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TITLE: Identification of G-Protein-Coupled Receptors (GPCRs) in Pulmonary Artery Smooth Muscle Cells as Novel Therapeutic Targets

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14. ABSTRACT
Pulmonary arterial hypertension (PAH) is associated with increased vascular resistance, sustained contraction, and enhanced proliferation of pulmonary arterial smooth muscle cells (PASMCs). The underlying idea of this project is that the currently limited treatments for PAH represent an unmet medical need for soldiers, veterans and those in the general population. We are testing the hypothesis that the discovery of "novel" (i.e., not previously recognized) G-protein-coupled receptors (GPCRs) and their functional activity in the PASMCs from subjects with PAH can reveal new insights into pathophysiology and new therapeutic targets for PAH. Our approach is to isolate PASMCs from PAH subjects and controls, to define the expression and function of their complement of GPCRs, with the goal of identifying GPCRs that have known physiologic agonists and are uniquely expressed and/or prominently up-regulated in PAH-PASMCs and to define their potential as novel therapeutic targets for PAH.

15. SUBJECT TERMS

Nothing listed

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Introduction

This project focuses on pulmonary arterial hypertension (PAH), which is associated with enhanced vasoconstriction and proliferation of pulmonary arterial smooth muscle cells (PASMCs). The limited, effective therapies for soldiers, veterans and those in the general population who have PAH represent an unmet medical need. Our overriding hypothesis is that previously unrecognized ("novel") G-protein-coupled receptors (GPCRs) expressed by PASMCs contribute to the pathophysiology and may be new therapeutic targets for PAH. To test this hypothesis, we isolate PASMCs from PAH subjects and controls, quantify the GPCR expression profile of the PASMCs and seek to identify (and then validate in signaling and functional studies) GPCRs that have known physiologic agonists and whose expression prominently increases in PAH-PASMCs. Our working hypothesis is that such GPCRs may be novel therapeutic targets for PAH.

Keywords

Pulmonary arterial hypertension (PAH), pulmonary artery smooth muscle cells (PASMCs), G-protein-coupled receptors (GPCRs), cyclic AMP, hypoxia.

Accomplishments

What were the major goals of this project?

Specific Aim 1/Task 1: The major task of Aim l is to isolate control PASMCs from humans, rats and mice and to identify the expression profile in these PASMCs of GPCRs with known physiologic agonists, to confirm expression of a subset of GPCRs by independent mRNA analyses, and to analyze expression and responses (functional activities) of receptor proteins. We will also determine if GPCR expression profile in PASMCs differs from that of human coronary artery and aortic smooth muscle cells.

la. Isolate and prepare primary cultures of PASMCs from lung samples obtained from 4 UCSD patients who do not have pulmonary hypertension (PH) **Completed 12/2015**

lb. Prepare RNA and cDNA from PASMCs and use Taqman GPCR arrays to identify and quantify expression of GPCRs with known physiologic agonists .**Completed 5/2017**

le. Prepare RNA and cDNA from commercially available coronary artery and aortic smooth muscle cells; use Taqman GPCR arrays to identify/quantify expression of GPCRs with known physiologic agonists. **Completed 12/2015**

ld. Isolate and culture PASMCs from 8 (4 male; 4 female) 3 -month old rats and mice, prepare RNA and cDNA and assess expression of GPCRs with known physiologic agonists by using Taqman GPCR arrays **In progress. To be completed by 2/2018**

le. Confirm mRNA expression by independent RT-PCR analyses of the 3 highest expressed PASMC-GPCRs that couple to Gs, Gi, Gq/G11, G12/13 or that are overall the 3 highest expressed GPCRs with known physiologic agonists from PASMCs, coronary artery and aortic smooth muscle cells **Completed 5/2017**

lf. Assess protein expression (immunoblotting, immunofluorescence and immunoprecipitation) with antibodies directed at 2 highly, preferentially expressed human, mouse and rat PASMC- GPCRs each that couple to Gs, Gi, Gq/11 or G12/13. **In progress. To be completed by 2/2018.**

lg. Characterize the function of 2 highly and preferentially expressed PASMC-GPCRs each that couple to Gs, Gi, Gq/qll and G12/13 by assessing signal transduction and cell physiology (cell cycle proteins [p21/p27],

