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TITRE

Connections between gastrins, iron and hypoxia in the development of colorectal cancer.

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ABSTRACT

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the second leading cause of cancer-related deaths in the world [1]. Growth factors contribute to the development of cancer and gastrins have been involved in the progression of gastrointestinal cancers. In particular, the non-amidated gastrins, progastrin and glycine-extended gastrin (Ggly), activate signaling pathways and stimulate proliferation, migration and cell survival, and consequently play a role in cancer development.

Gastrins bind to the trivalent metal ion iron, association of which is required for the biological activities of Ggly, and to other trivalent metal ions, including indium, ruthenium, gallium and bismuth. Circulating levels of gastrins are increased in mice or patients with iron-overload diseases like hemochromatosis. We have also previously demonstrated that hypoxia, or low levels of oxygen, increased gastrin expression in cell lines and iron absorption in mice. Based on these observations, we suggest that hypoxia, iron and gastrins may be tightly linked.

The overall aim of our studies was to investigate the interplay between gastrins and iron homeostasis in gastrointestinal cancers. Firstly, we aimed to characterize the role of gastrins in iron homeostasis under hypoxia. Another purpose was to identify if targeting the gastrin-iron complex could result in novel therapies for CRC.

In this work, we report that overexpression of progastrin affects the connection between iron homeostasis and oxygen availability and allows mice to cope better under hypoxic conditions. This observation could be explained by the ability of circulating gastrins to bind ferric ions and to transfer them to apotransferrin, which could lead to a more efficient mobilization of hepatic iron stores when progastrin is overexpressed. Similarly to hypoxia, Zn^{2+} ions block HIF1α hydroxylation resulting in the upregulation of cell survival proteins, so we have assessed the effects of Zn^{2+} ions *in vivo* by engineering a luciferase reporter construct into the human gastrin gene promoter in SW480 cells using the TALEN technology and by xenografting these cells in SCID mice. Gastrin promoter activity was stimulated by both Co^{2+} and Zn^{2+} ions *in vitro* and *in vivo*, and this activation was dependent on the MAPK but not the PI3K pathway.

We report as well that although gastrins bind to trivalent metal ions, metal ion-gastrin com-

plexes containing radioisotopes of indium, ruthenium and gallium failed to bind to the CCK2 receptor in AGS cells. These complexes should therefore act as inhibitors of gastrins preventing their biological activity. Blocking the biological activity of Ggly by displacing its essential binding to Fe^{3+} by treatment with Bi^{3+}, In^{3+} or Ru^{3+} ions was tested in $APC^{\Delta 14/+}$ mice, which spontaneously develop tumors in their intestines. Bi^{3+} treatment led to a significant decrease of intestinal tumors larger than 3mm in male mice but no effect was observed in the colon. As bismuth citrate is poorly absorbed and maintained in the circulation, increasing its dosage or changing the route of administration could intensify the benefits on tumor burden in $APC^{\Delta 14/+}$ mice.

These findings confirm a correlation between gastrins, hypoxia and iron homeostasis in CRCs. The effects of zinc on gastrin expression should be investigated further because of the effects of gastrins in CRCs and the implication of zinc in gene expression and in many diseases (diabetes, Alzheimer's and cancers). On the other hand, blocking the activity of gastrins either by displacing the bound ferric ions using another trivalent metal ion (bismuth, indium, ruthenium) or by preventing the binding of gastrins to their receptors is still relevant as potential therapies in the CRC context.

Contents

Acknowledgments ii

Abstract iii

List of Figures viii

List of Abbreviations xi

Introduction 1

1 Colorectal cancer 2

1.1 Epidemiology . . . 2

1.1.1 Incidence . . . 2

1.1.2 Risk factors . . . 2

1.1.3 Diagnosis . . . 4

1.2 Normal colon epithelium to carcinoma . . . 4

1.2.1 Anatomy of the colon . . . 4

1.2.2 Colon carcinogenesis . . . 6

1.3 Molecular characterization . . . 9

1.3.1 Chromosomal Instability (CIN) . . . 9

1.3.2 Microsatellite Instability (MSI) . . . 10

1.3.3 CpG island methylator phenotype (CIMP) . . . 10

1.4 Molecular prognostics . . . 11

1.4.1 APC . . . 11

1.4.2 KRAS-BRAF . . . 11

1.4.3 p53 . . . 12

1.4.4 CEA and CA19.9 . . . 12

1.5 Growth factors in CRC . . . 13

1.5.1 Epidermal Growth Factor (EGF) . . . 13

1.5.2 Vascular Endothelial Growth Factor (VEGF) . . . 13

1.5.3 Gastrins . . . 14

1.6 Treatments . . . 15
1.6.1 Surgery . . . 15
1.6.2 Radiotherapy . . . 15
1.6.3 Chemotherapy . . . 15
1.6.4 Targeted therapies . . . 17
1.6.5 Immunotherapies . . . 17

2 Gastrin family **18**
2.1 Cholecystokinin (CCK) and gastrin peptides . . . 18
2.1.1 Discovery and isolation . . . 18
2.1.2 Structure . . . 19
2.1.3 Synthesis . . . 20
2.1.4 Mechanisms of release . . . 21
2.2 CCK and gastrin receptors . . . 23
2.2.1 Description . . . 23
2.2.2 GPCR activation/inactivation cycle . . . 26
2.2.3 Signaling pathways . . . 28

3 Physiological roles of the CCK and gastrin peptides **32**
3.1 CCK . . . 32
3.2 Gamide . . . 32
3.2.1 Secretion of gastric acid . . . 33
3.2.2 Trophic action . . . 34
3.2.3 Glucagon gene expression . . . 34
3.2.4 Incretin effect . . . 35
3.3 Ggly . . . 35
3.3.1 Proliferation . . . 35
3.3.2 Effect on parietal cells . . . 36
3.4 Progastrin . . . 36
3.5 CTFP . . . 36

4 Gastrins and CRC **38**
4.1 Proliferation of gastrointestinal carcinoma cell lines . . . 38
4.1.1 Gastrin expression . . . 38
4.1.2 CCK2R positive cells . . . 39
4.1.3 Autocrine loops . . . 41
4.2 Adenoma-carcinoma sequence . . . 42
4.3 Hypergastrinemia . . . 43
4.4 Hypoxia . . . 44

4.4.1 Hypoxia-inducible factors (HIFs) . . . 44
4.4.2 Gastrin gene under hypoxia . . . 44
4.5 Potential gastrin-based therapies . . . 46
4.5.1 CCK2R antagonists . . . 46
4.5.2 Anti-secretory agents . . . 47
4.5.3 Anti-gastrin antibody therapy . . . 47

5 Gastrin and iron homeostasis 50
5.1 Iron homeostasis . . . 50
5.1.1 Description . . . 50
5.1.2 Regulation . . . 51
5.1.3 Transferrin saturation . . . 51
5.1.4 Role of acid secretion . . . 52
5.2 Gastrin and iron . . . 53
5.2.1 Gastrin-ferric ion complex . . . 53
5.2.2 Functional studies . . . 54
5.2.3 Impact on transferrin saturation . . . 54
5.3 Consequences in CRC . . . 55
5.3.1 Iron and CRC risk . . . 55
5.3.2 Gastrin contribution . . . 56
5.3.3 Inhibition of gastrin-iron complexes . . . 57

Results 59

6 Paper I 61
6.1 Introduction . . . 61
6.2 Summary . . . 72

7 Paper II 73
7.1 Introduction . . . 73
7.2 Summary . . . 84

8 Paper III 85
8.1 Introduction . . . 85
8.2 Summary . . . 95

9 Paper IV 96
9.1 Introduction . . . 96
9.2 Summary . . . 105

Conclusion 106

French summary **147**

List of Figures

1.1 Region-specific incidence age-standardized rates by sex for cancers of the colon and the rectum in 2018. . . . 3
1.2 Normal colorectal anatomy. . . . 5
1.3 Representative colonic epithelium. . . . 6
1.4 TNM staging system 7
1.5 Colorectal cancer development. . . . 8
1.6 The adenoma-carcinoma sequence (Vogelstein model). . . . 9
1.7 Examples of biomarkers for CRC diagnosis. . . . 11
1.8 Colon cancer surgery by anastomosis or colostomy. . . . 16

2.1 The homologous bioactive sequences of peptide in the gastrin family. . . . 20
2.2 Processing of the human progastrin in the antral G-cell. . . . 21
2.3 Gastrin release by G-cells. . . . 22
2.4 GPCRs structure. . . . 23
2.5 Human GPCRs classification. . . . 24
2.6 The GPCR cycle. . . . 27
2.7 Examples of G-protein- and β-arrestin-mediated downstream signaling pathways on GPCRs. . . . 28
2.8 Schematic description of the signaling pathways known to be activated by CCK1R and CCK2R. . . . 30

3.1 Schematic representation of the release of gastrin and its sites of action. . . . 33
3.2 Schematic diagram of CTFP involved in cell survival. . . . 37

4.1 Gamide, Ggly and progastrin ratios in normal colon and carcinomas. . . . 39
4.2 Posttranslational modifications of gene expression. . . . 39
4.3 CCK2R mRNA expression in colon cancer (grade I, II and III) and normal tissue. 40
4.4 Autocrine growth loops by progastrin. . . . 42
4.5 The anti-gastrin immunogen, Gastrimmune. . . . 48

5.1 Human iron homeostasis. . . . 51

5.2 Hypothetical model of the gastrin-ferric ion complex. 53
5.3 A possible dual role for gastrin in iron homeostasis and CRC. 57

List of Abbreviations

A2aR ..... Adenosine 2a Receptor
AC ..... Adenylyl Cyclase
AJCC ..... American Joint Committee on Cancer
AP-2 ..... Adaptor Protein 2
APC ..... Adenomatous Polyposis Coli
CA19.9 ..... Carbohydrate Antigen 19.9
cAMP ..... Cyclic Adenosine 3',5'-cyclic monophosphate
CBC ..... Crypt Base Columnar
CCK ..... Cholecystokinin
CEA ..... Carcinoembryonic antigen
CIMP ..... CpG Island Methylator Phenotype
CIN ..... Chromosomal Instability
CRC ..... Colorectal cancer
CSC ..... Cancer Stem Cell
CT ..... Computed Tomography
CTFP ..... C-Terminal Flanking Peptide
DAG ..... Diacylglycerol
DCYTB ..... Duodenal Cytochrome B
DFO ..... Desferrioxamine
DMT1 ..... Divalent Metal ion Transporter 1
dMMR ..... Defective Mismatch Repair
ECL ..... Enterochromaffin-like cells
EGFR ..... Epidermal Growth Factor Receptor
ERK ..... Extracellular-signal-Regulated Kinase
ER ..... Endothelium Reticulum
FAK ..... Focal Adhesion Kinase
FAP ..... Familial Adenomatous Polyposis
FDA ..... Food and Drug Administration
GABA ..... γ-Aminobutyric Acid
GBP ..... Gastrin-Binding Protein

GEF ..... Guanine-Nucleotide-Exchange Factor
Ggly ..... Glycine-extended form of gastrin
GHRH ..... Growth Hormone Releasing Hormone
GIPR ..... Gastric Inhibitory Polypeptide Receptor
GLP-1/2 ..... Glucagon-Like Peptide-1/2
GRK ..... G-protein-coupled Receptor Kinase
HB-EGF ..... Heparin-Binding EGF-like Growth Factor
HCA ..... Heterocyclic Amines
HDC ..... Histidine Decarboxylase
HIF ..... Hypoxia-Inducible Factors
HNPCC ..... Hereditary Non-Polyposis Colorectal Cancer
HRE ..... Hypoxia-Responsive Element
IEC ..... Intestinal Epithelial Cells
IP3 ..... Inositol 1,4,5-triphosphate
IRI ..... Ischemia/Reperfusion Injury
IUCC ..... International Union for Cancer Control
JNK ..... Jun NH2-terminal Kinase
LOH ..... Loss Of Heterozygosity
MAPK ..... Mitogen-Activated Protein Kinase
mGluR ..... Metabotropic Glutamate Receptor
MTC ..... Medullary Thyroid Carcinoma
NM ..... Nanomaterial
NMR ..... Nuclear Magnetic Resonance
NOC ..... N-nitroso Compounds
PAH ..... Polycyclic Aromatic Hydrocarbons
PAM ..... Peptidyl-α-Amidating Mono-Oxygenase
PC ..... Prohormone Convertase
PCR ..... Polymerase Chain Reaction
PDE ..... Phosphodiesterase
PET ..... Positron Emission Tomography
PiP2 ..... Phosphatidylinositol 4,5-biphosphate
PKC ..... Protein Kinase C
PKD ..... Protein Kinase D
PLC ..... Phospholipase C
PMN ..... Polymorphonuclear
PZ ..... Pancreozymin
RAIG ..... Retinoic Acid-Inducible orphan GPCRs
STAT ..... Signal Transducers and Activators of Transcription

TALEN ..... Transcription Activator-Like Effector Nucleases
Tf ..... Transferrin
TFF1 ..... Trefoil factor 1
TfR1 ..... Transferrin Receptor 1
TGFα ..... Transforming Growth Factor alpha
TNM ..... Tumor-lymph Node-Metastasis
USG ..... Ultrasonography
VEGF ..... Vascular Endothelial Growth Factor
WHO ..... World Health Organization

INTRODUCTION

Chapter 1

Colorectal cancer

1.1 Epidemiology

1.1.1 Incidence

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males after prostate and lung cancer, and the second in females after breast cancer. There were 1.8 million new cases and almost 1 million deaths in 2018 according to the GLOBOCAN database and the World Health Organization (WHO). The highest incidence rates of CRC are in Australia, New Zealand, Europe and North America, while Africa and South-Central Asia have the lowest rates (Figure 1.1) [2]. Although the prevalence of this disease has slowly increased since the 1960s, the mortality rate has decreased over the last few decades thanks to new treatments. People over 50 years old are the most at risk and this probability increases with the years. Around half of the patients die from CRC within five years. Approximately 65% of all CRCs are located in the colon and 35% in the rectum.

1.1.2 Risk factors

Risk factors in CRC are not well established yet but include age, family history and lifestyle. The majority of CRCs are sporadic rather than familial [3]; within the latest cases, only about 20% of the patients show a hereditary disposition [4]. The best known genetic syndromes are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC), also called Lynch syndrome [5]. FAP represents less than 0.1% of all CRC cases and is characterized by a mutation in the APC gene leading to a large number of adenomas. HNPCC accounts for about 1-3% of the total cases of CRC and is due to germline mutations in one of four different DNA mismatch repair genes. 90% of all identified mutations occur in the two genes MLH1 and MSH2, which should be the first tested in CRC patients [6].

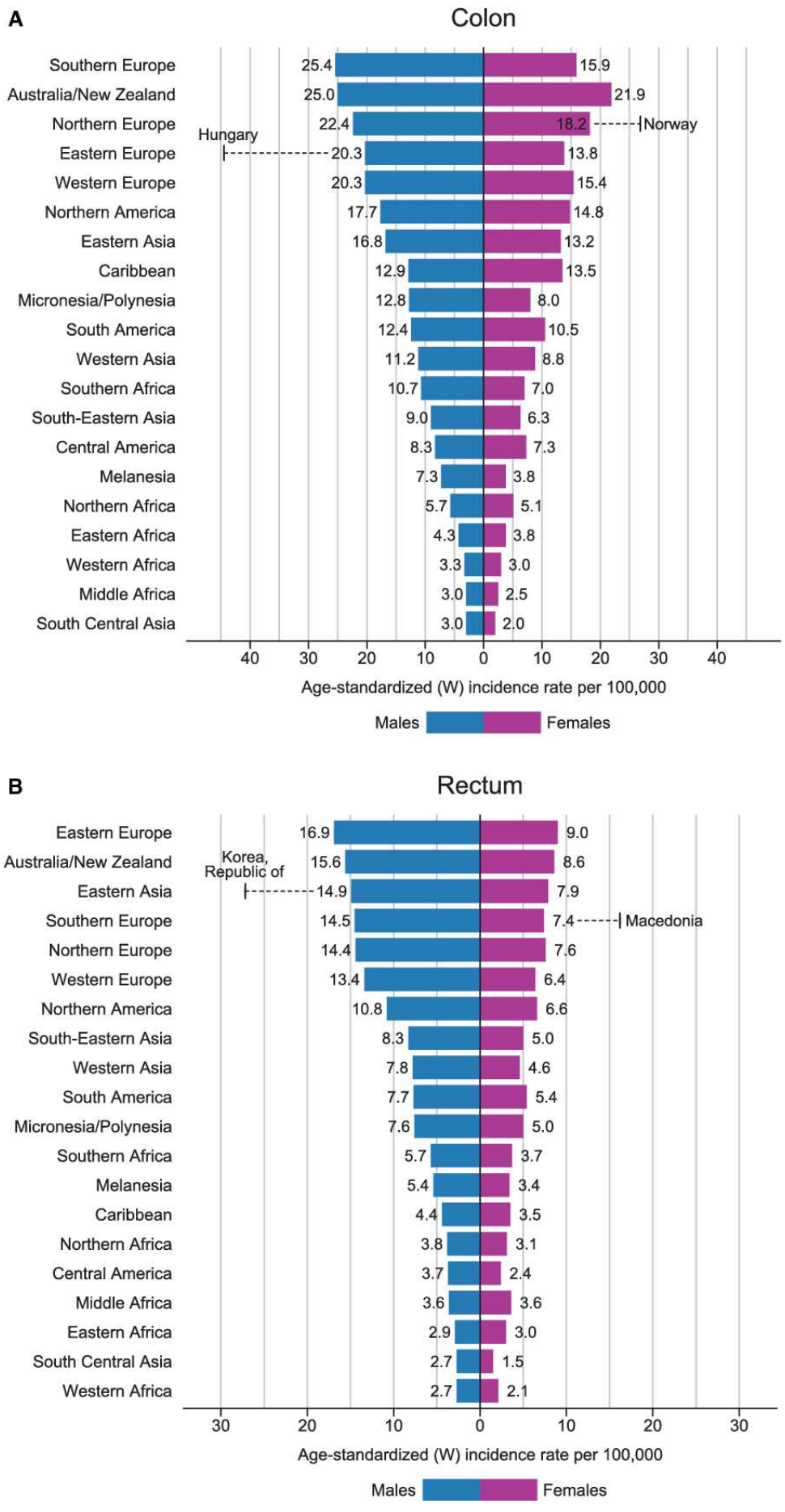

Figure 1.1: Bar chart of region-specific incidence age-standardized rates by sex for cancers of the colon (A) and the rectum (B) in 2018 (GLOBOCAN database).

Genetic changes are caused by age, lifestyle and environmental factors in sporadic cases. Dietary factors that may increase the risk of CRC are a low intake of fruit and vegetables, and/or a high intake of red meat, saturated fat consumption or excessive alcohol [7]. However, and despite various studies based on food habits, alcohol and tobacco consumption, physical activity and medication risk factors, their influence on the development of CRC remains unclear.

1.1.3 Diagnosis

The majority of colorectal cancer patients are diagnosed after the development of symptoms referable to the gastrointestinal tract, and manifest more advanced disease than asymptomatic patients [8]. Unfortunately, this is why bowel cancer is often very advanced if patients wait until symptoms show and why early screening have recently reduced CRC related deaths. Symptoms such as blood in the stools, bleeding anaemia, change in bowel habits, pain in the gastrointestinal tract and a palpable mass in the abdominal area must be checked by a specialist. The clinical examination should involve ascertainment of any familial history of the disease and abdominal and rectal palpation. Fecal occult blood test and measurement of non-enzymatic tumor markers are common diagnostic methods run in the laboratory but they unfortunately have some false negative results.

A more invasive method for diagnosis, but the most efficient one, is endoscopy [9] [10]. Colonoscopy allows screening of the entire colon and identification of precancerous polyps [11]. Most polyps grow over a decade or more through a series of mutations and are asymptomatic for the patient. Biopsy during colonoscopy allows the removal of these polyps prior to their progression to cancer. However, endoscopy often causes discomfort in patients and can also lead to complications such as a risk of perforation or bleeding from the large intestine [12]. Other imaging tests are then considered, including endorectal ultrasonography (USG), abdominal USG, computed tomography (CT), nuclear magnetic resonance (NMR) and positron emission tomography (PET), but these methods are applicable only for severe focal lesions [10].

1.2 Normal colon epithelium to carcinoma

1.2.1 Anatomy of the colon

The colon, or large intestine, is the distal part of the gastrointestinal tract and is about 1.5 meters long. The ileum (the last part of the small intestine) connects to the cecum (the first part of the colon). The rest of the colon is divided into four parts (Figure 1.2):

- The ascending colon goes superiorly on the right side of the abdomen from the right iliac fossa to the right lobe of the liver.

- The transverse colon is between the right and left colonic flexures. It is one of the longest parts of the large intestine.
- The descending colon is a retroperitonial organ which runs down the left side of the abdomen.
- The sigmoid colon is an S-shaped loop, which ends at the rectum.

The colon absorbs water, salt and some nutrients while it participates in potassium and chloride secretion, the compaction of feces, and in the movement of waste material toward the rectum [13]. The rectum, which is about 12 cm long, ends at the anal canal and is a storage reservoir [14].

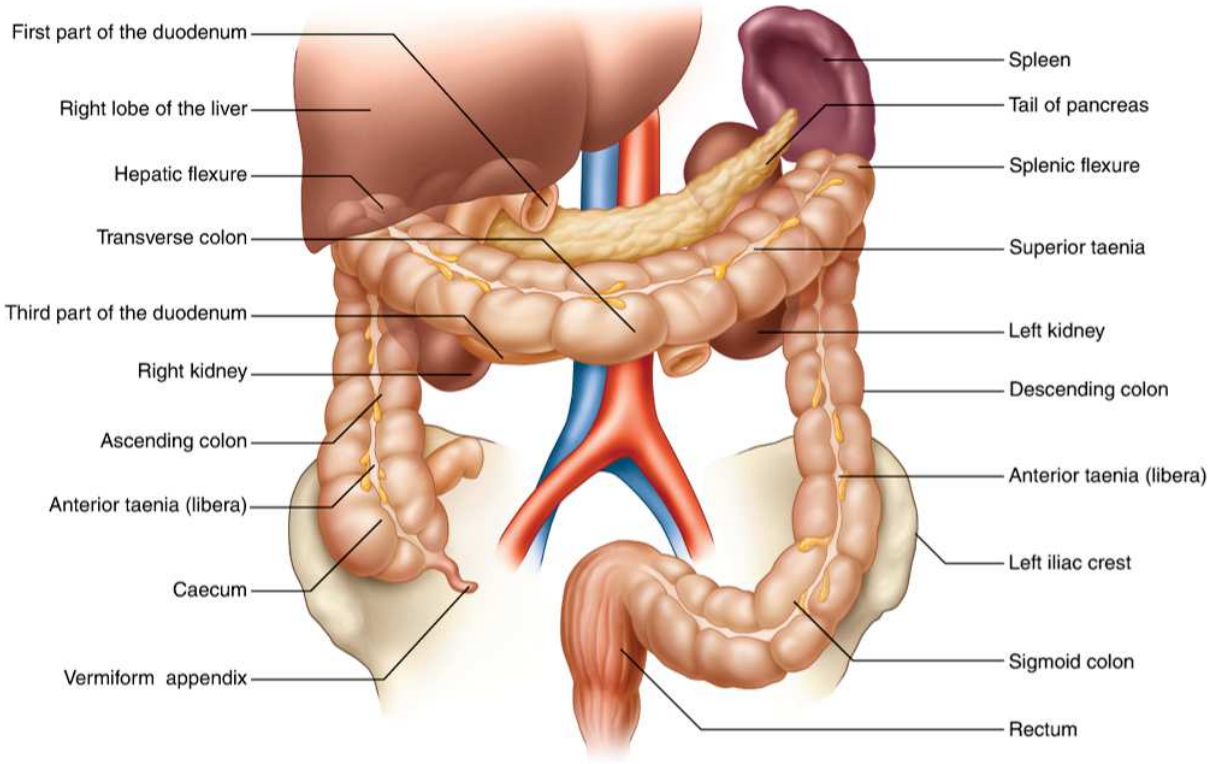

Figure 1.2: Normal colorectal anatomy [13].

The colon consists of four layers; the mucosa, the sub mucosa, the muscularis externa and the serosa [15]. Although the intestinal axis, between the small intestine and the colon, displays morphological differences, the intestinal epithelium remains similar along the tract. The small intestine is organized into millions of crypt-villus units while the colon lacks villi but has a relatively flat epithelial surface instead. The epithelium consists of invaginations called "crypts of Lieberkuhn" where the intestinal stem cells are found. (Figure 1.3). Stem cells slowly migrate through the transition zone towards the top of the crypts and they rapidly differentiate. The fully mature epithelial cells move up towards the top of the crypts where they undergo apoptosis

at the epithelial surface and are quickly replaced by advancing cells [16]. The crypt is a highly protective environment which allows division and proliferation of stem cells every day throughout life while minimizing the risk of DNA damage.

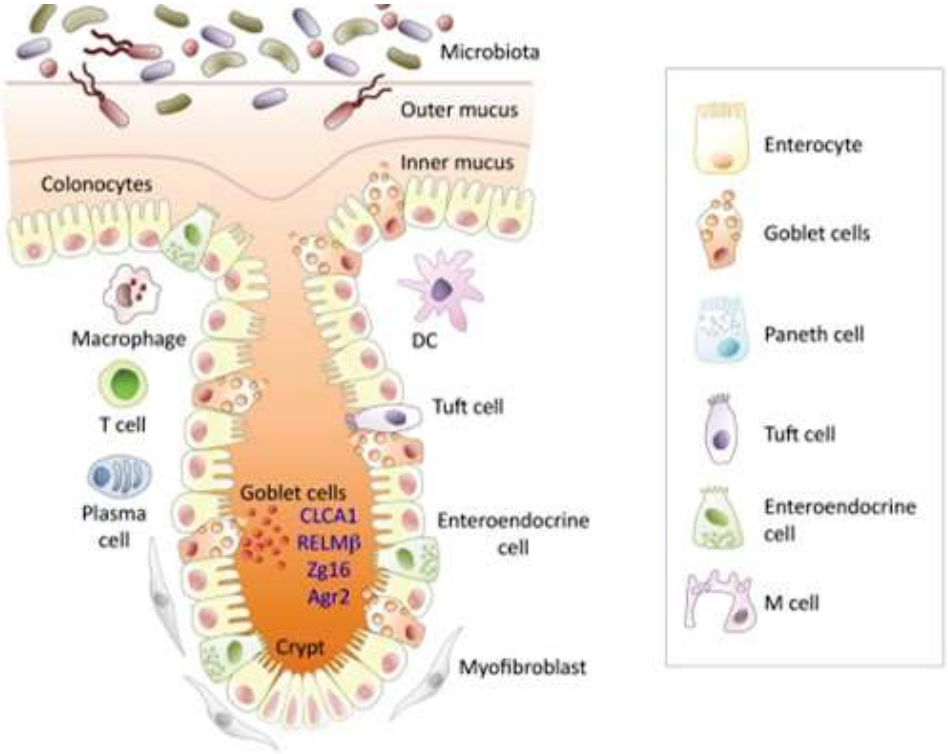

Figure 1.3: Representative colonic epithelium. Stem cells sit at the bottom of the crypts and they simultaneaously differentiate and migrate towards the top of crypt allowing a rapid intestinal cell turnover [16].

1.2.2 Colon carcinogenesis

Adenocarcinoma, which is the most common histological type of CRC, represents more than 90% of colorectal carcinomas and develops from the epithelial cells of the colorectal mucosa [17]. Neuroendocrine, squamous cell, adenosquamous and undifferentiated carcinomas are rare types of CRC and a number of histological variants including mucinous, signet ring cell, medullary and micropapillary occur as well [18]. CRCs are classified according to the tumor-lymph node-metastasis (TNM) staging system, which is widely used for therapeutic decisions and estimates of prognosis.

- The T refers to the size and extent of the primary tumor.
- The N refers to the number of nearby lymph nodes that have cancer.
- The M refers to whether the cancer has spread to other sites in the body by metastasis.

The TNM system is maintained by the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC), and is updated regularly based on new understanding of cancer prognosis to remain current and relevant to clinical practice. The newest version of the TNM classification was published in December 2016 as the 8th edition (Figure 1.4) [19].

Category		Criteria
Primary tumor (T)	TX	Primary tumor cannot be assessed
	T0	No evidence of primary tumor
	Tis	Carcinoma in situ / intramucosal adenocarcinoma (involvement of lamina propria with no extension through the muscularis mucosa)
	T1	Tumor invades the submucosa
	T2	Tumor invades the muscularis propria
	T3	Tumor invades through the muscularis propria into pericolorectal tissues
	T4	Tumor invades the visceral peritoneum or invades or adheres to adjacent organ or structure
	T4a	Tumor invades through the visceral peritoneum
	T4b	Tumor invades or adheres to adjacent organ or structure
Regional lymph nodes (N)	Nx	Regional lymph nodes cannot be assessed
	N0	No regional lymph node metastasis
	N1	Metastasis in 1–3 regional lymph nodes
	N1a	Metastasis in 1 regional lymph nodes
	N1b	Metastasis in 2–3 regional lymph nodes
	N1c	Tumor deposit(s), i.e. satellites, in the subserosa, or in nonperitonealised pericolic or perirectal soft tissue without regional lymph node metastasis
	N2	Metastasis in 4 or more regional lymph nodes
	N2a	Metastasis in 4–6 regional lymph nodes
	N2b	Metastasis in 7 or more regional lymph nodes
Distant metastasis (M)	cM0	No distant metastasis
	cM1	Distant metastasis
	cM1a	Metastasis confined to one organ [liver, lung, ovary, non-regional lymph node(s)] without peritoneal metastases
	cM1b	Metastasis in more than one organ
	cM1c	Metastasis to the peritoneum with or without other organ involvement

Figure 1.4: TNM staging system (8th edition) [19].

Both clinical parameters (obstruction, perforation) and the TNM staging system are combined to determine the progression of colorectal carcinogenesis as well as the outcome. Colorectal carcinogenesis is divided into several stages (Figure 1.5) [20]:

- Stage 0: Abnormal cells are found in the mucosa of the bowel wall. These abnormal cells may or may not become cancerous.
- Stage I: Cancer has formed and tumor invades the submucosa.
- Stage II: Cancer has reached the muscle layer (IIa) and the serosa (IIb) of the bowel wall and may grow into nearby tissues or organs (IIc).

- Stage III: Cancer has spread to local lymph nodes.
- Stage IV: Cancer metastasises and spreads to distant parts of the body such as the liver and lungs [21].

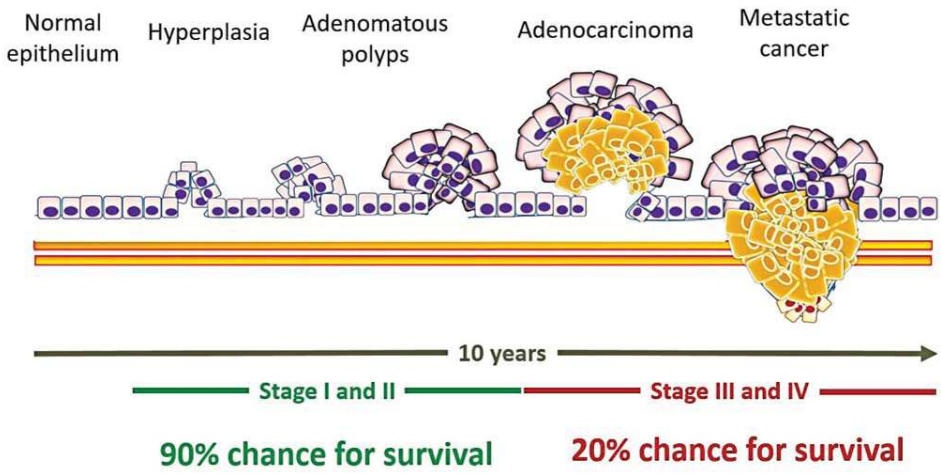

Figure 1.5: Colorectal cancer development. Progression from normal epithelium to late adenoma or metastatic cancer takes about 10 years from the formation of the first small polyps in the intestine. This period of time is divided to IV stages of cancer [20].

1.3 Molecular characterization

Colorectal tumors appear to arise as a result of the mutational activation of oncogenes coupled to mutational inactivation of tumor suppressor genes. Malignant tumors are the result of mutations in four to five genes [22] and the adenoma-carcinoma sequence shows the factors influencing the tumorigenesis (Figure 1.6) [23]. Three major pathways in CRC have been described: the chromosomal instability pathway (CIN), the microsatellite instability pathway (MSI) and the CpG island methylation phenotype pathway (CIMP).

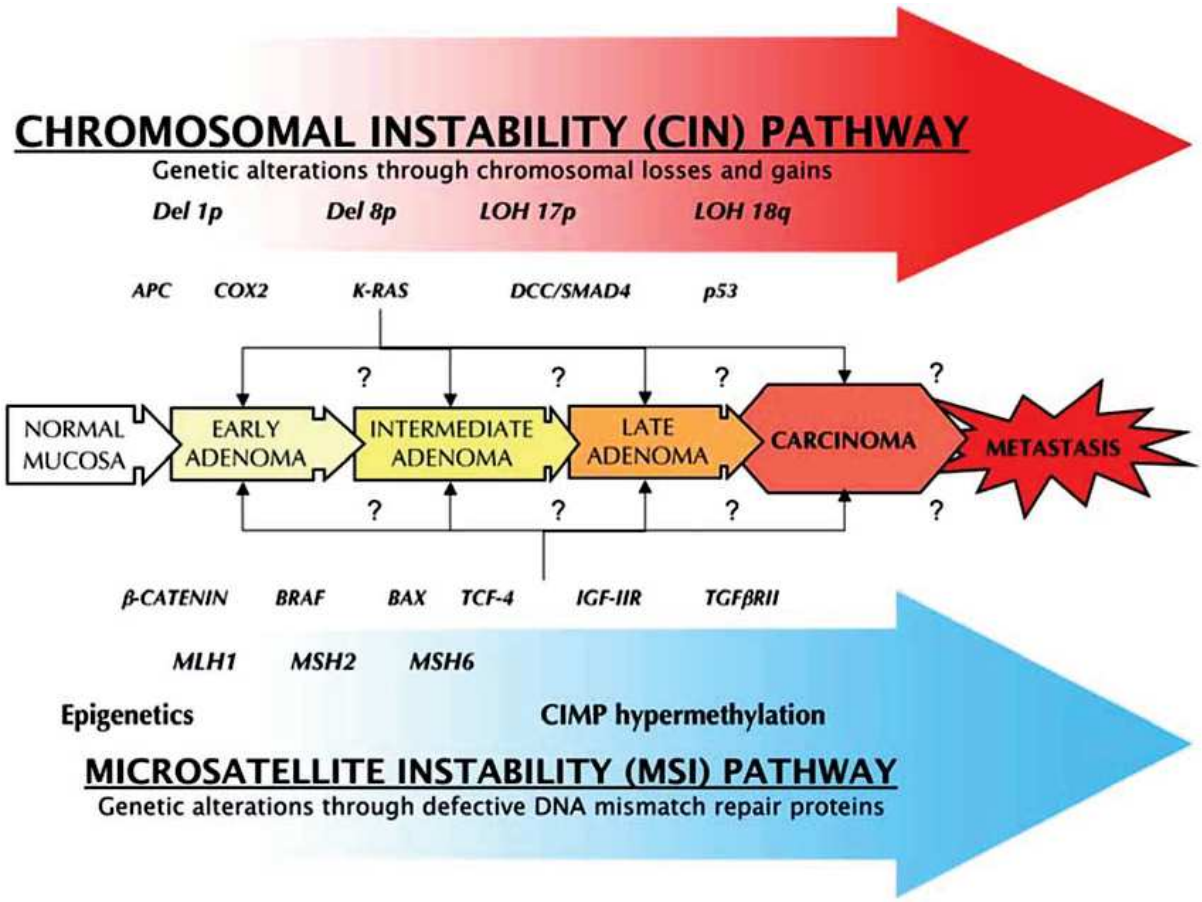

Figure 1.6: The adenoma-carcinoma sequence (Vogelstein model) [23].

1.3.1 Chromosomal Instability (CIN)

Chromosomal Instability (CIN), which results in genetic deletions, duplications and rearrangements, is the most common pathway of genomic instability. It occurs in approximately 60-70% of all CRC cases [24], and is lowest in stage I and highest in stage IV disease [25]. The mechanisms underlying CIN remain poorly understood but seem to reflect dysfunctional chromosome duplication or segregation during mitosis. Numerical CIN is characterized by loss or gain of whole chromosomes (aneuploidy), while structural CIN is defined by loss or gain of fractions of

chromosome [26].

Loss of mitotic fidelity in CIN results in genetic defects in genes such as APC, COX2, KRAS, DCC-SMAD4, p53, EGFR, as well as in loss of heterozygosity (LOH) [27]. Interestingly, a better understanding of the mechanisms and the cellular consequences of CIN could be exploited in cancer therapy [28] [29].

1.3.2 Microsatellite Instability (MSI)

Microsatellite Instability (MSI) occurs in approximately 15-20% of colorectal cancers and more than 95% of all Lynch syndrome cases. Microsatellites are small (1-6 base pairs) repetitive DNA sequences distributed throughout the entire genome (coding and non-coding regions) and representing about 3% of the human genome. Due to their repeated structure, they are inclined to a high mutation rate [30].

MSI indicates a defective mismatch repair (dMMR) system resulting in alterations in the repeat lengths because of a failure to correct nucleotide mismatches. Thus, MSI tumors share the same near diploid chromosome content as normal cells but with an increased nucleotide mutation rate.

MSI in sporadic CRC is mainly caused by hypermethylation of the promoter region of MLH1, resulting in transcriptional silencing, while in Lynch syndrome, MSI is caused by a germline mutation in the MSH2 gene [31] [32]. MSI is a significant genetic marker, which is used diagnostically for tumor detection and classification, and which could also be useful for drug development strategies [33].

1.3.3 CpG island methylator phenotype (CIMP)

CpG island methylator phenotype (CIMP) refers to the presence of concomitant hypermethylation of multiple genes resulting in gene silencing [34] [35], hence providing an alternative mechanism for loss of function of tumor suppressor genes [36]. It appears that decreased gene expression is only common to a subgroup of methylated genes in CRC. Cancer progression is associated with two types of methylation: type A methylation is age-related and appears in normal colorectal epithelial cells setting a predisposition state that precedes tumor formation in the colon [37], while type C methylation is exclusively found in CRC and not seen in normal colon [38].

Interestingly, overlap between MSI and CIMP occurs in approximately 12% of CRCs when the promoter of the MLH1 gene is methylated leading to MSI [39]. Methylated genes are used as epigenetic biomarkers nowadays therefore CIMP is a potential specific biomarker for CRC.

1.4 Molecular prognostics

CRC is a heterogeneous and complex disease due to the accumulation of different genetic and epigenetic alterations. An early diagnosis could lead to an increase in successful treatments and targeted therapies are absolutely necessary. Recently, studies have been focused on the identification of mutations in key genes involved in CRC progression such as APC, KRAS, BRAF or p53 and on identifying new predictive molecular markers such as CEA and CA-19.9 (Figure 1.7) [40].

Biomarkers	
BRAF mutations	Specific phenotype and metastasis; resistance to anti-EGFR mAb
KRAS mutations	Heterogeneity of CRC, resistance to anti-EGFR mAb
MSI	Resistance to 5-FU
APC mutations	Poorer overall survival
Micro-RNA	Early detection of CRC, prognostic stratification and therapy-response prediction
PI3KCA mutations	Poor prognosis and particular clinico-pathological characteristics, resistance to anti-EGFR mAb
Loss of PTEN	High tendency to develop metastasis, resistance to anti-EGFR mAb
p53 expression	Poor diagnosis
Loss of NDST4	Adverse prognosis, molecular predictor of metastasis
Loss of 18qLOH	Poor diagnosis
IGFR-IR	High levels in metastatic CRC, poor overall survival

Figure 1.7: Examples of biomarkers for CRC diagnosis [40].

1.4.1 APC

The Adenomatous Polyposis Coli (APC) gene is the most common mutated gene in FAP and this mutation is present in about 75% of all sporadic CRC. APC is a tumor-suppressor gene and its mutation triggers the activation of the Wnt signaling pathway in the earlier stages of CRC development. APC mutations activate the Wnt/β-catenin signaling pathway causing the stabilization and accumulation of beta-catenin protein in the cytoplasm, prior to its translocation to the cell nucleus. Wnt downstream effectors are overactivated and promote proliferation, migration, invasion and metastasis of cancerous cells [41] [42]. β-catenin mutations can trigger polyp formation even when APC is not mutated because it is downstream of APC.

A hypermethylated APC promoter was more frequent in adenoma than in normal epithelial cells and APC hypermethylation could be an important biomarker for early CRC diagnosis and potentially lead to personalized therapy [40].

1.4.2 KRAS-BRAF

The KRAS gene is a member of the RAS gene family, which is important in tumor cell proliferation, invasion and inhibition of apoptosis [43].

KRAS mutations, which are mainly found in codons 12 and 13 of exon 2, alter the GTPase

activity of KRAS leading to KRAS proteins being maintained in a GTP-bound active state. Active KRAS causes an increase in tumor cell proliferation and protects against apoptosis and promotes metastasis [44]. Approximately 50% of CRC have RAS gene mutations that occur in the early stages of CRC tumorigenesis. Some studies link KRAS mutations to a worse prognosis in CRC. BRAF is a downstream effector of the KRAS gene and BRAF mutations in CRC are found in exons 11 and 15 [45]. The common V600E substitution of valine-to-glutamic acid in the kinase domain results in abnormal activation of the MEK-ERK pathway [39] [46]. BRAF mutations are associated with the presence of defective MMR but some studies suggest that MMRs were due to an alternative mechanism [47].

Patients with either KRAS or BRAF mutations respond less well to EGFR-targeted monoclonal antibody therapies [48] [49]. Novel strategies targeting these mutations would help in CRC diagnosis and in discovering new treatments for patients with CRC to improve their poor survival.

1.4.3 p53

The p53 gene is located on the short arm of chromosome 7 and encodes a tumor suppressor protein, which is mutated in about half of all CRCs. p53 regulates the transcription of many genes involved in DNA repair, cell cycle interruption, apoptosis and metabolism following stress messages [50]. p53 dysregulation is significantly more frequent in carcinoma compared to adenoma, suggesting that the p53 protein may play a role in the transition from adenoma to carcinoma [51].

Conflicting data in the literature suggests that using p53 as a prognostic or predictive tool is still controversial. Some studies conclude that there is insufficient evidence to recommend the routine use of p53 for CRC prognosis [52], while others show that p53 mRNA could be a useful tool to predict survival in patients with stage III or rectal cancers [53].

1.4.4 CEA and CA19.9

Over the last 50 years, various markers for CRC have been used and measured in serum, tissue or stools [54]. Carcinoembryonic antigen (CEA) and carbohydrate antigen 19.9 (CA19.9) are the most common serum markers for CRC [55].

CEA is a glycoprotein overexpressed by adenocarcinomas and located mainly in colon, rectum, breast and lung [56]. CEA plays a role in adhesion between cells and in signal transduction. Although 90% of CRCs produce CEA, serum levels of CEA are not always elevated in CRC patients [57], and thus it should not be used for detection of primary CRC, especially early stage CRC. However, serum CEA levels or tumor CEA mRNA levels are good postoperative prognostic indicators for the evaluation of CRC progression, despite variations between patients

[58] [54].

CA19.9 is a carbohydrate produced by adenocarcinomas in the pancreas, stomach, gall bladder, colon, ovary and lung. It plays a role in cellular adhesion and in the process of tumor progression [59]. Measuring serum levels of CA19.9 for CRC prognosis has produced contradictory results in the literature but its detection could identify patients at high risk of cancer recurrence [60].

A combination of both CEA and CA19.9 levels is a better tool to monitor CRC patients and to potentially exclude a recurrence post operation [61].

1.5 Growth factors in CRC

Growth factors are peptides that stimulate cell growth, proliferation, migration, and healing as well as cellular differentiation. In addition, they regulate cellular functions in cancer cells playing a role in tumorigenesis and maintaining oncogenic mutations. Epidermal Growth Factor (EGF), Vascular Endothelial Growth Factor (VEGF) and gastrins are growth factors involved in CRC progression.

1.5.1 Epidermal Growth Factor (EGF)

EGF is a member of a family of cysteine-rich polypeptide growth factors. The EGF receptor (EGFR), which is a member of the ErbB family, is a receptor tyrosine kinase composed of an extracellular ligand-binding ectodomain, a transmembrane domain and an intracellular kinase domain. Following binding of a growth factor of the EGF family, receptor dimerization is essential for activation of the intracellular tyrosine kinase domain and phosphorylation of the C-terminal tail. EGFRs activate multiple signal transduction pathways, including the Ras/MAPK, PI3K/Akt, PLCγ1/PKC, STAT and PKC pathways, and therefore play essential roles in regulating cell proliferation, survival, differentiation and migration [62].

ErbB receptors are commonly overexpressed in many tumors leading to overactivation of downstream pathways, thereby promoting cancer cell proliferation, survival, invasion and metastasis. Studies have shown a higher expression levels of EGF and EGFR in colorectal cancer tissue than in surrounding mucosa [63] as well as a potential correlation between overexpression of EGFR and the stage of colon cancer [64].

1.5.2 Vascular Endothelial Growth Factor (VEGF)

VEGF is part of a family of growth factors and itself has several variants due to alternative splicing. It binds either of two VEGF receptors (VEGF receptor-1 and VEGF receptor-2), which are expressed on vascular endothelial cells and undergo ligand-induced dimerization. VEGF promotes angiogenesis in embryonic development and plays an important role in wound healing

in adults [65].

Changes in VEGF expression levels are involved in disease processes and VEGF was clearly identified as essential to tumorigenesis and disease progression in many human cancers, including breast, lung, gastrointestinal and urologic cancers. VEGF expression is upregulated by tumor cells through activation of oncogenes, such as Ras, p53, TGFβ and controlled by hypoxia [66]. Levels of circulating VEGF in patients could potentially be predictive of tumor status and prognosis, and may be used to monitor tumor response to anticancer therapies and to predict tumor relapse. Studies have demonstrated a role of VEGF in colon cancer, specifically in the stimulation of angiogenesis, and inhibition of VEGF led to a decrease of angiogenesis and a diminution of cancer growth [67].

1.5.3 Gastrins

Gastrins, first discovered as acid secretagogues, were recently shown to be growth factors for the gastrointestinal tract promoting cell growth and differentiation. A correlation between elevated serum gastrin levels and an increased risk of colorectal cancer has been reported [68], and this correlation suggests a potential role of gastrins in regulating the growth of normal colon cells as well as colon cancer cells [69].

1.6 Treatments

Treatments for CRC are chosen based on the stage of the cancer and its localization, as well as on the patient's health. Surgery is the only curative strategy and is often coupled with radiotherapy or chemotherapy. New approaches such as targeted therapies are currently emerging.

1.6.1 Surgery

Removing cancer by surgery is the main treatment for all stages of CRC and is the most important one in early stages aiming for a curative resection. Surgery in colon cancer can be performed either by open surgery or by using laparoscopy techniques with the same long-term results [70]. The surgeon can remove a cancerous polyp by polypectomy or he can perform a partial colectomy by removing the cancer and nearby tissue and then connecting the cut ends of the colon (anastomosis). He can also perform a colostomy by first removing the cancer and nearby tissue then by placing a temporary bag around the stroma to collect waste (Figure 1.8) [71].

In rectal cancer, total mesorectal excision is performed to remove the rectal tumor together with the surrounding mesorectal lymphovascular tissue [72]. Complete and precise removal of the rectum minimizes local recurrence and the 5-year survival increases by 53-87% [73] [74] [75]. After surgery most patients will receive adjuvant therapy, which can be radiotherapy or chemotherapy, to minimise cancer cell survival.

1.6.2 Radiotherapy

Radiotherapy is mainly used in the treatment of rectal cancer, and not much in colon cancer. It uses high-energy x-rays to destroy cancer cells or to stop them from growing. Radiotherapy can be performed externally with a machine outside the patient's body or internally by using a radioactive substance injected directly into or near the cancer. External radiation therapy is often used as palliative therapy to decrease symptoms and improve quality of life.

