Identification of Candidate G protein–coupled receptors: Role of purinergic receptor P2Y2 in Pulmonary Arterial Hypertension

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The family is one of nature's masterpieces.

Dedicated to

My parent: Viswanathan Kumarasamy and Malathi Viswanathan

My Grandmother: Saradhambal

who always picked me up on time and encouraged me to go on every adventure, especially this one.

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1 INTRODUCTION

1.1 Pulmonary arterial hypertension (PAH)

Pulmonary arterial hypertension is characterized by increased pulmonary vascular resistance (PVR) resulting in elevated pulmonary artery pressure (PAP) and leading to right-heart failure, and ultimately death if untreated [1, 2]. Pulmonary arterial hypertension is no longer an orphan disease [3], and 4 classes of drugs have been developed in the last 15 years [4, 5]. Breathlessness, fatigue, weakness, angina, and syncope are the symptoms of PAH [6]. The pathogenesis of PAH is characterized by pulmonary vasoconstriction, abnormal pulmonary vascular remodeling, and in-situ thrombosis [7].

1.1.1 Definition of PAH

Pulmonary arterial hypertension is defined by a mean PAP (mPAP) ≥ 25 mmHg at rest with a PVR > 3 wood units and an end-expiratory pulmonary artery wedge pressure (PAWP) ≤ 15 mmHg at the time of right-heart catheterization [8].

1.1.2 Idiopathic PAH (IPAH)

The emergence of PAH without a known risk factor or family context is known as IPAH. For patients with this sporadic disease, diagnosis is usually late due to the lack of a reliable screening program [9-11].

Idiopathic PAH is a progressive, nearly fatal condition with very few treatment options. Deregulated proliferation of pulmonary intimal endothelial cells and pulmonary arterial smooth muscle cells (PASMCs) results in progressive pulmonary vascular remodeling and subsequently elevated PAP during the IPAH progression. Specific targeted therapies have been developed using prostacyclin, endothelin-receptor antagonists, and phosphodiesterase 5 inhibitors. However, scientists are exploring new therapeutic modalities because of the insufficient efficacy and poor tolerability of these agents [12].

1.1.3 Epidemiology

Accurate diagnosis of PAH is difficult, and access to care is limited in many countries, which makes the global prevalence of PAH challenging to estimate. The global burden of PAH is likely greater than currently recognized, because risk factors, such as HIV, schistosomiasis, and sickle cell disease, are more prevalent in the developing world [13]. In developed countries, PAH cases will also likely increase as newer associations with PAH emerge, and

these include dialysis [14] and metabolic syndrome [15]. In addition, broad access to echocardiography results in earlier PAH diagnoses [16].

The epidemiology of PAH has been described by several registries [17-19]. In Europe, PAH occurs in 15 to 60 subjects per million of the population, resulting in 5 to 10 cases per million each year [18]. In sum, IPAH, heritable PAH, and drug-induced PAH comprise approximately half of registered PAH patients [17].

1.1.4 Updated clinical classification of PH

The first classification of pulmonary hypertension (PH) was proposed in 1973 [20]. A clinical taxonomy was established at the second World Symposium on PH (Evian, 1998) to categorize different classes of PH sharing similar pathological findings, hemodynamic characteristics, and management approaches (2).

There are five categories of PH: (1) PAH, (2) PH related to left-heart disease, (3) PH due to lung disease/hypoxia, (4) thromboembolic PH, and (5) miscellaneous variants. In the consecutive meetings of that global body, a series of changes were carried out. However, the general architecture and the philosophy of the clinical classification remained unchanged. According to new data published in recent years, certain modifications and updates, especially for the first type of PH, were proposed during the Fifth World Symposium in 2013 (Table 1) [16, 21].

Table 1. Updated classification of PH (Simonneau, 2013) [22]

1. PAH 1.1. Idiopathic PAH 1.2. Heritable PAH 1.2.1. Bone morphogenetic protein receptor type II (BMPR2) 1.2.2.Activin receptor-like kinase (ALK-1), Endoglin (ENG), SMAD Family Member 9 (SMAD9), Caveolin-1 (CAV1), Potassium Two Pore Domain Channel Subfamily K Member 3 (KCNK3) 1.2.3. Unknown 1.3. Drug- and toxin-induced 1.4. Associated with: 1.4.1. Connective tissue disease 1.4.2. HIV infection 1.4.3. Portal hypertension 1.4.4. Congenital heart diseases 1.4.5. Schistosomiasis **1′. Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis** **1″. Persistent PH of the newborn (PPHN)**
2. Pulmonary hypertension due to left-heart disease 2.1. Left ventricular systolic dysfunction 2.2. Left ventricular diastolic dysfunction 2.3. Valvular disease 2.4. Congenital/acquired left-heart inflow/outflow tract obstruction and congenital cardiomyopathies
3. PH due to lung disease and/or hypoxia 3.1. Chronic obstructive pulmonary disease 3.2. Interstitial lung disease 3.3. Other pulmonary diseases with mixed restrictive and obstructive pattern 3.4. Sleep-disordered breathing 3.5. Alveolar hypoventilation disorders 3.6. Chronic exposure to high altitude 3.7. Developmental lung diseases
4. **Chronic thromboembolic pulmonary hypertension (CTEPH)**
5. Pulmonary hypertension with unclear multifactorial mechanisms 5.1. Hematologic disorders: chronic hemolytic anemia, myeloproliferative disorders, splenectomy 5.2. Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis 5.3. Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders 5.4. Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure, segmental PH

1.1.5 Histology of PAH

Intimal hyperplasia, medial hypertrophy, occlusion of small arteries, in-situ thrombosis, adventitial proliferation or fibrosis, infiltration of inflammatory or progenitor cells, and angio-proliferative “plexiform” lesions are the histological features of PAH (Figure 1). In PAH, the distribution and prevalence of those histological abnormalities are heterogeneous within the lung. Of note, plexiform lesions, which are often located downstream from occluded arteries,

are only found in PAH, but not in other PH classes. Plexiform lesions express the transcription factors and growth factors (e.g., vascular endothelial growth factor [VEGF] and hypoxia inducible factor [HIF-1α]) typically seen in angiogenesis [23].

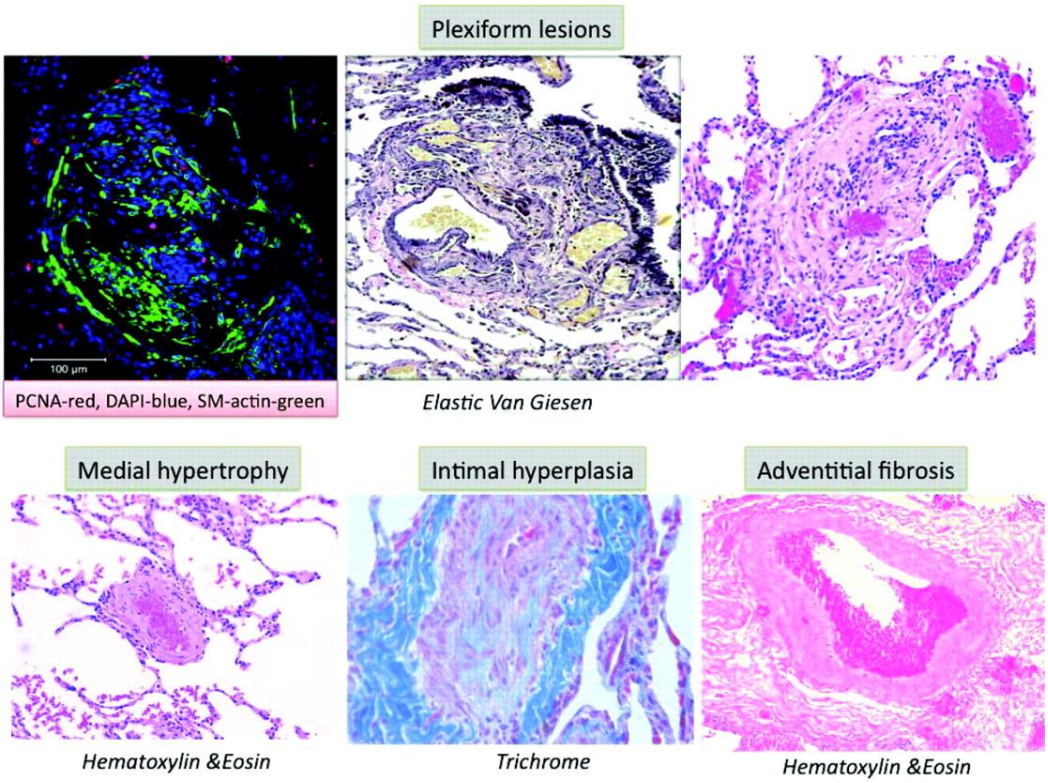


Figure 1. PAH histology. Plexiform lesions on the upper row. Evidence of cell proliferation in upper left-hand corner (proliferating cell nuclear antigen [PCNA] in red, smooth muscle [SM] actin in green, 4',6-diamidino-2-phenylindole (DAPI) nucleus staining in blue. Medial hypertrophy, intimal fibrosis, and adventitial proliferation on the bottom row (Archer, 2010) [24].

1.1.6 Pathogenesis and pathophysiology of PAH

The unexplained proliferation, migration, and survival of pulmonary vascular cells (i.e., smooth muscle cells (SMCs), myofibroblasts, and endothelial cells) within the pulmonary arterial wall implicates the complex nature of structural and functional changes in the pulmonary vasculature. Important discoveries have been made regarding factors that contribute to disease, such as inflammation, pulmonary endothelial dysfunction, aberrant cell proliferation in the vascular wall, and several gene mutations. Nonetheless, the causal patho-mechanisms of pulmonary vascular remodeling are still unclear [2, 6, 25].

The novel, cancer-like concept for PAH has emerged in recent years and has its roots in intriguing in-situ and in-vitro observations [26, 27]. More specifically, compared with endothelial cells found in the lungs of patients with congenital heart malformations, a

monoclonal expansion of endothelial cells has been found in IPAH [28]. In IPAH, there is evidence pointing towards the instability of short DNA microsatellite sequences within plexiform lesions [29]. Somatic chromosome abnormalities have been reported in the lungs of PAH patients, and in cultured cells from those individuals [30]. Pulmonary endothelial cells and vascular SMCs derived from PAH patients maintain their abnormal hyper-proliferative, apoptosis-resistant phenotype for a longer time than control cells when removed from their in-vivo environment [31-33]. More recently, pulmonary vascular cells derived from PAH patients exhibited an altered energy metabolism in situ and in vitro [34-39]. However, there crucial differences between PAH pathogenesis and carcinogenesis (Figure 2) [40, 41]. In PAH, neither invasion nor metastasis has been observed. Inspired by the cancer-like concept, researchers recently tested certain anti-proliferative and/or oncological drugs, typically used in the treatment of cancer, in PAH patients [42-46]. Tyrosine kinase inhibitors, such as imatinib, have been demonstrated to have potential benefits in PAH patients, but severe side effects raise many concerns regarding their use in clinical practice [48, 49]. Therefore, the nature of the similarities and differences between PAH pathogenesis and carcinogenesis needs further clarification [7].

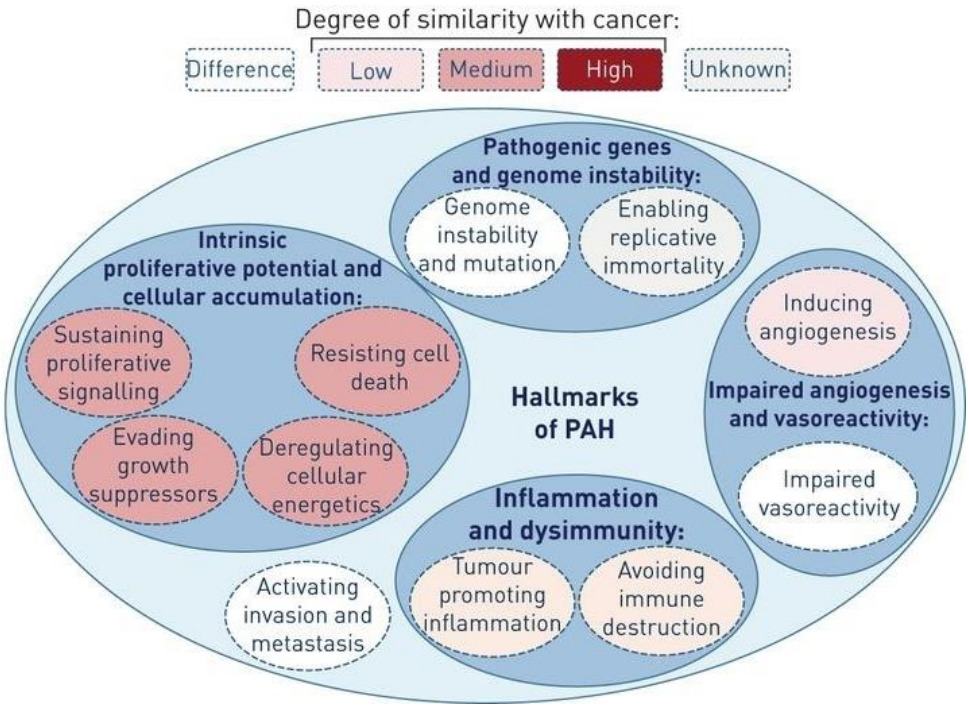


Figure 2. Degree of similarity between PAH and cancer. Pulmonary arterial hypertension shares several cancer characteristics, although the degree of similarity varies (Guignabert, 2013) [7].

1.1.7 Imbalance of vascular effectors

1.1.7.1 Prostacyclin and thromboxane A2

While levels of the potent vasodilator prostacyclin are reduced in PAH patients, thromboxane A2, a well-known vasoconstrictor, is elevated [47]. Prostacyclin also exhibits anti-proliferative effects on PASMCs [48]. In addition, less prostacyclin synthase was found in the small and medium-sized pulmonary arteries of IPAH patients than in healthy individuals [49].

1.1.7.2 Endothelin-1

Endothelin-1 is a key player in PAH, and it acts as a vasoconstrictor and as a mitogen [50, 51]. In PAH patients, plasma levels of endothelin-1 are significantly increased [52-54], and hemodynamic changes are directly influenced by this vascular effector.

1.1.7.3 Nitric oxide

Nitric oxide, synthesized by nitric oxide synthases (NOSs), inhibits vascular SMC proliferation and induces acute vasodilation. Nitric oxide deficiency is recognized because of decreased levels of the endothelial isoform of NOS in the pulmonary vasculature of PAH patients [55-57].

1.1.7.4 Serotonin

Serotonin (5-hydroxytryptamine [5-HT]) promotes SMC hyperplasia and vasoconstriction [58]. In IPAH, elevated plasma levels of serotonin have been reported, potentially due to upregulation of 5-HT transporters and 5-HT receptors in the pulmonary vasculature [59, 60].

1.1.7.5 Vascular endothelial growth factor (VEGF)

The production of VEGF and its receptors increases in lungs exposed to hypoxia [61]. In PAH, the main cause of plexiform lesions is disordered angiogenic responses, one of which is an increase in VEGF/VEGF receptor expression followed by activation of hypoxia-inducible factors in the lesion area [62].

1.1.7.6 Platelet-derived growth factor (PDGF)

Platelet-derived growth factor is elevated in PAH patients and has been proven to be a predominant inducer of PASMC proliferation and migration [63]. Moreover, increased PDGF receptor B expression has been observed in remodeled pulmonary arteries, and imatinib, a tyrosine kinase inhibitor [64], demonstrated promising effects in rodent models of PAH by

blocking PDGF signaling. Imatinib-treated PAH patients exhibited a significant decrease in PVR and cardiac output in a phase 2 study [44].

1.1.7.7 Transforming growth factor-beta (TGF-β) superfamily

In heritable PAH, the transforming growth factor-beta receptor family plays an important role [65]. The heritable type of PAH is primarily caused by a mutation of Bone morphogenetic protein receptor type II (BMPR2), which belongs to the TGF-β receptor family [66]. Approximately 45 BMPR2 mutations have been identified [67, 68]. Under normal conditions, BMPR2 and type 1 TGF-β receptor heterodimeric complexes are activated by their ligands (Bone morphogenetic protein (BMP) 2, 4, and 7) to suppress vascular SMC growth. Mutations in the BMPR2 kinase domain lead to dominant negative effects on receptor function [69]. Mutations in another member of the TGF-β receptor family, ALK1, are found in a rare group of patients with hereditary hemorrhagic telangiectasia and IPAH, where they result in Smad-dependent pro-growth signaling [67].

1.1.7.8 Role of cytokines and chemokines

Cytokines and chemokines (soluble cytokines that act as chemoattractant) are significant mediators of inflammation. Inflammatory cells of the innate immune system predominantly produce these types of mediators. The cellular components of the vascular wall or adventitia can also produce cytokines and chemokines [70]. Increased levels of Interleukins (IL-1, IL-6) [71, 72], CC-chemokine ligand 2/ Monocyte chemotactic protein 1 (CCL2/MCP-1), Chemokine C-C motif ligand 5/ Regulated on Activation, Normal T Cell Expressed and Secreted (CCL5/RANTES), and chemokine C-X3-C motif ligand 1 (CX3CL1/fractalkine) have been found in both the serum and tissue of PAH patients [73-75]. In addition, IL1-β, IL-6, and tumor necrosis factor α are predictive of outcome in PAH patients [72, 76].

1.1.8 Environmental and medical factors

1.1.8.1 Hypoxia

Hypoxia induces vasoconstriction in the pulmonary vasculature but stimulates vasodilation in systemic vessels. The acute effect of hypoxia is regulated by endothelin and serotonin derived from endothelial cells. Hypoxia causes dysfunction of ion-channel activity in SMCs [77]. Acute hypoxia leads to PASMC vasoconstriction due to plasma membrane depolarization and the accumulation of cytoplasmic calcium [78]. Chronic hypoxia results in structural remodeling concomitant with the proliferation and migration of vascular cells and extracellular matrix deposition.

1.1.8.2 Anorexigen aminorex fumarate

After the introduction of the anorexigen aminorex fumarate, an epidemic wave of IPAH was noted in Europe during the 1960s [79]. Though the prevalence of PAH rises with the period of use, an increase in pulmonary pressure can occur after 3 to 4 weeks of anorexigen exposure [80, 81].

1.1.8.3 Central nervous system stimulants

The risk of PAH increases with the use of central nervous system stimulants, such as methamphetamine and cocaine [82]. Of 20 heavy users of cocaine, the lungs of 4 individuals displayed medial hypertrophy of the pulmonary arteries, without evidence of foreign-body microembolization [83]. However, it is unclear if the stimulants alone can cause PAH [62].

1.1.9 Therapeutic approaches for PAH

Effective therapies targeting specific aberrant pathways have been developed for PAH, and these rely on an improved understanding of the pathobiological mechanisms of that disease. Agents that target prostacyclin, endothelin, and nitric oxide pathways have been shown to improve functional status and pulmonary hemodynamics, and possibly, to even slow disease progression (Figure 3). Several investigative approaches are in active development, and these focus on other potential pathways that have been proven to contribute to the pathogenesis of PAH [1].

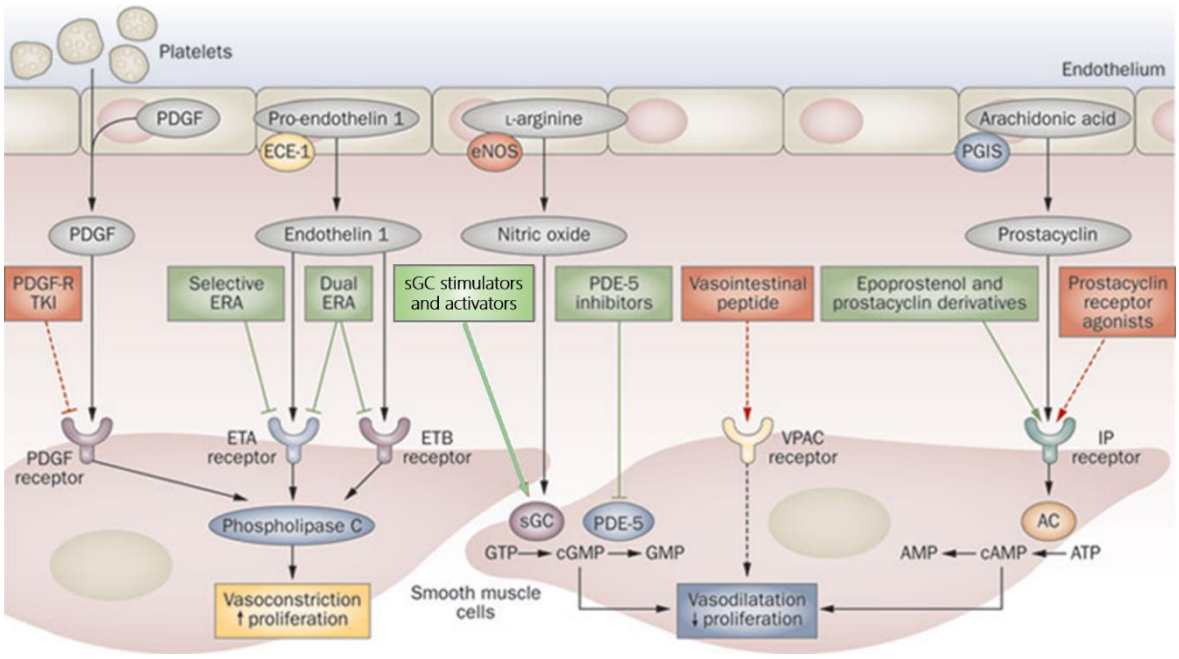


Figure 3. Therapeutic targets in PASMC. Licensed therapeutic agents (green boxes) and investigational (red boxes) treatment approaches for PAH. Arrows represent receptor stimulation, whereas terminated lines indicate receptor blockade. Modified from O'Callaghan (2011) [1].

1.1.9.1 Prostacyclins

Prostacyclin is a potent vasodilator involved in pulmonary and systemic circulation. Prostacyclin analogues in current clinical use include Epoprostenol, Beraprost, Treprostinil, and Iloprost. Depending on the individual half-life and mode of absorption, the route of administration varies depending on the mode of application (e.g., intravenous, subcutaneous, inhalational, or oral) [84].

1.1.9.2 Endothelin-receptor blockers

Endothelin has two receptors Endothelin receptor type A (ETA) and Endothelin receptor type B (ETB). Bosentan was the first oral drug to obtain Food and Drug Administration (FDA) approval for the treatment of PAH. That agent is a non-selective endothelin antagonist blocking both ETA and ETB. Recent studies have found that 1 and 2 year courses of bosentan improved survival rate [85, 86]. Ambrisentan, a relatively selective ETA antagonist, is also available for PAH treatment. Endothelin-receptor antagonists may be hepatotoxic and teratogenic; therefore, liver function tests and pregnancy tests in female patients receive regular monitoring. Macitentan is a dual endothelin-receptor antagonist with a receptor occupancy half-life 15 times greater than that of other endothelin-receptor antagonists. Due to the hydrophobic interaction of macitentan and the ETA receptor, the former's dissociation from the latter is slowed and effectively antagonizes Endothelin 1 (ET-1). Macitentan has also been associated with a minimized risk of liver enzyme changes (bile salt transport) and minimal edema retention [87].

1.1.9.3 Phosphodiesterase inhibitors

Nitric oxide stimulates soluble guanylate cyclase to catalyze guanosine monophosphate (GMP) to cyclic guanosine monophosphate (cGMP). This leads to the inhibition of calcium channels, thereby reducing intracellular calcium levels and ultimately resulting in vasodilation. Phosphodiesterases (PDEs), and especially PDE-5, degrade cGMP, through which the effects of nitic oxide are mediated. Moreover, PDE-5 is highly abundant in the lung and is upregulated in remodeled pulmonary arteries [88]. Hence, PDE-5 inhibition is among the first-line therapies for PAH. Sildenafil, which was initially approved for the treatment of erectile dysfunction, is a highly selective inhibitor of PDE–5. As effectual as inhaled nitric oxide, oral sildenafil has been demonstrated to improve pulmonary hemodynamics [88]. Tadalafil is also a PDE-5 inhibitor with an increased plasma half-life

(17.5 hours) as compared to sildenafil. If sildenafil requires 3 daily doses, then tadalafil requires 1 dose per day. Therefore, tadalafil is more convenient than sildenafil for the patient [89].

1.1.9.4 Combination therapy

Combination therapy is likely to be more effective than monotherapy, since it targets more than one pathway. The combination of sildenafil and endothelin-receptor antagonists (i.e., bosentan), is one of the most commonly used blends [90]. Sildenafil is a phosphodiesterase type 5 inhibitor (PDE5 inhibitor), and bosentan is endothelin-receptor antagonist. Their combination therapy leads to decreased plasma levels of sildenafil in patients. This might be due to the induction of cytochrome P450 3A4 enzyme (CYP3A4), which metabolizes sildenafil. However, the pharmacokinetics of these drugs are different. Sildenafil reaches a peak plasma level in 1.25 hours, whereas bosentan reaches its peak plasma level after 3 hours. The combination treatment of sildenafil and bosentan generated only a slight decrease in diastolic blood pressure compared to the separate application of each [91].

1.1.9.5 Soluble guanylate cyclase stimulator (sGC)

Soluble guanylate cyclase is the receptor for nitric oxide (NO). The binding of NO to sGC leads to the catalysis of guanosine monophosphate (GMP) to cGMP. In turn, cGMP activates protein kinase G and leads to a decrease in intracellular Ca^{2+} levels, resulting in vasodilation [92]. Riociguat is the only sGC stimulator that treats CTEPH and PAH [93]. In-vitro studies have revealed that Riociguat increases the catalytic rate 73 times and that it activates sGC in an NO-independent manner. Moreover, together with nitric oxide, Riociguat produces a 122-fold increase in sGC activity [92].

1.2 G protein-coupled receptors (GPCRs)

One of the largest protein super families in mammals is that of GPCRs. They are located in the cell membrane, and they sense a large variety of inputs and signals from the environment. Based on structural similarities, approximately 1,000 GPCRs are predicted in the human genome. Of these, 450 are non-olfactory receptors. Based on their structural and functional similarities, GPCRs can be grouped into six classes:

1) Rhodopsin (class A),
2) Secretin (class B),
3) Glutamate (class C),
4) Fungus pheromone (class D),

5) Cyclic adenosine monophosphate (cAMP) (class E),
6) Frizzled/smoothened (class F).

For many GPCRs, the natural ligand has not been identified, and these are called *orphan receptors*. In the pharmaceutical industry, GPCRs are the most investigated targets [98-100], since GPCR dysfunction plays role in multiple diseases, including neurological disorders, cancer, autoimmune diseases, and diabetes. Furthermore, GPCRs have a wide impact on numerous cellular processes, such as cell-cell communication, cell proliferation, and gene expression [47, 94-96]. In addition, recent studies have proposed that they regulate actin and contribute to other cell processes, such as migration, cell division, and membrane trafficking, via both traditional G protein signaling and non-canonical pathways [94, 97-99].

1.2.1 Canonical signaling of GPCRs

Also known as 7-transmembrane (TM) receptors, GPCRs have seven cross-membrane structures. These GPCRs are coupled to heterotrimeric G proteins, which are located on the cytoplasmic side of the plasma membrane (Figure 4). The G proteins are composed of Gα, Gβ, and Gγ subunits. When activated by their ligands, GPCRs undergo conformational changes and trigger guanosine diphosphate (GDP) to guanosine triphosphate (GTP) exchange in Gα, which is then activated and becomes disassociated from Gβγ [100]. In addition, GTPase can hydrolyze GTP into GDP, which results in a reversible process with Gα re-associated with Gβγ. When disassociated from its complex, Gα can trigger numerous signaling cascades via several effectors, such as adenylyl cyclase, protein kinase C (PKC),and Rho GTPases [101, 102]. Upon activation, those effectors produce cAMP; calcium; GMP; inositol-1,4,5-triphosphate (IP3); diacyl glycerol (DAG); and arachidonic acid. These group of cytoplasmic molecules are termed as *second messengers*. Receiving signals from the second messengers, downstream enzymes, intracellular receptors, and transcription factors ultimately control gene expression [103-105].

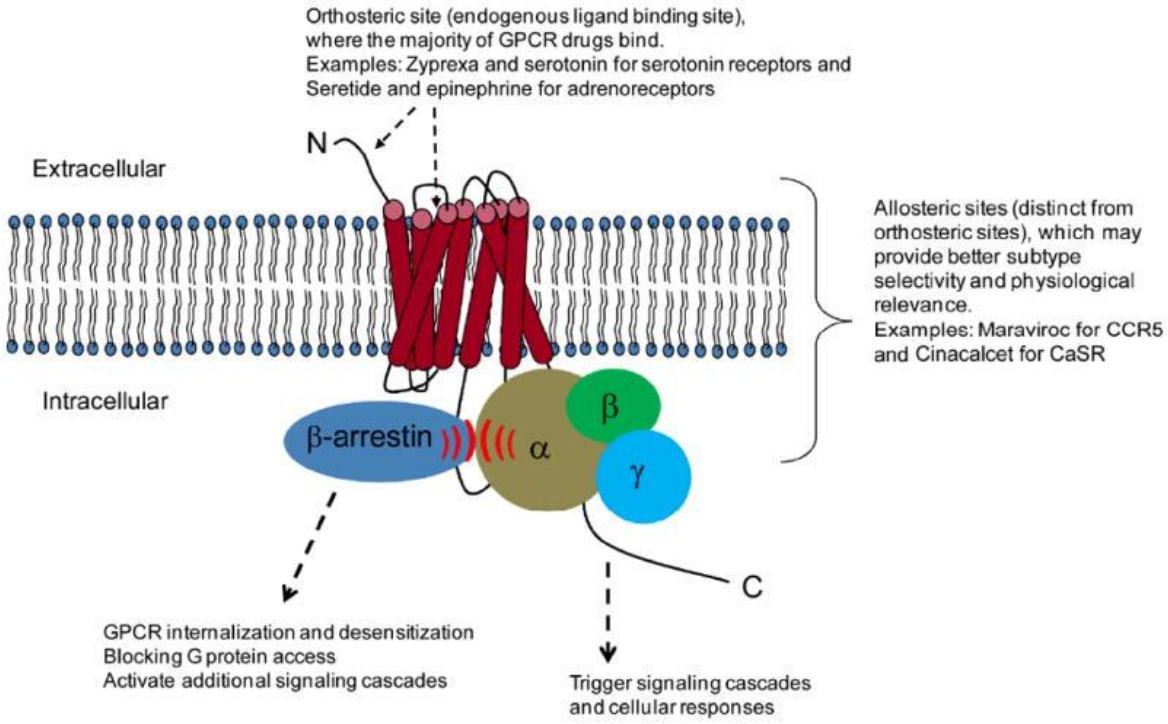


Figure 4. GPCR and possible ligand-binding sites. Sites at the outer surface of the receptor, and occasionally in the extracellular N-terminus region, are usually orthosteric sites. Sites located in any region of the receptor are allosteric sites and are distinct from the orthosteric ones. Finally, GPCRs demonstrate differential signaling behaviors when bound to G proteins vs arrestins (Zhang, 2013) [106].