DNA synthesis, caspase 3 activation, cell migration) **In progress. 25% completed. To be completed by 7/2018.**

Specific Aim 2/Task 2: The major task is to define the expression profile of GPCRs with known physiologic agonists in PASMCs from humans, rats and mice with PAH so as to identify alterations in GPCR expression that characterize PAH and to test if GPCRs with altered expression contribute to PAH pathophysiology The Sub-tasks are to:

2a. Isolate and prepare primary cultures of PASMCs from 4 patients each with primary and secondary PAH. **Primary (Group 1 PH) PAH: 9 cultures Completed 5/2017; Secondary (Groups 2-5 PH): 1 culture obtained. To be completed by 8/2018**

2b. Use RT-PCR and gene-specific primers with samples from at least 15 patients each with primary or secondary PAH to confirm the expression of GPCRs with known physiologic agonists of receptors with the most prominent differences in expression in PAH-PASMCs (compared to control PASMCs) and thereby, to identify the "signature" of the highest expressed such GPCRs, ones with the greatest differences compared to control-PASMCs and to determine the inter-subject variability in GPCR expression **In progress. RNA-seq completed for 10 samples. Plan is to complete by 8/2018**

2c. Assess protein expression and functional activity (as in Aim 1) for 2 GPCRs each that are Gs-, Gi-, Gq/11- and G12/13-coupled and whose expression is most prominently altered in PAH and most consistent in primary PAH-PASMC and secondary PAH-PASMC. **In progress. To be completed by 4/2018.**

2d. Set up rat models of PAH (monocrotaline, chronic hypoxia) and a mouse model of PAH (chronic hypoxia) with 4 animals each in each model, confirm PH by right ventricular hypertrophy and PA remodeling, and isolate PASMCs for culture, assay of expression of GPCRs with known physiologic agonists and functional analyses. **In progress. 50% complete. To be completed by 5/2018.**

Specific Aim 3/Task 3: The major task is to determine the therapeutic potential of "novel" GPCRs that we identify as preferentially expressed in PAH-PASMCs. We will use several criteria to choose GPCRs to target: ones with the largest changes (compared to control-PASMCs) in expression, similar changes in humans, rats and mice, with available pharmacological agents (in particular, for which FDA-approved drugs exist) and that we predict have a pathophysiological impact. The Sub-tasks are:

3a. Choose 2 Gi-coupled, Gq/11-coupled, 2 Gs-coupled and 1 G l2/13-coupled receptors to target using the criteria above and identify potential therapeutic agents **In progress. 40% completed. To be completed by 8/2018.**

3b. Use PAH-PASMCs and normal-PASMCs as controls and test antagonists of 2 Gi-coupled and 2 Gq/11-coupled GPCRs (chosen in Sub-task 1), with bosentan as a control, in concentration-response studies that assess effects on "basal" signal transduction/functional responses and on the responses to known receptor agonists. **In progress. 40% completed. To be completed by 8/2018.**

3c. Use PAH-PASMCs and normal-PASMCs as controls and test agonists of 2 Gs-coupled GPCRs (chosen in Sub-task I), with a prostacyclin agonist, as a control in concentration-response studies that assess effects on "basal" signal transduction/functional responses and on the responses to known receptor agonists. **In progress. 30% completed. To be completed by 8/2018.**

3d. Use PAH-PASMCs and normal-PASMCs as controls and test a G12/13-coupled GPCR antagonist (chosen in Sub-task I) in concentration-response studies that assess effects on "basal" signal transduction/functional responses and on the responses to a known receptor agonist. **To be completed by 8/2018.**

3e. Pending results of studies in Sub-tasks 2-4, test ability of drugs directed at the "new" GPCRs to produce additive (or perhaps synergistic) activity when used in combination with agents approved for the treatment of PAH (bosentan, prostacyclin agonist, PDE 5 inhibitor). **To be completed by 8/2018.**

3f. Test if commercially available anti-GPCR antibodies are able to antagonize Gi-, Gq/11- and G12/13-coupled GPCRs (chosen in sub-Task l) and evaluated as in Sub-Tasks 2-4. **To be completed by 8/2018.**

What was accomplished under these goals?