In rectal cancer, pre- and postoperative radiotherapies decrease the risk of recurrence and thus increase survival rates [76] [77]. However, treatment strategies have to be adjusted to the stage of cancer and doses have to be carefully chosen to avoid toxicity, including tissue damage or skin sunburn [78].

1.6.3 Chemotherapy

Chemotherapy is a drug therapy that kills cancer cells or slows their growth. While stage I and II CRCs are often treated by surgery, if the cancer cells in stage II have spread to nearby tissues, adjuvant chemotherapy is recommended. Stage III tumors are usually treated with surgery and

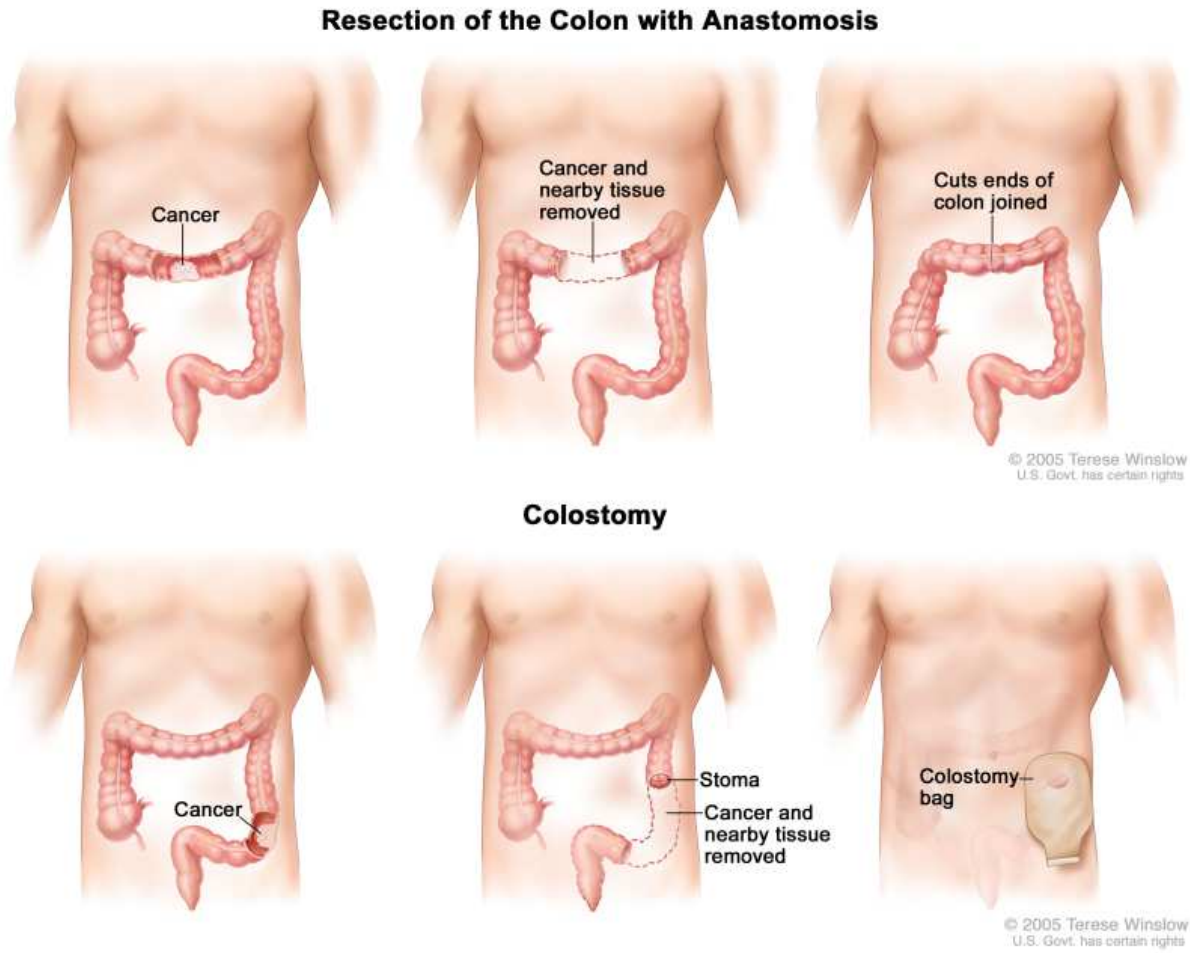

Figure 1.8: Colon cancer surgery by anastomosis or colostomy [71].

adjuvant chemotherapy to destroy metastases following surgery.

A number of chemotherapeutic options, approved by the U.S. Food and Drug Administration (FDA), are available for the treatment of CRC:

- Capecitabine (Xeloda)
- 5-Fluorouracil (5-FU)
- Irinotecan (Camptosar)
- Oxaliplatin (Eloxatin)
- Trifluridine (Lonsurf)

Combinations of several drugs are often given. For example 5-FU with folinic acid (Leucovorin) after surgery in stage III CRC reduces the recurrence rate by 30% [79], while 5-FU\Leucovorin\Oxaliplatin (FOLFOX4) increases the survival rate of CRC patients [80].

Chemotherapy can also be prescribed as a palliative treatment for patients with metastatic CRC and symptoms due to the tumor metastases [81]. Unfortunately, chemotherapy destroys healthy cells too and there are many side effects as it has cumulative effects and each round of treatment gets worse for the patients until it is either toxic or the tumors become resistant and continues growing.

1.6.4 Targeted therapies

Targeted therapies specifically target cancer genes, proteins and the tissue environment involved in cancer growth to destroy cancer cells with minimal side effects. The main studies have been done on EGFR and VEGF, which are proteins involved in the regulation of tumor cell growth, metastasis and angiogenesis. Unfortunately both healthy and cancer cells expressing EGFR or VEGF will be targeted. Bevacizumab, ziv-aflibercept and regorafenib target VEGF and cetuximab and panitumumab are EGFR inhibitors [82] [83]. They are currently used in treatment of metastatic CRCs, often in combination with conventional chemotherapeutic agents [84].

Although inhibiting tumor angiogenesis using antibodies against VEGF increases the effectiveness of chemotherapy, resistance to this therapy is a limiting factor [85]. In addition, patients with KRAS/NRAS/BRAF mutations are resistant to anti-EGFR monoclonal therapy.

1.6.5 Immunotherapies

Immunotherapy strategies rely on using the immune system to specifically fight cancer. Some tailor-made substances can be injected into patients to boost and to guide their immune system. Studies have mainly focused on immune checkpoint inhibitors, which block checkpoint proteins involved in the inactivation of T-cells, thus allowing the activated T-cells to kill cancer cells [86]. Nivolumab, an anti-PD-1 antibody, was the first checkpoint inhibitor used in combination with chemotherapy for patients with metastatic CRC in the absence of a BRAF mutation. Anti-PD-1 treatment has shown promising responses with increased survival rates in patients with dMMR\MSI-H metastatic CRC [87] [88], but unfortunately most CRC patients (95%) do not benefit from this immunotherapeutic strategy.

More studies on immunotherapies for treatment of CRC are ongoing, firstly to extend the use of immune checkpoint inhibitors to more CRC patients, and secondly to define their optimal use in terms of single or combination therapy, timing of initiation of treatment, and optimal duration of treatment.

Chapter 2

Gastrin family

The gastrin family, also called the gastrin/cholecystokinin family, includes the peptide hormones gastrin and cholecystokinin.

2.1 Cholecystokinin (CCK) and gastrin peptides

2.1.1 Discovery and isolation

William Bayliss and Ernest Starling showed that substances in the blood could stimulate pancreatic secretion, and that chemical substances were liberated from the cells of the duodenal and jejunal mucosa to secrete acid. They named the first hormone incretin in 1902. Hormones were then introduced as any substances from the blood having a messenger role between organs [89].

Gastrin was first discovered by John Edkins in 1905 by testing extracts of pyloric mucous membrane in anaesthetised cats and by observing the stomach activation that resulted in secretion of gastric juice [90]. After years of contradictory studies, Edkins's discovery was confirmed and gastrin was proven to be a distinct molecule from histamine [91]. The preparation of gastrin was simply an extraction from boiled hog pyloric mucosa with 0.1 N-hydrochloric acid in 95% methanol, followed by a neutralizing step to pH 5-5.5 to remove impurities and by a purification step via precipitation from water at pH 7 and dialysis. One unit of gastrin injected into anaesthetised cats released the secretion of 1 mL of acidic gastric juice in one hour [92]. In the 1960s, gastrin was isolated and purified, and its stimulation of gastric acid secretion was confirmed in conscious dogs and in humans. Gastrin was thus defined as an acid secretagogue. In 1964, two similar polypeptides, gastrin I and II, differing only in the presence of a sulphate group on the single tyrosine residue, were isolated from hog antral mucosa. While injected subcutaneously into conscious dogs, they strongly stimulated gastric acid secretion. Both gastrins are significantly more effective than histamine in dogs and gastrin II is more powerful in stimulating gastric acid secretion than histamine when injected into humans [93] [94].

CCK was discovered by Ivy and Oldberg in 1928 while testing intestinal extracts in dogs and was later also described as a hormone activating the gall bladder, leading to its contraction [95]. In 1943, another hormone called pancreozymin (PZ) was shown to be involved in the release of digestive enzymes from the pancreas [96]. The pancreozymin activity of both the cholecystokinin and the pancreozymin peptides is found in the same intestinal extracts and CCK-33, identified in 1968, has maximal activity [97]. CCK and PZ were thus identified as being the same molecule, officially called CCK.

2.1.2 Structure

The sequences of gastrin and cholecystokinin were both identified in the 1960s [98].

Chemical studies on both hormones, gastrins I and II, have revealed their heptadecapeptide amide structure, and that gastrin I is lacking the tyrosyl sulfate ester group compared to gastrin II [99]. The sequence of CCK-33 is structurally similar to gastrin as they share an identical COOH- terminal pentapeptide sequence: Gly-Trp-Met-Asp-Phe-CO-NH_2 (Figure 2.1) [100]. The last four amino acids represent the minimal structure necessary for their biological activity [101]. The similarity between gastrin and CCK has been explained by the presence of a common ancestor called cionin, purified from the neuronal ganglion of the protochordate *Ciona intestinalis* [102].

The complete homology of their biologically active sequences, and other structural similarities too, make gastrin and CCK members of the same hormone family. They are the only mammalian members of the gastrin family, which contains the peptides caerulein and phyllocaerulein identified from frog skin extracts, as well the neuropeptide cionin isolated from the protochordate. Interestingly, the insect peptides, the sulfakinins, have some homology in the bioactive site with the CCK family members.

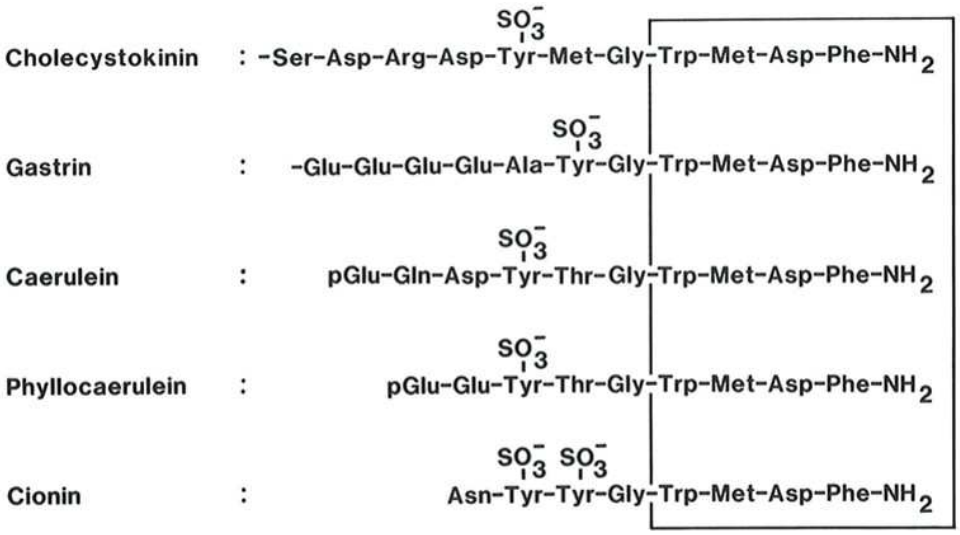

Figure 2.1: The homologous bioactive sequences of peptide in the gastrin family.

The gastrin family has a well preserved C-terminal sequence (boxed sequence) shared between mammalian peptides, CCK and gastrin, and some insects peptides, caerulein, phyllocaerulein and cionin [100].

2.1.3 Synthesis

cDNAs encoding CCK from human [103], pig [104] and rat [105], and gastrin from human [106] and pig [107] have been characterized in the 1980s.

In humans, the CCK gene is located on chromosome 3 and includes three exons separated by two introns [108]. The coding region for the CCK-33 sequence is in exon 3 [109]. PreproCCK, a precursor of 115 amino acid residues, a non-active molecule, is processed by series of cleavages, carboxyamidations and by O-sulfations of Tyr^{77}, Tyr^{91} and Tyr^{94}, resulting in several forms of CCK. CCK-58, -33, -22 and -8 are found in endocrine I-cells, as well as in human plasma, whereas neurons mostly release CCK-8 [110] [111]. CCK release is stimulated by ingestion of food, mainly due to potent secretagogues such as fat and proteins.

The gastrin gene is located on the long arm of chromosome 17 and exon 2 contains the region essential for gastrin function. The precursor, preprogastrin, is a peptide of 101 amino acid residues in human, which after cleavage of the signal peptide after residues 21 or 25 in the endoplasmic reticulum generates progastrin. Progastrin is translocated to the trans-Golgi network where it undergoes modifications (sulfation and phosphorylation) and cleavages by prohormone convertases (PC) at two specific sites. Cleavages at Arg36-Arg37 and Arg74-Arg75 release the 6-amino acid C-terminal flanking peptide (CTFP) and glycine extended gastrin-34-ArgArg (G34-Gly-ArgArg), which is followed by removal of the C-terminal arginines by carboxypeptidase E generating G34-Gly. Another PC cleavage of G34-Gly after Arg54-Arg55 followed by removal of

the C-terminal arginines by carboxypeptidase E generates G17-Gly, commonly called Ggly. Both glycine-extended peptides can be amidated by peptidyl-α-amidating mono-oxygenase (PAM) to give respectively G34-amide and G17-amide, called Gamide (Figure 2.2) [112] [113].

Because Gamide was originally assumed to be the only biologically active peptide, Gamide-34 and Gamide were referred to as the "classical gastrins" while progastrin and the COOH-terminal Gly-gastrins were the "non-classical gastrins".

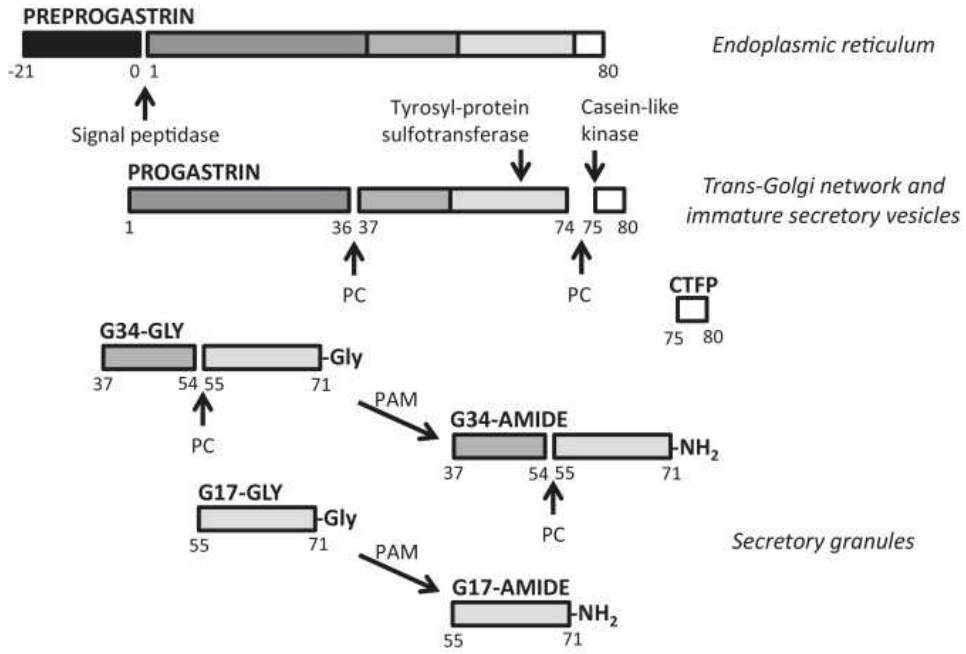

Figure 2.2: Processing of the human progastrin in the antral G-cell [112].

2.1.4 Mechanisms of release

Gastrin peptides are mainly produced by the antral G-cells, which are major endocrine cells in the stomach, in response to stimuli and then released into the blood. Smaller amounts are synthetized further down the intestinal tract, in the colon and in the fetal pancreas as well as in a few neurons, in the pituitary gland and in the spermatozoes [114]. In healthy humans, progastrin and glycine-extended gastrins represent less than 10% of circulating gastrins, and CTFP was originally found in the same quantities as Gamide in the circulation [115]. The observation that, following a meal, CTFP has a concentration 4-fold and 60-fold higher than Gamide in the antrum and in the circulation respectively, suggests differential secretion of CTFP and Gamide after cleavage from progastrin [116].

Studies have described the stimulation of gastrin release by bombesin [117] and the inhibition of bombesin-stimulated gastrin release by somatostatin [118] in isolated canine G-cells in primary

culture. Both bombesin and somatostatin receptors are expressed in antral G-cells and these peptides have a direct action on G-cells. cAMP and calcium/phosphatidylinositol-dependent mechanisms have been shown to be involved in G-cell activation. In the isolated human antral G-cell model, cAMP-, Ca^{2+}- and protein kinase C-dependent pathways participate in stimulus-secretion of human G-cells [119]. The presence of a calcium-sensing receptor on G-cells seems to be involved in the regulation of gastrin secretion via phospholipase C activation, non-selective cation channels and an influx of extracellular calcium [120]. The neurotransmitter GRP (the human analogue of the frog peptide bombesin) has a role in the regulation of acid secretion but not in secretion of gastrin after a meal by the G-cells as it was originally suggested [121]. To conclude, gastrin release is mainly repressed by acid secretion via the paracrine mediator somatostatin (Figure 2.3) [122].

Interestingly, in *H pylori* infection, the number of antral D-cells is reduced leading to a decrease in its inhibitory function against G-cells, which increases the secretion of serum gastrin [123].

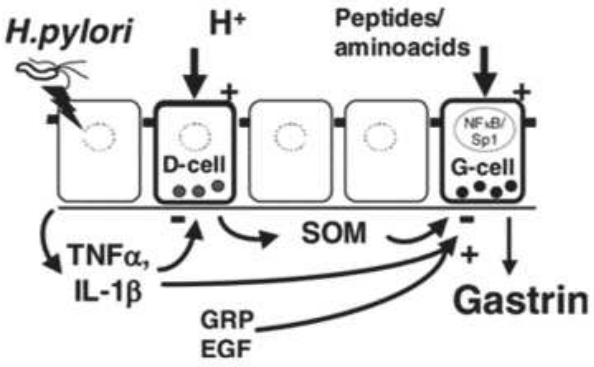

Figure 2.3: Gastrin release by G-cells [122].

2.2 CCK and gastrin receptors

There are two known receptors for peptides of the gastrin family, the CCK1R and the CCK2R, which can be differentiated by pharmacological studies. Both receptors are part of the G-protein-coupled receptor (GPCR) family. GPCRs belong to the largest and most diverse family of signaling proteins. Consequently, they are involved in an abundant number of cellular responses to hormones, metabolites, cytokines and neurotransmitters [124].

2.2.1 Description

GPCRs

At the end of the nineteenth century some studies showed the presence of receptors, but it was only in 1979 that Robert Lefkowitz purified the first receptor, the β2-adrenergic receptor, by affinity chromatography [125]. The similarities between the sequences of β2-adrenergic receptor and the reference membrane protein at the time, bovine rhodopsin, suggest the presence of a family of genes coding for membrane receptors [126]. This was the start of studies on GPCRs and there are now more than 800 known genes encoding for GPCRs.

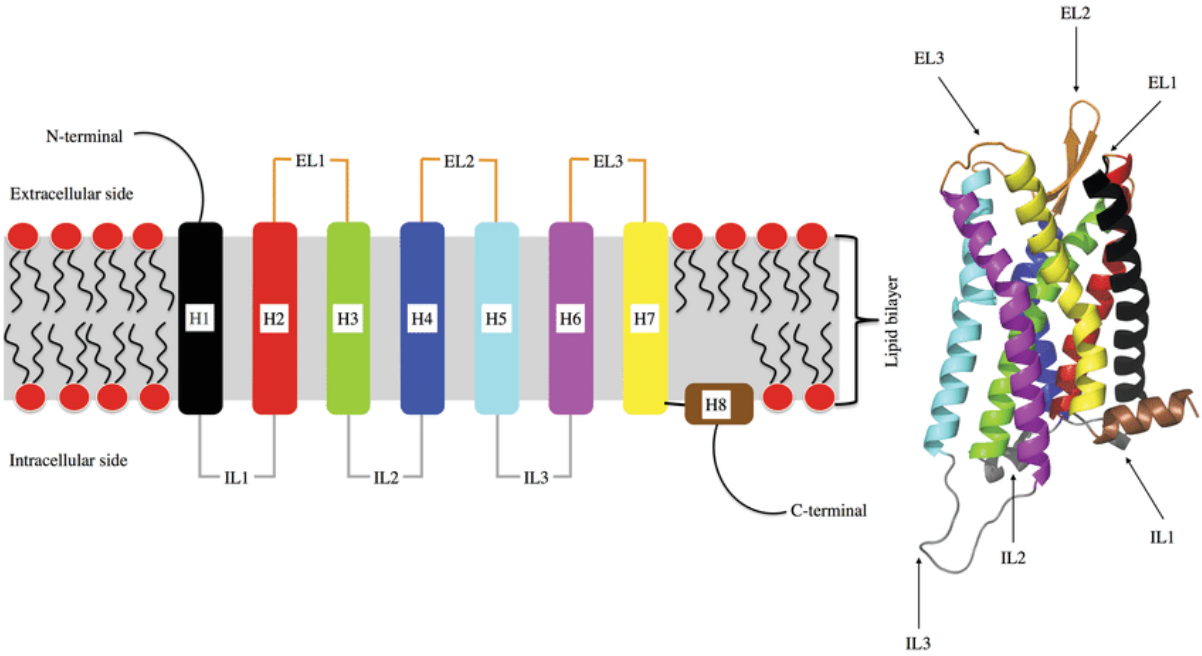

Figure 2.4: GPCRs structure
Left: Schematic view of the overall structure of a GPCR. Right: 3D structure of the reconstructed μOR structure [127].

In the 1950s, bovine rhodopsin was the first GPCR to be studied and isolated from sonicated bovine rod outer segment membranes. Circular dichroism showed that the structure of rhodopsin

contains about 30% α-helix. N- and C- terminal regions are on opposite sides of the membrane with most of the rhodopsin protein inserted within the membrane via seven transmembrane-spanning domains (Figure 2.4) [127]. Further studies have confirmed the seven transmembrane segments of bovine rhodopsin in 1983 [128] and its crystal structure was obtained in 2000 [129]. The crystal structure of the β-adrenergic receptor was obtained in 2007 and of the β1-adrenergic receptor and the adenosine receptor (A2aR) in 2008.

All GPCRs have a common structural signature of seven hydrophobic transmembrane (TM) α-helical segments of about 25-35 amino acid residues, and that is why they are known as seven-transmembrane domain receptors (Figure 2.4) [130].

The GPCRs are characterized by an extracellular N-terminus, seven transmembrane segments connected by three intracellular (IL1 to IL3) and three extracellular (EL1 to EL3) loops and an intracellular C-terminus. The EL1 and EL2 loops both contain a cysteine residue, which could participate in a disulfide bond. The carboxyl terminus, the intracellular loop between TM5 and TM6, and the amino terminus are the most variable parts of the GPCR structure, and these differences were used to classify several families of GPCRs. This classification is based on the sequence, the receptor-ligand binding, the structure and the amino acid residues [131]. Several classifications have been reported but the human GPCRs can be classified into five main classes according to the Kolakowski system (classes A to F). This system is based on sequence homology while the GRAFS classification system, which overlaps the Kolakowski system, is based on phylogenetic analysis. They both use pharmacological information to further characterize and group the GPCRs (Figure 2.5) [132].

Class	GRAFS Family	Note	# Human members
A	*Rhodopsin*	Incl. 390 olfactory and 5 vomeronasal 1 receptors	689
B	*Secretin*	Also referred to as B1	15
	Adhesion	Also referred to as B2	33
C	*Glutamate*	Incl. 3 Taste 1 receptors	22
D	—	Fungal mating pheromone receptors	0
E	—	cAMP receptors	0
F	*Frizzled*	—	11
T	*Taste 2*	Previously grouped with Class F but later redefined as a separate class evolved from class A	25
O	*Other*	7TM receptors not belonging to any of the above classes	6

Figure 2.5: Human GPCRs classification
Two overlapping classifications: the Kolakowski system (classes A-F) and the GRAFS system [132].

The human GPCRs can be classified into five main classes :

- Class A Rhodopsin: The rhodopsin-like family is the best characterized one and contains about 90% of all GPCRs. Olfactory receptors, small molecule binders and peptide binders are the three major branches for this class A family. These receptors have a short N-terminal tail and contain conserved regions such as the NPXXY motif in TM7, two cysteine residues linking IL1 and IL2 with a disulfide bond and the E/DRY motif at the border between TM3 and IL2 [133]. The rhodopsin receptor, the β2-adrenergic receptor and the CCK1R and CCK2R belong to the rhodopsin-like family.

- Class B Secretin: The secretin-like family contains receptors with structural similarities with the secretin receptor, such as the glucagon receptor, the gastric inhibitory polypeptide receptor (GIPR), the glucagon-like peptide-1/2 (GLP-1 and GLP-2) receptors and the growth hormone releasing hormone (GHRH) receptor. They have an extended amino-terminal domain with six conserved cysteine residues creating disulfide bonds and providing conformational constraints [134]. The sequence homology between these GPCRs and the class A GPCRs is only 12%.

- Class C Glutamate: The GPCRs from the class C family display a long amino-terminal domain forming two distinct lobes separated by a cavity where the agonist is able to bind. Another characteristic of this family is the presence of a short and highly conserved IL3. The metabotropic glutamate receptors (mGluR), the γ-aminobutyric acid (GABA) receptors and the retinoic acid-inducible orphan GPCRs (RAIG) receptors are all members of the class C family.

- Class F Frizzled: The Frizzled receptors mediate the actions of Wnt ligands and thus play a role in cell fate, proliferation and cell polarity [135]. Their IL loops are short compared to other GPCRs. This family contains the Frizzled receptors FZD1 to FZD10.

- Class T Taste 2: The GPCRs from the class T family are not well characterized but they have been isolated from taste receptor cells of the tongue and palate epithelium. They are implicated in the perception of bitterness [136].

CCK2R

CCKR receptors were discovered in 1978 using radiolabeled caerulein, a CCK analogue, in intact rat pancreatic acinar cells [137]. Another pharmacologically different CCK receptor was then found in the rat's brain. The first receptor was called CCKR-A for Alimentary and the second one CCKR-B for Brain, but these names were later changed to CCK1R and CCK2R, respectively. CCK1R binds to sulfated CCK with an affinity 500-1000 higher than gastrin while CCK2R binds to CCK and gastrin with similar affinity and does not require ligand sulfation for

binding [138]. Progastrin, Ggly and CTFP have poor affinity for CCK2R. Some studies have shown that Ggly stimulates the growth of the gastric cancer cell line AGS and the pancreatic acinar cell line AR4-2J similarly to Gamide, but through binding sites pharmacologically distinct from CCK1R and CCK2R [139] [140]. The gastrin-binding protein (GBP) is another receptor identified in porcine gastric mucosal membranes that binds to Ggly and Gamide [141] [142]. Several receptor subtypes have been implicated in growth effects by gastrin peptides and Annexin II was described as a potential gastrin/progastrin receptor in both normal and cancerous gastrointestinal cell lines [143]. A recent report has found the F1-ATPase as a candidate Ggly receptor [144].

The human CCK2R cDNA was cloned in 1993; it encodes a protein of 447 amino acids with a molecular weight of 48,419 daltons [145]. CCK2R, a member of the A family of GPCRs, has a typical seven-transmembrane α-helix structure and its N-terminal domain is involved in the translocation to the membrane. The sequence presents at least three consensus sites for N-linked glycosylation: Asn7, Asn30 and Asn205 [141]. CCK2R contains two conserved cysteine residues creating a disulfide bond between the first and second ELs and playing an essential role in receptor conformation and stabilization [146]. This receptor also contains the E/DRY motif at the intracellular end of helix III and the NPXXY motif in the transmembrane domain VII, which both are implicated in the regulation of receptor conformational states and in G-protein coupling/recognition. CCK2R is expressed by parietal cells, enterochromaffin-like (ECL) cells, some smooth muscle cells, neurons of the central and peripheral nervous system and pancreatic acinar cells.

2.2.2 GPCR activation/inactivation cycle

In the 1970s, a series of studies identified the first cAMP effector, protein kinase A [147], and the presence of a protein connecting the glucagon receptor to the adenylyl cyclase (AC) that catalyses the synthesis of cAMP [148]. This intermediate protein was described in 1977 and called Gs protein [149] [150]. The Gs protein was then purified and identified as a heterotrimeric molecule with Gα, Gβ and Gγ subunits.

A general model for the GPCR activation/inactivation cycle has since been proposed. Upon ligand binding to its receptor, the activated GPCR undergoes conformational changes, which facilitate interaction with one of the G proteins and activates them by exchanging GDP for GTP in the Gα subunit (Figure 2.6) [151]. Hence GPCRs function as guanine-nucleotide-exchange factors (GEFs). The GTP-bound Gα separates from GβGγ and the three subunits are then active and available to interact with downstream targets. Activated Gα triggers second messengers via effectors such as phospholipase C (PLC) or adenylyl cyclase. Independently the GPCR

is available for phosphorylation by G-protein-coupled receptor kinases (GRKs) stimulating its binding to β-arrestin [152]. This leads to GPCR internalization and the GPCR can then be recycled to the cell membrane or degraded by lysosomes.

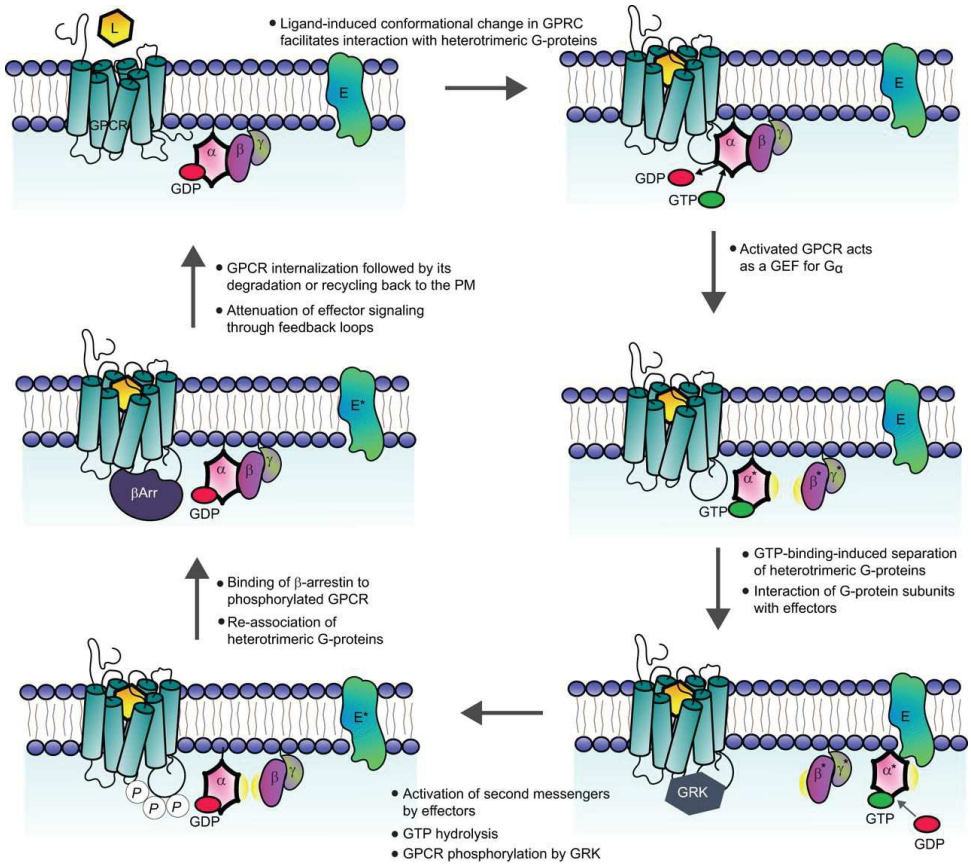

Figure 2.6: The GPCR cycle.
The GPCR cycle starts on the top left of the figure and goes from its basal state, the binding to its ligand and the protein G activation, the initiation of a second-messenger cascade, its internalization and recycling to the cell surface [151].

G-proteins, GRKs and arrestins are some of the signal transducers important to the GPCR cycle.

- G-Proteins: Heterotrimeric G-proteins are a group of GTPases with a common structure of three subunits. The Gα subunit, about 39 to 52 kDa, contains an intrinsic GTPase activity, which catalyses the hydrolysis of GTP to GDP, and a G$\beta\gamma$ unit that is noncovalently bound to the Gα subunit in its inactive state. Up to now, the G-protein family contains 23 Gα subunits (grouped into four main families Gαi/o, Gαs, Gαq /11 and Gα12), 6 β subunits and 12 γ subunits. There are many G-proteins and the different combinations of the three subunits bind to different kinds of receptors and activate various signaling pathways.

- GRKs: The G-protein-coupled receptor kinases represent a family of seven serine\protein

kinases. They have a well-conserved central catalytic domain and variable N-terminal and C-terminal domains. The amino terminus has binding sites that determine the GRK intracellular localization and thus confer its specificity to GPCRs [153]. GRKs phosphorylate serines and threonines located in the IL3 and the C-terminal domain of the ligand-activated GPCRs. However, this is not enough for a complete desensitization of the receptor [154].

- Arrestins: Arrestins, or β-arrestins, are proteins able to dissociate G-proteins from phosphorylated GPCRs and they consequently play a role in GPCR internalization and cell trafficking. They recruit proteins such as clathrin and AP-2 (adaptor protein 2), which are involved in GPCR endocytosis. Interestingly some studies have shown that not all of the GPCRs require β-arrestins for their endocytosis [155].

2.2.3 Signaling pathways

GPCR signaling pathways

Transmembrane signaling upon activation of a GPCR by its specific ligand is transduced by various proteins, which are controlled by the receptor. In the G-protein-mediated signaling system, the receptor, the G-protein and effectors can be regulated independently and can separately activate signaling pathways (Figure 2.7) [156].

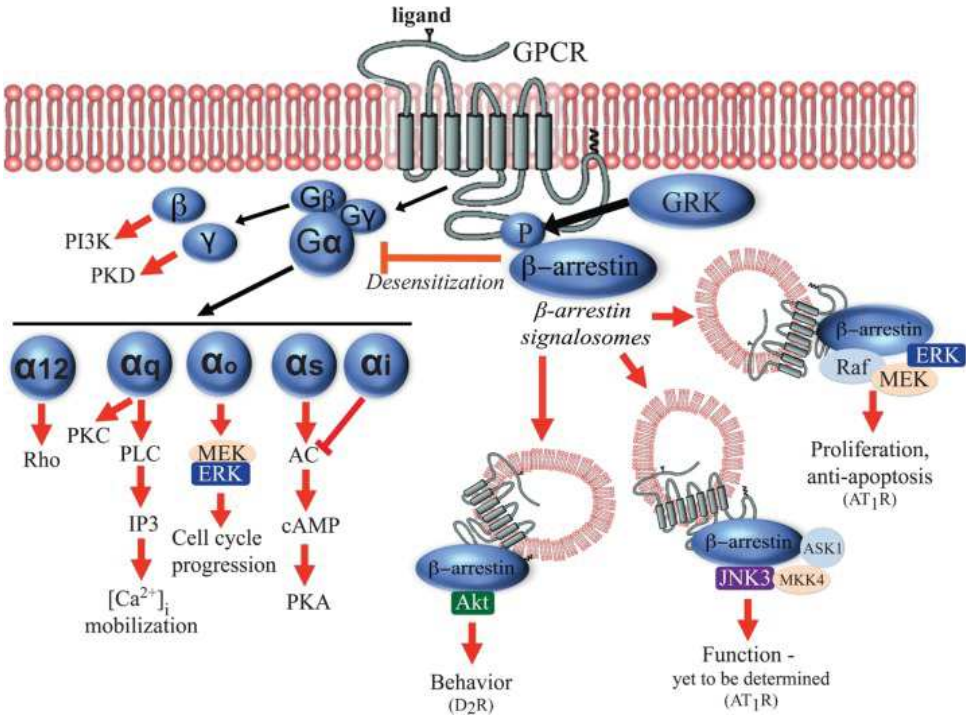

Figure 2.7: Examples of G-protein- and β-arrestin-mediated downstream signaling pathways on GPCRs [156].

After dissociation of the trimeric G-protein, the Gα subunit can interact with various effectors. Gαs activates adenylyl cyclase (AC) and thus the production of cAMP (cyclic adenosine 3',5'-cyclic monophosphate), while the Gαi subunit inhibits it. The Gαt subunit activates photoreceptor phosphodiesterase (PDE) resulting in membrane hyperpolarization, and the Gαq subunit activates phospholipase C (PLC) leading to calcium release and PKC activation [157] [158]. GβGγ stays as a unit and can mediate responses different from Gα. GβGγ can firstly inactivate the Gα subunit and activate the MAPK and JNK signaling pathways. The Gγ subunit can also activate or deactivate AC and activate PLCs or PI3K while the Gγ subunit can activate diverse kinases such as protein kinase D (PKD) [159].

β-arrestins play a role in GPCR endocytosis but are also involved in signaling, trafficking and ubiquitination of receptors [160]. Like G-proteins, β-arrestins bind MAPKs (Mitogen-Activated Protein Kinase), including ERK1/2 (Extracellular-signal-Regulated Kinase), but through distinct mechanisms [161]. ERK activation potentially leads to gene transcription promoting cell cycle progression [162]. β-arrestins play a role in activation of p38 and JNK2 (c-jun NH2-terminal kinase 2) and in inactivation of JNK3, which could be a useful tool against pro-apoptotic JNK3 activity [163] [164]. Some studies have reported a link between β-arrestins and receptor ubiquitination and degradation [165].

CCK2R signaling pathways

The CCK2R is coupled to Gαq, which is involved in many signaling pathways and consequently in many physiological and pathophysiological processes (Figure 2.8) [166].

Upon binding to Gq, the activated CCK2R activates the PLC pathway by binding of its C-terminal domain to the SH2 domain of the PLC isoform PLCγ1 [167]. PLC catalyses the hydrolysis of phosphatidylinositol 4,5-biphosphate (PiP2) to produce inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 acts as a second messenger in the cytoplasm through the IP3 receptor in the endothelium reticulum (ER) and by stimulating calcium release into the cytosol. Calcium will activate more proteins. DAG activates several protein kinase Cs involved in MAPKinase signaling via Raf. MAPKinases are a family of serine/threonine kinases including ERK1/2, JNKs, ERK5 and p38MAPK. They regulate transcription factors through the phosphorylation cascade Ras/Raf/ERK-kinases/ERK1/2 and so regulate proliferation, differentiation, survival and apoptosis. Several alternative signaling pathways have been described downstream of the activated CCK2R in different cell types. In AGS cells stably expressing CCK2R, after stimulation of CCK2R by gamide, ERK1/2 activation leads to the transcriptional regulation of the human trefoil factor (TFF1), which is involved in epithelial architecture and growth [168]. In CHO cells stably expressing CCK2R, p38MAPK is activated by CCK2R via the combination of PKC, intracellular calcium and Src mechanisms and plays a central role in proliferation [169]. In CHO cells, which stably express CCK2R, CCK2R activation leads to the

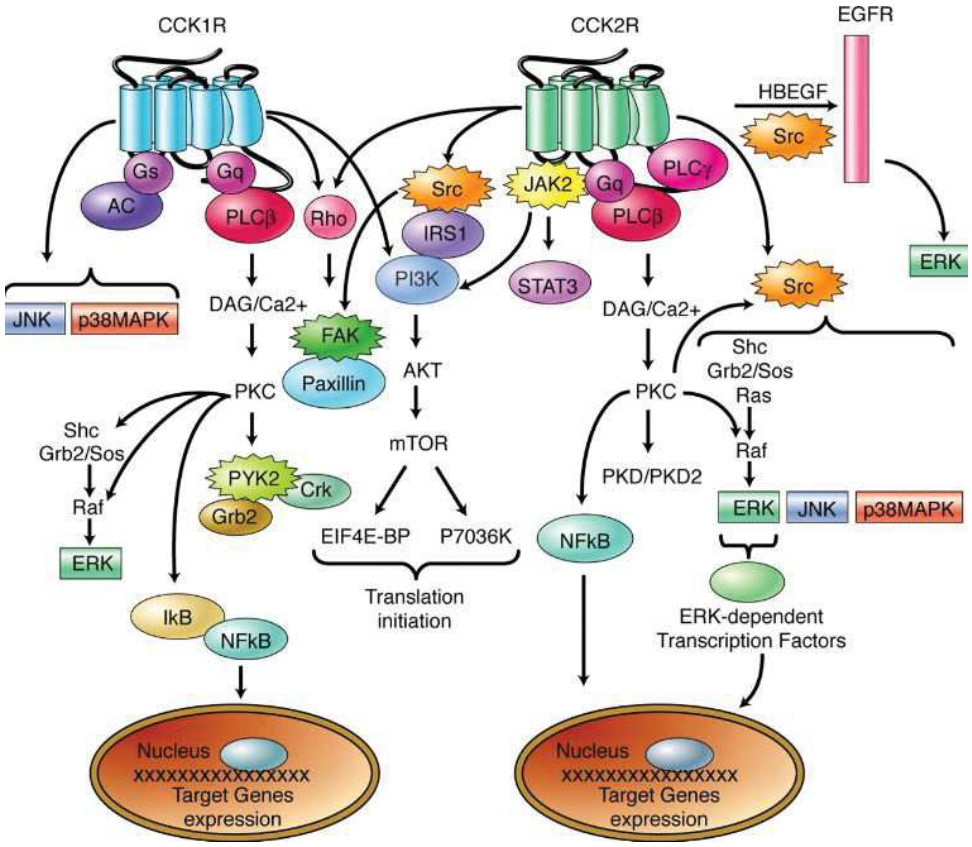

Figure 2.8: Schematic description of the signaling pathways known to be activated by CCK1R and CCK2R [166].

phosphorylation via the Src family kinases of the signaling adapter protein IRS-1, which recruits and activates the p85/p110 PI3-kinase [170]. The PI3-kinase/Akt signaling pathway is involved in the proliferative and anti-apoptotic actions of CCK2R, as well as in the regulation of cell adhesion and migration by the receptor [166]. In the gastrointestinal epithelium, maturation of proHB-EGF upon gamide stimulation leads to HB-EGF (Heparin-binding EGF-like growth factor) release. The HB-EGF receptor, EGFR, is phosphorylated and ERK1/2 pathways are activated.

The JAK/STAT pathway has been linked to GPCRs and its activation by Gαq. There are four kinases in this family, JAK1, JAK2, TYK2 and JAK3. They phosphorylate and activate the family of STAT (Signal Transducers and Activators of Transcription) proteins. The phosphorylated STAT protein dimerizes and translocates to the nucleus to bind to a specific gene. Gamide binding to the CCK2R activates the JAK2/STAT3 pathway via Gαq in AR4-2J and COS-7 cells, leading to proliferation [171].

Regulation of CCK2R expression

Expression of the CCK2R may be increased in sites where it is physiologically not expressed.

For example, CCK2R is expressed in the epithelium of Barrett's esophagus, where gastrins induce proliferation, but not in the normal esophageal epithelium [172]. CCK2R was seen in hypertrophied gastric mucosa and the observation that its expression was induced in a dose-dependent manner by gamide demonstrated that gamide autoregulates its own receptor [173]. CCK2R was also expressed in hypergastrinemic rats where it was involved in healing of mucosal tissue via cell proliferation enhanced through gastrin's trophic effects [174]. Another study on the pancreatic cell line AR4-2J, which expresses both CCK1R and CCK2R, has linked agonist-regulated short-term variation in CCK2R expression to its mRNA expression [175].

Chapter 3

Physiological roles of the CCK and gastrin peptides

Biological effects of CCK and gastrin peptides are completed via binding to their specific receptors.

3.1 CCK

CCK was discovered for its hormonal cholecystokinetic and pancreozymic actions but it has also since been shown to be a growth factor for the pancreas and a potent neurotransmitter in the central and peripheral nervous system [100]. In the gastrointestinal tract, CCK plays a role in the contraction and relaxation of the stomach, the sphincter of Oddi and the gallbladder. The latter contraction leads to the release of bile from the gallbladder into the duodenum. CCK stimulates the secretion of pancreatic enzymes, the secretion of intestinal enzymes such as alkaline phosphatase, disaccharidase and enterokinase, and the synthesis of digestive enzymes such as pancreatic amylase, chymotrypsinogen and trypsinogen [176] [177]. CCK has a role in gastrointestinal motility because of the presence of CCK nerves in the smooth muscle of the small intestine and the colon. In human, dogs and possums, CCK is a potent inhibitor of gastric emptying [178]. Following a meal, the release of CCK leads to satiety through gastric CCK1R receptors, relaying the effect into afferent vagal fibers [179]. Targeting CCK1R is of great interest in the prevention of obesity [180]. Contrary to gastrin's effect, CCK inhibits gastric acid secretion by releasing somatostatin from D-cells in the duodenum via CCK1R. Somatostatin inhibits acid secretion from the parietal cells and inhibits gastrin production [181].

3.2 Gamide

Gamide, which was first discovered as a hormone released by the upper digestive tract and stimulating gastric acid secretion, has a role in the metabolism of other tissues too.

3.2.1 Secretion of gastric acid

The gastric juice produced by the stomach is a highly acidic liquid with active proteolytic and lipolytic enzymes. It plays a role in digestion as well as in preventing bacterial infections by keeping the gastric pH below 4 [182].

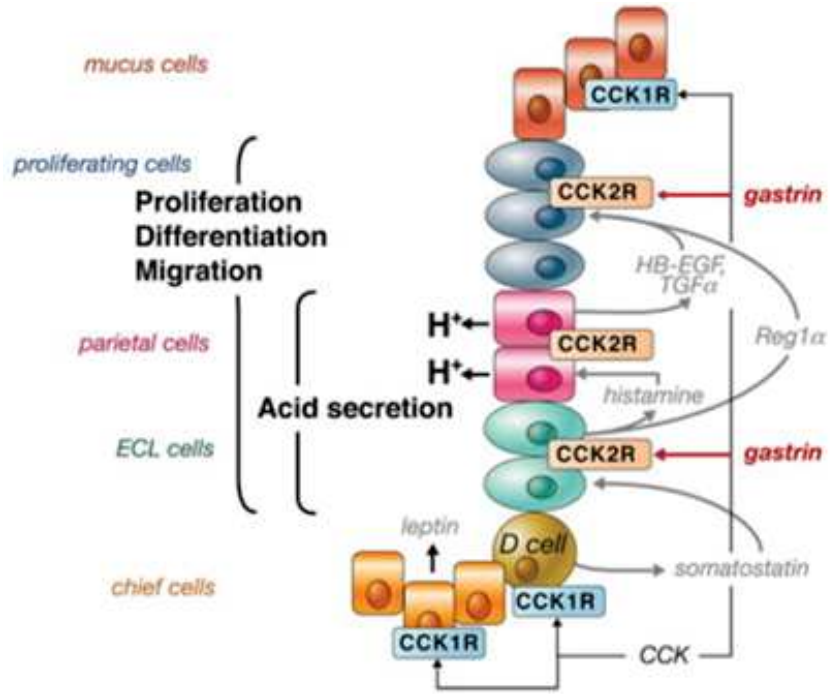

Figure 3.1: Schematic representation of the release of gastrin and its sites of action [166].

