UNIVERSITY OF CALIFORNIA, SAN DIEGO

G protein-coupled receptor expression and function in Pulmonary Artery Smooth Muscle Cells: Novel Targets in Pulmonary Arterial Hypertension

A Thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

by

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2013
EPIGRAPH

I used to walk down the street like I was a star... I want people to walk around delusional about how great they can be - and then to fight so hard for it every day that the lie becomes the truth.

*Stefani Joanne Angelina Germanotta (Lady Gaga)*
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LIST OF ABBREVIATIONS

cAMP = 3’,5’-cyclic adenosine monophosphate

PKA = Protein Kinase A

EPAC = Exchange Protein directly Activated by cAMP

PDE = Phosphodiesterase

FSK = Forskolin

IBMX = 3-isobutyl-1-methylxanthine

GPCR = G protein-coupled receptor

PAH = Pulmonary Arterial Hypertension

SPAH = Secondary Pulmonary Arterial Hypertension

IPAH = Idiopathic Pulmonary Arterial Hypertension

CH = Chronic Hypoxic

MCT = Monocrotaline

PASMC = Pulmonary Artery Smooth Muscle Cells

HEK 293 Cells = Human Embryonic Kidney 293 Cells

PAP = Pulmonary Arterial Pressure

RANTES = Regulated Upon Activation Normal T-Cell Expressed, and Secreted
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Pulmonary arterial hypertension (PAH) is characterized by increased pulmonary vascular resistance, in part due to increased proliferation of pulmonary artery smooth muscle cells (PASMC). Since the second messenger 3’5’-cyclic adenosine monophosphate (cAMP) decreases proliferation of PASMC, G protein-coupled receptors (GPCRs) that couple to Gαs are attractive targets for PAH. We used a TaqMan® GPCR array to identify the GPCRs expressed by PASMC isolated from normal subjects and from patients with PAH. The data revealed that human PASMC express >135 GPCRs, at least 50 of which regulate cAMP formation. We found that GPCR expression correlates with function e.g., of Gαs-coupled GPCRs with formation of cAMP and inhibition of cell proliferation (a functional response to receptor activation), thus documenting that we had identified physiologically relevant GPCRs. Our studies of PAH-PASMC with GPCR arrays revealed that PAH is associated with an increase (>2-fold) in the expression of 41 GPCRs. The greatest increase in GPCR expression was of two orphan receptors, namely
GPR113 and GPR75, whose expression was absent in normal PASMC. We also found the mRNA and protein expression of GPR113 and GPR75 were increased in animal models of PAH. Importantly, treatment of PAH-PASMC with a GPR75 antibody blunted the increased proliferation of PASMC and increased cellular cAMP levels. Taken together, the data in this thesis provide evidence that the GPCR microarray can identify GPCRs that contribute to the physiology of PASMC and can uncover new drug targets, such as GPR75 for PAH—a disease that requires therapies beyond those currently in use.
Chapter 1. Introduction

1.1. Pulmonary Arterial Hypertension

The pulmonary circulation is a low resistance, low pressure and highly compliant circulation, which allows for free gas exchange. Deoxygenated blood is pumped from the right ventricle through the pulmonary artery, where oxygen diffuses into blood and is exchanged for carbon dioxide in the hemoglobin of the erythrocytes. The oxygen-rich blood returns to the heart via the pulmonary veins to be pumped ultimately from the left ventricles into the systemic circulation. Normal (systolic/diastolic) pulmonary arterial pressure (PAP) is 24/9mmHg with a mean arterial pressure of 15mmHg, much lower than the average systolic/diastolic arterial pressures (120/80mmHg) in the systemic circulation. Abnormal vasoconstriction, pulmonary vascular remodelling and/or thrombosis in situ can lead to an increase in PAP and the development of pulmonary arterial hypertension (PAH), high blood pressure in the pulmonary circulation.

PAH is characterized by a mean PAP of greater than 25mmHg at rest or 30mmHg during exercise.\textsuperscript{1-2} PAH can occur secondary to a number of diseases (secondary PAH, SPAH), such as congenital heart disease, drug/toxin exposure, connective tissue disease or chronic obstructive pulmonary disease or can be primary or idiopathic (IPAH), which can be the result of a sporadic or familial genetic mutation or have an unknown cause.\textsuperscript{3} The female to male ratio for IPAH is about 2:1, suggesting that women may be predisposed to the disease and highlighting a possible role for estrogens in disease development.\textsuperscript{4} PAH is associated with increased vascular resistance due to sustained contraction and narrowing of the small pulmonary arteries (PAs): increased proliferation
of pulmonary artery smooth muscle cells (PASMC) contributes to remodeling of the PAs. Muscularization of peripheral arteries, medial hypertrophy of muscular arteries (which includes proliferation of fibroblasts and PASMC, endothelial cell swelling, and fragmented elastin), neointima formation (invasion of inflammatory cells), plexiform lesion formation (endothelial channel formation), and loss of small precapillary arteries contributes to the progression of PAH. Symptoms of PAH include shortness of breath with exercise, difficulty breathing at rest, dizziness and chest pain due to the excessive strain on the heart. The abnormally high pressure in the PA leads to right ventricular hypertrophy, which can ultimately lead to heart failure.

PAH has a poor prognosis and currently no cure. The goal of treating PAH with drugs is to reduce pressure and resistance in the PAs and to increase cardiac output. Current treatments for PAH include anticoagulants, vasodilators, and heart/lung transplantation. The vasodilators that are currently used clinically are calcium channel blockers, intravenous prostacyclin, inhaled nitric oxide (NO), endothelin receptor antagonists and cyclic nucleotide phosphodiesterase (PDE) 5 inhibitors. A major issue with the development of drugs for PAH is their lack of specificity for the pulmonary circulation. Vasodilators in the pulmonary circulation also tend to vasodilate the systemic circulation, leading to systemic hypotension. Discovery of pulmonary-selective targets are thus essential for the development of future therapies for the disease.
1.2. cAMP

3‘,5’-cyclic adenosine monophosphate (cAMP) is a ubiquitous intracellular second messenger that was discovered by Rall and Sutherland in 1958. Cyclic AMP has many effects: reduces inflammation and systemic blood pressure, can be pro-apoptotic in certain cell types (such as immature lymphoid cells) or anti-apoptotic in other cell types (such as epithelial cells), decreases platelet aggregation, inhibits fibrosis, causes bronchodilation, inhibits PASMC proliferation, and vasodilates the PA. The intracellular concentration of cAMP is determined by the activation of G protein-coupled receptors (GPCR) that stimulate or inhibit the activity of adenylyl cyclases (and thus, the synthesis of cAMP) and by PDEs, which hydrolyze cAMP. Nine membrane-bound isoforms of mammalian ACs have been characterized, each with their own tissue distribution and regulation; AC6, which is Gαs-coupled, is highly expressed in PASMC. Eleven PDEs have been characterized that hydrolyze cAMP to 5’-AMP, thus reducing its intracellular concentration (Figure 1.1). PDE1, PDE3 and PDE4 appear to control cAMP degradation in PASMC. The balance between formation by ACs and hydrolysis by PDEs controls the intracellular level of cAMP and the duration of its signaling. cAMP primarily activates two downstream effectors, protein kinase A (PKA) and exchange protein directly activated by cAMP (EPAC). Both of these effectors contribute to the antiproliferative and vasodilatory effects of cAMP in the PA.
Figure 1.1. Schematic of the cAMP pathway. Upon ligand binding of a Gα<sub>s</sub>-coupled GPCR, the heterotrimeric GDP-bound G protein exchange GDP for GTP on the Gα<sub>s</sub> subunit, thereby promoting subunit dissociation and activation of AC, which catalyzes cAMP formation from ATP. cAMP is degraded (hydrolyzed) by PDEs to 5’AMP. cAMP activates the downstream effectors, PKA and EPAC, which lead to vasodilation and inhibition of proliferation of PASMC. There are multiple ways to increase intracellular levels of cAMP in PASMCs: (1) activate Gα<sub>s</sub>-coupled GPCRs; block Gα<sub>i</sub>-coupled GPCRs, (2) increase AC expression or activity, (3) inhibit PDEs, and/or (4) activate PKA and EPAC or proteins that those effectors regulate.
1.3. Targeting cAMP in PASMC

As outlined above, cAMP relaxes PA smooth muscle and helps control pulmonary vascular tone.\(^{18-20}\) Increasing cAMP also inhibits PASMC proliferation.\(^ {21-23}\) A membrane permeable cAMP analog, 8Br-cAMP, reduced the percentage of cells in the S phase of the cell cycle after serum stimulation, by preventing cell cycle progression from G0/G1.\(^ {24,25}\) It has been proposed that cAMP decreases smooth muscle cell proliferation through both PKA and EPAC activation and inhibition of mitogenic pathways.\(^ {26-31}\)

Stimulation of PKA results in the phosphorylation of a number of proteins, thereby, regulating cellular processes and gene expression, which can produce vasodilation of the PA smooth muscle. PKA can phosphorylate Raf-1 on serines 43 and 621, thus inhibiting p42/p44 mitogen-activated protein kinase (MAPK) activation.\(^ {32,33}\) Inhibition of the phosphoinositide 3-kinase (PI3K) pathway by cAMP may also play a role in attenuating cell proliferation.\(^ {30}\)

EPAC-1 and EPAC-2 are cAMP-dependent guanine-nucleotide-exchange factors for the small GTPases RAP1 and RAP2, which are important mediators of cAMP signaling. EPACs have been associated with various cellular processes, such as integrin-mediated cell adhesion and cell-cell junction formation.\(^ {34}\) In a vascular injury mouse model, EPAC-1 was shown to be up-regulated during neointima formation and promoted vascular smooth muscle migration.\(^ {35}\) These data suggest that EPAC-1 regulates vascular remodeling upon vascular injury. EPAC-1 is decreased in PAH-PASMC.\(^ {31}\) Elevation of cAMP in response to β2-adrenergic receptor agonists or prostanoids activates both PKA and EPAC and can induce airway smooth muscle relaxation, inhibit airway smooth
muscle proliferation, and modulate cytokine secretion. EPAC induces airway smooth muscle relaxation through inhibition of RhoA and activation of Rac1. EPAC and PKA inhibit airway smooth muscle proliferation and cytokine secretion by signaling to PKB/Akt, p70S6K, ERK1/2, and NF-kB.31

There are multiple ways to increase intracellular levels or the function of cAMP in PASMCs and produce vasodilation and decreased proliferation: (1) activate Gαs-coupled GPCRs, block Gαi-coupled GPCRs, (2) increase expression or activity of ACs, (3) inhibit PDE expression or activity, and/or (4) activate downstream effectors of cAMP (PKA and EPAC) or by altering the expression or activity of proteins regulated by PKA and EPAC (Figure 1.1). GPCRs are attractive drug targets to raise cAMP in PASMC since they 1) localize on the plasma membrane, making them easily accessible to drugs; 2) are the largest receptor family, comprising of 3% of the human genome; 3) are the targets for over 30% of prescribed drugs, and 4) can be tissue-specific, which is beneficial in efforts to selectively target the pulmonary circulation.36,37 GPCRs are the most “upstream” component in the signal transduction pathway, thus the targeting of GPCRs benefits from the post-receptor amplification that occurs in this signaling pathway.

