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THE ROLE OF PARATHYROID HORMONE 1 RECEPTOR (PTH1R) SIGNALING IN GASTRIC EPITHELIAL HOMEOSTASIS

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THE ROLE OF PARATHYROID HORMONE 1 RECEPTOR (PTH1R) SIGNALING IN GASTRIC EPITHELIAL HOMEOSTASIS

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Molecular Biology and Biotechnology

Under the Supervision of Dr. Asma Al-Menhali

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Declaration of Original Work

I, Maram Maher Al-Hasan, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*The Role of Parathyroid Hormone 1 Receptor (PTH1R) Signaling in Gastric Epithelial Homeostasis*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Asma Al Menhali, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

In the stomach, the epithelial stem cells are responsible for glandular homeostasis. These stem cells continuously undergo cellular proliferation and differentiation to balance death of senescent cells. Studies conducted on gastric cancers showed significant increase in parathyroid hormone like hormone (PTHLH). Moreover, both PTHLH along with parathyroid hormone (PTH) act as endogenous ligands for parathyroid hormone 1 receptor (PTH1R) which belongs to family B of G- protein coupled receptors (GPCRs). This receptor functions by mediating many different signaling pathways and the most studied pathway is the stimulation of cyclic adenosine mono-phosphate (cAMP) as second messenger.

There are very little studies on understanding the normal function of gastric PTH1R. Specifically, how PTH1R and its ligands are associated with gastric cancer. The goal of this project is to investigate the expression of PTH1R in gastric epithelium, and to understand its possible function and signaling in maintaining gastric homeostasis. Firstly, gene expression analysis suggested that PTH1R is expressed *in vivo* in normal human stomach, and in mouse forestomach, corpus and antrum. *In vitro* studies on HeLa, and human embryonic kidney 293 cells (HEK293), also showed positive expression of PTH1R. However, mouse gastric epithelium progenitor cells (mGEP) and human gastric cancer cell line (AGS) showed no expression of PTH1R. To study the mechanism of PTH1R function, transfection of PTH1R plasmid was successfully conducted in mGEP and AGS cells. Next, we investigated the signaling pathways activated upon PTH1R stimulation. cAMP assay in transfected AGS cells treated with PTH1R agonist suggests activation of $G\alpha_s$ signaling pathway. On the other hand, transfected mGEP cells successfully activated ERK1/2 pathway. Morphological studies suggested difference in the cell morphology of transfected mGEP cells in which large cell to cell spaces was reported. Furthermore, cell viability results showed statistically a non-significant agonist and antagonist trend. Finally, several target genes were studied by Real-Time PCR. Low density lipoprotein receptor (*LDLR*) showed significant upregulation when comparing transfected and activated mGEP cells to control. However, calcium sensing receptor (*CaSR*), fibroblast growth factor 23 (*FGF23*), interleukin-6 (*IL-6*), and Na+/H+ exchanger regulatory factor 1 (*NHERF1*) did not show a significant change. This work highlights the importance of PTH1R in

the stomach which might open new perspectives in studying PTH1R signaling in gastric cancer patients. This might set new therapeutic modalities for gastric cancer, especially since PTH1R agonists are used in treatment of bone cancer and osteoporosis.

Keywords: Parathyroid hormone 1 receptor (PTH1R), parathyroid hormone like hormone (PTHLH), parathyroid hormone (PTH), gastric epithelium, stem cell.

Title and Abstract (in Arabic)

دور المستقبل PTH1R في توازن الخلايا المعدية

الملخص

خلايا بطانة المعدة هي المسؤولة عن الحفاظ على التوازن في المعدة. وتعد الخلايا الجذعية الموجودة في المعدة أساسية في تكاثر الخلايا وتمايزها. أظهرت الدراسات التي أجريت على سرطانات المعدة زيادة كبيرة في هرمون غدة الجار الدرقية مثل هرمون (PTHLH). علاوة على ذلك ، يعمل PTHLH مع هرمون الغدة الدرقية (PTH) كجزيئات لمستقبلات هرمون الغدة الدرقية (PTH1R) والتي تنتمي إلى عائلة B من مستقبلات البروتين (GPCRs). يعمل هذا المُسْتَقْبِل عن طريق التوسط في العديد من مسارات الإشارات المختلفة ، والمسار الأكثر دراسة هوالمسار المتعلق بتحفيز مسار cAMP كرسول ثاني.

هناك القليل من الدراسات حول فهم وظيفة PTH1R الطبيعية باللمعدة. على وجه التحديد ، كيف ترتبط PTH1R بسرطان المعدة. الهدف من هذا المشروع هو بحث تواجد PTH1R في أنسجة المعدة ، وفهم الوظيفة الممكنة للمستقبل PTH1R في الحفاظ على التوازن المعدي. يوحي تحليل التعبير الجيني بإستخدام PCR والكيمياء المناعية بأن PTH1R يتواجد في أجزاء معدة الفأر المختلفة. أشارت الدراسات المختبرية على الخلايا المعوية الفأرية (mGEP) و خلايا سرطان المعدة البشري (AGS) إلى عدم تواجد PTH1R. للتغلب على هذا التحدي تم إعداد خلايا mGEP و AGS والمعدلة وراثياً لتحتوي على مستقبلات ال PTH1R. ,وبالتالي تم تنشيط إشارات مسار ال cAMP في خلايا ال AGS و إشارات مسار ال ERK1/2 في خلايا mGEP. و بعد ذلك تَم قياس بقاء خلايا ال mGEP على قيد الحياة عند تعريض هذه الخلايا المعدّلة وراثياً للهرمون PTHrP(1-36) أو المُثبِت PTHrP(7-34). لوحظ زيادة طفيفة في قدرة الخلايا على البقاء على قيد الحياة بعد 24 ساعة من إستخدام PTHrP(1-36). كما تم دراسة تأثير PTHrP(1-36) على التعبير الجيني لعدد من الجينات مثل -*LDLR, CaSR, IL-6, NHERF1, FGF23*. تعد هذه الدراسة مهمة جدا في فهم دور المستقبل PTH1R الطبيعي في المعدة و قد يكون لها تأثير كبير في تطبيقات العلاج الكيميائي لمرضى السرطان.

مفاهيم البحث الرئيسية: المستقبلات, المعدة, خلايا بطانة المعدة, الخلايا الجذعية.

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Dedication

To my beloved parents, husband and daughter

Table of Contents

Title i
Declaration of Original Work ii
Copyright iii
Advisory Committee iv
Approval of the Master Thesis v
Abstract vii
Title and Abstract (in Arabic) ix
Acknowledgements x
Dedication xi
Table of Contents xii
List of Tables xiv
List of Figures xv
List of Abbreviations xvi
Chapter 1: Introduction 1
1.1 Overview 1
1.2 Stomach Anatomy 3
1.3 G- Protein Coupled Receptors (GPCRs) 6
1.4 Parathyroid Hormone 1 Receptor (PTH1R) 9
1.4.1 PTH1R Structure 10
1.4.2 Ligands 13
1.4.3 Major PTH1R Signaling Pathways 14
1.5 PTH1R in Health and Diseases 21
1.5.1 PTH1R in Stomach 21
1.5.2 PTH1R in Bones 21
1.5.3 PTH1R in Breast Cancer 23
1.5.4 PTH1R in Kidneys 24
1.6 PTH1R Mutations 25
1.7 PTH1R Target Genes 26
1.8 Thesis Objectives and Hypothesis 28
Chapter 2: Methods 30
2.1 Animals and Cells 30
2.2 RNA Isolation and Reverse-Transcription PCR 30
2.3 Histological analysis 31
2.3.1 Immunohistochemistry (IHC) 31
2.3.2 Immunocytochemistry (ICC) 32
2.4 Transformation and Transfection 32
2.5 Western Blot 34
2.6 cAMP Measurement 35
2.7 Cell Morphology Analysis 35
2.7.1 Cell Cytotoxicity 36

2.8 Quantitative Real-Time PCR 36
2.9 Statistical Analysis 36

Chapter 3: Results 40
3.1 PTH1R is Expressed in Human Stomach and Cell Lines. 40
3.2 PTH1R is Expressed in Normal Mouse Gastric Epithelial Tissues 41
3.3 The Transfection of mGEP and AGS Cell Lines with PTH1R Plasmid. 43
3.4 The Induction of cAMP in Transfected AGS Treated with PTH1R Agonist Suggests Receptor Activation via $G\alpha_s$ Signaling Pathway 45
3.5 No Induction of cAMP in Transfected mGEP Cells after PTHrP (1-36) Treatment 46
3.6 PTHrP (1-36) Caused Phosphorylation of ERK 1/2 in Transfected mGEP Cells 47
3.7 Effect of PTHrP (1-36) and PTHrP (7-34) on Transfected mGEP Cell Viability 49
3.8 Morphological Changes in mGEP Cells Treated with PTHrP (1-36) and PTHrP (7-34) 51
3.9 Expression of PTH1R Target Genes after PTHrP (1-36) Treatment 60

Chapter 4: Discussion 62

Chapter 5: Conclusion and Future Directions 67

References 71

List of Tables

Table 1.1: Classification of GPCRs classes and their functions 8
Table 1.2: Some PTH1R target genes. 29
Table 2.1: Oligonucleotides primers for the studied target genes 38

List of Figures

Figure 1.1: The mouse stomach anatomy 5
Figure 1.2: Diagram of a gastric gland from corpus (a), and antrum (b) 5
Figure 1.3: Human PTH1R structure 12
Figure 1.4: GPCR signaling pathways 19
Figure 1.5: GPCR endocytosis trafficking 20
Figure 2.1: Experimental design of the project 39
Figure 3.1: PTH1R mRNA is expressed in human stomach and other human cell lines 40
Figure 3.2: PTH1R gene is expressed in gastric tissue of wild type mouse 41
Figure 3.3: Immunohistochemical analysis of the corpus gastric gland showing PTH1R expression 42
Figure 3.4: mGEP cells expressing PTH1R after successful transfection 43
Figure 3.5: AGS cells expressing PTH1R after transfection 44
Figure 3.6: cAMP induction in PTH1R transfected AGS cells 45
Figure 3.7: No cAMP induction in PTH1R transfected mGEP cells 46
Figure 3.8: The activation of ERK1/2 pathway in PTH1R transfected mGE 48
Figure 3.9: Cellular viability of PTH1R transfected mGEP upon agonist and antagonist treatment for 24 and 48 hrs 50
Figure 3.10: Morphology of UT-mGEP and T-mGEP cells treated with PTHrP (1-36) after 6 hrs 52
Figure 3.11: Morphology of UT-mGEP and T-mGEP cells treated with PTHrP (1-36) after 12 hrs 53
Figure 3.12: Morphology of UT-mGEP and T-mGEP cells treated with PTHrP (1-36) after 24 hrs 54
Figure 3.13: Morphology of UT-mGEP and T-mGEP cells treated with PTHrP (1-36) after 48 hrs 55
Figure 3.14: Morphology of UT-mGEP and T-mGEP cells treated with PTHrP (7-34) after 6 hrs 56
Figure 3.15: Morphology of UT-mGEP and T-mGEP cells treated with PTHrP (7-34) after 12 hrs 57
Figure 3.16: Morphology of UT-mGEP and T-mGEP cells treated with PTHrP (7-34) after 24 hrs 58
Figure 3.17: Morphology of UT-mGEP and T-mGEP cells treated with PTHrP (7-34) after 48 hrs 59
Figure 3.18: Relative mRNA expression of PTH1R target genes in mGEP cells 61
Figure 5.1: Conclusive overall result model illustrating final findings 70

List of Abbreviations

AC	Adenylyl Cyclase
ATPase	Adenosine Triphosphate Enzyme
BMD	Bone Mineral Density
Ca^{2+}	Calcium Ion
cAMP	Cyclic Adenosine Monophosphate
CaSR	Calcium Sensing Receptor
CRM1	Chromosomal Maintenance 1
DNA	Deoxyribonucleic Acid
EB	Ezrin Binding Domain
ECL	Enterochromaffin-Like Cell
ERK1/2	Extracellular signal Regulated Kinases ½
FBS	Fetal Bovine Serum
F.S	Forestomach
FGF23	Fibroblast Growth Factor 23
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenases
GPCR	G-Protein Coupled Receptors
G-protein	Guanine Nucleotide Binding Protein
H+	Hydrogen Ion
H2	Histamine Receptor 2
HEK293	Human Embryonic Kidney Cells
HeLa	Henrietta Lacks Cells
HHM	Humoral Hypercalcemia of Malignancy
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IL-6	Interleukin-6

IP3	Inositol Triphosphate-3
JMC	Jansen's Metaphyseal Chondrodysplasia
ILDR	Low Density Lipoprotein Receptor
mGEP	Mouse Gastric Epithelial Progenitor Cells
mRNA	Messenger Ribonucleic Acid
NHERF1	Na+/H+ Exchanger Regulatory Factor 1
NLS	Nuclear Localization Sequence
OS	Osteosarcoma
OMIM	Online Mendelian Inheritance in Man
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
pERK1/2	Phosphorylated Extracellular Signal Regulated Kinases ½
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PTH	Parathyroid Hormone
PTH1R	Parathyroid Hormone 1 Receptor
PTH2R	Parathyroid Hormone 2 Receptor
PTHLH	Parathyroid Hormone Like Hormone
RNA	Ribonucleic Acid
RT-PCR	Real Time Polymerase Chain Reaction
T-mGEP	Transfected Mouse Gastric Epithelial Progenitor Cells
UT-mGEP	Untransfected Mouse Gastric Epithelial Progenitor Cells

Chapter 1: Introduction

1.1 Overview

Receptors in an organism are responsible for different cell responses. These communication elements are accountable for the cell to cell signal transmission over short or long distances across the body. Cells are equipped with unique protein structures located on their surface in order to read out the signals. Such signals lead to cell responses that can range from minute to major changes inside the cells. Receptors can be either activated by a specific signal to stimulate them, or can be inhibited by another signal. The stimulation or inhibition of the receptors can cause multiple signaling pathways to be activated; as such cells will be able to respond to this stimulus.

Parathyroid hormone 1 receptor (PTH1R) is a protein receptor found in multiple tissues of the body, dominantly in the kidney and bones [1,2], but its significant role in the stomach has not been fully identified [3]. PTH1R is a family B, G-protein receptor that contains three parts, an extracellular N terminal, seven transmembrane loops, and intracellular C terminal [2,4]. It has been reported that the main function of PTH1R is in bones and kidneys where it mediates extracellular Ca^{2+} homeostasis [3] and is important in bone growth and development [4]. In addition to these functions, PTH1R has been identified in the intestinal mucosa and in several carcinomas and adenocarcinomas [3,5].

PTH1R has two ligands, parathyroid hormone like hormone (PTHLH) or also known as parathyroid hormone related protein (PTHrP), as well as the parathyroid hormone (PTH). PTHLH is a hormone that plays a key role in bone formation and growth [6]. It stimulates calcium transport in many tissues, relaxes contracted smooth

muscles [7], and regulates various cellular events such as proliferation, differentiation and apoptosis [3]. However, it was also recognized and found to be highly expressed in hypercalcemia of malignancy, and many other types of cancers such as breast cancer [3,4,8]. Many studies were conducted and showed increased levels of PTHLH expression also in thyroid carcinomas [3,9], breast cancer [3], squamous cells tumors [3,8], and gastric cancers [3]. These studies highlighted the importance of PTHLH involvement in tumor proliferation and progression [3]. PTHLH is a complex hormone in which it can function as a paracrine, endocrine, and autocrine hormone [4]. It is well known that the PTHLH is expressed and found in the stomach [3]; however, very little research has been done on the expression of PTH1R in the stomach and its role in maintaining gastric homeostasis. The aim of this thesis is to investigate further the crucial function of PTH1R in gastric homeostasis.

The focus of this project will be mainly on studying the PTH1R expression and role in the gastric homeostasis. This will apply further knowledge on the regulation of this receptor in gastric cancers. To further understand the structure of this project's introduction, an overview of detailed stomach anatomy will be discussed first, followed by G- Protein Coupled Receptors (GPCRs) classification. Then, PTH1R with insights on its structure, ligands, and signaling pathways will be covered. Furthermore, PTH1R and its relation to health and multiple diseases associated with stomach, bones, breast cancer, and kidneys will be explained thoroughly followed by PTH1R mutations that cause diseases. Lastly, the importance of PTH1R in targeting specific genes and its relation in regulating system homeostasis will be discussed.

1.2 Stomach Anatomy

The stomach of the mouse is composed of three distinct parts, forestomach, corpus, and the antrum as shown in Figure 1.1. The forestomach is connected to the esophagus, the middle compartment is the corpus, and the antral distal part which connects the stomach to the small intestine is called antrum. The forestomach is a non-glandular tissue and is composed of stratified squamous epithelial cells. As for the corpus and antrum, they are glandular areas. Together, these two areas are responsible for secreting gastric mucins, hydrochloric acids, digestive enzymes, and hormones.

However, there are differences in the glands between the corpus and the antrum regions. The organization of these regions and glands is further illustrated in Figure 1.2. In the corpus, the glands are subdivided into four parts: pit, isthmus, neck, and base as illustrate in Figure 1.2a.

The isthmus region which contains mainly the progenitor cells, and as a result, it was noted to contain the highest mitotic activity [10]. The progenitor stem cells also have the ability to differentiate into other cells which can migrate to the top (pit) of the gland or in the lower part of the gland (neck or base). The parietal cells found mainly in the isthmus and neck are the source for gastric acid regulation. They secrete gastric acid which it plays an important role in food digestion. They can also produce several growth factors.

The pit, which is the top part of the gland, is an area that contains surface mucous cells that cover the lining of the stomach and secretes mucus to protect the stomach from strong acids and enzymes.

Below the isthmus region is the neck region which contains migrated parietal cells from isthmus, neck region also contains mucous neck cells, and Enterochromaffin-Like (ECL) endocrine cells which secrete histamine and can

stimulate parietal cells to secrete gastric acid. Lastly, the base region located at the very bottom of the gland, containing zymogenic cells which secrete pepsinogen. It is important to note that the origin of all these cells comes from gastric epithelial progenitor cells [11-13].

Moreover, the antrum gastric glands consist of surface mucous cells, progenitor cells, deep mucous cells, G cells which are endocrine cells secreting gastrin, and Lgr5+ stem cells as shown in details in Figure 1.2b [11,14,15]. The antrum gland contains highly proliferative regions below the pit region [10]. The gastric stem cells are responsible for maintaining the epithelial homeostasis inside the gland, due to their ability to differentiate and proliferate [11].

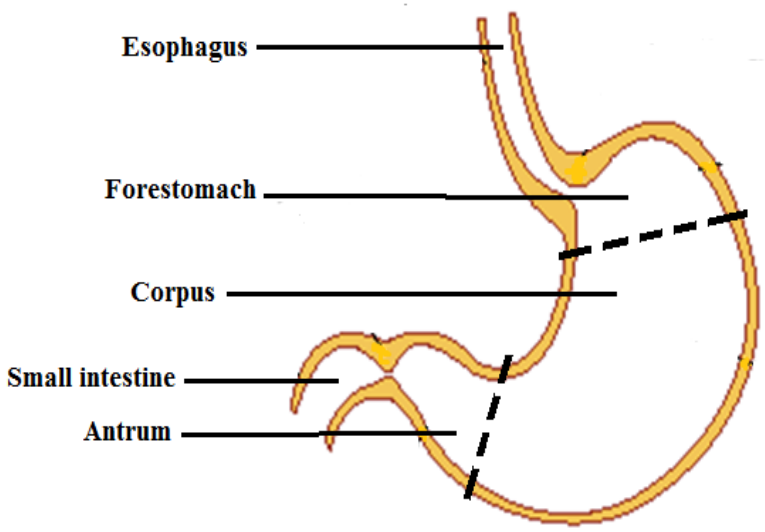


Figure 1.1: The mouse stomach anatomy

The mouse stomach is divided into three regions: forestomach, corpus and antrum. The esophagus connects to the forestomach, while the small intestine connects to the bottom part of the stomach called antrum. The mid-region of the stomach is called Corpus.

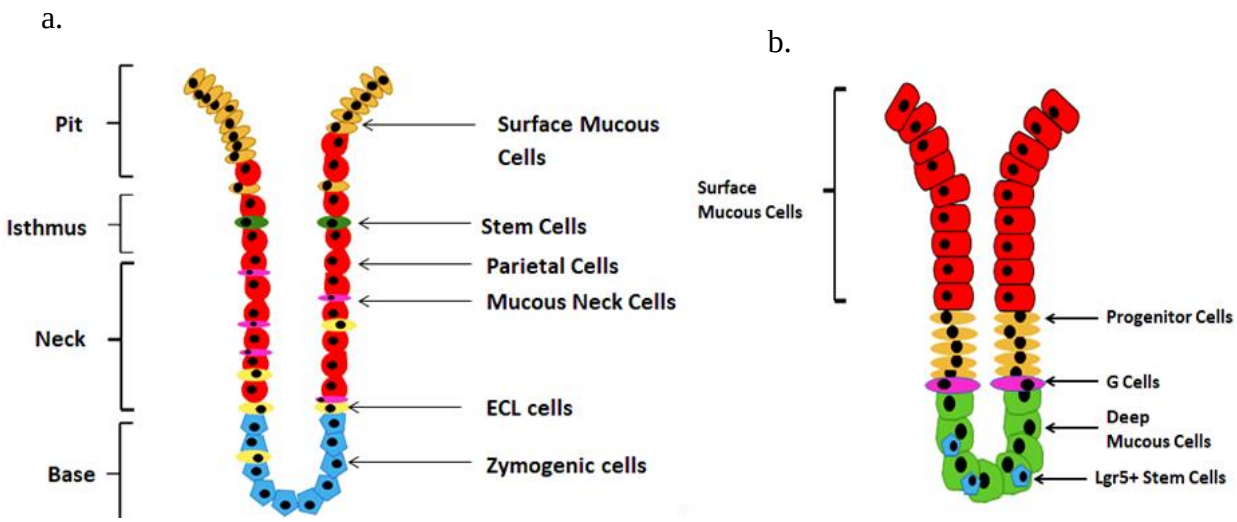


Figure 1.2: Diagram of a gastric gland from corpus (a), and antrum (b)

The corpus gastric gland is further divided into four regions; pit, isthmus, neck, and base. The antral gland is shorter and consists of Surface Mucous Cells also known as pit, and bottom portion known as base.

1.3 G- Protein Coupled Receptors (GPCRs)

GPCRs are the largest proteins in mammals and are categorized into six classes according to their structural and functional similarities. The six classes are; Class A, Class B, Class C, Class D, Class E, and Class F [16]. Table 1.1 lists the different classes, their names, and their biological functions and examples of each class. Class A named rhodopsin-like which is the largest group among the GPCRs. Class B named secretin-receptor family, which are regulated by peptide hormones. The Class B consists of glucagon receptors, parathyroid hormone receptor, and secretin receptors. Class C includes glutamate receptors and other receptors with similar structure. Class D, E, and F are important in stimulating multiple signaling pathways and regulate the activity of many genes [17].

GPCRs share a similar overall structure in which they contain an extracellular N terminal, seven hydrophobic transmembrane loops, and intracellular C terminal as shown in Figure 1.3. The N terminal is categorized as a site for ligand binding, while the seven transmembrane portions consist of alpha helices which alternate within the lipid bilayer membrane. The intracellular C terminal is bound to G-proteins inside the cell.

In the last three decades, GPCRs have been of growing interest due to their powerful actions in several biological events and pharmaceutical drug discoveries. Previous studies highlight the importance of GPCRs as biomarkers for early cancer diagnosis [16]. This illustrates the importance of GPCRs signaling target in drug discoveries, cancer treatments, cellular regulation and signal transduction.

The mechanism of action starts when the ligand binds to the receptor at a specific active site, this will cause the G-proteins to be activated and induce a conformational change. This will initiate the activation of series of biochemical

reactions within the cell. However, the active site in which the ligand binds to the receptor plays a critical role into which receptor signaling pathway to activate [17].

Upon the activation of GPCR, one of the three subtypes of heterotrimeric G-proteins located inside the cell will be activated, the three subunits are; Gα, Gβ, and Gγ as illustrated in Figure 1.4. These three subunits can further be classified into multiple subfamilies, for example Gα is additionally categorized as $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13.}$ These subfamilies will cause a cascade of further downstream protein activations and will therefore trigger a cellular response according to the ligands stimulus instruction [16].

Table 1.1: Classification of GPCRs classes and their functions

Class type		Functions	Examples
Class A	(Rhodopsin-like)	-Important in extracellular signals. -Most targeted class in pharmaceutical industry [18].	-C-C chemokine receptor type 1 -Angiotensin II -Melatonin receptors [18].
Class B	(Secretin receptor family)	-Activate adenylyl cyclase -Important drug targets in diabetes -large N-terminal extracellular domain[19].	Glucagon-like peptide 1 GLP-1 -Glucagon -PTH1R -Secretin [19].
Class C	(Metabotropic glutamate/ pheromone)	-Unique ligand recognition sites -Calcium sensing receptors -Involved in taste and odor receptors.[20].	-Metabotropic glutamate receptors (mGlu) - γ-aminobutyric acidB receptors (GABAB) - Ca2+- sensing receptors (CaSR) [20].
Class D	(Fungal mating pheromone receptors)	-Involved in the response to mating factors on the cell membrane[21].	- Pheromone a factor receptor Ste2, Ste3 [21].
Class E	(Cyclic AMP receptors)	-Important in regulating developmental genes.[22,23].	- cAMP receptor subtype (CAR1, CAR2, CAR3)[22,23].
Class F	(Frizzled/ Smoothened)	-Important in cell polarity, embryonic development, formation of neural synapses, and cell proliferation [24].	- Frizzled receptors - Smoothened receptor (SMO) [25].

1.4 Parathyroid Hormone 1 Receptor (PTH1R)

PTH1R has been categorized as a GPCR which was firstly discovered in 1991 in COS-7 kidney fibroblast like cells [26]. It was detected to be around 80kD in size and has two ligands PTH and PTHLH. Both of these ligands can bind to PTH1R at different sites and at different affinities which can activate different GPCR pathways. PTH1R was classified to be Class B of GPCR due to its high structural similarity to secretin and glucagon hormone family [3].

Its expression was mostly studied in bones and kidneys due to its important role in regulating calcium ion homeostasis in the blood through activation of adenylate cyclase and phospholipase C (PLC). However, PTH1R is also expressed in multiple other tissues and organs including adrenal glands, placenta, spleen and adipose tissue [3].

It has been recorded that the genomic location of PTH1R is 3p21.31 in human. In other terms, it is located on chromosome 3, region 2, band 1, and sub band 31, with 20 exon count. PTH1R mRNA codes for 593 amino acids in human as shown in Figure 1.3, and 591 amino acids for mouse. It is located in chromosome 9 with 21 exon count in mouse.

Defects and mutations in this receptor can cause Jansen's Metaphyseal Chondrodysplasia (JMC) in which bones of the arms and legs tend to develop and grow abnormally with unusual cartilage and bone formation [2]. Recent studies also show PTH1R mutations can cause failure of tooth eruption in some human patients [27]. Defects by PTH1R will be further explained in more details in another section later in chapter 1 under the title (PTH1R Mutations). However, very little studies consider studying PTH1R in the gastric system, therefore its role and importance in gastric homeostasis is not well known to this day.

1.4.1 PTH1R Structure

Structurally, PTH1R consists of extracellular N terminal, 7 transmembrane domains, and an intracellular C terminal. As the rest of class B family, PTH1R shares a sequence homology with other related receptors in this group such as secretin and glucagon [6]. This identifies that these receptors all share some similarities within their modes of action and signaling [6].

PTH1R is a receptor that allows for PTH (84 aa) and PTHLH (141 aa) to bind to it [28]. However, both molecules bind at different affinity and in different regions of the receptor [28]. On the other hand, PTH1R can be fully activated when small portions of the ligands bind to it, as such it does not require the full length of the ligand to completely bind to it [28]. An example is PTH (1-34) which is considered to be an agonist. It can bind to PTH1R from the N terminal and thus activating the receptor through $G\alpha_s$ pathway. PTH (1-34) was reported to bind at higher affinity resulting in prolonged activation and a longer lasting cAMP response of the receptor. Unlike PTHrP (1-36), which is another agonist, but it was reported to bind at a lower affinity to the receptor, however, it still activated $G\alpha_s$ pathway [28]. To block the activation of PTH1R, a peptide used is PTH (7-34) which has been identified to be an antagonist that inhibits the action of the receptor [28].

Even though the amino acid sequence of PTH1R is identified as illustrated in Figure 1.3, yet the 3-D protein structure has not been fully established according to protein data bank (PDB). However, what make PTH1R similar in structure to its other counterparts in Class B GPCRs is its four pairs of extracellular cysteine (C) specific amino acids illustrated in Figure 1.3 [29]. These residues can form disulfide bond which are conserved domain across all Class B [4,29]. This domain allows the receptor

to maintain its structure and function. Other residues also play an important role in activating different signaling pathways, for example; Lys388 is important in $G\alpha_s$ signaling [29].

In the PTH1R intracellular C terminal, a nuclear localization sequence (NLS) is present, which highlights the regulation of PTH1R localization in the nucleus [30]. Although the transport of GPCRs from the cell membrane into the cell by endocytosis is well known, the knowledge of transporting GPCRs into the nucleus is relatively new.

PTH1R was the third GPCR found to indeed translocate to the nucleus of the cell, which created a new range of studies regarding this phenomena [31]. Studies demonstrated that PTH1R nuclear import is actually possible [31]. Other studies confirmed PTH1R nuclear localization in rat liver, kidney, gut, ovary, and uterus [30].

The nuclear localization sequence of PTH1R was identified, which was reported in the cytoplasmic domain amino acids 471 – 488 of the receptor shown in Figure 1.3 [30]. It was in fact well documented in another study that PTH1R translocate to the nucleus when mouse osteoblast-like cells MC3T3-E1 are serum starved. However, in normal cell culture conditions PTH1R can be found both in cytoplasm and nucleus [31]. They further studied the mechanism of such action, and noted that treating the cells with PTH peptide induce PTH1R cytoplasmic localization. To aid in this mechanism, they reported a relation between Importin α and β, both of these transport regulatory proteins aid in the translocation of PTH1R to the nucleus. The Importin protein will interact with the nuclear localization sequence NLS found in the receptor, this will further cause the translocation of the complex into the nucleus. To reverse this mechanism, that is, to transport PTH1R from the nucleus back to the

cytoplasm, chromosomal region maintenance 1 CRM1 protein is involved, this process is named as nuclear export [31].

The importance of this shuttling can regulate various cellular process, gene expression and proliferation [31]. However, other studies linked this translocation of the receptor to happen during DNA synthesis and mitosis. This highlighted the possible importance of PTH1R during specific stages of the cell cycle [30].

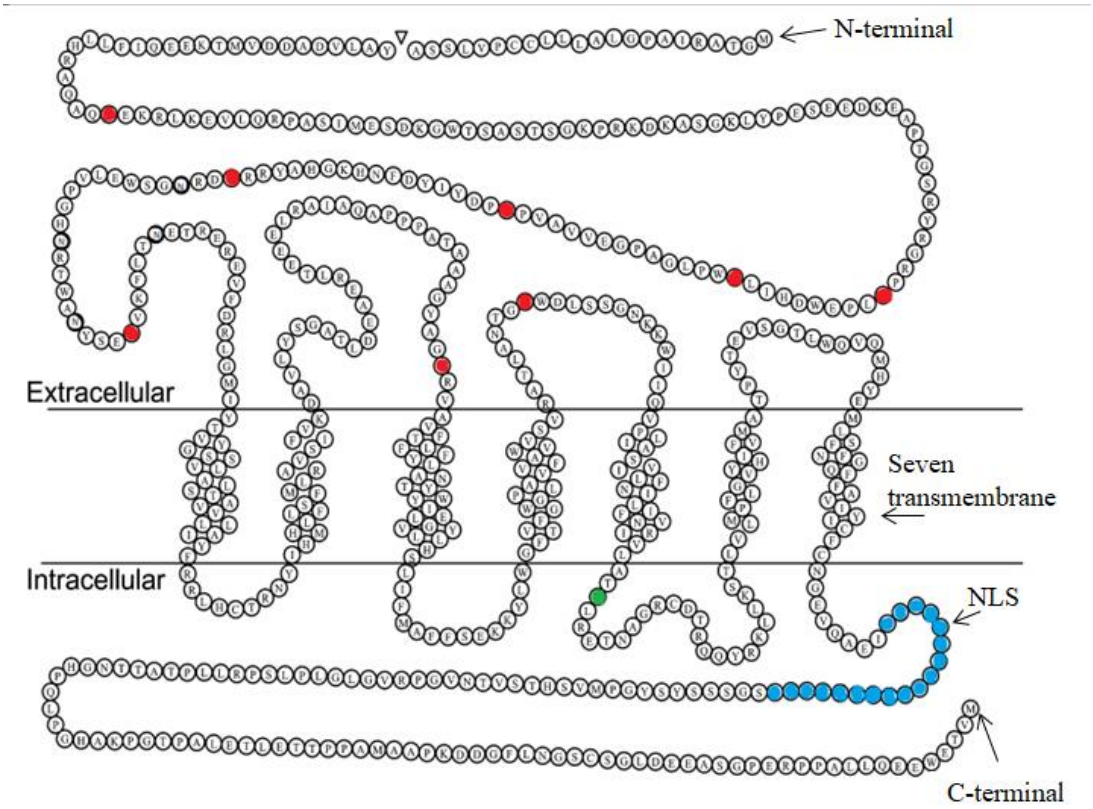


Figure 1.3: Human PTH1R structure

Human PTH1R Amino Acid sequence organization. The composition of different parts and regions of PTH1R, including N-terminal, seven trans-membrane loops, and C- terminal. Important sequence sites are: Nuclear localization sequence 471 – 488 highlighted in blue, four pairs of Cysteine highlighted in red, and Lysine 388 highlighted in green. Modified from [4].

1.4.2 Ligands

Parathyroid hormone (PTH) is secreted by the parathyroid gland which are four tiny glands found in the neck. PTH plays a crucial role in maintaining and regulating calcium ion homeostasis in the blood. When calcium ions in the blood decrease, this causes the PTH to stimulate bone cells, specifically osteoclasts which help in breaking down bones to release more calcium in the blood. In other words, PTH can raise calcium levels by increasing bone resorption which is breaking down of bone tissues to produce more calcium, as such, this will decrease renal excretion of calcium and will stimulate Vitamin D to absorb calcium from the intestine through its receptor VDR [32].

PTH can be regulated via two receptors PTH1R and PTH2R. However, PTH1R is deeply studied in bones and kidneys. Studies show that PTH treatments can increase bone mass when intermittently administered at low doses to patients with osteoporosis [28]. PTH role is very significant in bone regulation and maintain its homeostasis. PTH knockout mice showed substantial bone deformities and impaired bone formation [28,33].

Parathyroid hormone like hormone (PTHLH) was discovered as a cancer-derived hormone which was reported in humoral hypercalcemia of malignancy (HHM). The term derives since 1941 [28] when it was first reported as a case study in a patient with high plasma calcium accompanying a non-parathyroid cancer. During that time, it was known that excess secretion of PTH causes hyperparathyroidism which results in high calcium in the blood. However, hypercalcemia was also noted in other cancers such as lung, breast, and kidney cancers. PTHLH was then identified through immunochemical cross-reactivity with bovine PTH and immunohistology

which showed PTH like reactivity. By 1970, scientists concluded that the main cause of the hypercalcemia, and the tumor derived activity in some cancer patients did not originate from PTH itself, but from another hormone which very much resembles PTH, thus Parathyroid Hormone Like Hormone name appeared [34,35]. Recent studies to this date still support this idea, in which PTHLH is the reason for elevated calcium levels in blood, as well as tumor derived factor. Moreover, studies also show a similar N-terminal homology between PTH and PTHLH in the first 13 residues, which represents a possible agonist for both hormones to act on PTH1R [28].

Both of these ligands have been identified to be mediated through PTH1R. However, both can bind at different affinity and thus cause different signaling pathway to be triggered.

Compared to PTH, PTHLH is thought to be produced by many tissues and it is expressed throughout the gastrointestinal tract [7]. However, very limited number of studies are conducted involving PTHLH expression in the stomach [36,37], and it regulatory receptor PTH1R signaling pathway in maintaining gastric homeostasis.

1.4.3 Major PTH1R Signaling Pathways

In the case of PTH1R which is categorized as a GPCR Class B, two ligands can activate this receptor, PTH and PTHLH. Both of these hormones can further activate different PTH1R G proteins. The four possible activations are: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$. Figure 1.4 illustrates the multiple types of PTH1R activations. However, the most studied pathways for PTH1R are $G\alpha_s$ and $G\alpha_q$ [29].

cAMP signaling pathway

The activation of the $G\alpha_s$ pathway will cause a different cascade of downstream reactions. Firstly, the activation of adenylyl cyclase (AC), which will generate cyclic adenosine monophosphate (cAMP). Secondly, cAMP will activate protein kinase A (PKA) which will regulate several cellular proteins, and target specific genes [6].

To fully activate PTH1R, two highly studied agonists can be used, PTH (1-34) and PTH (1-36). The N-terminal of these agonists is fully capable of activating the receptor. Some studies suggest that other agonists like PTH (1-31) can stimulate AC and PLC pathways, however it cannot internalize PTH1R [38]. On the other hand, PTH (7-34) acts as an antagonist which causes a blockage or inhibition of PTH1R. Other peptide activators or inhibitors exist to this day, but they can cause other signaling pathways in GPCR to be activated. This broad range of peptide activators highlight an important focus on the specific ligand-receptor binding, which is selective and can trigger targeted signaling pathways in different organs and cells.

cAMP in the gastric system has important roles in regulating stomach homeostasis. Parietal cells found in the stomach are responsible in Gastric acid secretion. These cells contain vesicular tubular organelles that carry a proton pump H+/K+ ATPase which maintains H+ ion homeostasis in the stomach [39]. It has been reported that Histamine, gastrin, and acetylcholine act as gastric transmitters.

Histamine is a regulatory hormone responsible to activate Histamine receptor 2 (H2), this receptor is categorized as a GPCR which activates the $G\alpha_s$ signaling pathway [39]. This activation will initiate Adenylyl cyclase and protein kinase A which will cause the production of cAMP as a secondary messenger in the cell [40].

Acetylcholine and gastrin are also responsible in the induction of calcium ions. The intracellular increase in calcium ions triggers parietal cells to produce gastric acid

[39]. However, these parietal cell transmitters can be activated by different ligands and can be caused by different stimulus, whether it is increase in calcium ions or cAMP.

Some studies claim that certain basal levels of cAMP are crucial for the parietal cells to be stimulated by calcium ions [40]. On the other hand, some studies show that calcium ions are also important for the parietal cells to be activated through cAMP pathways [40]. Studies demonstrate a cross talk between calcium ions and cAMP pathways in general; however more studies are yet to be conducted to verify their effects on gastric acid secretion [41].

ERK1/2 signaling pathway

One of the highly studied pathways is the phosphorylation of extracellular signal regulated kinase (ERK1/2). It can be activated by many extracellular signals such as growth factor and hormones, or it can be activated by GPCR Gα, Gβ, and Gγ. This pathway is significant in many cellular actions including PTH and PTHLH involvement in differentiation, proliferation, survival and calcium transport [42]. Activation of this pathway has been reported in many endocrine cells such as; osteoblasts, bone cells, and kidney cells [42]. Other studies illustrates that activation of ERK1/2 in stomach cancer cells KATO III through GPCRs induced proliferation [43]. Some studies even suggested using drugs to inhibit gastric cancer cells from proliferating via inhibition of ERK1/2 [44]. However, it would be interesting to find out if PTH1R in the stomach has a role in this activation.

Some studies documented PTHLH amino terminal internalization into the cell after activating its receptor PTH1R [45]. Moreover, it has also been discovered that PTHLH contain a nuclear targeting signals located on amino acids 87-107 [45]. It has

been localized in the nucleus of some skeletal cells *in vivo*, and its role was significant in regulating differentiation and apoptosis *in vitro* [45]. Some studies even proposed the endocytosis of PTH1R after the ligation with its ligand PTHLH.

Studies investigated the possibility of actually whether or not PTH1R can be internalized from the cell membrane to the inside of the cell. The entire process of GPCR desensitization and resensitization is further explained in Figure 1.5. Briefly, when the ligand binds to the receptor, it will cause the phosphorylation and change in the conformation of the receptor. This mechanism can cause the uncoupling of Gα, Gβ, or Gγ subunit from the receptor, this in turn will cause β-arrestin to increase its affinity to the receptor, and will bind to the receptor causing desensitization. The receptor will further translocate inside the cell with β-arrestin, in which it can activate Extracellular signal Regulated Kinases ERK1/2 [38]. Furthermore, the receptor can either be broken down by lysosomes, or resensitized back to the cell membrane. This method is helpful in a way which will regulate the receptor activation, and thus prevent prolonged receptor activity and stimulation [38].

Desensitization of GPCR PTH1R alpha group $G\alpha_s$ and $G\alpha_q$ by β-arrestin was documented in some studies [38,42]. In a study conducted in 2005, they used human embryonic kidney (HEK293) cells to investigate PTH1R trafficking and activation of extracellular signal regulated kinases ERK1/2 by stimulating the receptor with PTH (1-34) peptide [42]. Consequently, the agonist peptide PTH (1-34) was able to induce a rapid activation of ERK1/2 in the first five minutes of stimulation unlike in PTH (1-36), in which they found no similar significant effects. They further proposed that PTH (1-34) agonist and PTH (7-34) antagonist were able to phosphorylate ERK1/2 and cause PTH1R internalization into the cell. However, PTH (7-34) antagonist promoted PTH1R internalization without activation of the receptor. They further suggested that

PTH1R can actually be internalized into the cell, using the aid of β-arrestin recruitment to the cell membrane to desensitize the receptor [42].

Another study conducted in 2004 [38], in which they removed a portion of PTH1R C terminal, and they reported that PTH1R was not able to get internalized into the cell. They concluded that PTH1R C terminal contain a sequence that is involved in receptor internalization [38].

The activation of ERK1/2 has been studied intensively in bone marrow cells and kidney cells, and it is associated with differentiation, proliferation, and calcium transport. However, very little studies were conducted on stomach to test PTH1R trafficking into the cell and activating ERK1/2.

PLC signaling pathway

Another pathway for PTH1R is $G\alpha_q$ which will activate phospholipase C (PLC) which will trigger downstream signaling involving inositol triphosphate (IP3) and protein kinase C (PKC) [29]. This pathway is further explained in Figure 1.4.

For the purpose of this project, I will be focusing and studying the $G\alpha_s$ pathway to highlight the importance of cAMP and ERK1/2 generation in gastric cells.

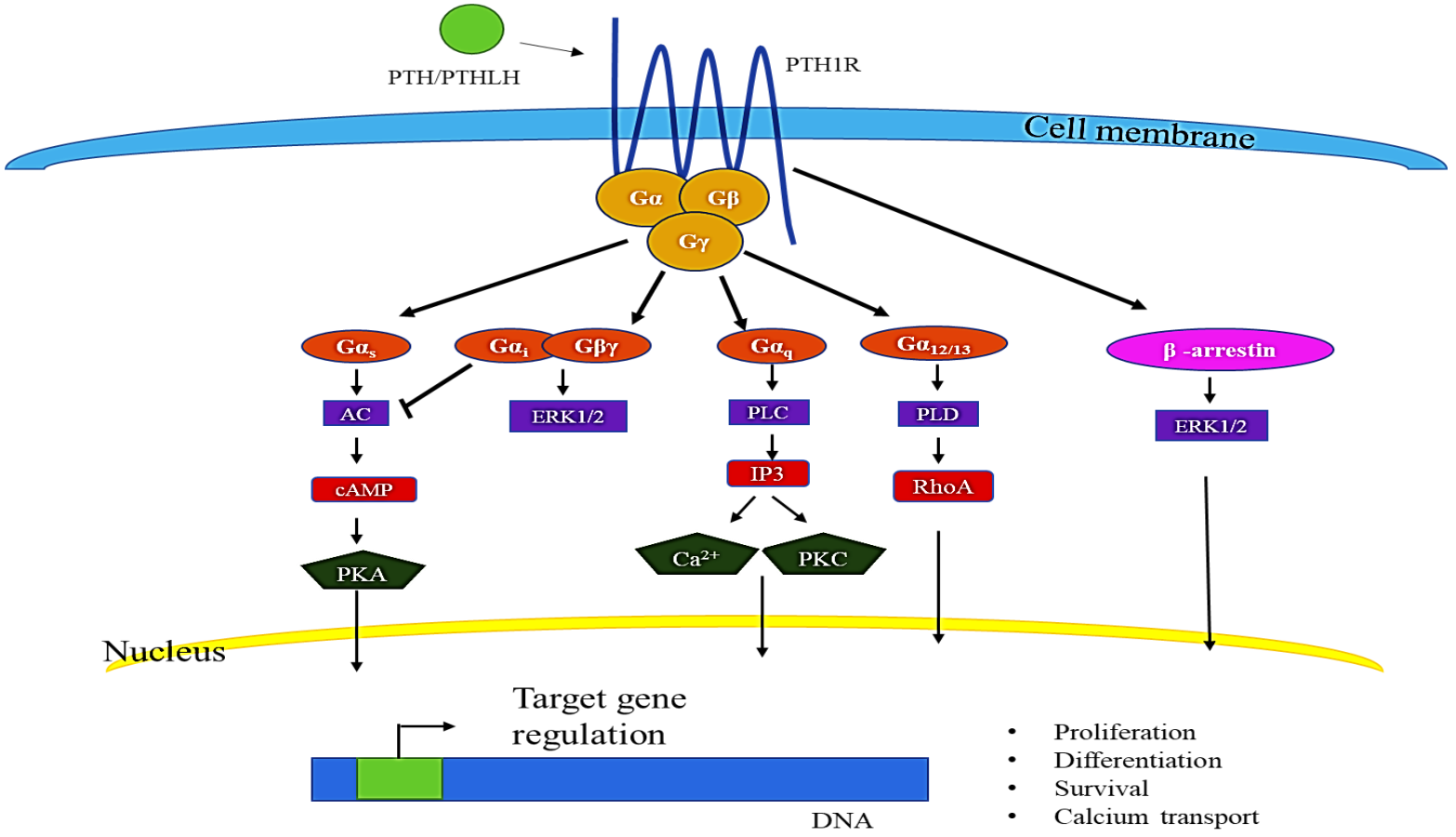


Figure 1.4: GPCR signaling pathways

GPCR multiple signaling pathways activations and secondary messengers involved in multiple downstream signaling. The focus of this project will be on $G\alpha_s$ and β-arrestin ERK1/2 signaling pathway.

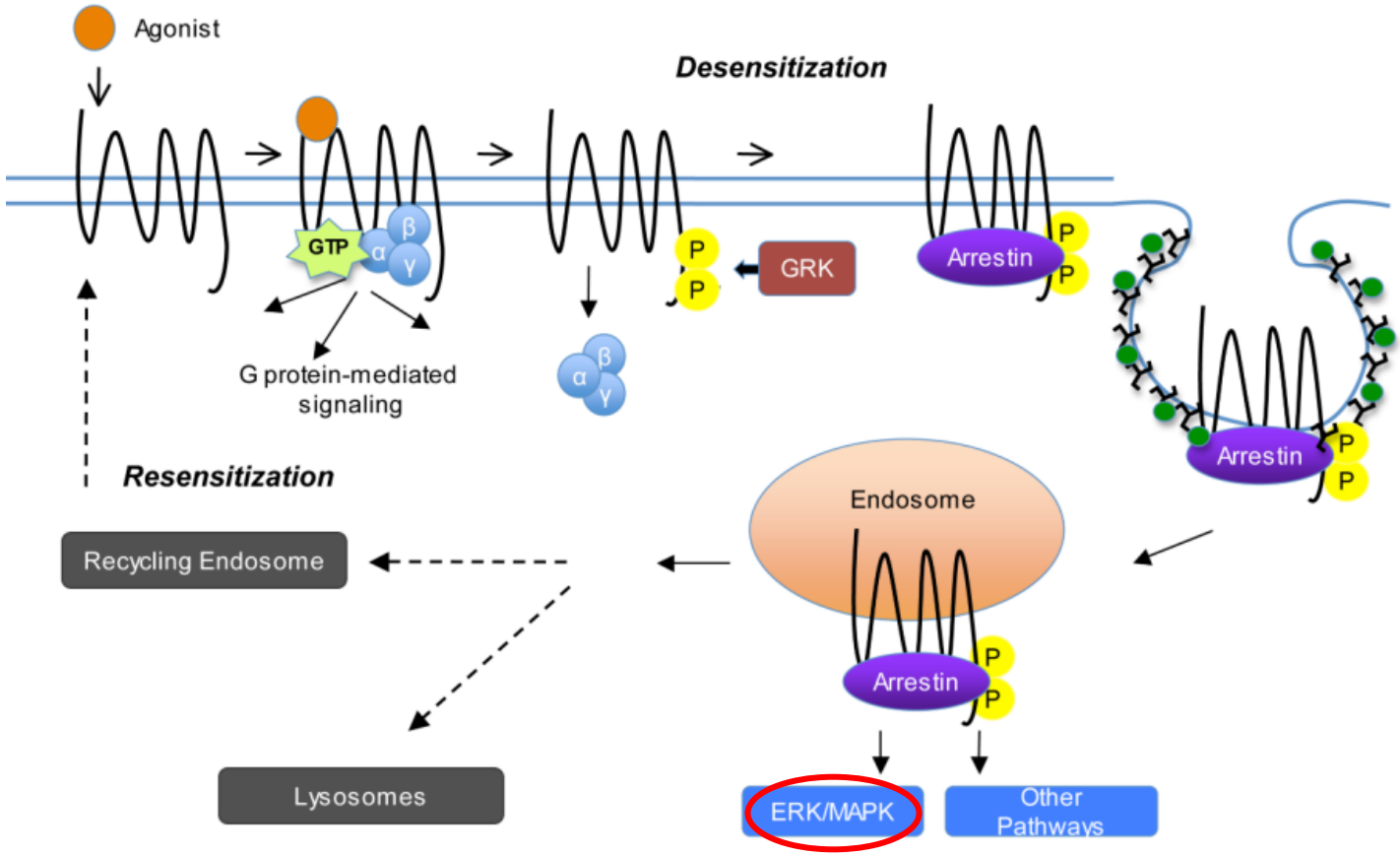


Figure 1.5: GPCR endocytosis trafficking

Detailed explanation of GPCR internalization into the cell by β-arrestin recruitment to the receptor. The diagram illustrates the desensitization and resensitization of the receptor causing the activation of ERK1/2. Modified from [46].

1.5 PTH1R in Health and Diseases

In this section PTH1R and its role in different organs and diseases such as in stomach, bone cancer, breast cancer, and kidneys will be discussed.

1.5.1 PTH1R in Stomach

There is limited number of studies available for PTH1R in the gastric system. In one family, a case study was conducted in which it linked a mutation of PTH1R and ATPase H+/K+ to loss of function in parietal cells which lead to gastric tumor, hypothyroidism and arthritis [47]. Another case study also identified PTHLH and PTH1R to be highly expressed and involved in gastric tumor and the metastasis of the tumor to other organs [48]. One study also mentioned the importance of PTH1R in relaxing gastrointestinal muscles [49]. It is important to note that PTH1R is not well studied in the gastric system and very limited information is available regarding this issue. As such, knowledge from other organs and researches will be important in studying the role of PTH1R in stomach.

1.5.2 PTH1R in Bones

Osteosarcoma (OS) is one of the most common cancer of bones [50]. It occurs mostly in children, and when diagnosed and localized, patients have a five years survival rate [50]. However, treatment for osteosarcoma is mainly administered drugs to control the proliferative cancer cells, or in other cases surgical resection.

PTH plays a major role in regulating bone cells and development of mature bones. It has been reported that PTH treatments increase bone mass. However, PTHLH has been reported to play a pivotal role in skeletal development [51].

Since both of these hormones can act on PTH1R, then this raise a potential on targeting this receptor to study its importance. It has been reported that intermittent PTH treatment can increase bone mass and inhibition of osteoblast apoptosis. PTH has also been reported to be used as a treatment for osteoporosis which enhances bone resorption [52]. However, long term and high doses of PTH can trigger and cause OS. This is further supported in other studies [52]. In one study, knockdown of PTH1R inhibited proliferation and tumor growth inhibition and increased differentiation *in vitro*. It is important to note that the knockdown of PTH1R did not affect the viability of cells in this study, however, it was able to impair the invasion of cells [50]. Other studies showed overexpression of PTH1R increased proliferation in OS cells [33,50,54].

Other *in vivo* studies demonstrated the anabolic bone formation effect of intermittent low-dose PTH (1-34) treatment on bones [55,56]. However, continuous high-dose treatment of PTH showed increase in bone turnover, resorption, and hypercalcemia.

Osteoporosis is defined as an imbalance between bone resorption by osteoclasts and bone formation by osteoblasts causing reduction in bone mineral density (BMD) [52]. PTH (1-34) and PTH (1-36) intermittent treatment have been established as an osteoporosis treatment to induce osteoblastic bone formation and increasing BMD [28,52,57]. It was also reported in 2002 that PTH (1-34) the first anabolic agent for treating osteoporosis in the United States [58]. However, due to the fact that PTH can activate multiple signaling pathways, and not precise exclusive anabolic actions, another analog PTHLH (1-34) has been recently approved in 2017 for osteoporosis treatment [58].

1.5.3 PTH1R in Breast Cancer

Breast cancer has been associated previously with skeletal metastases [59]. Some studies suggested a link between breast cancer and PTHLH and considered breast cancer mediation through PTHLH expression, especially since the identification of PTHLH in breast cancer in 1987 [59]. It is also important to note that elevated levels of PTHLH was reported in breast cancer patients and this induced tumor initiation, and progression, and cellular proliferation [60,58]. Another study reported silencing PTHLH in breast cancer cells affected over 200 genes expression tested by microarray analysis [60] .

In another epidemiology study, in which an increase in breast cancer was associated with type 2 diabetes, they investigated the role of PTH1R on apoptosis of breast cancer cells exposed to high levels of glucose [61]. It was reported that PTH1R expression increased in mice with diabetes. Furthermore, they also reported an increase in PTH1R in patients with breast cancer and diabetes. Additionally, they found that silencing PTH1R inhibited cell proliferation and promoted cell apoptosis induced by high levels of glucose [61].

Moreover, breast cancer patients have reported severe bone pain and hypercalcemia, which eventually lead to skeletal metastasis [58,60]. It is known that PTH1R regulate calcium homeostasis along with extracellular calcium-sensing receptor (*CaSR*). This highlights the important cross talk between breast cancer and bone cells by PTH1R. It was also proposed a future targeting of PTH1R and CaSR in order to control breast cancer tumor growth and inhibition on cell proliferation as well as the metastasis to the skeleton [58].

1.5.4 PTH1R in Kidneys

PTH1R has been reported to be highly expressed in the kidney for its effective role in maintaining its development maturation, structure and function by regulating calcium ions in the blood [62].

Calcium homeostasis in the blood plays a crucial role in kidneys. When the calcium level is low, this stimulates the chief cells in the parathyroid glands, and thus PTH secretion will increase and will allow it to bind to PTH1R. Furthermore, this will help in renal tubular reabsorption of calcium and in fact will increase Vitamin D synthesis to induce calcium absorption in the gut and hence facilitate osteoclast bone resorption to secrete calcium ions. In this mechanism, the body will be able to maintain systemic mineral ion homeostasis and calcium levels in the blood [63].

In a diabetic kidney, it was shown that PTHLH and PTH1R are overexpressed. It was reported that treating renal kidney cells with high glucose will increase the expression of PTHLH and PTH1R [1].

In one study on human embryonic kidney cells, they activated PTH1R and tested the apoptotic signaling pathways effects. They reported that PTH1R activated mitochondrial apoptosis pathways, and the overexpression of PTH1R led to an imbalance in the mitochondrial membrane. This highlighted the importance of PTH1R in apoptosis of kidney cells [64].

PTHLH and PTH1R have been under the investigation for their upregulation in renal injuries [65]. This, in fact, will cause the development of renal hypertrophy and diabetes. This in turn will cause an imbalance in the mineral ion homeostasis [65].

1.6 PTH1R Mutations

Mutations in PTH1R have been reported to be associated with multiple diseases such as; Jansen type metaphyseal chondrodysplasia, primary failure of tooth eruption, and Eiken skeletal dysplasia according to online Mendelian inheritance in man database (OMIM).

Jansen type metaphyseal chondrodysplasia is a mutation categorized as a heterozygous missense mutation in PTH1R gene in which it is categorized by short limbed dwarfism caused by impaired growth plates of bones and mineral ion imbalance. The mutation is thought to be in amino acids residue 223 in which Histidine was replaced with Arginine [2,66,67].

Primary failure of tooth eruption is a heterozygous splice site or nonsense mutation in PTH1R [62]. This disease is categorized as growth deficiency in teeth [27]. The mutation is reported in multiple places, for example it is noted in amino acid position 155, in which Glutamate undergoes Termination [68].

Eiken skeletal dysplasia is another bone disorder is which abnormal modeling of the bones and cartilage and severely delayed ossification is found in the hands, feet, and pelvis [48]. It is reported as a homozygous nonsense mutation in the c-terminal of PTH1R gene. Moreover, the specific truncation of the mutation on Arginine 485 to Termination [69]. This disorder is very similar to Jansen type metaphyseal chondrodysplasia, however, an important finding in one study in which they compared both disorders found that there was significant increase in vitamin D in patients with Jansen type metaphyseal chondrodysplasia [70] compared to Eiken patients [69]. However, no further discussion was related to highlight this issue.

1.7 PTH1R Target Genes

PTH1R is involved in several cross talks with other receptors. The activation or inhibition of PTH1R is involved in multiple regulatory actions downstream in the genomic pathway. Examples of some PTH1R direct and indirect target genes include: calcium sensing receptor (*CaSR*), fibroblast growth factor 23 (*FGF23*), interleukin-6 (*IL-6*), low density lipoprotein receptor (*LDLR*), and Na+/H+ exchanger regulatory factor 1 (*NHERF1*) as shown in Table 1.2.

The extracellular calcium sensing receptor, also known as *CaSR*, is a family C GPCR. Its major role is to detect minor alterations in blood calcium levels, and in turn indirectly induce PTH secretion and urinary calcium excretion [71]. Regulating calcium is a very complex process that involves many components to maintain its homeostasis. When calcium levels in the blood drops, this is sensed by *CaSR*, which in turn stimulates the parathyroid gland to secret PTH. This hormone will stimulate PTH1R to induce bone resorption which breaks down the bones to release calcium ions into the blood. Furthermore, PTH will also enhance Vitamin D synthesis in the renal system which will aid the intestines in calcium absorption [71-74]. This regulation mediates calcium homeostasis, and as such leads to feedback inhibition of PTH secretion.

The cross talk between *CaSR* and PTH1R has been reported previously in breast cancer [75]. PTHLH has been involved in breast cancer tumor progression and metastasis to the bones. It is reported that *CaSR* contributes to breast cancer cell proliferation [58].

CaSR was also found to be expressed in gastric tissues in parietal cells [76], and in gastric cancer. Studies illustrate the upregulation of *CaSR* expression [77]. For many years, calcium supplements were used as treatment for gastric cancer. However,

it has been reported that calcium activates *CaSR* and causes gastric cancer growth, unlike the case in colorectal and pancreatic cancer, in which calcium suppressed the tumor growth [77].

FGF23 has been reported to be induced and upregulated directly by PTH in bones when there is high phosphate and vitamin D levels in the blood [78,79]. Other studies demonstrated that PTH (1-34) treatment *in vitro* to osteocyte-like cells resulted in increased levels of *FGF23* in the first initial 2 hrs, but the levels returned back to normal by 4 hrs. [80] Another study conducted in which PTH1R was deleted from kidney in mice showed significant increase in *FGF23* and challenging the mice with PTH (1-34) caused an upregulation of *FGF23* [81]. However, removal of PTH1R from bones downregulated *FGF23* levels [81].

IL-6 is a pro-inflammatory cytokine studied in cases of hyperparathyroidism in which it is indirectly upregulated when PTHLH stimulates PTH1R in cancer cells [83]. IL-6 has been studies in patients with gastric cancer, and research shows that *IL-6* is involved in gastric cancer spread of tumor and invasion [84,85]. Another study related PTH treatment in osteoblastic cells and results showed an induction in *IL-6* [5,86,87].

LDLR is responsible for the uptake of cholesterol and is very important in maintaining plasma levels of lipoprotein. PTH is shown to induce *LDLR* when it binds to PTH1R [88]. Very limited number of research that related PTH1R and *LDLR* especially in gastric system.

NHERF1 consists of two domains, PDZ and carboxyl-terminal ezrin binding domain (EB). *NHERF1* acts as cytoplasmic adaptor protein and has the ability to bind and form different complexes with other molecules causing trafficking [89]. It is also involved in cancer progression such as in breast cancer and gastric cancer [90], and is

important in cellular localization [91,92]. *NHERF1* levels were detected at high amount in nucleus of cancer cells, thus altering some signaling pathways and second messengers [92]. *NHERF1* has been documented to directly interact with PTH1R and internalize with it when it is desensitized and cause endocytosis of the receptor into the cell [92,93,95].

1.8 Thesis Objectives and Hypothesis

Since there are limited studies focusing on PTH1R importance in the stomach, this project will investigate this issue further. This study is important as it will explore the expression and function of PTH1R in the stomach, and how this receptor signaling affects gastric homeostasis. This work might also set some impacts in the development of new therapeutic agents for gastric cancers.

There are two goals of my master thesis; the first is to find the expression and cellular localization of PTH1R in the mouse stomach, second, is to study the role of PTH1R signaling in gastric epithelial homeostasis *in vitro.*

Moreover, I am hypothesizing that PTH1R is expressed in the mouse stomach and its signaling is required for gastric epithelial homeostasis. To support my hypothesis, different experiments will be conducted involving PCR, Immunostaining, and western blot.

Table 1.2: Some PTH1R target genes

Target Gene		Function	Reference
CaSR	Calcium Sensing Receptor	To detect minor alterations in blood calcium levels.	[71,72]
FGF23	Fibroblast Growth Factor 23	Is a bone derived hormone regulating vitamin D activation in kidney.	[78,79]
IL-6	Interleukin-6	A pro-inflammatory cytokine which is studied in cases of hyperparathyroidism.	[83]
LDLR	Low Density Lipoprotein Receptor	Responsible for the uptake of cholesterol and is very important in maintaining plasma levels of lipoprotein.	[88]
NHERF1	Na+/H+ Exchanger Regulatory Factor 1	Acts as cytoplasmic adaptor protein and has the ability to bind and form different complexes with other molecules causing trafficking.	[79,80]

Chapter 2: Methods

2.1 Animals and Cells

C57BL/6J three month old mice (n=3) (2 males, 1 female) were kept in Specific Pathogen Free (SPF) facility located in the Faculty of Medicine and Health Sciences in United Arab Emirates University (UAEU). The mice were fed normal diet D10012Gi, Research Diet for three months, and then starved over night before dissection. Stomach tissue was collected to conduct IHC and Real-Time PCR analysis.

Cells used were Gastric Adenocarcinoma (AGS), stomach cancer cells NCI-N87, cervical cancer cells HeLa, cells obtained from American Type Culture Collection (ATCC), Human Embryonic Kidney (HEK293) generously provided by Dr. Ayoub (UAEU), and Mouse Gastric Epithelium Progenitor cells (mGEP) which are cells derived from FVB/N transgenic mouse expressing an oncogene, the simian virus (SV40) large T antigen gene [96] were generously provided by Dr. Karam (UAEU). mGEP and AGS cells were cultured in RPMI-1640 medium (Sigma), and HEK293 cells were cultured in DMEM medium (Gibco). Both mediums were supplemented with 10% Fetal Bovine Serum (FBS) and 100 U/ml/50μg/ml Penicillin-Streptomycin (Life Technologies). Cells were grown in a humidified Binder incubator at 37°C in 5% CO_2 atmosphere.

2.2 RNA Isolation and Reverse-Transcription PCR

For the mice stomach, total RNA was isolated from different stomach regions (forestomach, corpus, and antrum) using RNeasy Mini Kit (Qiagen). Normal human stomach total RNA sample was purchased from Origene. RNA isolation for the cells was also using RNeasy Mini Kit. RNA clean-up was also conducted using RNase-Free

DNase Kit (Qiagen). cDNA was then synthesized using iScript cDNA synthesis Kit (Bio-Rad) by using 1µg of RNA as recommended by the manufacturer.

RT-PCR was used to test the expression of PTH1R and other genes *in vivo* and *in vitro* by using GoTaq Flexi DNA Polymerase kit (Promega). Primers and product size used are listed in Table 2.1.

PCR product was then separated by gel electrophoresis using 1.5% agarose gel with Ethidium Bromide and bands were visualized using Gel Doc EZ Imager (bio-rad).

2.3 Histological analysis

Mouse gastric tissue collected was fixed by Bouin Fixative for 24 hrs., then washed with 70% ethanol and processed by using 5µm sections. Slides were then stained with the appropriate antibody and imaged using Olympus microscope IX83.

2.3.1 Immunohistochemistry (IHC)

Immuno-peroxidase staining was used by heat induced antigen retrieval, and all sections were pretreated with 3% H_2O_2 to inhibit endogenous peroxidase activity. Sections were incubated with 1% Bovine Serum Albumin for one hr. Followed by overnight 4°C staining with Rabbit Polyclonal to anti Parathyroid Hormone Receptor 1 (ab75150, abcam, dilution 1:100). Then, biotin-SP conjugated goat anti-rabbit was used and incubated for one hr. at room temperature. Followed by PBS wash, slides were then incubated with Extravidin-Peroxidase at dilution 1:1000 in blocking reagent at room temperature. Further PBS washing, and then DAB:UREA (1:1) was added for six minutes and washed with distilled water and counterstained with hematoxylin. Lastly, slides were dehydrated and then mountain DPX was used.

2.3.2 Immunocytochemistry (ICC)

mGEP and AGS cells were stained and imaged using Olympus microscope IX83. Cells were seeded on 6 well plate with coverslip inserted at a density of 200,000 cells/well using RPMI-1640 Full medium. Cells were then transfected and left till 70% confluent. Cells were rinsed with PBS and fixed with 3% Formaldehyde in 1xPBS for 20 minutes at room temperature, followed by PBS rinsing. Cells were then permeabilized using 0.1% triton x 100 PBS for 15 minutes at room temperature, following with PBS rinsing. Cells were blocked with 1% Bovine Serum Albumin (BSA) for one hr. at room temperature. Cells were incubated with primary antibody Rabbit Polyclonal to anti Parathyroid Hormone Receptor 1 (ab75150, abcam, dilution 1:50) in blocking reagent overnight. Next day, cells were rinsed by PBS containing 1% goat serum for 10 minutes and incubated with secondary antibody goat anti-rabbit IgG H&L Cy3 (ab6939) for one hr. at room temperature followed by PBS washing. Finally, DAPI with mounting media was placed on the microscopic slide and the coverslip was placed on top. Negative controls were used in the absence of primary antibody.

2.4 Transformation and Transfection

Two PTH1R dry plasmids were purchased from (cDNA Resource Center) weighing 5000ng, clone ID PTHR100000 and PTHR10TN00. Autoclaved water (50 µL) was added to each tube, 100ng/µL concentrations of the plasmids were used further for transformation. Plasmids were placed in 37°C water bath for 10 minutes.

Bacteria cells were used NEB 5-alpha competent *E. coli*. Plasmids and bacteria were mixed and placed 10 minutes on ice, followed by 45 seconds at 42°C in heating block, then five minutes on ice. 500 µL of SOC LB media was placed with each

plasmid and incubated for one hr. at 37°C shaking incubator. Next, tubes containing plasmids and bacteria were centrifuged at 5000 rpm for five minutes. Supernatant was removed, and the pellet was re-suspended in LB media. Mixture was plated on Agar petri-dish containing Soya, Bacteriological Agar, Kanamycin, and Ampicillin and left to dry for 10 minutes. Petri-dishes were then incubated at 37°C inverted for 24 hrs. until bacteria colonies are visible.

Next, 200 ml SOC LB media is prepared with 200µL of ampicillin and placed in a flask. One colony from the grown bacteria is taken using a tip and placed inside the flask and incubated at 37°C overnight in shaking incubator. Next day, samples were centrifuged at 6000g for 15 minutes. Supernatant was discarded, and the pellet was stored in -20°C.

For plasmid extraction and purification Qiagen Plasmid Maxi Kit was used as recommended by the manufacturer. Concentrations of the plasmids were further checked by NanoDrop 2000 from Thermo Scientific. Plasmids were diluted to make the concentration up to 1µg/µl. Gel electrophoresis was used to check DNA integrity using 1% gel.

For transfection, lipofectamin 2000 from (Invitrogen) and Opti-MEM reduced serum media from (Gibco) were used. When transfecting 6 well plate, cells need to be seeded until 70% confluent. Next, two complexes were made. First, 12µg of plasmid is diluted in 1,200µL OpTi-MEM, and second is 50µL lipofectamin is diluted in 1,200µL Opti-MEM. Solutions are incubated at room temperature for 5 minutes, then mixed together and incubated for 30 minutes at room temperature. Old media is discarded from the 6-well plate cells and new fresh media is added with the transfection-plasmid mix. Cells were kept for 24 hrs, then starved overnight and then

treated with different doses of agonist PTHrp (1-36) and antagonist PTHrp (7-34) (Bachem).

2.5 Western Blot

mGEP cells were cultured in 6 well plate at a density of 200,000 cells/well using RPMI-1640 medium supplemented with 10% FBS and 100U/ml/50µg/ml penicillin-streptomycin. Cells were transfected with PTH1R plasmid 2µg/well for 24 hrs, and then starved overnight for 12 hrs before introducing the agonist PTHrP (1-36) at 0.01µM and 0.1µM concentrations for 5 minutes. Cells were lysed using RIPA buffer containing phosphatase and protease inhibitors. Cells were incubated for one hr. at 4°C and then scrapped and centrifuged and the supernatant containing the protein was collected.

Resolving/separating gel 10% and 5% stacking gels, containing H_2O, 40% Acrylamide, 1.5M Tris (pH 8.8), 10% sodium dodecyl sulfate (SDS), 10% Ammonium Persulfate (APS), and Tetramethylethylenediamine (TEMED) were prepared.