1. Major activities

In this reporting period, we have made progress on several aspects. We have isolated PASMCs from male and female rats and mice (control and subjected to hypoxia) and an additional PAH model in mice (SUGEN + hypoxia). We have not-yet completed studies on the monocrotaline model in rats, but have been able to achieve RV hypertrophy in this model. We have isolated PASMCs and confirmed their cell type with immunohistochemistry and are obtaining additional cells to assure adequate RNA is available to characterize GPCR expression (which will be determined by RNA sequencing [RNA-seq]). We have also assessed target GPCR expression with qPCR. From the same mice used to isolate PASMCs, we were able to isolate cardiac tissue and cardiac cells- to examine possible targets for PAH therapy directed at the right ventricle (RV).

Using human PASMCs, we performed RNA-seq and identified multiple GPCRs with altered expression in PAH- compared to control-PASMCs. These results facilitated our generation of a list of potential GPCR targets, whose expression we confirmed by qPCR. For several putative GPCR targets, we utilized cAMP and Ca^{2+} assays to assess their signaling in response to agonists. In addition, we obtained 5 more patient samples and have and characterized their GPCR expression by RNA-seq.

2. Specific objectives

lb. Prepare RNA and cDNA from PASMCs and use Taqman GPCR arrays to identify and quantify expression of GPCRs with known physiologic agonists.

ld. Isolate and culture PASMCs from 8 (4 male; 4 female) 3 -month old rats and mice, prepare RNA and cDNA and assess expression of GPCRs with known physiologic agonists by using Taqman GPCR arrays.

le. Confirm mRNA expression by independent RT-PCR analyses of the 3 highest expressed PASMC-GPCRs that couple to Gs, Gi, Gq/G11, G12/13 or that are overall the 3 highest expressed GPCRs with known physiologic agonists from PASMCs, coronary artery and aortic smooth muscle cells.

2d. Set up rat models of PAH (monocrotaline, chronic hypoxia) and a mouse model of PAH (chronic hypoxia) with 4 animals each in each model, confirm PH by right ventricular hypertrophy and PA remodeling, and isolate PASMCs for culture, assay of expression of GPCRs with known physiologic agonists and functional analyses

3a. Choose 2 Gi-coupled, Gq/11-coupled, 2 Gs-coupled and 1 G l2/13-coupled receptor to target using the criteria above and identify potential therapeutic agents.

3c. Use PAH-PASMCs and normal-PASMCs as controls and test agonists of 2 Gs-coupled GPCRs (chosen in Sub-task I), with a prostacyclin agonist as a control in concentration- response studies that assess effects on "basal" signal transduction/functional responses and on the responses to known receptor agonists.

3. Significant results

In the reporting period, we conducted multiple experiments that addressed the above objectives. Our results include the following::

A. We isolated RNA from control- and IPAH-PASMCs and performed RNA-seq. These cells included PASMCs from PAH patients from a collaborator (Jason Yuan [University of Arizona]) and included 3 idiopathic PAH and 2 heritable PAH samples. From this analysis, we generated a list of potential targets that include 9 GPCRs whose expression is higher in PAH- than control-PASMCs. We confirmed these targets using qPCR as shown in **Figure 1**.

