JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

Title Page

Utilizing DREADD chemogenetic tools to identify beneficial GPCR signaling for fibrosis

Ji Zhang[1, #, §], Eyal Vardy[2, ¶, #], Eric S. Muise[3], Tzu-Ming Wang[4], Richard Visconti[2], Ashita Vadlamudi[2], Shirly Pinto[1, ¶], and Andrea M. Peier[2, §]

Departments of Cardiometabolic Diseases[1], Screening and Compound Profiling[2], GpGx[3], Translational Biomarkers[4], MRL, Merck & Co., Inc., 2000 Galloping Hill Road, Kenilworth, NJ 07033, USA

[¶]Kallyope Inc. 430 E. 29th Street, New York, NY 10016

[#] These authors contribute equally to the work.

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

Running title Page

Running Title: GPCR signaling in Fibrosis

§ To whom correspondence should be addressed:

Andrea M. Peier §

Departments of Screening and Compound Profiling, MRL, Merck & Co., Inc.

2000 Galloping Hill Road, Kenilworth, NJ 07033, USA

Email: andrea_peier@merck.com

Tel: 908-740-0148

Ji Zhang §

Departments of Cardiometabolic Diseases, MRL, Merck & Co., Inc.

2000 Galloping Hill Road, Kenilworth, NJ 07033, USA

Email: jzhang30@gmail.com

Tel: 650-496-4927

Number of text pages: 13

Number of tables: 1 in main text

Number of figures: 4 main figures

Number of references: 41 in main text, 18 in Table 1

Number of words in Abstract: 214

Number of words in Introduction: 743

Number of words in Discussion: 1,116

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

Non-standard abbreviations:

5-HT2B	5-Hydroxytryptamine receptor 2B
5-HT2C	5-Hydroxytryptamine receptor 2C
5-HT7	5-Hydroxytryptamine receptor 7
ALK5	Activin receptor-like kinase 5
αSMA	α-smooth muscle actin
BDKRB	Bradykinin receptor
BDL	Bile duct ligation
cAMP	Cyclic adenosine monphosphate
CCl4	Carbon tetrachloride
CB1	Cannabinoid receptor 1
CNO	Clozapine N-oxide
DREADD	Designed receptors exclusively activated by designer rrug
DRD1	Dopamine receptor 1
EP1	E-prostanoid receptors 1
EP2	E-prostanoid receptors 2
EP3	E-prostanoid receptors 3
EP4	E-prostanoid receptors 4
ESRD	End stage renal disease
GIRK	G protein-coupled inwardly-rectifying potassium channels
GPCR	G protein coupled receptor
GPER1	G protein coupled estrogen receptor 1
HSC	Hepatic stellate cells
IP_3	Inositol trisphosphate

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

IRES	Internal ribosome entry site
IPF	Idiopathic pulmonary fibrosis
LPAR1	Lysophosphatidic acid receptor 1
MC1R	Melanocortin receptor
MRTF-A	Myocardin-related transcription factor-A
NASH	Nonalcoholic steatohepatitis
NHLF	Normal human lung fibroblasts
OXTR	Oxytocin receptor
PAI-1	Plasminogen activator inhibitor-1
PAR2	Protease-activated receptor 2
PGE2	Prostaglandin E2
PKA	Protein kinase A
PLC-β	Phospholipase C-β
RPTEC	Renal proximal tubular epithelial cells
RT-qPCR	Quantitative reverse transcription PCR
S1PR3	Sphingosine-1-phosphate receptor 3
SRF	Serum-response factor
TBXA2R	Thromboxane A2 receptor
TGFβ	Transforming Growth Factor β
UUO	Unilateral ureter obstruction

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Fibrosis or accumulation of extracellular matrix is an evolutionarily conserved mechanism adopted by an organism as a response to chronic injury. Excessive fibrosis, however, leads to disruption of organ homeostasis and is a common feature of many chronic diseases. G protein-coupled receptors (GPCRs) are important cell signaling mediators and represent molecular targets for many FDA approved drugs. To identify new targets for fibrosis, we utilized a synthetic GPCR system named Designed Receptors Exclusively Activated by Designer Drugs (DREADDs) to probe signaling pathways essential for fibrotic response. We found that upon expression in human lung fibroblasts, activation of Gq and Gs-DREADDs abrogated the induction of TGFβ-induced fibrosis marker genes. Genome-wide transcriptome analysis identified dysregulation of multiple GPCRs in lung fibroblasts treated with TGFβ. To investigate endogenous GPCR modulating TGFβ signaling, we selected 13 GPCRs that signal through Gq or Gs and activated them by using specific agonists. We examined the impact of each agonist and how activation of endogenous GPCR affects TGFβ signaling. Among which, prostaglandin receptor agonists demonstrated the strongest inhibitory effect on fibrosis. Together, we have demonstrated that DREADDs system is a valuable tool to identify beneficial GPCR signaling for fibrosis. This study in fibroblasts has served as a proof-of-concept and allowed us to further develop in vivo models for fibrosis GPCR discovery.

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

Significance Statement

Fibrosis is the hallmark of many end-stage cardiometabolic diseases and there is an unmet medical need to discover new anti-fibrotic therapies, reduce disease progression, and bring clinically meaningful efficacy to patients. Our work utilizes DREADD chemogenetic tools to identify beneficial GPCR signaling for fibrosis, providing new insights into GPCR drug discovery.

Keywords GPCR, DREADDs, fibrosis, TGFβ, Gq, Gs

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Fibrosis is an evolutionarily conserved mechanism developed by an organism to survive chronic injury (Wynn and Ramalingam, 2012). Excessive fibrosis, however, leads to disruption of organ function and is a common feature of many chronic diseases. Many progressive forms of cardiometabolic diseases, such as end stage renal disease (ESRD), nonalcoholic steatohepatitis (NASH) and heart failure, are characterized by the presence of extensive fibrosis (Friedman et al., 2013). A deeper understanding of the complex biology underlying these disease associations would greatly help the development of newer transformative therapies. Despite the broad clinical impact, there are limited treatment options for fibrosis. In 2014, the FDA approved the use of Pirfenidone and Nintedanib in idiopathic pulmonary fibrosis (IPF) patients, which has kindled interests in developing more anti-fibrotic drugs for patients. To date, several agents have demonstrated activities in reducing fibrosis in patients, including Obeticholic acid, Cenicriviroc, NGM282, Liraglutide, and Pamrevlumab (FG-3019) (Neuschwander-Tetri et al., 2015; Armstrong et al., 2016; Ratziu et al., 2016; Friedman et al., 2018; Harrison et al., 2018). However, no new drug has yet been approved to stop fibrosis progression, relieve symptoms, or substantially improve patient survival. There is a huge unmet medical need to develop new anti-fibrosis therapies with clinically meaningful efficacy in patients.

GPCRs are important cell signaling molecules that respond to a variety of extracellular stimuli, including hormones, neurotransmitters, growth factors, chemokines, light, odor, taste, extracellular matrix, shear stress, etc. (Gilman, 1987; Hauser et al., 2017; Weis and Kobilka, 2018). GPCRs regulate nearly all physiological activities, such as blood pressure control, respiration, hormonal-neural regulation, and immune response (Premont and Gainetdinov, 2007). It has been estimated that GPCRs comprise approximately 34% of the molecular targets for FDA approved drugs and ~60 agents against novel GPCR targets are in clinical trials (Hauser et al., 2017). Several GPCR targeted therapies have been evaluated for fibrotic indications. The lysophosphatidic acid receptor LPAR1 has been found linking

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

pulmonary fibrosis to lung injury (Tager et al., 2008). A LPAR1 antagonist BMS-986020 was recently examined in IPF patients (NCT01766817) and found effective at slowing down the decline of lung function (Palmer et al., 2018). A drug-related liver toxicity precluded BMS-986020 from further clinical development. Cannabinoid receptor 1 (CB1) antagonism has also attracted a considerable amount of interests for the treatment of NASH due to its role in central orexigenic effect, energy expenditure and fibrosis (Mallat et al., 2013; Patsenker and Stickel, 2016). Neuronal adverse effects of the known CB1 inverse agonist Rimonabant have prompted the development of a newer peripherally restricted-agent (Sam et al., 2011). Nonetheless, the interest to develop new GPCR therapies with better safety profiles for fibrosis is clearly warranted.

The activation of GPCRs under different conditions can have different biological outcomes depending on the cellular context and conditions (Gilman, 1987; Weis and Kobilka, 2018). Identification of beneficial GPCR signaling pathways for fibrosis will enable more focused target identification and drug screening efforts. Upon activation, a GPCR binds to a heterotrimeric G protein. The α-subunit of the G protein is subsequently dissociated from the βγ subunits to mediate downstream signals (Weis and Kobilka, 2018). There are four main subtypes of α-subunit, Gq/11, Gs, Gi/o, and G12/13. Gq/11 protein couples GPCRs to Phospholipase C (PLC-β), which in turn hydrolyzes phosphatidylinositol 4,5-bisphosphate to second messengers diacyl glycerol and inositol trisphosphate (IP_3). IP_3 stimulates calcium release into the cytoplasm. Gs protein binds to adenylyl cyclase to produce cyclic adenosine monphosphate (cAMP) and activate protein kinase A (PKA) signaling. On the other hand, Gi/o protein primarily inhibits PKA signaling by inhibiting adenylyl cyclase activity and decreasing cAMP production. G12/13 protein regulates actin cytoskeletal remodeling in cells.

