells raised interesting questions. One hypothesis based on this result was that the IP receptor is indeed basally activated but is additionally coupled to the inhibitory G protein, G_i. The G_i protein inhibits adenylyl cyclase activity thus keeping intracellular levels of cAMP suppressed.

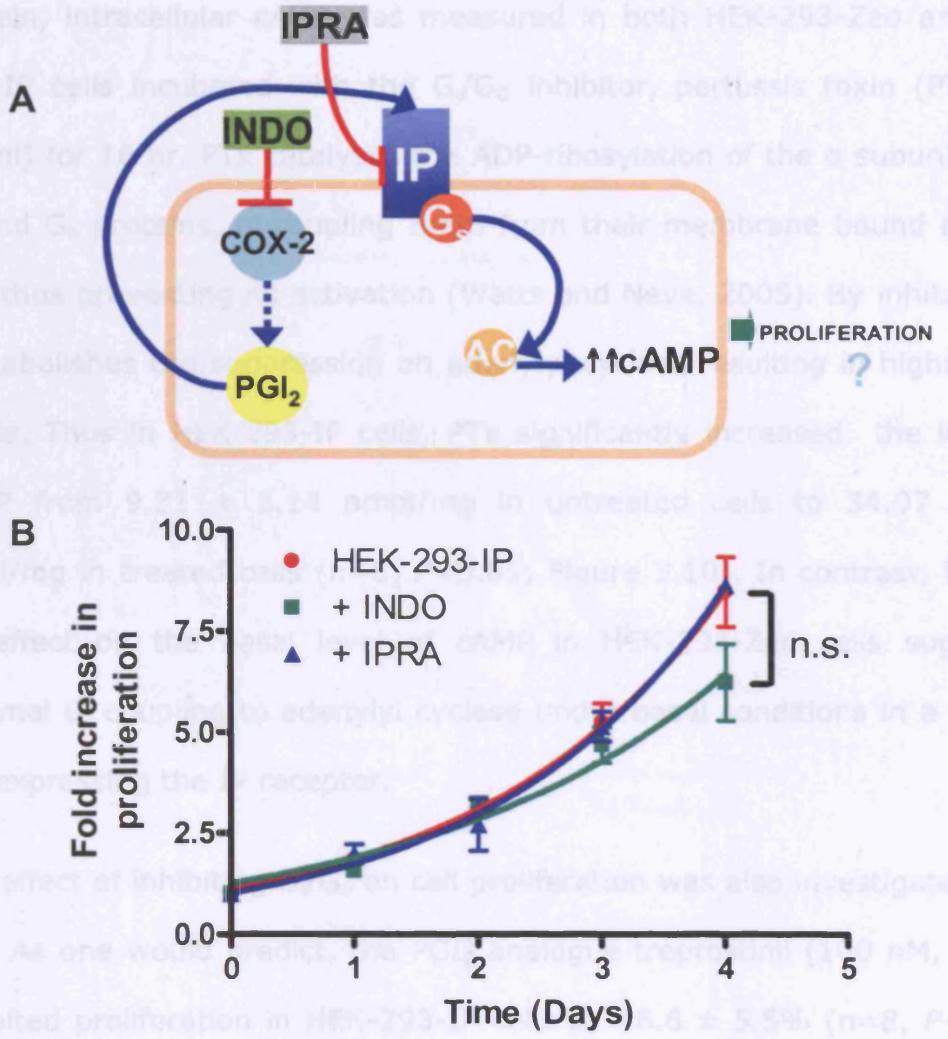

Figure 3.9 A. Schematics of the possible effect of the COX-2 antagonist indomethacin (INDO) and the IP receptor antagonist RO1138452 (IPRA) on the hypothesised basal activation of the IP receptor by endogenously produced PGI_2. (AC), adenylyl cyclase. **B**. Growth arrested HEK-293-IP cells were stimulated with 10% FBS and either left untreated (●) or were treated with INDO (■, 10 μM) or IPRA (▲, 1 μM). Cells were counted at different time points as shown. Data shown as mean ± S.E.M. (measurements in triplicate of 3-6 experiments). (n.s. = non significant)

In order to determine whether the IP receptor was indeed coupled to the G_i protein, intracellular cAMP was measured in both HEK-293-Zeo and HEK-293-IP cells incubated with the G_i/G_O inhibitor, pertussis toxin (PTx, 100 ng/ml) for 16 hr. PTx catalyses the ADP-ribosylation of the α subunit of the G_i and G_o proteins, uncoupling them from their membrane bound receptor and thus preventing its activation (Watts and Neve, 2005). By inhibiting G_i, PTx abolishes the suppression on adenylyl cyclase resulting in higher cAMP levels. Thus in HEK-293-IP cells, PTx significantly increased the levels of cAMP from 9.31 ± 3.14 pmol/mg in untreated cells to 34.07 ± 7.43 pmol/mg in treated cells (n=6, $P<0.05$; Figure 3.10). In contrast, PTx had no effect on the basal level of cAMP in HEK-293-Zeo cells suggesting minimal G_i coupling to adenylyl cyclase under basal conditions in a cell line not expressing the IP receptor.

The effect of inhibiting G_i/G_o on cell proliferation was also investigated using PTx. As one would predict, the PGI_2 analogue treprostinil (100 nM, 48 hrs) inhibited proliferation in HEK-293-IP cells by 46.6 ± 5.5% (n=8, $P<0.005$) compared to 10% FBS alone (Figure 3.11 B) but not in cells lacking the IP receptor (Figure 3.11 A). Furthermore, incubation with PTx in the absence of treprostinil also significantly inhibited the proliferation of HEK-293-Zeo cells by 24.8 ± 6.7% (n=12, $P<0.01$) and HEK-293-IP cells by 42.5 ± 9.7% (n=8, $P<0.01$). PTx further potentiated the inhibitory effect of treprostinil, though this was only significant in HEK-293-IP cells (74.8 ± 6.8%, n=8, $P<0.05$). The observation that PTx inhibits proliferation in HEK-293 cells both in the presence and absence of the IP receptor suggests that the effects of suppressing G_i/G_o coupling may not be wholly related to the IP receptor. However because significant potentiation to treprostinil does occur

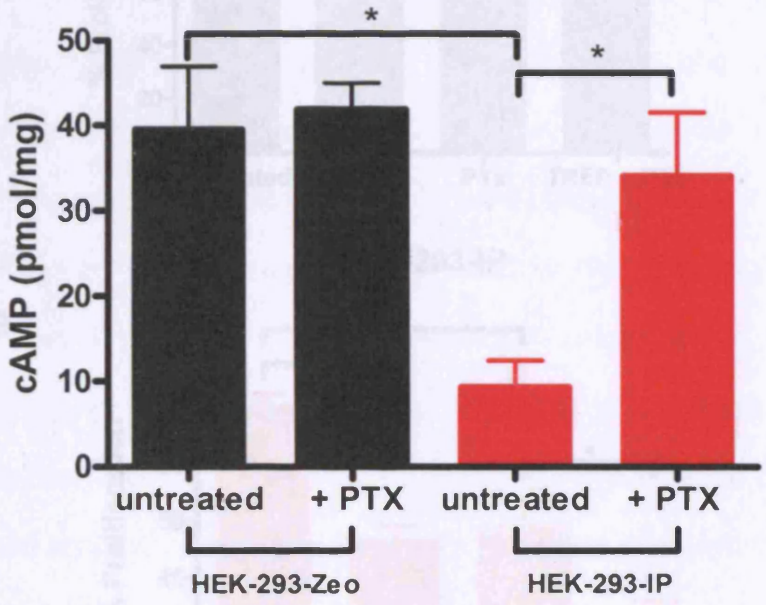

Figure 3.10 Effect of inhibiting G_i with pertussis toxin (PTx) on basal cAMP levels. HEK-293-Zeo (black bars) and HEK-293-IP (red bars) cells were grown in MEM + 10% FBS and either left untreated or stimulated with PTx (100 ng/ml) for 16 hrs. Cyclic AMP was extracted and measured using an enzyme immunolinked assay. Results expressed as mean pmol of cyclic AMP per mg of total protein ± S.E.M. (measurements in duplicate of 3 (HEK-293-Zeo) or 6 (HEK-293-IP) experiments. *=p<0.05.

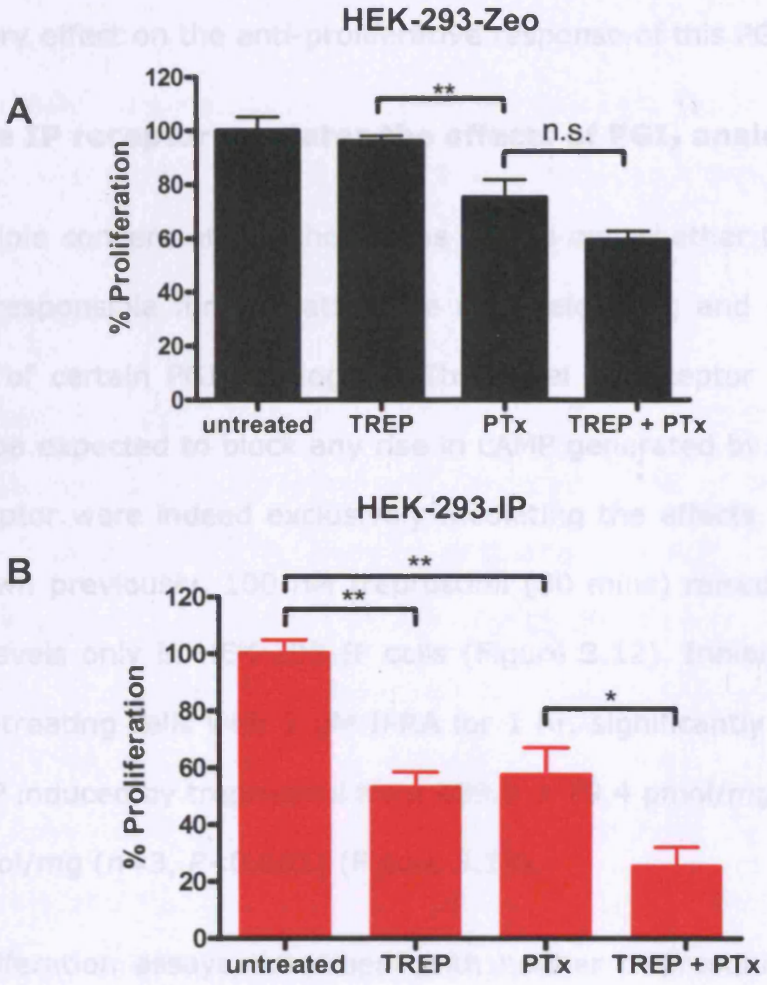

Figure 3.11 Comparison of the effects of treprostinil (TREP) and Pertussis Toxin (PTx) on HEK-293-Zeo (**A**) and HEK-293-IP (**B**) cells. Growth arrested cells were were stimulated with 10% FBS and either left untreated or were treated with TREP (100 nM), PTx (100 ng/ml), or a combination of both. Cells were pretreated with PTx for 16 hrs prior to stimulation with TREP. Cells were counted 48 hrs following treatment. Data expressed as mean % proliferative response relative to proliferation mediated by 10% FBS alone ± S.E.M. (n=8-12). * = $P<0.05$, ** = $P<0.01$, n.s.= not statistically significant.

in HEK-293-IP cells it suggests that G_i and/or G_o have some specific inhibitory effect on the anti-proliferative response of this PGI_2 analogue.

3.7 The IP receptor mediates the effects of PGI_2 analogues.

A principle concern of this thesis was to find out whether the IP receptor is solely responsible for mediating the cAMP-elevating and growth-inhibitory effects of certain PGI_2 analogues. The novel IP receptor antagonist, IPRA would be expected to block any rise in cAMP generated by treprostinil if the IP receptor were indeed exclusively mediating the effects of this analogue. As shown previously, 100 nM treprostinil (30 mins) raised the intracellular cAMP levels only in HEK-293-IP cells (Figure 3.12). Inhibiting the receptor by pre-treating cells with 1 μM IPRA for 1 hr, significantly reduced the rise in cAMP induced by treprostinil from 459.5 ± 79.4 pmol/mg (n=4) to 30.1 ± 4.5 pmol/mg (n=3, $P<0.001$) (Figure 3.12).

In proliferation assays, treatment with neither treprostinil nor carbacyclin (both at 1 μM for 48 hrs) inhibited proliferation of HEK-293-Zeo cells (Figure 3.13 A). The fact that the cell permeable analogue carbacyclin did not inhibit the proliferation of cells lacking the receptor suggests that there are no alternative intracellular targets (for example PPARs) present in HEK-293 cells that could mediate the anti-proliferative effects of this agent. Moreover, treprostinil completely blocked the proliferation of HEK-293-IP cells, whereas carbacyclin was significantly less effective ($P<0.05$), at the same concentration, inhibiting proliferation by only 69.3 ± 11.1% (n=12) relative to untreated control cells (Figure 3.13 B). Proliferation assay results are expressed as the mean % proliferation rate at 48 hrs in cells incubated in 10% FBS relative to the proliferation rate of cells kept in 0-0.1% FBS for

the same length of time. Normalisation was necessary because the growth response to FBS was variable. Figure 3.14 displays a representative assay where results are expressed in total cell number. Experiments were only included in our data analysis if there was between a two and a three fold increase in the proliferation rate between cells stimulated with 10% FBS and those cultured in 0-0.1% FBS.

In studying the cell proliferation responses of HEK-293-IP cells, the dose-response curves to treprostinil and carbacyclin (48 hr treatment) further indicate the difference in potency of these two PGI_2 analogues (Figure 3.15). Treprostinil inhibited FBS stimulated growth with an IC_{50} of 16 nM compared to carbacyclin, which has an IC_{50} of 116 nM. The effects of treprostinil on cell growth were long lasting as shown in Figure 3.16. HEK-293-IP cells were left to replicate with or without treatment of 1 μM treprostinil and counted every 24 hrs for 5 days. Under these conditions, proliferation was almost completely inhibited for the duration of the experiment, indicating that treprostinil remained effective for up to 5 days.

To further confirm the role of the IP receptor in PGI_2-regulated growth control, IPRA was used in proliferation assays. Treatment of HEK-293-IP cells with 100 nM IPRA for 48 hrs showed a small but non significant inhibition of proliferation (17.8 ± 8.3%, n=11, P=0.23). As expected, treatment with 100 nM treprostinil for 48 hrs greatly inhibited proliferation relative to untreated control by 77.9 ± 3.3% (n=11, P<0.001), an effect which was almost completely reversed in the presence of IPRA (n=11, P<0.001; Figure 3.17).

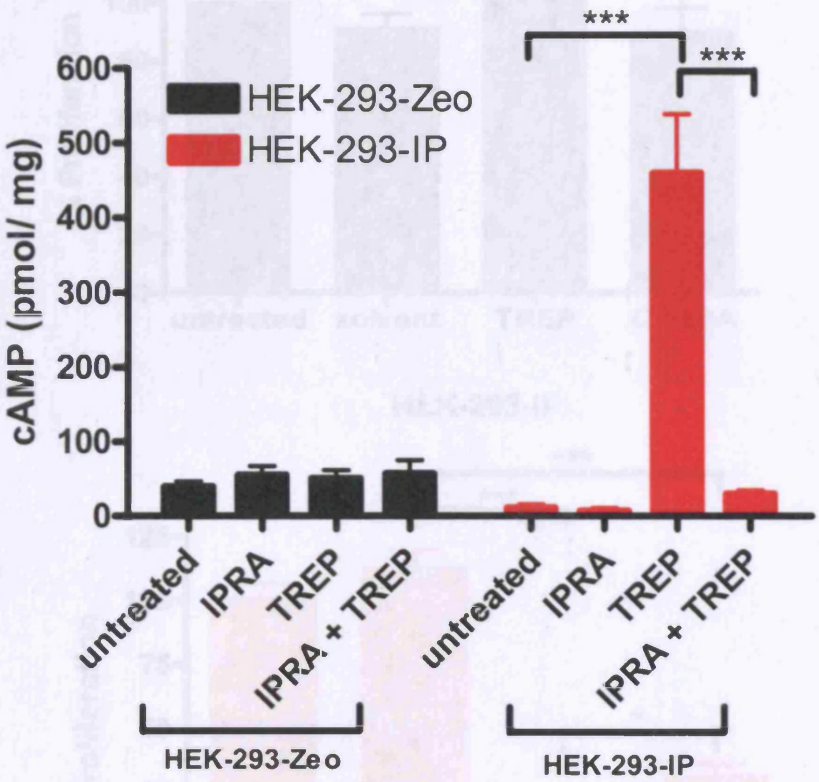

Figure 3.12 Cyclic AMP was measured in HEK-293-IP (black bars) or HEK-293-Zeo (red bars). In both cases, cells were stimulated with 10%FBS ± either TREP (100 nM) or IPRA (1 μM) or a combination. Cells were pretreated with IPRA for 1 hr prior to stimulation with TREP. Cyclic AMP was extracted 30 mins after TREP stimulation and measured using an enzyme immunolinked assay. Results expressed as mean pmol of cyclic AMP per mg of total protein ± S.E.M. (measurements in duplicate of 3-4 experiments). ***=p<0.001.

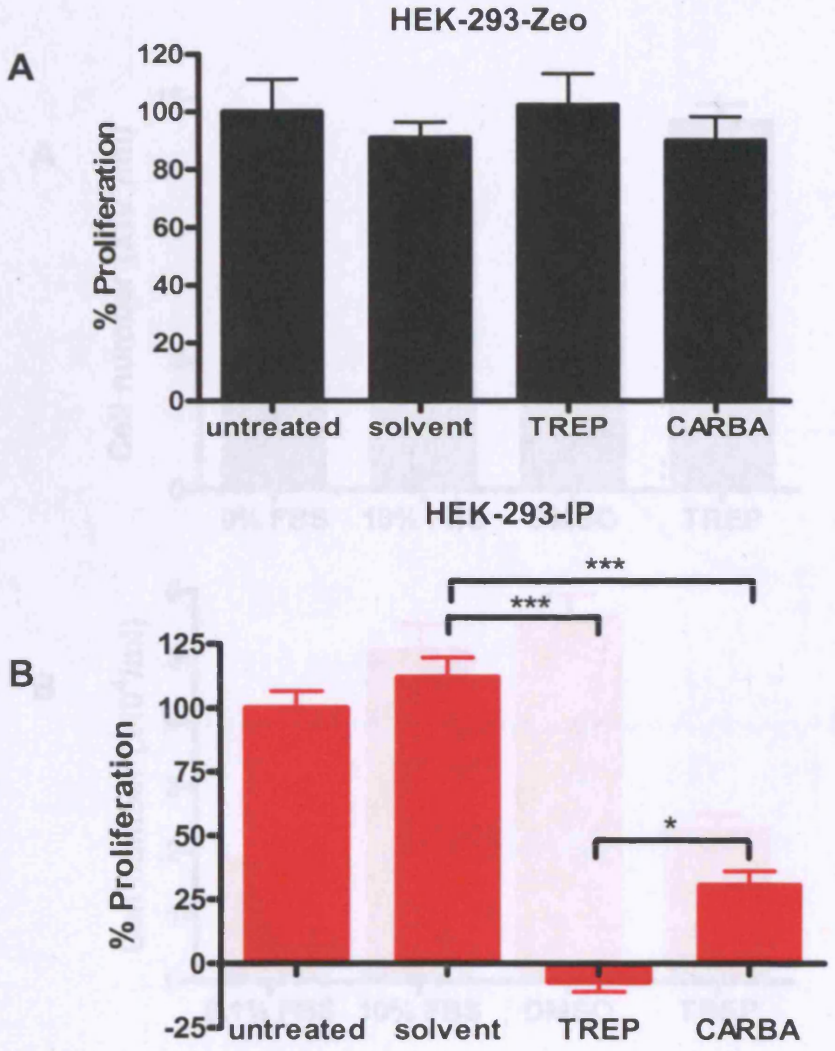

Figure 3.13 Comparison of the effects of treprostinil (TREP) and carbacyclin (CARBA) on HEK-293-WT (**A**) and HEK-293-IP (**B**) cells. Growth arrested cells were stimulated with 10% FBS and either left untreated (solvent control) or were treated with TREP (1 μM) or CARBA (1 μM). Cells were counted 48 hrs following treatment. Data expressed as mean % proliferative response relative to proliferation mediated by 10% FBS alone ± S.E.M. (n=12). * = $P<0.05$, *** = $P<0.001$

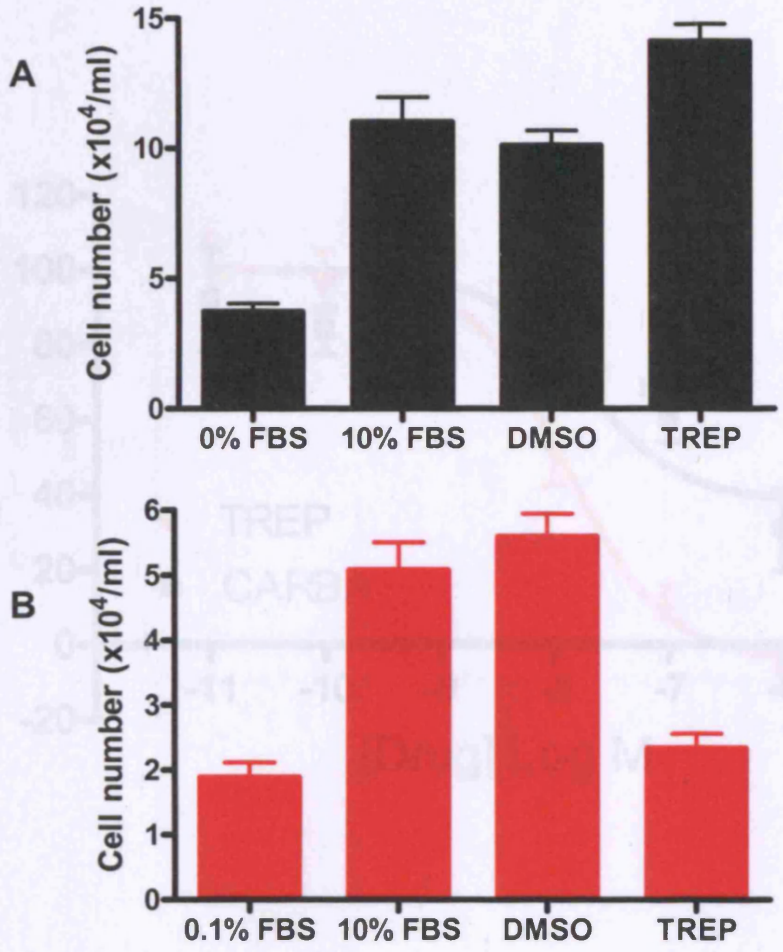

Figure 3.14 Representative proliferation assay comparing the effects of treprostinil (TREP) on HEK-293-WT (**A**) and HEK-293-IP (**B**) cells. Growth arrested cells were either stimulated with 0.-0.1% FBS or stimulated with 10% FBS in the absence or presence of DMSO (1:10000) or TREP (1 μM). Cells were counted 48 hrs following treatment. Data expressed as mean cell number (x10^4/ml) ± S.E.M. of one experiment performed in triplicate (n=3).

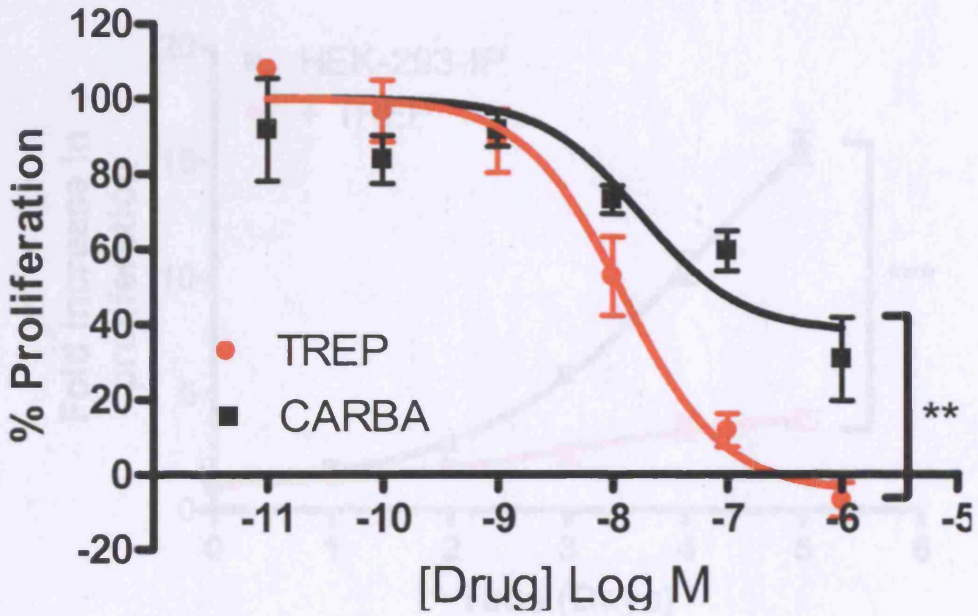

Figure 3.15 Dose response curves for treprostinil (TREP) and carbacyclin (CARBA). Growth arrested HEK-293-IP cells were stimulated with 10% FBS ± TREP (●) or CARBA (■) at different concentrations and counted after 48 hrs. IC_{50} for TREP = 16 nM, IC_{50} for CARBA = 116 nM Data expressed as mean % proliferative response relative to proliferation mediated by 10% FBS alone ± S.E.M. (measurements in triplicate of 3 separate experiments). ** = P<0.005.

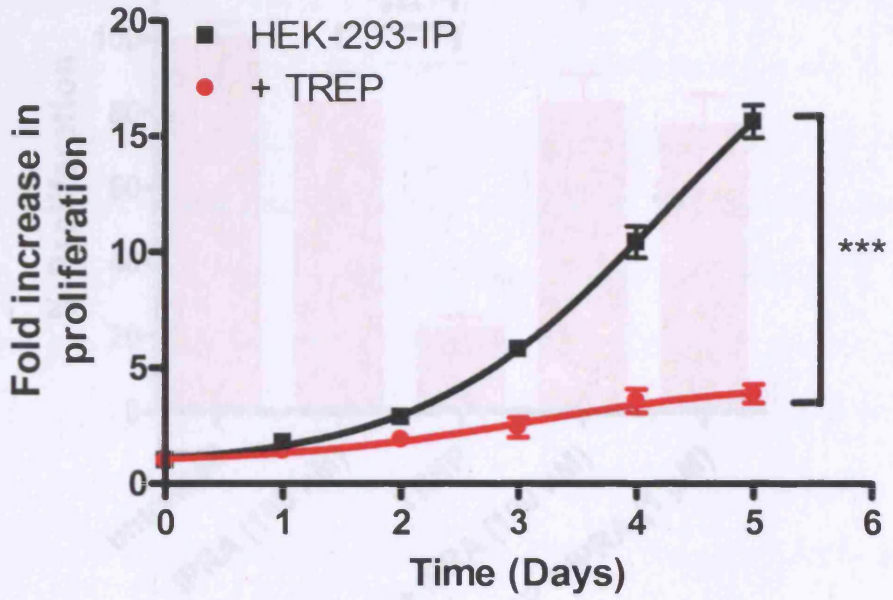

Figure 3.16. Proliferation curves of HEK-293-IP cells either left untreated (■) or treated with treprostinil (TREP, ●, 1 μM). Growth arrested cells were stimulated with 10% FBS ± TREP and counted at different time points as shown. Data shown as mean ± S.E.M. (measurements in triplicate of 4 experiments). *** = $P<0.001$

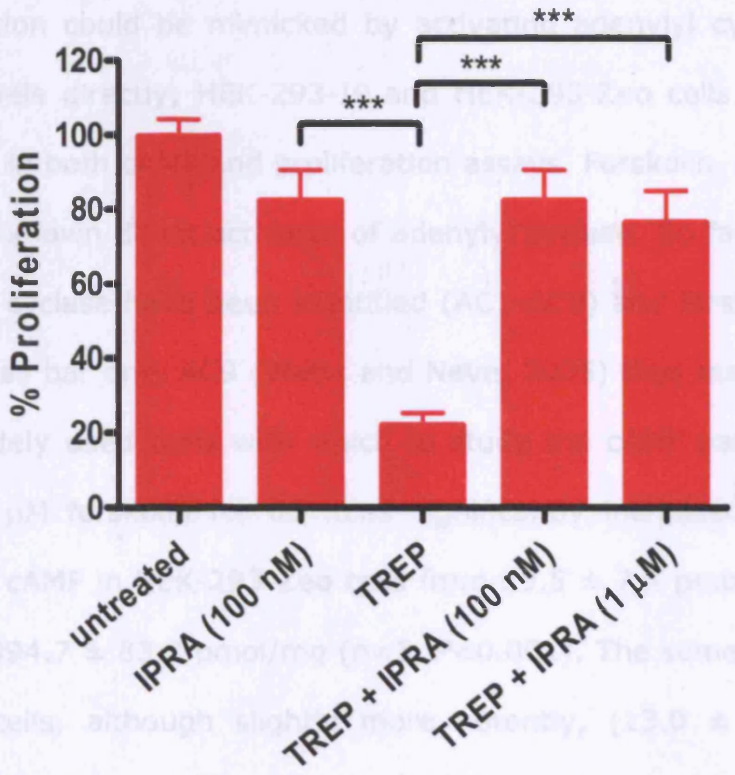

Figure 3.17 Growth arrested HEK-293-IP (red bars) cells were stimulated with MEM + 10% FBS and either left untreated or treated with TREP (100 nM), IPRA or a combination. Cells were pretreated with IPRA for 1 hr prior to stimulation with TREP. Cells were counted 48 hours following treatment. Data expressed as mean % proliferative response relative to proliferation mediated by 10% FBS alone ± S.E.M. (n=11). *** = $P<0.001$

3.8 Role of cAMP in regulating treprostinil effects on cell proliferation

To find out whether the effects of treprostinil on both cAMP levels and proliferation could be mimicked by activating adenylyl cyclase to increase cAMP levels directly, HEK-293-IP and HEK-293-Zeo cells were exposed to forskolin in both cAMP and proliferation assays. Forskolin, a plant diterpene, is a well known direct activator of adenylyl cyclase. So far nine isoforms of adenylyl cyclase have been identified (AC1-AC9) and forskolin can strongly activate all bar one, AC9 (Watts and Neve, 2005) thus making it one of the most widely used tools with which to study the cAMP pathway. Treatment with 10 μM forskolin for 30 mins significantly increased the intracellular levels of cAMP in HEK-293-Zeo cells from 39.5 ± 7.4 pmol/mg in untreated cells to 994.7 ± 83.2 pmol/mg (n=3, $P<0.001$). The same occurred in HEK-293-IP cells, although slightly more potently, (13.0 ± 5.7 pmol/mg in untreated cells vs 1353 ± 22.7 pmol/mg in forskolin-treated cells, n=3, $P<0.001$; Figure 3.18). When cells were co-treated for 30 mins with both treprostinil (100 nM) and forskolin (10 μM), the effects of these agents were not potentiated (Figure 3.18) seemingly as if the cAMP-elevating response to either agent is saturated in HEK-293-IP cells and maybe the two agents are activating the same pool of adenylyl cyclase.

Surprisingly, in HEK-293-Zeo cells forskolin, like treprostinil, did not have any significant effect on cell proliferation (Figure 3.19 A). Furthermore although forskolin stimulated a considerable increase in cAMP in both cell types, it only inhibited proliferation in cells expressing the IP receptor. As shown in Figure 3.19 B, treatment with 10 μM forskolin for 48 hrs

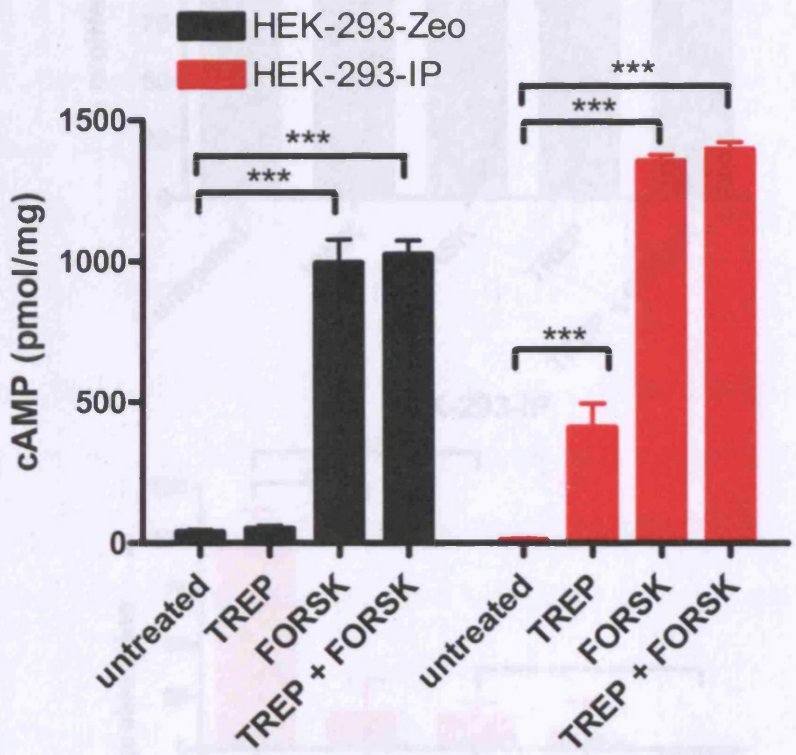

Figure 3.18 HEK-293-Zeo (black bars) and HEK-293-IP (red bars) cells were stimulated with MEM + 10% ± FORSK (10 μM) or TREP (100 nM) or a combination as shown. Cyclic AMP was extracted after 30 mins and measured using an enzyme immunolinked assay. Results expressed as mean pmol of cyclic AMP per mg of total protein ± S.E.M. (measurements in duplicate of 3 (HEK-293-Zeo) or 6 (HEK-293-IP) experiments. *** = P<0.001.

