

**PARATHYROID HORMONE-LIKE HORMONE (PTHLH):
A NOVEL PARIETAL CELL GROWTH FACTOR REGULATED BY GASTRIN**

by

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The Intention for Seeking Knowledge

By
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In the Name of Allah Most Gracious, Most Merciful

All praises and thanks be to Allah the Lord of all the worlds
And may the blessings and peace of Allah be upon our leader Muhammad, his
Family and his Companions

My intention is to learn and to teach,
and to remember and to remind,
and to give benefit and to gain benefit,
and to give profit and to gain profit,
and to encourage to hold fast to the Book of Allah and the Sunnah of His
Messenger,
and to call to guidance,
and to direct towards the good,
and to seek for the Countenance of Allah, His Pleasure, His Closeness and His
Rewards Glorified and Exalted is He

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2012

DEDICATION

To my family, with special thanks for your love, support and encouragement.

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ABSTRACT

Parietal cells play a fundamental role in stomach physiology, not only by creating a pathogen free environment through the production of gastric acid, but also by secreting essential growth factors thought to be important for cellular homeostasis of the gastric glands. The gastrointestinal hormone gastrin is known to be a critical regulator of parietal cell function as well as proliferation and differentiation of the gastric glands. High gastrin levels in the body are frequently associated with gastric hyperplasia, yet the mechanism has not been defined. Since parietal cells contain gastrin receptors, one hypothesis is that the proliferation effect of hypergastrinemia is due to gastrin stimulation of growth factor(s) from parietal cells. Recent gene expression microarray studies of mouse stomach mucosa identified parathyroid hormone-like hormone (Pthlh) as a potential new gastric growth factor. Pthlh is expressed widely and has been described to orchestrate key cellular events in different tissues, such as cell proliferation and differentiation. Although Pthlh is highly expressed in gastric tumors, its normal expression, function and regulation in the stomach have not been studied. In this dissertation I examined the physiological importance of Pthlh in the mouse stomach. I used pharmacologic and genetic mouse models as well as human gastric cancer cell lines to determine the cellular localization and regulation of this growth factor by the hormone gastrin. Analysis of Pthlh^{LacZ/+} reporter mice localized Pthlh to parietal cells in the gastric

corpus. Acute treatment of mice with gastrin rapidly and transiently increased Pthlh mRNA abundance. Accordingly, Pthlh expression was significantly reduced in gastrin-deficient mice. Together these data suggested that gastrin is a physiologic regulator of Pthlh in the gastric mucosa. To examine the mechanism, human gastric AGS-E cells treated with gastrin exhibited a robust induction of endogenous Pthlh mRNA via stimulation of the gastrin receptor (Cckbr). Pharmacologic inhibitor studies demonstrated that PI3K, PKC α , Erk1/2 and p38 MAPKs signaling were required for this induction. Furthermore, gastrin induced Pthlh mRNA isoforms that arose from different promoters. The rapid response and co-regulation of numerous molecular forms suggested that gastrin signaling targets the Pthlh mRNA stability elements in the 3'- untranslated region. Measurement of mRNA half-life confirmed that gastrin slowed the degradation of Pthlh transcripts.

The Hedgehog (Hh) signaling pathway is a key signaling pathway in gastrointestinal development, differentiation and homeostasis. Some studies suggested the regulation of Hh signaling components by gastrin. Since Pthlh gene expression regulation by the Hh signaling pathway is prominent in bone morphogenesis and mammary differentiation, we wanted to test the possible mediation of Hh pathway in the induction of Pthh gene expression by gastrin. There were no changes in the gastric expression of the Hh pathway components in both, gastrin mutant and acutely gastrin-treated mice. Several Hh mutants, including H⁺,K⁺-Cre/Shh^{-/-}, Gli2 and Gli3, demonstrated no change in Pthlh expression, suggesting that the Hh pathway does not regulate Pthlh gene expression in the stomach.

Last, to understand Pthlh function I analyzed Pthlh mutant mouse stomachs at E18.5 and found no major differences between mutant and control embryos. This suggested that Pthlh is not required for early stomach development. However, the report of high maternal Pthlh in amniotic fluid challenges this conclusion and the function of Pthlh in the stomach remained to be determined.

Collectively, this dissertation identified Pthlh as a novel parietal cell growth factor. It also identified Pthlh as a potential mediator of gastrin growth factor activity in the stomach.

CHAPTER 1

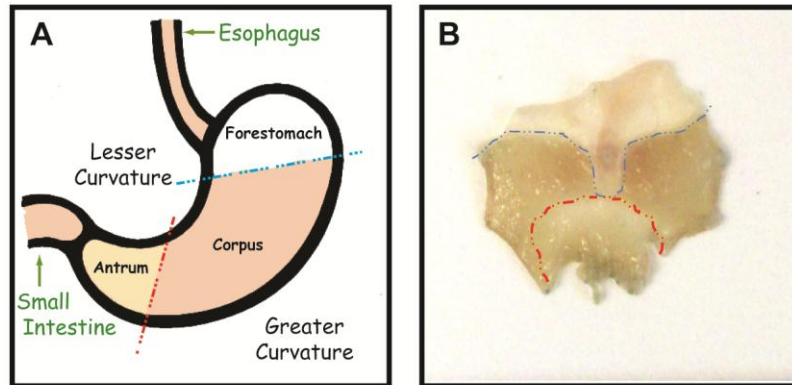
INTRODUCTION

1.1 THE STOMACH

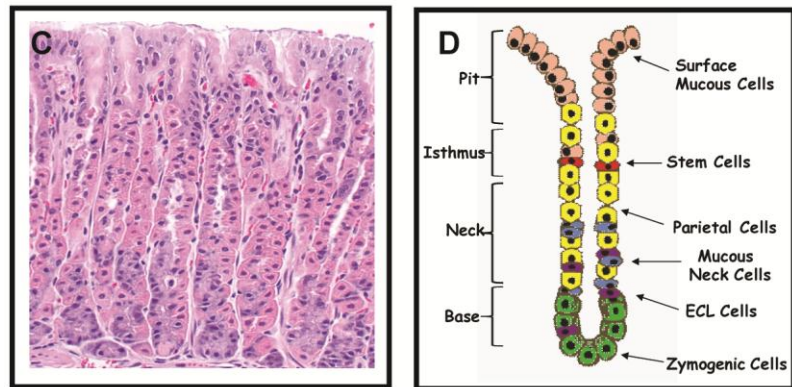
1.1.1 ANATOMY AND COMPARTMENTS

The stomach is a muscular and secretory organ of the upper digestive system. Upon macroscopic examination, three compartments of the adult mouse stomach can be distinguished by color and thickness. They include: forestomach, corpus and antrum (Figure 1-1, A and B). The forestomach is the most proximal compartment and is composed of squamous epithelium. The corpus and antrum, however, represent the glandular part of the stomach. The corpus, which is the middle compartment, is responsible for secreting gastric acid and digestive proteins, including pepsinogen. The corpus is also the thickest among the three compartments (Figure 1-1, C). The most distal region is the antrum, which has modest mucosal depth (Figure 1-1, E). The antrum is rich with endocrine cells responsible for secretion of the hormone gastrin. In the mouse stomach, the esophagus connects to the forestomach from the lesser curvature side, while the small intestine connects to the antrum.

Stomach Gross Anatomy



Corpus Gastric Gland



Antral Gastric Gland

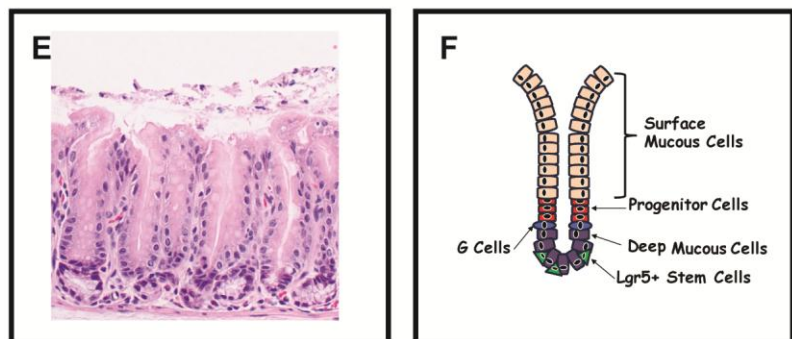


Figure 1 - 1 Mouse stomach anatomy

A, The stomach is divided to three regions: forestomach, corpus and antrum. B, adult mouse stomach opened along the greater curvature with transitions between forestomach and corpus (blue dashed line), and between corpus and antrum (red dashed line) highlighted. C-F, Hematoxylin and eosin stained sections and diagram of a gastric gland from corpus C-D, and antrum E-F.

1.1.2 GASTRIC GLAND STRUCTURE

The gastric glands form the functional units of the stomach. They are formed from evaginations of mesenchymal tissue covered with columnar epithelial cells. The mesenchymal layer is composed of connective and smooth muscle tissues, enteric nervous cells, immune cells, as well as blood vessels. The epithelial cellular composition and the architecture of the gastric gland are different in corpus and antrum (Figure 1-1 C-F).

In corpus, the gastric gland is divided into four regions: the pit, the isthmus, the neck and the base (Figure 1-1, D). The corpus epithelium is composed of several differentiated cell types, which each localizes differently along the gastric gland and has specific secretory functions: (i) Surface mucous cells, which are located in the pit region of the gastric gland and secrete mucous to protect the stomach epithelium from pathogens, strong acid and digestive enzymes present in the lumen; (ii) Mucous neck cells, located in the neck region, also secrete mucous and are zymogenic precursor cells; (iii) Zymogenic cells, located at the base, secrete pepsinogen, an acid-activated protease that contributes to digestion; (iv) Parietal cells, which migrate bi-directionally along the pit-base axis of the gastric gland, secrete hydrochloric acid (HCl) to help in food digestion and to make the gastric environment unfavorable for pathogens. The parietal cells have also been shown to be critical for proper cellular differentiation of the gastric epithelium, as will be discussed later; (v) Enterochromaffin-like cells (ECL), which secrete histamine, and as a result, stimulates parietal cell acid secretion. These endocrine cells are located

towards the base of the corpus gastric gland. All of these cells are differentiated from a stem/progenitor cell population located in the isthmus.

In antrum, the gastric gland epithelium (Figure 1-1, F) is composed of: (i) Surface mucous cells; (ii) Deep mucous cells; and (iii) G cells, which are the gastrin-producing endocrine cells. Gastrin hormone, as will be discussed later, is responsible for stimulating acid secretion from the corpus and has a potent growth effect in the corpus. The antral stem cell population is located in the base of the gland.

In addition to ECL and gastrin cells there are several endocrine cells that are located in both corpus and antrum and thought to be important for stomach physiology. These include: Enterochromaffin (EC) cells which secrete serotonin, D cells which secrete somatostatin and X (or A-like) cells which secrete ghrelin¹.

1.1.3 EPITHELIAL CELL HOMEOSTASIS: PROLIFERATION AND DIFFERENTIATION

The gastric epithelium is continuously replaced from stem cells located in the isthmus of the gastric gland². This dynamic process of continuous cell production and turnover of the gastric epithelium is fundamental for the organization of the epithelium and its capacity for protection, secretion and adaptation³. The stomach expresses several growth factors, including Heparin-Binding Epidermal-Like Growth Factor (HB-EGF)⁴, Sonic Hedgehog (Shh)⁵, Insulin Growth Factor Binding Protein 2 (Igfbp2)⁶, which are thought to be important for gastric epithelial cell differentiation and maturation.

In corpus, stem cells located in the isthmus of the gland (Figure 1-1, D) give rise to all of the differentiated cell types of the gastric mucosa. Elegant studies by Karam and Leblond have described the cell lineages in the adult mouse stomach^{2,3,7-9}. Lineage precursors differentiate to committed cells that express lineage specific markers during their differentiation program^{3,7-9} (Table 1-1). (i) Surface mucous cells are the most rapidly turned over cell type, with a lifespan of 3 days⁷, are marked by Muc5AC. (ii) Mucous neck cells spend 7 to 14 days in the gland neck region as they move towards the base and further differentiate into zymogenic cells⁸. Muc6 and TFF2 are specific markers for mucus neck cells. (iii) Zymogenic cells have a long lifespan of ~194 days⁸ and express intrinsic factor in the mouse stomach. (iv) Parietal cells are estimated to turnover in 54 days⁹, with the proton pump H⁺,K⁺-ATPase as a specific marker. (v) ECL cells secrete histamine, which stimulates parietal cell acid secretion. HDC is an ECL cell-specific marker.

Although the function and the characterization of the gastric corpus gland has been well studied, the pathways controlling cell proliferation versus differentiation or controlling cell numbers are not understood. As will be discussed below, several studies concluded that the hormone gastrin has a growth effect in the stomach; however the mechanism is yet to be defined. Moreover, changes in epithelial cell differentiation in different mouse models with loss or ablation of parietal cells, suggested that instructive messages are sent from parietal cells to other lineages in the stomach to regulate gastric epithelial cell homeostasis. These mouse models will be reviewed in the next section.

Table 1 – 1: Cell markers of differentiated mouse corpus epithelial cells

Cell Type	Marker
Surface mucous	Mucin 5, subtypes A and C (Muc5AC)
Mucous neck	Mucin 6 (Muc6) and Trefoil Factor 2 (TFF2)
Parietal	H ⁺ ,K ⁺ -ATPase, α (ATP4A) and β (ATP4B) subunits
Zymogenic	Intrinsic Factor (IF)
ECL	Histidine decarboxylase (HDC)
Tuft	Doublecortin-Like Kinase 1 (DCLK1) and Acetylated-α-Tubulin (acTub)
D	Somatostatin (SST)
X (or A-like)	Ghrelin (GHRL)

1.2 THE PARIETAL CELL

1.2.1 IMPORTANCE OF PARIETAL CELLS FOR GASTRIC HOMEOSTASIS

The parietal cell is a key component of a healthy stomach. The disruption or ablation of parietal cells is always associated with imbalanced gastric homeostasis. The basic function of the parietal cell is acid secretion, and this function is important for many reasons. First, gastric acid provides an environment that limits pathogen survival in the stomach. Studies in the gastrin-deficient mouse, which have low gastric acid, showed that low acid can cause bacterial overgrowth chronic gastritis and emergence of distal tumors that progressed to adenocarcinoma¹⁰. Second, the activation of digestive enzymes in the stomach occurs in parallel with gastric acidity. For example, the conversion of the zymogenic cell enzyme pepsinogen to its active form pepsin is dependent on low gastric luminal pH¹¹. Third, the processing and regulation of some gastric growth factors have been shown to be regulated by acid¹². Zavros *et al* demonstrated the requirement of acid produced by parietal cells for the processing of Shh from 45-kDa to its active 19-kD form¹². Last, gastric acid is also found to be critical for body bone density and calcium homeostasis^{13, 14}. Recent studies have made a connection between impaired gastric acidification, calcium absorption and osteoporosis in gastrin receptor-deficient mice^{13, 14}.

In the literature, the parietal cell has been described as “mitochondrion-laden, proton-secreting factories”¹⁵. Genes involved in energy production and acid secretion are highly expressed in parietal cells^{6, 15, 16}. In addition, recent studies

found the expression of novel factors thought to be important for gastric mucosal homeostasis^{6,16}, as it will be discussed in the next section.

1.2.2 PARIETAL CELL AND GROWTH FACTORS

In addition to acid secretion, parietal cells express and respond to several growth factors. The gastrin/cholecystokinin B receptor (Cckbr) and the epidermal growth factor (EGF) receptor have been found to be expressed in parietal cells^{17,18}. Studies also showed the ability of parietal cells to express a number of growth factors. A microarray analysis for parietal cell-enriched preparations showed that parathyroid hormone-like hormone (Pthlh), vascular endothelial growth factor B (Vegfb), and insulin-like growth factor-binding protein 2 (Igfbp2) are expressed in parietal cells¹⁶. Other microarray studies from wild type and gastrin-deficient mice confirmed the previous results, but also suggested for the first time the regulation of Pthlh, Igfbp2 and Vegfb by gastrin^{6,19}. These studies also suggested that targets of Wnt and Myc signaling, which are usually stimulated in proliferating cells, are regulated by gastrin in the parietal cell⁶. The Hedgehog ligands Shh and Indian hedgehog (Ihh) were also expressed by parietal cells^{5,6,20}. The expression of growth factors suggests a potential mechanism for parietal cell regulation of stomach growth.

The possibility that parietal cell growth factors are responsible for the proliferation/differentiation imbalance seen in mouse models with impaired parietal cell function is an intriguing hypothesis. Table 1-2 summarizes several

Table 1 – 2: Mouse models with reduced function/ablation of parietal cells

Mouse Model	Reference
Diphtheria toxin-transgenic mouse	16, 21
DMP777-treated mouse	22, 23
H ⁺ ,K ⁺ -ATPase-deficient mice	24, 25
Gastrin-deficient mouse	26, 27
Huntington interacting protein 1 related-deficient mouse	28, 29
Cholecystokinin B receptor-deficient mouse	30
Histamine receptor-deficient mouse	31
Parietal cell-specific Sonic hedgehog-deficient mouse	32

parietal cell loss/ablation models. In general, these models share the following phenotypes: (i) the differentiation program of zymogenic cells is blocked with accumulation of aberrant mucus neck cells referred to as spasmolytic polypeptide-expressing metaplasia (SPEM)³³; (ii) the expansion of surface mucous cells; (iii) increased proliferation, and (iv) high serum gastrin levels. In an elegant study, Keeley *et al* compared the postnatal development of the gastric glands in Huntington interacting protein 1 related (Hip1r)-deficient mice to wild type mice²⁸. The study found that the changes in zymogenic lineage differentiation and the expansion of mucous cells do not appear until after parietal cell loss in the Hip1r-deficient mouse. This study concluded that parietal cell loss was the initiating event in Hip1r-deficient mice, with secondary remodeling of the other cell types²⁸, suggesting that parietal cells are critical for maintenance of gastric epithelial cell homeostasis.

1.3 GASTRIN HORMONE

Gastrin, a key gastric hormone required for gastric homeostasis, is expressed by the antral G cells (Figure 1-1, F) and is processed from its precursor form to give the mature amidated-end products: gastrin-17 and gastrin-34³⁴. Gastrin is released into the bloodstream via an endocrine mechanism, where it travels back to its target cells in the gastric corpus (parietal cells and ECL cells). Gastrin-Cckbr receptor interaction induces gastric acid secretion^{27, 35} as well as gastric epithelial cell proliferation^{29, 36, 37}. This section will present an overview of: (i) gastrin's role in acid secretion; (ii) gastrin and its potential growth effects; (iii) Cckbr signaling

stimulation mediating the proliferative role of gastrin and; (iv) gastrin regulation of target gene expression.

1.3.1 STIMULATION OF ACID SECRETION

For many decades, gastrin has been known as the physiologic stimulator of gastric acid secretion. In response to meal ingestion, gastrin is released from the antrum and stimulates acid secretion from the parietal cells of the corpus³⁵. Gastrin acts *via* both direct and indirect mechanisms to activate gastric acid secretion³⁵. The direct pathway occurs when gastrin binds to its receptor on parietal cells, resulting in the stimulation of cellular phospholipase C (PLC), increased inositol trisphosphate (IP₃) and Ca²⁺ release from intracellular stores in the endoplasmic reticulum³⁸. This influx in Ca²⁺ has been demonstrated to be required for stimulation of acid secretion³⁹. Parietal cells then undergo a morphological change in which intracellular membrane organelles, known as “tubulovesicles”, migrate and fuse with the parietal cell apical membrane, increasing membrane surface area, and therefore enabling acid release via H⁺,K⁺-ATPase proton pump^{35, 40}. In the indirect pathway gastrin binds Cckbr on the ECL cell to release histamine, which is a potent acid secretagogue⁴¹. Parietal cells express the histamine receptor H₂, and therefore secrete acid when stimulated by histamine³⁵. Importantly, it is known that high gastric acid content inhibits gastrin release and as a result reduces stimulation of the corpus to secrete acid³⁵.

1.3.2 GASTRIN AND ITS POTENTIAL GROWTH EFFECTS

Understanding the pro-proliferative effects of gastrin on the stomach has been a very active area of research. It is currently unknown how gastrin promotes cellular proliferation, since Cckbr has only been defined on differentiated cells and not progenitor cells. Many different experimental approaches have been used to study gastrin as a growth factor in the stomach. These studies included gastrin treatment⁴²⁻⁴⁶, pharmacologic inhibition of acid to stimulate gastrin release^{36, 47}, antrectomy surgeries to delete the gastrin-secreting region^{36, 45}, fasting-refeeding models³⁷, and genetically engineered models^{27, 48}.

Treatment of mice with progastrin; an unprocessed form of gastrin peptide; showed induction of corpus total nucleic acids and protein, indicating that gastrin induces hyperplasia^{42, 44}. Administration of omeprazole, an acid secretion inhibitor, in chicken, hamsters and guinea pigs caused a significant increase in serum gastrin levels⁴⁷. Accordingly, the proventriculus/stomach size, weight and corpus mucosal thickness were increased⁴⁷. In chronic omeprazole treated rats, increased serum gastrin levels caused a significant, yet modest overall increase in proliferation rate³⁶. Interestingly, ECL-cell proliferation was clearly pronounced in these animals. Importantly, the omeprazole-induced increase in proliferation and ECL replication were not observed in antrectomized rats, suggesting that the trophic effect in the stomach is likely due to hypergastrinemia³⁶. Also, key work from Walsh and colleagues showed that the induction of gastric proliferation observed in rats after a cycle of fasting/re-feeding was abolished when rats were pre-treated with gastrin

neutralizing antibodies³⁷. Together, these studies suggested a critical role for gastrin in gastric epithelial cell proliferation.

More recent studies used genetically engineered mouse models in addition to microarray analyses to study gastrin function^{6, 19, 26, 27, 48}. The creation of the gastrin-overexpressing transgenic mouse (INS-gas) confirmed the sufficiency of gastrin to increase mucosal height and proliferation rates⁴⁸. Surprisingly, the gastrin-deficient mouse had normal mucosal thickness and normal proliferation rates^{26, 27}. The expression of parietal and ECL differentiation markers, however, was reduced in the gastrin-deficient stomach²⁶. These findings suggested that gastrin is not required for the proliferation and differentiation of the epithelial gastric cell lineages in the corpus, however, it is required for maturation of parietal and ECL cells^{26, 27}. These findings also suggested that other factors might mediate gastrin's effect on cell growth.

More recent studies have looked at genes regulated by gastrin using gene expression profiling microarray analyses of gastrin-deficient mouse stomach^{6, 19}. These studies showed that: (i) growth factors and mitogens like *Pthlh*, *Ihh*, *Igfb2*, as well as Wnt signaling targets are regulated by gastrin in parietal cells; (ii) cell lineage specific markers like H^+,K^+ -ATPase for parietal cells and HDC for ECL cells were regulated by gastrin, which agreed with previous studies characterizing gastric epithelial cells in gastrin-deficient mice^{6, 19, 26, 27}.

Chapter 2 of my thesis will present evidence demonstrating that *Pthlh* is regulated by gastrin.

1.3.3 GASTRIN SIGNALING VIA CCKBR

Gastrin hormone binds to the Cckbr receptor, which is a member of the G protein- coupled receptor (GPCR) super family. Cckbr is expressed broadly in normal and diseased gastrointestinal tissues⁴⁹⁻⁵³. In the mature stomach, Cckbr is expressed in parietal cells and ECL cells³⁵, and one study claims occasional expression of Cckbr in TFF2-expressing progenitor cells, although this has not been confirmed⁵².

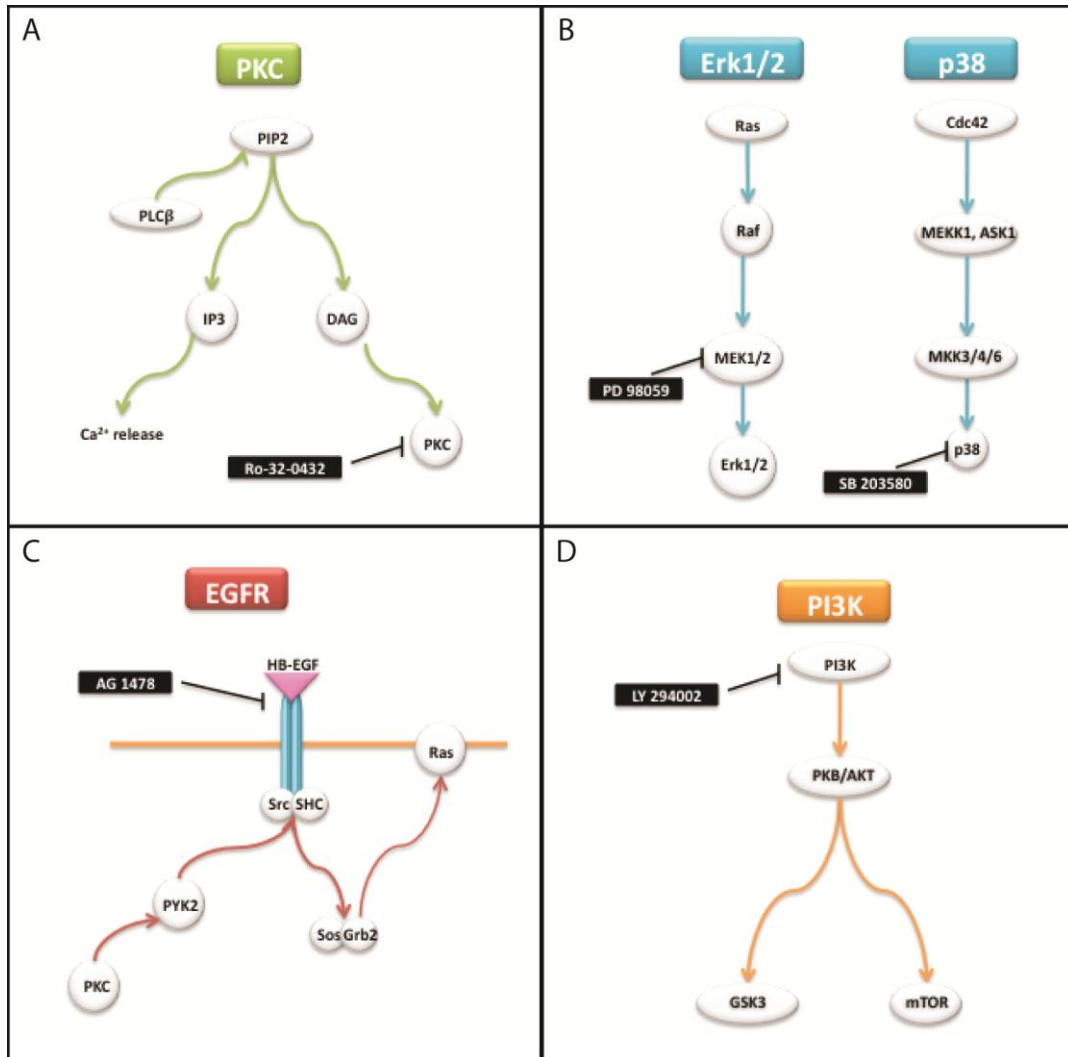
Although the importance of Cckbr expression in gastric acid secretion is well-studied, it is not completely clear how the activation of this receptor might induce stomach growth, especially with this pattern of expression in differentiated cell types (parietal and ECL), but not in stem/progenitor cells.

When Cckbr is stimulated by gastrin, a network of signaling pathways is activated (Figure 1 – 2). In this section, well-characterized pathways will be summarized, highlighting key known players. More details about Cckbr signaling are reviewed in the following references⁵⁴⁻⁵⁸. One thing to note is that activation of Cckbr signaling pathway(s) is highly dependent on the cellular context, thus Cckbr signaling activated in parietal cells, as an example, may be different than activated Cckbr on an ECL cell or a cancer cell.

Four main signal transduction pathways are activated to promote gastric epithelial cell proliferation when gastrin binds Cckbr. This includes:

1. *Ca²⁺ mobilization and activation of protein kinase C (PKC)*

Figure 1 - 2: Signaling pathways activated upon Cckbr stimulation by gastrin.



A, When gastrin binds to the G protein-coupled receptor Cckbr, activated PLC β stimulates plasma membrane protein PIP₂, and as a result the second messengers IP₃ and DAG are formed to cause Ca²⁺ release. In addition, Cckbr activation can stimulate the EGFR (B), MAPK (C) and PI3K (D) signaling pathways (see text). However, the precise mechanism of activation of these pathways is not completely defined. In addition to other unknown mediators, PKC has been shown to mediate the activation of EGFR and MAPK pathways. The small G protein Ras exchanges GDP with GTP when stimulated by the PKC or EGFR signals. This causes the activation of Raf, MEK1/2 and Erk1/2 kinases to affect downstream target genes. Ro-32-0432, AG 1478, PD 98059, SB 203580 and LY 294002 (black boxes) are commercially available inhibitors for PKC, EGFR, Erk1/2, p38 or PI3K signaling pathways, respectively.

The PKC pathway is highly active when gastrin stimulates Cckbr. When gastrin binds Cckbr, the dissociated $G_{\alpha q}$ protein from the Cckbr activates the β form of phospholipase C (PLC), which is required for the hydrolysis of phosphatidyl inositol 4, 5 biphosphate (PIP₂) in the plasma membrane to produce two second messengers: (i) inositol 1,4,5-triphosphate (IP₃) and (ii) 1,2-diacylglycerol (DAG). IP₃ rapidly and transiently releases Ca²⁺ from the intracellular stores after binding to the ligand-gated Ca²⁺ channels found on the endoplasmic reticulum. The second messenger DAG however, directly activates the protein kinase C (PKC).

II. Mitogen-Activated Protein Kinase Stimulation (MAPK)

Although it was first known to be activated by mitogens, the MAPK pathway can also be activated by hormones, neurotransmitters and stress. Stimulation of the MAPK pathway can result in cell growth, differentiation, survival, apoptosis and activation of the immune response⁵⁵. Classically, small G proteins are stimulated by substituting bound GDP for GTP. Then, a cascade of serine/threonine phosphorylation by three major kinases takes place: (MAPKKK), (MAPKK) and MAPK. Only Erk1/2 and p38 will be briefly discussed, due to their activation during induction of many genes regulated by gastrin (Table 1-3).

Erk1/2 is the most common MAPK signaling pathway that takes place in gastric epithelial cells. Typically, the membrane associated small G protein Ras is activated, causing the activation of the MAPKKK Raf, which phosphorylates the MEK1/2 MAPKK. This leads to the stimulation of the Erk1/2 MAPK, resulting in

Table 1 - 3: Genes and molecules regulated by signaling pathways downstream of Cckbr

Gene	PKC	Erk1/2	P38	EGFR	PI3K	Reference
Chromogranin A (CgA)	✓	✓	X	N.D.	X	59
Cyclooxygenase 2 (COX2)	N.D.	X	✓	N.D.	✓	60
Interleukin 8 (IL8)	N.D.	X	✓	N.D.	✓	60
Trefoil Family Factor 1 (TFF1)	✓	✓	N.D.	X	N.D.	61
Trefoil Family Factor 2 (TFF2)	✓	✓	N.D.	X	✓	52
Matrix Metalloproteinase 9 (MMP 9)	✓	✓	N.D.	X	N.D.	62
Histidine Decarboxylase (HDC)	✓	N.D.	N.D.	N.D.	N.D.	63
Myeloid Cell Leukemia Sequence (Mcl 1)	✓	✓	N.D.	N.D.	X	64
Nuclear Factor κ B (NF κ B)	✓	X	X	X	N.D.	65
Heparin-Binding Epidermal-Like growth Factor (HB-EGF)	✓	✓	N.D.	✓	✓	66

✓ = Regulated

X = Not regulated

N.D. =Not determined

activation of c-myc, Elk and histone H3, all of which are important for cell proliferation⁵⁵.

The MAPK/p38 is another key kinase mediating the regulation of different genes by gastrin. When human pancreatic cancer cells (AR4-2J), which express the Cckbr receptor, were stimulated by gastrin, the activated small G protein Cdc42 caused a series of phosphorylation events, leading to the activation of p38⁵⁷. Again, the direct activation between the GPCR Cckbr and the MAPK pathway is poorly understood. Different signaling pathways such as PKC, EGFR and PI3K can mediate their activation by gastrin.

III. Epidermal growth factor receptor signaling (EGFR)

Many hormones and growth factors signal *via* receptors with intrinsic tyrosine kinase motifs on their intracellular domains⁵⁴. EGF ligand binds to the receptor EGFR to activate the adaptor protein src homology/collagen (Shc). Subsequently, Shc binds to the protein complex growth factor-receptor binding protein 2 (Grb2) and son of sevenless (Sos). This Grb2/Sos complex activates the membrane small G protein Ras, which in turn activates the Erk1/2-MAPK pathway. Since Cckbr does not maintain intrinsic tyrosine kinase motifs, and because gastrin is known to regulate HB-EGF ligand, which is expressed along with its receptor, in parietal cells⁶⁶, the stimulation of proliferation by gastrin *via* this pathway is indirect.

IV. Phosphoinositide 3-kinase (PI3K)

This pathway is required for mitogenesis as well as cellular anti-apoptotic and metabolic regulation⁵⁵. As in the MAPK pathway, there are three key kinases: (i) PI3K, (ii) PKB/Akt, and (iii) GSK-3 and mTOR. Todisco *et al.* has shown that AR4-2J cells treated with gastrin activated GSK-3 protein *via* the Cckbr receptor⁵⁶. Additionally, COX2, IL8 and TFF2 genes are regulated by gastrin *via* the PI3K pathway^{52, 60}.

1.3.4 GASTRIN REGULATION OF GENE EXPRESSION

1.3.4.1 TRANSCRIPTIONAL REGULATION BY GASTRIN

In 1977, Enchos and Johnson *et al.* showed that a single injection of pentagastrin in rats significantly increased total corpus mRNA within one hour, followed by an increase in total corpus protein that peaked at 6 hours post-injection⁴². Subsequently, total corpus DNA peaked at 16 hours post-injection⁴². The sequence of these molecular events resembles the process of active cellular divisions taking place in a growing tissue, with the synthesis of RNA, protein and DNA taking place, correspondingly. Interestingly, pre-treatment of rats with actinomycin D, a potent mRNA synthesis inhibitor, prior to pentagastrin administration, eliminated protein synthesis⁴². This study clearly shows that increased protein synthesis is dependent on prior mRNA synthesis. It also questions the identities and effects of upregulated genes on gastric growth after gastrin stimulation.

Different studies have examined gastrin regulation of gene transcription by using promoter serial deletions or point mutations, or electrophoretic mobility shift

assays (EMSA) and Western blot analyses, as well as pharmacological inhibitors^{52, 59-64, 66-69}. Gastrin-regulated genes are listed in (Table 1 – 4) highlighting their *cis* and *trans* DNA elements. A careful look at the list suggests that diverse cellular functions can be regulated by gastrin. For example, the listed regulated genes are: (i) endocrine gene targets like Chromogranin A and Glucagon^{59, 60, 63, 67-69}, (ii) vesicular transport genes like VMAT2⁷⁰, (iii) mucosal protection and repair genes like TFF1 and TFF2^{52, 61}, (iv) inflammatory response genes like IL-8⁶⁰, (v) cell growth genes like HB-EGF⁶⁶ and cyclin D1^{71, 72}, (vi) and death genes like PAI-2⁷³. Furthermore, the *trans* elements are important proteins for early development as well as cancer cell development. Although there are still some *cis* and/or *trans* elements yet to be defined, we can appreciate the clear connection between the nature of the function of these regulated genes and the multi- effect of gastrin on the stomach in both normal and disease states.

1.3.4.2 POST-TRANSCRIPTIONAL REGULATION BY GASTRIN

Recently, Subramaniam et al⁶⁰ studied the ability of gastrin to regulate cyclooxygenase 2 (COX-2) and interleukin-8 (IL-8) genes at the post-transcriptional level. This study suggested that gastrin can stabilize the mRNAs of both genes and as a result protect the mRNAs from quick degradation, by increasing the half-life of the mRNAs. Moreover, this study suggested the involvement of the HuR protein, which binds the IL-8 and COX-2 mRNAs and protects them from degradation via the p38 MAPK pathway. This is the first study demonstrating the post-transcriptional regulatory role of gastrin.

Table 1 - 4 : Gastrin responsive elements

Gene	Cis-element	Transcription factor	Reference
Chromogranin A (CgA)	GCGGGGGGGCGG TGACGTAA	Egr1/Sp1 CREB	59, 67
Trefoil Factor Family 1 (TFF1)	GGCGGGAGGG	SP1/MAZ	61
Trefoil Factor Family 2 (TFF2)	CCAATA CCCTGTGG	N.D. N.D.	52
Heparin-Binding Epidermal-Like Growth Factor (HB-EGF)	CGGCGCCGGGCG	N.D.	66
Histidine Decarboxylase (HDC)	CCCTTTAAATAAAGGGCCACACTGG TGCCAGGGAGTGCGCAGGAC	N.D. N.D.	63 68
Vascular Monoamine Transporter-2 (VAMT2)	CCCCTCCGCC	AP2/Sp1	70
Cyclooxygenase-2 (Cox2)	N.D.	AP1	60
Interleukin-8 (IL-8)	N.D.	NF κ B	60
Glucagon	GAGTGGGTG	Egr1	69
Plasminogen Activator Inhibitor-2 (PAI-2)	TGGGAGGG	MAZ/ASC1	73
Cyclin D1	AACGTCACA	CREB/ β -catenin	71, 72

N.D. = Not determined

Chapter 2 of my dissertation will show evidence for gastrin stimulation of Pthlh mRNA expression.

1.4 THE HEDGEHOG SIGNALING PATHWAY

A key molecular signaling pathway involved in stomach development and gastric epithelial cell differentiation is the Hedgehog (Hh) pathway. Previous studies demonstrated that changes in Hh expression are associated with either congenital or pathogenic gastric conditions. For example, Hh has been shown to be crucial for normal mammalian gastric development⁷⁴⁻⁷⁶, required for gastric tumor growth¹⁸ and associated with human inflammatory gut diseases^{5, 77}. All of these actions take place through epithelial-mesenchymal interactions²⁰. There are three mammalian forms of Hh: Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). Shh and Ihh ligands demonstrate different yet overlapping expression patterns in the developing as well as the mature gastric epithelium^{20, 32, 74}. When Hh ligands bind to the patched (Ptc) receptor, the inhibitory action of Ptc on the membrane protein smoothed (Smo) is released. As a result, the glioma-associated oncogene homolog (Gli) transcription factors are activated and translocated to the nucleus to regulate transcription, including Ptc, Gli, hedgehog interacting protein (HIP1) and others. Gli1, Gli2 and Gli3 have different stimulatory and/or inhibitory effects on target gene expression in a tissue-specific manner^{75, 78-81}. Different studies have previously examined the expression of Hh signaling components in the stomach^{5, 20}. In the developing and adult stomach, Shh and Ihh were found to be expressed in the epithelium and Ptc and Gli1 were expressed in the mesenchyme²⁰.

1.4.1 GASTROINTESTINAL TRACT DIFFERENTIATION AND REGULATION BY HEDGEHOG

Several genetically-engineered mouse models demonstrated the importance of the Hh pathway for stomach development^{32, 74, 75}. Analysis of Shh-deficient (Shh KO) embryos at day E18.5 showed a reduction in stomach size, with reduced smooth muscle formation⁷⁴. Shh KO stomachs exhibit an impressive overgrowth of the gastric epithelium, with alterations in cellular identity suggesting intestinal transformation⁷⁴. This was demonstrated by histochemical staining with WFA lectin, normally specific for the brush border of enterocytes in the intestine⁷⁴. A striking finding with this mutant was that gastric overgrowth is not associated with increased proliferation rates⁷⁴. Subsequent studies done by Kim *et al* compared the stomachs of Shh, Gli2 and Gli3 knockouts to find which Gli is the critical transducer of the Hh signal at day E18.5⁷⁵. They found that although there was a mild Gli2 KO phenotype in the stomach, the Gli3 KO was phenotypically similar to the Shh KO. Gli3 mutants had glandular expansion with intestinal transformation of the stomach. The authors suggest that this finding supports a critical role for Hh and Gli3 in regulating glandular development in the stomach⁷⁵. Gli3 has been characterized as a transcriptional inhibitor^{80, 81} or activator^{78, 79, 82} depending on the cellular context. The finding that Gli3 null mice mimic the Shh-deficient embryos phenotype suggests that it acts as an activator in the stomach. Moreover, Kim *et al* related the epithelial overgrowth in Shh and Gli3 mutants to decreased apoptotic cell death⁷⁵. Also, histochemical analysis with different gastric cell markers demonstrated the presence of parietal cell, chief cell, neck and surface mucous cell

markers in the Hh mutants, thus challenging the original conclusion of intestinal transformation⁷⁵. This study concluded that Shh restrains the rapid growth of developing gastric glands through decreasing cellular apoptosis⁷⁵. Although these studies suggested the importance of Hh signaling for gut development and gastric cytodifferentiation, they did not identify a mechanistic pathway responsible for the gastric epithelial cell lineage expansion of parietal and zymogenic cells. These studies are limited because of qualitative analyses and the perinatal death of Hh pathway mutants.

In the adult stomach, Hh was first studied by treating mice with the Hh signaling inhibitor cyclopamine⁸³. Treated mice had decreased expression of putative Hh targets like the hepatocyte nuclear factor 3 β (HNF3 β) and bone morphogenetic protein 4 (BMP4)⁸³. Moreover cyclopamine-treatment caused increased proliferation, suggesting that Hh is a negative regulator of gastric gland epithelial cell proliferation⁸³. Recently, mice with a specific deletion of Shh in parietal cells ($H^+,K^+ \text{-Cre/Shh}^{-/-}$) were described to exhibit transformation of the gastric mucosa³². The $H^+,K^+ \text{-Cre/Shh}^{-/-}$ mouse showed multiple cellular changes: (i) expansion of surface mucous cells, (ii) increased proliferation rate, especially in the pit of the gland, (iii) nuclear localization of β - catenin, (iv) induction of cyclinD1, Wnt5a, Wnt3a, Gli1, Snail and Ihh, (v) impaired acid due to abnormal parietal cell tubulovesicles, and (vi) hypergastrinemia. These findings showed that parietal cell expression of Shh is critical for stomach homeostasis³².

1.4.2 REGULATION OF HEDGEHOG SIGNALING BY GASTRIN

There are three studies that suggest regulation of the Hh signaling pathway by gastrin. First, Jain *et al* described upregulation of *Ihh* expression in gastrin-deficient parietal cells⁶. Data from this study suggested the possible inhibitory role of gastrin on parietal cell *Ihh* expression. Recent research done by the Zavros laboratory using laser capture microdissection to identify roles might *Shh* follow in gastric cell proliferation and differentiation, showed that the hypergastrinemic H^+,K^+ -Cre/*Shh*^{-/-} mouse had high *Ihh* gene expression in the pit region of the corpus gastric glands³². This upregulation of *Ihh* in the gastric pit region was corrected when the mutant mice were treated with octreotide, a somatostatin analog, which inhibits, in addition to other actions, gastrin secretion³². These results suggest that high gastrin upregulates *Ihh* in gastric pit cells. *Ihh* is understudied in the stomach and it will be important to examine the normal localization of *Ihh* and whether gastrin regulates *Ihh* gene transcription. Last, Zavros *et al*, in an elegant study, discovered the role of gastrin and acid in *Shh* protein induction, processing, and association with tumor formation¹². This study aimed to examine whether gastrin regulates *Shh* expression in parietal cells using the gastrin mutant mouse and canine parietal cell primary cultures. Both *Shh* mRNA and protein isoforms were reduced in the gastrin mutant stomachs and normalized upon gastrin treatment. To test if this induction was acid dependent, canine parietal cells were treated with gastrin or omeprazole, and results showed that while the unprocessed 45kD *Shh* was normal in all treated groups, the active 19kD *Shh* was induced in the gastrin-treated cells, but not when gastrin and omeprazole were added together. Lastly, the study

showed that pepsin A and unprocessed Shh were highly expressed in human gastric tumors compared to normal tissue¹². These data suggest: (i) chronic gastrin treatment can induce the expression of Shh mRNA and protein; (ii) acid is required for the processing of the 45kD Shh to the active 19kD Shh; and (iii) that high unprocessed Shh protein and low pepsin A are associated with gastric tumor formation.

1.4.3 HEDGEHOG SIGNALING PATHWAY AND GASTROINTESTINAL CANCER

Hh signaling has been shown to be important for gastric development^{74, 75} and cellular transformation⁸⁴. Cancerous cells with aberrant Hh signaling can develop in different ways. For example, cancers can have constitutively activated Hh receptor, with no need of the ligand while others may activate the ligand in autocrine, juxtacrine or paracrine fashion⁸⁵. The analysis of the developing Hh mutants as well as the adult Shh mutant HKCre/Shh^{KO} showed transformation of the epithelium^{32, 74, 75}. Also, physiologic gastrin, acid and Shh protein processing events important for normal gastric homeostasis are found to be disrupted in human gastric tumors¹². Patients with low gastric acid content and high gastrin have been found to have aberrant Shh protein processing, which might be associated with gastric cancer development¹². Moreover, different human gastric cancer cell lines have been found to be responsive to Hh by expressing the Hh responsive gene encoding the Ptc receptor, including SNU1, SNU16, AGS and NCI-N87¹⁸. All of these studies suggest that Hh signaling plays an important role in controlling the mucosal cellular content and the structural organization of the stomach.

1.5 PARATHYROID HORMONE-LIKE HORMONE (PTHLH)

Pthlh was first known to be associated with hypercalcemia of malignancy, in which tumors increase serum calcium levels by promoting bone and renal tubular calcium resorption⁸⁶. Pthlh was named due to the similarity between the N-terminus of Pthlh and parathyroid hormone (Pth); 8 of the first 13 amino acids are identical and both can bind to the same receptor: parathyroid hormone 1 receptor (Pth1r)^{87, 88}. Although the terminology is based on biochemical and structural resemblance, Pthlh and Pth are different in many ways: (i) Pthlh has a wide temporal-spatial expression profile in the body, while Pth is limited to the parathyroid gland and central nervous system⁸⁹; (ii) Pthlh acts in autocrine, paracrine or intracrine fashion under normal physiological conditions, while Pth follows endocrine roles⁸⁹; (iii) While Pth regulates calcium homeostasis via renal and skeletal effects, Pthlh has a wide range of functions. It stimulates calcium transport in many tissues, relaxes contracted smooth muscles⁹⁰, and regulates various cellular events such as proliferation, differentiation and apoptosis^{88, 89, 91, 92}.

1.5.1 PTHLH GENE STRUCTURE AND TRANSCRIPTION

The human Pthlh gene consists of eight exons (Figure 1-3). Exon 5 codes the “prepro” region of the immature Pthlh protein, while exon 6a encodes the majority of the protein. The 5'-untranslated region has three different transcriptional starts: P1, P2 and P3⁹³⁻⁹⁶; P1 and P3 contain a TATA box while P2 contains a GC box⁹⁷.

There are distinct 3'-untranslated ends in exons 6b, 7 and 8 that are enriched with AU rich elements (ARE), which are important for the half-life of the mRNA. The

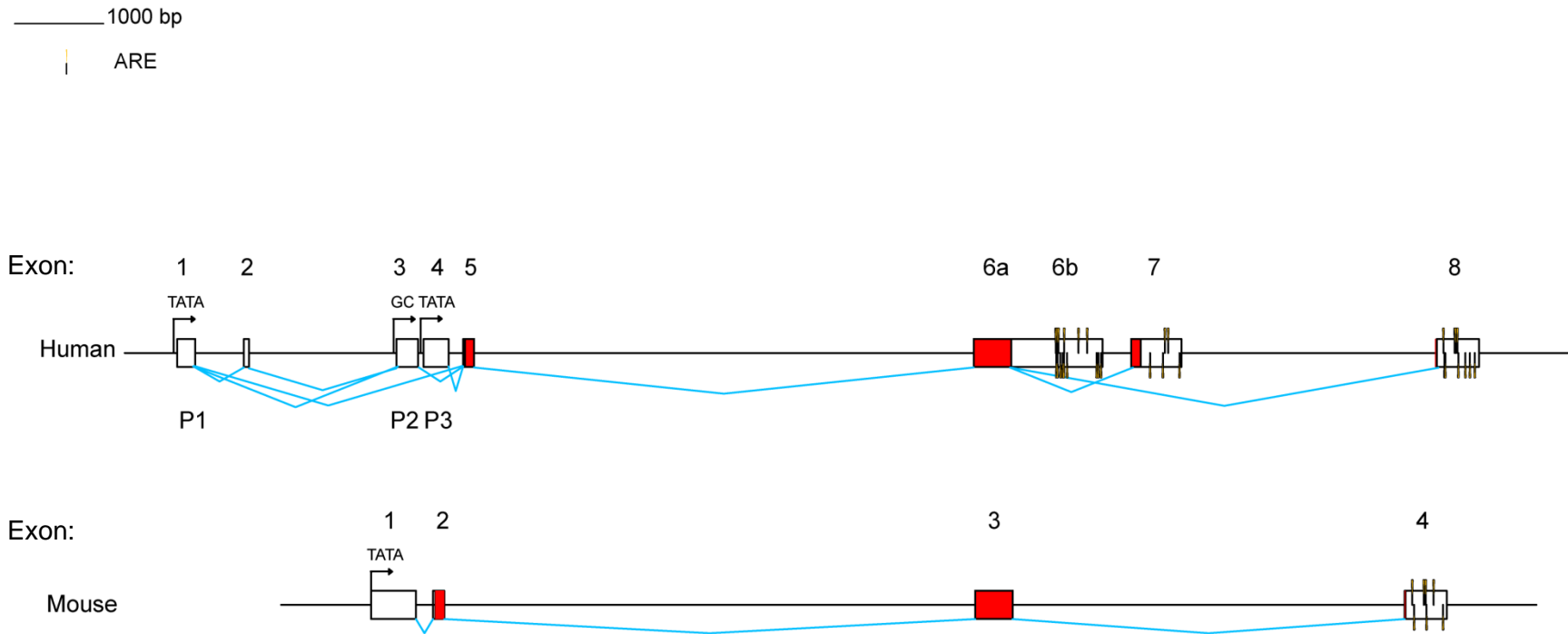


Figure 1 - 3: Pthlh gene structure.

The human Pthlh gene consists of 8 exons (untranslated: white boxes, translated: red boxes). The gene has 3 transcriptional starts (arrows) preceding exons 1, 3 and 4. Due to alternative splicing (blue lines), the human Pthlh gene can produce 15 mRNA isoforms, all of which include exons 5 and 6a, but differ in their 5' or 3' untranslated regions. Splicing from exon 6a to exon 7, 8 and 9 can give mRNAs, which when translated, produce 1-139aa, 1-173 or 1-141, respectively. The mouse Pthlh gene consists of 4 exons with one transcriptional start preceding exon1 and one 3'UTR with a single mRNA produced. The 3'UTR(s) of human and mouse Pthlh genes contain many AU-rich elements (ARE, vertical lines on the 3'UTR).

ARE motifs are targeted by proteins that regulate Pthlh mRNA stability⁹⁷. By alternative splicing, fifteen distinct mRNA isoforms can be formed, and due to the diversities in the 3' ends three initial translation products are formed: (1-139aa), (1-141aa) and (1-173aa)⁸⁸. These initial translation products then go through post-translational cleavages by prohormone convertase enzymes, and as a result a family of mature secretory peptides is formed, each peptide with its own physiological function^{87, 88} (Figure 1-4).

Pthlh is highly conserved between species, with the human and mouse peptide sequences differing by only 3aa among the first 111aa⁸⁸. Both mouse and rat genes have exons equivalent to the human exons 4, 5,6a and 8, although they differ in producing only one mRNA product (Figure 1-3). Also, the mouse and rat genes have one promoter which is equivalent to the human P3 transcriptional start site. There is some evidence that homology exists between human Pthlh and rodent Pthlh over the P2 and exon 3 of the human gene, but not exons 1 and 2^{87, 98}.

1.5.2 PTHLH BIOACTIVE PROTEINS

Pthlh complexity is not limited to its gene structure or gene transcription, but it also extends to the variety of bioactive proteins that are formed⁹⁷. In human, there are 3 initial post-translational products: 1-139 aa, 1-141 aa and 1-173 aa, each having different carboxyl-termini due to the alternative splicing of Pthlh gene transcription. The first 139aa are identical among the 3 initial products, and any proteins with amino acids sequences located between +140 and +173 are

considered unique, and to date there are no identified functional peptides resulted from the processing of the carboxyl-termini of 1-141aa or 1-173aa (Figure 1-4).

These immature peptides are preceded by 36 amino acids known to carry signal peptide and putative propeptide sequences. The signal peptidase enzyme cleaves this sequence in order to transfer Pthlh from the endoplasmic reticulum to the cytoplasm. The remaining sequence is thought to be cleaved at a dibasic motif located just before the first amino acid located at the position +1⁹⁷.

The immature peptides then undergo further processing by prohormone convertase processing enzymes⁹⁷. This is because of the enrichment of immature Pthlh peptides by mono-, di- and tetra-basic amino acids sequences (lysine and arginine), which are usually targeted by the processing enzymes; thus, leading to the production of mature peptides.

The identities of these of these prohormone processing enzymes are not completely known, however; there are several studies suggesting the expression of both Pthlh and the subtilisin family, which normally target dibasic and tetrabasic residues, in the same cells^{99,100}.

To date, only 3 bioactive Pthlh peptides have been identified as listed below:

I. *Pth-like*

This peptide is the most studied compared to the other Pthlh peptides. The first 13 aa are identical to the parathyroid hormone (Pth), and although the peptide sequence between (14-36) is different than in Pth, it results in a structure similar to

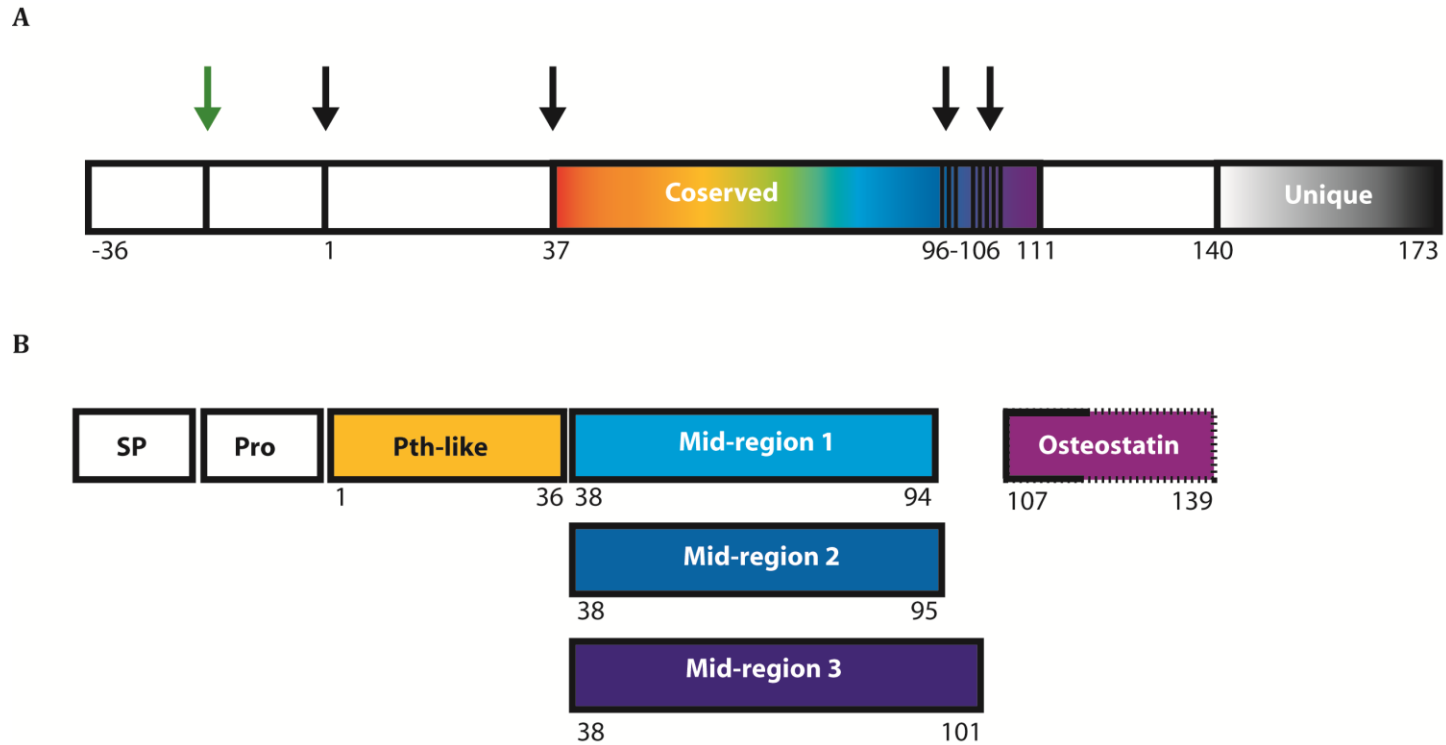


Figure 1 - 4: Pthlh functional domains and post-translational processing products

A, Functional domains of Pthlh among human and rodents includes a signal peptide (SP), a pro-peptide (Pro) and both together consume 36 amino acids preceding the immature Pthlh initial product(s). The peptide sequence between +37 to +111(rainbow shaded) is highly conserved among different species⁹⁷. However, and only in human, peptides with sequences located between +140 and +173 are unique and with unknown function(s). Pthlh peptide is enriched with basic amino acids which normally are processing targets for the signal peptidase (green arrow) and prohormone convertase enzymes (black arrows).B, Secretory forms of Pthlh that have been identified include: Pth-like, mid-region and the osteostatin Pthlh peptide. Both synthetic (107-111)aa and (107-139)aa had similar effect, thus the osteostatin protein had dashed carboxyl-terminus in the diagram (see text).

Pth and required for the binding of the Pth-like peptide to the receptor (Pth1r)⁹⁷. This peptide is known to act as a key bone resorption-enhancing factor¹⁰¹, smooth muscle relaxant ^{102, 103}, and inducer of proliferation⁹⁹ and differentiation in several tissues⁹¹. The arginine located at +37 is considered a cleavage site for a monobasic prohormone cleaving enzyme, although the identity of this enzyme is unknown.

II. Mid-region

This peptide has generated recent interest for several reasons. The mid-region peptide was first found to play a role in placental calcium transport¹⁰⁴. In addition, accumulating studies suggested the involvement of this peptide in the cellular death, proliferation, toxicity and invasiveness of human breast cancer cell lines¹⁰⁵. Although this peptide has been suggested to have multiple functions, the receptor of this peptide has yet to be identified.

At the biochemical level, this peptide is considered unique. It is rich with multiple basic sites located at the positions +88 and +106 which serves as (i) processing sites for different mid-region peptides (Figure 1-4), and a (ii) nuclear localization signal¹⁰⁶. There is evidence suggesting the nuclear localization¹⁰⁶ and the binding of Pthlh to chromatin¹⁰⁷; however the precise role and mechanism of this action is not clearly understood.

III. Carboxyl-terminal

Due to the presence of the presumed processing protease cleavage site at amino acids (102-106) and because of the high evolutionary conservation of the

peptide sequence (107-111) among human, rodent and chicken Pthlh, Fenton *et al* tested the effects of the synthetic Pthlh (107-111) and (107-139) on bone cells *in vitro*^{108, 109}. These 2 peptides showed potent inhibition of osteoclastic bone resorption^{108, 109}, but not the hPth (1-34)aa which resembles the Pthlh (1-36) in effect¹⁰⁸. The (107-111) aa peptide named osteostatin¹⁰⁹ has been shown to play a role in keratinocyte cell cycle¹¹⁰. Like the mid-region Pthlh, the receptor of this peptide is not yet identified.

1.5.3 PTHLH GENE EXPRESSION IN THE STOMACH

Cellular localization and regulation of Pthlh in the stomach is poorly understood. This is in part because of: (i) low steady-state cellular levels of Pthlh, (ii) and short Pthlh mRNA half-life caused by the numerous AREs located in the 3'UTR(s)¹¹¹. Previous studies that analyzed Pthlh mRNA expression in various tissues by RT-PCR showed highest expression in stomach¹¹², and other studies implied Pthlh expression in gastric mesenchyme¹⁰³ by immunohistochemistry, gastric gland isthmus region by *in situ* hybridization and immunohistochemistry, ECL cells by immunohistochemistry and microarray analysis^{113, 114} and parietal cells by microarray analyses^{6, 16}. The conclusions, regard Pthlh gastric expression between those studies, were conflicting. Thus, the creation of the Pthlh^{Lac Z/+} reporter mouse was advantageous, especially that the transgenic design eliminated the ARE regions, eliminating short mRNA half-life. The gastric expression, however, was not described¹¹¹. Chapters 2 and 3 address Pthlh localization in mature and developing stomach, respectively.

1.5.4 REGULATION OF PTHLH BY THE HEDGEHOG PATHWAY

Pthlh is well studied in endochondral bone development due to its importance in bone morphogenesis and growth^{115, 116}. Pthlh¹¹⁵ and the parathyroid hormone receptor (Pth1r)¹¹⁶-deficient embryos are small in size with short limbs. Also, they have domed skulls, short snouts and mandible-protruding tongues. These studies explained the short limbs to the role of Pthlh in delaying the movement of chondrocytes from proliferation to differentiation zones^{115, 116}. Although many studies suggested the association between Hh signaling^{80, 117-120} and Pthlh gene regulation and endochondral bone formation, whether Hh directly activates Pthlh has not yet been shown. Early studies found that treating embryonic mouse bone explants with Shh induced Pthlh expression, as well as delayed chondrocyte differentiation^{115, 116}. Furthermore, Shh pretreatment for either Pthlh or Pth1r-deficient bones did not rescue the premature chondrocyte differentiation^{115, 116}. These studies suggested that Hh regulates Pthlh expression. Chapter 4 of this thesis will address whether Hh regulates gastric Pthlh expression.

1.5.5 PTHLH FUNCTION IN STOMACH

The function of Pthlh in the stomach is poorly studied. This could be due to: (i) the Pthlh mutant mouse model is perinatal lethal^{115, 121}; (ii) the amniotic fluid surrounding the Pthlh null embryos during development is rich in Pthlh¹²²; (iii) and if the mutants were not lethal, there will be another challenge in which the mother's milk has high levels of Pthlh. Pthlh concentration in the mother's milk is 100,000 times higher than the physiological body serum concentration¹²³. These facts imply the central role of Pthlh in development. One way to study Pthlh function is by

examining the Pth1r mutants. The Pth1r embryos die between E9.5 and E18.5, depending on the mouse genetic background¹¹⁶. Studies done on bone development, which is the most studied organ, showed similar phenotypes in both Pthlh and Pth1r mutants, but it was more severe in the Pth1r mutant¹¹⁶. Pth1r mutants were smaller in size compared to Pthlh mutants, most likely due to the effect of maternal Pthlh which is complementing the absence of Pthlh in the Pthlh mutants but not in the Pth1r mutants¹¹⁶.

There are few studies which have suggested the importance of Pthlh in gastric smooth muscle relaxation. Ito *et al.*¹⁰³ examined the effect of Pthlh on rat gastric stress-induced hyper-contraction, which used a water immersion stress model¹⁰³. Pthlh inhibited gastric muscle contraction and Pthlh mRNA expression, but induced Pth1r expression. This suggests that Pthlh induces gastric relaxation¹⁰³. Possible roles of gastric Pthlh in gastric gland proliferation and differentiation is to be analysed.

1.5.6 PTHLH ASSOCIATION WITH GASTRIC CANCER

Aberrant Pthlh expression has been observed in different human cancers¹²⁴⁻¹²⁶. Increased levels of Pthlh expression in skeletal metastasis of human breast cancer¹²⁰ and in advanced stages of prostate cancers suggest the involvement of Pthlh in tumor proliferation and progression¹²⁷. Gastric cancer is a major cause of cancer death worldwide, and the most common malignant disease in Japan¹²⁸. Several studies showed that Pthlh is highly expressed in gastric tumors^{99, 128-134}. Although it is controversial whether increased Pthlh expression is associated with

hypercalcemia^{129-131, 135, 136}, it can be concluded from research on gastric cancer and its relation with Pthlh that: (i) high Pthlh serum is associated with adverse prognosis (reduced survival)^{99, 132, 135, 136, 133} (ii) strong Pthlh expression has been recognized in both poorly differentiated gastric tumor samples^{129, 130, 132, 133} and human gastric cancer cell lines^{99, 128, 129}; (iii) high Pthlh is associated with high prohormone convertase enzyme expression⁹⁹ which is important for the processing of the immature Pthlh protein; and (iv) gastric tumors with heterotopic ossification were also highly positive for Pthlh expression¹³⁴. The mechanism behind the aggressive role of Pthlh in gastric transformation is not yet known.

1.6 THESIS OVERVIEW

This thesis project aimed to describe the novel growth factor Pthlh in the stomach in three different areas: (i) defining the normal expression of Pthlh in the developing as well as the adult stomach; (ii) examining the function of Pthlh in the developing stomach; (iii) investigating the regulation of Pthlh gene expression in the stomach.

Chapter 2 investigates the regulation of Pthlh gene expression in the stomach by the hormone gastrin. Although there are numerous studies that indicate a potential proliferation effect of gastrin in normal and diseased stomachs, the mechanism is unknown. In this chapter, I used *in vivo* and *in vitro* models to explore gastrin regulation of Pthlh gene expression. I found that Pthlh is regulated by gastrin, with the observation of a significant reduction in Pthlh expression in gastrin-deficient mice. Additionally, wild-type mice that were acutely treated with

gastrin showed a rapid and transient induction of Pthlh gene expression. By analyzing different human gastric cancer cell lines, I found that the induction of Pthlh expression by gastrin occurs through the gastrin receptor Cckbr. Also, I screened different Pthlh mRNA isoforms found in the normal human stomach. Likewise, I identified Pthlh mRNA species induced by gastrin in the human gastric cancer cell line AGS-E which expresses the gastrin receptor Cckbr. Most importantly, my data indicate that the regulation of Pthlh by gastrin is at a post-transcriptional level by regulation of mRNA half-life.

Chapter 3 focuses on studying the function of Pthlh in the stomach. I examined the function of Pthlh in the embryonic stomach at E18.5 by taking advantage of the null mutation gene in homozygous Pthlh-LacZ embryos¹¹¹. I did not detect significant differences in Pthlh gene expression in the Pthlh mutant embryonic stomach, possibly due to the compensational effect of the maternal Pthlh found in the mother's amniotic fluid. The heterozygous mice were used to follow Pthlh expression in the stomach by following the LacZ knock in reporter expression¹¹¹. My results demonstrate that Pthlh is uniquely expressed in the acid secretory parietal cell of the mature mouse stomach (chapter 2), and in the squamous epithelial cells of the E18.5 mouse forestomach.

Chapter 4 studied the regulation of Pthlh gene expression by the Hh signaling pathway. The Hh/Pthlh signaling pathway, although not completely defined, has been well-studied in bone morphogenesis. Additionally, aberrant Pthlh expression and Hh signaling have been reported in human gastric cancer. Moreover, mice

treated chronically with gastrin display high Shh expression. Based on those studies, I hypothesized that Hh mediates the induction of Pthlh expression by gastrin. My data, however, contradicted this hypothesis. Gene expression of Hh signaling pathway components was not changed during acute gastrin treatment of wild-type mice, as was seen with Pthlh gene expression. Moreover, analysis of the embryonic stomachs of $H^+,K^+ \text{-Cre/Shh}^{-/-}$, Gli2 and Gli3 mutants did not show changes in Pthlh gene expression. Together, these data suggest that: (i) Hh is not mediating the transient induction of Pthlh expression by gastrin, and (ii) that the Hh pathway members Shh, Gli2 and Gli3 are not required for Pthlh expression.

Chapter 5 summarized the overall findings and underwent remaining questions with suggestions for possible future analyses can be performed to understand Pthlh role in normal and diseased stomach.

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CHAPTER 2

GASTRIN REGULATION OF PARATHYROID HORMONE-LIKE HORMONE (PTHLH) IN PARIETAL CELLS

2.1 ABSTRACT

Parietal cells play a fundamental role in stomach maintenance, not only by creating a pathogen free environment through the production of gastric acid, but also by secreting essential growth factors thought to be important for homeostasis of the gastric glands. The gastrointestinal hormone gastrin is known to be a critical regulator of parietal cell function as well as proliferation and differentiation of the gastric glands. Our previous gene expression microarray studies of mouse stomach mucosa identified parathyroid hormone-like hormone (Pthlh) as a potential new gastric growth factor. Although Pthlh was described to be highly expressed in gastric tumors, its normal expression, function and regulation in the stomach are unknown. In this study we used pharmacologic and genetic mouse models as well as human gastric cancer cell lines to determine the cellular localization and regulation of this growth factor by the hormone gastrin. Analysis of Pthlh^{LacZ/+} knock-in reporter mice localized Pthlh to parietal cells in the gastric corpus. Gastrin regulation was demonstrated by increased Pthlh mRNA abundance after acute gastrin administration and by reduced Pthlh expression in gastrin-deficient mice.

To examine the mechanism, we treated human gastric AGS-E cells with gastrin, observing a robust induction of endogenous Pthlh mRNA within 2 hours. Pharmacologic inhibitor studies demonstrated that PI3K, PKC α , Erk1/2 and P38 MAPK signaling were required for this induction. Analysis of mRNA half-life showed that gastrin stabilized the Pthlh transcripts. Furthermore, gastrin induced numerous Pthlh mRNA isoforms, including those from different promoters.

2.2 INTRODUCTION

Gastrin is known as the physiologic stimulator of gastric acid secretion¹. When a meal is ingested, gastrin is released to the blood stream from endocrine cells in the distal stomach (antrum) to target the proximal glandular mucosa (corpus) and stimulate gastric acid secretion¹. In the corpus, both the acid-secreting parietal cells and the histamine-secreting enterochromaffin-like (ECL) cells are the only cells known to express the gastrin receptor (Cckbr)¹ and thus respond directly to gastrin.

In addition to its central role in acid stimulation, gastrin is well known to act as a growth factor to stimulate gastric epithelial cell proliferation²⁻⁹. A single injection of pentagastrin, a synthetic form of gastrin peptide, into rats increased the total amount of corpus mRNA, protein and DNA consistent with stimulation of cellular division¹⁰. Moreover, hypergastrinemic human conditions¹¹ and mouse models¹² commonly exhibit gastric hyperplasia, although the mechanism is unknown.

Parietal cells acid secretion is essential in creating a gastric pathogen free environment, and therefore preserves a healthy gastric cellular integrity. It is

equally important to note that several proteins, thought to be important for homeostasis of the gastric gland, are expressed by parietal cells. These proteins include: (a) morphogens like sonic hedgehog (Shh)¹³ and Indian hedgehog (Ihh)^{14, 15}, (b) signaling pathway regulators like the insulin-like growth factor binding protein 2 (Igfbp2)¹⁶ and the secreted form of growth hormone receptor which acts as GH binding protein (GHR)¹⁶ and (c) growth factors such as vascular endothelial growth factor B (Vegfb)¹⁶, and parathyroid hormone-like hormone (Pthlh)^{15, 16}.

The growth factor Pthlh is broadly expressed in numerous tissues during development and adulthood, orchestrating key cellular events, such as cell proliferation and differentiation¹⁷. Unsurprisingly, imbalanced Pthlh gene regulation has been frequently observed in human cancers¹⁸, including stomach cancer¹⁹⁻²². Although the normal function and gene regulation of Pthlh is well studied in some tissues (for example, bone and mammary¹⁷), very little is known about the physiologic role and the regulation of Pthlh in the stomach. One study suggested the importance of Pthlh in promoting gastric smooth muscle relaxation²³, however the mechanism behind this process as well as whether Pthlh has other functions in the stomach are unknown.

This chapter investigates the normal gastric expression of Pthlh in the stomach. It also describes the regulation of Pthlh gene expression by gastrin.

2.3 MATERIAL AND METHODS

Mice

Pthlh^{LacZ/+} ²⁴ and gastrin-deficient²⁵ mice were on mixed CD-1 and C57BL/6 strain backgrounds, respectively. Mice were housed in ventilated and automated-watering cages under specific pathogen-free conditions. All animal procedures have been approved by the University of Michigan Committee on Use and Care of Animals. For all experiments, 1-4 month-old mice from both genders were used.

Gastrin Stimulation

Wild type mice were fasted overnight with free access to water before i.p. injection with vehicle (150 mM NaCl, pH 7) or 250µg/kg human gastrin I (Bachem) for 1, 2 or 4 hours before stomach collection for analysis.

Cells and Pharmacological Inhibitors

AGS-E human gastric cancer cell line stably transfected with the human Cckb receptor were obtained from Timothy Wang (Columbia University)²⁶. Cells were cultured in DMEM (Invitrogen) containing 10% fetal calf serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.292 mg/mL L-glutamine and 2mM sodium pyruvate (Invitrogen), and grown at 37°C with 5% CO₂. Puromycin (0.02mg/mL) was used occasionally during regular culture to maintain the Cckbr expressing construct in these cells. Unless otherwise mentioned, cells were seeded at 3x10⁵ cells /well in 6-well plates overnight, serum-starved for 24 hours, and then treated for 4 hours with serum-free media containing vehicle or 10⁻⁷M human gastrin. For Cckbr signaling experiments, cells were pre-treated 30 minutes before gastrin treatment with Cckbr pathway inhibitors: SB203580 (10⁻⁵ M), Ro-32-0432 (10⁻⁶ M), LY294002 (1.5x10⁻⁵ M), or AG 1478 (9x10⁻⁶ M) (Calbiochem), or PD98059 (5x10⁻⁷

M, Cell Signaling). Human gastric cell lines AGS and NCI-N87 were obtained from the American Type Culture Collection (ATCC # CRL-1739™ and CRL-5822™, respectively). MKN-45 (Riken, Japan) and 23132/87 cells were obtained from (DSMZ, Germany). AGS cells were cultured similarly as AGS-E cells. All other cell lines were cultured in RPMI-1640 media as recommended by the source.

Whole Mount X-gal Staining

The protocol previously described²⁷ for whole-mount X-gal staining was modified. Briefly, stomachs from *Pthlh^{LacZ/+}* mice were opened along the greater curvature, pinned flat on dental wax and fixed for 1 hour in 4% paraformaldehyde (PFA) in PBS. Tissues were washed 3 times (15 minutes) in X-gal wash buffer (0.1 M sodium phosphate pH 7.3 containing 2 mM MgCl₂ and 0.02% NP-40) and placed between 5 hours to overnight in X-gal (5-bromo-4-chloro-3-indyl-β-D-galactosidase; Roche) staining solution (1mg/mL X-gal in N,N dimethylformamide, 5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆·3H₂O) in X-gal wash buffer) at 37°C, protected from light. Tissue was washed in X-gal buffer and analyzed for LacZ expression.

Immunohistochemistry

Stomach cryosections (8μm) were dried at room temperature for 5 minutes, and then rinsed with 0.01% triton X-100 in PBS (TPBS). Sections were blocked with 10% goat serum in 0.1% TPBS for 30 minutes then stained with rabbit anti β-galactosidase (1:1000, gift from J. D. Engel, University of Michigan) overnight at 4°C. Slides were rinsed in 0.01% TPBS and incubated for 1 hour at room temperature with mouse monoclonal antibody against H⁺, K⁺- ATPase-α subunit (1:500, Medical

and Biological Laboratories, Nagoya). After washing in 0.01% TPBS, samples were incubated with appropriate secondary antibodies conjugated to Cy2 or Cy3 (1:200, 1:500, respectively, Jackson ImmunoResearch Laboratories, West Grove). Sections were mounted with ProLong Gold anti-fade reagent with 4', 6-diamidino-2-phenylindole DAPI (Invitrogen). Fluorescence images were captured with a cool snap digital camera mounted on a Nikon E800 upright fluorescence microscope.

RNA Isolation and Gene Expression Analysis

RNA was isolated from mouse stomachs or from cultured cells using TRIzol® (Invitrogen), as described¹⁵. Isolated RNA was purified and DNase-treated using the RNeasy Mini kit (Qiagen). 1µg RNA was reverse-transcribed using the iScript™ cDNA synthesis kit (Bio-Rad), as recommended by the manufacturer. Gene expression was measured using quantitative reverse transcription-PCR (qRT-PCR) analysis as described¹⁵ using the primers listed in (Table 2-1). Expression levels were determined for individual samples (in triplicate) and normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (Gapdh), which did not differ in control and experimental groups.

RT-PCR Analysis of Pthlh Isoforms

Pthlh gene structure (Figure 2-5, A) was drawn using GenePalette software²⁸. Normal human gastric RNA samples were purchased (Origene, Zyagen and Cell Applications, Inc.) and 1µg was transcribed using the iScript™ cDNA synthesis kit. PCR used the Go Taq Flexi DNA Polymerase kit (Promega). Primer sequences,

Table 2 - 1 : Oligonucleotid primers(5' to3') for quantitative reverse transcription polymerase chain reaction

Species	Gene	Forward	Reverse	Amplicon (bp)
Human	Gapdh	GAGTCCACTGGCGTCTTCACC	GAGGCATTGCTGATGATCTTGAGG	164
	Pthlh	GGTGTTCCTGCTGAGCTACGC	TCGTCGCCGTAATCTTGGATGG	136
	TFF2	TAACAGGACGAACTGCGGCTTC	GCACCAGGGCACTTCAAAGATG	244
Mouse	Gapdh	TCAAGAAGGTGGTGAAGCAGG	TATTATGGGGTCTGGGATGG	350
	Pthlh	GACGTACAAAGAACAGCCACTCA	TTTTTCTCCTGTTCTCTGCGT TT	81
	HK- α	TGTACACATGAGAGTCCCCTT G	GAGTCTTCTCGTTTTCCACACC	157
	HK- β	AAC AGA ATT GTC AAG TTC CTC	AGA CTG AAG GTG CCA TTG	140
	Pth1r	AAG TGG TGG CAG TAC CTT GTC GTCC	CCC GTT CCC GAG TCT CAT TGG	171
	Gastrin	GGACCAGGGACCAATGAGG	CCAAAGTCCATCCATCCGTAGG	173

product content, product size, as well as conditions used to amplify each Pthlh isoform are shown in (Tables 2-2 and 2-3). Reaction mixtures (50 μ l) contained 0.2 μ M of each forward and reverse primer, 0.2 mM dNTP, 1X reaction buffer, 2mM MgCl₂ and 0.05 units of Taq polymerase. Betaine²⁹ (1M, Sigma) was used in PCR reactions that amplified the P3 Pthlh isoform. Amplification was performed with 35 cycles of denaturation at 95° C for 30 seconds, annealing at 55° or 60° C (Table 2-3) for 20 seconds and extending at 72°C for 45 seconds. PCR products were run on a 1.7% agarose gel to evaluate mRNA isoforms. Amplicons were sequenced to validate the specificity of the different assays for different Pthlh isoforms.

Measurement of Pthlh mRNA Half-Life

AGS-E cells (10⁶) were grown overnight, then treated with vehicle or 10⁻⁷ M gastrin for 4 hours. Actinomycin D (10 μ g/mL DMSO, Sigma) to inhibit mRNA synthesis was added to the media and samples collected at 0, 2, 4, 8, 12 hours for RNA isolation. qRT-PCR values of Pthlh mRNA at each time point were normalized to Gapdh and compared to values at time 0.

Statistics

Data are presented as mean \pm SEM and analyzed by student's t-test or one way ANOVA (Graph Pad Prism) followed by Tukey post test. P < 0.05 was considered significant.

Table 2 - 2 : Oligonucleotid primers for reverse transcription polymerase chain reaction

Primer	Primer Sequence 5'-3'
F1	AGGTACCTGCTTTCTAATA
F3	TTCTCCGGCAGGTTTG
F4	CCACGAACCCAGGAGAACTGC
F5	GGAGACGATGCAGCGGAGAC
R6a	TTTCCTGCTCCTTGCGTTTCC
R6b	TTGCCCAGGTGTGAGAGTAAGG
R7	GGCAATAAAGTAGGGTCC
R8	CCAATGTGCAGTTTCATAGAGC
Pth1r F	GGCGTCCCTCACCGTAGC
Pth1r R	AGAGTAGAGCACAGCGTCCTTG
Gapdh F	GAGTCCACTGGCGTCTTACC
Gapdh R	GAGGCATTGCTGATGATCTTGAGG

The first 8 primers are for human Pthlh gene with F = forward, R = reverse and a number indicating Pthlh specific exon (see Figure 2 - 5).

Table 2 - 3 : Conditions and product size of each primers sets used to identify distinct pthlh transcripts by RT-PCR analyses

Transcript	Primer Set	Recognized Exons	Product Size (bp)	Annealing Temperature (C°)
All	F5-R6a	E5-E6a	419	
P1		E1-E2-E3-E5-E6a	915	
P1'	F1-R6a	E1-E3-E5-E6a	858	55°
P1''		E1-E5-E6a	615	
P2	F3-R6a	E3-E5-E6a	693	
P3	F4-R6a	E4-E5-E6a	648	
3'UTRa	F5-R6b	E5-E6a-E6b	901	
3'UTRb	F5-R7	E5-E6a-E7	981	60°
3'UTRc	F5-R8	E5-E6a-E8	691	

2.4 RESULTS

Pthlh is Expressed in Parietal Cells

To study gastric *Pthlh* expression, we first measured the endogenous *Pthlh* mRNA abundance by qRT-PCR in wild type mouse stomach. Analyzing tissue strips from proximal to distal stomach showed a gradient of expression of *Pthlh* in the glandular part of the stomach, with reduced expression in the direction toward antrum (Supplemental Figure 2-1, A and B). Interestingly, the *Pthlh* pattern of expression was consistent with the expression of the parietal cell marker H⁺, K⁺-ATPase α subunit in corpus compared to antrum (Figure 2-1, A and B and Supplemental Figure 2-1, B and C).

To identify which cells express *Pthlh*, we utilized *Pthlh*^{LacZ/+} reporter mice²⁴. Whole mount X-gal staining confirmed our qRT-PCR results, with LacZ expression restricted to the gastric corpus, in organization similar to the normal distribution parietal cell (Figure 2-1, C and D). Immunohistochemical analysis for the *Pthlh*^{LacZ/+} reporter mouse stomach demonstrated co-localization of antibodies for β -galactosidas and H⁺, K⁺-ATPase α , suggesting that *Pthlh* is localized to parietal cells (Figure 2-1, E- G).

Pthlh is Induced by Gastrin

Since the hormone gastrin regulates parietal cells¹⁵, we examined whether it might regulate *Pthlh* gene expression. Analysis of *Pthlh* expression in gastrin-deficient mice, showed a 5-fold reduction in mRNA abundance compared to control (Figure 2-2, A). This regulation was also demonstrated by a reduction of *Pthlh*^{LacZ/+}

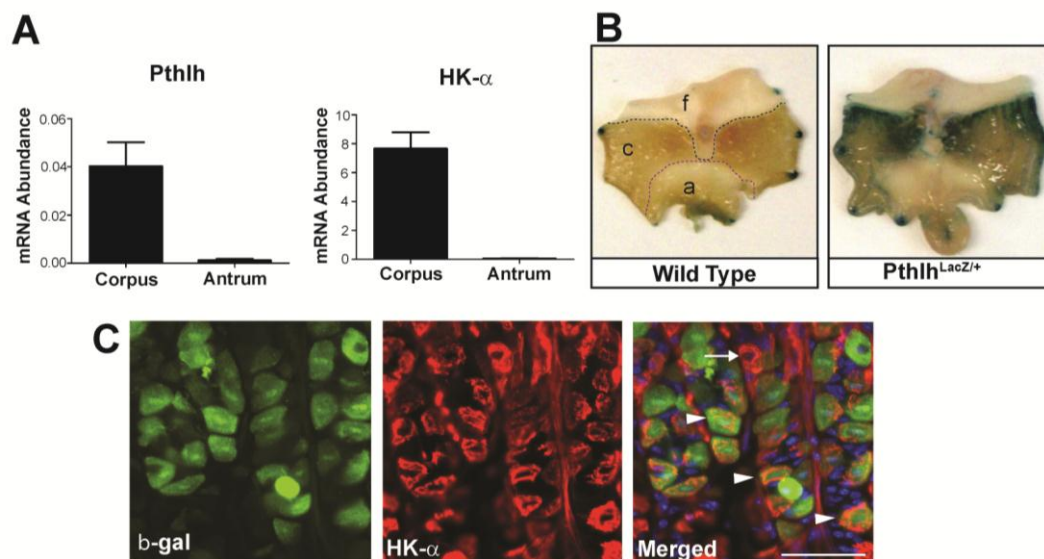


Figure 2 - 1: Parietal cells express *Pthlh*. A) *Pthlh* and the parietal cell marker H⁺, K⁺- ATPase α (HK- α) subunit mRNA abundance were measured by quantitative reverse transcription-PCR (qRT-PCR). Values were normalized to *Gapdh* as mean \pm SEM (N=3-5). B) Whole mount X-gal staining in *Pthlh^{LacZ/+}* reporter mouse stomach showed high LacZ expression near the squamoglandular junction compared to none in the wild type mouse (f:forestomach, c:corpus and a:antrum). Dark edge staining is an artifact due to pinning the tissue. C) Co-immunostaining of adult *Pthlh-LacZ* stomach cryo-sections with antibodies against β -galactosidase (β -gal) (green) and HK- α (red) showed colocalization in a subset of parietal cells. Arrowhead, β -gal-expressing parietal cell; arrow, non- β -gal-expressing parietal cell. Scale bar=50 μ m.

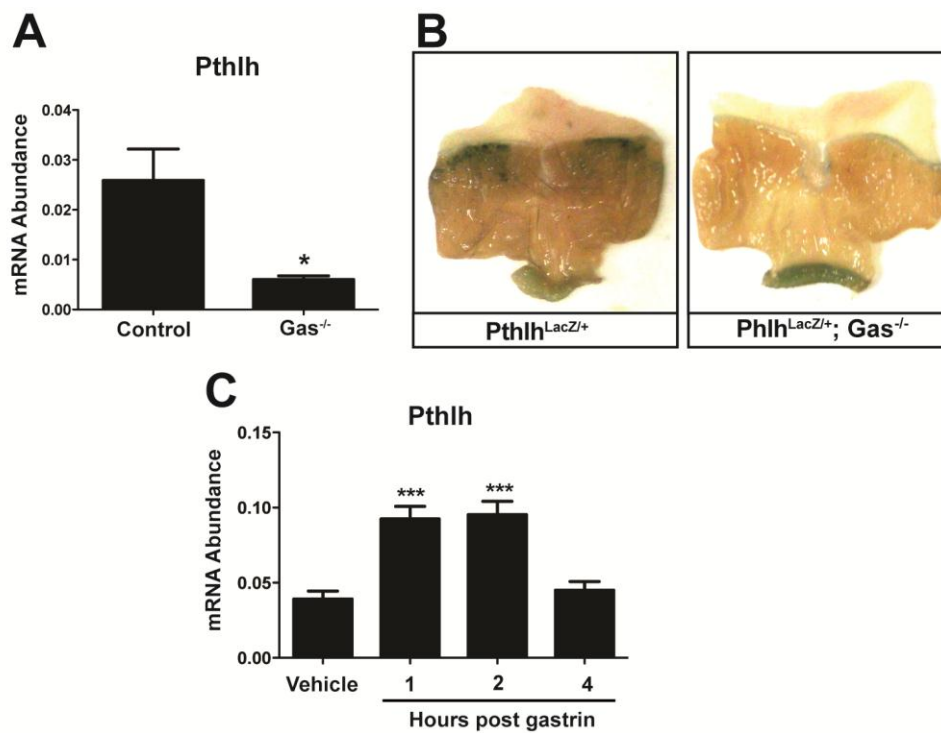


Figure 2 - 2: Gastrin stimulates *Pthlh* expression in vivo. A) *Pthlh* mRNA abundance was measured by qRT-PCR analysis of corpus from gastrin-deficient mice (Gas^{-/-}) compared to control littermates. Values were normalized to *Gapdh* and data are shown as mean ± SEM (N = 4, *P < 0.05). B) Whole mount X-gal staining of *Pthlh*^{LacZ/+} reporter mouse stomach on normal or Gas^{-/-} background. Staining at the pylorus is non-specific. C) qRT-PCR for *Pthlh* mRNA measured in corpus isolated from wild type mice 1, 2 or 4 hours after gastrin injection (250mg/kg). ***P < 0.001 vs. vehicle injected group. For A and C, data (mean ± SEM) are shown in reference to *Gapdh* expression measured in the same samples (N = 4).

reporter gene expression observed by X-gal staining (Figure 2-2, B). In addition, gastrin treatment of wild type mice demonstrated rapid and transient induction of Pthlh expression, which peaked at 1 and 2 hours post injection, returning to basal levels by 4 hours (Figure 2-2, C). Together these results demonstrated that gastrin can induce Pthlh expression *in vivo*.

To study possible mechanisms for this induction, we utilized the human gastric cancer cell line AGS-E²⁶. Treatment with gastrin for 2, 4, 6 or 12 hours showed a robust induction of Pthlh mRNA at all time points in comparison to vehicle (Figure 2-3 A, left). The highest induction of Pthlh expression (> 150- fold) was observed at 4 hours post-gastrin treatment. We also examined the response of the TFF2 gene, which had previously been shown to be induced by gastrin³⁰. Induction of TFF2 mRNA expression was delayed and longer-lasting than the pattern for Pthlh (Figure 2-3, A right). This result suggests that gastrin might follow distinct mechanisms to regulate the expression of both genes. These data suggested that gastrin can induce Pthlh expression rapidly and transiently.

To study if Pthlh mRNA induction by gastrin is mediated by the gastrin receptor Cckbr, we measured Pthlh gene expression in AGS cells compared to AGS-E, which differ in expression of the human Cckbr gene into AGS cells³¹. Gastrin treatment of AGS cells for 6 hours was not different from vehicle treated cells (Figure 2-3, B). In contrast, AGS-E cells exhibited a robust response to gastrin, suggesting that gastrin directly stimulates Pthlh expression *via* the Cckbr receptor.

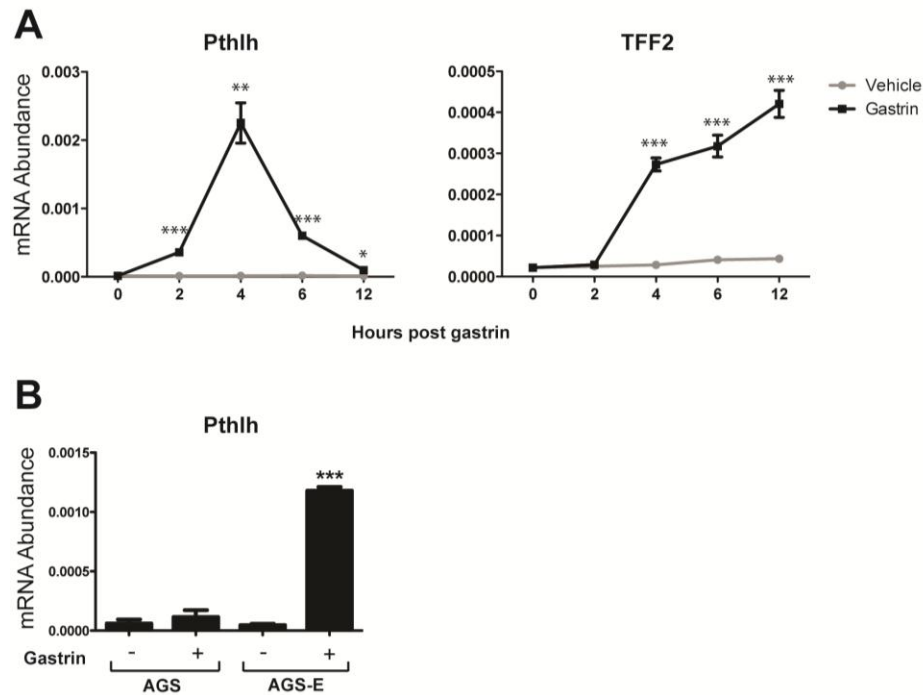


Figure 2 - 3: Gastrin stimulates *Pthlh* expression in vitro. A) qRT-PCR measurement of *Pthlh* (left) or *TFF2* (right) mRNA abundance in AGS-E cells treated with vehicle or gastrin for 0, 2, 4, 6 and 12 hours. * $P < 0.05$, ** $P < 0.005$, *** $P \leq 0.0005$ vs. vehicle-treated group of the same time point. B) qRT-PCR measurement of *Pthlh* mRNA abundance in AGS cells, which do not express the gastrin receptor *Cckbr*, or AGS-E cells after 6 hours treatment with gastrin. *** $P \leq 0.0005$ vs. vehicle-treated group of each cell line. All data (mean \pm SEM) are shown in reference to *Gapdh* expression measured in the same samples (N = 3).

Our earlier results showed that *Pthlh* is expressed by parietal cells (Figure 2-1, C). We wanted to test if the ECL cells express any *Pthlh* because *Cckbr* is also expressed in ECL cells. Immunohistochemical analysis for *Pthlh*^{LacZ/+} mouse stomach showed no co-localization between the pan-endocrine marker chromogranin A and β -galactosidase, suggesting that the *Pthlh* gene is not expressed in ECL cells (Supplemental Figure 2-2).

Gastrin stimulates the transcription of several genes by means of acid-dependent or acid-independent pathways^{32, 33}. Based on that, we tested whether acid mediates the induction of *Pthlh* by gastrin by analysis of mice treated with the proton pump inhibitor omeprazole. This analysis showed that *Pthlh* mRNA abundance was not changed in the omeprazole treated mice, suggesting that gastrin induction of *Pthlh* gene expression is acid-independent (Supplemental Figure 2-3).

Intracellular Signaling Pathways Mediating Gastrin Regulation of Pthlh

Cckbr stimulation is known to activate several downstream signaling pathways including the MAPK Erk1/2, MAPK p38, PI3K, PKC α and EGFR³⁴. Pre-treatment of AGS-E cells with pharmacologic inhibitors for Erk1/2, p38, PI3K or PKC α blocked gastrin induction of *Pthlh* mRNA expression (Figure 2-4, A-D). In contrast, the EGFR inhibitor only partially blocked the response (Figure 2-4, E). These results suggest that Erk1/2, p38, PI3K and PKC α mediate *Pthlh* mRNA induction by gastrin and that EGFR signaling has limited control on *Pthlh* expression activation.

Gastrin Induces Several Distinct Pthlh mRNA Isoforms

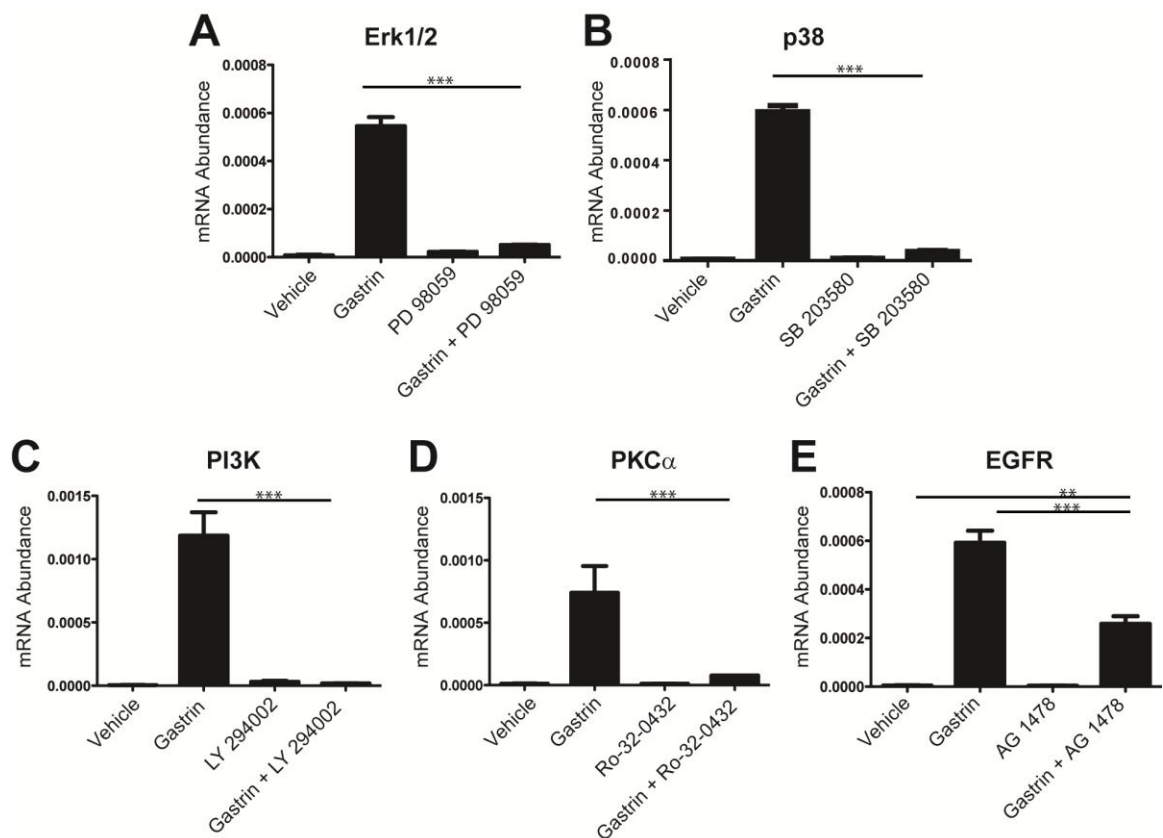


Figure 2 - 4: Cckbr signaling pathways mediate Pthlh mRNA induction by gastrin. qRT-PCR measurement of Pthlh mRNA abundance in vehicle, gastrin, Cckbr inhibitor or gastrin and Cckbr inhibitor. Values were normalized to Gapdh and data are shown as mean \pm SEM, ** P < 0.01, *** P < 0.001. (N=3).

It is known that the human *Pthlh* gene expresses many different *Pthlh* mRNA isoforms (Figure 2-5, A)³⁵. Extensive alternative splicing of *Pthlh* gene can give rise to mRNA species with differing 5' and 3' UTRs, that all express exons 5 and 6a. Because of this complexity, we wanted to know the normal expression of *Pthlh* mRNA isoforms in human stomach and to identify which *Pthlh* mRNA isoforms were regulated by gastrin in AGS-E cells. Thus, RNAs from normal human stomach and vehicle or gastrin-treated AGS-E cells were studied (Figure 2-5, B). Using RT-PCR analysis, normal human samples were found to express *Pthlh* transcripts specific to the promoters P2, P3 but not P1 as well as to the 3'UTR a and c but not b. Analysis of AGS-E cells after gastrin treatment showed that all regions were induced, including P1, P1', P2 and P3, as well as 3'UTR regions a, b and c (Figure 2-5, B). Interestingly, regions specific for P1 were highly amplified when AGS-E cells were gastrin treated compared to P2 and P3.

Given that *Pthlh* expression has been described to be highly expressed in gastric cancers, we next screened several human gastric cancer cell lines for *Pthlh* isoform expression. The human cervical cancer cell line HeLa was used as a positive control for most RT-PCR assays (Figure 2-5, C). NCI-N87, MKN-45 and 23132/87 gastric cell lines expressed the common *Pthlh* region with lower expression in 23132/78 cells. Analysis of *Pthlh* isoforms expression showed a similar pattern as normal stomach, with transcripts resulted from the activation of P2, P3 and/or containing the regions from the 3'UTRa and 3'UTRc. These studies show that *Pthlh* is expressed in normal human stomach and human gastric cancer

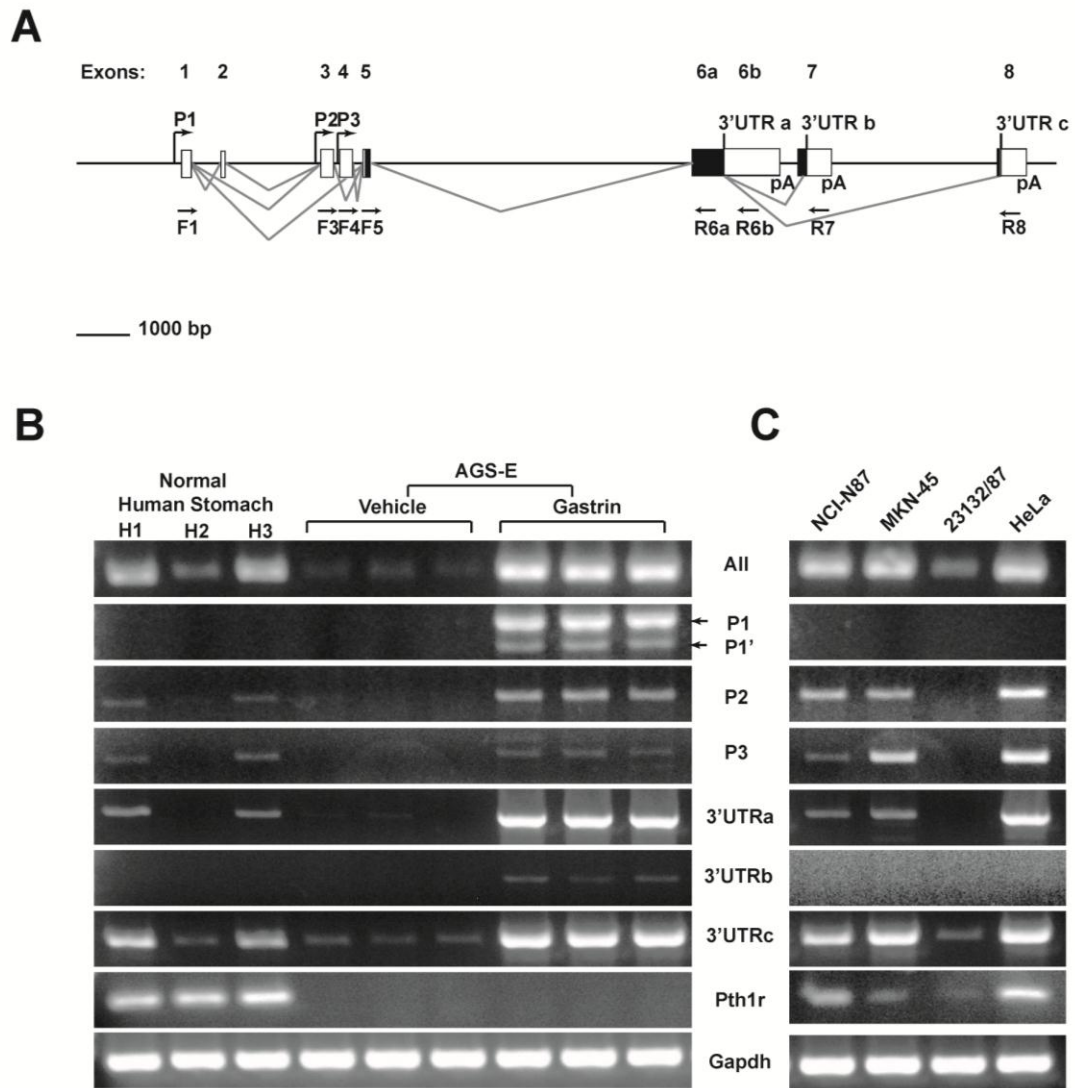


Figure 2 - 5: Gastrin induces different *Pthlh* mRNA isoforms. A) Diagram of human *Pthlh* gene structure. The *Pthlh* gene consists of eight exons (untranslated, white, translated, black) with three transcription starts (P1, P2 and P3). Alternative splicing gives rise to isoforms with different 3' untranslated regions: 3'UTR a, b and c. Primer sets were designed to amplify *Pthlh* mRNA species containing P1, P2, P3 and the 3'UTRs a, b or c regions (see Supplemental Tables 1 and 2). B) RT-PCR products for samples from normal human stomach samples H1, H2 and H3 as well as vehicle or gastrin-treated AGS-E cells. C) RT-PCR products for different human gastric cancer cell lines. HeLa cells were used as a positive control.

cells. Gastrin induction of Pthlh was not observed in other human cell lines, presumably because they do not express Cckbr (data not shown).

Gastrin Stabilizes Pthlh mRNA

Since our results showed rapid induction of Pthlh expression by gastrin (Figure 2-3, A), and because the Pthlh 3'UTRs are enriched with AU-rich elements (ARE)³⁵, we hypothesized that gastrin acts by stabilizing Pthlh mRNA. To test this hypothesis, Pthlh mRNA half-life was measured in AGS-E cells treated with vehicle or gastrin in the presence of actinomycin D for 0, 2, 4, 8 and 12 hours (Figure 2-6). We found that gastrin increased the half-life of Pthlh mRNA from 66 minutes in vehicle-treated cells to 144 minutes in gastrin treated-cells. This result confirms that gastrin regulates Pthlh gene expression at the post-transcriptional level.

2.5 DISCUSSION

This study described the normal pattern of expression of the novel gastric growth factor Pthlh by taking advantage of a genetically engineered mouse model in which the bacterial β -galactosidase reporter gene (LacZ) was inserted into the endogenous Pthlh locus²⁴. The reporter is expressed from the endogenous Pthlh promoter and shown to be an accurate readout of gene expression³⁶. In addition, the LacZ insertion creates a mutant allele and homozygous Pthlh^{LacZ/LacZ} mice are nulls (discussed in Chapter 3). By analyzing the Pthlh^{LacZ/+} mouse, we demonstrated that Pthlh expression is specific to parietal cells in the stomach. Interestingly not all of the parietal cells expressed the reporter gene, with expression most prevalent in

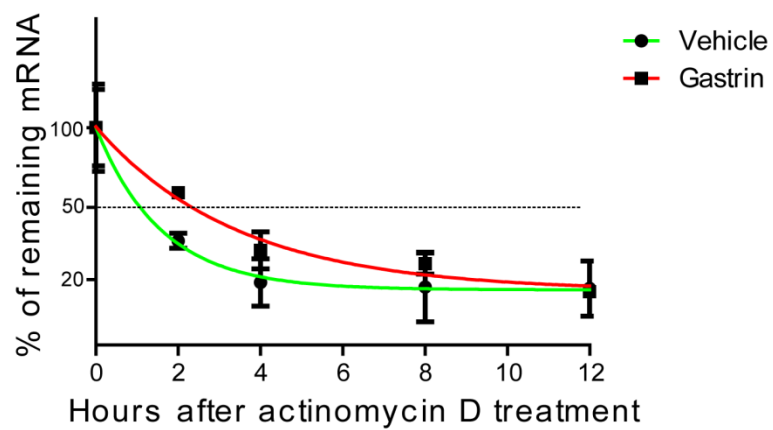


Figure 2 - 6: Gastrin stabilizes *Pthlh* mRNA. Exponential curve for remaining *Pthlh* mRNA measurements of AGS-E cells treated with vehicle or gastrin by qRT-PCR analysis. Cells were collected at 0, 2, 4, 8 and 12 hours after actinomycin D treatment.

parietal cells in the proximal glandular region, close to the squamous epithelium. This pattern of increased expression of other genes or cellular markers, especially in the first few glands of the proximal corpus, has been reported^{13,37}. For example the newly discovered gastric tuft cell marker doublecortin-like kinase 1 (DCLK1)³⁷ as well as the sonic hedgehog (Shh) growth factor¹³ were found to be highly expressed in this area. How this pattern of gene expression is important to stomach homeostasis is yet to be identified.

Parietal cells have been hypothesized to mediate the proliferating effect of gastrin in the stomach for several reasons. First, besides the ECL cell, the parietal cell is known to directly respond to gastrin *via* the Cckbr. Second, the parietal cell expresses several key gastric proteins that have been described in other tissues to regulate cellular proliferation or growth, including Sonic hedgehog (Shh)¹³, Indian hedgehog (Ihh)¹⁵, vascular endothelial growth factor B (Vegfb)^{15,16}, low molecular weight GH receptor which acts as GH binding protein (GHR)¹⁶ and insulin-like growth factor binding protein 2 (Igfbp2)^{15,16}. Lastly, gastrin receptors have not been defined on gastric progenitor cells, thus suggesting the proliferation effect is indirect. The discovery of Pthlh being expressed specifically by the parietal cell in addition to the induction of Pthlh expression by gastrin, are supportive facts to the commonly accepted hypothesis of parietal cells mediating the proliferative effect of gastrin in the stomach.

In this study we used both *in vivo* and *in vitro* systems to demonstrate that acute gastrin treatment induces Pthlh gene expression. Pthlh induction is rapid and

transient. Also, although different mRNA isoforms were induced by gastrin, isoforms with the P1 transcriptional start were pronounced compared to P2 and P3 (Figure 2-5, B). Based on the literature there is no clear explanation of the differential usage of Pthlh promoters, especially those studies that relied on cell lines. However, similar to my observations, some studies have confirmed the marked activation of the three promoters in human lung and/or breast tumors compared to normal cells^{18,38}. It will be important to identify which transcription factors might contribute to Pthlh expression in the stomach. A recent study in the C4-2 human prostate cancer cell line suggested regulation of Pthlh expression is mediated by the potential vitamin D response element (nVDRE) located at P1 promoter³⁹. Whether similar regulation occurs in parietal cells upon gastrin stimulation is yet to be explored.

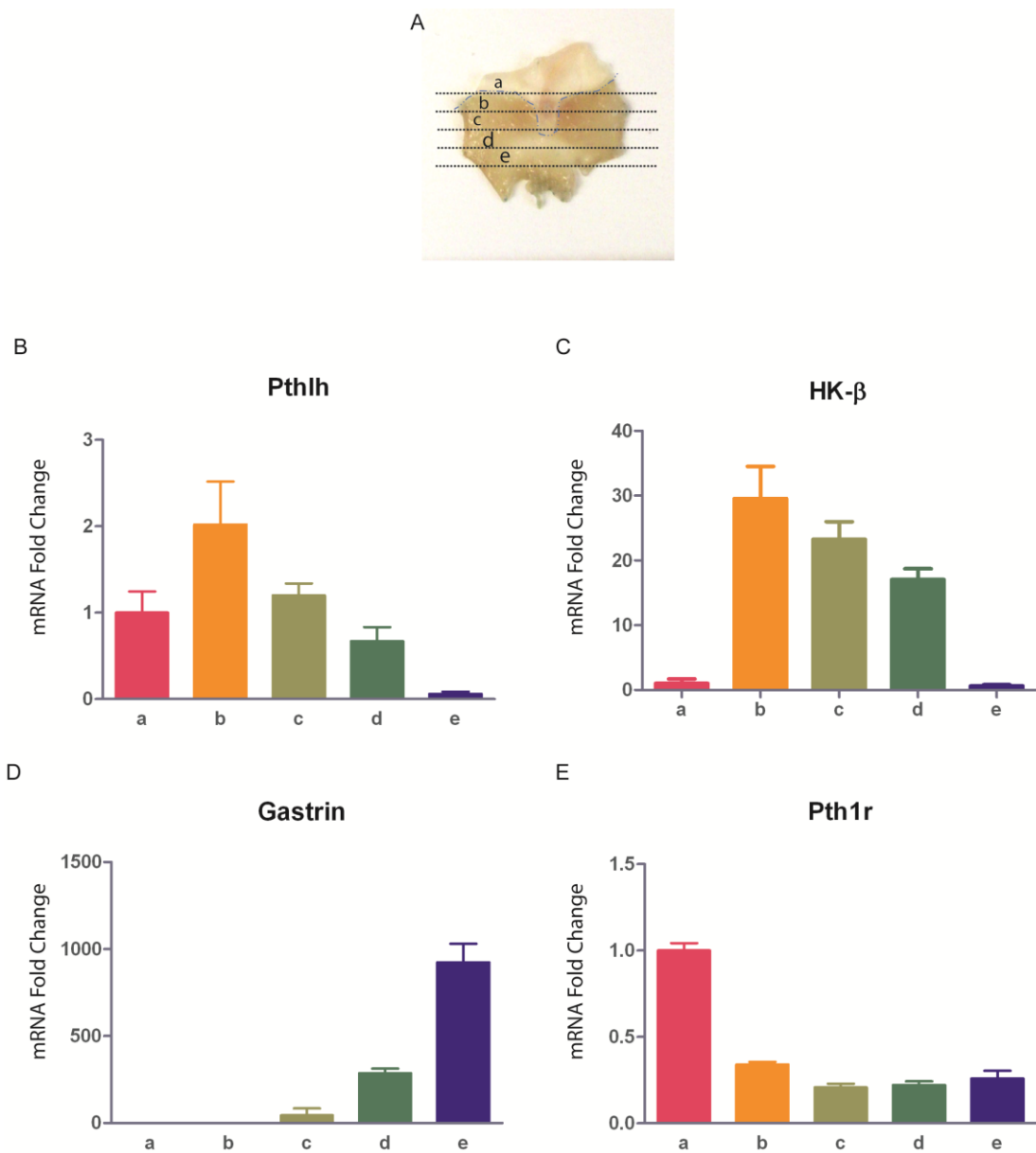
Pthlh 3'UTRs contain numerous AU-rich elements (ARE), which have been demonstrated to regulate Pthlh mRNA stabilization in human renal carcinoma cell lines⁴⁰. The rapid and transient nature of Pthlh induction by gastrin suggested a similar mechanism in effect to increase Pthlh mRNA abundance in the stomach. Our results confirm that the mechanism of gastrin induction of Pthlh expression is at least in part due to the increased Pthlh mRNA half-life after gastrin treatment. Other studies have described ARE binding proteins that can function to stabilize Pthlh mRNA, including HuR, or AUF1 binding proteins⁴⁰⁻⁴². The identity of ARE binding proteins involved in gastrin stabilization was not determined. However, a previous study had demonstrated that gastrin stabilizes interleukin-8 (IL-8) and cyclo-

oxygenase 2 (COX-2) mRNAs, which contain AREs, via the HuR binding protein⁴¹; thus it is likely that gastrin regulates Pthlh through the same mechanism.

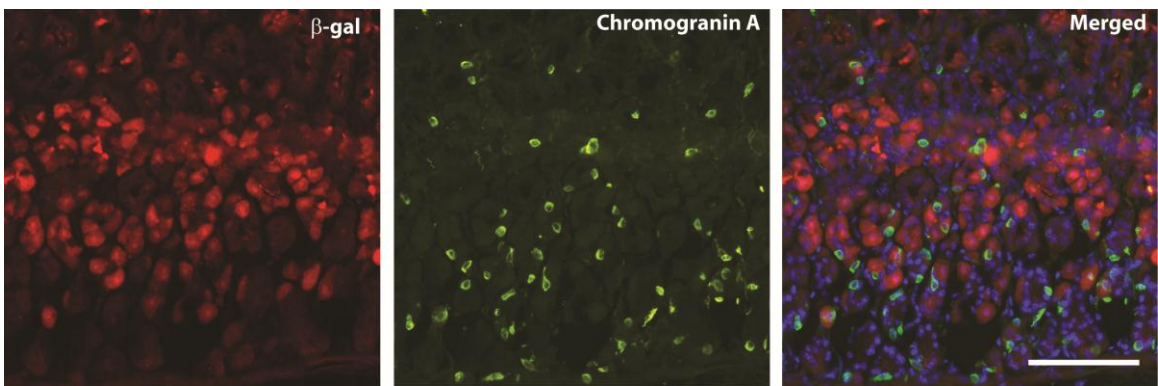
Although this study establishes Pthlh as a putative gastrin-regulated growth factor in the stomach, further studies are required to test whether Pthlh directly mediates the proliferative effects of gastrin on the gastric mucosa. It will be important to determine whether the Pthlh receptor Pth1r is expressed by the gastric progenitor cells. My preliminary results suggest that the receptor is more highly expressed in the mesenchymal compartment, but these studies did not exclude expression in epithelial cells (data not shown). It will also be interesting to find the growth/proliferation outcomes in human gastric cancer cell lines after Pthlh treatment. Analysis of Pthlh-deficient mouse mutants will be interesting to uncover the functional significance of this novel growth factor in the stomach. Accordingly an analysis of Pthlh null mice is described in Chapter 3.

Our study shows for the first time that Pthlh is also expressed in normal adult human stomach (Figure 2-5). It is unknown, however, if the cellular pattern of Pthlh expression in human stomach is similar to the mouse. Pthlh is known to be highly expressed in gastric cancer^{19, 22, 43-45}. Accordingly, my data demonstrated that Pthlh is expressed in several human gastric cancer cell lines (Figure 2-5, B and C).

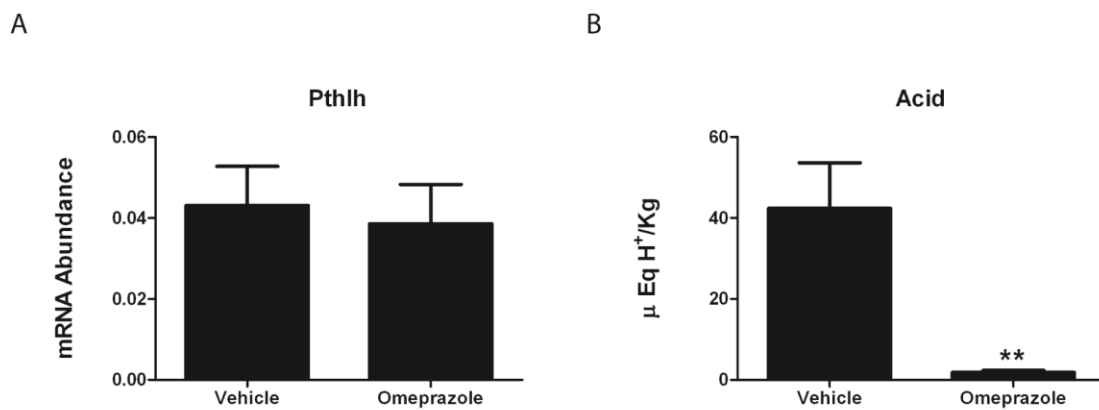
Overall, this study confirmed the Pthlh expression by parietal cell. It also demonstrated the stimulation of Pthlh gene expression by gastrin via post-transcriptional means. It will be essential to find the normal function of Pthlh especially that Pthlh is highly elevated in different cancers.



Supplemental Figure 2 - 1: The distribution of Pthlh mRNA and parietal cell marker expression is similar. A) Wild type mouse stomach was cut to strips where a: forestomach, b: proximal corpus, c: distal corpus, d: transition to antrum and e: antrum. (B-E) qRT-PCR analysis of endogenous Pthlh, H⁺,K⁺-ATPase β , gastrin and Pth1r in each stomach strip described in panel A. Values were normalized to Gapdh expression and reported as fold-change relative to forestomach. All data (mean \pm SEM) are shown in reference to Gapdh expression measured in the same samples (N=3).



Supplemental Figure 2 - 2: Endocrine cells do not express *Pthlh*^{LacZ/+}. Frozen stomach sections from were immunostained for β -gal (red) and the pan-endocrine cell marker chromogranin A (green, goat polyclonal, 1:100 dilution, Santa Cruz). Immunohistochemistry performed as described in Material and Methods sections. Scale bar=100 μ m.



Supplemental Figure 2 - 3: Induction of Pthlh gene expression is acid-independent.

A) qRT-PCR measurement of Pthlh mRNA abundance in vehicle or omeprazole-treated mice with a single daily i.p. injection of omeprazole (400 μmol/kg) for 4 days. Values were normalized to Gapdh. B) H⁺ concentration in stomach contents of vehicle and omeprazole-treated mice. Data are means ± SEM, ** P < 0.05. (N=5).

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CHAPTER 3
THE FUNCTION OF PARATHYROID HORMONE-LIKE HORMONE
IN THE DEVELOPING MOUSE STOMACH

3.1 SUMMARY

My studies described in chapter 2, demonstrated that Pthlh is a novel parietal cell growth factor that is regulated by gastrin. Pthlh is known to be expressed broadly in a number of tissues and has been described to orchestrate key cellular events, such as cell proliferation and differentiation. Although Pthlh is known to also be expressed in gastric tumors, its normal function in the stomach is unknown. In this chapter, I took advantage of the genetically engineered Pthlh^{LacZ/+} knock in mouse strain to study the function of Pthlh in the developing stomach by analyzing Pthlh^{LacZ/LacZ} nulls. Since these mice are perinatal lethal, this analysis focused on immature embryonic day 18.5 (E18.5) and postnatal day 0 (P0) stomach. This analysis showed that the stomach of Pthlh null mice was normal, with morphology and cell marker expression similar to wild-type. Our findings suggest that Pthlh mutant mouse model has no obvious gastric changes, which may be due to maternal compensation with Pthlh found in the amniotic fluid.

3.2 INTRODUCTION

In the stomach, regulation of gastric homeostasis is not fully understood. One important consideration is the growth factors secreted by parietal cell, which may preserve a healthy balance between proliferation and differentiation¹. Recent microarray studies identified parathyroid hormone-like hormone (Pthlh) as a novel growth factor expressed by gastric epithelial cells²⁻⁴. Pthlh was first identified as the causative factor for hypercalcemia of malignancy, in which tumors that secrete this hormone increase serum calcium levels by promoting bone and renal tubular calcium resorption through the activation of the parathyroid hormone receptor (Pth1r)⁵. In addition to association with malignancy, the wide expression of Pthlh in many systems during development and in mature tissues suggests a central role for Pthlh in physiological homeostasis^{6, 7}. Although Pthlh was known to be expressed in the stomach, little is known about the regulation or the function of Pthlh in this organ. For example, there were discrepancies about the cellular localization of Pthlh in the stomach with some studies suggesting that Pthlh is expressed in epithelial cells while others found mesenchymal expression of Pthh⁸⁻¹¹. Moreover, although Pthlh has been known to have a wide range of functions in many body systems, including calcium homeostasis, cell proliferation and cell differentiation⁷, the function of Pthlh in the stomach was poorly understood and limited to a possible involvement in gastric smooth muscle relaxation¹⁰.

Therefore, this chapter focused on identifying the normal gastric function of Pthlh by taking the advantage of Pthlh^{LacZ/+} and Pthlh^{LacZ/LacZ} mice¹².

3.3 MATERIALS AND METHODS

Mice

All experiments were performed according to protocols approved by the University of Michigan Committee on Use and Care of Animals. Mice were maintained in specific-pathogen-free barrier facilities under a 12-hour light/dark cycle. $Pthlh^{LacZ/+}$ mice (gift from A. Broadus), were maintained on mixed a CD-1 and C57BL/6 strain background. Stomachs at postnatal day 0 (P0) or at embryonic day E18.5 were obtained from intercrossing $Pthlh^{LacZ/+}$ mice. The morning of the day of presence of a vaginal plug was taken to be embryonic day 0.5 (E0.5). This mating gives rise to $Pthlh^{LacZ/LacZ}$ nulls, which were used for *Pthlh* functional studies, $Pthlh^{LacZ/+}$, which were used for *Pthlh* expression analysis, and wild type mice, which were used for controls.

Tissue Collection and Analysis of Gene Expression

For E18.5 embryonic stomachs, RNA was isolated from whole stomachs using the RNeasy Mini Kit according to the manufacturer's instructions. cDNA syntheses and qRT-PCR analyses were performed as described previously in chapter 2. Table 3-1 lists primers used to carry out the qRT-PCR analyses.

Table 3 - 1: Oligonucleotide primers (5' to 3') for quantitative reverse transcription polymerase chain reaction

Gene	Forward	Reverse	Size (bp)
Gapdh	TCA AGA AGG TGG TGA AGC AGG	TAT TAT GGG GGT CTG GGA TGG	350
Pthlh	GAC GTA CAA AGA ACA GCC ACT CA	TTT TTC TCC TGT TCT CTG CGT TT	81
H ⁺ ,K ⁺ -ATPase α	TGT ACA CAT GAG AGT CCC CTT G	GAG TCT TCT CGT TTT CCA CAC C	157
H ⁺ ,K ⁺ -ATPase β	AAC AGA ATT GTC AAG TTC CTC	AGA CTG AAG GTG CCA TTG	140
Gastrin	GGACCAGGGACCAATGAGG	CCAAAGTCCATCCATCCGTAGG	173
Pth1r	AAG TGG TGG CAG TAC CTT GTC GTCC	CCC GTT CCC GAG TCT CAT TGG	171
IF	CTT GGC CCT GAC CTG TAT GT	TAG GTT GCT CAG GTG TCA CG	191
Sm22α	CTC TAA TGG CTT TGG GCA GTT TGG	GCT CCT GGG CTT TCT TCA TAA ACC	90
Vimentin	ACT GCT GCC CTG CGT GAT GTG	GGT ACT CGT TTG ACT CCT GCT TGG	163
Desmin	AGA CCT TCT CTG CTC TCA ACT TCC	CTC GCT GAC AAC CTC TCC ATC C	122

X-Gal Staining

For regular X-gal staining, wild type or *Pthlh^{LacZ/+}* stomach and intestine cryosections (8 μ m) were dried at room temperature for 5 minutes, and then fixed in 4% PFA for 5 minutes before performing x-gal staining as described in chapter 2 with neutral red as a counterstain.

For whole mount X-gal staining, gastrointestinal tracts were dissected and fixed in 4% PFA for 1 hour before performing whole-mount X-gal staining, as described in chapter 2.

Immunohistochemistry Analyses

For E18.5 stomach analysis, pregnant female mice were injected 1.5 h before embryo collection with 5-bromo-2-deoxyuridine (BrdU 50 mg/kg ip; Sigma), freshly prepared in normal saline (0.9% NaCl, pH 7.4). Stomachs were dissected and fixed overnight in 4% PFA as described previously¹³. Proliferating cells were identified by immunostaining 4 μ m paraffin sections for BrdU (1:50 dilution of mouse monoclonal antibody; Dako).

For P0 null stomach analysis, stomachs were collected and fixed for 1 hour in 4% PFA then incubated overnight in 30% sucrose in 1XPBS. Gastric cell types were identified by immunostaining cryosections (8 μ m) with a mouse monoclonal antibody to the H⁺, K⁺-ATPase α subunit (1:500; Medical and Biological Laboratories) for parietal cells and a polyclonal antibody to gastric intrinsic factor (1:1,000 rabbit anti-human; gift from D. Alpers, Washington University St. Louis, St.

Louis, Missouri, USA) for zymogenic cells. Staining and secondary antibody use were done as previously described¹³. Sections were stained with biotin-conjugated secondary antibodies (1:200; Vector Labs) for 30 min at room temperature and visualized by using the Vectastain Elite ABC kit (Vector Laboratories), developed with DAB and counterstained with hematoxylin.

Statistics

Quantitative data are presented as mean \pm SEM and analyzed by student's t-test (Graph Pad Prism) with $P < 0.05$ considered significant.

3.4 RESULTS

The goal of this study was to determine the normal function of Pthlh in the mouse stomach by analyzing homozygous Pthlh^{LacZ/LacZ} mice. Pthlh mutants are perinatally lethal^{14, 15}, thus we initially examined Pthlh mutants at P0. The gross morphology of the stomach of P0 Pthlh mutants showed a dramatic reduction in size compared to control stomachs. (Figure 3-1, B). This difference was likely due to the inability of the Pthlh-null mice to feed, thus not stretching the stomach as in controls. Consequently, Pthlh-deficient gastric mucosa appeared to be expanded compared to normal stomach (Figure 3-1, C and D). To determine whether differentiated epithelial cells were formed, Pthlh mutant and control stomach sections were stained with H⁺,K⁺-ATPase and IF to assess parietal cells and zymogenic cells, respectively (Figure 3-1, E-H). This analysis showed that these differentiated cell types were formed in null stomach.

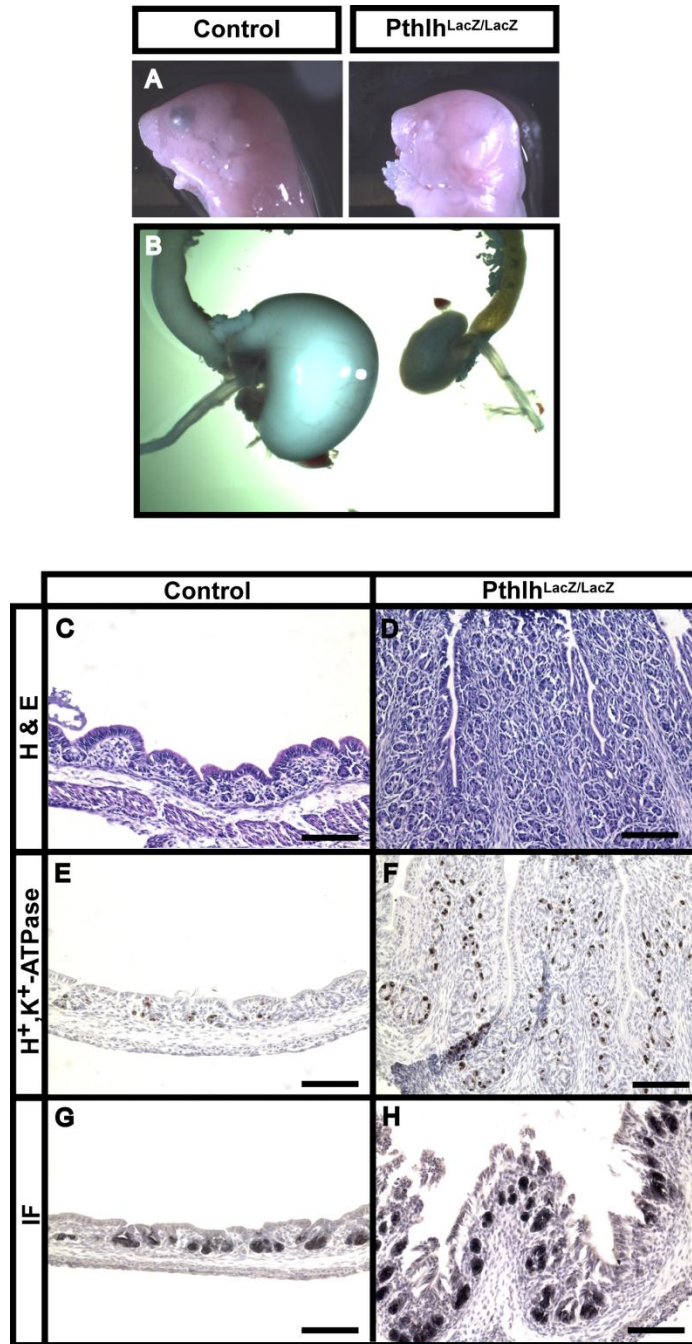


Figure 3 - 1: Pthlh mutant stomach phenotype at P0. A, the phenotypic appearance of Pthlh nulls compared to the controls. The Pthlh mutants have domed skulls, short snouts and mandible-protruding tongues. These skeletal abnormalities prevent the mutants from feeding. B, Pthlh-deficient P0 stomach is reduced in size compared to control. Hematoxylin and eosin, H⁺,K⁺-ATPase or intrinsic factor stained frozen sections for control (C, E and G) or Pthlh-deficient (D, F and G) P0 stomachs. Pthlh mutants at P0 appeared to have expanded mucosa compared to normal stomach. Scale bar = 100 μm.

Because the stomachs of control mice were filled with milk compared to *Pthlh*-deficient P0 (Figure 3-1, B), we examined *Pthlh* mutants at E18.5 to avoid any possible artifact resulting from gastric wall stretching. The first striking finding was that the dramatic reduction in stomach size seen in the *Pthlh* P0 nulls was eliminated and both groups had the same stomach size at E18.5 (Figure 3-2, A and B), thus suggesting that the apparent difference in mucosal morphology at P0 was due to stretching. By analyzing the stomach morphology with hematoxylin and eosin stained sections, no changes were observed in the E18.5 *Pthlh*-deficient prenatal mucosa compared to controls (Figure 3-2, A-D). To determine if epithelial cell proliferation was changed, gastric sections were stained with BrdU (Figure 3-2, E-F), with no change found in *Pthlh* mutant stomach. This was in agreement with the unchanged expression of the proto-oncogenes *c-Myc* and *N-Myc* known to be involved in promoting proliferation (Figure 3-3, D-E)^{4, 16}. Although *Pthlh* mRNA was absent in the *Pthlh*-deficient stomach, expression of *Pth1r* and the *Pthlh* target transcription factor SRY-box containing gene 4 (*Sox4*) were not changed¹⁷ (Figure 3-3, A-C).

To test if there are changes in gastric differentiation of *Pthlh* mutants, epithelial and mesenchymal marker expression was measured. Epithelial marker expression specific to parietal cells and zymogenic cells was first analyzed. There were no changes in H^+,K^+ -ATPase- α and IF expression by qRT-PCR and immunohistochemical analysis between *Pthlh* mutant and control stomachs (Figure 3-4, A-F). There were, however, modest mesenchymal changes in mesenchymal cell marker expression in mesenchymal cell marker expression with significant

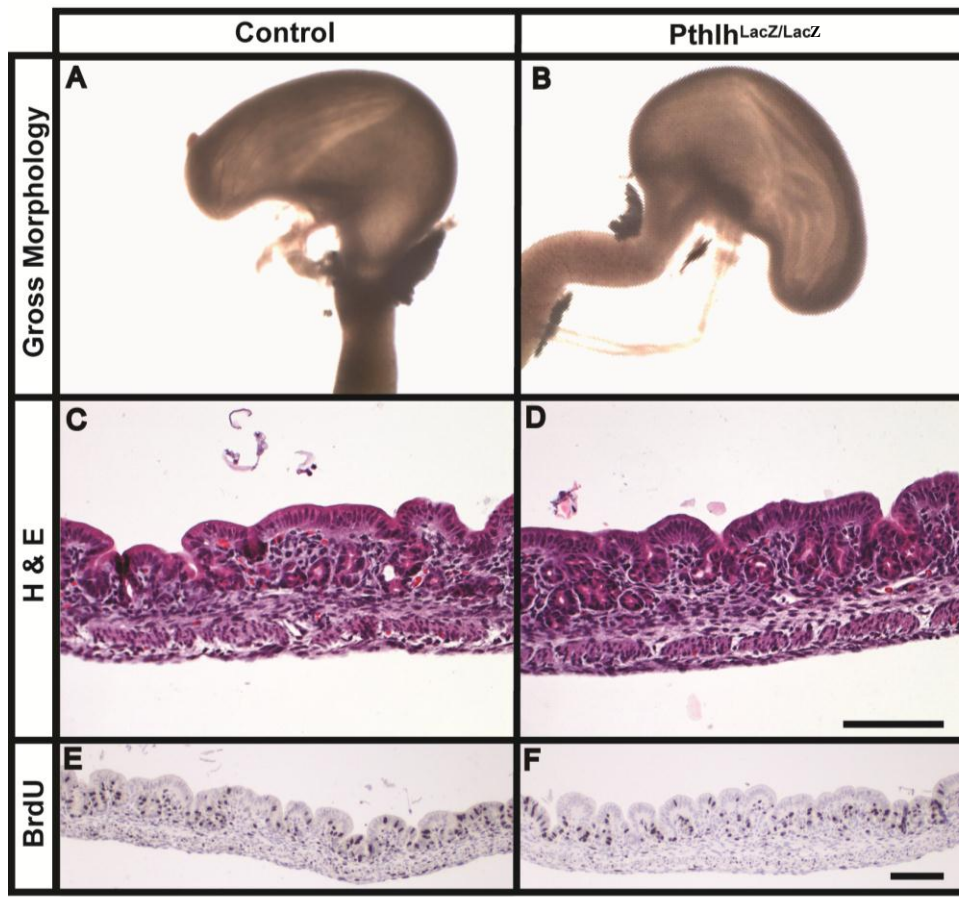


Figure 3 - 2: *Pthlh* mutant stomach at E18.5 does not differ from normal stomach. (A-B) Gross morphology, (C-D) Hematoxylin and eosin stained sections, and (E-F) BrdU stained sections of E18.5 control or *Pthlh* mutant stomachs. Scale bar = 100 μ m.

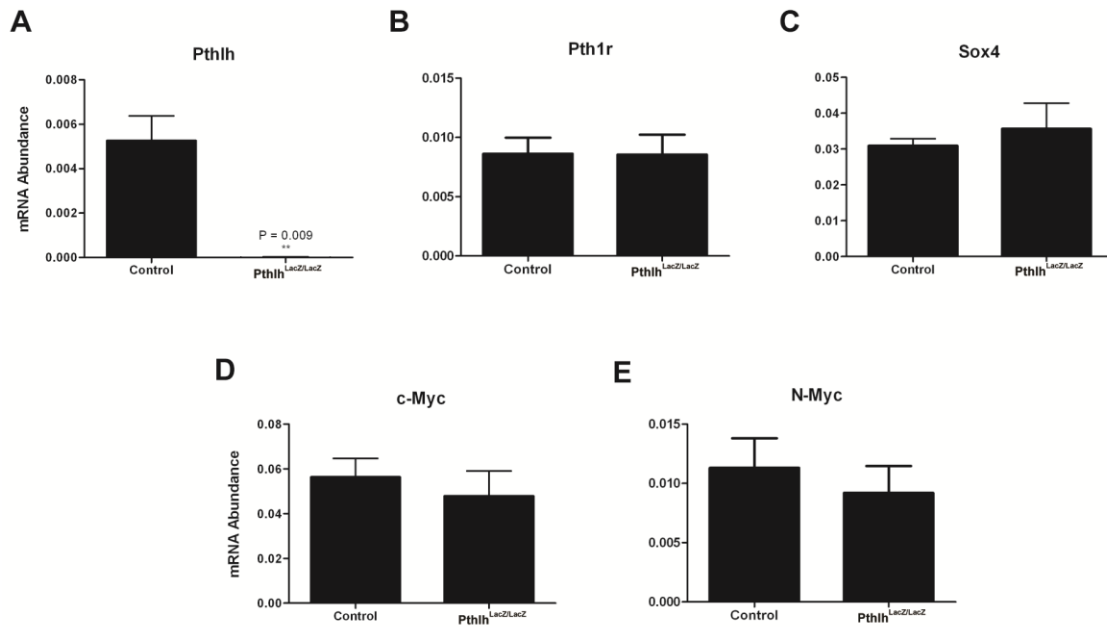


Figure 3 - 3: *Pthlh* mutant stomach at E18.5 does not differ from normal stomach. Total stomach RNA of controls or *Pthlh* mutants were analyzed by qRT-PCR for *Pthlh*, *Pth1r*, *Sox4*, *c-Myc* and *N-Myc* genes. ** P < 0.01 and all data (mean ± SEM) are shown in reference to *Gapdh* expression measured in the same samples (N = 5-6).

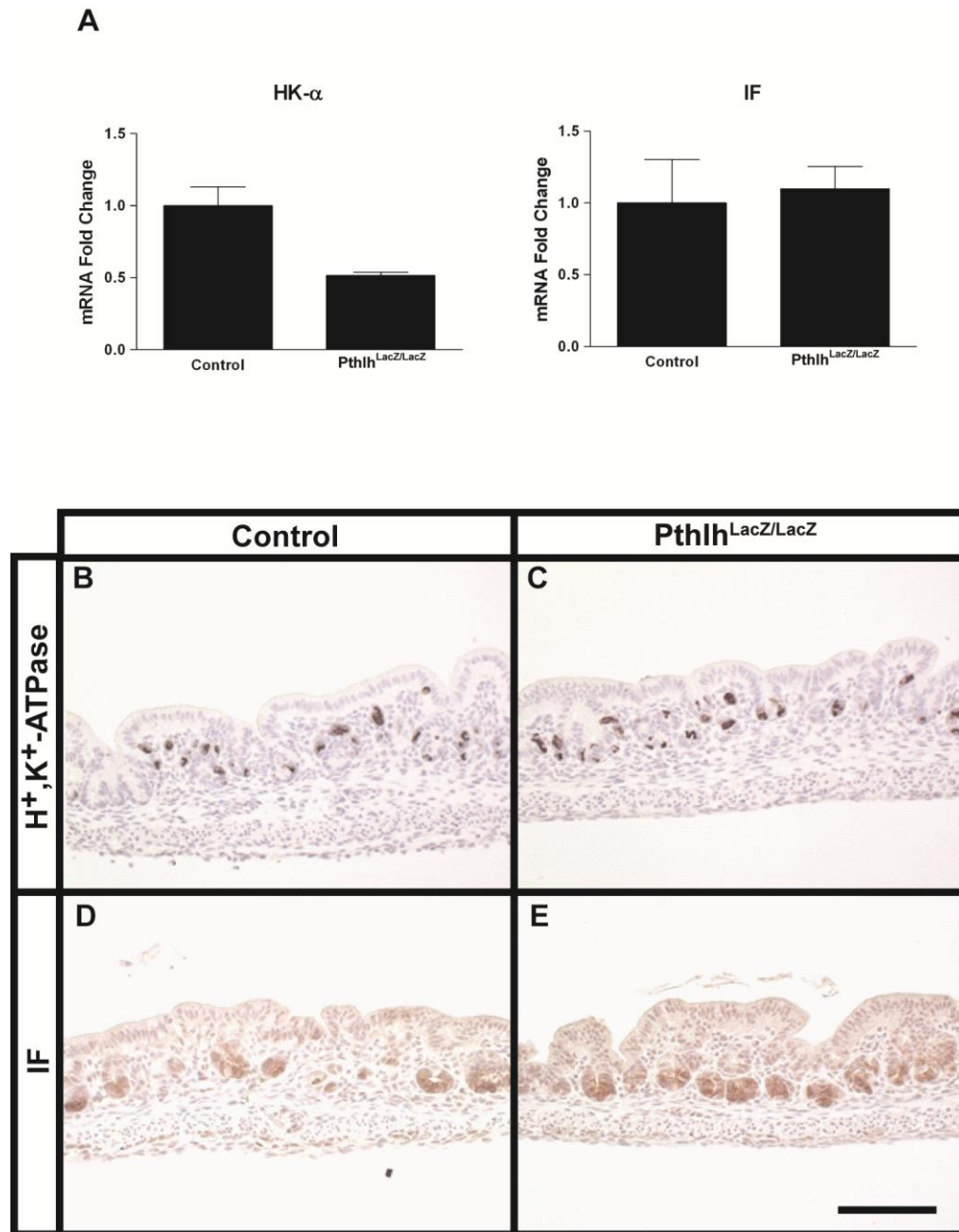


Figure 3 - 4: Differentiated epithelial gastric cell marker expression is not changed in E18.5 Pthlh mutant stomach. A, qRT-PCR analyses for H⁺,K⁺-ATPase- α or IF in control and Pthlh mutant stomach. ** P <0.01 and all data (mean \pm SEM) are shown in reference to Gapdh expression measured in the same samples (N = 5-6). Immunostaining for sections of Pthlh mutant or control stomach sections using H⁺,K⁺-ATPase (B-C) and intrinsic factor (D-E) antibodies.

reduction in the smooth muscle specific marker SM22 α . This result suggested that smooth muscle cells might be targets for Pthlh (Figure 3-5 A-C).

Since the analysis of Pthlh mutant stomach at E18.5 did not show major changes in the gastric mucosa, we wanted to know if Pthlh is expressed in stomach during fetal gastric development by analysis of LacZ expression. Pthlh was found to be restricted to the forestomach epithelium at E18.5. Thus Pthlh expression was not observed in the developing gastric glandular epithelium (Figure 3-6, B-C). In contrast, Pthlh was highly expressed in the developing intestinal villus-epithelium. (Figure 3-6, D-E), which implies the importance of Pthlh in intestinal development.