1.4. G protein-coupled receptors

GPCRs comprise a large protein family of 7 transmembrane receptors, which are guanine nucleotide exchange factors for heterotrimeric G proteins. Activation of these receptors occurs when ligands bind to the extracellular domain of the receptor, altering its
conformation and in turn, the activity of membrane-bound heterotrimeric guanine nucleotide (G) proteins: guanosine diphosphate (GDP)-bound Gα subunit and a Gβγ complex. Binding of agonist ligands to GPCRs promotes the exchange of the bound GDP for guanosine triphosphate (GTP) on the Gα-subunit, thus facilitating its dissociation from the receptor and the Gβγ heterodimer. The heterotrimeric G proteins are divided into four classes based on their α subunit: Gαs, Gαi, Gαq/11, and Gα12/13 (Figure 1.2). Gαs stimulates the activity of AC, which catalyzes the synthesis of cAMP from ATP while Gαi inhibits AC activity and thus decreases cAMP synthesis. Gαq/11 stimulates membrane-bound phospholipase C β (PLCβ), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). Gα12/13 regulates Ras homolog gene family, member A (RhoA), a low molecular weight GTPase which influences the actin cytoskeleton. In addition, the Gβγ heterodimer can activate PI3Kγ and also PLCβ.38,39
Figure 1.2. Schematic diagram of G protein-coupled receptor (GPCR)-dependent signaling in pulmonary artery smooth muscle cells (PASMC). Binding of agonist ligands to a GPCR catalyzes the exchange of bound GDP for GTP on the Gα-subunit, causing it to dissociate from the Gβγ-subunits. The Gα-subunit activates adenylyl cyclase (AC), which facilitates the conversion of ATP to 3',5'-cyclic adenosine monophosphate (cAMP). Cyclic AMP promotes vasodilation and decreases proliferation of PASMC. GPCRs that activate Gαi inhibit AC activity and decrease intracellular cAMP; Gαq/11 stimulates membrane-bound phospholipase C β (PLCβ), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG), which lead to increased intracellular Ca²⁺ and protein kinase C activity, respectively. Gα₁₂/₁₃ regulates Ras homolog gene family, member A (RhoA); Gβγ can activate phosphoinositide 3-kinase γ (PI3Kγ) and also PLCβ, all leading to PASMC vasoconstriction and increased proliferation.
Unbiased approaches have begun to identify GPCR expression in specific tissues. The quantification of RNA transcripts for 353 non-odorant GPCRs in 41 tissues from mice revealed new roles for a number of GPCRs in various tissues. Many orphan GPCRs (receptors whose endogenous agonist ligand is not known) and olfactory GPCRs are expressed in tissues, but their function has yet to be determined. Limited data are available regarding GPCR expression in individual cell types. Because the GPCR profile of PASMC has not been identified, key GPCRs that regulate the pulmonary circulation may have been overlooked. Profiling GPCR expression in PASMC from control, IPAH and SPAH patients thus has the potential to identify GPCRs that may contribute to the pathophysiology of PAH and that could be novel therapeutic targets for this disease.

1.5. Hypothesis and Goals

The goals of the project are to:

1. investigate the mRNA expression of GPCRs in PASMC through an unbiased approach using a GPCR real-time PCR array
2. validate results obtained from the arrays by measuring mRNA, and function of highly expressed Gαq/Gαi –coupled GPCRs,
3. investigate if PAH is associated with the altered expression of GPCRs which could be novel targets for the disease

My working hypothesis is that previously unrecognized GPCRs, in particular ones that regulate cellular cAMP concentration or that are uniquely expressed, may be novel
and innovative targets for PAH based on their regulation of muscle tone and proliferation in PAH-PASMC.
Chapter 2. Materials and Methods

2.1. Cell Culture

2.1.1. PASMC

PASMC (Control, IPAH, and SPAH) were isolated as previously described by Murray et al 2011 and grown in Lifeline Cell Technology™ media (containing L-glutamine, recombinant human (rh) Insulin, rh FGF-b, Ascorbic Acid, rh EGF, 1x penicillin and streptomycin, and 10% heat-inactivated fetal bovine serum [FBS]) in a humidified 37°C/5% CO₂ incubator. Cell number and viability was determined using 0.4% Trypan blue (Invitrogen; Carlsbad, CA), and a Bright-Line Hemacytometer (Reichart; Depew, NY). PASMC were passaged once and used in experiments when they reached 90% confluency.

2.1.2. Human embryonic kidney (HEK) 293 Cells

HEK 293 cells were cultured in Corning cellgro® Dulbecco’s Modification of Eagle’s Medium (DMEM) 1x (containing 4.5 g/L glucose, L-glutamine, and sodium pyruvate) with added 10% heat-inactivated FBS and 1x penicillin and streptomycin in a humidified 37°C/5% CO₂ incubator. Cell number and viability was determined using 0.4% Trypan blue (Invitrogen, and a Bright-Line Hemacytometer (Reichart). HEK 293 cells were passaged once and used in experiments when they reached 90% confluency.
2.2. Animal Models of PAH

2.2.1. Chronic Hypoxic Mouse Model

C57/BL6 mice (3 months old, male) were placed in a hypobaric chamber (0.5atm) ventilated at over 20 L/min for 6 weeks. CO$_2$ (1 - 5 %) was added to the air stream. Right ventricular hypertrophy was assessed to determine the development of PAH. It was evaluated as the ratio of the weight of the right ventricle to that of the left ventricle plus the septum (Fulton Index). Control mice had an average value of 0.24 and CH mice had an average value of 0.32. This model is commonly used and approved of being a valid model of PAH.$^{42,43}$ All animals were cared for in compliance with the guiding principles and approved by the UCSD Institutional Animal Care and Use Committee.

2.2.2. Chronic Hypoxic Rat Model

Adult Sprague–Dawley rats (250–300g, male) were placed in a hypobaric chamber (0.5atm) ventilated at over 20 L/min along with CO$_2$ (1-5 %) for 2-4 weeks. Right ventricular hypertrophy was assessed to determine the development of PAH. It was evaluated as the ratio of the weight of the right ventricle to that of the left ventricle plus the septum (Fulton Index). Control rats had an average value of 0.22 and CH rats had an average value of 0.32. This model is commonly used and approved of being a valid model of PAH.$^{42,43}$ All animals were cared for in compliance with the guiding principles and approved by the UCSD Institutional Animal Care and Use Committee.
2.2.3. Monocrotaline (MCT)-treated Rat Model

Adult Sprague–Dawley rats (250–300 g, male) were treated with MCT (60mg/kg) and sacrificed 2 weeks later. Right ventricular hypertrophy was assessed to determine the development of PAH. It was evaluated as the ratio of the weight of the right ventricle to that of the left ventricle plus the septum (Fulton Index). Control rats had an average value of 0.20 and MCT-treated rats had an average value of 0.34. This model is commonly used and approved of being a valid model of PAH. All animals were cared for in compliance with the guiding principles and approved by the UCSD Institutional Animal Care and Use Committee.

2.3. Transfection

2.3.1. Transfection of PASMC

PASMC (0.5-1 x 10^6) were suspended in 100 µL of Nucleofactor™ Solution (Amaxa™, Köln, Germany) and combined with 10 µg of plasmid DNA (e.g., GPR75 Plasmid Vector [using pCMV6-Entry Vector] or Empty Vector Control). The Amaxa™ Nucleofector™ Program A-033 was used for the transfection. The cells were then plated into a 6-well plate and incubated in a humidified 37°C/5% CO₂ incubator for 24-72 hrs.

2.3.2. Transfection of HEK 293 Cells

Prior to transfection (24 hrs), HEK 293 cells were plated into 6-well plates at 50-70% confluency in DMEM with 10% heat-inactivated FBS (no antibiotics). Mirus® TransIT-LT1 Transfection Reagent (7.5ul) was combined with 250 µL of Opti-MEM I Reduced-Serum Medium and 2.5 µg plasmid DNA (e.g., GPR75 Plasmid Vector [using
pCMV6-Entry Vector] or Empty Vector Control). The solution was incubated at room temperature for 30 min; the TransIT-LT1 Reagent: DNA complexes were then added drop-wise. Cells were incubated in a humidified 37°C/5% CO₂ incubator for 24-72 hrs.

2.4. Restriction Digest

2 µL of New England Biolabs® Inc. (NEB) Buffer 3 was added with 0.2 µL of Bovine Serum Albumin (BSA, 10mg/mL), 2 µL of plasmid DNA (.5µg/µL-1.5µg/µL), 1µL of New England Biolabs® Inc. Bgl II (10.00 U/mL) restriction enzyme, and 13.8µL H₂O. The digest was incubated at 37°C for 2 hrs and then visualized using ~500ng of digest on agarose gel electrophoresis to confirm the size of the insert.