The Designed Receptors Exclusively Activated by Designer Drugs (DREADDs) system has been recently developed to delineate G protein signaling underlying biological responses (Urban and Roth, 2015). DREADDs are synthetic GPCRs that have been engineered to respond to otherwise inert ligands such as

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

Clozapine N-oxide (CNO) (Urban and Roth, 2015). Different DREADDs have been engineered to selectively activate Gq, Gs or Gi pathways. By using the DREADD chemogenetic tools, we found that Gq and Gs signaling decreases cellular response to TGFβ. Whole-genome transcriptome analysis of human primary fibroblasts and fibrosis models revealed 13 highly-dysregulated GPCRs that signal through either Gq or Gs pathways with known agonists. We further showed that among those GPCRs, activation of the prostaglandin receptors reduced fibrotic marker expression in fibroblasts. The combination of DREADD chemogenetic tools with transcriptome analysis represents a powerful tool for identifying potential GPCR targets for fibrosis.

Materials and Methods

Cultured Cells and Reagents

Normal human lung fibroblasts (Lonza, #CC-2512, Lot # 0000343490) were cultured in fibroblast basal medium (Lonza, #CC-3131) with growth supplements (Lonza, #CC-4126). Human primary cardiac fibroblasts (Sciencell, #6300, Lot #5433) were cultured in FM2 media (Sciencell, #2331), supplemented with FBS (Sciencell, #0025), FGS-2 (Sciencell, #2382), and Penicillin-Streptomycin (Life Technologies, #15070-063). Human primary renal proximal tubule epithelial cells (Lonza, #CC-2553, Lot#0000362300) were cultured in renal epithelial cell growth basal medium (Lonza, #CC-3191) supplemented with REGM SingleQuots (Lonza, #CC-4127). Primary human hepatic stellate cells (Sciencell, #5300, Lot#10279) were cultured in Stellate Cell Medium (Sciencell, #5301) supplemented with FBS (Sciencell, #0010), stellate cell growth supplement (Sciencell, #5352), and Penicillin-Streptomycin solution (Sciencell, #0503). All cells were Incubated at 37^{o}C in the presence of 5% CO2.

hM3-Gq-DREADD, hM4-Gi-DREADD, hM3-Gs-DREADD, and KOR-Gi-DREADD were cloned into in a BacMam vector. The viruses were expressed and purified by Life Technologies. For TGFβ treatment, cells were plated and serum-starved for overnight. The next day, 5ng/ml of recombinant human TGFβ1

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

(BioLegends, #580702) was added to the culture medium and cells were incubated at 37^{o}C for the indicated time. For activation of DREADDs, cells were infected with control or DREADDs-expressing BacMam viruses at various titers for 24hrs. Cells were subsequently treated with Clozapine N-oxide (CNO) or Salvinorin B in the presence of recombinant human TGFβ1 for 24hrs. SB-525334, CNO, Salvinorin B, Bradykinin, Prostaglandin E2 (PGE2), Oxytocin, and Endothelin 3 were from Sigma. G-1, A68930, TCS2510, U46619, WAY267464, AC264613, CYM5541, BW723C86, AS19, and BMS470539 were from Tocris Biosciences. For inhibitor treatment, cells were treated with recombinant human TGFβ1 in the presence of vehicle DMSO (Sigma, #D2650) or compounds at various concentrations.

cAMP and Calcium signaling

At 24hr following BacMam viral infection, cells were treated with CNO at various concentration for 30 minutes. After removing media, cAMP stimulation or inhibition of basal levels of cAMP, were measured by cAMP Gs Dynamic kit (Cisbio, #62AM4PEB) or Gi kit (Cisbio, #62AM9PEB). For calcium measurement, cells were incubated with fluorescence dye from FLIPR Calcium 5 Assay Kit (Molecular Devices R8185) for 1 hour at 37°C. The plate was then set up in the FLIPR Tetra high-throughput cellular screen system, treated with CNO, and read immediately.

Rodent Fibrosis Models

Adult male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) and adult male Sprague Dawley/SD rats (Taconic, Rensselaer, NY) were housed in a temperature- and humidity-controlled facility with a 12-hour light: 12-hour dark cycle. Animals had ad libitum access to food (Purina Rodent Chow 5053, LabDiet, St. Louis, MO) and water. All procedures utilizing experimental animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at MRL, Kenilworth,

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

NJ. Mouse UUO kidney fibrosis and CCl4-induced liver fibrosis models, as well as rat bile-duct ligation models were carried out as previously described (Zhang et al., 2020).

Gene Expression Taqman Analysis

Total RNA was extracted from cultured cells or tissues following the instruction of RNeasy Mini QIAcube Kit (Qiagen, #74116). 2ug RNA was then reverse-transcribed using SuperScript VILO cDNA Synthesis kit (Invitrogen, #11754-050). 2ul diluted cDNA was added to quantitative real-time PCR mix with TaqMan Universal PCR Master mix (Applied Biosystems, #4364338) and Taqman probes (Life Technologies). PCR amplification (one cycle at 50°C for 2 minutes, one cycle at 95°C for 10 minutes and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute) was done in an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystem). Ct value of each sample was normalized to GAPDH level and quantified based on 2e-delta (deltaCT) method (comparing with no treatment or sham group).

RNA Isolation and Gene Expression Profiling

Gene expression analysis was performed as previously described (Schlessinger et al.). Briefly sequencing was performed using the Truseq stranded total RNA RiboZero library preparation kit (Illumina, #RS-122-2201) according to the manufacturer's instructions (Illumina, San Diego, CA). The resulting cDNA libraries were sequenced on an Illumina (HiSeqTM 4000) using a 50-base paired-end run. Alignment and differential gene expression analysis was performed in Omicsoft Array Studio version 9.0.8.92. Briefly, cleaned reads were aligned to the mouse and human B38 genome references, for the UUO kidney and primary cells respectively, using the Omicsoft Aligner with a maximum of 2 allowed mismatches. Gene level counts, and FPKM, were determined by the OSA algorithm as implemented in Omicsoft Array Studio and using Ensembl.R82 gene models. At least 90% of reads across all samples mapped to the respective genome (corresponding to 55-80 million reads). Differential gene expression

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

analysis was performed by the DESeq2 algorithm as implemented in Omicsoft Array Studio with the samples from the sham operated, or vehicle treated, animals serving as reference, yielding fold change and corrected p values (False Discovery Rate, Benjamini–Hochberg; FDR_BH). GO term enrichment analysis was performed using the PANTHER enrichment test (http://pantherdb.org). Heatmaps and box plots were created using Omicsoft Array Studio.

Data analysis

All data are presented as mean ± standard error of the mean (SE). One or Two-way ANOVA post hoc Tukey analysis was used for data comparisons as indicated in the figure legends, together with group size. All analyses were done using Prism 7 software. A p-value of <0.05 was statistically significant.

Results

Establishment of functional DREADDs expression system in primary human lung fibroblasts

DREADDs are synthetic GPCRs whose expression does not affect the biological system when not activated by their highly specific synthetic ligands. Gq-DREADD (hM3Dq) was created by directed molecular evolution of the human M3 muscarinic receptor (hM3) in yeast (Armbruster et al., 2007). When activated by the ligand CNO, it stimulated calcium release into the cytoplasm. Gi-DREADD (hM4Di) was designed based on the human M4 muscarinic receptor; it decreased cAMP signaling and opens K^+ channels (GIRKs) in response to CNO (Nawaratne et al., 2008). A chimeric hM3Dq β-adrenergic receptor DREADD was created as Gs-DREADD, which activated cAMP production and PKA signaling (Guettier et al., 2009). Unlike hM3Dq and hM4Di, Gs-DREADD has a modest degree of constitutive activity, which can lead to basal phenotypes in certain cellular contexts.

BacMam is a highly effective viral expression system that enables protein expression in a large variety of cells, including human primary cells (Ames et al., 2007). Gq/Gs/Gi-DREADDs were cloned into a bi-

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

cistronic BacMam vector that simultaneously expresses mCitrine fluorescent protein under the control of internal ribosome entry site (IRES) (**Figure 1A**). At 24hr after BacMam infection, the expression of DREADDs was confirmed by the co-expressed fluorescent signals in normal human lung fibroblasts (NHLFs) (**Figure 1B**). At the optimal viral titers, about 50% of cells were positive for fluorescence. To verify the function of exogenously-expressed DREADDs, we next examined calcium signaling in all cells. As shown in the top panel of **Figure 1C**, CNO treatment induced a dose-dependent spike of calcium release into the cytoplasm of Gq-DREADD expressing fibroblasts. cAMP production was induced by CNO in Gs-DREADD expressing fibroblasts (**Figure 1C, middle panel**). Conversely, activation of Gi-DREADD expressing fibroblasts by CNO led to an expected, modest reduction of the basal cAMP level (**Figure 1C, bottom panel**). These results indicated that BacMam-delivered DREADD expression system was functional in those cells.