There are 23 Gα, 5 Gβ, and 12 Gγ subunits in humans, as well as 37 regulators of G protein signaling (RGS) proteins [107]. Based on their functions, Gα proteins are classified into four categories: Gαs (stimulation), Gαi (inhibition), Gαq/Gα11, and Gα12/13. Each type signals through different pathways. In addition to the traditional mode of invoking signaling in the cytoplasm upon disassociation from Gβγ, Gαi can translocate into the nucleus in a complex with Gβγ and bind to chromatin [108], thereby regulating mitosis but not DNA synthesis [109]. Via their respective Gα proteins, GPCRs activate or inhibit several downstream effectors [110]:

1) Gαs activates adenylate cyclase and increases cAMP
2) Gαq/11 activates PLC and increases intracellular Ca2+ levels
3) Gαi/o inhibits adenylate cyclase
4) Gα12/13 and Gαq/11 activate Ras homolog gene family, member A (RhoA) GTPase
5) Gβγ activates mitogen-activated protein kinase (ERK)

1.2.2 GPCRs and effectors in membrane trafficking

While GPCRs are mostly localized to the plasma membrane, they also enter the cytoplasm through endocytosis, a process called internalization. The GPCR internalization route is mainly clathrin-dependent [111], and internalized GPCRs are either degraded in lysosome for permanent inactivation of such receptors or recycled back through recycling endosomes to the cell membrane for reactivation (Figure 5).

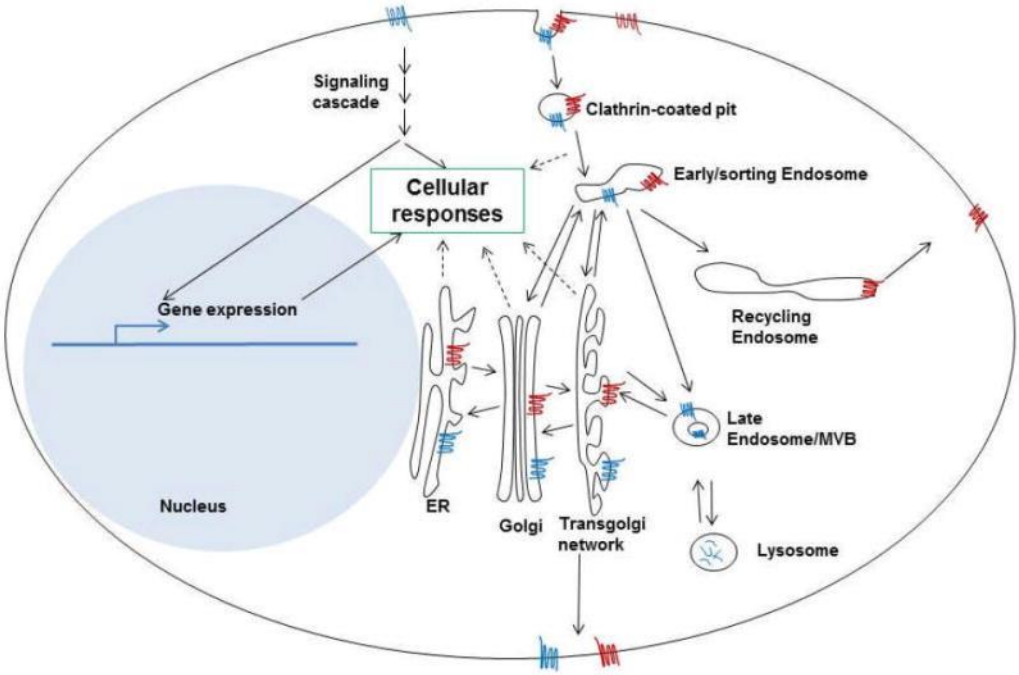


Figure 5. GPCR localizations and trafficking pathways in interphase cells. Two different GPCRs (blue and red) illustrate the different membrane trafficking pathways. The blue GPCR goes through the lysosomal degradation pathway, and the red GPCR moves through the recycling pathway. The two pathways can coexist for a given GPCR. Cells' responses to GPCRs can happen through external ligand-induced signaling cascades, as well as through internalized GPCRs (Zhang, 2013) [106].

1.2.3 Non-canonical signaling of GPCRs

The G protein-coupled receptors are expected to be more complex and diverse than predicted. For example, some GPCRs do not need all seven transmembrane domains. In addition, the same receptor can be activated by different agonists and cause multiple cellular responses [112, 113]. Furthermore, different agonist concentrations can result in distinct GPCR-mediated cellular responses [114]. At the same time, GPCR-based functional studies and drug designs are highly complex, because many GPCRs exist as dimers or oligomers. For G protein coupling, some GPCRs requires dimerization or oligomerization, which makes GPCR oligomerization an emerging area of research in need

of further investigation [115]. A few studies have indicated that odorant GPCRs are also expressed in non-olfactory organs, cells, and tissues. In humans and chimpanzees, many olfactory receptors are expressed in liver, heart, testis, lung, myocardial, and erythroid cells, as well as in the spleen, brainstem, colon, and prostate [116]. The potential functions of olfactory receptors in non-olfactory tissues remain largely unexplored. In light of the complex examples mentioned above, it is critical to evaluate and compare GPCR expression in different tissues, as well as in divergent cell types, to understand their roles in disease.

Independent of G proteins, GPCRs can also activate signaling cascades, for example, c-Jun N-terminal kinases (JNK), Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3 kinase), alpha serine/threonine-protein kinase (Akt), Ras homolog gene family, member A (RhoA), nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) and mitogen-activated protein kinase (MAPK), via β-arrestins [117, 118]. Upon ligand binding, GPCR kinases (GRKs) phosphorylate GPCRs and recruit β-arrestins. That process blocks G proteins from further interaction with GPCRs and therefore terminates or reduces downstream signaling [119]. Importantly, GPCR desensitization, sequestration, and intracellular trafficking are mediated by β-arrestins so that excessive receptor stimulation is terminated [120].

1.3 GCPRs as a therapeutic target in PAH

Many GPCRs are involved in several aspects of PAH. However, the depth and diversity of GPCR signaling requires nuanced study. In addition, the selectivity on pulmonary vasculature also poses therapeutic challenges, since in PAH, vascular remodeling is restricted to pulmonary circulation, whereas GPCRs are relatively ubiquitously expressed. The below figure illustrates GPCRs, their agonists or antagonists, and related therapeutic options in PAH.

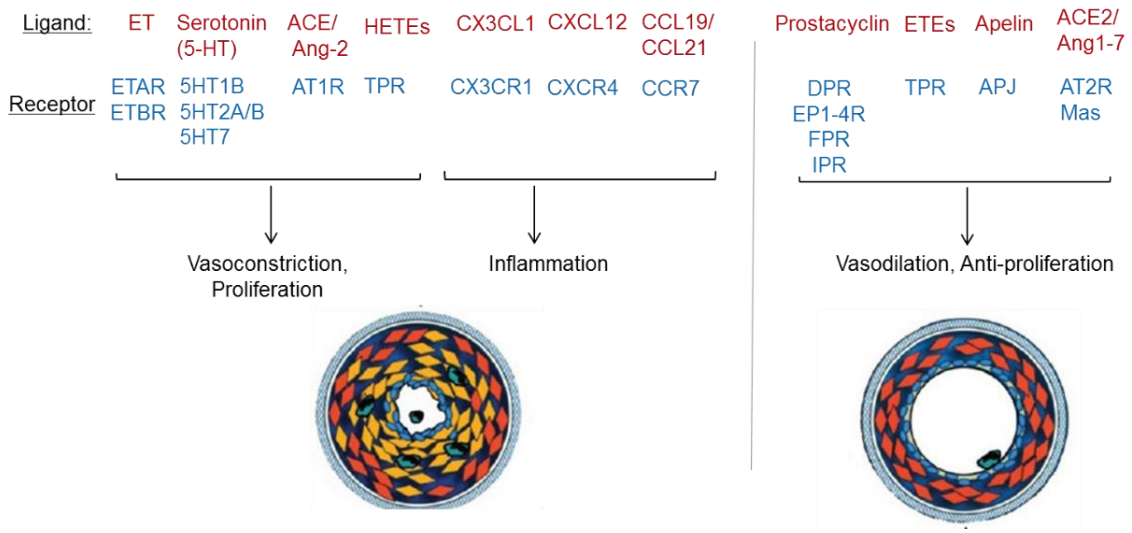


Figure 6. GPCRs and agonists as therapeutic targets studied in PAH. Ligands: ET: Endothelin, 5HT: 5-hydroxytryptamine (serotonin), ACE: Angiotensin-converting enzyme, Ang-2: Angiopoietin-2, HETEs: 5-hydroxyicosatetraenoic acid, CX3CL1: C-X3-C motif chemokine ligand 1, CXCL12: C-X-C motif chemokine ligand 12, CCL19: C-C motif chemokine ligand 19, CCL21: C-C motif chemokine ligand 21, ETEs:-eicosatetraenoate, ACE2: angiotensin I converting enzyme 2, Ang1-7: angiopoietins 1 to 7; **Receptors:** ETAR: endothelin receptor type A, ETBR: endothelin receptor type A, 5HT1B: 5-hydroxytryptamine (serotonin) receptor 1B, 5HT2A/B: 5-hydroxytryptamine receptor 2A/B, 5HT7: 5-hydroxytryptamine receptor 7, AT1R: angiotensin II receptor type 1, TPR: thromboxane receptor, CX3CR1: C-X3-C motif chemokine receptor 1, CXCR4: C-X-C motif chemokine receptor 4, CCR7: C-C motif chemokine receptor 7, DPR: prostaglandin D, EP1-4R: prostaglandin E2 receptors 1 to 4, FPR: formyl peptide receptor, IPR: prostaglandin I2 receptor, APJ: apelin receptor, AT2R: angiotensin type 2 receptor (Paulin, 2012) [121]

1.3.1 Endothelin signaling pathway

Activation of the ETA receptor mediates PASMC vasoconstriction [122]. ETA receptor is coupled to Gαq/11, Gα12/13, and Gαi subunits. By coupling to Gαq/11, ETA subsequently activates phospholipase C (PLC) and leads to increased intracellular Ca^{2+}. In contrast, the ETB receptor stimulation in pulmonary endothelial cells increases endothelial nitric oxide synthase (eNOS) activity via Ca^{2+} and subsequently produces nitric oxide, which is a vasodilator.

1.3.2 Prostanoid signaling pathway

Prostanoids include prostaglandin D2 (PGD2), prostaglandin E2 (PGE2), prostaglandin F2α (PGF2α); prostaglandin I2 (PGI2), and thromboxane A2 (TXA2), they can activate eight classical prostanoid receptors: prostaglandin D2 (PGD2) receptor, prostaglandin E2 receptors (EP 1–4), Prostaglandin F receptor (FP), prostaglandin I2 receptor (IP), thromboxane (TPR), prostaglandin D2 receptor 2 (PTGDR2/CRTH2), and peroxisome

proliferator-activated receptors (PPAR) [123]. Prostacyclin and its analogues, acting as vasodilators via adenylate cyclase activation and cAMP production, have been the first-line PAH medication for a decade. However, due to the diversity of ligands and receptors, the role of the prostanoid family in PAH is more complicated than what is currently understood. For example, mice with IP-receptor deficiency do not develop spontaneous PAH, but they are more susceptible to chronic hypoxia exposure and develop more severe pulmonary vascular remodeling. Those facts suggest a potential anti-proliferative effect [124]. On the other hand, U46619, a TPR antagonist, increases cytosolic Ca^{2+} and induces a contractile response in rat PASMCs [125]. Synthase inhibitor and TXA2-receptor antagonist have also been found to reduce monocrotaline-induced PH in rats [126].

1.3.3 Serotonin (5-HT) signaling pathway

Serotonin causes pulmonary vasoconstriction via its receptors expressed in the lung. These are the $5\text{-}HT_{1B}$ (Gαi/o-coupled), $5\text{-}HT_{2A/B}$ (Gαq11-coupled), and $5\text{-}HT_{7}$ (Gαs-coupled) receptors [127-131]. The $5\text{-}HT_{2B}$ receptor antagonist PRX-08066, an antagonist for both $5\text{-}HT_{2A}$ and $5\text{-}HT_{2B}$ receptors (terguride), and a novel $5\text{-}HT_{2B}$ and $5\text{-}HT_{7}$ receptor antagonist (C-122) have been demonstrated to prevent the progression of PH in the monocrotaline rat model [132-134].

1.3.4 Angiotensin signaling pathway

The renin-angiotensin system (RAS) is an important factor for endothelial dysfunction, as well as for the early stages of PAH [135]. The angiotensin-converting enzyme (ACE), angiotensin 2, or angiotensin receptor 1 (AT1R) coupled to Gαi/o or Gαq11 leads to vasoconstriction, proliferation, and fibrosis [136]. Whereas, ACE2, angiotensin 1-7, or angiotensin receptor 2 (AT2R) coupled to Gαi/o2/3, or a Mas receptor coupled to Gαq11, induces vasodilatation [137, 138]. Preclinical studies have demonstrated that inhibiting the angiotensin 2 AT1R axis or activating the angiotensin 1-7 AT2R axis [121] has beneficial effects in PAH animal models.

1.3.5 Apelin (APJ) signaling pathway

Apelin is the ligand for the GPCR receptor apelin receptor (APJ) [139]. Both apelin and APJ are highly expressed in the pulmonary endothelium [140, 141]. Moreover, apelin serum levels were significantly lower in PH patients than in healthy controls [142]. Exogenous apelin has been demonstrated to have a vasodilatory effect, and the disruption of apelin

signaling in mice leads to more severe PAH symptoms than those seen in wildtype mice [143].

1.3.6 β-chemokines (CC chemokines) and the C-C chemokine receptor type 7 (CCR7) signaling pathway

Inflammatory processes are believed to play a large part in both the initiation and progression of pulmonary vascular remodeling. These inflammation events, such as the peripheral infiltration of inflammatory cells, are mostly mediated by chemokines and cytokines. For example, T cells and antigen-presenting dendritic cells (DCs) are recruited to the lymph nodes and interact with each other via the activation of Gαi/o-coupled receptor C-C motif chemokine receptor 7 (CCR7), through CCR7 ligands (CCL19 and CCL21). Mice deficient in CCR7 develop mild PAH with increased perivascular leukocyte infiltration in the lungs [144]. In a subgroup of PAH patients, CCR7 gene expression is reduced in circulating mononuclear cells [145]. These findings suggest that CCR7 may play a significant role in PAH as an inflammatory mediator.

1.3.6.1 Fractalkine (CX3CL1), CX3C chemokine receptor 1 (CX3CR1) signaling pathway

A Gαi/o- and Gαq11-coupled receptor, CX3CR1 is upregulated in T cells, and an elevation of CX3CL1 (a ligand of CX3CR1) in plasma, lung parenchyma, and pulmonary artery endothelial cells has been reported in PAH patients [146]. Moreover, patients with scleroderma-associated PAH displayed CX3CR1 polymorphisms (249I and 280M), indicating a potential role for the CX3CL1/CX3CR1 axis in PAH [147]. In addition, CX3CL1 and CX3CR1 are upregulated in the PASMCs of a rat model, and CX3CL1 promotes PASMC proliferation. These findings suggest that CX3CL1 may also play a direct role in pulmonary artery remodeling, in addition to its conventional role in cell recruitment [75].

1.3.6.2 Chemoattractant cytokine (CLCX12), C-X-C chemokine receptor type 4 (CXCR4) signaling pathway

During the embryonic development of the cardiovascular, hemopoietic, and central nervous systems, CXCR4 and its ligand CXCL12 play a crucial role in the migration of progenitor cells. Furthermore, CXCR4 is involved in neo-intima formation, the mobilization of SM progenitor cell, and vascular remodeling [148]. Furthermore, CXCR4 inhibition by its antagonist AMD3100 significantly decreases hypoxia-induced PH in animal models [121].

1.4 Purinoceptor 2 (P2Y2) mediated signaling pathways

Purinergic P2Y receptors are heptahelical receptors expressed in eukaryotes. They are GPCRs coupled to heterotrimeric G proteins, and they recognize extracellular signaling molecules and activate intracellular signaling processes. Adenine nucleotides (adenosine triphosphate (ATP) and adenosine diphosphate (ADP)), uridine nucleotides (Uridine-5'-triphosphate (UTP) and uridine-5'-diphosphate (UDP)), and nucleotide sugars (UDP-glucose) are the ligands of the purinergic receptors [149]. Due to mechanical stress, infections, the activation of apoptosis, or a lack of oxygen, many cells release these nucleotides and nucleotide sugars. A purinergic receptor, P2Y2 is activated by ATP or UTP ligands. Purinoceptor 2 is coupled to three combinations of heterotrimeric G proteins (G$\beta\gamma$ with Gαq, Gαo, or G12) [150, 151]. Therefore, the downstream signaling caused by the activation of the P2Y2 receptor is based on the activation of Gα proteins, that is coupled to P2Y2 receptor. Activation of Gαo activates RhoA, Rac. Activation of G12 activates ras-related C3 botulinum toxin substrate, guanine nucleotide exchange factors (RacGEF). Activation of Gα16 activates phospholipase C-β1 (PLCβ) and mobilizes intracellular Ca2+ in gastric SMCs [152, 153].

1.4.1 P2Y2 receptor oligomerization

GPCRs can form homomeric, heteromeric, and oligomeric structures. Co-immunoprecipitation and fluorescence resonance energy transfer (FRET) analysis have provided several examples of P2Y receptor dimerization. In human embryonic kidney (HEK293) cells, the A1 adenosine receptor forms a heteromeric complex with P2Y1 and P2Y2 68,69. The ligand selectivity of the A1 receptor remains unaffected in the heteromeric receptor complex (A1-P2Y2). However, the stimulation of these receptors via their agonist interferes with Gi signaling and enhances the Gq signaling pathway [154].

1.4.2 P2Y2 receptor desensitization and trafficking

Both β-arrestin1 and β-arrestin 2 interact with the P2Y2 receptor. Depending on the ligand that activated P2Y2, the β-arrestin coupling differs. If the ATP ligand binds to P2Y2 and activate the receptor, the P2Y2 receptor then strongly couples to β-arrestin1 and weakly couples to β-arrestin 2. If the receptor is activated by UTP, the P2Y2 receptor strongly couples with both β-arrestin1 and β-arrestin2 [155].

1.4.3 P2Y2 receptor coupling to receptor and non-receptor tyrosine kinases

The P2Y2 receptors couple with actin-binding protein and filamin A via proline-rich (PxxP) motifs in the C-terminal tail. This is also necessary for transient activation of Proto-oncogene tyrosine-protein kinase (Src) [156]. Furthermore, several tyrosine kinase receptors, such as PDGF receptor (PDGFR) and epidermal growth factor receptor (EGFR), are activated by the P2Y2-dependent activation of Src [156]. In addition, studies of endothelial cells have revealed that the VEGF receptor-2 (VEGFR-2) is transactivated by P2Y2 receptors via the Src-dependent pathway. The P2Y2-dependent activation of VEGFR-2 leads to the increased expression of the vascular cell adhesion molecule VCAM-1, which promotes the binding of circulating leukocytes to endothelial cells and the vascular wall [157].

2 AIM OF STUDY

As the previous section has demonstrated, GPCRs are centrally involved in the pathobiology of PAH. Identifying dysregulated GPCRs in PAH may result in an expanded ability to target abnormal pulmonary vasoconstriction and remodeling, thus offering options for translational research. Therefore, the main objectives of this study were to:

1) Profile the entirety of GPCR expression in micro-dissected pulmonary vessels from donor and IPAH patients, and to identify highly and/or aberrantly expressed GPCR candidates for PAH studies. This analysis included cDNA isolation from pulmonary vessels. Cryo-sections of human lungs from donors, PAH patients are available from the Universities of Giessen and Marburg Lung Center (UGMLC) tissue bank. Pulmonary vessels within a diameter of 250µm-500µm were identified by laser-assisted microdissection. The expression of all GPCRs in the human samples was systematically analyzed and quantified using an established quantitative polymerase chain reaction (qPCR) array. The validation of GPCR expression and determination of GPCRs dysregulated in remodeled pulmonary vessels by alternative sets of gene-specific primers and probes.
2) Determine the functions of the most promising GPCR candidate(s) by applying pharmacological agonists, antagonists or respective siRNA, with a focus on the in-vitro effects of PASMCs. This goal was realized by evaluating intracellular cAMP levels, as well as cell proliferation (Bromodeoxyuridine (BrdU) incorporation), and migration (transwell), and matrix metalloproteinase (MMP) activity (zymography).
3) Define the signaling mechanisms of specific candidate(s) involved in the above cellular processes by exploring the downstream molecules regulating cell survival and motility using polymerase chain reaction (PCR) or immunoblotting.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals, reagents and kits

Names	Company
2-propanol	Sigma- Aldrich, Germany
Acetic acid	Sigma- Aldrich, Germany
Acrylamide	Roth, Germany
Ammonium persulfate (APS)	Sigma- Aldrich, USA
Bovine serum albumin (2 mg/ml)	Bio-Rad, USA
Bovine serum albumin powder	Serva, Germany
Bromophenol blue	Merck, Germany
Cell Proliferation ELISA, BrdU (colorimetric)	Roche, USA
Coomassie Brilliant Blue G	Sigma-Aldrich, USA
Crystal violet solution 2.3% w/v	Sigma-Aldrich, USA
Crystal Violet Solution	Sigma-Aldrich, USA
Cyclic AMP EIA Kit	Cayman Europe, Estonia
DC™Protein Assay	Bio-Rad, USA
DEPC water	Roth, Germany
DNeasy Blood & Tissue kit	Qiagen, Germany
Enhanced chemiluminescence (ECL) kit	Amersham, USA
Ethanol 70%	SAV LP, Germany
Ethanol 96%	Otto Fischhar, Germany
Ethanol 99.9%	Berkel AHK, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, USA
SuperSignal™ West Femto Maximum Sensitivity	ThermoFisher Scientific, Germany
Gelatin (porcine skin)	Sigma-Aldrich, USA
Glycerol	Sigma-Aldrich, USA
Glycin	Roth, Germany
iScript cDNA synthesis kit	Bio-Rad, USA
iTaqSYBR Green Supermix	Bio-Rad, USA
LightCycler® 480 Probes Master	Roche, Germany
Methanol	Sigma-Aldrich, USA
Non-fat milk powder	Roth, German

Ovation PicoSL WTA system V2 kit	NuGEN, USA
Paraformaldehyde (PFA) 3.7%	Sigma-Aldrich, USA
Positively charged glass slides	Langenbrinck, Germany
Precision Plus Protein Standards	Bio-Rad, USA
RIPA buffer	Santa Cruz, USA
RNAse-Away	Molecular BioProducts
RNeasy Plus Micro kit	Qiagen, Germany
RNeasy Plus Mini kit	Qiagen, Germany
SDS Solution, 20% w/v	AppliChem, Germany
Sodium chloride (NaCl)	Sigma-Aldrich, USA
Sodium Orthovanadat (Na3VO4)	Sigma-Aldrich, USA
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, USA
Tissue-Tek® O.C. T™ Compound	Sakura, Japan
Tris Base	Roth, Germany
Tris-HCl	Roth, Germany
Triton-X100	Sigma-Aldrich, USA
TRIzol® Reagent	Life Technologies, USA
Tween®20	Sigma-Aldrich, USA
UltraPure water	Cayman Europe, Estonia
Universal ProbeLibrary Probes	Roche, Germany
β-Mercaptoethanol	Sigma-Aldrich, USA

3.1.2 Cell culture medium and reagents

Fetal bovine serum	Biowest, Germany
HBSS (Hank´s buffered saline solution) PBS	PAN, Germany
Leukotriene D4 (LTD4)	Cyaman, USA
MRS 2768 tetrasodium salt	Tocris, USA
ON-TARGETplus siRNA	Dharmacon, USA
Opti-MEM® I Reduced Serum Medium	ThermoFisher Scientific, Germany
Platelet-activating Factor (PAF) C-16	Cyaman, Germany
SmBM Smooth Muscle Basal Medium	Lonza, Germany
SmGM-2 BulletKit (CC-3181 & CC-4149)	Lonza, Germany
Trypsin/EDTA	PAN, Germany
X-tremeGENE Transfection Reagent	Roche, Germany

3.1.3 Primers used for high-throughput screening of GPCRs

The primers for screening 408 human non-olfactory GPCRs were all purchased from Sigma, Germany. Their respective probes were purchased from Roche, Germany. Primers and probes were identified by Roche Universal Probe Library online. The primer sequences and probes for profiling and validation are given in appendix (Table 20, 21 App).

3.1.4 Candidate GPCR primers for quantitative real-time PCR in PASMC

GPCR primer oligonucleotides were all purchased from Metabion, Germany. The primer sequences for quantitative real-time PCR are listed below (Table 2).

Table 2. GPCRs human primer sequences for quantitative real-time PCR

Human gene	Forward primer	Reverse primer
CMKIR1	atc ttc ctg gtg gtg gtc tac	aca tgt tgt gga tga gaa gga
CXCR6	cta tgg gtt cag cag ttt caa	cag gct ctg caa ctt atg gta
CXCR7	acc tca tct tct cca tca acc	cgt gac ggt ctt cag gta gta
CYSTIR1	caa tgg ctt tgt gct cta tgt	tgg ctg tca taa aga aga tgc
DRD1	aag aag ttg gtc acc ttg gac	cat ctt act cat tgc cac ctc
EDNRB	aca tcg tca ttg aca tcc cta	aga gac cac cca aat caa aac
GOR 112	cac aat ctg tca gtg tga cca	gta tgt cac cac tgc aac tcc
GPR 112	ctc cat ttttct ggg agt tgc	cca ctg cag ctg tga tac aaa c
GPR 4	ctt tca cca gcc tca act gtg	gtt cct ctt gga ggt gag tgg
GPR109	gcc too aga gga aga taa cag	gac aat gtc cct tct tgg aat
GPR115	ttg ttg ctg tca aca ctc aga	cat aat ggt tcc aaa cag cag
GPR18	aaa agc tgt gaa cgt gct gaa	gtg gaa ggg cat aaa gca gac
GPR18	tca tca tga ttg ggt gct act	ccc agg gat tgt aac tgt tct
GPR21	tgg tcc ctt ctt tat cac tcc	cag ggt cga gta tag cca aat
GPR4	tgg gtt cat ctt cta cac caa	gga act tct caa agc aga agg
GPR97	aca cct act tcg ggc act act	aaa gag gaa ggt gat gag gaa
IGR7	cta tgg cac caa tgg agt atg	cag ttg ctg tta tgg cac ttt
IPAR2	cct ggt caa gac tgt tgt cat	gca aga gta cac agc agc att
LPART 1	atg ctc aca tct ttg gct atg	aac aaa acc aat cca gga gtc
NPY1R	atc aat ttc cat cgg act ctc	ttt ggt ttc act gga cct gta
NPY5R	cct tct caa gag aac cac tcc	tag tgg cat cca act aac agc
P2RY5	tac att ttcatc tgc gtc ctc	tgc ttc cgt aca tgt tgg tat
P2Y2	cat cct tgt ctg tta cgt gct	ggt aac ctt gta ggc cat gtt
PTAFR	ccg att gtt tac agc atc atc	aat ttg ggg agt atc cag ttg
PTAFR4	cat ctt act cat tgc cac ctc	ctg agc act gtc ttt ctc agg
PTGER4	ctg tgc gag tac agcacc ttc	gtt gga cgc ata gac tgc aaa
TAAR8	ggt gtg act gtg atg ctt ttc	tag acc agg ggatca gta acc
VIPR1	tct acc tgt aca ccc tgc ttg	cag tga gga gttgat ggt gtc

3.1.5 Small interfering RNA (siRNA)

ON-TARGETplus siRNA pools and Non-Targeting control pool (siRNA-scrambled) were designed and purchased from Dharmacon, USA. siRNA SMART pool is a mixture of 4 siRNA provided as a single reagent. The targeting sequences for respective GPCRs are provided in Appendix (Table 22 App.)

3.1.6 Antibodies

Table 3. Primary antibodies

Antibody	Source	Dilution	Company
Phospho- Myosin Light Chain 2 (Thr18/Se19) (#3674)	Rabbit	1:1000	Cell Signaling, USA
Myosin Light Chain 2 (D18E2) (#8505)	Rabbit	1:1000	Cell Signaling, USA
ROCK1 (C857)	Rabbit	1:1000	Cell Signaling, USA
GAPDH	Mouse	1:5000	Novus Biologicals, UK
ERK-1 (C16) (sc-93)	Rabbit	1:1000	Santa Cruz, USA
Phospho- ERK (E-4) (sc-7383)	Mouse	1:1000	Santa Cruz, USA
EPAC1 (5D3) (#4155)	Mouse	1:1000	Cell Signaling, USA

Table 4. Secondary antibodies: HRP-conjugated

Antibody	Source	Dilution	Company
anti-mouse IgG	Rabbit	1:40000	Sigma-Aldrich, USA
anti-rabbit IgG	Goat	1:50000	Pierce, USA

3.1.7 Equipment

Names	Company
Balance 1.0-3000g RP 3000	AugustSauter, Switzerland
Balance PCB 200-2 Precision	Kern, Germany
Balance XS205	MettlerToledo, Switzerland
BioDoc Analyzer	Biometra, USA
Cell culture incubator, Hera Cell	Heraeus, Germany
Centrifuge Roranta 460R	Hettich, Germany
Cytospin™ 4 Cytocentrifuge	Thermo Scientific, USA
Electrophoresis chamber	Bio-Rad, USA
Freezer (+4°C, -20 °C, -80 °C)	Bosch, Germany
Infinite® 200 microplate reader	Tecan, Switzerland
Inolab PH meter	WTW, Germany

Light microscope DM IL	Leica, Germany
LightCycler® 480 Instrument	Roche, Germany
Liquidator 96	Steinbrenner, Germany
Live imaging microscope DMI6000 B	Leica, Germany
Microtome RM2165	Leica, Germany
Mounting bath HI1210	Leica, Germany
Mounting heating plate HI1220	Leica, Germany
Multifuge centrifuge	Heraeus, Germany
Mx3000P qPCR System	Stratagene, USA
Pipetboy and pipettes	Eppendorf, USA
Power supply	Bio-Rad, USA
Precellys®24 homogenizer	BertinTech., France
Rotator Staurt®SB3	Bibby Scientific, U.K.
Shaker	Bruker, Belgium
Thermocycler T3000	Biometra, USA
Vortex machine	VWR, Germany
Water bath for cell culture	HLC, Germany
Water bath for tubes	Medingen, Germany
Western blot unit	Bio-Rad, USA

3.1.8 Other materials

Names	**Company**
6-well, 24-well, 96-well microplate	Corning, USA
96er PCR-plate for LC480	Steinbrenner, Germany
AGFA cronex 5 medical X-ray film	AGFA, Belgium
Chromatography column	Bio-Rad, USA
Cover glass 60 x 24 (0.13-0.18 mm)	Langenbrinck, Germany
Falcon tubes	BD Biosciences, USA
Film cassettes	Kodak, USA
Filter tips (10, 100, 1000µl)	Nerbe plus, Germany
Gel blotting paper	Whatman, USA
Glass pipettes, cell culture dishes, plates	Sarstedt, Germany
Glass slides Super Frost® Plus	R.Langenbrinck, Germany
Needles 26-20G (0.45-0.9mm)	Microlance™ 3 BD, Ireland
Nitrocellulose membrane	Bio-Rad, USA
Osmotic minipump (2 mL)	Durect Corparation, USA

Precellys bead mill sample tube	Berlin Tech., France
Radiographic films hypersensitive	Amersham, USA
Radiographic films	Santa Cruz, USA
Scalpels	Feather, Japan
Shandon™ Single Cytoslides™	Thermo Scientific, USA
Syringes 1, 2, 5, 10, 25 ml	B. Braun, Germany
Tips (10, 100, 1000 µl)	Eppendorf, USA
Tissue culture chamber slide	BD Falcon, USA
Transwell permeable support	Corning, USA

3.1.9 Software

Software	**Company**
Excel and Word 2013	Microsoft, USA
Fluorescence (LAS AF) Microscope Software	Leica, Germany
i-Control	Tecan, Austria
Leica Application Suite Advanced	Leica, Germany
Leica QWin imaging software	Leica, Germany
LightCycler® 480 Software	Roche, Germany
Magellan v.6.3	Tecan, Austria MS®
MxPro™ QPCR software	Agilent Technologies, USA
Prism® v6.05	GraphPad statistics, USA

3.2 Methods

3.2.1 Patients' characteristics

Human lung tissues were obtained from donors and patients with IPAH undergoing lung transplantation. After ex-plantation, lung tissues were formalin fixed and paraffin embedded according to common tissue-processing protocols. The study protocol for tissue donation was approved by the ethics committee of the University Hospital Giessen, in accordance with national law and international guidelines (AZ 31/93). Written informed consent was obtained from each individual patient or the patient's next of kin.