2.5. Real-time PCR

2.5.1. Primer Design

Primers for each GPCR were designed using the NCBI Entrez search engine (http://www.ncbi.nlm.nih.gov/sites/entrez, Bethesda, MD) and the Primer3 online primer-designing program (http://frodo.wi.mit.edu/, MIT, Cambridge, MA) using standard settings. Multiple primer pairs were chosen for each GPCR (ValueGene, San Diego, CA) and stored at a concentration of 200µM.

2.5.2. Real-time PCR Protocol

mRNA was extracted from 5 million Control (Ctrl)-, IPAH-, and SPAH-PASMC and/or from isolated mouse/rat lungs using RNeasy (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized using Superscript III Reverse
Transcriptase kit (Invitrogen), as per the manufacturer’s instructions. Real-time PCR was performed using 8 ng cDNA, 0.5 µM forward and reverse primers, and qPCR Mastermix Plus for Sybr Green I (Eurogentec, San Diego, CA) and an Opticon 2 RT-PCR machine (MJ Research, Waltham, MA). The RT-PCR program and RT-PCR primers are shown in Table 2.1 and Table 2.2, respectively. Primer efficiency was calculated for each primer set before use. Samples were compared using the relative cycle threshold (C<sub>t</sub>) method, normalizing to 28S or 18S rRNA.

2.5.3. TaqMan® GPCR array

GPCR expression was determined using a TaqMan® GPCR array (Life Technologies), according to manufacturer’s instructions, with cDNA pooled from Ctrl- (n=3), IPAH- (n=3), and SPAH-PASMC (n=3) and the TaqMan® Universal PCR Master Mix. GPCR expression was normalized to that of 18S rRNA.
Table 2.1. Real-time PCR protocol.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>2 min</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>10 min</td>
</tr>
<tr>
<td>3</td>
<td>95</td>
<td>15 sec</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>30 sec</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>1 min</td>
</tr>
<tr>
<td>6</td>
<td>Plate read.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Construct melting curves for samples by heating the plate from 60°C to 95°C. Read plate every 0.2°C, holding the temperature for 1 sec.</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2. RT-PCR primers. All primers are for human genes unless otherwise stated (m=Mouse, r=Rat).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’→3’)</th>
<th>Reverse primer (5’→3’)</th>
<th>T&lt;sub&gt;m&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADORA2B</td>
<td>ACCCAGAGGACAGCAATGA</td>
<td>CAGAGCTCCATCTTCAGCC</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>BDKRB2</td>
<td>TCCAGGGAGAGAATTTTG</td>
<td>AGTACCAGGGAGCGACTT</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>GA</td>
<td></td>
</tr>
<tr>
<td>CALCRL</td>
<td>CGATATGCAAAATGGTCTCA</td>
<td>TTCCTTAAGAGCTGGACTG</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>CA</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>GPR113</td>
<td>TCTCCAACATGTCCCATCAC</td>
<td>ATGGAAGTCGAGCCACATCT</td>
<td>60°C</td>
</tr>
<tr>
<td>GPR124</td>
<td>CTCAGGTCCAGTCTTCTCA</td>
<td>CTTCTGCCTAAACGGGCAC</td>
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<td>G</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>GPR176</td>
<td>GCAGGGCCAGTTTTTAATG</td>
<td>TGTACCGCCAGTTCACAC</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>GPR75</td>
<td>GACCTGTACTTTTTCTACTGG</td>
<td>GCTGTCACTCCACAATGAG</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>CG</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>GPRC5B</td>
<td>ATGTGCTGGCGTTTTCAGA</td>
<td>AGGCCAGCTGGAGCGTC</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>LPAR1</td>
<td>CCAGGAGTCAGCAGATGA</td>
<td>TGTCTGGCAGATTTCTGG</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>mGPR113</td>
<td>AAGAGGCTCTGTGGGACTG</td>
<td>TACCACTTTGGCCAGTAAG</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>mGPR75</td>
<td>AGGAGCAAGATGCAGGAA</td>
<td>CACCTTCGTGCTTCTTCA</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>OXTR</td>
<td>CACGAGTTCTGGGAAGGAGG</td>
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<td>60°C</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>PAR1</td>
<td>GGGGATCTAAGGTGGCATT</td>
<td>CCGCCTGCTTCAGTCTGT</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>rGPR113</td>
<td>TGGTCAGATGAAAGGTGTGA</td>
<td>CCGGTAATAACCAGGCA</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>GA</td>
<td></td>
</tr>
<tr>
<td>rGPR75</td>
<td>GCGGTCAACCTCTCTCTACTG</td>
<td>GCACCAGAGACTTTCTCTTT</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>S1P2</td>
<td>GCAAACAGAGGATGACGATG</td>
<td>GGAGTACCTGAACCCAACA</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>VIPR1</td>
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<td>CTGGGTATGCTGCTG</td>
<td>60°C</td>
</tr>
<tr>
<td>18S</td>
<td>GTAACCCCGTTGAACCCCAT</td>
<td>CCATCCAAATCGGTAGTAGC</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>28S</td>
<td>GCCTAGCAGCAGACTTTAGA</td>
<td>AAATCACATCGGTCACAC</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>AC</td>
<td></td>
</tr>
</tbody>
</table>
2.6. cAMP Radioimmunoassay

Control-PASMC, PAH-PASMC or HEK 293 cells were seeded at 30,000 cells/well in a 24-well plate. After 24 hrs the cells were serum starved for 2 hrs and incubated in the absence or presence of 3-isobutyl-1-methylxanthine (IBMX, 200µM), a competitive non-selective PDE inhibitor, for 30 min at 37°C. Agonists/antagonists of interest were then added (1-10 µM) to the cells for a further 30 min, after which the cells were treated with/without forskolin (FSK, 1µM, a direct activator of AC, which enhances Gαs-GPCR promoted cAMP formation and helps demonstrate Gαi-GPCR activation) for 10 min at 37°C. 10µM FSK was used as a positive control. After the incubations, the media was aspirated and 150 µL of 7.5% trichloroacetic acid (TCA) was added to each well.

Assay tubes were filled with 1 mL of 10 mM sodium acetate buffer (pH 4.75) and a standard curve was constructed by serial dilution of stock 5µM cAMP (Millipore, Billerica, MA). An appropriate amount of sample was added to the assay tubes and the samples were acetylated by addition of 20 µL triethylamine (Sigma) and 10µl acetic anhydride (Sigma). 100 µL of the sodium acetate buffer was added to each well of a 96-well filter plate to prepare the plate and then was removed by vacuum. 50 µL of diluted, acetylated sample was added to each well along with 25 µL diluted antibody [1:1000, 6 µL primary cAMP antibody (Millipore) in 6 mL γ-globulin buffer (100mg human γ-globulin/100mL 50mM NaAc pH 4.75)] and 25 µL diluted 125I radioactivity [16 µL acetylated adenosine 3’,5’-cyclic phosphoric acid, 2’-O-succinyl [125I]-iodotyrosine methyl ester (PerkinElmer) in 3 mL γ-globulin buffer so that 0.001 mCi of 125I could be
added to each sample. The 96-well filter plate containing the sample was incubated overnight at 4°C.

Following overnight incubation, 50 μL secondary antibody (Biomag® Goat anti-Rabbit IgG 8-4300D, Qiagen) was added to each well and the plate was incubated for 1 hr at 4°C. The wells were then washed with 100 μL of 12% polyethylene glycol in 10 mM sodium acetate (pH 6.2) three times. The base of the plate was then removed with a MultiScreen Punch Kit (Millipore) into fresh assay tubes and then counted on the WIZARD2 Automatic Gamma Counter (PerkinElmer).

2.7. [³H]Thymidine Incorporation Assay

Ctrl- and PAH-PASMC or HEK 293 cells were seeded at 30,000 cells/well on a 6-well plate (Greiner Bio-One, Monroe, NC) for 24 hr and then serum-starved for a further 24 hrs. GPCR agonists/antagonists in the absence or presence of 200 μM IBMX and [³H] thymidine (1 μCi/ml) were added to the cells for 24-48 hr. Following incubation, cells were washed with a large volume of cold phosphate buffer saline (PBS) and then twice with a large volume of cold 7.5% TCA. The precipitated material was dissolved with 0.5 M NaOH and combined with 3 mL of scintillation fluid (Ecoscint O, National Diagnostics) and radioactivity was determined using a liquid scintillation counter (Beckman Coulter LS 1801).
2.8. Protein Analysis

2.8.1. Cell lysis and protein determination

Cells were washed with cold PBS on ice and lysed in 80 μL – 150 μL of lysis buffer (Novagen Cytobuster protein extraction reagent). Protein concentration was determined using a Bio-Rad Protein Assay Dye Reagent according to the manufacturer’s instructions.

2.8.2. Tissue lysis and protein determination

Lungs and hearts were isolated from control and PAH animals and 10 mg of tissue was homogenized using a glass homogenizer in 400 μL of 1x lysis buffer (Cell Biolabs, Inc.). Samples were then centrifuged at 1200 rpm for 10 min at 4°C and the supernatant was collected. Protein concentration was determined using a Bio-Rad Protein Assay Dye Reagent Concentrate, according to the manufacturer’s instructions.

2.8.3. Western Blots

7.5 μl of Nupage® LDS Sample Buffer 4x (Invitrogen), 0.75 μl of reducing agent (2-mercaptoethanol for electrophoresis >= 98%, Sigma®), and an appropriate amount of lysis buffer were added to purified protein samples (to give a final protein concentration of 1-5 μg protein / 20 mL). Samples were loaded onto pre-cast 4-12% gel (Invitrogen) and run for 1 hr at 200V, 40mA, and 25W. Gels were incubated in 10% methanol transfer buffer for 10 min and protein was transferred to polyvinylidene fluoride (PVDF) membrane using the iBlot (Invitrogen). Membranes were then blocked in 5% milk or BSA (in PBS Tween [PBST], dependent upon antibody) for 1 hr. Primary antibody
[1:1000 dilution in 1% milk/BSA] was then added and the membrane was incubated overnight. The following day, it was washed 3 times (10 min/wash) with PBST at RT. Secondary anti-rabbit or anti-mouse antibody (1:5000 or 1:3000 respectively in 1% milk/BSA, AbCam) was added and incubated for 1 hr. The membranes were washed 3 additional times (10 min/wash) with PBST at RT and ECL luminescence (GE Healthcare) was added for detection.