Activation of Gq and Gs signaling inhibits the expression TGFβ-induced fibrosis marker genes

Among many factors that regulate fibrosis, TGFβ plays an essential role in both myofibroblast activation and extracellular matrix remodeling (Akhurst and Hata, 2012); it has often been used to induce fibrotic response in cultured cells. To gain novel insight into the GPCR pathways modulating TGFβ, we next established a cell-based assay to examine TGFβ response (**Figure 2A**). At 24hr after TGFβ treatment, RT-qPCR analysis of fibrotic marker genes, such as α-smooth muscle actin (αSMA), PAI-1, and Collagen 1A1, in NHLFs revealed a robust and statistically significant response to TGFβ, which was almost completely abrogated in the presence of SB-525334, a TGFβ type I receptor (ALK5) inhibitor (**Figure 2A**).

To test the effect of DREADD activation on fibrosis markers in those cells, we infected the NHLFs with DREADDs-expressing BacMAM viruses and treated NHLFs with various concentration of CNO in the presence of TGFβ. After treatment of 24hr, the cells were collected for mRNA extraction and RT-qPCR

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

analysis. TGFβ induced transcription of αSMA, PAI-1, and Collagen 1A1 (Col1A1) was suppressed by activation of Gq and Gs DREADDs . CNO had no effect on these markers in NHLFs infected with Gi DREADD. A clear dose dependent CNO effect was observed for all markers in Gs-DREADD expressing NHLFs; while for Gq-DREADD expressing cells, a clear dose dependent effect was only observed for αSMA, only a minimal effect on Col1A1, and a saturated significant effect on PAI-1 (**Figure 2B**).

RNA-seq analysis identifies dysregulated GPCRs in NHLFs upon TGFβ treatment.

Notably, many GPCRs function through Gq and Gs signaling, such as 5HT2 receptors and prostaglandin receptors (Fribourg et al., 2011). To identify endogenous GPCRs that modulate TGFβ signaling, we resorted to our previous genome-wide transcriptome analysis of human primary cells treated with TGFβ to uncover differentially-expressed GPCRs under TGFβ stimulation (manuscript accepted at Cell Reports Medicine, NCBI GEO accession number GSE152250) (Zhang et al., 2020). Upon TGFβ treatment, human primary cardiac fibroblasts, NHLFs, hepatic stellate cells (HSC), and renal proximal tubular epithelial cells (RPTEC) were collected for RNA-seq analysis. Among the four cell types, the expression of many non-sensory GPCR genes was significantly changed upon TGFβ treatment when compared to the vehicle group (**Figure 3A**). To delineate this further, a volcano plot visualization of GPCR expression fold change in NHLFs is shown in **Figure 3B**. We highlighted 13 GPCRs that signal through Gq or Gs pathways and have known biological functions relevant to fibrosis and commercially-available agonists (**Table 1**). Among the selected GPCRs, S1PR3, HTR2B, BDKRB1, BDKRB2, TBXA2R, PTGER2, and GPER1 were significantly decreased upon TGFβ treatment in lung fibroblasts, whereas DRD1, PAR2 (F2RL1), and PTGER4 were significantly up-regulated (**Figure 3B**). Furthermore, we examined the expression of this set of GPCRs in UUO (unilateral ureter obstruction) kidney, CCl4 (carbon tetrachloride) liver, and BDL (bile duct ligation) liver fibrosis models. As shown in the heatmap of **Figure 3C**, the expression of many

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

GPCRs were reduced in primary cells treated with TGFβ, but were augmented in fibrotic tissues, suggesting additional layers of complexity for the regulation of GPCR.

Reversal of fibrosis by prostaglandin receptors in NHLFs.

We have found a subset of Gq or Gs-coupled GPCRs whose expression was differentially regulated in lung fibroblasts treated with TGFβ. We next sought to examine whether activation of those endogenous GPCRs would alter TGFβ signaling. To this end, we obtained 14 potent and selective agonists for those GPCRs with reported EC50 at low nM range (**Table 1**). NHLFs were first treated with CNO at various concentration. Calcium signal and cAMP production were determined to ensure selective G protein pathway activation. As shown in **Figure 4A**, dose-dependent calcium release was detected in cells treated with Bradykinin and Oxytocin at a statistically significant level. These results suggested that BDKRB1/2 and OXTR receptor are the major Gq-coupled endogenous receptors in NHLFs. Moreover, cAMP production was substantially elevated in cells treated with A68930 (DRD1 agonist), Prostaglandin E2 (EP agonist), and TCS2510 (EP4 agonist), consistent with their role in Gs-mediated signaling (**Figure 4B**). U46619 treatment induced a dose-dependent increase of cAMP level, albeit with lower overall signals, indicating an alternative signaling pathway than the previously reported Gq signaling. WAY267464 (OXTR agonist), AC264613 (PAR2 agonist), CYM5541 (S1PR3 agonist), and BW723C86 (5-HT2B/C agonist) treatment led to a slight reduction of cAMP, indicative of Gi activation. Those observations were inconsistent with their reported Gq pathway.

Subsequently, we measured the expression of various fibrosis marker genes in those cells treated with 100nM or 1uM of compounds in the presence of TGFβ (**Figure 4C**). As expected, TGFβ substantially induced αSMA gene expression (top panel, orange bar). The addition of Bradykinin, A68930 (DRD1 agonist), U46619 (TBXA2R agonist), and Oxytocin were not able to suppress αSMA expression. However, treating cells with Prostaglandin/PGE2 and TCS2510 (EP4 agonist) dramatically reduced αSMA level at

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

both concentrations. Endothelin-3 treatment inhibited αSMA expression at the lower concentration (100nM) but not the higher concentration (1uM). The impact of agonist treatment on TGFβ-induced Col1A1 gene expression were also examined. PGE2, TCS2510 (EP4 agonist), and Oxytocin led to a dose-dependent inhibition of Col1a1 expression (**Figure 4C**, middle panel). Among which, PGE2-mediated inhibition of Col1A1 is more substantial. PAI-1 is another important TGFβ target gene. RT-qPCR analysis revealed that PGE2 and Oxytocin potently suppressed PAI-1 induction upon TGFβ treatment (**Figure 4C**, bottom panel). Overall, our results found the prostaglandin agonist activates Gs signaling and has the strongest anti-fibrotic effect in NHLFs.

Discussion

In the present study, we utilized DREADD chemogenetic tools to probe GPCR signaling pathway(s) essential for fibrotic response in fibroblasts. We discovered that activation of either Gq or Gs pathway inhibits TGFβ-induced fibrosis marker gene expression. Stimulation of the Gi DREADD did not affect transcription of these marker genes. However, the current experiments cannot completely rule out the possibility that low level of hM4D expression is the reason we could not see a response. The reduction of basal levels of cAMP we measured, although statistically significant, was small and may suggest low level of expression.

To identify endogenous Gq/Gs-coupled GPCR in fibroblasts, we performed datamining of our fibrosis profiling dataset, in which whole-genome transcriptome analyses were carried out in human primary cardiac fibroblasts, NHLFs, hepatic stellate cells, and renal proximal tubular epithelial cells treated with TGFβ, as well as UUO kidney, CCl4 liver, and BDL liver fibrosis models (Zhang et al., 2020). Among the highly differentially-expressed GPCRs, we selected 13 receptors that were signaling through Gq or Gs and have known agonist available. We further verified the expression and activation of those GPCRs by agonist stimulation in the fibroblasts. Among the 13 GPCRs, activation of prostaglandin receptors using

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

the pan-agonist prostaglandin, generated the strongest anti-fibrotic effect. Activation of only one of the prostaglandin receptors (EP4) using TCS2510 resulted in a reduced antifibrotic effect, suggesting the requirement of multiple prostaglandin receptors for optimal anti-fibrotic effect.

Although we refer to the signaling pathways of the DREADDs based on the Gα subunit they interact with, the signaling pathways activated by them are likely more complicated and include components of βγ signaling, arrestin signaling and potentially other down-stream molecules that may affect transcription factors in a cumulative manner. The definition of receptors as coupled to Gq or Gs does not necessarily mean that this is the only Gα protein they interact with, but it is the most common one. In many cases these definitions relay on the cellular effects seen by activation of a specific receptor based on cAMP or Ca^{2+} response. The endogenous receptors may have different effects on some of the signaling pathways due to different coupling preference or alternative signaling pathways that were not monitored in the current experiment.

Signal intensity in cellular system is another factor that needs to be considered when discussing downstream physiological effects. It is important to understand that in an artificial system over-expressing a GPCR, the signal intensity is often higher than what is generated by activation of GPCRs naturally-expressed in those cells, due to stochiometric considerations of the signal producing process. Both the intensity and subcellular location of the signal may play a role in the type and amount of physiological response. Even when considering the limitation of the DREADD system, our results have demonstrated that it is a valuable tool to investigate beneficial GPCR signaling for fibrosis and other physiological phenomena.

Many studies have investigated the molecular mechanisms underlying fibrosis and a wide range of targets have been tested for drug discovery and clinical development (Wynn and Ramalingam, 2012; Friedman et al., 2013; Duffield, 2014; Nanthakumar et al., 2015). Regardless of disease etiology, fibrosis in the heart, lung, kidney, liver, and skin share many similar features, such as increases in myofibroblast

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

activation, collagen production and inflammation, suggesting the existence of common core pathways/mechanisms among all fibrotic tissues. Our study focused on probing common GPCR signaling in the fibroblasts and identified Gs signaling as an important pathway for fibrosis, consistent with previously reported modulation of TGFβ signaling by cAMP (Schiller et al., 2010). DREADDs are synthetic GPCRs activated by synthetic small molecules that exhibit no biological activity at native receptors, so that it can be engineered to transduce a specific signaling in response to otherwise inert ligands *in vitro* and *in vivo*. Identification of GPCR signaling pathways that have desired outcomes in a given phenotypic assay along with transcriptional analysis of the disease state may greatly facilitate target identification and enable more focused drug discovery efforts.

About 34% of the FDA approved drugs are targeting against GPCRs (Hauser et al., 2017). Fibrosis is associated with end-stage organ damage and worsening disease progression in NASH, ESRD, heart failure, and IPF patients. There is a huge unmet medical need to develop new fibrosis therapies which bring clinically meaningful efficacy to patients. The interest in discovering new GPCR target for fibrosis is conceivable. Our work highlighted the importance of prostaglandin signaling in the fibroblasts. PGE2 is a locally-active eicosanoid that modulates vascular tone, coagulation homeostasis, and inflammation. PGE2 binds to and activates family of GPCR receptors, EP1 (E-prostanoid receptors 1), EP2, EP3, and EP4, in an autocrine or paracrine manner (Elwakeel et al., 2019). It has been shown that lung fibroblasts isolated from IPF patients have diminished expression of PGE2 (Wilborn et al., 1995). PGE2 inhibits fibroblast proliferation and myofibroblast activation via EP2 and PKA-dependent, Smad-indepenedent pathway (Lama et al., 2002; Kolodsick et al., 2003; Thomas et al., 2007; Wettlaufer et al., 2016). Additionally, PGE2 has been found working through serum-response factor (SRF)- MRTF-A (myocardin-related transcription factor-A) to regulate αSMA expression (Penke et al., 2014). EP2 agonist butaprost attenuates fibrosis in UUO kidneys and human kidney slices (Jensen et al., 2019). Recent high-throughput myofibroblast phenotypic screen assay also finds EP2 and EP4 agonists beneficial for the

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

treatment of lung fibrosis (Sieber et al., 2018). The role of PGE2 in inflammation is complexed, pro-inflammatory in the early stages of inflammation but promoting the resolution of inflammation at the later time point (Calder, 2009). The pleiotropic effect of PGE2 limits its applications in broader patient population and chronic usage. Side effects of PGE2 systemic administration include headache, uterine contraction, gastrointestinal disturbance, and diarrhea. As such, a substantial amount of effort has been put into developing subtype-selective prostanoid receptor agonists to obtain better risk/benefit profile (Flesch et al., 2013). EP4 agonist ONO-4819CD has been tested in a phase 2 ulcerative colitis trial, but failed to achieve statistically significant therapeutic effect (Nakase et al., 2010). To date, no EP2, EP4, or EP2/4 dual agonist (antagonist) has been approved for clinical use. Developing locally-delivered PGE2 modulator, such as via inhalation and topical administration, may represent a viable approach for the treatment of fibrosis.

Our work has demonstrated that delineating beneficial GPCR signaling pathway for fibrosis is valuable for new target identification. In our proof-of-concept study, we focused on in vitro cell-based system. DREADDs chemogenetic tools have been previously used to manipulate neuronal activity and behavior in animals (Vardy et al., 2015). As such, it is conceivable that a similar approach can be used to probe GPCR signaling in multiple fibrosis animal models. Combining with transcriptome analysis of GPCR family, it is possible to identify novel GPCR target(s) for fibrosis.

Acknowledgements

The authors thank Jennifer Cho for help with the RNA-seq, Gayathri Swaminath and Christopher Novotny for their critical reviews of the manuscript.

Author contributions

Participated in research design: Zhang, Vardy, Pinto, and Peier

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

Conducted experiments: Zhang, Vardy, and Vadlamudi

Contributed new reagents or analytic tools: Visconti

Performed data analysis: Muise and Wang

Wrote or contributed to the writing of the manuscript: Zhang, Vardy, Muise, Wang, and Visconti

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

Reference

Akhurst RJ and Hata A (2012) Targeting the TGFbeta signalling pathway in disease. *Nat Rev Drug Discov* **11**:790-811.

Ames RS, Kost TA and Condreay JP (2007) BacMam technology and its application to drug discovery. *Expert Opin Drug Discov* **2**:1669-1681.

Armbruster BN, Li X, Pausch MH, Herlitze S and Roth BL (2007) Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proc Natl Acad Sci U S A* **104**:5163-5168.

Armstrong MJ, Gaunt P, Aithal GP, Barton D, Hull D, Parker R, Hazlehurst JM, Guo K, team Lt, Abouda G, Aldersley MA, Stocken D, Gough SC, Tomlinson JW, Brown RM, Hubscher SG and Newsome PN (2016) Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. *Lancet* **387**:679-690.

Billot X, Chateauneuf A, Chauret N, Denis D, Greig G, Mathieu MC, Metters KM, Slipetz DM and Young RN (2003) Discovery of a potent and selective agonist of the prostaglandin EP4 receptor. *Bioorg Med Chem Lett* **13**:1129-1132.

Bologa CG, Revankar CM, Young SM, Edwards BS, Arterburn JB, Kiselyov AS, Parker MA, Tkachenko SE, Savchuck NP, Sklar LA, Oprea TI and Prossnitz ER (2006) Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol* **2**:207-212.

Bozyk PD and Moore BB (2011) Prostaglandin E2 and the pathogenesis of pulmonary fibrosis. *Am J Respir Cell Mol Biol* **45**:445-452.

Calder PC (2009) Polyunsaturated fatty acids and inflammatory processes: New twists in an old tale. *Biochimie* **91**:791-795.

Coleman RA, Humphrey PP, Kennedy I, Levy GP and Lumley P (1981) Comparison of the actions of U-46619, a prostaglandin H2-analogue, with those of prostaglandin H2 and thromboxane A2 on some isolated smooth muscle preparations. *Br J Pharmacol* **73**:773-778.

DeNinno MP, Schoenleber R, MacKenzie R, Britton DR, Asin KE, Briggs C, Trugman JM, Ackerman M, Artman L, Bednarz L and et al. (1991) A68930: a potent agonist selective for the dopamine D1 receptor. *Eur J Pharmacol* **199**:209-219.

Deschamps AM and Murphy E (2009) Activation of a novel estrogen receptor, GPER, is cardioprotective in male and female rats. *Am J Physiol Heart Circ Physiol* **297**:H1806-1813.

Duffield JS (2014) Cellular and molecular mechanisms in kidney fibrosis. *J Clin Invest* **124**:2299-2306.

Elwakeel E, Brune B and Weigert A (2019) PGE2 in fibrosis and cancer: Insights into fibroblast activation. *Prostaglandins Other Lipid Mediat* **143**:106339.

Flesch D, Merk D, Lamers C and Schubert-Zsilavecz M (2013) Novel prostaglandin receptor modulators--part II: EP receptor modulators; a patent review (2002 - 2012). *Expert Opin Ther Pat* **23**:233-267.

Fribourg M, Moreno JL, Holloway T, Provasi D, Baki L, Mahajan R, Park G, Adney SK, Hatcher C, Eltit JM, Ruta JD, Albizu L, Li Z, Umali A, Shim J, Fabiato A, MacKerell AD, Jr., Brezina V, Sealfon SC, Filizola M, Gonzalez-Maeso J and Logothetis DE (2011) Decoding the signaling of a GPCR heteromeric complex reveals a unifying mechanism of action of antipsychotic drugs. *Cell* **147**:1011-1023.

Friedman SL, Ratziu V, Harrison SA, Abdelmalek MF, Aithal GP, Caballeria J, Francque S, Farrell G, Kowdley KV, Craxi A, Simon K, Fischer L, Melchor-Khan L, Vest J, Wiens BL, Vig P, Seyedkazemi S, Goodman Z, Wong VW, Loomba R, Tacke F, Sanyal A and Lefebvre E (2018) A randomized, placebo-controlled trial of cenicriviroc for treatment of nonalcoholic steatohepatitis with fibrosis. *Hepatology* **67**:1754-1767.

Friedman SL, Sheppard D, Duffield JS and Violette S (2013) Therapy for fibrotic diseases: nearing the starting line. *Sci Transl Med* **5**:167sr161.

Gardell LR, Ma JN, Seitzberg JG, Knapp AE, Schiffer HH, Tabatabaei A, Davis CN, Owens M, Clemons B, Wong KK, Lund B, Nash NR, Gao Y, Lameh J, Schmelzer K, Olsson R and Burstein ES (2008) Identification and characterization of novel small-molecule protease-activated receptor 2 agonists. *J Pharmacol Exp Ther* **327**:799-808.

Gilman AG (1987) G proteins: transducers of receptor-generated signals. *Annu Rev Biochem* **56**:615-649.

Gruber CW, Koehbach J and Muttenthaler M (2012) Exploring bioactive peptides from natural sources for oxytocin and vasopressin drug discovery. *Future Med Chem* **4**:1791-1798.

Guettier JM, Gautam D, Scarselli M, Ruiz de Azua I, Li JH, Rosemond E, Ma X, Gonzalez FJ, Armbruster BN, Lu H, Roth BL and Wess J (2009) A chemical-genetic approach to study G protein regulation of beta cell function in vivo. *Proc Natl Acad Sci U S A* **106**:19197-19202.

Harrison SA, Rinella ME, Abdelmalek MF, Trotter JF, Paredes AH, Arnold HL, Kugelmas M, Bashir MR, Jaros MJ, Ling L, Rossi SJ, DePaoli AM and Loomba R (2018) NGM282 for treatment of non-alcoholic steatohepatitis: a multicentre, randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet* **391**:1174-1185.

Hauser AS, Attwood MM, Rask-Andersen M, Schioth HB and Gloriam DE (2017) Trends in GPCR drug discovery: new agents, targets and indications. *Nat Rev Drug Discov* **16**:829-842.

Jensen MS, Mutsaers HAM, Tingskov SJ, Christensen M, Madsen MG, Olinga P, Kwon TH and Norregaard R (2019) Activation of the prostaglandin E2 EP2 receptor attenuates renal fibrosis in unilateral ureteral obstructed mice and human kidney slices. *Acta Physiol (Oxf)*:e13291.

Jo E, Bhhatarai B, Repetto E, Guerrero M, Riley S, Brown SJ, Kohno Y, Roberts E, Schurer SC and Rosen H (2012) Novel selective allosteric and bitopic ligands for the S1P(3) receptor. *ACS Chem Biol* **7**:1975-1983.

Kang L, McIntyre KW, Gillooly KM, Yang Y, Haycock J, Roberts S, Khanna A, Herpin TF, Yu G, Wu X, Morton GC, Tuerdi H, Koplowitz B, Walker SG, Wardwell-Swanson J, Macor JE, Lawrence RM and

Carlson KE (2006) A selective small molecule agonist of the melanocortin-1 receptor inhibits lipopolysaccharide-induced cytokine accumulation and leukocyte infiltration in mice. *J Leukoc Biol* **80**:897-904.

Kolodsick JE, Peters-Golden M, Larios J, Toews GB, Thannickal VJ and Moore BB (2003) Prostaglandin E2 inhibits fibroblast to myofibroblast transition via E. prostanoid receptor 2 signaling and cyclic adenosine monophosphate elevation. *Am J Respir Cell Mol Biol* **29**:537-544.

Lama V, Moore BB, Christensen P, Toews GB and Peters-Golden M (2002) Prostaglandin E2 synthesis and suppression of fibroblast proliferation by alveolar epithelial cells is cyclooxygenase-2-dependent. *Am J Respir Cell Mol Biol* **27**:752-758.

Mallat A, Teixeira-Clerc F and Lotersztajn S (2013) Cannabinoid signaling and liver therapeutics. *J Hepatol* **59**:891-896.

Markovic T, Jakopin Z, Dolenc MS and Mlinaric-Rascan I (2017) Structural features of subtype-selective EP receptor modulators. *Drug Discov Today* **22**:57-71.

Nakase H, Fujiyama Y, Oshitani N, Oga T, Nonomura K, Matsuoka T, Esaki Y, Murayama T, Teramukai S, Chiba T and Narumiya S (2010) Effect of EP4 agonist (ONO-4819CD) for patients with mild to moderate ulcerative colitis refractory to 5-aminosalicylates: a randomized phase II, placebo-controlled trial. *Inflamm Bowel Dis* **16**:731-733.

Nanthakumar CB, Hatley RJ, Lemma S, Gauldie J, Marshall RP and Macdonald SJ (2015) Dissecting fibrosis: therapeutic insights from the small-molecule toolbox. *Nat Rev Drug Discov* **14**:693-720.

Nawaratne V, Leach K, Suratman N, Loiacono RE, Felder CC, Armbruster BN, Roth BL, Sexton PM and Christopoulos A (2008) New insights into the function of M4 muscarinic acetylcholine receptors gained using a novel allosteric modulator and a DREADD (designer receptor exclusively activated by a designer drug). *Mol Pharmacol* **74**:1119-1131.

Neuschwander-Tetri BA, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, Chalasani N, Dasarathy S, Diehl AM, Hameed B, Kowdley KV, McCullough A, Terrault N, Clark JM, Tonascia J, Brunt EM, Kleiner DE, Doo E and Network NCR (2015) Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. *Lancet* **385**:956-965.

Palmer SM, Snyder L, Todd JL, Soule B, Christian R, Anstrom K, Luo Y, Gagnon R and Rosen G (2018) Randomized, Double-Blind, Placebo-Controlled, Phase 2 Trial of BMS-986020, a Lysophosphatidic Acid Receptor Antagonist for the Treatment of Idiopathic Pulmonary Fibrosis. *Chest* **154**:1061-1069.

Patsenker E and Stickel F (2016) Cannabinoids in liver diseases. *Clin Liver Dis (Hoboken)* **7**:21-25.

Penke LR, Huang SK, White ES and Peters-Golden M (2014) Prostaglandin E2 inhibits alpha-smooth muscle actin transcription during myofibroblast differentiation via distinct mechanisms of modulation of serum response factor and myocardin-related transcription factor-A. *J Biol Chem* **289**:17151-17162.

Perez-Garcia GS and Meneses A (2005) Effects of the potential 5-HT7 receptor agonist AS 19 in an autoshaping learning task. *Behav Brain Res* **163**:136-140.

Porter RH, Benwell KR, Lamb H, Malcolm CS, Allen NH, Revell DF, Adams DR and Sheardown MJ (1999) Functional characterization of agonists at recombinant human 5-HT2A, 5-HT2B and 5-HT2C receptors in CHO-K1 cells. *Br J Pharmacol* **128**:13-20.

Premont RT and Gainetdinov RR (2007) Physiological roles of G protein-coupled receptor kinases and arrestins. *Annu Rev Physiol* **69**:511-534.

Ratziu V, Harrison SA, Francque S, Bedossa P, Lehert P, Serfaty L, Romero-Gomez M, Boursier J, Abdelmalek M, Caldwell S, Drenth J, Anstee QM, Hum D, Hanf R, Roudot A, Megnien S, Staels B, Sanyal A and Group G-IS (2016) Elafibranor, an Agonist of the Peroxisome Proliferator-Activated

Receptor-alpha and -delta, Induces Resolution of Nonalcoholic Steatohepatitis Without Fibrosis Worsening. *Gastroenterology* **150**:1147-1159 e1145.

Revankar CM, Cimino DF, Sklar LA, Arterburn JB and Prossnitz ER (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* **307**:1625-1630.

Ring RH, Schechter LE, Leonard SK, Dwyer JM, Platt BJ, Graf R, Grauer S, Pulicicchio C, Resnick L, Rahman Z, Sukoff Rizzo SJ, Luo B, Beyer CE, Logue SF, Marquis KL, Hughes ZA and Rosenzweig-Lipson S (2010) Receptor and behavioral pharmacology of WAY-267464, a non-peptide oxytocin receptor agonist. *Neuropharmacology* **58**:69-77.

Sam AH, Salem V and Ghatei MA (2011) Rimonabant: From RIO to Ban. *J Obes* **2011**:432607.

Schanstra JP, Neau E, Drogoz P, Arevalo Gomez MA, Lopez Novoa JM, Calise D, Pecher C, Bader M, Girolami JP and Bascands JL (2002) In vivo bradykinin B2 receptor activation reduces renal fibrosis. *J Clin Invest* **110**:371-379.

Schiller M, Dennler S, Anderegg U, Kokot A, Simon JC, Luger TA, Mauviel A and Bohm M (2010) Increased cAMP levels modulate transforming growth factor-beta/Smad-induced expression of extracellular matrix components and other key fibroblast effector functions. *J Biol Chem* **285**:409-421.

Schlessinger K, Li W, Tan Y, Liu F, Souza SC, Tozzo E, Liu K, Thompson JR, Wang L and Muise ES Gene expression in WAT from healthy humans and monkeys correlates with FGF21-induced browning of WAT in mice. *Obesity (Silver Spring, Md* **23**:1818-1829.

Sieber P, Schafer A, Lieberherr R, Le Goff F, Stritt M, Welford RWD, Gatfield J, Peter O, Nayler O and Luthi U (2018) Novel high-throughput myofibroblast assays identify agonists with therapeutic potential in pulmonary fibrosis that act via EP2 and EP4 receptors. *PLoS One* **13**:e0207872.

Sumner MJ, Cannon TR, Mundin JW, White DG and Watts IS (1992) Endothelin ETA and ETB receptors mediate vascular smooth muscle contraction. *Br J Pharmacol* **107**:858-860.

Tager AM, LaCamera P, Shea BS, Campanella GS, Selman M, Zhao Z, Polosukhin V, Wain J, Karimi-Shah BA, Kim ND, Hart WK, Pardo A, Blackwell TS, Xu Y, Chun J and Luster AD (2008) The lysophosphatidic acid receptor LPA1 links pulmonary fibrosis to lung injury by mediating fibroblast recruitment and vascular leak. *Nat Med* **14**:45-54.

Thomas PE, Peters-Golden M, White ES, Thannickal VJ and Moore BB (2007) PGE(2) inhibition of TGF-beta1-induced myofibroblast differentiation is Smad-independent but involves cell shape and adhesion-dependent signaling. *Am J Physiol Lung Cell Mol Physiol* **293**:L417-428.