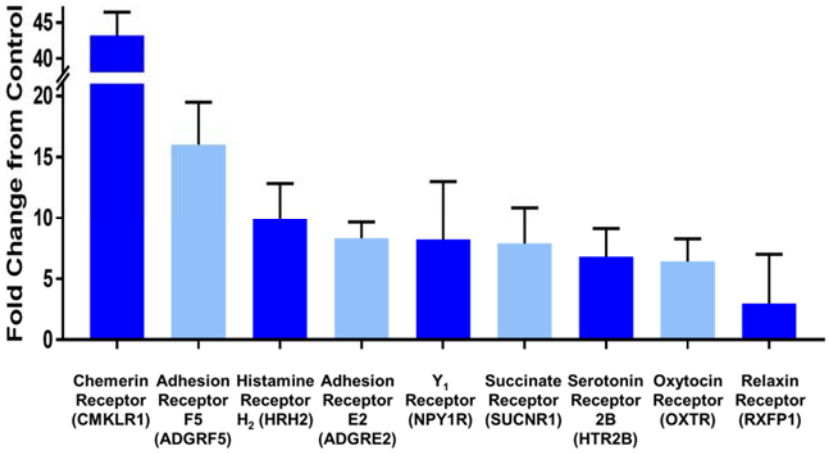

Figure 1: *qPCR of target GPCRs expressed higher in PAH-PASMCs compared to control.*

B. Using agonists of the target GPCRs, we measured second messenger activity, i.e., cAMP assays (DiscoverX) and Ca^{2+} assays (Molecular Devices) to measure by PASMCs. We found that the cells showed increased cAMP accumulation with the addition of adenosine (as a positive control) and moderate Gi activity (but greater in PAH-PASMCs) in response to succinate (the SUCNR1 ligand) or ML290 (a RXFP1 ligand) (**Figure 2**). The PASMCs responded with an increase in intracellular Ca^{2+}, indicative of signaling via Gq, in response to the addition of oxytocin (a ligand for OXTR) or histamine (histamine receptor agonist) (**Figure 3**), but not in response to succinate, chemerin, or bradykinin (data not shown).

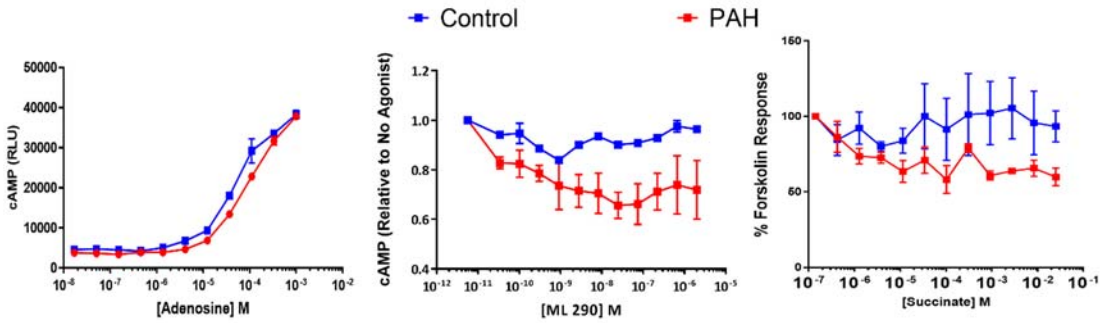

Figure 2*: cAMP accumulation in response to agonists for target GPCRs.*

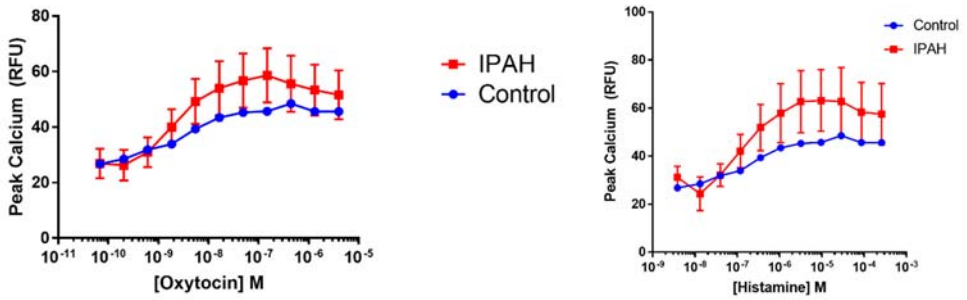

***Figure 3**: Intracellular calcium in response to agonists for target GPCRs.*

C. RNA-seq data were further analyzed for differential gene expression using EDGER; stringent parameters were used to reduce the false discovery rate (FDR) of GPCRs as PAH targets. Several samples had distinct clustering of gene expression compared to others (**Figure 4**, IPAH1 – 3). Based on that result and other data showing that agonists lacked signaling activity in those cells, we excluded them and generated a revised list of target GPCRs that were consistently overexpressed in PAH- compared to control-PASMCs. The list includes PTGER2, LPAR3, and GPR126, which had higher expression in the PAH-PASMCs compared to control-PASMCs (FDR<0.1) and were also low-expressed in aortic and coronary SMCs (**Figure 5**).

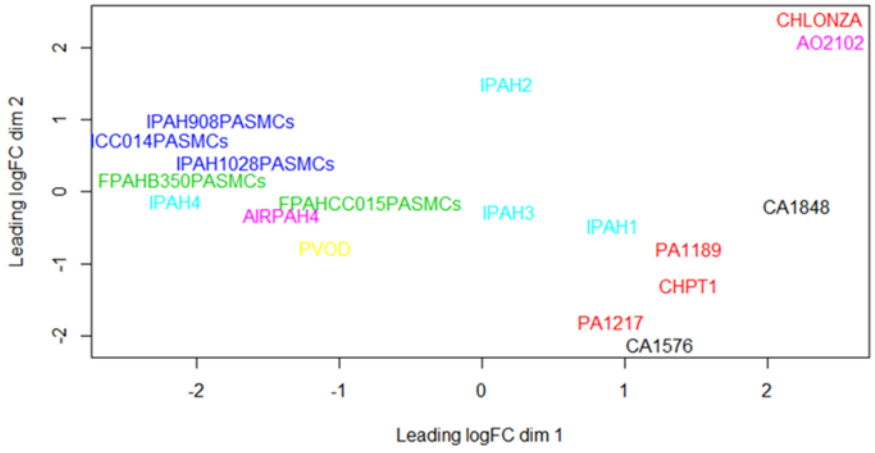

***Figure 4**: Clustering of gene expression of smooth muscle cells (SMCs) derived from human tissues.*

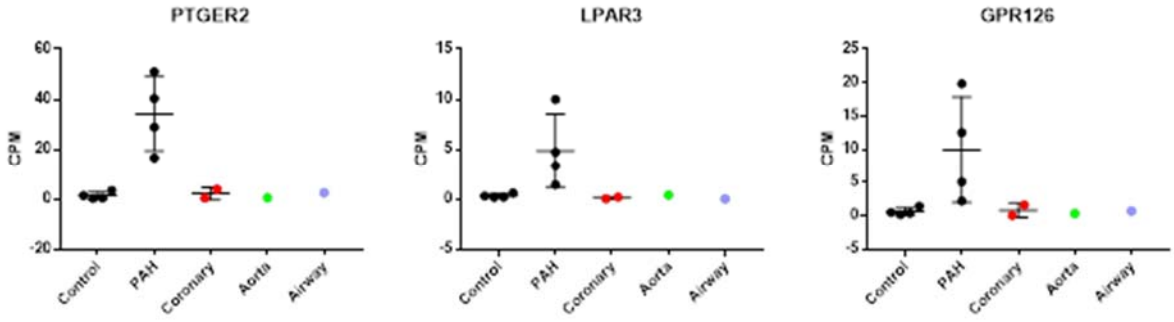

***Figure 5**: Target GPCRs increased in PAH compared to control PASMCs.*

D. Further analysis of differentially-expressed genes from RNA-seq revealed many genes with significantly altered expression in PAH-PASMCs (**Figure 6**). Using EDGER, we found that 2378 genes were altered with a FDR <0.1: 985 were increased at least 2-fold, and 1086 were decreased at least 2-fold in IPAH samples compared to control-PASMCs. These genes were used for a gene ontology analysis (GO, Panther) for biological processes. **Table 1** lists the 10 processes with the highest overrepresentation (P<0.05). Of note, all of the up-regulated processes relate to proliferation, a hallmark of PAH-PASMCs.

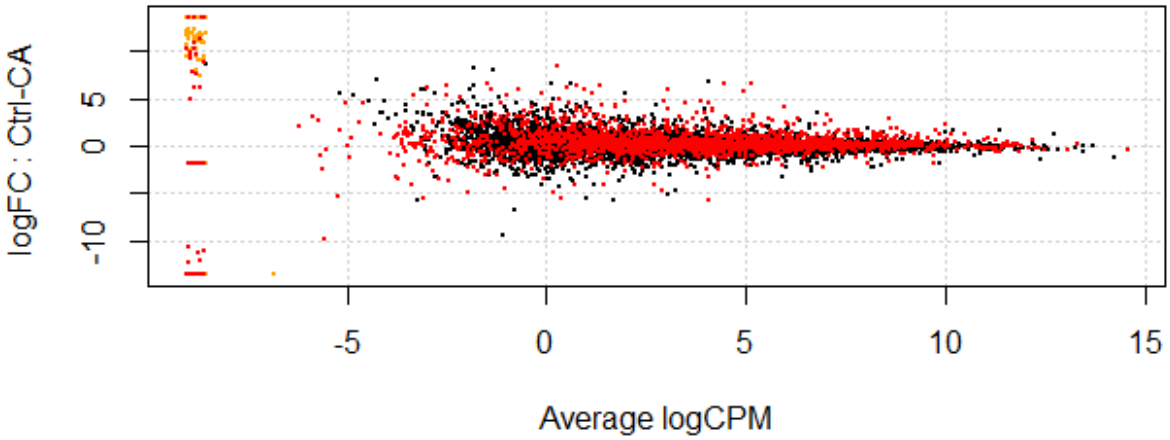

***Figure 6**: Smear plot of genes with significantly altered expression in PAH. Each dot represents a gene, red dots indicate genes with false discovery rate (FDR) <0.1.*

***Table 1**: Gene ontology (GO) analysis for the 10 most up-regulated and down-regulated processes in IPAH.*

GO Biological Processes Up-regulated in IPAH	**GO Biological Processes Down-regulated in IPAH**
Positive regulation of DNA-directed DNA polymerase activity	PERK-mediated unfolded protein response
DNA unwinding involved in DNA replication	Positive regulation of transcription from RNA polymerase II promoter in response to endoplasmic reticulum stress
Lagging strand elongation	Intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress
CENP-A containing nucleosome assembly	Positive regulation of vascular endothelial growth factor production
Kinetochore assembly	Lipopolysaccharide-mediated signaling pathway
Protein localization to kinetochore	Cellular response to glucose starvation
Telomere maintenance via semi-conservative replication	IRE1-mediated unfolded protein response
DNA replication initiation	Cardiac muscle tissue morphogenesis
Regulation of mitotic cell cycle spindle assembly checkpoint	Type I interferon signaling pathway
Mitotic chromosome condensation	Regulation of response to endoplasmic reticulum stress

E. We have made additional progress on rat and mouse models of PAH. We isolated PASMCs from mice exposed to hypoxia (10% O_2) with or without weekly injections of SU5416 (SUGEN) for 3 weeks. PASMCs were isolated after the mice were at room air (~21% O_2) for 3 additional weeks. PASMCs were isolated from mice and stained for smoothelin to confirm their SMC identity. The mice had increased right ventricle (RV) weight relative to the left ventricle (LV) and septum (S) compared to normoxic, control micel, as shown in **Figure 7** (Fulton index: RV/(LV + S)). The three groups provide a graded model of PAH development, as the SUGEN injections produced a higher degree of RV hypertrophy.