2.9. Immunofluorescence

Ctrl- and PAH-PASMC were grown on sterile 12mm coverslips pre-coated with poly-D-lysine. Cells were washed twice with PBS at 37°C, then 1 mL of fixative (2% paraformaldehyde) was added for 10 min. Aldehyde groups were then quenched by incubation with 100 mM glycine (in PBS, pH 7.4) for 10 min. Cells were washed twice with PBS and then with blocking buffer (1% BSA/PBS/0.05% Tween) for 30 min at room temperature and then with primary antibody in that buffer and incubated (1:250) overnight at 4°C. Cells were then washed 3 times with wash medium (PBS/0.1% Tween20) for 5 min each at room temperature. Blocking buffer was then added containing secondary antibodies (1:250) for 60 min at room temperature. Cells were washed 6 times for 5 min each in wash medium at room temperature and then incubated with 4’,6-diamidino-2-phenylindole (DAPI, 1:5000) for 20 min in the dark and, mounted on slides with 10 µL of gelvatol. Slides were left to dry overnight at 4°C prior to viewing by confocal microscopy.
2.10. Deglycosylation Assay

1-20 μg of lysate was combined with 1 μL of 10X Glycoprotein Denaturing Buffer (New England Biolabs Inc.) and H₂O in 10 μL total volume. The sample was denatured by heating at 100°C for 10 min. The reaction volume was increased to 20 μL by adding 2 μL of 10X G7 Reaction Buffer, 2 μL of 10% NP-40, H₂O and 1-2 μL PNGase F (500,000 U/mL, New England Biolabs Inc.) and then incubated at 37°C for 1 hr.

2.11. Statistical analysis

Values are expressed as mean ± SEM. Statistical significance was determined via an unpaired or paired Student t test or an Anova when applicable. A value of P < 0.05 was considered statistically significant.
Chapter 3. Results Part I

3.1. Quantification of GPCR Expression in Ctrl-, IPAH-, and SPAH-PASMC

Using an unbiased approach, a TaqMan® GPCR array, we defined GPCR expression in Ctrl-, IPAH-, and SPAH-PASMC. We sought to use this approach to identify GPCRs that are higher or uniquely expressed in patients with PAH among the 384 genes that were analyzed (29 housekeeping genes + 355 non-chemosensory GPCRs). We found that Ctrl-PASMC (n=3) express 135 GPCRs (including 56 orphan receptors), IPAH-PASMC (n=3) express 115 GPCRs (51 orphan receptors) and SPAH-PASMC (n=3) express 81 GPCRs (32 orphan receptors) (Table 3.1). The non-orphan expressed GPCRs in each cell type were classified further and separated according to their linkage to specific G proteins (Gαs/Gαi/Gαq/11/Gα12/13, Table 3.2) by using the 2011 BJP (British Journal of Pharmacology) Guide to Receptors and Channels and the IUPHAR Database of Receptors and Ion Channels as references. The results for the three highest expressed Gαs-, Gαi-, Gαq/11-, Gα12/13-linked GPCRs from Ctrl-, IPAH-, and SPAH-PASMC are shown in Tables 3.3 - 3.6. The values are shown as ΔCt, whereby the cycle threshold (Ct) for each GPCR RNA is normalized to that of 18S rRNA; a lower Ct value thus indicates higher expression. Categorizing the receptor expression in each group allowed us to determine the expression of GPCRs that regulate cAMP and highlighted potential therapeutic targets, since increases in cAMP accumulation decrease proliferation and vasodilate PASMC.\textsuperscript{18-20,26-31}
Table 3.1. GPCR expression in Ctrl-PASMC (n=3), IPAH-PASMC (n=3), and SPAH-PASMC (n=3).

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Total GPCRs</th>
<th>Undetectable GPCRs</th>
<th>Expressed GPCRs</th>
<th>Expressed orphan GPCRs</th>
<th>Expressed non-orphan GPCRs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl-PASMC</td>
<td>355</td>
<td>220</td>
<td>135</td>
<td>56</td>
<td>79</td>
</tr>
<tr>
<td>IPAH-PASMC</td>
<td>355</td>
<td>240</td>
<td>115</td>
<td>51</td>
<td>64</td>
</tr>
<tr>
<td>SPAH-PASMC</td>
<td>355</td>
<td>274</td>
<td>81</td>
<td>32</td>
<td>49</td>
</tr>
</tbody>
</table>

Table 3.2. GPCRs in Ctrl-PASMC (n=3), IPAH-PASMC (n=3), and SPAH-PASMC (n=3) classified by their G protein-coupling. (Many GPCRs have multiple coupling so may appear in more than 1 category)

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Ga&lt;sub&gt;1&lt;/sub&gt;-coupled</th>
<th>Ga&lt;sub&gt;12/13&lt;/sub&gt;-coupled</th>
<th>Ga&lt;sub&gt;4/11&lt;/sub&gt;-coupled</th>
<th>Ga&lt;sub&gt;12/13&lt;/sub&gt;-coupled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl-PASMC</td>
<td>23</td>
<td>36</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>IPAH-PASMC</td>
<td>19</td>
<td>31</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>SPAH-PASMC</td>
<td>13</td>
<td>22</td>
<td>20</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 3.3. Three highest expressed Gα<sub>s</sub>-linked GPCRs in Ctrl-PASMC (n=3), IPAH-PASMC (n=3), and SPAH-PASMC (n=3). ΔC<sub>t</sub> values averaged and normalized with 18S (lower values represent higher expression).

<table>
<thead>
<tr>
<th>Ctrl-PASMC</th>
<th>IPAH-PASMC</th>
<th>SPAH-PASMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ga&lt;sub&gt;s&lt;/sub&gt;-coupled GPCR</td>
<td>ΔC&lt;sub&gt;t&lt;/sub&gt;</td>
<td>Ga&lt;sub&gt;s&lt;/sub&gt;-coupled GPCR</td>
</tr>
<tr>
<td>ADORA2B</td>
<td>18.7</td>
<td>ADRB2</td>
</tr>
<tr>
<td>VIPR1</td>
<td>19.2</td>
<td>P2RY11</td>
</tr>
<tr>
<td>CALCRL</td>
<td>20.2</td>
<td>PTGIR</td>
</tr>
</tbody>
</table>

Table 3.4. Three highest expressed Gα<sub>i</sub>-linked GPCRs Ctrl-PASMC (n=3), IPAH-PASMC (n=3), and SPAH-PASMC (n=3). ΔC<sub>t</sub> values averaged and normalized with 18S (lower value represents higher expression).

<table>
<thead>
<tr>
<th>Ctrl-PASMC</th>
<th>IPAH-PASMC</th>
<th>SPAH-PASMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ga&lt;sub&gt;i&lt;/sub&gt;-coupled GPCR</td>
<td>ΔC&lt;sub&gt;t&lt;/sub&gt;</td>
<td>Ga&lt;sub&gt;i&lt;/sub&gt;-coupled GPCR</td>
</tr>
<tr>
<td>LPAR1</td>
<td>15.2</td>
<td>LPAR1</td>
</tr>
<tr>
<td>OXTR</td>
<td>16.1</td>
<td>CHRM2</td>
</tr>
<tr>
<td>PAR1</td>
<td>17.2</td>
<td>SSTR1</td>
</tr>
</tbody>
</table>
Table 3.5. Three highest expressed Gα<sub>q/11</sub>-linked GPCRs Ctrl-PASMC (n=3), IPAH-PASMC (n=3), and SPAH-PASMC (n=3). ΔC<sub>t</sub> values averaged and normalized with 18S (lower value represents higher expression).

<table>
<thead>
<tr>
<th></th>
<th>Ctrl-PASMC</th>
<th>IPAH-PASMC</th>
<th>SPAH-PASMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gα&lt;sub&gt;q/11&lt;/sub&gt;-coupled GPCR</td>
<td>ΔC&lt;sub&gt;t&lt;/sub&gt;</td>
<td>Gα&lt;sub&gt;q/11&lt;/sub&gt;-coupled GPCR</td>
<td>ΔC&lt;sub&gt;t&lt;/sub&gt;</td>
</tr>
<tr>
<td>OXTR</td>
<td>16.1</td>
<td>PAR1</td>
<td>14.1</td>
</tr>
<tr>
<td>BDKRB2</td>
<td>16.2</td>
<td>BDKRB2</td>
<td>14.1</td>
</tr>
<tr>
<td>PAR1</td>
<td>17.2</td>
<td>LPAR1</td>
<td>16.1</td>
</tr>
</tbody>
</table>

Table 3.6. Three highest expressed Gα<sub>12/13</sub>-linked GPCRs Ctrl-PASMC (n=3), IPAH-PASMC (n=3), and SPAH-PASMC (n=3). ΔC<sub>t</sub> values averaged and normalized with 18S (lower value represents higher expression).