3.2.2 Microdissection of pulmonary vessels and cDNA synthesis

Laser-assisted microdissection of pulmonary vessels from human lung tissue was performed as described below. Human lung tissue cryosections were mounted on glass slides. After brief staining with hemalaun and eosin (Table 5), pulmonary vessels (diameter

range: 50 – 100 µm), which are placed near the bronchus were microdissected from the sections with the use of the Laser Microbeam System (Leica, Germany). Around 100-150 vessels per donor or per patient were initially collected to 25 µl of RLT lysis buffer (Table 6), later 275 µl of RLT lysis buffer was added and stored at -80°C. Total cellular RNA from microdissected pulmonary vessels were isolated with the micro RNeasy kit according to the manufacturer's instructions (Qiagen, Valencia, CA). The total RNA obtained was reverse transcribed and amplified to cDNA by RT-PCR using Ovation PicoSL WTA System V2 kit according to the manufacturer's instructions (NuGEN, USA) (Table 7). The cDNA quality and concentration was measured by NanoDrop spectrophotometer.

Table 5. Hematoxylin and eosin staining

Steps	**Duration**
3 x Xylol	10 mins
Ethanol 99.6% (2 times), Ethanol 96%, Ethanol 70%	5 mins (each)
Distilled water	2 mins
Haemalaun, acidic	20 mins
Tap water	5 mins
Ethanol 96%	1 min
Eosin-Y alcoholic	4 mins
Distilled water	rinse
Ethanol 96% (2 times), Ethanol 99.6%	5 mins (each)
Isopropanol 99.8%	5 mins
3 x Xylol	5 mins

Table 6. Lysis buffer recipe

Components	**Volume**
Qiagen RLT buffer	1 ml
ß-Mercaptoethanol	10 µl

Table 7. NuGEN Reverse transcription – Thermocycler program

First stand cDNA synthesis	
Primer annealing	65°C – 2 mins, hold at 4°C
First strand synthesis	4°C – 2mins 25°C – 30 mins 42°C – 15 mins 70°C – 15 mins, hold at 4°C

Second stand cDNA synthesis	
Second strand synthesis	4°C – 1 min 25°C – 10 mins 50°C – 30 mins 80°C – 20 mins, hold at 4°C
SIPA amplification	
SIPA amplification	4°C – 1 min 47°C – 75 mins 95°C – 5 mins, hold at 4°C

3.2.3 High-throughput (HT) screening of GPCRs

Gene specific primers for 408 GPCR genes and their corresponding probes were identified using the Roche Probe Finder software.

3.2.3.1 Oligo resuspension and storage

The lyophilized primers were thawed and centrifuged to spin down the lyophilized powder from cap. The primers were re-suspended (stock concentration: 100 µM) in respective volumes of sterile nuclease-free water as mentioned in the ordering list obtained from company (Sigma-Aldrich, Germany). The re-suspended primers were incubated at 37°C in shaker for 20 minutes. The primers were diluted to a working concentration ratio of 1:10

3.2.3.2 Plate preparation and storage

Each well of 96-well plate loaded with gene specific exon spanning primers with their probes and stored at -20°C.The daughter plates are prepared from mother plate (10X primer-probe mix) by using automated pipetting system (Liquidator 96, Steinbrenner Laborsysteme GmbH) and stored at -20°C.

3.2.3.3 High-throughput (HT) screening procedure

Each plate consists of 48 set of gene specific primers and probes. These plates were thawed, centrifuged and loaded with master mix (LightCycler 480 Probe Master, Roche Germany) along with 3 ng of cDNA (Table 8). The multiwell plate is sealed with LightCycler® 480 foil and centrifuged for 2 mins at 1500xg. High-throughput quantitative real-time PCR was performed with the LightCycler® 480 Instrument (Table 9). The quality of the RNA is tested by Rn 18S primer and the quality test for genomic DNA contamination in cDNA samples were tested using intron spanning primer of GPR133. The cDNA levels of each

gene were normalized with respective genomic DNA levels and copy number of genes was calculated based on absolute quantification. The genomic DNA levels were obtained for same set of genes in each plate. From 408 GPCRs around 45 candidates were identified and such dysregulated GPCR expression were validated in remodeled pulmonary vessels (same set of donors and IPAH patients) by alternative sets of gene-specific primers and probes.

Table 8. HT- qPCR reaction components

Reaction components	**Volume per reaction**
Primer-probe mix, 10X conc.	1 µl (Final conc.: primer= 1 µM, probe= 0.2 µM)
LightCycler 480 Probe Master, 2X conc.	10 µl
Water PCR grade	4 µl
cDNA/gDNA (0.6 ng/µl)	5 µl (3 ng)
Total volume	**20 µl**

Table 9. HT- qPCR- LightCycler® 480 program

Program		**Cycle**	**Temperature**	**Time**
Pre-incubation		1	95°C	5 mins
Amplification	Denaturation	45	95°C	10 secs
	Annealing		60°C	30 secs
	Extension		72°C	1 sec
Cooling		1	40°C	30 secs

3.2.4 Human pulmonary arterial smooth muscle cells (PASMCs) isolation and cell culture

Human pulmonary arterial smooth muscle cells (PASMCs) of donors were purchased from Lonza (Basel, Switzerland). PASMCs were isolated from IPAH patients by explant method. After removal of pulmonary endothelium and adventitia from distal pulmonary arteries, the media layer was cut into 1mm^3 pieces. The artery pieces were cultured on cell-culture dishes in smooth muscle growth medium (SmGM-2) from Lonza (Basel, Switzerland). Cells were incubated in humidified atmosphere of 95% air with 5% CO_2 at 37°C and allowed to migrate until a monolayer of PASMCs formed.

3.2.4.1 Cryopreservation

At 80% confluency cells were washed with DPBS buffer. Trypsinization was performed by adding pre-warmed Trypsin 1X. FBS was added to stop the trypsin activity. The cells were centrifuged at 1000 rpm, 20°C for 5 minutes. The pellet was then resuspended in cooled

freezing cryopreservation medium (SmBM medium: FCS: DMSO) and kept in cell-freezing container for 24 hours at -80°C, later transferred to liquid nitrogen and stored for future use.

3.2.4.2 Seeding and splitting

The appropriate volume of medium was pre-incubated at 37°C, 5% CO_2 for 30 minutes. The cryopreserved cells were thawed and seeded to the plates containing pre-incubated medium. The cells were incubated at 37°C, 5% CO_2 until the cell confluency reaches to 80-90%. The cells were trypsinized and cell pellets were re-suspended with fresh SmGM-2 growth medium. The cell count and viability was determined using hemacytometer and trypan blue. The cells were seeded at a seeding density of $1*10^4$ cells/cm^2.

3.2.5 Polymerase chain reaction (PCR)

3.2.5.1 RNA isolation

The total RNA from PASMCs were isolated by using Qiagen RNeasy Plus Mini/Micro kit. The procedures were followed according to the manufacturer's instructions. Finally, RNA was dissolved in DEPC-water and stored at -80°C. The quality and concentration of total RNA was measured by NanoDrop spectrophotometer.

3.2.5.2 Reverse transcription - polymerase chain reaction (RT-PCR)

cDNA was synthesized by RT-PCR using Bio-Rad iScript cDNA synthesis kit according to the manufacturer's instructions. 0.5-1 µg RNA in 20 µl master mix was used per reaction (Table 10). The reverse transcription was performed in thermocycler and the program is shown in Table 11.

Table 10. RT-PCR components

Components	**Volume per reaction**
RNA (0.06 µg/µl)	15 µl
5x iScript reaction mix	4 µl
iScript reverse transcriptase	1 µl
Total Volume	**20 µl**

Table 11. RT-PCR thermocycler program

Program	**Temperature**	**Time**
Annealing	25°C	5 mins
Incubation	42°C	30 mins
Thermal inactivation of reverse transcriptase	85°C	5 mins
Hold at 4°C		

3.2.5.3 Quantitative realtime- polymerase chain reaction (qPCR)

The exon-spanning primer pairs for human GPCR genes were designed using the Primer3 software tool and are shown in Table 2. Nucleotide blast was used to cross check primers for specificity and homology. The product size is within the range of 150 bp - 200 bp. qPCR was performed on a Mx3000P® QPCR system machine using iTaq™ SYBR® Green Supermix kit and procedures were followed according to manufacturer's instructions (Table 12, 13). The nuclease free water instead of cDNA was used as negative control.

Table 12. Quantitative PCR reaction components

qRT- PCR reaction component	Volume per reaction	Final concentration
cDNA template	Variable	0.2 µg
Forward primer 10 µM	0.5 µl	0.2 µM
Reverse primer 10 µM	0.5 µl	0.2 µM
iTaq SYBR Green supermix (2X)	10 µl	1X
Nuclease-free water	Variable	-
Total volume	**20 µl**	**-**

Table 13. Quantitative PCR program

qPCR program		Temperature	Time	Cycle
Polymerase Activation & DNA denaturation		95°C	10 mins	1
Amplification	Denaturation	95°C	30 secs	40
	Annealing	58°C	30 secs	
	Extension	72°C	30 secs	
Denaturation		95°C	1 min	
Melt-Curve analysis		55-95°C (0.5°C increment)	2-5 secs/step	1
Hold		4°C	Indefinite	-

The dissociation curve was generated by MxPro™ QPCR software, and used to ensure a single product amplification. Threshold cycle (Ct values) was determined for each gene. Hypoxanthin phosphoribosyl transferase (HPRT) was used as housekeeping gene, all Ct values of target genes were normalized to HPRT by using the formula,

$$\Delta Ct = Ct_{reference\ gene} - Ct_{target\ gene}$$

The mRNA levels were compared between donor and IPAH patients or between siRNA-scrambled and siRNA-target gene. Each reaction was run in duplicate and repeated three times independently.

3.2.6 RNA interference by siRNA (GPCRs)

Transient transfection of siRNAs (Table 22 App.) was performed with X-tremeGENE siRNA transfection reagent according to the manufacturer's instruction.

3.2.6.1 siRNA re-suspension and storage

siRNA vials were briefly centrifuged to ensure the siRNA pellet was collected at the bottom of the tube. 5 nmol of siRNA was re-suspended in 100 µl of RNase free water to obtain a stock concentration of 50 µM. The siRNAs were aliquoted and stored at -20°C.

3.2.6.2 Transient transfection of siRNA in PASMC

Donor PASMCs were cultured to 30-50% confluency in antibiotic-free SmGM-2 medium. The cells were pre-incubated with opti-MEM medium at 37°C, 5% CO2 for 30 minutes. In mean time siRNA and transfection reagent were diluted in opti-MEM medium and mixed within 5 min after dilution. 3:1 ratio of X-tremeGENE siRNA transfection reagent (µl) to siRNA (µg) was used. The mixture of siRNA and transfection reagent were incubated at room temperature (RT) for 20 mins. After incubation 50 nM siRNA was transfected to PASMCs in opti-MEM medium for 4 hours at 37°C, 5% CO2. Later the opti-MEM medium was replaced by fresh SmGM-2 growth medium and cultured for 48 hours. The RNA interference was well established and repeated at least three times. The knockdown efficacy was checked by quantitative PCR. The siRNA knockdown of GPCRs were used to study the proliferation and migration of PASMCs.

3.2.7 BrdU cell proliferation assay

PASMCs proliferation was determined using Cell Proliferation ELISA, BrdU (colorimetric) assay based on manufacture instructions (Roche, Germany). The cellular proliferation detection of this kit is based on the BrdU incorporation into the genomic DNA of proliferating cells. Cells were grown in 96-well tissue-culture microplates (initial seeding density 1×10^4 cells / well). After 24 hours of 50 nM siRNA transfection followed by 24 hours' starvation and 24 hours' stimulation (FCS/PDGF/ligands), the cells were incorporated with BrdU labelling for 4 hours. Labelling medium is removed by flicking off and tapping the plate, and the cells were fixed with 200µl of FixDenat solution for 0.5 hrs. FixDenat was removed by

flicking off and tapping the plate, 100µl of anti-BrdU-POD antibody working solution (1:100) was added and incubated in RT for 90 mins. The anti-BrdU-POD antibody then bound to the BrdU incorporated into the newly synthesized cellular DNA. After 3 times washing with 1XPBS washing buffer, the immune complexes were detected by addition of 100 µl of substrate. The reaction product was quantified by measuring the absorbance at 370nm with the reference at 492 nm using TECAN spectrophotometer (ELISA reader).

3.2.8 Wound healing - in vitro scratch assay

PASMC were seeded on 24-well plates. Transfected cells were serum-starved for 24 hours and scratches were made using a p10 pipette tips in the center of each well. At this stage cells were at complete confluency. After scratch cells were carefully washed with 1X PBS twice and freshly prepared basal medium (SmBM) with or without stimulators (FCS, PDGF), with or without ligands (MRS2768, PAF, LTD4). The closure of the scratch was monitored by live cell imaging and the images were captured every 1 hour until 24 hours by Leica real-time microscope system. The distances of migration from the scratch to the growing edges were measured at five different points by Leica Application Suite Advanced Fluorescence (LAS AF) Microscope software and percentage of closure was calculated.

3.2.9 Transwell migration assay

PASMCs were transfected with 50 nM of siRNA-scrambled or siRNA-P2Y2 for 24 hours and serum-starved for 24 hours. Cells were trypsinized and 1×10^4 cells were plated in 100 µl of SmBM medium with or without P2Y2 ligand 10µM MRS2768, in the 6.5-mm transwell inserts with 8.0-µm pore size polycarbonate membrane. The lower chambers were filled with 600 µl of SmBM medium with or without stimulator (5% FCS or 30 ng/ml PDGF) and with or without P2Y2 ligand 10 µM MRS2768. The migration of cells through the membrane was analyzed after 10 hours of incubation at 37°C, and 5% CO2. PASMCs on the upper side of the insert were removed by using cotton swab. Migrated PASMCs on the lower side of the insert were fixed for 10 mins with 3.7% PFA. Cells were washed by merging the insert in water. Then cells were stained for 30 mins with 0.1% crystal violet in 20% methanol. The cells were washed 2 times with tap water and the inserts were dried in RT. The percentage of migrated cells were calculated by counting the cells under 20-fold magnification using Leica DM6000 B microscope.

3.2.10 cAMP enzyme immunoassay (EIA)

Intracellular adenosine 3',5' cyclic monophosphate (cAMP) content of PASMCs were determined by a competitive non-acetylated EIA. The assay procedures were followed according to the manufacturer's instructions of cAMP EIA Kit (Cyaman, Europe, Estonia). PASMCs (1.6 x 10^5 / well of 6-well plate) were transfected with siRNA-scrambled and siRNA-P2Y2 for 24 hours followed by serum-starvation for 24 hours. Then the cells stimulated for 24 hours with P2Y2 ligand (MRS 2768). At this stage cells were 80% confluent.

3.2.10.1 Sample preparation

Cells were washed with 1XPBS twice and lysed with 0.1 M HCL at RT for 20 mins on a shaker. Cells were scraped with a cell scraper and the cell mixture was dissociated with 24 g needle and 1 ml syringe by pipetting up and down. The cell suspension was further centrifuged at 1000 x g for 10 mins at 4°C. The supernatants were collected to clean Eppendorf tubes and stored at -80°C. All samples were diluted with equal volume of UltraPure water (50 µl sample in 50 µl water).

3.2.10.2 Assay procedure

The standards (S1 to S8) range from 750 pmol/ml to 0.3 pmol/ml, were prepared by manufacturer's instructions. 50 µl of diluted protein samples and standard solutions were incubated with 50 µl of tracer and 50µl of antibody at 4°C for 18 hours. Then the plate was washed 5 times with washing buffer and 200 ml of Ellman's solution was added to each well using a multi pipette. The plates were incubated in the dark at room temperature for 90 to 120 mins on an orbital shaker.

3.2.10.3 Reading the plate and calculation

The absorbance was read at between 405 - 420 nm using TECAN spectrophotometer (ELISA reader). The cAMP concentrations were calculated by the online available Cayman EIA Double workbook. The cAMP concentration of the samples was provided as pmol/mL protein. The standard curve was made as a plot of %B/B0 (%Bound/Maximum Bound) for standards (S1-S8) versus cAMP concentrations using a linear (y) and a log (x) axes. Each sample were performed in duplicate and repeated twice.

3.2.11 Western blotting

3.2.11.1 Protein isolation

Cells were washed with 1XPBS and lysed with RIPA buffer. The RIPA buffer composed of PMSF, proteinase inhibitor cocktail and sodium orthovanadate and was freshly prepared before use. 2×10^6 PASMCs in 250 µl RIPA was centrifuged under 12000 rpm for 30 min at 4°C and the supernatants were collected and stored at -80°C.

Table 14. RIPA buffer recipe

Components of RIPA buffer	Final concentration
RIPA buffer	1x
Protease inhibitor cocktail	1x
Sodium orthovanadate	1%
PMSF	1%

3.2.11.2 Protein concentration analysis

Bio-Rad DC protein assay was used to determine the protein concentration. The procedures were followed according to manufacturer's instructions. It is based on Lowry assay with slight modifications. Bovine serum albumin (BSA) was used as standard (0.25, 0.5, 1, 2 mg/ml). The protein samples were pre-diluted to fall in the range of standards. The protein concentrations of each sample were estimated in duplicates. Protein concentrations were determined by measuring absorbance at 750 nm using TECAN spectrophotometer (ELISA reader). Final protein concentrations were calculated using Magellan™ software. The protein samples were stored at -80°C

3.2.11.3 SDS-PAGE gel electrophoresis

Protein samples were equalized to fixed concentration. The protein samples were denatured by adding 5X SDS loading buffer (Table 15) at a ratio of 4:1 (v/v) and by incubating at 100°C for 10 mins. The denatured protein samples were stored at -20°C.

Table 15. 5×SDS loading buffer recipe

Components	Final concentration
Tris-HCl (2 M, pH 6.8)	375 mM
SDS	10% (w/v)

Glycerol	50% (v/v)
β-Mercaptoethanol	12.5% (v/v)
Bromophenol blue	0.02% (w/v)

Polyacrylamide gels were prepared by filling the space between glass plates with 10% of resolving gel mixture (Table 16) and gel was solidified for 30 mins. Later 6% of stacking gel mixture (Table 17) was added and comb was inserted. The gel was allowed to polymerize for 1 hour. Protein samples and rainbow marker were loaded in the wells of the gel. The gel electrophoresis was carried out in running buffer (Table 18) at 100-130 v for 2-3 hours for protein to separate well based on their molecular size.

Table 16. Resolving gel (10%) components

Resolving gel (10%) component	Volume	Final concentration
Tris-Cl (1.5 M, pH 8.9)	1.875 ml	375 mM
Acrylamid 30% (w/v)	1.5 ml	10% (w/v)
SDS 10% (w/v)	75 µl	0.1% (w/v)
APS 10% (w/v)	37.5 µl	0.05% (w/v)
TEMED	7.5 µl	0.1% (w/v)
H2O	3.5 ml	-

Table 17. Stacking gel (6%) components

Stacking gel (6%) component	Volume	Final concentration
Tris-Cl (0.5 M, pH 6.8)	0.625 ml	375 mM
Acrylamid 30% (w/v)	0.5 ml	10% (w/v)
SDS 10% (w/v)	25 µl	0.1% (w/v)
APS 10% (w/v)	12.5 µl	0.05% (w/v)
TEMED	2.5 µl	0.1%
H2O	1.34 ml	-

Table 18. Running buffer components

Running buffer component	Final concentration
Tris-HCl	25 mM
Glycine	192 mM
SDS	10% (w/v) 0.1% (w/v)

3.2.11.4 Immunoblotting

Immunoblotting was performed by wet transfer method and the proteins on the SDS-PAGE were transferred to nitrocellulose membrane at 100V for 1 hour in blotting buffer (Table 19).

The membranes were removed from the blotting chamber and blocked with 5% non-fat milk or 5% BSA at RT for 1 hour. The membranes were washed with 1XTBS and incubated overnight at 4°C with primary antibodies, that were diluted in 1XTBST (Table 20) with 5% BSA. After overnight incubation, the membranes were washed with 1XTBST for 15 mins (3 times, 5 mins each). The membranes were incubated for 1 hour at RT with horse radish peroxidase (HRP) conjugated secondary antibodies (diluted in TBST with 5% BSA). After secondary antibody incubation, membranes were washed 3 times for 5 mins with 1XTBST.

Table 19. Blotting buffer recipe

Blotting buffer	Final concentration
Tris-HCl	50 mM
Glycine	40 mM
Methanol	20% (v/v)

Table 20. TBST buffer recipe

TBST buffer (pH 7.6) component	Final concentration
Tris-HCl	20 mM
NaCl	150 mM
Tween	0.1% (v/v)

3.2.11.5 Developing and densitometry analysis

The membranes were developed using substrates such as, enhanced chemiluminescence (ECL) kit or SuperSignal™ West Femto Maximum Sensitivity and by X-ray films. The protein band density was measured using BioDoc densitometry analyzer. The protein expression levels of target proteins were normalized to loading control GAPDH.

3.2.12 Zymography

3.2.12.1 Sample preparation from medium

Cell culture medium (400 µl) was centrifuged shortly (to remove cell debris) and supernatant was collected. To this supernatant 100 µl of 5X SDS buffer (without ß-mercaptoethanol) was added. The protein samples were stored in -20°C.

Table 21. Preparation of 5X SDS

Reagents	For 10 ml
Tris-Cl	3.75 ml
SDS	1 g
Glycerol	5 ml
PMSF	0.5 ml
H_2O	0.75 ml
Bromophenol blue	spatula

3.2.12.2 Sample preparation from cells

Cells were washed with PBS and 500 µl of 1X SDS buffer (without ß-mercaptoethanol) was added. The cell culture plates were kept on ice for 5 mins. Cell lysates were collected by cell scrapper and transferred to fresh Eppendorf tube. 100 µl of this cell lysate was transferred to a new Eppendorf tube (this was used for running loading control GAPDH) together with 25 µl of ß-mercaptoethanol and boiled for 10 mins at 100°C. The remaining samples were stored at -20°C.

3.2.12.3 Loading and running gel electrophoresis

The protein samples were loaded in the gel. The gel electrophoresis was carried out in running buffer (Table 18) at 100-130 v for 2-3 hours for protein to separate well based on their molecular size. The gel was carefully removed from the glass plated. Gel was washed 2 times for 30 mins with wash buffer (2.5% triton X-100, 0.02% NaN_3 mixed in water) and one time with incubation buffer (50mM Tris-HCl of pH7.6, 5mM $CaCl_2$, 0.02% NaN_3) for 20 mins. After washing the gel was incubated with fresh incubation buffer for 48 hours at 37°C. Then buffer was removed and gel was fixed and stained with coomassie blue solution (0.1% m/v coomassie, 30% methanol, 10% acetic acid, 60% H_2O) for 1.5 hours.

Afterwards the gel was distained with 1st wash buffer (30% methanol, 10% acetic acid, 60% H_2O) for 30 mins, followed by 2nd wash buffer (20% methanol, 10% acetic acid, 70% H_2O) for 1 hour and finally with 3rd wash buffer (10% methanol, 5% acetic acid, 85% H_2O) for 2 hours. The MMPs protein bands were viewed under BioDoc analyzer and photographed.

Table 22. Zymography gel recipe

Components	Stacking gel (2.5 ml)	Separating gel (6 ml)
30 % Acrylamide	500 µl	2 ml
1.5M Tris pH 8.9	-	1.5 ml
1M Tris pH 6.8	625 µl	-
H_2O	1.34 ml	1.8 ml
10% SDS	25 µl	60 µl
10% ammonium persulfate (APS)	12.5 µl	30 µl
TEMED	2,5 µl	6 µl
1% Gelatin	-	0.6 ml

3.2.13 P2Y2 ligand

MRS2768 is selective ligand/agonist for P2Y2 (EC50 = 1.89 µM). Displays no affinity for human P2Y4 or P2Y6 receptors. MRS2768 chemical name is Uridine-5'-tetraphosphate δ-phenyl ester tetrasodium salt. In vitro experiments were carried out using MRS2768 agonist. MRS2768 aliquots were stored at -80°C for long storage and -20°C for one month.

3.2.14 Statistical analysis

GraphPad Prism® v6.05 was used for all statistical analysis. Data are expressed as mean ±SEM. Significance was calculated using unpaired t-test to compare between two test groups or one-way ANOVA Dunnett test to analyze multiple test groups. The significance was considered when P value was < 0.05. Graphs are represented in column, grouped or contingency method of GraphPad Priam tool.

4 RESULTS

4.1 Gene expression profiling of GPCRs in human pulmonary vessels

4.1.1 High-throughput screening of 408 GPCRs with first set of primers

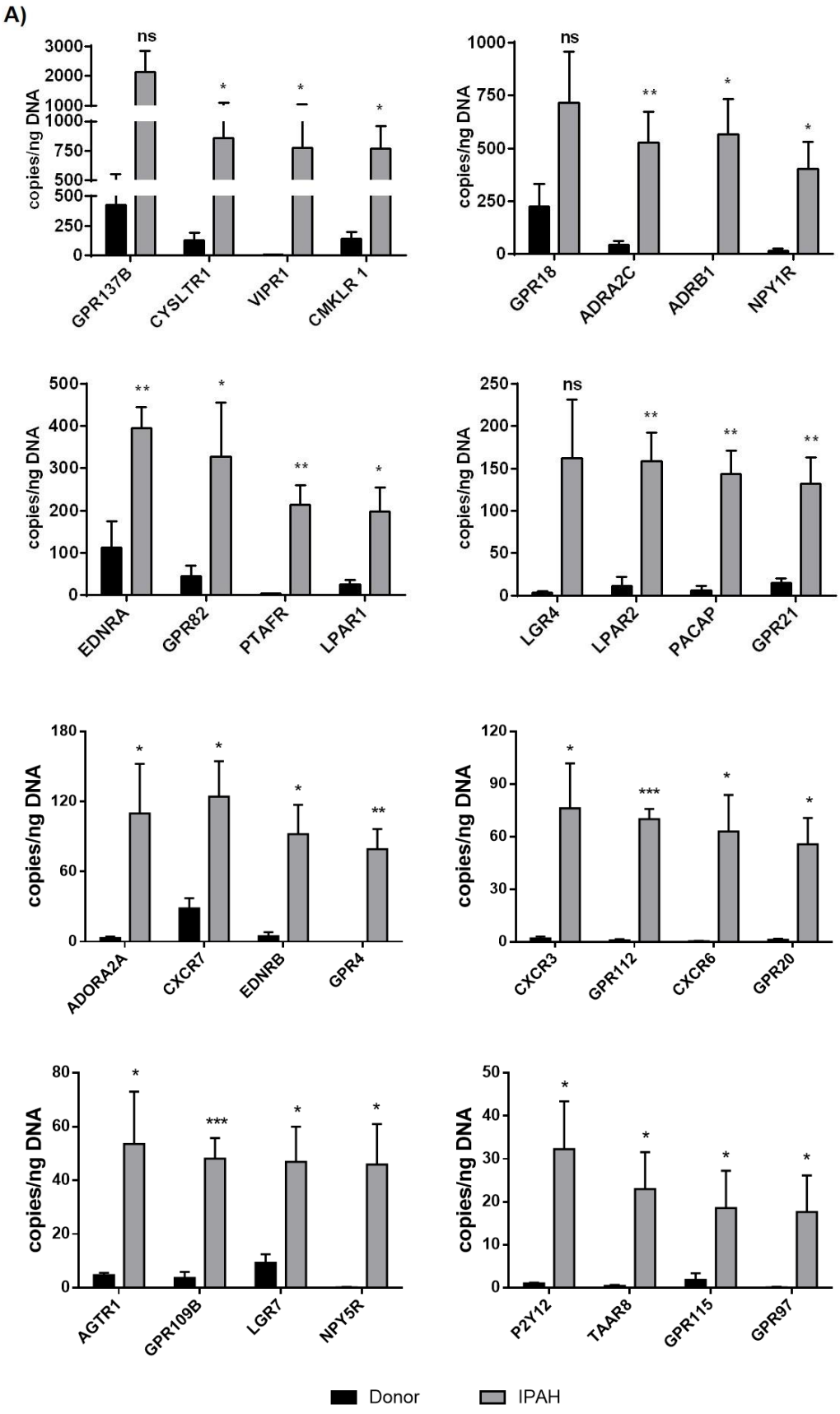
The expression of 408 GPCRs in laser micro-dissected human pulmonary vessels from donors (n-5) and IPAH patients (n=7) was investigated via a customized high-throughput PCR array. The mRNA expression level was represented as the absolute number of copies, which was normalized with genomic DNA from the respective GPCR gene. Initially, 40 candidates were identified from among 408 GPCRs (Figure 7).

4.1.1.1 Upregulated GPCRs in pulmonary vessels from IPAH patients versus donors

The high-throughput screening revealed 32 GPCRs that were upregulated in the pulmonary vessels from the IPAH patients compared to donors (Figure 7A). These included the leukotriene receptor (CYSLTR1), vasoactive intestinal peptide receptor (VIPR1), chemokine receptors (CMKLR1, CXCR7, CXCR3, CXCR6), adrenoceptors (ADRA2C, ADRB1), neuropeptide Y receptors (NPY1R, NPY5R), endothelin receptors (EDNRA, EDNRB), adenylate cyclase-activating polypeptide receptor (PACAP), platelet-activating factor receptor (PTAFR), lysophosphatidic acid receptors (LPAR1, LPAR2), leucine-rich repeat (LGR7), adenosine receptor (ADORA2A), angiotensin II receptor (AGTR1), purinergic receptor (P2Y12), trace amine receptor (TAAR8), and 10 orphan receptors (GPR137B, GPR18, GPR82, GPR21, GPR4, GPR112, GPR20, GPR109B, GPR97, GPR115). Of note, GPR109B has recently been deorphanized and is also known as niacin receptor 1 [158-160].

4.1.1.2 Downregulated GPCRs in pulmonary vessels from IPAH patients versus donors

The high-throughput screening revealed 8 GPCRs were downregulated in the pulmonary vessels of IPAH patients compared to donors (Figure 7B), and these included a prostaglandin E receptor (PTGER4), frizzled receptor (FZD1), serotonin receptor (HTR2B), sphingosine receptors (S1PR3, S1PR1), purinergic receptor (P2Y2), dopamine receptor (DRD1), and one orphan receptor (GPR110).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EDNRA
GPR82
PTAFR
LPAR1
LGR4
LPAR2
PACAP
GPR21
ADORA2A
CXCR7
EDNRB
GPR4
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CXCR3
GPR112
CXCR6
GPR20
AGTR1
GPR109B
LGR7
NPY5R
P2Y12
TAAR8
GPR115
GPR97
Donor
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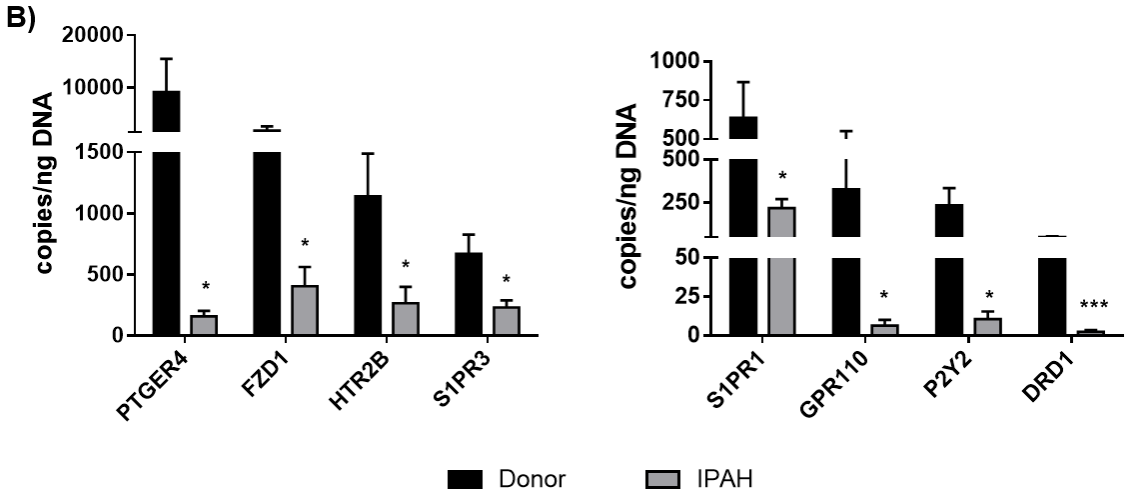


Figure 7. High-throughput screening of GPCRs in laser micro-dissected pulmonary vessels from IPAH patients and donors. The mRNA levels of GPCRs in the laser micro-dissected pulmonary arteries (diameter range: 50-100µm) from donors (black bars) and IPAH patients (grey bars) were acquired by high-throughput PCR array. The high-throughput screening data are represented in copies per ng of DNA after normalization to genomic DNA levels of the respective GPCR genes. **(A)** Upregulated GPCR gene expression (upper panel) in pulmonary arteries from IPAH patients compared to donors. Abbreviations for upregulated genes in IPAH patients: leukotriene receptor (CYSLTR1), vasoactive intestinal peptide receptor (VIPR1), chemokine receptors (CMKLR1, CXCR7, CXCR3, CXCR6), adrenoceptors (ADRA2C, ADRB1), neuropeptide Y receptors (NPY1R, NPY5R), endothelin receptors (EDNRA, EDNRB), adenylate cyclase-activating polypeptide receptor (PACAP), platelet-activating factor receptor (PTAFR), lysophosphatidic acid receptors (LPAR1, LPAR2), leucine-rich repeat (LGR7), adenosine receptor (ADORA2A), angiotensin II receptor (AGTR1), purinergic receptor (P2Y12), trace amine receptor (TAAR8), and 10 orphan receptors (GPR137B, GPR18, GPR82, GPR21, GPR4, GPR112, GPR20, GPR109B, GPR97, GPR115). **(B)** Downregulated GPCR gene expression (lower panel) in the pulmonary arteries of IPAH patients compared to donors. Abbreviations for downregulated genes in IPAH patients: prostaglandin E receptor (PTGER4), frizzled receptor (FZD1), serotonin receptor (HTR2B), sphingosine receptors (S1PR3, S1PR1), purinergic receptor (P2Y2), dopamine receptor (DRD1), and one orphan receptor (GPR110). All values are expressed as the mean ± SEM of n=5 in the donor group and n=7 in the IPAH patient group. *P < 0.05, **P < 0.01, ***P < 0.001 versus donors.

4.1.2 Validation of dysregulated GPCR expression by alternative sets of primers

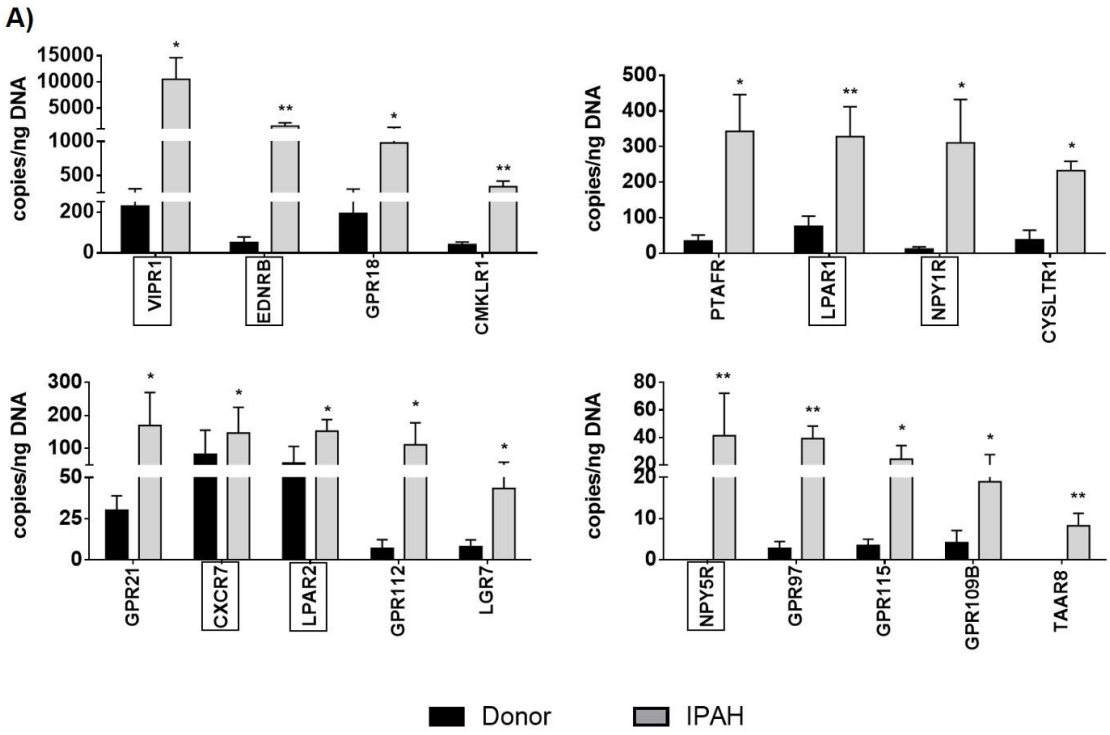
The expression of the 40 candidate GPCRs was validated with different primer sets using the same PCR array system employed for screening. Only 22 GPCRs demonstrated an expression pattern consistent with that seen for the previous array of IPAH patients versus donors. More specifically, there were 19 upregulated GPCRs (including 5 orphan receptors) and 3 downregulated candidates (Figure 8). The data confirmed the known dysregulation of certain GPCRs in PAH, which are marked with black boxes in Figure 8. Examples included VIPR1 and EDNRB. However, most of the candidates have not been previously reported in the field of PAH, and so they deserve further investigation.

4.1.2.1 Validation of upregulated GPCRs in the pulmonary vessels of IPAH patients

The consistently and significantly upregulated GPCR mRNA expression level in the IPAH patients (Figure 8A) involved the following GPCRs: vasoactive intestinal peptide receptor VIPR1, chemokine receptors (CMKLR1, CXCR7), platelet-activating factor receptor PTAFR, leukotriene receptor CYSLTR1, neuropeptide Y receptors (NPY1R, NPY5R), lysophosphatidic acid receptors (LPAR1, LPAR2), endothelin receptor EDNRB, trace amine receptor TAAR8, leucine-rich repeat LGR7, niacin receptor GPR109B, and 5 orphan receptors (GPR18, GPR21, GPR97 GPR112, GPR115).

4.1.2.2 Validation of downregulated GPCRs in the pulmonary arteries of IPAH patients

The downregulated GPCR mRNA expression level in the IPAH patients (Figure 8B) involved the following GPCRs: prostaglandin E receptor PTGER4, purinergic receptor P2Y2, and dopamine receptor DRD1.



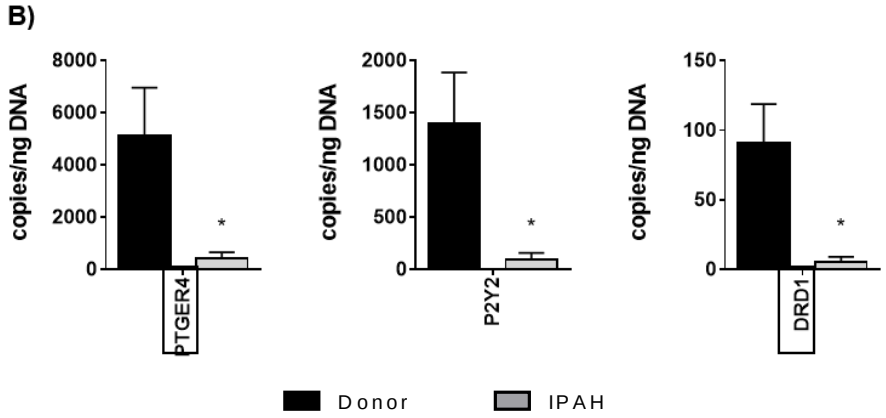


Figure 8. Validation of GPCRs with different primer sets in laser micro-dissected pulmonary vessels from IPAH patients and donors. The mRNA levels of the candidate GPCRs were validated using different sets of primers, and the mRNA levels were determined via a high-throughput PCR array in laser micro-dissected pulmonary arteries (diameter range: 50-100µm) from donors (black bars) and IPAH patients (grey bars). The validation data are represented in copies per ng of DNA after normalization to genomic DNA levels of the respective GPCR genes. The GPCRs already known in the PAH field are marked with black boxes. **(A)** Upregulated GPCR gene expression (upper panel) in the pulmonary arteries of IPAH patients compared to donors. Abbreviations for upregulated genes in IPAH patients: vasoactive intestinal peptide receptor VIPR1, chemokine receptors (CMKLR1, CXCR7), platelet-activating factor receptor PTAFR, leukotriene receptor CYSLTR1, neuropeptide Y receptors (NPY1R, NPY5R), lysophosphatidic acid receptors (LPAR1, LPAR2), endothelin receptor EDNRB, trace amine receptor TAAR8, leucine-rich repeat LGR7, niacin receptor GPR109B, and 5 orphan receptors (GPR18, GPR21, GPR97 GPR112, GPR115). **(B)** Downregulated GPCR gene expression (lower panel) in the pulmonary arteries of IPAH patients compared to donors. Abbreviations for downregulated genes in IPAH patients: prostaglandin E receptor PTGER4, purinergic receptor P2Y2, and dopamine receptor DRD1. All values are expressed as the mean ± SEM of n=5 in the donor group and n=7 in the IPAH patient group. *P < 0.05, **P < 0.01 versus donors.

4.2 Screening for candidate GPCRs in the PASMCs of donors and IPAH patients via real-time PCR

The abnormal proliferation, migration, and survival of PASMCs within the pulmonary arterial wall is a key pathological feature of vascular remodeling in IPAH. Therefore, it was important to investigate the expression patterns of the GPCR candidates identified by high-throughput array in the human PASMCs. The relative mRNA expressions of 22 candidate GPCRs were examined in cultured PASMCs from donors and IPAH patients using quantitative PCR.

4.2.1 Expression of the upregulated candidate GPCRs in the PASMCs of donors and IPAH patients

The qPCR results revealed upregulation of VIPR1 and LGR7 mRNA expression in the PASMCs of IPAH patients compared to the donor PASMCs. However, CYSLTR1, which

exhibited upregulation in the pulmonary vessels from IPAH patients (high-throughput array), was found to be downregulated in the PASMCs from IPAH patients relative to those of the donors. No significant differences between the PASMCs from the IPAH patients and the donors were noted for the 16 remaining GPCRs (Figure 9).

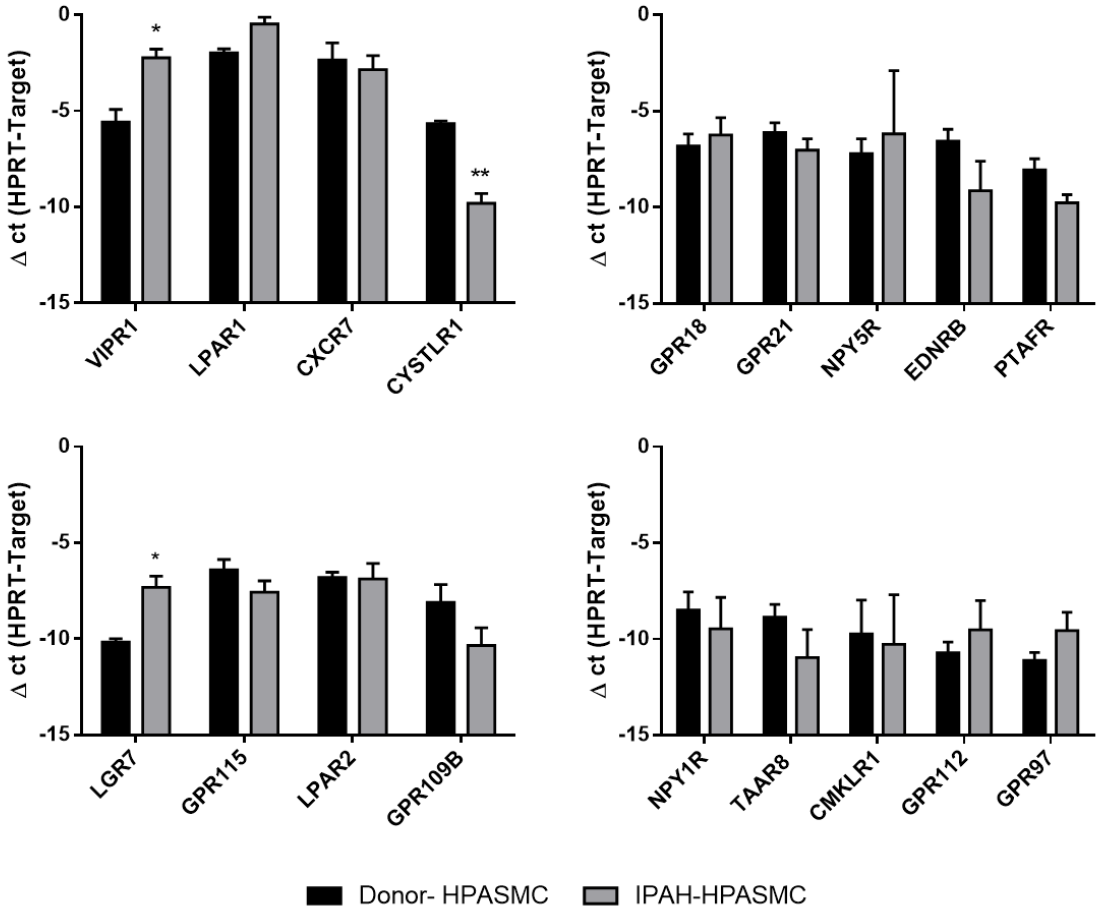


Figure 9. Expression of upregulated candidate GPCRs in the PASMCs of IPAH patients and donors as identified by quantitative PCR. The relative mRNA expressions of the candidate GPCRs in cultured PASMCs from IPAH patients (grey bars) and donors (black bars) were analyzed using quantitative PCR (qRT-PCR). The mRNA expression of each GPCR is represented as the delta Ct value after normalization with house-keeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT). Vasoactive intestinal peptide receptor (VIPR1) and leucine-rich repeat (LGR7) were significantly upregulated in the cultured PASMCs from the IPAH patients. A leukotriene receptor (CYSLTR1) was significantly downregulated in the cultured PASMCs from the IPAH patients. All values are expressed as the mean ± SEM of n=5 in the donor PASMCs and n=6 in the IPAH PASMCs. *P < 0.05, **P < 0.01 versus donor PASMC.

4.2.2 Expression of the downregulated candidate GPCRs in the PASMCs of donors and IPAH patients

The qPCR results revealed downregulation of the purinergic receptor (P2Y2) and dopamine receptor (DRD1) in the PASMCs of the IPAH patients compared to those from the donors

(Figure 10). In contrast, mRNA expression of the prostaglandin E receptor (PTGER4) was not detected in the PASMCs from either the IPAH patients or the donors, indicating that the PTGER4 might only be expressed in other vasculature compartment cell types.

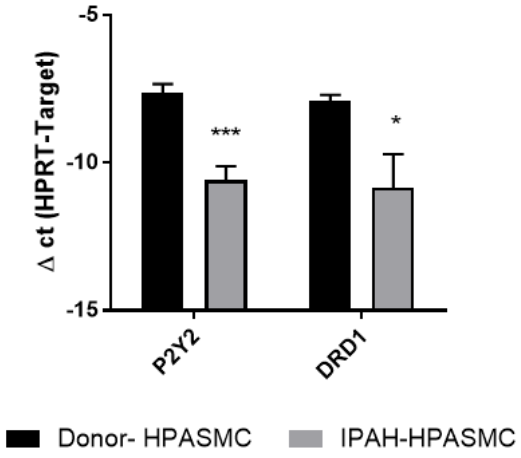


Figure 10. Expression of downregulated candidate GPCRs in PASMCs from IPAH patients and donors as identified by quantitative PCR. The relative mRNA expressions of the candidate GPCRs in cultured PASMCs from IPAH patients (grey bars) and donors (black bars) were analyzed using quantitative PCR (qRT-PCR). The mRNA expression of each GPCR is represented as the delta Ct value after normalization with house-keeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT). The purinergic receptor (P2Y2) and dopamine receptor (DRD1) were significantly downregulated in the cultured PASMCs from the IPAH patients. All values are expressed as the mean ± SEM of n=5 in the donor group and n=6 in the IPAH patient group. *P < 0.05, ***P < 0.001 versus donor PASMCs.

4.3 si-RNA knockdown of GPCRs in PASMCs

Six GPCRs that have not been well studied in PAH were selected and further examined in terms of their functional role in human PASMCs. To that end, the transient knockdown of GPCRs (GPR 21, P2Y2, PTAFR, CYSLTR1, LGR7, GPR115) was established in cultured PASMCs from donors. All 6 selected GPCRs were successfully knockdown by 50 nM siRNA. Around 83-96% knockdown of GPCR mRNA is achieved, and no changes occurred when the cells were treated with scramble siRNA (Figure 11).

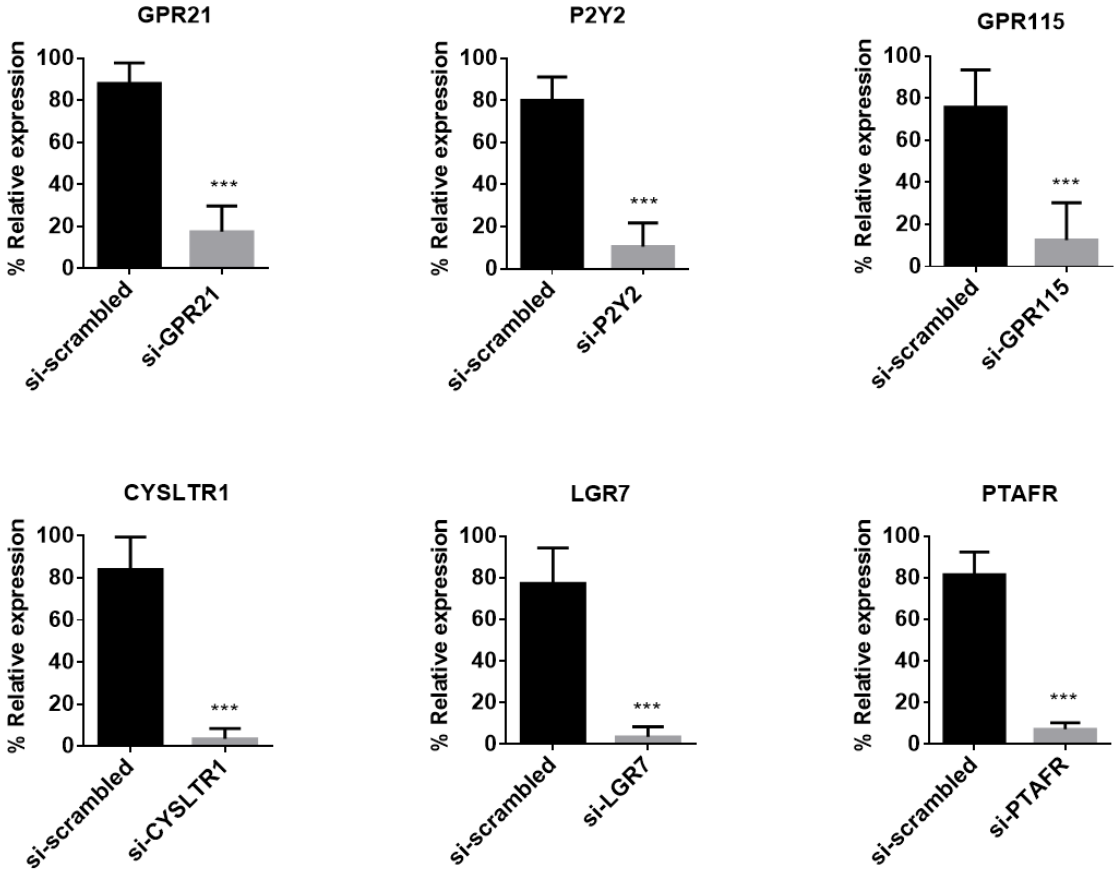


Figure 11. Knockdown of GPCRs by specific siRNAs. Human PASMCs from donors were transiently transfected with 50nM of si-GPCRs for 48 hours. Human PASMCs treated with si-scramble served as a negative control. Relative mRNA levels of GPCRs in human PASMCs after 48 hours of transfection were determined by quantitative PCR (qRT-PCR). The mRNA expression of each GPCR is indicated as a percentage of si-scramble after normalization to HPRT. mRNA expression of respective GPCRs after si-scramble transfection are represented in black bars. mRNA expression of respective GPCRs after respective si-GPCRs transfection are represented in grey bars. The knockdown efficiency of the respective GPCR gene expressions were as follows: si-GPR21= 83.32%, si-P2Y2= 88.50%, si-GPR115= 96.45%, si-CYSLTR1= 96.43%, si-LGR7= 96.43%, and si-PTAFR= 92.83%. All values are expressed as the mean ± SEM of n=4 in each group. ***P <0.001 versus si-scramble. Abbreviations: orphan GPCRs (GPR21, GPR115), purinergic receptor (P2Y2), leukotriene receptor (CYSLTR1), leucine-rich repeat (LGR7), platelet activating factor receptor (PTAFR), and small (or short) interfering RNA (si-RNA).

4.4 Proliferation of PASMCs after the siRNA knockdown of GPCRs

To examine the functional role of GPCRs in the proliferation of PASMCs, specific GPCRs were knocked down using siRNA transfection. The proliferation of PASMCs was determined after serum stimulation. No differences in PASMC proliferation occurred between the non-transfected (mock) and scrambled siRNA. The serum-stimulated (+FCS) proliferation of

PASMCs was slightly inhibited after the siRNA knockdown of GPR115, LGR7, and PTAFR (Figure 12). Knockdown of the P2Y2 receptor, which was downregulated in both the pulmonary vessels and PASMCs from the IPAH patients, did not produce any differences in the proliferation of the donor PASMCs (Figure 12). Under the serum-starved condition, PASMC proliferation was constant and at a basal level in both all siRNA knockdown scenarios and mock controls.

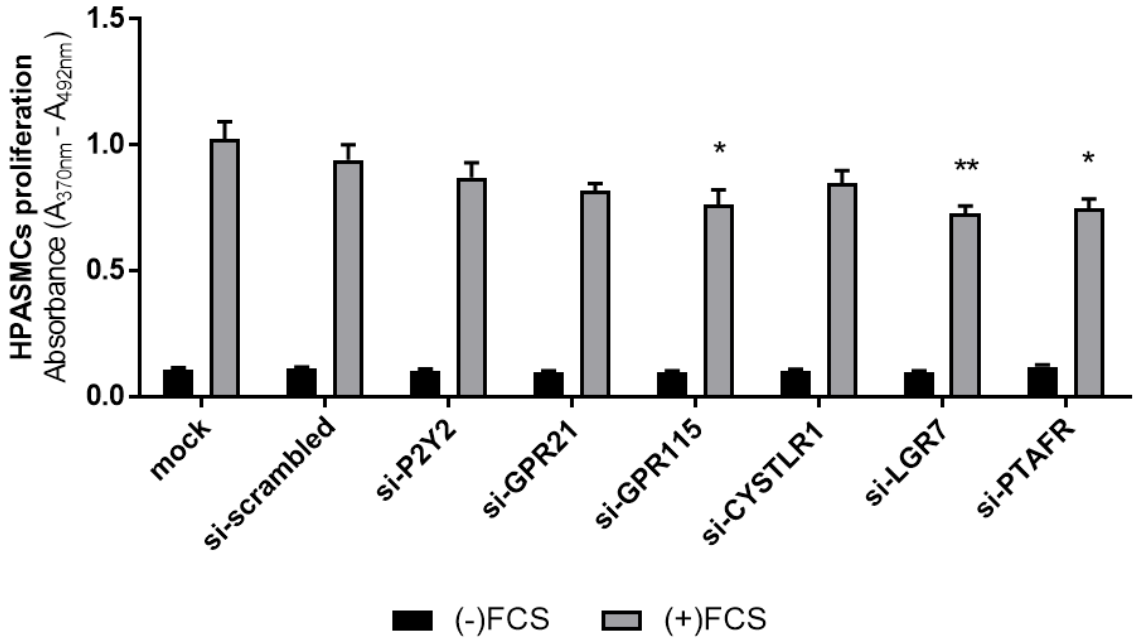


Figure 12. Effect of GPCR siRNA knockdown on human PASMC proliferation. Human PASMCs from donors were serum-starved for 24 hours and stimulated with 10% fatal calf serum (FCS) to induce cellular proliferation. The black bars indicate serum starvation (-FCS), the grey bars indicate serum stimulation (10% FCS). The siRNA for P2Y2, GPR21, GPR115, CYSLTR1, LGR7, and PTAFR was transfected for 24 hours to knockdown the respective GPCR gene expression. Before the end of stimulation, BrdU incorporation was performed for 4 hours to determine the rate of human PASMC proliferation. Knockdown of GPR115, LGR7, and PTAFR gene expression led to significant inhibition of serum-stimulated (10% FCS) human PASMC proliferation. All values are expressed as the mean ± SEM of n=6 in each group. *P < 0.05, **P < 0.01 versus si-scrambled. Abbreviations: orphan G protein-coupled receptor (GPR115), leucine-rich repeat (LGR7), platelet-activating factor receptor (PTAFR), small (or short) interfering RNA (si-RNA).

4.5 Functional role of the purinergic receptor (P2Y2) in human PASMCs

Purinergic receptor P2Y2 was the most downregulated in the pulmonary vessels and PASMCs from IPAH patients. Hence, P2Y2 was further studied using human PASMCs to better understand its functional role in IPAH and pulmonary vascular remodeling.

4.5.1 Effect of a P2Y2 agonist (MRS2768) on the basal proliferation of PASMCs

The knockdown of the P2Y2 receptor did not influence PASMC proliferation under serum-starved or serum-stimulated conditions as compared to the scrambled siRNA (Figure 12). Therefore, it was necessary to investigate whether the activation of the P2Y2 receptor by its ligand influences PASMCs proliferation. To that end, the PASMCs were serum-starved for 24 hours and treated with increasing concentrations of P2Y2 receptor-specific agonist MRS2768 (uridine-5'-tetraphosphate δ-phenyl ester tetrasodium salt) for 24 hours. The basal proliferation of the PASMCs is decreased when treated with 5, 10, and 50µM of MRS2768 compared with the non-treated control (Figure 13). However, at higher dosages (100 µM and 200 µM), MRS2768 had no effect on PASMC proliferation. This might be due to P2Y2 receptor desensitization triggered by exposure to an excess amount of the agonist.

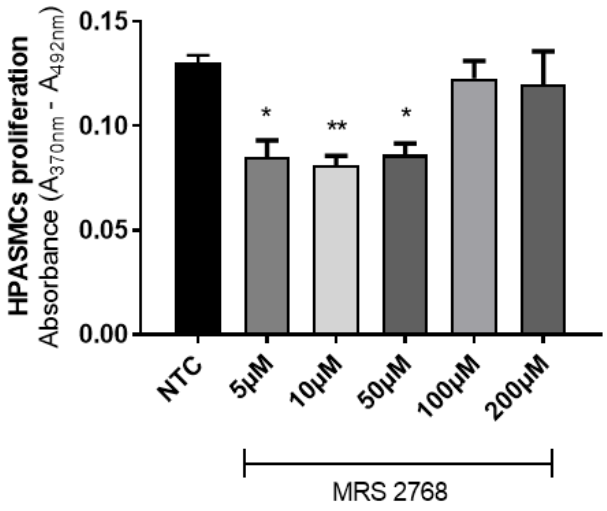


Figure 13. Anti-proliferative effect of selective P2Y2 agonist MRS2768 in human PASMCs. Human PASMCs from donors were serum-starved for 24 hours and treated for 24 hours with increasing concentrations of MRS2768. During the last 4 hours of stimulation, BrdU incorporation was performed to determine the rate of human PASMC proliferation. Human PASMC proliferation was inhibited by 34.65%, 37.71%, and 33.91% when treated with different concentrations of MRS2768 (5µM, 10µM, and 50µM) respectively. All values are expressed as the mean ± SEM of n = 4 in each group *P < 0.05, **P < 0.01 versus non-treated cells (NTC).

4.5.2 The P2Y2 ligand MRS2768 inhibited PASMC proliferation induced by serum and PDGF

Uncontrolled PASMCs proliferation is a pathological feature of progressive pulmonary vascular remodeling, and so the effect of MRS2768 on donor PASMC proliferation induced by serum or PDGF was determined. The results indicated that MRS2768 (10 µM) inhibited serum- and PDGF-induced PASMC proliferation by 28% and 25%, respectively, as compared with the NTCs (Figure 14 A and B).

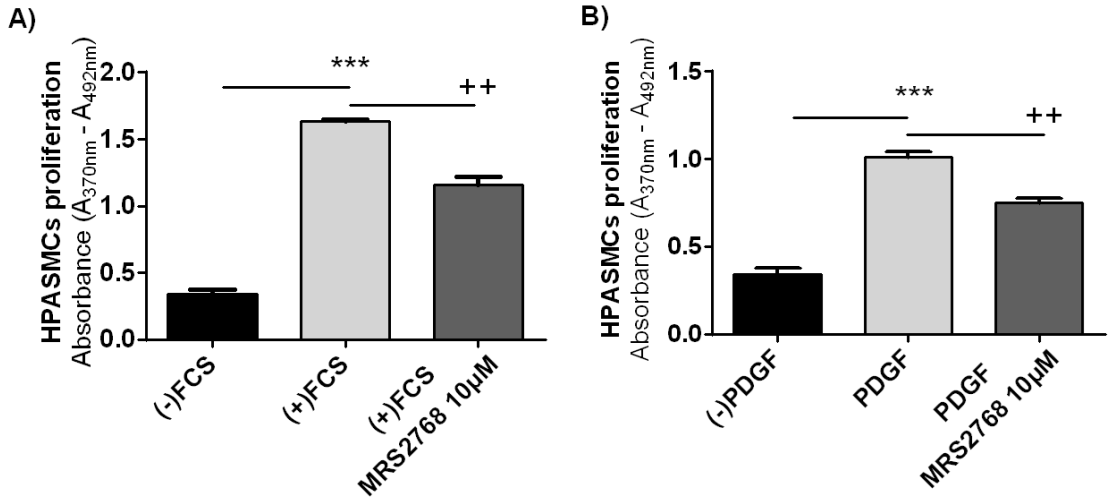
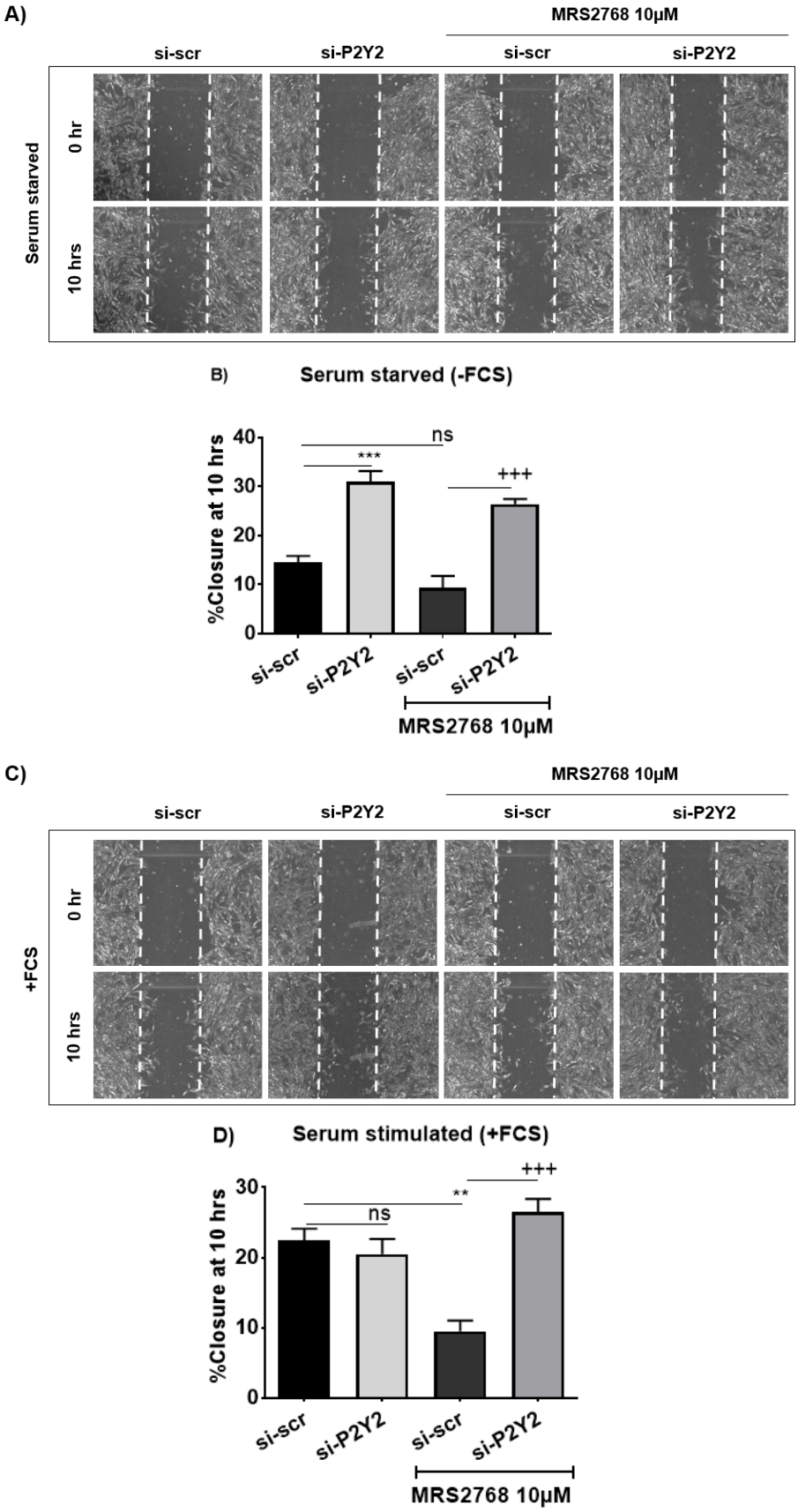


Figure 14. MRS2768 (10 µM) attenuated FCS- and PDGF-induced proliferation of human PASMCs. Proliferation of donor PASMCs induced by **A)** 10% fatal calf serum (FCS) and **B)** 30ng/ml platelet-derived growth factor (PDGF). The PASMCs underwent serum starvation for 24 hours and were subsequently stimulated with 10% FCS or PDGF for 24 hours in the presence or absence of 10 µM of MRS2768. In the last 4 hours of stimulation/treatment, BrdU incorporation was performed. The BrdU proliferation results indicated a 473% increase in serum-induced (10% FCS) PASMC proliferation and a 292% increase in PDGF-induced PASMC proliferation relative to the serum-starved (-FCS) PASMCs. Moreover, MRS2768 (10µM) significantly attenuated serum-induced (10%FCS) and PDGF-induced (30ng/ml) PASMC proliferation, resulting in boosts of 10% and 25%, respectively. All values are expressed as the mean ± SEM of n = 3 in each group, ***P < 0.001 versus serum-starved (-FCS); ++P < 0.01 versus serum-stimulated (+FCS)/PDGF-stimulated.

4.5.3 The P2Y2 ligand MRS2768 inhibited PASMC migration induced by serum and PDGF

Uncontrolled PASMC migration is another pathological feature of progressive pulmonary vascular remodeling. The effect of PASMC migration was determined by scratch wound healing assay, with human PASMCs from donors treated with 10µM of MRS2768 (ligand activates P2Y2 receptor) after the transfection of si-P2Y2 (P2Y2 receptor gene knockdown) and si-scrambled (presence of P2Y2 receptor). Real-time microscopy monitoring revealed that the knockdown of the P2Y2 receptor increased wound closure by 50% and 35% relative to the si-scrambled control in serum-starved (-FCS; Figure 15 A and B) and PDGF-stimulated (Figure 15 E and F) PASMCs, respectively. While MRS2768 via P2Y2 inhibited PASMC wound closure by 20% under the basal condition (-FCS; Figure 15 A and B) compared with MRS2768 non-treated si-scr PASMCs. MRS2768 via P2Y2 significantly inhibited serum-induced (+FCS) by 65% (Figure 15 C and D) or PDGF-induced PASMC wound closure by 50% (Figure 15 E and F). In addition, MRS2768 failed to attenuate basal, or serum- or PDGF-induced, PASMC wound closure after the knockdown of P2Y2 receptor as compared to MRS2768-treated si-scrambled PASMCs (Figure 15 A-F).


A)
MRS2768 10μM
si-scr
si-P2Y2
si-scr
si-P2Y2
Serum starved
0 hr
10 hrs
B)
Serum starved (-FCS)
%Closure at 10 hrs
40
30
20
10
0
ns
***
+++
si-scr
si-P2Y2
si-scr
si-P2Y2
MRS2768 10μM
C)
MRS2768 10μM
si-scr
si-P2Y2
si-scr
si-P2Y2
+FCS
0 hr
10 hrs
D)
Serum stimulated (+FCS)
%Closure at 10 hrs
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si-scr
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si-P2Y2
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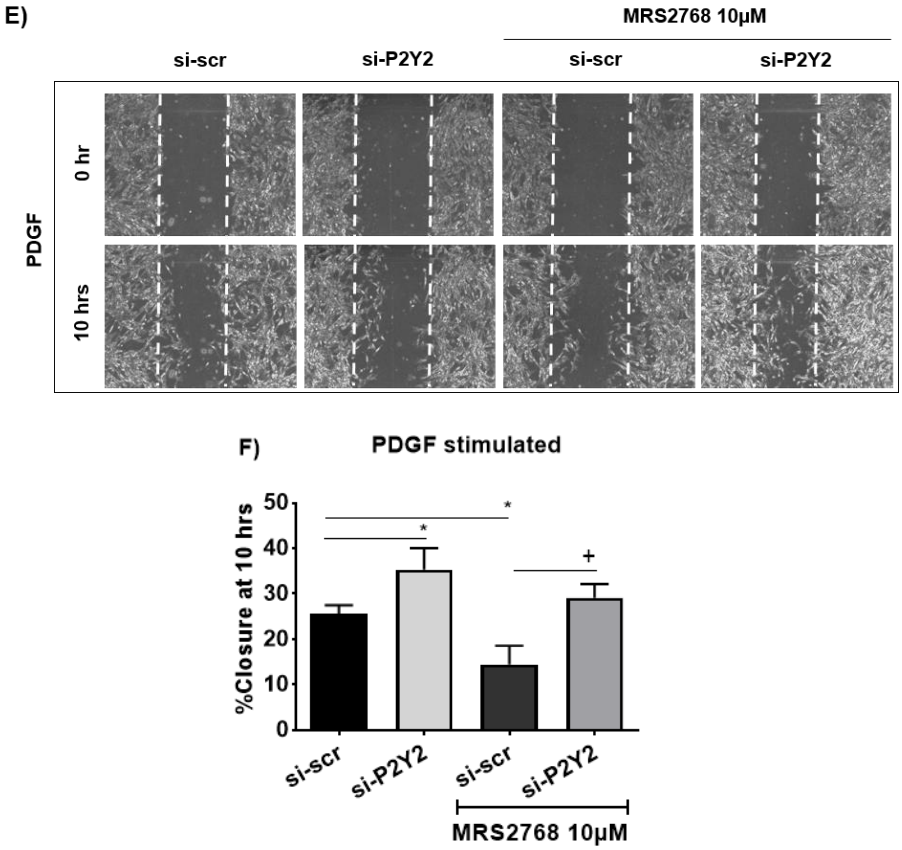

Figure 15. MRS2768 inhibited human PASMC wound healing induced by FCS and PDGF. Human donor PASMCs were transfected by si-scr (scrambled) and si-P2Y2 receptor. They were then serum-starved for 24 hours and a scratch was made in the center of each well. Following the scratch, the PASMCs were either **(A, B)** serum-starved (-FCS), **(C, D)** stimulated with 5% fetal calf serum (FCS), or **(E, F)** stimulated with 30ng/ml platelet-derived growth factor (PDGF) for 10 hours. Wound healing was monitored from the 0 hour to 10 hours using a live-cell imaging microscope, and images of each scratch were captured every hour, with the wound in the same position. Wound closure after 10 hours is expressed as a percentage of closure at the 0 hour. Representative live-cell images **(A, C, E)** at 0 hours (dotted line) and 10 hours are shown. Graphical representations are given in **B, D, and F,** respectively. When the P2Y2 receptor gene expression was knocked down, the migration of donor PASMCs increased in the serum-starved condition **(A, B)** and the PDG stimulation condition **(E, F)** (si-scr versus si-P2Y2). The MRS2768 (10µM) attenuated serum-induced **(C, D)** and PDGF-induced **(E, F)** migration in the presence of endogenous P2Y2 receptor expression (si-scr versus si-scr MRS2768 10µM). In contrast, MRS2768 (10µM) failed to attenuate basal, or serum- or PDGF-induced, PASMC migration when the P2Y2 receptor gene expression was knocked down (si-scr MRS2768 10µM versus si-P2Y2 MRS2768 10µM). All values are given as the mean ± SEM of n= 5 per group. *P < 0.05, **P < 0.01, ***P < 0.001 versus si-scrambled; +P < 0.05, +++P < 0.001 versus si-scrambled treated with MRS2768 (10µM). Abbreviations: non-significant (ns), purinergic receptor (P2Y2), small (or short) interfering RNA (si-RNA), and siRNA scrambled (si-scr).

Additionally, cell migration was validated by transwell migration assay, and the results demonstrated a 45% and 25% increase in serum-starved (-FCS) and PDGF-induced

migration, respectively, after P2Y2 knockdown in the PASMCs relative to the si-scrambled transfected PASMCs. In contrast, MRS2768 inhibited FCS- and PDGF-induced migration by 25% and 35%, respectively (Figure 16), and it failed to attenuate basal (-FCS), serum-induced (+FCS), or PDGF-induced PASMC migration when P2Y2 was knocked down. (Figure 16). The transwell migration assay presented results consistent with the scratch wound closure results. These data strongly suggest that MRS2768 suppresses FCS- and PDGF-induced PASMC migration through the P2Y2 receptor.

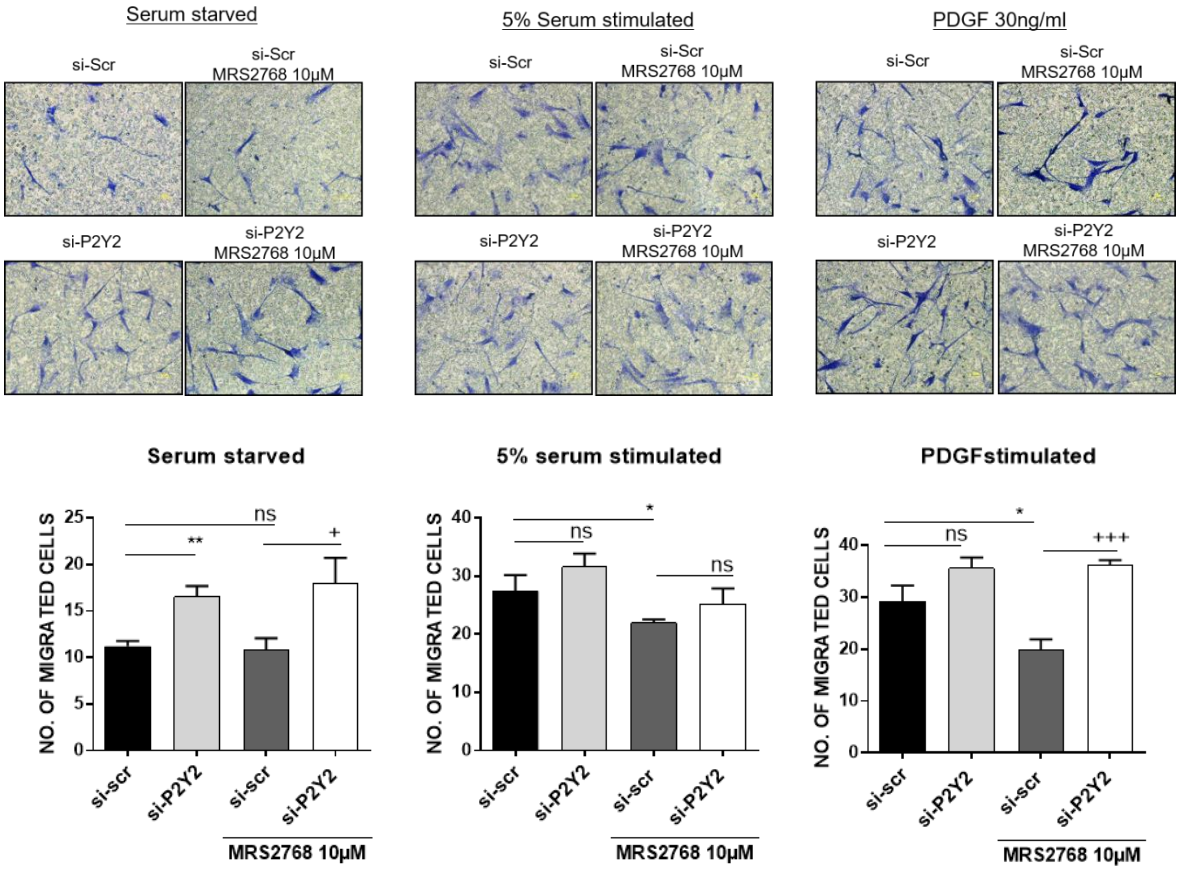

Figure 16. MRS2768 inhibited human PASMC migration induced by FCS and PDGF. Transwell migration of donor PASMCs under serum-starved (-FCS) conditions was induced by 5% fetal calf serum (FCS) and 30ng/ml platelet-derived growth factor (PDGF). The migration rate after 10 hours is expressed in terms of the number of migrated cells. Representative images of cells on transwell membranes were taken using light microscopy (20X). The graphical representation of each group is depicted below. When the P2Y2 receptor gene expression was knocked down, the migration of donor PASMCs increased in serum-starved condition. The MRS2768 (10μM) attenuated serum-induced or PDGF-induced migration in the presence of endogenous P2Y2 receptor expression (si-scr versus si-scr MRS2768 10μM). In contrast, MRS2768(10μM) failed to attenuate basal or PDGF-induced PASMC migration when the P2Y2 receptor gene expression was knocked down (si-scr MRS2768 10μM versus si-P2Y2 MRS2768 10μM). All values are given as the mean ± SEM of n= 5 per group. *P < 0.05, **P < 0.01 versus MRS 2768 untreated si-scrambled; +P < 0.05, +++P < 0.001 versus si-scrambled

treated with MRS2768. Abbreviations: non-significant (ns), purinergic receptor (P2Y2), small (or short) interfering RNA (si-RNA), and siRNA scrambled (si-scr).

4.5.4 P2Y2 ligand MRS2768 inhibited matrix metalloproteinases 2 (MMP-2) in PASMCs

In addition, MMP-2 is involved in PASMC migration [161]. The MMP activity was examined by gelatin zymography. The zymography results did not reveal any changes in active MMP-2s (62 kDa) in the PASMCs after P2Y2 knockdown (si-P2Y2) as compared with the control (si-scrambled). When treated with the P2Y2 ligand (MRS2768) in the presence of its receptor, active MMP-2 levels decreased compared to the non-MRS2768 treated condition, although the difference was not significant. In contrast, MRS2768 failed to reduce active MMP-2 levels and significantly increased the amount of active MMP-2 in the knockdown of the P2Y2 receptor (Figure 17). Taken together, the data revealed that MRS2768 slightly reduced MMP2 activity in the presence of P2Y2. Furthermore, in the case of P2Y2 receptor knockdown, MRS2768 failed to attenuate MMP2 activity in the PASMCs. Therefore, MRS2768 and P2Y2 might play a role in migration involving MMP2 activity.

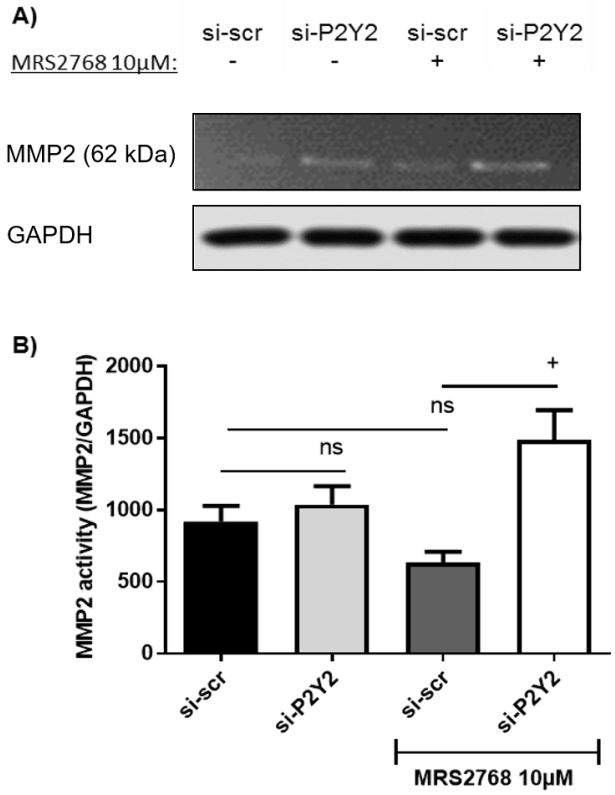

Figure 17. Matrix metalloproteinases 2 (MMP-2) activity in human PASMCs analyzed using gelatin zymography. The level of active MMP-2 is determined in human donor PASMCs culture medium either in the presence of P2Y2 receptor or knockdown-induced absence of the P2Y2 receptor both with or without MRS2768. **(A)** Gelatin zymography gels representing MMP2 (above) protein expression from the PASMC culture medium and a western blot representing GAPDH (below) protein expression in the PASMCs. **(B)** Densitometry and

quantification of MMP2 zymography with GAPDH serving as the loading control. Zymography analysis revealed that MRS2768 slightly reduced MMP2 activity in the presence of P2Y2 (si-scr versus si-scr MRS2768 10µM) and failed to reduce MMP2 activity when the P2Y2 receptor expression was knocked down (si-scr MRS2768 10µM versus si-P2Y2 MRS2768 10µM). The enzyme activities of MMP-2 are expressed as the mean ± SEM of n= 3 per group. +P < 0.05 versus si-scrambled treated with MRS2768 10µM. Abbreviations: non-significant (ns), purinergic receptor (P2Y2), small (or short) interfering RNA (si-RNA), and siRNA scrambled (si-scr).

4.5.5 P2Y2 ligand MRS2768 increased intracellular cAMP in PASMCs

Cyclic adenosine monophosphate plays a significant role in vascular SMC relaxation and proliferation. Hence, the intracellular cAMP level was analyzed by enzyme immunoassay (EIA). The human donor PASMCs were transfected with either si-scrambled or si-P2Y2 and treated with or without MRS2768 (10µM). The results demonstrated a basal intracellular cAMP level of 2.99 (pmol/mL protein) in the si-scrambled transfected PASMCs and 25.23 (pmol/mL protein) in the MRS2768-treated (10µM) si-scrambled transfected PASMCs. When the P2Y2 receptor gene expression was knocked down (si-P2Y2), MRS2768 failed to increase the cAMP levels in the PASMCs (Figure 18). These results confirmed that MRS2768 activates cAMP production via the P2Y2 receptor.

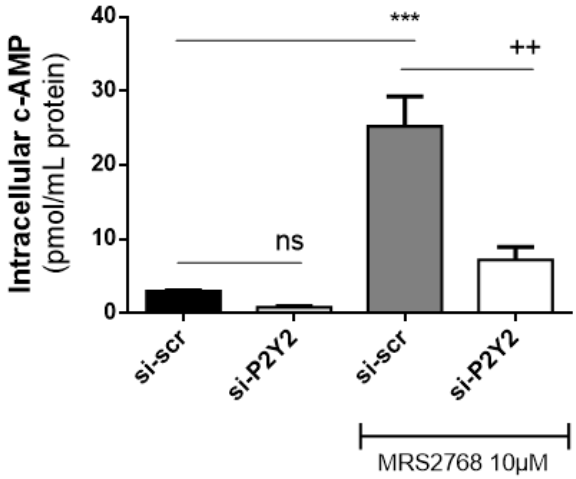

Figure 18. The P2Y2 ligand MRS2768 resulted in the accumulation of intracellular cAMP in human PASMCs. Donor PASMCs were transfected with si-scr or si-P2Y2, starved for 24 hours, and treated with MRS2768 (10µM) for 24 hours to perform cAMP EIA assay. The cAMP contents of the PASMCs' lysates are given as pmol/mL protein. The MRS2768 (10µM) activated cAMP production via the P2Y2 receptor (si-scr versus si-scr MRS2768 10µM). The MRS2768 (10µM) failed to increase cAMP production when the P2Y2 receptor expression was knocked down (si-scr MRS2768 10µM versus si-P2Y2 MRS2768 10µM). All values are given as the mean ± SEM of n= 3 per group. **P < 0.01 versus si-scrambled untreated; +P < 0.05, +++P < 0.001 versus si-scrambled treated with MRS2768 10µM. Abbreviations: non-significant (ns), purinergic receptor (P2Y2), small (or short) interfering RNA (si-RNA), and siRNA scrambled (si-scr).

4.6 Downstream signaling pathways of P2Y2

4.6.1 MRS2768 increased exchange protein directly activated by cAMP (EPAC) in PASMCs

The EPAC group is a newly described class of guanine nucleotide exchange factors (GEFs) that contain a cAMP-binding domain(s). Cyclic adenosine monophosphate was demonstrated to inhibit the rho-associated coiled-coil-containing protein kinase 1 (ROCK1) pathway through EPAC [162]. Western blot analysis from cultured human PASMCs from donors revealed that MRS2768 (10µM) increased EPAC protein expression both in serum-starved (-FCS) and serum-stimulated (+FCS) conditions (Figure 19).

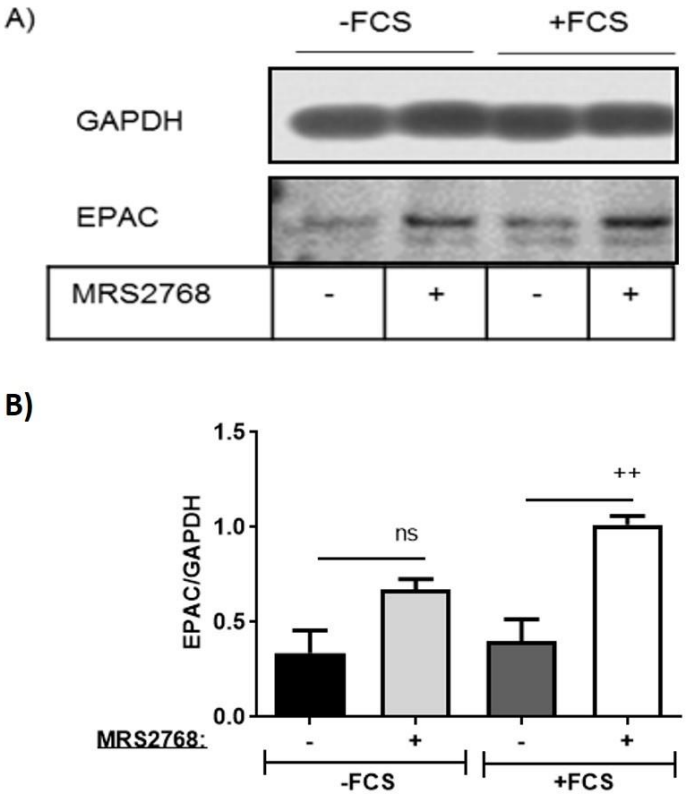

Figure 19. MRS2768 (10µM) increased EPAC protein expression in human PASMCs. Protein expression of EPAC is determined using western blot. GAPDH expression is used as the loading control. **(A)** Representative western blot of EPAC and GAPDH. **(B)** Densitometry analysis of EPAC protein expression. The experiment was performed after 24 hours of serum-starvation, followed by FCS stimulation (10%FCS) for 24 hours, in the absence or presence of MRS2768 (10µM). The MRS2768 increased EPAC protein expression in both serum-starved (-FCS) and serum-stimulated (+FCS 10%) conditions, although the results were only significant in the latter case. All values are given as the mean ± SEM of n = 3 per group. ++P < 0.01 versus NTCs. Abbreviations: non-significant (ns), exchange protein directly activated by cAMP (EPAC), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), fatal calf serum (FCS), and serum starvation (-FCS).

4.6.2 MRS2768 attenuated ROCK1 and myosin light chain 1 (MLC1) phosphorylation in PASMCs

Activation of the RhoA and ROCK1 pathway led to MLC phosphorylation, resulting in vascular SMC contraction and migration. Previous studies have reported that ROCK1 is inhibited by cAMP via EPAC [163]. It is already known from our previous results, that 10µM of MRS2768 activates cAMP (Figure 18) and increases EPAC protein expression (Figure 19). Western blot analysis from the cultured human donor PASMCs revealed that MRS2768 (10µM) significantly reduced ROCK protein expression (Figure 20 A and B) and serum-induced (+FCS) MLC phosphorylation (Figure 20 C and D). In contrast, no significant differences regarding the protein expression of ROCK1 and MLC phosphorylation in serum-starved (-FCS) PASMCs (Figure 20) were found. Taken together, these results suggest that MRS2768 activates the EPAC/cAMP cascade and block ROCK/MLC activation which reduces vascular SMC migration.

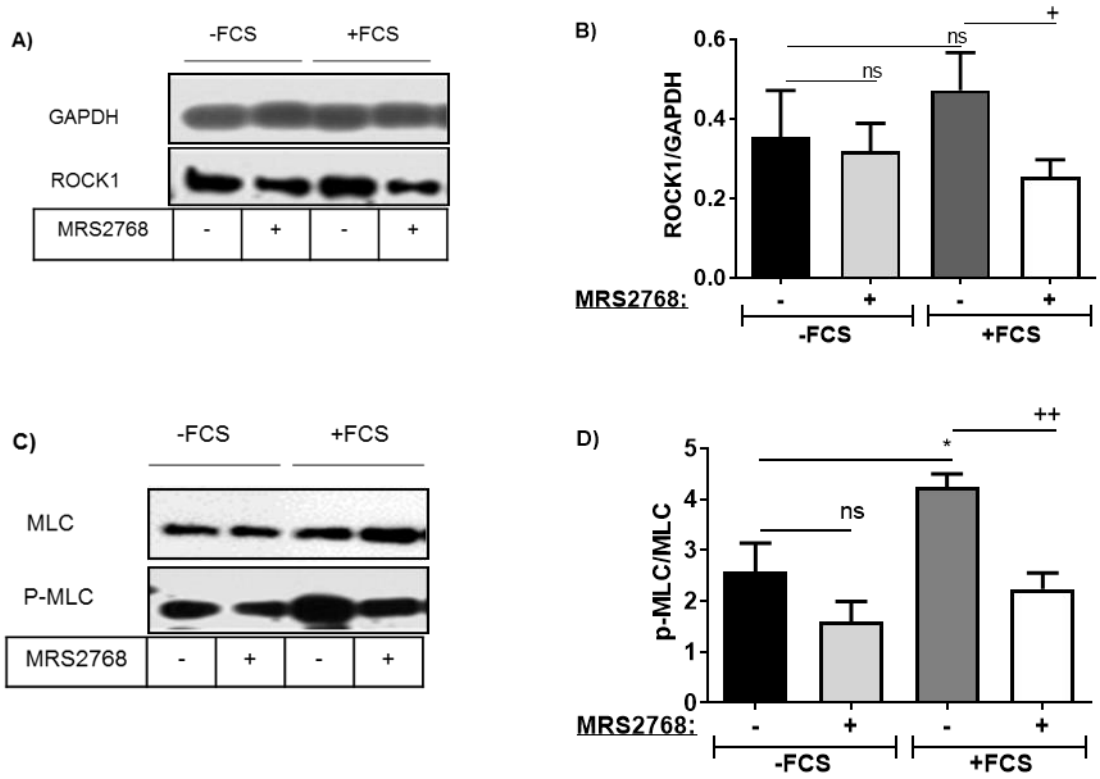

Figure 20. MRS2768 attenuated ROCK1 expression and MLC1 phosphorylation in human PASMCs. (A, B) Western blot analysis of ROCK1 protein expression, with GAPDH serving as the loading control. **(C, D)** Western blot analysis of phospho-MLC1 expression, with MLC1 serving as total protein. **(B, D)** Densitometry analysis of ROCK1 and p-MLC1 protein expression. The experiment was performed on donor PASMCs stimulated with or without 10% FCS for 24 hours in the absence or presence of MRS2768 (10µM). The MRS2768 significantly reduced ROCK protein expression and MLC phosphorylation induced by serum (+FCS 10%) in the PASMCs. All values are given as the mean ± SEM of n = 3 per group. *P < 0.05 serum stimulated

versus serum starved, +P < 0.05, ++P < 0.01 versus NTCs. Abbreviations: non-significant (ns), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), rho-associated, coiled-coil-containing protein kinase 1 (ROCK1), myosin light chain 1 (MLC1), fatal calf serum (FCS), and serum starvation (-FCS).

5 DISCUSSION

This study provided evidence that GPCRs play a pathological role in PAH. Purinoceptor 2 receptor gene expression was downregulated in the pulmonary arteries of PAH patients and highly expressed in pulmonary arteries from donors. MRS2768 was the ligand for the P2Y2 receptor by which P2Y2 was activated, and it mediated several downstream signaling pathways. Via the P2Y2 receptor, MRS2768 mediated anti-proliferative and anti-migration effects in human donor PASMCs stimulated by serum or PDGF. Furthermore, this study defined the downstream signaling of the P2Y2 receptor and found that that receptor and its ligand MRS2768 increased cAMP production. By elevating cAMP, MRS2768 activated the cAMP direct downstream effector, EPAC. Via cAMP and EPAC, MRS2768 decreased ROCK1 protein expression, resulting in the reduced phosphorylation of pMLC. This reduction in the phosphorylation pMLC might explain the decreased PASMC migration.

5.1 GPCR expression profile in the pulmonary vasculature of IPAH patients

Depending on the cell type and organ, GPCRs have a broad spectrum of pathophysiological functions. They have been intensively studied in many diseases, including obesity, cardiovascular disease, inflammation, cancer, diabetes, and Alzheimer's disease. The human genome contains approximately 800 GPCR genes, of which roughly 450 are non-olfactory receptors. Approximately 50% of GPCRs are orphan receptors with unknown agonists and modes of action.

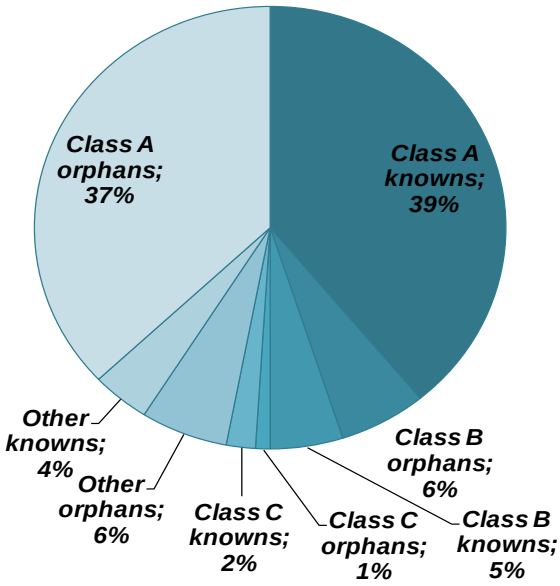

Figure 21. Classification of GPCRs based on orphan receptors and known receptors. The rhodopsin (class A) receptor family comprises 76% of all GPCR receptors. Around 10% of GPCRs belong to secretin (class

B) receptor family. A further 3% of GPCRs belong to the glutamate (Class C) receptor family, among which 1% are orphans. In addition, 10% of GPCRs belong to other families, such as cAMP receptors, fungus pheromone receptors, and frizzled receptors. This class represents 6% of orphan receptors (Murray, 2011) [110].

About several aspects of PAH etiology, convincing evidence supports the involvement of several GPCRs. However, in terms of their depth and breadth, cellular functions, and signaling pathways, those GPCRs are not yet completely understood. That is also why ongoing therapeutic strategies targeting such GPCRs have being facing challenges. Moreover, in PAH, the potential role of many orphan GPCRs, as well as those of their agonists and antagonists, remains to be discovered [121]. Given the increasing interest in targeting GPCRs to treat PAH and other diseases, this study provided initial profiles of all non-olfactory receptors in the pulmonary vasculature by performing a GPCR array. Dozens of GPCRs were expressed differentially in the pulmonary vessels of IPAH patients versus donors.

5.1.1 Micro-dissection of pulmonary arteries from donors and IPAH patients

Human tissue from IPAH patients' lungs and non-tumorous donor lungs were cut into small pieces and cryopreserved. Following cryopreservation, a thin layer of tissue was cut and mounted on glass slides. The tissue slides were briefly stained with hematoxylin and eosin. After staining, the pulmonary arteries of diameter ranging between 50-100 µm were micro-dissected using the laser microbeam system (Leica, Germany). Pulmonary arteries from IPAH patients exhibited plexiform lesions (PLs) in some cases, and they also revealed a thickening in the intima, medial hypertrophy, and a reduced lumen diameter.

5.1.2 Upregulated GPCRs in the pulmonary vasculature of IPAH patients

Data from the high-throughput screening showed that VIPR1, chemokine receptors (CMKLR1, CXCR7, CXCR6), platelet-activating factor receptor PTAFR, leukotriene receptor CYSLTR1, neuropeptide Y receptors (NPY1R, NPY5R), lysophosphatidic acid receptors (LPAR1, LPAR2), endothelin receptor EDNRB, trace amine receptor TAAR8, leucine-rich repeat LGR7, niacin receptor GPR109B, and 5 orphan receptors (GPR18, GPR21, GPR97 GPR112, and GPR115) were highly expressed in the remodeled pulmonary vasculatures of the IPAH patients.

5.1.2.1 Vasoactive Intestinal Peptide Receptor

The agonist of VIPR1 is the vasoactive intestinal peptide (VIP), and its role has been well studied in the treatment of PAH. Specifically, VIP is an agonist for both VIPR1 and VIPR2,

also known as pituitary adenylate cyclase-activating peptide receptor type1 (VPAC1) and type 2 (VPAC2), respectively. While VIPR1 is widely expressed in the central nervous system, liver, lung, intestine, and T-lymphocytes, VIPR2 is widely expressed in the CNS, pancreas, skeletal muscle, heart, kidney, adipose tissue, testis, and stomach [164]. Decreased VIP levels and increased VIP receptor expression in the pulmonary vasculature have been reported in clinical settings [165].

Consistent with previous analyses, this study found that VIPR1 was significantly upregulated in pulmonary vessels and PASMCs from IPAH patients. Hilarie et al. also demonstrated that VIP receptors are expressed in rat tissue sections from pulmonary arteries and cultured SMCs from those arteries. Moreover, they also found that antagonists of VIP receptors restored PASMC proliferation and reduced cAMP levels mainly by blocking VIPR2 [164]. Kort et al. found that VIP knockout mice developed severe PAH [166]. Those researchers also found that when VIP knockout mice were treated with VIP, vascular and right ventricular remodeling were attenuated [166]. Despite all the encouraging preclinical studies demonstrating the vasodilative and anti-proliferative effects of VIP, VIP inhalation did not prove effective in a recent phase 2 clinical trial [167]. The role of VIP and VIP receptors in PAH requires further clarification, and thus, further investigation.

5.1.2.2 Chemokine receptors

An accumulating body of evidence suggests that inflammation plays a significant role in the pathogenesis of PAH. In IPAH patients, increased perivascular infiltration of inflammatory cells, such as T and B cells, and macrophages was found [168]. A mononuclear inflammatory infiltrate that primarily included T cells, macrophages, and B cells was found around the remodeled vessels and plexiform lesions in PAH patients [76, 169-172]. In IPAH patients, perivascular mast cells [173] and DCs were also present [174]. Cytokines, such as interleukin (IL-1-β, IL-6, and IL-8) [72, 175], and chemokines (CCL2/MCP-1, CCL5/RANTES, and CX3CL1/fractalkine) were elevated in serum from IPAH patients [76].

The high-throughput screening revealed the elevated expression of CXCR7 in the pulmonary vessels of IPAH patients relative to those of donors, and that finding agrees with previous studies pointing to increased CXCR7 expression in the pulmonary vasculatures of chronic hypoxia-induced PH mice. Newly deorphanized, CXCR7 is a receptor for SDF-1, CXCL11, and CXCL12. Studies from Karen C. Young et al. have also suggested that the antagonist (CC-X771) of CXCR7 attenuates chronic hypoxia–induced PH in mice [176].

However, no significant difference in cultured PASMCs was noted between IPAH patients and donors. Costello et al. found CXCR7 elevation in the pulmonary endothelium of IPAH patients, and its agonist, CXCL12, promotes pulmonary endothelial cell proliferation [177]. Thus, it is reasonable to speculate that increased CXCR7 expression in pulmonary vessels, as observed in this study, may be primarily attributed to endothelial cells, and not to PASMCs.

Other chemokine receptors were also upregulated in the pulmonary vessels of IPAH patients, and these included CMKLR1 and CXCR6. The former is well known for its multifunctional effects in reducing immune responses and enhancing adipogenesis and angiogenesis [178]. In addition, CXCR6 expression has been studied in non-small cell lung carcinoma. Via metalloproteinases, CXCR6 supports metastatic processes [179]. It also plays a critical role in renal injuries and fibrosis through regulating macrophages, T-cell infiltration, and the bone marrow-derived accumulation of fibroblasts [180]. However, the mRNA analysis found no significant differences concerning CMKLR1 and CXCR6 in the PASMCs of IPAH patients and donors. The roles of CMKLR1 and CXCR6 in PAH remains unclear.

5.1.2.3 Leukotriene receptor CYSLTR1

In previous studies, cysteinyl leukotrienes (CysLTs) were recognized as causing hypoxic pulmonary vasoconstriction, bronchoconstriction, decreased lung compliance, and pulmonary edema [181-183]. The role of leukotrienes remains poorly understood in PAH, but it has been well studied in pulmonary airway diseases. Elevated levels of leukotrienes in neonates with hypoxemia and PH were first reported in 1983 [184]. An increase in leukotriene receptor agonists, such as LTB4, LTC4, and LTE4, has also been reported in the bronchoalveolar lavage fluid (BALF) and blood of infants with persistent PH. Studies on CysLTs in airways might lead to further insights concerning the role of leukotrienes in the development of PH and PAH [185].

This study revealed an upregulation of CYSLTR1 in pulmonary vessels from IPAH patients. In contrast, CYSLTR1 was downregulated in PASMCs from IPAH patients as compared with donors. Therefore, expression of CYSLTR1 must be studied in other pulmonary vasculature cell types (e.g., endothelial cells, fibroblasts, and immune cells). The involvement of leukotriene receptors has yet to be determined.

5.1.2.4 Neuropeptide Y receptors

Recent findings from Kwapiszewska et al. revealed an increase in the expression of the NPY1 receptor within the pulmonary arterial wall of IPAH patients as compared with donors, but no changes in NPY1 were observed in the serum or pulmonary arteries [186]. In addition, the study also identified the upregulation of the NPY1 receptor in different PAH animal models.

Correlating to those findings, our study's high-throughput screening showed an upregulation of NPY1R and NPY5R in pulmonary vessels from IPAH patients compared to donors. However, no significant difference in NPY1R and NPY5R mRNA expression in cultured PASMCs was observed between IPAH patients and donors. Kwapiszewska's study also indicated that activating the NPY/Y1 axis leads to vasoconstriction, and more importantly, pulmonary vascular remodeling [186]. Therefore, neuropeptide Y receptors may present a promising target for PAH, and so they are worth further investigation.

5.1.2.5 Lysophosphatidic acid receptors

Lysophosphatidic acid, the ligand for lysophosphatidic acid receptors, is a bioactive lipid molecule synthesized by plasma. In rodents, the local administration of LPA promotes systemic arterial remodeling [187-189]. Smyth et al. found that when LPA is absent, the endothelin system takes over and supports vascular remodeling [190]. The same vasoconstrictive and phenotypic changes are stimulated in cultured SMC by LPA and ET-1 [191].

This high-throughput screening study found increased expression of LPAR1 and LPAR2 in laser micro-dissected pulmonary arteries from IPAH patients. However, no significant upregulation of LPAR1 and LPAR2 was noted in the cultured PASMCs from the IPAH patients. The LPAR1 and LPAR2 gene expression was equal in the cultured PASMCs from the IPAH patients and donors.

5.1.2.6 Endothelin receptors

Endothelin receptors are the best known GPCRs in PAH. Endothelin is the ligand for both the ETA and ETB receptors. Endothelin (ET1) is a vasoconstrictor expressed by endothelial cells and SMCs, and it plays an important role in the pathobiology of PAH [52]. The protein levels of ET1 are elevated in the plasma of IPAH patients [192]. Endothelin binding to ETA receptor leads to Gq activation. Gq activates PLC, leads to the formation of IP3, and finally,

an increase in intracellular Ca2+ levels in the SMC [195]. The persistently elevated intracellular Ca2+ levels lead to vasoconstriction, even after removing ET1 (the ligand required for ETA receptor activation) [196]. In chronic hypoxia-induced rats, ET-1 and ET receptors levels are increased. Monocrotaline induced in ETB-deficient transgenic rats demonstrates higher PVR levels and increased neo-intimal lesions similar to those of PAH patients [193]. Muller et al. found that when ET1 receptors (ETA and ETB) were blocked, inflammatory signaling was inactivated [194]. Bosentan is a potent antagonist/blocker for ETA and ETB receptors, and it inhibits NF-kappaB and AP1 activation. The genes that are regulated by NF-kappaB and AP1, such as intercellular adhesion molecule 1(ICAM-1), VCAM-1, and transferrin (TF), are also further blocked by bosentan.

The high-throughput screening confirmed the increase in ETA and ETB (also known as EDNRA and EDNRB, respectively) in a remodeled pulmonary vasculature. However, pulmonary vasculature is differentially regulated by ET-1 depending on the receptors and cell types. Many studies have indicated that in PASMCs, ETA mediates vasoconstriction and vascular remodeling, whereas ETB promotes vasodilatation in endothelial cells by increasing nitric oxide production. In addition, ETB mediates endothelial cells' reuptake of ET-1, thereby clearing circulating ET-1 [195, 196]. Therefore, in a clinical setting, an ETA-specific antagonist, such as ambrisentan, may be a better option than a dual-receptor antagonist.

5.1.2.7 Leucine-Rich repeat LGR7

Well studied in airway diseases, LGR7 is relaxin family peptide receptor-1 that shares the ligand, relaxin, with LGR8 [197]. Tang et al. have demonstrated that LGR7 protects against airway fibrosis during homeostasis but fails to protect against fibrosis associated with chronic allergic airway disease [198].

Our high-throughput screening study found the upregulation of LGR7 in both pulmonary vessels and PASMCs from IPAH patients as compared to donors. The receptor itself has not previously been implicated in PAH. However, highly elevated relaxin levels have been reported in serum from PAH patients, although not in diastolic heart failure-induced PH patients [199]. Interestingly, an earlier study found a therapeutic effect of recombinant relaxin on hypoxia-induced PAH in rats [200]. These controversial observations encourage further studies addressing the potential role of LGR7 and relaxin in PAH.

5.1.2.8 Other candidate GPCRs

The recently deorphanized niacin receptor GPR109B [159, 201] and several orphan receptors (trace amine receptor TAAR8, GPR18, GPR21, GPR97 GPR112, and GPR115) were also upregulated in the pulmonary vessels from the IPAH patients, according to this study's screen data. However, none of these receptors exhibited differential expression in the cultured PASMCs of IPAH patients versus donors. Defining the role of these dysregulated orphan receptors in PAH may prove rather challenging until their ligands are discovered.

5.1.3 Downregulated GPCRs in the pulmonary vasculature of IPAH patients

High-throughput screening by means of GPCR arrays revealed 3 significantly downregulated GPCRs, namely, PTGER4, P2Y2, and DRD1.

5.1.3.1 Prostaglandin E receptor

One of the earliest and most effective treatments for PH is iloprost. It activates prostacyclin receptors (IP receptor) and increases cAMP, resulting in vasodilation and less remodeling of the pulmonary arteries [48]. Prostacyclin signaling is altered in PAH. Prostaglandin I2 (prostacyclin) receptor (IP) protein expression is low in IPAH lung tissues [202]. In addition, prostacyclin is a potent vasodilator and exhibits anti-inflammatory properties via the IP receptor [203].

Our previous studies have indicated that the prostacyclin analog iloprost reverses vascular remodeling in monocrotaline-induced PH rats [204, 205]. Our previous findings clarified that other prostanoid receptors, such as prostaglandin E2 receptor 2 (EP2/PTGER2) and prostaglandin E2 receptor 4 (EP4/PTGER4) are stably expressed in lung tissue from IPAH patients and monocrotaline-induced PH rats [48]. Moreover, EP2 and EP4 receptors are coupled to the Gαs protein, and therefore, receptor activation leads to both an increase in cAMP levels in SMCs and relaxation [206]. Iloprost, in the absence of its receptor prostaglandin I2 (prostacyclin) receptor (IP), acts via EP4/PTGER4 and mediates vasodilation in PH [48].

Building on our previous findings that IP receptor expression is reduced in remodeled PASMCs and lung homogenates from IPAH patients [48], our high-throughput GPCR array revealed decreased PTGER4 mRNA expression in the laser micro-dissected pulmonary arteries of IPAH patients versus donors.

5.1.3.2 Dopamine receptors

Dopamine receptors are expressed in the vascular wall. Activation of a dopamine receptor by its ligand (dopamine) results in vasodilation [207]. Dopamine plays an important role in epithelial sodium transport, and it interacts with vasoactive hormones, thereby playing a crucial role in the pathogenesis of systemic hypertension [208]. Kummer et al. demonstrated that pulmonary arterial endothelial cells and VSMCs produce a substantial pool of dopamine [209].

Our current findings from our high-throughput GPCR array revealed reduced DRD1 (a dopamine receptor subtype) levels, in laser micro-dissected pulmonary arteries and cultured PASMCs from IPAH patients, suggesting a vasoconstrictive potential of the pulmonary arteries in PAH patients.

5.1.3.3 Purinergic receptor P2Y2

Vasodilatory mediators, such as nitric oxide, that are released from the endothelium determine the blood pressure. Offermanns et al. evaluated human and bovine endothelial cells and found that the purinergic receptor P2Y2 mediated activation of [Ca2+] transients, activation of the eNOS, phosphorylation of PECAM-1 and VEGFR-2, and activation of SRC and AKT. A lack of P2Y2 in endothelial cells reduced eNOS activation, deceased vasodilation, and resulted in hypertension in those mice [210].

Our current findings from the high-throughput GPCR array revealed the reduced expression of purinergic receptor P2Y2 in laser micro-dissected pulmonary arteries and cultured PASMCs from IPAH patients.

5.2 Effect of GPCRs on human PASMC proliferation

G protein-coupled receptors play several roles in cellular functions, among which SMC proliferation is essential to PAH pathogenesis. Histological lung biopsy studies have revealed that medial vascular wall remodeling is mainly due to PASMC proliferation [211]. Therefore, we were interested in the role of GPCRs in PASMC proliferation. Loss-of-function experiments employing siRNA targeting GPR115, LGR7, and PTAFR found a mild inhibitory effect on serum-induced PASMC proliferation.

Previous findings have noted that the ligand of LGR7 (relaxin) regulates fibroblast proliferation, differentiation, and collagen deposition in cardiac fibrosis. Relaxin is known to reduce fibroblast proliferation mediated by angiotensin II and TGF-beta [212]. Studies on Sertoli cells have indicated that relaxin increases cell numbers and the expression of PCNA [213]. In our current study, we found that the knockdown of LGR7 gene expression resulted in the inhibition of serum-induced human donor PASMC proliferation. However, determining the exact mechanism and role of relaxin/LGR7 in PASMC proliferation requires further investigation.

Platelet-activating factor, a potent phospholipid mediator, and its receptor (PTAFR) have been well studied in cardiovascular diseases. Platelet-activating factor, released by either inflammatory cells or the endothelium, has been shown to promote atherosclerosis by activating MMPs [214], while PTAFR is present in vascular SMCs. Moreover, PTAFR has been reported in the pathogenesis of PPHN. Ibe et al. have stated that hypoxia and hyperoxia increase proliferation via PTAFR signaling in PASMCs from newborn ovine [215]. In our current study, we found that the knockdown of PTAFR gene expression resulted in the inhibition of serum-induced human donor PASMC proliferation. The downstream mechanisms through which PTAFR regulates PASMC proliferation have yet to be investigated.

Our BrdU proliferation assay also revealed an orphan receptor (GPR115) with a role in PASMC proliferation. The knockdown of GPR115 gene expression reduced serum-induced donor PASMC proliferation. Since GPR115 is an orphan receptor, further investigation of its functional role in PAH could be possible after deorphanization.

Although the knockdown of P2Y2, CYSLTR1, and GPR21 did not have any effect on serum-induced PASMC proliferation, that finding does not rule out the possibility that activation of CYSLTR1 and GPR21 by their specific ligands contributes to the pro-proliferative and anti-apoptotic effects of other vascular cell types. Therefore, further studies are necessary to address the potential role of dysregulated GPCRs.

5.3 Role of purinergic receptor P2Y2 in PAH

Activation of P2Y receptors by ATP and UTP has been repeatedly reported in carcinogenesis, endothelial regulation, and blood flow regulation [216]. However, evidence on its role in vascular remodeling is very limited. In our study, expression profiling of GPCRs

suggested a remarkable dysregulation of P2Y2 in pulmonary vessels and PASMCs from PAH patients. The role of P2Y2 is completely unknown in PAH, and its part in vascular cell functioning has yet to be investigated.

5.3.1 Effect of P2Y2 agonist MRS2768 on PASMC proliferation

In PAH, PASMCs play an important role in pulmonary vascular remodeling mediated by strong proliferation and migration [217]. Several growth factors and tyrosine kinase receptors (PDGF receptor) are involved in the process of SMC proliferation [217].

In this study, we observed that MRS2768 reduced PASMC proliferation at lower doses (5µM to 50µM). However, higher doses of MRS2768 failed to influence PASMC proliferation, perhaps due to the desensitization of the P2Y2 receptor when exposed to excessive amount of its ligand, MRS2768.

Earlier studies have indicated that via Raf1 (serine/threonine kinase) and MAPK, PDGF leads to an increase in smooth muscle proliferation [218, 219], which could be inhibited by a cAMP-mediated PKA mechanism [220]. The PDGF-β receptor is activated by the PDGF ligand, which results in Ras-Raf mediated ERK1/2 activation [221]. In turn, ERK1/2 activation leads to the transcription of protooncogenes (c-fos, c-myc) and matrix metalloproteinases (MMP2 and MMP9), which result in cell proliferation [221]. Previous studies from our team have demonstrated the degradation of the extracellular matrix by MMPs and elastase, followed by vascular SMC proliferation [64]. Lung homogenates from monocrotaline rat models exhibited a significant increase in MMP2 and MMP9 expression [64]. The effects of PDGF receptor activation and increased MMP levels were reversed after PDGF receptor inhibition by STI571 [64].

More interestingly, we also observed that MRS2768 could moderately attenuate the PDGF- and serum-induced proliferation of donor PASMCs. This brings raises the question of whether the P2Y2 receptor engages in crosstalk with other receptor families that interfere in PASMC proliferation. In particular, P2Y2 is known to transactivate non-tyrosine kinase receptors, as well as several receptor tyrosine kinases [149]. The crosstalk may explain why MRS2768 partially inhibits PASMC proliferation induced by both PDGF and serum.

5.3.2 Effect of P2Y2 agonist MRS2768 on PASMC migration

Increased PASMC migration into the media and the intima is another pathological feature of PASMCs in PAH [222, 223]. Several growth factors, including PDGF, activate the MAPK pathway, which then leads to an increase in MMP expression and cell migration [224]. In contrast, the MAPK mechanism is inhibited by c-AMP via PKA [220].

In atherosclerosis, the role of PDGF in SMC proliferation and migration has received substantial attention [225]. Endothelial cell and PASMC migration result in plexiform lesions and neointima formation [172, 226, 227]. Perros et al. revealed the overexpression of PDGF and PDGF receptors in the pulmonary arterial walls of PAH patients [63]. They also found phosphorylated PDGFR-β (activated PDGF receptors) in vascular lesions and cellular proliferation in the lung tissues of PAH patients [63]. Specifically, the PDGFR-β receptor was overexpressed in hyperproliferating PASMCs [63]. Perros et al. found increased PASMC migration upon the activation of PDGF signaling [63].

In turn, PDGFR activation results in the activation of several pathways such (e.g., PI3K, Ras-MAPK, and PLC) that result in the proliferation, differentiation, and migration of VSMCs [228]. In hypoxia-induced PH in rats, elevated mRNA expression of PDGF-B and PDGF-A was noticed [229]. Rosenkranz et al. found that under hypoxic conditions, normal human PASMCs displayed an increased expression of PDGF-B and heightened PASMC proliferation and migration [230].

Therefore, we were interested in studying the role of P2Y2 receptors in PDGF- and serum-induced human PASMC migration. Initially, we studied the role of P2Y2 in donor PASMC migration by performing a scratch wound healing assay to monitor the wound closure rate. We later validated our results on PASMC migration by conducting a transwell migration assay. These experiments on the migration of donor PASMCs demonstrated that P2Y2's loss of function due to siRNA knockdown promoted spontaneous cell migration without growth factors (when the PASMCs were serum-starved). In contrast, MRS2768 could attenuate the PDGF- or serum-induced migration of donor PASMCs via the P2Y2 receptor. In the case of P2Y2 receptor knockdown, MRS2768 failed to attenuate the PDGF- or serum-induced migration of donor PASMCs. Our data implied that MRS2768, via P2Y2, is a potential agent that acts against the PDGF- or other growth factor- (serum-) mediated migration of human donor PASMCs.

In line with our discovery, studies from Lehmann et al. have stated that P2Y2 activation by ATP delays the growth factor-induced wound healing of keratinocyte monolayers [231]. They also suggested that the anti-migration effect of P2Y2 on keratinocytes is mediated by blocking growth factor-induced ERK and AKT phosphorylation. On the other hand, other study has suggested that uridine adenosine tetraphosphate (Up4A) induces VSMC migration by directly activating its receptor, P2Y2, and transactivating the PDGF receptor [232]. These controversial findings are not surprising, since the cellular functions of GPCRs are highly dependent on the cell type and ligand type.

5.3.3 MRS2768 regulates MMP2 in human PASMCs via the P2Y2 receptor

An MMP imbalance is involved in extracellular matrix turnover, which in turn affects cell migration and proliferation in the vascular wall [233, 234]. Studies from Lafuma et al. have demonstrated an overexpression of MMP2 total protein and elevated MMP2 activity in SMCs from IPAH patients. They also found that tissue inhibitor of metalloproteinase (TIMP) was dysregulated in cultured PASMCs from IPAH patients [235]. Elevated MMP2 activity is responsible for the migrative and proliferative phenotype of PASMCs from IPAH patients [235]. Studies of adult respiratory distress syndrome (ARDS) have indicated that the active MMPs in the epithelial lining fluid degrade the extracellular matrix (ECM) [236]. Specifically, MMPs cleave elastin, and elastin fragments have been observed in patients with congenital heart defects and PAH [237, 238].

Studies on IPAH associated with human herpesvirus 8 (HHV8) infection have indicated that a viral GPCR (vGPCR) activated Src and focal adhesion kinase (FAK) and promoted the angiogenesis of human pulmonary arterial endothelial cells (HPAECs) [239]. In addition, MMP2 activation is inhibited or blocked by membrane type 1 MMP (MT1-MMP) neutralizing antibody and TIMP2 [239]. Shan et al. found that Scr activation by vGPCR led to pro-MMP2 activation by regulating MT1-MMP and TIMP2 expression [239].

Studies from Ravindra et al. revealed the crosstalk between EGFR (tyrosine kinase receptor) and chemokine receptors (GPCRs: CCR3 and CXCR4) [240]. Activation of CCR3 and CXCR4 chemokine receptors by their respective ligands, eotaxin-1 (CCL11) and stromal cell-derived cell factor (CXCL12), leads to the phosphorylation or activation of the EGFR receptor and increases the synthesis of pro-MMP2/active MMP2 in arterial SMCs [240].

It is known that MMPs play a significant role in the migration of PASMCs and that GPCRs are involved in the regulation and activation of MMPs. Our findings indicated that the P2Y2 receptor (a GPCR receptor) is involved in regulating the migration of PASMCs. Thus, we were interested in studying the role of P2Y2 and its ligand, MRS2768, in regulating MMP activation. The zymography experiment revealed that MRS2768 diminished MMP2 activity in human donor PASMCs in culture medium.

5.3.4 P2Y2 agonist MRS2768 increases cAMP production in human PASMCs

Prostacyclins, prostaglandins, and thromboxanes are pulmonary vasoactive mediators [241]. Prostacyclins activate GPCRs that bound to Gαs proteins, resulting in the hydrolyzation of GTP to GDP [241]. Later, GTP activates adenylate cyclases (ACs) and converts ATP to cAMP [241]. Prostanoids (prostacyclin analogues), such as epoprostenol, treprostinil, and iloprost, are used for the treatment of IPAH [242].

Cyclic adenosine monophosphate is a universal second messenger that regulates various biological processes, such as vascular cell differentiation, proliferation, migration, and apoptosis, and so it governs vascular toning and remodeling. It is degraded by the PDE enzymes superfamily [243]. According to our previous findings, PDE1 is upregulated in PASMCs and lung homogenates from IPAH patients and PAH animal models (hypoxic mice and monocrotaline rats) [244]. Our studies from 2011 revealed that PDE10 A expression was upregulated in PASMCs from PAH induced by monocrotaline (MCT) in rats. Upon PDE10A knockdown, the intracellular cAMP levels increased, which resulted in decreased PASMC proliferation [245].

Our current findings revealed that the P2Y2 receptor and its ligand, MRS2768, regulate the proliferation and migration of human PASMCs. Moreover, cAMP is the second messenger of GPCR, which regulates PASMC proliferation. Thus, we wanted to examine the effect of MRS2768 on intracellular cAMP levels. The results of our cAMP-EIA assay revealed that MRS2768 elevated intracellular cAMP levels in human donor PASMCs, whereas it failed to increase cAMP levels when P2Y2 receptor expression was knocked down by siRNA. These findings suggest that via P2Y2, MRS2768 increases cAMP levels in human PASMCs.

5.3.5 MRS2768 increases EPAC expression in human PASMCs

The direct downstream effectors of cAMP are PKA and EPAC [246]. In pulmonary artery SMCs, cAMP activates PKA and suppress PASMC proliferation [241]. Murray et al. found

decreased EPAC1 gene and protein expression in PASMCs isolated from IPAH patients [247]. Murray et al. also showed that overexpression of EPAC1 by adenovirus transfection decreased serum-induced proliferation of PASMCs isolated from IPAH patients [247].

It was recently demonstrated that beraprost (prostacyclin analogue), increases cAMP levels and decreases PDGF-induced human VSMC migration via EPAC [248]. Fluorescence resonance energy transfer studies by McKean et al. found that beraprost activates EPAC but not PKA. In addition, EPAC inhibits VSMC migration by activating Ras-related protein 1 (Rap1) and by inhibiting Ras homolog gene family member A (RhoA) [248]. Compared with the well-studied cAMP/PKA pathway, the functions and downstream targets of the more recently identified cAMP/EPAC pathway are not yet fully understood [246].

Based on the above-mentioned studies, EPAC is a direct downstream effector of cAMP, and it regulates VSMC proliferation and migration. In our study, MRS2768 increases cAMP levels in human PASMCs. Furthermore, MRS2768 regulated the proliferation and migration of human PASMCs. Therefore, we studied the effect of the P2Y2 receptor and its ligand, MRS2768, on EPAC protein expression in human PASMCs. Western blot analysis revealed that MRS2768 significantly increased EPAC protein expression in serum-starved and serum-stimulated human donor PASMCs. Hence, we believe that the MRS2768-mediated regulation of PASMC proliferation and migration may function via activation of cAMP and its downstream protein, EPAC.

5.3.6 MRS2768 inhibits ROCK and decreases MLC phosphorylation in human PASMCs

In SMCs, the activation of GPCR coupled to Gq/11 or G12/13 converts RhoA-GDP to RhoA-GTP. Activated Rho-GTP binds to ROCK at the Ras-binding domain (RBD-domain). This leads to the stimulation of ROCK1 activity. The phosphorylation of MLC is known to promote cell migration and increased cell contractility [249]. Myosin light chain is regulated by two enzymes, MLC kinase (MLCK) and MLC phosphatase (MLCP). A ligand binding to a GPCR coupled to Gq/11 or G12/13 G-proteins results in the activation of phosphoinositide phospholipase C- beta (PLC-β). Phosphoinositide phospholipase β increases intracellular Ca2+ and MLCK activity, which in-turn increases the phosphorylation of MLC [249]. Furthermore, the activation of the RhoA/ROCK1 cascade leads to the inactivation of MLCP, which is responsible for MLC dephosphorylation [250, 251]. Decreased MLCP activity and increased MLCK activity lead to a surge in MLC phosphorylation, which results in SMC

contraction and migration [252]. Studies from Oldenburger et al. have demonstrated that EPAC can inhibit RhoA and ROCK1 cascades [253]. Rho-kinases (ROCK-1 and ROCK-2) play a key role in vascular cell proliferation and vasoconstriction [254]. In the lung homogenates of IPAH patients, Guilluy et al. found an increase in Rho kinase expression and RhoA activity [255]. Therefore, it was essential to study the effects of MRS2768 and the P2Y2 receptor on ROCK protein expression and MLC phosphorylation.

Expression analysis revealed that MRS2768 significantly increased EPAC protein expression and led to a simultaneous reduction in ROCK protein expression. The decrease in ROCK protein levels led to an increase in MLCP, which boosted MLC levels and decreased the MLC phosphorylation level. Based on these results, we believe that P2Y2 receptor signaling inhibits ROCK activity via EPAC and further decreases MLC phosphorylation and PASMC migration.

6 CONCLUSION

The data from our screening experiment demonstrated a clear difference in GPCR gene expression in the pulmonary vasculature of IPAH patients versus donors. However, of the dysregulated genes from the array data, only a few are regulated in the same direction in the PASMCs of IPAH patients, with VIPR1, LPAR1, and LGR7 being upregulated, and P2Y2 and DRD1 being downregulated. The selective knockdown of candidate GPCRs. including GPCR115, LGR7, and PTAFR, had a significant but mild inhibitory effect on PASMC proliferation. However, further studies are needed for each dysregulated GPCR candidate to understand their importance in PAH.

Further, P2Y2 receptor gain-and-loss-of-function studies in human PASMCs indicated that MRS2768 inhibited serum- and PDGF-induced PASMC proliferation and migration, and MMP2 activation, in a P2Y2/cAMP/EPAC-dependent manner, counteracting the RhoA/ROCK/MLC pathway activated by growth factors. These findings imply that P2Y2 is a potential therapeutic target for PAH.

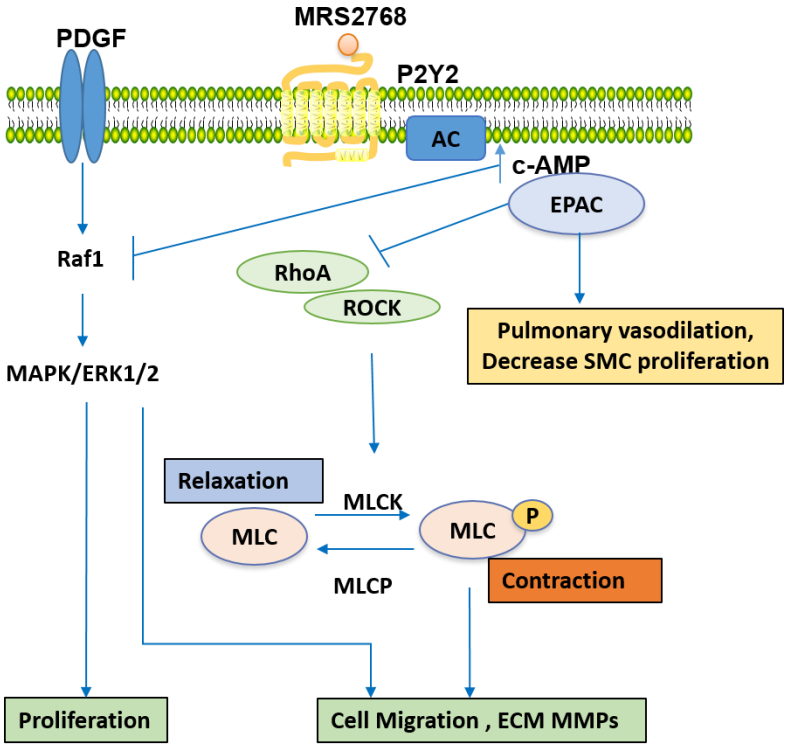

Figure 22. Schematic diagram of the proposed mechanism by which P2Y2 and MRS2768 play a role in PAH. Abbreviations: MLC (myosin light chain), MLCK (myosin light-chain kinase), MLCP (myosin light-chain phosphatase), RhoA (Ras homolog gene family member A), ROCK (Rho-associated protein kinase), AC (adenylyl cyclase), cAMP (cyclic adenosine monophosphate), EPAC (exchange factor directly activated by cAMP), ECM (extracellular matrix), MMPs (matrix metalloproteinases), and P2Y2 (purinergic receptor).

7 OUTLOOK FOR THE FUTURE

The role of GPCRs in the pathogenesis of PAH has been well recognized [121]. Yet, GPCRs are among the most complicated proteins, since they comprise largest protein family in the human genome/proteome. In this study, approximately 100 GPCRs were found to be differentially expressed in IPAH patients' pulmonary arteries. Of these GPCRs, only a few were known to play a role in PAH pathogenesis. Therefore, further investigation of the functions of these differentially expressed GPCRs in PAH in needed.

All types of vascular cells, including endothelial cells, SMCs, fibroblasts, progenitor cells, and inflammatory cells, contribute to the pathogenesis of PAH [2]. There is clear evidence suggesting that GPCRs regulate various cellular responses depending on the cell type [121]. Therefore, it remains critical to study the function of dysregulated GPCRs in other vascular cell types to broaden our understanding of GPCR functions in PAH. For example, the current study only examined PASMCs to shed light on the cellular functions mediated by P2Y2. An obvious follow-up study would be an analysis of the role of P2Y2 in pulmonary endothelial cells and intima formation in PAH. Studies on systemic circulation have demonstrated that the P2Y2s located in systemic endothelial cells regulate blood pressure by transactivating VEGFR/eNOS/NO signaling [210]. It would be interesting to investigate whether P2Y2 has a similar effect on pulmonary circulation.

To further explore the therapeutic potential of P2Y2 signaling, in-vivo studies using animal PH models are necessary. For example, PH animal models with P2Y2 knockout or MRS2768 treatment should be studied regarding their functional (hemodynamics assessed by invasive and non-invasive methods) and structural (histomorphometry) relevance. In addition, the in-vitro effects on proliferation and migration in PAECs and PASMCs isolated from these animal models should be considered.

Finally, the dysregulated orphan GPCRs are also of great interest, even though our lack of knowledge of their ligands poses an obstacle. A recently deorphanized example from our candidate list is GPR109B [159]. A challenging but not impossible task, deorphanizing such receptors via the large-scale screening of agonist/antagonist banks is another important goal.

8 SUMMARY

Idiopathic pulmonary arterial hypertension is a progressive, chronic, and usually lethal lung disease of unknown causes with limited therapeutic options. Pulmonary arterial hypertension is characterized by aberrant pulmonary vasoconstriction and abnormal pulmonary vascular remodeling. Several lines of evidence suggest that deregulated GPCRs may represent a key factor in the progression of PAH.

Specifically, GPCRs are centrally involved in the pathobiology of PAH. Identifying dysregulated GPCRs in PAH may pave the way to targeting abnormal pulmonary vasoconstriction and remodeling, thereby resulting in options for translational research. Therefore, the purpose of the present study was to identify and analyze both known and orphan GPCRs potentially involved in the development of PAH.

An analysis of GPCR expression was performed in laser micro-dissected pulmonary vessels from donors and IPAH patients using a quantitative GPCR high-throughput expression screening system. The quantitative GPCR expression analysis revealed that a large number of GPCRs were upregulated in the pulmonary vasculature of PAH patients, and these included VIPR1, chemokine receptors (CMKLR1, CXCR7, CXCR6), a leukotriene receptor (CYSLTR1), neuropeptide Y receptors (NPY1R, NPY5R), lysophosphatidic acid receptors (LPAR1, LPAR2), an endothelin receptor (EDNRB), a trace amine receptor (TAAR8), a leucine-rich repeat (LGR7), a niacin receptor (GPR109B), and 5 orphan receptors (GPR18, GPR21, GPR97 GPR112, and GPR115). The purinergic receptor (P2Y2) and dopamine receptor (DRD1) were downregulated.

Further, the mRNA expression of relevant GPCR hits was analyzed in human PASMCs derived from donors and PAH patients. The qPCR results revealed the upregulation of VIPR1 and LGR7 in PASMCs from IPAH patients. In contrast, P2Y2 and DRD1 were downregulated in the PASMCs of IPAH patients.

The potential role of candidate GPCRs in PASMCs was previously determined by siRNA knockdown or ligand stimulation. The proliferation of PASMCs determined using BrdU incorporation assay indicated that the knockdown of GPR115, LGR7, and PTAFR significantly reduced serum-induced PASMC proliferation. On the other hand, the knockdown of P2Y2 did not result in any significant changes in PASMC proliferation.

Furthermore, the P2Y2 ligand MRS2768 reduced both PDGF- and serum-induced PASMC proliferation. In addition, MRS2768 limited both serum- and PDGF-induced PASMC migration. The knockdown of P2Y2 by siRNA resulted in a spontaneous increase in PASMC migration under serum-starved conditions. MRS2768 also decreased MMP2 activation in PASMCs. Therefore, MRS2768 and its receptor P2Y2 play significant role in migration.

Moreover, upon MRS2768 stimulation, P2Y2 promoted cAMP (a potent vasodilator) production in PASMCs. In addition, MRS2768 activated the cAMP/EPAC-mediated inhibition of RhoA/ROCK1 and reduced the MLC phosphorylation. Due to its role in decreasing MLC phosphorylation, MRS2768 might thereby reduce PASMC vasoconstriction.

overall, this study implies that more GPCRs are differentially expressed in the pulmonary vasculatures of PAH patients than in those of donors. Most interestingly, P2Y2 might play a key role in pulmonary vascular remodeling by regulating PASMC proliferation and migration.

9 ZUSAMMENFASSUNG

Die Pulmonal Arterielle Hypertonie (PAH) ist eine fortschreitende, chronische und meist tödliche Lungenerkrankung mit unbekannten Ursachen und limitierten therapeutischen Optionen. Die Erkrankung ist durch eine erhöhte pulmonale Vasokonstriktion und einen abnormalen pulmonalen Gefäßwandumbau charakterisiert. Es gibt Hinweise, dass fehlregulierte G-Protein-gekoppelte Rezeptoren (GPCRs: G-protein-coupled receptors) eine Rolle bei der Krankheitsentstehung und dem Verlauf spielen.

GPCRs sind maßgeblich an der Pathobiologie der PAH beteiligt. Die Identifikation von deregulierten GPCRs in der PAH könnte helfen, diese abnormale pulmonale Vasokonstriktion und pulmonalen Gefäßumbau (Remodeling) besser zu verstehen und eine neue zielgerichtete Therapie zu ermöglichen. Auf Grund dessen liegt das Ziel heutiger Studien in der Identifikation und Analyse von bekannten, sowie seltenen GPCRs, die potentiell in der Entwicklung der PAH involviert sind.

Die Analyse der GPCR-Expression erfolgte in dieser Doktorarbeit mittels eines quantitativen Hochdurchsatzverfahrens in Laser-mikrodissektierten pulmonalen Gefäßen von Donoren und PAH-Patienten. Die quantitative GPCR mRNA Expressionsanalyse zeigte eine Vielzahl von hochregulierten GPCRs in den pulmonalen Blutgefäßen von IPAH-Patienten im Vergleich zu den pulmonalen Blutgefäßen der Donoren. Zu diesen hochregulierten GPCRs zählen: VIPR1 (Vasoactive intestinal peptide receptor), Chemokinrezeptoren (CMKLR1, CXCR7, CXCR6), CYSLTR1 (Cysteinyl Leukotriene receptor 1), Neuropeptid Y Rezeptoren (NPY1R, NPY5R), Lysophosphatidsäure Rezeptoren (LPAR1, LPAR2), EDNRB (Endothelin receptor type B), TAAR8 (Trace amine-associated receptor 8), LGR7 (Leucine-rich repeat-containing G-protein coupled receptor), GPR109B (Niacin receptor 2) und fünf seltene (orphane) Rezeptoren (GPR18, GPR21, GPR97 GPR112, and GPR115). Im Gegensatz dazu fanden wir P2Y2 (purinergic receptor P2Y2) und DRD1 (dopamine receptor D1) herunterreguliert.

Im nächsten Schritt wurde die mRNA-Expression von dysregulierten GPCRs in humanen glatten pulmonalarteriellen Muskelzellen (PASMCs: pulmonary arterial smooth muscle cells) von Donoren und PAH-Patienten verifiziert. Die Resultate der qPCR zeigten eine erhöhte Expression von VIPR1 und LGR7, sowie eine verminderte Expression von P2Y2 und DRD1 in PASMCs von PAH-Patienten.

Die potentielle Rolle verschiedener GPCRs in glatten Muskelzellen wurden über einen siRNA knockdown oder eine Liganden-Stimulation untersucht. Die siRNA mediierte Ausschaltung (knockdown) von GPR115, LGR7 und PTAFR führte zu einer signifikanten Reduktion der Serum-induzierten Proliferation von PASMCs. Im Gegensatz dazu führte der knockdown von P2Y2 zu keiner signifikanten Veränderung der PASMC-Proliferation. Eine Stimulation mit dem P2Y2 Liganden MRS2768 zeigte hingegen eine Reduktion der Serum-induzierten PASMC-Proliferation. Der siRNA-vermittelte knockdown von P2Y2 in PASMCs erhöhte die Migration, während die Stimulation mit MRS2768 die Migration reduzierte. Eine Erklärung dafür kann die durch MRS2768 reduzierte Matrixmetalloproteinase (MMP) 2 Aktivität sein.

Zusätzlich erhöhte die MRS2768-vermittelte P2Y2-Stimulation die cAMP-Produktion und führte über eine EPAC-vermittelte Inhibierung der RhoA/ROCK1 Aktivierung zu einer verringerten MLC-Phosphorylierung.

Zusammengefasst lässt sich eine veränderte Expression von mehreren GPCRs in den pulmonalen Gefäßen von PAH-Patienten im Vergleich zu Donoren feststellen. Dabei scheint insbesondere P2Y2 über die Regulation von Proliferation und Migration von PASMCs eine wichtige Rolle für den pulmonalen Gefäßumbau zu spielen.

10 APPENDIX

Table 23 App. Primers sequence and respective probes used for High-throughput screening of 408 non-olfactory GPCRs

Human gene	Probe	Forward primer	Reverse primer
Rho	60	atggcagttctccatgctg	tgacgtagagcgtgaggaag
OPN1SW	69	gcccctcaactacattctgg	cagggaagacagagaagatgc
OPN1MW	53	gaactggatcctggtgaacct	cctggttcacaacgctgata
OPN1LW	49	tggtcactgcatccgtctt	caccaggatccagttcagc
OPN4	18	cgctacctggtaatcacacg	ccagggcatagagccaaac
OPN3	79	ctccgcactcccactcac	gaccccgaagagggacac
RRH	74	ggggtcagtagcattggctat	cgtatccaaatttccaacttcc
RGR	87	tgaccatcttctctttctgcaa	tcaggctgatcccactgtc
OPN5	35	gtagggggccaggttttc	aacacgatcacagccgttg
ADRA1A	16	tatccattggacccctgttc	ggcagatggtctcgtcctc
ADRA1B	34	gccaagagaaccaccaagaa	gctccttggagttggacatc
ADRA1D	3	cctggtggtgtccgtagg	cggtgataccgcagaagc
ADRA2A	49	ccaaaacctcttcctggtgt	ccagcgagaaagggatgac
ADRA2B	28	tggtcatcctggctgtgtt	agcgacaccaggaacaggt
ADRA2C	49	cacagaacctcttcctggtgt	cgagaagggcatgaccag
ADRB1	20	gaccgcctcttcgtcttct	gcagtagatgatggggttgaa
ADRB2	62	caggaagccatcaactgcta	ggcttggttcgtgaagaagt
ADRB3	56	gcctccaacatgccctac	cgaagagcatcacgagaaga
HRH1	62	cctgagcactatctgcttggt	ctcactccgtacggcataca
HRH2	64	agctttggcaaggtcttctg	aggctgtgcagagcatcac
HRH3	58	cctgagctgggagtacctgt	agaactcggcatagcagtgg
HRH4	7	gcaatacaattgcttccaaaatg	tgttccctttggtgaagagc
DRD1	86	gggtggcctttgacatcat	cacgctgatcacacagaggt
DRD2	67	caggagctggagatggagat	ggctgtaccgggtcctct
DRD3	76	cctgcagactaccaccaactact	gtggccaccagcaagtct
DRD4	28	ccttcttcgtggtgcacat	agggcgctgttgacgtag
DRD5	80	gaacctgtgcgtcatcagc	gagtcatcttgcgcttgtagc
CHRM1	23	tagctgggcagtgctacatc	atggctgtgccaaaggtg
CHRM2	83	tccttataagacatttgaagtggtgt	cccgataatggtcaccaaac
CHRM3	22	caagcttccgggtcacag	aggttgtccgatgagggtaa
CHRM4	62	acgccacctttaaaaagacct	agtgccgatgttccgatact

CHRM5	2	aaagcagcccagacactgag	tatacggggtccatgtgatg
HTR1A	71	atcatcatgggcaccttca	gcagaagggcagaacaagag
HTR1B	9	ggtcaccgacctgcttgt	gtgacagtgtacatggtgctga
HTR1D	79	actgaaccagtcagcagaagg	gtggcattcagggatctgtt
HTR1E	2	ccccccttcgacaatgatcta	cgttccctggtgctagagat
HTR1F	5	tttgttcccttgcagtcaca	caatgctgaagggcatcac
HTR2A	51	cagcggtcgatccatagg	ttgctcattgctgatggact
HTR2B	8	tttggcgatttcatgctctt	caatcataattgcaagaggtgtg
HTR2C	47	gcaagttgagaatttagagttaccag	tcacacactgctaatcctttcg
HTR4	49	ttcatggtcaacaagcccta	ccatgaggagaaatgggatg
HTR5A	1	tggtgctcttcgtgtactgg	cttcggatatgggtgagacg
HTR6	4	ctgccttttgtccttgtgg	gatcctgcagtaggtgaagcata
HTR7	59	tcagccaggactttggctat	catgacggacatggggata
TAAR1	50	gttggagtgtccctgctgtt	tcttcagcgcctttgaagtt
TAAR2	79	tctccatggccatcactg	ttctccaccgatctgatcatacta
TAAR9	49	caatgtcgccatggtgttta	gcttggctggctgtactttc
TAAR6	9	ctggctgtgtttggaaacct	gagtgcagctgcttgaaatg
TAAR8	34	tgtgtcgggaatttgcatc	aacacagcaccgctgtacg
TAAR2	79	tctccatggccatcactg	ttctccaccgatctgatcatacta
TAAR5	71	gctgtgggcatatacctcttg	gctgtcgaccatcgtgtcta
MTNR1A	49	agcttagcggtggcagac	atcgacatcagcaccaacg
MTNR1B	49	tcttggtgagtctggcattg	aggattagcgggtaggggta
GPR50	13	ccaccctaagccccattc	tggtgggtagaggcagattt
ADORA1	89	actgcatcaccctcttctgc	ggcatagacaatggggttca
ADORA2A	72	tgctcatgctgggtgtctat	catctgcttcagctgtcgtc
ADORA2B	80	ccgagacttccgctacactt	tgacatctgcttggcagaga
ADORA3	29	caagagaagctttgtgctgga	tagcctcggcaccagtattt
MC1R	85	acacctggaggggaagaact	aggaagcaggaaggagtcgt
MC2R	9	gtcatctggacgttctgcac	ggcacatgatgggagaagat
MC3R	9	actcggagagcaaaatggtc	aggagcatcatggcgaag
MC4R	44	cgggttgggatcatcataag	tgatgaacaaaatgcctgaaa
MC5R	86	ggcatccatgtgcagctta	ggcgtagaagatggtgacgta
CNR1	20	catggacatagagtgtttcatgg	gtgagggacaggactgcaa
CNR2	72	ctgttcattggcagcttgg	agctgcatgcaaagaccac
GPR55	69	cctggctgtcttcgtggt	caggaactgcaggaagaacc
PTGFR	17	aggcagatctcatcatttgga	tgggctccaacaaatacagg
$TBXA_2R$	3	gatcttcttcggcctgtcc	ccaggtagcgctctgagg
PTGDR	7	tctaccgacggcacatcac	aaaggtagcgcgcagaaag

PTGIR	27	cgtccatgctcatcctcttt	cagctgcgcgtagaggtag
PTGER1	28	cgctatgagctgcagtaccc	agaggccagcaagcagtg
PTGER2	41	tgcacctacttcgctttcg	cgaagagcatgagcatcgt
PTGER3	45	aacctttttcttcgcctctgc	ggttgcaggaaaaggtgact
PTGER4	68	gtggtgctgtgcaagtcg	cccacataccagcgtgtaga
GPR26	4	gtcttcactggcgccttc	cttgaggtacgtgcagcaga
GPR78	79	tgctatccaacgcactggt	ggtggcccagagacagatt
S1PR1	42	accgctcttcatcctgctc	aggatgtcacaggtcttcacc
S1PR2	61	ccactcggcaatgtacctgt	acgcctgccagtagatcg
S1PR3	69	cttacgacgccaacaagagg	aaggcaatgagccagcac
S1PR4	19	tttgctgggctggaactg	aagaggatgtagcgcttgga
S1PR5	44	cttccacgctcccatgtt	gccccgacagtaggatgtt
LPAR1	9	cttgccacagaatggaacac	ggccaacatgatgaagatacaa
LPAR2	45	cccaaggatgtggtcgtg	gcagattggtcagcagcac
LPAR3	13	ctggaattgcctctgcaac	tccagaaaacaaggtaactcctg
LPAR4	31	gtcctcagtggcggtatttc	gtggtggttgcattgttgac
LPAR5	4	cctgctggctaacctcgtc	gccagcgtgctgttgtag
GPR3	39	tctactgcctgctgggtgat	ggcagggagcaaggtaagat
GPR6	18	gggtctctggagctgtcct	cacgtcccacggattcac
GPR12	52	ggagaacatctcggctgct	gagctcaggctctggctcta
GPR21	60	cgcttcagcagccagagt	gcatagcgcttatcaggaca
GPR22	29	tcgaataggcacaagattttca	gcctcatgttgtgtggttagag
GPR52	51	gtggcattgtgaatgtgtcc	accacactgtagtggccaaa
GPR119	76	tctactgcgacatgctcaaga	gcatgttccatctttcgaatc
GPR148	6	acctacacctccattctgtgc	gaaacagttggcaatgagagc
GPR153	46	accagcgagcgcttctac	aggaagcagacgccaaag
GPR162	4	cttctctacccctcgggaac	atgtcatcagaggatgaccactt
GPR27	74	ggagctgagggactgcttc	tttcaggtcgcagggatg
GPR85	27	ggacttttcatggtcctggag	tccaaatcctgctagccaat
GPR173	15	ccgtcaacccaattgtctg	tgagtcctcaggcacttcttg
GPR61	73	ctgctgttgactttcgaatcc	ctgctccaggaactcagagg
GPR62	3	gatcctggcagctgtcgt	caggtgcgccaggtagag
GPR161	27	tctggcttcactcgctcat	aaactccacggatgaccaac
PSP24	49	cgcctacgtggtcacctt	cacagcatgacgccaaag
PSP24B	24	gcctggacatgatgcctaa	tatccgtcgctttgtgtgac
GPR101	82	ccagtcgtcgtaacagcaac	cagctttgcactggtagcac
AVPR1A	61	gtgctggcggtgactttc	tgcagagccagcagtacg
AVPR1B	66	cgctacctggctgtctgtc	gatgagcaggtaggtggactg

AVPR2	43	ccgtggctctgttccaag	tacttcacggcccgacac
OXTR	77	gtgccgcctggtcaagta	cagcagcaggtaggtggag
EDNRA	56	acaacacagaccggagcag	ggagtgcttctaagggtggtc
EDNRB	14	gcgaaacggtcccaatatc	gatgtcaatgacgatgtgcag
GPR37	36	ccaggagcagagtgtgaagac	gctctccttggccagtaaaa
GPR37L1	37	tctcctctctgggagtcacg	cacgtggaagcggtcaat
HCRTR1	27	caggctgcagtcatggaat	actgagaagagccgtgtgc
HCRTR2	8	tttctctggctgatgtgctc	cagtgatatccacgaccagtg
GPR103, QRFPR	50	tcagtgacctgctcatcacc	ccagccagttgtcggaaa
NPFF1 (GPR147)	71	atggtggcgctgttcttc	agctgcccgtagtcgatg
NPFF2 (GPR74)	53	ggtttccaagaagctttcca	aagcttccataggctttgctc
NPSR1	1	aagcaagccagggtcctc	cctcttcccaaatatgatcagg
TACR1	44	caatcagttcgtgcaaccag	caccacaatgaccgtgtagg
TACR2	88	taatgccatcgtcatctgga	cgatgaagtagttggtgactgtg
TACR3	59	ctggcgtatggtgtggtg	caggatgatccagatgacga
NTSR1	44	cagagcacggtgcattacc	cagcagggtgagcaggtc
NTSR2	62	ttcctgaatggggtcacagt	gaagtggacggcacttgg
NMBR	9	gcaacatcatgctggtgaag	gccgccaggttagagatg
GRPR	63	ttccaaacctgttcatttcca	atccactggagcacacgtta
BRS3	58	caagctctgtggtttctaacga	tgcttctattcctggagagttgt
CCKAR	88	tctcaggaacccccatttc	cagtagatgatggggttgacg
CCKBR	74	aggcctgcctggaaactt	agggtcctcatcgggaag
NPY1R	15	tgctacctgcaaccacaatc	atggggttgacacaagtgg
NPY2R	76	tgcgcacagtaaccaacttt	gacacagagtgttcaccaaaaga
PPYR1	9	catgaagcaggtcaatgtgg	cagccagagcacagcaaag
NPY5R	48	actgctgccactcggaat	tcatctacactgcttttatagtcatcc
GPR178	75	gcgacacaaagggtggag	agggtactcggcgtccat
GPR38	77	tgaccgtgatgctgatcg	catgctgcccaggtacaag
GHSR	6	ggccttctccgatctgct	ggtactgccagaggcgaac
GPR39	19	ccgagacctccaatatgtcc	gatgctggactggaacacg
GNRHR	59	ccaacctgttggagactctga	ccagcataccattggactgtaa
GNRHRII	55	gcctcctcaatgctccttt	gttcttggtgccctcttcg
GPR150	70	caggcaacaccacagtgc	caccagcaggaagtccatc
PRPR	88	ctgctgctggtcacctacct	ctgacacccggacgtaaga
NMUR1	83	tgtggcacaactacccctc	aaacagtagcgtgcggaaat
NMUR2	15	ggcggtctctgacctcct	ttgcgccacatctcatagac
GPR73	85	ggcttcttacaatggcggta	ggcatcccaattgtcttga

GPR73L1	12	ccacggatgaattatcaaacg	gaatggacaccatccagacc
GPR83	5	catcatctctgtggcctacg	tggtcacatcgccaatcata
GPR19	15	gaccttctcatcagcgttgc	tccaccttccagtggtgaac
GPR75	41	aatgacttagtgcaggaatatgacag	taaacggaggggactggaat
OPRM1	36	cttgcgcctcaagagtgtc	tccttcgaagattcctgtcct
OPRK1	58	gggacttctgctttccactg	tttcggactctgctagtgctc
OPRD1	45	gtgtgcatgctccagttcc	gaacacgcagatcttggtca
OPRL1	38	gcctcaaccccatcctcta	gcagatgcacagcagaactt
SSTR1	42	gatgatggtggtgatggtgt	accagctgcaccacgtaga
SSTR2	76	cctgacagtcatgagcatcg	ccacttggccgacttgat
SSTR3	72	cgacgagctcttcatgctg	ccagtaggacagggcgttc
SSTR4	13	gacaacttccgccgattct	tccagcaccttccaggag
SSTR5	29	gcgtcaaccagttcaccag	gtgcaccactgccaggta
GPR7	4	ggtgctgcccatcaacat	cacatgagctccccgaag
GPR8	9	ccccactggtcatcagtatgt	aggcacgagttggcgtag
GALR1	62	aagaaggcctacgtggtgtg	ggcatagcagaagcagatga
GALR2	38	atggacatctgcaccttcgt	gtaggtcaggccgagaacc
GALR3	41	atcctgtgcttctggtacgg	gaggcaggagttggcgtag
GPR54	3	ctcgtgccgctcttcttc	acgtagatgaccagcgagttc
MCHR1	26	cttggtcctgtcggtgaag	tctgtcctctcctcgtcagc
MCHR2	18	tgtctcagctatgccagcag	ggatttgaggcagacgtttc
CCR1	76	tcacccatgagtgtgagca	gcgatcacctccgtcact
CCR2	76	ggggtggtgacaagtgtgat	aaagatgattcctgggacagaa
CCR3	58	tgagaagctggaaagaaccag	aatagagagttccggctctgc
CCR4	84	ttcgtgttttccctcccttt	acctagcccaaaaacccact
CCR5	7	tgcatcaaccccatcatcta	agaagcgtttggcaatgtg
CCR6	68	aaaggcatctatgccatcaact	gtaccggtccatgctaatgc
CCR7	25	gtcttcctgtcggcacatc	ggagaaggtggtggtggtc
CCR8	64	tgcctcctgtttgtattcagtct	cagaccacaaggaccaggat
CCR9	31	gccatgttcatctccaactg	ggtgacctggaagcagatgt
CCR10	41	ctttgctacaaggccgatg	gtcagggagacactgggttg
CXCR1	3	gcgtcacttggtcaagtttgt	gggcagggacagattcatag
CXCR2	59	aaactggcggatgctgtta	gggtgaatccgtagcagaac
CXCR3	86	tgctggtgctgacactgc	agccaaagacccactggac
CXCR4	79	attgggatcagcatcgactc	caaactcacaccttgcttg
CXCR5	6	gccatcctggtgacaagc	tccaggaagatgacgatgtg