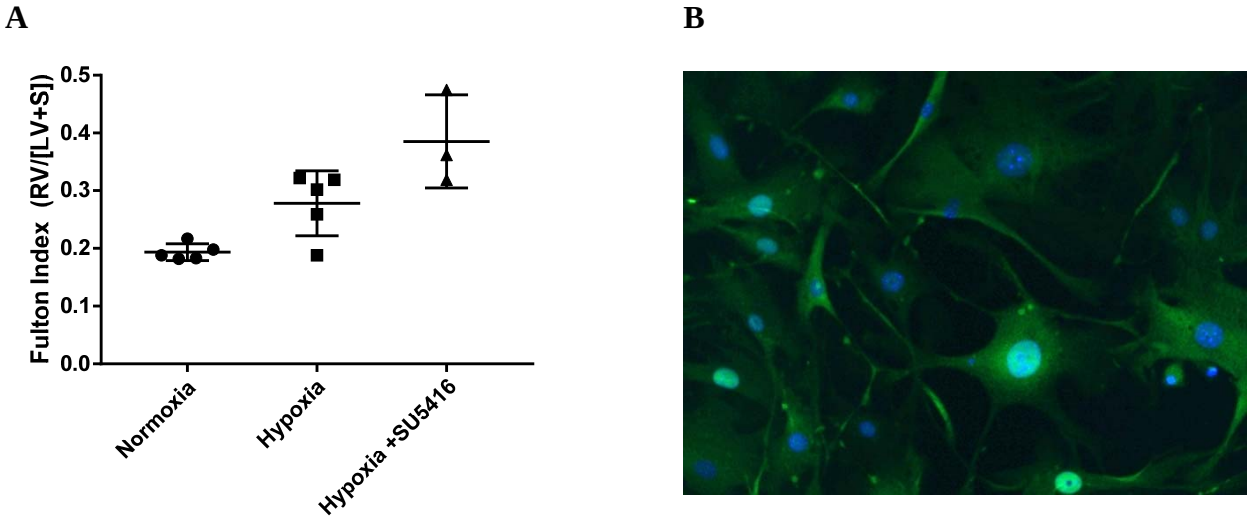

Figure 7*: (A) Fulton index of mice exposed to hypoxia with or without weekly SUGEN (SU5416) injections for three weeks compared to normoxia. (B) PASMCs isolated from mice and probed by immunocytochemistry for smoothelin, a SMC marker).*

What opportunities for training and professional development has the project provided?

Mathew Gorr, PhD, a post-doctoral fellow, has devoted most of his efforts to this project. He has received support from an NHLBI-funded Cardiology Training Grant and has participated in training grant activities, including having made two presentations to faculty and trainees about his findings on this project and has assisted as an organizer of a one day retreat for participants on this Training Grant. Additionally, he has presented findings from this project in two talks and two posters at the 2017 Experimental Biology meeting, one resulting in a Postdoc Award from ASPET (American Society for Pharmacology and Experimental Therapeutics). Additionally, this project has provided training for three volunteer-based UC San Diego undergraduate students, Esther Wu, Jason Liao and Abinaya Muthusamy, all of whom have subsequently enrolled in independent research elective courses to continue work on this project..

How were the results disseminated to communities of interest?

Poster and oral presentations from this project were given at the 2017 Experimental Biology meeting (April 2017). Abstracts are attached below.

What do you do plan to do during the next report period to accomplish the goals?

We will undertake analysis of additional samples using RNA-seq to assess GPCR expression and relate the expression of GPCRs to that of other RNAs, whose expression may change with PAH. We seek to increase the number of subjects for studies in Aims 1 and 2 and in particular to determine if there is a “PAH-specific

GPCR expression profile". We will expand the studies related to PTGER2, LPAR3 and GPR126 as potential contributors to the pathophysiology of PAH and perhaps therapeutic targets. The studies will include protein analysis (using antibodies and proteomic techniques), and assessment of functional activities, including signaling events, DNA synthesis and effects on cell growth and death. Additionally, we will complete studies with mouse and rat models of PAH, including examining the GPCR profile of isolated PASMCs. Pending the outcome of those studies and available time, we will also initiate studies of other GPCRs with reduced expression in PAH-PASMCs that also have high expression (and thus may be therapeutic targets). .

Impact

What was the impact on the development of the principal discipline(s) of the project?

Our findings strongly suggest that multiple types of vascular smooth muscle cells, including PASMCs, coronary artery SMCs and aortic SMCs, express >70 GPCRs, each of which has the potential to regulate cellular signaling and function. GPCRs with known agonists (the focus of this project), may have previously unappreciated effects in normal and diseased PASMCs. Of particular note is our discovery of multiple GPCRs whose expression consistently differs between PAH-PASMCs and control-PASMCs. GPCRs with increased expression in PAH-PASMCs may contribute to the pathophysiology of PAH and may thus be therapeutic targets for PAH.

What was the impact on other disciplines?

The notion that individual cell types express many more types of GPCRs than were previously known is potentially important for the regulation of cells and tissues in health and disease. The results have impact on cell biology, biochemistry, physiology, pharmacology and pathology, as well as clinical medicine, especially if the newly recognized GPCRs can further understanding of pathophysiology and be used to aid in diagnosis, assessment of prognosis and/or be therapeutic targets in disease states.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

CHANGES/PROBLEMS

None.

PRODUCTS

Publications, conference papers and presentations during this reporting period:

MW Gorr, K Sriram, PA Insel. GPCRs in Pulmonary Arterial Smooth Muscle Cells as Novel Targets in Pulmonary Arterial Hypertension. Experimental Biology, April 2017, San Diego, CA. *Winner of ASPET Postdoc Award.* The FASEB Journal 31 (1 Supplement), 664.11-664.11

MW Gorr, K Sriram, PA Insel Transcriptomic Analysis of the Right and Left Ventricle in Normoxia and Hypoxia (a Model of Pulmonary Arterial Hypertension). Experimental Biology, April 2017, San Diego, CA. The FASEB Journal 31 (1 Supplement), 884.8-884.8

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Paul A. Insel
Project Role: Principal Investigator
Researcher Identifier: 402799
Nearest person month worked: 2
Contribution to Project: Directed all phases of the project
Funding Support: This project, a contract from Bristol-Myers Squibb and two NIH AG grants

Name: Krishna Sriram
Project Role: Post-doctoral fellow
Researcher Identifier: 791836
Nearest person month worked: 4
Contribution to Project: Performed RNA-Seq analysis; analyzed RNA-seq, GPCR data
Funding Support: This project and a contract from Bristol-Myers Squibb

Name: Matthew Gorr
Project Role: Post-doctoral fellow
Researcher Identifier: 306535
Nearest person month worked: 12
Contribution to Project: Set up animal models for PAH, obtained lung samples, prepared and cultured PASMCs, isolated RNA and prepared cDNA, performed GPCR data analysis, undertook protein and functional studies of GPR91
Funding Support: This project and a T32 NHLBI Cardiology Training Grant

Name: Amy Chinn
Project Role: Graduate Student
Researcher Identifier: 015032
Nearest person month worked: 1
Contribution to Project: Assisted with GPCR analysis
Funding Support: This project and a NIGMS Pharmacological Sciences Training Grant

Name: Kris Haushalter
Project Role: Post-doctoral fellow
Researcher Identifier: 351184
Nearest person month worked: 2
Contribution to Project: Assisted with GPCR analysis
Funding Support: This project and NIH R21 AG grant

Name: Shu Zhou Wiley
Project Role: Post-doctoral fellow
Researcher Identifier: 007388
Nearest person month worked: 2
Contribution to Project: Assisted with GPCR analysis
Funding Support: This project and Bristol-Myers Squibb contract

Has there been a change in the active other support of the PD/PI or senior/key personnel since the last reporting period? Yes

Collaborative Research Agreement (PI: P. Insel) 6/1/2014-5/31/2018 1.20 calendar

Bristol-Myers Squibb

“Novel therapeutic targets in cardiac and extra-cardiac fibroblasts for the treatment of fibrotic diseases”

Major goals: To identify GPCRs of cardiac and lung fibroblasts as possible drug targets for tissue fibrosis.

R21AG053568 (PI: P. Insel) 9/30/2016-4/30/2018 0.96 calendar

NIH/NIA

“Aging and G-protein-regulated MMP14”

Major goals: To assess G-protein-mediated activation of MMP14, its enhanced activity in aging, and consequences of this activation in heart cells from young and old mice.

R21AG52914 (PI: P. Insel) 4/1/2017-3/31/2019 0.96 calendar

NIH/NIA

“Caveolin 3 and cardiac fibrosis”

Major goals: To determine the role of cardiac myocyte-expressed caveolin-3 in regulating fibrotic activity of cardiac fibroblasts with aging in mice.

What other organizations were involved as partners?

None

8. SPECIAL REPORTING REQUIREMENTS

None

9. APPENDICES

None