<table>
<thead>
<tr>
<th></th>
<th>Ctrl-PASMC</th>
<th>IPAH-PASMC</th>
<th>SPAH-PASMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gα&lt;sub&gt;12/13&lt;/sub&gt;-coupled GPCR</td>
<td>ΔC&lt;sub&gt;t&lt;/sub&gt;</td>
<td>Gα&lt;sub&gt;12/13&lt;/sub&gt;-coupled GPCR</td>
<td>ΔC&lt;sub&gt;t&lt;/sub&gt;</td>
</tr>
<tr>
<td>LPAR1</td>
<td>15.2</td>
<td>PAR1</td>
<td>14.1</td>
</tr>
<tr>
<td>S1PR2</td>
<td>17.2</td>
<td>LPAR1</td>
<td>16.1</td>
</tr>
<tr>
<td>PAR1</td>
<td>17.2</td>
<td>S1PR2</td>
<td>16.1</td>
</tr>
</tbody>
</table>

3.2. Highest expressed GPCRs in Ctrl-PASMC confirmed by independent real-time PCR

The highest expressed GPCRs, determined by the TaqMan® GPCR array, were confirmed by real-time PCR to ensure validity of the array data. We confirmed the expression of the 3 highest expressed Gα<sub>ε</sub>-, Gα<sub>ε</sub>-, and Gα<sub>q/11</sub>-coupled GPCRs and orphan receptors in Ctrl-PASMC (Table 3.7). Data from the individual real-time PCR studies generally correlated well with values from the microarray. The overall r<sup>2</sup> value was calculated to be 0.70 (Figure 3.1).
Table 3.7. Highest expressed GPCRs in Ctrl-PASMC confirmed by independent real-time PCR.

<table>
<thead>
<tr>
<th>Microarray</th>
<th>Real-time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gas-coupled</strong></td>
<td><strong>Gas-coupled</strong></td>
</tr>
<tr>
<td>Adenosine A2B Receptor</td>
<td>Adenosine A2B Receptor</td>
</tr>
<tr>
<td>VIP Receptor 1</td>
<td>VIP Receptor 1</td>
</tr>
<tr>
<td>Calcitonin Receptor-Like</td>
<td>Calcitonin Receptor-Like</td>
</tr>
<tr>
<td><strong>Gai-coupled</strong></td>
<td><strong>Gai-coupled</strong></td>
</tr>
<tr>
<td>LPAR1</td>
<td>LPAR1</td>
</tr>
<tr>
<td>Oxytocin Receptor</td>
<td>Oxytocin Receptor</td>
</tr>
<tr>
<td>PAR1</td>
<td>PAR1</td>
</tr>
<tr>
<td><strong>Gaq-coupled</strong></td>
<td><strong>Gaq-coupled</strong></td>
</tr>
<tr>
<td>Oxytocin Receptor</td>
<td>Oxytocin Receptor</td>
</tr>
<tr>
<td>Bradykinin Receptor B2</td>
<td>Bradykinin Receptor B2</td>
</tr>
<tr>
<td>PAR1</td>
<td>PAR1</td>
</tr>
<tr>
<td><strong>Orphan Receptor</strong></td>
<td><strong>Orphan Receptor</strong></td>
</tr>
<tr>
<td>GPRC5B</td>
<td>GPRC5B</td>
</tr>
<tr>
<td>GPR124</td>
<td>GPR124</td>
</tr>
<tr>
<td>GPR176</td>
<td>GPR176</td>
</tr>
</tbody>
</table>

Lower ΔCt = Higher Expression
Figure 3.1. Microarray ΔCt values compared to independent real-time PCR ΔCt values ($r^2=0.70$).
Chapter 4. Results Part II: Validation of the GPCR array

To further validate the TaqMan® GPCR array, we performed functional studies on the Goαs-coupled GPCRs that were expressed at high, intermediate and low levels in Ctrl-PASMCs and determined if agonist-induced cAMP accumulation of these GPCRs correlated with their mRNA expression. The highest, intermediate and lowest Goαs-coupled GPCRs tested were the adenosine 2B receptor (A2BR, ΔCt = 18), the vasoactive intestinal peptide receptor (VIPR1, ΔCt = 19), the prostaglandin I receptor 2 receptor (PGI2R, ΔCt = 20), the prostaglandin E receptor 2 receptor (EP2R, ΔCt = 22) and the gastric inhibitory polypeptide receptor (GIPR, ΔCt = 24). cAMP accumulation (fmol cAMP / cell / 10 min treatment with agonist) in response to receptor agonists, CV1808 (A2BR, 1µM: 0.6 fmol), VIP (VIPR1, 1µM: 0.2 fmol), epoprostenol (PGI2R, 10µM: 0.4 fmol), butaprost (EP2R, 1µM: 0.4 fmol) and GIP (GIPR, 1µM: 0.2 fmol) correlated with receptor mRNA expression (r²=0.31, Figure 4.1). The concentrations of agonists used were previously shown to produce a maximal response under the conditions tested.
Figure 4.1. Higher mRNA expression in PASMC of Ga\(_s\)-coupled GPCRs correlates with cAMP accumulation by GPCR-selective agonists (\(r^2=0.31\)).

We tested the ability of the agonists shown in Figure 4.1 to decrease proliferation of PASMC (assayed by \([^3\text{H}]\) thymidine incorporation, which measures DNA synthesis) and found that agonist-induced cAMP accumulation correlated with anti-proliferative effect (Figure 4.2).
We further investigated cAMP formation and anti-proliferative response mediated by the Vasoactive Intestinal Peptide (VIP) receptor, which was the second highest expressed Gα<sub>s</sub>-coupled GPCR in Ctrl-PASMC and since little was known regarding its role in PASMC (Table 3.7). We found (Figure 4.3) that the VIPR1 agonist VIP dose-dependently increased cAMP and that this increase in cAMP corresponded to a decrease in the proliferation of PASMC. A lower concentration of VIP increased cAMP accumulation without altering PASMC proliferation; these data suggest a threshold of cAMP is needed before it affects PASMC proliferation. We confirmed that VIPR1 is expressed on the membrane of PASMC by performing immunoblot with different subcellular fractions (Figure 4.3A).
We also investigated the expression and function of the oxytocin receptor, the second highest expressed Gαi-coupled GPCR in PASMC (Table 3.7). We found that it, too, is expressed on the membrane of Ctrl-PASMC and that its agonist oxytocin decreased cAMP levels and increased proliferation of PASMC (Figure 4.4). Akin to the findings for VIP, lower concentrations of oxytocin decreased cAMP levels than were able to increase proliferation of PASMC.
Figure 4.4. A.) OXTR is expressed on the membrane in Ctrl-PASMC. B.) Oxytocin decreases forskolin-stimulated cAMP levels in Ctrl-PASMC in a concentration-dependent manner. C.) Oxytocin increases proliferation of Ctrl-PASMC in a concentration-dependent manner.
Chapter 5. Results Part III: Altered expression of GPCRs in PAH-PASMC

The altered expression of GPCRs in PAH-PASMC could provide insight into the possible mechanisms that contribute to the development and progression of the disease as well as defining possible targets for future therapy. Using the data derived from the TaqMan® GPCR array we sought to identify GPCRs that showed the greatest differences in expression between Ctrl- and PAH-PASMC or GPCRs that were expressed in one cell type but not another (uniquely expressed GPCRs). GPCR expression was compared between Ctrl- and PAH-PASMC (both idiopathic and secondary).

Figures 5.1 shows Venn diagrams that depict increases in GPCR expression (>2-fold) and decreases in GPCR expression (<0.5-fold) in IPAH- and SPAH-PASMC compared to Ctrl-PASMC. The number in each circle indicates the number of GPCRs that increased/decreased in expression in either IPAH or SPAH while the number in the overlapping area of both circles indicates shared GPCRs that increase/decrease in expression in both forms of PAH. We identified 66 GPCRs with increased expression in IPAH-PASMC compared to Ctrl-PASMC, 16 GPCRs with increased expression in SPAH-PASMC compared to Ctrl-PASMC, and 41 GPCRs with increased expression in both IPAH and SPAH compared to control cells. IPAH thus has 107 (66 + 41) GPCRs with increased expression while SPAH has 57 (16 + 41) GPCRs with increased expression compared to Ctrl-PASMC.
Figure 5.1. A.) Venn Diagrams depicting increases in mRNA expression (>2-fold) in IPAH- and SPAH-PASMC compared to Ctrl-PASMC. B.) Venn Diagrams depicting decreases in mRNA expression (<0.5-fold) in IPAH- and SPAH-PASMC compared to Ctrl-PASMC.

Tables 5.1-5.4 show the 3 GPCRs that have the greatest increase or decrease in expression in IPAH-/SPAH-PASMC compared to Ctrl-PASMC. These GPCRs could be novel therapeutic targets due to their altered expression in the diseased cells.

**Table 5.1.** The 3 GPCRs with the greatest increase in mRNA expression in IPAH compared to Ctrl-PASMC.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full Name</th>
<th>Fold Increase</th>
<th>Principal Transduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADRA1D</td>
<td>Adrenergic α-1D</td>
<td>549</td>
<td>Gαq/11</td>
</tr>
<tr>
<td>CHRM2</td>
<td>Cholinergic muscarinic 2</td>
<td>290</td>
<td>Gαi</td>
</tr>
<tr>
<td>ADRB2</td>
<td>Adrenergic β-2</td>
<td>287</td>
<td>Gαs</td>
</tr>
</tbody>
</table>

**Table 5.2.** The 3 GPCRs with the largest decrease in mRNA expression in IPAH compared to Ctrl-PASMC.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full Name</th>
<th>Fold Decrease</th>
<th>Principal Transduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXTR</td>
<td>Oxytocin Receptor</td>
<td>-3.70</td>
<td>Gαq/11, Gαi</td>
</tr>
<tr>
<td>LPHN1</td>
<td>Latrophilin 1</td>
<td>-3.64</td>
<td>Class B Orphan</td>
</tr>
<tr>
<td>GPRC5B</td>
<td>GPCR, family 5CB</td>
<td>-3.61</td>
<td>Class C Orphan</td>
</tr>
</tbody>
</table>
Table 5.3. The 3 GPCRs with the greatest increase in mRNA expression in SPAH compared to Ctrl-PASMC.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full Name</th>
<th>Fold Increase</th>
<th>Principal Transduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4</td>
<td>Chemokine (C-X-C motif) 4</td>
<td>69.3</td>
<td>Gα_i</td>
</tr>
<tr>
<td>SSTR1</td>
<td>Somatostatin receptor 1</td>
<td>35.7</td>
<td>Gα_i</td>
</tr>
<tr>
<td>CHRM5</td>
<td>Cholinergic muscarinic 5</td>
<td>35.4</td>
<td>Gα_{q/11}</td>
</tr>
</tbody>
</table>

Table 5.4. The 3 GPCRs with the largest decrease in mRNA expression in SPAH compared to Ctrl-PASMC.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full Name</th>
<th>Fold Decrease</th>
<th>Principal Transduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPR161</td>
<td>GPCR 161</td>
<td>-7.26</td>
<td>Class A Orphan</td>
</tr>
<tr>
<td>GPR153</td>
<td>GPCR 153</td>
<td>-3.63</td>
<td>Class A Orphan</td>
</tr>
<tr>
<td>PTGFR</td>
<td>Prostaglandin f receptor</td>
<td>-3.51</td>
<td>Gα_{q/11}</td>
</tr>
</tbody>
</table>

We found multiple GPCRs that are uniquely expressed in PAH-PASMC compared to Ctrl-PASMC: 26 GPCRs were uniquely expressed in either IPAH or SPAH compared to control. Of these 26 GPCRs, only 2 were expressed in both IPAH- and SPAH-PASMC: GPR75 and GPR113, both of which are orphan receptors (Tables 5.5 & 5.6). IPAH was associated with a much greater number of uniquely expressed GPCRs than was SPAH.
Table 5.5. Uniquely expressed GPCRs in IPAH-PASMC compared to control-PASMC.