Tintner R, Manian P, Gauthier P and Jankovic J (2005) Pleuropulmonary fibrosis after long-term treatment with the dopamine agonist pergolide for Parkinson Disease. *Arch Neurol* **62**:1290-1295.

Urban DJ and Roth BL (2015) DREADDs (designer receptors exclusively activated by designer drugs): chemogenetic tools with therapeutic utility. *Annu Rev Pharmacol Toxicol* **55**:399-417.

Vardy E, Robinson JE, Li C, Olsen RHJ, DiBerto JF, Giguere PM, Sassano FM, Huang XP, Zhu H, Urban DJ, White KL, Rittiner JE, Crowley NA, Pleil KE, Mazzone CM, Mosier PD, Song J, Kash TL, Malanga CJ, Krashes MJ and Roth BL (2015) A New DREADD Facilitates the Multiplexed Chemogenetic Interrogation of Behavior. *Neuron* **86**:936-946.

Weis WI and Kobilka BK (2018) The Molecular Basis of G Protein-Coupled Receptor Activation. *Annu Rev Biochem* **87**:897-919.

Wettlaufer SH, Scott JP, McEachin RC, Peters-Golden M and Huang SK (2016) Reversal of the Transcriptome by Prostaglandin E2 during Myofibroblast Dedifferentiation. *Am J Respir Cell Mol Biol* **54**:114-127.

Wiernas TK, Griffin BW and Sharif NA (1997) The expression of functionally-coupled B2-bradykinin receptors in human corneal epithelial cells and their pharmacological characterization with agonists and antagonists. *Br J Pharmacol* **121**:649-656.

Wilborn J, Crofford LJ, Burdick MD, Kunkel SL, Strieter RM and Peters-Golden M (1995) Cultured lung fibroblasts isolated from patients with idiopathic pulmonary fibrosis have a diminished capacity to synthesize prostaglandin E2 and to express cyclooxygenase-2. *J Clin Invest* **95**:1861-1868.

Wynn TA and Ramalingam TR (2012) Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med* **18**:1028-1040.

Zhang J, Muise ES, Han S, Kutchukian PS, Costet P, Zhu Y, Kan Y, Zhou H, Shah V, Huang H, Saigal A, Akiyama TE, Shen X-L, Cai T-Q, Shah K, Carballo-Jane E, Zycband E, Yi L, Imbriglio J, Smith E, Devito K, Conway J, Ma L-J, Hoek M, Sebhat IK, Peier AM, Talukdar S, McLaren DG, Previs SF, Jensen K and Pinto S (2020) Molecular Profiling Reveals A Common Metabolic Signature of Tissue Fibrosis. *Cell Reports Medicine*.

Footnotes

This work did not receive any outside funding support.

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

Figure legends

Figure 1. Expression of DREADDs in normal human lung fibroblasts (NHLFs). A) Schematic representation of DREADD-expressing BacMam plasmids. Different DREADDs were cloned into the BacMam vector followed by IRES-mCitrine to enable quick assessment of infectivity. B) Expression of DREADDs-containing BacMam viruses in NHLFs. mCitrine-positive cells are shown after 24hour of infection. Representative images from 3 independent experiments were obtained. C) The overexpressed DREADDs were functional in NHLFs upon CNO induction, as shown by calcium release and cAMP signaling assays. N=3, Mean ± SD.

Figure 2. Activation of Gq and Gs signaling inhibits TGFβ-induced fibrosis marker genes in lung fibroblasts. A) αSMA, Col1A1, and PAI-1 genes were marked up-regulated after TGFβ treatment (5ng/ml for 24hr) in NHLFs, which was almost completely reversed by the co-administration of ALK5 inhibitor, SB-525334 (10uM). N=3, Mean ± SD. *p < 0.05, **p<0.005, ***p < 0.001, compared to no CNO treatment, One-way ANOVA post hoc Tukey test. 3 independent replicates were obtained. B) Activation of Gq and to a lesser extent Gs by CNO inhibit αSMA, Col1A1, and PAI-1 expression in NHLFs. N=3, Mean ± SD. *p < 0.05, **p < 0.005, **p<0.001, compared to no CNO treatment, One-way ANOVA post hoc Tukey test. 3 independent replicates were obtained.

Figure 3. RNA-seq analysis identifies dysregulated GPCRs in NHLFs upon TGFβ treatment. A) Expression profile of non-sensory GPCRs in human primary cells treated with vehicle or with TGFβ (5ng/ml). The color gradient represents fold change compared to vehicle-treated cells (-3.0 to 3.0-fold). NHLF, normal human lung fibroblasts; HSC, human primary hepatic stellate cells; RPTEC, renal proximal tubular epithelial cells. N=3 for each time point or treatment group. B) Volcano plot of GPCR expression in

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

NHLFs. C) Gene expression ratios of selected GPCRs in human primary cells and fibrotic tissues. From left: NHLFs, cardiac fibroblasts, hepatic stellate cells (HSC), RPTEC, UUO kidney, CCl4 liver, and BDL liver. Heatmap was generated by using Morpheus software (https://software.broadinstitute.org/morpheus/). The color gradient represents fold change compared treatment (TGFβ 24hr, surgery, or CCl4 treatment) to control (-10 to 10-fold). Grey, below detection limit.

Figure 4. Reversal of fibrosis by prostaglandin receptors in NHLFs. A) Dose-titration of various agonists to identify functional GPCRs for Gq activation in NHLFs. N=3, Mean ± SD. *p < 0.05, **p<0.005, ***p < 0.001, compared to no compound treatment group, Two-way ANOVA post hoc Tukey test. 3 independent replicates were obtained. B) Dose-titration of various agonists to identify functional GPCRs for Gs or Gi activation in NHLFs. N=3, Mean ± SD. *p < 0.05, **p<0.005, ***p < 0.001, compared to no compound treatment group, Two-way ANOVA post hoc Tukey test. 3 independent replicates were obtained. C) Inhibition of TGFβ-induced fibrosis marker genes by prostaglandin receptor agonists in NHLFs. N=3, Mean ± SD. *p < 0.05, **p<0.005, ***p < 0.001, compared to TGFβ but no compound treated-group (orange bar), Two-way ANOVA post hoc Tukey test. 3 independent replicates were obtained.

Table 1. Selected GPCRs that function through Gq and/or Gs pathways. Their agonists were reported with EC_{50} value.

GPCR	Signaling	Functions	Agonists	EC50 or Kd
GPER1 (G protein-coupled estrogen receptor 1, Gpr30)	Gq	activated by estradiol and functions via Gq signaling (Revankar et al., 2005). A selective agonist G1 is beneficial in myocardial ischemia reperfusion injury model (Bologa et al., 2006; Deschamps and Murphy, 2009)	G1	2nM (Bologa et al., 2006)
BDKRB1 (Bradykinin receptor B1)	Gq	BDKRB1 is a Gq-coupled GPCR, while BDKRB2 signals through Gq and Gi. In vivo activation of BDKRB2 attenuated kidney fibrosis (Schanstra et al., 2002)	Bradykinin	3.26nM (Wiernas et al., 1997)
DRD1 (Dopamine receptor D1)	Gs	pleuropulmonary fibrosis was linked to the long-term use of dopamine agonist Pergolide in Parkinson patients (Tintner et al., 2005)	A68930	2.5nM (DeNinno et al., 1991)
PTGER2 (Prostaglandin E2 receptor 2)	Gq	PGE2 is a potent bioactive eicosanoid that strongly inhibits fibroblast proliferation, migration, and collagen secretion (Bozyk and Moore, 2011)	PGE2	PTGER1, 25nM; PTGER2, 13nM; PTGER3, 3nM; PTGER4, 3nM (Markovic et al., 2017)
PTGER4 (Prostaglandin E2 receptor 4)	Gs	TCS2510 is a highly selective EP4 agonist	TCS2510	2.5nM (Billot et al., 2003)
TBXA2R (Thromboxane A2 receptor)	Gq/Gs/$G_{12/13}$	U46619 is a potent and stable TBXA2R agonist	U46619	35nM (Coleman et al., 1981)
OXTR (Oxytocin receptor)	Gq	Oxytocin is a peptide hormone and neurotransmitter signaling through OXTR and Gq pathways (Gimpl and Fahrenholz, 2001). WAY-267464 is a potent, selective, non-peptide OXTR agonist.	Oxytocin WAY267464	9.0-10.8nM (Gruber et al., 2012) 9.0nM (Ring et al., 2010)
EDNRB (Endothelin receptor B)	Gq	Endothelin 3 is a vasoconstrictor that preferentially binds to EDNRB	Endothelin 3	1nM (Sumner et al., 1992)
F2RL1 (Protease-activated receptor 2, PAR2)	Gq/Gi	PAR2 is implicated in lung, kidney, liver, and cardiac fibrosis (Palygin et al., 2016; Shearer et al., 2016; Sun et al., 2017; Friebel et al., 2019).	AC264613	~31nM (Gardell et al., 2008)
S1PR3 (Sphingosine-1-phosphate receptor 3)	Gq	S1PR3 KO mice has attenuated inflammation and fibrosis upon bleomycin-induced lung injury (Murakami et al., 2014).	CYM5541	72~132nM (Jo et al., 2012)

HTR2B (5HT2B receptor)	Gq	Serotonin fibrosis in human and treatment of Fenfluramine, a serotonin releasing agents, causes cardiac valve fibrosis in obese patients (Roth, 2007). 5-HT2B activation mediates valvular fibroblasts activation.	BW723C86	5-HT2B, 1.1nM; 5-HT2C, 931.3nM (Porter et al., 1999)
HTR7 (5HT7 receptor)	Gs	AS-19 is a potent 5-HT7 receptor agonist	AS19	0.853nM (Perez-Garcia and Meneses, 2005)
MC1R (Melanocortin receptor 1)	Gs	MC1R is anti-inflammatory and has been implicated in several inflammatory diseases.	BMS470539	16.8nM (Kang et al., 2006)

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

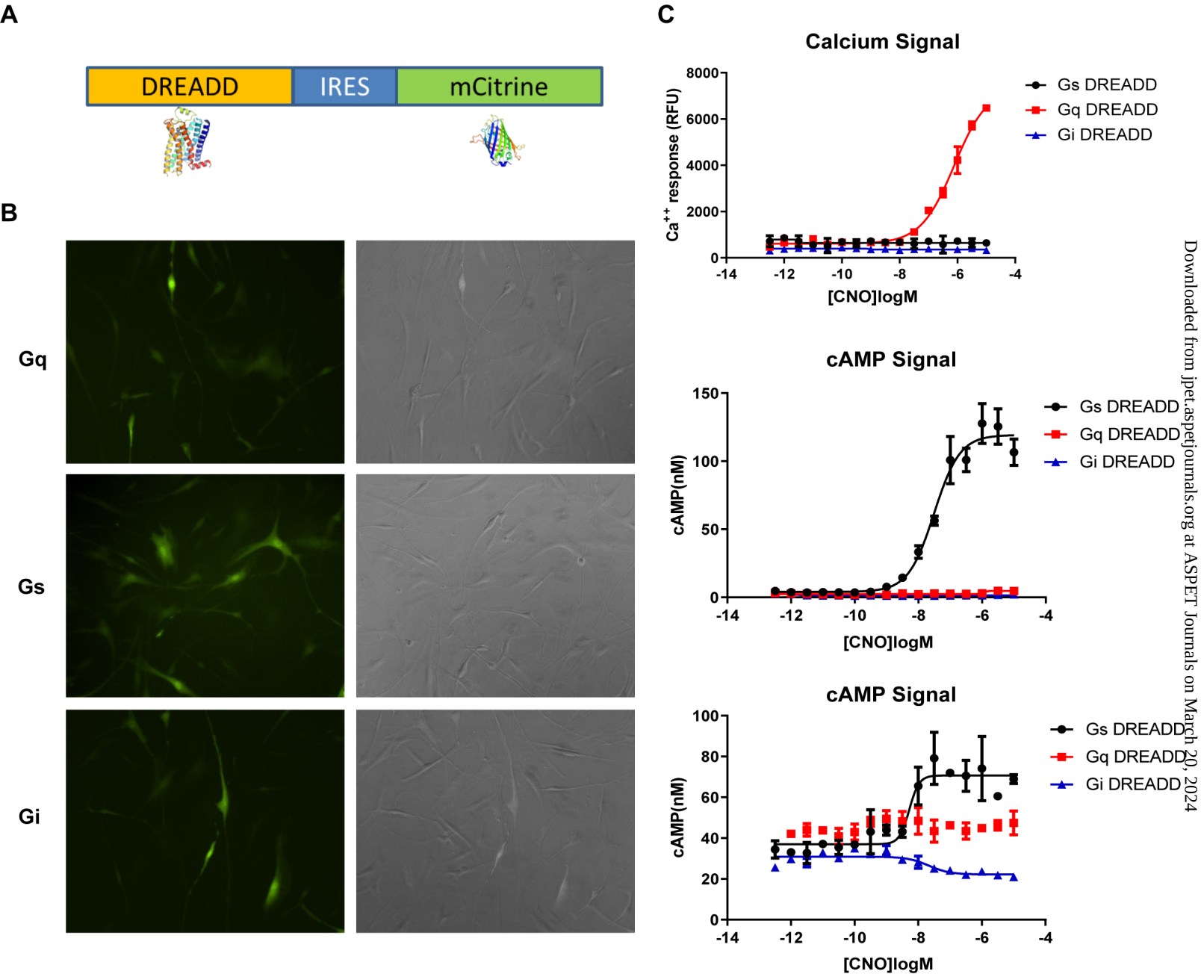


Figure 1

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

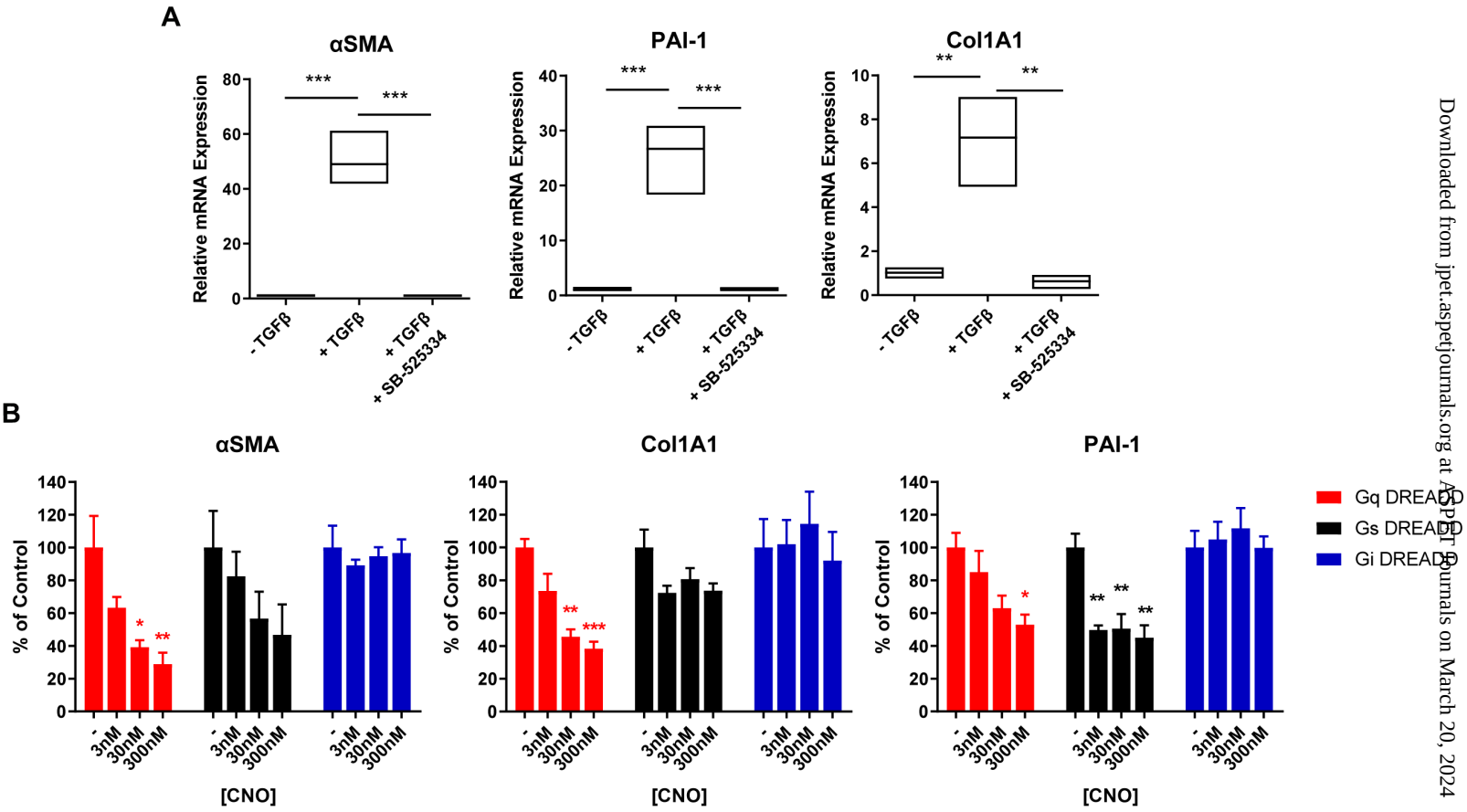


Figure 2

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.

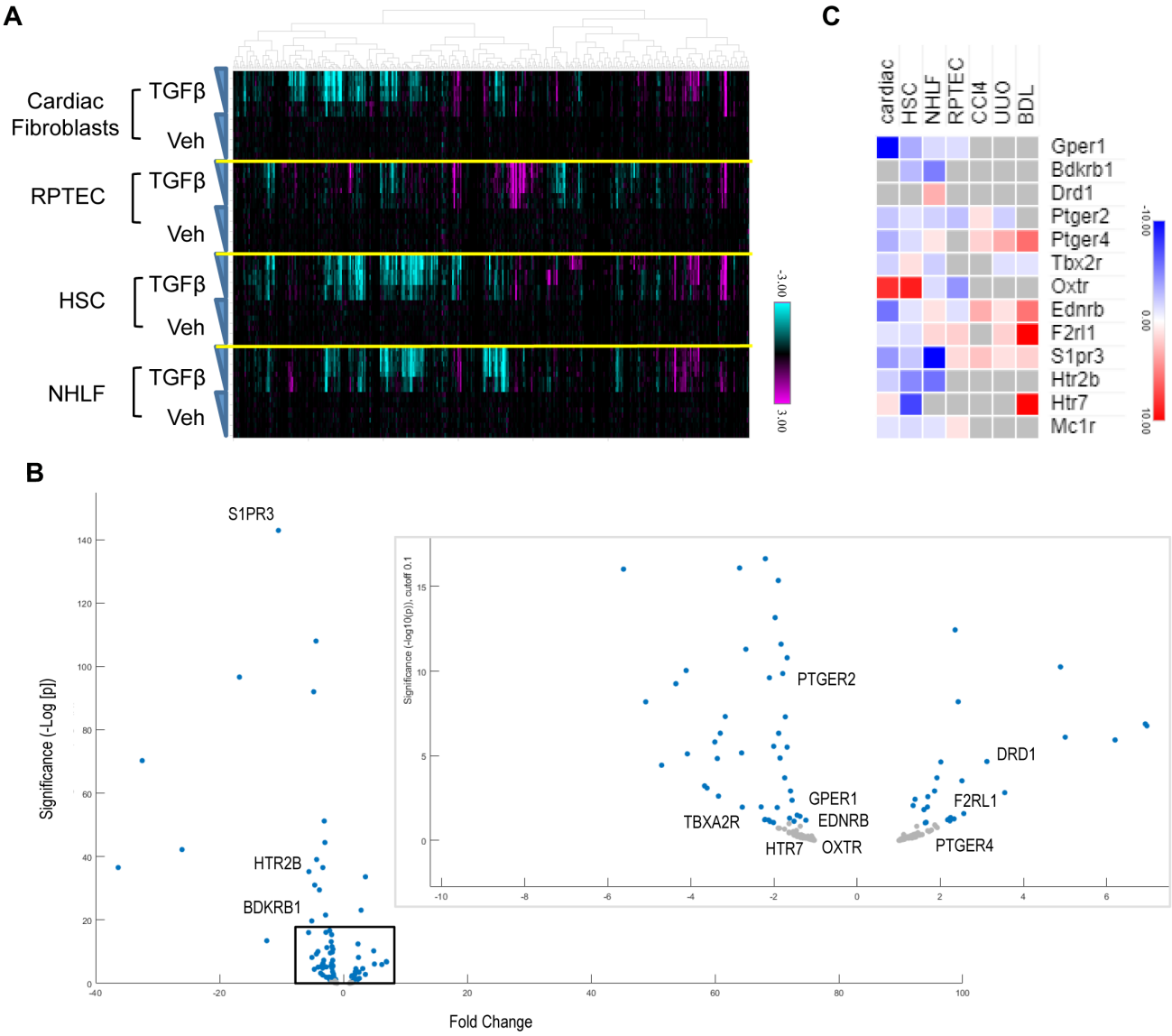
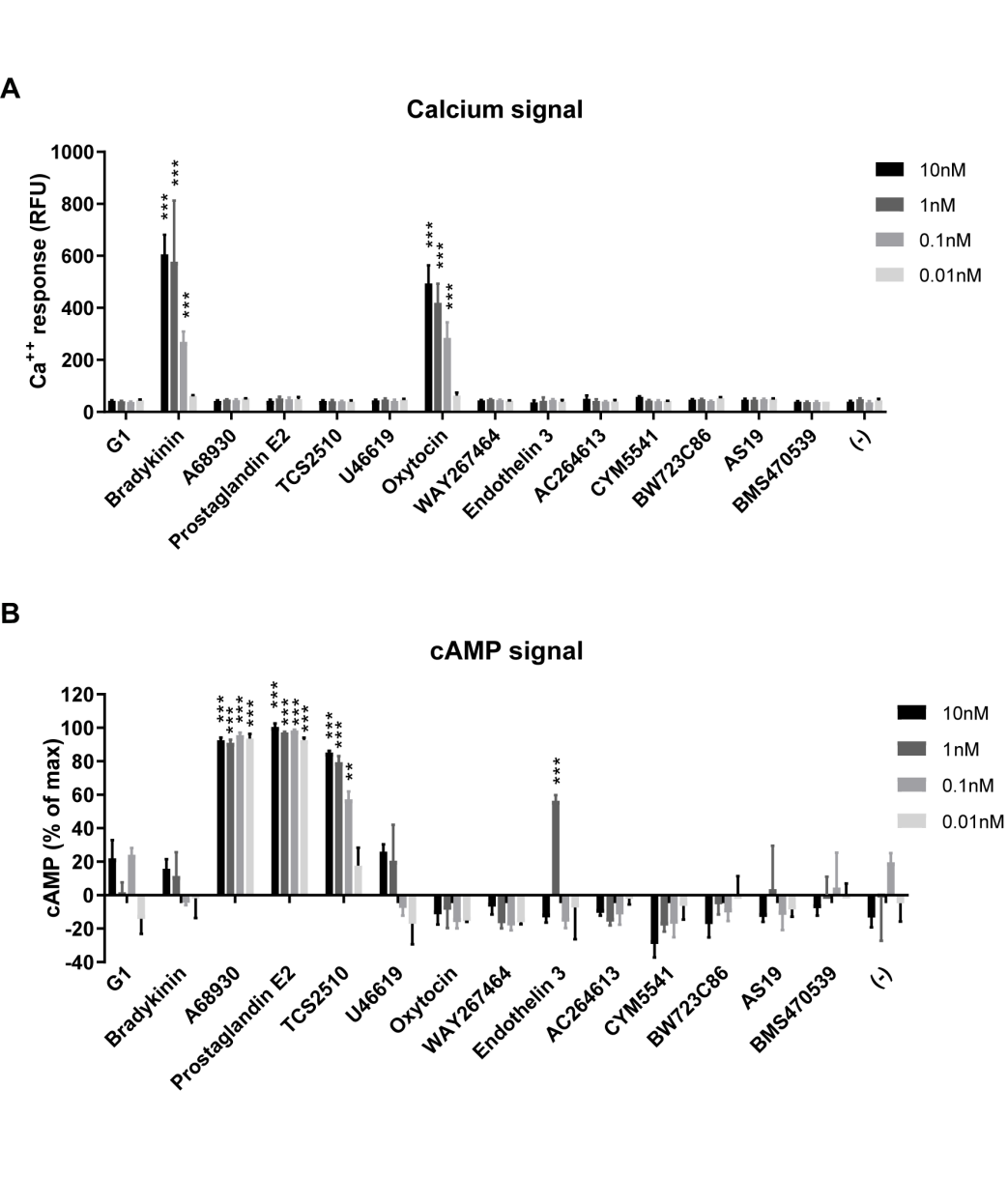


Figure 3

JPET Fast Forward. Published on August 26, 2020 as DOI: 10.1124/jpet.120.000103
This article has not been copyedited and formatted. The final version may differ from this version.



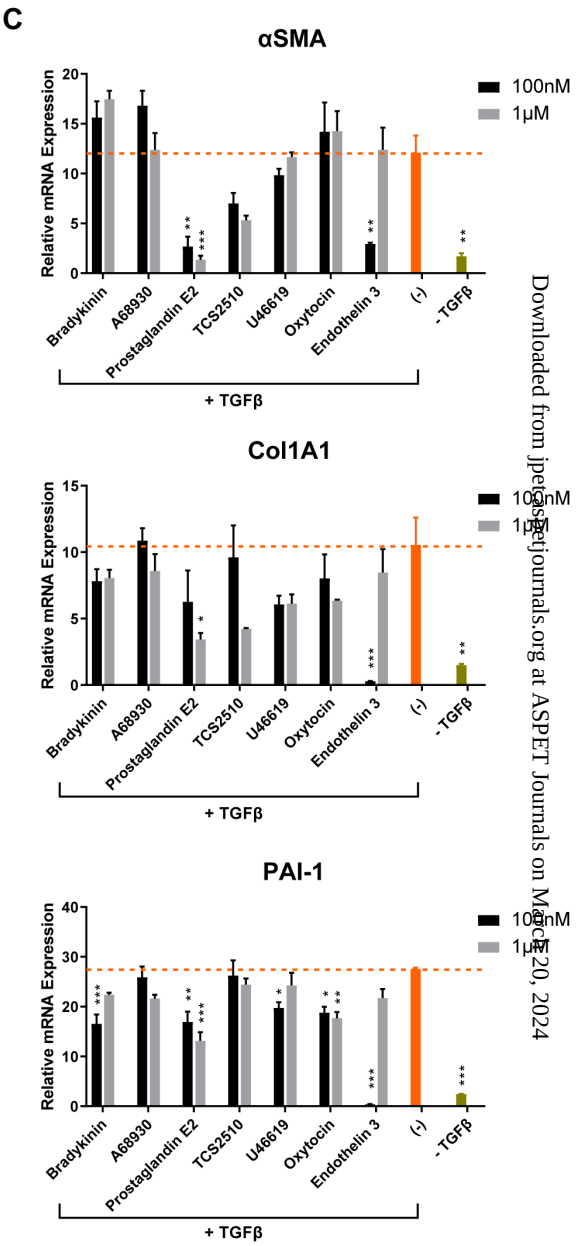


Figure 4