3.5 DISCUSSION

Pthlh is a growth factor that is expressed widely in different tissues in both developmental and postnatal tissues. Pthlh is known to have numerous functions in normal tissues as well as to be highly expressed in many cancers^{6, 18, 19}. This chapter aimed to find the function of Pthlh in the stomach by analysis of Pthlh null mice¹². Although the Pthlh null stomach at P0 showed apparent mucosal expansion (Figure 3-1, C-H), the E18.5 null stomach showed no major differences between mutants and control stomach. The Pthlh mutants have skeletal abnormalities that affect the newborn mouse milk suckling. My data suggests that the mucosa of the control P0 stomach is thinner and stretched in organization compared to the mutants due to the lack of feeding. Furthermore, Pthlh is known to be high in both the mother's amniotic fluid²⁰ as well as mother's milk²¹. Thus loss of Pthlh in the developing stomach may be compensated for by maternal sources. Pthlh mutants did not show any changes in stomach size, gastric mucosa organization or differentiated epithelial

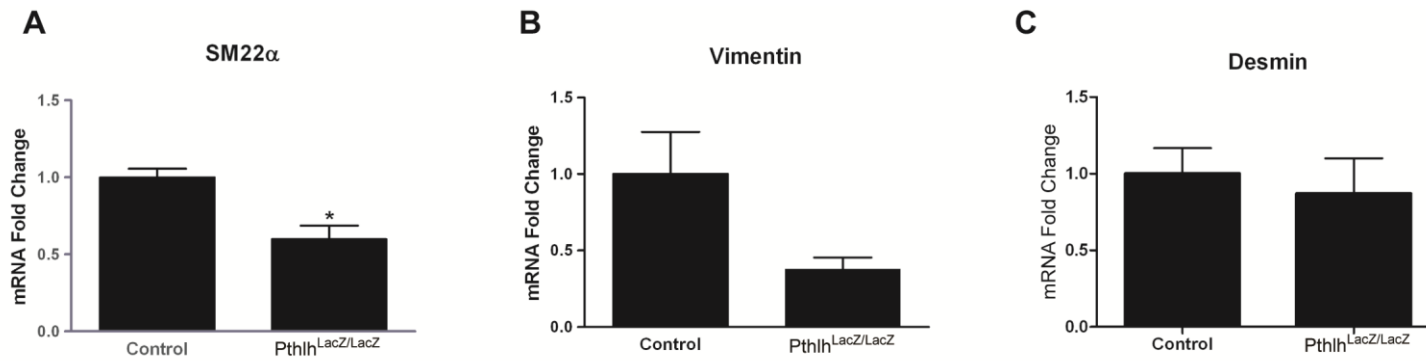


Figure 3-5 Differentiated mesenchymal gastric cell marker expression had minor changes in *Pthlh* mutant stomach. qRT-PCR analyses for SM22 α , vimentin and desmin in control and *Pthlh* mutant stomach. ** P < 0.01 and all data (mean \pm SEM) are shown in reference to *Gapdh* expression measured in the same samples (N = 3-6).

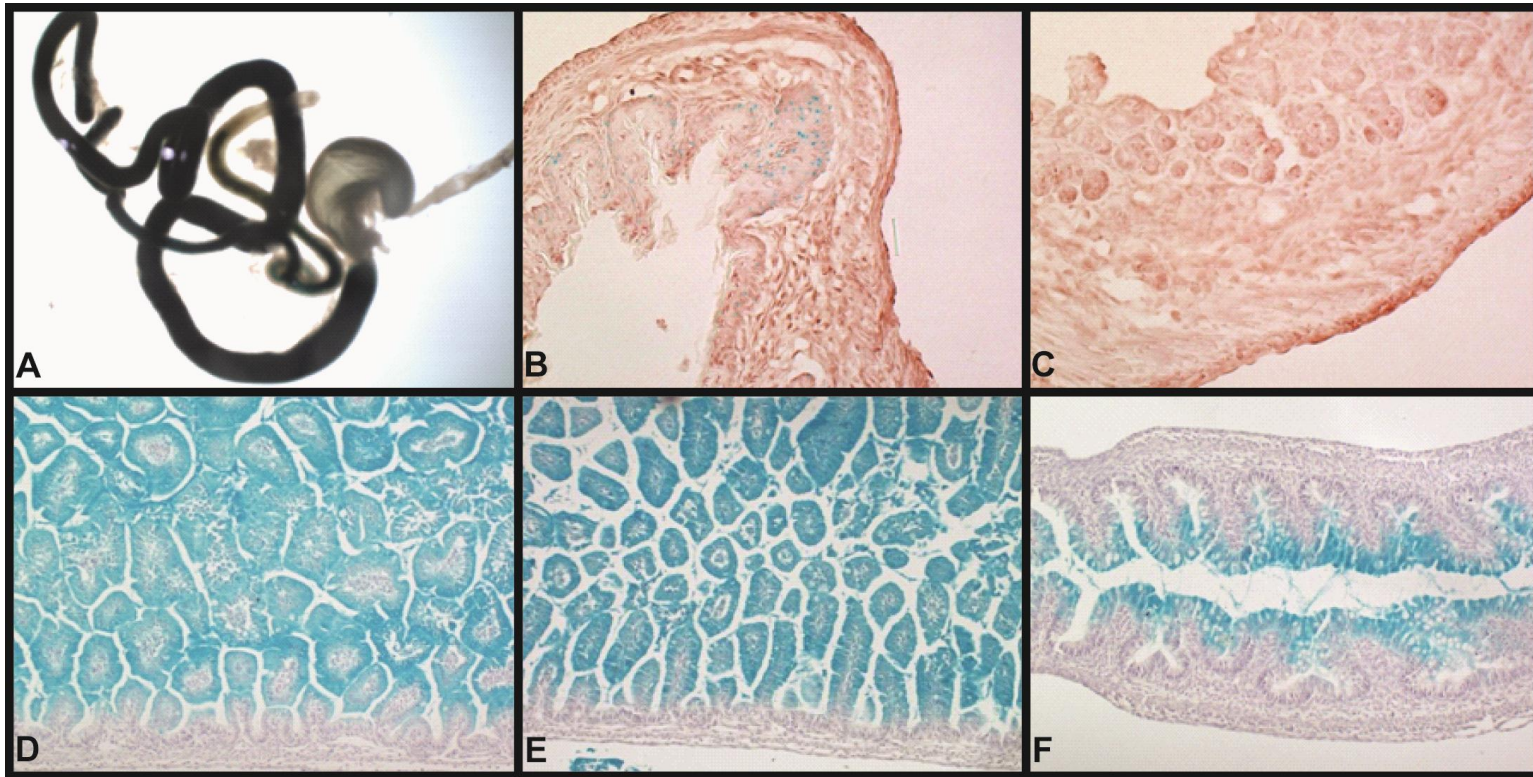


Figure 3 - 6: *Pthlh* expression in the gastrointestinal tract at E18.5. Whole mount X-gal staining for E18.5 (A) gastrointestinal tract, (B) forestomach, (C) glandular stomach, (D) proximal intestine, (E) distal intestine, and (F) colon of a *Pthlh*^{LacZ/+}embryo.

cellular compositions of the gastric gland. The only change was very minor and was seen in the reduction of the mesenchymal smooth muscle cell marker SM22 α (Figure 3-5, A). This result might suggest that Pth1r is expressed in the mesenchyme. Since maternal expression of Pthlh in amniotic fluid and milk complicates the analysis of developing stomach, the best way to study Pthlh function, is by analyzing the Pth1r mutant. The receptor mutant generally shows more severe phenotypes compared to the Pthlh mutant²². The last reason for not seeing a phenotype in the Pthlh null mouse stomach is that my data shows that there is no expression of Pthlh in the glandular mucosa (Figure 3-6, A-C). Pthlh is only expressed in epithelial cells of the forestomach in the developing stomach. As we saw previously in chapter 2, this expression changes in the adult stomach, and Pthlh was mainly expressed in the mature stomach corpus with little expression in forestomach. Pthlh was highly expressed in the developing intestinal mucosa with specific expression on the villus-epithelial cells. However, the gastric pattern of endogenous mRNA Pthlh expression increases between P0 and mature stomach (Figure 3 – 7). This pattern agrees with a previous study that showed gastrin and H⁺,K⁺-ATPase expression did not reach mature levels until several weeks after birth (Keeley and Samuelson, 2010). Overall, my data suggest that Pthlh-null mouse is an imperfect model to study Pthlh function in the developing stomach. Possible alternative models are summarized in Chapter 5.

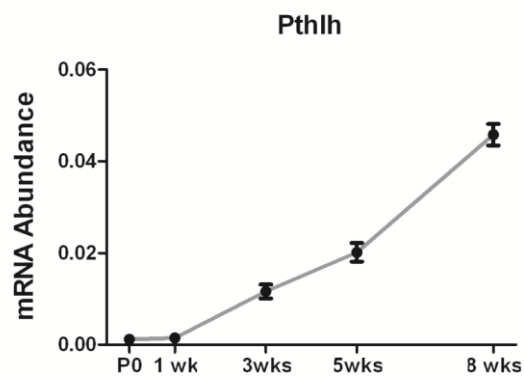


Figure 3 - 7: *Pthlh* mRNA expression increases gradually during stomach maturation. qRT-PCR measurement of *Pthlh* mRNA abundance in whole stomachs (P0 and 1 week) or corpus (3, 5 and 8 weeks) of wild type mice. Data (means \pm SEM) are shown in reference to *Gapdh* expression measured in the same samples ($N=3$).

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CHAPTER 4
HEDGEHOG SIGNALING DOES NOT MEDIATE GASTRIN INDUCTION
OF PTHLH GENE EXPRESSION

4.1 SUMMARY

Although *Pthlh* gene expression is known to be regulated by the Hedgehog (Hh) signaling pathway in different systems, such as bone morphogenesis and mammary cell differentiation, whether similar regulation takes place in the stomach is unknown. My results from chapter 2 showed that gastrin regulates *Pthlh* expression in the parietal cells. Gastrin has been previously shown to regulate *Shh* and *Ihh* ligand expression in the stomach. Thus this chapter aimed to test the role of the Hh pathway in mediating the induction of *Pthlh* gene expression by gastrin. Levels of both Hh ligand and target gene expression by qRT-PCR were not changed in both gastrin-deficient and gastrin-injected mouse stomachs. Moreover, I found that *Pthlh* gene expression is not changed in different Hh mutant mouse models, such as the developing stomachs of *Gli2* and *Gli3* mutants, as well as the parietal cell-specific *Shh*-deficient mature stomach. These data suggest that the Hh pathway does not mediate the induction of *Pthlh* expression by gastrin and is not required for *Pthlh* gene expression.

4.2 INTRODUCTION

Parietal cells express several growth factors with central roles in stomach homeostasis. Chapter 2 demonstrated how the growth factor Pthlh is specifically expressed by parietal cells in mature stomach. It also briefly touched upon the role of gastrin in stimulating Pthlh mRNA transcripts expression *via* the Cckbr. The precise mechanism, however, is undetermined.

The Hedgehog (Hh) signaling pathway is a key signaling pathway in gastrointestinal development, differentiation and homeostasis¹⁻⁴. The Hh ligands are normally expressed by gastric epithelial cells, while the Hh targets are expressed by mesenchymal cells⁵. Analysis of several Hh mutants showed abnormal gastric mucosal architecture and reduced stomach size^{2,3}. Moreover, studies which examined human gastric tumors suggested an association between aberrant Shh protein processing and gastric tumor formation⁶.

In the stomach, different studies have implied the regulation of gastric Hh expression by gastrin. In parietal cell-specific, Shh-deficient mice, the increased Ihh expression in the surface pit cells was due to high gastrin levels in these mice⁷. Moreover, one report has demonstrated that gastrin treated mice have high Shh mRNA and protein expression⁶.

In bone and mammary tissues, different studies strongly suggested the induction of Pthlh mRNA expression by Hh⁸⁻¹¹. Therefore, the goals of this chapter were: first, to find if Hh regulates Pthlh gene in the stomach. Second, to investigate whether Hh signaling mediates gastrin-induced Pthlh gene expression in parietal

cells. To achieve these goals, gastrin as well as Hh mutant mouse models were tested.

4.3 MATERIALS AND METHODS

Mice

All experiments were performed according to protocols approved by the University of Michigan Committee on Use and Care of Animals. Mice were maintained in specific-pathogen-free barrier facilities under a 12-hour light cycle. Mice were generated as described previously: gastrin mutant¹², Shh^{LacZ/+} reporter mouse¹³, and Pthlh^{LacZ/+} reporter mouse¹⁴ (gift from A. Broadus). The Gli1^{LacZ/+} reporter mouse¹⁵, Gli2^{lacZ/+} knock in mutant¹⁵ and the extra toes-J spontaneous mutation Gli3^{xtj/+} mutant¹⁶ were obtained from the Jackson Laboratories (stock numbers 002448, 007922 and 000026, respectively). Adult heterozygous Shh^{LacZ/+} and Gli1^{LacZ/+} reporter mice were used to study normal gastric expression while the adult gastrin-deficient mouse, and E18.5 Gli2^{lacZ/LacZ} or Gli3^{xtj/xtj} homozygous null embryos were used to measure Pthlh mRNA abundance in the stomach.

Immunohistochemistry Analysis

Frozen stomach sections (8µm) were co-immunostained with rabbit anti β-galactosidase antibody (1:1000, gift from J. D. Engel) followed by the mouse anti H⁺,K⁺-ATPase-α (1:500, Medical and Biological Laboratories) or simultaneously with rat anti keratin-8 (1:50, Developmental studies), followed by incubation with appropriate secondary antibodies conjugated to Cy2 or Cy3, as described in the Materials and Methods section of chapter 2.

Tissue collection and gene expression analysis

For adult mice, stomachs were dissected and opened along the greater curvature. The corpus was then isolated and stored at -80°C until the time of RNA isolation. Trizol (Invitrogen) was used to isolate RNA as recommended by the manufacturer. RNA was DNase-treated and purified using the RNeasy Mini Kit (Qiagen). For E18.5 embryonic stomachs, RNA was isolated using the RNeasy Mini Kit, according to the manufacturer's instructions. Gene expression was measured by quantitative reverse transcription-PCR (qRT-PCR) analysis, using cDNA (2 µl) that was reverse-transcribed from 1 µg of RNA template (iScript cDNA Synthesis kit, Bio-Rad). qRT-PCR was performed as described previously¹⁷ with SYBR green dye and primers listed in (Table 4-1). Expression levels were determined with triplicate assays per sample and normalized to the expression of Gapdh, which remained constant for both control and experimental groups.

Corpus RNA samples from Shh loxP¹⁸ crossed to H⁺,K⁺-Cre¹⁹ were a gift from Y Zavros (University of Cincinnati).

Statistics

Data are presented as mean ± SEM and analyzed by Student's t-test or one way ANOVA (Graph Pad Prism) followed by Tukey post-test. P < 0.05 was considered significant.

Table 4 - 1: Oligonucleotide primers(5' to 3') for quantitative reverse transcription polymerase chain reaction

Gene	Forward	Reverse	Size (bp)
Gapdh	TCA AGA AGG TGG TGA AGC AGG	TAT TAT GGG GGT CTG GGA TGG	350
Pthlh	GAC GTA CAA AGA ACA GCC ACT CA	TTT TTC TCC TGT TCT CTG CGT TT	81
H ⁺ ,K ⁺ -ATPase α	TGT ACA CAT GAG AGT CCC CTT G	GAG TCT TCT CGT TTT CCA CAC C	157
Shh	ATG TTT TCT GGT GAT CCT TGC T	ATC GTT CGG AGT TTC TTG TGA T	200
Ihh	ACC ATT GAG ACT TGA CCA GGT T	TGG AGT AAG CAG TCC AAA GAC A	172
Ptc1	CTG CTG TGG TGG TGG TAT TC	GGC TTG TGA AAC AGC AGA AA	120
Gli1	TTG GGA TGA AGA AGC AGT TG	GGA GAC AGC ATG GCT CAC TA	145
Gli2	ACC CAA CTC AGC AGC AGT AGC	GCT CCG CTT ATG AAT GGT GAT GG	114
Gli3	CCC TCC GCT CCT GTG TCT CTT C	CAG ACT CGC TTG TGG GCT TGC	214

4.4 RESULTS

We first wanted to confirm the normal expression of Hh members in the gastric mucosa. Analysis of *Shh^{LacZ/+}* and *Gli1^{LacZ/+}* mice confirmed the epithelial expression of *Shh* and the mesenchymal expression of *Gli1* in adult stomach, as described previously (Figure 4-1)⁵. To test the hypothesis that *Pthlh* induction by gastrin is mediated by Hh, we first wanted to investigate if gastrin modulates the Hh signaling pathway in the stomach by analysis of gastrin-deficient mice. Although both *Pthlh* and the parietal cell marker *H⁺,K⁺-ATPase- α* mRNA abundance were significantly reduced in the gastrin mutant, the various Hh signaling components were unchanged, including the ligands *Shh* and *Ihh*, and targets: patched 1(*Ptc1*), Glioma-associated oncogenes(*Gli1*, *Gli 2* and *Gli 3*) (Figure 4-2, A-H). These results suggest that this pathway is not regulated by gastrin.

We next tested this hypothesis by acutely injecting wild-type mice with gastrin. Hh expression was measured at 1, 2 or 4 hours after gastrin treatment to examine if the rapid and transient induction in *Pthlh* gene expression is preceded by an activation of Hh signaling. Analysis of the Hh pathway components by qRT-PCR showed that the gene expression of Hh ligands and targets did not change (Figure 4-3, A-F). This result suggested that induction in *Pthlh* mRNA by gastrin is not associated with a stimulation of Hh signaling.

To make sure that Hh is not inhibited by *Pthlh*, as has been described in bone growth and morphogenesis²⁰, we measured the expression of Hh pathway components in the E18.5 *Pthlh* mutant stomach, again observing that Hh signaling

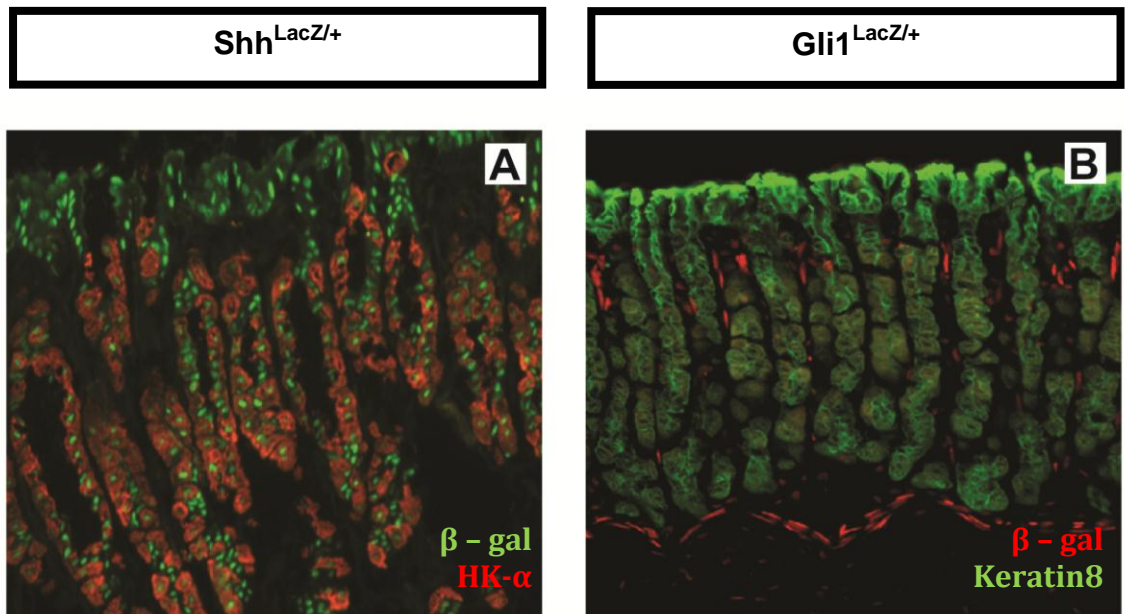


Figure 4 - 1: Hh expression in adult stomach. Co-immunostaining adult $Shh^{LacZ/+}$ (A) and $Gli1^{LacZ/+}$ (B) stomach sections using β -gal and H^+,K^+ -ATPase α or keratin-8 antibodies showed that Shh is epithelial and Gli1 is mesenchymal. Note the nuclear localization of LacZ in Shh and Gli1 strains.

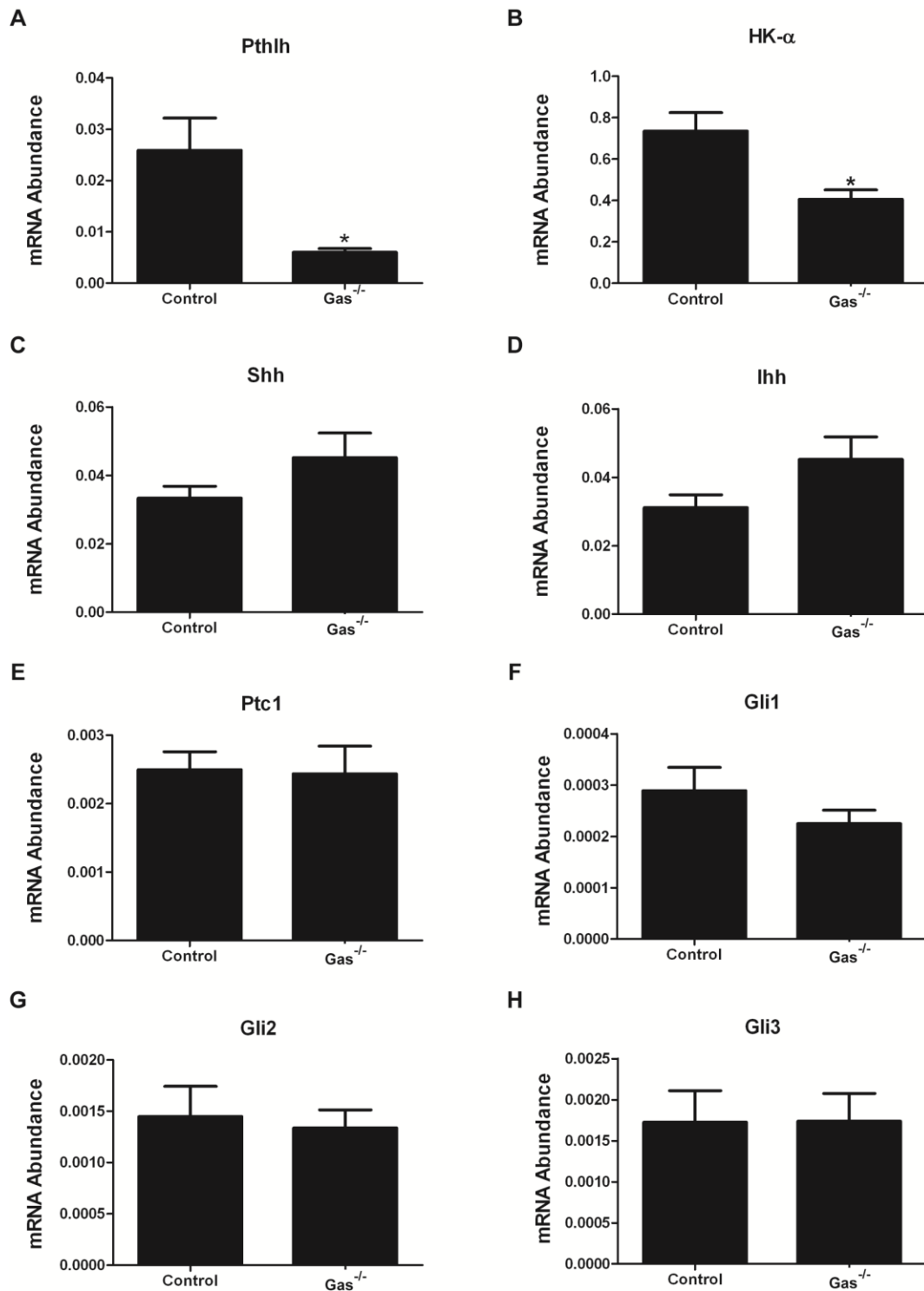


Figure 4 - 2: Hh signaling pathway is not altered in gastrin-deficient mice. qRT-PCR measurement of Pthlh, H⁺,K⁺-ATPase α , Shh, Ihh, Ptc1 and Gli1-3 gene expression of the gastrin-deficient corpus compared to littermates. Data (mean \pm SEM) are shown in reference to Gapdh expression measured in the same samples (N = 4-5; * P < 0.05).

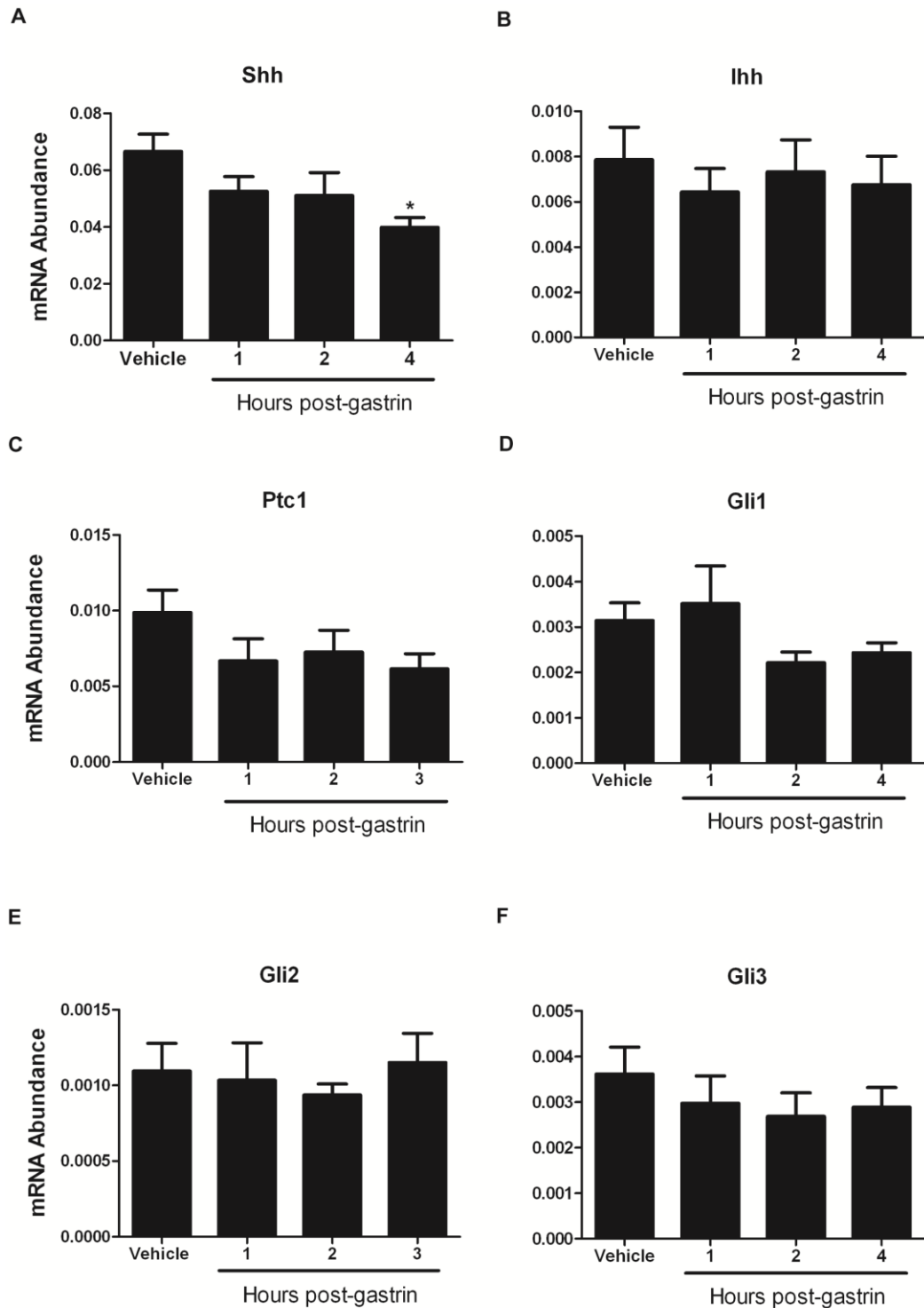


Figure 4 – 3: Hh signaling pathway is not altered in gastrin-treated mouse. qRT-PCR measurement of corpus Shh, Ihh, Ptc1 and Gli1-3 gene expression of gastrin-injected mice (250µg/Kg) at 1, 2 or 4 hours compared to vehicle-treated mice. Data (mean ± SEM) are shown in reference to Gapdh expression measured in the same samples (N = 5; * P< 0.05).

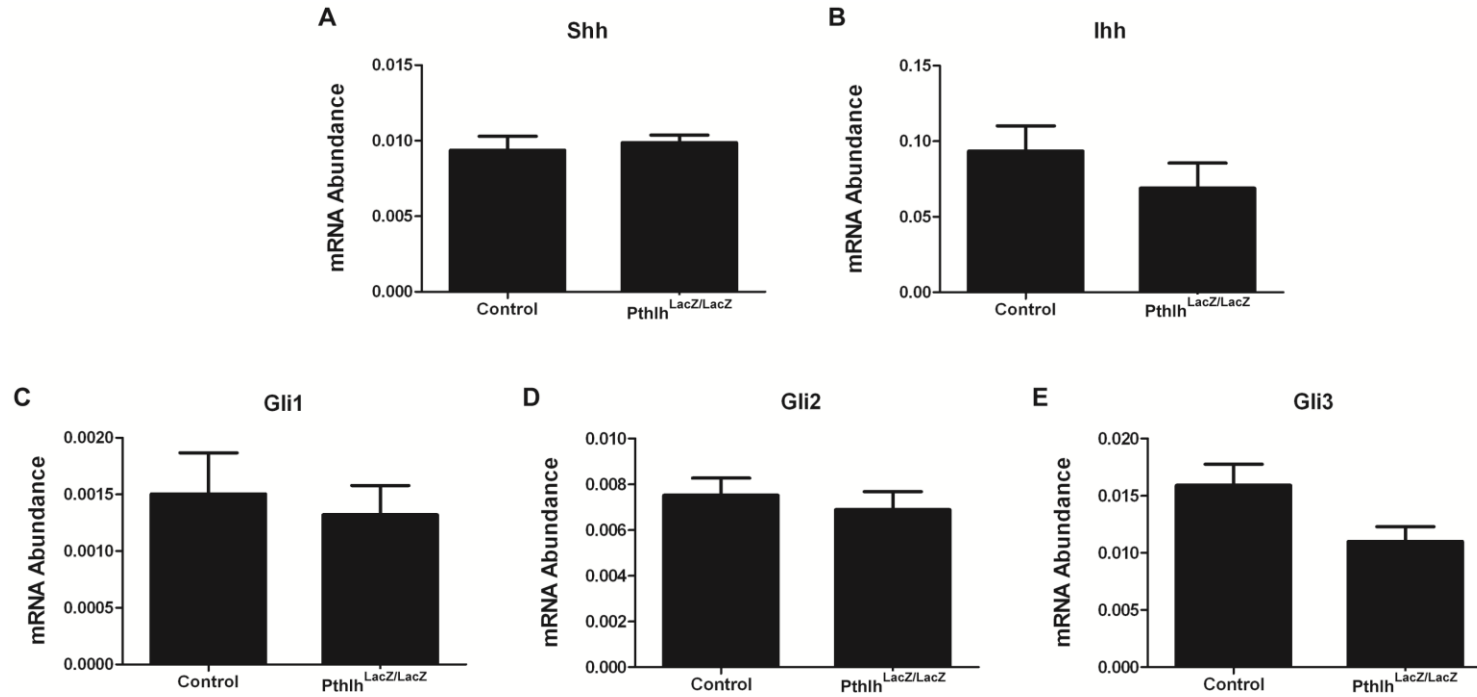


Figure 4 - 4: Hh signaling pathway is not altered in Pthlh mutant stomach. qRT-PCR measurement of Shh, Ihh, Ptc1 and Gli1-3 gene expression of the E18.5 Pthlh-deficient stomach compared to littermates. Data (mean \pm SEM) are shown in reference to Gapdh expression measured in the same samples (N = 5-6; * P < 0.05).

activity was not changed (Figure 4-4, A-F). Lastly, three different Hh mutant mouse models were tested to investigate if disruption of the Hh pathway affects Pthlh in the stomach. There was no change in Pthlh mRNA expression in the adult parietal cell-specific Shh-deficient corpus $H^{+},K^{+}\text{-Cre/Shh}^{-/-}$ (Figure 4-5). Moreover, measurements of Pthlh gene expression in E18.5 stomachs from the Gli2 and Gli3 mutants did not differ from controls (Figure 4-5, B and C).

Together, these data demonstrate that Hh signaling does not regulate Pthlh gene expression in the stomach.

4.5 DISCUSSION

This study examined five different mouse models to answer three questions: (1) does gastrin regulate the Hh signaling pathway? (2) is Hh signaling required for Pthlh gene expression in the stomach? and (3) does Pthlh regulate Hh signaling in the stomach? To answer the first question, both gastrin mutants and gastrin-treated mice were examined. This study demonstrated that the Hh signaling pathway activity was not gastrin-regulated. These results were surprising since Zavros *et al* previously found that gastrin induced Shh mRNA and protein expression in both wild-type and gastrin mutant mice⁶. However, there are some differences in the methods used in that study compared to our study. Our study acutely examined responses within hours of G-17 gastrin-treatment, while Zavros *et al* found Shh expression was induced after infusion with a mixture of G-17 and G-17gly for 14 days. The chronic treatment and mixture of gastrin forms might be required for Hh induction.

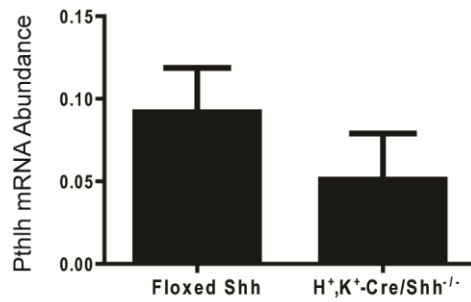


Figure 4 - 5: Pthlh mRNA expression in adult Hh mutant mouse model. qRT-PCR measurement of Pthlh gene expression of the adult H⁺,K⁺-Cre/Shh^{-/-} stomach compared to control littermates. Data (means ± SEM) are shown in reference to Gapdh expression measured in the same samples (N = 4).

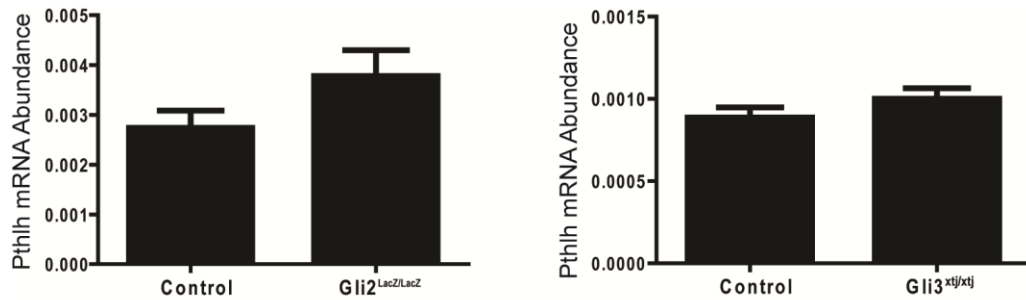


Figure 4 - 6: Pthlh mRNA expression in Hh mutant mouse model. qRT-PCR measurement of Pthlh gene expression of E18.5 Gli2 or Gli3 mutant whole stomachs compared to control littermates. Data (means ± SEM) are shown in reference to Gapdh expression measured in the same samples (N = 4).

To answer the second question, H^+,K^+ -Cre /Shh^{-/-} adult stomach corpus, as well as E18.5 stomachs of Gli2^{LacZ/LacZ} and Gli3^{xtj/xtj} mutants, were analyzed. We did not see a significant change in the Pthlh mRNA abundance in the H^+,K^+ -Cre /Shh^{-/-} mice. This could be due to the complexity of the phenotypes associated with this mouse model⁷, or simply that deleting Shh in parietal cells does not affect Pthlh expression. Although these mice have lower levels of total corpus Shh, Ihh and Gli1 were increased, suggesting activated Hh signaling⁷. Moreover, this mouse model is hypergastrinemic due to aberrant tubulovesicles in parietal cells⁷. All of these factors together make it difficult to predict how Pthlh gene regulation might be affected by the Hh pathway.

The unchanged Pthlh mRNA expression in the Gli2 and Gli3 mutants suggests that these transcription factors are not required for Pthlh gene expression regulation, probably due to the absence of Pthlh expression in embryonic glandular stomach, as I demonstrated in the previous chapter. Although other studies focused on bone morphogenesis^{10, 11, 20, 21} and mammary gland metastasis^{8, 9} showed induction in Pthlh by different Hh signaling members such as Shh, Ihh and Gli2, until now their regulation was not understood due to the absence of a consensus Gli binding site on the Pthlh gene promoter⁸. This might suggest the presence of non-canonical Gli binding sites in the Pthlh promoter or indirect regulation of this gene. Another important point is that Hh target genes have been found to be primarily expressed in mesenchymal cells⁵. Since the induction of parietal cell Pthlh expression by gastrin is very rapid, it will be harder for Hh to mediate this induction if the site of Hh activation is limited to the mesenchyme⁵.

Lastly, our results confirmed that Pthlh is not required for the stimulation of Hh signaling pathway in the stomach, as described previously in bone²⁰. Analyses in this chapter clearly suggest that Hh pathway is not mediating the induction of Pthlh by gastrin in the stomach.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation aimed to study the novel parietal cell growth factor Pthlh in the stomach in several ways, including normal gastric expression, gene regulation and gene function. Although this study examined different aspects of this growth factor in the stomach, there are still several remaining areas to be examined and questions to be answered. It is important that many of these questions are not limited to stomach, but they extend to understanding Pthlh function in other organs and systems as well. As mentioned in Chapter 1, human Pthlh has a complex gene structure, giving rise to numerous transcripts, which are translated and processed to produce a number of different mature proteins. So far, only three possible bioactive protein products have been identified to result from post translational processing, and not all of these Pthlh proteins have a recognized receptor. Currently there is nothing known about which of these peptides might be expressed in the stomach.

This chapter will focus on summarizing key conclusions from my research as well as exploring possible future experiments that can be done to answer the remaining questions about the role of Pthlh in the stomach.

I. Pthlh Gastric Expression

By analyzing the $Pthlh^{LacZ/+ 1}$ reporter mouse, I found that *Pthlh* was found to be expressed normally in both developing and mature stomach but in distinct stomach compartments. At day E 18.5, *Pthlh* expression is limited to the squamous epithelial cells in the forestomach. In the mature stomach, however, *Pthlh* is specifically expressed in parietal cells in the corpus. This change in expression pattern between different region of the stomach has been observed with other genes such as the *Shh*² and *DCLK1*³; however the reason for this differential expression during gastric development is unknown. Moreover, the *Pthlh*-positive parietal cells are more abundant in the few first glands near the squamo-glandular junction. This region has attracted the attention of many studies lately, due to the large number of the newly discovered tuft cell lineage³, high expression of key gastric homeostatic signaling pathway proteins such as *Shh*⁴, and because of the formation of tumors in this junction (personal communication, Andrzej A. Dlugosz). A microarray study of these few glands during development and/or in normal human and mouse gastric cancer models may help to uncover the specific properties of this interesting region.

Knowing that *Pthlh* produces at least three proteins: (Pth-like, mid-region and the carboxyl-end, reviewed in Figure 1 - 4), it will be important to generate and test antibodies specific for each *Pthlh* protein-product by immunohistochemical analysis or other approaches to find if they exhibit different expression patterns in the stomach. Different radio-immunoassays (RIA)⁵ have been developed against

Pthlh (1-34)⁶, (37-67)⁷ and (109-138)⁸ regions using rabbit antiserum. Protein extracts from isolated parietal cells⁹ can be analyzed for specific Pthlh-peptides to measure the expression of each Pthlh peptide in parietal cells.

II. Pthlh Gastric Function

This project had limitations in determining the normal gastric function of Pthlh due to the perinatal lethality of Pthlh-deficient mouse. In addition, the maternal Pthlh compensation for absent Pthlh in Pthlh-deficient embryos is likely to compensate for Pthlh loss in the embryo. In spite of these limitations, I observed minor differences in the expression of some mesenchymal markers specific to myofibroblast and smooth muscle cells in the Pthlh-deficient developing stomach. Immunohistochemical analysis using actin smooth muscle alpha (SM22 α) or desmin antibodies followed by morphometric analysis for both normal and Pthlh null stomachs might give a better sense about whether Pthlh affects mesenchymal cell number. However, an *in vitro* organ culture system may be required to understand the specific contribution of maternal versus fetal Pthlh for stomach development.

Another way to study the function of Pthlh in mature stomach is to target Pthlh expression in parietal cells by crossing the H⁺,K⁺-Cre¹⁰ mouse to the floxed Pthlh transgenic mouse¹¹. The resulting parietal cell-specific Pthlh-deficient mouse will avoid perinatal lethality as well as the difficulties that arise from existence of maternal Pthlh sources found in the developing Pthlh-deficient embryos. This approach would escape the health complications (lack of tooth eruption, skeletal defects and short lifetime) found in the commonly used “rescued” Pthlh-deficient

mouse, in which the whole body lacks Pthlh, but the Pthlh receptor (Pth1r) is constitutively activated in the bone growth plate¹². The stomach parietal cell specific H⁺,K⁺-Cre/Pthlh^{-/-} mouse will allow critical analysis of Pthlh function in the mature stomach. Immunohistochemical analysis with specific cell lineage makers (Table 1-1) can be used to evaluate the role of parietal cell Pthlh on mature gastric gland differentiation. Proliferation measurements by BrdU staining followed by morphometric analysis, can be used to test if Pthlh stimulates proliferation, as has been suggested in gastric tumors^{13,14}.

Since Pthlh^{LacZ/LacZ} null mice combine phenotypes resulting from the absence of all bioactive Pthlh proteins, it will be interesting to analyze other mouse models that specifically target each of the potential bioactive Pthlh proteins. The Pth1r-deficient embryos¹⁵, which deletes the Pth-like protein receptor (Pth1r), can be studied to understand the function of the Pth-like Pthlh in the stomach. However, although these mice are specific to a single Pthlh protein, Pth1r null die by mid-gestation¹⁵, and this would not be useful for study of the stomach because gastric development is not complete until after birth¹⁶. The new mouse model Pthrp^{Δ/Δ17}, however, can live longer (5-20 days). The Pthrp^{Δ/Δ} mouse is designed to permanently eliminate the expression of mid-region and C-terminus Pthlh proteins by replacing the DNA sequence responsible for the production of Pthlh-(67–137) aa with a floxed Neo cassette. The Pthrp^{Δ/Δ} mice demonstrate severe skeletal defects as well as hematopoiesis¹⁷; the stomach has not been analyzed.

Although the receptors for some of the Pthlh proteins are not yet identified, there are several commercially available Pthlh reagents that are currently available to understand the specific role of each Pthlh bioactive protein. My research suggested the expression of Pth1r in developing and mature stomach as well as in several human gastric cancer cell lines, as demonstrated in Chapter 2. To find the role of Pthlh for proliferation and differentiation, wild type mice, mouse gastric epithelial cell primary cultures, and human gastric cell lines can be treated with any of the Pthlh synthetic proteins: Pthlh (1-37) agonist¹⁸, Pthlh (4-37) antagonist¹⁹ or Pthlh (107-139) agonist^{20, 21}. These experiments can be followed up to analyze the Pthlh effect on: (i) cell proliferation, using Ki67, BrdU immunohistochemistry and proliferation assays, (ii) differentiation, using qRT-PCR, western blots and immunohistochemistry specific for each gastric cell lineage, and (iii) apoptosis, using cleaved-caspase-3 and TUNEL staining. Using these Pthlh synthetic proteins is advantageous because it will help define the function of Pthlh in the stomach, and because it will avoid the Pthlh protein specificity problem found in different Pthlh-deficient mouse models.

III. Pthlh Gene Regulation

One of the main aspects of this study was to understand how Pthlh expression is regulated in the stomach. My study showed that Pthlh is transiently and rapidly induced by the gastric hormone gastrin in both *in vitro* and *in vivo* systems, as described in Chapter 2. It also showed that this induction is mediated by the gastrin receptor Cckbr, through activation of key signaling pathways, including

MAPK ErK1/2, MAPK p38, PKC- α , PI3K and the EGFR pathways. My analysis was limited to analysis of Pthlh mRNA abundance after gastrin and/or inhibitor treatments. These pathways can be further studied by measuring key phosphorylated proteins in each of the signal transduction pathways. The PKC pathway deserves special attention due to its fast response and stimulation of Ca²⁺, and because there is evidence for Ca²⁺ mediated induction of Pthlh^{22, 23}. There are several isoforms of PKC which can be activated by either Ca²⁺ or other regulators, therefore characterizing which PKC isoforms are involved in Pthlh induction by gastrin will help to understand the mechanism. Several commercially available inhibitors for PKC- α , PKC- β I, PKC- β II, PKC- δ , and PKC- ϵ in addition to adenoviruses that overexpress different PKCs isoforms have been described²⁴ and can be tested to understand the regulation of PKC signaling pathways in Pthlh gene induction by gastrin. The use of specific PKC adenoviruses is more valuable than pharmacological inhibitor due to their high isoform specificity.

My results suggested that gastrin activates several Pthlh mRNA species, which are transcribed under the control of the P1, P2 and P3 promoters. Further research is needed to identify response elements controlling Pthlh gene expression that are associated with P1, because it was robustly expressed upon gastrin administration. Several studies suggested the control of Pthlh by hypoxia-responsive elements (HRE)²⁵, negative vitamin D response element (nVDRE)²⁶ as well as Ets/Sp1 transcription factors binding sites^{27, 28}. These response elements can be tested by making serial deletions or point mutations for each Pthlh promoter tagged to a luciferase construct, followed by measurements for the luciferase

expression in the presence of vehicle versus gastrin. Chromatin immunoprecipitation (ChIP) assay can be then applied to identify if specific transcription factors bind to the *Pthlh* gene to show that these transcription factors directly target the gene.

At the post-transcriptional level, I found that gastrin can stabilize *Pthlh* mRNAs and increase their half-life, as described in Chapter 2. The specific mechanism behind this phenomenon is currently unknown. The *Pthlh* mRNAs 3'UTR contain AU-response elements (AREs), which are usually targets for ARE binding proteins²⁹ to affect mRNA stability. Gastrin has been shown to stabilize both *IL-8* and *Cox-2*³⁰ genes via the ARE-binding proteins HuR and TTP³¹. The HuR protein was also found to bind and stabilize *Pthlh* mRNAs in human renal cell carcinomas³². It will be important to determine whether HuR is mediating the stabilization of *Pthlh* by gastrin. This can be tested in AGS-E cells by knocking down HuR with specific siRNA and measuring *Pthlh* expression after gastrin treatments. In addition, the cytoplasmic translocation of HuR from the nucleus to the cytoplasm can be tested by immunohistochemistry for HuR after AGS-E treatment with gastrin. RNA-binding assays can be used to identify and confirm the binding between HuRs and ARE elements in 3'UTRs^{32, 33}. The recent study by the Wang laboratory³⁰ demonstrated HuR protein induction and cytoplasmic localization in gastrin treated AGS-E cells. This induction was found to be mediated by p38 MAPK. Accordingly, my data proved that *Pthlh* mRNA induction by gastrin is mediated by p38 as well. Moreover, my preliminary analysis suggested that HuR protein is expressed in the mouse gastric epithelial cells, including the parietal cell (data not shown).

Does Pthlh mediate the proliferation effect of gastrin in the stomach?

This question is yet to be answered. Although gastrin has been shown to induce gastric proliferation both after meal ingestion³⁴ as well as with various hypergastrinemic conditions⁹, the mechanism is not understood. Analysis of adult mice with parietal specific deletion of Pthlh ($H^+,K^+-Cre/Pthlh^{-/-}$) could be used to address the importance of Pthlh for gastrin effects. It will also be important to find if the gastric proliferating cells express Pthlh receptor(s), but before that the identification of gastric stem cells markers will be essential. The identification and/or the manipulation of human gastric cell lines that can be responsive to both gastrin and Pthlh will be critical in understanding the mechanism of proliferation induced by gastrin via Pthlh. These studies will help especially in the clinical field, when we know that gastric cancer is one of the leading causes of death.

This project used several *in vitro* and *in vivo* systems to uncover many aspects of parietal cell growth factor (Pthlh) in the stomach. Importantly, my dissertation localized Pthlh to the parietal cells, and demonstrated the induction of Pthlh gene expression by gastrin in normal gastric tissues and in gastric cancer cell lines. There are however many remaining questions on the function of Pthlh and the precise regulation by gastrin. Further study is needed to understand the role of Pthlh in both healthy and diseased stomach.

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