In the last 50 years, many studies have been done on the three gastric acid secretagogues (i.e., acetylcholine, gastrin and histamine) to understand their interactions and their exact role in gastric acid secretion. In 1970, gastrin was finally described as a potent gastric acid secretagogue upon food intake, while the role of histamine was actually questioned [183] until the identification of the histamine-2 (H-2) receptor two years later. In the late 1970s, a stimulatory effect of acetylcholine and histamine, but not gastrin, on acid secretion was demonstrated in isolated oxyntic glands and parietal cells [184]. The same effect was shown *in vivo* confirming the idea that acetylcholine and histamine, but not gastrin, have a direct effect on parietal cells [185]. However, the administration of increasing doses of Gamide to totally isolated and vascularly perfused rat stomachs produces a dose dependent histamine release as well as the release of correlated quantities of acid secretion [186]. This result demonstrated that the acid-stimulatory effect of gastrin was mediated by histamine release [187]. To summarize, upon binding to CCK2R in the stomach, Gamide activates the release of histamine, which in turn stimulates parietal cells and thus activates the secretion of gastric acid (Figure 3.1) [166] [122]. When the rate of gastric acid secretion is high enough (pH decreasing to about 2), gastrin secretion is

inhibited by somatostatin [188].

3.2.2 Trophic action

A link between tumors in pancreas producing gastrin and the release of higher quantities of gastric acid by thickened oxyntic mucosa was recognised in the 1950s and suggested a trophic effect of gastrin on oxyntic mucosa [189]. Gastrin has a trophic action by stimulating DNA synthesis in various tissues and by stimulating proliferation and differentiation of gastric cells [190]. These characteristics were further studied when gastric acid secretion started to be associated with histamine release from ECL cells [191] [192]. It was proved that gastrin, injected subcutaneously through implanted osmotic minipumps to rats, directly mediates trophic effects on ECL cells, with a concentration dependence similar to the functional effects [193]. Gastrin controls proliferation of ECL cells in rats [194] as well as in mice [195]. Interestingly, in the absence of gastrin or CCK2R, parietal cells and ECL cells are either reduced in number or defective in maturation, leading to a lack of the acid-secretory responses to gastrin, histamine or acetylcholine [196]. Moreover, histamine can have a trophic effect itself [197] and the regenerating (Reg) protein, expressed in gastric ECL, is involved in mediating the trophic effect of gastrin on the oxyntic mucosa [198].

3.2.3 Glucagon gene expression

Important changes in peptide maturation are manifested during neonatal development. Although the gastrin gene is mainly expressed in endocrine cells in humans, its expression in fetal mammals is mostly in pancreatic islet cells. A correlation between the development of pancreatic islets, which occurs mainly in neonatal pancreas, and the presence of gastrin at the same moment was suggested. Gastrin in neonatal rat pancreas is totally sulfated and disappears from the pancreas not long after birth. Studies on mice expressing the human gastrin gene driven from the rat insulin I promoter demonstrated that gastrin overexpression did not have an impact on islet mass but, when TGFα (Transforming Growth Factor alpha) was overexpressed too, a twofold increase was seen. Both gastrin and TGFα act in synergy to stimulate pancreatic islet growth [199]. A few weeks after birth, pancreatic gastric mRNA levels decrease rapidly while at the same time CCK increases in the intestine [200] and other mRNAs (e.g., glucagon, insulin, somatostatin) reach their maximum quantities in pancreatic islet cells [199]. Additionally, the CCK2R gene is expressed almost exclusively in glucagon-producing cells in human pancreatic islets in both fetal and adult pancreas. Gastrin and CCK apply a direct regulation of glucagon-producing cell activity through the CCK2R [201] and gastrin has a role in glucose homeostasis via stimulation of glucagon secretion [202]. Together with these results, an essential transcription factor, Erg-1, was shown to be phosphorylated after gastrin stimulation and was identified as one of the gastrin-regulated transcription factors [203]. Gastrin clearly contributes to activation

of the glucagon gene and glucagon secretion during the embryonic period [204].

3.2.4 Incretin effect

In the 1960s, it was suggested that gastrin acts potentially like an incretin. Antral extracts containing gastrin and purified porcine gastrin II both increase the insulin-releasing activity in dogs to a level similar to that induced by secretin or pancreozymin [205]. In humans, gastrin potentiates the secretion of insulin by glucose [206]. Interestingly, gastrin is transiently present in fetal pancreatic islets when β-cells are proliferating. Gastrin alone does not increase β-cell mass, but acts in combination with TGFα [207]. Also, in diabetic NOD mice, only the combination of gastrin and GLP-1 reduces glycemia back to normal. An increase in pancreatic insulin content, β-cell mass and insulin-positive cells in pancreatic ducts, as well as a decrease of β-cell apoptosis, were observed [208]. Another study showed that a novel GLP-1-gastrin dual agonist, ZP3022, controlled glycemia in db/db mice better than the GLP-1 agonists (e.g., exendin-4 and liraglutide). A preventive effect on the β-cells was also seen [209].

Studies on the incretin effect of gastrin provide conflicting data [210] [211] but there is clear evidence of an interaction between gastrin and gut hormones.

3.3 Ggly

3.3.1 Proliferation

Similarly to Gamide, Ggly induces cell proliferation and migration of various cell lines *in vitro* through binding to its receptor.

The elevated serum Ggly in patients with colorectal carcinoma suggests that the process of progastrin maturation fails to produce mature Gamide, thus the main peptides produced are progastrin and Ggly [212]. Ggly is also present in colon carcinoma cell lines. Ggly induces pancreatic AR4-2J cell proliferation with the same potency as Gamide [140]. Proliferation of the immortalized mouse colon cell line YAMC was inhibited by a gastrin antisense mRNA. These observations have led to the idea of an autocrine loop composed of an extracellular component, involving a specific receptor for Ggly, and an intracrine component, potentially involving the 78kDa gastrin-binding protein, and identified in the inhibition of proliferation of non-tumorigenic gastrointestinal cells [213]. The presence of Ggly receptors on human colon cancer cells has been described to mediate Ggly trophic effects via a MEK-independent mechanism suggesting an impact in colon cancer cell growth [214].

Also, Ggly has been reported to induce colonic mucosal proliferation *in vivo*. Gastrin-deficient mice, continuously injected with Ggly for two weeks, have elevated Ggly levels, a 10% increase in colonic mucosal thickness and an 81% increase in colonic proliferation compared to mice treated

with placebo. The proliferative zone of the colonic crypt increases, which suggests that Ggly plays a crucial role in intensifying the risk for developing colorectal cancer [215]. Studies in rats have confirmed that Ggly acts as a promoter of colorectal carcinogenesis [216].

3.3.2 Effect on parietal cells

Although the level of Ggly in plasma is similar to Gamide, Ggly does not increase acid secretion when it is intravenously infused in rats. However, when Ggly is administrated together with Gamide, the acid-secretory response is stimulated [217]. Ggly does not activate intracellular Ca^{2+} release but instead stimulates tyrosine kinases and activates PI3-kinases and JNK. After prolonged treatment, Ggly potentiates the acid secretory action of Gamide through the H^+, K^+-ATPase associated with tyrosine-kinase activation *in vitro* [218]. Ggly has a role in differentiation or maturation of parietal cells as the administration of Ggly prevents the loss of parietal cell function observed when only Gamide is present [219]. Ggly regulates the capacity of the parietal cells to be stimulated by secretagogues and thus acts as a modulator of parietal cell function.

3.4 Progastrin

Progastrin has been identified as a growth factor in the gastrointestinal tract. Progastrin is not processed to the mature amidated form in transgenic mice overexpressing the human progastrin gene (hGAS mice) in the liver. Consequently, serum progastrin is elevated and the proliferation of colonic mucosa is increased [220]. Proliferative effects of recombinant human progastrin6-80 on the mouse colonic cell line YAMC are also consistent with a role for progastrin in colonic cell growth, independently of both CCK1R and CCK2R [221]. Also, progastrin exerts proliferative actions on murine colonic epithelia in the absence of other forms of gastrin and the COOH-terminal 26-amino acid residues of the human preprogastrin are responsible for these effects [222]. Additionally, progastrin has anti-apoptotic effects. Studies have demonstrated that progastrin is associated with a decrease of DNA fragmentation in intestinal epithelial cells (IEC) *in vitro*. This is correlated with an upregulation of ATP due to the Cox Vb gene being a potential target of progastrin [223]. Anti-apoptotic effects of progastrin may contribute to the increase in cell growth of normal and cancerous intestinal epithelial cells.

3.5 CTFP

CTFP is present in the antrum of various species including human [224] [225] [115]. The quantity of antral CTFP was significantly higher than Gamide in sheep, while this difference was lower in pig, rat and dog. The concentration of circulating CTFP is higher in human plasma than the other forms of gastrin, and this was observed in sheep too [116] [226]. Although CTFP does not induce acid secretion [227], it markedly effects proliferation and migration via the MAPK

pathway in normal and diseased gastrointestinal cells [116]. Moreover, CTFP inhibits apoptosis through the PI3-kinase pathway by stimulating Bcl-xL and by decreasing active caspase 3 (Figure 3.2) [228]. Interestingly, after meal-stimulated secretion of Gamide and CTFP, acid secretion increases while the quantity of Gamide decreases, but the concentration of CTFP remains high for at least 4 times longer than Gamide [229].

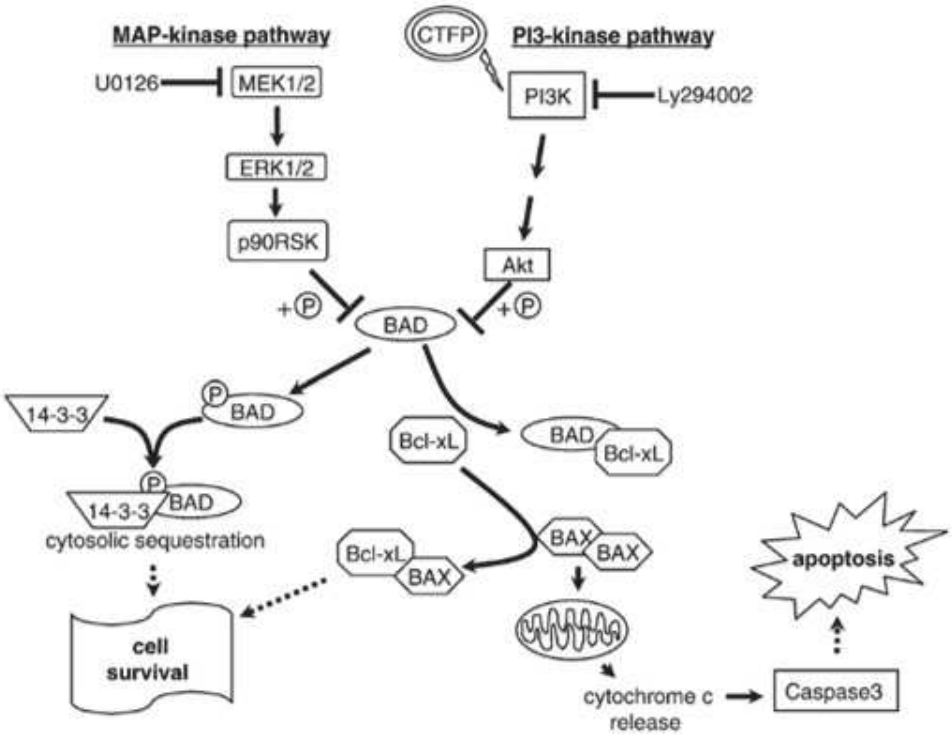

Figure 3.2: Schematic diagram of CTFP involved in cell survival [228].

Chapter 4

Gastrins and CRC

Most human colon cancers express the gastrin gene and almost all colorectal polyps and carcinomas synthesize progastrin. Although Gamide is found in lower concentrations than in the antrum, immature forms of gastrin (progastrin, Ggly and CTFP) are highly expressed in carcinomas. Gastrin maturation is weakened in tumors because Gamide is not stored in secretory granules.

Gastrins clearly play a role in the development of colorectal carcinoma but in contradictory studies a correlation between patients with colorectal adenocarcinoma and hypergastrinemia was not always observed [230] [231].

4.1 Proliferation of gastrointestinal carcinoma cell lines

4.1.1 Gastrin expression

Gastrin mRNA was identified in the human colon cancer cell line HCT116 and the human colorectal carcinoma cell line LIM1215 by polymerase chain reaction (PCR) [232]. Gamide was secreted in similar quantities from colorectal tumors and normal tissue, while progastrin and CTFP were found in higher concentrations in tumors [233]. In colonic tissue, Ggly was 10-fold more abundant than Gamide while progastrin was 700-fold more abundant (Figure 4.1) [234]. Interestingly, the sequence of the gastrin gene was identical whether it was expressed in tumor cell lines or normal cells. A study on five human colon carcinoma cell lines, twelve solid colon carcinomas and normal colonic tissue, confirmed the incomplete maturation of progastrin due to posttranslational events such as translational attenuation [212].

	normal colon	carcinoma	p-value (normal vs. cancer)
G-gly/gastrin	11.7±1.84	27.1±7.78	p=0.007
Progastrin/gastrin	706±211	1,390±570	p=0.067
Progastrin/G-gly	73.3±28.4	48.4±9.21	p=0.17

Note: Results expressed as mean ± S.E.M., fmol/g wet tissue, n=15 matched pairs.

Figure 4.1: Gamide, Ggly and progastrin ratios in normal colon and carcinomas [234].

Posttranslational maturation of progastrin varies during development and can be divided into three different processes (Figure 4.2) [235]:

- Attenuation: cells fail to translate mRNA to the mature and fully functionally protein. There is either no processing of progastrin or a partial processing leading to its processing intermediates.
- Maturation: progastrin is processed to its mature form Gamide.
- Inactivation: mature protein loses its biological activity due to proteolytic fragmentation or derivatization of the active site.

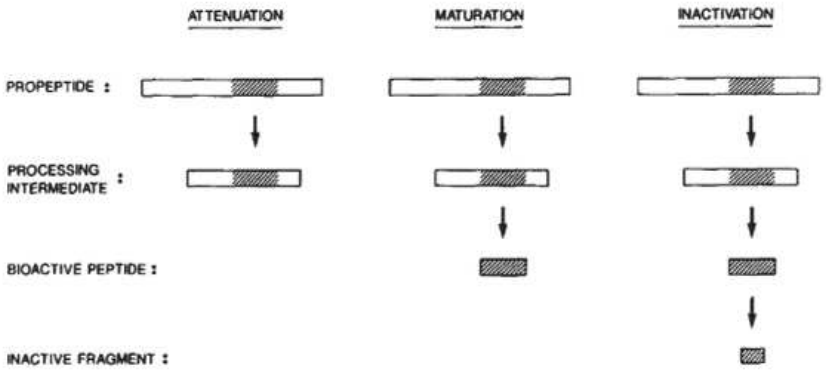

Figure 4.2: Posttranslational modifications of gene expression can be divided into three processes: attenuation, maturation and inactivation [235].

In CRC patients, progastrin, Gamide and Ggly levels were detected in 100%, 69% and 44% of tumors respectively, and circulating non-amidated gastrins were increased [236].

4.1.2 CCK2R positive cells

Many controversial studies on CCK2R expression in gastric and colorectal cancers have been reported and this may be explained by the use of tissue homogenates [237] [238] [239]. Malignant cells may grow into surrounding non-malignant tissue and it is impossible to distinguish and

separate them before any receptor binding assays. Resected tumors contain both malignant and non-malignant tissue including mucosa, smooth muscle cells, nerves and blood vessels.

Some studies showed that CCK2R is often upregulated in human colon cancers. CCK2R mRNA was expressed in 38% of human colorectal cancers, 13% of normal colonic tissue and co-expressed with gastrin in 32% of tumors (Figure 4.3) [240]. CCK2R was expressed in carcinoma cells as well as polymorphonuclear (PMN) cells within the tumor stroma. Increased expression of CCK2R in CRC cells compared to normal colonic tissue may be due to changes during the passage from polyps to invasive adenocarcinomas.

	Cancer (n = 79)			Normal
	GI (n = 38)	GII (n = 21)	GIII (n = 20)	tissue (n = 15)
mRNA expression	34%	19%	65%	13%
	(13/38)	(4/21)	(13/20)	(2/15)
Total	38%			13%
	(30/79)			(2/15)

Figure 4.3: CCK2R mRNA expression in colon cancer (grade I, II and III) and normal tissue [240].

An isoform of CCK2R cDNA lacking the N-terminal extracellular domain (ΔCCK2R) was firstly isolated from the human stomach [241]. Eight out of thirteen gastrointestinal tumor cell lines co-expressed wild-type CCK2R and ΔCCK2R, including eight colorectal cell lines [242]. However, as the N-terminal domain confers on the CCK2R its high affinity for gastrin, the truncated isoform was not involved in growth promotion in CRC. Another CCK2R splice variant, isolated from human colon cancer [243], has retained intron IV during RNA processing resulting in a longer IL3 (69 amino acid residues) and an increased affinity for Ggly. A spontaneous single point mutation (V287F) in IL3 showed a decrease in gastrin-induced MAPK p44/p42 signaling in CCK2R-expressed Colo 320 cells. This mutation did not increase the affinity for Ggly, indicating that mutated CCK2R-V287F is not the receptor for non-amidated forms of gastrins [240].

Upregulation of CCK2R in gastrointestinal cancer cell lines influences signaling pathways. Gamide-bound CCK2R stimulated the activation of the focal adhesion kinase (FAK) signaling pathway in colon cancer cells [244]. FAK plays a role in cell adhesion, cell motility and apoptosis and this pathway is involved in the process leading to metastatic cells. Consequently, CCK2R overexpression promoted the development of colon cancer via the FAK pathway [245] [246].

The alternative gastrin CCK-C receptor is expressed almost as often as gastrin mRNA, and binds progastrin-derived peptides with low affinity. The CCK-C receptor may be involved in the autocrine loop in CRC.

A distinct gastrin receptor subtype, F1-ATPase, selectively recognizing Ggly with high affinity was described in both normal and colon cancer cells (HT29, HCT116) [140] [144]. Annexin II was as well described as a potential gastrin/progastrin receptor in gastrointestinal cells [143]. The role of these putative receptors in CRC still remains to be established.

4.1.3 Autocrine loops

Gastrins act as an autocrine growth factor in gastric and colonic carcinoma cell lines. Pre-immunization of rats with the immunogen Gastrimmune (Figure 4.5), composed of the N-terminal domain of Gamide linked to diphtheria toxoid, raised antibodies neutralizing the effects of both Gamide and Ggly *in vivo*. Consequently, the growth of the Ggly-expressing rat colonic cell line, DHDK12, was inhibited and the tumor reduced [247]. Studies using antisense gastrin mRNA are the most convincing ones regarding the involvement of progastrin-derived peptides in an autocrine loop. The human colon cancer cell lines (Colo320 and HCT116) both express gastrin mRNA and had their proliferation and tumorigenicity reduced *in vitro* and *in vivo* when transfected with an antisense gastrin mRNA [248]. Proliferation of the immortalized mouse colon cell line YAMC was decreased when expressing an antisense gastrin mRNA, and Ggly, but not Gamide, could reverse this inhibition [213].

Proliferation in several colonic and non-colonic cell lines was inhibited by the cholecystokinin receptor antagonist, L-364,718, with no reversal effects observed in the presence of Gamide or sulfated CCK-8. The gastrin receptor antagonist L-365,260 did not affect cell proliferation [249]. These observations suggested the presence of an alternative autocrine loop involving non-amidated gastrins. The proliferation of YAMC cells was inhibited in the presence of an antibody selective for Ggly, but not with an antibody selective for Gamide. These cells expressed a specific receptor binding to Ggly resulting in an autocrine loop (Figure 4.4) [250].

It may be concluded that progastrin, and to a lesser extent Ggly, act as autocrine growth factors in CRC.

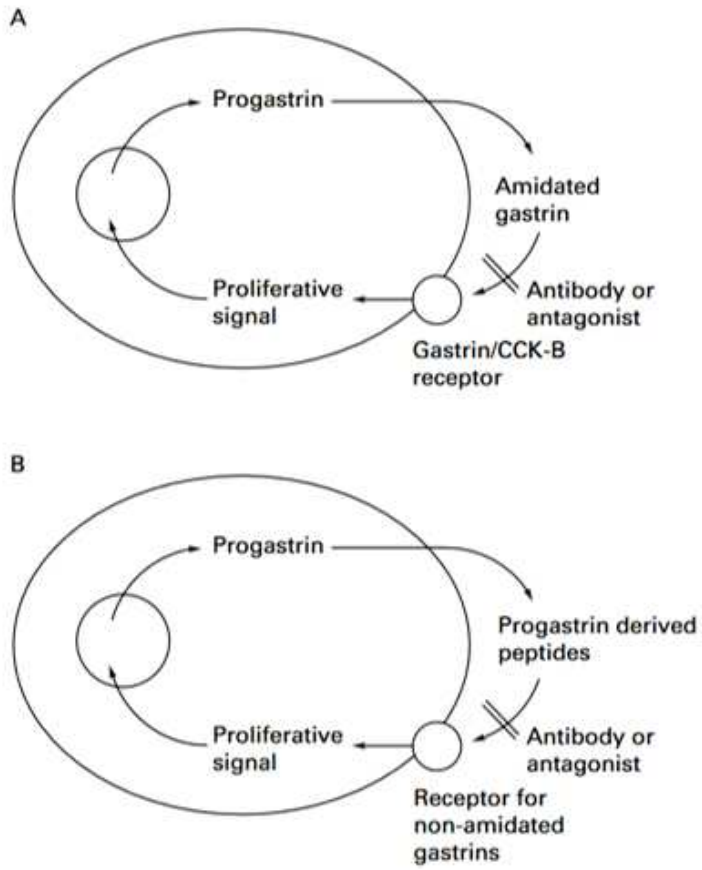

Figure 4.4: Autocrine growth loops by progastrin. Two classes of autocrine growth loops by progastrin are possible.
A- Class I involving CCK2R. B- Class II involving non-amidated gastrin receptors [250].

4.2 Adenoma-carcinoma sequence

Gastrins are poorly expressed in normal colonic epithelium and increased expression in CRC occurs during the adenoma-carcinoma sequence.

Gastrin mRNA activation occurs in the early stages of the adenoma-carcinoma sequence as it is expressed in small tubular adenomas. Gastrins were expressed in colonic polyps and hyperproliferative normal epithelium in animal models displaying APC mutations, but not in control animals [251]. These observations suggested that the APC gene is involved in gastrin transcription. The APC gene is located on chromosome 5q21 supporting the idea of APC controlling gastrin transcription. A correlation between gastrin expression levels and KRAS mutations has been demonstrated in pancreatic cancers [252] and a link between the expression of gastrins and p53 in gastric cancer cells [253]. A correlation between gastrins and key gene mutations should be investigated in CRC.

4.3 Hypergastrinemia

The correlation between hypergastrinemia and CRC is controversial. Hypergastrinemia triggered high proliferation of colonic mucosa *in vivo* [220] and gastrin gene knock-out mice had reduced proliferation of the colonic mucosa [254]. These observations suggested that hypergastrinemia could play a role in tumorigenesis.

Hypergastrinemia is the consequence of several clinical conditions such as pernicious anemia, Zollinger-Ellison syndrome or *Helicobacter pylori* infection [255] [256]. Studies showed that hypergastrinemia induced by the proton pump inhibitor omeprazole, with a serum gastrin level five times higher than the normal level, did not influence tumor growth or the survival of tumor-bearing animals (in both mice [257] and rats [258]). Chronic hypergastrinemia in rats, maintained by daily gavage of omeprazole, displayed a significant increase in gastrin concentrations but a decrease of tumor incidence compared to the control mice. Patients on chronic proton pump inhibitor therapy did not present with changes in the size of adenomatous polyps, but had a significant reduction in the development of hyperplastic polyps, compared to untreated patients. Omeprazole has a potential protective effect against the progression of CRC [259].

Zollinger-Ellison syndrome (ZES) is a good model to study chronic hypergastrinemia in humans because patients have increased concentrations of all forms of gastrins in their plasma. Similarly to the studies using omeprazole treatment, patients with ZES did not have an increased rate of colonic polyps or cancer [260]. Although there is a profound chronic hypergastrinemia, patients did not have a higher risk of developing CRC.

However, a long-term study including 250 cases of incident CRC, has tested frozen sera (collected between 1964 and 1969) for *H. pylori*, immoglobulin G, Gamide and Ggly. The number of CRC cases with Gamide levels above normal ones was significant and hypergastrinemia was thus associated with increased risk of colorectal malignancy. Moreover, high gastrin levels were strongly associated with *H. pylori* infection [68]. In the same way, $APC^{Min-/+}$ mice with multiple intestinal neoplasia after treatment by oral administration of omeprazole had serum gastrin levels 5-6 times higher than untreated mice. This hypergastrinemia led to reduced survival rates and the addition of gastrimmune, the gastrin immunogen, reversed the survival effect, as well as the increased proliferation. In this model, hypergastrinemia promoted adenoma progression [261].

To conclude, whether hypergastrinemia plays a role in the progression of colonic cancer or colonic cancer leads to hypergastrinemia is still controversial but both concepts may be correct.

4.4 Hypoxia

Hypoxia, or low levels of O2, is a common characteristic of many solid tumors due to poor vasculature. Hypoxia stimulates the transcription of approximately 1.5% of genes in tumor cells [262], inducing both tumor growth and angiogenesis via the transcription factor hypoxia-inducible factor 1 (HIF-1α). Studying HIF-1α has recently been of great interest to understand its involvement in human cancer progression.

4.4.1 Hypoxia-inducible factors (HIFs)

HIF-1 expression was first identified in Hep3B cells in the late 1990s and was defined as a protein regulating the expression of the erythropoietin gene in the presence of low oxygen [263]. Actually, the HIF-1 protein is ubiquitously expressed amongst mammalian cells and regulates the expression of key target genes.

The HIF-1 protein is a heterodimeric transcription factor composed of two subunits, the 120 kDa inducible alpha subunit (HIF-1α) and the 91-94 kDa constitutively expressed beta subunit (HIF-1β). There are two additional isoforms of HIF-1α, HIF-2α, which is expressed in vascular endothelial cells, the kidney, heart, lung and the epithelium of the small intestine and is involved in regulating gene transcription [264], and HIF-3α, which is poorly understood but may play a role in reducing the transcriptional activity of HIF-1α and HIF-2α [265].

HIF-1α is synthetized independently of the oxygen level but its degradation is regulated by O2-dependent prolyl hydroxylation leading to ubiquitinylated HIF-1α, which is quickly degraded by the proteasome [266]. However, under hypoxic conditions, HIF-1α is not degraded, but dimerizes with the HIF-1β subunit and can induce expression of its target genes by binding to the HIF-responsive element (HRE) regulatory regions [267].

HIF-1 plays a role in gene regulation, upregulating the expression of various important growth factors such as VEGF, TNF-α, and IGF-2, which in turn promote growth in tumor cells under hypoxia. Conversely, activated growth factors have been shown to be implicated in increased HIF-1α mediated transcription [268].

4.4.2 Gastrin gene under hypoxia

Gastrin gene is overexpressed in response to hypoxia in rats [269], newborn calves [270] and humans [271].

Exogenous Ggly increased VEGF, the most potent angiogenic factor, at both mRNA and protein levels in different colon cancer cell lines (HT29, DLD-1 and Lovo). A decrease in VEGF expression as well as cell growth and AKT phosphorylation was observed when autocrine progastrin production was blocked by gastrin siRNA. The accumulation of HIF-1α seen in response

to hypoxia in DLD-1 and HT29 cells was not found in the presence of Ggly. Also, treatment with the PI3K inhibitor LY294002 reversed the Ggly-induced increase in VEGF mRNA and protein, so Ggly acts via the PI3K/AKT pathway. Similar observations were made in *in vivo* experiments with an increase in VEGF expression, but no HIF-1 accumulation in transgenic mice overexpressing Ggly. Gastrin precursors thus play a role in the process of angiogenesis by promoting the expression of the pro-angiogenic factor VEGF via the PI3K pathway but independently of hypoxia [272].

Both gastrin promoter activity and HIF-1α expression were increased in a dose-dependent manner by the hypoxia mimetic cobalt chloride ($CoCl_2$) in AGS and AGS-CCK2R cells. However, deletions of some to all of the putative HIF binding sites (HBS) in the gastrin promoter did not change the gastrin promoter activity after treatment by 1% O_2 or $CoCl_2$, compared to the full length construct. Activation of the gastrin gene did not involve binding of HIF-1α to HRE. The absence of changes in gastrin promoter activity in gastrointestinal cells transfected by HIF knockdown shRNA confirmed that HIF-1α did not play any role in hypoxic activation of the gastrin gene [273]. However, hypoxia stimulated gastrin gene expression in LoVo cells via binding of HIF-1α to the gastrin promoter. Additionally, non-amidated gastrins were shown to mediate a hypoxia-induced resistance to apoptosis in LoVo cells [274].

Chemical hypoxia led to greater effects on gastrin promoter activity, mRNA and protein levels than with true hypoxia. Similarly to $CoCl_2$, several other metal ions have been evaluated for their capacity to induce activation of gastrin activity. Indeed, Zn^{2+} ions, but not Ca^{2+}, Ni^{2+}, Mg^{2+} nor Fe^{3+} ions, promoted gastrin gene expression at the promoter, mRNA and protein levels in AGS, DLD-1, HCT116, HT29, SW480 and LNCaP cell lines. *In vivo* studies confirmed the upregulation of the gastrin gene in mice following treatment with Zn^{2+} ions. Activation of the gastrin gene by Zn^{2+} ions was partly via the MAPK and PI3K pathways and dependent on an E-box mechanism, which may induce an epithelial-mesenchymal transition (EMT) [275].

Although Zn^{2+} ions are already used as anti-ulcer drugs in humans [276], further studies on the correlation between zinc homeostasis and gastrins are required to understand their role in the EMT and in various pathological conditions.

4.5 Potential gastrin-based therapies

Gastrins act as autocrine growth factors, therefore gastrins and their receptors could both be targeted.

4.5.1 CCK2R antagonists

Proglumide, derived from glutamic acid, was the first CCK2R antagonist developed in 1968 and led to a decrease of gastric acid secretion [277]. It blocked both CCK1R and CCK2R subtypes and is now mainly used in the treatment of stomach ulcers. Numerous antagonists were then described such as benzotript, an N-acyl derivative of tryptophan, and L-365,260, a derivative of aspercillin, with higher potency and specificity for CCK2R. YM022 and YF476 are both high affinity CCK2R antagonists.

The non-selective antagonists proglumide and benzotript decreased the monolayer growth of six human colon carcinoma cell lines [278]. However, they both displayed low potency for CCK2R and high concentrations were needed to displace Gamide [279]. Proglumide and benzotript also inhibited the gastrin-stimulated proliferation of gastrointestinal tumor cells. In addition, incubation with excess Gamide could not reverse the inhibition of proliferation of MKN45G and AGS cell lines by the antagonists [280]. These results suggested that the low affinity CCK2R antagonists proglumide and benzotript exerted their irreversible and non-competitive growth inhibition of cancer cells via another receptor [281]. Indeed, covalent cross-linking of ^{125}I-gastrin to CCK-C receptor was inhibited by both proglumide and benzotript, but not by CCK-1R-selective or CCK2R-selective antagonists. The antiproliferative effects of proglumide and benzotript were mediated by the CCK-C receptor [282]. Clinical trials with proglumide did not show any survival benefit in patients with advanced gastric cancer [283] or advanced colorectal cancer [284].

The selective CCK2R antagonist L-365,260 bound to CCK2R on AR4-2J with 7.5-fold less potency than Gamide but reduced Gamide-stimulated mitogenesis. L-365,260 treatment decreased the basal growth of LoVo and C146 colon cancer cell lines, but this growth went quickly back up to basal levels. *In vivo*, the increased growth of AR4-2J xenografts stimulated by Gamide was blocked by co-administration of oral L-365,260 [285]. This inhibitor could be used to slow down the proliferation of CCK2R positive and Gamide-sensitive gastrointestinal tumors.

The antagonists YM022 and YF476, displaying a high and selective affinity for CCK2R, effectively decreased acid secretion via CCK2R without affecting histamine-induced acid secretion in dogs or rats [286] [287]. Also, a single subcutaneous injection of YM022 or YF476 reduced histidine decarboxylase (HDC) activity in ECL cells and blocked CCK2R in the stomach of rats for four and eight weeks respectively [288].

Most colorectal carcinomas do not express CCK2R and the existence of several receptor subtypes

suggested that CCK2R selective antagonists will not be used as a general treatment for CRCs.

4.5.2 Anti-secretory agents

Somatostatin acts as an inhibitor of the gastrin autocrine loop because it decreases gastrin release by G-cells by inhibiting transcription of the gastrin gene [289]. The inhibition of gastrin's biological activity by the somatostatin analogs, octreotide and RC160, has been tested in gastrointestinal cancers.

Both gastrin gene expression in the human colon cell line HCT116 and basal growth of the human gastric cell line MKN45G (up to 73% compared to control) were reduced by octreotide [280]. Basal and gastrin-activated growth of *in vivo* gastrointestinal tumors were inhibited by octreotide too [290]. Although a low dose of octreotide was ineffective in patients with advanced colorectal cancer or pancreatic cancer, higher dose treatment slightly increased median survival rate [291].

RC160 is a potent inhibitor of the growth of pancreatic cancers *in vitro* [292] as well as *in vivo* with a significant reduction of the incidence and growth of hepatic metastases in nude mice (using colon cancer cell lines 320 DM and WidR) [293]. In patients with pancreatic cancer, the benefits of long-term administration of RC160 were not relevant but somatostatin analog therapy will potentially be more effective in combination with other treatments including cytotoxic drugs, growth inhibitory factors and immunomodulators [294].

A recent review regrouped the clinical studies using somatostatin analogs to reduce the secretory functions of tumoral cells and showed the absence of a significant objective tumor response or survival advantage in gastrointestinal cancers [295].

4.5.3 Anti-gastrin antibody therapy

Antibodies against gastrins could be a straightforward way to target gastrin-stimulated carcinogenesis. In the late 1980s, the first study testing an antigastrin, specific to the gastrin C-terminus, observed a concentration-dependent inhibition of cell proliferation in HCT116 cells. Addition of excess Gamide decreased this inhibition proving that the antibodies specifically inhibited the effects of gastrin [278]. *In vivo* work showed a decrease in growth of the human colorectal xenograft C523 following infusion of the animals with anti-gastrin antibodies [296]. However, anti-gastrin therapies will be facing unavoidable problems clinically, such as long-term administration, the high concentration and the presence of several gastrin peptides, which would have to be treated simultaneously.

Another approach is the use of gastrin immunogens such as Gastrimmune, also called G17DT. It is an immuno-conjugate composed of the amino terminal part of Gamide linked via a spacer to

diphtheria toxoid, and thus generates antibodies recognizing both Gamide and Ggly (Figure 4.5) [251]. Rats preimmunized with Gastrimmune before injection of the rat colorectal carcinoma cell line DHDK12 had reduced tumor areas and weights and thus the secreted antibodies inhibited *in vivo* tumor growth [247]. Antibodies were raised by Gastrimmune in pigs and Gamide-induced acid secretion was inhibited but no antiproliferative effects on the gastrointestinal mucosa were detected. The secreted antibodies specifically targeted Gamide but not gastrin-34. A clinical phase I/II study in patients with advanced colorectal cancer revealed the safety and efficacy of Gastrimmune immunization. An excess of secreted antibodies was measured allowing competition for gastrin binding with CCK2R-positive colorectal cancer cells, but no tumor regression was detected [297]. G17DT is currently a vaccine in phase III trials for advanced pancreatic cancer, used alone or in combination with gemcitabine. It is also in a phase II/III trial for advanced stomach cancer in combination with 5-fluorouracil and cisplatin, and in a late phase II trial for advanced CRC in combination with irinotecan (https://www.drugbank.ca).

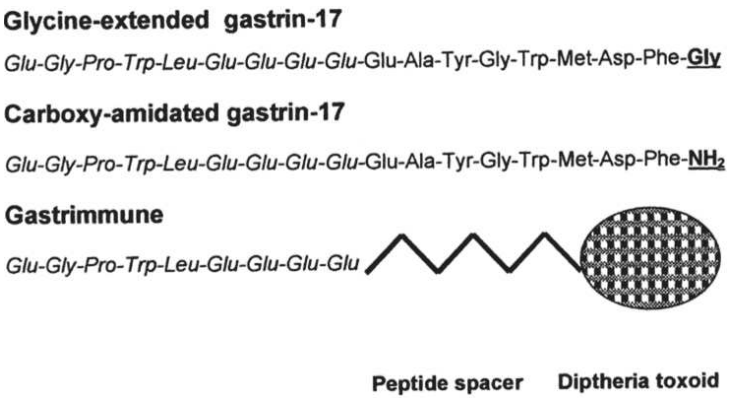

Figure 4.5: The anti-gastrin immunogen, Gastrimmune [251].

A recent study targeting progastrin with antibodies has shown a positive outcome on CRC development. Indeed, anti-progastrin monoclonal antibodies, selectively binding to progastrin, reduced SW620 cell growth up to 40%. The best humanized antibody, Hz8CV2, could detect both native and recombinant progastrin. *In vitro*, Hz8CV2 triggered a dose-dependent diminution of cell growth in SW620 and T84 cells as well as an increase of apoptosis and cell death. Hz8CV2 induced a decrease of cancer stem cell (CSC) frequency with an inhibition from 42 to 62% depending on the CRC cell line. Similarly, humanized anti-progastrin antibodies inhibited CSC frequency in subcutaneously xenografted BALBc/nude mice, especially when combined with chemotherapy. Tumor cells from Hz8CV2-treated mice had reduced migratory and invasive properties. Continuous long-term progastrin antibody treatment in combination with sequential chemotherapy cycles *in vivo* showed a chemosensitivity for SW620 and HT29 tumors as well as a longer survival for T84 tumors. Important observations are the inhibition of the Wnt signaling

pathway and the APC mutation-induced tumorigenesis. To conclude, anti-progastrin antibodies decreased cell growth and increased cell death, and more importantly improved chemosensitivity and survival in combination with chemotherapy *in vivo*. Wnt signaling pathway and tumorigenesis were decreased so anti-progastrin antibodies represent a potential therapy for KRAS-mutated patients [298].

Chapter 5

Gastrin and iron homeostasis

Several lines of evidence, including a correlation between gastric acid and the absorption of dietary iron, and the high affinity binding of two ferric ions to gastrins implicated a role of gastrins in iron homeostasis.

5.1 Iron homeostasis

5.1.1 Description

Humans have internal mechanisms regulating iron concentrations within a physiologic range by transporting, storing or utilizing iron; these mechanisms are especially important following a meal and thus an increase of iron uptake.

Dietary iron is described as heme or non-heme iron. It is easier for the body to absorb iron when it is tightly bound to heme (i.e., heme iron), but this process is poorly understood. Non-heme iron is more accessible as it is kept in a soluble form by the low pH of the stomach leading to its absorption. In the small intestine, non-heme iron crosses the brush-border membrane of the enterocytes through the divalent metal ion transporter 1 (DMT1), which acts as a proton-dependent iron importer of Fe^{2+} after iron reduction by duodenal cytochrome B reductase (DCYTB) (Figure 5.1) [299] [300]. Iron can either be stored in ferritin complexes in the enterocyte, as well as in the liver and macrophages, or be exported out of the cells if it is required by the body. Iron traverses the basolateral membrane of the enterocytes via the iron exporter ferroportin (FPN) [301]. Iron is transported in the bloodstream and to the organs via the iron carrier serum transferrin (Tf), which binds to cell surface transferrin receptor 1 (TfR1) with high affinity, and to TfR2 with low affinity. Internalization of transferrin is followed by the release of iron via a decrease in endosomal pH [302]. If the cell requires iron for its metabolic functions, it will reach the sites of utilization such as the mitochondria.

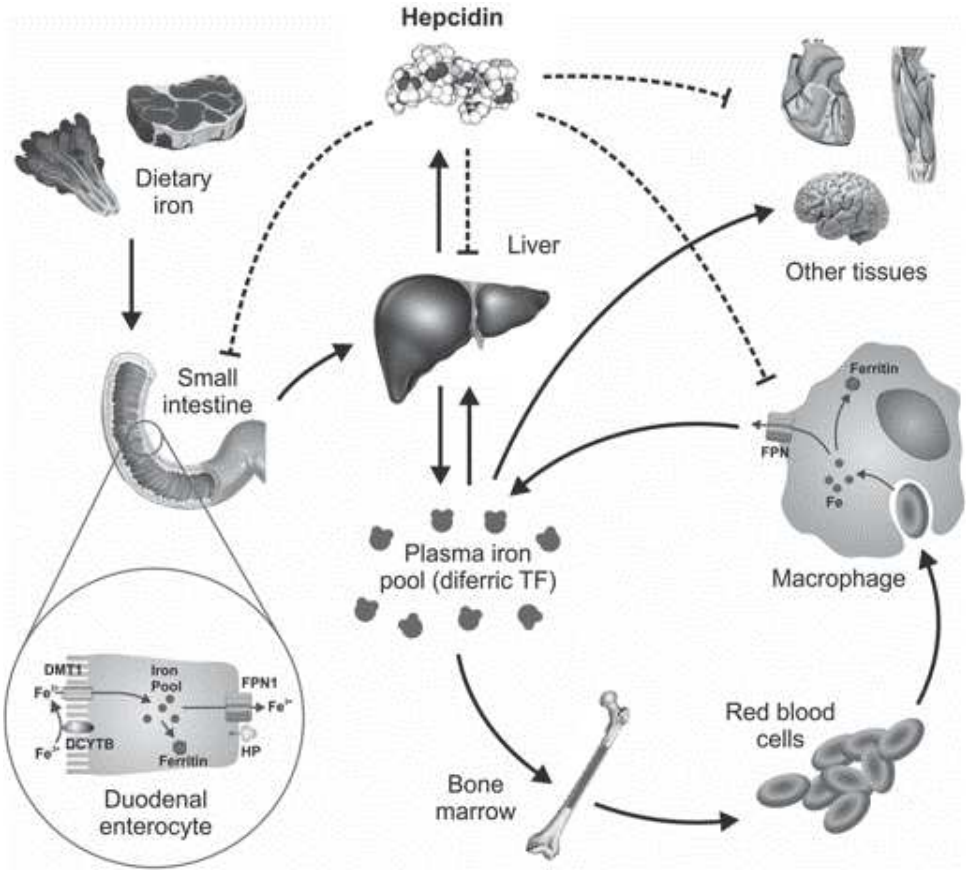

Figure 5.1: Human iron homeostasis [299].

5.1.2 Regulation

Release of iron from duodenal enterocytes into the bloodstream is controlled by hepcidin [303]. This hormone is mainly secreted by the liver and its circulating concentration fluctuates, increasing in the presence of elevated serum iron, iron overload and inflammation, and decreasing if there is a high erythropoietic demand, for example in hypoxia or iron deficiency [304] [305].

Hepcidin inhibits duodenal iron absorption, hemoglobin iron recycling from macrophages and iron mobilization from hepatic stores by binding to FPN, which is then internalized. Blocking iron export could reduce iron uptake in these cells [306]. The hepcidin-ferroportin interaction has a central role in iron homeostasis.

5.1.3 Transferrin saturation

Serum transferrin is one of the main actors in iron homeostasis because it carries absorbed iron from the intestines, and stored iron from the tissues, to cells requiring iron for their metabolic functions. Iron-loaded transferrin binds mainly to TfR1, which is ubiquitously expressed in cells, to trigger cellular iron uptake [307]. TfR2, mostly expressed in hepatocytes, is more involved in the regulation of iron homeostasis as it is upregulated when transferrin saturation increases

[308]. A study reported that a mutation in the mouse transferrin gene led to a production of transferrin lower than 1% compared to the normal level of serum transferrin. These mice were born seriously anemic and died within 14 days of birth unless treated [309]. These observations confirmed the importance of transferrin in iron metabolism.

TfR2 mutations in hemochromatosis patients suggested that TfR2 acted as a sensor of circulating iron-loaded transferrin and activated hepcidin in response to elevated transferrin saturation. TfR2 and HFE, a protein generally mutated in hemochromatosis, interacted in cells and an increase of Fe2-TF could be sensed by HFE affecting the regulation of hepcidin expression *in vivo* [310]. Mice with deletions of both HFE and TfR2 genes had more severe iron loading and a reduction in hepcidin expression compared to mice lacking either HFE or TfR2. This study suggested that TfR2 and HFE acted via different pathways and so their effects on hepcidin could potentially be independent [311]. Intraperitoneal injection of iron in TfR2-null mice did not stimulate hepcidin expression, which confirmed that TfR2 is required for hepcidin production in response to iron [312].

5.1.4 Role of acid secretion

Gastric acid secretion is involved in iron absorption and consequentially in the bioavailability of non-heme iron. 60 years ago a study reported that iron-deficiency anemia was a long-term consequence of partial gastrectomy [313]. Another work showed that patients with achlorhydria, the absence of hydrochloric acid in their gastric secretions, had a clearly reduced dietary iron absorption, at least in iron-deficient subjects [314]. Iron absorption in patients with achylia gastrica, the partial or complete absence of gastric juice, but in the absence of anemia, was decreased compared to control. The defective absorption was corrected by the administration of normal, but not of neutralized, gastric juice suggesting that pH plays a role in the effect by gastric juice. Acidity may dissolve and ionize food iron, and may maintain soluble forms of iron, which could explain the correlation between pH and iron absorption [315].

Treatment of rats with the proton pump inhibitor omeprazole resulted in a reduction of the absorption of dietary iron (by 43%) and ferrous iron (by 50%) when the animals were fed an iron-deficient diet, but not a normal diet [316]. Following unsuccessful studies on patients, a correlation between omeprazole and iron deficiency anemia has finally been reported. Omeprazole triggered a profound hypochlorhydria in iron-deficient patients, which decreased the absorption of orally administrated iron and consequently altered the optimal therapeutic dose and benefits. Patients on long-term therapy with omeprazole developed iron deficiency anemia [317].

5.2 Gastrin and iron

5.2.1 Gastrin-ferric ion complex

In the 1990s, it was suggested that gastrin could be involved in iron uptake from the gastrointestinal lumen as a chelator of metal ions to luminal albumin and transferrin [318]. Both Ggly and Gamide bind two ferric ions in aqueous solution. Fluorescence quenching experiments reported that truncated Ggly(1-11) had a similar affinity for ferric ions compared to the intact form of Ggly and suggested that one or more of the five glutamic acid residues at positions 6-10 (Glu_5 sequence) were important for iron binding. Interestingly, the binding of ferric ions to Ggly was stronger at pH 2.8 than to the commonly used ferric chelator nitrilotriacetic acid. As the resting pH of the gastric juice is about 2, that confirmed the potential role of progastrin-derived peptides in iron uptake [319].

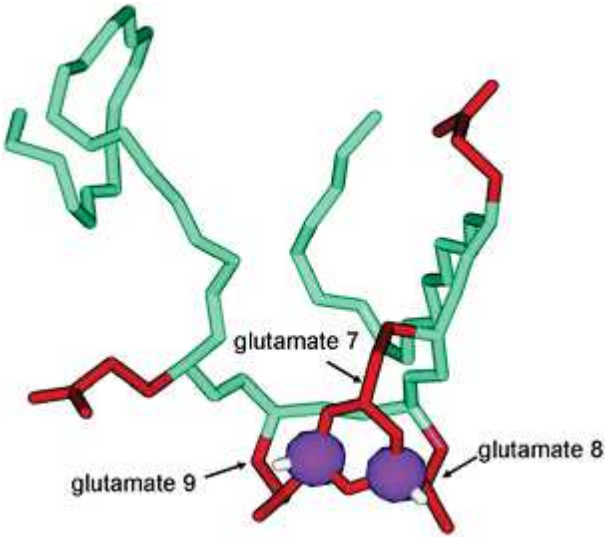

Figure 5.2: Hypothetical model of the gastrin-ferric ion complex. The side chains of the glutamate residues are shown in red, the peptide backbone in green and the ferric ions in purple [320].