<table>
<thead>
<tr>
<th>Gαs-coupled</th>
<th>ΔCt</th>
<th>Gαq-coupled (cont)</th>
<th>ΔCt</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPAR3</td>
<td>16</td>
<td>GPR92</td>
<td>20</td>
</tr>
<tr>
<td>ADCYAP1R1</td>
<td>19</td>
<td>FPRL2</td>
<td>21</td>
</tr>
<tr>
<td>DRD5</td>
<td>20</td>
<td>GALR2</td>
<td>21</td>
</tr>
<tr>
<td>MC5R</td>
<td>21</td>
<td>GPR43</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GRM5</td>
<td>21</td>
</tr>
<tr>
<td>Gαi-coupled</td>
<td></td>
<td><strong>Orphan Receptor</strong></td>
<td></td>
</tr>
<tr>
<td>LPAR3</td>
<td>16</td>
<td>GPR63</td>
<td>16</td>
</tr>
<tr>
<td>PTGER3</td>
<td>16</td>
<td>LPHN3</td>
<td>19</td>
</tr>
<tr>
<td>P2RY13</td>
<td>19</td>
<td>GPR113</td>
<td>19</td>
</tr>
<tr>
<td>P2AFR</td>
<td>20</td>
<td>GPR75</td>
<td>19</td>
</tr>
<tr>
<td>RLN3R1</td>
<td>20</td>
<td>MRGPRE</td>
<td>19</td>
</tr>
<tr>
<td>GRM4</td>
<td>21</td>
<td>GPR45</td>
<td>20</td>
</tr>
<tr>
<td>GALR2</td>
<td>21</td>
<td>GPR35</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BAI3</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCR2</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPR142</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GPR171</td>
<td>21</td>
</tr>
</tbody>
</table>

*Lower ΔCt = Higher Expression*

Table 5.6. Uniquely expressed GPCRs in SPAH-PASMC compared to control-PASMC.

| Gαi-coupled | ΔCt | Gαq-coupled | ΔCt |
| HTR1D        | 20  | P2RY2        | 20  |
| HTR1B        | 21  | **Orphan Receptor** |     |
|              |     | GPR113    | 20  |
|              |     | GPR75     | 20  |

*Lower ΔCt = Higher Expression*
Chapter 6. Results Part IV: Expression of GPR113 in PASMC from PAH patients and animals with experimental PAH

We assessed the protein expression of GPR113 in PAH-PASMC compared to control cells (Figure 6.1) as it was one of the two GPCRs that was uniquely expressed in PAH cells. In each PAH-PASMC patient there was at least a 4-fold increase in protein expression of GPR113 compared to control. To determine whether this expression was species specific, we evaluated results in the chronic hypoxic (CH) rat and monocrotaline (MCT)-treated rat model. These models are commonly used and approved of being a valid model of PAH and shows vascular remodeling as seen in Supplemental Figure 9.1.\textsuperscript{42,43} We found similar results in the two animal models of PAH compared to control (Figures 6.2-6.5) in terms of mRNA expression.

![GPR113 protein expression](image)

**Figure 6.1.** GPR113 protein expression in PAH-PASMC compared to Ctrl-PASMC (a representative blot).
Figure 6.2. GPR113 mRNA expression in CH rat lungs (n=4) compared to control (n=4). C_t values normalized to 18S. The change in GPR113 mRNA expression is statistically significant (P<0.05), according to Student’s t-test.
Figure 6.3. Fold-change in GPR113 mRNA expression normalized to 18S RNA in CH rat lungs (n=4) compared to control (n=4). The change in GPR113 mRNA expression is statistically significant (P<0.05), according to Student’s t-test.
Figure 6.4. GPR113 mRNA expression in MCT-treated rat lungs (n=3) compared to control (n=3). Ct values normalized to 18S RNA. The change in GPR113 mRNA expression is statistically significant (P<0.01), according to Student’s t-test.
Figure 6.5. Fold-change in GPR113 mRNA expression normalized to 18S RNA in MCT-treated rat lungs (n=3) compared to control (n=3). The change in GPR113 mRNA expression is statistically significant (P<0.01), according to Student’s t-test.
Chapter 7. Results Part V

7.1. GPR75 has increased mRNA and protein expression in PAH

GPR75 is the other GPCR whose mRNA is uniquely expressed in both IPAH- and SPAH-PASMC. Consistent with this result, we found that in each SPAH-PASMC patient there was at least an 8-fold increase in protein expression of GPR75 compared to control and in each IPAH-PASMC patient there was at least a 15-fold increase compared to control (Figure 7.1).

![GPR75 protein expression in Ctrl-, SPAH-, and IPAH-PASMC](image)

**Figure 7.1.** GPR75 protein expression in Ctrl-, SPAH-, and IPAH-PASMC (a representative blot).

To visualize GPR75 expression, we performed immunofluorescence using a GPR75 N-terminal binding antibody (without permeabilizing the cells). Figure 7.2 shows a prominent increase in GPR75 expression, in IPAH-PASMC compared to Ctrl-PASMC.
Figure 7.2. Immunofluorescence revealed increased GPR75 expression in IPAH-PASMC compared to control. Secondary antibody was tagged with FITC for visualization of GPR75 (green). DAPI was used for visualization of the nucleus (blue). Cells were not permeabilized. Image taken at 60x magnification.

To investigate if this increase in GPR75 expression with PAH-PASMC also occurs in animal models of PAH, we investigated its expression in lungs from CH mice, CH rats, and MCT-treated rats. Figures 7.3-7.9 show GPR75 mRNA and protein expression increases in each of the animal models of PAH, compared to controls. We also found that GPR75 expression is increased in the heart from the CH rat hearts compared to controls (Figure 7.10); this may be due to receptor glycosylation (as will be discussed below).
Figure 7.3. GPR75 protein expression in CH mouse lungs compared to control (a representative blot).

Figure 7.4. GPR75 protein expression in CH rat lungs compared to control (a representative blot).

Figure 7.5. GPR75 protein expression in MCT-treated rat lungs compared to control (a representative blot).
Figure 7.6. GPR75 mRNA expression in CH rat lungs (n=4) compared to control (n=4). Ct values normalized to 18S RNA. The change in GPR75 mRNA expression is statistically significant (P<0.05), according to Student’s t-test.
Figure 7.7. Fold-change in GPR75 mRNA expression normalized to 18S RNA in CH rat lungs (n=4) compared to control (n=4). The change in GPR75 mRNA expression is statistically significant (P<0.05), according to Student’s t-test.
Figure 7.8. GPR75 mRNA expression in MCT-treated rat lungs (n=3) compared to control (n=3). C_t values normalized to 18S RNA. The change in GPR75 mRNA expression is statistically significant (P<0.05), according to Student’s t-test.
Figure 7.9. Fold-change in GPR75 mRNA expression normalized to 18S RNA in MCT-treated rat lungs (n=4) compared to control (n=4). The change in GPR75 mRNA expression is statistically significant (P<0.05), according to Student’s t-test.

Figure 7.10. GPR75 protein expression in control (n=2) and CH (n=2), cardiac left ventricle (LV) and right ventricle (RV).
7.2. The function of GPR75

GPR75 is an orphan receptor whose endogenous ligand is currently unknown and about which little information is available. Because its mRNA and protein expression is increased in PAH-PASMC and in the lungs of PAH animal models compared to control, we sought to determine if this increase altered cell physiology. We used a commercially obtained antibody that has been generated against the N-terminal of the receptor and tested if this antibody might “block” the receptor. We reasoned that this antibody but not an antibody directed at cytoplasm-exposed domains, e.g., intracellular loops or the C-terminal, might block GPR75. Immunoglobulin (IgG) was used as a control. We investigated cAMP accumulation and proliferation in PASMC to determine the role of GPR75 in the function of PAH-PASMC. The antibody was added at 1µg/mL for 2 hrs prior to 10 min stimulation with forskolin (FSK). The N-terminal GPR75 antibody did not significantly alter FSK-induced cAMP accumulation in Ctrl-PASMC compared to the IgG-control (Figure 7.11). However, in IPAH-PASMC, GPR75 antibody significantly increased FSK-induced cAMP accumulation compared to IgG-control. These data suggest that the N-terminal GPR75 antibody selectively “blocks” this receptor on IPAH-PASMC and that GPR75 is coupled to Go. We also saw lower cAMP levels in IPAH-PASMC compared to Ctrl-PASMC which has been shown in previous literature.¹¹
Figure 7.11. cAMP accumulation in Ctrl- (n=3) and IPAH-PASMC (n=3) in the presence and absence GPR75 antibody (1µg/mL). All cells received 10 min forskolin stimulation. There is no significant change in cAMP accumulation in Ctrl-PASMC treated with IgG control or GPR75 antibody, but in IPAH-PASMC, the GPR75 antibody increased cAMP accumulation, this increase in cAMP accumulation is statistically significant (P<0.05), according to Student’s t-test. The decrease in cAMP in IgG treated IPAH-PASMC compared to Ctrl-PASMC is statistically significant (P<0.01), according to Student’s t-test.