CXCR6	63	tgggttcagcagtttcaatg	ttgctgaactgcaggaagtct
CXCR7	54	gagcacagcatcaaggagtg	agaagggaacggcaaagc
CX_3CR1	35	ccttctggtggtcatcgtgt	aagcgtctccaggaaaatca
XCR1	89	ctggtgaccatggctgtgt	gaagatggtgtcgaggatgg
ADMR	89	acctgggcattgtcctgtc	aggtgtagtccagcgtgacc
BDKRB1	24	gccaacttctttgccttcac	cggcccacaaagacataaat
BDKRB2	4	acatgctcctgaatgtcgtg	catcgtgcagaaggtgatga
AGTR1	66	gtagccaaagtcacctgcatc	cgatggattatagctggcaaa
AGTR2	5	ggtttctagcatatacatcttcaacct	gcccatagaggaagagtagcc
AGTRL1	40	cacctggtgaagacgctgta	aggaagaggtcaaagtcacagg
FPR	62	tctctccccacgaacatctc	ccaggaagagatagccagca
FPRL1	81	gcacagtcaccaccatctgt	gtggccgtgaaagaaaagtc
FPRL2	42	attgctctggaccgctgtat	actcatggtgcgatggttct
C3AR	4	gtcctcaacatgtttgccagt	aagacagcgatccaggctaa
GPR77	72	gctctcagtgccgacctct	gctgaaccgtagaccaccag
C5R1	12	ttggggtttagccctgct	aagtactcctcccggaccac
BLTR	71	gctcatcatcctgaccttcg	cctcagccaggttcacca
BLTR2	74	ggccttggccttcttcag	gtgaggaaacggggacct
GPR44	21	catcctggagcagatgagc	gtcgatgtagcggatgctg
GPR14	24	gcaaccctcaacagctcct	cagcagagtcccaatggtg
GPR31	38	tgctgtttgctctgtgcttt	tggaagatgtgcatcaggac
GPR25	3	ctctacctggcggccttc	cagcagccacaccacaaa
GPR15	51	ctgtgccagcatctggttt	tccctggacagaagagtaggc
GPR32	73	gggcctgcaaactctacatc	ggaggcagttactggcaaag
CMKLR 1	69	gcagtggcagatttcctgtt	catggcggcataggtgata
GPR1	64	tgaacatgtttgccagtgttt	aagacaggatggatcaagtgg
CCRL1	13	cctgaatttggctgtagcaga	ccacccatgaactgcattaac
CCRL2	32	tgcaaatgagaaaaacactaaggt	tggcaaaaacaagcttgaaa
MAS1	62	ttcgctatgcccatgagact	tcccaaaggtcgaccaatac
MAS1L	3	actctactcattagattcctgtgctg	tctgcaccaccgcataga
MRGPRD	3	aggcaacagcatggtgatct	atgcagaaggggttcctgt
MRGPRE	35	catcctcctcaccgtcctc	acagccagtagatgccgaag
MRGPRF	45	gcacgtttgccgactacat	cacgccggtaaggaacat
MRGPRG	62	gcgtggtcctctttgtctg	ccaggacgatgccgtaga
MRGPRX1	87	cctgtggcccatctggta	gacacacaccaccgctga
MRGPRX2	37	tgatcccggtcttcctgat	gagcacaaacccgtttccta
MRGPRX3	70	cctgtggcccatctggta	gagcaggacacacatgactgat

MRGPRX4	1	ggacaagcctgaggtggata	atggccccaatctgcttc
LHR	84	tgcaatctctcctttgcagac	ggtttgggaatcaactgagg
TSHR	63	tggggtgttcgtctccac	tgcgttgaatatccttgcag
FSHR	29	gactcaggctaggggtcaga	cgtcatgtcaaatcctctgc
LGR4	43	tgggaatctatactggcatccta	ccaccaaatgccaaattca
LGR5	20	gtcatcgcagcagtgaaca	tgccaaaagtgaacgcatc
LGR6	24	tcagggcccctagcctat	gcctgggtagaatcacagga
LGR7	58	tttcgtggagaatacaataagcat	tggccaaagatcctacaagc
LGR8, GPR106	56	gatggagagcgtgcagtg	gtcagtagcagaacagagacttcg
SALPR	17	ttttctccaccacggtcaa	agcaacttgtccgggaaac
GPR100R	6	ctctgtccgcatcctggt	agagagtgaccacatggttgg
P2Y1	26	atgttctgtgtccccttggt	aaatcaaagctctcacaattaatcc
P2Y2	41	taacctgccacgacacctc	ctgagctgtaggccacgaa
P2Y4	64	catcacccgcaccatttact	ttcagtactcggcagtcagc
P2Y6	42	ctgcccacagccatcttc	gctgaggtcatagcagacagtg
P2Y11	17	gttggtggccagtggtgt	ccgcatgatgtggtaggg
P2Y12	48	catgattctgacttttccattca	tgacacacaaaagttctcagtgg
P2Y13	21	ttttcttgaccggcatcc	agctggggatgtgaacaaac
P2Y14	84	cttgcggtatatgaaagaattcac	aagaaataaataatagggtccaagca
F2R	68	atctgggctttggccatc	acctggatggtttgctcct
F2RL1	13	gagccatgtctatgccctgt	cgatgcagctgttaagggtag
F2RL2	46	ccatttgtgcttatcatctactgc	ctatgatcgtatgcattaagtgtcc
F2RL3	64	aacctctatggtgcctacgtg	gatccacgcagctgttgag
P2RY8	89	gtggccttttacgcaaacat	ctccacgctgatacaggtca
OXGR1	40	ggtaacctgttactatatgtggtggtc	ttgcatctcactgttgagca
SUCNR1	38	tcatgcggaatgtgaggat	gttgatgacgacctgagtgc
GPBAR1	23	tgcagctcccaggctatc	cagcaggagcccatagactt
CYSLT1	71	tgcagaagtccgtggtcata	ggagagggtcaaagcaacaa
CYSLT2	21	tcatggcttcctcaataatgc	tgatgtgacactgccgttct
PTAFR	22	gtgggagctgcatcctactt	agcactgtcgggcactgt
GPR109B	38	ggtggccatcgtctttgt	gtgcaggagccagaagatgt
GPR109A	50	gagcgtcgagctcacagg	accggagttggccattaac
GPR81	83	gggagcatcgtgttccttac	ggggtggaccactttgaa
TG1019	27	atgagacctggcgctttg	ttggtggacagcatgaagag
GPR68	77	gccccggtggtctatgtta	gtagagggacaggcagttgg
GPR4	44	ccgccatccctctacatct	ccacagagccaggcagtt
GPR132	13	aggtactgcagggcaacg	tgtacagcagctcgcagagt

GPR65	51	atacctggaataaagacaactggact	gaaaagcactccctttgcac
GPR18	29	acccttcgaatgttttattatgc	ttgggtaaaacactgtgagagc
GPR35	86	cgctctgggtgttctgct	cgccaggttggtcatgtag
GPR40	20	gtgtcacctgggtctggtct	ccagggaggtgttgctgt
GPR120	13	gctcatctggggctattcg	gggacgactcggaagaaga
GPR41	43	cacccactgtggtacaagacc	ggccacactcaccagacc
GPR43	19	ttcaccgataaccagttggac	gggatgaagaagagcaccag
GPR84	46	tgcatcaaccctgtgctct	aaatggagccatatgcttgg
GPR30	71	gtggtgctggtcttcttcgt	gaggtgcacgctgatgaa
TRHR	41	gagacagtcagtgaactgaacca	attctaaggccaccactgctc
EBI2	84	ttccacttataatcattctcatctgc	tgggttttgtttggcagttc
P2RY5	74	cacaaacatttgttaattgctcagt	cagagagtgattgggtacattgtc
P2RY10	34	atggctttccaagggatca	gactgcagcacacatgaaca
GPR17	81	gtctacgtgctgcactaccg	tgaggcaggaggtgatgc
GPR20	18	tctttgcgctgactgtcct	ccggtaaacacgctgatga
GPR34	16	gccattgcagacctcctact	tgtccacttgttttggttaatatgat
GPR55	71	agcagaaagcctgcatctaca	ggaaggagaccacgaagaca
GPR82	88	cttccatggctttaccaatca	tgtttccaaaaacaccaacaat
GPR87	40	gccatatccaggtacatccac	gctttcggcttgactgactt
GPR92	4	cctgctggctaacctcgtc	gccagcgtgctgttgtag
GPR171	76	tcaaagccaaagaggctacac	ggataggatcaaagcacaggtt
GPR174	89	gacggctttcaagacaagattt	tcacaaaggattttgcatgg
GPR182	89	acctgggcattgtcctgtc	aggtgtagtccagcgtgacc
SCTR	31	gcatcatggccaactactcc	gcgaggagtgtgtgaaggtag
GHRHR	29	gccaccatgaccaacttca	ccaggaggcagttcaggtag
GIPR	22	cccagatcgtgacccagta	aggagactgtgcaggtagacg
PTHR1	30	acacaagtgtgaccaatgtcg	ggtggcagtgggcagtag
PTHR2	15	tcactttacctggctatgtctgg	tggtctcctcgtggaaagag
CALCR	17	ctggcgacatcccaatttac	ggttgttggctggttcattc
CALCRL	82	tggcgacctgaaggaaag	agtgcataaggatgtgcatga
CRHR1	10	ggtggcaggacaagcact	gcttgatgctgtgaaagctg
CRHR2	9	cggaatgtgattcactggaa	cagcaggaaccacatgaca
GCGR	49	gtggcagccaggattcat	ggggctctcagccaatcta
GLPR1	56	ttgagcacttgcacatcca	tgctggtgggacacttga
GLPR2	46	cattactttgtgggtgccaat	agcagcgtgtggaggtaga
VIPR1	16	gggctggaaccccaaata	catggaaacctgcgtgct
VIPR2	7	gattacagggtctgcggttc	ggtctccgtttgcaggaag

PACAP	11	gcggagatcaagcgaaaat	gaagtccacagcgaagtaacg
CASR	26	cacagcagcaacgatctca	cgctgccaaagatgacct
GABBR1	63	agacagggtcatcgaccaac	cggttctccttctccaacag
GABBR2	16	cacacggaagccaactcat	atccacgccaatgtagcc
GRM1	49	tctgcaataccagcaacctg	tcatgatgaggagtccattgtag
GRM2	3	gttcccagtgcaccagaag	acggtgctcattgacagga
GRM3	75	ctggttccgggacttctg	gcctgtggttgcgtttgt
GRM4	3	aagtgtgacatctcggacctg	ggtgcacgtgaccatgag
GRM5	20	agaccaaccgtattgcaagg	gaatctgggctttttggtaca
GRM6	57	tacgccatcaccttcctcat	gagcagggcagagtagctga
GRM7	72	cctgggctgtgattcctg	tgacaaagatggtggcaatg
GRM8	80	gtggtgtttgcattgctcag	tgtttctagcaggcgtttga
GPRC6A	48	tgactccttggccatcctac	tgccaacaaccagaacaaat
TAS1R1	37	tgtgtatgccacgctgaga	tctccttggagctctatgtggt
TAS1R2	44	ggtttcagcttcgcctacat	gatgaacttggcctcgttgt
TAS1R3	71	cccaggtgttacctgctcat	cccctcccaggaagaactc
GPR156	20	ggagaccaagtccccatga	atctgagccgcctctttct
GPRC5A	11	ccttcaccttctgtggttcc	gcatcgtgaggtagatgtgg
GPRC5B	15	atgagagctcaccaggtgct	agaggccaccgaggtgat
GPRC5C	3	tttgtgcaggacaccaagaa	ccccagaaggaagaatacctg
GPRC5D	18	gtctgcattgctctggtcac	gcagagctcagggacgat
GPR158	53	cgggtggctggttactctt	ttccgggaccaggttagg
GPR179	43	cctgggagctggatgataac	ctctacctgtccccagactca
CELSR1	76	aatgtggccgtgtacaacct	ctaaggggagtgggactgc
CELSR2	15	tttgagcggggaaatgaac	cttcagctcacccgtgga
CELSR3	7	gggtcagggagcctatcttc	gactattccgggcgcttt
BAI1	12	cgtgcgcacctaccagtt	ctctccacgcccaggtag
BAI2	23	ccacaccctgtccaatgc	agtgcaaatcggcctcag
BAI3	17	tacccctgaatgagcagaca	gattgcagacttgggtggtt
CD97	89	caagaagcaagccgaactg	gagaggcgtctgagttggac
EMR1	77	catctgtgcctttggttatgg	gctgcacactggcagagat
EMR2	42	cagcactgtgtggccagt	ccattggaaaggttcttgct
EMR3	71	gtgtctactggaagagcacagg	ttgttcacgtgtatcaggaagc
ELTD1	65	tgccgacctgtaaaagtgtg	gcaagatttggcagacttcat
GPR97	3	agaaccggaagaaggtgctc	caacccccatgtcacacc
GPR110	18	tctggatgctcatgcttgg	tgtgatggaacacgaggatg
GPR111	55	atttttgaaaagatcagcaagtca	agagtgccagccaacacac

GPR112	43	cccaactaggcttattgagacc	gatgtcaattctgtttcagctgtc
GPR113	21	cttcgccttcagcttaccc	aggaaacgtgggtccaaac
GPR114	74	gccttggccttcttttcttt	ttaagatggtgaagaggaacagc
GPR115	3	tcagcaaaaatgttgccatc	gctattccaaaaccccaggt
GPR116	46	ggtccactcagaaagccatt	tgatgaccgagatggcaag
GPR123	27	ccacttcctggacatggtct	gaagagtcccagggtcacg
GPR124	75	gtgtgcagctgcttgtacg	ggcacagtggtgagtgaaga
GPR125	65	tgtggtattagagagttctgcacagt	ctgaagtcacctttgttgtttacc
GPR126	69	ccctgctgttcctgaatctc	caatgcaaagtccatccaca
GPR127	33	cacgggcagattcaagaag	ttctggggtccaactattgc
GPR128	7	cttccaaagtgttgatgttgct	tggcaatgacctgaggaaa
GPR133	22	cttcttcctgagtgccttcg	ttgatcaccatgctgtagaggt
GPR144	13	tggtgttcgacgagaggac	gtgagtacacgcggtcagc
GPR64	10	ctatgccccaaacccatgt	cacggtgggagaggaaaat
LPHN1	28	tccgtgtacgtggatgatga	gcattggtgttgaaggcata
LPHN2	10	ctggatcacaggaaggaagc	ggtggaattttggttgtagaaact
LPHN3	8	cgctgccctgttacatttct	acccccctccaggaacatc
GPR56	18	aaggtgcacatgaacctgct	gctgagcaggaagctcgt
GPR98	18	cagcacagccttttcacg	ctgggtggacttgctgattt
GPR157	17	ctgtccaccttcgccaac	ggtacaagtagagcgcaatgg
FZD1	69	cggcaagaccctcaactc	ccttgtttgctgttggtgag
FZD2	1	ggtgtcggtggcctacat	gagaagcgctcgttgcac
FZD3	78	gccatatcctcgacttgtgg	actggggctccttcagttg
FZD4	64	gaagccattgaaatgcacag	gttttcactgcggggatg
FZD5	49	tcttcacgctgctctacacg	ggtagtgctgctcgtacaggt
FZD6	14	cgtctatgagcaagtgaacagg	tggtactgacgacaatgatcaga
FZD7	3	ggacatcgcctacaaccag	gcgtcctcttggttcgtg
FZD8	39	ctctgcttcgtgtccacctt	gaagcgctccatgtcgat
FZD9	27	ccacggcagctatttcca	ggatgacgatggtcttgagc
FZD10	74	ttggacctccaagactctgc	tccggctcttcttctttaacc
SMOH	24	cattcctcgactgcctcag	tccctgtcggcaagagtc
TAS2R1	7	tcctgtccttcctgatcctc	aaagatgaaccttctgatgtgaaa
TAS2R3	49	agctctattctgtctttaggggaat	cctcttctttctgaagtgttcagtc
TAS2R4	35	caattgcaaaacttgggtca	cccaggctgaacagaatcc
TAS2R5	88	cagtccaaattggcttaacattc	agggataccgaatgctgct
TAS2R7	10	ttgcctactatttgtcctttctca	gctaattccgtctctggcata
TAS2R8	41	gccagttcctctcatccact	tccagtgcaccaccatatca
TAS2R9	63	gggccataaaggcagtgat	caagaaagactgggtagtacacga

TAS2R10	76	tgcgagaaaacaaactgctg	aatgagtgaccccagggata
TAS2R13	20	tgctaggaaccttggtcttctt	ccaagttgtgtttctttcatatcg
TAS2R14	5	acaggcctctctgtcagtgc	tgagggctccccatcttt
TAS2R16	46	gcagcctaattgttgcagtg	acaggcatcagccttctgac
TAS2R19	73	tgactgggttaacacacgaaag	ctggagaccaccagagcag
TAS2R20	73	atttcattgcctgggtcaag	gagactgccagagcagcaat
TAS2R38	64	tgaaggtctataccagaaactctcg	agacttgagggctttaatgtgg
TAS2R39	27	attcttaaatttttgtagcctgtgg	attggcaatcttcacaaagtagaa
TAS2R40	67	tttccaggctcttgctacaga	caattcggaatagcagactgaa
TAS2R41	40	ggccttcttcgtgttgctc	agcacaatgaagccattcg
TAS2R42	30	cttcccccactccctttc	agcataacaatcattccgttca
TAS2R43	12	cagcaccaaggtccacataa	ttccagacttccaaaactcca
TAS2R44	9	ctccaaccttatttttcttcacttaaa	gttgacaagccaaaaatagtaaagg
TAS2R45	41	gccaagctattggattcagc	cttgtttccccaaatcagga
TAS2R46	12	gatctcaagatcccagcatga	tggacagaaagtaaatggcaca
TAS2R47	75	tctgtccatgatcatatcagtttgt	tggcagaacatgaagacagg
TAS2R48	73	tgactgggttaacacacgaaag	ctggagaccaccagagcag
TAS2R49	73	atttcattgcctgggtcaag	cagagcaaaccaactctgga
TAS2R50	75	cctaatcgtttcggtttgga	cagccttgctaaccatgaca
TAS2R60	87	tctagctttccagtgggacttc	ctgagccaggtagaggacca
VN1R1	21	gcaacagactctcctgcagac	gagctcaccaggaccatga
VN1R2	2	tgtaggaacaatctctaccccaac	ctcaccaatgcaaggatgc
VN1R4	2	atccaggtgggcaaaactta	taccaacatgcacacgatcc
DARC	68	cctggcaattttgcactgt	gcctggtggcagaatagg
GPR88	3	gcagctaccgcctgctac	agaggagggacaccgtgag
GPR135	3	cgtcctcatcatgatcgtctt	gcagcaccaggaagcagta
GPR137	74	cttgcccttctggcttctc	ggttcataagcgtcaaggtga
GPR139	86	cctctgtgcatcacgtcct	agcagggcaccaggtagac
GPR141	85	cgttgctgtgattctgttgg	cgtagcttctgcaccatcaa
GPR142	52	gaagaaaagcctgggcaag	ggtgacctcgaaggatcg
GPR146	3	gctctggacgccacactatc	ttccctcgcgagatgatg
GPR152	83	gcacagagcttgatgatgagg	ccaccaggaagaccgtgt
GPR160	9	gcattcagagttactggctgtc	cccaacaggttatgaaagctaca
GPR151	49	cagccaagagcctgacaatc	ctgggtcacttgcatacatga
GPR149	13	ccccggttacttccaatttc	gaggccctggcataaataca
GPR143	64	ttgtctttggccttctacgg	tccttcctgggagactgaaa
GPR172A	8	cactgactgcccttctggtc	ggcaacagcagcagaagac

GPR172B	3	gcccatcagctgttctcag	attggtcacggcactggt
GPR137B	38	ccttcgtcttctggctgct	cagcgtgagggtgaaaaact
GPR137C	38	ctgcacttcttcccccact	gcgtggagaactggagaca
TM7SF3	68	gagaggctctatggccgatt	gcaaaggcgttctctctcc
GPR175	36	tcctacaaatgccaagtggac	cgtagggctggggtaggt
GAPDH	82	gcatcctgggctacactga	ccagcgtcaaaggtggag
GPR133	29	ccagctctgcataccaaaca	acctctcagggtttccgagt

Table 24 App. Primer sequence and probes used for GPCR array validation

Human gene	Probe	Forward primer	Reverse primer
VIPR1	25	atgtgcagatgatcgaggtg	tgctgcagcctattgtctcat
GPR137B	38	ccttcgtcttctggctgct	cagcgtgagggtgaaaaact
ADRA2C	64	gtgctgttttgcacctcgt	ctgcgtcaccgaccagtag
CMKLR1	76	ttgagttccccatctctcgt	agcaggatattttcccatgc
CYSLT1	34	agcagtagcagatctactttgtgtg	agccaaatgcctttgtgaac
ADRB1	71	gcagaaggcgctcaagac	cacgttggccaggaagaa
GPR18	29	acccttcgaatgttttattatgc	ttgggtaaaacactgtgagagc
NPY1R	53	tgctacctgcaaccacaatc	atggggttgacacaagtgg
LGR4	75	ttggggttgtgactcttatgc	ctttctcctgtgccacactg
LPAR1	69	ccccacagaaggctcagac	ggtcattgctgtgaactcca
AGTR1	23	gctttccattatgagtcccaaa	aaaggaaacaggaaacccagta
TAS2R4	64	tcctgctgaagcggaatatc	ggtgaaagcagaaatcagca
ADORA2A	90	atcatctggcccagggtag	tcaggaaggggcaaactct
GPR21	8	ttcctgatttggctatactcgac	tatccaggtttgccccagt
GPR4	45	cacaacctgctccgcttt	ttcctcttggaggtgagtgg
GPR112	58	ttcctaagcccacactggac	ccccagtggatatgagtgga
PTAFR	37	cctgccactttggattgtct	gcacaggaatttggggagta
GPR109B	4	gaatggcactgcaaatgtgt	tgagcagaacaggatgatgc
CXCR3	63	agactggcggggacagtt	tgtgattgagtctgatttagtctgtg
TAAR8	83	tgatgggctggaggaattag	aacacccagccttgacttaca
LGR7	50	ttccccttgattcagtggtc	accgatggaacagctcgtaa
GPR115	71	gcgaaggaccttgtatttcttct	tccatgaaagtccttagcacag
DRD1	8	ccttggctgtgtcagatctctt	gccagaagccagcaatct
P2Y12	27	tttgcctaacatgattctgacc	ggaaagagcatttcttcacattct
CXCR6	49	tgcatcactgtggatcgttt	cttggcttgctggttgtagg
LPAR2	24	tcggcttcttctataacaacagtg	ccacgaccacatccttgg

NPY5R	21	tctcaagagaaccactccagaa	gggacccctggtatgaactt
EDNRB	53	gcacatcgtcattgacatcc	attcccacggaggctttc
GPR110	19	ccccagaactgctaccttca	tggctgaggttgttgagatg
GPR20	58	cgtcctcggttacttcctca	gtccacgcagatgcaggt
CXCR7	4	cccttctccattatcgctgt	gcttctcctggtcactggac
PACAP	13	agagctgtggcttaccagatg	cccccagagtttgaggtatg
CCR7	58	tgtgggcatctggatactagc	gctcctctggaggtcactgt
HCRTR1	1	actggtgcggaactggaa	gcttcacttcagccaggaag
ADRA1A	84	cccgggtgagaagtaaaagc	tggttggaacttgatggttct
GPR97	63	cccaagtcagagcaccaca	attcttgagatgcggagtgg
PTGER4	44	gtccaacgtgctcttttgc	accaggtgtctgggtactgc
FZD1	2	accagcaaccctcagcac	ggtaggagggcaccttgag
HTR2B	31	tgggcagctcttctgatactc	tgaaacagccagaataacaagg
S1PR2	34	gctcatctcgctggtcct	gcacagcacataatgcttgg
S1PR1	3	ccgcctcttcctgctaatc	gcagttccagcccatgatag
P2RY5	69	ctggcgtgtggttaactgtg	ccctgagagtgggtagactga
P2Y2	27	ctacaggtgccgcttcaac	cgttcagacacagcccaag
EDNRA	78	aaaggaaagagaaaacagatggaa	gcatgtctgaaacgtgttgttt
S1PR3	66	tcctctacgcacgcatctac	tccgagttgttgtggttgg

Table 25 App. ON-TARGETplus siRNA pools for Human GPCRs

Catalog Number	**Human GPCR siRNA - SMARTpool**	**Target sequences**
L-005556	GPR21 (2844)	ucgaaucacuaguguauuu
		agacuacgccuguguauuu
		cgugguccccuucuuuauca
		ccucuuaauggaugucaua
L-005533	GPR112 (139378)	gcacauuccucuacacuau
		ugacaaagauccuuacugu
		gcaaacaucuacauuggau
		aggcacaccuucagaaaaua
L-005536	GPR115 (221393)	gcucaaggaauccguaaga
		gauccaagauucacuaaa
		ccaaaucgaugaccgacaa
		uggcuuaacugggacaaua
L-005467	CMKLR1 (1240)	ugagaggacuucuaugaau
		cagccaaccugcaugggaa
		cggcaugcuuugauccuca
		cauagaagcuuuaccaaga
L-005475	CYSLTR1 (10800)	ggucuugcauuauguguca
		uaugucaaccucuauugua

		ucuaugaucucuguuguag
		agucagccuuccaaguaua
L-005649	RXFP1 (59350)	caacgagacucaauuccua
		caacugaaauacggaauca
		guauuaauuuggccgcauu
		ggauggagaguacucauug
L-005552	GPR18 (2841)	ggugcuacuuggucauuau
		gucaugcuauaccguaauu
		gaaccacgguaaccaucua
		gugggagucuggauaauga
L-005709	PTAFR (5724)	gcuuugagcauuacgagaa
		uagcaccaacugugucuua
		gauacacucucuucccgau
		gacaugcucuucuugauca
L-005570	GPR4 (2828)	ccugcaagcucuuuggguu
		ggaugaaccucuaucgggu
		uaccacagcucacuggcuu
		cgagaccgcuacaaccaca
L-005474	CXCR6 (10663)	ggucucacaucaauggaaa
		cugggcaucuacacuauua
		ggucauauccaucuucuac
		ggcaaugucuuuaaucucg
L-006688	HCAR3 (338442)	ucaaauaaccauuccaaga
		agaaguugcugauccagaa
		cguucgugauggacuacua
		cgccagggcagcaucauau
L-003688	P2RY2 (5029)	cgagaacacuaaggacauu
		cgacagaacugacaugcag
		gga augcguccaccacaua
		ugccgcu gcuggucuauua
D-001810	Non-targeting Control	ugguuuacaugucgacuaa
		ugguuuacauguuguguga
		ugguuuacauguuuucuga
		ugguuuacauguuuuccua

11 LIST OF ABBREVIATIONS

5-HT = 5 hydroxytryptamine

AC = adenylate cyclase

ACE = angiotensin converting enzyme

Akt = Protein kinase B (PKB)

AT1R = Angiotensin receptor 1

AT2R = Angiotensin receptor 2

BALF = bronchoalveolar lavage fluid

BMPR- bone morphogenic protein receptor type II

cAMP = cyclic AMP

cAMP = cyclic AMP

CAV1 = caveolin-1

CCL19 and CCL21 = CC-chemokine ligand 19 and 21

CCR7 = CC-chemokine receptor

cGMP = cyclic GMP

CHF = congestive heart failure,

CMKLR1 = Chemokine receptor-like 1

CXCR6 = Chemokine (C-X-C motif) receptor 6

CXCR7 = Atypical chemokine receptor 3

CYSTLR1, CysLTs = Cysteinyl leukotriene receptor 1

DAG = diacyl glycerol

DCs = dendritic cells

DRD1 = Dopamine receptor D1

ECE-1 = endothelin converting enzyme 1

ECL = enhanced chemiluminescence

EDNRB = Endothelin receptor type B

EETs = epoxyeicosatrienoic acids

EETs = Epoxyeicosatrienoic acids

EGF = epidermal growth factor

EIA = enzyme immunoassay

ENG = endoglin

eNOS = endothelial nitric oxide synthase

EPAC = exchange protein directly activated by cAMP

ERA = endothelin receptor antagonists

ERK = mitogen-activated protein kinase 1

ETA = endothelin receptor type A

ETB = endothelin receptor type B

FRET = Fluorescence Resonance Energy Transfer

FGF = fibroblast growth factor

GDP = guanosine diphosphate

GMP = guanosine monophosphate

GPCRs = G protein-coupled receptors

GPR109B = Hydroxycarboxylic acid receptor 3

GPR112 = G protein-coupled receptor 112

GPR115 = G protein-coupled receptor 115

GPR18 = G protein-coupled receptor 18

GPR21 = G protein-coupled receptor 21

GPR4 = G protein-coupled receptor 4

GPR97 = G protein-coupled receptor 97

GRK = G protein-coupled receptor kinase

GTP = guanosine triphosphate

HBSS = Hank´s buffered saline solution

HETEs = hydroxyecosatetraenoic acids

HIV = human immunodeficiency virus

HRP = horse radish peroxidase

IP = prostaglandin I2

IP = prostaglandin I2 (prostacyclin) receptor

IP3 = inositol-1,4,5-triphosphate

JNK = c-Jun N-terminal kinases

LAS AF = Leica Application Suite Advanced Fluorescence

LGR7 = Relaxin/insulin-like family peptide receptor1

LIM = Lin-11, Isl-1, and Mec-3 protein

LO = lipoxygenase

LPAR1 = Lysophosphatidic acid receptor 1

LPAR2 = Lysophosphatidic acid receptor 2

LTB4, LTC4, and LTE4 = Leukotrienes B4, C4 and E4

LTD4 = Leukotriene D4

MAPK = Mitogen-activated protein kinases

Mins = minutes

mPAP = mean pulmonary artery pressure

mTOR1 = mammalian target of rapamycin complex 1

NF-κB = Nuclear factor-κB

NPY1R = Neuropeptide Y receptor Y1

NPY5R = Neuropeptide Y receptor Y5

P2Y2 = Purinergic receptor P2Y

PAF = Platelet-activating Factor

PAH = pulmonary arterial hypertension

PAP = pulmonary artery pressure

PCNA = proliferating cell nuclear antigen

PCP = pulmonary capillary pressure

PDE-5 = phosphodiesterase type 5

PDGF = platelet derived growth factor

PDGF-R TKI = PDGF receptor tyrosine kinase inhibitors

PFA = Paraformaldehyde

PGIS = prostaglandin I synthase

PI3 kinase = Phosphoinositide 3-kinase

PKC = protein kinase C

PLC = phospholipase C

PPARs = peroxisome proliferator-activated receptors

PTAFR = Platelet-activating factor receptor

PTGER4 = Prostaglandin E receptor 4 (subtype EP4)

PVR = pulmonary vascular resistance

qPCR = quantitative realtime polymerase chain reaction

RAS = Renin–angiotensin system

RGS = regulators of G protein signaling

RhoA = Ras homolog gene family, member A

ROCK1 = Rho kinase 1

R-SMAD = receptor-regulated Smads

scr = scrambled

SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sec = seconds

sGC = soluble guanylate cyclase

siRNA = small interfering RNA

SMCs = smooth muscle cells

TAAR8 = Trace amine associated receptor 8

TBS = tris-buffered saline

TBST = tris-buffered saline with tween 20

TGF-β = transforming growth factor beta

TKR = tyrosine kinase receptor

TM = transmembrane

TP = thromboxane receptor

VEGF = vascular endothelial growth factor

VIPR1 = Vasoactive intestinal polypeptide receptor 1

VPAC = vasointestinal peptide receptor

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15 DECLARATION

„Ich erkläre: Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe.

Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht.

Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

Gießen, 2017

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Gayathri Viswanathan

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