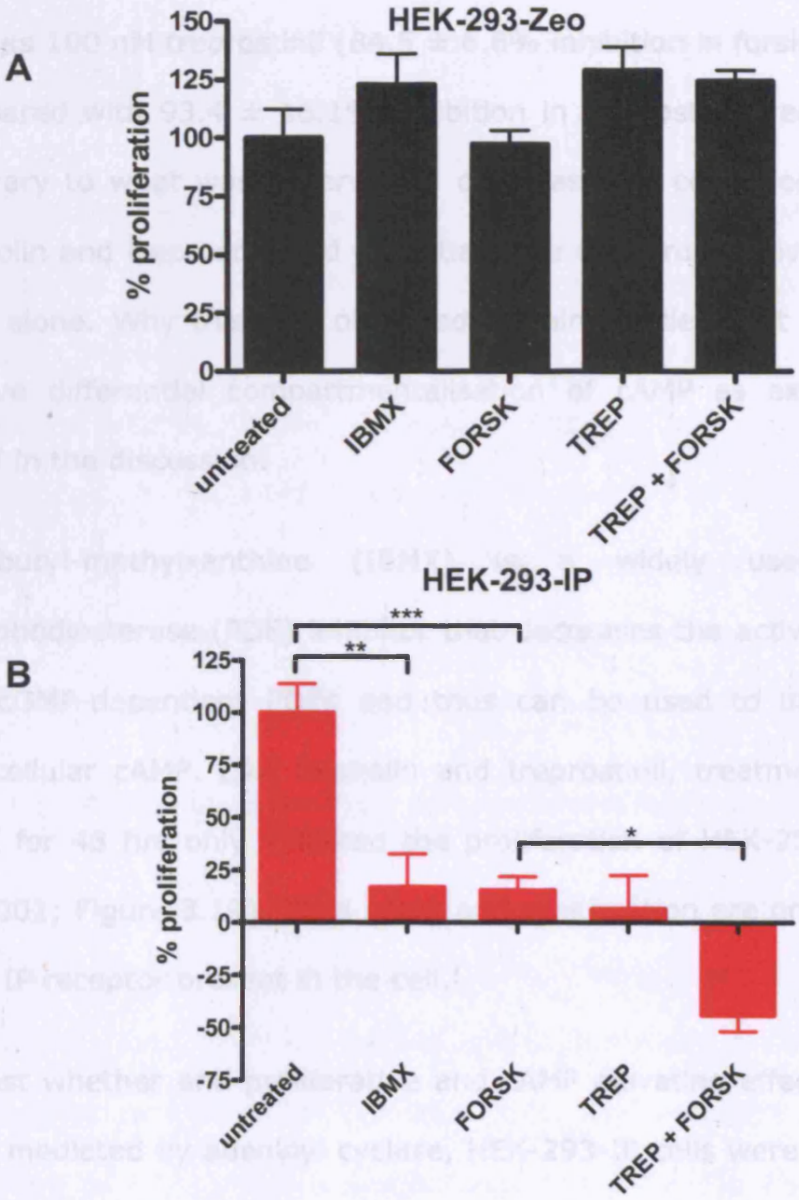

Figure 3.19 HEK-293-Zeo (A) and HEK-293-IP (B) cells were grown in MEM + 10% FBS and either left untreated or stimulated with FORSK (10 μM), IBMX (10 μM), TREP (100 nM) or a combination as shown. Cells were counted 48 hours following treatment. Data expressed as mean % proliferative response relative to proliferation mediated by 10% FBS alone ± S.E.M. (n=6). * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

completely inhibited the proliferation of HEK-293-IP cells to about the same level as 100 nM treprostinil (84.5 ± 6.6% inhibition in forskolin treated cells compared with 93.4 ± 16.1% inhibition in treprostinil treated cells, n=6). Contrary to what was observed in cAMP assays, combined treatment with forskolin and treprostinil did potentiate the anti-proliferative effect of either drug alone. Why this was observed remains unclear but could potentially involve differential compartmentalisation of cAMP as explained in more detail in the discussion.

3-isobutyl-methylxanthine (IBMX) is a widely used non-selective phosphodiesterase (PDE) inhibitor that decreases the activity of both cAMP and cGMP-dependent PDEs and thus can be used to increase levels of intracellular cAMP. Like forskolin and treprostinil, treatment with 100 μM IBMX for 48 hrs only inhibited the proliferation of HEK-293-IP cells (n=6, $P<0.001$; Figure 3.19). Thus cAMP and proliferation are only linked if there is an IP receptor present in the cell.

To test whether anti-proliferative and cAMP elevating effects of treprostinil were mediated by adenylyl cyclase, HEK-293-IP cells were pre-treated with the adenylyl cyclase antagonist, 2'5'- dideoxyadenosine (DDA) prior to stimulation with treprostinil. DDA inhibits adenylyl cyclase by binding to the inhibitory binding domain (P site). Treatment with 100 μM DDA alone for 1.5 hrs unexpectedly significantly increased the levels of cAMP from 13.4 ± 5.4 pmol/mg to 45.0 ± 7.2 pmol/mg, (n=3, $P<0.05$; Figure 3.20). Pre-treatment with DDA for 1 hr prior to stimulation significantly inhibited the treprostinil-induced rise in cAMP, reversing it from 528.6 ± 55.3 pmol/mg to 193.9 ± 50.3 pmol/mg (n=3, $P<0.01$). However, DDA did not appear to

suppress the response to treprostinil completely although this was not statistically significantly different from treatment with DDA alone (P=0.099; Figure 3.20).

In proliferation assays, DDA alone had a small inhibitory effect on HEK-293-IP cells (33.4 ± 3.2% inhibition compared to untreated controls, n=8, P<0.001; Figure 3.21). When cells were pre-treated with DDA prior to stimulation with treprostinil, DDA significantly inhibited the anti-proliferative effect of treprostinil, proliferation being similar to that found with DDA alone (n=8, P<0.001). From our data it appears that adenylyl cyclase mediates about 75% of the anti-growth response, although incomplete inhibition may reflect the small rise in cAMP observed in the presence of DDA.

3.9 Role of PKA in regulating treprostinil effects on cell proliferation

It is generally assumed that the high levels of cAMP generated by PGI_2 analogue induced activation of adenylyl cyclase will activate PKA and inhibit cell proliferation. Therefore the role of PKA in the anti-proliferative effects of IP receptor activation in HEK-293 cells was assessed by blocking the activation of PKA and seeing what effect this had on treprostinil-induced inhibition of proliferation in HEK-293-IP cells. Two PKA inhibitors were used; H-89 and the more PKA specific agent, Rp-MB-cAMPS. Treatment for 48 hrs with either 10 μM H-89 or 100 μM Rp-MB-cAMPS alone resulted in a small but non-significant increase in proliferation of HEK-293-IP cells compared to the same cells grown in 10% FBS containing mono-butyryl (a control for Rp-MB-cAMPS treatment). The marked inhibitory effect of 100 nM treprostinil on cell proliferation was significantly reduced by pre-treating cells for 30 mins with H-89 (n=12, P<0.001) or with Rp-MB-cAMPS (n=18,

$P<0.01$; Figure 3.22) suggesting that PKA is indeed involved in mediating the effects of treprostinil. Both antagonists, however, failed to restore proliferation of HEK-293-IP cells to the level observed in cells treated with the antagonists alone ($P<0.05$). This suggests that there may be an additional PKA-independent component to the antiproliferative effects of treprostinil. Similarly to DDA, PKA antagonists appeared to reverse the anti-proliferative effects by approximately 75%, suggesting that a further 25% of the response must be mediated by a cAMP-PKA independent mechanism.

3.10 Summary

- A HEK-293 stably transfected cell line expressing the IP receptor was generated and characterised and used to study IP receptor signalling pathways.
- A novel specific antibody for the IP receptor was developed and characterised and used to investigate the localisation of this receptor in HEK-293 cells
- The novel antibody was shown to be more specific than other antibodies used in the literature.
- Physical presence of the IP receptor slows the proliferation rate of HEK-293 cells and is crucial in mediating the anti-proliferative effects of PGI_2 analogues.

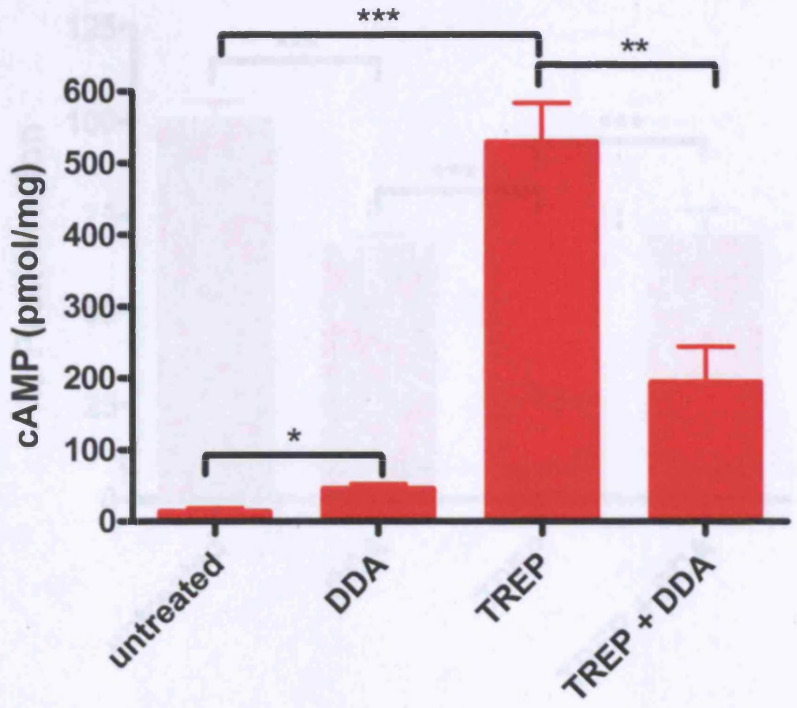

Figure 3.20 HEK-293-IP cells were stimulated with MEM + 10% FBS ± 2'5'-DDA (100 μM) or TREP (100 nM) or a combination as shown. Cells were pretreated with 2'5'-DDA for 1 hr prior to stimulation with TREP. Cyclic AMP was extracted 30 mins after treatment with TREP and measured using an enzyme immunolinked assay. Results expressed as mean pmol of cyclic AMP per mg of total protein ± S.E.M. (measurements in duplicate of 3 experiments). *=p<0.05, ** = P<0.01, *** = P<0.001

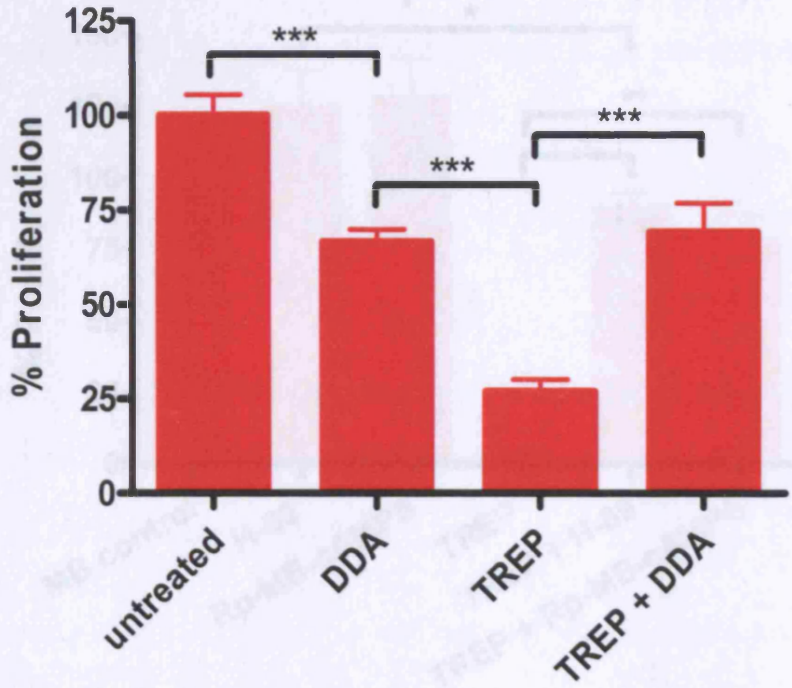

Figure 3.21 Growth arrested HEK-293-IP (red bars) cells were stimulated with MEM + 10% FBS and either left untreated or treated with TREP (100 nM) or 2'5'-DDA (100 μM) or a combination as shown. Cells were pretreated with 2'5'-DDA for 1 hr prior to stimulation with TREP. Cells were counted 48 hours following treatment. Data expressed as mean % proliferative response relative to proliferation mediated by 10% FBS alone ± S.E.M. (n=8). *** = P<0.001

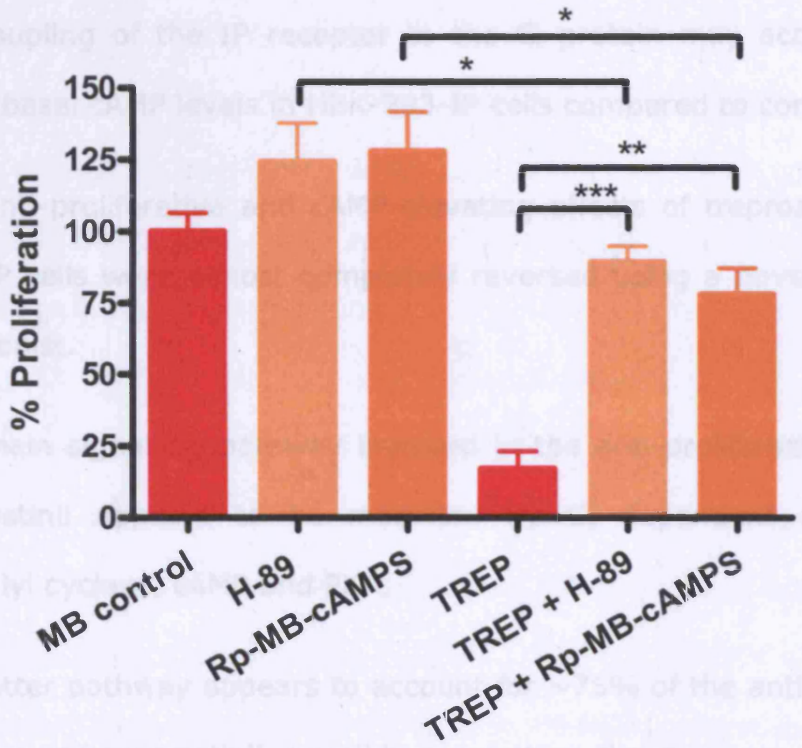

Figure 3.22 Growth arrested HEK-293-IP cells were stimulated with MEM + 10% FBS and either left untreated or treated with H-89 (10 μM), Rp-MB-cAMPS (100 μM), TREP (100 nM) or a combination as shown. Cells were pretreated with H-89 nor Rp-MB-cAMPS for 30 mins prior to stimulation with TREP. Cells were counted 48 hours following treatment. Data expressed as mean % proliferative response relative to proliferation mediated by 10% FBS + mono butyryl (MB) alone ± S.E.M. (n=12-18). * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$

- Presence of the IP receptor alters the proliferative response of HEK-293 cells to cAMP-elevating agents.
- Pre-coupling of the IP receptor to the G_i protein may account for the lower basal cAMP levels in HEK-293-IP cells compared to control cells.
- The anti-proliferative and cAMP-elevating effects of treprostinil in HEK-293-IP cells were almost completely reversed using a novel IP receptor antagonist.
- The main signalling pathway involved in the anti-proliferative effects of treprostinil appears to be mediated by G_s dependent activation of adenylyl cyclase, cAMP and PKA.
- The latter pathway appears to account for ~75% of the anti-proliferative response to treprostinil, possibly suggesting the presence of additional cAMP-PKA independent mechanism/s.

3.11 Discussion

3.11.1 Establishment and characterisation of the HEK-293-IP cell line

In native smooth muscle cells it is likely to be harder to dissect the signalling pathway of the IP receptor because PGI_2 analogues have affinity for other prostanoid receptors, which are present in these cells, and until very recently there was no IP receptor antagonist to analyse the role of these different pathways. A HEK-293 cell line stably expressing the IP receptor (HEK-293-IP) was generated in this project to better understand the role of the IP receptor in isolation. RT-PCR and immunohistochemistry confirmed the presence of the receptor in these cells. In parallel, a HEK-293-Zeo cell line was generated to control for any non-specific effects of stable transfection. This was done by stably transfecting cells with a plasmid encoding resistance to the antibiotic zeocin. RT-PCR in these cells as well as in wild type cells (HEK-293-WT) showed virtual absence of the receptor. The functionality of the receptor was shown by using a cAMP assay and assessing whether stimulation of cells increased intracellular cAMP levels in an IP receptor antagonist reversible manner. As predicted this occurred in the HEK-293-IP cells and not in the HEK-293-Zeo cells, again validating the model used in these studies.

Although the HEK-293 cell line does not possess the same sophisticated level of biochemical processes and interactions as native pulmonary artery smooth muscle cells do (of particular interest to this project), they retain many advantages that make them ideally suited to this study. This cell line has been used extensively as an expression tool since it was generated over

25 years ago (Graham *et al.,* 1977). The principal reason for using a HEK-293 cell line in this project was the ability to study the properties of our protein of interest, the IP receptor, in isolation from other receptors of the same, or different families which may be activated by PGI_2. Other characteristics which make it such a popular tool are its fast and easy reproduction and maintenance; the relative ease and high efficiency of transfection that can be achieved using a wide range of methods and the faithful translation and processing of proteins (Thomas and Smart, 2005).

The HEK-293-IP cell line is also a valuable tool which helped us characterise novel antibodies. Immunohistochemistry was performed using an anti-IP receptor antibody developed and generated during this project. This antibody (C1) was raised against a peptide at the C-terminal of the protein providing a different target to the Komhoff *et al* (N1) and the Cayman antibodies. The antibody staining was abundantly visualised at the membrane as well as in the cytoplasm of stably transfected HEK-293 cells. The pattern of expression was in fact analogous to that shown by Smyth *et al* (1998) in HEK-293 cells transfected with a hemagglutinin (HA)-tagged human IP receptor construct (HA-hIP) and stained with an anti-HA antibody. The similarity between the IP receptor localisation described in HEK-293-HA-hIP (Smyth *et al.,* 1998) and in HEK-293-IP cells in the present study further strengthens the validity of our newly developed C1 antibody. The abundant presence of staining in the cytoplasm may be attributed to the nature of over-expressing the IP receptor in these cells. Because of the large quantity of receptor produced in these cells, receptor density at the plasma membrane may be saturated hence many receptors may be excessively internalised.

Based on using our HEK-293-IP cell line, the C1 antibody appeared to be selective for the IP receptor, whereas this did not appear to be the case for either the N1 or the Cayman antibody. In the study by Komhoff *et al* (1998) they assessed the specificity of the N1 antibody by performing Western immunoblotting on membrane fractions from human platelets. Platelets are a good positive control as they are known to abundantly express IP receptor which functions as a counteracting pathway for the pro-aggregatory actions of thromboxane (Oliva and Nicosia, 1987). The study detected a diffuse band with an apparent molecular mass of about 52 kDa (Komhoff *et al.*, 1998) although as mentioned before the predicted molecular weight of the IP receptor from the open reading frame of its cDNA is 41 kDa (Nakagawa *et al.*, 1994). The study attributed the discrepancy between the apparent and the predicted molecular weight to glycosylation of the IP receptor. However no negative control showing lack of a band in a cell extract known to lack the IP receptor was shown. In terms of immunohistochemistry the study did confirm specificity by pre-absorbing the purified N1 antibody with an excess of the peptide to which it was raised against, and showed labelling could be blocked by pre-incubation. This does not entirely exclude non-specific binding of the antibody to other proteins exhibiting a similar epitope. Moreover, instead of the predicted membrane localisation, expression was predominately observed around the nuclear region, which could potentially point to lack of specificity.

The Cayman antibody has also not been studied extensively and no immunohistochemistry appears to have been previously performed using this antibody. The company claims that the antibody detects a band at 67 kDa on a Western immunoblot and again attributes the higher molecular

weight than predicted by the gene product to post-translational modifications.

It appears that the short immunohistochemical study provided in this thesis is to date the most controlled comparison of these three antibodies. The HEK-293-Zeo cell line serves as a good negative control to run alongside our HEK-293-IP cells. By targeting the internal terminus of the IP receptor protein, this antibody may also be more useful in the event of the extracellular portion of the receptor being more susceptible to degradation by proteases and general cell culture maintenance. A further commercially available antibody available from Santa Cruz (Santa Cruz, California) was used in a very recently published study on the role of the IP receptor in angiogenesis in the endometrium (Smith *et al.,* 2006). Like C1 the Santa Cruz antibody also targets the C-terminal of the receptor although the company does not provide the amino acid sequence of the peptide to which the antibody was raised against. Staining of endometrial sections with this antibody shows plasma membrane localization of the IP receptor with some cytoplasmic immunoreactivity also present (Smith *et al.*, 2006). This is again very similar to the expression picked up with C1 possibly indicating that the two are designed to recognise very similar receptor regions. However, even with the late advent of a similar antibody, the new tool developed in this study will prove extremely useful in future studies to better understand the expression and localisation of the IP receptor.

Having established the stable HEK-293 cell lines, the first phenotypical difference noted between the two cell types was that HEK-293-IP cells had a lower rate of proliferation than cells lacking the receptor. It was

hypothesised that this lower rate may be caused by basal constitutive activation of the IP receptor, leading to the classical anti-mitogenic effects of cAMP known to occur in many cell types including vascular smooth muscle cells (Indolfi *et al.*, 1997; Fukumoto *et al.*, 1999; Wharton *et al.*, 2000; Clapp *et al.*, 2002). We presumed that this constitutive activation may be brought about by endogenously produced PGI_2. This appeared not to be the case, as was shown by the lack of effect of the IP receptor antagonist (IPRA) on the basal rate of proliferation. In addition, blocking the putative endogenous production of PGI_2 with the non-selective COX antagonist indomethacin had no effect on HEK-293-IP growth. By blocking COX, indomethacin inhibits the production of all prostanoids including those which have opposing effects to PGI_2 such as the mitogenic prostanoid TXA_2. However, this should have no effect in a HEK-293 cell line system due to the absence of the required prostanoid receptors in these cells. A study by Abramovitz *et al* (2000) found no significant specific binding of a variety of prostanoid receptor ligands (e.g. PGE_2, PGD_2, PGF_2, iloprost, and the thromboxane receptor agonist, U46619) to membranes prepared from mock-transfected HEK-293 cells.

3.11.2 Multiple G Protein coupling to the IP receptor

Basal intracellular cAMP levels were lower in HEK-293-IP cells than in HEK-293-Zeo, again suggesting the absence of tonic G_s activity. However, upon treatment with PTx, which irreversibly inhibits G_i and G_o activity, cAMP levels in HEK-293-IP cells approached levels close to those measured in HEK-293-Zeo. This strongly suggests that the IP receptor can also couple to G_i. Although PTx is able to act on both G_i or G_o, the former is a more likely

candidate in light of a study in HEK-293 cells transiently transfected with a CFP-IP receptor construct and a YFP-tagged G_o protein. The study demonstrated that in the absence of agonists, the IP receptor coupled to G_s but found no evidence that this occurred with G_o (Nobles *et al.,* 2005). Although the study showed that the IP receptor is indeed coupled to G_s, it must not be assumed that this association renders the G_s protein active. In contrast there may be constitutive activation of G_i pre-coupled to the IP receptor in the HEK-293-IP cells which represses adenylyl cyclase activity, thus maintaining low levels of cAMP as we found in this project.

Constitutive activation of G_i by the IP receptor has never been described; however there is ample experimental evidence in the literature supporting the existence of receptors tightly associated to their respective G protein and ensuing constitutive activation in the absence of ligand. This is the case for the CB_1-canabbinoid receptor (Vasquez and Lewis, 1999), the melatonin (Mel_{1a}) receptor (Roka *et al.,* 1999) and the vasoactive intestinal peptide (VCAP1) receptor (Martin, 2002). Vasquez and Lewis suggested that the CB-1 receptor, because of its tight pre-association with G_i/G_o in the absence of ligand, sequesters a proportion of the available G_i/G_o protein pool. This leads to a reduction of available G_i/G_o protein, restricting activation by other G_i/G_o coupled receptors. The study demonstrated that expression of the G_i/G_o-coupled CB1 receptor attenuated the ability of α2-adrenergic receptors (α2AR) and somatostatin receptors to activate G_i/G_o in superior cervical ganglion neurons (Vasquez and Lewis, 1999). Similarly, expression of the G_s-coupled serotonin receptor, $5\text{-}HT_7$ (5-hydroxytryptamine-$_7$), attenuated adenylyl cyclase activation by other G_s coupled receptors expressed endogenously in HEK-293 cells, an effect shown to be

independent of agonist binding (Andressen *et al.,* 2006). It is possible therefore that a similar mechanism may underlie the observation that HEK-293-IP cells have a slower proliferative rate than wild type cells. Although in need of extensive further investigation, it is plausible to think that because of its high affinity and potential pre-coupling to G_i, the over-expressed IP receptor may be sequestering the pool of G_i necessary for the activation of ERKs by growth factors and other proliferative pathways.

G protein coupling of the IP receptor has been the subject of intensive debate. Namba and colleagues (Namba *et al.,* 1994) showed that the murine IP receptor (mIP), when stably expressed in Chinese hamster ovary (CHO) cells, coupled independently to G_s and G_q. Iloprost increased cAMP and IP_3 levels; the latter was insensitive to pertussis toxin as well as to a 24 hr pre-treatment with cholera toxin, which degrades G_s (Namba *et al.,* 1994). Degrading G_s, nevertheless did result in a loss of the iloprost-induced rise in cAMP. In other studies, where the human IP receptor has been expressed in HEK-293 cells, independent coupling to G_s and G_q was demonstrated but no coupling to G_i was observed (Miggin and Kinsella, 2002). Previously, Lawler and colleagues (Lawler *et al.,* 2001) demonstrated that the mIP receptor, when expressed in HEK-293 cells, could only couple to G_q or G_i after coupling to G_s had first occurred and that such a process required PKA-mediated phosphorylation of the receptor. This is in contrast to studies in CHO cells where G_q coupling was not dependent on G_s and PKA (Chow *et al.,* 2003). Some of the conflicting data may be explained by the fact that IP receptor coupling to different G proteins may be a cell-type dependent phenomenon.

In native cells, there does appear to be a clear role for G_i/G_o involvement in mediating or modulating responses to PGI_2 analogues. A PTx-sensitive G protein appears to mediate the vasodilatory effects of iloprost in the porcine cerebral circulation (Zucker and Leffler, 1998) and be required for the antiproliferative effects of cicaprost in mouse aorta (Kothapalli *et al.*, 2003). The former study concluded that the IP receptor is linked to PLC, activating a component of adenylyl cyclase through a PKC-dependent phosphorylation. In rat tail artery the relaxant responses to cicaprost were substantially potentiated after pre-treatment with PTx (Orie *et al.,* 2006), an effect similar to what was observed in HEK-293-IP cells, suggesting that Gi/Go coupling is not necessary for treprostinil-mediated inhibition of proliferation but rather is exacting some inhibitory influence on the responses to this agent.

3.11.3 Role of the IP receptor in mediating the anti-proliferative effects of treprostinil

The present study demonstrates that the IP receptor alone can mediate the anti-proliferative effects of PGI_2 analogues. The effects of treprostinil could be significantly reversed by the novel specific IP receptor antagonist, RO1138452. In addition, both treprostinil and carbacyclin could only inhibit proliferation of HEK-293 cells transfected with the IP receptor. The IC_{50} for the anti-proliferative effects of treprostinil was found to be approximately 16 nM. This value is similar to that observed previously in native human pulmonary artery smooth muscle cells where treprostinil inhibited serum-induced proliferation with an IC_{50} of 4.2 nM (Clapp *et al.,* 2002) supporting a common mechanism likely to be through the IP receptor. These results

are in agreement with data from IP receptor knockout mice whereby cicaprost was not able to inhibit the proliferation of cultured aortic smooth muscle cells from these mice even though it retained a strong anti-mitogenic effect in cells from wild type mice (Kothapalli *et al.*, 2003). It would appear that the IP receptor is essential to mediate other PGI_2-dependent cellular functions too since cicaprost could no longer produce hypotension or inhibit platelet aggregation in IP receptor knockout mice (Murata *et al.,* 1997).

It has been suggested that the PGI_2 analogues may have anti-proliferative effects independent of the IP receptor. Both carbacyclin and iloprost have been shown to bind and activate the PPARα and PPARδ nuclear receptors (Hertz *et al.*, 1996; Forman *et al.*, 1997). Carbacyclin, however did not inhibit proliferation in HEK-293-WT cells thus excluding an IP-receptor independent mechanism. In addition, treprostinil was actually more potent than carbacyclin in HEK-293-IP cells, and it is generally thought that the former is much less cell-permeable. These observations suggest that drug cell-permeability does not confer an anti-proliferative advantage and strengthens the fact that these agents function primarily by activating the cell surface IP receptor.

Proliferation assays were performed in the presence of foetal bovine serum as we felt that serum, compared to treatment with just one growth factor, triggers proliferative pathways more akin to what would be observed *in vivo*. Serum in fact contains a plethora of hormone factors to stimulate cell growth and proliferation including platelet derived growth factor (PDGF) and insulin-like growth factor (IGF-1). There are therefore a number of different

pro-proliferative pathways which are activated by serum, mainly the PI3 kinase, the STAT and the MAPK-ERK pathways. In addition to growth factors, serum contains transport and binding proteins which take care among other things of the supply of hormones, minerals and lipids. A frequent critique of utilising serum is that batches can vary greatly in the amount and composition of growth factors and that there may be non-specific effects due to the presence of other serum proteins. To control this as much as possible, the same batch of FBS was used for the majority of the experiments in this thesis.

3.11.4 Role of cAMP in cell proliferation

In the present study we have confirmed that the anti-proliferative effects of treprostinil are primarily mediated by adenylyl cyclase as demonstrated by the reversal of the anti-mitogenic effects of the former with the antagonist, 2'5'-dideoxyadenosine (DDA). Treprostinil-induced cAMP-elevation could also be significantly reversed with both IPRA and DDA; in both cases this correlated with a reversal of its growth effects in HEK-293-IP cells. These observations are in accordance with previous studies in the literature which demonstrated the reversal of PGI_2 analogue-induced inhibition of proliferation with DDA in human PASMC (Wharton *et al.*, 2000; Clapp *et al.*, 2002) and rat VSMC (Phillips *et al.*, 2005). In addition activating adenylyl cyclase directly with forskolin also inhibited proliferation in these cells as did inhibiting phosphodiesterases with IBMX. These results support a classical G_s pathway for treprostinil and an anti-proliferative role for cAMP.

Forskolin activated adenylyl cyclase and increased cAMP in both HEK-293-IP and control cells although in the former cell type forskolin-induced cAMP-

elevation was significantly higher than in HEK-293-Zeo cells. This may be attributed to a process termed heterologous sensitisation. Acute activation of G_i-coupled receptors inhibits adenylyl cyclase and attenuates cAMP accumulation; in contrast prolonged activation of G_i, as may occur in HEK-293-IP cells in the absence of ligand, typically sensitises adenylyl cyclase to subsequent activation by forskolin or G_s-coupled receptors (Watts and Neve, 2005). This was first demonstrated by Sharma and colleagues for the $G_{i/o}$ coupled morphine-activated δ opioid receptors (Sharma *et al.*, 1975). Later Thomas and Hoffman (Thomas and Hoffman, 1987) proposed a model in which the persistent activation of G_i-coupled receptors induces heterologous sensitisation in a PTX-sensitive, G protein-dependent fashion, resulting in enhanced cAMP responsiveness. The mechanism remains unknown but possibly involves alterations in G_s, G_i or adenylyl cyclase.