After obtaining the results in Figure 7.11, we investigated the effect of the GPR75 antibody on the proliferation of PAH-PASMC. In order to confirm that our N-terminal GPR75 antibody blocks GPR75 we compared its effects to that of a GPR75 antibody targeted to cytoplasmic-exposed domains of the receptor. Figure 7.12 shows that proliferation of Ctrl-PASMC is not significantly changed by addition of control IgG or either the N-terminal-targeted or cytoplasmic domain-targeted GPR75 antibodies.
However, in IPAH-PASMC there is a statistically significant decrease in proliferation upon addition of the N-terminal GPR75 antibody, but not the cytoplasmic-domain binding GPR75 antibody or the IgG-control (Figure 7.13). Because the cytoplasmic-domain binding GPR75 antibody had no effect on the proliferation of PAH-PASMC, this result provides evidence that the N-terminal binding antibody is having its effect by binding to and blocking activation of the GPR75 receptor on IPAH-PASMC.

![Figure 7.12](image)

**Figure 7.12.** Proliferation ([^3]H]Thymidine incorporation) of Ctrl-PASMC (n=4) in the presence of IgG control (1µg/mL), cytoplasmic-domain targeted or N-terminal-targeted GPR75 antibody.
Figure 7.1. Proliferation ([3H]Thymidine incorporation) of IPAH-PASMC (n=4) in the presence of IgG control, cytoplasmic domain-binding GPR75 antibody and N-terminal binding GPR75 antibody. The N-terminal GPR75 antibody, but not the cytoplasmic domain-targeted antibody, decreased proliferation compared to the IgG control. This decrease in proliferation is statistically significant (P<0.05), according to Student’s t-test.

In order to further investigate the function of GPR75, we overexpressed the receptor in heterologous cells so as to mimic its increased expression in IPAH-PASMC. Primary cells such as PASMC are difficult to transfect due to their membrane integrity; therefore we performed initial experiments in Human Embryonic Kidney (HEK) 293 cells. HEK 293 cells were transfected with a pCMV6-Entry Vector (4.9kb) alone or vector with a GPR75 insert (1.6kb); the plasmid is shown in Supplemental Figure 9.2. The size and sequence of GPR75 was verified by restriction digest (restriction map of
GPR75 is shown in Supplemental Figure 9.3) using restriction enzyme \textit{Bgl II} and performing agarose gel electrophoresis (Supplemental Figure 9.4). Figure 7.14 shows the expression of GPR75 in HEK 293 cells by western blot after 24, 48, and 72 hrs. The 72 hr time point showed the largest increase in GPR75 compared to cells transfected with empty vector.

**Figure 7.14.** Transfection of GPR75 plasmid (2.5µg) into HEK 293 cells. Fold-change in GPR75 expression normalized to GAPDH compared to empty vector control. Maximal fold-change occurred 72-hr after transfection.

HEK cells that overexpressed GPR75 (72 hrs-transfected cells) had significantly lower cAMP levels compared to cells transfected with the empty vector control (Figure
These data provide further evidence that GPR75 is Gαi-coupled because overexpression lowered basal and forskolin-stimulated levels of cAMP.

\[ \text{cAMP accumulation (fmol / µg protein)} \]

\[ \text{Empty Vector} \quad \text{GPR75 Vector} \quad \text{Empty Vector} \quad \text{GPR75 Vector} \]

**Figure 7.15.** cAMP accumulation in GPR75 vector-transfected HEK 293 cells (n=3) compared to empty vector-transfected cells (n=3). Cells transfected with the GPR75 construct had lower basal levels of cAMP and lower cAMP levels if incubated with forskolin (FSK, 10µg/mL) for 10 min. These decreases in cAMP levels are statistically significant (P<0.01), according to Student’s t-test.

GPR75 overexpressed HEK 293 cells also show a significant increase in proliferation compared to empty vector–transfected cells (Figure 7.16).
Figure 7.1. $[^3]$H-Thymidine incorporation in GPR75 overexpressed HEK 293 cells (n=6). Such cells show an increase in proliferation compared to empty vector-transfected cells (n=6). This increase in proliferation is statistically significant (P<0.01), according to Student’s t-test.

Despite the relative difficulty of transfecting primary cells, we were able to successfully transfect the PASMC using electroporation, however functional studies have yet to be done with these PASMC (Supplementary Figure 9.5).
7.3. GPR75 is glycosylated

Detection of GPR75 by Western Blot often revealed, double or triple bands (Figure 7.17).

![Western Blot of GPR75](image)

**Figure 7.17.** Western Blot of GPR75 overexpressed HEK 293 cells. The middle band is the protein’s true weight (59 kDa).

Such patterns often occur as a consequence of receptor glycosylation. To provide evidence that GPR75 is glycosylated we performed a deglycosylation assay to investigate if its migration pattern changed. Figure 7.18 shows a Western Blot of CH rat lung, SPAH-PASMC, and GPR75 vector-transfected HEK 293 cell lysate before and after the addition of Peptide-N-Glycosidase F (PNGase F, an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins). Figure 7.18 shows a lower band that appears in the presence of PNGase F The upper most band is likely a more heavily glycosylated receptor that is not fully deglycosylated, the middle band being the
glycosylated receptor (at the appropriate 59 kDa size) while the lowest band likely represents a deglycosylated form of the receptor.
**Figure 7.18.** Deglycosylation of GPR75 in CH Rat lung, SPAH-PASMC, and GPR75 Overexpressed (O/E) HEK 293 cell protein lysate. Were treated without (-) and with PNGase F added (+, 1 µL at 500,000 U/mL).

These data suggest that GPR75 is glycosylated. Interestingly the GPR75 receptor seems to change in glycosylation state in PAH, as shown for IPAH-PASMC and CH rat hearts (Figure 7.1 and 7.10).
Chapter 8. Discussion

PAH is characterized by increased pulmonary vascular resistance, in part due to vasoconstriction and increased proliferation of PASMC. Finding unique GPCRs in PAH-PASMC through expression profiling could be useful for uncovering new targets in the disease. Unbiased approaches have begun to identify GPCR expression in specific tissues. The quantification of RNA transcripts for 353 non-odorant GPCRs in 41 tissues from mice revealed new roles for a number of GPCRs in various tissues.\textsuperscript{40} Many orphan GPCRs (receptors whose endogenous agonist ligand is not known) and olfactory GPCRs are expressed in tissues, but their function has yet to be determined.\textsuperscript{41} Limited data are available regarding GPCR expression in individual cells, which can express >100 GPCRs.\textsuperscript{44} Because the GPCR profile of PASMC has not been identified, key GPCRs that regulate pulmonary circulation may have been overlooked.

Data from our lab has indicated that microarrays, such as those by Affymetrix, that assess total cellular mRNA are not optimal for detecting the expression of GPCRs, therefore we used a specific TaqMan® GPCR array to investigate GPCR expression in control- SPAH- and IPAH-PASMC. Microarrays are one of the leading methods to identify differentially expressed genes, however their reliability in detecting differences in RNA expression hinges on many factors. These factors include RNA extraction, probe labeling, hybridization conditions, as well as array production. Due to such limitations in reliability, mRNAs identified as differentially expressed on the gene array need to be validated with other methods.\textsuperscript{45} We validated results obtained from our GPCR array by independent real-time PCR to confirm the relative expression of GPCRs identified from
the array and to focus future studies. Real-time PCR is quantitative, requires a low amount of RNA, is relatively inexpensive, and provides rapid results.\cite{45,46} Our data provide evidence that the GPCR array, with data confirmed by independent real-time PCR (using primers designed in the lab), provides a reliable tool (based on C\textsubscript{t} values) to determine GPCR expression in cells (Table 3.7).

Since mRNA expression may not correlate with protein expression or function, we initiated efforts to provide a proof-of-principle that our approach can identify functional GPCRs in PASMC. We found that receptor expression correlated with cAMP production and function (e.g., a decrease in proliferation). We initially focused on the vasoactive intestinal peptide receptor 1 (VIPR1, G\alpha\textsubscript{s}-coupled) and the oxytocin receptor (G\alpha\textsubscript{i}-coupled) since they were both highly expressed in PASMC and limited data are available regarding their role in PASMC. We found that VIPR1 protein is expressed in the membrane fraction of PASMC and that its activation by VIP (0.1-300 nM, 10 min) increased cAMP accumulation and decreased PASMC proliferation by roughly 50\% (0.1 nM-10 \mu M VIP for 24 hr, Figure 4.3). In parallel, we found that oxytocin receptor protein is expressed on membranes of PASMC and that oxytocin dose-dependently (0.1 nM-10 \mu M, 10min) decreased cAMP accumulation and promoted PASMC proliferation (in cells grown in serum free media over 24 hr (Figure 4.4)).

Expression and function of VIPR1 in PASMC is consistent with recent findings indicating that VIP could be important in pulmonary artery remodeling and even beneficial in the treatment of PAH. VIP (-/-) mice spontaneously develop moderate
pulmonary remodeling, variants in the VIP gene occur in IPAH and chronic inhalation of VIP was shown to improve the hemodynamics and exercise capacity in a small (n=8) cohort of PAH-patients.\textsuperscript{47-49} The expression and function of oxytocin receptor in PASMC corresponds to previous studies in ewes that found oxytocin (0.2 units/kg) increases mean PAP and pulmonary vascular resistance (PVR). Interestingly cytokines, such as IL-6, that are upregulated in PAH increase the expression of the oxytocin receptor.\textsuperscript{50} Thus, our preliminary data validate the use of a GPCR-array as an initial approach to discover highly expressed or unrecognized GPCRs in PASMC that may contribute to the physiology and pathophysiology of the pulmonary vasculature.