The team has previously determined the structure of Ggly in aqueous solution by NMR spectroscopy and have investigated the effect of ferric ions on the Ggly NMR spectrum (Figure 5.2) [320]. Glutamate 7 acted as one of the ligands for the first ferric ion binding site and Glutamate 8 and Glutamate 9 acted as ligands for the second ferric ion binding site. Mutant Ggly peptides, with one or several of the glutamates replaced by alanines, confirmed these observations by fluorescence quenching. Only truncated forms of Ggly containing the Glu_5 sequence triggered IMGE-5 proliferation and migration. Glutamate 7 was especially important as GglyE7A and GglyE6-10A were inactive. In addition, treatment with the iron chelator DFO fully blocked the biological activity of Ggly in IMGE-5 cells but reduced Gamide-induced IMGE-5 cell pro-

liferation by only 60%. It was suggested that Glutamate 7 acts as a bridging ligand for both ferric ions and that Glutamate 8 and Glutamate 9 each interact with one of the ferric ions [320] [321]. Further studies revealed that the heptapeptides LE_5A and E_5AY each bound two ferric ions, and that iron binding was essential for proliferation activity. They are the shortest fully active fragments of Ggly [322]. Taken together, these observations confirmed that the biological activity of Ggly requires binding of ferric ions to Glutamate 7.

5.2.2 Functional studies

DFO blocked the activation of proliferation in rat and mouse colonic mucosa by non-amidated gastrins, but did not affect the binding of Gamide to the CCK2R or the biological activity of Gamide. These observations confirmed that ferric ions were not essential for the biological activity of Gamide and that CCK2R and the Ggly receptor are distinct [323].

On another note, Gamide interacted with the iron transporter transferrin in extracts of porcine gastric mucosa by covalent cross-linking [324]. Gamide bound to the iron-free transferrin, apotransferrin, but not to diferric transferrin, and Ggly also interacted with apotransferrin. The addition of the chelator EDTA blocked the interaction between apotransferrin and both Gamide and Ggly, confirming the presence of gastrin-ferric ion complexes [325].

Patients with HFE-related hemochromatosis had an increase of circulating Gamide and Ggly [326]. Although $HFE^{-/-}$ mice had high concentrations of gastrins in the gastric mucosa and plasma, the number of parietal cells and the pH of the luminal contents was not affected. Increased concentrations of circulating gastrins may be an explanation of the fact that patients with HFE mutations have a higher risk of CRC [327].

Gastrin KO mice, which lack all forms of gastrin, displayed a significant reduction of acid secretion but a week of gastrin infusion reversed these results and led to metaplasia and polyp development. Amongst thirteen down-regulated genes in gastrin KO mice, hepcidin mRNA was decreased to 40% compared to WT mice (100%) but increased back to 130% following subcutaneous Gamide treatment [328]. A sixfold decrease in intestinal iron absorption was measured in Gastrin KO mice and a fourfold and fivefold increase in DMT-1 mRNA was found in gastrin KO mice and CCK2R KO mice, respectively, compared to WT mice. However, gastrin KO mice had a 20% reduction in transferrin saturation while CCK2R KO mice showed a 50% increase [329].

5.2.3 Impact on transferrin saturation

Our team has proposed a mechanism to explain the correlation between fluctuating concentrations of circulating Gamide and changes in transferrin saturation [329]. After export of ferric ions from the enterocyte to the bloodstream by FPN, circulating Gamide or Ggly may accom-

pany the uptake of ferric ions by apotransferrin. Dissociation of Gamide after iron transfer could explain the lack of evidence regarding the binding of Gamide to diferric transferrin [330]. In addition, hGas mice lacking the CCK2R had an increase in transferrin saturation, which showed that Gamide acted independently of the CCK2R. Consequently, Gamide plays a catalytic role in the binding of iron by transferrin leading potentially to changes in hepcidin gene expression [331].

5.3 Consequences in CRC

Iron homeostasis and gastrins have both been reported to play a role in the development of CRCs.

5.3.1 Iron and CRC risk

In early 2000, a review collected results from several experimental techniques on whether iron increases CRC risk in humans. Firstly, a link between both dietary and body iron stores and an increase of CRC risk was described in a small group of patients [332]. A positive correlation between body iron stores and the development of precancerous lesions in the colon, such as colonic adenomas and polyps, was shown [333]. Increased transferrin saturation was significantly associated with CRC development, especially among men [334]. It was reported that patients with hemochromatosis and also carrying a mutation in the transferrin receptor gene had their risk to develop CRC increased more than eightfold compared to healthy individuals [335]. While some studies have reported a positive association between transferrin saturation and CRC risk and between serum ferritin levels and the formation of colorectal adenomatous polyps [333] [336], other studies, however, described inverse correlations between transferrin saturation [337] or ferritin levels [338] and CRC risk. These contradictory data may be due to the fact that transferrin saturation and ferritin vary according to both age and gender in healthy individuals. Diurnal and day-to-day variation of up to 25% in both transferrin saturation and ferritin concentration were reported [339].

Dietary iron and body iron stores impact iron homeostasis and so contribute to the development of CRCs. Red meat is a major dietary source of iron, especially in Western societies, and has a high quantity of myoglobin and hemoglobin, which both comprised of heme containing a central iron atom. Dietary heme has been suggested to damage the colonic mucosa via an unknown intermediate resulting in a compensatory hyperproliferation of the epithelium and consequently an increase in risk for CRC development [340]. A study reviewing the epidemiological literature on meat and CRC reported that high intakes of red meat and of processed meat were correlated with increased risk of CRC [341]. However, the exact mechanisms explaining the association between high intake of red meat and CRC risk are still not clear, but can possibly be related to

several factors such as N-nitroso compounds (NOCs), heterocyclic amines (HCAs), polycyclic aromatic hydrocarbons (PAHs), heme iron from red meat and bile acids [342].

At the cellular level, iron absorption may occur in the colon too, which suggests the presence of colonic iron transport and effects on cell proliferation and cancer development. Levels of iron, transferrin receptor mRNA and ferritin mRNA expression were higher in colonic epithelial cells in iron-loaded rats [343]. A study on 11 human colorectal cancers reported a correlation between progression to CRC and highly expressed iron import proteins. Inhibition of iron export due to decreased expression and aberrant localization of hephaestin and FPN, respectively, was observed too. Cell proliferation may be triggered by the retention of iron by tumors inducing proliferation and repressing cell adhesion [344].

Genetic factors, including the previously mentioned mutations in the HFE gene, can influence body iron stores and lead to CRC development. A correlation between iron and colorectal carcinogenesis was suggested leading to studies on the effect of iron in mutant APC-containing cell lines (Caco-2 and SW480). APC mutations are present in all FAP patients and in almost all sporadic CRC patients. Indeed, in cell models carrying an APC mutation, both inorganic iron and heme-induced Wnt signaling was increased, as well as c-MYC and Nkd1 mRNA expression and cell proliferation. The oncogene c-MYC has been shown to induce both TfR1 and the iron regulatory protein 2 (IRP2) expression and to suppress the iron storage protein ferritin [345]. Increased TfR1 and a loss of ferritin expression were associated with elevated intracellular iron deposition in the development of CRC [344]. Although iron alone was insufficient to induce carcinogenesis, it could increase colorectal tumor incidence and tumor multiplicity in the background of APC loss [346].

5.3.2 Gastrin contribution

Both gastrin and iron are involved in the development of CRC and a synergistic contribution has been suggested. Our team has presented potential mechanisms underlying the involvement of gastrins in iron homeostasis and CRC (Figure 5.3) [347]. Gastrins have two roles, on gastric acid regulation in the stomach and in the availability of iron in the circulation. Following a meal, circulating Gamide stimulates acid secretion by mobilizing histamine from ECL cells in the gastric glands. Ggly potentiates Gamide-stimulated acid release, which most probably requires binding to Fe^{3+} ions like the other biological activities of Ggly. The concentration of soluble Fe^{3+} ions going to the duodenum is enhanced by the acid release. After reduction by DCYTB, ferrous Fe^{2+} ions enter the enterocyte via DMT1 and are exported into the bloodstream by ferroportin. Hepcidin is the main iron regulator and controls ferroportin. Circulating Fe^{2+} ions are re-oxidized to Fe^{3+} ions by hephaestin. Circulating Ggly and Gamide bind to Fe^{3+} ions, catalyze the iron-loading of apo-transferrin and consequently increase the tissue availability of iron. In CRCs, the same process will be observed following the production of non-amidated

gastrins by the tumor and the enhanced availability of Fe-transferrin will result in a stimulation of tumor growth.

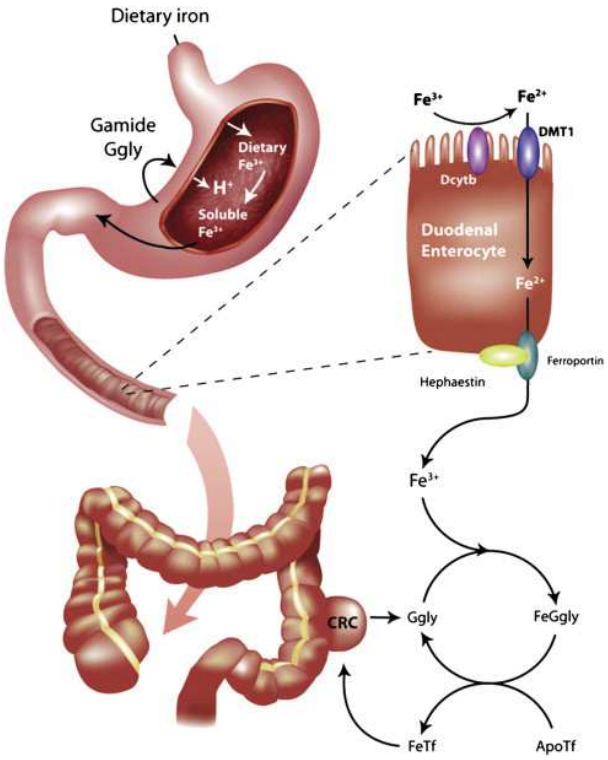

Figure 5.3: A possible dual role for gastrin in iron homeostasis and CRC [347].

5.3.3 Inhibition of gastrin-iron complexes

The association between iron and non-amidated gastrins and their role as growth factors in CRC has opened new therapeutic options based on chelating agents and iron displacement. The advantage of these strategies is the absence of effect on the activity of Gamide.

Chelating agents

Gastrins bind two ferric ions and the binding of a ferric ion to Glutamate 7 is essential for Ggly biological activity. The GglyE7A mutant was unable to stimulate proliferation and migration in gastric epithelial IMGE-5 cells and treatment with the iron chelator DFO completely inhibited Ggly-stimulated cell proliferation and cell migration *in vitro* [321]. Injection of DFO for four weeks into rats, which had undergone a colostomy, partially reversed the Ggly-stimulated proliferation in the defunctioned rectal mucosa. Mice overexpressing progastrin or Ggly showed a significant reduction of crypt height and proliferation index in the colon when treated with DFO for four weeks, compared to WT FVB/N mice. Interestingly, DFO had no significant effect on iron status in mice [348] or in rats [349] and thus it did not influence iron stores. These obser-

vations could then be explained by the chelator DFO displacing ferric ion from non-amidated gastrins and consequently inhibiting their biological activity [349].

Using iron chelators to block the binding of iron to non-amidated gastrins in the gastrointestinal tract and thus to inhibit cell proliferation was suggested as a novel therapeutic option in CRCs.

Iron displacement

Trivalent bismuth ions competed with ferric ions for binding to lactoferrin [350] and transferrin [351] suggesting the option of using gastrin-bismuth complexes to influence gastrin bioactivity. Indeed, our team showed by fluorescence quenching that Ggly bound two bismuth ions at pH 4 but with an affinity 10-fold lower than for ferric ions. Similar to our study on binding of ferric ions to Ggly, Glutamate 7, Glutamate 8 or Glutamate 9 acted as bismuth ion ligands and both ferric ions and bismuth ions competed for the same metal ion binding sites in Ggly. *In vitro*, treatment with bismuth ions decreased Ggly-induced inositol phosphate production and cell proliferation in HT29 cells as well as migration of IMGE5 cells. In similar conditions, Gamide bioactivity was not affected by treatment with bismuth ions [323].

Further studies by the team indicated that the Ggly-bismuth complex can still bind two ferric ions so bismuth ions and ferric ions do not compete for the same ion binding sites in Ggly. One of the crucial Ggly glutamates (Glutamate 7, Glutamate 8 or Glutamate 9) may bind to both a bismuth and a ferric ion. Also, spectroscopic data clearly defined a different conformation for the Ggly-Bi complex compared to the Ggly-Fe complex and an absence of binding to the Ggly receptor. *In vivo*, stimulatory activities of non-amidated gastrins were fully reversed by treatment with bismuth ions in three animal models including rats, which had had a colostomy and were infused by Ggly, MTI/Ggly mice overexpressing Ggly and hGas mice overexpressing progastrin [352].

Bismuth ions represent a safe and promising candidate for the inhibition of the proliferative effects of non-amidated gastrins, and should be tested in a model of spontaneous intestinal cancers *in vivo* such as the $APC^{\Delta 14/+}$ mice.

RESULTS

Our group has previously identified a correlation between gastrins and iron homeostasis as well as a potential contribution of the gastrin-iron association to the development of CRC.

Iron is an important element in the body as low iron levels can lead to anemia and high iron levels may result in iron overload and organ toxicity. The body maintains the balance between iron intake and excretion by modulating intestinal uptake, recycling iron from old red blood cells, storing it in tissues and transporting it via the transferrin protein.

Gastrins bind to iron and this association is required for Ggly biological activities such as cell proliferation and migration, and consequently in the progression of gastrointestinal cancer cells. Circulating levels of gastrins are increased in mice or patients with iron overload diseases like hemochromatosis. We have also demonstrated that hypoxia, or low levels of oxygen, increased gastrin expression in cell lines and iron absorption in mice. Based on these observations, we suggest that hypoxia, iron and gastrins may be tightly linked.

The overall aim of these studies was to investigate the interplay between gastrins and iron homeostasis in gastrointestinal cancers. We asked the following two questions: Do gastrins play a role in iron homeostasis under hypoxia? Could targeting the gastrin-iron complex result in novel therapies for CRC?

Specific aims:

- To determine the physiological significance of the upregulation of gastrins in response to hypoxia *in vivo* (paper I).
- To investigate the activation of the gastrin gene promoter by zinc ions in colon cancer cells *in vitro* and *in vivo* (paper II).
- To complex Gamide with the radioactive isotopes of indium, ruthenium or gallium and to test their abilities to bind to CCK2R in order to potentially use them for the detection of tumors expressing the CCK2R (paper III).
- To displace binding of Fe^{3+} to Ggly by using Bi^{3+}, In^{3+} or Ru^{3+} and to analyse the effects of this competition on the development of intestinal tumors in $APC^{\Delta 14/+}$ mice

(paper IV).

Chapter 6

Paper I

Increased gastrin gene expression provides a physiological advantage to mice under hypoxic conditions. Am J Physiol Gastrointest Liver Physiol 2014.

6.1 Introduction

The mature form of gastrins, Gamide, was firstly discovered for its ability to stimulate acid secretion. Gamide binds to the CCK2R. The immature forms of gastrins, progastrin and Ggly, also play a role in cell proliferation and migration, and in stimulation of cell survival. However, the receptors and mechanisms involved have not been described yet.

Hypoxia, or a low oxygen level, is a characteristic of many solid tumors. Hypoxia increases gene transcription [262] but there is significant heterogeneity in response between different cell types. These transcriptional modifications can lead to more aggressive cancer cells, and are associated with resistance to standard treatments [353]. Although there are few data on gastrins and hypoxia, the gastrin gene is upregulated by hypoxia in the human gastric carcinoma cell line AGS by HIF-independent mechanisms [273]. The mRNAs for most peptides have their translation stopped under hypoxia but the translation of the gastrin mRNA is persistent in several gastrointestinal cancer cell lines. Hypoxia increases expression of the gastrin gene in rats [269], newborn calves [270] and humans [354] [271]. Knockdown of the gastrin gene in LoVo cells (a human colorectal cancer cell line) intensifies apoptosis *in vitro* and necrosis *in vivo* [274].

Our group has previously demonstrated by fluorescence quenching that Gamide and Ggly bound two ferric ions, via a region of five consecutive glutamate residues, with high affinity in an aqueous solution [319]. A complex between recombinant human progastrin6-80 and two ferric ions was demonstrated [355] and this binding inhibited the cleavage of progastrin to Ggly by prohormone convertase 1 *in vitro* [356]. The inhibition of Ggly activity by the iron chelator desferrioxamine (DFO) or the complete lack of activity of the Glu7 mutant (GglyE7A) showed that ferric ion binding was essential for Ggly biological activity *in vitro* [321]. Similarly, DFO reduced crypt

height and proliferation index in the colonic mucosa of mice overexpressing Ggly [349]. However, the biological activity of Gamide was not dependent on binding of ferric ions [357]. Bismuth ions are also trivalent and displaced ferric ions from the iron binding sites of lactoferrin and transferrin so they could act in a similar way with Ggly. The production of inositol phosphate in the human colorectal carcinoma cell line HT29 and the migration of the gastric epithelial cell line IMGE5 in response to Ggly activation were significantly decreased by bismuth ions [323].

A correlation between gastrins and changes in iron homeostasis in response to hypoxia was strongly suggested. The aim of this study was to determine if upregulation of gastrins plays a role in cell protection in response to hypoxia. hGas mice, which overexpress human progastrin in the liver, were maintained either in normoxia or hypoxia and their health and body weights were compared to mice of the parental FVB/N strain. Physiological parameters such as full blood count, serum iron and ferritin concentrations, tissue iron concentrations and expression of genes encoding for a regulator of iron homeostasis were determined.

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Increased gastrin gene expression provides a physiological advantage to mice under hypoxic conditions

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Laval M, Baldwin GS, Shulkes A, Marshall KM. Increased gastrin gene expression provides a physiological advantage to mice under hypoxic conditions. *Am J Physiol Gastrointest Liver Physiol* 308: G76–G84, 2015. First published November 13, 2014; doi:10.1152/ajpgi.00344.2014.—Hypoxia, or a low concentration of O_2, is encountered in humans undertaking activities such as mountain climbing and scuba diving and is important pathophysiologically as a limiting factor in tumor growth. Although data on the interplay between hypoxia and gastrins are limited, gastrin expression is upregulated by hypoxia in gastrointestinal cancer cell lines, and gastrins counterbalance hypoxia by stimulating angiogenesis in vitro and in vivo. The aim of this study was to determine if higher concentrations of the gastrin precursor progastrin are protective against hypoxia in vivo. hGAS mice, which overexpress progastrin in the liver, and mice of the corresponding wild-type FVB/N strain were exposed to normoxia or hypoxia. Iron status was assessed by measurement of serum iron parameters, real-time PCR for mRNAs encoding critical iron regulatory proteins, and Perls' stain and atomic absorption spectrometry for tissue iron concentrations. FVB/N mice lost weight at a faster rate and had higher sickness scores than hGAS mice exposed to hypoxia. Serum iron levels were lower in hGAS than FVB/N mice and decreased further when the animals were exposed to hypoxia. The concentration of iron in the liver was strikingly lower in hGAS than FVB/N mice. We conclude that increased circulating concentrations of progastrin provide a physiological advantage against systemic hypoxia in mice, possibly by increasing the availability of iron stores. This is the first report of an association between progastrin overexpression, hypoxia, and iron homeostasis.

gastrin; hypoxia; iron

THE GASTROINTESTINAL PROHORMONE progastrin is produced in G cells of the gastric antrum and processed in a series of steps to the mature amidated form (Gamide) (12). Gamide acts on the cholecystokinin 2 receptor (CCK2R) to maintain and stimulate gastric acid secretion (2, 24). The nonamidated gastrins progastrin and glycine-extended gastrin (Ggly) also have biological activities linked to the development and progression of cancer by activating signaling pathways, stimulating proliferation, migration, and cell survival (11, 16, 30). However, the identity of their receptors remains under debate. Several recent studies provide evidence of previously unrecognized targets of progastrin-derived peptides, including matrix remodeling (11), angiogenesis (8, 10), and macrophage differentiation (17).

Previously, our group found that Gamide and Ggly bound two ferric ions (6). Iron binding was critical for Ggly bioactivity in vitro (27) and in vivo (13, 22) but was not necessary for activation of CCK2R by Gamide (29). Bismuth ions can block Ggly activity by displacing iron (22, 28). Recombinant human progastrin also binds ferric ions with high affinity (4), and the observation that binding of iron was able to block cleavage of synthetic progastrin to Ggly by prohormone convertase 1 indicates that iron binding may regulate progastrin processing in vivo (9).

Several lines of evidence suggest that gastrins may be involved in the regulation of iron homeostasis (20). Increased concentrations of circulating gastrins were observed in mice and humans with the iron overload disease hemochromatosis (32). Furthermore, saturation of transferrin, the main iron transport protein in the body, was reduced in gastrin-deficient mice but increased in humans with hypergastrinemia caused by multiple-endocrine neoplasia (23). The observation that Gamide and Ggly bind to apotransferrin, but not to diferric transferrin, in vitro at physiological pH values (5, 21, 25) suggests that gastrins may catalyze the loading of apotransferrin with iron. The interrelationship between gastrins and iron homeostasis appears to be physiologically important, as hematopoiesis was disturbed in gastrin-deficient mice subjected to dietary iron deficiency (19).

Hypoxia, or reduced O_2, increases expression of the gastrin gene in rats (36), newborn calves (26), and humans (1, 7, 18, 31). Translation of the gastrin mRNA continues in several gastrointestinal cancer cell lines during hypoxia, while translation of other peptides is inhibited because of the presence of an internal ribosomal entry site (15). The gastrin gene is upregulated by hypoxia in the human gastric carcinoma cell line AGS by a mechanism independent of hypoxia-inducible factors (35). In fact, gastrins may play a protective role during hypoxia, as knockdown of the gastrin gene in the human colorectal cancer cell line LoVo increased apoptosis in vitro and necrosis in xenografts in vivo (34).

Thus there is abundant circumstantial evidence that gastrins may be involved in the compensatory changes in iron homeostasis in response to hypoxia. The aim of this study was to investigate the physiological significance of the upregulation of gastrins in response to hypoxia. The effect of hypoxia on the body weight and health of hGAS mice, which overexpress progastrin in the liver, was compared with the effect of hypoxia on mice of the parental FVB/N strain. The mechanisms of the effects were investigated by measurement of hematological parameters, critical regulatory genes by PCR, and tissue iron concentrations by Perls' stain and atomic absorption spectrometry. The results are the first report of a link between progastrin overexpression, hypoxia, and iron homeostasis.

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MATERIALS AND METHODS

Mouse strains. hGAS mice, which overexpress human progastrin in the liver, were developed on the FVB/N background and kindly provided by Professor T. Wang (Columbia University, New York, NY) (33). Wild-type FVB/N mice were obtained from the Walter and Eliza Hall Institute of Medical Research (Melbourne, VIC, Australia). All mice were fed a commercial pellet diet supplemented with sunflower seeds and hydrogel (rodent recovery gel) and allowed free access to water. Mice [8–12 wk old, ≥10 mice/group (≥5 males and 5 females)] were acclimatized to hypoxic or normoxic containers 3 days prior to the start of the experiment. Hypoxia [10% O_2, automatically adjusted by an electronic O_2 controller (ProOx model 110, Biospherix, Redfield, NY)] was obtained by injection of compressed N_2 into an almost-airtight container. A similar container that allowed the circulation of normal air was used to obtain normoxia (21% O_2). Both chambers were used under normobaric conditions and were opened once a day to release any accumulated CO_2 and to weigh mice and evaluate their health. Sickness scores were determined using a combination of clinical observations, such as mouse activity, breathing, eating, drinking, movement, body condition, body weight, dehydration, and vocalization. After 10 days, the animals were given an anesthetic overdose of isoflurane before exsanguination by cardiac puncture. Fresh blood (250 μl) was placed into tubes containing EDTA as an anticoagulant. Tissue was collected and flash-frozen in liquid nitrogen or fixed in 10% formalin. All animal work was approved by the Austin Health Animal Ethic Committee (AEC2013-04957).

Full blood examination. Hematological parameters were determined by the Austin Pathology Laboratory (Heidelberg, VIC, Australia) using an automated hematological analyzer (Advia 120, Bayer, Tarrytown, NY).

Tissue staining. Paraffin-embedded, formalin-fixed liver and spleen sections were stained with hematoxylin and eosin or Perls' Prussian blue according to standard techniques. Stained tissue sections were imaged using a Coolscope (Nikon, Lidcombe, NSW, Australia) and analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD).

Iron measurements. Serum was prepared from the remaining mouse blood (~700 μl) for measurement of serum iron, ferritin, and transferrrin concentrations. Serum iron levels were quantitated by the Austin Pathology Laboratory using the Roche/Hitachi cobas c system (Castle Hill, NSW, Australia), and ferritin levels were detected using a Roche ferritin kit. Total transferrin concentrations were determined using an iron/total iron-binding capacity (TIBC) reagent set (Pointe Scientific, Canton, MI). Total serum iron and TIBC were calculated according to the manufacturer's protocol. Transferrin saturation was calculated with the following equation: transferrin saturation = (total serum iron/TIBC) × 100. Hepatic iron levels were quantitated by Regional Laboratory Services (Benalla, VIC, Australia) using atomic absorption spectrometry on liver mouse samples after digestion in nitric/perchloric acid.

Serum erythropoietin quantification. Serum concentration of erythropoietin (EPO) was determined with an EPO ELISA kit (R & D systems, Minneapolis, MN) according to the manufacturer's instructions.

Dmt-1 and Hamp mRNA quantification. Total RNA was isolated from ~100 mg of frozen liver or duodenum of all mice using TRIzol reagent (Life Technologies, Melbourne, VIC, Australia) according to the manufacturer's instructions. Total RNA (5 μg) was used for cDNA synthesis with the SuperScript II First Strand Synthesis system (Life Technologies). The resulting cDNA transcripts of hepatic or duodenal mRNA were used for real-time PCR amplification using a PRISM 7700 sequence detector (Applied Biosystems, Melbourne, VIC, Australia) and TaqMan chemistry. The following primers were used: 5′-AGCACCACCTATCTCCATCAACA-3′ (forward) and 5′-GCTTTCTTCCCCGTGCAA-3′ (reverse) for *Hamp*, 5′-CTGCAGCTCTGTAGTCT-3′ for *Hamp* MGB probe, 5′-GCGGCCAGTGATGAGTGAGT-3′ (forward) and 5′-ATGCCACCGGCAATCCT-3′ (reverse) for *Dmt-1*, and 5′-CAGCCTATTCCATTGGAA-3′ for *Dmt-1* MGB probe. Gene expression was quantitated relative to 18S RNA expression.

Statistics. Values are means ± SE. Significance was determined by one-way ANOVA followed by Student's *t*-test with a Holm-Sidak correction. Statistics were analyzed using SigmaStat and graphed using SigmaPlot (Jandel Scientific, San Rafael, CA).

RESULTS

To test the hypothesis that overexpression of the gastrin precursor progastrin protects mice against systemic hypoxia, responses of hGAS mice and mice of the corresponding wild-type strain (FVB/N) to normoxia (21% O_2) and hypoxia (10% O_2) were compared.

Increased progastrin protects mice against hypoxia. hGAS and FVB/N mice lost significantly more weight and had significantly higher sickness scores under hypoxia than normoxia (Fig. 1). However, FVB/N mice lost weight at a faster rate and had higher sickness scores than hGAS mice. Mice with gastrin gene overexpression appeared to cope better under hypoxic conditions than control mice under the same conditions.

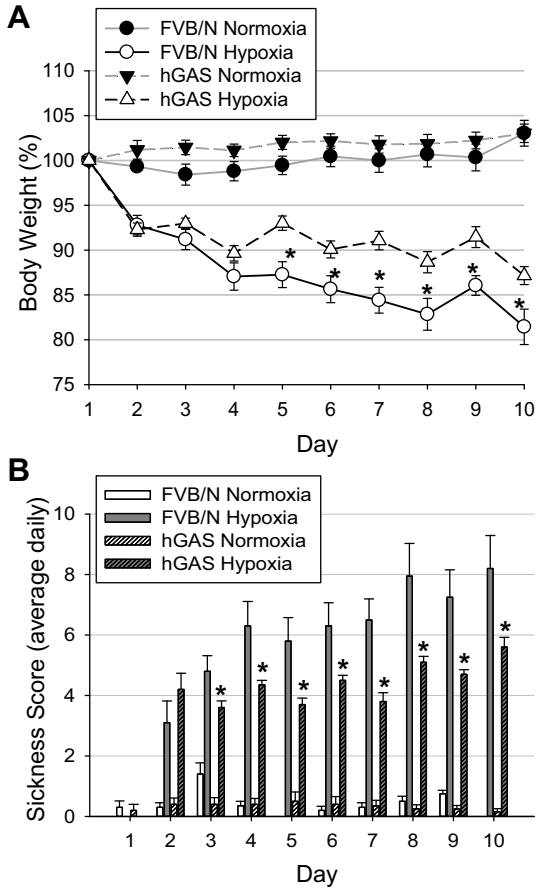

Fig. 1. Hypoxia induced weight loss and increased sickness scores. *A*: total body weight was recorded daily for FVB/N and hGAS mice exposed to hypoxia (10% O_2) or normoxia (21% O_2). Values for each group were normalized to 100% on *day 1*. *B*: sickness scores were higher for FVB/N than hGAS mice exposed to hypoxia. There was no difference between FVB/N and hGAS mice exposed to normoxia. *$P < 0.05$.

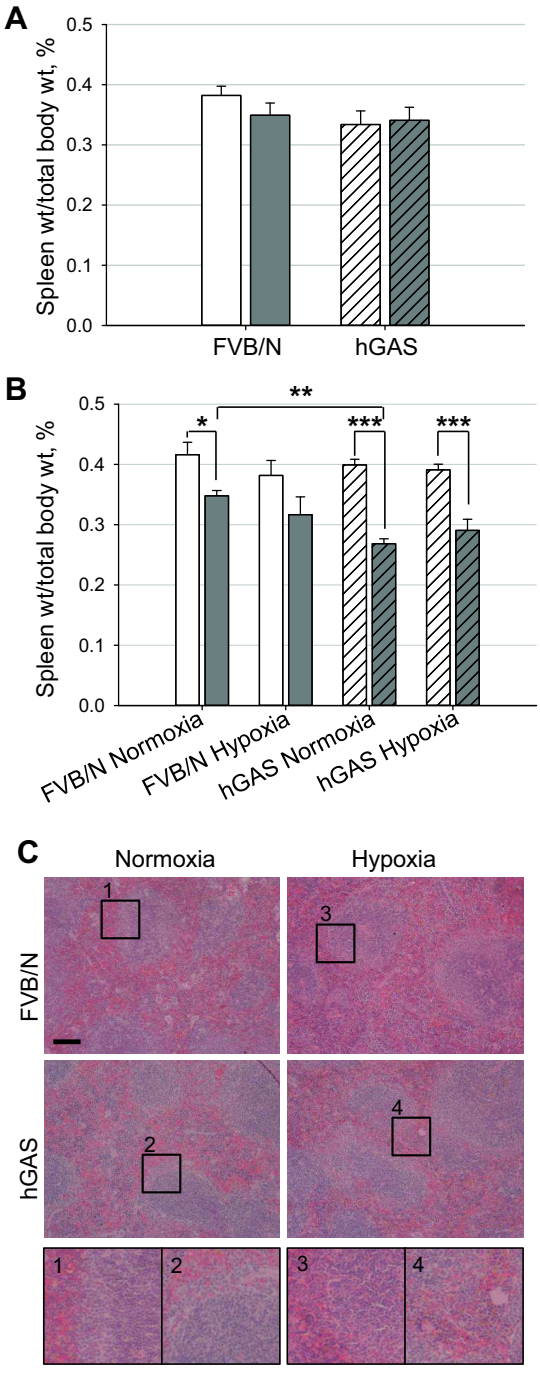

Fig. 2. Spleen weight and histology during hypoxia. *A*: spleen size was determined as a percentage of total body weight for FVB/N and hGAS mice exposed to normoxia (white) and hypoxia (gray) for 10 days. *B*: spleen size for female (white) and male (gray) mice in *A*. *C*: spleen morphology was not substantially different between groups. *Top*: representative hematoxylin-eosin-stained section for each group. *Bottom*: magnified regions from within each image, as indicated by matching numbers. Scale bar = 100 μm. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

hGAS mice have significantly lighter spleens and livers than FVB/N mice. Previous work from our group demonstrated that dietary iron deficiency in gastrin-deficient mice could cause gross splenomegaly (19). To determine whether O_2 status in combination with altered gastrin expression could alter this tissue, the weight and histological sections of the spleen were examined. No difference was found between hGAS and wild-type FVB/N mice in spleen weight, expressed as a percentage of total body weight, when data from male and female animals

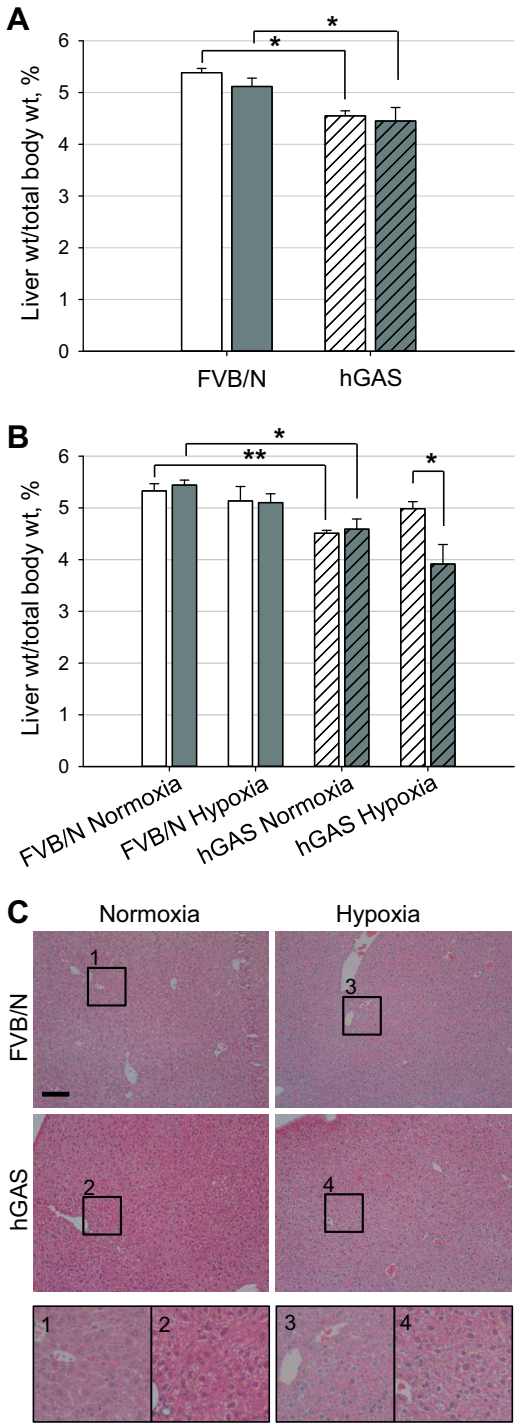

Fig. 3. Liver weight and histology during hypoxia. *A*: liver size was determined as a percentage of total body weight for FVB/N and hGAS mice exposed to normoxia (white) or hypoxia (gray) for 10 days. *B*: liver size for female (white) or male (gray) mice in *A*. *C*: liver morphology was not substantially different between groups. *Top*: representative hematoxylin-eosin-stained section for each group. *Bottom*: magnified regions from within each image, as indicated by matching numbers. Scale bar = 100 μm. $*P < 0.05$; $**P < 0.01$.

Table 1. *Hematological profile of hGAS on FVB/N background and wild-type FVB/N mice under normoxia or hypoxia*

	Normoxia		Hypoxia	
Peripheral Blood	FVB/N	hGAS	FVB/N	hGAS
RBC, 10^{12}/l	8.09 ± 0.20 (11)	8.67 ± 0.20 (12)	8.48 ± 0.35 (16)	8.91 ± 0.83 (10)
Hct, l/l	0.42 ± 0.01 (11)	0.42 ± 0.01 (12)	0.44 ± 0.02 (16)	0.46 ± 0.02 (10)
MCH, pg	15.50 ± 0.16 (11)	14.85 ± 0.20 (12)	15.85 ± 0.31 (16)	14.74 ± 0.19** (10)
MCV, fl	50.64 ± 0.36 (11)	48.58 ± 0.54** (12)	48.88 ± 0.26# (16)	47.10 ± 0.35*# (10)
WBC, 10^9/l	2.00 ± 0.28 (11)	3.03 ± 0.24** (12)	1.86 ± 0.21 (16)	1.99 ± 0.31# (10)
Neutrophils	12.59 ± 3.50 (8)	11.38 ± 1.90 (12)	10.65 ± 1.88 (15)	6.88 ± 2.30 (6)
Lymphocytes	81.80 ± 2.66 (10)	83.28 ± 1.84 (12)	70.91 ± 2.50## (16)	76.58 ± 2.77 (9)
Monocytes	3.30 ± 1.13 (10)	3.80 ± 1.19 (12)	14.76 ± 2.67## (16)	9.96 ± 2.60 (9)
Eosinophils	0.09 ± 0.09 (9)	0.09 ± 0.07 (12)	0.49 ± 0.33 (16)	0.00 ± 0.00 (6)

Values are means ± SE of number of mice shown in parentheses. Serum samples were collected from hGAS and FVB/N wild-type mice under normoxia (21% O_2) or hypoxia (10% O_2). Hct, hematocrit; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; RBC, red blood cells; WBC, white blood cells. Significantly different from FVB/N: $*P < 0.05$, $**P < 0.01$; significantly different from normoxia: $\#P < 0.05$, $\#\#P < 0.01$.

were combined (Fig. 2*A*). However, when the sexes were analyzed separately, spleens from normoxia-exposed FVB/N and normoxia- or hypoxia-exposed hGAS male mice were significantly lighter than spleens of female mice under the corresponding conditions (Fig. 2*B*). Spleens from male hGAS mice were also lighter than spleens from male FVB/N mice under normoxia, but splenic architecture did not appear to be affected (Fig. 2*C*). hGAS mice had significantly lighter livers than FVB/N mice, regardless of O_2 levels (Fig. 3*A*). When the sexes were analyzed separately, no difference in hepatic weight was observed for either strain under hypoxia, except livers were lighter in male than female hGAS mice exposed to hypoxia (Fig. 3*B*). No morphological difference was observed between the liver sections (Fig. 3*C*).

Hypoxia-exposed hGAS mice have smaller red blood cells than hypoxia-exposed FVB/N mice. Hematopoiesis was also disturbed when gastrin-deficient mice were fed a low-iron diet (19). Full blood examinations were therefore done on all animals to see if increased circulating concentrations of progastrin altered blood parameters (Table 1). While differences in the number of red blood cells or hematocrit were not observed, mean corpuscular volume was significantly decreased in both strains under hypoxia compared with normoxia and in hGAS mice compared with FVB/N mice under hypoxia. However, no difference in mean corpuscular volume was observed between strains under normoxia. White blood cell numbers were increased in hGAS mice compared with FVB/N mice under normoxia but were significantly reduced in hGAS mice under hypoxia compared with normoxia.

Serum iron and total ferritin concentrations are reduced in hGAS mice under hypoxia. To determine if the selective advantage observed in hGAS mice was caused by changes in iron status, concentrations of hemoglobin, serum iron, serum ferritin, and transferrin saturation were measured. As expected, hemoglobin was significantly increased in FVB/N and hGAS mice exposed to hypoxia, but no differences were observed between strains (Fig. 4*A*). Interestingly, serum iron levels were

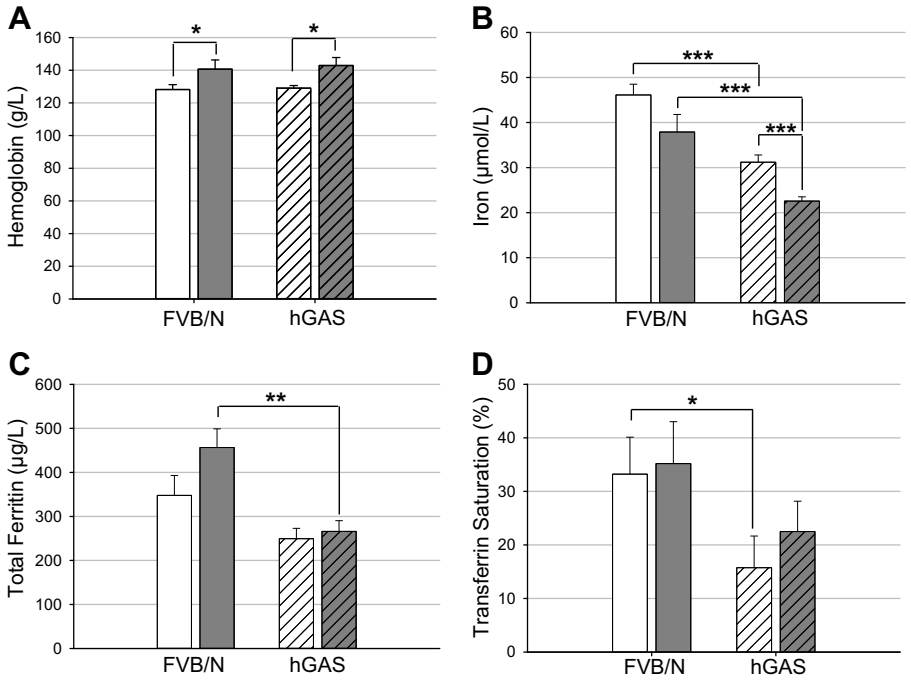

Fig. 4. Serum iron parameters. Serum was collected from FVB/N (solid bars) or hGAS (hatched bars) mice treated for 10 days with normoxia (white) or hypoxia (gray), and hemoglobin (*A*), serum iron (*B*), serum ferritin (*C*), and transferrin saturation (*D*) were measured. Hemoglobin was increased in both strains and total iron was decreased in hGAS mice during hypoxia. Serum iron was significantly reduced in hGAS mice compared with FVB/N mice exposed to normoxia or hypoxia, while transferrin saturation was significantly reduced in hGAS mice compared with FVB/N mice exposed to normoxia. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

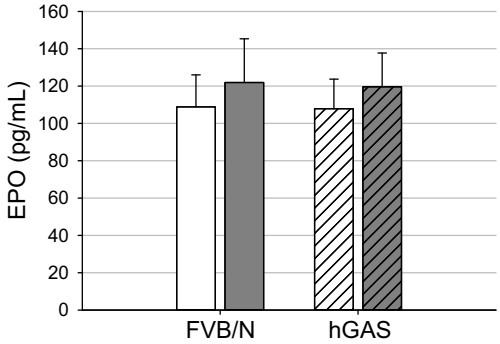

Fig. 5. Erythropoietin (EPO) concentrations are unchanged in hGAS mice exposed to hypoxia for 10 days. EPO concentrations were determined for FVB/N (solid bars) and hGAS (hatched bars) mice exposed to normoxia (white) or hypoxia (gray). No significant difference was observed.

lower in hGAS than FVB/N mice and decreased further when the animals were exposed to hypoxia (Fig. 4*B*). The serum concentration of ferritin, a major iron storage protein, was also decreased in hGAS mice compared with FVB/N mice (Fig. 4*C*). Saturation of transferrin, the main iron transport protein, was decreased in hGAS mice compared with FVB/N mice (Fig. 4*D*).

EPO is not increased by short-term hypoxia. EPO is upregulated in response to an increased need for erythropoiesis (3). As gastrin-deficient ($Gas^{-/-}$) mice fed a low-iron diet developed splenomegaly, likely as a result of significantly increased circulating concentrations of EPO (19), EPO concentrations were measured in hGAS and FVB/N mice under normoxia and hypoxia. No significant differences in EPO concentrations were observed between strains or treatment groups (Fig. 5).

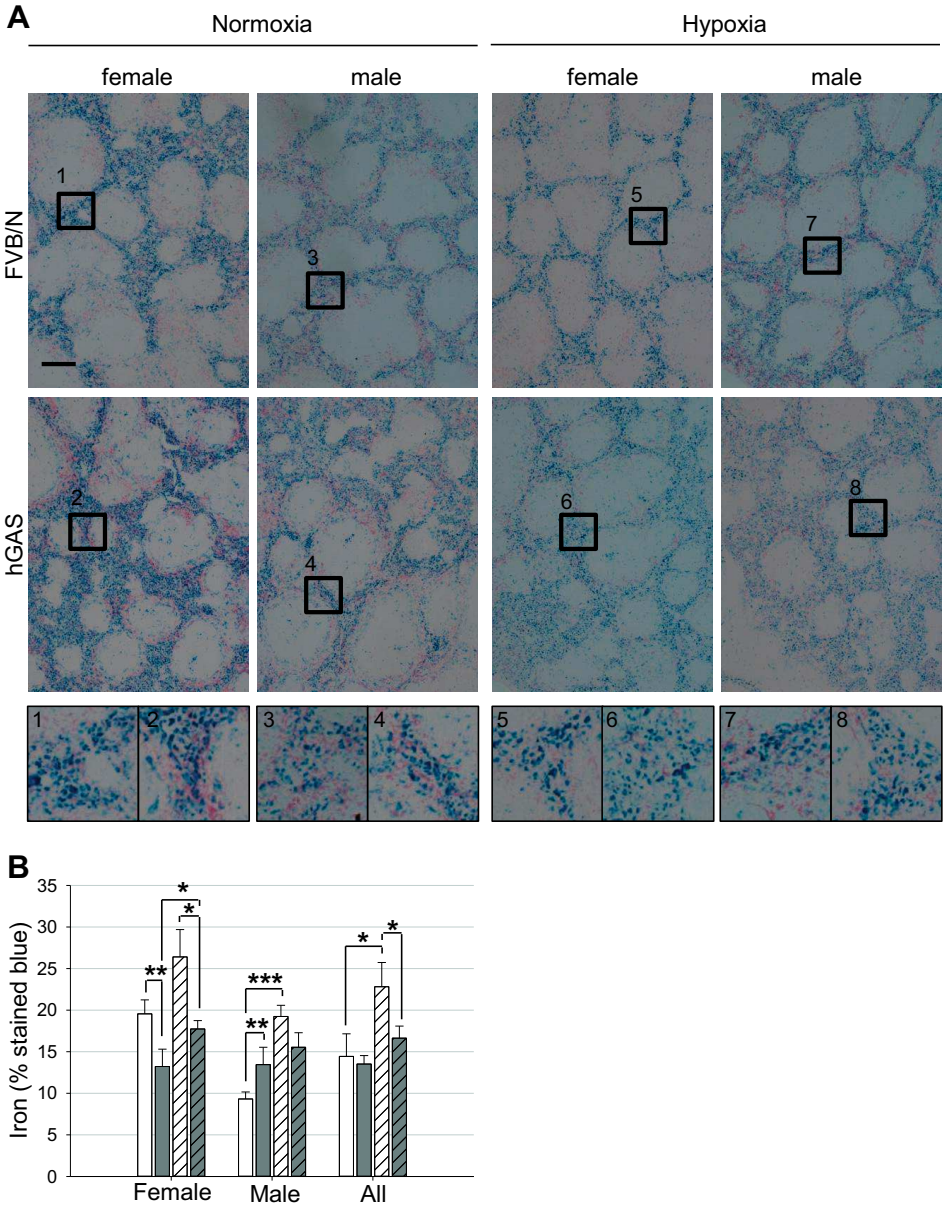

Fig. 6. Iron staining in the spleen. *A*: representative images of spleen sections stained with Perls' Prussian blue (*top*). Blue areas contain iron; pink areas are counterstained with hematoxylin. *Bottom*: magnified regions from within each image, as indicated by matching numbers. Scale bar = 200 μm. *B*: quantification of Perls' Prussian blue staining using Image-Pro Plus. Area of blue staining for FVB/N (solid bars) or hGAS (hatched bars) mice exposed to normoxia (white) or hypoxia (gray) is expressed as a percentage of total area. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Iron concentrations are reduced in the liver, but not the spleen, of hGAS mice. Iron concentrations in the spleen and liver were examined using Perls' Prussian blue stain (Fig. 6*A*), and staining was quantitated using Image-Pro Plus. Iron concentrations were significantly greater in the spleen of hGAS than FVB/N mice under normoxia (Fig. 6*B*). The increase in normoxic hGAS mice was reversed under hypoxia. Surprisingly, Perls' Prussian blue staining revealed a strikingly reduced amount of liver iron in hGAS mice compared with FVB/N mice (Fig. 7, *A* and *B*). Quantification of iron in liver samples using atomic absorption spectrometry verified significantly less liver iron in hGAS than FVB/N mice, and the difference was independent of O_2 status (Fig. 7*C*).

Hepcidin, but not DMT-1 concentrations, are increased in hGAS mice. Quantitation of the expression of *Hamp* [the gene coding for hepcidin, the master regulator of iron homeostasis (3)] by real-time PCR revealed the expected reduction in hepcidin mRNA expression in FVB/N mice exposed to hypoxia (Fig. 8*A*). Concentrations of hepcidin mRNA were higher in hGAS than FVB/N mice under normoxia (Fig. 8*A*), and, in contrast to FVB/N mice, no reduction in hepcidin mRNA expression was observed under hypoxia in hGAS mice. As duodenal iron uptake involves the divalent metal ion transporter DMT-1 (3), expression of the *Dmt-1* gene was also measured. No difference was found for *Dmt-1* mRNA expression in either strain under hypoxia or normoxia (Fig. 8*B*).

DISCUSSION

Hypoxia increases expression of the gastrin gene in rats (36) and newborn calves (26). In humans, exposure to high altitude and, hence, a lower O_2 concentration also increased the amounts of circulating gastrins (1, 7, 18, 31). To investigate the

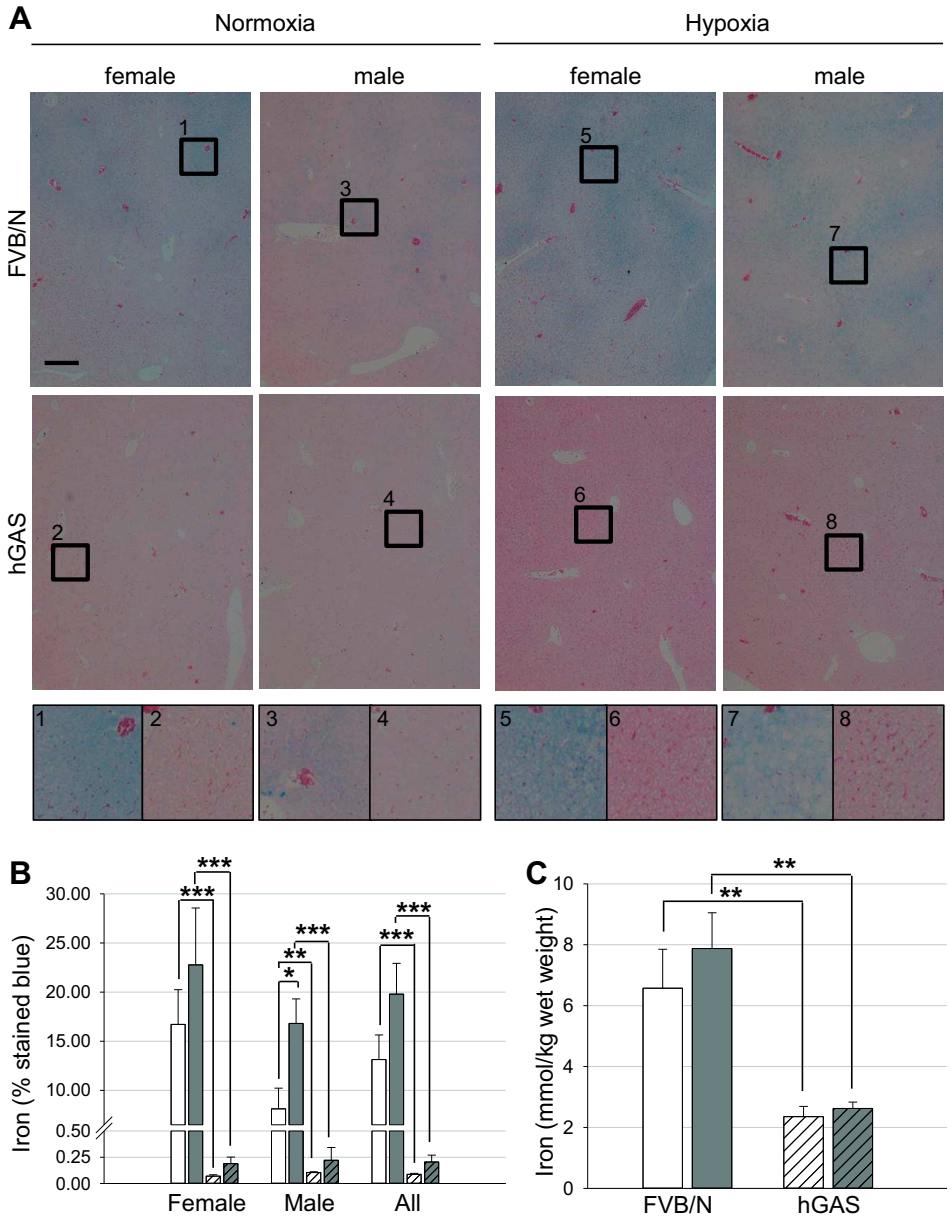

Fig. 7. Livers of hGAS mice are iron-deficient. *A*: representative images of liver sections after staining with Perls' Prussian blue (*top*). Blue areas contain iron; pink areas are counterstained with hematoxylin. *Bottom*: magnified regions from within each image, as indicated by matching numbers. Scale bar = 200 μm. *B*: quantification of Perls' Prussian blue staining using Image ProPlus. Area of blue staining for FVB/N (solid bars) or hGAS (hatched bars) mice exposed to normoxia (white) or hypoxia (gray) is expressed as a percentage of total area. *C*: iron concentrations in formalin-fixed mouse liver were determined by atomic absorption spectrometry. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

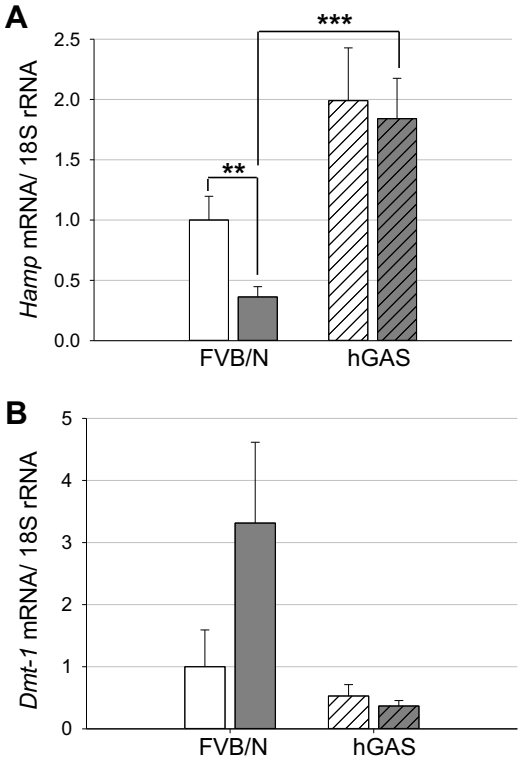

Fig. 8. Iron regulation is altered in hGAS mice. *Hamp* (*A*) and divalent metal ion transporter *Dmt-1* (*B*) mRNA levels were determined in FVB/N (solid bars) and hGAS (hatched bars) mice exposed to normoxia (white) or hypoxia (gray). Increase in *Hamp* mRNA in response to hypoxia in FVB/N mice was not seen in hGAS mice. $^{**}P < 0.01$; $^{***}P < 0.001$.

hypothesis that increased gastrin gene expression would confer a selective benefit on O_2-stressed animals, hGAS mice, which overexpress the gastrin precursor progastrin in the liver, and mice of the corresponding wild-type FVB/N strain were exposed to normoxia or hypoxia, and physiological responses were investigated.

A higher concentration of progastrin decreased the amount of weight loss in animals under hypoxia and reduced daily sickness scores but was not able to restore health to normoxic control levels (Fig. 1). However, elevated progastrin concentrations still provided a significant selective health advantage in mice exposed to hypoxia. These data provide convincing evidence that gastrins may play a protective role in low-O_2 conditions. Previously, our studies with $Gas^{-/-}$ mice showed that gastrins protected against the adverse effects of a low-iron diet. Thus the health of $Gas^{-/-}$ mice deteriorated when the animals were fed a low-iron diet for 6 wk, and the mice developed severe anemia (19). The protective effects of gastrins were not the result of activation of the CCK2R, as $Cck2r^{-/-}$ mice had lower sickness scores (i.e., were healthier) than wild-type controls fed a low-iron diet (19). We therefore predict that the selective advantage in hGAS mice would also be independent of the CCK2R.

Kovac et al. (19) also reported that $Gas^{-/-}$ mice fed a low-iron diet developed splenomegaly, likely as a result of significantly increased circulating concentrations of EPO. Therefore, the effects of hypoxia on the spleen and liver of mice with normal or increased circulating gastrin concentrations were examined. Although the spleens from male animals were generally lighter than those from female animals and the spleens from male hGAS mice under normoxia were significantly lighter than the spleens of male FVB/N mice under the

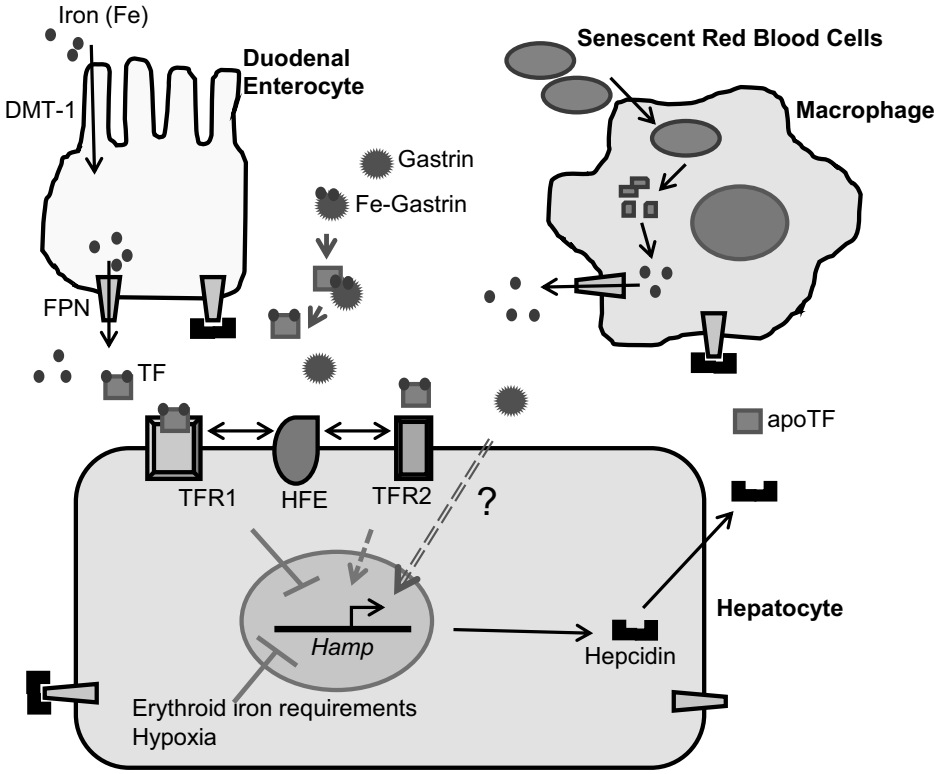

Fig. 9. Model of iron regulation. Basic mechanisms of iron homeostasis are well understood (3). Iron is absorbed into duodenal enterocytes via DMT-1 and exported via ferroportin (FPN) into the circulation, where it binds apotransferrin (apoTF). Transferrin (TF) binds to target cell TF receptors (TFR1 or TFR2), which signal the *Hamp* gene, which encodes the hepcidin protein. Binding of hepcidin to FPN on enterocytes, macrophages, and hepatocytes causes internalization of the hepcidin-FPN complex and, hence, blocks efflux of iron into the circulation. The hereditary hemochromatosis protein HFE interacts with TFR1 and TFR2 but dissociates from TFR1 when iron-bound TF binds, leading to an increased interaction between HFE and TFR2 and, potentially, promoting expression of hepcidin. The present study provides evidence that increased circulating concentrations of progastrin protect against systemic hypoxia in mice, likely by increasing the availability of iron stores. This conclusion is consistent with our previous suggestion (20) that circulating gastrins bind iron and catalyze loading of TF with iron.

same conditions, no significant differences in spleen weight were noted between strains when the sexes were combined (Fig. 2). Consistent with this observation, circulating concentrations of EPO were also unchanged (Fig. 5). In contrast, the livers of hGAS mice were significantly lighter than the livers of FVB/N mice, and this difference was independent of O_2 status (Fig. 3). No differences were observed in spleen or liver architecture after exposure of either strain to hypoxia.

Subtle changes were noted in serum iron parameters. hGAS mice had significantly lower serum iron under normoxia and hypoxia (Fig. 4*B*), lower ferritin concentrations under hypoxia (Fig. 4*C*), and lower transferrin saturation under normoxia (Fig. 4*D*) than wild-type FVB/N controls. Despite this evidence of slightly impaired iron status, the hGAS mice maintained a normal circulating hemoglobin concentration, which could still be increased in response to hypoxia (Fig. 4*A*).

A striking and previously unreported finding was significantly lower concentrations of iron in the liver of hGAS mice than wild-type FVB/N mice (Fig. 7). The initial observation of a low level of Perls' staining (Fig. 7, *A* and *B*) was confirmed by atomic absorption spectrometry (Fig. 7*C*). Previous work from our laboratory showed that gastrins can bind two ferric ions (4, 6, 27). The observations that gastrins bind to apotransferrin, but not to diferric transferrin (21, 25), led to the hypothesis that gastrins were able to catalyze the loading of apotransferrin with ferric ions (20). This hypothesis provides a satisfactory explanation for the finding that serum transferrin saturation is decreased in $Gas^{-/-}$ mice and increased when circulating gastrin concentrations are increased, as in $Cck2r^{-/-}$ mice and in humans with multiple-endocrine neoplasia type 1 (23).

At first sight, the significantly reduced transferrin saturation in hGAS mice (Fig. 4*D*) may appear to be inconsistent with the above-described hypothesis. However, the reduced liver iron (Fig. 7) and serum ferritin (Fig. 4*C*) concentrations suggest that, in the first 8–12 wk of their life, the hGAS mice may have already successfully mobilized most of the readily available iron from their livers and from other body stores. Further studies of iron homeostasis during the early development of hGAS mice might shed light on this issue.

Concentrations of *Hamp* mRNA in the liver were higher in hGAS than FVB/N mice exposed to normoxia (Fig. 8*A*). This observation is consistent with our previous report that *Hamp* mRNA concentrations were lower in the livers of juvenile $Gas^{-/-}$ mice than wild-type controls (23). In this context, it is also interesting to note that in a microarray study, Friis-Hansen et al. (14) showed that $Gas^{-/-}$ mice had reduced concentrations of *Hamp* mRNA in the gastric mucosa compared with wild-type mice and that Gamide infusion for 1 wk partly reversed the reduction. In contrast to FVB/N mice, no reduction in hepcidin mRNA expression was observed in the livers of hGAS mice exposed to hypoxia. Presumably, the fact that most of the readily available iron has already been mobilized from their livers is, by some as yet undefined mechanism, reported to the *Hamp* gene to prevent an increase in its expression.

The basic mechanisms of iron homeostasis (Fig. 9) are well understood (3). Duodenal enterocytes take up ferrous ions from the lumen of the gut via DMT-1. Ferrous ions are exported across the basolateral membrane of enterocytes into the circulation by ferroportin, oxidized to ferric ions, and bound to apotransferrin for transport throughout the body. Iron-bound transferrin binds to transferrin receptor (TFR) 1 (TFR1), and the complexes are internalized to provide target cells with their iron requirements. We have developed a model (20) of how progastrin-derived peptides may participate in the control of iron homeostasis (Fig. 9). Circulating gastrins, such as Gamide, Ggly, and progastrin, bind ferric ions (4, 6, 27). Gamide and Ggly also bind apotransferrin, but not diferric transferrin (5, 21, 25). Our model proposes that gastrins catalyze the uptake of ferric ions by apotransferrin.

We previously reported that hypoxia-inducible gastrin gene expression protects against chronic hypoxia in the human colorectal cancer cell line LoVo in vitro and in vivo (34). The viability of LoVo cells, in which gastrin expression had been reduced by shRNA knockdown, was diminished after exposure to hypoxia (1% O_2) in vitro because of loss of resistance against hypoxia-induced apoptosis, and the effect was partly reversed by treatment with nonamidated, but not amidated, gastrin. Furthermore, necrosis in LoVo xenografts in mice exposed to continuous hypoxia for 21 days was significantly increased by knocking down gastrin gene expression. Thus nonamidated gastrins also appear to play a crucial role in pathophysiological responses to hypoxia. The corollary of these observations is that nonamidated gastrin antagonists, such as bismuth ions (28), may be useful as a novel treatment for carcinoma of the colon.

This study investigated the effects of altered O_2 concentrations on mice with normal or increased levels of nonamidated gastrins. A novel physiological connection between progastrin status, O_2 deprivation, and iron homeostasis was discovered. The surprising finding that hGAS mice cope with hypoxic conditions better than FVB/N control mice appears to be a consequence of more efficient mobilization of hepatic iron stores, as manifested by a significant reduction in hepatic iron concentrations.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.L. and K.M.M. performed the experiments; M.L. and K.M.M. analyzed the data; M.L. and K.M.M. prepared the figures; G.S.B., A.S., and K.M.M. developed the concept and designed the research; G.S.B. and A.S. edited and revised the manuscript; G.S.B. and A.S. approved the final version of the manuscript; K.M.M. interpreted the results of the experiments; K.M.M. drafted the manuscript.

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6.2 Summary

hGas mice overexpressing human progastrin in the liver, and the corresponding wild-type mice (FVB/N), were maintained either in normoxia (21%) or hypoxia (10%) for 10 days. hGas mice had lower sickness scores and lost weight more slowly than control mice under hypoxia. Spleens and livers were significantly lighter in hGas mice than control mice, regardless of O_2 levels but histological analysis did not show any difference in liver or spleen sections. Although hGas mice had smaller red blood cells, but more white blood cells than FVB/N control mice under hypoxia, there was no significant difference in EPO concentrations between strains or with short-term hypoxia treatment. hGas mice had less iron and ferritin, an iron storage protein, in serum than FVB/N control mice and the iron transport protein transferrin was less saturated in mice overexpressing progastrin. In addition, hGas mice had reduced concentrations of iron in the liver. Interestingly, the regulator of iron homeostasis, hepcidin, had its gene expression increased in hGas mice compared to FVB/N mice in normoxic conditions but not under hypoxia. To conclude, this study highlights the fact that gastrins provide significant health benefits to mice under hypoxia and potentially play a role in the control of iron homeostasis by regulating hepatic iron storage. A novel physiological connection between progastrin, hypoxia and iron homeostasis was discovered and should be further investigated.

Chapter 7

Paper II

Activation by zinc of the human gastrin gene promoter in colon cancer cells *in vitro* and *in vivo*. Metallomics 2015.

7.1 Introduction

Gastrin was originally discovered for its ability to stimulate acid secretion and defined as a hormone, which is secreted by the antral G-cells. The mRNA of the 101-residue gastrin precursor preprogastrin has the signal peptide cleaved to form progastrin (80 residues). Following multiple enzymatic processes in the trans-Golgi network, several gastrin intermediates are generated, including glycine-extended gastrin17 (Ggly) and amidated gastrin (Gamide). Although, Gamide was considered for a long time to be the only bioactive gastrin, Gamide, Ggly and progastrin are now all implicated in the progression of cancers of the stomach and colon [358] [359].

Hypoxia stimulates tumor growth and has been shown to increase transcription of about 1.5% of genomic genes. The HIF-1α protein is synthetized in response to hypoxia-induced cellular changes and regulates hypoxia-inducible genes by binding to the hypoxia-responsive element (HRE) within the regulatory sequences of target genes. The upregulation under hypoxia of the gastrin expression at the promoter, mRNA, and protein levels in both human gastric cancer (AGS) and colorectal cancer (LoVo) cells has previously been identified by our group [273]. Although both the hypoxia mimetic cobalt chloride and true hypoxia increased gastrin gene expression, the increase was independent of HIF-1α. Analysis of the effects of various metal ions led us to discover a stimulation of gastrin gene expression (90-fold) by exogenous Zn^{2+} ions in carcinoma cell lines such as AGS, DLD-1, HCT116, HT29 and SW480 [275]. Consequently, zinc ions potentially play a role in the regulation of gastric acidity via induction of gastrin expression. Initial studies with the zinc chelator TPEN suggested that zinc ions may partly regulate the acidity within the tubulovesicular and luminal compartments of the gastrin gland. However, additional studies diverge and have reported that gastric acid secretion was either increased

[360] or reduced by Zn^{2+} [361].

In previous work, transient transfection of plasmids expressing the luciferase gene activated by variants of the human gastrin promoter was used to show upregulation of the gastrin gene by Zn^{2+}. However, stably transfected cells were required for *in vivo* work. We have used the transcription activator-like effector nuclease (TALEN) technology to engineer a luciferase reporter construct knocked-in at the translation start site of the endogenous human gastrin gene promoter in SW480 colon cancer cells ($Gast^{luc}$ SW480 cells). This strategy had the advantages of avoiding variation in gene copy number or site of integration effects, as well as of controlling the exact genomic location of the promoter. The aim was to characterize the activation of the gastrin promoter by zinc ions *in vitro* and most importantly *in vivo* using the engineered $Gast^{luc}$ SW480 cells. Bioluminescence measured in cell culture or in tumor xenografts in SCID mice reflected the activity of the gastrin promoter. Activation of signaling pathways such as MAPK and PI3K, as well as free zinc concentrations, were assessed.

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Activation by zinc of the human gastrin gene promoter in colon cancer cells *in vitro* and *in vivo*†

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Over-expression of growth factors can contribute to the development and progression of cancer, and gastrins in particular have been implicated in accelerating the development of gastrointestinal cancers. Previously our group showed that hypoxia, cobalt chloride (a hypoxia mimetic) and zinc chloride could activate the expression of the gastrin gene *in vitro*. To characterise activation of the gastrin promoter by zinc ions further *in vivo*, TALEN technology was used to engineer a luciferase reporter construct into the endogenous human gastrin gene promoter in SW480 colon cancer cells. Gastrin promoter activity in the resultant $Gast^{luc}$ SW480 colon cancer cells was then measured by bioluminescence in cell culture and in tumour xenografts in SCID mice. Activation of intracellular signalling pathways was assessed by Western blotting. Activation of the gastrin promoter by zinc ions was concentration dependent *in vitro* and *in vivo*. Zinc ions significantly stimulated phosphorylation of ERK1/2 (MAPK pathway) but not of Akt (PI3K pathway). We conclude that the endogenous gastrin promoter is responsive to zinc ions, likely *via* activation of the MAPK pathway.

Introduction

The hormone gastrin was originally identified as a stimulant of acid secretion.[1] The circulating forms of 17 and 34 residues share the same C-terminal tetrapeptide amide as the related hormone cholecystokinin (CCK), and C-terminal amidation is essential for secretory activity. Gastrin is synthesized as a 101-residue precursor (preprogastrin) which, on removal of a 21-residue signal peptide, yields progastrin (80 residues). Proteolytic processing in the antral G cell generates a number of intermediate, non-amidated progastrin-derived peptides, including glycine-extended $gastrin_{17}$ (Ggly), and amidation of glycine-extended gastrins yields amidated gastrin (Gamide). Gamide, acting through the cholecystokinin 2 receptor (CCK2R), is the major hormonal regulator of gastric acid secretion. Gamide, Ggly and progastrin have all been implicated in accelerating the development of cancers of the stomach or colon.[2,3]

We demonstrated for the first time that hypoxia up-regulated gastrin gene expression in the human gastric adenocarcinoma cell line AGS.[4] The increase in gastrin was independent of hypoxia-inducible factor 1 (HIF1), but could be reproduced by the hypoxia mimetic cobalt chloride. Comparison of the effect of other metal ions led us to the unanticipated but remarkable discovery that exogenous Zn^{2+} ions also induced gastrin gene expression by as much as 90-fold in carcinoma cell lines of various origins including gastric (AGS) and colorectal (DLD1, HCT116, HT29, SW480).[5] As gastrin is a major regulator of gastric acidity, this observation raised the possibility that zinc ions may regulate gastric acidity *via* induction of gastrin expression. Previously the zinc chelator TPEN has been used to show that acidity within the tubulovesicular and luminal compartments of the gastric gland may be regulated, at least in part, by zinc ions.[6,7] However, other workers have reported that treatment with Zn^{2+} ions may either increase[8] or reduce gastric acid secretion.[9,10]

To decipher the mechanism responsible for increased gastrin gene expression by Zn^{2+} ions previously we used a traditional reporter gene technology, with transient transfection of plasmids expressing the luciferase gene activated by variants of the human gastrin promoter.[5] However some of these vectors can give rise to aberrant reporter activity because of the presence of cryptic transcription factor binding sites within the plasmid,[11] and *in vivo* work requires the creation of stably transfected cells.

An alternative to using a reporter vector is to replace the endogenous gene of interest with a reporter gene at the original locus.[12] Advantages of this approach include the absence of variation in gene copy number or site of integration effects,

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‡ These authors have contributed equally to this work.

as well as control over the precise genomic location of the promoter, which will contain its own regulatory elements that may not always be seen with cloned gene promoters in vectors.[13] In the present study gastrin reporter Gast^{luc} SW480 cells were generated by knocking-in a firefly luciferase reporter coding sequence at the translation start site of the endogenous gastrin gene in wild type SW480 colon cancer cells. The aim of these experiments was to use the Gast^{luc} SW480 cells to investigate the role of zinc in gastrin promoter activation *in vitro* and most importantly *in vivo* in mice with established xenografts. The intracellular mediators of the effects of zinc have also been assessed.

Material and methods

Cell culture

The human colorectal cancer cell line SW480 was obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Carlsbad, CA, USA) supplemented with 7.5% heat-inactivated fetal bovine serum (FBS, Thermo Scientific, Waltham, MA, USA) and 2 mM of L-glutamine (Thermo Scientific). Cells were grown at 37 °C in a humidified incubator with 95% air and 5% carbon dioxide.

TALEN construction

TALEN sequences were designed using the ZiFiT Targeter software package (http://zifit.partners.org/ZiFiT/). TALENs were constructed using the Joung Lab TAL Effector Engineering Reagents TALEN kit available from ADDgene (www.addgene.org/TALEN/) combined with the REAL (Restriction Enzyme and Ligation) assembly method.[14,15] ESI,† Fig. S1 details the construction method used.

Targeting plasmid construction

The 3′ targeting arm was cloned into the pBSII ks+ plasmid. The luciferase gene was connected to the 5′ targeting arm by PCR and subsequently cloned into the 3′ arm-pBSII ks+ plasmid. LoxP-puro-loxP was added as a removable selection marker (ESI,† Table S1 lists primers used for cloning).

Generation of Gast^{luc} SW480 cells

TALEN plasmids were co-transfected with the targeting plasmid encoding the 3′ and 5′ targeting arms at equal concentrations (500 ng each) into SW480 cells (1.5×10^6) in 100 μl total volume using the Neon Transfection System (Life Technologies). The electroporated cells were diluted into 15 ml of DMEM and seeded into 150 × 20 mm culture dishes, which were incubated at 37 °C for 24 h before selection with puromycin (0.5 μg ml^{-1}) (Gibco, Life Technologies). Selected clones were screened by PCR for random integration (primers: P1 and P2) or targeted integration (primers: P1 and P3) (ESI,† Table S1) and integration was confirmed by Southern blot using Probe A (Fig. 1). Probe A comprised of 401 base pairs located upstream from exon 1 of the human gastrin gene on chromosome 17:39,867,075-39,867,475 (UCSC genome browser Feb 2009 GRCh37/hg19). The human gastrin gene, including UTRs is located on chromosome 17:39,868,578-39,872,221. The gastrin gene promoter is located on chromosome 17:39,867,277-39,868,577.

Gastrin promoter assays

Expression of firefly luciferase was driven by the endogenous gastrin gene promoter. Briefly gastrin wild type Gast^{wt} SW480 and luciferase expressing Gast^{luc} SW480 cells were separately seeded into a 24 well plate (1.0×10^5 cells in 1 ml) and treated with $CoCl_2$ (Sigma-Aldrich, Sydney, Australia) or $ZnCl_2$ (Sigma-Aldrich) in serum free medium for 16 h. Luciferase activity was determined using the Dual Luciferase® reporter assay system (Promega). Cells were lysed with 80 μl per well 1× reporter lysis buffer and firefly luciferase activity was measured with a Fluostar Optima plate reader (BMG Labtech, Mornington, Australia). Relative luciferase activity was normalized to the total protein content of each well determined with a Bradford protein assay kit (Bio-Rad, Gladesville, Australia). LY294002 (Sigma-Aldrich) and UO126 (Cell Signaling, Danvers, MA, USA) were used to inhibit the PI3 kinase and MAP kinase pathways, respectively. Cells were incubated with inhibitors for 30 min prior to metal ion treatment (10 μM) for 16 h.

Protein quantification

Cells (4×10^5 per well) were seeded in 2 ml per well in a 6 well plate and treated with inhibitors or metal ions as described above. Western blots were performed as described previously.[5] The indicated antibodies (Cell Signaling) were diluted 1 : 2000 in Tris buffered saline tween 20 (TBST)–5% BSA for pMAPK (9101S, p-p42/44 MAPK), tMAPK (9102L, p44/42 MAPK (ERK1/2)), pAkt (4060S, S473 DgE mAb), tAkt (4685, pan 11E7) and 1 : 20 000 in TBST–5% BSA for GAPDH (2118L).

In vivo bioluminescence

Animal care and experiments followed the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes endorsed by the National Health and Medical Research Council. All procedures were approved by the Animal Ethics Committee (Austin Health, Melbourne, Australia, project number 2014/5203). Xenografts were initiated by subcutaneous injection of 5×10^6 Gast^{luc} SW480 cells in 100 μl Dulbecco's phosphate buffered saline (DPBS) in the right flank of SCID mice. Tumours were grown to an average volume of 30–50 mm^3 prior to treatment. Mice were then treated with saline (control), 5 mg kg^{-1} $ZnCl_2$, 10 mg kg^{-1} $ZnCl_2$ or 30 mg kg^{-1} $CoCl_2$ on days 2, 8 and 10, and imaged on days 1, 3, 9 and 11. Bioluminescence arising from stimulation of the gastrin promoter was detected using the IVIS® Spectrum for Bioluminescence (Thermofisher Scientific). For imaging, mice were injected i.p. with D-luciferin (150 mg kg^{-1} body weight, ThermoFisher Scientific) prior to induction of anaesthesia with isoflurane. The mice were then transferred onto the imaging platform IVIS and nose cones were placed on the animals to maintain anaesthesia with an isoflurane/oxygen mixture. Images were taken 6 min post luciferin injection for 16 min, with an image every 2 min, to obtain optimal signal data. Data was acquired and analysed with Living Image 4.4 software (Thermofisher Scientific). Bioluminescent counts were converted to radiant units, and expressed as a

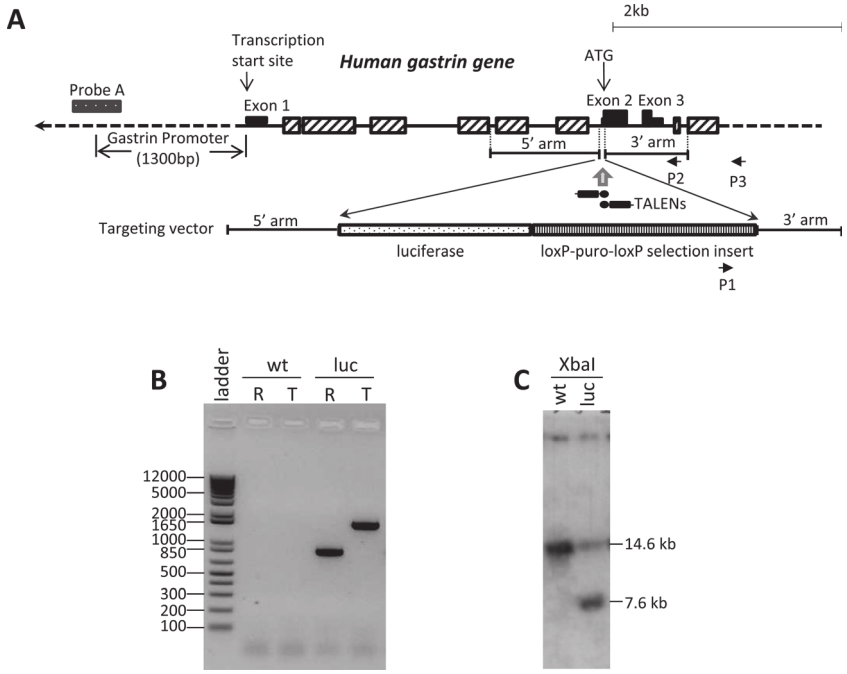

Fig. 1 Targeting and screening for a gastrin promoter-linked luciferase. (A) Targeting plan for incorporating the firefly luciferase gene at the gastrin translation start site at the 5′ end of exon 2 to be translated by the gastrin promoter. Black boxes represent gastrin exons and hatched boxes represent repetitive DNA that makes targeting the human gastrin gene more difficult. TALENs (not to scale) were designed to cleave the gastrin gene at the start of exon 2, promoting DNA repair with the targeting vector. Targeted clones were generated with puromycin selection. Luciferase has a stop site to prevent any random tags being added. The removable loxP-puro-loxP cassette contains its own promoter and stop site. The remaining portion of the gastrin gene has no promoter and is out of frame, and will therefore not be expressed. (B) PCR screening was used to determine random, R (828 bp, primers P1 and P2), or targeted, T (1526 bp, primers P1 and P3), integration of the vector using the primers P1–P3 (arrows, not to scale) in panel A. PCR products were only derived from the cDNA of luciferase ($Gast^{luc}$) clones and not from the cDNA of wild type ($Gast^{wt}$) cells. (C) Southern blot of genomic DNA restriction digested with XbaI from $Gast^{wt}$ (wt) and $Gast^{luc}$ (luc) cells. Probe A (panel A) used in combination with XbaI genomic DNA digestion detects a single band of 14.6 kb from wild type $Gast^{wt}$ SW480 cells and a second band of 7.6 kb from $Gast^{luc}$ targeted SW480 cells.

percent increase compared to untreated control for each treatment group. Our preliminary studies included a dose of 30 mg kg^{-1} zinc chloride, however it adversely affected the health of the mice and therefore was not used further.

MTT cell proliferation assay

Cell proliferation was measured using a colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Briefly, 5×10^4 cells per well were plated into two 24 well plates in DMEM with 7.5% FBS. Next day a MTT assay was performed on one plate to determine the relative cell numbers prior to the start of zinc treatment. The values were recorded as the 0 h absorbance reading. Cells in the second plate were treated with various concentrations of zinc either in the presence or absence of serum and incubated for a further 24 h, after which a second MTT assay was performed. Following the solubilization of formazan crystals in acidified isopropanol the absorbance, which is directly proportional to cell numbers, was measured at a wavelength of 570 nm with background subtraction at 620 nm. Data are expressed as a percentage of the 0 h reading taken just prior to zinc treatment.

Zn^{2+} quantification

Relative zinc concentrations in tissue and serum were determined using a zinc quantification kit (Abcam, Melbourne, Australia) according to the manufacturer's instructions. In brief, tumours (50 mg) were sonicated in 250 μl EDTA-free lysis buffer: 7% TCA (1 : 1) or 50 μl of 7% TCA was added to 100 μl of serum. Samples were centrifuged and 50 μl of each sample was transferred to a 96 well plate. Zinc reaction mix (100 μl per well) was added and the plate incubated for 10 min prior to reading at 570 nm on the Fluostar Optima Plate Reader (BMG Labtech, Australia).

Intracellular free Zn^{2+} measurements

$Gast^{luc}$ SW480 cells (2.5×10^3 cells in 100 μl per well) were seeded onto black 96 well plates and incubated in 5% CO_2 for 24 h at 37 °C. $ZnCl_2$ was added and cells were incubated for an additional 0, 2 or 24 h. FluoZin-3 (AM, cell permeant, F-24195, Life Technologies) was added to a final concentration of 5 μM (50 μl per well) and the 96 well plates were covered with foil to protect them from light. Samples were equilibrated for 30 min before the dye was removed and replaced by Hank's balanced salt solution (HBSS) for 15 min. The resulting fluorescence was recorded on a MicroLumi XS luminometer (Harta Instruments, Gaithersburg, MA, USA). The minimum (F_{min}) and maximum (F_{max}) fluorescence was determined by incubating with zinc chelator *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN) (10 μM) or $ZnCl_2$ (500 μM), respectively. The dissociation constant (K_d) of FluoZin-3 for zinc is 15 nM according to

the manufacturer's instruction manual. Free zinc (nM) was calculated as:

$$\frac{(F - F_{\min})}{(F_{\max} - F)} \times 15$$

Statistics

Data are presented as the average of the mean + SEM. One way ANOVA and student's *t*-test were used to determine significance as appropriate using SigmaStat and graphed using SigmaPlot (Jandel Scientific, San Rafael, CA, USA).

Results

Generation of the knock-in gastrin reporter Gast^{luc} SW480 colon cancer cell line

A knock-in cell line was constructed using wild type human SW480 colon cancer cells to monitor gastrin expression. The gene targeting strategy is shown in Fig. 1A. The coding region of the firefly luciferase reporter protein was expressed at the start site of translation of the endogenous gastrin gene using specifically designed TALENs (ESI,† Fig. S1). This strategy eliminated chromosomal position effects and copy number concerns in cells stably transfected with a reporter vector. Targeting specificity was determined by PCR and Southern blot. Both PCR using external and internal primers (Fig. 1B) and Southern blot (Fig. 1C) with an external probe produced bands consistent with correct targeting event. The removable loxP-puromycin-loxP cassette contained its own promoter and stop site and was used as a selection marker. The remaining portion of the gastrin gene had no promoter or start site and was out of frame, and therefore not expressed.

Zinc and cobalt induce gastrin promoter activity in the Gast^{luc} SW480 colon cancer cells

The induction of the luciferase reporter gene driven by the endogenous gastrin promoter in the gastrin knock-in SW480 colon cancer cells (Gast^{luc} SW480) was examined by assaying luciferase activity.[4] Consistent with our previous report that the gastrin promoter can be stimulated by the divalent metal ions zinc and cobalt,[4,5] treatment of Gast^{luc} SW480 cells with zinc chloride for 16 h increased the relative luciferase activity, which is directly proportional to gastrin promoter activation. Maximal stimulations of 22 ± 3-fold and 68 ± 7-fold were observed following treatment of Gast^{luc} SW480 cells with 50 μM $ZnCl_2$ (Fig. 2A) or 250 μM cobalt chloride (Fig. 2B), respectively. Zinc at 50 μM was approximately 4 times more potent than cobalt chloride at the same dose.

$ZnCl_2$ mediated gastrin promoter activation is dependent on the MAPK pathway

Activation of the gastrin promoter by zinc was dependent on the MAP kinase and PI3 kinase pathways in gastric cancer AGS cells.[5] In order to establish which signalling pathway was involved in the stimulation of gastrin expression by Zn^{2+} ions in colon cancer cells, the gastrin promoter activity was measured after pre-treatment of Gast^{luc} SW480 cells for 30 min with either the PI3K inhibitor LY294002 (10 μM) or the MAPK inhibitor U0126 (10 μM),

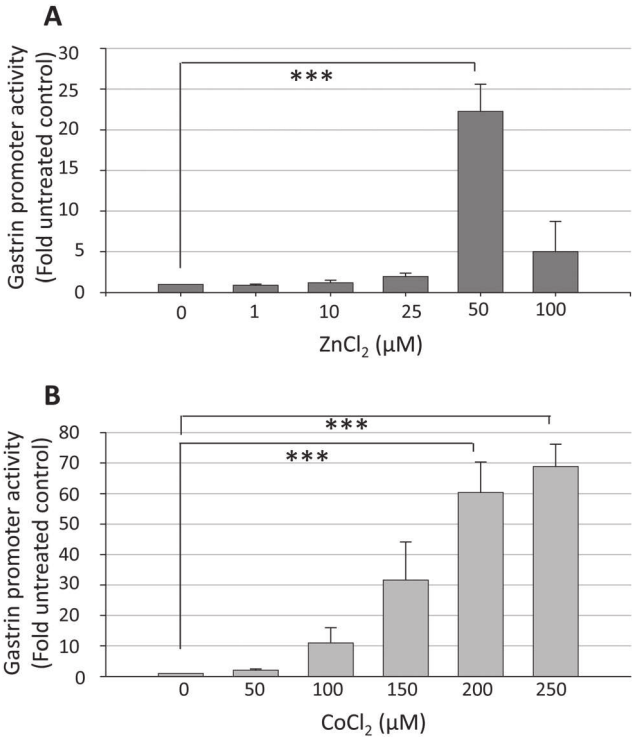

Fig. 2 Activation of the endogenous gastrin promoter. Gast^{luc} SW480 cells that express luciferase under the control of the endogenous gastrin promoter were stimulated with the indicated concentrations of (A) $ZnCl_2$ or (B) $CoCl_2$ for 16 h. Both treatments resulted in concentration-dependent activation of the gastrin promoter, shown as the fold increase in luciferase activity normalised to that of untreated cells (0 μM $ZnCl_2$ or $CoCl_2$). The average + SEM are shown, $n \geq 3$ independent experiments with multiple replicates; ***, $p < 0.001$.

followed by treatment with either 50 μM $ZnCl_2$ or 250 μM $CoCl_2$ for 16 h in the presence of the same inhibitors. As shown in Fig. 3A treatment with the MAPK inhibitor U0126 (10 μM) significantly reduced the zinc- or cobalt-stimulated gastrin promoter activity to 31 ± 9% and 14 ± 3%, respectively, compared to cells treated with zinc or cobalt only (100%). In contrast, the PI3K inhibitor LY294002 had no effect on cobalt-stimulated gastrin promoter activity, but reduced the zinc-stimulated gastrin promoter activity to only 73 ± 2% compared to cells treated with zinc only (100%). To confirm that Zn^{2+} ions activate phosphorylation of Akt (a downstream target of PI3K) and of ERK1/2 (a downstream target of MAPK) in Gast^{luc} SW480 cells, the expression of phospho-Akt and phospho-ERK1/2 was measured by Western blotting. Treatment of Gast^{luc} SW480 cells with 50 μM $ZnCl_2$ increased phosphorylation of ERK1/2 (Fig. 3B) in a time-dependent manner, with a significant decrease in the expression of phospho-Akt (Fig. 3C).

Serum reduces the stimulation of gastrin promoter activity by zinc

Many metals including zinc bind to serum albumin[16,17] and the biological effects of extracellular Zn^{2+} ions are significantly reduced in the presence of serum in cell culture media.[18] Consistent with this observation, the 16 ± 6-fold increase in gastrin promoter activity stimulated by 50 μM $ZnCl_2$ was completely abolished in

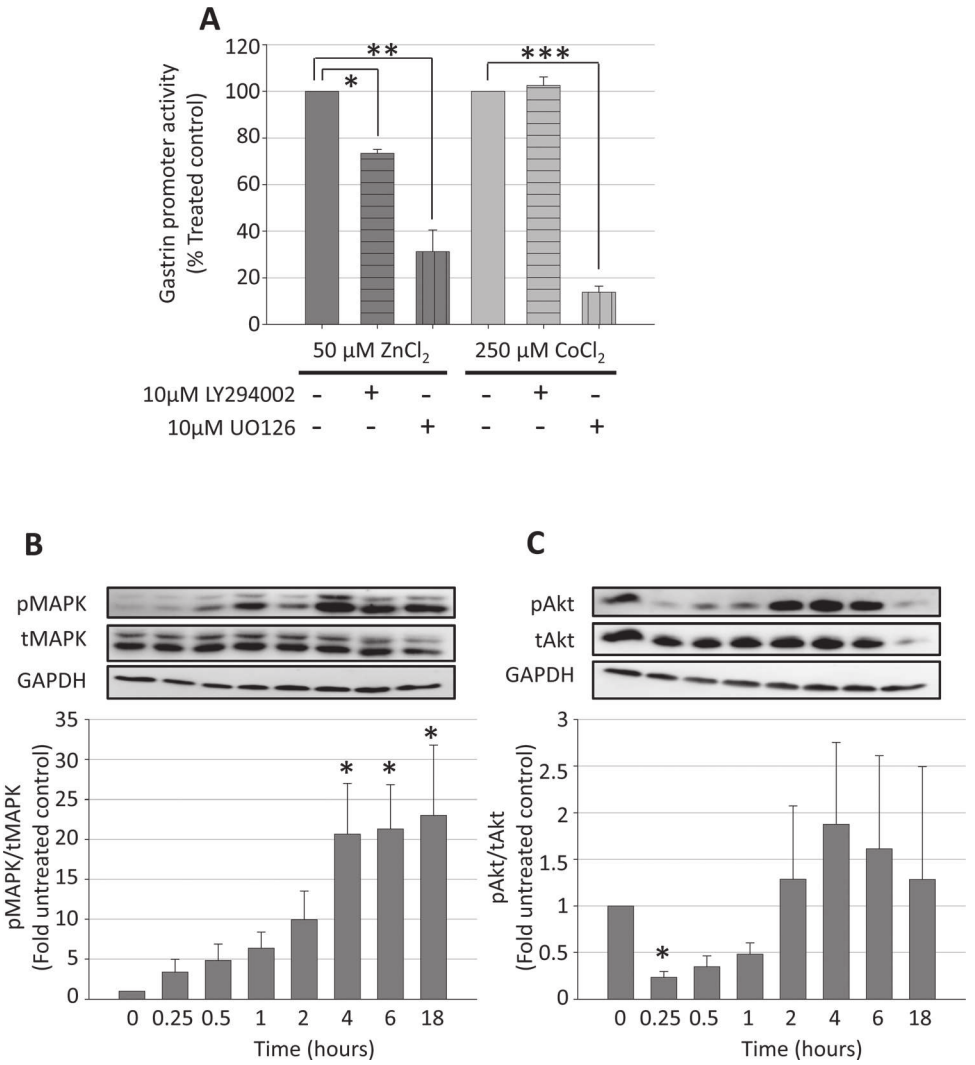

Fig. 3 Gastrin promoter activation by $ZnCl_2$ is dependent on the MAPK pathway. (A) Gastrin promoter activity was measured in $Gast^{luc}$ SW480 colon cancer cells following treatment with 50 μM $ZnCl_2$ for 24 h in the presence of either a PI3K inhibitor (10 μM LY294002) or a MAPK inhibitor (10 μM U0126). $ZnCl_2$ and $CoCl_2$ stimulated the gastrin promoter activity by 22 ± 3-fold and 68 ± 7-fold, respectively, compared with untreated control. Phosphorylation of ERK1/2 (B), a mediator of the MAPK pathway, and of Akt (C), a mediator of the PI3K pathway, was measured by Western blot as described in Materials and methods following the treatment of $Gast^{luc}$ SW480 cells with 50 μM $ZnCl_2$ for the times indicated. Band densities were determined and are presented as the ratio of densities of phosphorylated to total protein. The average + SEM are shown, $n \geq 3$ independent experiments with multiple replicates; *, $p < 0.05$; ***, $p < 0.001$ compared to control (no inhibitor).

the presence of 7.5% FBS (Fig. 4A). Interestingly treatment of $Gast^{luc}$ SW480 cells with 100 μM $ZnCl_2$ in serum-free medium resulted in only a 7 ± 3-fold increase in gastrin promoter activity due to significant toxicity. However, the presence of 7.5% FBS not only abolished toxicity induced by 100 μM $ZnCl_2$, but also significantly increased the gastrin promoter activity by 25 ± 3-fold. $ZnCl_2$ at 200 μM was toxic to the $Gast^{luc}$ SW480 cells either in the presence or absence of 7.5% FBS (Fig. 4A). As observed previously,[18] serum albumin may provide an important extracellular "zinc buffer" which in turn mitigates zinc toxicity whilst providing safe free zinc concentrations for cellular uptake.

Effect of zinc on cell proliferation

To determine whether exposure of $Gast^{luc}$ SW480 cells to zinc had any effect on proliferation, relative cell numbers were determined by MTT cell proliferation assay following the incubation of gastrin reporter $Gast^{luc}$ SW480 cells with various concentrations of zinc for 24 h. As seen in Fig. 4B, the relative cell numbers in either serum free or 7.5% FBS-containing medium increased to 217 ± 5% and 281 ± 8%, respectively, compared to the numbers at 0 h (100%). The 143 ± 5% increase in the relative cell numbers following the treatment of $Gast^{luc}$ SW480 with 50 μM $ZnCl_2$ for 24 h in the absence of serum suggests that cells are still able to proliferate in the presence of 50 μM $ZnCl_2$ albeit at a slower rate than untreated cells (217 ± 5%). This observation suggests that the induction of the gastrin promoter by 50 μM $ZnCl_2$ is independent of cell death induced by heavy metal toxicity. Interestingly only 12 ± 2% cells survived following the treatment of $Gast^{luc}$ SW480 with 100 μM $ZnCl_2$ for 24 h in the absence of serum. However, when serum was

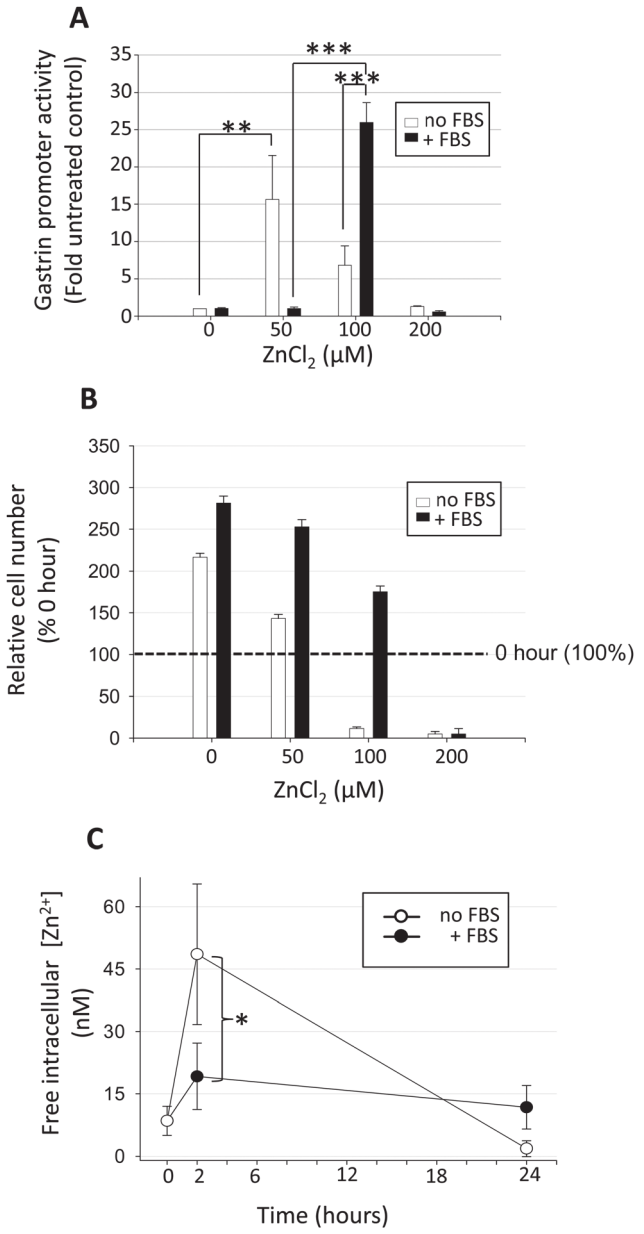

Fig. 4 Impact of serum on zinc-induced gastrin promoter activation and intracellular increases in Zn^{2+}. (A) Gastrin promoter activity was measured in $Gast^{luc}$ SW480 colon cancer cells following treatment with 50, 100 or 200 μM $ZnCl_2$ in culture medium with or without fetal bovine serum (FBS). (B) Relative cell numbers were determined using the MTT assay following the incubation of $Gast^{luc}$ SW480 with various concentrations of zinc either in the presence or absence of serum for 24 h. Following the solubilization of formazan crystals in acidified isopropanol the absorbance, which is directly proportional to cell numbers, was measured at a wavelength of 570 nm with background subtraction at 620 nm. Data are expressed as a percentage of the 0 h reading taken just prior to zinc treatment. All data are shown as means ± SEM of $n \geq 3$ independent experiments. (C) Intracellular free zinc (Zn^{2+}) was determined using the Fluozin3 fluorescent probe in cells treated with 50 μM $ZnCl_2$ in culture medium with or without fetal bovine serum (FBS). Fluorescence was measured and the free Zn^{2+} concentration (nM) was calculated as described in Material and methods. Minimum (F_{min}) and maximal (F_{max}) fluorescence was determined by addition of 10 μM TPEN and 500 μM $ZnCl_2$ respectively. The average ± SEM are shown, $n \geq 3$ independent experiments with multiple replicates; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

included with the zinc treatment the relative cell numbers increased to 176 ± 7%.

Measurement of free zinc in $Gast^{luc}$ SW480 cells

To understand the relationship between intracellular Zn^{2+} and activation of the gastrin promoter, the increase in intracellular free Zn^{2+} was measured using FluoZin-3 in $Gast^{luc}$ SW480 cells incubated with 50 μM $ZnCl_2$ in culture medium in the presence or absence of 7.5% FBS (Fig. 4C). Incubation of $Gast^{luc}$ SW480 cells for 2 h in serum-free medium containing 50 μM $ZnCl_2$ led to a 7 ± 1-fold increase in intracellular free Zn^{2+} concentration compared to untreated cells. In contrast after incubation of $Gast^{luc}$ SW480 cells for 2 h in medium containing 7.5% FBS and 50 μM $ZnCl_2$, the increase in intracellular free Zn^{2+} concentration was only 3 ± 1-fold. Surprisingly there was no significant change in the intracellular free Zn^{2+} following the incubation of $Gast^{luc}$ SW480 cells in 50 μM $ZnCl_2$ for 24 h either in the presence of absence of serum compared to untreated cells.

Dose-dependent effect of zinc on gastrin promoter activation *in vivo*

To investigate whether zinc ions induce gastrin promoter activity *in vivo*, $Gast^{luc}$ SW480 cells were established as xenografted tumours in SCID mice. SCID mice bearing $Gast^{luc}$ SW480 tumours were injected intraperitoneally with either saline or $ZnCl_2$ at three different doses (5 mg kg^{-1}, 10 mg kg^{-1} and 30 mg kg^{-1}) or with $CoCl_2$ at 30 mg kg^{-1}. Bioluminescence after injection of D-luciferin was then measured on the IVIS as described in the Methods section. Preliminary treatment of mice with 30 mg kg^{-1} $ZnCl_2$ was toxic and this treatment was not studied further. As shown in Fig. 5B, there was a 700 ± 140% increase in gastrin promoter activity following a single injection of 10 mg kg^{-1} $ZnCl_2$. In contrast $CoCl_2$ at 30 mg kg^{-1} induced a smaller increase of 249 ± 64% in gastrin promoter activity compared to the saline control. Injecting the mice twice more with either 10 mg kg^{-1} $ZnCl_2$ or 30 mg kg^{-1} $CoCl_2$ did not result in any further increase in gastrin promoter activity. However, three injections of $ZnCl_2$ at 5 mg kg^{-1} did not activate gastrin promoter activity. Gastrin promoter activity in mice treated with $ZnCl_2$ at 10 mg kg^{-1} was still present up to 5 days post treatment (Fig. 5C). Relative zinc concentrations were determined in xenografted tumours of $Gast^{luc}$ SW480 cells and in serum 24 h post treatment of SCID mice with 10 mg kg^{-1} $ZnCl_2$ or saline. As shown in Fig. 5D, there was no significant difference in zinc concentrations in either the xenografted tumours or the serum 24 h post injection and this finding is consistent with the *in vitro* data (Fig. 4C).

Discussion

Although gene expression studies using promoter reporter constructs transfected either transiently or stably have been critical for our understanding of physiological and pathophysiological processes, a number of studies have raised concerns regarding their use.[11,19,20] Unfortunately in stable cell lines established using these plasmids random genomic integration

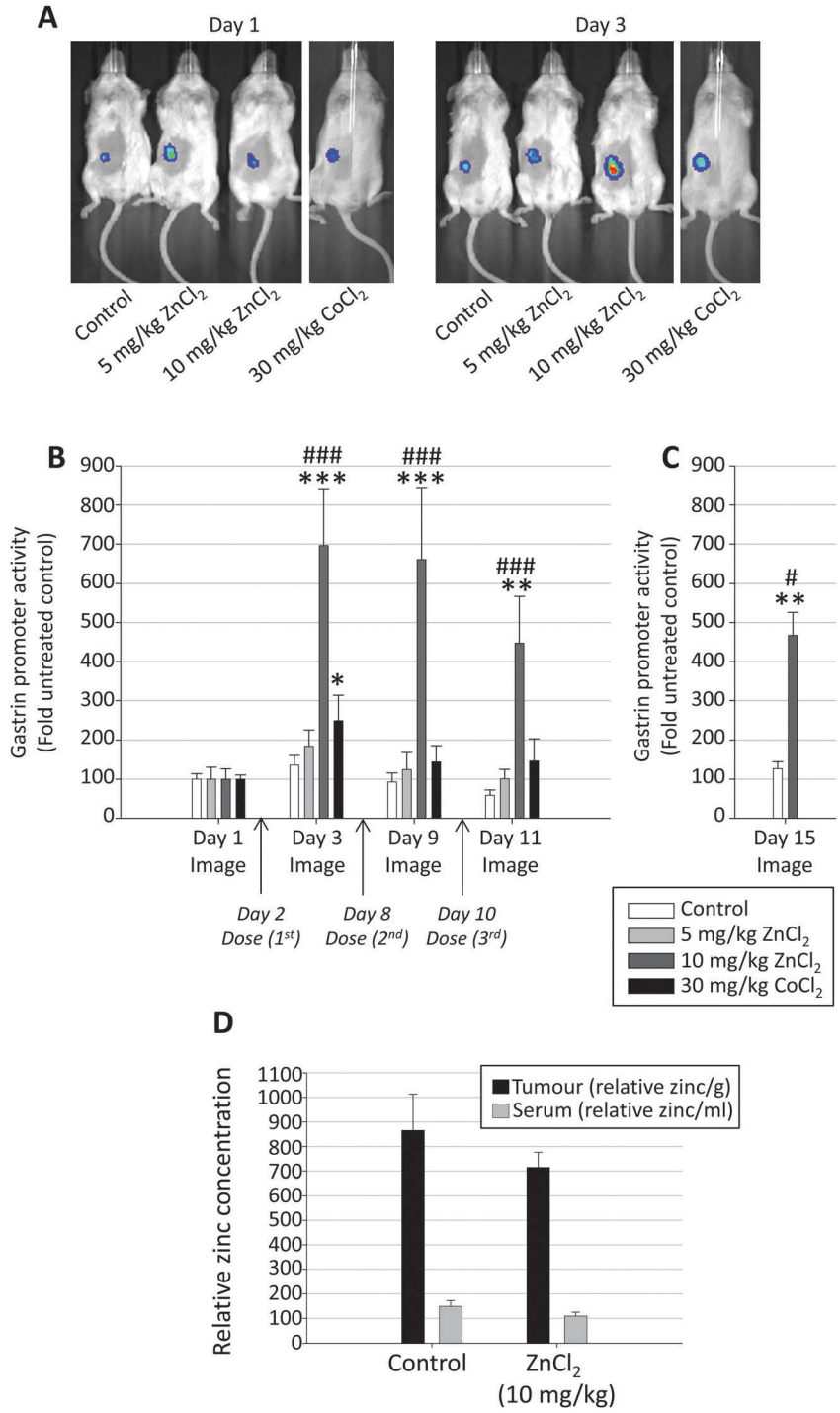

Fig. 5 $ZnCl_2$ stimulates the gastrin promoter *in vivo*. SCID mice bearing $Gast^{luc}$ SW480 xenografted tumours were given intraperitoneal injections of $ZnCl_2$ (5 or 10 mg kg^{-1}) or $CoCl_2$ (30 mg kg^{-1}) on days 2, 8 and 10 and imaged for bioluminescence the following day. Animals received no treatments prior to imaging on day 1. (A) Representative images of bioluminescence observed in mice before and after the first injection. (B) Relative luciferase activity was determined as a percent increase from the untreated control for each treatment group. (C) Luciferase levels were stable up to 5 days post zinc stimulation, as there was no decrease in activity after the last injection on day 10. The average + SEM are shown, $n \geq 5$ mice per treatment; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared to untreated on day 1 in the same treatment group, set to 100%. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$, compared to the control group on the same day. Mice treated with $ZnCl_2$ (10 mg kg^{-1}) had significantly more bioluminescence than mice treated with 5 mg kg^{-1} $ZnCl_2$ or 30 mg kg^{-1} $CoCl_2$ on days 3 and 9, and more bioluminescence than mice treated with 5 mg kg^{-1} $ZnCl_2$ on day 11. (D) Relative zinc concentrations were determined in tumour and serum 24 h after injection with vehicle (control) or 10 mg kg^{-1} $ZnCl_2$ using an Abcam zinc quantification kit (Ab102507). No significant difference was observed between control and zinc-treated mice. The average + SEM are shown, $n \geq 5$ mice per treatment.

might not create an accurate read out for the activation of the gastrin gene expression as vector incorporation site and the number of integration event within the genome may results in differential expression of gastrin compared to expression from its endogenous locus. Thus a more precise approach is required. Gene targeting in mammalian cells is constrained by two limitations: the low rate of homologous recombination in the commonly used somatic human cancer cells and the high rate of random (non-targeted) integration of the vector DNA.[21] Furthermore the presence of repetitive sequences as in the human gastrin gene makes targeting difficult. However with the use of genome editing technologies such as TALEN or CRISPR it is now possible to generate knock-out or knock-in mutants of commonly used cancer cells.[22] In this study TALENs have been used to generate a knock-in gastrin reporter $Gast^{luc}$ SW480 colon cancer cell line in which the endogenous human gastrin gene sequence has been replaced by the firefly luciferase coding sequence.

Previously we had shown that treatment of wild type SW480 cells with exogenous zinc induced the expression of gastrin mRNA.[5] In the present study we have confirmed that gastrin promoter activity is induced by both zinc (Fig. 2A) and cobalt (Fig. 2B) in $Gast^{luc}$ SW480 colon cancer cells. Induction of the gastrin gene at the mRNA and promoter level was abrogated to a similar extent by inhibitors of both MAPK (U0126) and PI3K (LY294002) pathways in AGS gastric cancer cells.[5] However in $Gast^{luc}$ SW480 colon cancer cells inhibition of the MAPK pathway led to a more potent inhibition of zinc induced gastrin promoter activation as compared to the inhibition of the PI3K pathway. The reduced effect of the PI3K inhibitor is consistent with the observation that zinc ions were unable to significantly induce phosphorylation of Akt (a downstream target of the PI3K pathway) in these cells.

The finding that serum blocked the zinc-induced activation of the gastrin promoter is consistent with the hypothesis that the increase in intracellular free zinc ions is indispensable for activation of gastrin promoter activity. The observation that the intracellular increase in free zinc ions following the treatment of $Gast^{luc}$ SW480 colon cancer cells with exogenous zinc ions was significantly lower in the presence of serum (Fig. 4C) is in agreement with a previously published study by Haase and co-workers in which the uptake of exogenous zinc ions was reduced in the presence of 1% FBS and was completely abrogated in the presence of 10% FBS.[18] Interestingly, in our study the increase in intracellular free zinc ions was only transient as the 7-fold difference in intracellular free zinc ions observed between zinc-treated and untreated cells at 2 hours completely disappeared by 24 hours (Fig. 4C), although the activation of the gastrin promoter was still apparent 24 hours following the addition of zinc ions (Fig. 5). The transient nature of the increase in free zinc ions may be due to an increased expression of zinc transporters, leading to an increase in efflux of zinc ions.[23,24]

In vitro studies have now convincingly confirmed that serum reduces the zinc-mediated biological effects such as the increase in phosphorylation of p38[18] or activation of the gastrin promoter (Fig. 4A) by decreasing the zinc uptake. These studies have raised the possibility that blood proteins such as serum albumin may decrease zinc uptake and in turn reduce the zinc-mediated biological effects *in vivo.* Low doses of zinc may not reach the biological threshold and, at higher doses, zinc may become ineffective due to zinc toxicity (Fig. 4A and B). To determine whether there was a biologically relevant relationship between zinc dose and promoter response *in vivo*, xenografts of the gastrin reporter $Gast^{luc}$ SW480 cells were established in SCID mice. A single i.p. injection of zinc ions (10 mg kg^{-1}) produced a 7 fold increase in promoter activity while $CoCl_2$ at 30 mg kg^{-1} resulted in a smaller 2.5 fold increase as measured by whole animal bioluminescence imaging. Both the timing and amount of zinc ions appear to be critical as three injections of zinc at 5 mg kg^{-1}, cumulating to a total of 15 mg kg^{-1}, failed to elicit activation of the gastrin promoter, while one injection of zinc at 10 mg kg^{-1} elicited maximal gastrin promoter activity after 24 hours. These observations highlight the fact that the individual dose of zinc ions, as well as the time at which the end effect (biological activity) is measured, are both very important factors to consider when investigating zinc-dependent effects *in vivo*.

Even though there was no significant difference in relative zinc concentrations in either the xenografted tumour or the serum 24 h post saline or zinc injection, the stimulation of gastrin promoter activity in mice treated with 10 mg kg^{-1} $ZnCl_2$ was still present up to 5 days after zinc treatment, even though the luciferase protein is stable for only 3–4 hours (Promega). Therefore, while the initial activation of the gastrin promoter is directly dependent on an increase in intracellular free zinc ions, in the longer term zinc ions may also activate other growth factors or proteins which then in turn sustain the longer term activation of gastrin promoter activity in an autocrine positive feed forward mechanism.[25,26]

Taken together the current study using colonic SW480 cells and our previously published study[5] using gastric AGS cells demonstrate that the induction of gastrin gene expression by Zn^{2+} ions is both a reproducible and a general phenomenon. However, the relationship between Zn^{2+} ions and gastric acidity is unclear, as Zn^{2+} ions have been reported to either increase[8] or reduce gastric acid secretion.[9,10] Using the zinc chelator TPEN it has been shown that acidity within the tubulovesicular and luminal compartments of the gastric gland may be regulated, at least in part, by zinc ions.[6,7] Our observation that treatment with Zn^{2+} increases both intracellular and secreted gastrin in the gastric carcinoma cell line AGS,[5] if replicated in antral G cells *in vivo*, would be expected to result in a CCK2R-mediated increase in gastric acid secretion. However, Kirchhoff and co-workers have shown direct inhibitory effects of Zn^{2+} on gastric acid secretion[10] and therefore further work will be required to establish the relative importance of Zn^{2+}-induced gastrin expression and of the direct inhibitory effects of Zn^{2+} on the parietal cell.

A key mechanism of a cell's ability to adapt to low oxygen (hypoxia) involves a set of hypoxia-inducible transcription factors (HIFs) that induce more than 200 functionally diverse genes involved in cell survival.[27] $CoCl_2$ is often used as a hypoxia-mimicking agent, by virtue of its ability to induce the expression of HIFs including HIF1α and HIF2α. However, chemical hypoxia induced by $CoCl_2$ appears to differ from true hypoxia[28] as,

although both Co^{2+} and hypoxia (1% O_2) induce HIF1α and HIF2α, the overlap in gene expression profiles is very limited.[28,29] Interestingly our previous studies have shown that the induction of gastrin expression by either Co^{2+} or Zn^{2+} ions is independent of either HIF1α or HIF2α induction[4,5] and is mediated by an as yet unidentified E-box binding transcription factor. These findings taken together suggest that the effect of $CoCl_2$ on gene expression may be mediated at least in part by intracellular zinc ions. A critical implication of these results is that data obtained using $CoCl_2$ may need to be re-examined to determine if results previously attributed to HIFs could, in part, be due to Zn^{2+}.

In summary our study using luciferase reporter $Gast^{luc}$ SW480 colon cancer cells have shown that both zinc and cobalt ions activate the endogenous gastrin gene promoter *in vitro* and *in vivo* and that activation is dependent on the MAPK but not the PI3K pathway. The biological roles of zinc are of similar significance to those of iron and calcium.[30] Zinc-initiated changes in gene expression have been implicated in the etiology of a number of diseases including diabetes, Alzheimer's and cancer,[31] and a better understanding of the zinc–gastrin axis is highly relevant in this context.

Disclosure summary

The authors declare no conflicts of interest.

Acknowledgements

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7.2 Summary

The stable Gast^{luc} SW480 cell line expressing the firefly luciferase reporter protein under the control of the gastrin gene promoter was successfully generated using the gene editing tool TALENs. The gastrin gene activity was stimulated by both $ZnCl_2$ and $CoCl_2$ in Gast^{luc} SW480 cells with zinc being four times more potent than $CoCl_2$ at the same dose. Treatment with either the PI3K inhibitor LY294002 or the MAPK inhibitor U0126 reduced the zinc-stimulated gastrin promoter activity to 73% and 31% respectively. In addition, $ZnCl_2$ induced a time-dependent increase of phosphorylation of ERK1/2, but not of Akt, in Gast^{luc} SW480 cells, which confirmed that $ZnCl_2$ mediates gastrin promoter activation via the MAPK pathway. The presence of serum (7.5% FBS) in *in vitro* experiments blocked the zinc-induced activation of the gastrin promoter and inhibited the increase of intracellular free Zn^{2+} concentrations when the Gast^{luc} SW480 cells were incubated with 50 μM $ZnCl_2$. Although the significant increase of intracellular free zinc between zinc-treated and untreated cells at 2 hours totally disappeared by 24 hours, the activation of the gastrin promoter remained present for 24 hours following zinc treatment. The bioluminescence measured in SCID mice bearing Gast^{luc} SW480 tumors showed a 7-fold increase in gastrin promoter activity after the intraperitoneal injection of a single dose of 10 mg/kg of $ZnCl_2$, and the increase was still apparent after 24 hours. A smaller 2.5 fold increase was observed in tumors after treatment with 30 mg/kg $CoCl_2$. While a dose of 30 mg/kg of $ZnCl_2$ was toxic for the mice, injections of 5 mg/kg of $ZnCl_2$ did not activate the gastrin promoter activity. These observations suggested that both the timing and the concentrations of injected $ZnCl_2$ were critical for the activation of the gastrin promoter activity *in vivo* and raised questions on the role of zinc in the activation of gene expression correlated with diseases such as diabetes, Alzheimer's and cancer. A better understanding of the zinc-gastrin axis is thus highly relevant.

Chapter 8

Paper III

Complexes of gastrin with In^{3+}, Ru^{3+} or Ga^{3+} ions are not recognised by the cholecystokinin 2 receptor. J Biol Inorg Chem 2017.

8.1 Introduction

Amidated gastrin17 (Gamide; ZGPWLEEEEEAYGWMDF-amide) was identified originally as a hormone activating gastric acid secretion following a meal [362]. Gamide also plays a role in the regulation of growth and differentiation of the gastric mucosa, which consequently makes it a key element in the development of gastrointestinal cancers [363] [364]. The non-amidated progastrin-derived peptide glycine-extended gastrin (Ggly) has very low affinity for the CCK2 receptor (CCK2R) compared to Gamide and does not impact acid secretion. However, *in vivo* studies showed that short-term administration of Ggly to rats increased colonic mucosa proliferation as well as the number of aberrant crypt foci in colorectal mucosa [216]. Ggly is thus involved in CRC development.

Metal ions play a role in several biological processes, such as iron in mitochondrial oxidation or calcium in protein stabilization. Gamide and Ggly both bind two trivalent ferric ions with high affinity. NMR spectroscopy identified that the Glu_5 sequence of Ggly was essential for metal ion binding and that the first ferric ion binds to Glutamate 7 while the second one binds to Glutamate 8 and Glutamate 9. Glutamate 6 and Glutamate 10 were not directly involved in ferric ion binding. As assessed by fluorescence quenching, the stoichiometry of ferric ion binding to mutated Ggly peptides, which had one or more Glutamates replaced by Alanines, confirmed the previous results. *In vitro* assays with the mutant peptides identified Glutamate 7 as a key player in cell proliferation and migration. The substitution of Glutamate 7 to Alanine 7, treatment with desferrioxamine (DFO) or competition with bismuth ions totally blocked Ggly-stimulated cell proliferation and migration [323]. Binding of the first ferric iron to Glutamate 7 of Ggly was also necessary for receptor binding [321]. However, the biological activity of Gamide was not

affected by the mutation Glu7Ala or by treatment with DFO, confirming that ferric ions were not essential for the activation of the CCK2R by Gamide [357].

CCK2Rs, classified as G protein-coupled receptors, were identified in medullary thyroid carcinomas, in small cell lung cancers, in astrocytomas and in stromal ovarian cancers, but more occasionally in gastroenteropancreatic tumors, breast, endometrial and ovarian adenocarcinomas [365]. Most CCK2R-positive tumors also express gastrin mRNA. Therefore, metal chelate-conjugated gastrin derivatives have been of great interest for the diagnosis of CCK2R-positive tumors. Peptides successfully targeting CCK2R positive tumors, such as the minigastrin analog MGo, were however highly retained in kidneys or displayed poor *in vivo* stability [366]. Radiolabeled minigastrin peptides are synthetized under severe conditions such as pH, incubation time and temperature, which could for example modify tryptophan residues and hence impact the binding of the peptide to the CCK2R [367].

We recently described the binding of Gamide and Ggly to other trivalent metal ions including gallium (Ga), indium (In) and ruthenium (Ru). Complexes of Gamide with isotopes of trivalent metal ions could be used for the diagnosis of CCK2R positive tumors as similar strategies with gastrin analogs and chelated isotopes of In and Ga are used clinically to detect tumors by single photon emission computed tomography (SPECT) and positron emission tomography (PET) [366]. The aim of the study was to create complexes of Gamide with radioactive isotopes of In, Ru or Ga, to purify them on Sep-Pak C18 cartridges or by anion exchange HPLC and to test their ability to bind to the CCK2R. Binding assays were done on CCK2R-expressing AGS cells by competing radiolabeled-metal ion-Gamide complexes with ^{125}I-CCK-8.

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ORIGINAL PAPER

Complexes of gastrin with In^{3+}, Ru^{3+} or Ga^{3+} ions are not recognised by the cholecystokinin 2 receptor

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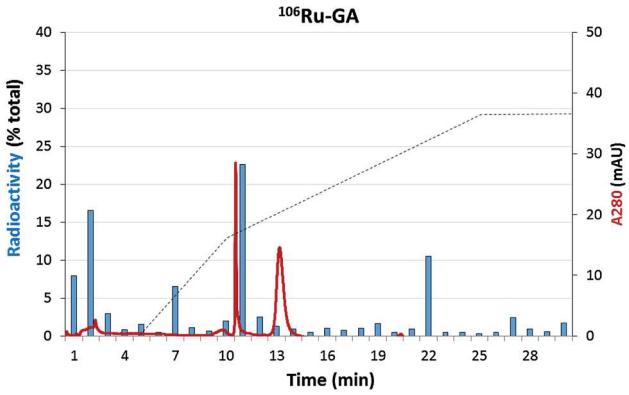
Abstract The peptide hormone gastrin (Gamide) binds trivalent metal ions, including indium (In), ruthenium (Ru) and gallium (Ga), with high affinity. Complexes of gastrin with chelated isotopes of In and Ga have previously been used for the location of tumours expressing the cholecystokinin 2 receptor (CCK2R). The aim of the present study was to purify the complexes of Gamide with radioactive isotopes of In, Ru or Ga and to investigate their ability to bind to the CCK2R. The radioactive Gamide complexes were purified on Sep-Pak C18 cartridges or by anion exchange HPLC. Binding to the CCK2R was assessed with a stably transfected clone of the gastric carcinoma cell line AGS. The ^{106}Ru-Gamide complex could be eluted from the C18 cartridge; the ^{111}In-Gamide and ^{68}Ga-Gamide complexes bound irreversibly. All three complexes were successfully purified by anion exchange HPLC. The failure to detect binding of the ^{111}In-Gamide, ^{106}Ru-Gamide and ^{68}Ga-Gamide complexes to the CCK2R suggests that formation of these complexes will not be useful for the detection of tumours expressing this receptor, but may instead provide alternative ways to block the actions of Gamide as a growth factor or a stimulant of gastric acid secretion.

Graphical abstract The complexes between the hormone gastrin and radioactive ^{111}In, ^{106}Ru or ^{68}Ga ions were purified by anion exchange HPLC using a NaCl gradient. The failure to detect binding of the complexes to the cholecystokinin 2 receptor suggests that metal ion treatment may provide novel approaches to block the biological actions of gastrin.



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Keywords Gastrin · Gallium · Indium · Iron · Ruthenium

Abbreviations

ACN	Acetonitrile
CCK2R	Cholecystokinin 2 receptor
DFO	Desferrioxamine
Gamide	Amidated gastrin17
Ggly	Glycine-extended gastrin17
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
PBS	Phosphate-buffered saline
PET	Positron emission tomography
RPMI	Roswell Park Memorial Institute medium
SEM	Standard error of the mean
SPECT	Single photon emission computed tomography

Introduction

Amidated gastrin17 (Gamide; ZGPWLEEEEEAYGWMDF-amide) is a gut hormone that stimulates gastric acid secretion [1], and also regulates growth and differentiation of the gastric mucosa [2]. Gamide is thought to play a role in the development of gastrointestinal cancers, affecting proliferation, angiogenesis [3] and apoptosis [4]. The effects of Gamide are mediated through the cholecystokinin 2 receptor (CCK2R) [5, 6]. Glycine-extended gastrin (Ggly; ZGPWLEEEEEAYGWMDFG) is an immature precursor of Gamide with negligible affinity for the CCK2R and no direct effect on acid secretion. However, Ggly can also stimulate cell proliferation, migration, and survival and is implicated in the development of colorectal cancer [7, 8].

The CCK2R is a G-protein-coupled 7 transmembrane domain receptor, which is expressed on several tumour types. Reubi and co-workers [9] have demonstrated that almost all medullary thyroid carcinomas and ovarian stromal carcinomas, and more than half of astrocytomas and small cell lung carcinomas, are CCK2R positive. The use of metal chelate-conjugated gastrin derivatives for the diagnosis of CCK2R-positive tumours has been explored [10, 11], but the harsh conditions required for complex formation may result in some oxidative damage or modification to the peptide [12], which may potentially interfere with the ability of these modified gastrins to bind to CCK2Rs.

Gamide and Ggly both bind two ferric ions [13], and for both peptides the first ferric ion binds to Glu7 and the second to Glu8 and Glu9 [14, 15]. The biological activity of Ggly as a stimulant of cell proliferation and migration is dependent on the presence of ferric ions, and thus can be completely blocked either by the substitution Glu7Ala, by competition with bismuth ions, or by treatment with the iron chelator desferrioxamine (DFO) [14], [16, 17]. In contrast, ferric ions are not required for the activation of the CCK2R by Gamide, as the resultant biological activity is unaffected by the substitution Glu7Ala or by treatment with DFO [18].

We recently reported that Gamide and Ggly also bind other trivalent metal ions, including gallium (Ga), indium (In) and ruthenium (Ru). In the case of Gamide, the order of affinities at the first metal ion-binding site was In ($K_d1 = 6.5 \times 10^{-15}$ M) > Ru ($K_d1 = 2.6 \times 10^{-13}$ M) > Fe ($K_d1 = 3.0 \times 10^{-10}$ M) > Ga ($K_d1 = 3.3 \times 10^{-7}$ M). As complexes of gastrin with chelated isotopes of In and Ga are in clinical use for tumour location by single photon emission computed tomography (SPECT) and positron emission tomography (PET), respectively [10], the possibility arose that complexes of Gamide with appropriate trivalent metal ions could be used for diagnosis of CCK2R-expressing tumours. The aim of the present study was to utilise radioactive isotopes of indium, ruthenium and gallium to investigate the purification of their Gamide complexes and their subsequent ability to bind to the CCK2R.

Experimental procedures

Peptides and chemicals

Human amidated gastrin was synthesised by Auspep (Clayton, Australia) to a purity of 88%. The impurities consisted of water and salts. All other consumables were obtained from Sigma Aldrich (Castle Hill, Australia), unless noted otherwise. Radiolabelled indium chloride (^{111}In, 5 mCi in 0.5 ml 50 mM HCl) was purchased from Landauer Australasia Pty Ltd (Sydney, Australia). Radiolabelled ruthenium chloride (^{106}Ru, 1 mCi in 0.5 ml 6 M HCl) was purchased from Eckert & Ziegler Isotope Products (Valencia, CA). Radiolabelled gallium (^{68}Ga, 500 µCi in 100 µl 0.05 M HCl, 0.25 M NaAc) was prepared with a pharmaceutical grade ^{68}Ge/^{68}Ga generator (Eckert & Ziegler Isotope Products) according to the manufacturer's instructions. Solutions of metal ions (Aldrich, St. Louis, MO, USA) were prepared in 10 mM HCl.

Cell culture

The human gastric cancer cell line, AGS, was obtained from ATCC. AGS cells stably transfected with the cholecystokinin 2 receptor (CCK2R) were kindly provided by Professor Andrea Varro, University of Liverpool, UK [19]. Cells were maintained in RPMI 1640 medium (Life Technologies, Mulgrave, Australia) supplemented with 8%

foetal bovine serum and 20 mM HEPES, 0.4% penicillin/streptomycin, and grown at 37 °C in 5% CO_2.

Sep-Pak purification

The ^{111}In-Gamide, ^{106}Ru-Gamide and ^{68}Ga-Gamide complexes were made by incubating 38 nmol of ^{111}In, 250 pmol of ^{106}Ru or 700 pmol of ^{68}Ga with 250 pmol of Gamide in 50 mM Na^+ acetate, pH 4, for 30 min at room temperature. The reaction mixtures were then loaded onto Sep-Pak C18 reverse phase cartridges (Waters Corporation, Milford, MA, USA), which had been activated by precycling with 10 ml of buffer A [100 mM Na^+ acetate (pH 4)], followed by 10 ml of buffer B (100 mM Na^+ acetate (pH 4), 50% acetonitrile (ACN)) and 10 ml of buffer A. After loading, the Sep-Pak cartridge was washed with 10 ml of buffer A to remove unbound ^{111}In, ^{106}Ru or ^{68}Ga, and eluted with buffer B in 5 serial fractions (1 ml each). The amount of radioactivity in each collected fraction was measured either with a β-counter (Packard, Meriden, CT, USA) or a γ-counter (Perkin Elmer, Waltham, MA, USA).

HPLC purification

The ^{111}In-Gamide, ^{106}Ru-Gamide and ^{68}Ga-Gamide complexes (made as in 2.3, but not run on a Sep-Pak column) were purified by HPLC on either reverse phase or anion exchange columns. Reverse phase HPLC used a C4 column at a flow rate of 1 ml/min with a gradient from 10 mM Na^+ acetate, 100 mM NaCl (pH 4) to 10 mM Na^+ acetate, 100 mM NaCl (pH 4) + 70% ACN over 30 min. The anion exchange HPLC used a Protein-Pak Q 8HR column (Waters, Rydalmere, Australia) at a flow rate of 1 ml/min with a gradient from 10 mM Na^+ acetate (pH 4) to 10 mM Na^+ acetate (pH 4) + 1 M NaCl over 30 min. Fractions were collected every minute and the amount of radioactivity was measured either with a β-counter or a γ-counter. Peptide peaks (line) were observed as an increase in 280 nm absorbance. Radioactivity (%, bars) was determined based on the total amount loaded onto the column as 100%.

Stability of ^{106}Ru-Gamide complex over time

The Sep-Pak purified ^{106}Ru-Gamide complex was diluted with an equal volume of 50 mM Na^+ HEPES, pH 7.6, and divided into 4 identical aliquots, which were loaded onto new C18 Sep-Pak cartridges after 0, 24, 48 h and 5 days, respectively. The ^{106}Ru-Gamide complex was eluted as described above, and the radioactivity eluted in fractions 1 and 2 at each time point was expressed as a percentage of the 0 h point and used to calculate the stability of ^{106}Ru-Gamide by curve fitting to the equation for exponential decay.

Binding assay

AGS wildtype cells (WT) and AGS cells stably expressing the CCK2R (AGS-CCK2R) were seeded at a density of 5×10^5 cells/well in 6-well plates. The following day, cells were rinsed with binding buffer (20 mM Na^+ Hepes, 10 mM KCl, 1.25 mM benzamidine, 20 μM $CaCl_2$, 80 μM $MgCl_2$, pH 7.4) and pre-treated with binding buffer or 100 nM Gamide in binding buffer for 15 min at 37 °C. ^{111}In-Gamide and ^{106}Ru-Gamide were purified by anion exchange HPLC as described above; purification of ^{68}Ga-Gamide was not attempted because of the short half-life of ^{68}Ga (68 min). ^{111}In-Gamide (48,300 cpm/well), ^{106}Ru-Gamide (4350 cpm/well), ^{68}Ga-Gamide (81,600 cpm/well), or sulphated cholecystokinin 8 (CCK8) labelled at the N-terminus with ^{125}I-labelled-Bolton and Hunter reagent (20,000 cpm/well, Amersham Biosciences, Castle Hill, Australia) was added to separate wells and incubated for 30 min at 37 °C. The cells were washed twice with PBS and lysed with 500 μl of 1 M NaOH. The amount of bound radioactivity was determined using either a β-counter or a γ-counter.

Statistics

Each experiment was repeated at least 3 times. All values are expressed as mean ± standard error. Statistical significance was determined by Student's *t* test using SigmaStat (Jandel Scientific, San Rafael, CA, USA).

Results

Selective binding of indium, ruthenium or gallium ions by Gamide or Ggly was previously demonstrated using absorption spectroscopy and extended X-ray absorption fine structure (EXAFS) spectroscopy [15]. The formation of these non-radioactive complexes suggested that the radioactive forms of these trivalent metals (M^{3+}) bound to Gamide could be utilised to identify tumours expressing the CCK2R. To accomplish this task, these complexes were formed as previously described and then purified and tested for stability as detailed below.

Purification of the ^{106}Ru-Gamide complex using C18 Sep-Pak cartridges

Radioactive isotopes of Indium (^{111}In), Ruthenium (^{106}Ru) and Gallium (^{68}Ga) were used to quantitate

M^{3+}-Gamide complex formation. Purification of the ^{111}In-Gamide, ^{106}Ru-Gamide and ^{68}Ga-Gamide complexes was initially attempted with C18 Sep-Pak reverse phase cartridges, by elution of the M^{3+}-Gamide complex with acetonitrile (ACN). Although no elution of ^{111}In-Gamide (Fig. 1a) or ^{68}Ga-Gamide (Fig. 1c) from the C18 Sep-Pak cartridges was detected, ^{106}Ru-Gamide was clearly bound (Fig. 1b) and was differentially eluted with 50% ACN in fractions 1 and 2. Unbound ^{106}Ru passed through the cartridge in the initial flow through. The ^{111}In-Gamide and ^{68}Ga-Gamide complexes remained bound to the C18 resin as no additional radioactivity was observed in fraction 2 in the presence of Gamide, and as these cartridges remained radioactive after elution.

Purification of ^{111}In-Gamide, ^{106}Ru-Gamide and ^{68}Ga-Gamide complexes using HPLC

As the ^{111}In-Gamide and ^{68}Ga-Gamide complexes were not eluted from the C18 Sep-Pak cartridges, purification of all three complexes was attempted by C4 reverse phase HPLC. The main absorbance peak for Gamide in the absence of added metal ions eluted at 21 min (Fig. 2a). In the presence of metal ions (Fig. 2b–d), unbound metal ions eluted at 4–6 min, the Gamide peak at 21 min was no longer present, and no additional radioactive peaks were eluted. Therefore, the ^{111}In-Gamide, ^{106}Ru-Gamide and ^{68}Ga-Gamide complexes presumably remained bound to the C4 resin.

Subsequently, the ^{111}In-Gamide, ^{106}Ru-Gamide and ^{68}Ga-Gamide complexes were purified by anion exchange HPLC. The main absorbance peak for Gamide in the absence of added metal ions eluted at 14.5 min. In the presence of Ru^{3+} ions, a new absorbance peak, with associated radioactivity, appeared at 11 min (Fig. 3c). A new early absorbance peak also appeared in the presence of In^{3+} (Fig. 3b) or Ga^{3+} (Fig. 3d) ions, although in these cases the peak was smaller, and the associated radioactivity tailed extensively. The earlier radioactive peaks eluting in the breakthrough at 1–2 min represent unbound metal ions. The correlation between radioactivity and peptide absorbance indicated that all three M^{3+}-Gamide complexes formed and could be purified. Therefore, appropriate fractions were collected and used to test their stability (in the case of ^{106}Ru-Gamide) and ability to bind selectively to cells expressing the CCK2R.

The ^{106}Ru-Gamide complex was stable for over 5 days

Determination of the long-term stability of the M^{3+}-Gamide complexes was only possible for ^{106}Ru

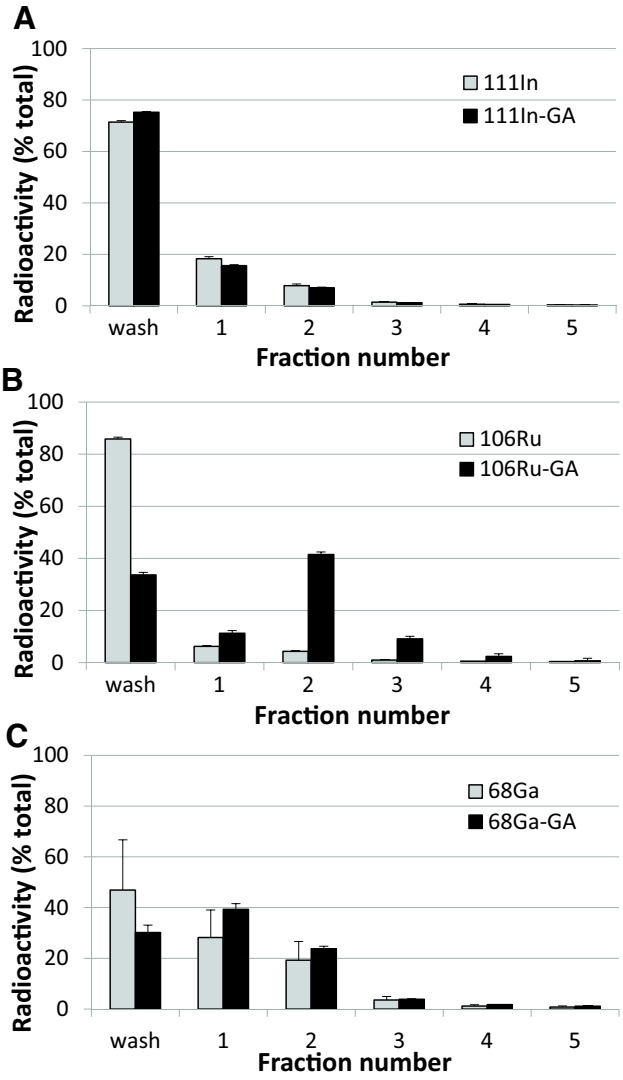

Fig. 1 Purification of the ^{106}Ru-Gamide complex on Sep-Pak C18 cartridges. The reaction mixtures containing ^{111}In-Gamide (**a**), ^{106}Ru-Gamide (**b**) or ^{68}Ga-Gamide (**c**) complexes were passed through C18 Sep-Pak cartridges and the elution of radioactivity was compared to ^{111}In, ^{106}Ru and ^{68}Ga alone, respectively. After passage of the complexes, the cartridges were first washed with 10 ml 100 mM Na^+ acetate (pH 4) to remove unbound ^{111}In, ^{106}Ru or ^{68}Ga, and then eluted with 5 × 1 ml 100 mM Na^+ acetate (pH 4) + 50% ACN. The ^{106}Ru-Gamide complex was eluted with 50% ACN in fractions 1 and 2, and the radioactivity in each eluted fraction is expressed as a percentage of the total eluted radioactivity. The ^{111}In-Gamide and ^{68}Ga-Gamide complexes remained bound to the C18 resin as no additional radioactivity was observed in fraction 2 in the presence of Gamide, and the cartridges remained radioactive after elution

($t_{½}$ = 367 days), as the short half-lives of ^{111}In (2.8 days) and ^{68}Ga (68 min) were limiting. After 30-min incubation, the ^{106}Ru-Gamide complex was purified on a C18 Sep-Pak reverse phase cartridge, divided into four aliquots, and each aliquot was subsequently re-purified by the same method after various incubation times at room

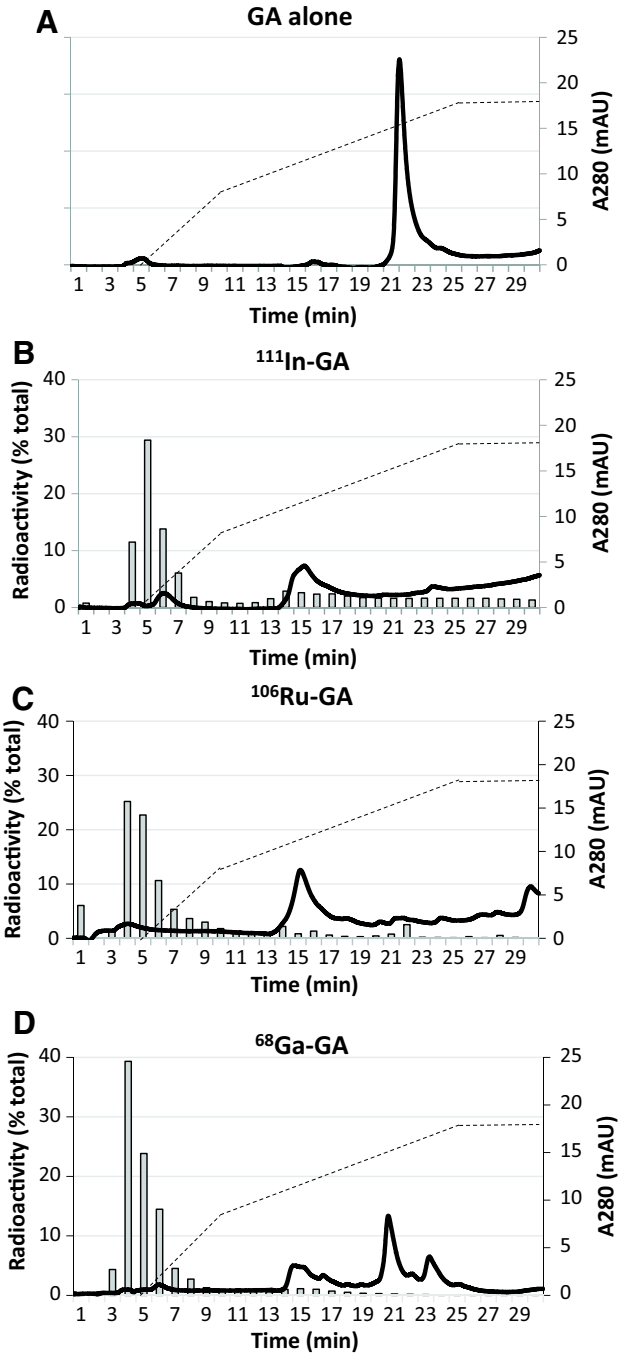

Fig. 2 Purification of the ^{111}In-Gamide, ^{106}Ru-Gamide and the ^{68}Ga-Gamide complexes by C4 HPLC. Because the ^{111}In-Gamide and ^{68}Ga-Gamide complexes remained bound to the C18 resin (Fig. 1), Gamide alone (**a**) or the ^{111}In-Gamide (**b**), ^{106}Ru-Gamide (**c**) or ^{68}Ga-Gamide (**d**) complexes were run on a C4 reverse phase HPLC column. A gradient from 0 to 70% ACN in 10 mM Na^+ acetate, 100 mM NaCl, pH 4 was used over 30 min [*dotted line*, 70% ACN was increased from 0 to 40% over 5 min (5–10 min), at which point it was gradually increased to 100% over 20 min (10–30 min)]. The main absorbance peak (*line*) for Gamide in the absence of added metal ions eluted at 21 min (**a**). In the presence of metal ions (**b**–**d**) unbound metal ions eluted at 4–6 min, the Gamide peak at 21 min was no longer present, and no additional radioactive peaks (*bars*) were eluted. Therefore, the ^{111}In-Gamide, ^{106}Ru-Gamide and ^{68}Ga-Gamide complexes presumably remained bound to the C4 resin

temperature. The half-life of the ^{106}Ru-Gamide complex obtained by curve fitting assuming exponential decay was 11.1 days at room temperature and pH 7.6 (Fig. 4).

^{111}In-Gamide, ^{106}Ru-Gamide and ^{68}Ga-Gamide complexes do not bind to the CCK2R on cultured cells

Human gastric cancer cells deficient for (AGS-WT), or stably transfected with a plasmid expressing the CCK2R (AGS-CCK2R), were used to determine if any of the purified M^{3+}-Gamide complexes were selectively bound by the CCK2R. Since their introduction by the Varro and Dockray group, this pair of cell lines has become a frequently used model for studies of CCK2R binding [19]. Binding to AGS-WT and AGS-CCK2R cells was assessed using ^{111}In-Gamide, ^{106}Ru-Gamide and ^{68}Ga-Gamide diluted into binding buffer in the presence or absence of unlabelled Gamide. Sulphated cholecystokinin 8 labelled at the N-terminus with ^{125}I-Bolton and Hunter reagent (^{125}I-CCK8) was used as a positive control. As expected the amount of ^{125}I-CCK8 radioactivity bound to the CCK2R on AGS-CCK2R cells decreased in the presence of unlabelled Gamide (Fig. 5d). The absence of any such competition with ^{111}In-Gamide, ^{106}Ru-Gamide or ^{68}Ga-Gamide (Fig. 5a–c) in the presence of unlabelled Gamide indicated that none of the radioactive Gamide-metal ion complexes bound to the CCK2R. Presumably, the association of Gamide with ^{111}In, ^{106}Ru or ^{68}Ga prevented recognition of the complex by the CCK2R.

Discussion

Metal chelate-conjugated gastrin derivatives, such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-minigastrin radiolabelled with either ^{111}In or ^{68}Ga, have previously been used for the diagnosis of CCK2R-positive tumours [10]. However, the harsh conditions (pH 4.5, 98 °C, 15 min) required to incorporate these metal ions may potentially result in oxidative damage or modification to the peptide [12]. Based on the previous work demonstrating high binding affinity of certain trivalent metals to Gamide under relatively mild conditions (pH 4, room temperature, 30 min) [15], we set out to purify M^{3+}-Gamide complexes that might be used to identify cells and tumours that express CCK2Rs.

The ^{106}Ru-Gamide complex was purified on a C18 Sep-Pak cartridge (Fig. 1). In contrast to the ^{111}In-Gamide and ^{68}Ga-Gamide complexes, the radioactivity recovered from

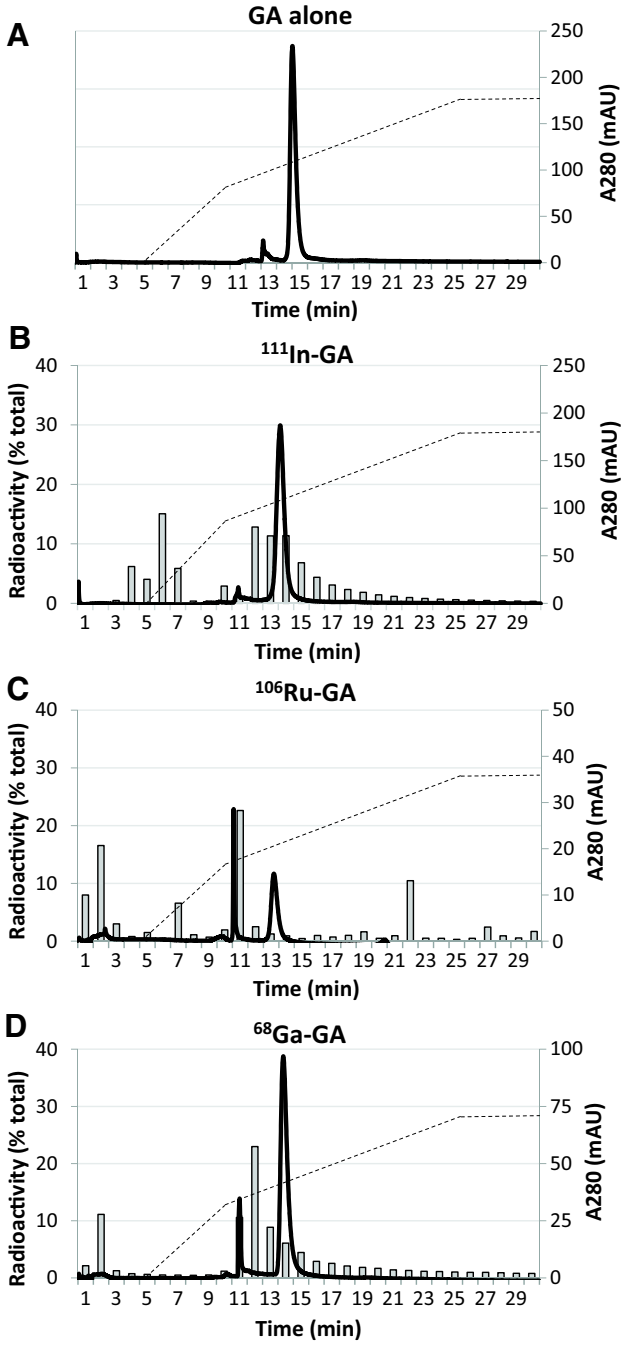

Fig. 3 Purification of the ^{111}In-Gamide, ^{106}Ru-Gamide and the ^{68}Ga-Gamide complexes by anion exchange HPLC. Gamide alone (**a**) or the ^{111}In-Gamide (**b**), ^{106}Ru-Gamide (**c**) and ^{68}Ga-Gamide (**d**) complexes were run on an anion exchange HPLC column using a gradient from 10 mM Na^+ acetate (pH 4) to 10 mM Na^+ acetate (pH 4) + 1 M NaCl over 30 min [*dotted line*, 1 M NaCl was increased from 0 to 40% over 5 min (5–10 min), at which point it was gradually increased to 100% over 20 min (10–30 min)]. The main absorbance peak (*line*) for Gamide in the absence of added metal ions eluted at 14 min (**a**). In the presence of Ru^{3+} ions a new peak, with associated radioactivity (*bars*), appeared at 11 min (**c**). The new early peak also appeared in the presence of Ga^{3+} (**d**) or In^{3+} (**b**) ions, although in these cases the peak was smaller, and the associated radioactivity tailed extensively. The earlier radioactive peaks eluting in the breakthrough at 1–2 min represent unbound metal ions

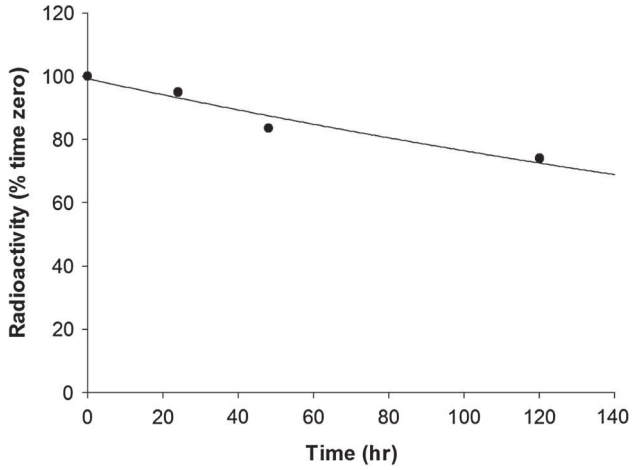

Fig. 4 Stability of ^{106}Ru-Gamide complex. The Sep-Pak-purified ^{106}Ru-Gamide complex was diluted with an equal volume of 50 mM Na^+ HEPES, pH 7.6, and divided into 4 aliquots, which were incubated for 0, 24, 48 h or 5 days at room temperature, and loaded onto new C18 Sep-Pak cartridges. The remaining ^{106}Ru-Gamide complex was eluted as described above, and the radioactivity eluted in fractions 1 and 2 at each time point was expressed as a percentage of the 0 h point. The half-life of the ^{106}Ru-Gamide complex obtained by curve fitting assuming exponential decay was 11.1 days at room temperature and pH 7.6

the C18 Sep-Paks was always less than what was added to the cartridges, which remained radioactive after elution. These observations suggest that the ^{111}In-Gamide and ^{68}Ga-Gamide complexes may have remained bound to the cartridges, even in the presence of 50% acetonitrile. Similarly, none of the M^{3+}-Gamide complexes were eluted from the C4 HPLC column under conditions that eluted Gamide (Fig. 2). This observation is not unexpected as the neutralisation of the negative charge of Gamide (6−) by the presence of the positive charge (6+) of two M^{3+} ions would be predicted to increase the hydrophobicity of the complex, causing it to bind more tightly to the column.

In contrast all three M^{3+}-Gamide complexes were successfully purified by anion exchange HPLC (Fig. 3). As expected from the neutralisation of the negative charge of Gamide (6−) by the presence of the positive charge (6+) of two M^{3+} ions, the M^{3+}-Gamide complexes eluted from the anion exchange column earlier than Gamide when using a salt gradient.

No binding of the ^{111}In-Gamide, ^{106}Ru-Gamide or ^{68}Ga-Gamide complexes to the CCK2R was detected. This observation was unexpected, as several lines of indirect evidence had previously suggested that binding of Fe^{3+} ions was not essential for the recognition of Gamide by the CCK2R. For example, the minimum peptide structure required for CCK2R binding was the C-terminal tetrapeptide amide [20], which lacks the pentaglutamate sequence implicated in the binding of Fe^{3+} ions [14]. Furthermore

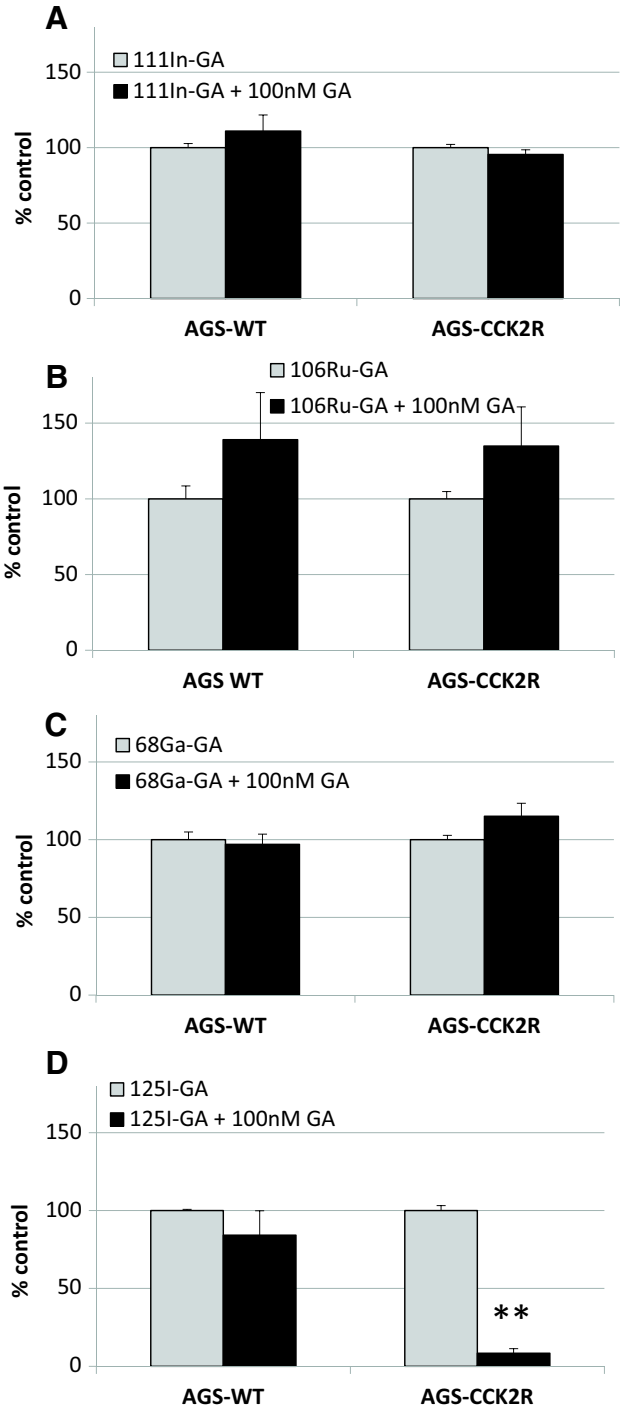

Fig. 5 The ^{111}In-Gamide, ^{106}Ru-Gamide and ^{68}Ga-Gamide complexes do not bind to the CCK2R. ^{111}In-Gamide and ^{106}Ru-Gamide were purified by anion exchange HPLC as described in "Experimental procedures"; purification of ^{68}Ga-Gamide was not attempted because of the short half-life of ^{68}Ga (68 min). Binding of the ^{111}In-Gamide (**a**, 48,300 cpm/well), ^{106}Ru-Gamide (**b**, 4350 cpm/well), or ^{68}Ga-Gamide (**c**, 81,600 cpm/well) complexes to wild type (WT) or the CCK2R expressing AGS cells in the presence, or absence, of 100 nM unlabelled Gamide was determined as described under "Experimental procedures". Binding of ^{125}I-CCK8 (**d**, 20,000 cpm/well) in the presence, or absence, of 100 nM unlabelled Gamide was assessed as a positive control. Although the presence of 100 nM unlabelled Gamide reduced the binding of ^{125}I-CCK8 to the CCK2R by more than 90% (**$p < 0.01$), no evidence was obtained for binding of M^{3+}-Gamide complexes to the CCK2R

removal of the first Fe^{3+} binding site by the E7A mutation had no significant effect on CCK2R binding or on biological activity [18], and CCK2R binding and biological activity were unaffected by the Fe^{3+}-selective chelator desferrioxamine [18]. In the case of ^{106}Ru-Gamide, the failure to detect binding does not appear to be the result of dissociation, as the complex was shown to be very stable at neutral pH, with a half-life of 11.1 days (Fig. 4). Similarly, disruption of the ^{111}In-Gamide and ^{106}Ru-Gamide complexes by the 1 M NaCl gradient used in anion exchange chromatography appears unlikely, as the extremely high stability constants of the In^{3+} (6.5×10^{-15}) and Ru^{3+} (2.6×10^{-13}) complexes were actually determined in the presence of 100 mM NaCl [15], and the concentration of NaCl required for elution was less than 500 mM (Fig. 3).

The failure of the ^{111}In-Gamide, ^{106}Ru-Gamide or ^{68}Ga-Gamide complexes to bind to the CCK2R is presumably due to the differences in their structures caused by differences in their ionic radii and coordination state. Indeed a previous EXAFS study has shown that the structure of Ru_2Gamide differs from Fe_2Gamide, with a direct Ru-Ru bond instead of the bridging water molecule between the two ferric ions [15]. One intriguing possibility is that the aspartic acid in the C-terminal moiety of gastrin might interact with indium, ruthenium or gallium ions with consequent inhibition of binding to the CCK2R. In the case of ferric ions and bismuth ions, our NMR data provided convincing evidence that only the glutamic acid residues at positions 7, 8 and 9 of glycine-extended $gastrin_{17}$ were involved in metal binding [14]. Our EXAFS data indicated the presence of carboxylate ligands, but did not discriminate between aspartic and glutamic acid residues in the case of indium, ruthenium and gallium ions [15]. However, the binding data presented in the same paper were consistent with competitive inhibition, in which ruthenium (or indium) and ferric ions competed for the same two metal ion-binding sites on gastrin. Although attempts to crystallise the gastrIn-metal ion complexes have so far been unsuccessful, X-ray crystallography may ultimately be required to identify precisely which gastrin residues are acting as metal ion ligands.

On the other hand, there is now abundant evidence that Fe^{3+} ions are essential for the biological activity of non-amidated gastrins such as progastrin and Ggly [14, 16, 17]. However, the identity of the receptors for non-amidated gastrins is still controversial [21–23], and the use of ^{111}In-Ggly, ^{106}Ru-Ggly or ^{68}Ga-Ggly complexes may help to resolve that debate. As non-amidated gastrins stimulate

proliferation in the normal colorectal mucosa and accelerate the development of colorectal cancer [7, 8], these receptors may become important targets for improvements in cancer diagnosis and therapy.

This study also suggests a new method of blocking the biological actions of amidated gastrins. Occupation of the metal ion-binding site of amidated gastrins by indium, ruthenium or gallium ions prevents the binding of Gamide to the CCK2R, and so renders the peptide inactive. The high affinity of Gamide for indium and ruthenium ions suggests the possibility that low doses of these ions could be used as specific inhibitors for the treatment of excessive acid production in patients with conditions such as gastrointestinal ulcers, gastro-oesophageal reflux or gastric carcinoid. The same approach might also be used to inhibit G-amide stimulated growth and differentiation of the gastric mucosa, and of gastric cancer [2].

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8.2 Summary

Radiolabeled-metal ion-Gamide complexes were prepared by incubation of ^{111}In, ^{106}Ru or ^{68}Ga with Gamide in 50 mM Na^{+} acetate, pH 4.0, for 30 minutes at room temperature. Although ^{111}In-Gamide and ^{68}Ga-Gamide could not be purified using Sep-Pak C18 reverse phase cartridges, ^{106}Ru passed through the cartridge while ^{106}Ru-Gamide was retained and could be eluted with acetonitrile (ACN). ^{111}In-Gamide, ^{68}Ga-Gamide and ^{106}Ru-Gamide complexes were all purified by anion exchange HPLC, but not by C4 reverse phase HPLC. However, none of the radioactive Gamide-metal ion complexes competed with ^{125}I-CCK8 for the CCK2R. As previously shown that Gamide did not require the binding of Fe^{3+} to recognize the CCK2R, the association with ^{111}In, ^{106}Ru or ^{68}Ga presumably blocked the recognition of Gamide by the CCK2R. We have demonstrated in a previous EXAFS study that Ru_2-Gamide is structurally different to Fe_2-Gamide so an interaction between the aspartic acid in the C-terminal tail of Gamide and indium, ruthenium or gallium ions could explain the absence of binding to the CCK2R. Ggly activity requires binding to Fe^{3+} ions. Developing ^{111}In-Ggly, ^{106}Ru-Ggly or ^{68}Ga-Ggly complexes could be used to block Ggly-stimulated cell proliferation, thus inhibiting the development of CRC. Additionally, radiolabelled-metal ions-Ggly complexes could help to characterize receptors for non-amidated gastrins.

Chapter 9

Paper IV

Oral trivalent bismuth ions decrease, and trivalent indium or ruthenium ions increase, intestinal tumor burden in $APC^{\Delta 14/+}$ mice. Metallomics 2018.

9.1 Introduction

Gastrin peptides are derived from progastrin and secreted after maturation by antroduodenal G-cells, leading to the major gastrin forms in tissue and plasma, gastrin-34 and gastrin-17 (Gamide). Gamide stimulates gastric acid secretion via specific binding to its receptor CCK2R. The immature progastrin-derived forms, progastrin itself and glycine-extended gastrin (Ggly), are not involved in acid secretion but stimulate colon cell division, migration and survival [213]. Consequently, they play a role in the development of CRC by stimulating cell growth and progression of colonic polyps and adenocarcinomas [368]. Upregulation of gastrin gene expression in CRC leads to high concentrations of immature gastrins in the serum of patients [369]. Although Gamide acts via the CCK2R, progastrin and Ggly do not, and several candidates for the Ggly receptor have been suggested, including annexin II and the F1-ATPase-α subunit [143] [144].

APC (adenomatous polyposis coli) mutant mice are a good model for CRC as about 80% of all human colon tumors have an inactivating mutation in the APC tumor suppressor gene [370]. When $APC^{min-/+}$ mice, which spontaneously develop intestinal tumors, were crossed with mice overexpressing Ggly (MTI/Ggly) or gastrin knockout mice (GASKO), tumor size and number were increased and tumor incidence was significantly reduced respectively [69]. $APC^{\Delta 14/+}$ mice, which have APC exon 14 deleted, displayed more spontaneous tumors in their large intestine compared to $APC^{min-/+}$ mice and are therefore a better model for human familial adenomatous polyposis (FAP) [371]. $APC^{\Delta 14/+}$ mice represent a useful model to study the molecular mechanisms of colorectal tumorigenesis.

A correlation between excess iron and CRC risk has been reported [372]. In $APC^{min-/+}$ mice, iron deficiency triggered apoptosis of APC-deficient cells while an excess of iron increased pro-

liferation. Also, tumor number and size correlated with dietary iron status, which indicated a critical role for iron in the fate of APC-deficient cells [373]. Both gastrin KO mice and hypergastrinemic CCK2R KO mice had reduced basal gastric acid secretion but unchanged iron homeostasis. When they were fed an iron deficient diet, gastrin KO mice developed severe anemia as well as splenomegaly. The expression of Hamp mRNA, encoding the iron-regulatory peptide hepcidin, was down-regulated in both gastrin KO mice and hypergastrinemic CCK2R KO mice when challenged by iron deficiency [374]. Hypoxia studies reported that hGas mice, overexpressing progastrin in the liver, coped better under hypoxia than FVB/N control mice due to a better mobilization of hepatic iron stores [375].

Our team has shown that Ggly binds to two ferric ions. Glutamate 7 is critical for binding the first ferric ion and Glutamate 8 and Glutamate 9 are important for binding the second ferric ion. Studies with Ggly fragments and mutants revealed the importance of the Glu_5 sequence for Ggly-induced proliferation of IMGE-5 cells. The Glu7Ala mutation and treatment with the iron chelator desferrioxamine (DFO) both inhibited Ggly, but not Gamide, biological activity [321]. The observation that the affinity of Fe^{3+} ions for Ggly was significantly reduced in the presence of Bi^{3+} *in vitro* suggests that Bi^{3+} ions can displace the binding of Fe^{3+} ions to Ggly [352]. Moreover Ggly-induced proliferation in the rectal mucosa was reduced to control values in rats receiving oral bismuth plus Ggly treatments. Gastrins bind to trivalent In^{3+} and Ru^{3+} ions and the affinity of the first site for both of these ions is much higher than for ferric ions [376]. Binding to ferric ions is necessary for Ggly biological activity so we hypothesized that Bi^{3+}, In^{3+} and Ru^{3+} could displace Fe^{3+} and act as competitive inhibitors of the Ggly receptor. The aim of this study was to define the effects of blocking ferric ion binding to Ggly using Bi^{3+}, In^{3+} or Ru^{3+} ions on the incidence and size of intestinal tumors in $APC^{\Delta 14/+}$ mice. The mice were treated by oral gavage with PBS, bismuth citrate, indium citrate or ruthenium citrate three times a week, up to 20 weeks of age. Animals were checked and weighed every day and after sacrifice by isoflurane treatment, full blood determination was performed and the intestines were collected to count tumors.

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Oral trivalent bismuth ions decrease, and trivalent indium or ruthenium ions increase, intestinal tumor burden in $Apc^{\Delta 14/+}$ mice†

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Immature forms of the peptide hormone gastrin have been implicated in the development of colorectal cancer (CRC). The biological activity of glycine-extended gastrin (Ggly) is dependent on the binding of Fe^{3+} ions *in vitro* and *in vivo*. The aim of the present study was to determine the effect of blocking Fe^{3+} ion binding to Ggly, using Bi^{3+}, In^{3+} or Ru^{3+} ions, on the development of intestinal tumors in $APC^{\Delta 14/+}$ mice. $APC^{\Delta 14/+}$ mice were treated orally with Bi^{3+}, In^{3+} or Ru^{3+} ions for up to 60 days, serum trace metals were analyzed by inductively coupled plasma mass spectrometry, and the incidence and size of intestinal tumors were assessed. Bi^{3+} treatment significantly decreased the number of tumors larger than 3 mm in male mice. In^{3+} or Ru^{3+} treatment significantly increased the tumor burden in all animals and In^{3+} increased the number of tumors larger than 3 mm or 5 mm in male mice alone. The fact that binding of In^{3+} or Ru^{3+} ions to Ggly was orders of magnitude stronger than the binding of Bi^{3+} ions implies that the inhibitory effect of Bi^{3+} ions is not a consequence of a reduction in Ggly activity. However, further testing of higher doses of Bi^{3+} ions for longer periods as an oral treatment for intestinal tumors is warranted.

Introduction

Gastrins are a collective term for mature and immature peptides derived from the gastrin gene. Amidated gastrin (Gamide), the fully processed gut hormone, activates the cholecystokinin 2 (CCK2) receptor to stimulate gastric acid secretion and maintain the gastric mucosa.[1,2] Immature forms of gastrin (*e.g.*, progastrin and glycine-extended gastrin (Ggly)) are also biologically active independently of the CCK2 receptor and act primarily in the colon to stimulate cell division,[3–6] migration, and survival.[1,4] Immature gastrins are found in colonic polyps and adenocarcinomas,[7] and have been implicated in the development of colorectal cancer (CRC). Thus greater concentrations of immature gastrins are found in the circulation of CRC patients,[8,9] and hypergastrinemic patients have a 4-fold risk of developing CRC.[8,10,11] Although several candidates for the Ggly receptor have been identified (*e.g.* annexin II,[12] F1-ATPase α-subunit[13]), small molecule inhibitors have not as yet been developed.

Most human sporadic CRCs contain an inactivating mutation in the *APC* (adenomatous polyposis coli) tumor suppressor gene.[14] APC mutant mice provide a model of spontaneous intestinal cancers *in vivo* without the use of carcinogens.[14,15] Koh *et al.* crossed $APC^{min-/+}$ mice that spontaneously develop intestinal tumors, primarily in the small intestine, with either mice overexpressing glycine-extended gastrin (MTI/Ggly) or gastrin knockout mice (GASKO).[16] The data showed that mice prone to develop intestinal tumors had enhanced tumor size and numbers when Ggly was over-expressed and significantly reduced tumor incidence when all gastrins were absent. The development of $APC^{\Delta 14/+}$ mice, with a deletion of exon 14 engineered into one allele of their *Apc* tumor suppressor gene, has provided a better model of familial adenomatous polyposis, as the proportion of spontaneous tumors developing in their large intestine is greater than with $APC^{min-/+}$ mice.[17]

Strong links between excess iron and CRC have also been reported.[18,19] Study of $APC^{min/+}$ mice with either iron-deficient, normal or excess iron diets found that high iron drove intestinal tumorigenesis, as tumor number and size correlated with dietary iron status.[20] Using GASKO mice and CCK2 receptor knockout mice (which have high circulating gastrin concentrations), Kovac *et al.* found that gastrins have a direct role in hematopoiesis under iron deficiency.[21] GASKO mice on a low iron diet had impaired erythropoiesis and developed splenomegaly. Hypoxia studies indicated that gastrins may play a protective role under low oxygen conditions, possibly *via* catalysis of iron binding to transferrin.[22,23]

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Work to elucidate the mechanism of action of gastrins revealed that ferric ion binding to glutamates 7 and 8/9 in the sequence QGPWLEEEEEAYGWMDF was essential for biological activity of Ggly, but not Gamide.[24–26] Bioactivity of Ggly was diminished or destroyed by deleting these glutamates or by treating with the chelator desferrioxamine (DFO).[25,27] Trivalent Bi^{3+} ions can displace ferric ions from the iron-binding sites on gastrins, with a concomitant reduction in the biological activity of Ggly *in vitro*.[28,29] *In vivo*, Ggly-induced stimulation of proliferation in the defunctioned rectal mucosa of rats could be abrogated with oral Bi^{3+} treatments, and oral Bi^{3+} also decreased proliferation rates in the colonic mucosa in both hGAS (over-expressing progastrin) and MTI/Ggly mice (over-expressing glycine-extended gastrin).[29] In addition, DFO restored the increased colorectal mucosa proliferation rates in mice overexpressing progastrin (hGAS) or Ggly (MTI/Ggly) to the levels observed in wild-type control mice.[30]

Recently trivalent In^{3+} and Ru^{3+} ions have been shown to form stable, high affinity complexes with gastrins.[26,31] Ru^{3+} and In^{3+} ions were both bound to the iron-binding site of gastrins orders of magnitude more tightly than ferric ions. Because the biological activity of Ggly was dependent on the presence of ferric ions, the possibility arose of using Bi^{3+}, In^{3+} or Ru^{3+} ions as competitive inhibitors of the biological activity of Ggly. As inhibitors of the Ggly receptor are not yet available, the aim of the present study was to determine the effect of blocking ferric ion binding to Ggly using Bi^{3+}, In^{3+} or Ru^{3+} ions on the incidence and size of intestinal tumors in $APC^{\Delta 14/+}$ mice.

Experimental

Reagents and dosage

All consumables were obtained from Sigma Aldrich (Castle Hill, Australia), unless noted otherwise. Bismuth citrate, indium citrate and ruthenium citrate were all suspended in phosphate-buffered saline (ThermoFisher Scientific, Waltham, MA, USA), and the concentrations described below are based on the metal ion. The dose of Bi^{3+} used was derived from previous work in which 141 mg Bi^{3+} per kg per day was active, but not toxic, in mice treated daily for 4 weeks.[29] This dose is 70-fold less concentrated than the single 50% lethal dose (LD50) of 10 000 mg per kg in mice. A single Bi^{3+} dose of 2000 mg per kg (1/5 of LD50), or Bi^{3+} doses of 1000 mg per kg per day (1/10 of its LD50) repeated daily for 28 days in rats, also showed no adverse effects.[32,33] A similar study with oral In^{3+} in rats also demonstrated that a single dose of 2000 mg per kg (1/5 of its LD50 of 10 000 mg per kg in mice), or an In^{3+} dose of 1000 mg per kg per day (1/10 of its LD50) repeated daily for 28 days in rats, had no adverse effects.[33,34] Based on this study, the same concentration was used for In^{3+} as for Bi^{3+}. The same logic was used to derive the Ru^{3+} dose of 6.5 mg per kg per day based on a concentration 70-fold less than the reported LD50 of 463 mg per kg in mice.[35]

Mouse model

$APC^{\Delta 14/+}$ mice (C57Bl/6 background) were kindly provided by Dr Julie Pannequin (National Centre for Scientific Research, Institute of Functional Genomics, Montpellier, France). These mice, which have had a heterozygous deletion of exon 14 engineered into one allele of their *Apc* tumor suppressor gene, provide a model of familial adenomatous polyposis (autosomal dominant disease), with spontaneous tumors developing in their small and large intestines.[17,36]

$APC^{\Delta 14/+}$ mice (8–10 weeks old) were divided into four treatment groups and orally gavaged with 0.1–0.15 mL of PBS (16 female and 17 male mice), bismuth citrate (9 female and 8 male mice, 141 mg Bi^{3+} per kg per day), indium citrate (7 female and 8 male mice, 141 mg In^{3+} per kg per day) or ruthenium citrate (7 female and 8 male mice, 6.5 mg Ru^{3+} per kg per day) once a day, 3 times a week, up to 20 weeks of age. Animals were weighed and sickness scores taken daily. Mice were humanely sacrificed at the end of the experiment by isoflurane inhalation and blood was collected by cardiac puncture. Intestines were collected, inverted on wooden skewers, formalin fixed and prepared as Swiss Rolls for counting of intestinal tumors as described.[37] All animal work was approved by the Austin Health Animal Research Ethics Committee (AEC13/4957 and AEC15/5307).

Hematological parameters

Full blood examination was performed as previously described[22] by the Austin Pathology Laboratory (Heidelberg, VIC, Australia) using an Advia 120 automated hematological analyzer (Bayer, Tarrytown, NY, USA). Serum trace metals were analyzed by inductively coupled plasma mass spectrometry (ICPMS) (Biometals Facility, Florey Institute of Neuroscience and Mental Health, University of Melbourne). Serum from mice was diluted with 1/10 1% (v/v) nitric acid and measured with an Agilent 7700 series ICPMS instrument under routine multi-element operating conditions using a helium gas reaction cell. A certified standard solution containing 200 ppb of Yttrium (Y89) was used as an internal control.

Statistics

Survival statistics were performed using log-rank and cox-regression (SPSS version 22.0, IBM, Armonk, NY, USA). All other statistics were analyzed using either student's *t*-test or one-way ANOVA (SigmaStat, Jandel Scientific, San Rafael, CA, USA). A P value < 0.05 was considered significant.

Results

Treatment increased metal ion concentrations in the serum

Metal salts were given by oral gavage for up to 60 days at the following doses: Bi^{3+}, 141 mg per kg per day; In^{3+}, 141 mg per kg per day; Ru^{3+}, 6.5 mg per kg per day. At the end of the experiment, concentrations of each metal ion in the serum were measured by ICPMS. All metals were absorbed as the serum concentrations increased significantly in the treated animals (Fig. 1). However, the ratio of Bi^{3+} absorbed to the amount given orally was low (all: 1/980; female: 1/681; male: 1/1,986). The ratios of In^{3+} and Ru^{3+} absorbed to the amounts given orally were closer to unity (In^{3+}, all: 1/35; female: 1/92; male: 1/25 and Ru^{3+}, all: 1/27; female: 1/64; male: 1/18).

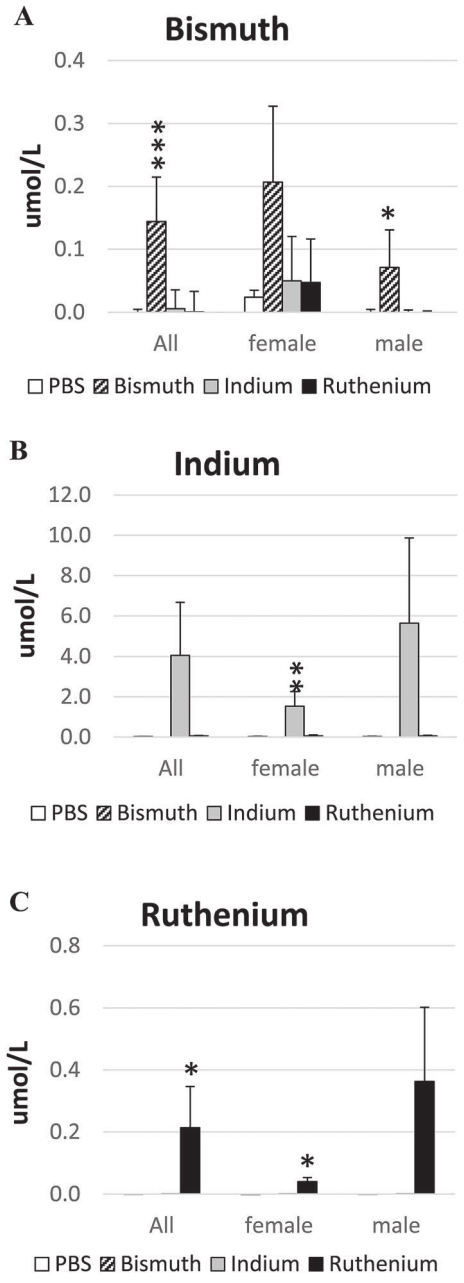

Fig. 1 Serum metal ion analyses. The concentrations of Bi^{3+} (A), In^{3+} (B) or Ru^{3+} (C) in the serum from $APC^{\Delta 14/+}$ mice treated with PBS (control), bismuth citrate, indium citrate or ruthenium citrate were analyzed by inductively coupled plasma mass spectrometry (ICPMS). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Effect of metal ions on the health of $APC^{\Delta 14/+}$ mice

$APC^{\Delta 14/+}$ mice develop intestinal bleeding and anemia similar to $APC^{min/+}$ mice. However, $APC^{\Delta 14/+}$ mice may show earlier signs of decreased health as they develop tumors in both their small and large intestines, while $APC^{min/+}$ mice typically develop tumors only in their small intestines.[17] $APC^{\Delta 14/+}$ mice in all treatment groups (PBS, Bi^{3+}, In^{3+} or Ru^{3+}) had increased sickness scores over time (Fig. S1, ESI†). Male mice appeared to be more sensitive with greater sickness scores (Fig. S1C, ESI†) and greater average weight loss (Fig. S2C, ESI†) than females. Overall, neither Bi^{3+} nor In^{3+} affected mouse survival (Fig. 2A); mice treated with Ru^{3+} died 2.0 times faster than PBS mice ($P = 0.079$) and the survival of Ru^{3+}-treated animals was less than Bi^{3+}- or In^{3+}-treated animals.

$APC^{\Delta 14/+}$ mice have hypoxemia

Anemia is a common outcome of the $APC^{\Delta 14/+}$ mutation due to intestinal bleeding from large polyps. The PBS control and all three treatment groups had significantly decreased hemoglobin concentrations (139 ± 3), hematocrits (0.47 ± 0.01) and red cell counts (9.2 ± 0.2) compared to the values shown in the brackets

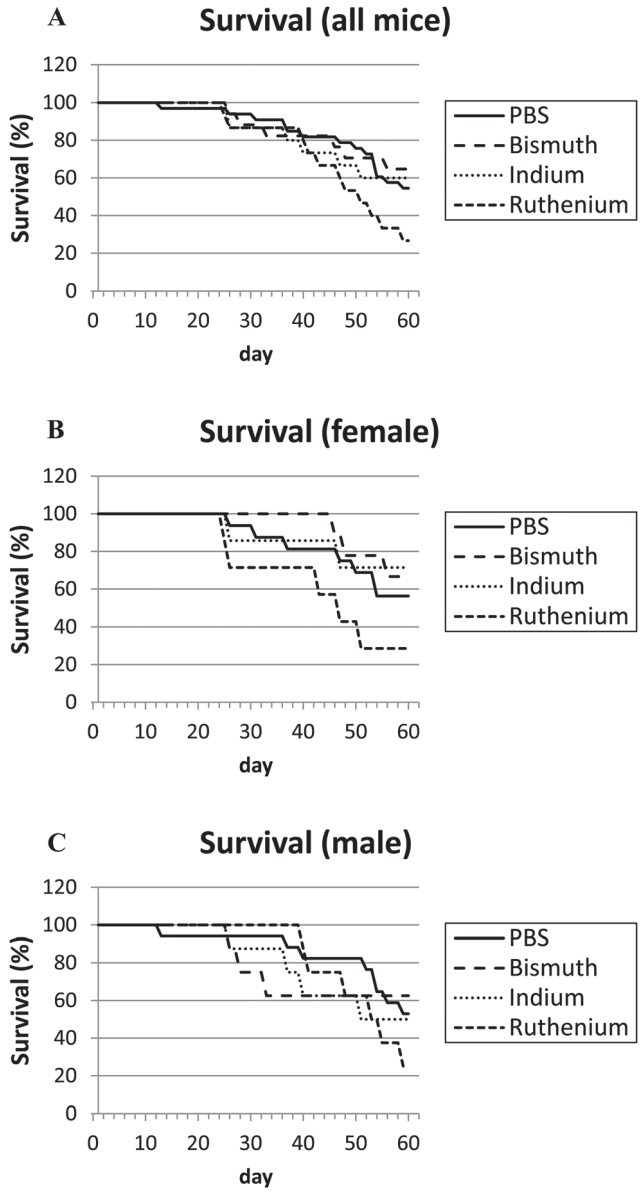

Fig. 2 Survival plots. $APC^{\Delta 14/+}$ mice were treated with PBS (control), bismuth citrate, indium citrate or ruthenium citrate over 60 days. Survival graphs are shown for all (A), female only (B) and male only (C) mice. The difference between PBS- and Ru^{3+}-treated mice neared significance as cox-regression analysis indicated Ru^{3+}-treated mice died 2.0 times faster than PBS-treated mice ($P = 0.079$).

for untreated C57Bl/6 mice of a similar age (our unpublished data) (Fig. 3A–C). Males, particularly those treated with Ru^{3+}, appeared to be more affected. Serum iron concentrations were not significantly different to each other or to the value for untreated wild type animals (26.2 ± 1.5), but In^{3+} and Ru^{3+} appeared to increase or decrease iron levels respectively (Fig. 3E). Treatment with In^{3+} significantly increased WCC compared to the other groups (Fig. 3D), in agreement with previous findings.[34,38] Platelet numbers were significantly reduced in Bi^{3+}-treated $APC^{\Delta 14/+}$ mice, to values closer to those observed in untreated wild-type mice (Fig. 3F).

Bi^{3+} treatment decreased, and In^{3+} or Ru^{3+} treatment increased, tumor burden in the small intestine

Intestinal tumor size and numbers were determined in the small and large intestines from $APC^{\Delta 14/+}$ mice treated with PBS,

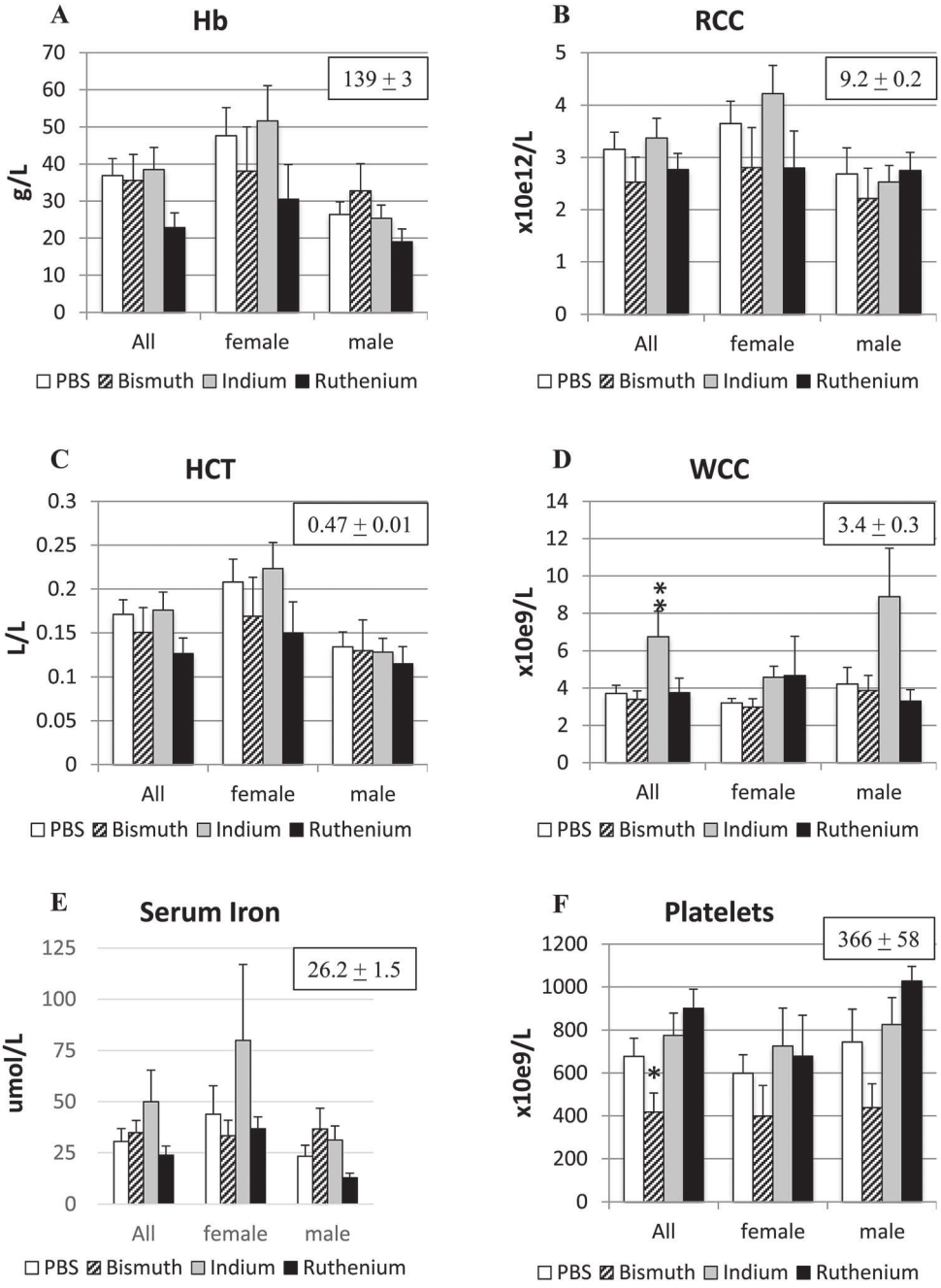

Fig. 3 Hematological parameters. Blood samples from $APC^{\Delta 14/+}$ mice treated with PBS (control), bismuth citrate, indium citrate or ruthenium citrate were analyzed for hemoglobin (A), red blood cell count (B), hematocrit (C), white blood cell count (D), and platelet count (F) by the Austin Pathology Laboratory (Heidelberg, VIC, Australia). Serum iron levels (E) were analyzed by ICPMS. Boxed numbers are the corresponding mean ± SEM values from untreated wild-type C57Bl/6 mice of similar ages. *$P < 0.05$; **$P < 0.01$.

Bi^{3+}, In^{3+} or Ru^{3+}. Bi^{3+} treatment significantly decreased the number of tumors larger than 3 mm in male mice (Fig. 4C). In^{3+} or Ru^{3+} treatment significantly increased the tumor burden in all animals and in male mice alone (Fig. 4A and C). No significant differences were observed in the colon (Fig. 5).

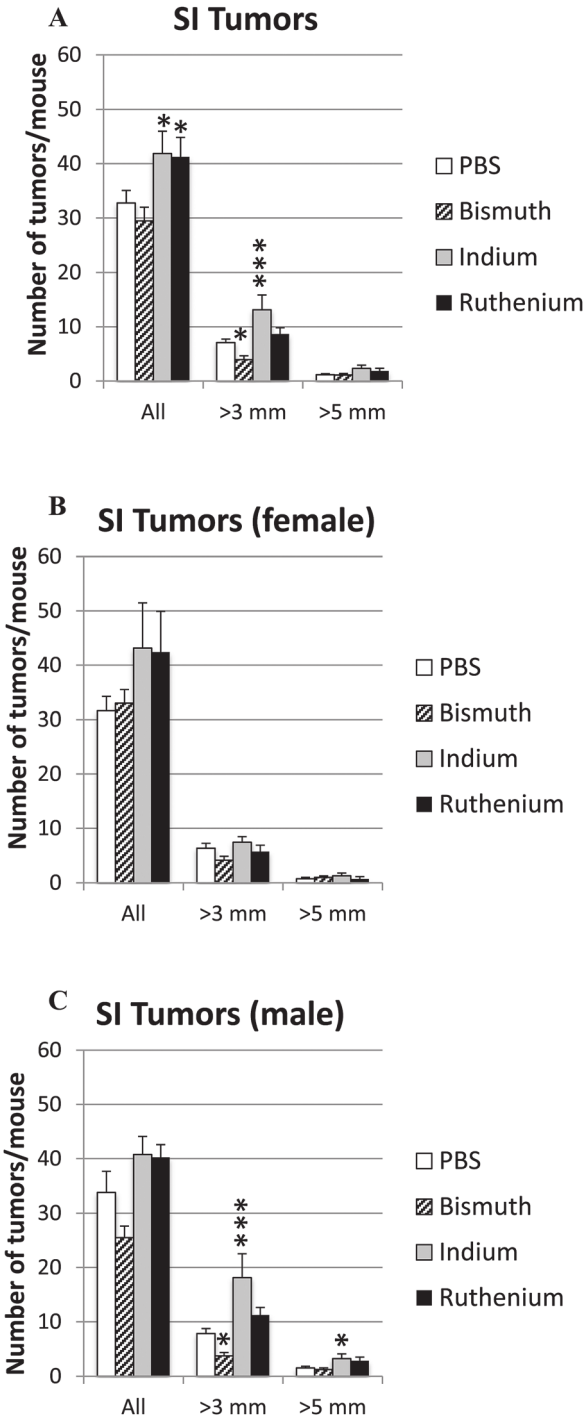

Fig. 4 Tumor burden in small intestine. $APC^{\Delta 14/+}$ mice were treated with PBS, Bi^{3+}, In^{3+} or Ru^{3+}, and the total number and number of tumors over 3 mm or 5 mm in the small intestine were counted for all mice (A), female only (B) and male only (C). Significance was determined compared to the PBS-treated mice. $*P < 0.05$; $***P < 0.001$.