Although treprostinil appears to inhibit proliferation via a classical cAMP dependent mechanism, cAMP levels do not exclusively correlate with growth in all cases observed in this project. This is demonstrated by the following observations: 1) HEK-293-Zeo cells have a higher basal level of cAMP which is actually associated with a higher rate of proliferation. 2) Forskolin increases cAMP in HEK-293-Zeo cells but does not inhibit their proliferation. 3) The higher level of cAMP induced by forskolin compared to treprostinil does not correlate with a more pronounced inhibition of proliferation in HEK-293-IP cells; the two agents in fact display roughly the same level of inhibition. The same was observed in human pulmonary artery smooth muscle (HPASM) cells where the cAMP-elevating properties of PGI_2 analogues did not correlate with their anti-proliferative effects (Clapp *et al.*, 2002). The explanations for these observations are not clear but suggest

that the mere presence of the IP receptor modulates the response to cAMP elevating agents.

Cyclic AMP is one of the most studied second messenger signalling molecules. In many cases it is thought to be anti-proliferative and this effect has been attributed mainly to the ability of cAMP to inhibit the extracellular signal-related kinase (ERK) signalling pathway, the activity of which appears essential for the proliferation of many cell types, although other targets may also mediate these effects (reviewed in Dumaz and Marais, 2005). Cyclic AMP, via the activation of PKA, suppresses ERK signalling through its ability to target the serine/threonine-specific kinase C-RAF, a mechanism involving the uncoupling of RAS signalling to C-RAF (Figure 3.23)(Dumaz and Marais, 2005). In addition cAMP has also been shown to inhibit vascular smooth muscle cells by up-regulating p21, an inhibitor of G1 cyclin-dependent kinases, and the tumour suppressor gene p53, which has been shown to induce cell cycle arrest at the G1/S border (Hayashi *et al.,* 2000).

However cAMP can also be pro-proliferative as has been shown in different cell types, including rat pheochrymocytoma PC12 cells, melanocytes and thyroid cells (Dumaz and Marais 2005) as well as in polycystic kidney epithelial cells (Belibi *et al.*, 2004). The mechanism here is again thought to involve the ERK signalling pathway but in contrast to targeting C-RAF, cAMP is thought to act via B-RAF and this is linked to stimulation of proliferation. However, models to explain ERK activation are incomplete and may involve RAS, Rap1, Src , PKA and 14-3-3 as well as B-RAF (Figure 3.23) (Dumaz and Marais, 2005).

Cyclic AMP signalling however is not as simple as the set of linear cascades described above might suggest. There are nine isoforms of adenylyl cyclases, eight phosphodiesterase families that hydrolyse cAMP, three PKA catalytic and four regulatory subunits and over thirty different AKAPs. Other than PKA, there are a number of other cAMP effectors, among these there are two Epacs (exchange proteins directly activated by cAMP), up to six cAMP-gated channels and cAMP-response-element-binding-protein (CREB) (Dumaz and Marais, 2005). Furthermore, the consequences of biological signalling have been shown to depend on cell type and specific cellular contexts. Suffice it to say, that the cAMP signalling pathway is exceptionally intricate and complex, and our understanding of its regulation

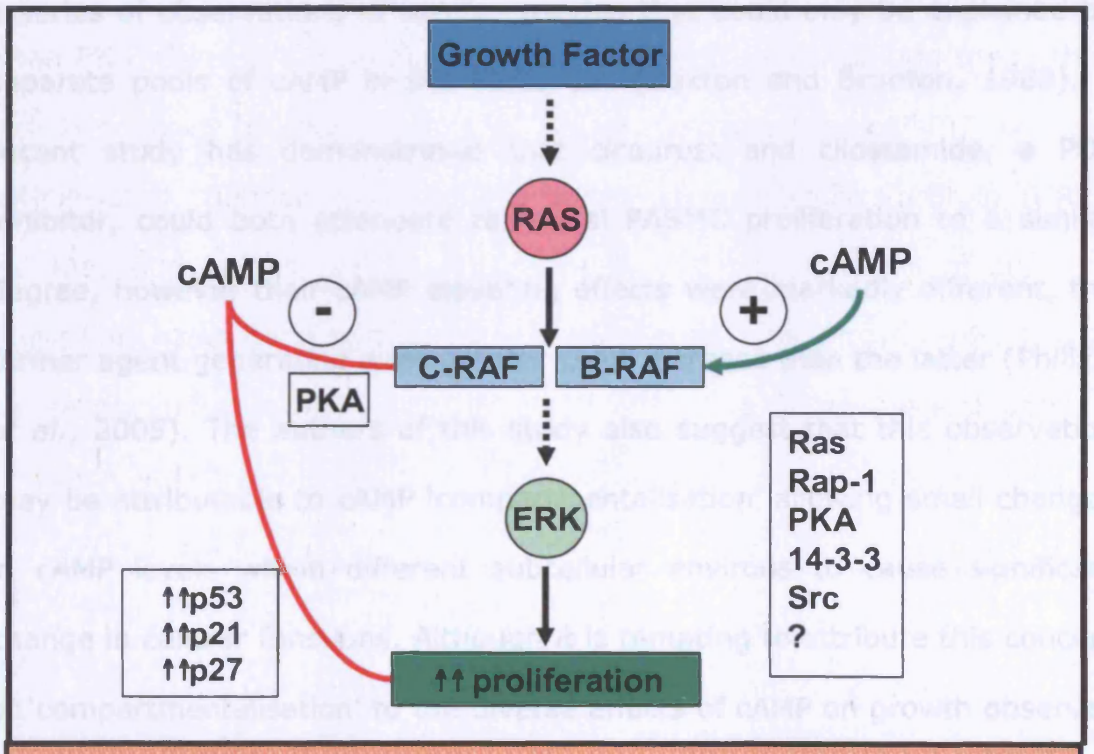

Figure 3.23 The diverse effects of cAMP on cell proliferation can depend on the responses of C-RAF and B-RAF. The inhibition of the ERK mitogenic pathway by cAMP is linked to C-RAF inhibition. In contrast ERK activation by cAMP is attributed to B-RAF. The latter mechanism is still poorly understood but may involve Ras, Rap1, Src, PKA and 14-3-3.

is far from complete. The global measurement of intracellular cAMP, therefore, probably trivialises the complexity of the cAMP signalling pathway.

Evidence is accumulating about the compartmentalisation of adenylyl cyclase and cAMP signalling into micro-domains within the cell and such a possibility would help explain some of the apparent inconsistencies in the cAMP responses observed in this project. It has been shown that cAMP kinetics are quite transient at the membrane compared with a gradual accumulation of cAMP in the cytoplasm and that these different cAMP localisations may have distinct signalling consequences (Cooper, 2005). This theory was first proposed in 1983 by Buxton and Brunton when they made a series of observations in cardiomyocytes that could only be explained by separate pools of cAMP in the same cell (Buxton and Brunton, 1983). A recent study has demonstrated that cicaprost and cilostamide, a PDE inhibitor, could both attenuate rat distal PASMC proliferation to a similar degree, however their cAMP elevating effects were markedly different, the former agent generating a far greater cAMP increase than the latter (Phillips *et al.*, 2005). The authors of this study also suggest that this observation may be attributable to cAMP 'compartmentalisation' allowing small changes in cAMP levels within different subcellular environs to cause significant change in cellular functions. Although it is tempting to attribute this concept of 'compartmentalisation' to the diverse effects of cAMP on growth observed in this project further investigations will be required.

3.11.5 Role of PKA in cell proliferation

We addressed the question of whether PKA was involved in mediating the anti-proliferative effects of IP receptor activation by treprostinil. As the main effector of cAMP it is generally thought that PKA mediates its anti-proliferative effects. Research by Indolfi and colleagues (1997) demonstrated that inhibition of PKA reversed the neointimal formation induced by aminophylline, a phosphodiesterase inhibitor drug (Indolfi *et al.*, 1997) and a more recent study has shown it is crucial in mediating PGI_2 analogue induced differentiation of smooth muscle cells (Fetalvero *et al.*, 2006). Although Phillips and colleagues have recently elucidated PKA's involvement in inhibiting the anti-proliferative effects of cicaprost on PDGF-induced growth (Phillips *et al.,* 2005) ours is the first study to investigate the role of PKA in treprostinil-driven inhibition of serum-induced proliferation. Both PKA antagonists H-89 and Rp-MB-cAMPS reversed the anti-proliferative effects of treprostinil in HEK-293-IP cells. Again this strongly supports a role for cAMP and ensuing PKA activity in producing these effects.

Both PKA antagonists however did not appear to fully reverse the growth inhibition of treprostinil. Cells pre-treated with PKA antagonists prior to stimulation with treprostinil failed to proliferate as much as cells treated with PKA antagonist alone. There are several possible explanations. Firstly, the concentration of antagonists used may not have been fully blocking PKA activity. The doses used however, were the maximal doses used in experiments reported in the literature. Secondly the antagonists may have some non-specific effects on other kinases. H-89 is often quoted as being a

relatively specific blocker of PKA because of its lack of interaction with adenylyl cyclase or any phosphodiesterases (Hidaka and Kobayashi, 1992). However, a study by Davies and colleagues (Davies *et al.,* 2000), found that H-89 affected several other types of protein kinases when they investigated the supposed specificity of a wide range of protein kinase inhibitors. H-89 in fact was found to inhibit 8 protein kinases out of the 24 that were tested by more than 80%. In addition there were three kinases (MSKI, S6K1 and rho-kinase II (ROCKII)) that were inhibited with a similar or greater potency than for PKA. This highlights the fact that care must be taken when interpreting results using this agent. Rp-MB-cAMPS is however, a more specific PKA antagonist being the Rp isomer of a cAMP analogue. This antagonist displayed the same effects as H-89, strengthening the validity of the results.

The most likely hypothesis is that additional PKA-independent pathways may be operating including signalling pathways activated by the IP receptor coupling to other G proteins. Indeed, some studies question the role of the cAMP/PKA pathway in mediating PGI_2 effects altogether. According to a study by Kothapalli and colleagues (2003), cicaprost inhibits proliferation by inhibiting cyclin A expression. This occurs as a result of cicaprost inhibiting CRE occupancy by CREB as well as inhibiting the expression of the cell cycle regulator, cyclinE-cdk2. Furthermore the study shows that the actions of cicaprost are completely reversed in cells treated with PTX suggesting that in this context the IP receptor is mainly signalling through G_i to inhibit proliferation. This however does not concur with the observations of the present study where PTX in fact potentiated the effects of treprostinil on cell growth suggesting that G_s and the ensuing activation of adenylyl cyclase

and not G_i is responsible for the anti-proliferative effects of treprostinil in our system.

Epacs are a family of cAMP-regulated guanine nucleotide exchange factors (cAMPGEFs) that mediate diverse cAMP-dependent cellular processes, including integrin-mediated cell adhesion, vascular endothelial cell barrier formation and cardiac myocyte gap junction formation but is independent of PKA signalling (reviewed in Holz *et al.*, 2006). The latter property of Epacs suggests they could have a potential role in mediating some of the PKA-independent effects of PGI_2 analogues we have observed in HEK-293-IP cells. In future studies their role could be tested with novel Epac-selective cAMP analogues (ESCAs) which fail to mimic cAMP at low concentrations.

In conclusion, we have shown that the IP receptor is crucial in mediating the anti-proliferative effects of treprostinil and that the majority of these effects appear to work via the classical G_s-adenylyl cyclase-PKA pathway. Further study is necessary to elucidate the signalling pathways downstream from PKA and the significance of potential PKA independent pathways. The possibility of a role for the nuclear receptor PPARγ in the cAMP-PKA dependent mechanism or even as a mediator of a cAMP-PKA independent pathway was the subject of further investigation.

Chapter 4

Can PGI_2 analogues activate PPARγ?

Consequence for cell proliferation

4.1 Introduction

PGI_2 and its stable analogues classically mediate their biological effects by binding to the IP receptor activating adenylyl cyclase and triggering signalling through the cAMP/PKA cascade. This study so far has shown that IP receptor-mediated anti-proliferative effects largely involve cAMP but may also be regulated by pathways independent of cAMP and PKA.

In addition to activating cell surface receptors PGI_2 is also a ligand for PPARs, a family of nuclear transcription factors that bind to specific PPREs in the promoter region of target genes to regulate their expression (Lim and Dey, 2002; Wise, 2003). The PPAR family comprises of three isoforms, α, δ (β) and γ, which are activated by a broad spectrum of ligands, including hypolipidemic agents and derivatives produced by the cyclo-oxygenase and lipoxygenase pathway to regulate biological processes such as lipid metabolism and insulin sensitivity (Willson and Wahli, 1997; Desvergne and Wahli, 1999; Lehrke and Lazar, 2005). Because of the complex interaction of prostaglandins with PPARs, the function of endogenous regulation by PGI_2 has been hard to tease out. Nonetheless, PGI_2 signalling through PPARδ is thought to have important roles in embryo implantation (Lim *et al.,* 1999), tumourgenesis (Gupta *et al.,* 2000, Fukumoto *et al.,* 2005) and apoptosis (Hatae *et al.,* 2001). It is less clear if PGI_2 is a major endogenous ligand for PPARα and PPARγ.

Stable PGI_2 analogues like iloprost and carbacyclin ($cPGI_2$), also act as PPAR ligands, directly binding to and causing transcriptional activation of PPARα and PPARδ *in vitro* (Forman *et al.*, 1997). The latter may in part mediate the anti-proliferative effects of treprostinil in lung fibroblasts (Ali *et al.,*

2006). Surprisingly no information exists about whether these analogues can regulate PPARγ. Interestingly, the relatively selective IP receptor agonist, cicaprost does not bind or promote activation of either PPARα or PPARδ (Forman *et al.,* 1997). Thus, cicaprost has often been used experimentally to distinguish between mechanisms involving cell surface IP receptors and PPARs (Wise, 2003; Lim and Dey, 2002). Moreover, PPAR activation is readily observed in cells not expressing the IP receptor (Hatae *et al.,* 2001; Hertz *et al.,* 1996; Forman *et al.,* 1997), and this has led to the assumption that this receptor plays little role in directly regulating PPAR function. However, PPARs are substrates for several kinases, including PKA, which can phosphorylate all isoforms and enhance PPAR activity both in the absence and presence of ligands (Lazennec *et al.*, 2000). This raises the intriguing possibility that the IP receptor may contribute to the regulation of PPARs.

The aim of this study was therefore to investigate whether PGI_2 analogues could regulate PPARγ activity. Given its wide expression in vascular tissue and the immune system, and its broad role as a suppressor of inflammation, tissue injury and cell proliferation, particularly in the lung (Standiford *et al.,* 2005), PPARγ could represent an important therapeutic target for PGI_2 analogues. Using a cell-based reporter gene assay, the effects of PGI_2 analogues on PPARγ activity were examined, and the role of the IP receptor was assessed using the IP receptor antagonist (RO1138452; IPRA) in HEK-293 cell lines stably expressing either the IP receptor or the empty plasmid. The protocol was optimised to take into account non-specific effects of pharmacological agents on plasmid vectors as well as to better control for transfection efficiency and experimental error (see section

2.7.1). Pharmacological tools were used to further dissect the mechanism of PPARγ activation and proliferation was assessed using the same protocol used extensively in chapter 3.

RESULTS

4.2 Effect of rosiglitazone on PPARγ activation

A reporter gene expressing the ligand-binding domain (LBD) of PPARγ (Gelman *et al.,* 1999) was transiently transfected into HEK-293-IP cells and activity measured using a luciferase-based assay. To test the functionality of the PPARγ construct in our system, we investigated the effect of the specific PPARγ agonist, rosiglitazone. At 1 μM this agent significantly increased luciferase levels by 4.86 ± 0.58 fold compared to untreated control (n=12, $P<0.001$), an effect essentially abolished by pre-treatment with the selective PPARγ antagonist, GW9662 (1 μM; n=12, $P<0.001$; Figure 4.1). The antagonist alone had no effect on luciferase levels (Figure 4.1).

4.3 Effect of PGI_2 analogues on PPARγ activation

It has previously been reported that both PPARα and PPARδ can be activated by PGI_2 analogues (Forman *et al.*, 1997), though it is not known whether such agents can activate PPARγ. We therefore investigated the effects of PGI_2 analogues on the PPARγ reporter gene assay. Both treprostinil and carbacyclin significantly increased PPARγ activity by 1.97 ± 0.14 fold and 2.52 ± 0.13 fold respectively (n=12, $P<0.001$) at a concentration of 1 μM (Figure 4.2). Furthermore activation by both analogues was significantly reversed using 1 μM GW9662 (treprostinil,

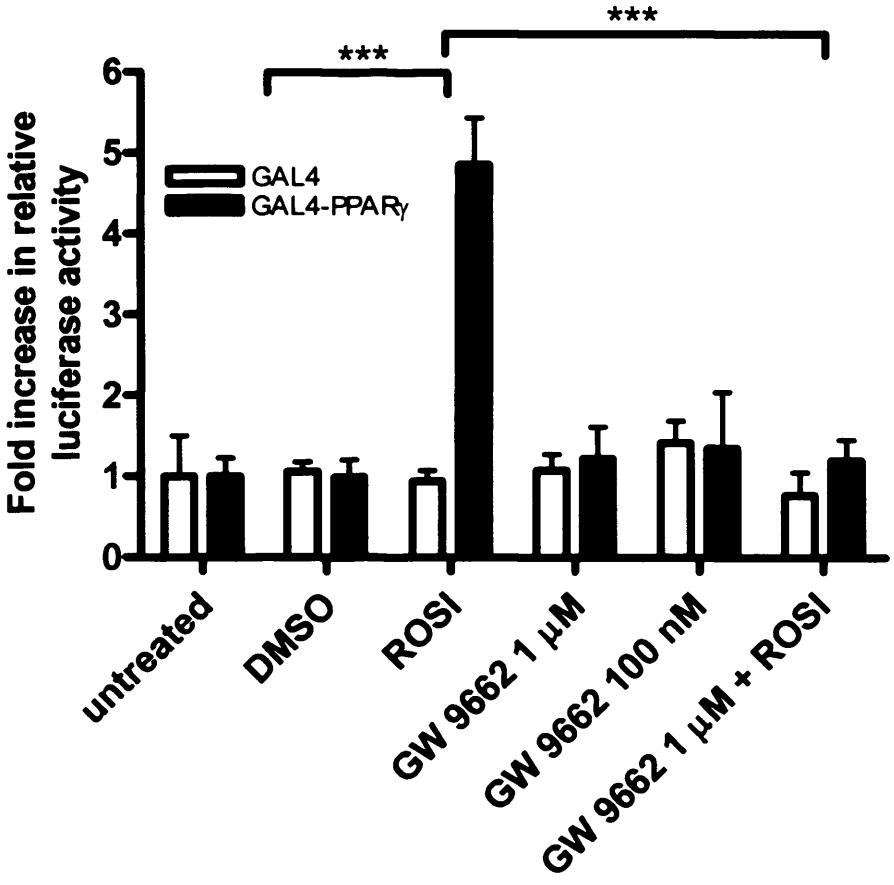

Figure 4.1 HEK-293-IP cells were transiently transfected with pGAL5TKpGL3, pMLUC-2 and either GAL4-pcDNA3 (control – white bars) or GAL4-hPPARγ-pcDNA3 (reporter – black bars). After 48 hr, cells were stimulated with 10% FBS in the absence of drugs (untreated) or presence of rosiglitazone (ROSI, 1 μM), GW-9662 or a combination as shown. Cells were pre-treated with GW9662 (PPARγ antagonist) for 1 hr prior to stimulation with ROSI. Luciferase activity was determined after 24 hr and normalised to *Renilla* activity. Results are expressed as mean fold increase in luciferase activity relative to untreated control ± s.e.m. (n=12, 3 separate transfections). *** = $P<0.001$.

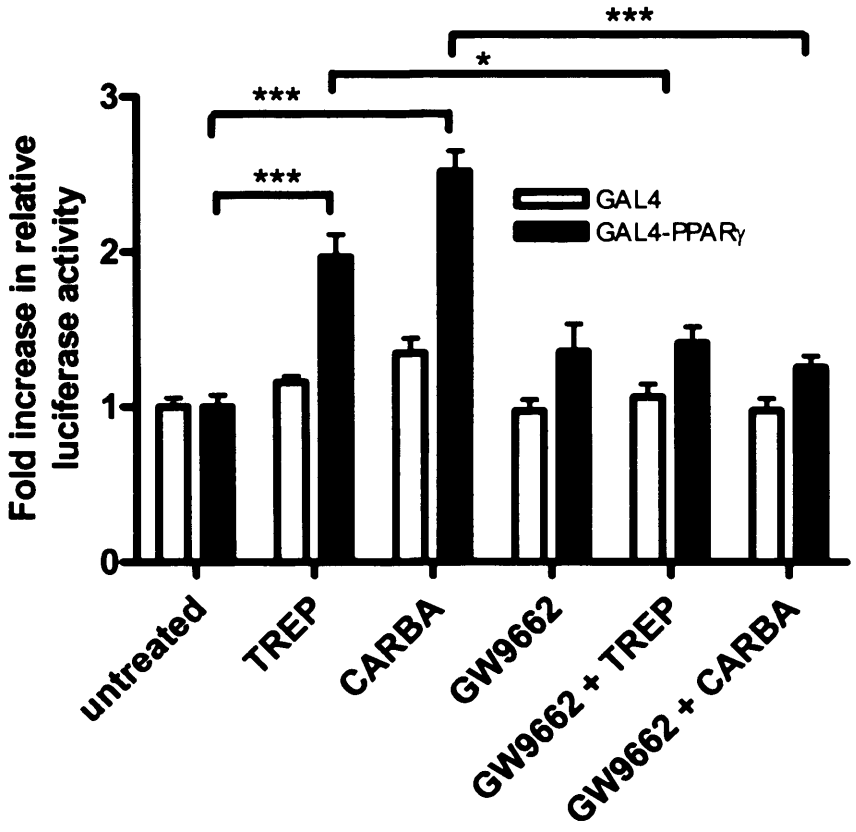

Figure 4.2 Transiently transefected (as in Figure 4.1) HEK-293-IP cells were stimulated with 10% FBS (untreated) ± either treprostinil (TREP), carbacyclin (CARBA), GW9662 (all at 1 μM) or a combination. Cells were pre-treated with GW9662 for 1 hr prior to the addition of agonists. Luciferase activity was determined 24 hr after FBS stimulation and normalised to *Renilla* activity. Results are expressed as mean fold increase in luciferase activity relative to untreated control ± s.e.m. (n=12, 3 separate transfections). *=$P<0.05$, *** = $P<0.001$.

n=12, $P<0.05$ and carbacyclin, n=12, $P<0.001$; Figure 4.2) supporting the notion that PGI_2-mediated increase in luciferase activity is PPARγ specific. Treprostinil induced-PPARγ activation was also dose-dependent, with concentrations as low as 100 nM causing activation, an effect which peaked at around 10 μM (Figure 4.3). In addition, the relatively selective IP receptor agonist, cicaprost (1 μM) significantly increased luciferase activation by 2.2 ± 0.46 fold compared to untreated (n=16, $P<0.05$; Figure 4.4 C).

4.4 Role of the IP receptor in PGI_2 mediated PPARγ activation

Activation of PPARs *via* the conventional route occurs through binding of an agonist to the LBD and this mechanism appears to account for the effects of carbacyclin and iloprost on PPARα and PPARδ (Forman *et al.,* 1997). To test whether PPARγ activation by treprostinil, carbacyclin and cicaprost occurs *via* either direct ligand binding or is mediated through activation of the IP receptor, we examined the effect of the IP receptor antagonist, RO1183452 (IPRA), in cells containing or lacking the IP receptor. Treprostinil, carbacyclin and cicaprost all failed to significantly activate PPARγ at 1 μM in HEK-293-IP cells pre-treated with 1 μM IPRA for 1 hr (Figure 4.4 A, B & C). This antagonist was not an inhibitor of PPARγ activity, since it did not inhibit rosiglitazone activation; on the contrary there was a significant increase in luciferase activity from 8.05 ± 0.83 fold to 13.17 ± 2.84 fold in cells treated with IPRA (n=12, $P=0.023$; Figure 4.5). Furthermore, in cells lacking the IP receptor, neither carbacyclin nor treprostinil activated PPARγ even at the higher dose of 10 μM. This

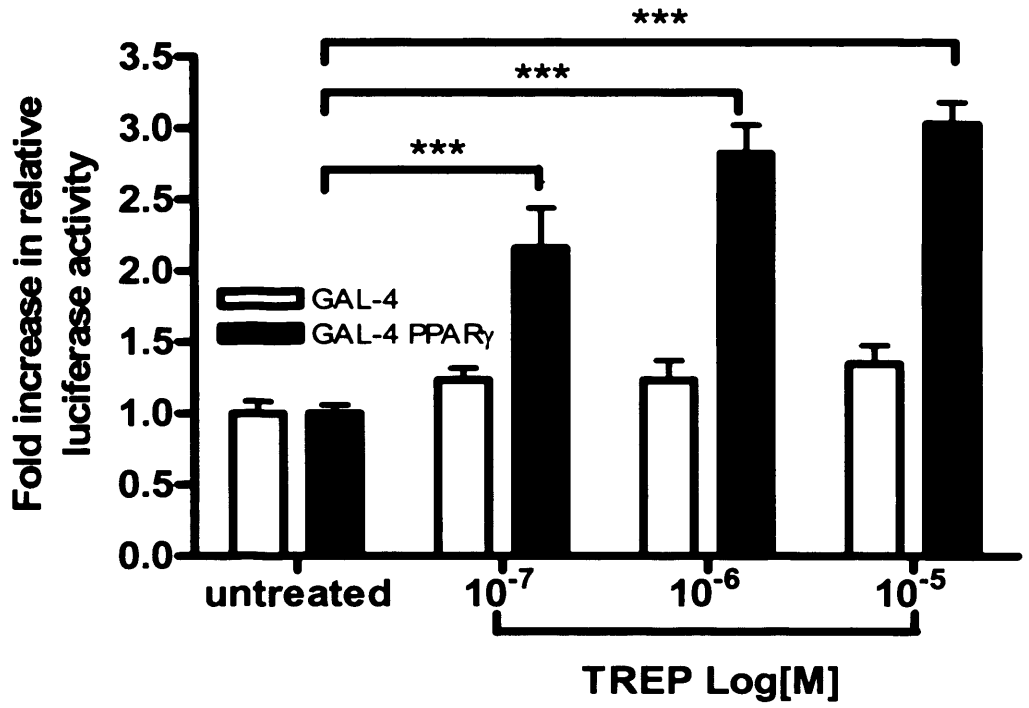

Figure 4.3 Transiently transefected (as Figure 4.1) HEK-293-IP cells were stimulated with 10% FBS (untreated) ± treprostinil (TREP) at varying concentrations as shown. Activation of PPARγ transcription was assessed by measuring relative light units after 24hr of stimulation using a dual luciferase assay system. Luciferase activity was determined 24 hr after FBS stimulation and normalised to *Renilla* activity. Results are expressed as mean fold increase in relative luciferase activity above untreated control as ± s.e.m. (n=12, 3 separate transfections). *** = $P<0.001$,

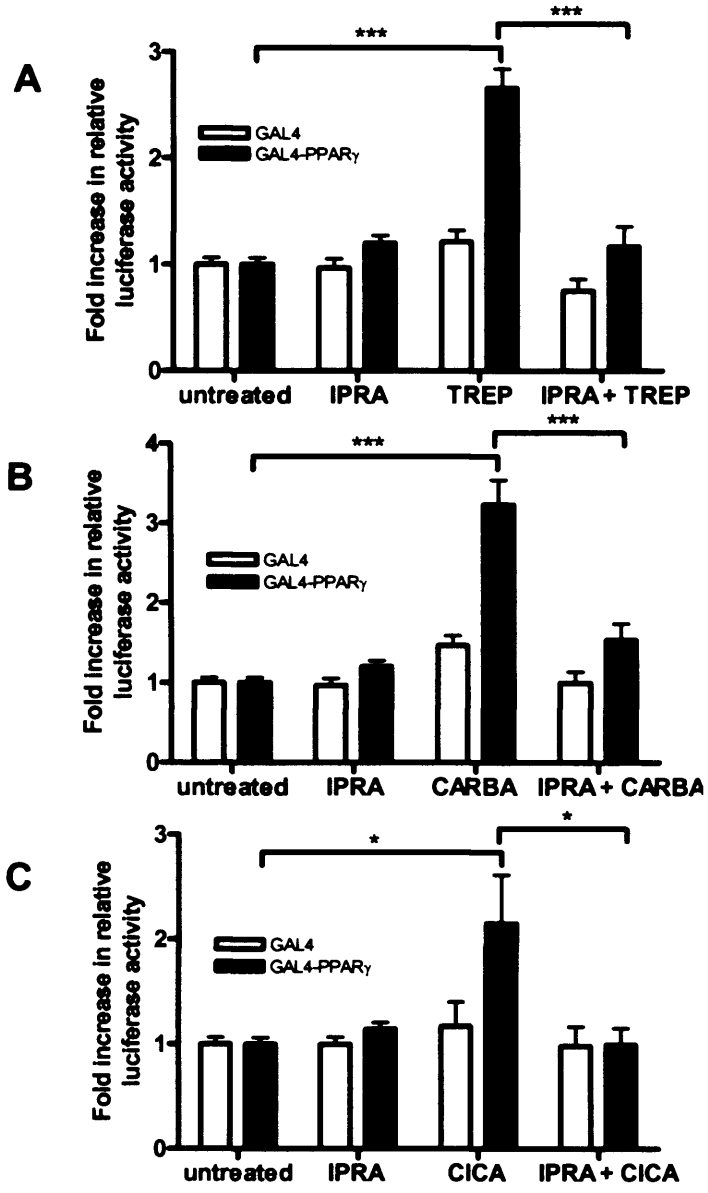

Figure 4.4 Transfected HEK-293-IP cells were stimulated with 10%FBS (untreated) ± either treprostinil (TREP, **A**), carbacyclin (CARBA, **B**), cicaprost (CICA, **C**), IPRA (all at 1 μM) or a combination where cells were pre-treated with antagonist for 1 hr. Luciferase activity was determined 24 hr after stimulation and normalised to *Renilla* activity. Results are expressed as mean fold increase in luciferase activity relative to untreated control ± s.e.m. (n=12-16, 3-4 separate transfections). *=$p<0.05$, ***=$p<0.001$.

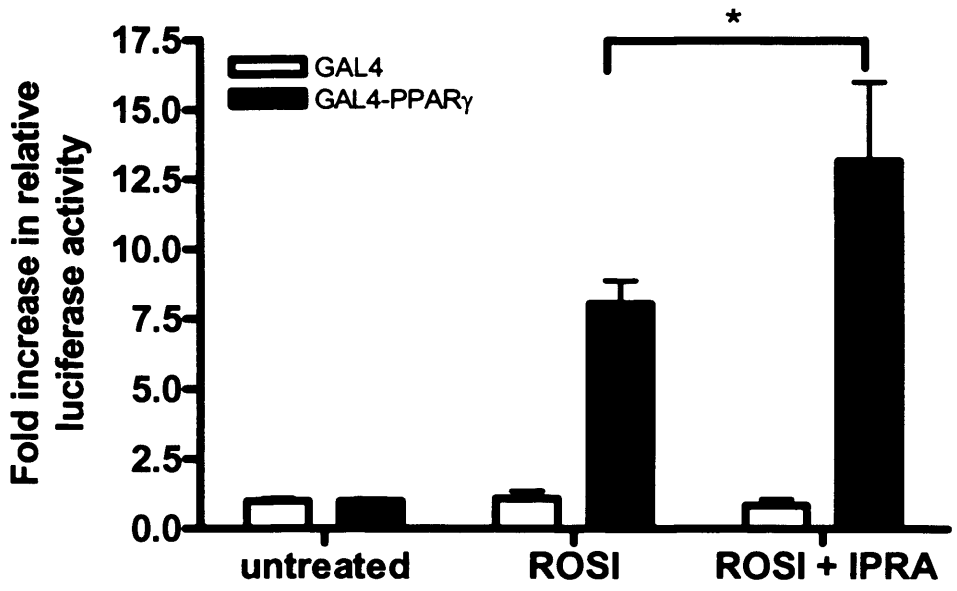

Figure 4.5 Transfected HEK-293-IP cells were stimulated with 10%FBS (untreated) ± either rosiglitazone (ROSI), IPRA (all at 1 μM) or a combination as shown (cells pre-treated with antagonist for 1 hr). Luciferase activity was determined 24 hr after stimulation and normalised to *Renilla* activity. Results are expressed as mean fold increase in luciferase activity relative to untreated control ± s.e.m. (n=12, 3 separate transfections). * = $P<0.05$.

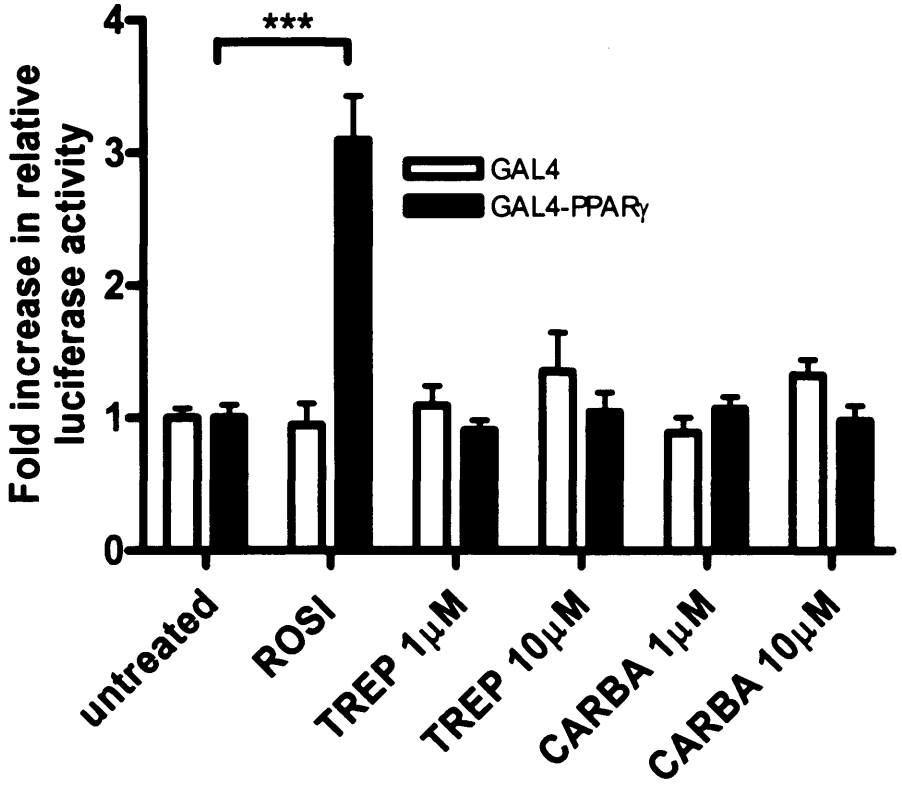

Figure 4.6 Transfected HEK-293-Zeo cells were stimulated with 10%FBS alone (untreated) ± rosiglitazone (ROSI, 1 μM), treprostinil (TREP) or (CARBA). Luciferase activity was determined 24 hr after stimulation and normalised to *Renilla* activity. Results are expressed as mean fold increase in relative luciferase activity from untreated control ± s.e.m. (n=12, 3 separate transfections). ***=$p<0.001$.

occurred despite the effectiveness of rosiglitazone at activating PPARγ (Figure 4.6).

4.5 Role of cAMP and PKA in PGI_2 mediated PPARγ activation

Having established that PGI_2 analogues can activate PPARγ through the IP receptor, we assessed whether activation involved the cyclic AMP pathway, possibly *via* a PKA-dependent phosphorylation of PPARγ. Forskolin, a direct adenylyl cyclase activator failed to have any significant effect on luciferase activity in either HEK-293-Zeo or HEK-293 IP cells (Figure 4.7 A & B), even though it dramatically increased the levels of intracellular cAMP in both cell types (see Figure 3.21 B, chapter 3). This suggests that cAMP alone cannot directly activate PPARγ. In addition, neither the adenylyl cyclase inhibitor, 2'5'-dideoxyadenosine (DDA) nor the selective PKA antagonist Rp-cAMPS (both at 100 μM) significantly reduced activation of PPARγ by treprostinil in HEK-293 IP cells (Figure 4.8).

The alternative, less specific PKA antagonist H-89 had a different effect to Rp-cAMPS. When HEK-293-IP cells were treated with 10 μM H-89 alone for 24 hrs, the basal luciferase activity was lower relative to untreated control (0.47 ± 0.07 fold, n=12, $P<0.01$; Figure 4.9) indicating that H-89 is having an effect on its own, maybe even blocking a kinase needed to drive optimal mTK promoter activity. When cells were pre-treated for 1 hr with H-89 prior to the addition of 1 μM treprostinil, the activating effect of this agent (2.04 ± 0.12 fold increase) was abolished (n=12, $P<0.001$) with luciferase levels dropping slightly below control levels to 0.73 ± 0.09 fold (Figure 4.9). As discussed in chapter 3, H-89, although marketed as a specific and potent PKA antagonist, can also inhibit a number of other kinases with the same

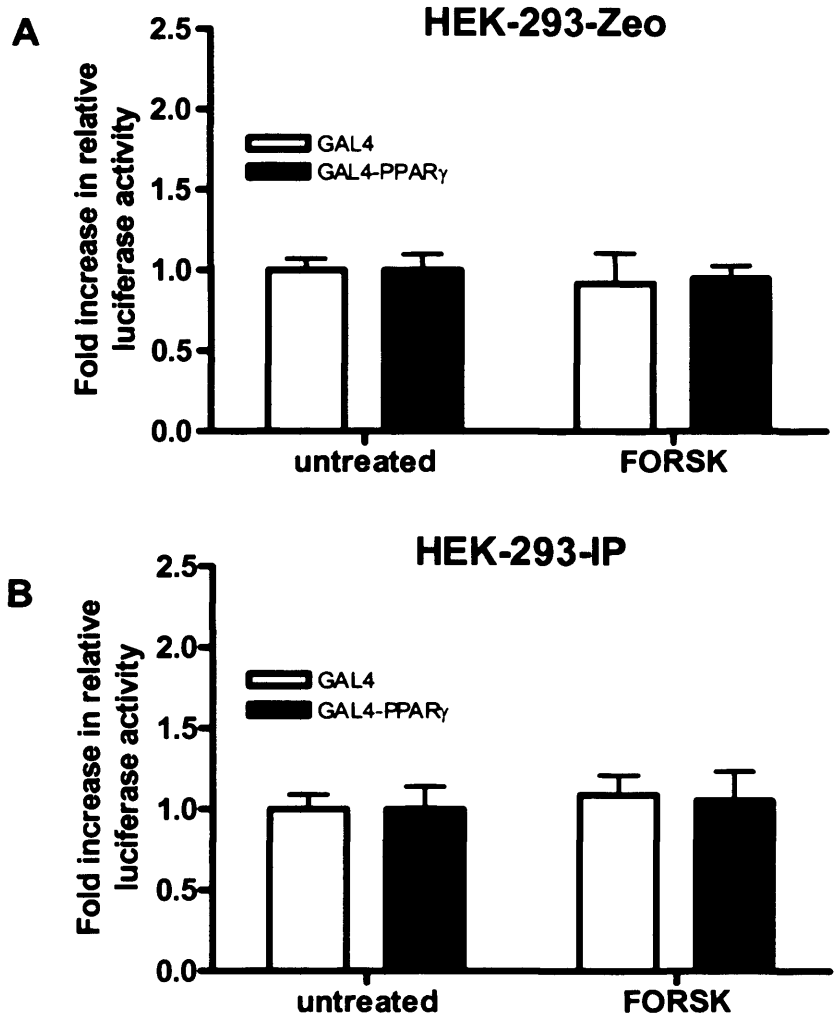

Figure 4.7 Transfected HEK-293-Zeo cells (**A**) or HEK-293-IP cells (**B**) were stimulated with 10% FBS alone (untreated) or in the presence of forskolin (FORSK, 10 μM). Luciferase activity was determined 24 hr after stimulation and normalised to *Renilla* activity. Results are expressed as mean fold increase in relative luciferase activity from untreated control ± s.e.m. (n=12, 3 separate transfections).

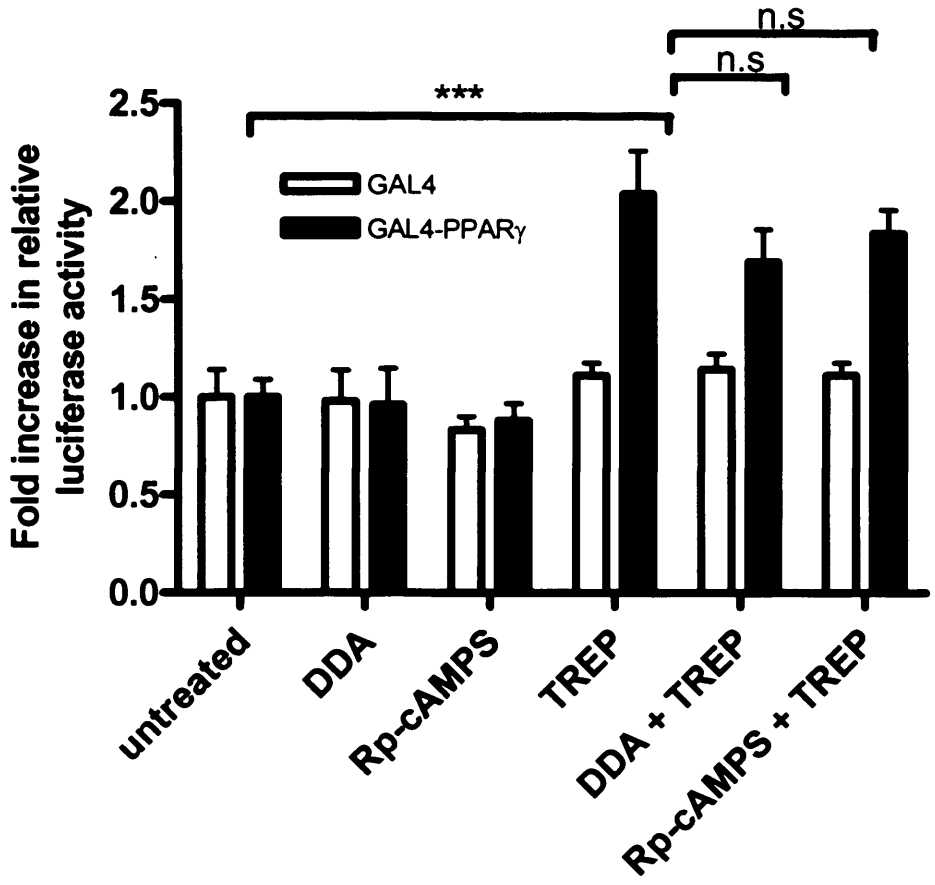

Figure 4.8 Transfected HEK-293-IP cells were stimulated with 10%FBS alone (untreated) or in the presence of treprostinil (TREP; 1 μM), 2'5'-DDA (DDA; 100 μM), the PKA antagonist, Rp-cAMPS (100 μM) or in combination as shown (cells pre-treated with antagonist for 1 hr). Luciferase activity was determined 24 hr after stimulation and normalised to *Renilla* activity. Results are expressed as mean fold increase relative to luciferase activity from untreated control ± s.e.m. (n=12, 3 separate transfections). *** = $P<0.001$.

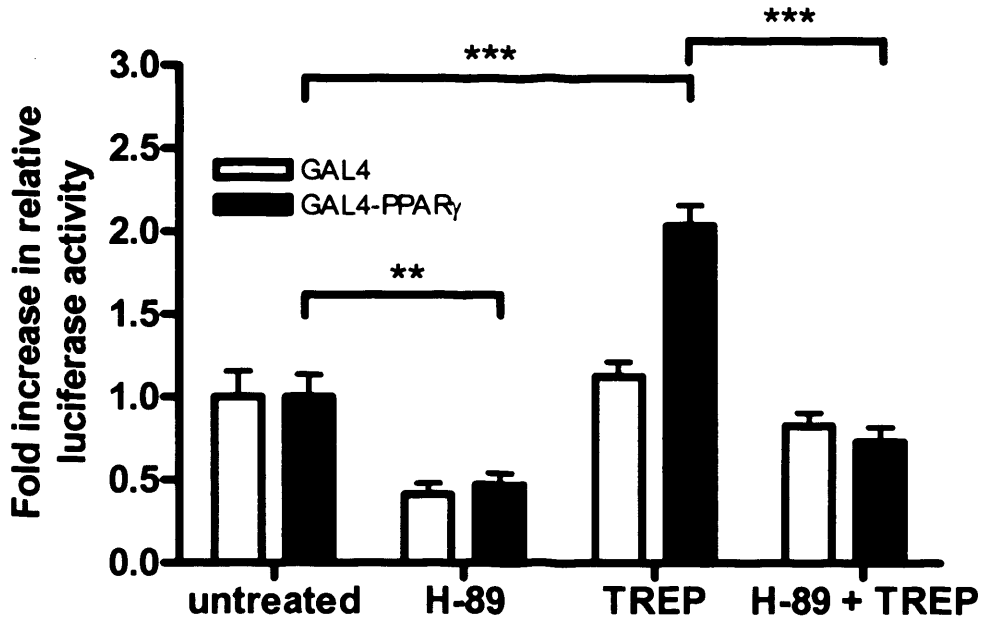

Figure 4.9 Transfected HEK-293-IP cells were stimulated with 10% FBS alone (untreated) ± H-89 (10 μM), treprostinil (TREP, 1 μM), or a combination as shown. Cells were pre-treated with H-89 for 45 mins prior to stimulation with TREP. Luciferase activity was determined 24 hr after stimulation and normalised to *Renilla* activity. Results are expressed as mean fold increase in luciferase activity relative to untreated control ± s.e.m. (n=12, 3 separate transfections). ** = $P<0.01$, *** = $P<0.001$.

potency as for PKA (Davies *et al.,* 2000). Taken together, these data suggest that the cAMP/PKA pathway is not a major mediator of PPARγ activation by PGI_2 analogues.

4.6 An investigation into other putative mediators of PGI_2-mediated PPARγ activation

4.6.1 Staurosporine-sensitive signalling

Although for the reasons shown above, it is unlikely that PKA is responsible for PPARγ activation by PGI_2 analogues, the fact that H-89 did have such a strong inhibitory effect on treprostinil-induced luciferase activity, leads us to assume that activation of PPARγ is mediated *via* a kinase-dependent phosphorylation mechanism. PKCα and PKCβII have been shown to phosphorylate PPARα in human liver cells at serines 179 and 230 which increases ligand-induced PPARα transcriptional activity (Blanquart *et al.*, 2004). Both serine sites are present in the D-E-F region, the same as present in our GAL4 construct. In addition it is an attractive hypothesis to think that a similar mechanism to that described with PKC and PPARα may occur in our system.

Staurosporine is a widely used cell permeable, potent (IC_{50} = 5 nM) but non-selective inhibitor of PKC (Tamaoki *et al.,* 1986). To test the role of this kinase, HEK-293-IP cells were treated with 1 μM staurosporine, a concentration that should be sufficient to fully inhibit PKC. Treatment of HEK-293-IP cells with 1 μM staurosporine alone for 24 hrs had no effect on luciferase activity (n=16, P=0.81; Figure 4.10). Pre-treatment with 1 μM staurosporine for 1 hr prior to stimulation with 1 μM treprostinil had no

effect on treprostinil-mediated luciferase increase (n=16, $P<0.73$; Figure 4.10). Conversely, when the dose of staurosporine was increased to 10 μM, the treprostinil-induced luciferase increase (2.19 ± 0.31 fold) was completely inhibited, with the levels falling below those of the untreated control (0.47 ± 0.15 fold, n=16, $P<0.001$; Figure 4.10). Thus, the effects observed with 10 μM are not likely to be PKC dependent but rather one of many kinases that staurosporine can inhibit. Similar to the conclusions one draws from the results obtained with H-89, we still hypothesise that the mechanism involves a kinase, though it is unlikely to involve PKC, PKA, CaMKII, or myosin light chain kinase (MLCK), all of which should effectively be inhibited at 1 μM.

4.6.2 AMP-activated kinase

As is the case with IP receptor and PPARγ activation, AMP-activated kinase (AMPK), a metabolite-sensing protein serine/threonine kinase, is known to inhibit proliferation at the G1 stage of the cell cycle (Igata *et al.*, 2005) and more recently has been shown to be activated by thiazolidinediones (Han and Roman, 2006; LeBrasseur *et al.*, 2006).

To test whether AMPK could underlie PPARγ activation, HEK-293-IP cells were first treated with the AMPK agonist, AICAR (100 μM). This agent failed to significantly activate PPARγ (n=12, $P=0.24$; Figure 4.11), suggesting that AMPK alone cannot increase PPARγ activity. To test whether AMPK was responsible for treprostinil-mediated PPARγ activation, cells were pre-treated for 1 hr with two inhibitors, quercetin (20 μM) and Ara-A (100 μM). Interestingly, AMPK is also potently inhibited by H-89 (Davies *et al.*, 2000). Pre-treatment with these two antagonists produced two very different

results. Quercetin had no significant effect on treprostinil induced PPARγ activation (n=12, *P*=0.87). In contrast, Ara-A significantly inhibited PPARγ activation by treprostinil, reversing luciferase levels from 2.03 ± 0.13 fold to 1.24 ± 0.19 (n=12, *P*<0.005; Figure 4.11). In light of these results the role of AMPK in this mechanism has not been proven.

4.6.3 G_i/G_o

As discussed in chapter 3, it is possible that the IP receptor may be pre-coupled to G_i protein mediating some of the effects of receptor activation. To test whether G_i specifically had a role in mediating treprostinil-induced activation of PPARγ, luciferase activity was measured in HEK-293-IP cells with or without pre-treatment with PTx, an agent which prevents downstream signalling of G_i/G_o. Treatment of HEK-293-IP cells with 100 ng/ml of PTx for 40 hr alone had no effect on luciferase activity (n=16, *P*=0.61; Figure 4.12). When cells were pre-treated for 16 hrs with PTx before the addition of 1 μM treprostinil, there was a small but non significant increase in luciferase activity from 2.19 ± 0.31 fold in the absence to 3.15 ± 0.56 fold (n=16, *P*=0.1263) in the presence of PTx. These results suggest that activation of G_i/G_o is not a pre-requisite for PPARγ activation by treprostinil (Figure 4.12).

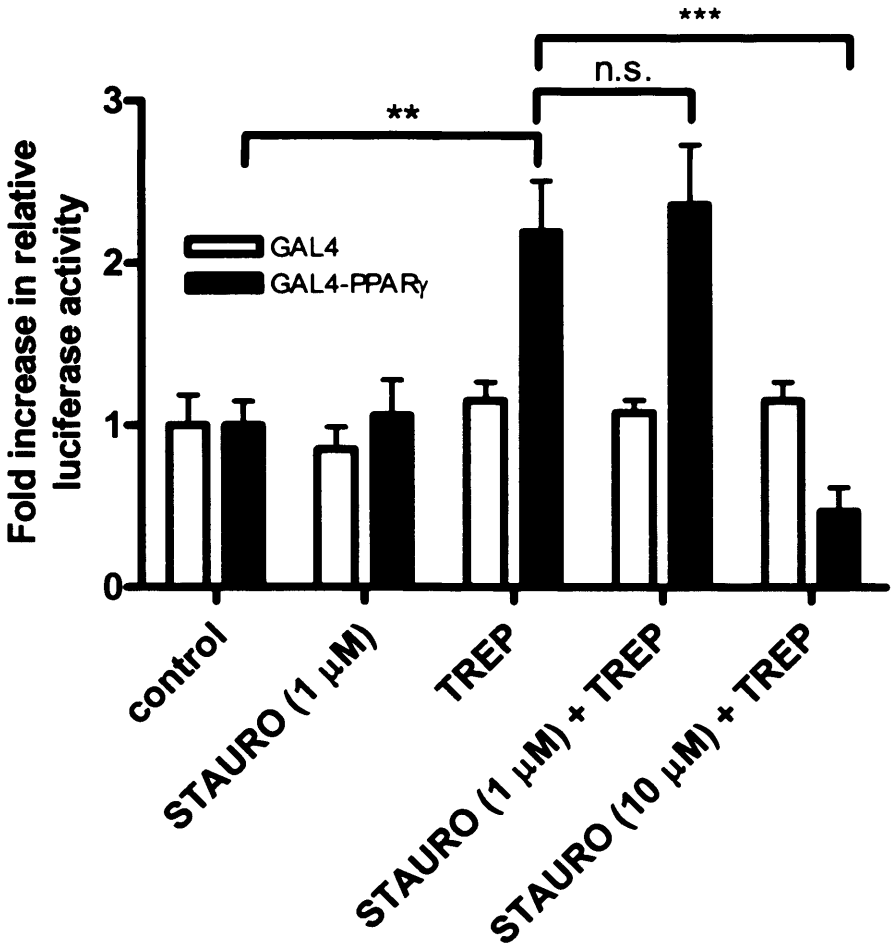

Figure 4.10 Transiently transfected (as before) HEK-293-IP cells were stimulated with 10% FBS alone (untreated) ± treprostinil (TREP, 1 μM), staurosporine (STAURO, 1 or 10 μM) or a combination as shown. Cells were pre-treated with STAURO for 1 hr prior to stimulation with TREP. Luciferase activity was determined 24 hr after stimulation and normalised to *Renilla* activity. Results are expressed as mean fold increase in luciferase activity relative to untreated control ± s.e.m. (n=12, 3 separate transfections). ** = $P<0.01$, *** = $P<0.001$.

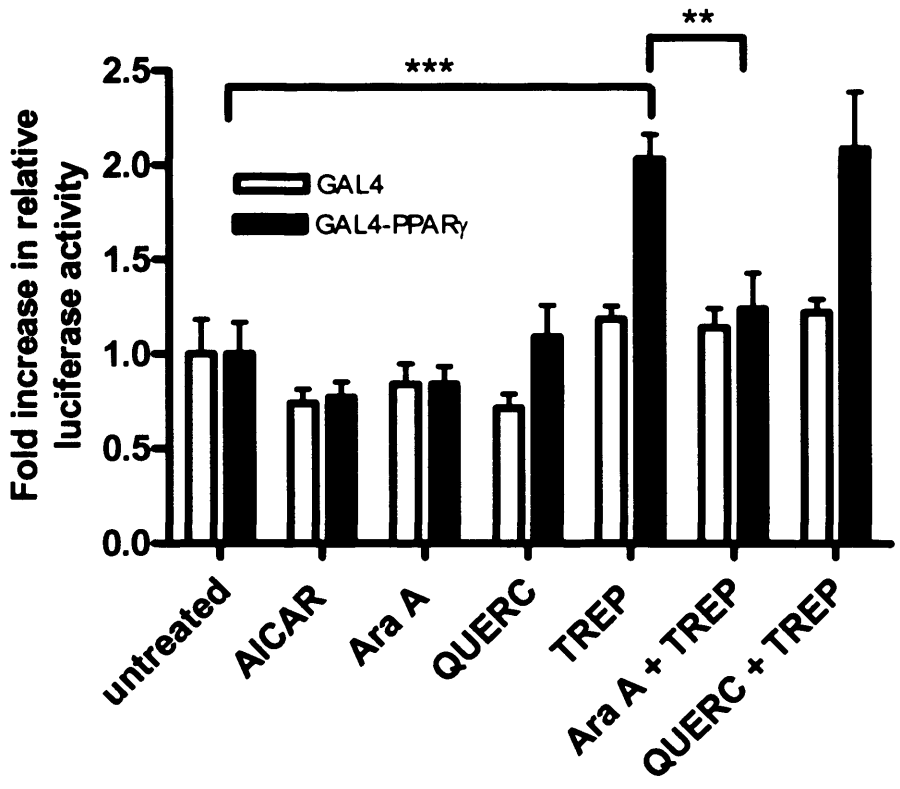

Figure 4.11 Transfected HEK-293-IP cells were stimulated with 10% FBS alone (untreated) ± AICAR (100 μM), Ara A (100 μM) quercetin (QUERC, 20 μM), treprostinil (TREP, 1 μM) or a combination as shown. Cells were pre-treated with antagonists for 1 hr prior to stimulation with TREP. Luciferase activity was determined 24 hr after stimulation and normalised to *Renilla* activity. Results are expressed as mean fold increase in luciferase activity relative to untreated control ± s.e.m. (n=12, 3 separate transfections). ** = $P<0.01$, ***=$P<0.001$.

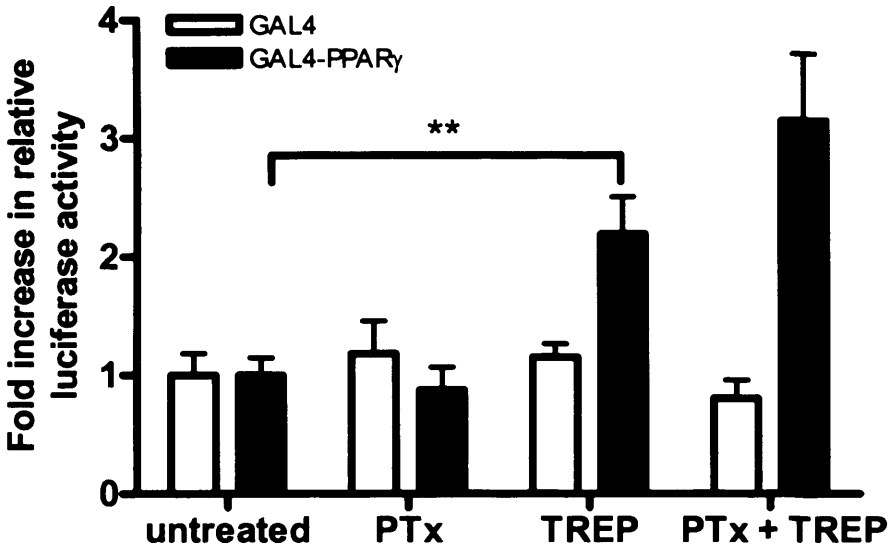

Figure 4.12 Transfected HEK-293-IP cells were stimulated with 10% FBS alone (untreated) ± treprostinil (TREP, 1 μM), pertussis toxin (PTx, 100 ng/ml) or a combination as shown. Cells were pretreated with PTx for 16 hr prior to stimulation with TREP. Luciferase activity was determined 24 hr after stimulation and normalised to *Renilla* activity. Results are expressed as mean fold increase in luciferase activity relative to untreated control ± s.e.m. (n=16, 4 separate transfections). ** = $P<0.01$.

4.7 Role of PPARγ in cell proliferation

To test whether PPARγ underlies any of the anti-proliferative effects of treprostinil, HEK-293-IP cells were pre-treated with the relatively specific PPARγ antagonist GW9662 prior to treatment with the PGI_2 analogue. We wished to determine whether PPARγ could account for the remainder of the PKA-independent effects of treprostinil on cell growth (Figure 4.13 A). Pre-treatment of HEK-293-IP cells with 1 μM GW9662 for 1 hr significantly ($P<0.05$) reversed the anti proliferative effect of 100 nM treprostinil by about 23%. As shown in Figure 4.13 B, growth was 29.6 ± 3.6% (n=9) of the normal response to 10 % FBS in the absence of GW9662, rising to 44.4 ± 4.3% (n=9) in the presence of GW9662. These results suggest that PPARγ may indeed have a role in mediating, at least in part, the anti-proliferative effects of treprostinil.

To test the effect of direct activation of PPARγ on cell proliferation, both HEK-293-IP and HEK-293-WT were treated with varying doses of rosiglitazone and counted after 48 hr. Surprisingly the presence of the IP receptor markedly altered the response to rosiglitazone in the two cell types. In HEK-293-IP cells, this agent inhibited proliferation in a dose-dependent (0.1-100 μM) manner (n=24, $P<0.001$; Figure 4.14). At high doses of above 10 μM we cannot exclude that the pronounced anti-proliferative effects of rosiglitazone may be due to toxic effects of the drug, a severe limitation of the study is in fact the absence of data analysing cell death following treatment with these agents. In HEK-293-WT cells, rosiglitazone markedly increased proliferation over and above that observed

in the presence of 10% FBS at doses below 30 μM and only inhibited proliferation at the highest dose (100 μM) (Figure 4.14). For example, at 10 μM, rosiglitazone increased proliferation by 84.5 ± 19.5% over untreated control cells (n=18, $P<0.001$).

To determine the potential role of PPARα and PPARδ on cell growth, the effect of agonists of these PPARs were also assessed. Somewhat similar to rosiglitazone, the PPARδ agonist L165,041 had differential effects in the two cell types. In HEK-293-IP cells, this agent had no significant effect on growth over a wide concentration range (1-1000nM). In contrast, in HEK-293-WT, the PPARδ agonist increased cell proliferation, although the relationship was bell shaped (Figure 4.15). On the other hand, the PPARα agonist GW7647 appeared to have no consistent effect on growth in either cell type (Figure 4.16). Thus it would appear that the only PPAR able to inhibit the growth of HEK-293-IP cells, was the γ isoform.

4.8 Summary

- The PGI_2 analogues treprostinil, carbacyclin and cicaprost activate PPARγ in a luciferase based reporter gene assay.
- PPARγ activation by PGI_2 analogues was IP receptor dependent and likely to involve a phosphorylation mechanism.
- The mechanism of PPARγ activation does not appear to involve the cAMP/PKA or PKC pathway.
- It is yet unclear if AMPK has a role in mediating treprostinil induced PPARγ activation.

- G_i/G_o activation is not a pre-requisite for PPARγ activation by treprostinil.
- PPARγ activation may account for the PKA independent anti-proliferative effects of treprostinil
- The IP receptor modulates the growth responses of PPARγ and PPARδ agonists.

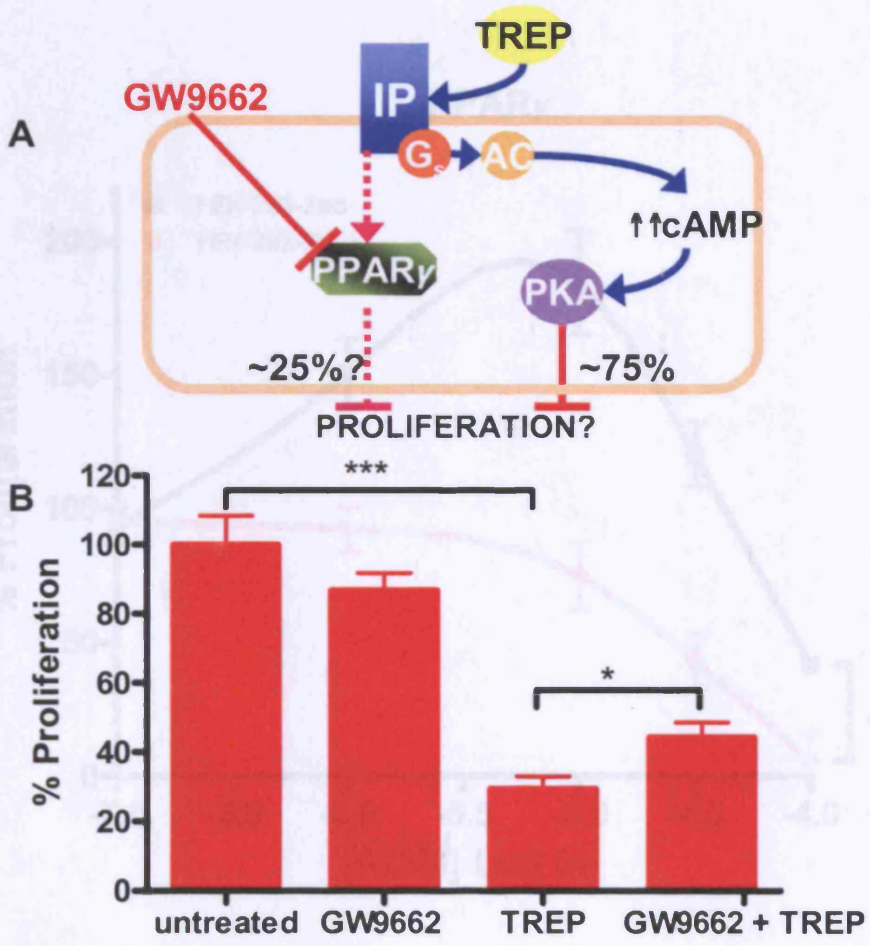

Figure 4.13 **A**. Schematics illustrating the inhibition of PPARγ with GW9662 and the possible effect on TREP induced inhibition of proliferation. It also illustrates the hypothesis that PPARγ may account for the remaining anti-proliferative effects which do not appear to be mediated by the PKA pathway. **B**. Growth arrested HEK-293-IP cells were stimulated with MEM + 10% FBS and either left untreated or treated with GW9662 (1 μM), TREP (100 nM) or a combination as shown. Cells were pretreated with GW9662 for 1 hr prior to stimulation with TREP. Cells were counted 48 hours following treatment. Data expressed as % proliferation relative to proliferative response mediated by 10% FBS alone and expressed as mean ± S.E.M. (n=9). * = $P<0.05$.

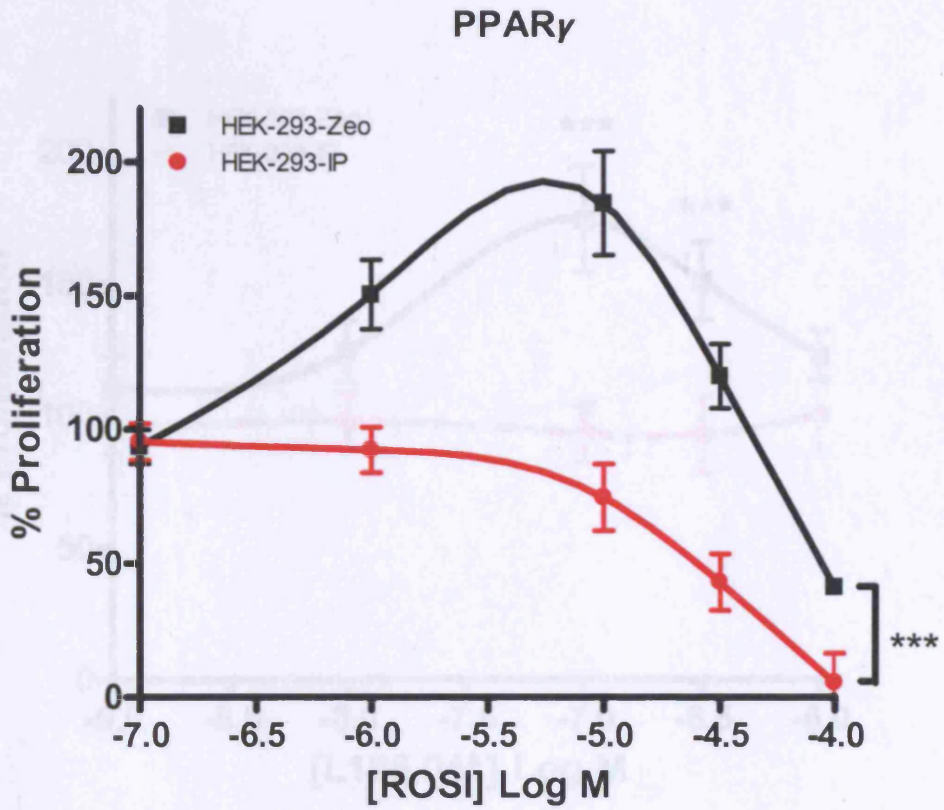

Figure 4.14 Effect of Rosiglitazone (ROSI) on cell proliferation. Growth arrested HEK-293-Zeo (■) and HEK-293-IP (●) cells were stimulated with 10% FBS ± the PPARγ agonist, ROSI at the concentrations shown. Cells were counted after 48 hr and data shown as % cell proliferation relative to the proliferative response mediated by 10% FBS alone and expressed as mean ± S.E.M. (n=18). *** = $P<0.001$

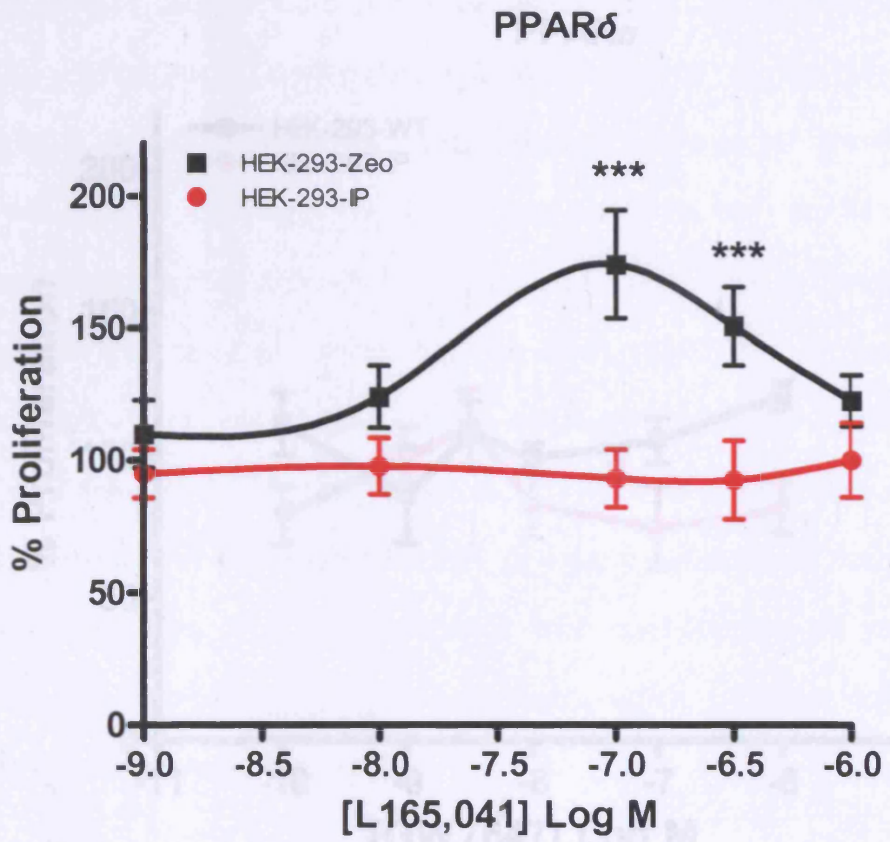

Figure 4.15 Effect of L165,041 on cell proliferation. Growth arrested HEK-293-Zeo (■) and HEK-293-IP (●) cells were stimulated with 10% FBS ± the PPARδ agonist, L165,041, at different concentrations and counted after 48 hrs. Data expressed as mean % proliferative response relative to proliferation mediated by 10% FBS alone ± S.E.M. (n=18).

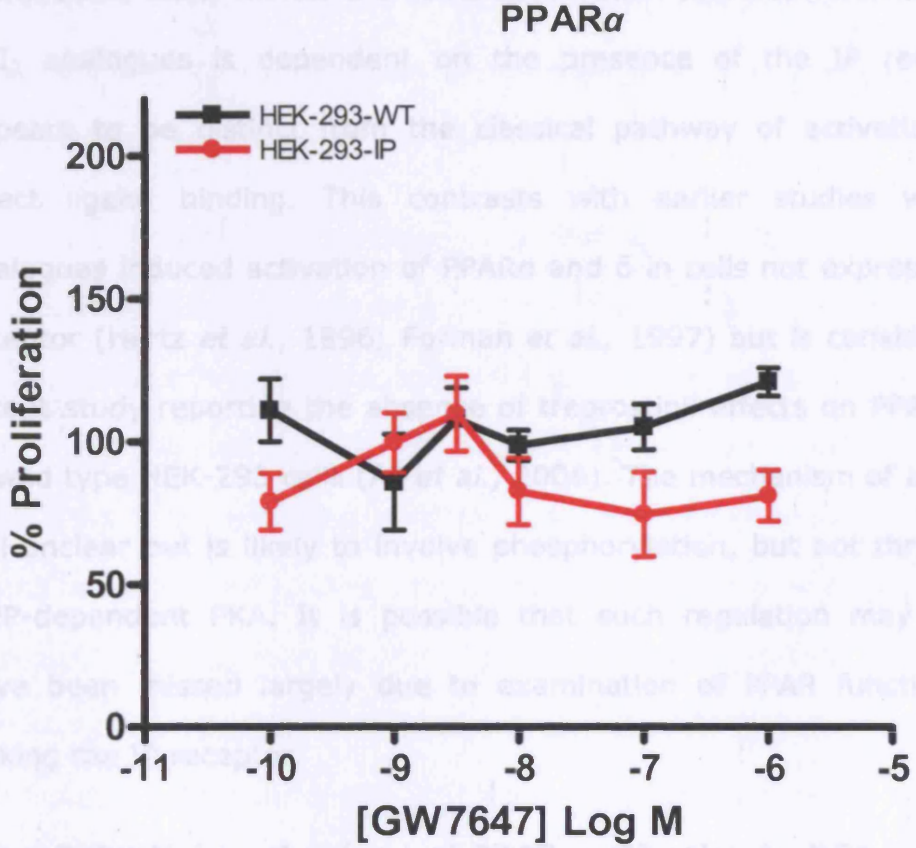

Figure 4.16 Effect of GW7647 on cell proliferation. Growth arrested HEK-293-Zeo (■) and HEK-293-IP (●) cells were stimulated with 10% FBS ± the PPARα agonist, GW7647, at different concentrations and counted after 48 hrs. Data expressed as mean % proliferative response relative to proliferation mediated by 10% FBS alone ± S.E.M. (n=18).

4.9 Discussion

The present study makes the novel observation that activation of PPARγ by PGI_2 analogues is dependent on the presence of the IP receptor and appears to be distinct from the classical pathway of activation through direct ligand binding. This contrasts with earlier studies where PGI_2 analogues induced activation of PPARα and δ in cells not expressing the IP receptor (Hertz *et al.*, 1996; Forman *et al.,* 1997) but is consistent with a recent study reporting the absence of treprostinil effects on PPARγ activity in wild type HEK-293 cells (Ali *et al.,* 2006). The mechanism of activation is still unclear but is likely to involve phosphorylation, but not through cyclic AMP-dependent PKA. It is possible that such regulation may previously have been missed largely due to examination of PPAR function in cells lacking the IP receptor.

4.9.1 Potential mechanisms of PPARγ activation by PGI_2 analogues.

The construct used in the luciferase assays contained the Gal4 DNA binding domain (DBD) fused to the C terminal part of the PPARγ containing the D (hinge region), E (the ligand binding domain; LBD) and F domains (Gelman *et al.,* 1999). Thus, for treatment with a cell permeable analogue like carbacyclin, one might predict PPARγ activation to occur in control HEK-293 cells if the primary mechanism was indeed direct binding. Clearly this was not the case, with concentrations as high as 10 μM failing to significantly activate PPARγ, despite previous data showing near maximal binding and activation of PPARα and PPARδ with respect to their selective ligand

activators (Forman *et al.*, 1997). Interestingly, cicaprost, which does not bind to or activate either α or δ isoforms (Forman *et al.*, 1997) was also an effective activator of our PPARγ construct, supporting the notion that receptor expression is crucial. However, it is still not possible to exclude the possibility that the IP receptor is required to promote ligand binding by these agents, at least for this PPAR isoform.

There is a growing body of evidence indicating that PPARs can be activated *via* phosphorylation (Diradourian *et al.*, 2005; Gelman *et al.*, 2005) and involve cell surface receptor activation. In rat adipocytes, both PPARα and PPARγ are phosphorylated following treatment with insulin, an effect associated with enhanced transcriptional activity (Shalev *et al.*, 1996; Zhang *et al.*, 1996). This insulin-dependent phosphorylation has been attributed to activation of the mitogen activated protein kinase (MAPK) pathway, involving serine residues contained within the N-terminal ligand-independent activation domain (AF-1) of PPARα (Juge-Aubry *et al.*, 1999) and PPARγ (Zhang *et al.*, 1996). Likewise, the eicosanoid, prostaglandin $F_2\alpha$ ($PGF_2\alpha$), phosphorylates PPARγ through a mechanism dependent on the FP receptor and the MAPK pathway (Reginato *et al.*, 1998). Such phosphorylation by the FP receptor leads to an inhibition of adipogenesis in 3T3-L1 cells through suppression of PPARγ activity (Reginato *et al.*, 1998).

Most of the PPAR phosphorylation sites described are located in the N-terminal (A/B domain; LBD). Since the construct used in this project lacks this domain, the data gathered here suggests there could be a phosphorylation site or a ligand independent activation site located in the C-terminal (D, E or F domain; DBD) to promote ligand-independent

transcriptional activity. Alternatively, IP receptor activation could simply enhance endogenous ligand binding to the LBD, as is the case with phosphorylation of the A/B domain in PPARγ (Shao *et al.,* 1998) or phosphorylation of PPARα serine residues 179 and 230 by PKC (Blanquart *et al.,* 2004). Another possibility is the promotion of recruitment or dissociation of co-factors. This may occur either via phosphorylation of PPARγ or the co-activators themselves (Diradourian *et al.*, 2005).

Given the ability of PKA to activate PPARγ, both in the presence and absence of exogenous ligands (Lazennec *et al.,* 2000) and the established coupling of the IP receptor to G_s (Namba *et al.,* 1994), it was considered that IP receptor-dependent activation of PPARγ would be reliant on cAMP and PKA activity. Cyclic AMP is known to regulate PPARδ-mediated pre-adipocyte proliferation and plays an important role in PPARδ mediated gene transactivation (Hansen *et al.,* 2001); however the exact mechanism remains unclear but may involve PKA. The authors of the latter study suggest that PKA's ability to influence heterodimerisation (Bhat *et al.,* 1994; Rochette-Egly *et al.,* 1995; Chen *et al.*, 1999), DNA binding (Sugawara *et al.*, 1994) and transactivation (Taneja *et al.*, 1997; Sadar, 1999) of other nuclear receptors may also mediate cAMP-dependent effects on PPARδ. In addition it has been shown that increased cAMP levels diminish or abolish interaction between nuclear hormones and co-repressors (Wagner *et al.,* 1998; Lavinsky *et al.,* 1998), another way in which the cAMP-PKA signalling mechanism may affect PPAR activation.

Surprisingly cAMP did not appear to play a major role in the IP receptor-dependent activation of PPARγ. In this study forskolin failed to activate

PPARγ and both the adenylyl cyclase antagonist, DDA, and the specific PKA antagonist, Rp-cAMPS failed to significantly reverse the PPARγ-activating effect of treprostinil.

By using truncated PPARα constructs, Lazennec and colleagues (2000) demonstrated that the AF-2 domain containing the LBD (as is present in our GAL4-PPARγ construct) was the most important domain for transactivation by PKA activators. However, there are PKA phosphorylation sites mapped across the whole of the PPARγ structure, the main site being located in the DBD. One can therefore not entirely exclude PKA-dependent effects if other truncated PPARγ forms of the GAL4 construct were to be used. Moreover, it is possible that several domains of PPAR need to be phosphorylated in order for PKA to activate PPARγ. In addition, the small (though not significant) reversal in activation caused by DDA and Rp-cAMPS suggests that there may be a small fraction of the effect dependent on a cAMP/PKA-dependent pathway.

It is not altogether surprising that cAMP does not appear to mediate the effects of PGI_2 analogues on PPARγ activity, since previous data from this and other laboratories also describe a distinctly cAMP-independent pathway that mediates the vascular relaxant responses to PGI_2 analogues in blood vessels (Turcato and Clapp, 1999; Tanaka *et al.*, 2004, Orie *et al.*, 2006). Furthermore, the IP receptor is known to couple to multiple G-proteins including G_i/G_o and G_q (Wise and Jones, 2000), which may contribute in some way to PPARγ activation. However, we can rule out an involvement of G_i/G_o as pertussis toxin, rather than inhibiting the effects of treprostinil, actually potentiated PPARγ activation somewhat. Likewise, the relaxant

responses to cicaprost were significantly enhanced following PTx treatment (Orie *et al.,* 2006), possibly suggesting that the IP receptor couples to Gi/G_o to counterbalance the effects of G_s stimulation.

The PKA antagonist, H-89, not only significantly reduced basal PPARγ activity in HEK-293-IP cells but also prevented treprostinil activation of PPARγ. However, in light of data obtained with forskolin, DDA and RpCAMPS, and the fact that this agent has effects on a broad spectrum of kinases, we conclude that the effects observed with H-89 are probably not via PKA. Similarly 1 μM staurosporine, which should adequately block PKA (IC_{50} 15 nM) (Meyer *et al.,* 1989), also failed to inhibit the effects of treprostinil. Only the high, non-specific dose of 10 μM, had a dampening effect on treprostinil induced PPARγ activation. Like H-89, staurosporine can inhibit a broad spectrum of kinases including PKC (5 nM), PKG (18 nM), CaMKII (20 nM), MLCK (21 nM) and ERK1 (1.5 μM) (Gschwendt *et al.,* 1994; Meggio *et al.*, 1995). It is logical to suggest that treprostinil-induced PPARγ activation is regulated by a phosphorylation mechanism and the latter may also be responsible for a basal level of activation. Whether phosphorylation occurs directly on the nuclear receptor or whether it works by regulating co-repressors or co-activators remains to be established.

AMP-activated kinase (AMPK) is a metabolite-sensing protein serine/threonine kinase (Hardie and Carling, 1997) which acts as a cellular fuel sensor and is an important mediator of exercise-induced glucose uptake in skeletal muscle (Hardie *et al.,* 2003). It is mainly activated under stress conditions that are associated with an increased AMP/ATP ratio such as heat shock and hypoxia (Kemp *et al.,* 2003). A number of signalling

pathways have been reported to be regulated by AMPK through direct phosphorylation (Motoshima *et al.,* 2006). There has been a growing body of evidence showing that activation of AMPK by the widely used AMP mimetic aminoimidazole-4-carboxamide ribonucleoside (AICAR) causes cell cycle arrest in a number of cell lines such as hepatoma HepG2 cells (Imamura *et al.,* 2001), mouse embryonic fibroblasts (Jones *et al.*, 2005) and human aortic smooth muscle cells (Igata *et al.,* 2005).

The mechanism by which AMPK inhibits cell proliferation is still not entirely understood but it is thought to involve the accumulation of the p53 tumour suppressor *via* a phosphorylation of its Ser-15 residue and subsequent upregulation of the CDKI $p21^{CIP}$ (Motoshima *et al.,* 2006) as well as upregulation of $p27^{KIP}$ (Rattan *et al.,* 2005). Interestingly as with prostacyclin treatment and PPARγ activation, AMPK activation, in serum or PDGF-BB stimulated smooth muscle cells, inhibits proliferation by causing cell cycle arrest at the G1 phase (Igata *et al.*, 2005). In addition thiazolidinediones have been shown to activate AMPK although it is unclear whether this is through a PPARγ-dependent or independent mechanism (Han and Roman, 2006; LeBrasseur *et al.,* 2006).

AMPK was tested as a potential mediator of the pathway studied in this investigation. The reversal of treprostinil-mediated effects with the antagonists Ara-A and H-89 (also able to inhibit this kinase) made it an eligible candidate for PPARγ activation. Against this, was the observation, that a third antagonist, quercetin, did not follow suit nor did the AMPK agonist, AICAR increase PPARγ activity. Together these data provide mixed evidence both for and against a possible role for this kinase. Again, there

are problems with agent specificity. H-89, quercetin, and Ara-A all have a number of other targets. The latter is in fact marketed by Sigma-Aldrich as an adenylyl cyclase antagonist. We cannot yet exclude AMPK as a mediator and testing with a further antagonist, iodotubercidine could shed more light on the involvement of this kinase.

Thus the nature of the signalling pathway involved in PPARγ activation, remains to be fully elucidated. Currently we can exclude the direct role of both PKA and PKC whereas the role of AMPK warrants further investigation to resolve conflicting results. A mediator we are interested in looking at in future studies is the extracellular regulated kinase (ERK). ERK is able to phosphorylate PPARγ and although direct phosphorylation sites have been located in the DBD (absent in our expression construct), one could hypothesise that other residues which can be activated by the ERK pathway might be present in the LBD of this nuclear receptor (Diradourian *et al.*, 2005). There is evidence in the literature for dose-dependent cicaprost phosphorylation of ERK (Chu *et al.,* 2004). The authors of this study attribute ERK phosphorylation to PKC *via* IP receptor coupling to G_q as demonstrated by the reversal of cicaprost effects with 1 μM staurosporine, a concentration that has no effect in our investigation. They exclude the involvement of PKA by demonstrating lack of reversal with Rp-cAMPS. It is however interesting that they do see a significant reversal with H-89, highlighting again the lack of specificity of this antagonist. What is of interest, however, is that they cannot fully exclude mediation by G_s. In fact forskolin could not upregulate ERK phosphorylation, akin to our not seeing any effect on PPARγ activation with this cAMP-elevating agent, whereas, surprisingly, the specific Epac agonist CPTM-cAMP could cause ERK

phosphorylation. Epacs can stimulate ERK via activation of Rap-GTPase (Wang *et al.,* 2006). As we can see in Figure 3.20 (chapter 3) we were able to inhibit most but not all treprostinil-dependent cAMP elevation with DDA. Therefore it could be possible that some cAMP may still be elevated in the presence of DDA and this could be free to activate Epacs rather than PKA. This may lead to ERK phosphorylation and subsequently PPARγ phosphorylation and activation. This hypothesis could be tested pharmacologically by treating cells with cholera toxin (commercially available), the novel antagonist YM-254890 (Takasaki *et al.,* 2004) and PD98059 (commercially available) to block G_s, G_q and ERK respectively. In addition a molecular approach could be taken and the three mediators could be inhibited using siRNA, or in the case of G_q, by overexpressing a carboxyl-terminal peptide of $G\alpha_q$ (G_qI).

Figure 4.17 summarises our current understanding of the signalling pathway responsible for IP receptor dependent PPARγ activation.

4.9.4 Effect of PPARγ on cell proliferation

The effect of PPAR ligands on HEK-293 cell proliferation produced some unexpected findings. What was evident was that the presence of the IP receptor altered the proliferative response to rosiglitazone and L165,041. PPARγ ligands are generally known to inhibit proliferation of a number of different cell types including vascular smooth muscle cells (Law *et al.*, 1996; Sarafidis and Lasaridis, 2006) but have also been shown to be pro-mitogenic by being able to increase mitogenic factors such as vascular endothelial growth factor (VEGF) (Yamakawa *et al.*, 2000) or inducing formation of colon polyps (Saez *et al.,* 1998). In this project, rosiglitazone

appeared to enhance serum-induced proliferation of HEK-293-WT cells but at the highest dose used it actually inhibited proliferation. Whether or not at this dose it was causing cell death or apoptosis was not established in this study and is a significant limitation of the work presented here. However there was no visible cell death observed when manually counting cells stained with trypan blue (Invitrogen) or in the shape of the distribution curves obtained from the PDA500 cell counting analysis system (data not shown).

In contrast, in HEK-293-IP cells, the PPARγ ligand had a more distinct inhibitory effect across a wide concentration range. This response is similar to what is found in smooth muscle cells (Law *et al.*, 1996; Ward *et al.*, 2004). Inhibition of proliferation was only significant at concentrations of 10 μM or above raising the question of whether these are non-PPARγ specific effects of the drug. However Ward *et al* (2004) have shown that the effect of 10 μM rosiglitazone could be totally reversed using 1 μM GW9662. Furthermore evidence shows that proliferation inhibition by thiazolidinediones can be replicated using the unrelated PPARγ ligand, 15d-PGJ_2 (Sasaguri *et al.*, 1992; *Ward et al.*, 2004), supporting the notion that the effect is most probably PPARγ dependent. In future studies we would like to use GW9662 or RNAi to block this PPAR isoform to establish the extent of PPARγ involvement in our system. How PPARγ is regulating cell proliferation is still unclear. Both troglitazone and rosiglitazone can attenuate the mitogen-induced degradation of the cdk inhibitor $p27^{Kip1}$ in smooth muscle cells causing G_1 arrest (Sherr and Roberts, 1999). An alternative hypothesis is that activation of PPARγ may be having an effect on the synthesis of PGI_2. In support of this, a study has shown that PPARγ

activation can mediate the induction of COX-2 expression and synthesis, the precursor of PGI_2 (Kalajdzic *et al.*, 2002). This hypothesis could be tested in our cell model by measuring prostacyclin levels in treated versus untreated cells.

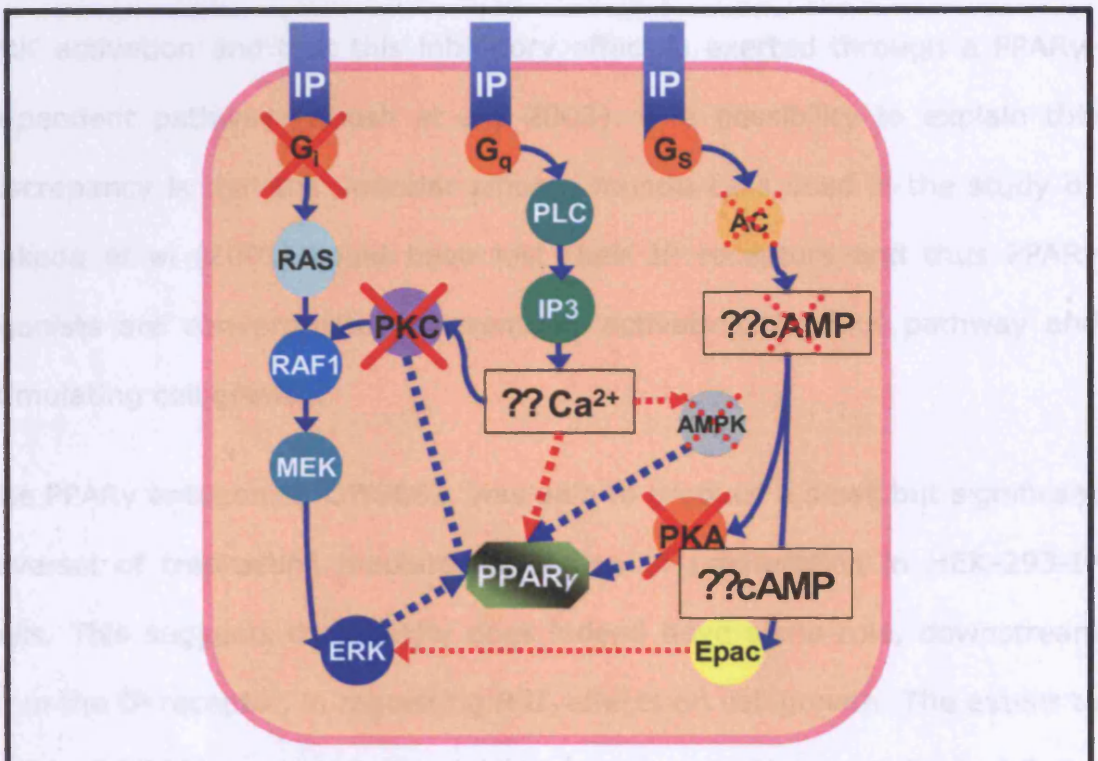

Figure 4.17 Potential signalling mechanisms regulating IP receptor-dependent PPARγ activation. Solid blue lines indicate known direct association. Dotted blue lines indicate a potential phosphorylation of PPARγ. Red dotted lines indicate an indirect association. Red solid cross indicates strong evidence against mediation; Red dotted cross indicates some negative data but necessitates further investigation.

In contrast to the findings that PPARγ activation inhibits smooth muscle cell proliferation, a study by Takeda et al (2001) observed that thiazolidinediones as well as 15d-PGJ_2 have a pro-proliferative effect on vascular smooth muscle cells in the absence of growth stimuli. They suggest that this was caused by a rapid induction of ERK phosphorylation leading to upregulation of cyclin D1 levels; such a mechanism is known to

underlie the effect of various growth factors including PDGF and bFGF. These observations are in direct contrast with studies by Ward et al (2004) and Ghosh et al (2003) who found no significant effect on ERK activity with thiazolidinediones. In fact the latter study suggested that ciglitazone reduces mesangial cell proliferation by inhibiting pathways downstream of ERK activation and that this inhibitory effect is exerted through a PPARγ-dependent pathway (Ghosh *et al.,* 2003). One possibility to explain this discrepancy is that the vascular smooth muscle cells used in the study by Takeda et al (2001) could have lost their IP receptors and thus PPARγ agonists are converted to preferentially activating the ERK pathway and stimulating cell growth.

The PPARγ antagonist, GW9662, was able to produce a small but significant reversal of treprostinil mediated inhibition of proliferation in HEK-293-IP cells. This suggests that PPARγ does indeed have some role, downstream from the IP receptor, in regulating PGI_2 effects on cell growth. The extent to which GW9662 reversed the inhibition mirrored the magnitude of the effects that could not be blocked by the PKA antagonists, H-89 and Rp-cAMPS (see section 3.9). It is tempting to speculate that PPARγ could indeed account for the PKA independent effects of treprostinil. In light of these results and those showing that activation of PPARγ by treprostinil is IP receptor-dependent but PKA-independent, the PPARγ pathway may be a separate mechanism which works in parallel to the classical cAMP/PKA pathway to mediate the anti-proliferative effects of PGI_2.

HEK-293-WT cells also had a pro-proliferative response to the PPARδ ligand L165,041 an effect which was absent in HEK-293-IP cells. PPARδ ligands

have been shown to promote proliferation of breast, prostate and hepatocellular cancer cell lines (Glinghammar *et al.,* 2003; Stephen *et al.,* 2004) and enhance proliferation of vascular smooth muscle cells (Zhang *et al.,* 2002). Since PPARδ is associated with proliferation and tumour progression it might follow that HEK-293 cells, which are derived from an immortalised cell line, should respond this way. Absence of the response in HEK-293-IP cells may be taken as an indication that the IP receptor dampens down PPARδ activity or that the cellular consequence of PPARδ activation is altered by the presence of the IP receptor. Further work is required to distinguish between these possibilities.

These findings are important in furthering our understanding of how prostacyclin and PPARs interact to regulate cellular function. The idea that the IP receptor is crucial for PPARγ activation by PGI_2 may have physiological and therapeutic implications. PPARγ is known to be important for cell differentiation and the regulation of cell growth, so that in diseases where there is loss of the IP receptor this could alter the control of these processes. It remains to be established whether the novel IP receptor-dependent PPARγ signalling mechanism described in the present study is functionally relevant in native cells. Activation of PPARγ could contribute to the beneficial effects of PGI_2 analogues in the treatment of diseases such as pulmonary arterial hypertension and peripheral vascular disease.

Chapter 5

An investigation into the mechanisms underlying the anti-proliferative effects of PGI_2 analogues in PASMC derived from control and IPAH patients.

5.1 Introduction

Idiopathic Pulmonary Arterial Hypertension (IPAH) is a progressive and incurable disease leading to right heart failure and death. Untreated, the median survival from the time of diagnosis is 2.6 years in adults (D'Alonzo *et al.,* 1991) and only 10 months in children (Sandoval *et al.,* 1995; Barst *et al.*, 1999). To date, the most efficacious treatment is a continuous intravenous infusion of epoprostenol, the sodium salt of prostacyclin, which improves pulmonary vascular resistance, haemodynamics, exercise tolerance and survival in patients who do not respond to vasodilatory therapy (Higenbottam *et al.*, 1993; Barst *et al.*, 1994; Barst *et al.*, 1996; McLaughlin *et al.*, 1998).

The disadvantage of using epoprostenol is its short half life *in vivo* (2-3 mins) meaning that continuous epoprostenol infusion is required in the clinical setting. After long-term use, complications arise from the indwelling central venous catheter, often giving rise to infection. This has led to the development of more stable PGI_2 analogues, such as iloprost and treprostinil both of which show clinical benefit in the treatment of PAH. Iloprost has a half-life of 20-25 mins and can be delivered by inhalation. Using an ultrasonic nebuliser, minute particles (3-5 pm) of iloprost can reach the smallest airways and alveoli which are close to the intra-acinar arteries, a site of pulmonary vascular resistance in PAH (reviewed in Howard and Morrell, 2005). Iloprost may offer some advantages in that the drug will produce fewer systemic side affects, though several dosings are required per day, raising the issue of peak and trough effects of the drug. Treprostinil can be delivered subcutaneously, has a relatively long biological half-life of

~2 hrs and is stable at room temperature. The major problem with its use subcutaneously is site pain so that an i.v. preparation has been developed and recently approved for clinical use (reviewed in Howard and Morrell, 2005).

Prostacyclin and its stable analogues are thought to slow the progression of pulmonary vascular disease and possibly even reverse the abnormal remodelling process, although there is no *in vivo* proof that this happens in humans. Inhaled iloprost has however, recently been shown to cause regression of PAH structural changes in a rat model of monocrotaline-induced PAH (Schermuly *et al. b,* 2005). Since this is a model presenting with medial thickening in the absence of significant lesion formation, it is difficult to assess what effect if any, these agents might have in advanced disease. In the clinical setting, both epoprostenol and the stable analogues lose their effectiveness in PAH patients suggesting that these agents alone cannot halt the disease progression in humans. In such patients, lung or heart-lung transplantation becomes the only option left (Haworth, 2002; Radley-Smith and Aurora, 2006). In these circumstances, the clinician cannot be certain that the patient continues to derive any benefit from PGI_2 when they are clearly deteriorating and, in any event, benefit might be attributable to the inotropic effect on the heart rather than directly affecting the pulmonary vasculature (personal communication, Professor S.G. Haworth).

It has been shown that *in vitro* replication of normal pulmonary artery smooth muscle cells (PASMCs) is reduced by PGI_2 analogues (Clapp *et al.,* 2002). In the same study it was observed that treprostinil had a greater and

more potent inhibitory effect on cell proliferation when compared to either iloprost, beraprost or cicaprost. There is, however, no certainty that PASMCs from patients with IPAH respond in the same manner, as no studies have been done using these cells, particularly in children in whom disease progression is faster than in adults. The antiproliferative effects of PGI_2 analogues in PASM cells are generally thought to be mediated through the IP receptor (Coleman *et al.,* 1994), though lack of a specific IP receptor antagonist until fairly recently (Clark *et al.*, 2004) has hampered such confirmation. Supporting data does however come from studies showing lack of anti-proliferative effects of cicaprost on smooth muscle cells from $IP^{-/-}$ KO mice (Kothapalli *et al.,* 2003). The equivalent data in human cells has yet to be reported.

In terms of mechanism, PGI_2 analogues inhibit pulmonary arterial smooth muscle proliferation by a largely cAMP-dependent mechanism (Wharton *et al.,* 2000; Clapp *et al.,* 2002), though the pathway beyond this is much less clear and has not benefited from much investigation. A very recent study, has confirmed the importance of PKA in mediating iloprost-induced differentiation of vascular smooth muscle cells from a synthetic, proliferative phenotype to a quiescent, contractile phenotype (Fetalvero *et al.*, 2006). Similarly, I found PKA antagonists inhibited analogue anti-growth responses in HEK-293 cells expressing the IP receptor (chapter 3). Furthermore, as shown in chapter 4, PGI_2 analogues can also activate PPARγ *via* the IP receptor, a mechanism largely independent of PKA. Whether this mechanism is relevant in VSMC has yet to be determined.

In the present study we hypothesised that PASM cells in children with IPAH might not respond normally to prostacyclin analogues because either the IP receptor density had decreased and/or the receptor had become dysfunctional. Therefore, the expression and function of the IP receptor were examined by RT-PCR, immunohistochemistry and cAMP immuno-linked assays in PASMC grown from the explanted lungs of children with IPAH, all of whom had been on long term treatment with intravenous epoprostenol.

The role of the prostacyclin receptor in the regulation of cell proliferation in both control and IPAH PASMC was also studied and the downstream signalling pathways investigated using a range of pharmacological tools. Interestingly, distal PASM cells isolated from arteries of less than 1 mm in external diameter appear to be more susceptible to the anti-proliferative effects of PGI_2 analogues than cells from proximal arteries with an external diameter greater than 8 mm (Wharton *et al.,* 2000). Hence PASM cells from both control and IPAH patients were obtained from small peripheral vessels in the present study (see section 2.2.3).

In the present study I was able to confirm the presence of the IP receptor in treated end-stage pulmonary vascular disease, albeit at a lower expression level to normal cells. To my surprise I found that the signal transduction pathways regulating the effects of the PGI_2 analogue switched from a mechanism involving cAMP in normal PASMC to one largely bypassing the IP receptor or cAMP in IPAH cells.

RESULTS

5.2 Control and IPAH human PASM cells have different proliferative rates.

The first phenotypical difference noted between control and IPAH distal PASM cells was their markedly distinct proliferative rates. All three control cell isolates grew at a slower rate than IPAH cells (Figure 5.1). When the data are combined it is easy to see a significant difference in the proliferative rate (Figure 5.2). After 5 days of culture in DMEM:F-12 media containing 10% FBS, IPAH cells had grown 22.1 ± 1.1 fold (n=9) compared to a 10.5 ± 1.2 fold increase in control cells (n=13, $P<0.001$). This equates to IPAH cells having a proliferative rate double that of control cells.

This striking difference in proliferation rates between the two cell types is akin to that observed between HEK-293-IP and HEK-293-Zeo cells as described in chapter 3. This result led to the hypothesis that IPAH cells may lack or have a significantly decreased expression of the IP receptor causing, as in HEK-293-Zeo, an increase in cell proliferation in response to serum.

5.3 IP receptor expression in control and IPAH cells

Using specific primers to amplify a 204bp sequence of the IP receptor as previously described, RT-PCR was performed on cDNA produced by 5µg of total RNA to confirm presence of IP receptor mRNA in the three control and IPAH cell isolates. A band of the appropriate size can be seen in the lanes for all control and IPAH cells (Figure 5.3 A & B) confirming the presence of the IP receptor in both cell types. Primers for the human GAPDH were used to amplify a 363 bp of this house-keeping gene in all cDNA samples.

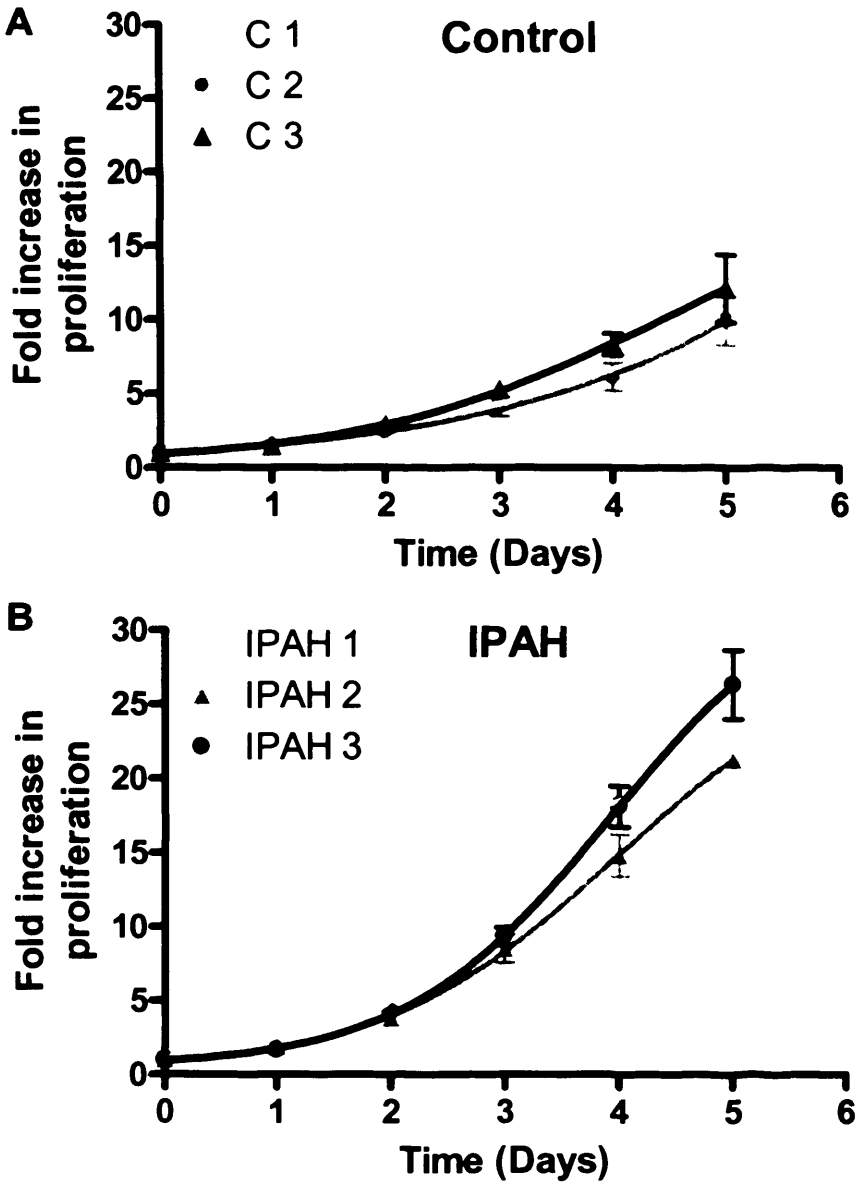

Figure 5.1 Distal HPASM cell isolates from three control (**A**) and three IPAH (**B**) patients were growth-arrested and subsequently stimulated with 10% FBS for varying times as indicated. Cells were counted at time 0 and then at 24 h intervals. Results are expressed as mean fold increase in proliferation from time 0 ± s.e.m. of at least 3 separate experiments performed in triplicate.

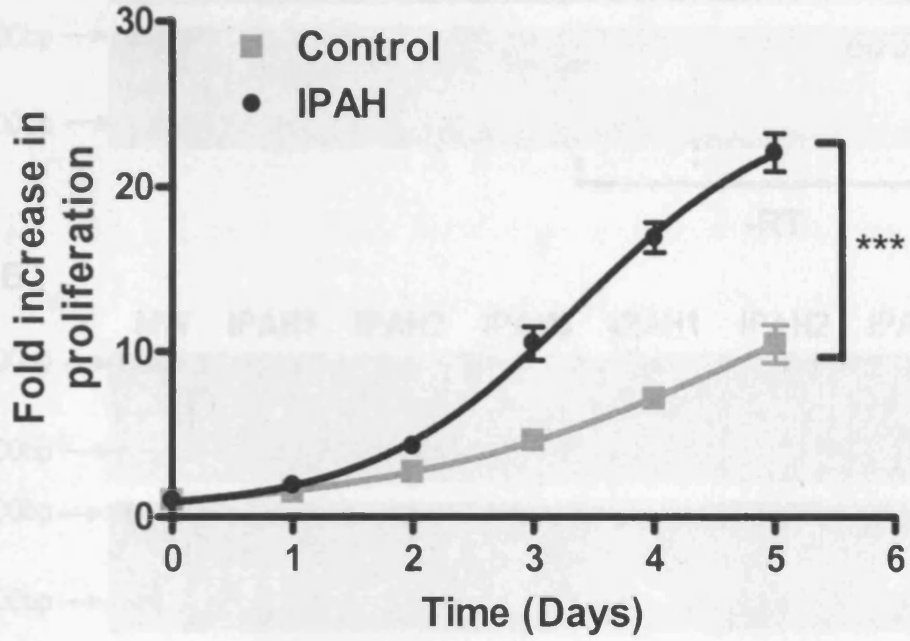

Figure 5.2 Comparison of the proliferative rates of control (■) and IPAH (●) cells growing in DMEM:F12 containing 10% FBS. Cells were counted at time 0 and then at 24 h intervals. Results are expressed as mean fold increase in proliferation from time 0 ± s.e.m. of the combined proliferative rates of three isolates per cell type (see figure 5.1), n=9-13, ***=P<0.001.

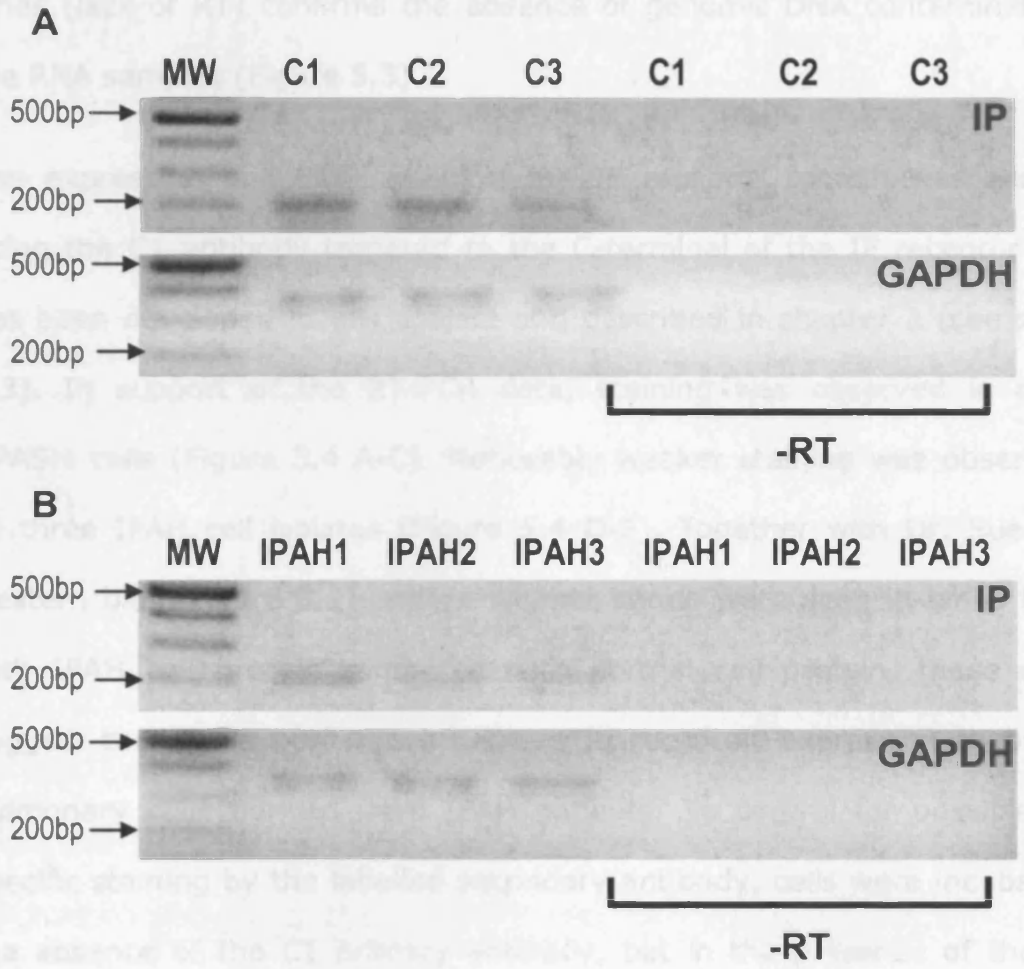

Figure 5.3. PCR was performed on reverse transcriptase cDNA products of 5μg of total RNA from control (**A**) and IPAH (**B**) PASM cells using IP receptor specific primers to amplify a 204 bp region of the receptor sequence and GAPDH primers to amplify a 363 bp band of this house-keeping gene as control. Reverse transcriptase controls (-RT) were negative in all cases. The gel is a representative result which has been reproduced at least three times using different RNA preparations.

The presence of a correct sized band of approximately the same intensity was observed in all lanes (Figure 5.3). Lack of bands in the negative control lanes (lack of RT) confirms the absence of genomic DNA contamination in the RNA samples (Figure 5.3).

The expression and localisation of the IP receptor protein was assessed using the C1 antibody targeted to the C-terminal of the IP receptor which has been developed in this project and described in chapter 3 (see section 3.3). In support of the RT-PCR data, staining was observed in normal HPASM cells (Figure 5.4 A-C). Noticeably weaker staining was observed in all three IPAH cell isolates (Figure 5.4 D-F). Together with Dr. Sue Hall's western blot (Figure 3.2), where weaker bands were seen in lanes loaded with IPAH cell protein compared with normal cell protein, these results suggest that while both types express IP receptor, expression is lower in pulmonary cells derived from IPAH patients. To control for possible non-specific staining by the labelled secondary antibody, cells were incubated in the absence of the C1 primary antibody, but in the presence of the FITC labelled secondary. Under these conditions, no staining was seen in either control or IPAH cells (Figure 5.5) confirming the absence of non specific secondary binding.

5.4 Effects of treprostinil on cAMP elevation in control and IPAH cells

After confirming that both control and IPAH cells express the IP receptor, the functionality of this receptor was tested by measuring cAMP levels in both cell types stimulated with treprostinil with or without pre-treatment with the IP receptor antagonist (IPRA). We chose a 30 mins stimulation

period since we had previously observed this to be the peak cAMP elevation with treprostinil in HPASM cells (Clapp *et al.,* 2002).

In control cells, cAMP was significantly elevated following treatment with 100 nM treprostinil in isolate 1 (204.8 ± 37.0 pmol/mg vs 511.1 ± 20.8 pmol/mg with treprostinil n=3 $P<0.001$) and isolate 3 (130.0 ± 23.4 pmol/mg vs 465.1 ± 37.5 pmol/mg with treprostinil, n=3, $P<0.001$) (Figure 5.6). No cAMP elevation was observed upon treatment with treprostinil in isolate 2 (310.3 ± 29.7 pmol/mg vs 347.3 ± 37.2 with treprostinil, n=3, $P=0.58$) (Figure 5.6). In the two responsive cell types, pre-treatment with 1 μM IPRA for 1 hr fully reversed the cAMP elevating effects of treprostinil restoring them back to levels found in untreated cells (n=3, $P<0.001$) (Figure 5.6).

As with the normal HPASM cells, only two out of the three cell isolates of IPAH cells responded to treprostinil treatment with a significant increase in intracellular cAMP. In IPAH-1, cAMP rose from 157.9 ± 57.0 pmol/mg to 653.6 ± 160.0 pmol/mg (n=3, $P<0.05$) and in IPAH-2 from 196.1 ± 44.0 pmol/mg to 794.0 ± 27.3 pmol/mg upon treatment with 100 nM treprostinil (n=3, $P<0.001$) (Figure 5.7). Pre-treatment of the responsive cells with IPRA prevented the cAMP rise produced by treprostinil, with levels returning back to near-basal levels (Figure 5.7). Thus in both control and IPAH cell types only two out of the three isolates appear to have a functional IP receptor. However, we cannot rule out the possibility that higher concentrations or longer treatments with treprostinil may have given rise to a significant elevation in cAMP in these "seemingly" unresponsive cells.

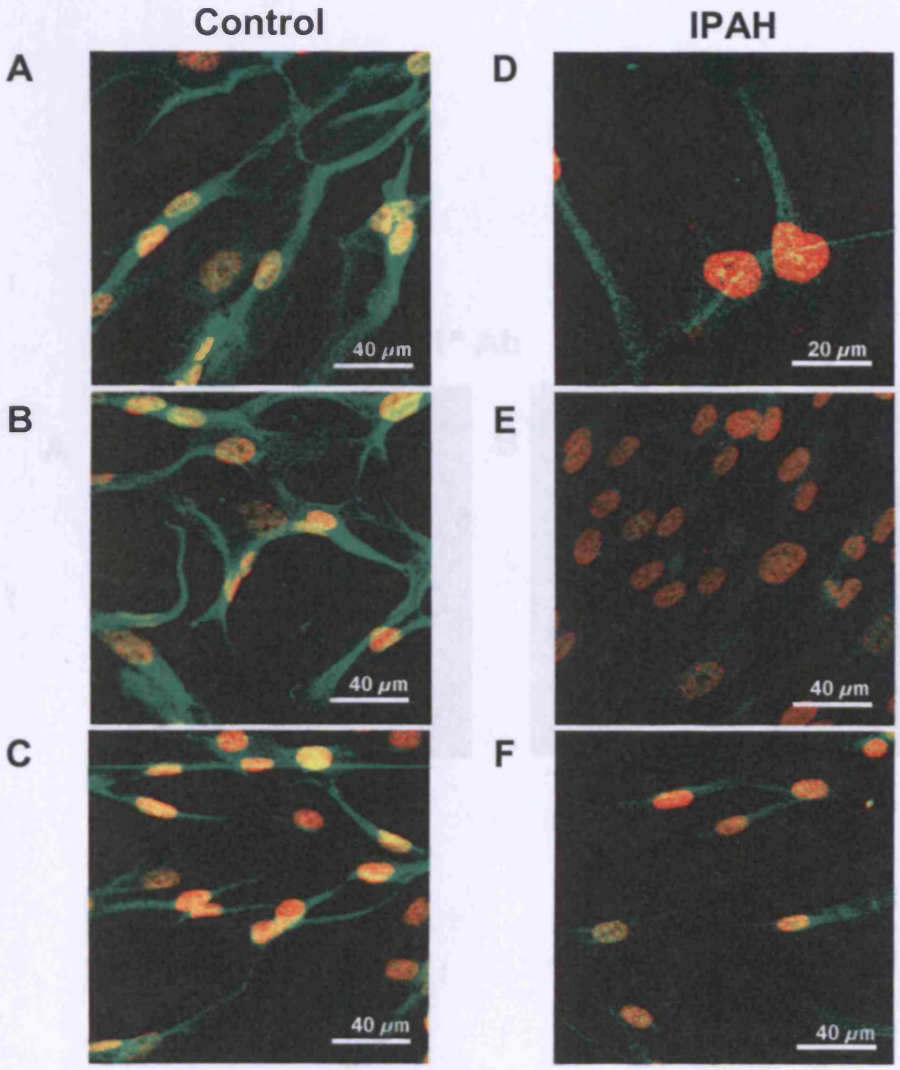

Figure 5.4 Control HPASM cells 1 (**A**), 2 (**B**), 3 (**C**) and IPAH HPASM cells 1 (**D**), 2 (**E**), 3 (**F**) stained with the C1 antibody. Cells were grown in DMEM:F12 + 10% FBS for 48 hours and fixed in 4% paraformaldehyde prior to staining with the C1 primary antibody. FITC conjugated Alexa fluor 488 (green) was used as a secondary antibody and TO-PRO-3 (red) was used to stain nuclei. Cells were visualised using a confocal microscope with a X60 magnification water immersion lens.

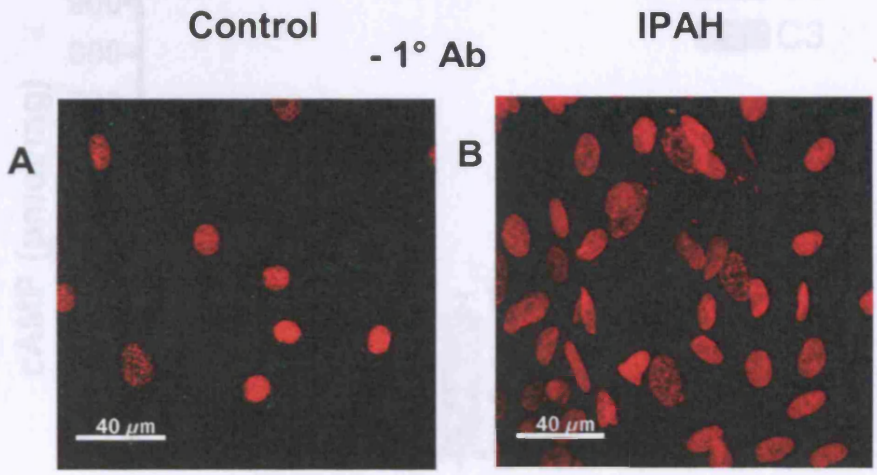

Figure 5.5 Immunohistochemistry controls. Control (**A**) and IPAH (**B**) PASM cells were fixed in 4% paraformaldehyde and immuno-stained with FITC conjugated Alexa fluor 488 (green) and TO-PRO-3 (Red) in the absence of primary antibody (both 1:300 dilution).

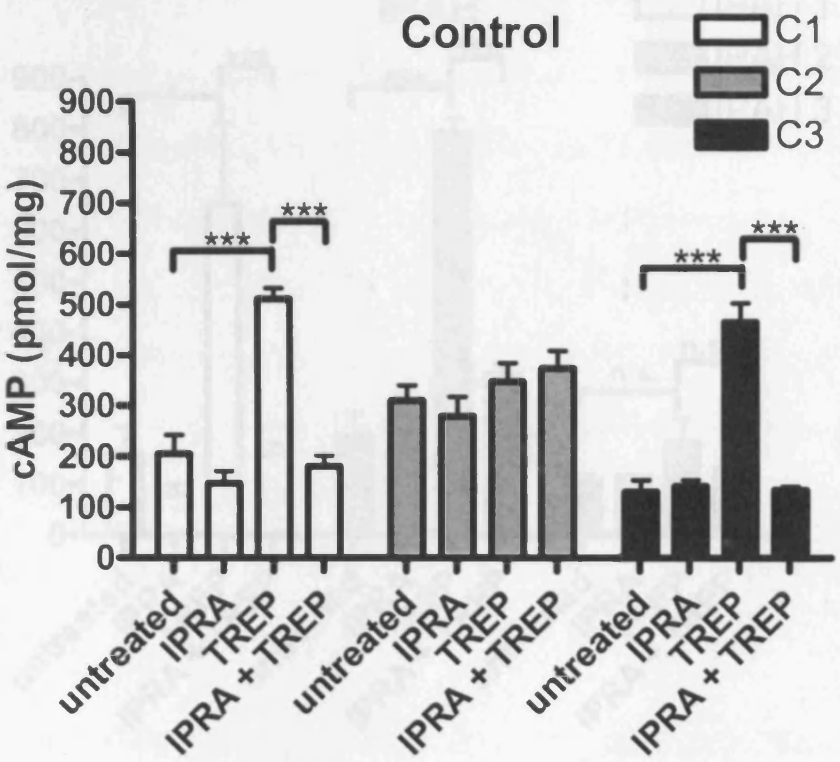

Figure 5.6 Cyclic AMP was measured in three control distal HPASM cell isolates. Cells were stimulated with 10% FBS ± treprostinil (TREP; 100 nM) or IP receptor antagonist (IPRA;1 μM) or a combination. Cells were pretreated with IPRA for 1 hr prior to stimulation with TREP. Cyclic AMP was extracted 30 mins after TREP stimulation and measured using an enzyme immunolinked assay. Results expressed as mean pmol of cyclic AMP per mg of total protein ± S.E.M. (n=3). ***=p<0.001.

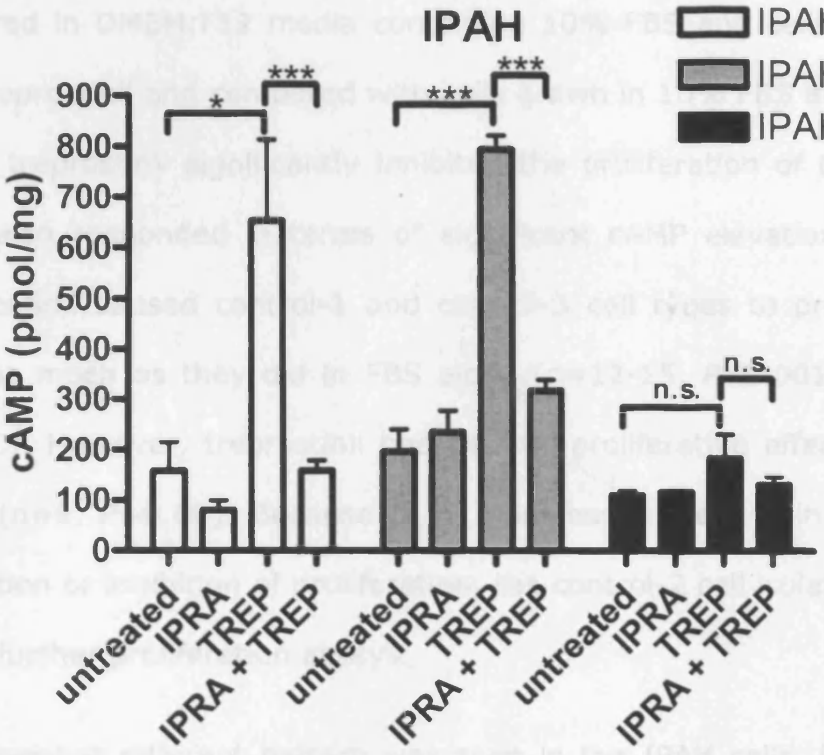

Figure 5.7 Cyclic AMP measurements in three IPAH distal HPASM cell isolates. Cells were stimulated for 30 min with 10% FBS ± either treprostinil (TREP;100 nM) or IP receptor antagonist (IPRA; 1 μM) or a combination. Cells were pretreated with IPRA for 1 hr prior to stimulation with TREP. Cyclic AMP was extracted and measured using an enzyme immunolinked assay. Results expressed as mean pmol of cyclic AMP per mg of total protein ± S.E.M. (n=3).* = $P<0.05$, ***=$P<0.001$.

5.5 Effects of treprostinil on control and IPAH cell proliferation

To further test IP receptor functionality, the effects of treprostinil on cell proliferation were assessed in all isolates of both cell types. Cells were cultured in DMEM:F12 media containing 10% FBS and cells treated with 1 μM treprostinil and compared with cells grown in 10% FBS alone. In control cells, treprostinil significantly inhibited the proliferation of the two isolates that also responded in terms of significant cAMP elevation. After 5 days treprostinil caused control-1 and control-3 cell types to proliferate almost half as much as they did in FBS alone (n=12-15, $P<0.001$) (Figure 5.8 A and C). However, treprostinil had no anti-proliferative effects on control-2 cells (n=9, $P=0.69$). Because of its non-responsiveness in terms of cAMP elevation or inhibition of proliferation, the control-2 cell isolate was excluded from further proliferation assays.

A somewhat different pattern was seen in the IPAH cells. Despite the fact that no significant cAMP elevation was observed in IPAH-3, cell proliferation was still inhibited by around 50% after 5 days treatment with treprostinil (n=12, $P<0.001$), with near similar effects observed in the other two isolates (Figure 5.9). The results obtained with IPAH-3, contrasts to what was observed in control cells where cAMP elevation and proliferation inhibition were correlated. Thus the difference in IP receptor signalling between control and IPAH cells necessitated further investigation.

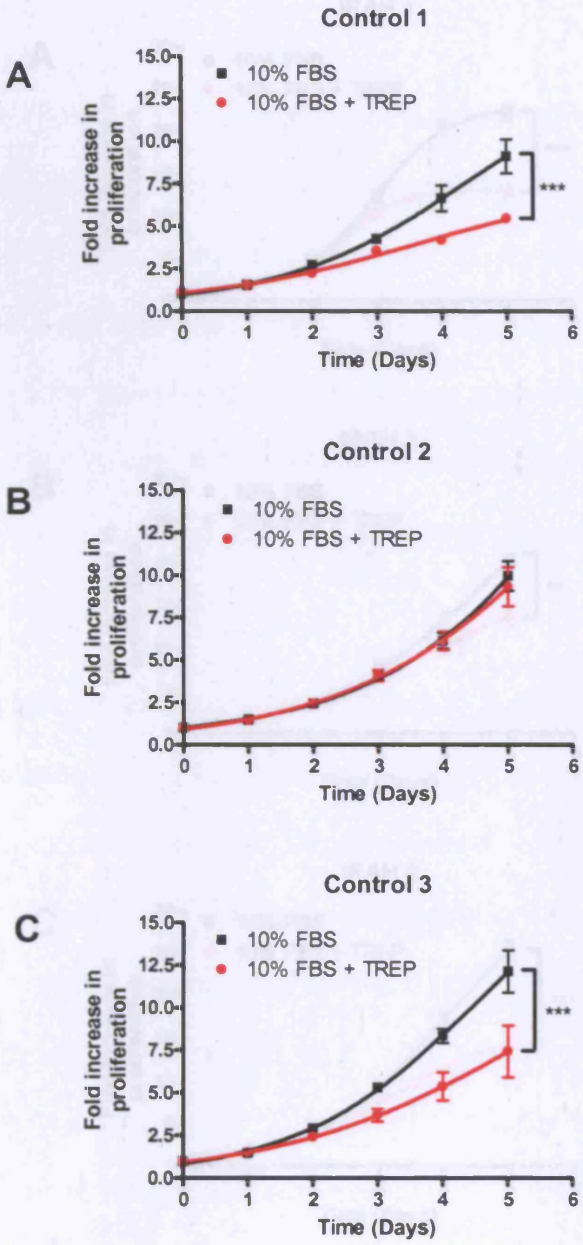

Figure 5.8 Proliferation rates of three normal distal HPASM cell isolates, 1 (**A**), 2 (**B**) and 3 (**C**), either left untreated (■) or treated with treprostinil (TREP, ●, 1 μM). Growth arrested cells were stimulated with 10% FBS ± TREP and counted at different time points as shown. Data shown as mean ± S.E.M. (n=9-15). *** = P<0.001.

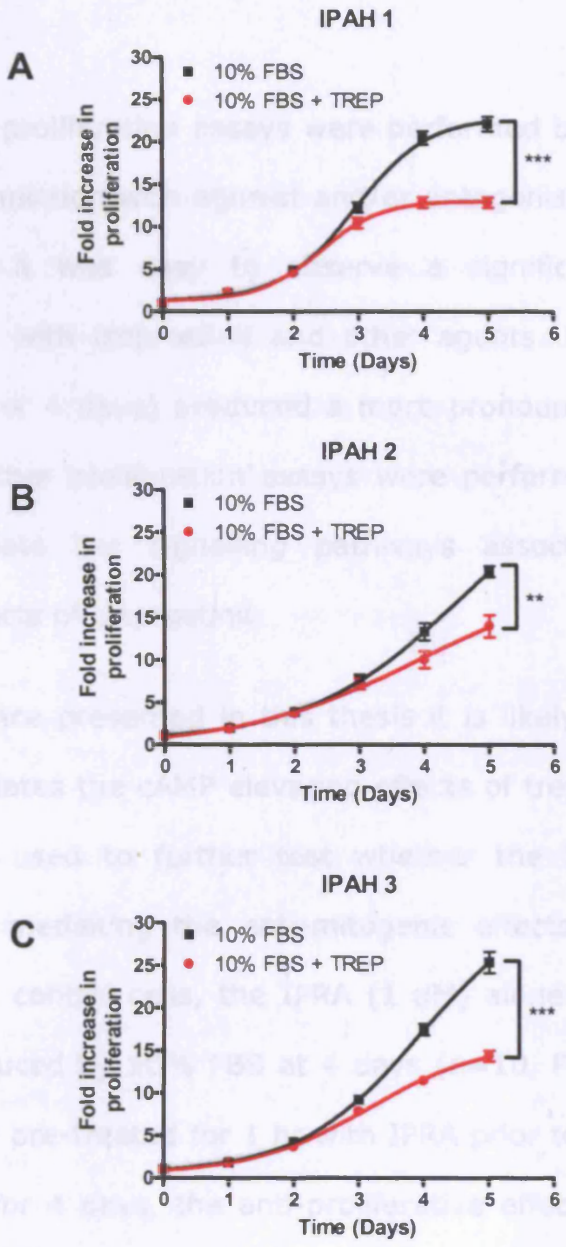

Figure 5.9 Proliferation rates in response to serum of three IPAH distal HPASM cell isolates, 1 (**A**), 2 (**B**) and 3 (**C**), either left untreated (■) or treated with treprostinil (TREP, ●, 1 μM). Growth arrested cells were stimulated with 10% FBS ± TREP and counted at different time points as shown. Data shown as mean ± S.E.M. (n=12).** = P<0.01, *** = P<0.001.

5.6 PGI_2 analogue inhibition of proliferation in control PASM cells: role of cAMP.

In HEK-293 cell proliferation assays were performed by counting cells at 48 hr following stimulation with agonist and/or antagonists (see chapter 3). At this time-point it was easy to observe a significant and pronounced inhibitory effect with treprostinil and other agents. In PASM cells a time point of 96 hr (or 4 days) produced a more pronounced effect than at 48 hours. Thus further proliferation assays were performed at the latter time point to elucidate the signalling pathways associated with the anti-proliferative effects of treprostinil.

Based on evidence presented in this thesis it is likely that the IP receptor exclusively mediates the cAMP elevating effects of treprostinil. The receptor antagonist was used to further test whether the IP receptor was also responsible for mediating the anti-mitogenic effects of this analogue in HPASM cells. In control cells, the IPRA (1 μM) alone had no effect on cell proliferation induced by 10% FBS at 4 days (n=10, P=0.56) (Figure 5.10). When cells were pre-treated for 1 hr with IPRA prior to stimulation with 100 nM treprostinil for 4 days, the anti-proliferative effects of this agent were significantly reversed from 34.5 ± 3.7% to 14.2 ± 5.1% (n=10, P=0.007) (Figure 5.10). This suggests that the IP receptor plays a major role in meditating the antiproliferative effects of treprostinil. Likewise, the effects of 100 nM iloprost, which inhibited proliferation by 31.5 ± 3.0% (n=6, $P<0.001$), were almost totally abolished by pre-treatment with IPRA (Figure 5.11). This suggests that the EP_1 and EP_3 receptors, for which iloprost has an equally strong binding affinity (reviewed in Hata and Breyer, 2004), are

unlikely to play a role in mediating the antiproliferative effects of iloprost in control HPASM cells.

To test whether IP receptor-dependent inhibition of proliferation with PGI_2 analogues was driven by increased levels of intracellular cAMP, control cells were treated with the adenylyl cyclase inhibitor, 2'5'-DDA. Treatment with 100μM DDA alone had no significant effect on cell proliferation (n=9, P=0.37). However, pre-treatment with DDA for 1 hr prior to stimulation with 100 nM treprostinil, significantly reversed treprostinil-induced inhibition of proliferation from 45.9 ± 4.0% to 22.0 ± 7.8% (n=9, P<0.05) (Figure 5.12). Thus, as one would expect, by blocking cAMP production the effects of treprostinil on proliferation are dampened suggesting that adenylyl cyclase is indeed an important mediator in this pathway.

5.7 Role of IP receptor and cAMP-dependent pathway in meditating PGI_2 analogue inhibition of proliferation in IPAH cells

In contrast to the results obtained in control cells, pre-treatment with IPRA did not significantly reverse the inhibition of proliferation caused by treprostinil in IPAH cells (n=9, *P*=0.67) (Figure 5.13). This is surprising given the extent to which the IPRA reduced cAMP levels elevated by treprostinil in these cells. Similarly, treatment with 100 nM iloprost inhibited cell proliferation by 33.8% ± 2.7% (n=9, *P*<0.001) and this inhibition was again not sensitive to pre-treatment with IPRA (n=9, *P*=0.38) (Figure 5.14). Taken together these results suggest that, contrary to what is observed in control cells, IPAH cells possess almost an exclusively IP-receptor independent mechanism for the inhibition of proliferation by PGI_2 analogues.

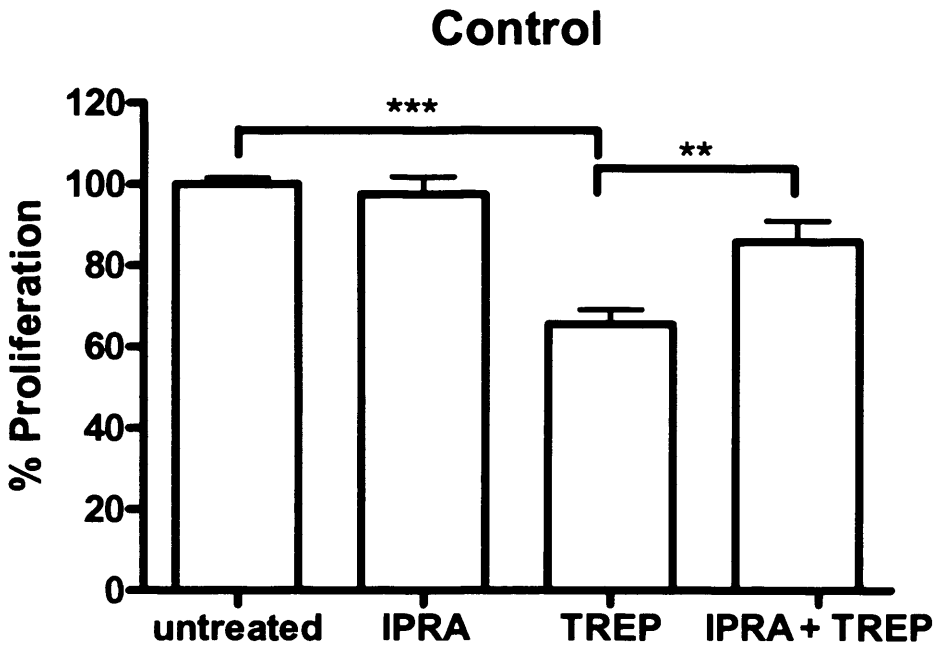

Figure 5.10 Growth arrested control distal HPASM cells (2 isolates) were stimulated with 10% FBS ± TREP (100 nM) or the IP receptor antagonist (IPRA; 1 μM) or a combination. Cells were pretreated with IPRA for 1 hr prior to stimulation with TREP. Cells were counted 96 hrs and data expressed as mean % cell proliferation relative to proliferation mediated by 10% FBS alone ± S.E.M. (n=9). ** = $P<0.01$, *** = $P<0.001$.

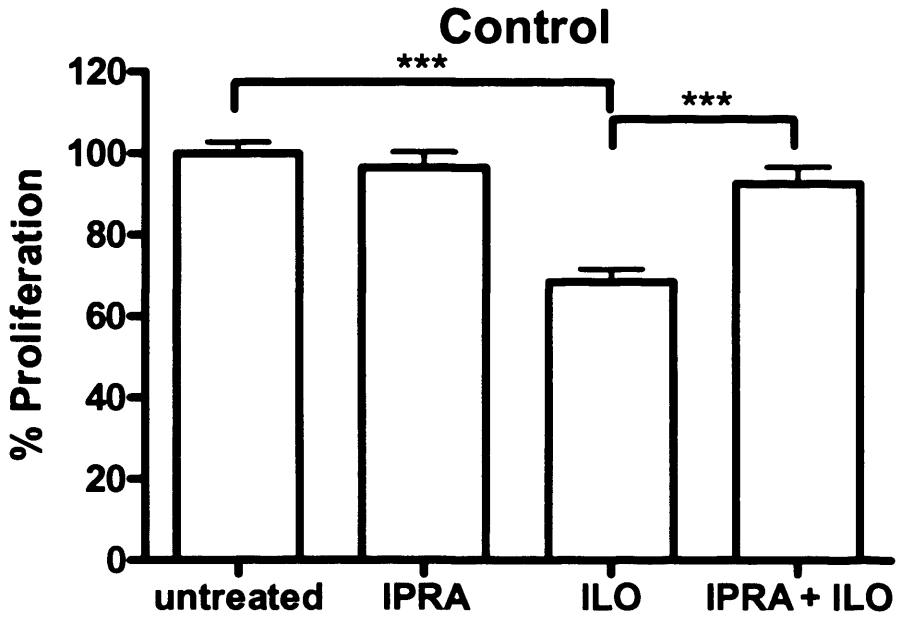

Figure 5.11. Growth arrested control distal HPASM cells (2 isolates) were stimulated with DMEM:F12 + 10% FBS and either left untreated or treated with iloprost (ILO, 100 nM) or IPRA (1 μM) or a combination as shown. Cells were pretreated with IPRA for 1 hr prior to stimulation with ILO. Cells were counted 96 hrs following treatment. Data expressed as mean % proliferative response relative to proliferation mediated by 10% FBS alone ± S.E.M. (n=6). *** = $P<0.001$.

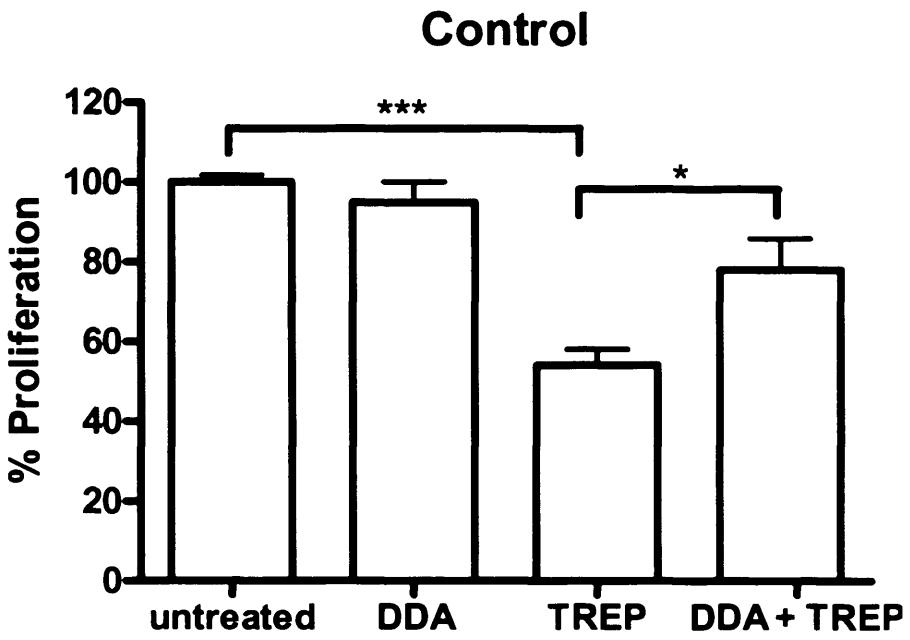

Figure 5.12 Growth arrested control distal HPASM cells (2 isolates) were stimulated with 10% FBS ± TREP (100 nM) or the adenylyl cyclase antagonist, DDA (100 μM) or a combination. Cells were pretreated with DDA for 1 hr prior to stimulation with TREP. Cells were counted 96 hrs following treatment. Data expressed as % cell proliferation relative to proliferative response mediated by 10% FBS alone and shown as mean ± S.E.M. (n=9). * = $P<0.05$, *** = $P<0.001$.

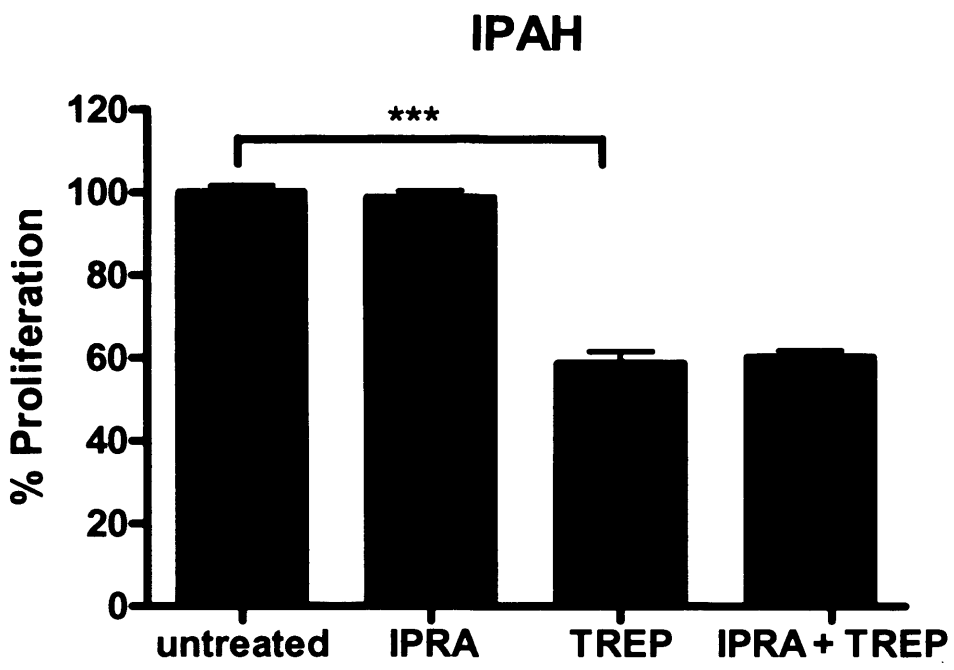

Figure 5.13 Growth arrested IPAH distal HPASM cells (3 isolates) were stimulated with 10% FBS ± TREP (100 nM) or IPRA (1 μM) or a combination as shown. Cells were pretreated with IPRA for 1 hr prior to stimulation with TREP. Cells were counted 96 hrs following treatment. Data is expressed as % cell proliferation relative to the proliferative response mediated by 10% FBS alone and shown as mean ± S.E.M. (n=9). ** = $P<0.01$, *** = $P<0.001$.

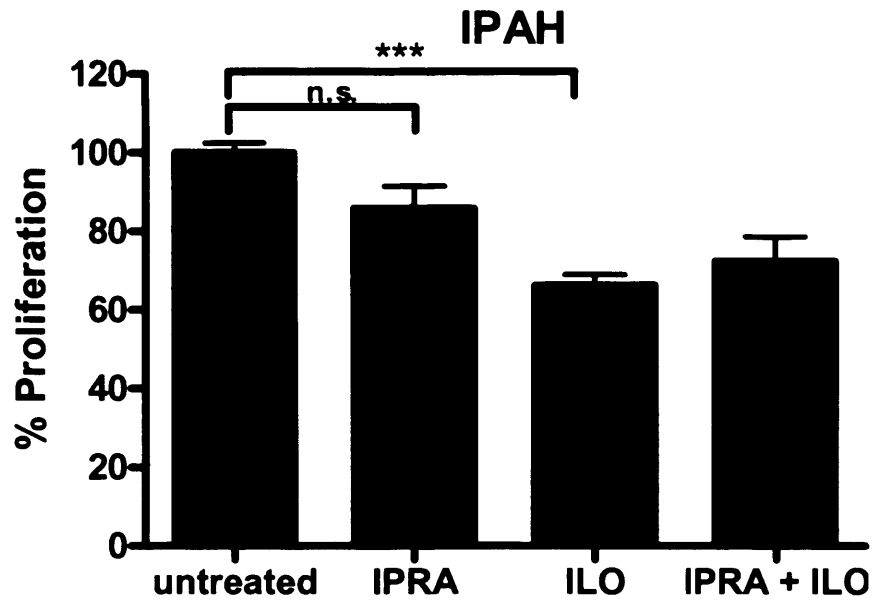

Figure 5.14 Growth arrested IPAH distal HPASM cells (3 isolates) were stimulated with 10% FBS in the presence or absence of iloprost (ILO, 100 nM), IPRA (1 μM) or a combination. Cells were pretreated with IPRA for 1 hr prior and counted 96 hrs following treatment. Data are expressed as % cell proliferation relative to the proliferatative response mediated by 10% FBS alone and shown as mean ± S.E.M. (n=9). *** = $P<0.001$.

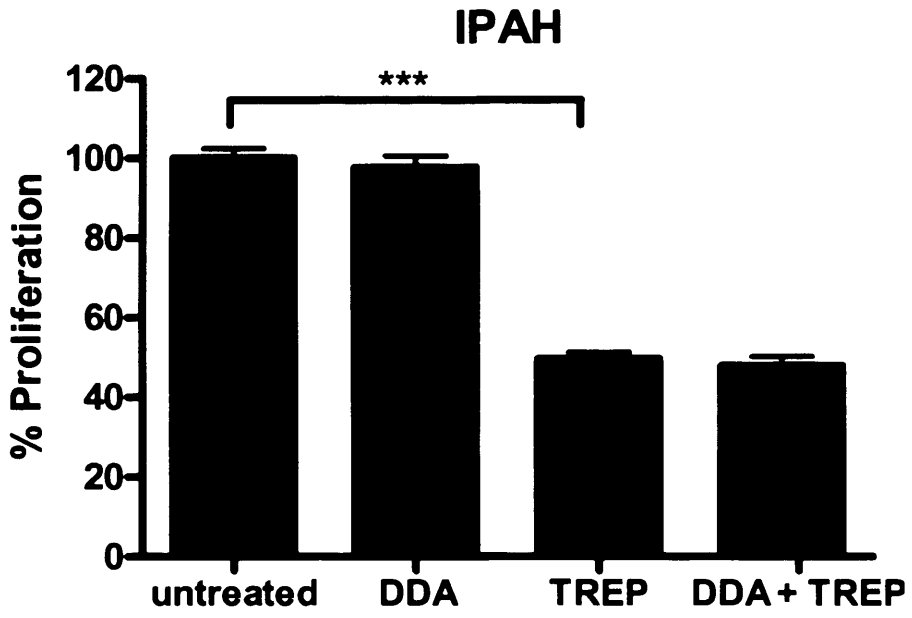

Figure 5.15 Growth arrested IPAH distal HPASM cells (3 isolates) were stimulated with 10% FBS and either left untreated or treated with TREP (100 nM), the adenylyl cyclase inhibitor, DDA (100 μM) or a combination. Cells were pretreated with DDA for 1 hr prior to stimulation with TREP and cells counted 96 hrs later. Data is expressed as % cell proliferation relative to proliferative response mediated by 10% FBS alone and shown as mean ± S.E.M. (n=9). * = $P<0.05$, *** = $P<0.001$.

This observation was further strengthened by the fact that the adenylyl cyclase inhibitor, DDA was unable to reverse the treprostinil induced inhibition of proliferation in IPAH cells (n=9, P=0.53) (Figure 5.15). Hence the IP receptor-independent mechanism, preferentially utilised by treprostinil in IPAH cells does not seemingly involve cAMP. An interesting point to note is that, although isolate 3 was distinct from the other two isolates in terms of not being able to elevate cAMP in response to treprostinil, it behaved similarly in all proliferation studies; hence all three isolates were included experimentally.

5.8 Role of PPARγ in the regulation of cell proliferation in control and IPAH PASM cells

In the HEK-293-IP stable line, PPARγ was found to mediate in part the anti-proliferative effects of treprostinil, possibly being responsible for the part of the response not sensitive to adenylyl cyclase or PKA inhibitors. To assess whether control and IPAH cells exhibited distinct proliferative responses upon direct activation of PPARγ akin to the differences observed between HEK-293-IP and HEK-293-Zeo cells, both HPASM cell types were treated with varying concentrations of rosiglitazone and counted after 4 days. Rosiglitazone had an inhibitory effect on the proliferation of both control and IPAH cells with essentially no difference in potency between the two, with the control IC_{50} being 35.1 μM compared to 43.3 μM in IPAH cells (Figure 5.16). Thus the effects of rosiglitazone are very similar to those seen previously with the same drug in HEK-293-IP cells.

To test whether PPARγ could contribute, even if only partially, to the antiproliferative mechanism of PGI_2 analogues, both control and IPAH cells

were pre-treated for 1 hr with 1 μM GW9662 prior to a 4 day stimulation with 100 nM treprostinil. In control cells, GW9662 alone had no effect on cell proliferation (n=9, P=0.95; Figure 5.17), although the antagonist was able to cause a small but significant reversal of the antiproliferative effects of treprostinil (n=12, P<0.05; Figure 5.17). In IPAH cells however, the reversal with GW9662 was noticeably greater. The antagonist was able to reverse the anti-proliferative effects of treprostinil by more than 50% from a 33.5 ± 1.8% to a 13.2 ± 3.7% inhibition (n=15, P<0.001; Figure 5.18). This set of results highlights once more the different mechanism used by treprostinil in the two cell types to inhibit growth, and fully supports an important role for PPARγ in IPAH cells.

5.9 Summary

- IPAH PASM cells have a higher proliferative rate compared to control PASM cells.
- Both control and IPAH PASM cells express IP receptor mRNA and protein, though the expression of this receptor is reduced in IPAH, with its localisation, as determined by immunohistochemistry appearing to be predominantly cytoplasmic with little evidence of the receptor being present at the membrane.
- The PGI_2 analogues, treprostinil and iloprost, can inhibit the proliferation of both control and IPAH cells but the mechanism by which this occurs appears to be markedly different.

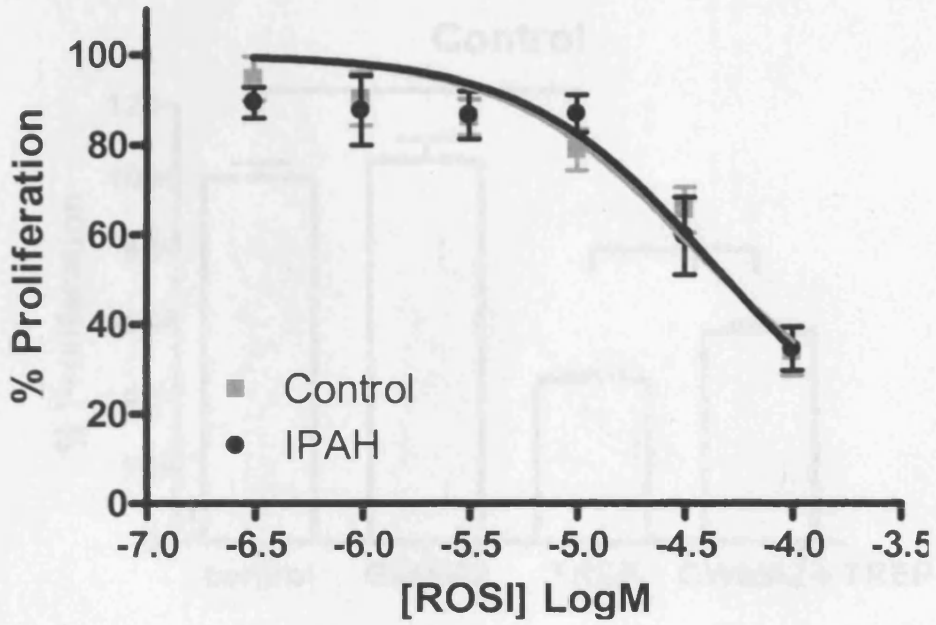

Figure 5.16 Concentration-dependent effect of rosiglitazone (ROSI) on cell proliferation. Growth arrested control (2 isolates, ▪) and IPAH (3 isolates, ●) distal HPASM cells were stimulated with 10% FBS ± ROSI and counted after 96 hrs. Data are expressed as % cell proliferation relative to proliferative response mediated by 10% FBS alone and shown as mean ± S.E.M. (n=9-12). Data was fitted using a sigmoidal dose-response formula (see equation 2.1).

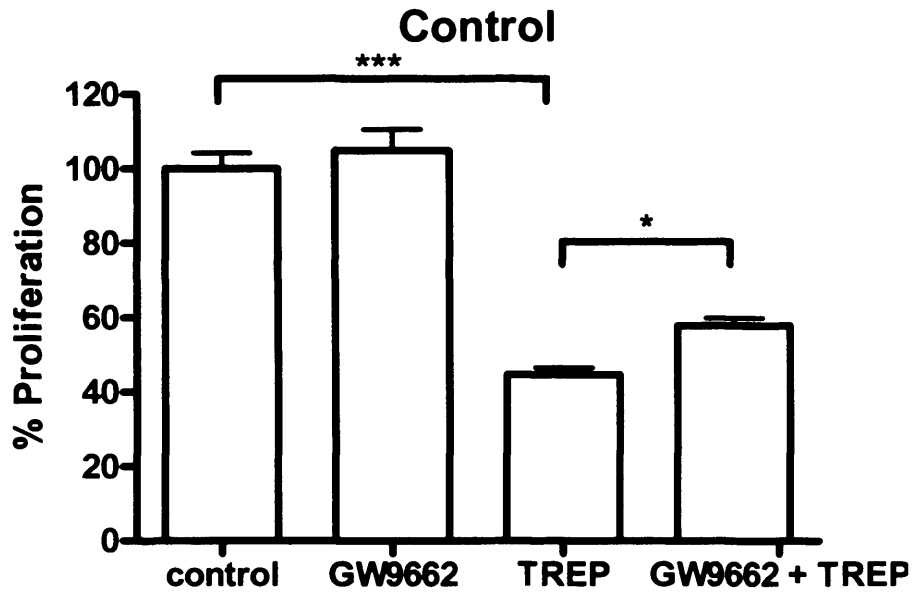

Figure 5.17 Growth arrested control distal HPASM cells (2 isolates) were stimulated with 10% FBS and either left untreated or treated with TREP (100 nM), the PPARγ antagonist, GW9662 (1 μM) or a combination. Cells were pretreated with GW9662 for 1 hr prior to stimulation with TREP and cells counted at 96 hrs. Data is expressed as % cell proliferation relative to proliferative response mediated by 10% FBS alone and shown as mena ± S.E.M. (n=12), * = P<0.05, *** = P<0.001.

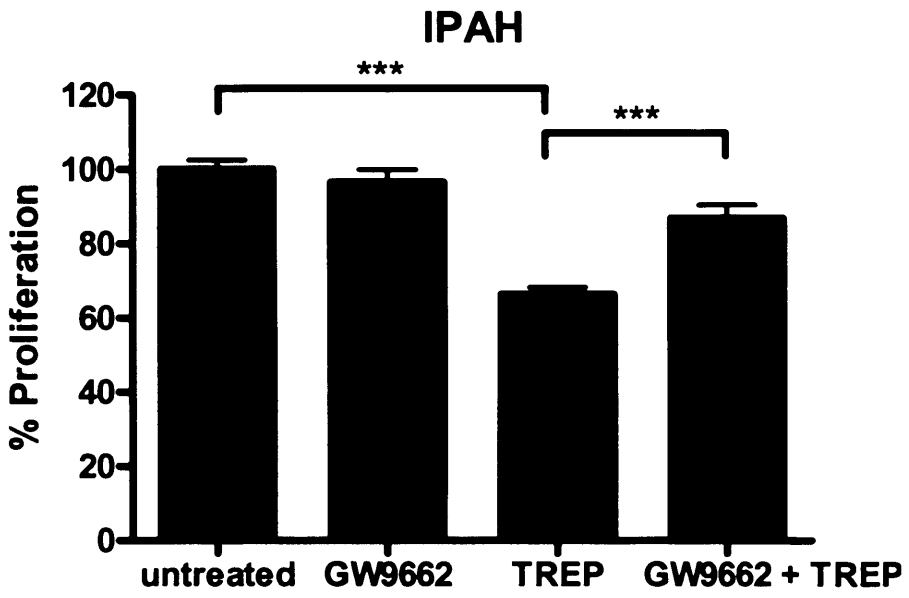

Figure 5.18 Growth arrested IPAH distal HPASM cells (3 isolates) were stimulated with 10% FBS and either left untreated or treated with TREP (100 nM), the PPARγ antagonist, GW9662 (1 μM) or a combination. Cells were pretreated with GW9662 for 1 hr prior to stimulation with TREP and cells counted at 96 hr. Data is expressed as % cell proliferation relative to proliferative responses mediated by 10% FBS alone and shown as mean ± S.E.M. (n=15), *** = $P<0.001$.

- In responsive control cells the IP receptor elevates cAMP in response to treprostinil and mediates the majority of anti-proliferative effects of PGI_2 analogues.

- In IPAH cells treprostinil appears to function through an IP receptor and cAMP-independent pathway despite its ability to elevate cAMP through the IP receptor in two out of the three isolates.

- PPARγ is an important mediator of the anti-proliferative effects of treprostinil in IPAH cells but appears to only have a minor role in control cells.

- More numbers of both normal and IPAH PASMC are needed to confirm these results and ensure that these conclusions apply to all/most children with IPAH.

- The fact that the IPAH patients from which the HPASM cells were derived were juvenile and the normal patients were adult is a significant limitation of the study as the physiology of smooth muscle cells as well as the functionality and expression of the IP receptor and other potentially relevant cellular mechanism may be affected by age.

5.10 Discussion

5.10.1 IP receptor expression in control and IPAH pulmonary artery smooth muscle cells

The present study has shown that PASM cells derived from IPAH patients have a significantly faster proliferative rate relative to control PASM cells. A similar difference was described between HEK-293 cells with or without the receptor (see section 3.5). This initial observation led to the hypothesis that IPAH PASM cells may have a lower expression of the IP receptor resulting in an altered proliferative phenotype. The present study has confirmed the presence of IP receptor mRNA and protein in both control and IPAH PASM cells. However, although staining with the C1 antibody was observed in both cell types, the degree of expression of this receptor and its localisation appeared to be different. Expression was noticeably weaker in IPAH cells compared to control cells when stained at the same time and under similar experimental conditions. This observation is further strengthened by recent western blotting data (Sue Hall, UCL unpublished observations). In both cell types, our C1 antibody picked up a band of 60 kDa, concomitant with a glycosylated active form of the IP receptor (Smyth *et al.,* 1998). However, the signal was 40% more intense in control pulmonary cells compared to IPAH cells. Thus it is possible that down-regulation of the IP receptor in IPAH PASM cells could affect the growth characteristics of these cells. Reduced expression of the IP receptor in IPAH leading to abnormal growth characteristics may be consistent with data from studies in IP receptor knockout mice. Mice lacking the receptor displayed essentially normal pulmonary arteries, however when exposed to chronic hypobaric hypoxia,

they developed a greater degree of pulmonary hypertension, right ventricular hypertrophy and pulmonary vascular remodelling compared to wild type mice (Hoshikawa *et al.,* 2001). This suggests that the IP receptor can modulate the response to remodelling stimuli, but that loss of the IP receptor *per se* is not sufficient to trigger the disease.

Down regulation of the receptor might be due to internalisation or desensitisation of the receptor. The expression of the receptor was essentially confined to the cytosol in IPAH cells, possibly reflecting more internalisation of the receptor, maybe as a result of chronic treatment of the lung with epoprostenol for several years prior to cell culture.

Alternatively, the receptor may be more susceptible to desensitisation in IPAH as a result of long-term agonist stimulation. The IPAH patients from whom PASM cells originated had all been receiving long term continuous epoprostenol infusion prior to transplantation. The prototypical model for GPCR regulation involves three key mechanisms (reviewed in Bohm *et al.,* 1997). The first phase of desensitization occurs rapidly after agonist stimulation and is caused by agonist-induced receptor phosphorylation mediated by second messenger kinases, such as PKA and PKC, and this eventually uncouples the receptor from its G protein. This short-term desensitisation is followed by sequestration of the receptor away from the membrane. Eventually, more prolonged stimulation causes a net down-regulation of the receptor.

The human IP receptor, when overexpressed in HEK293 cells, exhibits rapid agonist-induced desensitization occurring within minutes and this has been shown to involve phosphorylation of the C-terminal by PKC (Smyth *et al.*,

1998). In contrast, native cells naturally expressing the IP receptor reveal a much slower time course of desensitization (Krane *et al.,* 1994; Zucker *et al.,* 1998). In fibroblasts, iloprost-induced sequestration was observed but no role for PKC phosphorylation was found in the desensitisation mechanism (Nilius *et al.,* 2000) whereas in rat PASMC, PKA rather than PKC, appears to be the mediator responsible for the uncoupling of the IP receptor and G_s leading to desensitisation (Sobolewski *et al.,* 2004). In light of this it could be an attractive hypothesis to suggest that prolonged stimulation with PGI_2 (long-term epoprostenol treatment) may have a role in causing disfunctionality of the IP receptor by causing excessive sequestration and internalisation.

As discussed in chapter 3, there are few commercially available antibodies against the IP receptor, and most of these have been developed in the last 3-4 years. This has meant IP receptor protein expression and localisation in smooth muscle cells and indeed in other cell types has been scantily investigated. To my knowledge this is the first time expression and cellular localisation of the IP receptor protein have been studied in human PASM cells in detail. In agreement with our study, data published in an abstract form claims to show reduced expression of the IP receptor in remodelled vessels obtained from adults with pulmonary hypertension (Mason *et al.,* 1999).

In our experiments, downregulation of the IP receptor was not concomitant with a downregulation of cAMP elevating effects. If anything treprostinil-mediated cAMP-elevation, in terms of fold increase, was more pronounced in IPAH than in control cells. This observation suggests that the number of IP

receptors present on the cell-surface may not be a measure of how much cAMP can be generated; it may be that the re-sensitisation process, whereby the re-coupling of the G protein to the receptor occurs after un-coupling (reviewed in Bohm *et al.*, 1997), is more rapid in the fewer IP receptors present in IPAH cells. It should be pointed out that the cAMP levels and elevation observed in the normal, control SMCs in the present study differ significantly from those previously described in the same cell type. The basal level of cAMP I measured is significantly higher in our experiments than those formerly observed in our laboratory (Clapp *et al.,* 2002) and the fold increase upon stimulation with a PGI_2 analogue is lower than that described by Wharton *et al* (2000), Clapp *et al* (2002) or Growcott *et al* (2006). The reason for this discrepancy is not immediately obvious but may be a result of differing experimental and assay conditions. For example IBMX was not added in our extraction step, we use a different assay to measure cAMP which may have a different sensitivity and furthermore we did not perform a time course to establish the peak cAMP elevation time. However, a time of 30 mins should be very close to maximal cAMP-elevation as reported by both Wharton *et al* (2000) and Clapp *et al* (2002).

Our studies indicating dysregulation of the prostacyclin pathway in the PASMCs of patients with IPAH is supported by the work of other investigators showing that PGI_2 synthase (PGIS) is decreased in PASM cells obtained from adults with IPAH (Tuder *et al.,* 1999). Thus it is tempting to speculate that the whole prostacyclin system may be down-regulated in pulmonary hypertension and that the level of IP receptor expression may dictate the level of PGIS expression or vice versa. Obviously, this will require further investigation and could be tested by measuring PGIS mRNA

levels in our HEK-293-Zeo and HEK-293-IP cell model. Reduction in IP receptor expression may explain why short term administration of PGI_2 induces beneficial effects which become less pronounced after more chronic use.

5.10.2 Mechanisms responsible for the anti-proliferative effects of PGI_2 analogues in control PASM cells

Data in the present study show clearly that the PGI_2 analogues treprostinil and iloprost inhibit proliferation in both control and IPAH PASM cells. However, the mechanism by which they do so is markedly different. In control PASM cells PGI_2 analogues act through the IP receptor and the response follows the classical pathway; by activating adenylyl cyclase *via* G_s and elevating intracellular levels of cAMP to inhibit proliferation. This mechanism has been described before in these cells (Wharton *et al.,* 2000; Clapp *et al.,* 2002) and is in agreement with the results obtained with HEK-293-IP cells, showing strong involvement of cAMP. However, there appears to be a small part of the regulatory mechanism which cannot be inhibited by DDA, suggesting the presence of cAMP-independent pathways. Although this result is consistent with previous studies (Wharton *et al.,* 2000; Clapp *et al.,* 2002; Phillips *et al.*, 2005) and this thesis (see section 3.8), we have not yet tested whether a combination of both adenylyl cyclase and PKA antagonists may be required to observe full reversal. Furthermore only one adenylyl cyclase antagonist was used which may or may not be able to inhibit all isoforms. Clapp *et al* (2002), have previously shown that DDA in combination with another adenylyl cyclase inhibitor, SQ22536, did produce

greater inhibition of iloprost–induced growth suppression than either inhibitor given alone.

In the studies presented in chapter 4, it was shown that treprostinil-induced PPARγ activation was dependent on the presence of the IP receptor, but was activated largely independently of cAMP and PKA and that this transcription factor was responsible for mediating part of the anti-proliferative effects of this PGI_2 analogue (see section 4.7). In agreement with published studies performed investigating VSMC (Sasaguri *et al.*, 1992; Benson *et al.*, 2000; Ward *et al.*, 2004), the present study also shows that activation of PPARγ inhibits PASM cell proliferation in control cells. Furthermore, as in HEK-293-IP cells, this nuclear receptor does seem to have a small but significant effect in mediating the inhibition of proliferation caused by treprostinil. Thus, as initially hypothesised, we conclude that in control smooth muscle cells, the PGI_2 analogue-induced signalling pathway is mainly mediated by IP-receptor-dependent cAMP elevation with a small role for PPARγ. The role of the IP receptor in PPARγ activation in these cells will need to be confirmed by replicating the PPARγ reporter gene assays and using siRNA to "knockdown" the IP receptor in these cells, a protocol we would like to optimise in the near future.

The concentrations of rosiglitazone at which we could observe inhibition of cell proliferation in both PASM and HEK-293 cells were relatively high with 10 μM being the lowest concentration to have significant anti-proliferative effects. Although this is in line with what has been previously reported in smooth muscle cells with both rosiglitazone (Ward *et al.*, 2004) and troglitazone (Benson *et al.*, 2000) it is inconsistent with the binding ability

of rosiglitazone at the LBD of PPARγ (IC_{50} = 9 nM in human adipocytes) (Young *et al.,* 1998) as well as our observation that 1 μM rosiglitazone is enough to generate a big increase in PPARγ activation in our reporter gene assay (see section 4.2). It therefore remains to be determined whether the anti-proliferative effects of rosiglitazone are exclusively mediated by PPARγ or may be in part dependent on non-specific effects of the drug. Evidence against the latter comes from the observation that the PPARγ antagonist, GW9662 (1 μM) can fully reverse the inhibitory effect of 10 μM rosiglitazone in smooth muscle cells (Ward *et al.*, 2004).

5.10.3 Mechanisms responsible for the anti-proliferative effects of PGI_2 analogues in IPAH PASM cells

In the present study we show for the first time the retained effectiveness of PGI_2 analogues in inhibiting the proliferation of cells from IPAH patients. This observation is of great relevance given the widespread clinical use of these analogues and our lack of knowledge about their effects on medial proliferation in advanced disease. Unexpectedly the signalling mechanism observed in control cells does not appear to be responsible for the anti-proliferative effects of PGI_2 analogues in IPAH cells. While treprostinil retains its ability to increase cAMP and inhibit proliferation in both control and IPAH PASMCs, the former is IP receptor dependent while the latter is unaffected by blocking either adenylyl cyclase or the IP receptor. Instead, the PPARγ antagonist, GW9662, appeared to reverse a large part of the anti-proliferative effects. Thus we conclude that although the IP receptor is present in IPAH cells, the signalling pathway mediating the anti-proliferative

effects of PGI_2 analogues switches to a mechanism primarily dependent on PPARγ.

At first glance, this concept contrasts with a study describing decreased PPARγ gene and protein expression in PAH lungs (Ameshima *et al.,* 2003). The authors of this study conclude that PPARγ is abundantly expressed in control human lung tissue, particularly in endothelial cells, but is reduced or absent in the angiogenic plexiform lesions of pulmonary hypertensive lungs, suggesting a down-regulation of the PPARγ signalling system in this disease. However their data was specifically focused on endothelial cells and because PPARγ was shown to be absent in smooth muscle cells from control lungs, they did not assess PPARγ expression in PAH smooth muscle cells. In fact the authors actually state that the PPARγ-negative cells in the plexiform lesions analysed are not smooth muscle cells as these lack smooth muscle cell actin staining (Ameshima *et al.,* 2003). In contrast, our lab has shown that the medial smooth muscle cells of peripheral arteries stain well for PPARγ in lung sections from IPAH patients with weak or undetectable staining in intimal proliferative cells (Falcetti *et al.*, 2005). Consequently an attractive hypothesis may be that cellular localisation/expression of PPARγ alters in disease; it may be predominantly expressed in endothelial cells in normal pulmonary arteries, and in smooth muscle cells in IPAH. This shift in localisation may be key to the role of PPARγ in mediating the inhibition of proliferation observed in IPAH PASM by PGI_2 analogues. Again, as with downregulation of the IP receptor, this shift in PPARγ localisation may be a result of long-term treatment with PGI_2 analogues.

When the mechanism of treprostinil-mediated PPARγ activation was studied in chapter 4, we concluded that treprostinil was acting through the IP receptor to activate PPARγ. This could still be applicable to control cells where most of the anti-proliferative properties are mediated by the IP receptor; however in IPAH cells this does not appear to be the case given the lack of effect with the IP receptor antagonist. One hypothesis is that the IP receptor is not exclusive in mediating receptor-dependent activation of PPARγ; other membrane-bound receptors could activate the same signalling cascade upon stimulation. Likely candidates for this role are the EP class of receptors due to their known affinity for PGI_2 analogues. All analogues have some affinity for EP_3 receptors. In addition iloprost and carbacyclin also have effects at the EP_1 receptor. However its ability to bind EP_1 is species specific, only occurring in rat and mice and is largely absent in humans (Sharif and Davis, 2002)

Out of the four EP receptor subtypes, EP_2 and EP_3 are widely expressed in smooth muscle, including pulmonary artery SMCs (Jones *et al.,* 1997) and are therefore of most interest to this study. Similar to the IP receptor, the EP_2 receptor subtype is classified as a "relaxant" receptor given its ability to mediate vasodilation by signalling through the G_s protein and causing intracellular cAMP elevation (reviewed in Hata and Breyer, 2004). EP3 receptors classically mediate vascular contraction and aggregation of human platelets by signalling through G_i (Sugimoto *et al.,* 1992). Different splice variants of EP_3 can also couple to G_s and G_q and cause cAMP or IP_3 generation respectively (Irie *et al.,* 1993; Namba *et al.,* 1993). The broad spectrum of signalling options is akin to that observed with the IP receptor whereby post-translational modification of the C-terminal is thought to

modulate its coupling to multiple signalling pathways (Hata and Breyer, 2004). Interestingly, as discussed earlier, although iloprost treatment leads to agonist-induced IP receptor-desensitisation this does not occur with PGE_1 a known activator of the EP_3 receptor (Zucker *et al.,* 1998). Taking these observations together we hypothesise that the EP_3 receptor may be an alternative target for PGI_2 analogues in cells where the IP receptor has lost its ability to regulate cell proliferation and warrants further investigation.

Although we observe IP receptor-dependent PPARγ activation in HEK-293-IP cells (chapter 4), it is possible that in PASM cells a direct ligand binding mechanism is responsible for the treprostinil-induced activation of PPARγ. However, given the complete inability of treprostinil to activate PPARγ in HEK-293-Zeo cells, this hypothesis seems unlikely.

In conclusion this study has highlighted important differences in the signalling mechanisms utilised by PGI_2 analogues in IPAH and control PASM cells. Although the IP receptor appears to be markedly down-regulated in IPAH, PGI_2 analogues are still efficacious at inhibiting cell proliferation although treprostinil did not reduce the proliferation rate to normal, indicating that the PASMCs in IPAH have escaped normal growth regulatory mechanisms. The IP-receptor independent signalling mechanism in IPAH cells warrants further study to uncover potential novel targets which may prove to be beneficial in the treatment of pulmonary hypertension.

Chapter 6

General Discussion and Conclusion

6.1 Characterisation of novel tools to analyse IP receptor expression and function

The key objective of this thesis was the study of the IP receptor and its role in mediating the anti-proliferative effects of PGI_2 analogues. This was of interest because, whilst it is assumed that PGI_2 analogues work through the IP receptor they are also known to have binding affinity at other PG receptors (Abramovitz *et al.,* 2000), as well as acting as ligands for PPARs (Forman *et al.,* 1997). Due to the lack of experimental tools with which to study the role of this receptor, it is not known to what extent other pathways may mediate the effects of PGI_2 analogues.

At the start of the project, pharmacological antagonists had yet to be developed. There were no commercially available siRNA kits for knockdown of the receptor and, in any case, there were no IP receptor-specific antibodies with which to test the reliability of protein knockdown. An IP receptor knockout mouse was generated in 1997 (Murata *et al.,* 1997) and smooth muscle cells from IP null mice have subsequently been used to demonstrate that the IP receptor mediates the anti-proliferative properties of cicaprost (Kothapalli *et al.,* 2003). However, as described previously, there are marked differences not only in the affinity of PGI_2 analogues for human and mouse prostanoid receptors, but also in the nature of G-protein coupling of the IP receptor in the two species. Thus the IP knockout mouse may not be an ideal model for those of us working on the human receptor, studying its role as a target for the PGI_2 analogues used in the treatment of IPAH.

A major contribution made by this project has been the generation and characterisation of a number of novel tools for the study of the human IP receptor. HEK-293 cell lines were generated which stably expressed the human IP receptor (HEK-293-IP) using the empty zeocin resistance plasmid (HEK-293-Zeo) as a control. We also developed a novel antipeptide antibody (C1 antibody), raised against a portion of the intra-cellular C-terminal of the IP receptor. Comparing the staining obtained with this and other IP antibodies, such as the commercially available Cayman antibody and the antibody raised against the N-terminal sequence described by Komhoff et al (1998) (N1 antibody), we concluded that the C1 antibody is the most specific one available to date, as indicated by the presence of strong staining in HEK-293-IP and its virtual absence in HEK-293-Zeo cells. The stable transfected HEK-293 line was an invaluable tool with which to study the specificity of these receptors, as HEK-293-IP cells could be considered strong positive controls while HEK-293-Zeo acted as negative controls. The C1 antibody was subsequently used to assess localisation and protein expression in PASMC from control and IPAH patients. RT-PCR performed using custom designed primers further strengthened the validity of both the HEK-293-IP cell line and the C1 antibody as IP receptor specific tools by again highlighting the presence of the functional receptor only in HEK-293-IP cells. Currently we are optimising a protocol for use of the C1 antibody in Western Blotting. Preliminary blots are promising and the antibody appears as expected to pick up a band at around 60 kDa in HEK-293-IP cells but only a very weak band in HEK-293-Zeo cells. With these promising results, in collaboration with Dr. Sue Hall (Institute of Child

Health, UCL, London), the antibody is being used to further investigate the expression of the IP receptor in PASMC from control and IPAH patients.

The present study is one of the first to utilise the newly developed IP receptor antagonist RO1138452 (IPRA) (Roche, Palo Alto) and we show that it is able to fully reverse the anti-proliferative and cAMP-elevating effects of treprostinil in HEK-293-IP cells. The investigators responsible for developing this agent as an analgesic have demonstrated that it is highly selective for the IP receptor over other prostanoid receptor families. This antagonist has in fact been shown to have a low binding affinity for EP_1, EP_3, EP_4 and TP receptors (Bley *et al.,* 2006) and in various functional systems has been shown not to block DP_1, EP_2, EP_3, EP_4 and TP receptors (Jones *et al.,* 2006). For example, at a dose of 1 μM it does not affect PGD_2-dependent inhibition of platelet aggregation, PGE_2-induced relaxation of rabbit mesenteric artery, or relaxation of guinea pig trachea induced by the EP_2 receptor agonist butaprost (Jones *et al.,* 2006). This potent antagonist is therefore an extremely useful tool with which to selectively block the IP receptor pharmacologically, a feat which would otherwise necessitate using siRNA or an IP receptor knockout cell line.

6.2 What can we learn from the IP receptor HEK-293 stable line?

Results obtained with the HEK-293-IP cell line (see chapter 3) have shown that the physical presence of the IP receptor is sufficient to slow down cell proliferation but that this response is unlikely to depend on endogenous PGI_2 generation. The presence of the receptor also alters the proliferative response to cAMP elevating agents, such as forskolin and IBMX as well as PPARγ and PPARδ agonists. In addition it is crucial in mediating the anti-

proliferative effects of PGI_2 analogues. The latter observation was demonstrated both by the lack of effect in HEK-293-Zeo control cells as well as by the reversal of the anti-proliferative effects of treprostinil with IPRA. The IP receptor is therefore crucial in regulating the proliferative responses to a wide range of stimuli even in the absence of receptor stimulation.

Using an adenylyl cyclase inhibitor and two structurally dissimilar inhibitors of PKA, it was established that the anti-proliferative effects of treprostinil were mostly mediated by the cAMP-PKA mechanism in accordance with the classical G_s-coupling paradigm of the IP receptor. However, full reversal could not be achieved with this set of antagonists, possibly indicating the presence of additional IP receptor-dependent but cAMP/PKA-independent mechanisms accounting for approximately 25% of total treprostinil effects; effects which may be mediated by PPARγ. In conclusion, it is becoming more apparent that cAMP-independent effects play an important role in the response to PGI_2 analogues.

The main advantage of using a HEK-293 stable line to dissect IP receptor-dependent mechanisms was the fact that we were able to study the receptor in relative isolation from prostanoid and other types of receptors. However a frequent critique of using this approach, is that HEK-293 cells may lack many other cellular components including ion channels and scaffold proteins (Thomas and Smart, 2005), potentially altering the pathway/mechanism to one which may not be relevant in native cells. However PGI_2 analogues exhibited a very similar mode of action in HEK-293-IP cells and normal, control PASMC, highlighting the validity of using this expression system as a tool in this investigation. Expressing the IP

receptor in HEK-293 cells altered their proliferative rate to one closely emulating that presented in control PASMC, which appeared to express similar amounts of IP receptor message, immuno-staining and protein levels.

Like HEK-293 cells, IPRA was able to reverse treprostinil-induced proliferation inhibition while DDA was only able to reverse the effects by approximately 75%, a very similar split between cAMP-dependent and cAMP-independent effects to that observed in HEK-293-IP cells. This 25% magnitude of IP receptor-dependent but cAMP-independent mechanism(s) mediating the anti-proliferative properties of PGI_2 analogues is reflected in other studies in native VSMC (Wharton *et al.,* 2000; Clapp *et al.*, 2002; Phillips *et al.*, 2005). Differences in the proliferative response to treprostinil between HEK-293-IP and control PASMC were similar in terms of the fold increase in treprostinil-induced cAMP-generation. Inhibition of proliferation by treprostinil was markedly less pronounced in IPAH PASMCs, possibly because the IP receptor was over-expressed in HEK-293-IP cells, resulting in a greater-than-physiological amount of receptor at the membrane leading to an amplified signal upon ligand stimulation. However, the pattern of C1 antibody staining as well as relative mRNA expression appeared to be very similar between the two cell types. It is not unreasonable therefore to suggest that in this case the reduced level of sophistication of HEK-293 cells may have a role in masking some of the mitogenic processes which may still be occurring in PASMC.

6.3 IP receptor dependent activation of PPARγ

One of our initial hypotheses was that PGI_2 analogues could activate PPARγ. This thesis describes for the first time a novel and unexpected IP receptor-dependent activation of PPARγ by PGI_2 analogues, which appears to be distinct from the known ligand binding effects of these analogues to the other two PPAR isoforms (Hertz *et al.*, 1996; Forman *et al.*, 1997). Using a luciferase-based reporter gene assay, we have shown that the PGI_2 analogues treprostinil, carbacyclin and cicaprost can activate PPARγ only in HEK-293 cells that express the receptor. Furthermore, this activation was fully reversed by the IP receptor antagonist. This regulation may previously have been missed due to PPAR function often being studied in cells not expressing the receptor.

Surprisingly, pharmacological inhibition of adenylyl cyclase or PKA was unable to reverse treprostinil-induced PPARγ activation, although it remains to be determined whether the concentration at which antagonists were used was sufficient to completely block target function. Moreover, treatment of both HEK-293-IP and control PASMC with a PPARγ antagonist reversed the anti-proliferative effects of treprostinil by an amount not dissimilar to that accounted for by potential cAMP/PKA-independent mechanisms as discussed previously. Taken together these results indicate that PPARγ does indeed have a role in mediating the anti-proliferative effects of treprostinil but that it is not downstream of cAMP or PKA and is therefore potentially regulating the non-cAMP effects of this agent observed in both HEK-293-IP and control PASMC. There are a number of limitations associated with the protocol used in this project to investigate PPARγ activation. The expression construct

used in the luciferase assays contains only the LBD portion of PPARγ hooked up to the GAL4 DBD, which upon stimulation will bind to the GAL4 response element on the reporter construct and lead to transcription of the luciferase gene (Figure 2.8). This is an important consideration to take into account when planning future experiments using a reporter gene assay to study this agent. The advantage of this system is that it is totally specific for PPARγ rather than relying on non-isoform specific activation of a PPRE-luciferase construct. However, the fact that it does not represent the full length of the protein may mask some effects, especially the ligand independent effects we are interested in unravelling, such as phosphorylation mechanisms of other regions of the protein. In addition it does not take into account endogenous levels of PPARγ as only the stimulation of transiently transfected expression vector will be picked up by this assay. Optimisation of this luciferase assay protocol was lengthy due to the fact that treprostinil appears to have non-specific effects on multiple promoters present in many commercially available construct plasmids.

The mechanism of PPARγ activation beyond IP receptor activation is still unclear and will require extensive further investigation both in native cells and in the HEK-293 cell model. Data obtained with various pharmacological tools ruled out the involvement of cAMP, PKA, PKC and G_i although we still hypothesise that the mechanism of IP-receptor-dependent PPARγ activation is primarily driven by a phosphorylation event, as two relatively broad spectrum kinase inhibitors, H-89 and staurosporine could reverse the PPARγ activating effects at a non PKA/PKC-specific concentration of 10 μM.

A critique of looking at PPARγ activation in HEK-293 cells is that while it is an efficient experimental system, as discussed previously, it is also an artificial system. Ultimately we would like to be able to replicate the PPARγ activation results in native PASMC by optimising a method of transfecting these cells. I have attempted this a number of times using the nucleofector™ method of transfection (Amaxa, Cologne, Germany), but I have not yet been able to get more than a 30-40% transfection rate, which is not a high enough efficiency for the assay to give strong and reproducible luciferase readings in a 96-well-plate format. If transfection efficiency is the rate limiting step, the solution may lie in using more cells by scaling up to a 24- or even a 12-well-plate; this remains to be tested in future studies.

In collaboration with Dr. Vidya Mohammed-Ali at UCL, we are investigating whether this IP receptor-dependent mechanism of PPARγ activation is present in pre-adipocytes and is of relevance in promoting the process of adipogenesis. The rationale behind studying this mechanism in these cells is that they have been shown to abundantly express PGIS (Negrel and Ailhaud, 1981), PPARγ and the IP receptor (Borglum *et al.,* 1999). In addition both prostacyclin and carbacyclin have been shown to promote their terminal differentiation into adipocytes (Catalioto *et al.,* 1991; Aubert *et al.,* 1996). It is hypothesised that this response depends in part on the presence of the IP receptor (Wise, 2003) and subsequent upregulation of C/EBPs, possibly via a CREB dependent mechanism (Aubert *et al.*, 2000), in addition to direct ligand binding to PPARs, including PPARγ (Massiera *et al.*, 2003). Whether PGI_2 analogues can cause adipogenesis via PPARγ as described by our novel IP receptor-dependent pathway therefore warrants further investigation.

PPARγ-dependent mechanisms mediating proliferation inhibition necessitate further study. A hypothesis we would like to test is whether stimulation of the IP receptor by PGI_2 may activate PPARγ to down-regulate endothelin-1 thus decreasing proliferation. Endothelin-1 production is known to be increased in HPASMC by the mitogenic stimuli present in serum and cicaprost has been shown to inhibit the release of this vasoconstrictor in these cells (Wort *et al.,* 2001). Further evidence to support this hypothesis comes from the fact that rosiglitazone can abrogate the increase in endogenous production of endothelin-1 in the mesenteric vasculature of hypertensive deoxy-corticosterone acetate (DOCA)-salt rats, which overexpress ET-1, and can prevent hypertrophic vascular remodelling and progression of hypertension (Iglarz *et al.,* 2003). To test this hypothesis the production of ET-1 could be measured before and after PGI_2 analogue treatment in HEK-293-IP cells and compared with HEK-293-Zeo cells. If production of ET-1 was indeed inhibited after PGI_2 analogue treatment in the presence of the IP receptor then the role of PPARγ could be tested using the antagonist GW9662.

6.4 Altered PGI_2 analogue signalling mechanism in IPAH cells

While PPARγ could account for some of treprostinil's anti-proliferative effects in HEK-293-IP and control PASMC, its role in IPAH PASMC appeared more pronounced. We found that IPAH PASMC had a reduced expression of the IP receptor and a faster replication rate, akin to the difference between HEK-293-IP and HEK-293-Zeo. Interestingly and importantly the analogues could still significantly inhibit proliferation of these cells although they could not restore the replication rate of IPAH cells back to that observed in normal

control cells; indicating that treatment with PGI_2 analogues alone is possibly not sufficient to reverse or impede medial thickening occurring in this disease. PGI_2 analogue-induced inhibition was not dependent on the IP receptor and did not correlate with the analogue induced increase in cAMP *via* the IP receptor. On the other hand, the PPARγ antagonist was able to reverse the majority of treprostinil effects suggesting that in IPAH PASMC, a switch in the signalling mechanism occurs from a classical IP receptor and cAMP-driven pathway to a PPARγ-dependent but IP receptor and cAMP independent mechanism.

As discussed in chapters 4 and 5, we have demonstrated an IP receptor dependent mechanism of PPARγ activation by PGI_2 analogues in our HEK-293-IP cell model and we may still be observing a similar receptor-mediated mechanism in IPAH cells. The most probable candidate for this role is another prostanoid receptor, the most likely being the EP_3 receptor given its known affinity for PGI_2 analogues as well as its ability to couple to multiple G proteins (Sugimoto *et al.,* 1992; Irie *et al.*, 1993; Namba *et al.*, 1993) and its known expression in the pulmonary artery (Jones *et al.*, 1997). In future work we would like to test the involvement of the EP_3 receptor in mediating the anti-proliferative properties of treprostinil in IPAH cells. This hypothesis could be tested by blocking this receptor with the novel highly specific antagonist L-798,106 (Merck Frost, Quebec, Canada). In addition one could generate HEK-293 cells stably expressing other prostanoid receptors and perform luciferase-based reporter assays, and upon optimisation of the same assay in PASMC, the same experiments could be performed in both control and IPAH PASMC.

There are a number of limitations related to our investigation of IPAH PASMC. Because lung transplantation is a rare occurrence we were only able to obtain PASMCs from 3 children. It is obvious that results would be vastly strengthened with an increased number of isolates to test the concepts explored in this thesis. Furthermore we studied the PASMCs from children with IPAH and experimental data should ideally have been compared with data obtained from the PASMCs of children with a normal pulmonary vasculature.

6.5 Significance of loss or down-regulation of the IP receptor

Loss or down-regulation of the IP receptor appears to modulate growth and alters the response to cAMP-elevating agents. Currently we have very preliminary data (n=1) from a proteomics based screening assay (Kinetworks™, Kinexus, Vancouver, Canada) to establish semi-quantitatively if there are differences in cell-cycle protein expression as a consequence of the presence of the IP receptor. Of note is the up-regulation of both the tumour-suppressor p53 and the cyclin dependent kinase inhibitor $p27^{Kip1}$ in HEK-293-IP versus HEK-293-Zeo, both proteins are usually up-regulated during cell-cycle arrest. Although still premature and in need of confirmation, these results add some credence to the importance of this receptor in terms of growth modulation.

Loss of the IP receptor does not cause pulmonary hypertension *per se* as demonstrated in IP receptor knockout mice. It does, however, hasten the development of pulmonary hypertension under hypoxic conditions (Hoshikawa *et al.,* 2001). In addition IP receptor mRNA expression is reduced in spontaneously hypertensive rats (SHR) compared to control

Wistar-Kyoto rats (WKY). Specifically it was suppressed at the pre-hypertensive state (Numaguchi *et al.*, 1999). Whether an early loss or down-regulation of the IP receptor contributes to increased cell proliferation in the pre-symptomatic patient leading to medial thickening of the pulmonary arteries, or conversely is a consequence of other initiating factors, remains to be determined.

Loss or down regulation of the IP receptor could lead to a mere loss of receptor function, but could lead to upregulation/downregulation of other mechanisms, some of which may have deleterious rather than having beneficial compensatory effects. Recently, the importance of the physical presence of the IP receptor to limit the deleterious effects of the thromboxane (TP) receptor was demonstrated both *in vitro* and *in vivo*. Deletion of the IP receptor exacerbated TP receptor-dependent smooth muscle cell proliferation following arterial injury in mice (Cheng *et al.*, 2002). In contrast, the presence of the IP receptor significantly enhanced cAMP generation in response to TPα receptor isoform activation in both transfected HEK-293 cells and aortic smooth muscle cells (Wilson *et al.*, 2004). It would appear that the presence of the IP receptor facilitates this effect *via* the formation of an IP/TPα heterodimer rather than by activating the cAMP-PKA cascade (Wilson *et al.*, 2004). The presence of the IP receptor therefore has a specific role in promoting a "PGI_2-like" cellular response to TXA_2 and the isoprostanes. Thus it is possible that in IPAH, where the IP receptor appears to be down-regulated, PASMCs could be more responsive to the pro-proliferative responses mediated by TP receptors, thereby increasing the replication rate.

Lack of the IP receptor may provide an additional pro-proliferative pathway in the form of direct PPARδ activation. PGI_2 analogues can bind to and activate PPARδ (Hertz *et al.,* 1996; Forman *et al.,* 1997). Although the role of this nuclear isoform in controlling cell proliferation is the subject of much debate, PPARδ has been reproducibly identified as a biological target for PGI_2 during angiogenesis, a process requiring smooth muscle cell proliferation (Lim *et al.,* 1999; Lim and Dey, 2000; Pola *et al.,* 2004). It may be possible therefore that loss of the IP receptor may enhance this angiogenic pathway for PGI_2 analogues.

An important consideration in the present study is that IPAH PASMC were derived from patients who had previously undergone long-term epoprostenol treatment. The observed down-regulation of the IP receptor therefore may not be associated with the disease itself but rather a consequence of desensitisation caused by long-term agonist stimulation. It is known that internalisation and reduced GPCR expression can occur following long-term agonist stimulation (reviewed in Bohm *et al.,* 1997). In future studies we would like to test whether the reduced expression of the IP receptor in IPAH cells is a result of long-term treatment and whether this course of events could contribute to the switch in the signalling pathway which we have demonstrated. A model of long-term treatment might be established in an *in vivo* model of pulmonary hypertension and PASMC from these animals could be compared in terms of IP receptor expression and PGI_2 signalling mechanisms. It is however notoriously difficult, if not impossible to produce an animal model of human obliterative pulmonary vascular disease. The role of the IP receptor could be further investigated by knocking down the IP receptor (using siRNA) in normal PASMC, to test

whether this would lead to the same switch in mechanism and increase in cell proliferation that was observed in IPAH cells. Conversely we could over-express functional IP receptor in IPAH PASMC and test whether this could rescue some of the "normal" characteristics observed in the control PASMC we studied.

6.6 Conclusion

To conclude, this thesis has developed novel tools for the study of the prostacyclin receptor and has established that this receptor is important for the control of cell proliferation and the response to cAMP-elevating agents. In "normal" cells, IP receptor stimulation by PGI_2 analogues leads to inhibition of cell proliferation *via* a predicted elevation of cAMP although a fraction of the anti-proliferative effects of PGI_2 analogues appear to be mediated by PPARγ in a potentially PKA-independent manner. In IPAH cells a downregulation of the IP receptor was observed in Dr. Sue Hall's western blot, but PGI_2 analogues are still efficacious at inhibiting cell proliferation. However, the regulation of growth control switches to an IP receptor- and cAMP-independent pathway and PPARγ becomes an important mediator, further establishing the importance of PPARs as regulators of cell proliferation. A limitation of the present thesis, which will need to be addressed in future studies, is the absence of data on the contribution of apoptosis and cell death to the effects of IP-receptor and PPARγ activation.

Further work will be necessary to elucidate the receptor and signalling pathways involved in regulating the anti-proliferative effects of PGI_2 analogues in IPAH cells, remembering that the replication rate still remains significantly greater than normal. The data obtained in the present studies,

particularly those showing PGI_2 analogue dependent activation of PPARγ in a reporter gene assay justifies exploring PPARγ as a potential therapeutic target for the treatment of IPAH, probably in combination with existing therapies including prostacyclin analogues, endothelin receptor antagonists and phosphodiesterase inhibitors.

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