Proteins were then transferred from the gel to the Polyvinylidene difluoride (PVDF) membrane for 90 minutes at 100 Volts, followed by blocking using 5% skimmed milk. ERK1/2 and pERK1/2 antibody from (cell signaling technology) were used at dilutions 1:1000 in 5% BSA, and 1:2000 in 5% skimmed milk respectively. Membranes were incubated at 4°C overnight. Membranes were then incubated with cell signaling technology secondary antibody anti-rabbit for ERK1/2 and anti-mouse for pERK1/2 for 45 minutes. SuperSignal™ ELISA Femto Substrate from (ThermoFisher Scientific) was used as substrate. Bands were detected using BioRad Universal Hood II Gel Doc System.

2.6 cAMP Measurement

cAMP measurement was done by two different kits. cAMP-Glo Max Assay obtained from Promega, and cAMP-Gs dynamic kit from CisBio.

For the Promega-cAMP, kit, AGS cells were seeded and transfected in 96 well plate. Preparation of the reagents including induction buffer, detection buffer and kinase reagent were prepared according to the manufacturer's instructions. Reagents were added to the treated cells, and plate was read using plate reader- Glomax from Promega.

Cisbio-cAMP kit was also used to detect levels of cAMP after treating the cells with agonist. Cells were seeded and transfected, stimulation buffer, Anti-cAMP cryptate (donor) was added along with cAMP d2 (acceptor) and incubated for 25 minutes at 37°C before reading the plate using TriStar2 S LB 942 from Berthold Technologies. All reagents were prepared according the manufacturer's instructions.

2.7 Cell Morphology Analysis

mGEP cells were cultured in 6 well plate at a density of 200,000 cells/well using RPMI-1640 medium supplemented with 10% FBS and 100U/ml/50μg/ml penicillin-streptomycin. Cells were transfected with PTH1R plasmid 2ug/well for 24 hrs., and then starved overnight for 12 hrs. before introducing the treatment at different concentration. Agonist PTHrP (1-36) and antagonist PTHrP (7-34) concentrations were 0.01, 0.05, 0.1, 0.5, 1, 1.5, 2.5, 5, and 10 μM. Treated cells morphological changes were monitored at different times (6, 12, 24, and 48 hrs). Imaging was done using Olympus microscope IX83. Experiment was conducted in triplicates.

2.7.1 Cell Cytotoxicity

mGEP cells were seeded in triplicate in 96-well plate at a density of 5,000 cells/well using regular RPMI-1640 medium supplemented with 10% FBS and 100U/ml/50μg/ml penicillin-streptomycin. Cells were transfected with PTH1R plasmid for 24 hrs., cells were then starved overnight for 12 hrs. with serum free media. Cells were then treated with different concentration of agonist PTHrP (1-36) and antagonist PTHrP (7-34). Concentrations were 0.01, 0.05, 0.1, 0.5, 1, 1.5, 2.5, 5, and 10 μM. Reading were taken after 24 hrs, and 48 hrs. Controls used were Un-transfected cells and transfected cells not treated. The effect of the drugs on cell viability was determined using Cell Cytotoxicity Kit (Abcam 112118) according to the manufacturer's instructions. Experiment was conducted four times.

2.8 Quantitative Real-Time PCR

QRT-PCR QuantiStudio 5 (Applied Biosystems(ABI)) was used to determine the effect of transfected mGEP cells treated with (1μM) PTHrP (1-36) on different target genes. SYBR green dye was used for quantification of cDNA. Table 2.2 shows the different target gene primers used for the reaction. PCR reaction volume of 20μl were used for 40 amplification cycles. Samples were loaded in triplicates and values were calculated using ΔΔCT Method and normalized to GAPDH gene expression level.

2.9 Statistical Analysis

Statistical analysis was conducted using Graphpad Prism 7.0.3 in order to calculate the significance and draw the graphs. Data analyzed via one-way or two-way ANOVA test, while comparing the data as means +/- SD followed by Dunnett's

multiple comparisons post-test. Significance was indicated using asterisks *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Table 2.1: Oligonucleotides primers for the studied target genes

	Species	Gene	Forward	Reverse	Product size (bp)
1	Mouse	PTH1R	5'-GTGGCAGTACCTTGTCCCG-3'	5'-CGGTCAAATACCTCCCGTTC-3'	179
2	Mouse	CaSR	5'-AGCAGGTGACCTTCGATGAGT-3'	5'-ACTTCCTTGAACACAATGGAGC-3'	100
3	Mouse	LDLR	5'-TGACTCAGACGAACAAGGCTG-3'	5'-ATCTAGGCAATCTCGGTCTCC-3'	118
4	Mouse	FGF23	5'-ATGCTAGGGACCTGCCTTAGA-3'	5'-AGCCAAGCAATGGGGAAGTG-3'	100
5	Mouse	IL-6	5'-CCAAGAGGTGAGTGCTTCCC-3'	5'-CTGTTGTTCAGACTCTCTCCCT-3'	118
6	Mouse	NHERF1	5'-CCAGTTTATCCGTCTGGTAGAAC-3'	5'-CACCTCCACCAATCGGTCTC-3'	79
7	Mouse	PTHLH	5'-GACGTACAAAGAACAGCCACTCA-3'	5'-TTTTTCTCCTGTTCTCTGCGTTT-3'	81
8	Mouse	GAPDH	5'-TCAAGAAGGTGGTGAAGCAGG-3'	5'-TATTATGGGGGTCTGGGATGG-3'	350
9	Human	PTH1R	5'-AAGGACGATGGGTTCCTCAAC-3'	5'-TTTGGTCCATCTGTCCATCCA-3'	161
10	Human	GAPDH	5'-GAGTCCACTGGCGTCTTCACC-3'	5'-GAGGCATTGCTGATGATCTTGAGG-3'	164

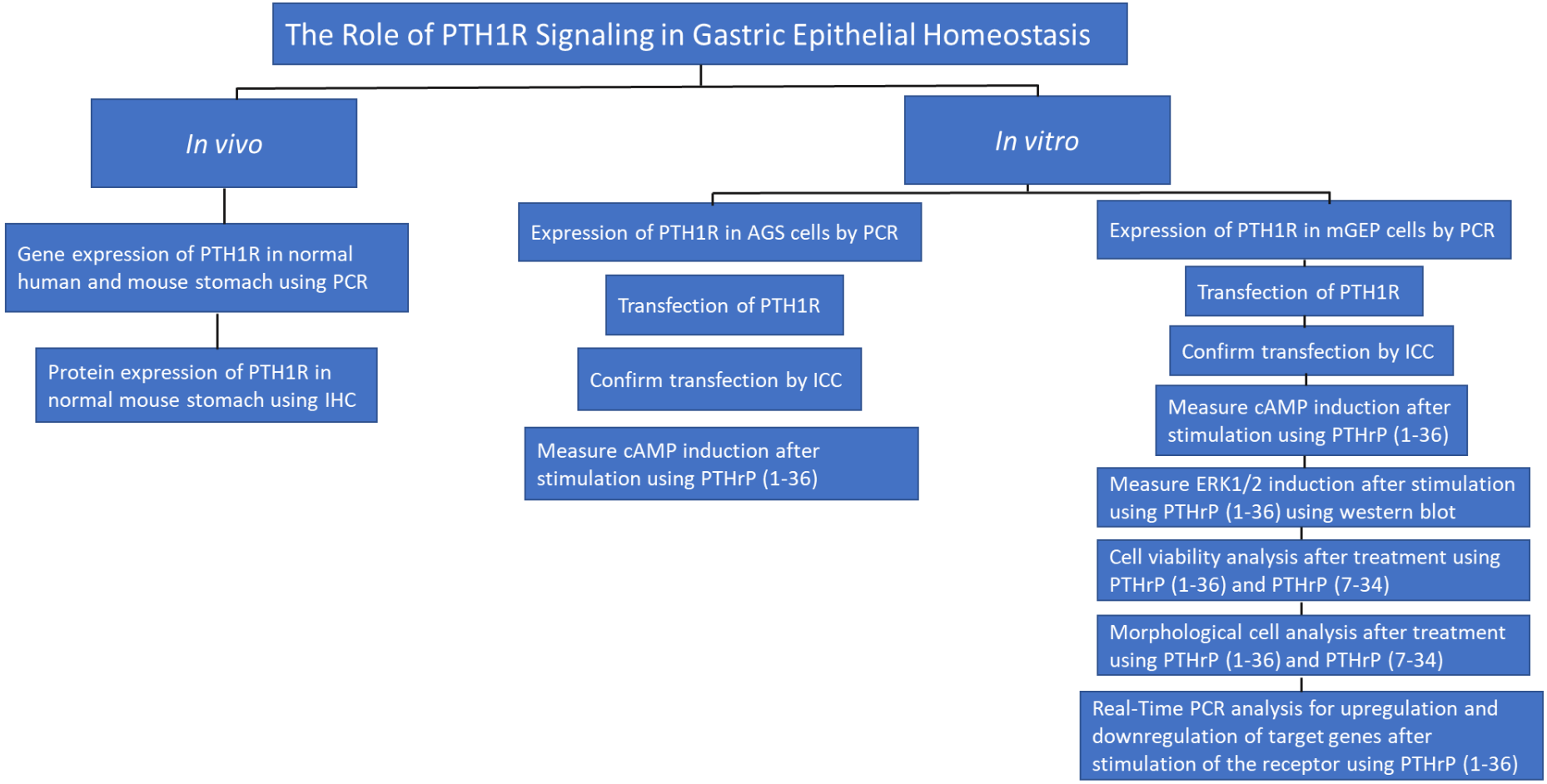


Figure 2.1: Experimental design of the project

Chapter 3: Results

3.1 PTH1R is Expressed in Human Stomach and Cell Lines

RNA analysis for normal human stomach, and multiple human cell lines such as HeLa and HEK293 showed positive gene expression of PTH1R using PCR. However, AGS, and NCI-N87 which are two cancer stomach cell lines did not indicate an expression for PTH1R as shown in Figure 3.1.

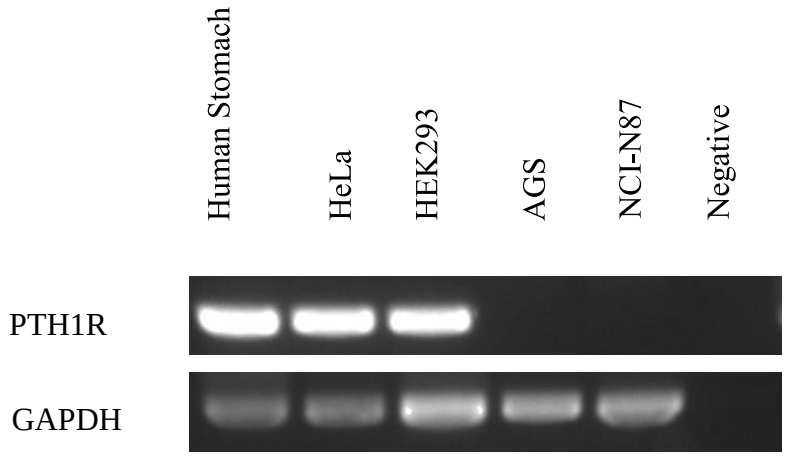


Figure 3.1: PTH1R mRNA is expressed in human stomach and other human cell lines

RT-PCR detected expression of PTH1R in normal human stomach, HeLa cells which are derived from cervical cancer, HEK293 which are Human Embryonic Kidney cells. No expression for PTH1R was detected in AGS and NCI-N87 which are two stomach cancer cells.

3.2 PTH1R is Expressed in Normal Mouse Gastric Epithelial Tissues

RNA analysis using normal mouse stomach three distinct regions (forestomach, corpus, and antrum) showed positive gene expression of PTH1R. However, mGEP cells did not show any expression using PCR Figure 3.2 (A).

To further confirm this result at the protein level, IHC was conducted on the corpus gland of wild type mouse. Staining results showed a positive expression of PTH1R in the base of the corpus gland. Kidney tissue was used as positive control. PTHLH gene expression was detected in mGEP cells as shown in Figure 3.2 (B).

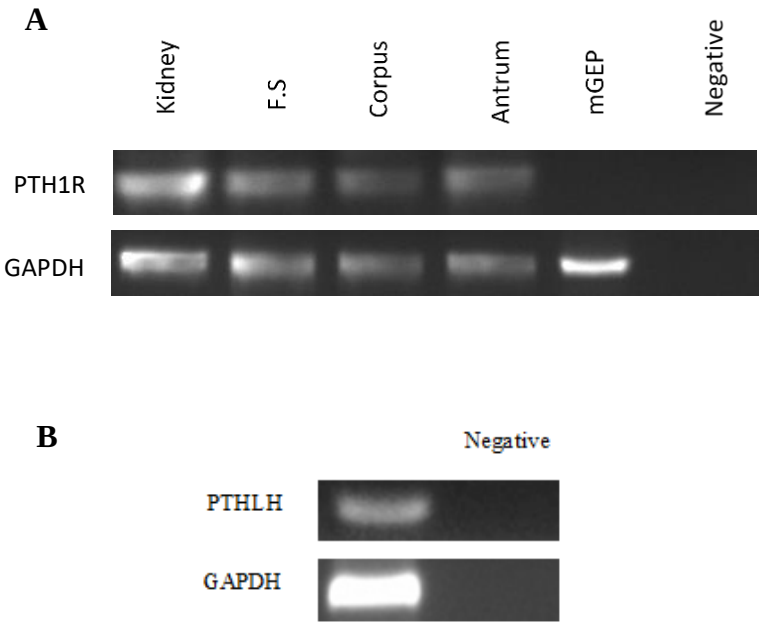


Figure 3.2: PTH1R gene is expressed in gastric tissue of wild type mouse

(A) RT-PCR detected expression of PTH1R gene in gastric epithelial tissue of C57BL/6J wild type mice (n=3) at the age of 3 months. The tissues were collected from different regions of the stomach tissue (Forestomach, Corpus, and Antrum). (B) RT-PCR detected expression of PTHLH gene in mGEP cells.

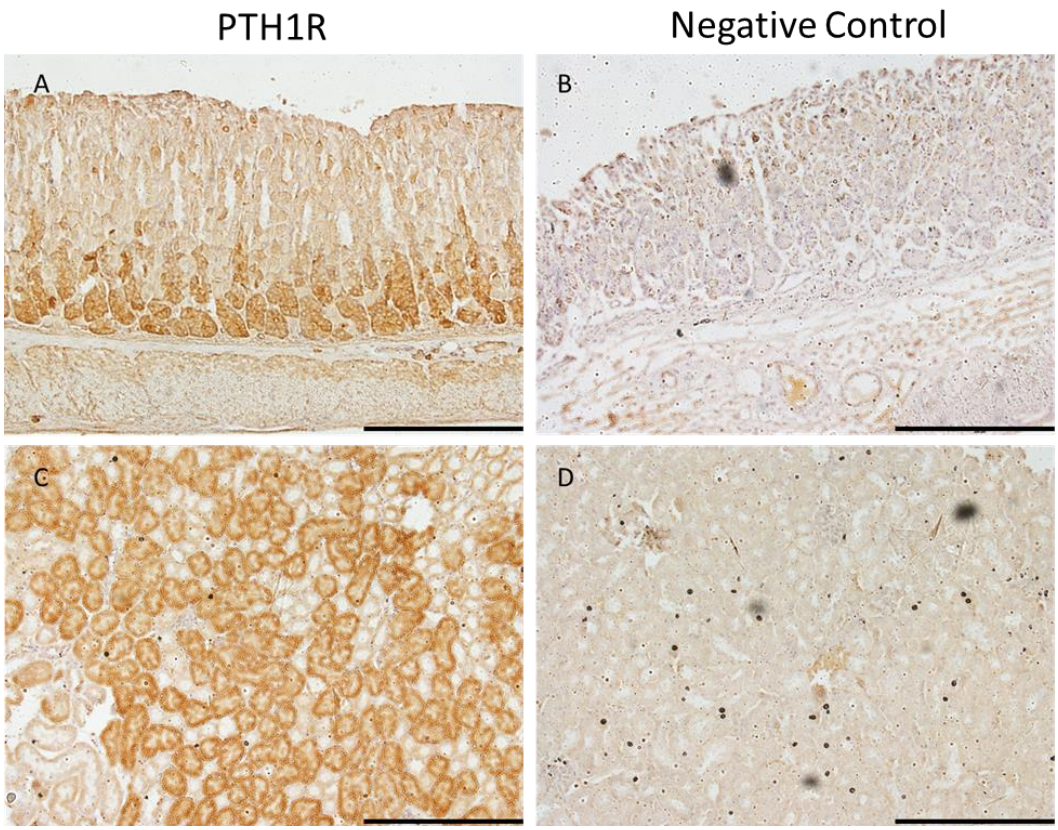


Figure 3.3: Immunohistochemical analysis of the corpus gastric gland showing PTH1R expression

Wild type C57BL/6J mice (n=3), (age 3 months) were sacrificed and the collection of stomach and kidney was done. (A and B) represent sections in mouse Corpus tissue. (C and D) represent mouse kidney sections. The staining for PTH1R was conducted using Rabbit polyclonal to anti-Parathyroid Hormone Receptor 1 (dilution 1:150) and biotin-SP conjugated goat anti-rabbit IgG (H+L). Scale bar: 200μm.

3.3 The Transfection of mGEP and AGS Cell Lines with PTH1R Plasmid

To overcome the limitation that PTH1R was not expressed in stomach cell lines mGEP and AGS. Transient transfection of PTH1R plasmid was conducted to force an expression of the target receptor PTH1R by the cells. To confirm that PTH1R plasmid was successfully transfected into mGEP (Figure 3.4) and AGS cells (Figure 3.5), Immunocytochemistry was conducted. It was noted the cellular localization of PTH1R to be expressed in both nuclear and cytoplasmic in mGEP and AGS cell lines.

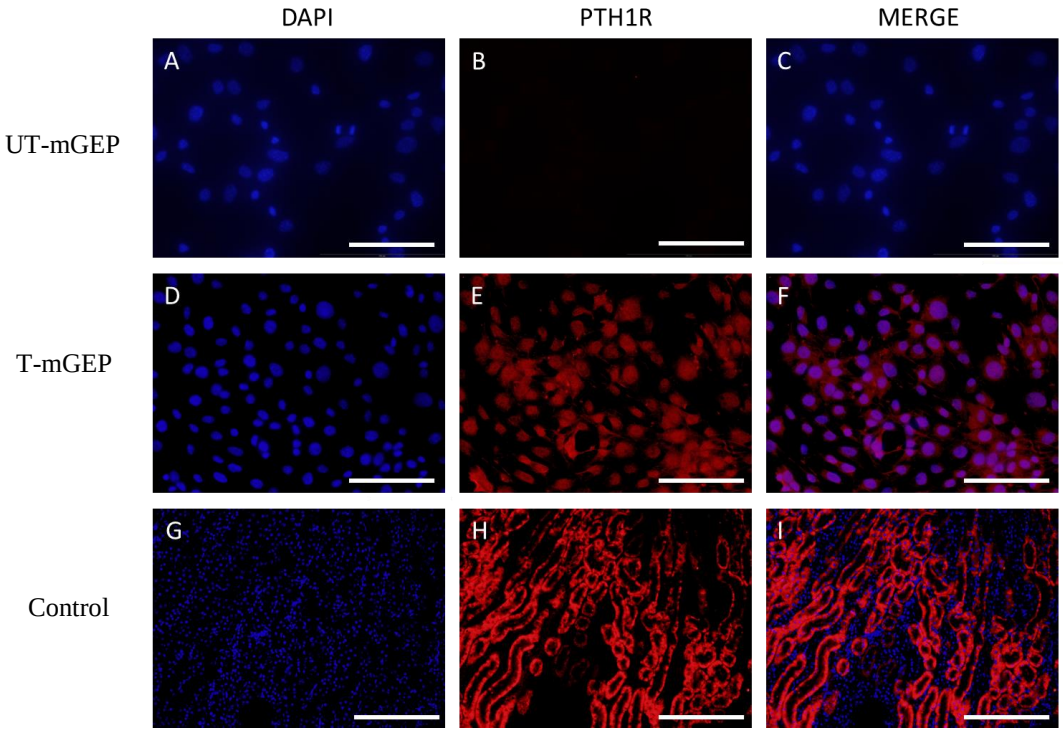


Figure 3.4: mGEP cells expressing PTH1R after successful transfection

(A, B, C) represents un-transfected mGEP. (D, E, F) represents PTH1R transfected in mGEP. (G, H, I) represents mouse kidney as the control. Staining was done using Rabbit Polyclonal to anti Parathyroid Hormone Receptor 1 (ab75150, abcam, dilution 1:100). Scale bar (A to F) is 100µm. Scale bar (G to I) is 200µm.

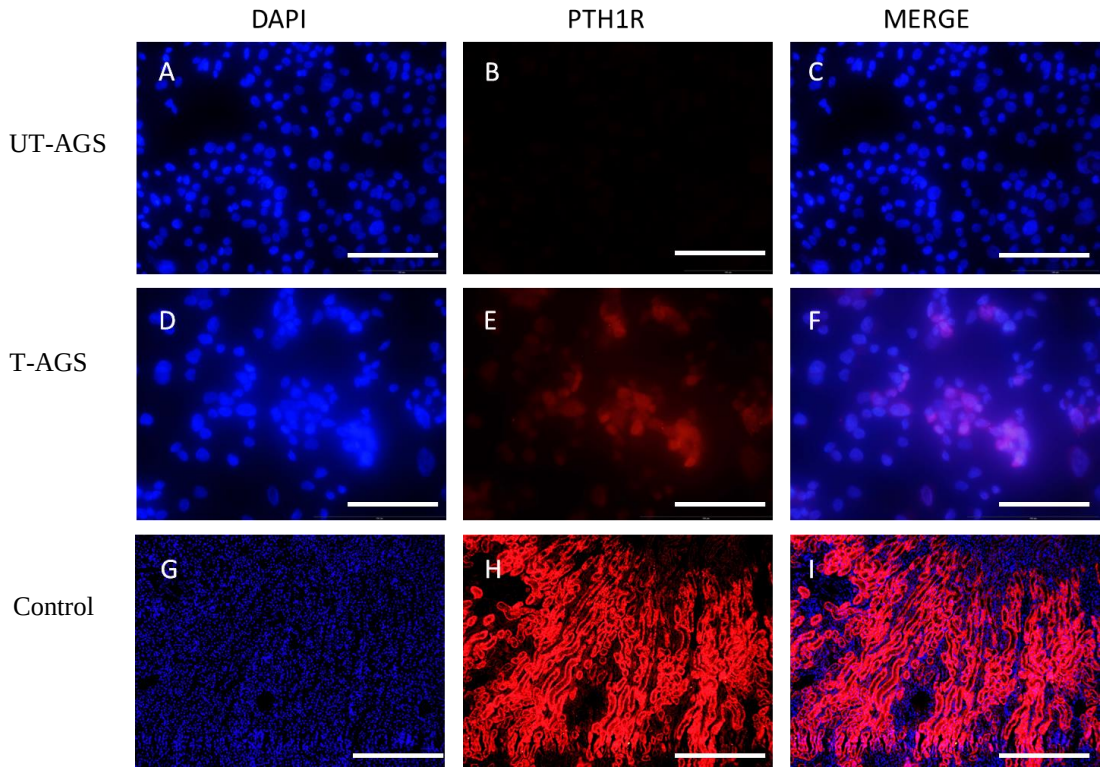


Figure 3.5: AGS cells expressing PTH1R after transfection

(A, B, C) represents un-transfected AGS cells. (D, E, F) represents PTH1R transfected in AGS. (G, H, I) represents mouse kidney section as positive control. Staining was done using Rabbit Polyclonal to anti Parathyroid Hormone Receptor 1 (ab75150, abcam, dilution 1:100). Scale bar (A to F) is 100µm. Scale bar (G to I) is 500µm.

3.4 The Induction of cAMP in Transfected AGS Treated with PTH1R Agonist Suggests Receptor Activation via $G\alpha_s$ Signaling Pathway

To study the effect of PTH1R agonist PTHrP (1-36) in gastric cells; AGS cells were transfected with PTH1R and treated with 1μM of its agonist PTHrP (1-36). Forskolin was used as a positive control stimulator for cAMP production. Results support the activation of $G\alpha_s$ signaling pathway by PTH1R as the accumulation of cAMP increased after treating the cells with PTHrP (1-36) as shown in Figure 3.6.

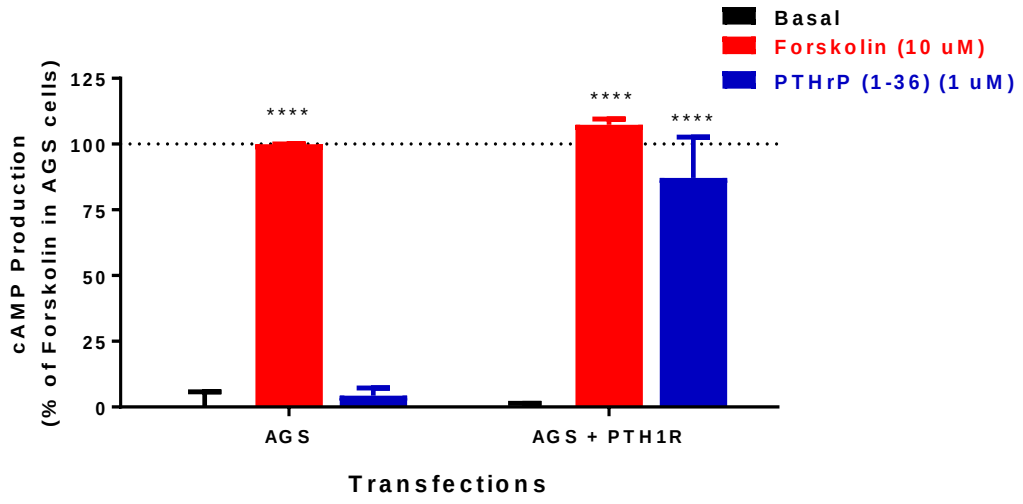


Figure 3.6: cAMP induction in PTH1R transfected AGS cells

AGS cells were transfected and treated with PTHrP (1-36) agonist 1μM. Forskolin 10μM was used to stimulate the production of cAMP as a positive control. Data represents positive stimulation of cAMP after treating transfected AGS with PTHrP agonist. Two-way ANOVA test was used for data analysis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.5 No Induction of cAMP in Transfected mGEP Cells after PTHrP (1-36) Treatment

Since there was a positive induction of cAMP after treating the transfected AGS cells with agonist PTHrP (1-36), we tried investigating if mGEP cell line will be able to induce cAMP and activate $G\alpha_s$ pathway thus having similar effect on signaling pathway like AGS cell line. There was no induction of cAMP in PTHrP (1-36) treated and transfected mGEP cells, unlike AGS cells. Results show a positive cAMP response to Forskolin, but not to PTHrP (1-36) shown in Figure 3.7.

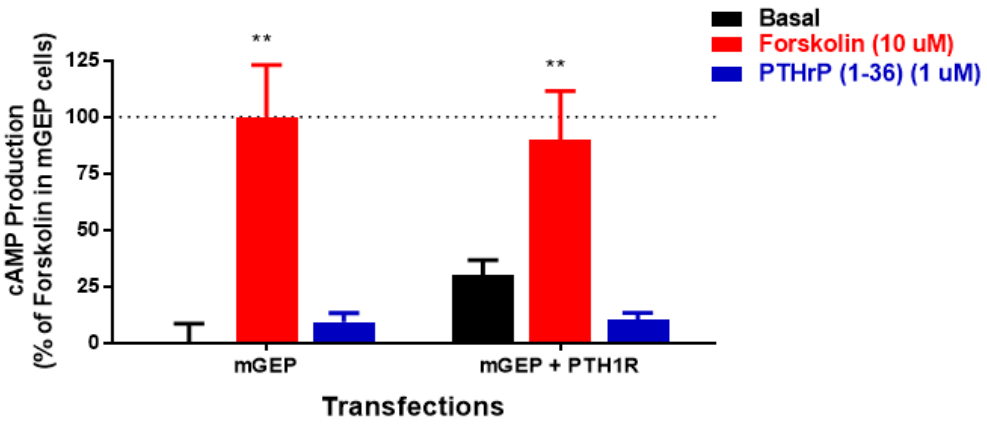


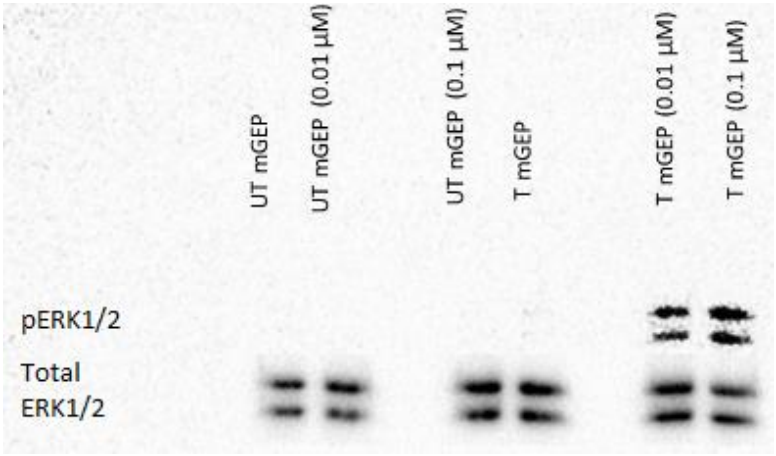
Figure 3.7: No cAMP induction in PTH1R transfected mGEP cells

mGEP cells were transfected and treated with PTHrP (1-36) agonist 1µM. Forskolin 10µM was used to stimulate the production of cAMP as a positive control. Data represents no stimulation of cAMP after treating transfected mGEP cells with PTHrP agonist. Two-way ANOVA test was used for data analysis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.6 PTHrP (1-36) Caused Phosphorylation of ERK 1/2 in Transfected mGEP Cells

Transfected mGEP cells did not show any response in activating the $G\alpha_s$ signaling pathway by the induction of cAMP after PTHrP (1-36) treatment. Another possibility was to test the activation and phosphorylation of ERK pathway after treating the transfected mGEP cells with PTHrP (1-36). Transfected mGEP cells were treated with different concentrations of PTHrP (1-36) agonist for five minutes. Concentrations used (0.01 μM and 0.1 μM). Results indicate a rapid activation and phosphorylation of ERK1/2 in transfected mGEP cells as shown in Figure 3.8.

Phosphorylated ERK1/2 was compared to total ERK1/2 and the fold increase was calculated. Results indicate 22 fold increase of phosphorylated ERK1/2 compared to the total ERK1/2 in the transfected mGEP treated with 0.01μM PTHrP (1-36) for five minutes. Furthermore, results showed significant 36 fold increase of phosphorylated ERK1/2 compared to Total ERK1/2 in the transfected mGEP treated with 0.1μM PTHrP (1-36) for five minutes.



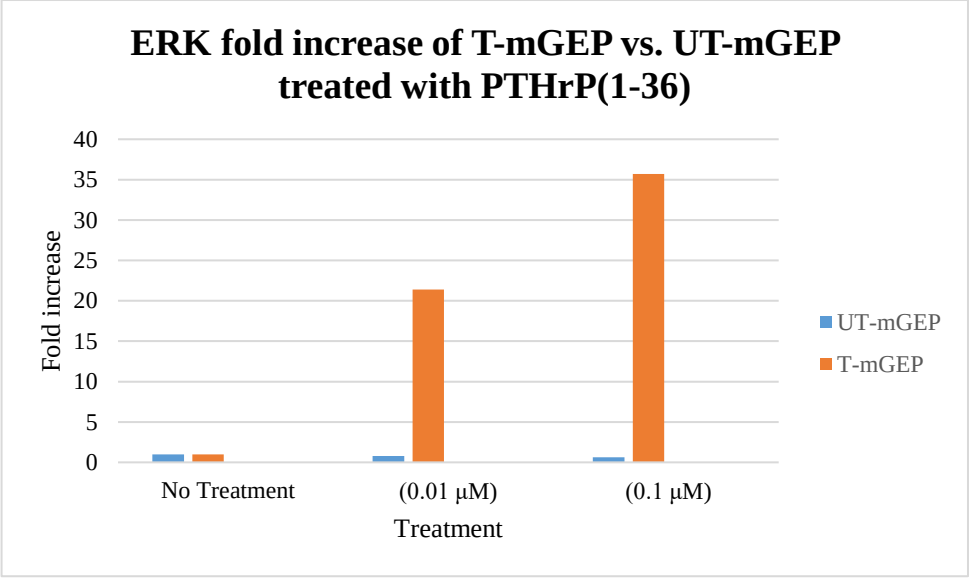


Figure 3.8: The activation of ERK1/2 pathway in PTH1R transfected mGEP

mGEP cells were transiently transfected for 48 hrs with PTH1R (2µg/well). Serum starved cells were treated with PTHrP (1-36) at two different doses (0.01 µM) and (0.1 µM) for five minutes before lysis. Bar graph represents the fold increase of pERK1/2 compared to Total ERK1/2. Highest fold increase was reported at concentration 0.1µM after PTHrP (1-36) treatment.

3.7 Effect of PTHrP (1-36) and PTHrP (7-34) on Transfected mGEP Cell Viability

The effect of different concentrations of PTHrP (1-36) and PTHrP (7-34) at different times was tested on PTH1R transfected mGEP cell line shown in Figure 3.9. Concentrations varied from 0.01, 0.05, 0.1, 0.5, 1, 1.5, 2.5, 5, and 10 μM. Although repeated several times, the results demonstrated inconsistency among the groups of each treatment (experiment repeated at least three times for both time points). There was a trend with slight increase after treatment with agonist at 24 hrs. However, this change is not significant.

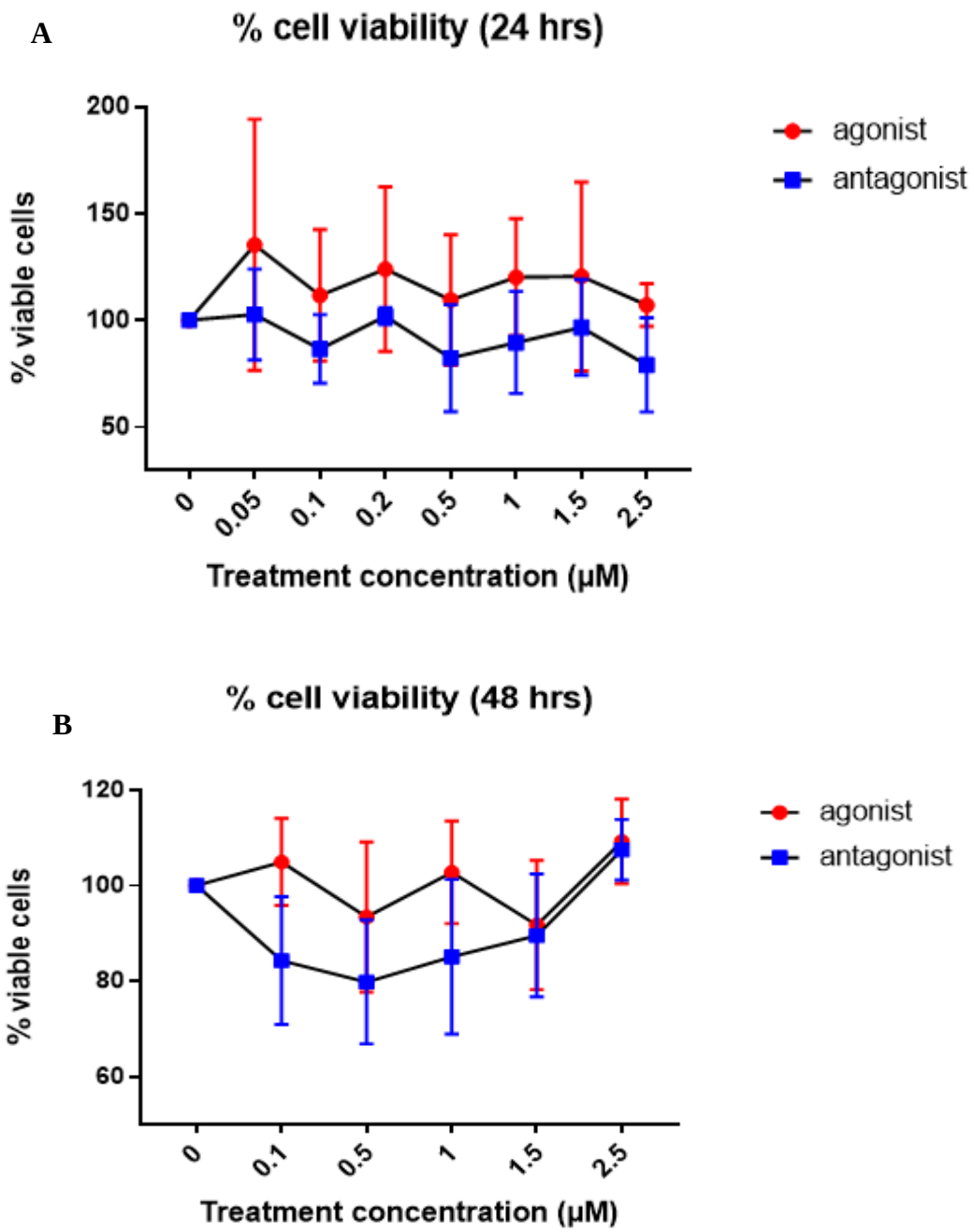


Figure 3.9: Cellular viability of PTH1R transfected mGEP upon agonist and antagonist treatment for 24 and 48 hrs

PTHrP (1-36) and PTHrP (7-34) did not show any consistent significance or results recorder at 24 hrs. and 48 hrs. Statistical analysis for cell viability data was performed using Two-way ANOVA test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.8 Morphological Changes in mGEP Cells Treated with PTHrP (1-36) and PTHrP (7-34)

Morphological observations of mGEP cells treated with PTHrP (1-36) as shown from (Figure 3.10 to Figure 3.13) and PTHrP (7-34) as shown from (Figure 3.14 to Figure 3.17) for different times (6, 12, 24, and 48 hrs.) and different doses (0.01, 0.1, 0.2, 0.5, and 1 μM). Results show change in shape and structure between untransfected and transfected cells in different conditions. Different doses of each treatment did not show drastic morphological changes except for long connections and cell membranes connecting one cell to another. Rounding and shrinking of cells were observed in all treatments at different doses.

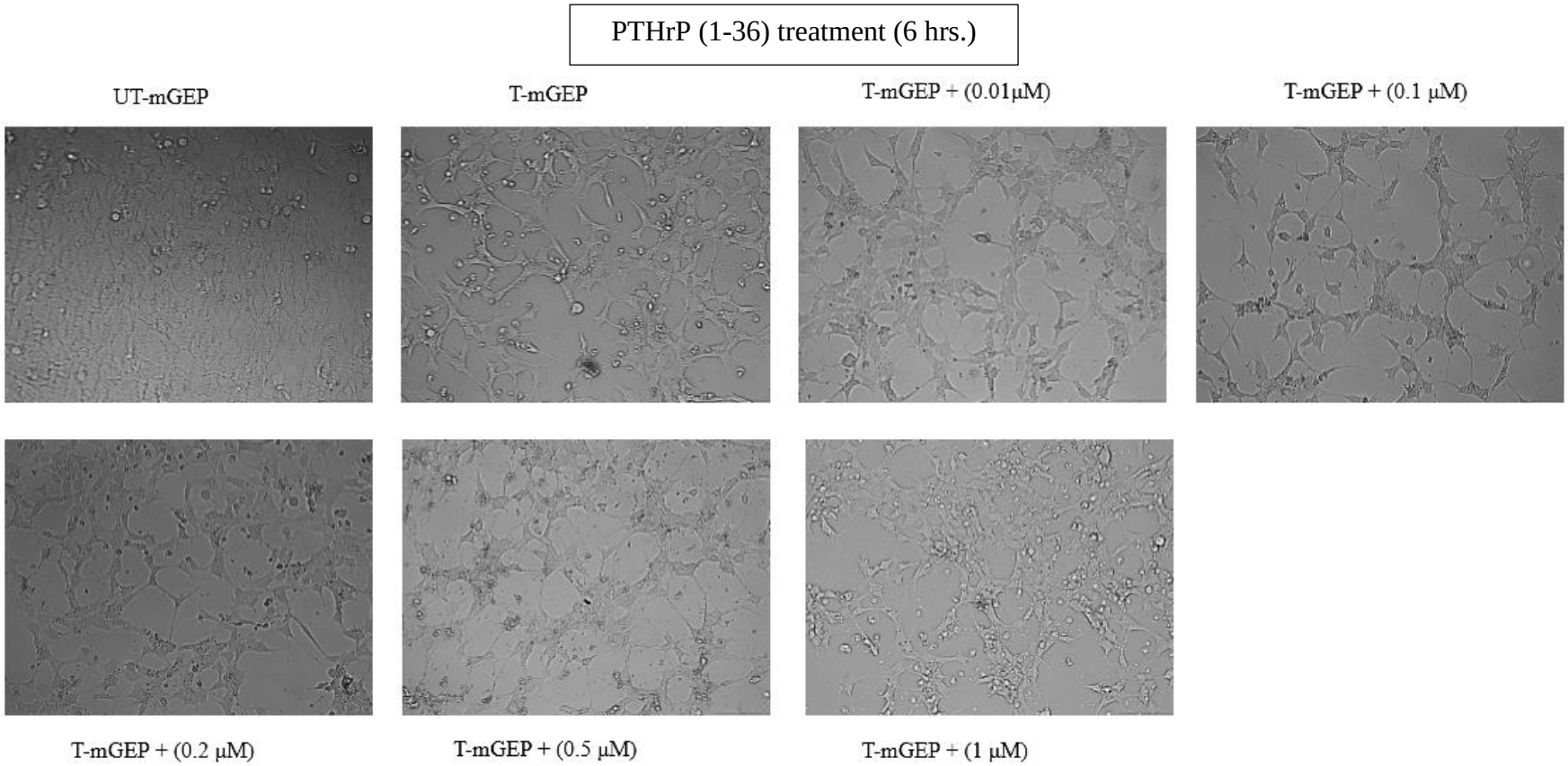


Figure 3.10: Morphology of UT-mGEP and T-mGEP cells treated with PTHrP (1-36) after 6 hrs

The observed morphology of T-mGEP cells showed larger spaces between cells and clustering at different concentrations of PTHrP (1-36) treatment for the first 6 hrs.

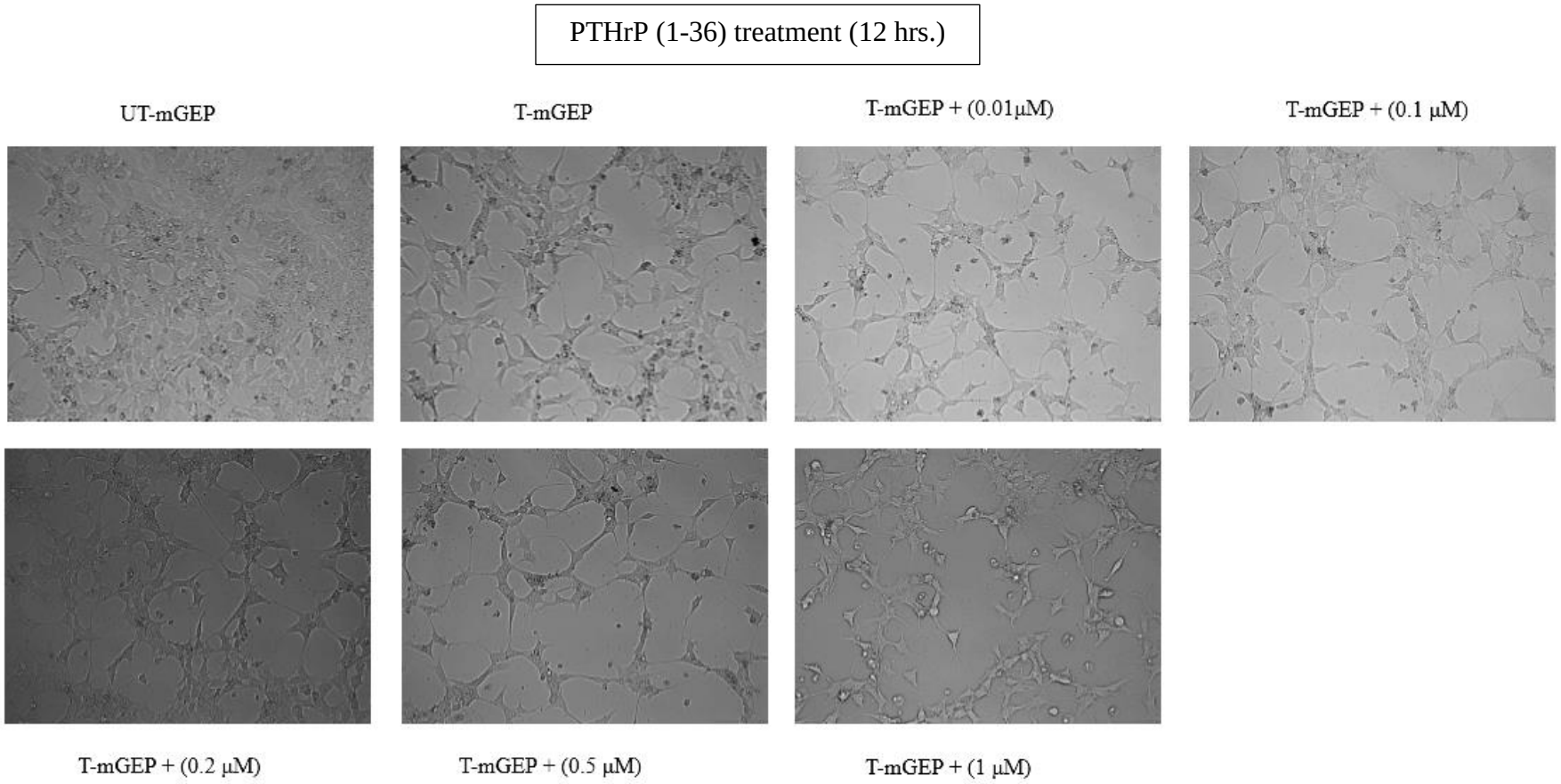


Figure 3.11: Morphology of UT-mGEP and T-mGEP cells treated with PTHrP (1-36) after 12 hrs

The observed morphology of T-mGEP cells showed larger spaces between cells and clustering at different concentrations of PTHrP (1-36) treatment for 12 hrs.

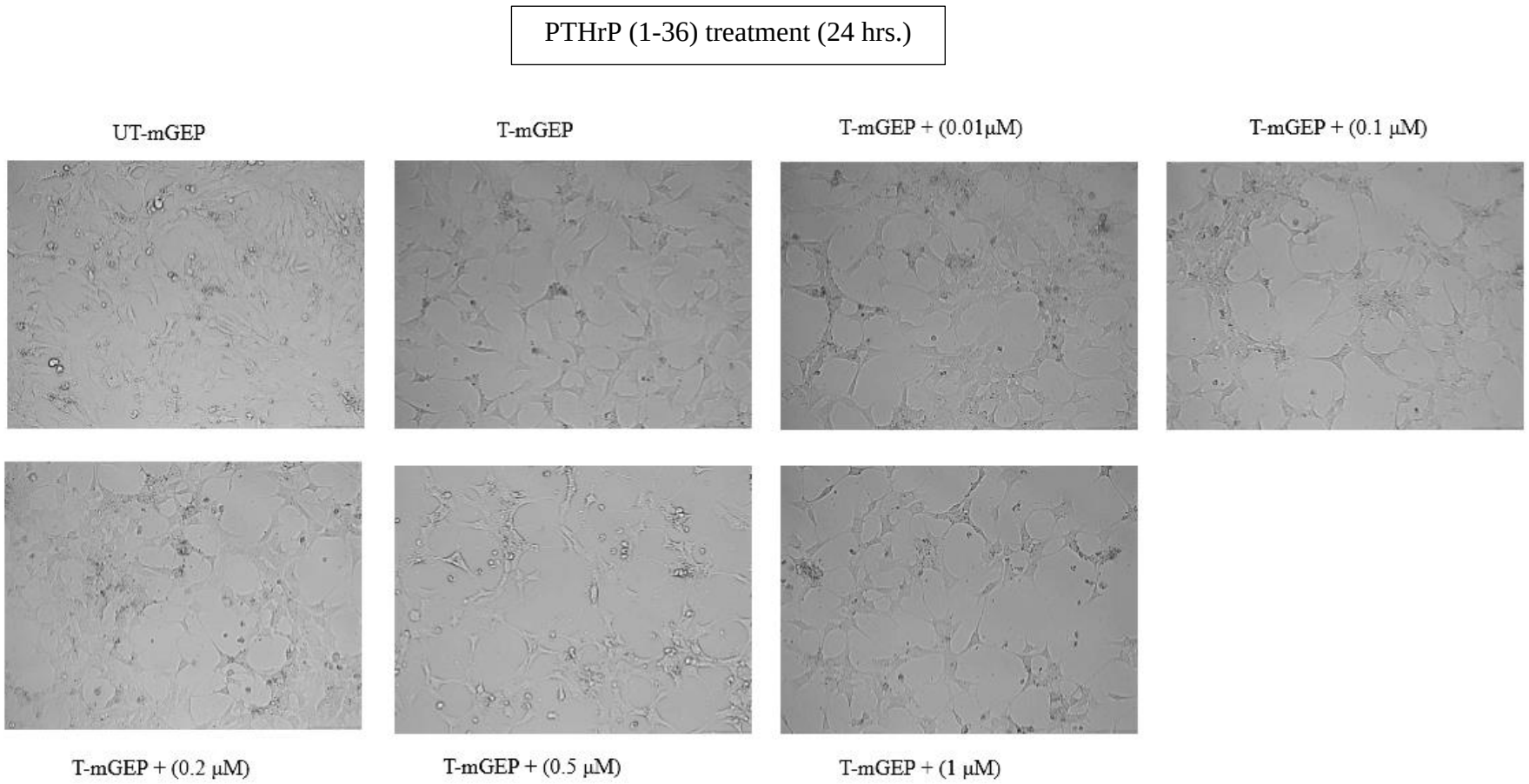


Figure 3.12: Morphology of UT-mGEP and T-mGEP cells treated with PTHrP (1-36) after 24 hrs

The observed morphology of T-mGEP cells showed larger spaces between cells and clustering at different concentrations of PTHrP (1-36) treatment for 24 hrs.

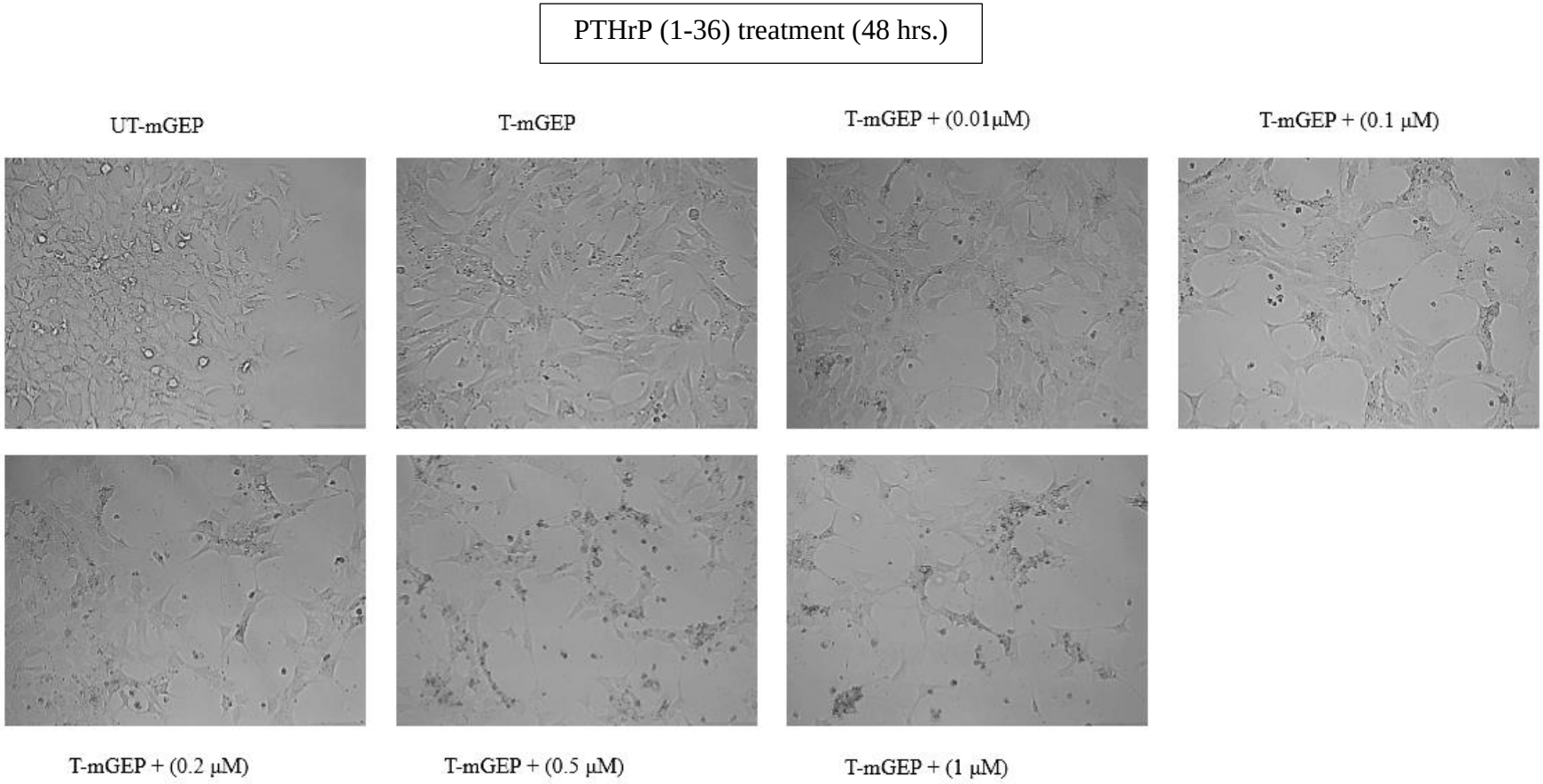


Figure 3.13: Morphology of UT-mGEP and T-mGEP cells treated with PTHrP (1-36) after 48 hrs

The observed morphology of T-mGEP cells showed larger spaces between cells and clustering at different concentrations of PTHrP (1-36) treatment for 48 hrs.

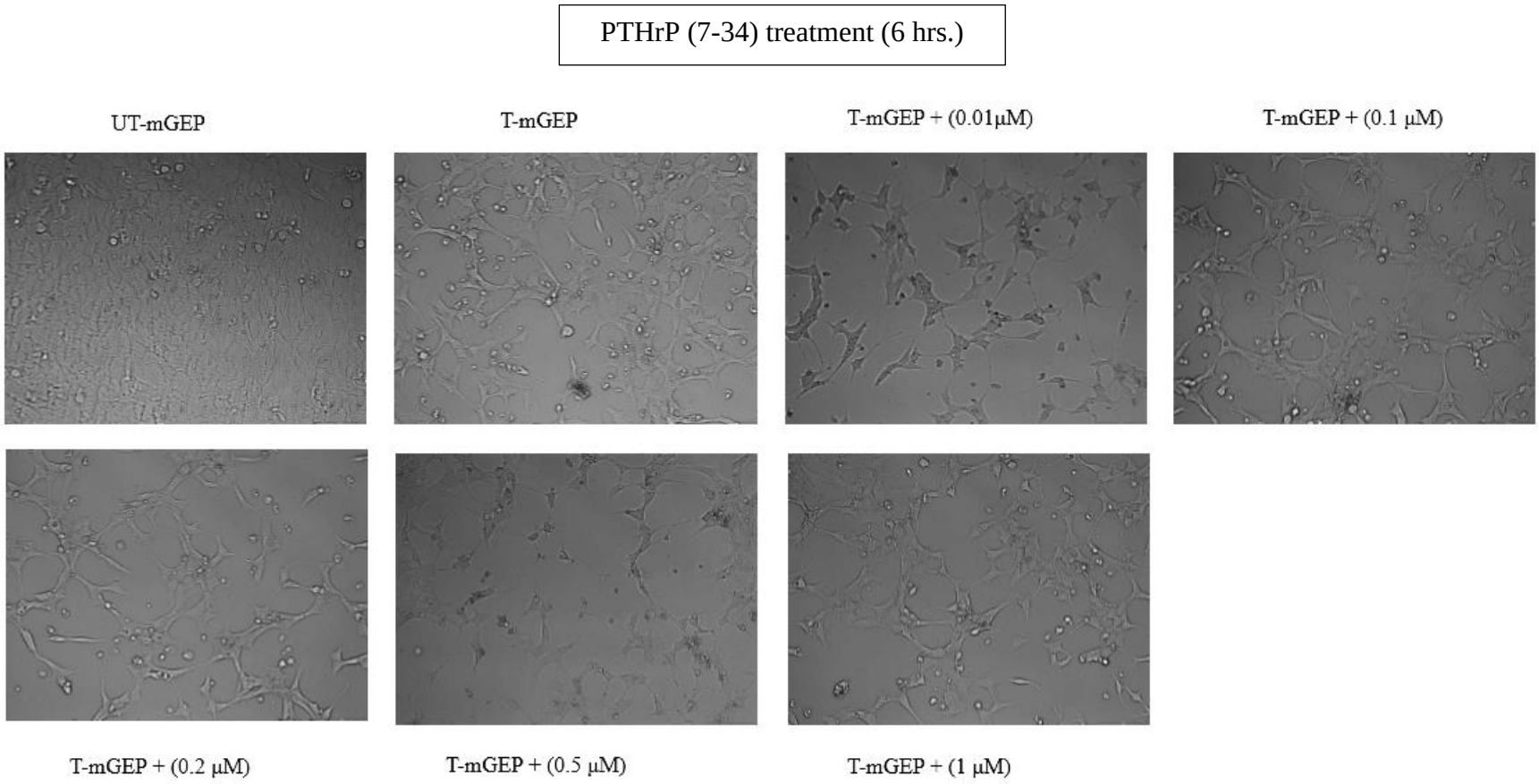


Figure 3.14: Morphology of UT-mGEP and T-mGEP cells treated with PTHrP (7-34) after 6 hrs

The observed morphology of T-mGEP cells showed larger spaces between cells and clustering at different concentrations of PTHrP (7-34) treatment for 6 hrs.

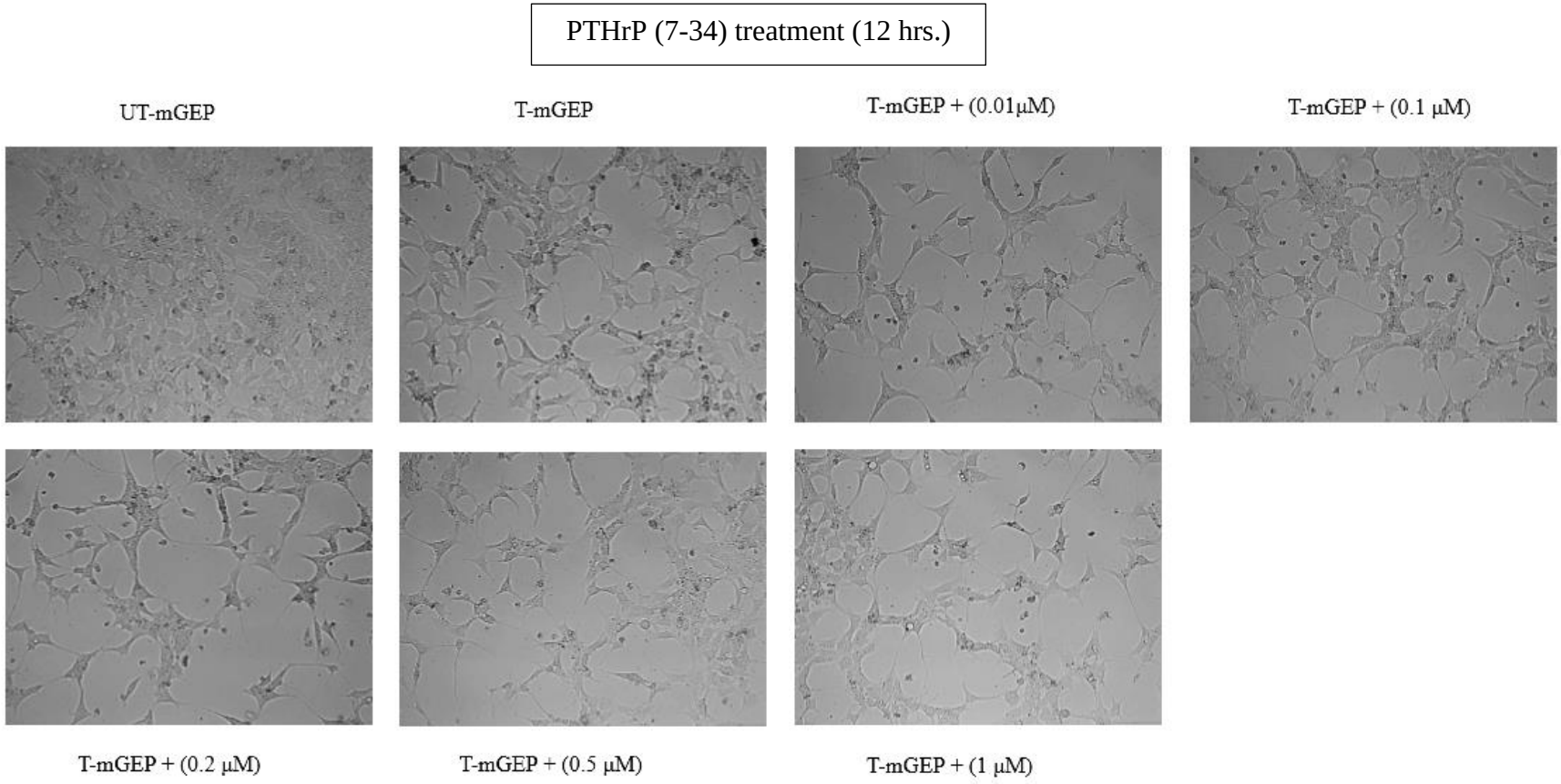


Figure 3.15: Morphology of UT-mGEP and T-mGEP cells treated with PTHrP (7-34) after 12 hrs

The observed morphology of T-mGEP cells showed larger spaces between cells and clustering at different concentrations of PTHrP (7-34) treatment for 12 hrs.

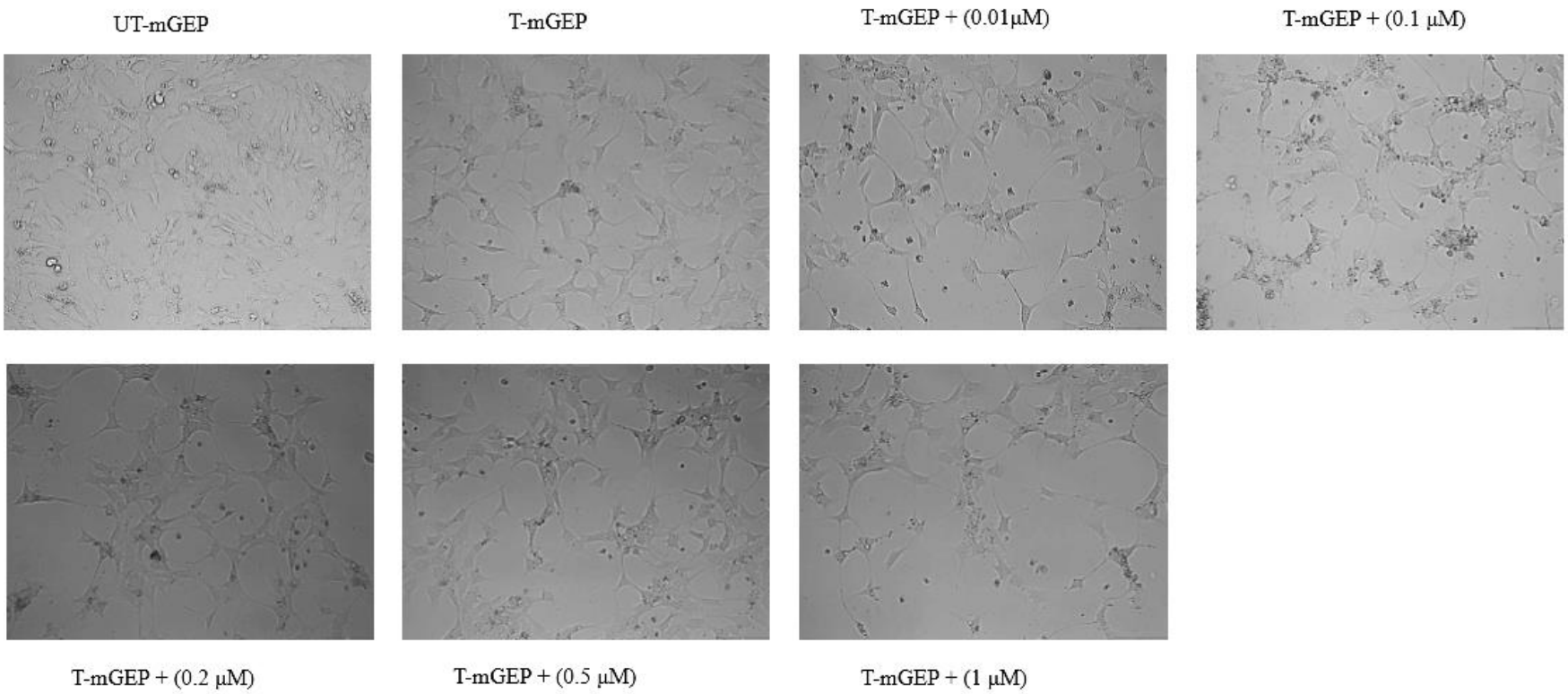


Figure 3.16: Morphology of UT-mGEP and T-mGEP cells treated with PTHrP (7-34) after 24 hrs

The observed morphology of T-mGEP cells showed larger spaces between cells and clustering at different concentrations of PTHrP (7-34) treatment for 24 hrs.

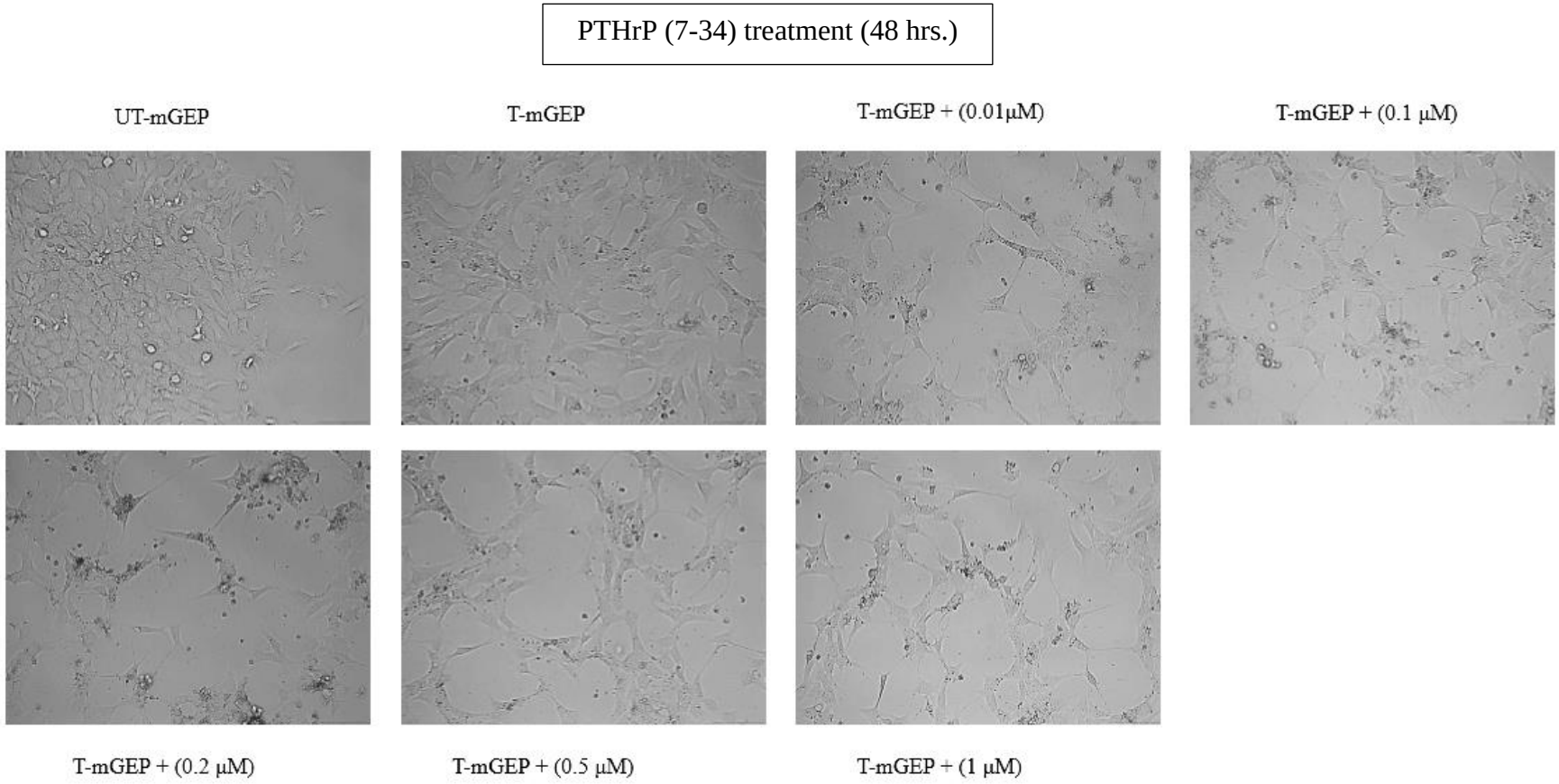


Figure 3.17: Morphology of UT-mGEP and T-mGEP cells treated with PTHrP (7-34) after 48 hrs

The observed morphology of T-mGEP cells showed larger spaces between cells and clustering at different concentrations of PTHrP (7-34) treatment for 48 hrs.

3.9 Expression of PTH1R Target Genes after PTHrP (1-36) Treatment

Expression of PTH1R target genes were examined using Real Time-PCR after transfecting mGEP cells for 24 hrs., starved overnight, and then treated with PTHrP (1-36) for five minutes before extracting the RNA. The control cells used were transfected cells not treated with PTHrP (1-36).

In the case for *CaSR, FGF23, IL-6,* and *NHERF1,* while comparing transfected mGEP to transfected and treated mGEP with PTHrP (1-36), there is no significance change in the gene expression. However, for the target gene *LDLR*, there was significant increase in transfected and treated mGEP cells in comparison with transfected mGEP cells.

On the other hand, while comparing untransfected mGEP to transfected mGEP, there was a significant reduction of gene expression in *CaSR, FGF23, IL-6,* and *NHERF1* in transfected mGEP. However, *LDLR* gene expression was upregulated in transfected mGEP in comparison to untransfected mGEP (Figure 3.18).

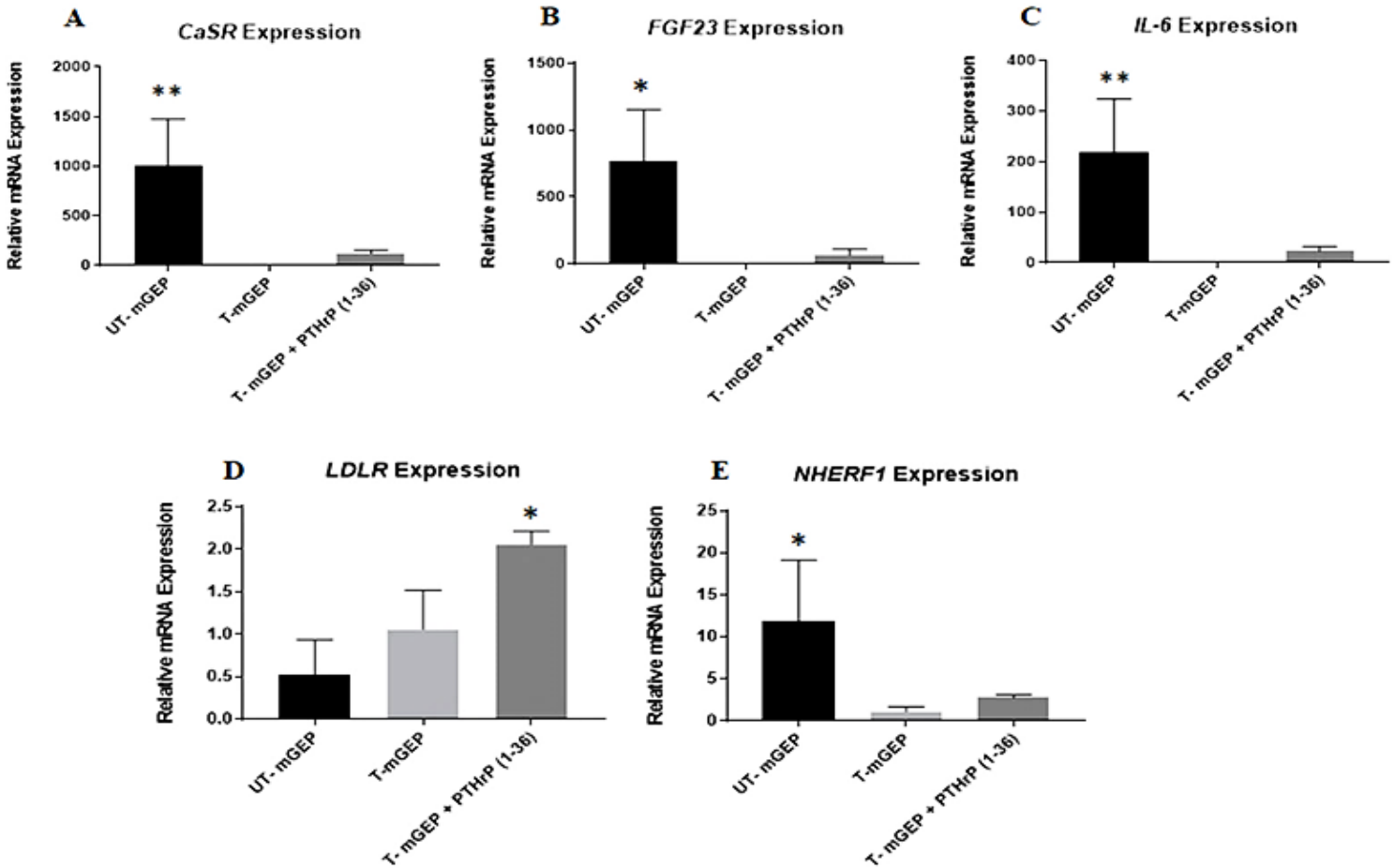


Figure 3.18: Relative mRNA expression of PTH1R target genes in mGEP cells

RNA expression of PTH1R target genes was analyzed by Real Time-PCR in Untransfected mGEP, Transfected mGEP, Transfected and treated mGEP with PTHrP (1-36) for five minutes. Results were compared to the control which is Transfected mGEP. One-way ANOVA test was used for data analysis. *p<0.05, **p<0.01, ***p<0.001,****p<0.0001.

Chapter 4: Discussion

PTH1R regulates calcium ion homeostasis in bones and kidneys, as supported by many extensive researches [29]. However, very limited studies focused on other regulatory and functional effects of PTH1R in the gastric system.

In the present study, the expression of PTH1R in gastric epithelial cells both *in vivo* and *in vitro* was examined. Even though the expression was detected in normal human and mouse stomach as shown in Figure 3.1 and 3.2 respectively, the expression was not detected in cell lines representative of mouse gastric progenitor cells, and human gastric cancer cells.

The absence of PTH1R expression in cells was managed by transfecting PTH1R into the cells. To confirm successful transfection, the cells were stained against PTH1R antibody, and the localization of the receptor was detected to be cytoplasmic and nuclear as shown in Figure 3.4 and Figure 3.5. Although PTH1R is a GPCR, which consists of large sequence of amino acids for its protein structure embedded in the cell membrane, yet studies reported its ability to localize into the cytoplasm and nucleus via its NLS which is shown in the diagram Figure 1.3, this is consistent with other studies [30,31] .

PTH1R acts on multiple signaling pathways when it is stimulated [29]. In our study, we found that the human gastric cancer cell line activated $G\alpha_s$ as shown in Figure 3.6. However, mouse gastric progenitor cells failed to induce $G\alpha_s$ Figure 3.7, and as such another signaling pathway was examined, and we confirmed the activation of ERK1/2 as shown in Figure 3.8. ERK1/2 is a known pathway for many endocrine cells and its importance in various cellular processes including proliferation has been documented in a number of studies [42,97]. Also, ERK1/2 has been reported to be

overexpressed in patients with gastric cancer, this pathway represents an ideal target for generating therapeutic drugs for patients with gastric cancer as discussed in many articles [97,98].

In a study, they reported that when one signaling pathway is activated, it might cause inhibition of other signaling pathways [38]. This concept is relatively new in GPCR, and is referred to as biased GPCR signaling [99]. Since the activation of different signaling pathways for PTH1R is studied, this shows that different activations might depend on cell type, ligand, and condition of the cell [100]. For example a study was conducted on HEK293 cells using a different PTH1R ligand, PTH (1-34) and was reported to induce ERK1/2, but when they used a synthetic and modified ligand, PTH (1-36) which solely acts on $G\alpha_s$ pathway, it failed to induce ERK1/2 and only activated $G\alpha_s$ [42]. In PTH1R, biased agonism involving β-arrestin that acts independently from GPCR mechanism is a new therapeutic target to improve osteoporosis therapy [101,102].

Another result emerged at this point which is the presence of PTHLH in mGEP cells as shown in Figure 3.2B. We proposed a hypothesis which states that as soon as the mGEP cells are transfected with PTH1R, the cell will uptake its endogenous PTHLH and activate the receptor. The prolonged overstimulation and exposure of PTH1R to PTHLH will cause the receptor to reach its threshold and stop responding to any further PTHLH or PTH treatment. As such the transfection is transient for a time duration of 48 hrs, resulting in receptor desensitization and will thus activate ERK1/2 pathway. The desensitization of GPCRs has been documented in other studies as well which matches well with the proposed explanation above [38,42,103]. This result was further confirmed when we transfected mGEP cells and noted cAMP production without adding any treatment from our side, illustrated in Figure 3.7.

However, whether or not the ligand PTHLH did internalize into the cell along with the receptor, needs to be further examined since it was previously reported that PTHLH contains an NLS as well [45].

Collectively, our results can be explained by receptor desensitization. In the case of AGS cells, they do not express PTHLH as a ligand for PTH1R as reported in one study [36], neither do they express the receptor PTH1R based on our finding shown in Figure 3.1. Upon transfecting and treating the cells with PTHrP (1-36), the receptor was activated causing an induction in $G\alpha_s$ pathway producing cAMP as shown in Figure 3.6.

Using this proposed hypothesis, we can explain the results found in cell viability as well, which resulted in inconsistent results as shown in Figure 3.9. However, the morphological changes of transfected mGEP treated with different concentrations of agonist and antagonist, as shown from Figure 3.10 showed larger cell to cell spaces and longer connections between cells. This needs to be further examined and investigated.

As for the target genes, no significant change was observed in *CaSR, FGF23, IL6* and *NHERF1* between PTH1R-transfected mGEP and transfected and PTHrP (1-36) treated mGEP. This is also consistent with our previous findings in which we found no significant change between transfected mGEP cells and transfected and treated mGEP cells. Moreover, the significant change for the above mentioned target genes were while comparing PTH1R-transfected mGEP with untransfected mGEP. However, it is important to note that there was a significant upregulation of *LDLR* in treated and PTH1R transfected mGEP in comparison with transfected mGEP.

Results showed a down regulation of *CaSR, FGF23, IL-6* and *NHERF1* in transfected mGEP cells compared to untransfected mGEP cells. This can be explained

in a negative feedback loop, in which it is supported by other studies [77], increased calcium levels in the blood can be sensed by *CaSR* which will downregulate and inhibit PTH1R from activating bone resorption to secrete more calcium in the blood. We report a similar finding in which after 48 hrs of transfection, the negative feedback loop for transfected mGEP cells caused the downregulation of the target genes in order to maintain gastric calcium homeostasis as shown in Figure 3.18.

IL-6 and *NHERF1* both showed a downregulation in PTH1R transfected mGEP cells in comparison to untransfected mGEP cells. *IL-6* is previously reported to be upregulated by PTHLH in cancer patients [80]. *NHERF1* is thought to inhibit PTH1R desensitization [79,80]. My results could be explained as that in transfected mGEP cells expressing PTH1R, *NHERF1* is being used up by the cells in order to inhibit PTH1R desensitization. This can be further illustrated in a slight increase in *NHERF1* gene expression in transfected and PTHrP (1-36) treated mGEP cells.

LDLR showed a significant upregulation in PTH1R transfected and treated mGEP cells in comparison to only PTH1R transfected cells. It is known from previous research that PTH can induce *LDLR* [80]. *LDLR* is known to act via the Wnt signaling pathway [104]. It has been also reported that PTH1R can act on the Wnt pathway along with the frizzled GPCRs to regulate bone formation [105]. The Wnt pathway is a pharmaceutical target to treat many different cancers [106]. Briefly, Wnt pathway functions by activating either canonical or non-canonical pathway. In the case of non-canonical pathway, it is activated by LDLR and frizzled receptors. This will be followed by a cascade of stimulations leading to activation of different Wnt target genes which plays important roles in cellular regulation [106]. This shows that PTH1R activation might suppress and downregulate other target genes in order to activate the Wnt pathway, which is also reported in many gastric cancers [107]. The mechanism

of action and more details on this crosstalk which highlights the importance of cholesterol and lipoprotein with PTH1R activation needs to be further investigated, especially in the gastric system.

Chapter 5: Conclusion and Future Directions

The present study aimed to determine the normal expression *in vivo* on mice and the, function and role of PTH1R in gastric epithelium *in vitro* on mGEP (mouse) and AGS (human) cells. These aims were established by further studying the signaling pathway of PTH1R and the mechanism of induction in the gastric epithelial cells. Overall findings are shown in figure 5.1.

Our *in vivo* results showed positive expression of PTH1R on both transcriptional and translational levels in the forestomach, corpus, and antrum of the gastric epithelium of wild type mice. Another important *in vivo* result was the expression of PTH1R in normal human stomach.

Our *in vitro* results were subdivided into two, where we tested the signaling pathway of AGS and mGEP mouse cell lines. In the case of AGS, cAMP was induced in PTH1R transfected and PTHrP (1-36) treated cells. mGEP cells, on the other hand, showed expression of the ligand PTHLH, and PTH1R transfected and PTHrP (1-36) treated mGEP cells did not induce cAMP, in fact pERK1/2 was reported which is highly linked to receptor desensitization.

Target genes were also investigated in this study, and while comparing untransfected mGEP cells to transfected mGEP cells, significant downregulation was reported in *CaSR, FGF23, IL6, and NHERF1*. On the other hand, upregulation of *LDLR* in PTH1R transfected and treated mGEP cells in comparison with transfected mGEP. Collectively, these results showed the importance of PTH1R in maintaining and regulating gastric epithelium.

PTH1R is not highly studied in the gastric glands. One of our interesting results is the expression of PTH1R in normal mouse stomach. However, the exact localization

of the receptor needs to be further investigated by conducting immune co-staining of different types of gastric cells and PTH1R. Especially, since previous findings illustrate cAMP importance in regulating parietal cells to secrete gastric acid. It will be novel to identify the exact location of PTH1R and whether or not it is expressed in parietal cells, since these cells also express PTHLH. This might propose a functional mechanism to identify PTHLH autocrine regulatory role in maintaining gastric homeostasis.

PTH1R is a GPCR which was identified with NLS in the C terminal as previously reported; however, no further research examined how this sequence functions in the stomach and its role in comparison to normal receptor activation. Investigating this issue by creating a PTH1R C terminal knock out might highlight new modified signaling mechanism in response to specific ligand-binding sites. Moreover, PTHLH contains NLS as well, it would be interesting to examine by co-precipitation if PTHLH will internalize with PTH1R in any specific signaling system.

Our morphological study showed difference between transfected mGEP and untransfected mGEP cells. A future study aiming the use of electron microscopy to detect the changes inside the cell will be beneficial in understanding the morphological changes.

In this study, the only two pathways studied were $G\alpha_s$ and ERK1/2. It is vital to study the other signaling pathways as the receptor can act otherwise in different organs and systems using different agonists. Also, one of the aims in future directions could be testing the phosphorylation of ERK1/2 using western blot at different time points after treating the cells with PTHrP (1-36). In this study, PTHrP (1-36) agonist was used to stimulate PTH1R, however, other agonists exist such as PTH (1-34), and it would be interesting to examine it for similar or different results.

Since the focus of this project was on PTH1R in the gastric gland, very little studies were available to compare our results to. More research needs to be conducted on gastric cancer and the signaling pathway that is activated when PTH1R expression is upregulated.

To overcome the endogenous PTHLH effect on cells and its alteration of the results, it will be a better option to use AGS transfected cells since it does not express PTHLH, this will lead to more consistent results.

The target genes investigated in this thesis were *CaSR, FGF23, IL6, LDLR,* and *NHERF1* by Real-Time PCR. It is important to investigate more target genes in the future to be able to examine the overall change in the gastric system. For this project, only 0.1 µM which is a small concentration of PTHrP (1-36) was used in treating transfected mGEP cells for five minutes only. Other concentrations and different times should be further studied. Creating a detailed time and dose study to confirm the hypothesis of desensitization in the RT-PCR experiment will be helpful. Also, treating the transfected mGEP cells with an antagonist PTHrP (7-34) should restore the gene expression of the target genes.

Finally, PTH1R *in vitro* study on gastric cell lines showed change and induction of several signaling pathways and target genes. Conducting further studies on the importance of PTH1R in gastric glands *in vivo* would be very interesting, either by knockdown of PTH1R, or treating wild type mice of different ages with PTH1R agonists and antagonists for different times and concentrations.

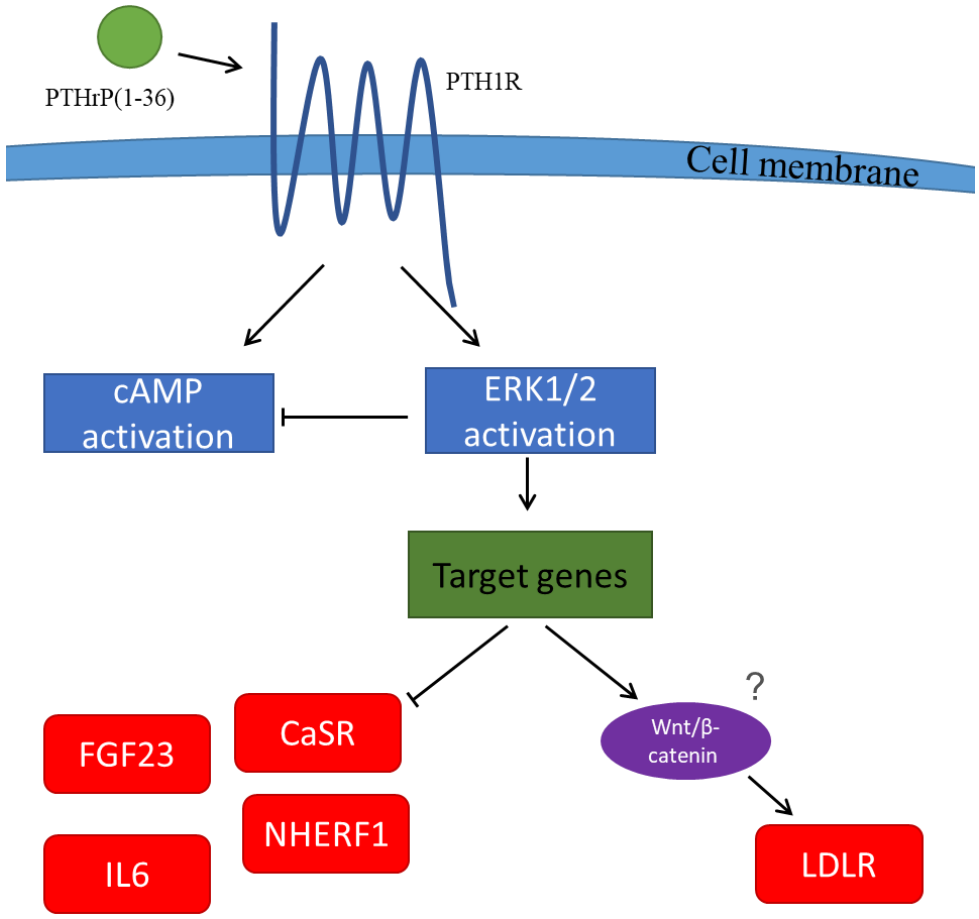


Figure 5.1: Conclusive overall result model illustrating final findings

References

[1] A. Izquierdo *et al.*, “The parathyroid hormone-related protein system and diabetic nephropathy outcome in streptozotocin-induced diabetes,” *Kidney Int. Lond.*, vol. 69, no. 12, pp. 2171–2177, 2006.

[2] M. Mannstadt, H. Jüppner, and T. J. Gardella, “Receptors for PTH and PTHrP: their biological importance and functional properties,” *Am. J. Physiol.-Ren. Physiol.*, vol. 277, no. 5, pp. F665–F675, 1999.

[3] T. L. Clemens *et al.*, “Parathyroid hormone-related protein and its receptors: nuclear functions and roles in the renal and cardiovascular systems, the placental trophoblasts and the pancreatic islets,” *Br. J. Pharmacol.*, vol. 134, no. 6, pp. 1113–1136, 2001.

[4] R. C. Gensure, T. J. Gardella, and H. Jüppner, “Parathyroid hormone and parathyroid hormone-related peptide, and their receptors,” *Biochem. Biophys. Res. Commun.*, vol. 328, no. 3, pp. 666–678, 2005.

[5] D. Zindel *et al.*, “Identification of key phosphorylation sites in PTH1R that determine arrestin3 binding and fine-tune receptor signaling,” *Biochem. J.*, vol. 473, no. 22, pp. 4173–4192, 2016.

[6] J.-P. Vilardaga, G. Romero, P. A. Friedman, and T. J. Gardella, “Molecular basis of parathyroid hormone receptor signaling and trafficking: a family B GPCR paradigm,” *Cell. Mol. Life Sci. CMLS*, vol. 68, no. 1, pp. 1–13, 2011.

[7] M. Ito, M. Nakashima, T. Nakayama, S. Shikuwa, A. Ohtsuru, and I. Sekine, “Preventive effects of parathyroid hormone-related peptide on stress-induced gastric hypercontraction in the rat,” *J. Gastroenterol. Hepatol.*, vol. 17, no. 12, pp. 1260–1266, 2002.

[8] W. Chang *et al.*, “Parathyroid Hormone-Like Hormone is a Poor Prognosis Marker of Head and Neck Cancer and Promotes Cell Growth via RUNX2 Regulation,” *Sci. Rep. Nat. Publ. Group Lond.*, vol. 7, pp. 41131-41143, 2017.

[9] M. Nakashima *et al.*, “Expression of parathyroid hormone-related peptide in human thyroid tumours,” *J. Pathol.*, vol. 175, no. 2, pp. 227–236, 1995.

[10] W. Hoffmann, “Stem Cells, Self-Renewal and Cancer of the Gastric Epithelium,” *Current Medicinal Chemistry*, 30-Nov-2012. [Online]. Available: http://www.eurekaselect.com/105285/article. [Accessed: 16-Mar-2019].

[11] S. M. Karam, “Lineage commitment and maturation of epithelial cells in the gut,” *Front. Biosci. J. Virtual Libr.*, vol. 4, pp. D286-298, 1999.

[12] Z. Cheng, D. Garvin, A. Paguio, P. Stecha, K. Wood, and F. Fan, “Luciferase Reporter Assay System for Deciphering GPCR Pathways,” *Curr. Chem. Genomics*, vol. 4, pp. 84–91, 2010.

[13] W. N. Dare, C. A. Oyinbo, and A.-H. M. Izunya, “Study of the Structure of the Gastric Mucosa in the Mouse (Cell Population),” *J. Life Sci. Biomed.*, vol. 2, no. 5, pp 182-186, 2012.

[14] S. M. Karam and C. P. Leblond, “Identifying and counting epithelial cell types in the ‘corpus’ of the mouse stomach,” *Anat. Rec.*, vol. 232, no. 2, pp. 231–246, 1992.

[15] S. M. Karam, “Dynamics of epithelial cells in the corpus of the mouse stomach. IV. Bidirectional migration of parietal cells ending in their gradual degeneration and loss,” *Anat. Rec.*, vol. 236, no. 2, pp. 314–332, 1993.

[16] G.-M. Hu, T.-L. Mai, and C.-M. Chen, “Visualizing the GPCR Network: Classification and Evolution,” *Sci. Rep.*, vol. 7, no. 1, pp. 15495–15510, 2017.

[17] V. V. Gurevich and E. V. Gurevich, “Molecular Mechanisms of GPCR Signaling: A Structural Perspective,” *Int. J. Mol. Sci.*, vol. 18, no. 12, pp. 2519–2536, 2017.

[18] X. Cong, J. Topin, and J. Golebiowski, “Class A GPCRs: Structure, Function, Modeling and Structure-based Ligand Design,” *Curr. Pharm. Des.*, vol. 23, no. 29, pp. 4390–4409, 2017.

[19] A. Bortolato, A. S. Doré, K. Hollenstein, B. G. Tehan, J. S. Mason, and F. H. Marshall, “Structure of Class B GPCRs: new horizons for drug discovery,” *Br. J. Pharmacol.*, vol. 171, no. 13, pp. 3132–3145, 2014.

[20] L. Chun, W. Zhang, and J. Liu, “Structure and ligand recognition of class C GPCRs,” *Acta Pharmacol. Sin.*, vol. 33, no. 3, pp. 312–323, 2012.

[21] M. Eilers, V. Hornak, S. O. Smith, and J. B. Konopka, “Comparison of Class A and D G Protein-Coupled Receptors: Common Features in Structure and Activation,” *Biochemistry*, vol. 44, no. 25, pp. 8959–8975, 2005.

[22] P. S. Klein, T. J. Sun, C. L. Saxe, A. R. Kimmel, R. L. Johnson, and P. N. Devreotes, “A chemoattractant receptor controls development in Dictyostelium discoideum,” *Science*, vol. 241, no. 4872, pp. 1467–1472, 1988.

[23] R. L. Johnson, C. L. Saxe, R. Gollop, A. R. Kimmel, and P. N. Devreotes, “Identification and targeted gene disruption of cAR3, a cAMP receptor subtype expressed during multicellular stages of Dictyostelium development.,” *Genes Dev.*, vol. 7, no. 2, pp. 273–282, 1993.

[24] H.-C. Huang and P. S. Klein, “The Frizzled family: receptors for multiple signal transduction pathways,” *Genome Biol.*, vol. 5, no. 7, pp. 234–241, 2004.

[25] C. Wang *et al.*, “Structural basis for Smoothened receptor modulation and chemoresistance to anticancer drugs,” *Nat. Commun.*, vol. 5, pp. 4355–4375, 2014.

[26] H. Juppner, M. Freeman, and et al, “A G Protein-Linked Receptor for Parathyroid Hormone and Parathyroid Hormone-Related Peptide,” *Sci. Wash.*, vol. 254, no. 5034, pp. 1024–1026, 1991.

[27] H. Subramanian *et al.*, “PTH1R Mutants Found in Patients with Primary Failure of Tooth Eruption Disrupt G-Protein Signaling,” *PLoS ONE*, vol. 11, no. 11, 2016.

[28] T. J. Martin, “Parathyroid Hormone-Related Protein, Its Regulation of Cartilage and Bone Development, and Role in Treating Bone Diseases,” *Physiol. Rev.*, vol. 96, no. 3, pp. 831–871, 2016.

[29] T. J. Gardella and J.-P. Vilardaga, “International Union of Basic and Clinical Pharmacology. XCIII. The Parathyroid Hormone Receptors—Family B G Protein–Coupled Receptors,” *Pharmacol. Rev.*, vol. 67, no. 2, pp. 310–337, 2015.

[30] E. K. Patterson *et al.*, “Expression of PTH1R constructs in LLC-PK1 cells: Protein nuclear targeting is mediated by the PTH1R NLS,” *Bone*, vol. 41, no. 4, pp. 603–610, 2007.

[31] B. W. Pickard, A. B. Hodsman, L. J. Fraher, and P. H. Watson, “Type 1 Parathyroid Hormone Receptor (PTH1R) Nuclear Trafficking: Regulation of PTH1R Nuclear-Cytoplasmic Shuttling by Importin-α/β and Chromosomal Region Maintenance 1/Exportin 1,” *Endocrinology*, vol. 148, no. 5, pp. 2282–2289, 2007.

[32] J. S. Martins, M. de O. Palhares, O. C. M. Teixeira, and M. Gontijo Ramos, “Vitamin D Status and Its Association with Parathyroid Hormone Concentration in Brazilians,” *J. Nutr. Metab.*, vol. 2017, 2017.

[33] D. Nikitovic *et al.*, “Parathyroid hormone/parathyroid hormone-related peptide regulate osteosarcoma cell functions: Focus on the extracellular matrix (Review),” *Oncol. Rep.*, vol. 36, no. 4, pp. 1787–1792, 2016.

[34] G. J. Strewler, R. D. Williams, and R. A. Nissenson, “Human renal carcinoma cells produce hypercalcemia in the nude mouse and a novel protein recognized by parathyroid hormone receptors.,” *J. Clin. Invest.*, vol. 71, no. 3, pp. 769–774, 1983.

[35] A. H. Tashjian, L. Levine, and P. L. Munson, “IMMUNOCHEMICAL IDENTIFICATION OF PARATHYROID HORMONE IN NON-PARATHYROID NEOPLASMS ASSOCIATED WITH HYPERCALCEMIA,” *J. Exp. Med.*, vol. 119, no. 3, pp. 467–484, 1964.

[36] A. Al Menhali, T. M. Keeley, E. S. Demitrack, and L. C. Samuelson, “Gastrin induces parathyroid hormone-like hormone expression in gastric parietal cells,” *Am. J. Physiol. - Gastrointest. Liver Physiol.*, vol. 312, no. 6, pp. G649–G657, 2017.

[37] J. C. Mills, A. J. Syder, C. V. Hong, J. L. Guruge, F. Raaii, and J. I. Gordon, “A molecular profile of the mouse gastric parietal cell with and without exposure to Helicobacter pylori,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 98, no. 24, pp. 13687–13692, 2001.

[38] W. B. Sneddon *et al.*, “Ligand-Selective Dissociation of Activation and Internalization of the Parathyroid Hormone (PTH) Receptor: Conditional Efficacy of PTH Peptide Fragments,” *Endocrinology*, vol. 145, no. 6, pp. 2815–2823, 2004.

[39] N. Sahoo *et al.*, “Gastric acid secretion from parietal cells is mediated by a Ca2+ efflux channel in the tubulovesicle,” *Dev. Cell*, vol. 41, no. 3, pp. 262-273.e6, 2017.

[40] Z.-Q. Li and S. Mårdh, “Interactions between Ca2+- and cAMP-dependent stimulatory pathways in parietal cells,” *Biochim. Biophys. Acta BBA - Mol. Cell Res.*, vol. 1311, no. 2, pp. 133–142, 1996.

[41] C. S. Chew, “Parietal cell protein kinases. Selective activation of type I cAMP-dependent protein kinase by histamine.,” *J. Biol. Chem.*, vol. 260, no. 12, pp. 7540–7550, 1985.

[42] C. A. Syme, P. A. Friedman, and A. Bisello, “Parathyroid Hormone Receptor Trafficking Contributes to the Activation of Extracellular Signal-regulated Kinases but Is Not Required for Regulation of cAMP Signaling,” *J. Biol. Chem.*, vol. 280, no. 12, pp. 11281–11288, 2005.

[43] Y. Pazos, C. J. P. Alvarez, J. P. Camiña, and F. F. Casanueva, “Stimulation of extracellular signal-regulated kinases and proliferation in the human gastric cancer cells KATO-III by obestatin,” *Growth Factors*, vol. 25, no. 6, pp. 373–381, 2007.

[44] W. Yan, S. Chen, Y. Zhao, and X. Ye, “Fisetin inhibits the proliferation of gastric cancer cells and induces apoptosis through suppression of ERK 1/2 activation,” *Oncol. Lett.*, vol. 15, no. 6, pp. 8442–8446, 2018.

[45] M. M. Aarts, A. Rix, J. Guo, R. Bringhurst, and J. E. Henderson, “The nucleolar targeting signal (NTS) of parathyroid hormone related protein mediates endocytosis and nucleolar translocation,” *J. Bone Miner. Res. Off. J. Am. Soc. Bone Miner. Res.*, vol. 14, no. 9, pp. 1493–1503, 1999.

[46] S. L. Ritter and R. A. Hall, “Fine-tuning of GPCR activity by receptor-interacting proteins,” *Nat. Rev. Mol. Cell Biol.*, vol. 10, no. 12, pp. 819–830, 2009.

[47] O. Calvete, M. Herraiz, J. Reyes, A. Patiño, and J. Benitez, “A cumulative effect involving malfunction of the PTH1R and ATP4A genes explains a familial gastric neuroendocrine tumor with hypothyroidism and arthritis,” *Gastric Cancer*, vol. 20, no. 6, pp. 998–1003, 2017.

[48] I. M. al et, “Gastric cancer associated with overexpression of parathyroid hormone-related peptide (PTHrP) and PTH/PTHrP receptor in relation to tumor progression. - PubMed - NCBI.” [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/9213256. [Accessed: 16-Apr-2019].

[49] I. M. and O. A, “[Parathyroid hormone-related peptide (PTHrP) and PTH/PTHrP receptor in the gastrointestinal tract]. - PubMed - NCBI.” [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/8920682. [Accessed: 16-Apr-2019].

[50] P. W. M. Ho *et al.*, “Knockdown of PTHR1 in osteosarcoma cells decreases invasion and growth and increases tumor differentiation in vivo,” *Oncogene N. Y.*, vol. 34, no. 22, pp. 2922–2933, 2015.

[51] K. Motomura *et al.*, “Osteogenic action of parathyroid hormone-related peptide (1-141) in rat ROS cells.,” *Endocr. J.*, vol. 43, no. 5, pp. 527–535, 1996.

[52] M. J. Horwitz, M. B. Tedesco, C. Gundberg, A. Garcia-Ocana, and A. F. Stewart, “Short-Term, High-Dose Parathyroid Hormone-Related Protein as a Skeletal Anabolic Agent for the Treatment of Postmenopausal Osteoporosis,” *J. Clin. Endocrinol. Metab.*, vol. 88, no. 2, pp. 569–575, 2003.

[53] J. L. Vahle *et al.*, “Skeletal Changes in Rats Given Daily Subcutaneous Injections of Recombinant Human Parathyroid Hormone (1-34) for 2 Years and Relevance to Human Safety,” *Toxicol. Pathol.*, vol. 30, no. 3, pp. 312–321, 2002.

[54] M. Maycas, J. A. Ardura, L. F. de Castro, B. Bravo, A. R. Gortázar, and P. Esbrit, “Role of the Parathyroid Hormone Type 1 Receptor (PTH1R) as a Mechanosensor in Osteocyte Survival,” *J. Bone Miner. Res.*, vol. 30, no. 7, pp. 1231–1244, 2015.

[55] D. Yang, R. Singh, P. Divieti, J. Guo, M. L. Bouxsein, and F. R. Bringhurst, “Contributions of parathyroid hormone (PTH)/PTH-related peptide receptor signaling pathways to the anabolic effect of PTH on bone,” *Bone*, vol. 40, no. 6, pp. 1453–1461, 2007.

[56] R. L. Jilka, “Molecular and cellular mechanisms of the anabolic effect of intermittent PTH,” *Bone*, vol. 40, no. 6, pp. 1434–1446, 2007.

[57] J. Feng *et al.*, “A novel human parathyroid hormone (1-34) analog for the treatment of osteoporosis,” *Peptides*, vol. 30, no. 6, pp. 1173–1180, 2009.

[58] Y. Yang and B. Wang, “PTH1R-CaSR Cross Talk: New Treatment Options for Breast Cancer Osteolytic Bone Metastases,” *International Journal of Endocrinology*, 2018. [Online]. Available: https://www.hindawi.com/journals/ije/2018/7120979/. [Accessed: 25-Feb-2019].

[59] N. J. Bundred, W. A. Ratcliffe, R. A. Walker, S. Coley, J. M. Morrison, and J. G. Ratcliffe, “Parathyroid hormone related protein and hypercalcaemia in breast cancer.,” *BMJ*, vol. 303, no. 6816, pp. 1506–1509, 1991.

[60] A. Dittmer *et al.*, “Parathyroid Hormone-related Protein Regulates Tumor-relevant Genes in Breast Cancer Cells,” *J. Biol. Chem.*, vol. 281, no. 21, pp. 14563–14572, 2006.

[61] H. Liang, Y. Zhong, Y. Huang, and G. Chen, “Type 1 receptor parathyroid hormone (PTH1R) influences breast cancer cell proliferation and apoptosis induced by high levels of glucose,” *Med. Oncol.*, vol. 29, no. 2, pp. 439–445, 2012.

[62] K. Aya, H. Tanaka, Y. Ichinose, M. Kobayashi, and Y. Seino, “Expression of parathyroid hormone-related peptide messenger ribonucleic acid in developing kidney,” *Kidney Int.*, vol. 55, no. 5, pp. 1696–1703, 1999.

[63] N. Amizuka *et al.*, “Cell-Specific Expression of the Parathyroid Hormone (PTH)/PTH-Related Peptide Receptor Gene in Kidney from Kidney-Specific and Ubiquitous Promoters,” *Endocrinology*, vol. 138, no. 1, pp. 469–481, 1997.

[64] M. Müller, S. Gagiannis, P. P. Nawroth, M. Brune, and T. Schilling, “Activation of the receptor for parathyroid hormone and parathyroid hormone related protein induces apoptosis via the extrinsic and intrinsic signaling pathway,” *Int. J. Mol. Med.*, vol. 24, no. 3, pp. 373–380, 2009.

[65] M. Romero *et al.*, “Novel Role of Parathyroid Hormone-Related Protein in the Pathophysiology of the Diabetic Kidney: Evidence from Experimental and Human Diabetic Nephropathy,” *Journal of Diabetes Research*, 2013. [Online]. Available: https://www.hindawi.com/journals/jdr/2013/162846/. [Accessed: 20-Mar-2019].

[66] J. Shimomura-Kuroki, M. Farooq, T. Sekimoto, N. Amizuka, and Y. Shimomura, “Characterization of a PTH1R missense mutation responsible for Jansen type metaphyseal chondrodysplasia,” *Odontology*, vol. 105, no. 2, pp. 150–154, 2017.

[67] S. Nampoothiri *et al.*, “Jansen Metaphyseal Chondrodysplasia due to Heterozygous H223R-PTH1R Mutations With or Without Overt Hypercalcemia,” *J. Clin. Endocrinol. Metab.*, vol. 101, no. 11, pp. 4283–4289, 2016.

[68] E. Decker *et al.*, “PTHR1 loss-of-function mutations in familial, nonsyndromic primary failure of tooth eruption,” *Am. J. Hum. Genet.*, vol. 83, no. 6, pp. 781–786, 2008.

[69] S. Duchatelet, E. Ostergaard, D. Cortes, A. Lemainque, and C. Julier, “Recessive mutations in PTHR1 cause contrasting skeletal dysplasias in Eiken and Blomstrand syndromes,” *Hum. Mol. Genet.*, vol. 14, no. 1, pp. 1–5, 2005.

[70] M. Bastepe *et al.*, “A form of Jansen’s metaphyseal chondrodysplasia with limited metabolic and skeletal abnormalities is caused by a novel activating parathyroid hormone (PTH)/PTH-related peptide receptor mutation,” *J. Clin. Endocrinol. Metab.*, vol. 89, no. 7, pp. 3595–3600, 2004.

[71] F. M. Hannan, V. N. Babinsky, and R. V. Thakker, “Disorders of the calcium-sensing receptor and partner proteins: insights into the molecular basis of calcium homeostasis,” *J. Mol. Endocrinol.*, vol. 57, no. 3, pp. R127–R142, 2016.

[72] C. M. Gorvin, “Insights into calcium-sensing receptor trafficking and biased signalling by studies of calcium homeostasis,” *J. Mol. Endocrinol.*, vol. 61, no. 1, pp. R1–R12, 2018.

[73] C. S. Maria *et al.*, “Interplay between CaSR and PTH1R Signaling in Skeletal Development and Osteoanabolism,” *Semin. Cell Dev. Biol.*, vol. 49, pp. 11–23, 2016.

[74] Y. Xue *et al.*, “The calcium-sensing receptor complements parathyroid hormone-induced bone turnover in discrete skeletal compartments in mice,” *Am. J. Physiol. - Endocrinol. Metab.*, vol. 302, no. 7, pp. E841–E851, 2012.

[75] W. Kim *et al.*, “Calcium-Sensing Receptor (CaSR) Promotes Breast Cancer by Stimulating Intracrine Actions of Parathyroid Hormone-Related Protein,” *Cancer Res.*, vol. 76, no. 18, pp. 5348–5360, 2016.

[76] M. M. Dufner *et al.*, “The calcium-sensing receptor acts as a modulator of gastric acid secretion in freshly isolated human gastric glands,” *Am. J. Physiol.-Gastrointest. Liver Physiol.*, vol. 289, no. 6, pp. G1084–G1090, 2005.

[77] R. Xie *et al.*, “Calcium Promotes Human Gastric Cancer via a Novel Coupling of Calcium-Sensing Receptor and TRPV4 Channel,” *Cancer Res.*, vol. 77, no. 23, pp. 6499–6512, 2017.

[78] I. Kaneko, R. K. Saini, K. P. Griffin, G. K. Whitfield, M. R. Haussler, and P. W. Jurutka, “FGF23 gene regulation by 1,25-dihydroxyvitamin D: Opposing effects in adipocytes and osteocytes,” *J. Endocrinol.*, vol. 226, no. 3, pp. 155–166, 2015.

[79] V. Lavi-Moshayoff, G. Wasserman, T. Meir, J. Silver, and T. Naveh-Many, “PTH increases FGF23 gene expression and mediates the high-FGF23 levels of

experimental kidney failure: a bone parathyroid feedback loop," *Am. J. Physiol.-Ren. Physiol.*, vol. 299, no. 4, pp. F882–F889, 2010.

[80] V. M. Knab *et al.*, "Acute Parathyroid Hormone Injection Increases C-Terminal but Not Intact Fibroblast Growth Factor 23 Levels," *Endocrinology*, vol. 158, no. 5, pp. 1130–1139, 2017.

[81] N. Ide *et al.*, "In vivo evidence for an interplay of FGF23/Klotho/PTH axis on the phosphate handling in renal proximal tubules," *Am. J. Physiol.-Ren. Physiol.*, vol. 315, no. 5, pp. F1261–F1270, 2018.

[82] Y. Fan *et al.*, "Parathyroid hormone 1 receptor is essential to induce FGF23 production and maintain systemic mineral ion homeostasis," *FASEB J.*, vol. 30, no. 1, pp. 428–440, 2016.

[83] S. Takahashi *et al.*, "Elevation of circulating plasma cytokines in cancer patients with high plasma parathyroid hormone-related protein levels," *Endocr. Relat. Cancer*, vol. 10, no. 3, pp. 403–407, 2003.

[84] T. Ashizawa *et al.*, "Clinical significance of interleukin-6 (IL-6) in the spread of gastric cancer: role of IL-6 as a prognostic factor," *Gastric Cancer*, vol. 8, no. 2, pp. 124–131, 2005.

[85] M. Ikeguchi *et al.*, "Serum interleukin-6 and -10 levels in patients with gastric cancer," *Gastric Cancer*, vol. 12, no. 2, pp. 95–100, 2009.

[86] A. ValÍn, C. Guillén, and P. Esbrit, "C-Terminal Parathyroid Hormone-Related Protein (PTHrP) (107–139) Stimulates Intracellular Ca2+ through a Receptor Different from the Type 1 PTH/PTHrP Receptor in Osteoblastic Osteosarcoma UMR 106 Cells," *Endocrinology*, vol. 142, no. 7, pp. 2752–2759, 2001.

[87] N. S. Datta and A. B. Abou-Samra, "PTH and PTHrP signaling in osteoblasts," *Cell. Signal.*, vol. 21, no. 8, pp. 1245–1254, Aug. 2009.

[88] M. Wan *et al.*, "Parathyroid hormone signaling through low-density lipoprotein-related protein 6," *Genes Dev.*, vol. 22, no. 21, pp. 2968–2979, 2008.

[89] D. Wheeler, J. L. Garrido, A. Bisello, Y. K. Kim, P. A. Friedman, and G. Romero, "Regulation of Parathyroid Hormone Type 1 Receptor Dynamics, Traffic, and Signaling by the Na+/H+ Exchanger Regulatory Factor-1 in Rat Osteosarcoma ROS 17/2.8 Cells," *Mol. Endocrinol.*, vol. 22, no. 5, pp. 1163–1170, 2008.

[90] A. Mangia *et al.*, "The potential predictive role of nuclear NHERF1 expression in advanced gastric cancer patients treated with epirubicin/oxaliplatin/capecitabine first line chemotherapy," *Cancer Biol. Ther.*, vol. 16, no. 8, pp. 1140–1147, 2015.

[91] M. Centonze, C. Saponaro, and A. Mangia, “NHERF1 Between Promises and Hopes: Overview on Cancer and Prospective Openings,” *Transl. Oncol.*, vol. 11, no. 2, pp. 374–390, 2018.

[92] G. Du *et al.*, “The cellular distribution of Na+/H+ exchanger regulatory factor 1 is determined by the PDZ-I domain and regulates the malignant progression of breast cancer,” *Oncotarget*, vol. 7, no. 20, pp. 29440–29453, 2016.

[93] W. B. Sneddon *et al.*, “Activation-independent parathyroid hormone receptor internalization is regulated by NHERF1 (EBP50),” *J. Biol. Chem.*, vol. 278, no. 44, pp. 43787–43796, 2003.

[94] B. Wang, Y. Yang, A. B. Abou-Samra, and P. A. Friedman, “NHERF1 Regulates Parathyroid Hormone Receptor Desensitization: Interference with β-Arrestin Binding,” *Mol. Pharmacol.*, vol. 75, no. 5, pp. 1189–1197, 2009.

[95] M. J. Mahon, “The parathyroid hormone 1 receptor directly binds to the FERM domain of ezrin, an interaction that supports apical receptor localization and signaling in LLC-PK1 cells,” *Mol. Endocrinol. Baltim. Md*, vol. 23, no. 10, pp. 1691–1701, 2009.

[96] V. S. Farook, M. Alkhalaf, and S. M. Karam, “Establishment of a gastric epithelial progenitor cell line from a transgenic mouse expressing the simian virus 40 large T antigen gene in the parietal cell lineage,” *Cell Prolif.*, vol. 41, no. 2, pp. 310–320, 2008.

[97] Y. Fujimori, M. Inokuchi, Y. Takagi, K. Kato, K. Kojima, and K. Sugihara, “Prognostic value of RKIP and p-ERK in gastric cancer,” *J. Exp. Clin. Cancer Res.*, vol. 31, no. 1, pp. 30–38, 2012.

[98] E. Ihara, H. Akiho, K. Nakamura, S. R. Turner, and J. A. Macdonald, “MAPKs represent novel therapeutic targets for gastrointestinal motility disorders,” *World J. Gastrointest. Pathophysiol.*, vol. 2, no. 2, pp. 19–25, 2011.

[99] A. K. Shukla, G. Singh, and E. Ghosh, “Emerging structural insights into biased GPCR signaling,” *Trends Biochem. Sci.*, vol. 39, no. 12, pp. 594–602, 2014.

[100] D. Gesty-Palmer and L. M. Luttrell, “‘Biasing’ the parathyroid hormone receptor: a novel anabolic approach to increasing bone mass?,” *Br. J. Pharmacol.*, vol. 164, no. 1, pp. 59–67, 2011.

[101] B. N. Bohinc and D. Gesty-Palmer, “Biased agonism at the parathyroid hormone receptor: a demonstration of functional selectivity in bone metabolism,” *Mini Rev. Med. Chem.*, vol. 12, no. 9, pp. 856–865, 2012.

[102] K. M. Appleton *et al.*, “Biasing the parathyroid hormone receptor: relating in vitro ligand efficacy to in vivo biological activity,” *Methods Enzymol.*, vol. 522, pp. 229–262, 2013.

[103] S. Rajagopal and S. K. Shenoy, “GPCR desensitization: Acute and prolonged phases,” *Cell. Signal.*, vol. 41, pp. 9–16, 2018.

[104] M. A. Chiurillo, “Role of the Wnt/β-catenin pathway in gastric cancer: An in-depth literature review,” *World J. Exp. Med.*, vol. 5, no. 2, pp. 84–102, 2015.

[105] G. Romero, W. B. Sneddon, Y. Yang, D. Wheeler, H. C. Blair, and P. A. Friedman, “Parathyroid Hormone Receptor Directly Interacts with Dishevelled to Regulate β-Catenin Signaling and Osteoclastogenesis,” *J. Biol. Chem.*, vol. 285, no. 19, pp. 14756–14763, 2010.

[106] N. Krishnamurthy and R. Kurzrock, “Targeting the Wnt/beta-catenin Pathway in Cancer: Update on Effectors and Inhibitors,” *Cancer Treat. Rev.*, vol. 62, pp. 50–60, 2018.

[107] J. Mao *et al.*, “Roles of Wnt/β-catenin signaling in the gastric cancer stem cells proliferation and salinomycin treatment,” *Cell Death Dis.*, vol. 5, no. 1, pp. e1039, 2014.