The GPCR microarray, in addition to profiling GPCR expression in a specific cell type, can also identify uniquely expressed receptors. The highest or uniquely expressed GPCRs in PAH-PASMC are potential therapeutic targets for PAH. Interestingly the highest expressed GPCRs in many of the cells studied in the lab are orphan receptors, which have no known endogenous ligands. Orphan receptors can be difficult to study as no agonists or antagonists are available for functional studies. The best methods to investigate the role and signaling of these receptors are by siRNA, overexpressing the receptors, or using an antibody that can bind receptors and block function.

We identified 2 GPCRs that are uniquely expressed in both IPAH-PASMC and SPAH-PASMC compared to control, namely GPR113 and GPR75. Both might be therapeutic targets for PAH. As orphan receptors, GPR113 and GPR75 lack an identified endogenous agonist. Limited research has been done on GPR113: only 5 articles have
been published regarding this receptor. The gene for GPR113 maps to chromosome 2p23.3 and makes up a rather large 1,079 amino acid protein (116,341 Da). GPR113 is an adhesion GPCR belonging to family 2B, a family characterized by receptors having long N-terminal extracellular domains. Its expression was thought to be restricted to a subset of taste receptor cells. It has a GPCR proteolytic site (GPS) domain in its N-terminus and long Ser/Thr-rich regions forming mucin-like stalks. GPR113 has a hormone binding domain and one epidermal growth factor (EGF) domain. Unfortunately none of the antibodies I tested appeared to allow assessment of the function of GPR113. More research must be done on GPR113 to identify blocking antibodies that can be used to define its role in PAH. In past studies GPR113 was found to be upregulated >5-fold in small bowel neuroendocrine tumors compared to normal tissues and thus, perhaps it may be associated with other disease states.

We also discovered that PAH is associated with increased expression of GPR75. To date only 4 articles have been published regarding GPR75. The gene for GPR75 maps to chromosome 2p16 and encodes a 540 amino acid protein (59,359 Da). GPR75 is highly expressed in the retina and central nervous system. It has been proposed that Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES, Chemokine Ligand 5 [CCL5]) may be a ligand for GPR75. Upon treatment with RANTES, an increase in inositol trisphosphate (IP3), and stimulation of Ca\(^{2+}\) mobilization was noted and treatment with U73122 (a PLC inhibitor) blocked Ca\(^{2+}\) mobilization, suggesting that GPR75 couples to Go\_q/11. RANTES is a chemokine that recruits leukocytes into inflammatory sites and also induces the proliferation and
activation of natural-killer cells. Interestingly, levels of RANTES are increased in patients with PAH; RANTES mRNA expression, detected by competitive RT-PCR, in lung biopsies from patients with severe PAH was dramatically increased compared to control subjects.\textsuperscript{58}

PAH-PASMC are known to produce lower basal levels of cAMP than do Ctrl-PASMC, but we wanted to target GPR75 and assess its effect on cAMP accumulation in both Ctrl- and IPAH-PASMC.\textsuperscript{11} We used a commercially available N-terminal antibody as a type of antagonist to “block” receptor activation. Antibodies that target intracellular domains cannot be used for this purpose with intact cells as they would need to get into the cell to be functional. We found that an N-terminal GPR75 antibody increased cAMP accumulation and decreased proliferation of PAH-PASMC, but not control-PASMC, which had lower expression of GPR75. The results shown in this thesis suggest that GPR75 is a G\textsubscript{\alpha\text{i}}-coupled GPCR, in addition to being previously shown as a G\textsubscript{\alpha\text{q/11}}-coupled.\textsuperscript{57} Our results are quite clear-cut. Blockade of the receptor increases cAMP levels (Figure 7.11, which is characteristic of an inhibited G\textsubscript{\alpha\text{i}}-coupled GPCR). When GPR75 is blocked, PASMC proliferation also decreases (Figure 7.13). Overexpression of the receptor in HEK293 cells lowers basal cAMP levels and increases cell proliferation (Figure 7.15-7.16), results which provides further evidence of G\textsubscript{\alpha\text{i}}-coupling.

Figure 8.1 is a schematic of GPR75 signaling, either via RANTES\textsuperscript{57} or when blocked by an N-terminal binding GPR75 antibody. Previous literature has shown that antibodies can block the activity of membrane proteins. For example, blocking IL-17A
with anti-IL-17A antibody can protect against lung injury-induced pulmonary fibrosis.\textsuperscript{59} Although antibodies are more expensive to develop than small molecules, they tend to have a longer duration of action. It has been suggested that antibody therapeutics might be possible to develop against \textasciitilde88 GPCRs, some of which would require agonistic antibodies.\textsuperscript{60} Such therapeutic antibodies have shown the greatest success in inflammatory diseases, although some success has been seen in cardiovascular diseases.\textsuperscript{60} It has been shown that sometimes targeting the receptor can be more successful than targeting the ligand with an antibody. This has been shown by the observed lack of efficacy when targeting MIP1-\textalpha{} or RANTES (CCL5) as opposed to targeting the receptors CCR1 and CCR5.\textsuperscript{61} The same is true for CXCL8 (IL-8) and its receptors CXCR1 and CXCR2.\textsuperscript{62} Ligand levels can increase to overcome antibody blockade of such ligands easier than expression of receptors can increase. Also there is a redundancy of some GPCRs for multiple ligands.\textsuperscript{63} Antibodies directed towards GPCRs can play a therapeutic role not only by altering signaling pathways, such as those involved in proliferation and vasodilation in PAH, but also by serving as carriers for targeted toxin therapy.\textsuperscript{60} We propose that an anti-GPR75 antibody could be a potential therapeutic treatment for patients with IPAH and SPAH based on the much higher GPR75 expression in PAH-PASMC relative to Ctrl-PASMC. Blocking GPR75 is predicted to block whichever G protein or other pathways this receptor uses to perturb cell function and in the setting of PAH, to promote vasodilation and decreased PASMC proliferation. Further research into the expression of GPR75 in other cell types within the pulmonary and systemic circulation could help predict adverse effects. Drug and/or small molecule screening could also be beneficial to determine potential ligands for the orphan GPCRs
that we believe may have an important role in the pathogenesis of PAH: GPR113 and
GPR75 or perhaps other orphans highlighted in Tables 5.5 & 5.6.

GPR75 has multiple glycosylation sites. Glycosylation is a key factor for the
activity of a receptor and hence could contribute to the progression of PAH. Previous
research has suggested that GPR75 has 3 putative N-glycosylation sites (at position 2, 12,
and 25). Our results with PNGase F strongly suggest that this receptor is glycosylated
(Figure 7.18). Receptor glycosylation is often necessary to transport the receptor to the
cell surface and can stabilize receptors on the cell surface. Glycosylated receptors, in
particular changes that occur after the high mannose glycosylated form, are thus
considered more “mature.” Glycosylation can play a role in receptor-ligand binding as
ligands may bind based on receptor glycosylation. Glycosylation can also be essential
for conformational changes required for G protein coupling and subsequent cAMP
signaling. Some GPCRs such as relaxin receptor RXFP1 have been shown to lead to
blunted cAMP levels without proper glycosylation of the receptor. Glycosylation of
GPR75 thus might contribute to its signaling activity, including the inhibition of cAMP
accumulation. Using deglycosylation strategies, such as the drug tunicamycin (which
blocks the synthesis of all N-linked glycoproteins), one might be able to prevent such
glycosylation events from occurring and thus, the receptor from being active in PAH-
PASMC. Tunicamycin is known to prevent N-glycosylation events and the secretion of
glycoproteins from cells, such as recombinant human lysyl oxidase-like 2 (rhLOXL2)
from Drosophila S2 Cells.
In summary, data in this thesis show that a GPCR RT-PCR array is a useful tool to profile GPCR expression in cells and to highlight GPCRs that are involved in regulation of PASMC, cells in the pulmonary circulation. This technique has revealed GPCRs that could contribute to the pathophysiology of PAH but have previously been “missed.” Perhaps profiling GPCRs in patients with PAH could aid in development of personalized medicine by revealing uniquely expressed GPCRs in IPAH vs SPAH (Tables 5.5 & 5.6). Using a GPCR microarray to test patient samples may be a way to identify GPCRs (and thus GPCR agonist/antagonists) that would be most effective for treating PAH in a specific patient. The discovery of previously unrecognized GPCRs in PAH has the potential to generate high reward basic research by identifying targets and ultimately drugs that might rapidly enter clinical trials since many GPCR agonists/antagonists are already approved. Orphan GPCRs, such as GPR75, for which antibodies could be developed, could be a novel treatment for PAH; such antibody therapeutics, which are used in other diseases, could lead to a paradigm shift in the treatment of PAH.
Figure 8.1. Schematic portraying potential GPR75 signal transduction pathway. The left panel represents a PAH-PASMC without use of a blocking antibody. Thus, this shows RANTES activating GPR75 leading to Ga₁- and Ga_q/11-coupling. The outcome of this response is vasoconstriction and increased PASMC proliferation. The right panel represents a PAH-PASMC treated with N-terminal binding anti-GPR75 antibody. This antibody will bind and block the receptor, thus blunting the activation from RANTES. In turn, the Ga₁- and Ga_q/11-coupled pathways will not be activated leading to higher intracellular cAMP levels and thus, vasodilation and decreased proliferation of PASMC.
Figure 9.1. CH Rat lung slice showing vascular remodeling (B) compared to control (A). Image taken at 40x magnification.
Figure 9.2. pCMV6-Entry Vector schematic taken from OriGene Technologies, Inc.
Figure 9.3. GPR75 Restriction Map (from New England Biolabs, Inc).

Figure 9.4. Restriction Digest with Bgl II confirming GPR75 DNA insert in pCMV6-Entry Vector.
Figure 9.5. Western Blot of GPR75 overexpression (10µg) in PASMC.
References:


