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Expression Profile and Activation of Components for a Novel Signaling Module Mediating EGFR Transactivation

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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The thesis of Rebecca Marrie Lynch is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-chair

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Chair

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LIST OF ABBREVIATIONS

GPCR = G protein-coupled receptor

MMP = matrix metalloprotease

MT1-MMP = Membrane Type-1 Matrix Metalloprotease

Hb-EGF= Heparin-binding Epidermal Growth Factor

EGFR= Epidermal Growth Factor Receptor

PBST= 1X Phosphate Buffered Saline Tween-20

UT= untreated

EGF= epidermal growth factor

BK = bradykinin

AG 1478/EGFRinh = inhibitor of EGFR

MMP14inh = inhibitor of MT1-MMP

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ABSTRACT OF THE THESIS

Expression Profile and Activation of Components for a Novel Signaling Module Mediating EGFR Transactivation

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Master of Science in Biology

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Paul A. Insel, Chair
Julian Schroder, Co-Chair

Activation of G protein-coupled receptors (GPCRs) mediates the transactivation of epidermal growth factor receptor (EGFR)-dependent signaling pathways, yet the mechanisms underlying this phenomenon are not fully understood. EGFR transactivation can occur via protease "shedding" of EGFR ligands (e.g., heparin-binding EGF [hb-EGF]) from the cell surface. Work in the Insel laboratory has shown the direct activation

of MT1-MMP (membrane type-1 matrix metalloprotease) via GPCRs and heterotrimeric G proteins and in support of the hypothesis that transactivation of EGFR might occur via components of a novel, membrane-associated signaling module (GPCR/MT1-MMP/HB-EGF/EGFR). In order to identify and quantify GPCRs that might regulate this proposed mechanism, I conducted targeted GPCR arrays using NIH 3T3 and HeLa cells. Next, I assessed the expression of the components in the putative signaling module by using whole cell lysates and isolated membranes from various cell types (NIH 3T3 fibroblasts, HeLa cells, and primary rat cardiac myocytes). I found that these signaling components localize to the plasma membrane and that bradykinin, a GPCR agonist, can activate EGFR in isolated HeLa cell membranes. This response was attenuated by AG1478, an inhibitor of EGFR, and by Peptide G, a selective inhibitor of MT1-MMP. Taken together, these findings provide evidence in support of the hypothesis that components of a plasma membrane-delimited signaling module that mediates EGFR transactivation can be activated by GPCR stimulation. Because over-activation of EGFR has been implicated in a variety of disease states, such as the progression of cancer and cardiovascular disease, further understanding of this mechanism may lead to the discovery of novel therapeutic targets.

Chapter 1. Introduction:

1.1 G Protein-Coupled Receptors

G protein-coupled receptors (GPCRs) are the largest family of membrane signaling receptors, with 900 GPCRs encoded by the human genome (1). These receptors are involved in a large number of physiological responses, from transduction of vision, taste, and smell, to regulation of the immune system, cardiovascular system, and digestive tract. GPCRs have a variety of ligands, including light, odorants, neurotransmitters, hormones, ions, and large proteins (2), and are an attractive drug target because of their relative abundance, their location on the plasma membrane, and their functional selectivity. In fact, 40% of all prescription pharmaceuticals on the market target a GPCR (3).

GPCRs are seven transmembrane receptors that are attached to an intracellular, heterotrimeric G protein complex. This complex consists of an α subunit and a ß/γ dimer (2). When a ligand binds to a GPCR, the receptor undergoes a conformational change, allowing the α subunit to exchange GDP for GTP, which activates the G protein complex and allows it to dissociate from the ß/γ subunits, a functional dimer. Canonical G protein signaling occurs through 4 main Gα protein families: Gs, Gi, Gq, and G12/13 pathways. Signaling mediated by the Gß/γ dimer, however, is not as well understood. Gß/γ has multiple effectors, including ion channels and enzymes, and it can regulate activity of many signaling pathways through protein-protein interactions (4).

Previous studies have also shown that G proteins can lead to activation of other signaling molecules, such as matrix metalloproteases (MMPs) (5). This activation has functional significance because dysregulation of MMPs can contribute to cancer, inflammation, and adverse cardiac remodeling (6).

1.2 Matrix Metalloproteases

MMPs are zinc-dependent endopeptidases from the metzincin superfamily (7). They are activated by a cysteine switch, whereby a cysteine residue dissociates from the zinc atom and exposes the active site (8). MMPs are primarily involved in extracellular matrix (ECM) degradation and remodeling, which plays an important role in embryonic development, reproduction, and tissue remodeling. A variety of disease states, such as cancer, arthritis, and cardiovascular disorders, are linked to changes in the ECM, which is regulated by MMPs and other enzymes (7). Additionally, MMPs can activate pro-inflammatory cytokines, chemokines, other MMPs, and various proteins to regulate inflammation (9).

MMPs were first discovered in 1962 by Jerome Gross and Charles M Lapiere who found that these enzymes lead to collagen degradation in tadpole tails. Since then, more than twenty-five different MMPs have been identified. Within the MMP family, there are several membrane-tethered MMPs (MT-MMPs), which are located on the plasma membrane of cells. MMP 14 (also known as MT1-MMP) is one such membrane-associated protein that cleaves ECM components, but it can also cleave and activate other

MMPs and signaling molecules (10). MT1-MMP has been shown to cleave and release from the plasma membrane heparin-binding epidermal growth factor (hb-EGF), in a process known as hb-EGF "shedding." Hb-EGF then binds to and activates the EGF receptor (EGFR) (11), which leads to the regulation of various downstream signaling cascades, as will be described below. Mutations that lead to EGFR overexpression and over-activation have been implicated in a variety of disease states, such as cardiovascular disorders and cancer. Further understanding of the mechanisms of MT1-MMP activity and hb-EGF shedding could yield further insight into disease mechanisms and may provide novel therapeutic targets.

1.3 Epidermal Growth Factor Receptor

The EGFR is a receptor tyrosine kinase in the ErbB family of proteins that spans the cell's plasma membrane. EGFR is highly expressed in epithelial tissue, such as the epithelial cells of the GI tract, respiratory system, cardiovascular system, reproductive system, and others (11). Activation of EGFR can lead to cell growth, differentiation, and proliferation. Receptor activation occurs through binding of ligands (such as hb-EGF, TGF-α, EGF, and others) to its extracellular domain, leading to formation of a homodimer and autophosphorylation on the cytoplasmic tail at tyrosine residues 992, 1045, 1068, 1148, and 1173 (1) (12).

EGFR has been implicated in wound healing and oncogenesis (13). It has also been shown to play a role in the development in the epithelial cells of the skin, kidney,

brain, and GI tract (14). EGFR signaling helps to form the semilunar valves of the heart (15). Previous studies have shown that GPCRs can enhance EGFR activity through EGFR transactivation. Ulrich *et al* were the first to show that treatment with GPCR agonists, such as lysophosphatidic acid, endothelin 1, and thrombin, led to increased phosphorylation of EGFR. This phosphorylation was attenuated when cells were pre-treated with AG1478, an inhibitor of EGFR tyrosine kinase activity (16).

1.4 Membrane-Delimited EGFR Transactivation Mechanism

The work of Ullrich *et al*, and subsequently others, led to the idea that GPCRs mediate EGFR transactivation through a 'triple-membrane-passing-signal' mechanism (17) (18) (2) (16) (Figure 1). In this mechanism, ligand binding to the GPCR leads to activation of intracellular signaling components, such as protein kinase C, calcium, Src, and arrestins (2). Such components can then activate a membrane-bound MMP or an ADAM (a disintegrin and metalloprotease), which cleave EGFR ligands from the plasma membranre. This "shedding" allows the ligand to bind and activate EGFR, leading to activation of further downstream signaling pathways, such as the MAPK and PI3K-AkT pathways (2). However, the precise mechanisms of GPCR-mediated EGFR transactivation are not fully understood.

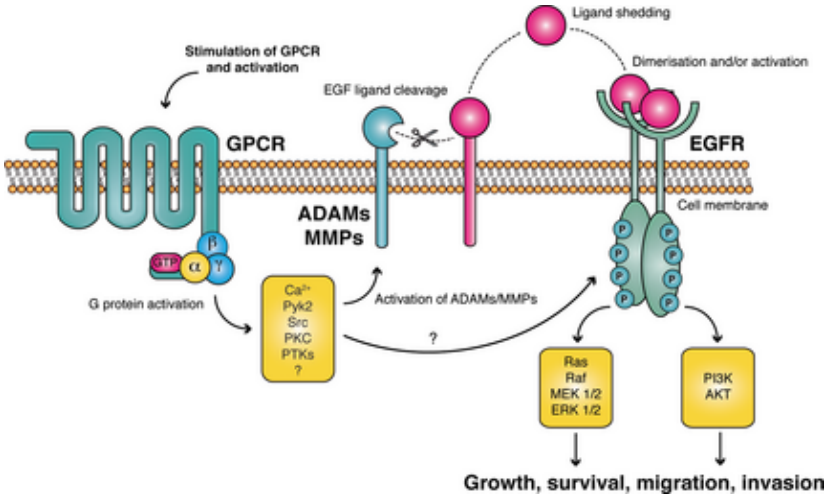

Figure 1. GPCR-mediated transactivation of EGFR. In specific cell types and under certain conditions, GPCR stimulation leads to activation of second messenger molecules, including PKC, Src, Pyk2, PTKs, Ca^{2+} and other unknown molecules. Transactivation of the EGFR by GPCR signaling occurs through two possible mechanisms: the 'triple-membrane-passing-signaling' paradigm, whereby second messengers activate membrane-bound MMPs such as the ADAM family members, which cleave EGF ligands that are able to bind to the EGFR and other EGFR family members and promote dimerization and activation of the EGFR. This promotes the subsequent activation of signaling cascades including the MAPK pathway and the PI3K–AKT pathway, ultimately leading to cellular responses including growth, migration, invasion and survival. ERK, extracellular signal-regulated kinase; GTP, guanosine triphosphate; MEK, mitogen activated protein kinase kinase; PI3K, phosphatidylinositide 3-kinase; PKC, protein kinase C; PTK, protein tyrosine kinase; Pyk2, protein tyrosine kinase 2. (2)

In contrast to this 'triple-membrane-passing-signal' mechanism, we propose that a direct, membrane-delimited mechanism of GPCR-mediated EGFR transactivation can occur through a specific membrane-bound MMP (Figure 2). In our proposed model, agonist stimulation of GPCRs acts via heterotrimer G proteins to directly activate MT1-MMP, which then can cleave EGFR ligands, in particular hb-EGF, which binds to and activates EGFR, promoting downstream signaling (19). . Although previous studies

have shown that MMPs become activated through the actions of intracellular components, the membrane-delimited model is akin to other membrane actions of heterotrimeric G Proteins and perhaps is mediated by the G ß/γ dimer (19). Further understanding of this new membrane-delimited mechanism could lead to new insights regarding cell regulation and the discovery of novel therapeutic targets.

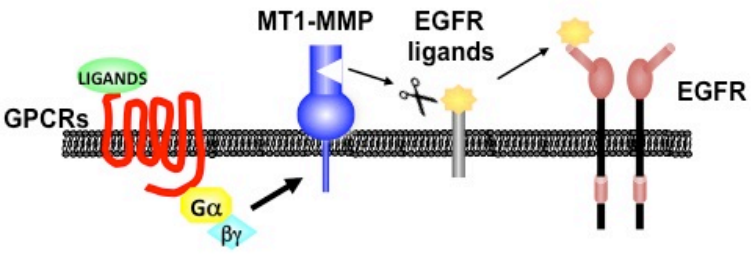

Figure 2. Proposed mechanism of GPCR-mediated EGFR transactivation. Upon ligand binding and GPCR activation, the Gα and Gß/γ subunits can dissociate from the plasma membrane and directly activate MT1-MMP. Activated MT1-MMP goes on to cleave EGFR ligands from the plasma membrane, in a process known as "ectodomain-shedding." The EGFR ligand, specifically heparin-binding epidermal growth factor (Hb-EGF), can then bind to its receptor, activating downstream signaling pathways. Activation of EGFR can lead to increased proliferation, differentiation, and cell growth.

1.5 Bradykinin

In my efforts to find a GPCR that mediates EGFR transactivation through the membrane-delimited mechanism, I decided to target the bradykinin receptor. I chose bradykinin because previous studies have shown that stimulation of the bradykinin receptor can lead to EGFR transactivation (20). There are two types of bradykinin receptors, B1 and B2. Prior studies have shown that the bradykinin B2 receptor is highly expressed in NIH 3T3 (Table 3.1) and HeLa cells (21), mouse and human cell lines, respectively, that are widely used for cell biological and signal transduction studies.

Physiologically, bradykinin helps to lower blood pressure by causing vasodilation but in addition, it can lead to contraction of smooth muscle in the bronchus and gut (22). Additionally, studies have shown that bradykinin mediates the pathophysiological processes of inflammation and pain, and increased bradykinin release often accompanies tissue damage (23).

As noted above, the effects of bradykinin are mediated through two distinct receptors, the B1 receptor and the B2 receptor. The B2 receptor is expressed in a wide range of tissues, including sympathetic nerves, vascular smooth muscle, and immune cells. The receptor primarily mediates vasodilation and other effects of the autonomic nervous system (24). The B2 receptor is thought to be Gq-linked (25). The B1 receptor, on the other hand, is expressed at lower levels in normal tissues, but receptor synthesis increases following tissue damage or after exposure to inflammatory agents (24). In fact, bradykinin has been described as the most potent endogenous pain-inducing substance known (23). The B1 receptor is thought to be Gi and/or Gq linked (25).

1.6 NIH 3T3 Cells, HeLa cells, and Rat Cardiac Myocytes

I conducted experiments in three different cell types: NIH 3T3 cells, adult rat cardiac ventricular myocytes, and HeLa cells. Below is a brief background on these three cell types.

NIH 3T3 cells are derived from mouse embryonic fibroblasts, and have been an immortalized cell line since 1962 (26). Fibroblasts help to synthesize the extracellular matrix and secrete collagen, and are the main connective tissue-production cell in the body (27). I chose to use NIH 3T3 cells because they are easy to grow and transfect and have a fibroblast phenotype.

HeLa cells are epithelial cells derived from a human patient with cervical cancer, Henrietta Lacks. They are the oldest immortalized human cell line, dating back to 1952(28). HeLa cells have been instrumental in the development of the polio vaccine, and research studies related to cancer, AIDS, gene mapping, and a large number of other scientific discoveries (29). I chose to use HeLa cells because they grow relatively rapidly, are a human cell line, and because I found that Western blot analysis readily detects expression of EGFR and phosphorylated-EGFR.

Cardiac myocytes are contractile cells in the heart that make up the atria and ventricles (30). Adult rat cardiac ventricular myocytes were chosen because they are primary cells that closely mimic the physiology of cells *in vivo*. I also worked with rat cardiac ventricular myocytes because over-activation of MMPs have been linked to adverse cardiac remodeling and cardiovascular diseases (10). Moreover, MMP-dependent EGFR transactivation occurs in the heart (31).

1.7 Goals and Hypothesis

1. To define the repertoire of GPCR expression in NIH 3T3 cells and HeLa cells.

2. To determine the baseline expression for components of a novel membrane-delimited signaling mechanism for EGFR transactivation (GPCR/MT1-MMP/hb-EGF/EGFR).

3. To functionally validate this novel signaling pathway by measuring GPCR-mediated EGFR transactivation via Western blot analysis.

Overall Hypothesis: GPCR agonists (e.g., bradykinin) can activate a novel signaling pathway mediating EGFR transactivation via components that localize to the plasma membrane.

2. Materials & Methods

2.1. Reagents

Epidermal Growth Factor, human: Sigma, E9644
Bradykinin: Sigma, B3259
AG 1478: Calbiochem, 658552
MT1-MMP Inhibitor: Calbiochem, 444295
Adenosine 5'-Triphosphate (ATP): Sigma, FLAAS

Antibodies used:

MT1-MMP/MMP14 Rabbit Primary Antibody (EP1264Y): Abcam, ab51074
Hb-EGF Rabbit Primary Antibody: Abcam, ab16783
EGF Receptor (D38B1) Rabbit Primary Antibody: Cell Signaling, 4267
Phospho-EGF-Receptor (Tyr 1068) (D7A5) Rabbit Primary Antibody: Cell Signaling, 3777
Anti-rabbit IgG, HRP-linked Antibody: Cell Signaling, 7074

2.2. Cell Culture

NIH 3T3 and HeLa cell lines were grown in T-75 flasks with 10 mL of Dulbecco's Modified Eagle Medium (DMEM), 10% FBS (Fetal Bovine Serum) and 2% Penicillin/Streptomycin. Cells were kept at 37˚ C and 10% CO2. Cells were split using Trypsin:EDTA (Gemini Bioproducts).

2.3. Isolation of Adult Rat Ventricular Myocytes

Ethical approval for the care and use of animals was granted by the University of California at San Diego Institutional Animal Care and Use

Committee. Rat cardiac ventricular myocytes were isolated from adult (8–10 weeks), male Sprague-Dawley rats. Briefly, the rats were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection. The heart was excised and digested with collagenase II (Worthington Biochemical, Lakewood, NJ) via a modified reverse-Langendorff apparatus. Cardiac myocytes were separated from cardiac fibroblasts by gravity separation and the cell membranes were isolated as described in Section 2.3.

2.3. Membrane Isolation

2.3.1. Crude Membrane Isolation

HeLa cells and NIH 3T3 cells were removed from T-75 plates using trypsin. The enzymatic reaction was stopped by addition of DMEM media and cells were subsequently spun down to form a pellet. Rat cardiac myocytes were spun down in cold PBS to also form a pellet. For all three samples, the supernatant was removed, and the pellet was washed with ice-cold PBS and spun at 233 G for 3 minutes at 4˚C using a Beckman Coulter Allegra™ X-12R Centrifuge. After two PBS washes, 3-5 mL of homogenizing buffer was added to the pellet and homogenized using trituration and a glass homogenizer. (Homogenizing Buffer: 357.5 mg of HEPES, 23.8 mg of MgCl2, 19.02 mg of EGTA, and 15.43 mg of DTT were added to 50 mL of ultrapure water, and the pH of the solution was adjusted to 7.5.) The mixture was spun at 300 G for 5 minutes at 4˚C. The pellet was discarded and the supernatant was collected and centrifuged for 10 min at 5,000 G and 4˚C. Supernatant was discarded, and the

pellet was mixed with fresh homogenizing buffer. Cells were then incubated on ice for 10 minutes in homogenizing buffer containing 500 mM KCl. Following incubation, the mixture was centrifuged for 10 min at 50,000 G at 4˚C.

2.3.2. 101 Bio® Plasma Membrane Protein Extraction

Plasma membranes were isolated according to manufacturer's instructions (101 Bio®). Briefly, HeLa cells and NIH 3T3 cells were removed from T-75 plates using trypsin and DMEM media and subsequently spun down to form a pellet. Rat cardiac myocytes were spun down in cold PBS to also form a pellet. For all three samples, the supernatant was removed, and the pellet was washed with cold PBS and spun at 233 G for 3 minutes at 4˚C. After two PBS washes, 1 mL of Buffer A was added to the pellet, and homogenized in a glass homogenizer for 1 minute. The mixture was spun at 14,000 G for 1 minute and 4˚ C through a filter cartridge to purify the sample. Next, the sample was re-homogenized by vortexing and spun at 3,000 G for 1 minute (4˚ C) to pellet out the cell nuclei. The supernatant was placed in a fresh 1.5 mL microfuge tube, and spun at 16,000 G for 30 minutes (4˚ C). Supernatant was removed, and 200 µL of Buffer B was added to the pellet and homogenized by trituration and vortexing. The mixture was spun at 10,000 G for 5 minutes at 4˚ C. Supernatant was then transferred to a fresh microfuge tube, and brought up in 1 mL of PBS with Phosphatase-Inhibitor Cocktail 2 (Phic2, 1:100). The sample was spun at 16,000 G for 30 minutes at 4˚C. The pellet was brought up in 0.5-1 mL of PBS + Phic2 and protein content was measured.

2.4. Protein Analysis

2.4.1. Bradford Protein Assay

Membrane samples were added to a 96-well plate, and protein concentration was analyzed using the Pierce Bradford protein assay kit according to the manufacturer's instructions. Results were analyzed using a Beckman Coulter DTX 800 Multimode Detector.

2.4.2. Western Blots

Following the Bradford protein assay, plasma membrane samples were added at a ratio of 29 µl of sample: 10 µl of 4X sample buffer (Invitrogen): 1 µl beta-mercaptoethanol (Sigma). 20 µl of each sample were loaded onto a pre-cast 4% to 12% PAGE gel (Invitrogen) and run for 1 hr 10 min at 200 V, 40 mA, and 25 W. Gels were incubated in 10% methanol transfer buffer for 10 min and protein transferred to a PVDF membrane for 90 min at 110 V, and 380 mA. Membranes were then incubated in 4% BSA in PBST for 1 hr and primary rabbit antibody was added to membranes and incubated overnight. Membranes were washed 3x (5 min/wash) with PBST. Next, secondary anti-rabbit antibody (1:5000 in 1% BSA, AbCam) was added to the membrane and incubated for 1 hr. Membranes were washed 5x-6x (5 min/wash) and developed with ECL luminescence (GE Healthcare)

2.5. TaqMan® GPCR array

GPCR expression in NIH 3T3 cells and HeLa cells was determined using a TaqMan® GPCR array (Life Technologies), according to manufacturer's instructions. cDNA from each cell type was placed into a 384-well microfluidic card along with TaqMan® Universal PCR Master Mix. The plate was centrifuged to dispense the sample mix into the individual wells and run on a Real-Time PCR System. GPCR expression was assessed in comparison to the housekeeping gene 18S rRNA and other housekeeping genes using Real-Time PCR.

Chapter 3. Results:

3.1. Quantification of GPCR Expression in NIH 3T3 cells and HeLa cells

I determined the GPCR expression profile in NIH 3T3 cells and HeLa cells using an unbiased TaqMan® GPCR array. I used this approach to define the repertoire of GPCR expression in each of these two cell lines The arrays assess 384 genes: 29 housekeeping genes + 355 non-chemosensory (not odorants, tastants, visual) GPCRs. The cycle threshold (CT) value was determined for each cDNA and normalized to the average of four replicates of the most highly expressed gene, the 18S rRNA, a housekeeping gene. Thus, expression was quantified as ΔCT (sample CT- 18S CT). Genes that did not reach threshold by 40 cycles were considered to be undetected, and assigned a generic ΔCT value of (40 - 18S CT). The ΔCT value and gene expression are inversely related, such that lower CT values indicate higher GPCR expression, while higher CT values indicate lower expression of a particular GPCR.

3.2. GPCR Expression in NIH 3T3 cells

The GPCR array revealed that NIH 3T3 cells express 208 non-chemosensory GPCR genes. The CT for the four 18S rRNA replicates was determined to be 11.08. Using the IUPHAR Database of Receptors and Ion Channels as a reference, the expressed GPCRs were classified further and separated either as orphan receptors (n=46) or by their specific G protein-coupled signaling pathway: Gi (n=83), Gq (n=44), Gs (n=27), G12/13 (n=13) (Figure 3.1.). A GPCR orphan receptor, by definition, is a receptor that does not yet have an identified endogenous ligand (32). The twenty most highly expressed

GPCRs by NIH 3T3 cells are identified in Table 3.1, and the five most highly expressed orphan receptors are shown in Table 3.2. Of note, orphan receptors, the smoothened receptor, two frizzled receptors and adhesion GPCRs represent over half of the 20 highest expressed GPCRs in NIH 3T3 cells (Table 3.1). Although orphan GPCRs have no known agonist, their putative linkage to heterotrimeric G proteins has been determined in some cases (for example, GPRC5A and GPRC5B). I also identified the five most highly expressed receptors with known ligands (and their associated ligands) for each signaling pathway: Gi (Table 3.3), Gq (Table 3.4), Gs (Table 3.5), and G12/13 (Table 3.6).

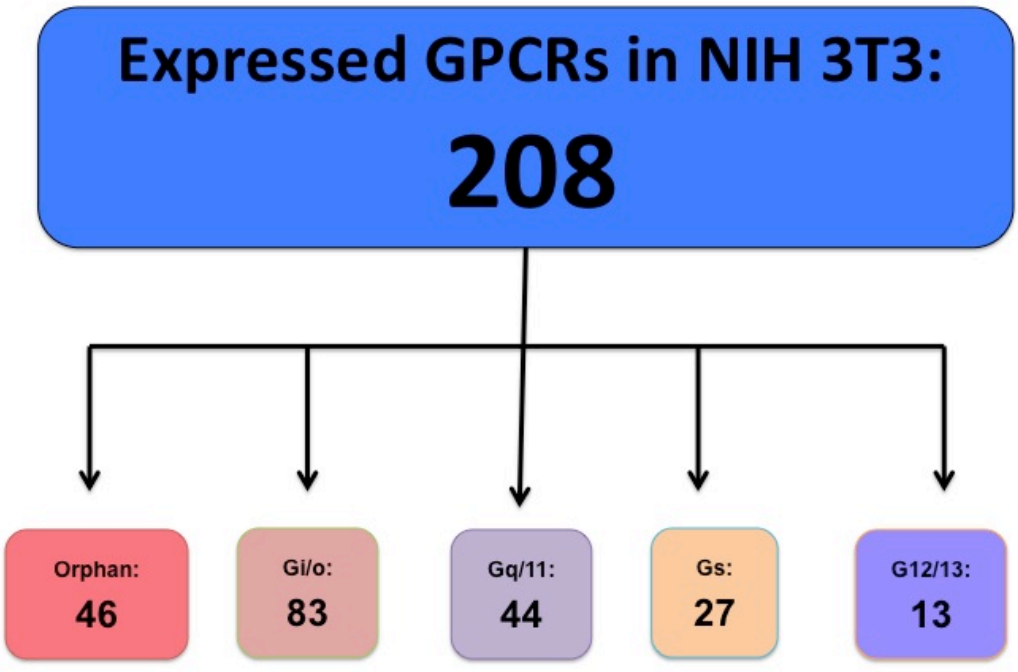

Figure 3.1. Total GPCR expression in NIH 3T3 cells. Relative abundance of orphan receptors and GPCRs from each signaling pathway.

Table 3.1. Twenty Highest Expressed GPCRs in NIH 3T3 Cells

	GPCR:	**Delta CT:**	**Class/Linkage:**
1	F2R/PAR1	13.73	Gi, G12/13, Gq/G11
2	SMO	13.88	Gi/Go, G12/G13
3	EDG2	14.55	Gi/Go, Gq/G11, G12/G13
4	LPHN2	14.73	Adhesion Class
5	GPR137B	15.02	Orphan
6	GPRC5A	15.23	Class C Orphan
7	GRM8	15.67	Gi/Go
8	GPRC5B	15.86	Class C Orphan
9	GPR124	16.18	Adhesion Class
10	LENG4	16.20	Unknown
11	FZD1	16.24	Gi/Go
12	GPR125	16.25	Adhesion Class
13	GPR137	16.74	Orphan
14	GABBR1	16.92	Unknown
15	HTR7	16.96	Gs
16	HTR1b	17.03	Gi/Go
17	EDG4	17.08	Gi/Go, Gq/G11, G12/G13
18	FZD7	17.08	Gs, Gi/Go
19	LPHN1	17.16	Gq/G11
20	TM7SF3	17.19	Gi/Go

Table 3.2. Five Highest Expressed Orphan Receptors in NIH 3T3 cells

	GPCR:	**Delta CT:**	**Name:**
1	GPR137B	15.0	Orphan
2	GPRC5A	15.2	Class C Orphan
3	GPRC5B	15.9	Class C Orphan
4	GPR124	16.2	Adhesion Class GPCR
5	GPR125	16.3	Adhesion Class GPCR

Table 3.3. Five Highest Expressed Gi-linked Receptors in NIH 3T3 cells

	GPCR:	**Delta CT:**	**Name:**	**Ligand:**
1	F2R/PAR1	13.7	Protease-activated receptor 1	Thrombin
2	SMO	15.9	Smoothened Frizzled receptor 1	*constitutively active
3	EDG2	14.5	Lysophospholipid (LPA1) receptor	LPA
4	HTR1B	17	5-hydroxytryptamine (HT1B) receptor	5-hydroxytryptamine (Serotonin)
5	EDG4	17.1	Lysophospholipid (LPA2) receptor	LPA

Table 3.4. Five Highest Expressed Gq-linked Receptors in NIH 3T3 cells

	GPCR:	**Delta CT:**	**Name:**	**Ligand:**
1	F2R/PAR1	13.7	Protease-activated receptor 1	Thrombin
2	EDG2	14.5	Lysophospholipid (LPA1) receptor	LPA
3	EDG4	17.1	Lysophospholipid (LPA2) receptor	LPA
4	LPHN1	17.2	Latrophin-1-receptor	α-latrotoxin
5	BDKRB2	18.3	Bradykinin receptor B2	Bradykinin

Table 3.5. Five Highest Expressed Gs-linked Receptors in NIH 3T3 cells

	GPCR:	**Delta CT:**	**Name:**	**Ligand:**
1	P2RY5	17.2	Lysophospholipid (LPA6) receptor	LPA
2	ADORA2B	17.8	Adenosine receptor 2B	Adenosine
3	ADRB1	19.6	ß-1 adrenergic receptor	Epinephrine, norepinephrine
4	MC4R	19.7	Melanocortin receptor	adrenocorticotropic hormone (ACTH)
5	GPBAR1	19.8	Bile acid receptor	Cholic Acid

Table 3.6. Five Highest Expressed G12/G13 Receptors in NIH 3T3 cells

	GPCR:	**Delta CT:**	**Name:**	**Ligand:**
1	F2R/PAR1	13.7	Protease-activated receptor 1	Thrombin
2	SMO	15.9	Smoothened Frizzled receptor 1	*constitutively active
3	EDG2	14.5	Lysophospholipid (LPA1) receptor	LPA
4	EDG4	17.1	Lysophospholipid (LPA2) receptor	LPA
5	P2YR5	17.2	Lysophospholipid (LPA6) receptor	LPA

3.3. GPCR Expression in HeLa cells

GPCR array analysis of HeLa cells revealed the expression of 115 GPCRs, and the experimental CT for the four 18S rRNA replicates was set at 13.7. The twenty most highly expressed GPCRs by HeLa cells are listed in Table 3.7. The expressed GPCRs were further classified as either orphan receptors (n=36, the five most highly expressed of which are shown in Table 3.8) or according to their G protein-coupled signaling pathway: Gi/o (n=32), Gq/11) (n=27), Gs (n=15), or G12/13 (n=8) (Figure 3.2), using the IUPHAR Database of Receptors and Ion Channels as a reference. The twenty most highly expressed GPCRs in HeLa cells are listed in Table 3.7, and the five most highly expressed orphan GPCRs in Table 3.6. Next, I present information regarding the ligands and GPCRs for the five highest expressed Gi (Table 3.7), Gq (Table 3.8), Gs (Table 3.9), and G12/13 (Table 3.10)-coupled GPCRs. For HeLa cells, most of the 20 highest expressed GPCRs, including 11 of the 13 mostly highly expressed receptors are orphan GPCRs or frizzled receptors.

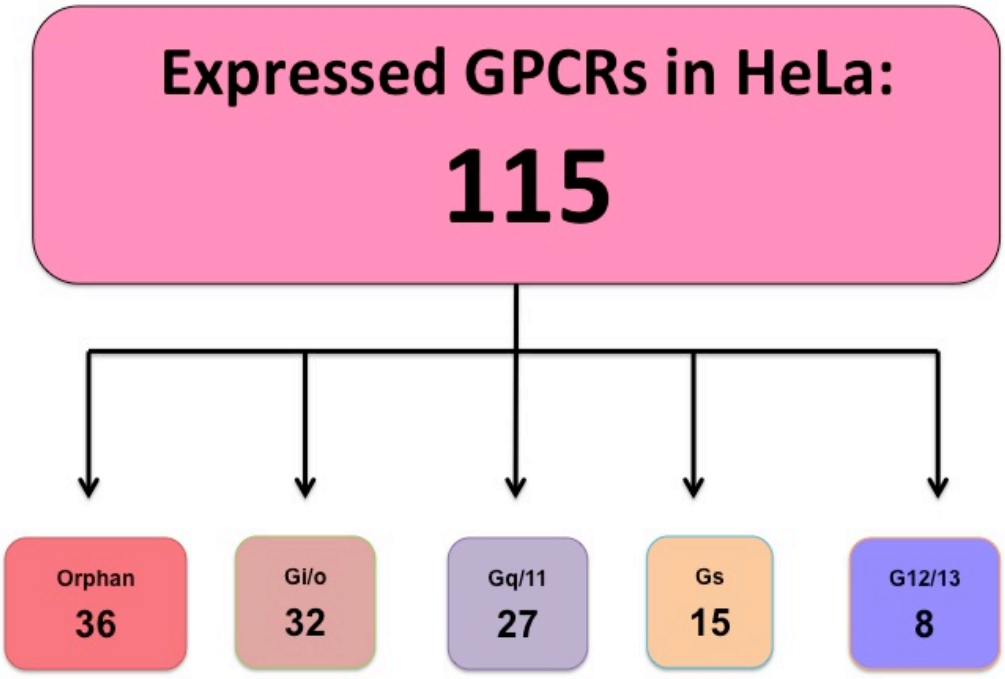

Figure 3.2. Total GPCR expression in HeLa cells. Relative abundance of orphan receptors and GPCRs from each signaling pathway.

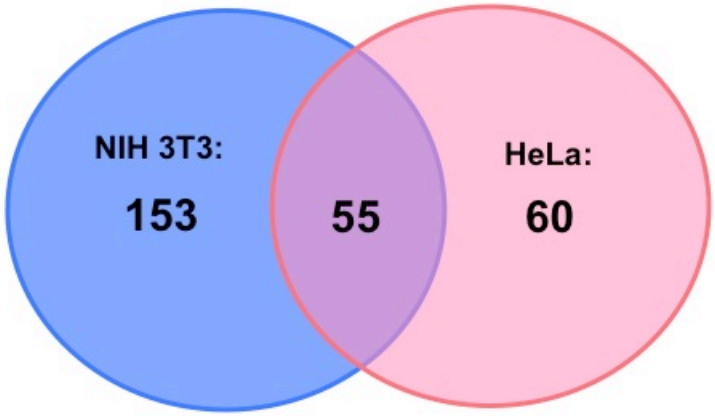

Figure 3.3 Comparison of GPCR Expression in NIH 3T3 and HeLa cells. Of the 208 expressed receptors for NIH 3T3 cells, 153 were unique to NIH 3T3, and 55 were shared with HeLa. Of the 115 expressed HeLa GPCRs, 60 were unique to HeLa.

Table 3.7. Twenty Highest Expressed GPCRs in HeLa Cells

	GPCR:	**Delta CT:**	**Class/Linkage:**
1	GPRC5A	13.36	Class C orphan
2	CD97	13.39	Class B orphan
3	GPR56	14.08	Class B orphan
4	LANCL1	14.09	Orphan
5	FZD6	14.32	$G_{i/o}$
6	SSTR1	14.56	$G_{i/o}$
7	LGR4	14.96	Class A orphan
8	GPR153	15.22	Class A orphan
9	GPR137A	15.31	Orphan
10	TBXA2R	15.86	$G_{q/11}$
11	FZD2	15.88	$G_{i/o}$
12	OPN3	16.01	Class A orphan
13	GPR126	16.04	Class B orphan
14	F2R/PAR1	16.20	$G_{q/11}$, $G_{i/o}$, $G_{12/13}$
15	GPR125	16.21	Class B orphan
16	FZD5	16.27	Unknown
17	GPR	16.91	Class A orphan
18	HTR1D	16.94	$G_{i/o}$
19	EMR2	17.13	Class B orphan
20	FZD4	17.19	$G_{12/13}$

Table 3.8. Five Highest Expressed Orphan Receptors in HeLa cells

	GPCR:	**Delta CT:**	**Name:**
1	GPRC5A	13.4	Class C Orphan
2	CD97	13.4	Adhesion Class GPCR
3	GPR56	14.1	Adhesion Class GPCR
4	LANCL1	14.1	Orphan
5	LGR4	15	Class A Orphan

Table 3.9. Five Highest Expressed Gi-linked Receptors in HeLa cells

	GPCR:	**Delta CT:**	**Name:**	**Ligand:**
1	FZD6	14.3	Frizzled receptor 6	Wnt-5A
2	SSTR1	14.6	Somatostatin receptor	Cortistatin
3	FZD2	15.9	Frizzled receptor 2	Wnt-5a
4	F2R/PAR1	16.2	Protease-activated receptor 1	Thrombin
5	HTR1D	16.9	5-hydroxytryptamine receptor	5-hydroxytryptamine

Table 3.10. Five Highest Expressed Gq-linked Receptors in HeLa cells

	GPCR:	**Delta CT:**	**Name:**	**Ligand:**
1	TBXA2R	15.9	Prostanoid tp receptor	I-BOP
2	F2R/PAR1	16.2	Protease-activated receptor 1	Thrombin
3	EDNRA	17.5	Endothilin receptor	Endothilin-3
4	F2RL1/PAR2	18.3	Protease-activated receptor 2	Trypsin
5	OXTR	18.8	Oxytocin Receptor	Oxytocin, Vasopressin

Table 3.11. Five Highest Expressed Gs-linked Receptors in HeLa cells

	GPCR:	**Delta CT:**	**Name:**	**Ligand:**
1	ADORA2B	17.5	Adenosine receptor 2b	Adenosine
2	PTGER2	18.4	Prostanoid ep2 receptor	Prostaglandin D2
3	ADRB2	19.3	ß-2 adrenergic receptor	Epinephrine, norepinephrine
4	VIPR2	19.6	VPAC2 receptor	Vasoactive intestinal peptide (VIP)
5	ADRB1	19.8	ß-1 adrenergic receptor	Epinephrine, norepinephrine

Table 3.12. Five Highest Expressed G12/G13 Receptors in HeLa cells

	GPCR:	**Delta CT:**	**Name:**	**Ligand:**
1	F2R/PAR1	16.2	Protease-activated receptor 1	Thrombin
2	EDG2	19	LPA1 receptor	Lysophosphatidic acid (LPA)
3	GPR92	19.6	LPA5 receptor	Lysophosphatidic acid (LPA)
4	EDG3	19.8	S1P3 receptor	Sphingosine-1-phosphate
5	P2YR	21.1	Purinergic (P2Y) receptor	ATP

Chapter 4. Expression of the Signaling Components in the Proposed Membrane-delimited Mechanism for GPCR-EGFR Transactivation

I determined the expression of various signaling components using Western blot analysis of cell lysates and isolated cell membranes from NIH 3T3 cells, HeLa cells, and rat cardiac ventricular myocytes. First, I assessed expression of MT1-MMP in the lysates and cell membranes of the three cell types (Figure 4.1). The results indicate that MT1-MMP localizes to the plasma membrane in all three cell types. HeLa cell blots showed higher relative expression of MT1-MMP in the isolated cell membranes compared to the whole cell lysates.

Second, I evaluated expression of hb-EGF in the lysates and membranes of the three cell types (Figure 4.2). In blots of both the lysates and membranes, doublets of hb-EGF are present. The EGF-like domain of hb-EGF contains six cysteine residues that form three disulfide bonds (33). Doublets might represent incomplete cleavage of the disulfide bonds by the denaturing 4X Buffer. The Western blots demonstrate that hb-EGF localizes to the plasma membrane in all three cell types. HeLa cells show higher relative expression of hb-EGF in isolated cell membranes compared to whole cell lysates. NIH 3T3 cells also show higher relative expression of hb-EGF in membranes compared to whole cell lysates, although rat cardiac myocytes do not seem to follow this trend. I attribute this to the fact that rat cardiac myocytes are primary cells, which make them difficult to work with and potentially yielding inconsistent results

Next, I assessed the baseline expression of EGFR and Phosphorylated-EGFR (pEGFR; Tyr1068) in the two cell lines and rat cardiac myocytes (Figures 4.3 and 4.4). Of the three cell types, HeLa cells showed the highest expression of EGFR and pEGFR, in both the cell membranes and the whole cell lysates. Thus, all future pharmacological experiments were conducted in HeLa cells.

I also assessed the basal expression of the signaling molecules Src and ß-arrestin in rat cardiac myocytes. As mentioned previously, Src and ß-arrestin are known second messengers in the 'triple-membrane-passing-signal.' Src is a non-receptor tyrosine kinase named after the Src gene, a proto-oncogene (34). In contrast, ß-arrestin is a subtype of the arrestin family, a small class of proteins that regulate signal transduction (35). It was first discovered that ß-arrestins help to terminate GPCR-mediated signaling through receptor internalization. More recently, however, an addtional function of ß-arrestins has come to light: ß-arrestins can serve as scaffolding proteins for many signaling mechanisms, such as the MAPK pathway (36). Although these signaling molecules are not associated with our proposed mechanism, I looked at basal expression of Src and ß-arrestin in lysates and cell membranes in order to see if Src or ß-arrestin localize to the plasma membrane. While Src and ß-arrestin are present in whole cell lysates, they are absent from isolated cell membranes and thus seem highly unlikely to contribute to our proposed membrane-delimited EGFR transactivation mechanism.

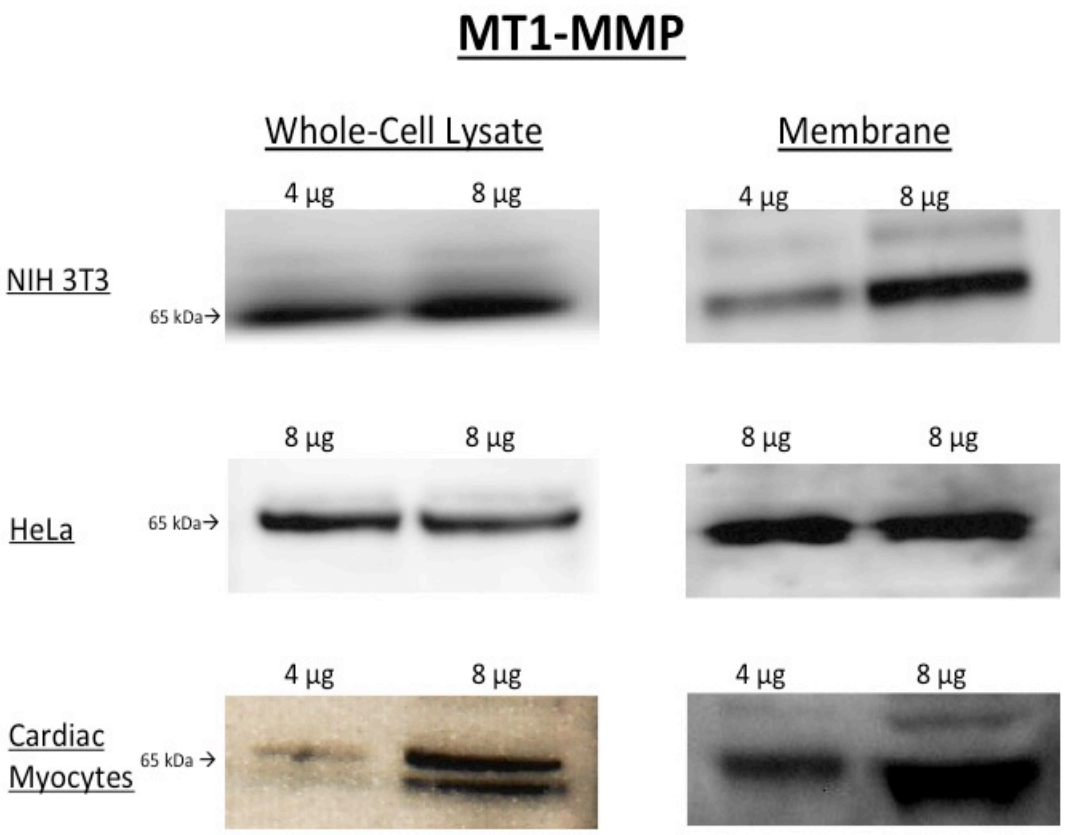

Figure 4.1. Protein expression of MT1-MMP MT1-MMP expression in lysates and membranes from NIH 3T3 cells, HeLa cells, and rat cardiac myocytes.

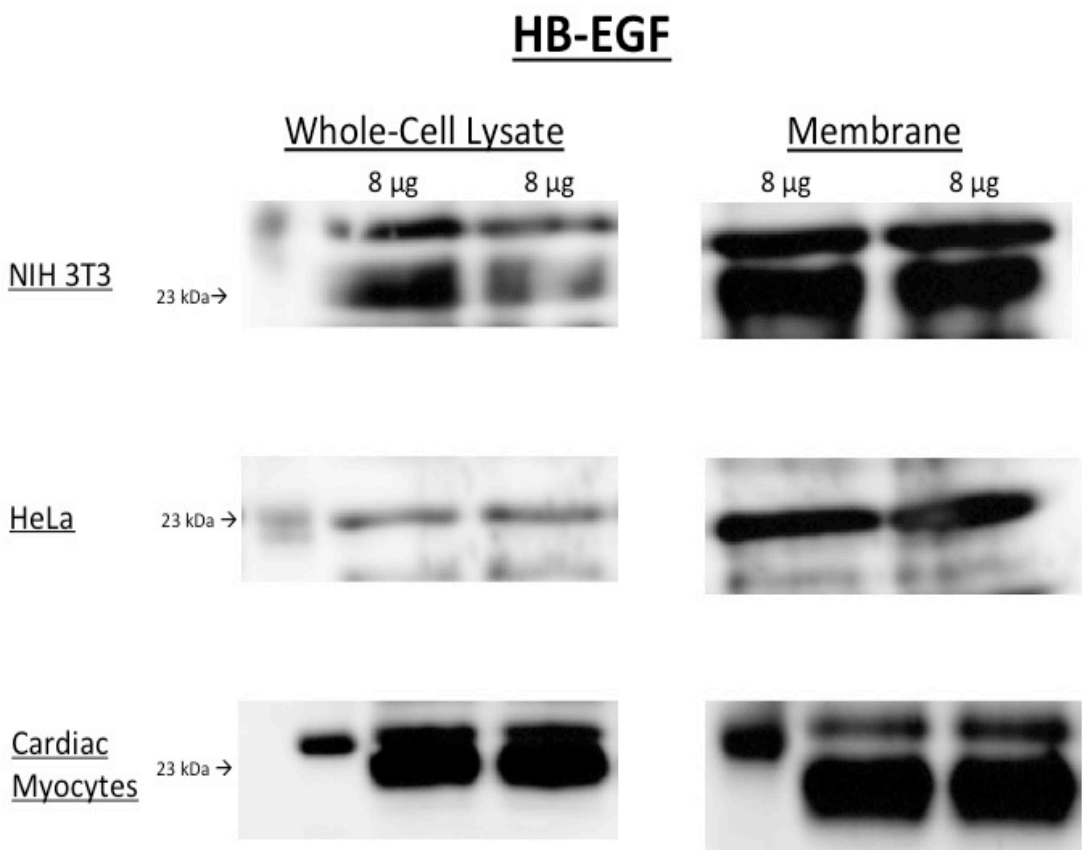

Figure 4.2. Protein expression of Hb-EGF Hb-EGF expression in lysates and membranes from NIH 3T3 cells, HeLa cells, and rat cardiac myocytes.

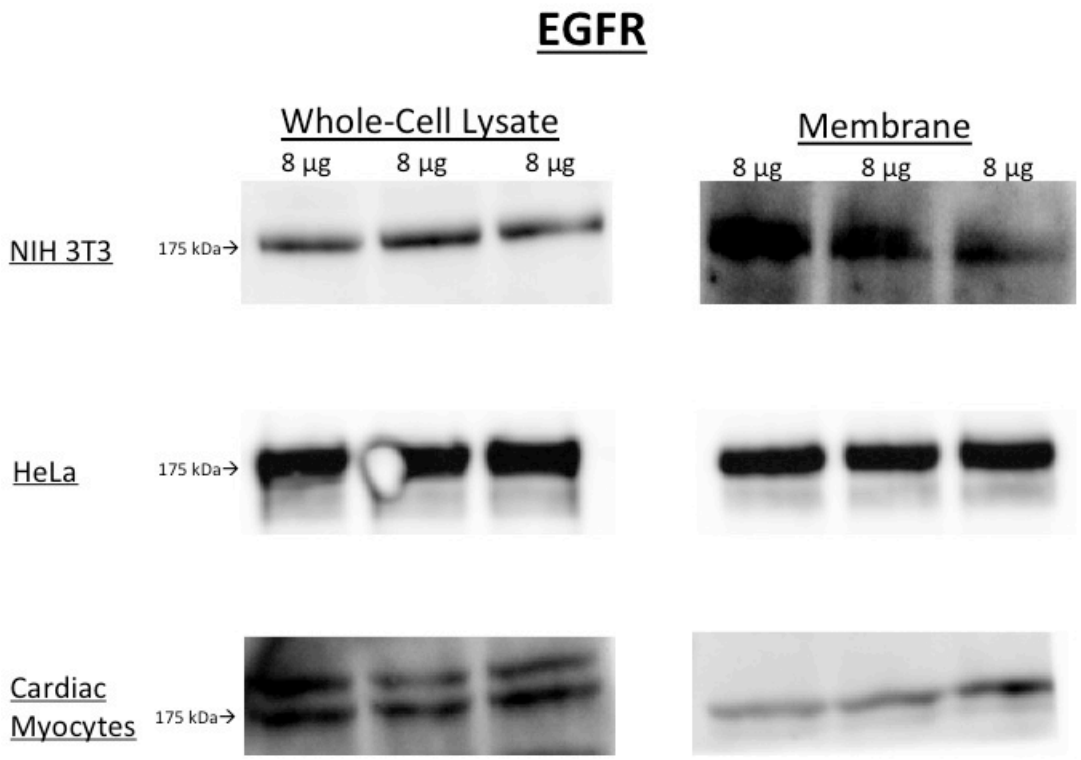

Figure 4.3. Protein expression of EGFR EGFR expression in lysates and membranes from NIH 3T3 cells, HeLa cells, and rat cardiac myocytes.

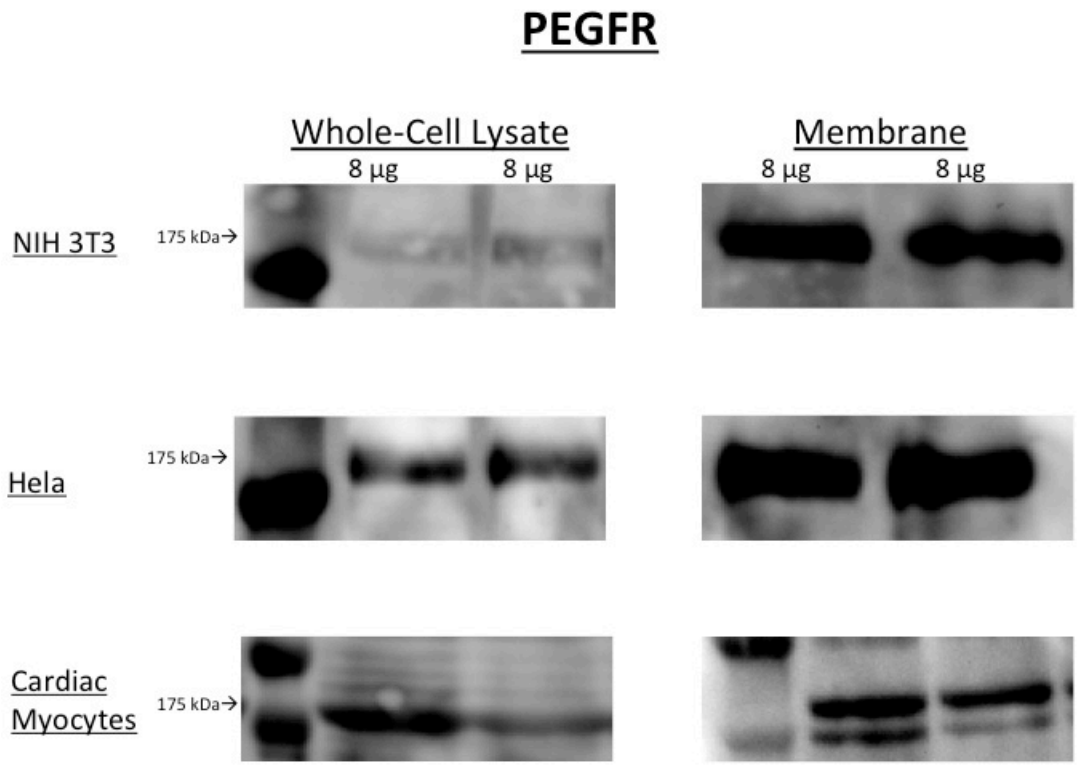

Figure 4.4. Protein expression of pEGFR pEGFR expression in lysates and membranes from NIH 3T3 cells, HeLa cells, and rat cardiac myocytes.

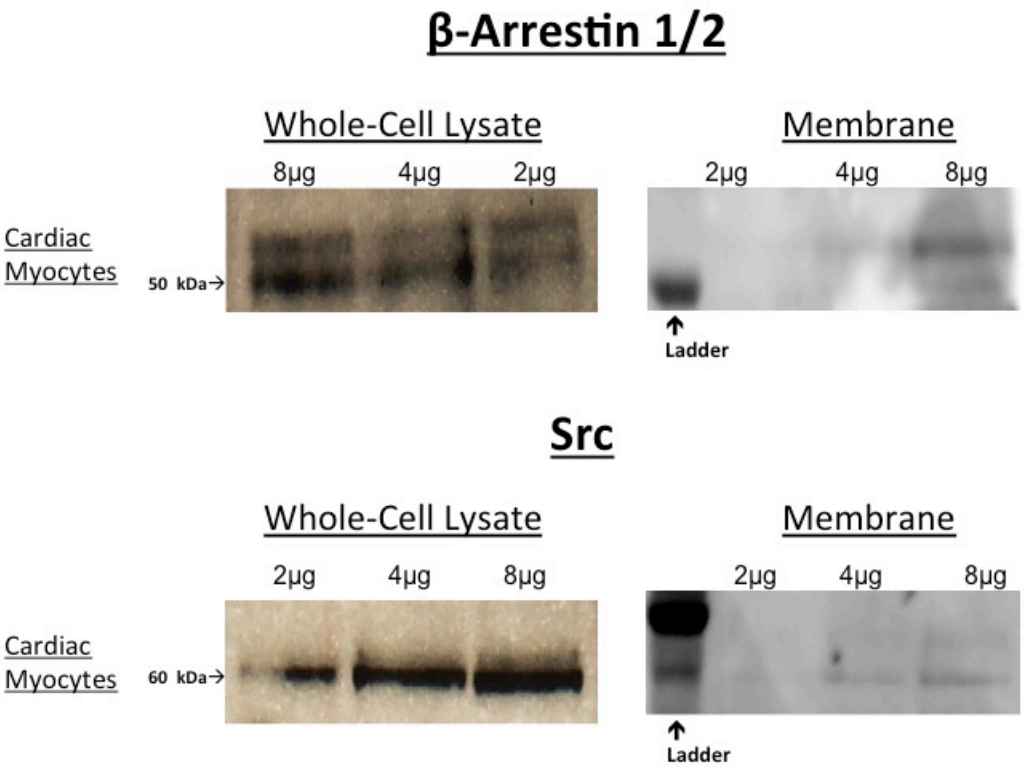

Figure 4.5. Protein expression of ß-Arrestin 1/2 and Src ß-Arrestin 1/2 and Src expression in lysates and membranes from rat cardiac myocytes.

Chapter 5. GPCR agonist-mediated EGFR transactivation

In order to assess EGFR activation, I probed for pEGFR via Western blot and quantified results using densitometry with normalization to total EGFR. HeLa membranes and HeLa lysates were treated with recombinant human EGF (100 nM, 10 min on ice) as a positive control (Figure 4.1). I sought to induce EGFR transactivation by adding the GPCR agonist bradykinin (Figure 4.2). Bradykinin (BK) was added to HeLa membranes and lysates at a concentration of 100 nM for 10 min on ice.

pEGFR expression increased following treatment with EGF and bradykinin compared to untreated samples. However, the increases in the membranes were unexpectedly lower than the increases seen in the whole cell lysates. I attribute this to the fact that the tyrosine kinase activity of EGFR uses ATP as a substrate for phosphorylation, and that isolated cell membranes lack the ATP stores present in the whole cell. To compensate for the ATP deficit, I added ATP (30 μM) to the membrane samples. Membrane samples were also treated at 37˚ C to mimic physiologic conditions. These two factors increased pEGFR in the membranes compared to the whole cell lysates. Therefore, membranes were treated at 37˚C with ATP (30 μM) added for all subsequent experiments.

In order to optimize EGF-mediated increases in pEGFR, I ran an EGF time-course. Membranes were treated at 37˚ C and 100 nM for 1 minute, 5 minutes, 10 minutes, and 30 minutes, respectively (Figure 5.3). I observed the highest expression of

pEGFR following the 5-minute pre-treatment. Thus, HeLa cell membranes were treated with EGF for 5 minutes for all subsequent experiments.

In addition to observing EGFR activation, I also tested for the inhibition of the receptor phosphorylation by measuring decreases in pEGFR expression in response to treatment with several inhibitors. One of the inhibitors I used was Tyrphostin AG-1478, a protein tyrosine kinase (PTK) inhibitor specific for EGFR. AG-1478 was added to membrane samples for 20 minutes at 37˚ C, at a concentration of 2 μM (Figure 5.4). Peptide G, an inhibitor that specifically blocks activity of MT1-MMP, was also used (37). Peptide G was added to membrane samples for 20 minutes at 37˚ C, at a concentration of 250 μM. Pooled data from various Western blot experiments show that EGF and bradykinin significantly increase pEGFR expression compared to controls and that AG 1478 and Peptide G attenuate this bradykinin-induced increase. Although the pooled data for AG1478 and Peptide G are not statistically significant, they show a general trend towards inhibition. Additionally, previous studies in the lab have shown that treatment of the membranes with GTPγS, a non-hydrolyzable analog of GTP that activates the G Protein, leads to increased MT1-MMP activation. I added GTPγS to the isolated HeLa cell membranes at a concentration of 1 μM for 10 minutes at 37˚ C (Figure 5.4), but these results need to be optimized.

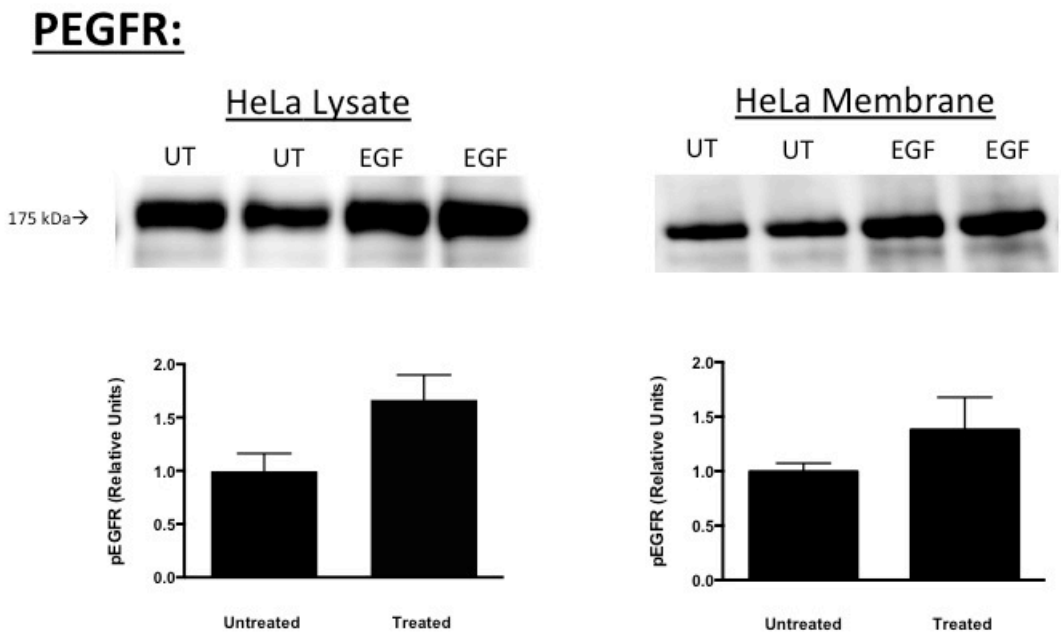

Figure 5.1. HeLa lysates and membranes treated with recombinant human EGF. Samples were treated for 10 minutes on ice at a concentration of 100 nM. Densitometry was performed on the pEGFR blot, and normalized to total EGFR.

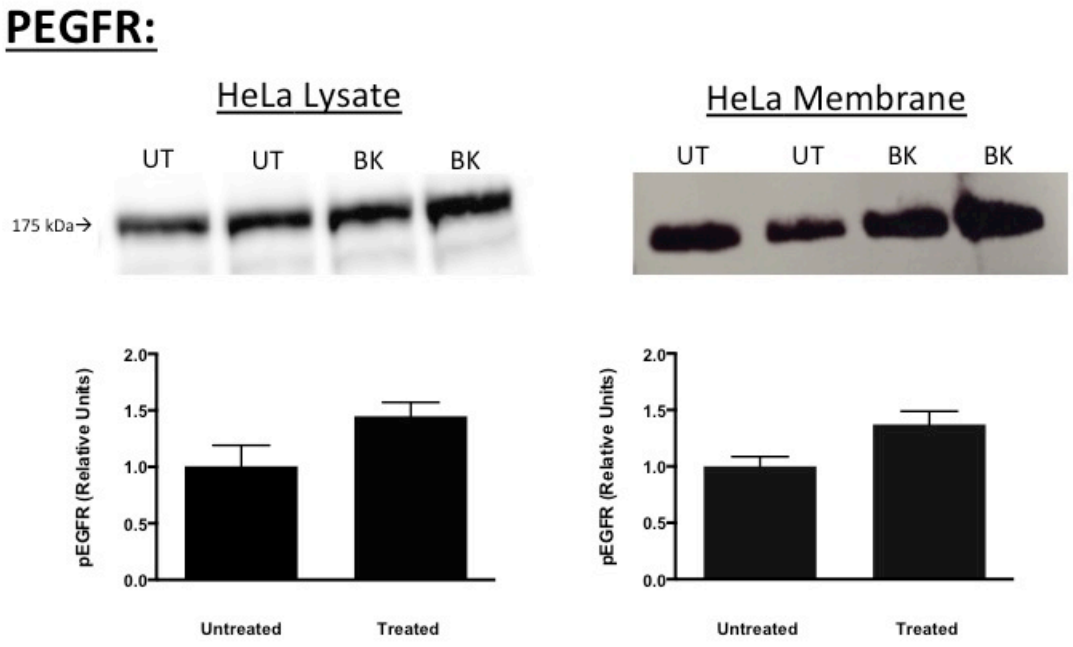

Figure 5.2. HeLa lysates and membranes treated with the GPCR agonist bradykinin. Samples were treated for 10 minutes on ice at a concentration of 100 nM. Densitometry was perfomed on the pEGFR blot and normalized to total EGFR

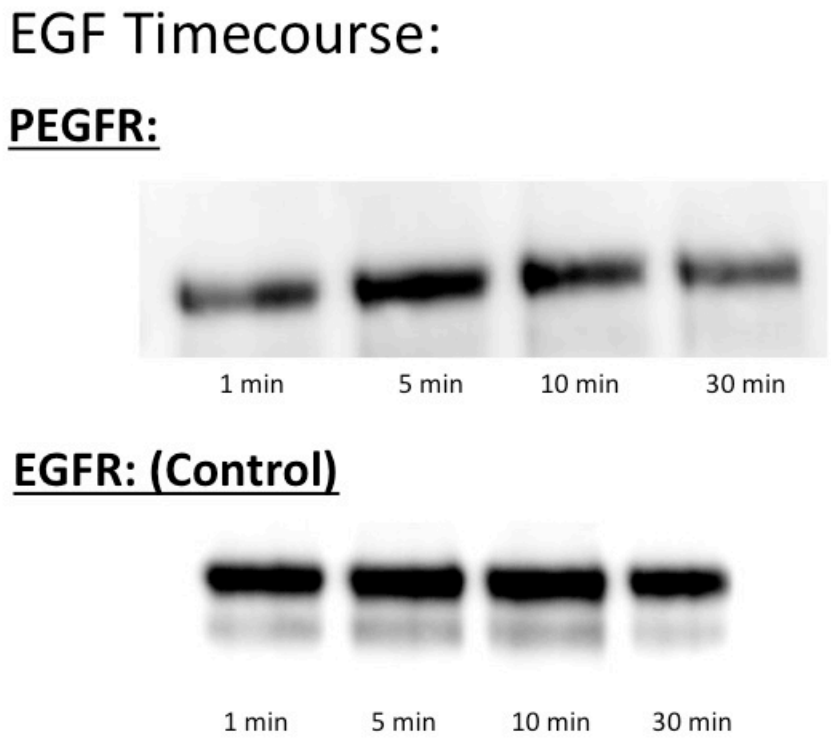

Figure 5.3. Time course of EGF phosphorylation. EGF was added for 1 minute, 5 minutes, 10 minutes, and 30 minutes at a concentration of 100 nM at 37˚ C. pEGFR showed the highest expression after the 5 minute EGF treatment time.

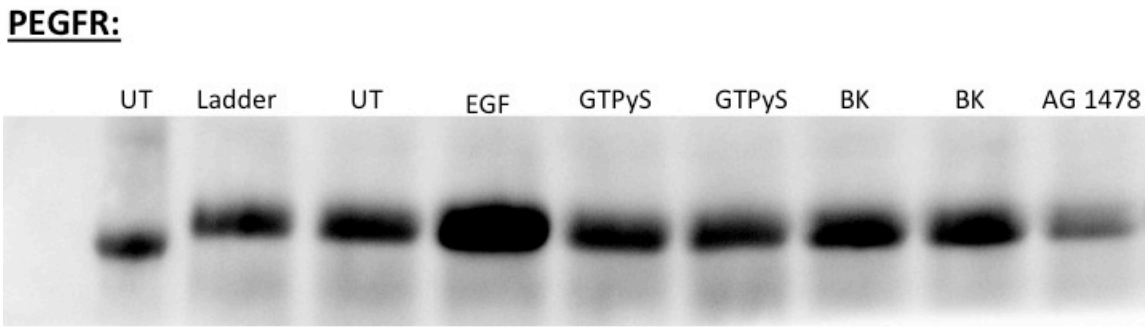

Figure 5.4. EGFR phosphorylation of **HeLa membranes treated with EGF, GTPγS, Bradykinin (BK), and the EGFR inhibitor AG 1478**. EGF was added for 5 minutes at 37˚ C at a concentration of 200 nM. GTPγS was added for 15 minutes at 37˚ C at a concentration of 200 μM. Bradykinin was added for 10 minutes at 37˚ C at a concentration of 100 nM. AG 1478 was added for 20 minutes at 37˚ C at a concentration of 2 μM, followed by treatment with GTPγS. pEGFR was measured using densitometry and normalized to total-EGFR.

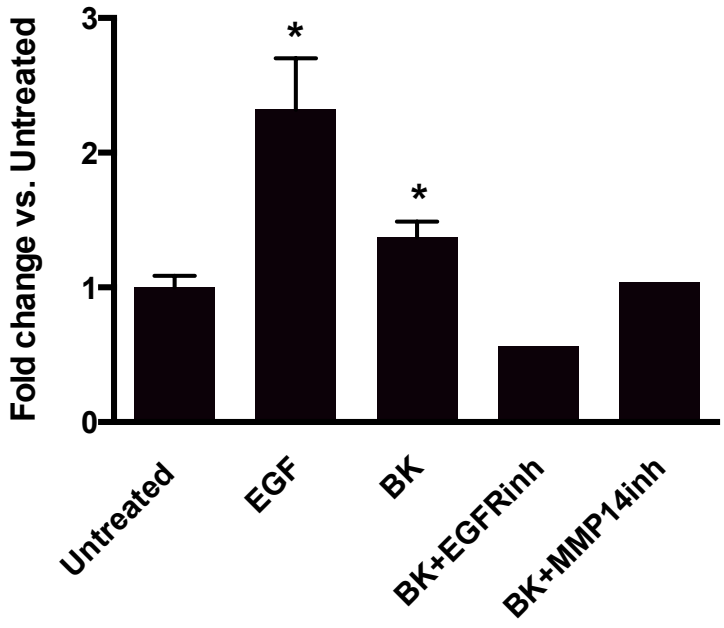

Figure 5.5. EGFR phosphorylation: **Pooled data of HeLa membranes treated with EGF, Bradykinin (BK), AG 1478, and Peptide G.** EGF was added for 5 minutes at 37˚ C at a concentration of 200 nM. Bradykinin was added for 15 minutes at 37˚ C at a concentration of 100 nM. AG 1478 and Peptide G were added for 20 minutes at 37˚ C at concentrations of 2 μM and 250 μM, respectively. Inhibition was followed by treatment with GTPγS. pEGFR was normalized to total EGFR for each treatment group. The fold differences compared to untreated are shown above. * $p < 0.05$; $n \geq 4$.

Chapter 6. Discussion

NIH 3T3 cells and HeLa cells are two cell lines that have been used extensively in many types of experiments. However, there are no published data regarding the GPCR expression profile of these two cell types. Taqman GPCR arrays provide an unbiased approach to assess overall expression of non-chemosensory GPCRs. Targeted GPCR arrays are also more useful to detect and quantify GPCRs than non-targeted arrays, because generic cDNA arrays tend to show many false-positives and false-negatives for GPCRs expressed at lower levels (38). Before choosing a GPCR to study further, a GPCR array allows the experimenter to first ask: what is the relative abundance of a particular GPCR? Although other experimental methods exist for identifying and quantifying GPCRs, such as functional assays and radioligand binding assays, they are biased approaches where one chooses a receptor of interest and ignores less well-known receptors that could be regulators of cell function and perhaps be novel therapeutic targets. For example, orphan GPCRs are a largely underappreciated class of receptors that could bring new insights into physiological mechanisms, disease states, and create new drug targets. In fact, of the 400 non-olfactory GPCRs encoded by the human genome, at least 100 GPCRs are still considered to be orphans (39). These orphan receptors represent an unexplored frontier of GPCR cell signaling and drug discovery.

Orphan GPCRs represented a large subset of the receptors detected in both the NIH 3T3 and HeLa cells: 57 orphans of the 208 GPCRs in NIH 3T3 cells (~27%), and 36 orphans of the 115 GPCRs in HeLa cells (~33%). Additionally, numerous GPCRs are

among the most highly expressed GPCRs in both cell types (Tables 3.1 and 3.7) and of the five most highly expressed orphan GPCRs in each cell type, the Class C Orphan, GPRC5A, was shared between NIH 3T3 and HeLa cells. GPRC5A may interact with retinoic acid, which plays a critical role in development, cellular growth, and differentiation (40). GPRC5A has been reported to be dysregulated in many human cancers, as well as other diseases, such as Chronic Obstructive Pulmonary Disease (COPD) (41). It is interesting that this receptor is the most highly expressed orphan GPCR in both cell types even though they originate from different species and different tissue types.

Of the most highly expressed Gi and Gq-linked receptors in NIH 3T3 and HeLa cells, only one receptor, protease-activated receptor 1 (PAR1, also known as F2R) is shared in the two cell types. Protease-activated receptors are highly expressed in platelets, endothelial cells, myocytes, and neurons (42). The PAR1 ligand, thrombin, is a key intermediate in the coagulation process. Because thrombin also plays an essential role in wound healing, perhaps it affects cell and tissue remodeling through our proposed mechanism. However, studies in our lab have thus far shown that addition of a synthetic analog of thrombin, Trap-6, does not increase MT1-MMP activity above basal levels (data not shown). While it is clear that thrombin plays a role in adverse cardiac remodeling (43), this effect may occur by a mechanism distinct from our proposed membrane-delimited (GPCR/MT1-MMP/Hb-EGF/EGFR) mechanism. The Bradykinin 2 receptor (BDKRB2), on the other hand, is highly expressed in NIH 3T3 cells (Table 3.4),

and while it was not highly expressed in our HeLa cell GPCR array, previous studies have shown that HeLa cells may also express the Bradykinin 2 Receptor (21).

Overall, Gi and Gq-linked receptors showed higher expression in both cell types compared to Gs and G12/G13-linked receptors. However, the cell lines both had relatively high expression of the two Gs-linked receptors, adenosine receptor 2B (ADORA2B) and ß-2 adrenoreceptor (ADRB2). ADORA2B affects bronchoconstriction (as seen in asthma) and vasodilation. Targeting adenosine receptors in the brain has been shown to help treat Alzheimer's disease, Parkinson's disease, drug addiction, and other pathologies (44). Adenosine receptors are also abundant in the heart, kidney, lungs, and immune system (45). Although the membrane-delimited mechanism of EGFR transactivation is thought to be Gi or Gq-linked (17), the high expression of ADORA2B in both cell types and its association to many pathological states highlight the adenosine 2B receptor as a receptor to assess for regulation of this mechanism in future research. Additionally, if EGFR transactivation is Gi-linked, perhaps Gs could have an inhibitory effect on EGFR transactivation, akin to their opposite effects on adenylyl cyclase activity.

The other shared Gs-linked receptor with high expression was the ß-2 adrenergic receptor. ß-adrenergic receptors mediate responses to norepinephrine (the neurotransmitter of the sympathetic nervous system) and epinephrine, (released into the circulation from the adrenal medulla), causing smooth muscle relaxation, increased heart contractility (inotropy), and increased glycogenolysis (46). Previous studies have shown

that activation of adrenergic receptors, such as ADRB2, can lead to activation of MAP kinase pathways (47), and that this occurs through post-receptor mechanisms, such as Src and ß-arrestin, as opposed to the membrane-delimited mechanism.

The two most highly expressed receptors in the G12/13 pathway for both cell types are PAR1, which was discussed above, and EDG2, also known as lysophospholipid receptor 1 (LPA1). LPA1 activation induces a range of cellular responses, including: cell proliferation and survival, cell migration, cytoskeletal changes, Ca^{2+} mobilization, and activation of MAP-K and Rho pathways, to name a few (21)

Taken together, the GPCR array data indicate that there is a good deal of overlap among the highly expressed G protein-coupled receptors in mouse 3T3 and human HeLa cell lines. This is significant because it shows that endogenous GPCRs have remained largely unchanged in the 50-60 million years since the two species diverged (48). Additionally, GPCR arrays provide an unbiased approach to define the relative abundance and differential expression of the non-chemosensory GPCRs in different cell types. The array data highlight the fact that orphan receptors make up a large percentage of the expressed GPCRs; de-orphanizing those receptors could provide further insight into their roles in physiology and disease states. The GPCR expression profiles of NIH 3T3 and HeLa cells can be used to evaluate the role of newly identified receptors in the function of those cells and perhaps as a means to identify those receptors as potential

therapeutic targets. As such, the GPCR expression data complement the large body of knowledge already collected on these two cell lines.

While finding the expression of previously unrecognized GPCRs is an important result, this finding only represents a piece of the puzzle. A receptor gains significance when its downstream signaling pathway becomes elucidated and it is incorporated into an identified mechanism. GPCR arrays were one of many tools I used to search for a GPCR pathway target in our proposed signaling mechanism.

Although previous studies have shown that G protein-coupled receptors can induce EGFR transactivation through an intracellular second-messenger (2) (18), we sought to show that EGFR transactivation can also occur in a direct, membrane-delimited manner. Other data from our laboratory have suggested that agonist stimulation of GPCRs causes direct activation of MT1-MMP via a pertussis-toxin sensitive (i.e., Gi/o-dependent) mechanism that may involve the $G_{\beta\gamma}$ dimer (19). Once activated, MT1-MMP cleaves the membrane-anchored proHb-EGF, releasing it as soluble Hb-EGF. Hb-EGF is then able to bind to EGFRs and initiate downstream signaling pathways that affect growth, differentiation, proliferation, and modulation of apoptosis.

I assessed the protein expression of Src and ß-arrestin in the lysates and membranes of rat cardiac myocytes since Src and ß-arrestin are components implicated in the triple-membrane-passing mechanism for transactivation. ß-arrestin was initially discovered based on its role in desensitization of GPCRs (36). However, more recent

studies have shown that ß-arrestin can serve as a scaffolding protein for numerous signaling networks. Upon GPCR activation, ß-arrestin is recruited to the plasma membrane and serves as a scaffold for the activation of MAP kinases, such as ERK, JNK, or p38, which affect cell cycle progression, transcriptional regulation, and apoptosis (36). ß-arrestin can also recruit Src, a non-receptor tyrosine kinase, to the plasma membrane, leading to the activation of ERK (47). My results indicate that while Src and ß-Arrestin are present in whole cell lysates, they are absent from the isolated cell membranes of rat cardiac myocytes. This result implies that neither ß-Arrestin nor Src are involved in our membrane-delimited mechanism for EGFR transactivation. Other data show that a Src inhibitor does not block the activation of MT1-MMP activity by GTPγS (19).

I found that the signaling components MT1-MMP, Hb-EGF, EGFR, and pEGFR all localize to the plasma membranes of the cells I studied (NIH 3T3 cells, HeLa cells, and rat cardiac myocytes). These signaling components were also present in the whole cell lysates, although they showed relatively lower expression compared to that seen in the isolated cell membranes. Those results thus help validate my hypothesis that MT1-MMP, Hb-EGF, EGFR, and pEGFR all localize to the plasma membrane. Upon careful examination of baseline protein expression in the three cell types, I found the cleanest and highest expression of these signaling components in HeLa cells.

After observing baseline expression of the signaling molecules in the isolated cell membranes, I tested whether GPCR agonist stimulation results in EGFR activation. I used recombinant EGF as my positive control for the activation of the receptor tyrosine

kinase and treated HeLa membrane samples with bradykinin, a known GPCR agonist that mediates inflammation and the mechanism of pain. I found that treatment of membranes with either EGF or bradykinin increased the level pEGFR. The EGF- and bradykinin-promoted increases in pEGFR were not initially as robust in the isolated membranes as in the whole-cell lysates, a finding that I found resulted, at least in part, from a lack in the membranes of ATP, a substrate for the receptor kinase. I thus subsequently added 30 μM ATP to the membranes to compensate for this ATP deficit. I also conducted subsequent experiments with membranes at 37˚ C, in order to mimic physiological conditions. Both of these changes increased the EGF- and bradykinin-promoted activation of EGFRs in the isolated cell membranes.

In addition to the EGFR activation experiments, I measured inhibition of EGFR activation by adding several pathway inhibitors. I tested AG 1478, an inhibitor of EGFR, as a positive control, and Peptide G, an inhibitor of MT1-MMP. Although the pooled data for the inhibitors is not statistically significant, the general trend indicates that the inhibitors attenuate bradykinin-induced increases in EGFR activation, thus implying that the agonist response can be blocked by the addition of an inhibitor.

Taken together, these results provide new information regarding the GPCRs that are expressed by two well-known cell types, and new evidence to support the hypothesis that GPCR activation can lead to EGFR transactivation via MT1-MMP in a membrane-delimited manner. The signaling components of this mechanism-- MT1-MMP, Hb-EGF, EGFR, and pEGFR (Figure 2)--localize to the plasma membrane, while Src and ß-arrestin

are absent from the isolated cell membranes. The GPCR agonist, bradykinin, increases EGFR activation, which is attenuated by the EGFR inhibitor AG 1478 and the MT1-MMP inhibitor Peptide G. Because GPCR-mediated EGFR transactivation has been associated with various functional responses and disease states, such as cardiovascular disease and cancer, further understanding of this mechanism may help elucidate aspects of cellular regulation and new ways to treat abnormal response in disease settings.

Chapter 7. Future Directions

To enhance our current understanding of this membrane-delimited mechanism of GPCR-mediated EGFR transactivation, additional experiments are required. For example, one could test other GPCR agonists (besides bradykinin) in isolated membranes to determine whether other agonists can activate EGFR in this novel signaling mechanism. Possible agonists include: isoproterenol, angiotensin II, phenylephrine, histamine, LPA, and adenosine. Additionally, Overland and Insel have shown that adding GTPγS (a non-hydrolyzable analog of GTP) to isolated cell membranes leads to increased MT1-MMP activity (19). Future experiments could test if addition of GTPγS to isolated cell membranes affects EGFR activation by GPCR agonists, an effect observed in the interaction of GTP and GTP analogs in activating other effectors of heterotrimeric G proteins (for example, adenylyl cyclases and phospholipase C-β). Preliminary studies have been conducted on this topic but need to be optimized.

Additional experiments with the inhibitors AG 1478 and Peptide G a need to be employed to make the general trend of decreased EGFR activation statistically significant and to confirm this result with other inhibitors and approaches (such as in membranes that have knockdown of MT1-MMP). Also, isolated membranes need to be treated with the inhibitors alone, to see if EGFR activation decreases below baseline levels. In addition to the aforementioned inhibitors, inhibitors such as Gallein (an inhibitor of G ß/γ) could be employed to see if inhibition of G ß/γ decreases EGFR activation.

Finally, because many experiments were conducted in NIH 3T3 cells, HeLa cells, and rat cardiac ventricular myocytes, one could run a GPCR array on the myocytes as well. It would be interesting to see if the receptors shared between the NIH 3T3 cells and the HeLa cells were also present in the rat cardiac myocytes. However, rat cardiac myocytes are primary cells that may have different patterns of GPCR expression than do the cell lines from other tissues and species.

Taken together, significant strides have been made in elucidating the mechanisms of membrane-delimited EGFR-transactivation, but much remains unknown. Further identification of the regulators in this signaling module could provide new insights regarding cell regulation and potentially could identify therapeutic targets for diseases such as cancer and cardiovascular disease.

Chapter 8. Appendix

8.1. Expressed GPCRs in NIH 3T3 Cells:

	GPCR:	**Delta CT:**	**Class/Linkage:**
1	F2R	13.73	Gi, G12/13, Gq
2	SMO	13.88	Gi/Go, G12/G13
3	EDG2	14.55	Gi/Go, Gq/G11, G12/G13
4	LPHN2	14.73	Adhesion Class
5	GPR137B	15.02	Orphan
6	GPRC5A	15.23	Class C Orphan
7	GRM8	15.67	Gi/Go
8	GPRC5B	15.86	Class C Orphan
9	GPR124	16.18	Adhesion Class
10	LENG4	16.20	Unknown
11	FZD1	16.24	Wnt-Signaling
12	GPR125	16.25	Adhesion Class
13	GPR137	16.74	Orphan
14	GABBR1	16.92	Unknown
15	HTR7	16.96	Gs
16	HTR1b	17.03	Gi/Go
17	EDG4	17.08	Gi/Go, Gq/G11, G12/G13
18	FZD7	17.08	Gs, Gi/Go
19	LPHN1	17.16	Gq/G11
20	TM7SF3	17.19	Gi/Go
21	P2RY5	17.20	Gs, Gi/Go, G12/G13
22	Gpr161	17.52	Class A Orphan
23	FZD2	17.65	Gi/Go
24	GPR108	17.80	Orphan
25	ADORA2B	17.80	Gs
26	BDKRB2	18.19	Gs, Gi/Go, Gq/G11
27	GPR153	18.27	Class A Orphan
28	ADRA1B	18.53	Gq/G11
29	RAMP2	18.56	Unknown
30	GPR135	18.57	Class A Orphan
31	ADRA2C	18.61	Gi/Go
32	P2RY2	18.61	Gq/G11
33	LTB4R2	18.80	Gi/Go
34	SSTR1	18.85	Gi/Go
35	MRGPRF	18.89	Class A Orphan

36	HRH2	19.04	Gq/G11
37	GPR63	19.07	Orphan
38	GPR56	19.08	Gq/G11, G12/G13
39	GPR4	19.10	Gs, Gi/Go, Gq/G11, G12/G13
40	GPR39	19.20	Gq/G11
41	MTNR1A	19.25	Gi/Go
42	GPR123	19.30	Adhesion Class
43	RXFP3	19.30	Gi/Go
44	CXCR3	19.31	Gi/Go
45	FZD10	19.45	Gi/Go
46	CCR2	19.47	Gi/Go
47	GPR75	19.53	Gq/G11
48	GPR62	19.56	Orphan
49	EDG6	19.57	Gi/Go, G12/13
50	FZD3	19.59	Gs
51	RXFP4	19.66	Gi/Go
52	ADRB1	19.67	Gs
53	MC4R	19.67	Gs
54	GPR175	19.68	Orphan
55	GPR44	19.72	Gi/Go
56	PPYR1	19.79	Gi/Go
57	HRH1	19.80	Gq/G11
58	GPBAR1	19.82	Gs
59	GPR81	19.85	Gi/Go
60	GPR61	19.89	Orphan
61	MC1R	19.93	Gs
62	CHRM4	20.13	Gi/Go
63	MRGPRA2	20.15	Unknown
64	BDKRB1	20.15	Gi/Go, Gq/G11
65	ADRA2B	20.17	Gi/Go
66	FPR1	20.20	Gi/Go
67	AGTRL1	20.20	Gi/Go
68	GPR6	20.24	Gs, Gi/Go
69	FZD9	20.24	Wnt-Signaling
70	CHRM3	20.29	Gq/G11
71	GPR82	20.33	Orphan
72	GPR119	20.37	Class A Orphan
73	CNR1	20.41	Gi/Go
74	CCR3	20.44	Gi/Go
75	PTAFR	20.48	Gi/Go, Gq/G11
76	C5AR1	20.50	Gi/Go
77	CX3CR1	20.55	Gi/Go
78	GALR2	20.58	Gq/G11

79	GPR77	20.65	Orphan
80	GPR85	20.67	Orphan
81	GPR87	20.69	Orphan
82	MRGPRG	20.70	Class A Orphan
83	GPR152	20.73	Orphan
84	GPR35	20.73	Orphan
85	GPR21	20.75	Orphan
86	P2RY6	20.77	Gq/G11
87	GPR45	20.78	Orphan
88	MRGPRA4	20.79	Unknown
89	CCR8	20.80	Gi/Go
90	MTNR1B	20.81	Gi/Go
91	GPR92	20.88	Gq/G11, G12/G13
92	SSTR3	20.93	Gi/Go
93	GPR20	20.93	Class A Orphan
94	CHRM1	20.95	Gq/G11
95	GPR173	20.98	Orphan
96	FZD8	21.01	Wnt-Signaling
97	TAAR1	21.05	Gs
98	IL8RB	21.05	Gi/Go
99	XCR1	21.12	Gi/Go
100	CCR9	21.16	Gi/Go
101	FPR	21.17	Unknown
102	ADMR	21.19	Class A Orphan
103	LGR6	21.23	Class A Orphan
104	ADRB2	21.23	Gs
105	HTR1A	21.24	Gi/Go
106	MRGPRB5	21.31	Unknown
107	MAS1	21.32	Gi/Go, Gq/G11
108	GPR149	21.37	Orphan
109	CMKOR1	21.40	Class A Orphan
110	GPR176	21.41	Orphan
111	GPR3	21.44	Gs
112	GPR151	21.48	Class A Orphan
113	TAAR9	21.52	Class A Orphan
114	FPR-RS2	21.53	Gi/Go
115	SSTR5	21.57	Gi/Go
116	FPR-RS7	21.57	Unknown
117	EDG1	21.60	Gi/Go
118	CHRM5	21.64	Gq/G11
119	CCR1	21.64	Gi/Go
120	TACR2	21.67	Gs, Gq/G11
121	MC2R	21.68	Gs
123	GPR1	21.71	Class A Orphan

124	GPR146	21.73	Class A Orphan
125	MC3R	21.80	Gs
126	GPR34	21.81	Gi/Go
127	MRGPRB2	21.82	Orphan
128	GPR109A	21.82	Gi/Go
129	P2RY13	21.83	Gi/Go
130	CCR4	21.87	Gi/Go
131	CMKLR1	21.88	Gi/Go
132	GPR132	21.88	Class A Orphan
133	PRlHR	21.90	Unknown
134	DRD1A	21.92	Gs
135	GPR30	21.98	Gq/G11
136	CCRL1	22.00	Gi/Go
137	FPRL1	22.02	Gi/Go
138	GPR55	22.10	Gq/G11, G12/G13
139	NPY6R	22.11	Unknown
140	GPR150	22.13	Orphan
141	CHRM2	22.15	Gi/Go
142	LTB4R1	22.16	Gi/Go, Gq/G11
143	FPR-RS6	22.23	Unknown
144	GPR162	22.26	Orphan
145	NPBWR1	22.34	Gi/Go
146	EBI2	22.37	Gi/Go
147	Celsr1	22.38	Adhesion Class
148	GPR65	22.39	Gs
149	DARC	22.49	Unknown
150	EDG3	22.54	Gi/Go, Gq/G11, G12/G13
151	GPR12	22.55	Class A Orphan
152	CCR6	22.70	Gi/Go
153	MRGPRX2	22.72	Gi/Go, Gq/G11
154	DRD5	22.73	Gs
155	GPR68	22.78	Gi/Go, Gq/G11
156	CXCR6	22.85	Gi/Go
157	MRGPRE	22.86	Class A Orphan
158	KISS1R	22.99	Gq/G11
159	GPR84	23.01	Gi/Go
160	GPR116	23.07	Adhesion Class
161	GPR19	23.08	Orphan
162	GPR171	23.15	Orphan
163	CALCRL	23.29	Unknown
164	GPR23	23.29	Gs, Gi/Go, Gq/G11, G12/G13
165	HTR1D	23.31	Gi/Go
166	P2RY14	23.38	Gi/Go
167	NPY2R	23.44	Gi/Go

168	CCRL2	23.50	Unknown
169	EDG8	23.52	Gi/Go, G12/G13
170	MRGPRD	23.58	Gi/Go, Gq/G11
171	BAI1	23.66	Adhesion Class
172	MRGPRB4	23.76	Unknown
173	NPY5R	23.80	Gi/Go
174	P2RY4	23.83	Gq/G11
175	AVPR2	23.84	Gs
176	P2RY10	23.87	Class A Orphan
177	AGTR1B	23.98	Gi/Go, Gq/G11
178	MRGPRA8	23.99	Unknown
179	FFAR1	24.09	Gq/G11
180	GPR18	24.12	Gi/Go
181	FFAR2	24.12	Gq/G11
182	GPR141	24.15	Orphan
183	LPHN3	24.25	Adhesion Class
184	RHO	24.38	Unknown
185	GPR114	24.44	Adhesion Class
186	CYSLTR1	24.55	Gq/G11
187	SUCNR1	24.59	Gi/Go, Gq/G11
188	GPR41	24.60	Orphan
189	OXTR	24.67	Gq/G11
190	GPR33	24.79	Gi/Go
191	HTR1F	24.84	Gi/Go
192	GPR88	24.85	Orphan
193	AGTR1A	24.87	Gi/Go, Gq/G11
194	MRGPRA5	24.95	Unknown
195	GIPR	25.04	Gq/G11
196	MRGPRB3	25.27	Unknown
197	OXGR1	25.33	Gq/G11
198	PTHR2	25.54	Unknown
199	MRGPRA3	25.76	Unknown
200	C3AR1	26.01	Unknown
201	CYSLTR2	26.54	Gq/G11
202	MRGPRB1	27.12	Unknown
203	P2RY12	27.47	Gi/Go
204	GPR27	27.49	Gs
205	PTGDR	27.63	Gs
206	GPR128	27.71	Adhesion Class
207	GPR112	27.90	Adhesion Class
208	FZD6	28.20	Gi/Go, Gq/G11

8.2. Expressed GPCRs in HeLa cells:

	Name:	Delta CT:	Class/Linkage:
1	GPRC5A	13.36	class C orphan
2	CD97	13.39	class B orphan
3	PGK1	13.81	
4	GPR56	14.08	class B orphan
5	LANCL1	14.09	
6	RARS	14.15	
7	FZD6	14.32	G protein independent mechanism
8	SSTR1	14.56	$G_{i/o}$
9	LGR4	14.96	class A orphan
10	SENP3	15.09	
11	GPR153	15.22	class A orphan
12	PHGDH	15.31	
13	C11ORF4	15.31	Other 7TM proteins
14	TBXA2R	15.86	$G_{q/11}$
15	FZD2	15.88	$G_{i/o}$
16	OPN3	16.01	class A orphan
17	GPR126	16.04	class B orphan
18	F2R	16.20	$G_{q/11}/G_{i/o}/G_{12/13}$
19	GPR125	16.21	class B orphan
20	FZD5	16.27	
21	HPRT1	16.47	
22	LONPL	16.55	
23	GPR	16.91	class A orphan
24	HTR1D	16.94	$G_{i/o}$
25	GUSB	17.12	
26	EMR2	17.13	class B orphan
27	FZD4	17.19	FZD_4/canonical WNT signalling
28	FZD1	17.39	FZD/beta-catenin signalling
29	EDNRA	17.48	$G_{q/11}$
30	ADORA2B	17.53	G_s
31	VIPR1	17.60	G_s
32	GPR124	17.65	class B orphan
33	BAI2	17.79	class B orphan
34	LANCL2	17.98	
35	HDAC3	17.99	
36	GPR37	18.05	$G_{i/o}$
37	LPHN1	18.20	class B orphan
38	F2RL1	18.34	$G_{q/11}/G_{i/o}$
39	PTGER2	18.42	G_s
40	GPR75	18.52	Orphan
41	MATK	18.54	
42	HMBS	18.75	
43	OXTR	18.81	$G_{q/11}/G_{i/o}$

44	GPR135	18.82	class A orphan
45	FZD3	18.87	
46	EDG2	19.00	$G_{q/11}/G_{i/o}/G_{12/13}$
47	CELSR3	19.00	class B orphan
48	CELSR1	19.14	class B orphan
49	FZD8	19.18	
50	ADRB2	19.26	$G_s/G_{i/o}$
51	FZD7	19.33	
52	GPR161	19.36	class A orphan
53	ADRA1B	19.44	$G_{q/11}$
54	GPR92	19.55	$G_{q/11}/G_{12/13}$
55	VIPR2	19.58	G_s
56	GPR173	19.64	class A orphan
57	EBI2	19.66	$G_{i/o}$
58	ADRB1	19.73	$G_s/G_{i/o}$
59	EDG3	19.80	$G_{q/11}/G_{i/o}/G_{12/13}$
60	GPR160	19.81	class A orphan
61	GPR39	20.07	$G_{q/11}/G_s/G_{12/13}$
62	LTB4R2	20.11	
63	TACR1	20.23	$G_s/G_{q/11}$
64	GPR63	20.25	class A orphan
65	GPR22	20.33	$G_{i/o}$
66	GPR21	20.39	$G_{q/11}$
67	C5R1	20.77	$G_{q/11}/G_{i/o}$
68	GPR7	20.92	$G_{i/o}$
69	GPR115	20.97	class B orphan
70	MC1R	20.97	G_s
71	NPY1R	20.99	$G_{i/o}$
72	PTGER1	21.00	$G_{i/o}/G_{q/11}$
73	LGR6	21.04	class A orphan
74	P2RY2	21.05	$G_{q/11}/G_{i/o}/G_{12/13}$
75	P2RY11	21.28	$G_{q/11}/G_s$
76	GPR150	21.31	class A orphan
77	LGR5	21.34	class A orphan
78	GPR133	21.35	class B orphan
79	CELSR2	21.40	class B orphan
80	GPR20	21.42	$G_{i/o}$
81	GPR82	21.45	class A orphan
82	CCR10	21.47	$G_{i/o}$
83	GPR147	21.50	$G_{i/o}$
84	ELTD1	21.51	class B orphan
85	VN1R1	21.51	
86	LGR7	21.58	$G_s/G_{i/o}$
87	CCR1	21.81	$G_{i/o}$

88	EDG8	21.95	$G_{i/o}/G_{12/13}$
89	GPR52	22.10	class A orphan
90	CYSLTR1	22.12	
91	FZD9	22.13	
92	GPR146	22.19	class A orphan
93	PTGFR	22.22	$G_{q/11}/G_s$
94	FZD10	22.29	
95	LTB4R	22.33	
96	PPYR1	22.50	$G_{i/o}/G_{q/11}$
97	CHRM2	22.51	$G_s/G_{q/11}/G_{i/o}$
98	CHRM4	22.64	$G_{i/o}$
99	CHRM1	22.70	$G_{q/11}$
100	GPR3	22.74	class A orphan
101	GALR2	22.77	
102	GPR1	23.01	class A orphan
103	EDG4	23.03	$G_{q/11}/G_{i/o}/G_{12/13}$
104	NTSR2	23.13	$G_{q/11}$
105	CHRM3	23.15	$G_{q/11}$
106	GPRC5B	23.26	class B orphan
107	NPY6R	23.79	
108	GPR34	23.91	$G_{i/o}$
109	GIPR	24.13	G_s
110	ADRA2A	24.27	$G_{i/o}$
111	GPR152	24.69	class A orphan
112	HCRTR1	25.22	$G_{q/11}/G_s/G_{i/o}$
113	GPR171	26.18	class A orphan
114	LPHN3	26.20	class B orphan
115	ADCYAP1R	26.25	G_s

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