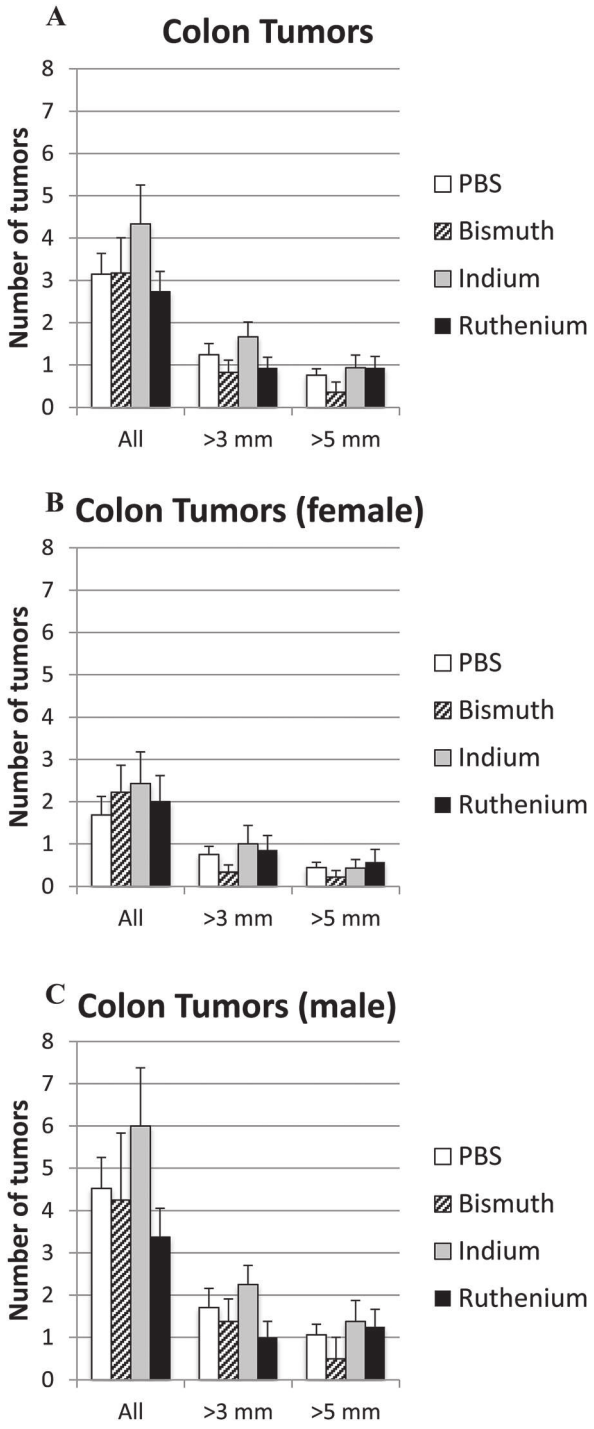

Fig. 5 Tumor burden in colon. $APC^{\Delta 14/+}$ mice were treated with PBS, Bi^{3+}, In^{3+} or Ru^{3+}, and the total number and number of tumors over 3 mm or 5 mm in the colon were counted for all mice (A), female only (B) and male only (C). No significance difference was detected.

Discussion

Previous work has revealed that $APC^{min-/+}$ mice, which are prone to develop intestinal tumors, had enhanced tumor size and numbers when Ggly was over-expressed and significantly reduced tumor incidence when all gastrins were absent.[16] The observation that Bi^{3+}

ions blocked the binding of Fe^{3+} ions to gastrins, and thereby decreased the bioactivity of Ggly *in vivo* and *in vitro*,[28,29] suggested that Bi^{3+} treatment might also decrease tumor burden in APC mice. Further work revealed that the binding of In^{3+} or Ru^{3+} ions to gastrins was orders of magnitude stronger than the binding of Bi^{3+} ions.[26] The effect of blocking ferric ion binding to Ggly using Bi^{3+}, In^{3+} or Ru^{3+} ions on the incidence and size of intestinal tumors in $APC^{\Delta 14/+}$ mice was therefore investigated.

Bi^{3+} treatment significantly decreased the number of small intestinal tumors larger than 3 mm in male mice (Fig. 4C). No reduction in tumor numbers was observed in the colon, or on tumors larger than 5 mm in either small intestine or colon, possibly because of the smaller numbers involved. The mechanism is likely to involve competition by Bi^{3+} for Fe^{3+} ion uptake and/or inhibition of intracellular processes involving iron. The effectiveness of Bi^{3+} treatment might possibly be improved by increasing the dosage or route of administration, as bismuth citrate was poorly absorbed and/or retained in the circulation (Fig. 1), in agreement with previous reports on its poor absorption.[39,40] The observation that Bi^{3+} did not affect circulating iron concentrations or most blood parameters at the dosage used (Fig. 3) suggests that greater dosages might be tolerated, although the significant reduction in platelet counts following Bi^{3+} treatment suggests that a cautious approach would be appropriate.

In contrast to Bi^{3+}, both In^{3+} and Ru^{3+} treatments significantly increased tumor numbers in the small intestine of $APC^{\Delta 14/+}$ mice. Again no effect was observed on tumors in the colon, possibly because of the smaller numbers involved. The fact that binding of In^{3+} or Ru^{3+} ions to gastrins was orders of magnitude stronger than the binding of Bi^{3+} ions,[26] therefore implies that the inhibitory effect of Bi^{3+} ions is not a consequence of a reduction in Ggly activity. The failure to observe an effect does not appear to be caused by poor absorption as the absorption ratios ($\mu mol\ L^{-1}$ in the serum: amount given orally) for In^{3+} and Ru^{3+} were greater than for Bi^{3+}. Furthermore both In^{3+} and Ru^{3+} treatments were effective in other respects. Thus In^{3+} treatment significantly increased white cell counts, in agreement with previous reports that In^{3+} can stimulate the immune system,[34,38] and Ru^{3+} treatment decreased survival. The latter effect may be caused by displacement by Ru^{3+} of circulating iron from transferrin and hemoglobin,[41,42] as ruthenium is in the same column of the periodic table as iron.

Conclusions

Bi^{3+} treatment significantly decreased the number of tumors larger than 3 mm in male $APC^{\Delta 14/+}$ mice. Comparison of the effects of Bi^{3+}, In^{3+} and Ru^{3+} treatment on tumor numbers suggests that the inhibitory effect of Bi^{3+} ions is unlikely to be gastrin-dependent. However, further testing of higher doses of Bi^{3+} ions for longer periods as an oral treatment for intestinal tumors is warranted. The use of In^{3+} or Ru^{3+} ions for preventative reduction of intestinal tumor burden appears inadvisable based on the present findings, and their use in other tumor treatments should be approached with caution, especially if the patient is anemic.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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9.2 Summary

$APC^{\Delta 14/+}$ mice treated by gavage with Bi^{3+}, In^{3+} and Ru^{3+} for up to 60 days had an increase of the metal ion concentrations in the serum with a ratio of In^{3+} and Ru^{3+} absorbed close to the ratio of the amounts given orally. The amount of absorbed Bi^{3+} was low compared to the amount given to the mice. $APC^{\Delta 14/+}$ mice in all treatment groups developed tumors in both their small and large intestines and their health decreased over time. Although Bi^{3+} and In^{3+} treated mice had the same sickness score as PBS-treated mice, Ru^{3+} treatment affected mouse survival and mice died 2.0 times faster than control mice. $APC^{\Delta 14/+}$ mice generally develop internal bleeding from large polyps and thus anemia. All the mice, including PBS-treated mice, had significantly lower hemoglobin concentrations, hematocrits and red cell counts compared to the values in untreated C57Bl/6 mice. No significant differences were found in serum iron concentrations between PBS control mice and all treated mice. The numbers of tumors in both the small and large intestines were counted and the tumors categorized by size. Although In^{3+} and Ru^{3+} increased the tumor burden in all animals, Bi^{3+} treatment significantly decreased the number of tumors larger than 3mm in male mice. No changes were observed in the colon. These observation, added to the fact that bismuth citrate was poorly absorbed, suggested it is unlikely that Bi^{3+} blocked the binding of Fe^{3+} to gastrins but that the mechanism involved could imply intracellular processes involving iron. The benefits of Bi^{3+} treatment on cancer progression could be enhanced by increasing the dosage or changing the route of administration.

CONCLUSION

Gastrins represent a group of mature (Gamide) and immature (progastrin and Ggly) peptides derived from the gastrin gene. Gamide is a peptide hormone that stimulates secretion of gastric acid following a meal and that also functions as an autocrine growth factor for the gastrointestinal mucosa. Proliferation in the colonic mucosa was inhibited in gastrin knockout mice but stimulated in mice overexpressing progastrin or Ggly [215] [220]. Studies have implicated gastrins, and most importantly the immature forms of gastrins, in the development of colorectal carcinoma; but the mechanisms involved were not clear. However, we have previously suggested a correlation between gastrins and iron homeostasis, which could be a key element in colorectal cancer progression [347]. Another property to investigate in CRCs is the ability of gastrins to bind two ferric ions as well as other trivalent metal ions, including indium, ruthenium, gallium and bismuth [319] [323].

In the first study, we were interested in the role of gastrins under hypoxic conditions, which occur in many solid tumors. Gastrin expression is upregulated by hypoxia in gastrointestinal cancer cell lines [273] and gastrins may play a protective role against hypoxia [274]. To investigate the protective role of progastrin under hypoxia, hGas mice, which overexpress progastrin in the liver, were exposed to normoxia or hypoxia (Paper I [375]). Both hGas and control FVB/N mice had higher sickness scores and lost more weight under hypoxia than normoxia but the mice with gastrin gene overexpression were healthier than the control mice. Interestingly, hGas mice had significantly lower concentrations of liver iron and serum ferritin than FVB/N mice. As already described in mountaineers under acute high-altitude hypoxia, the hepcidin levels were reduced while the Dmt-1 levels were increased in the FVB/N mice in hypoxic conditions [377]. However, hGas mice had higher hepcidin levels compared to control mice, but showed no further increase under hypoxic conditions, and they showed no increase in Dmt-1 levels when under hypoxia. Although there is a tight connection between iron regulation and oxygen homeostasis via the oxygen-responsive blood hormone erythropoietin [378], our results indicate that progastrin also affects the iron-oxygen association to help the mice cope better under hypoxic conditions. We have therefore suggested that there was a more efficient mobilization of hepatic iron stores when progastrin is overexpressed and that could be explained by the ability of circulating gastrins to bind ferric ions and transfer them to apotransferrin. A recent study has demonstrated that

Gamide reduced cardiac ischemia/reperfusion injury (IRI) in both *in vitro* and *in vivo* experiments via the PI3K/AKT, ERK1/2 and STAT3 pathways. Gamide increased recovery of cardiac function and reduced cardiac necrosis-induced IRI in patients demonstrating Gamide has a protective role against hypoxia and could potentially be used as a therapeutic strategy in IRI [379].

Hypoxia upregulates a number of specific genes, including growth factors, and the transcription factor HIF1α mediates metabolic adaptation, angiogenesis, erythropoiesis, cell growth, survival and apoptosis [380]. Study of the mechanisms involved in gene upregulation under hypoxic conditions is highly relevant in understanding cancer progression. Similar to hypoxia, Zn^{2+} ions block HIF1α hydroxylation resulting in the upregulation of cell survival proteins [381].

Both chemical hypoxia, such as that induced by $CoCl_2$, and treatment with Zn^{2+} ions upregulate gastrin gene expression *in vitro*. In our second study, the effects of Zn^{2+} ions *in vivo* have been assessed by engineering a luciferase reporter construct into the human gastrin gene promoter in SW480 cells using TALEN technology and by xenografting these cells in SCID mice (Paper II [382]). Gastrin promoter activity was stimulated by both $CoCl_2$ and Zn^{2+} ions *in vitro* and *in vivo*, and this activation was dependent on the MAPK but not the PI3K pathway. In addition, although the seven-fold increase of intracellular free zinc ions in zinc-treated cells compared to untreated cells disappeared by 24 hours, the activation of the gastrin promoter was still present 24 hours following treatment by zinc ions. Similar observations were seen *in vivo* with the gastrin promoter still activated up to five days after zinc treatment. The presence of a positive autocrine loop could explain the long-term activation of gastrin promoter activity via zinc ion-activated growth factors or proteins. The ability of Zn^{2+} ions to upregulate HIF1α and have a protective role against hypoxia was investigated in the case of IRI in the kidney. Preconditioning with subcutaneous Zn^{2+} ions significantly reduced the acute kidney injury in rats in a dose-dependent manner [383] and in a sheep model of IRI [384]. Zinc ions play a role in the immune and antioxidant response and in apoptosis, and thus have both beneficial and detrimental effects. The immunosuppressive effect could be dangerous. Understanding further the correlation between gastrins and zinc homeostasis could be as important as the association of gastrins and iron homeostasis in the progression of CRCs, especially since a potential role of zinc in colorectal carcinogenesis has been reported [385].

The third study was focused on the ability of gastrins to bind trivalent metal ions and the option to use metal ion-gastrin complexes as an alternative to metal chelate-conjugated gastrin derivatives to detect CCK2R-positive tumors. Gamide complexes with radioactive isotopes of indium, ruthenium and gallium were purified by HPLC and their ability to bind to the CCK2R was tested (Paper III [386]). The three complexes, ^{111}In-Gamide, ^{106}Ru-Gamide and ^{68}Ga-Gamide, unexpectedly failed to bind to the CCK2R in AGS cells. As the complexes were stable over time, this failure could be due to structural changes or to an interaction of the aspartic

acid in the C-terminal domain of gastrin with indium, ruthenium and gallium ions blocking the binding to the CCK2R. In this context it is important to note that the C-terminal tetrapeptide amide WMDFamide is the minimum requirement for binding to the CCK2R. New minigastrin analogs, which have substitutions in the C-terminal part of the peptide and DOTA at the N-terminus for radiolabelling with ^{111}In, have recently been developed and displayed better enzymatic stability, lower kidney intake and increased tumor targeting *in vivo* [387]. Another CCK2R-binding radiolabeled peptide called CP04, which is a ^{111}In-DOTA-labeled gastrin analog, was selected for clinical evaluation because of the positive results of a preclinical study. Administration of ^{111}In-CP04 to humans was safe and medullary thyroid carcinomas (MTCs) were efficiently detected [388]. Targeting CCK2Rs thus represents a promising strategy in CRC diagnosis and treatment and, although the Gamide-complexes developed in our study failed to bind to the CCK2R, they could be used to block the biological actions of Gamide. Indium and ruthenium, which displace ferric ions from the metal ion binding site of Gamide, are potentially specific inhibitors of Gamide acting as a growth factor or a stimulant of gastric acid secretion.

Blocking the biological activity of Ggly by displacing its essential binding to Fe^{3+} by treatment with Bi^{3+}, In^{3+} or Ru^{3+} ions is also a promising strategy to reduce the progression of gastrointestinal cancer cells. $APC^{\Delta 14/+}$ mice, which develop spontaneous tumors in their large intestine, were treated orally with Bi^{3+}, In^{3+} or Ru^{3+} ions for up to 60 days (Paper IV [389]). Although In^{3+} or Ru^{3+} treatments increased tumor burden in the small intestine of $APC^{\Delta 14/+}$ mice, Bi^{3+} treatment led to a significant decrease of tumors larger than 3mm in male mice. No effect was observed in the colon. As bismuth citrate is poorly absorbed and maintained in the circulation, increasing its dosage or changing the route of administration could increase the benefits on tumor burden in $APC^{\Delta 14/+}$ mice. Bismuth remains a promising treatment in CRC because it is known to be safe for humans as organobismuth compounds are already used for treatment of gastritis [390], dyspepsia [391], ulcers and *H. pylori* infection [392]. Bismuth-based nanomaterials (NMs) hold great potential as a tool in cancer diagnosis and therapy especially because of their unique intrinsic properties, including strong X-ray attenuation making Bi a good element for CT contrast agent and narrow band gap conferring semiconductor properties and so a photothermal response to irradiation useful for photothermic therapies [393]. The performances of Bi-based NMs have been greatly improved recently but still face challenges such as developing a safer Bi-based NM for radiation applications or further testing the safety of theranostic Bi-based NMs before they can be used in clinical stages. The strategy to displace the binding of Fe^{3+} to Ggly by using metal ions, and especially Bi^{3+}, is still relevant in CRCs but studies on ways to improve its absorption should be done before new *in vivo* studies are attempted.

To conclude, the studies presented here bring new and critical insights into the correlations between gastrins, hypoxia and iron homeostasis in CRCs. Immature forms of gastrins, which have

been implicated in cancer cell proliferation and angiogenesis, are upregulated by hypoxia and by the divalent metal ion zinc. However, a high expression of progastrin has a protective role on mice under hypoxia. The effects of zinc on gastrin expression should be investigated further because of the effects of gastrins in CRCs and the implication of zinc in gene expression and in many diseases (diabetes, Alzheimer's and cancers). On the other hand, blocking the activity of gastrins either by displacing the bound ferric ions using another trivalent metal ion (bismuth, indium, ruthenium) or by preventing the binding of gastrins to their receptors are still relevant as potential therapies in the CRC context.

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French summary

Le cancer du côlon

Le côlon se situe après l'intestin grêle et forme la dernière partie du système digestif. Il peut mesurer jusqu'à 1,5 mètres et est composé de 4 parties : le côlon ascendant, le côlon transverse, le côlon descendant et le côlon sigmoide. Son principal rôle est d'absorber l'eau, le sel et certains nutriments, de maintenir l'équilibre hydrique et de déplacer les déchets vers le rectum. Le côlon est composé de plusieurs couches, de l'extérieur vers l'intérieur : une enveloppe séreuse, des muscles lisses, une sous-muqueuse vascularisée et innervée et une muqueuse. Des études ont montré que le gène cible de la voie Wnt intestinale, LGR5, est un marqueur spécifique des cellules souches intestinales.

L'adénocarcinome, qui représente plus de 90% des cancers colorectaux, se développe à partir des cryptes de la muqueuse intestinale. Un système de classification des tumeurs colorectales, internationalement standardisé et basé sur l'extension locale (T), ganglionnaire (N) et métastatique (M) de la tumeur, permet d'évaluer le stade tumoral témoignant de l'état d'avancement du cancer. Le pronostic de la tumeur est ainsi établi et un protocole thérapeutique adapté au stade du cancer et au patient peut être déterminé.

Le cancer colorectal est classé au 3ème rang des cancers les plus diagnostiqués, après les cancers de la prostate et des poumons, et au 2ème rang des cancers les plus meurtriers. Les cancers du côlon sont majoritairement sporadiques puisque seulement 20% des patients ont des prédispositions héréditaires. Celles-ci regroupent notamment la maladie génétique de la polypose adénomateuse familiale (FAP), dont les patients développent de nombreux adénomes au niveau du côlon et du rectum, et le syndrome de Lynch. Des mutations germinales du gène APC (Adenomatous Polyposis Coli) et des mutations constitutionnelles d'un gène MMR (MisMatch Repair) en sont respectivement les causes. L'âge, le mode de vie, les habitudes alimentaires, telles qu'un régime alimentaire pauvre en fruits et légumes mais riche en viande rouge, en acides gras saturés et une grande consommation d'alcool, seraient des facteurs influençant le risque de développement du cancer du côlon. La maladie étant le plus souvent asymptomatique, le diagnostic est réalisé alors que le cancer est déjà à un stage avancé. La vérification de l'historique familial, la palpation abdominale et rectale ainsi que la vérification de la présence de sang dans

les selles sont les 1ers examens médicaux réalisés lors d'un diagnostic du cancer du côlon. Ceux-ci étant suivis par des méthodes de diagnostic plus efficaces telles que la coloscopie ou des tests d'imagerie (CT, PET, NMR).

Les tumeurs malignes sont le résultat de mutations de 4 à 5 gènes, regroupant des altérations génétiques affectant des gènes suppresseurs de tumeurs et des oncogènes. Les trois principaux mécanismes moléculaires décrits dans les cancers colorectaux sont l'instabilité chromosomique (CIN), l'instabilité microsatellitaire (MSI) et l'instabilité épigénétique (CIMP), correspondant à la séquence adénome-cancer montrant les facteurs influençant la tumorigenèse. Les cancers de type CIN, représentant 60-70% des cancers colorectaux, sont le résultat de délétions, duplications ou réarrangements génétiques conduisant à une aneuploïdie; les cancers de type MSI ont des mutations dans les gènes du système de réparation MMR et des insertions/délétions de bases de l'ADN ; et les cancers de type CIMP présentent une hyperméthylation des îlots CpG de l'ADN. Les principales altérations génétiques et épigénétiques impliquées dans le cancer du côlon sont notamment la mutation du gène APC, entrainant l'activation de la voie de signalisation Wnt favorisant la prolifération, la migration et l'invasion des cellules cancéreuses et la mutation du gène KRAS, causant également une augmentation de la prolifération des cellules cancéreuses et favorisant le développement de métastases. Des mutations des gènes BRAF et p53 jouent également un rôle dans la progression des cancers colorectaux. Les facteurs de croissance, également impliqués, stimulent la croissance, la prolifération et la migration cellulaire ainsi que la différenciation cellulaire jouant par conséquent un rôle dans la tumorigenèse. Le facteur de croissance épidermique (EGF-epidermal growth factor) active de nombreuses voies de signalisation, telles que les voies Ras/MAPK, PI3K/Akt, STAT et PKC, et la surexpression de son récepteur ErbB dans certaines tumeurs entraine une suractivation de la transduction du signal et par conséquence une stimulation des cellules cancéreuses, notamment dans les stades avancés du cancer du côlon. Le facteur de croissance de l'endothélium vasculaire (VEGF- vascular endothelial growth factor), surexprimé dans les cellules cancéreuses via les oncogènes Ras, p53, TGFβ et par l'hypoxie, est impliqué dans les cancers du côlon. La gastrine, découverte pour ses capacités à stimuler la sécrétion d'acide gastrique après un repas, est également un facteur de croissance impliqué dans la croissance cellulaire du système intestinal. Un lien entre des taux élevés de gastrine dans le sérum de patients et leurs risques d'avoir un cancer du côlon a été identifié. Les traitements actuels du cancer du côlon sont basés sur l'avancée du cancer et sa localisation. La chirurgie est la stratégie principale pour tous les stades du cancer du côlon, et particulièrement pendant les stades précoces visant une résection pouvant entrainer la guérison du patient. La radiothérapie est principalement utilisée pour les cancers rectaux et la chimiothérapie peut être adjuvante et prescrite avant une opération pour réduire la taille de la tumeur ou en complément de la radiothérapie pour augmenter les effets positifs du traitement. Dans la majorité des cas, la chimiothérapie est prescrite après une résection chirurgicale afin

de limiter les risques de rechute. La thérapie ciblée et des études sur l'inhibition des protéines EGF et VEGF ont abouti à des molécules dont certaines sont déjà utilisées comme traitement du cancer du côlon. L'immunothérapie est également prometteuse dans ce type de cancers avec par exemple des anticorps contre la protéine PD-1 qui sont utilisés en complément de la chimiothérapie. Cependant, les immunothérapies actuelles n'apportent pas d'effets positifs à la majorité des patients présentant un cancer du côlon.

La famille de la gastrine

La famille de la gastrine regroupe la protéine de la gastrine, découverte en 1905 et décrite pour sa capacité à activer l'estomac et la sécrétion d'acide gastrique, et la cholécystokinine (CCK), découverte en 1928 en tant qu'hormone stimulant la vésicule biliaire. Leur séquence a été identifiée dans les années 1960 et présente un pentapeptide terminal commun, Gly-Trp-Met-Asp-Phe-CO-NH2, représentant la structure minimale nécessaire à l'activité biologique de la Gastrine et de la CCK. La gastrine et la CCK présentent plusieurs formes de différentes longueurs dû aux étapes de maturation que ces protéines subissent afin d'atteindre leur forme mature. Le précurseur de la gastrine, appelé la préprogastrine, contient 101 acides aminés chez l'homme et génère la progastrine après clivage du peptide signal puis les gastrines à glycine étendue (G34-Gly et G17-Gly) et le CTFP (C Terminus Flanking Peptide) après plusieurs modifications (sulfatation et phosphorylation) et clivages successifs. L'α-amidation carboxyterminale des protéines G34-Gly et G17-Gly aboutit à l'obtention de deux peptides de gastrine bioactifs de 34 et 17 acides aminés. Le peptide mature de 17 acides aminés est communément appelé la gastrine. La préproCCK, qui contient 115 acides aminés, subit une série de clivages protéiques et de sulfatations aboutissant à plusieurs formes matures de la CCK, CCK-58, -33, -22 et -8. Chez l'homme, l'expression du gène de la gastrine est localisée au niveau des cellules G de la muqueuse antrale pylorique et du duodénum, et la sécrétion de la CCK a lieu au niveau de la muqueuse du duodénum et des cellules I. Les peptides de la famille de la gastrine se lient au récepteur RCCK1 et RCCK2, l'activant et permettant la transduction de signaux. Ces récepteurs sont des récepteurs couplés à la protéine G (RCPG), la plus large et diverse famille de protéines de signalisation, impliqués dans de nombreuses réponses cellulaires suivant leur activation par des hormones, des métabolites, des cytokines ou des neurotransmetteurs. Les RCPGs se définissent par une extrémité N-terminale extracellulaire, sept segments transmembranaires antiparallèles hydrophobes reliés par trois boucles intracellulaires (IL1 à IL3) et par trois boucles extracellulaires (EL1 à EL3), et une extrémité C-terminale intracellulaire. Ils sont regroupés pour leurs homologies de séquence et leurs caractéristiques pharmacologiques via plusieurs systèmes de classification, la classification GRAFS et le système Kolakowski. Le RCCK1 se lie à la CCK sulfatée avec une affinité 500-1000 fois plus élevée que la gastrine, alors que le RCCK2 se lie avec la même affinité au CCK et à la gastrine. Cependant, la progastrine, le Ggly et le CTFP ont tous une très faible affinité

pour le RCCK2 et certaines études ont mis en évidence l'implication de d'autres récepteurs, le GBP (gastrin-bound protein) ou le F1-ATPase. Les RCPGs sont activés après liaison avec leur ligand leur conférant des changements conformationnels et facilitant leur interaction avec les protéines G. La protéine G activée peut alors initier la transduction du signal intracellulaire induite par le récepteur, aboutissant le plus souvent à la transcription de gènes. Le RCCK2 est un récepteur couplé aux protéines Gq, qui vont activer la voie de la phospholipase C produisant des inositols triphosphates (IP3) et du diacylglycérol (DAG). Ces messagers secondaires vont respectivement stimuler la libération de calcium dans le cytosol et activer différentes protéines kinase C (PKC) impliquées dans la signalisation des MAPKinases via la protéine Raf. La cascade de phosphorylation Ras/Raf/ERK kinases/ERK1/2 conduit à la régulation de la prolifération, la différenciation, la survie ou l'apoptose cellulaire et à l'activation de facteurs de transcription.

Effets physiologiques

La CCK est une hormone qui permet la contraction et la relaxation de l'estomac et de la vésicule biliaire puis la libération de la bile dans le duodénum. La CCK agit également comme facteur de croissance pour le pancréas et comme neurotransmetteur pour le système nerveux central et périphérique. De plus, la CCK inhibe la sécrétion d'acide gastrique en promouvant la libération de la somatostatine par les cellules D du duodénum via le RCCK1. Le rôle initial de la gastrine est de stimuler la sécrétion d'acide gastrique après la prise d'un repas pour aider sa digestion. En effet, des études ont démontré que la liaison de la gastrine au RCCK2 active la sécrétion d'histamine qui à son tour stimule les cellules pariétales et active la sécrétion d'acide gastrique. Cette sécrétion est inhibée par la somatostatine lorsque la quantité suffisante d'acide gastrique est atteinte. La gastrine exerce une action trophique puisqu'elle stimule la synthèse d'ADN dans plusieurs tissus et qu'elle stimule la prolifération et la différenciation des cellules de la muqueuse gastrique. Chez les souris et les rats, la gastrine contrôle la prolifération des cellules ECL. De plus, au niveau du pancréas, la gastrine et la CCK réguleraient directement la stimulation de la production de glucagon via le RCCK2 et la gastrine aurait un rôle dans l'homéostasie du glucose. La gastrine aurait un effet incrétine mais la littérature reste conflictuelle à ce sujet. Ggly stimule la prolifération et la migration cellulaire et la présence de son récepteur dans les cellules humaines du cancer de côlon lui confère un effet trophique via un mécanisme indépendant de la voie MEK suggérant un potentiel impact sur la progression du cancer du côlon. Des études sur les rats ont confirmé que Ggly agit comme un promoteur de la carcinogenèse colorectale. La progastrine est un facteur de croissance de l'appareil digestif puisque sa surexpression entraîne une augmentation de la prolifération des cellules de la muqueuse gastrique. La progastrine a également des effets anti-apoptotiques contribuant à la prolifération des cellules cancéreuses.

La gastrine et le cancer du côlon

Le gène de la gastrine est exprimé dans les lignées cellulaires de cancer du côlon (telles que les cellules HCT116 ou LIM1215) mais alors que la gastrine est sécrétée à des quantités similaires dans les cellules normales et les tumeurs colorectales, les formes immatures de la gastrine (progastrine, Ggly et CTFP) sont retrouvées à des concentrations plus élevées dans les tumeurs. La maturation de la progastrine n'est pas complète dû à des événements post-translationnels pendant la séquence adénome-carcinome et par la présence de mutations du gène APC. Le récepteur RCCK2 est souvent surexprimé chez les patients atteints de cancer du côlon et des études *in vitro* ont montré que sa stimulation par la gastrine stimulait l'activation des voies de signalisation de l'adhérence focale (FAK-Focal adhesion kinase) dans les cellules cancéreuses promouvant le développement du cancer du côlon et sa progression en métastases. La gastrine est un facteur de croissance agissant de façon autocrine dans les lignées cellulaires de carcinomes colorectaux et la progastrine et Ggly auraient potentiellement le même rôle. L'hypergastrinémie, ou la production excessive et anormale de gastrine, est la conséquence de certaines conditions cliniques telles que l'anémie pernicieuse, le syndrome de Zollinger-Ellison ou l'infection à Helicobacter pilori et pourrait être corrélée avec le développement de cancers du côlon. Cependant, la littérature reste controversée à ce sujet. L'hypoxie, ou un taux faible d'oxygène, est une caractéristique commune de beaucoup de tumeurs du fait d'une pauvre vascularisation. L'hypoxie stimule la transcription d'environ 1.5% de gènes dans les cellules tumorales entrainant la croissance tumorale et l'angiogenèse via le facteur de transcription HIF-1α (Hypoxia-inducible factor 1). Le gène de la gastrine est surexprimé en réponse à l'hypoxie chez les rats et chez l'humain. Des études ont montré que les précurseurs de la gastrine jouaient un rôle dans l'angiogenèse en promouvant l'expression du facteur pro-angiogénique VEGF via la voie des PI3K mais indépendamment de l'hypoxie. L'hypoxie chimique, $CoCl_2$, et les ions Zn^{2+} agissent également sur le gène de la gastrine. L'activation du gène de la gastrine par les ions zinc est partiellement faite via les voies MAPK et PI3K et dépendant du mécanisme E-box. Etudier la corrélation entre l'homéostase du zinc et la gastrine serait nécessaire pour comprendre leurs rôles au niveau de la transition épithélio-mésenchymateuse (TEM) et dans certaines pathologies. Au vu de l'implication de la gastrine dans le développement des cancers du côlon, des thérapies basées sur cette protéine ont émergé. Alors que l'antagoniste non spécifique du RCCK2, Proglumide, ne prolonge pas la survie des patients présentant des cancers gastriques ou colorectaux avancés, l'antagoniste spécifique du RCCK2, L-365,260, diminue l'action mitogénique de la gastrine et ralentit la prolifération des tumeurs gastrointestinales. Le RCCK2 étant très peu exprimé dans les carcinomes colorectaux, des analogues de la somatostatine ont été testés pour réduire la libération de la gastrine par les cellules G avec des résultats peu concluants cliniquement. Des anticorps ciblant la gastrine et Ggly via l'utilisation d'immunogènes gastriques, le Gastrimmune par exemple, permettraient de réduire la prolifération des cellules cancéreuses. Cette stratégie est actuellement en essais

cliniques. De façon similaire, des anticorps ciblant la progastrine ont montré une diminution de la prolifération cellulaire et une augmentation de la mort cellulaire de lignées cellulaires de cancer du côlon.

La gastrine et l'homeostasie du fer

Le fer est un élément important du corps humain et sa concentration, élevée après un repas, est maintenue à des valeurs physiologiques grâce à son utilisation et à des mécanismes de transport et de stockage. Dans l'intestin grêle, le fer non héminique est réduit en fer ferreux par la ferriréductase (Dcytb) puis traverse la membrane des hépatocytes via le transporteur spécifique DMT1. Le fer est soit retenu sous forme de ferritine puis éliminé, soit libéré par l'entérocyte via le transporteur ferroportine. Ce dernier est bloqué et dégradé par l'hepcidine, une hormone synthétisée par le foie, afin de contrôler la sortie du fer de l'entérocyte. L'interaction hepcidine-ferroportine ainsi que la transferrine ont des rôles centraux dans l'homéostasie du fer. La sécrétion d'acide gastrique est également impliquée dans l'absorption du fer et par conséquent dans la biodisponibilité du fer non héminique. Les patients sous omeprazole, un inhibiteur de la pompe à protons utilisé pour réduire la production d'acidité gastrique, développent une déficience en fer. La gastrine et Ggly se lient à deux ions ferriques en solution aqueuse et l'activité biologique de Ggly, au contraire de la gastrine, nécessite sa liaison au Glutamate 7 des ions ferriques. Le traitement par la déféroxamine (DFO), un chélatant du fer, bloquant l'action proliférative des gastrines immatures le confirme. D'autre part, le traitement avec le chélateur EDTA bloque l'interaction entre l'apotransferrine et la gastrine ou Ggly confirmant la présence de complexes gastrine-ion ferriques. Chez les souris dont le gène de la gastrine a été inactivé, une réduction significative de la sécrétion d'acide gastrique a été observée, ainsi qu'une diminution de l'expression du gène de l'hepcidine. De plus, la gastrine joue un rôle catalytique dans la liaison du fer par la transferrine menant potentiellement à des changements d'expression du gène de l'hepcidine. Dans les années 2000, un journal a collecté les résultats d'études sur les effets du fer sur les cancers colorectaux chez l'homme et une corrélation positive entre le fer stocké et le développement de lésions précancéreuses dans le côlon a été établie. De plus, une augmentation de la saturation de la transferrine est associée de façon signifiante au développement des cancers du côlon. L'apport excessif de fer alimentaire, notamment par la viande rouge, augmenterait les risques de cancer du côlon mais les mécanismes impliqués ne sont pas clairs. Les facteurs génétiques tels que les mutations du gène HFE et du gène APC peuventégalement influencer le fer et son stockage menant au développement des cancers colorectaux. Une contribution synergique de la gastrine et du fer dans le développement des cancers du côlon a été suggérée. Les tumeurs sécrètent des gastrines immatures qui se lient aux ions Fe^{3+} et catalysent la charge de fer des apotransferrines entrainant une plus grande disponibilité de complexes Fe-transferrine et par conséquent la progression des tumeurs. La synergie gastrine-fer dans le contexte de cancer du

côlon est devenue une nouvelle cible thérapeutique. La première stratégie repose sur l'utilisation de chélateurs du fer qui bloqueraient la liaison du fer aux gastrines immatures au niveau du tractus gastro-intestinal, ce qui par conséquent inhiberait l'activation de la prolifération des cellules cancéreuses par les gastrines. L'autre stratégie est de déplacer le fer de sa liaison avec Ggly pour limiter ses effets et, par exemple, nous avons précédemment démontré *in vitro* que l'ajout d'ions bismuth diminuait la prolifération et la migration cellulaire en réponse à Ggly. Les ions bismuth, considérant leur manque de toxicité, représentent un candidat prometteur dans l'inhibition des effets prolifératifs des formes immatures de la gastrine et devraient être testé dans des souris modèles de cancers intestinaux spontanés, les souris $APC^{\Delta 14/+}$.

Résultats scientifiques

Dans la 1ère étude, nous nous sommes intéressés au rôle de la gastrine en réponse à des conditions hypoxiques, retrouvées très souvent dans les tumeurs solides. Pour cela, des souris hGas, surexprimant la progastine dans le foie, ont été mises en conditions de normoxie (21% d'O_2) ou d'hypoxie (10% d'O_2) pendant 10 jours, et leur poids ainsi que leur état de santé général (leur mouvement, leur activité, leur déshydratation et leur respiration) ont été déterminés quotidiennement et comparés aux souris contrôles. Les souris hGas semblent généralement moins malades et ont perdu moins de poids que les souris contrôles sous hypoxie. Leur rate et leur foie étaient significativement plus légers chez les souris hGas mais leur taux de fer et de ferritine dans le sérum est plus faible comparé aux souris contrôles avec également une saturation plus faible de la transferrine. De façon plus intéressante, les souris hGas avaient une concentration de fer dans le foie plus basse, mais l'expression du gène hepcidine, un régulateur de l'homéostasie du fer, était plus élevée comparé aux souris contrôles. Cette étude décrit de façon signifiante le fait que la gastrine permet aux souris sous hypoxie de rester en meilleure santé et pourrait potentiellement jouer un rôle dans le contrôle de l'homéostasie du fer en régulant le stockage du fer hépatique. Dans un deuxième volet, nous avons caractérisé l'activation du promoteur du gène de la gastrine par les ions Zn^{2+} dans des cellules de cancer du côlon *in vitro* et *in vivo*. Nous avions précédemment montré la régulation positive du gène de la gastrine par le Zn^{2+} en utilisant des transfections transitoires. Pour l'étude *in vivo*, nous avons créé une lignée cellulaire stable, appelée $Gast^{luc}$, en intégrant le gène reporter de la luciférase au promoteur du gène de la gastrine humaine endogène dans les cellules de cancer du côlon SW480 et en utilisant la technologie des TALENs (Transcription activator-like effector nucleases). Cette stratégie permet d'éviter la variabilité du nombre de copies et de contrôler le lieu exact de l'intégration génomique. La bonne intégration de la luciférase au gène de la gastrine a été vérifiée en quantifiant la bioluminescence émise par la luciférase après activation du gène de la gastrine par les ions Zn^{2+}. Le gène de la gastrine est activé par $ZnCl_2$ et, plus faiblement par $CoCl_2$, dans les cellules Gastluc SW480. Lorsque les cellules sont traitées par l'inhibiteur de la voie des MAPKs, U0126,

l'activité du promoteur de la gastrine, stimulée par $ZnCl_2$, est réduite de 70% donc Zn^{2+} active le promoteur de la gastrine via la voie des MAPKs. Pour l'étude *in vivo*, les cellules $Gast^{luc}$ ont été injectées en sous cutanée à des souris immunodéficientes (SCID) qui ont alors développé des tumeurs. Les souris ont été traitées par injection intrapéritonéale de différentes concentrations de $ZnCl_2$ ou de $CoCl_2$ ou par le placébo les jours 2, 8 et 10 après la xénogreffe de GastLuc SW480 et des images de la bioluminescence ont été prises les jours 1, 3, 9 et 11. Bien que le traitement des souris par une injection intrapéritonéale de 30 mg/kg de $ZnCl_2$ ait été toxique, et évidemment exclue de l'étude, et des doses répétées de 5mg/kg de $ZnCl_2$ inefficaces, une dose unique de 10 mg/kg de $ZnCl_2$ a augmenté de 7 fois l'activité du promoteur de la gastrine. Cette activation était toujours présente 24 heures après le traitement. Il semblerait que la concentration de $ZnCl_2$ injectée ainsi que la fréquence soient critiques pour l'activation du promoteur de la gastrine *in vivo* et cette étude soulève alors la question du rôle du zinc dans l'activation de l'expression de gènes impliqués dans des maladies telles que le diabète, Alzheimer et le cancer. Dans un troisième volet, et en se basant sur nos précédents résultats décrivant la liaison de la gastrine et de Ggly à d'autres ions métalliques trivalents que le fer tels que le gallium (Ga), l'indium (In) et le ruthénium (Ru), nous avons émis l'hypothèse que des complexes de la gastrine formés avec des isotopes radioactifs du gallium, de l'indium ou du ruthénium et capables de se lier au récepteur RCCK2 pourraient être utilisés pour la détection de tumeurs exprimant le RCCK2. Nous avons créé ces complexes en incubant ^{68}Ga, ^{111}In ou ^{106}Ru avec la gastrine dans une solution d'acétate de sodium, à pH4 et à température ambiante pendant 30 minutes. Bien que les complexes ^{111}In-gastrine et ^{68}Ga-gastrine n'ont pas pu être purifiés en utilisant des mini-colonnes Sep-pak, ^{106}Ru seul a traversé la colonne mais le complexe ^{106}Ru-gastrine a été retenu puis élué avec de l'acétonitrile. Les trois complexes ^{111}In-gastrine, ^{68}Ga-gastrine et ^{106}Ru-gastrine ont été tous purifiés par la chromatographie échangeuse d'anions. Cependant, la compétition de ces complexes gastrine-ion métallique avec ^{125}I-CCK pour le RCCK2 était inexistante suggérant que l'association avec ^{111}In, ^{68}Ga et ^{106}Ru bloquerait la capacité de la gastrine à reconnaitre et à se lier au récepteur RCCK2. Nous avions précédemment montré par spectroscopie d'absorption X (EXAFS) que Ru_2-gastrine était structurellement différent de Fe_2-gastrine, et par conséquent l'interaction entre l'acide aspartique du domaine C-terminal de la gastrine et l'indium, le gallium et le ruthénium pourrait expliquer l'absence de liaison au récepteur RCCK2. Contrairement à la gastrine qui ne nécessite pas Fe^{3+} pour reconnaitre le RCCK2, Ggly a besoin des ions ferriques pour se lier au récepteur. Il pourrait alors être judicieux de développer les complexes ^{111}In-Ggly, ^{68}Ga-Ggly et ^{106}Ru-Ggly pour bloquer la stimulation de la prolifération cellulaire par Ggly, et ainsi inhiber le développement de cancer du côlon. De plus, la formation de complexes Ggly-ions métalliques radioactifs permettrait de caractériser les récepteurs spécifiques des formes immatures de la gastrine. Notre dernière étude s'est portée sur le déplacement des ions ferriques liées à Ggly en utilisant les ions bismuth (Bi^{3+}), indium (In^{3+}) ou ruthénium (Ru^{3+}) et les conséquences de cette compétition quant au développement

de tumeurs intestinales chez les souris APC$^{\Delta 14/+}$, qui sont un modèle de cancérogenèse colique spontanée. L'affinité des ions Fe^{3+} pour Ggly étant réduite en présence de Bi^{3+} *in vitro* et sachant que Ggly nécessite de se lier à Fe^{3+} pour son activité biologique, nous avons proposé l'utilisation des ions Bi^{3+}, In^{3+} et Ru^{3+} comme inhibiteurs du récepteur du peptide Ggly. Les souris APC$^{\Delta 14/+}$ ont été traitées oralement avec du placébo (PBS), du citrate de bismuth, du citrate d'indium ou du citrate de ruthénium trois fois par semaine jusqu'à l'âge maximal de vingt semaines. Après le sacrifice des souris, une augmentation de la quantité d'ions métalliques dans le sérum a été observée avec une quantité de In^{3+} et de Ru^{3+} absorbée proche de la quantité donnée oralement, comparé à une faible quantité de Bi^{3+} absorbée. Les souris ont toutes développé des tumeurs au niveau de l'intestin grêle et du gros intestin et leur état de santé s'est détérioré au fil des jours. Cependant, le traitement au Ru^{3+} a impacté négativement l'état de santé des souris qui sont mortes deux fois plus vite que les souris contrôles ou traitées avec Bi^{3+} ou In^{3+}. Le degré d'anémie ainsi que la concentration de fer dans le sérum étaient identiques chez toutes les souris. Bien que In^{3+} et Ru^{3+} aient augmenté le nombre de tumeurs chez les souris, le traitement au Bi^{3+} a quant à lui diminué de façon signifiante le nombre de tumeurs supérieures à 3mm chez les mâles. Pas de différence a été observée pour le côlon. Ces résultats ne confirment pas que le bismuth empêche la liaison du fer à la gastrine mais laisse penser à une implication de processus intracellulaires en lien avec le fer. La charge tumorale chez les souris APC étant diminuée, le traitement au bismuth pourrait être plus bénéfique en augmentant sa dose ou en optimisant sa voie d'administration.

Conclusion

La gastrine est une hormone qui stimule la sécrétion d'acide gastrique après un repas mais qui agit également comme un facteur de croissance autocrine au niveau de la muqueuse gastro-intestinale. En effet, la prolifération de la muqueuse colique est inhibée chez les souris dont le gène de la gastrine a été délété et stimulée lorsque la progastrine ou Ggly sont surexprimés. Les formes immatures de la gastrine seraient particulièrement impliquées dans le développement de carcinomes colorectaux. L'étude approfondie des formes immatures de la gastrine par notre équipe a permis de clarifier plusieurs points dans le contexte de cancer du côlon. Premièrement, nous avons démontré que la progastrine avait un rôle protecteur chez les souris mises en conditions d'hypoxie, en affectant l'association fer-oxygène et suggérant alors une mobilisation plus efficace du fer stocké par les hépatocytes par la progastrine. Nous avons ensuite testé l'hypoxie chimique en utilisant le $CoCl_2$ ainsi que les ions zinc qui, tout comme l'hypoxie, bloquent l'hydroxylation de la protéine HIF1α. L'activité du gène de la gastrine a été activée par le $CoCl_2$ et le $ZnCl_2$ *in vitro* et *in vivo* et de façon dépendante de la voie des MAPKs, mais indépendante de la voie PI3K. La présence d'une boucle autocrine positive pourrait expliquer l'activation à long terme du promoteur de la gastrine par des facteurs de croissance activés par les ions zinc. Des études

cliniques ont également démontré que la gastrine mature et le $ZnCl_2$ avaient chacun un rôle de protection contre l'hypoxie en diminuant les risques d'ischémie/reperfusion cardiaque et rénale respectivement. Dans un deuxième temps, nos recherches se sont tournées vers la capacité de la gastrine à se lier à des ions métalliques trivalents. La création de complexes entre la gastrine et des isotopes radioactifs d'indium, de ruthénium et de gallium pour permettre de détecter les tumeurs exprimant le RCCK2 n'a pas abouti puisque, contre toute attente, ces complexes ne se sont pas liés au récepteur. Cependant, ils représentent des inhibiteurs spécifiques bloquant l'activité biologique de la gastrine et pouvant par conséquent être utilisés pour réguler son activité de facteur de croissance ou de stimulant de l'acide gastrique. D'autre part, même si le déplacement de la liaison essentielle des ions ferriques au Ggly par les ions bismuth n'a pas été observé, la charge tumorale chez les souris porteuses de la mutation APC et ayant des tumeurs spontanées dans l'intestin a néanmoins été réduite. Les ions bismuth étant déjà utilisés dans le traitement de certaines pathologies, il serait pertinent d'approfondir cette étude en amélioriant son absorption et en optimisant son dosage. Des corrélations entre la gastrine, l'hypoxie et l'homéostasie du fer dans les cancers du côlon sont indéniables et poussent à la réflexion quant à des études plus approfondies sur le rôle exact des formes immatures de la gastrine et sur la caractérisation de leurs récepteurs.

Marie LAVAL

Connections between gastrins, iron and hypoxia in the development of colorectal cancer.

RÉSUMÉ

La gastrine, un facteur de croissance, et notamment la progastrine et la gastrine à glycine étendue (Ggly), est impliquée dans la progression du cancer colorectal. Nous avons déjà montré que le fer était indispensable à l'activité biologique de Ggly et que l'hypoxie augmentait l'expression de la gastrine *in vitro* et l'absorption du fer *in vivo*.

Le but était d'explorer l'intéraction entre la gastrine, l'homéostasie du fer et l'hypoxie dans les cancers colorectaux. Une surexpression de la progastrine a permis aux souris de mieux supporter des conditions hypoxiques dû à un changement de connection entre l'homéostasie du fer et la disponibilité en oxygène. L'activité du promoteur de la gastrine est quant à elle stimulée par les ions Co^{2+} et les ions Zn^{2+} *in vitro* et *in vivo*.

D'autre part, les complexes gastrine-ion métallique (indium, ruthénium ou gallium) ne se lient pas au récepteur RCCK2 *in vitro* et représentent de potentiels inhibiteurs de la gastrine. Les ions Bi^{3+} quant à eux diminuent le nombre de tumeurs intestinales supérieures à 3mm chez les souris $APC^{\Delta 14/+}$.

Une corrélation entre la gastrine, l'hypoxie et l'homéostasie du fer dans les cancers colorectaux est confirmée.

ABSTRACT

Gastrins, which are growth factors, and especially progastrin and glycine-extended gastrin (Ggly), are involved in the progression of colorectal cancer. We have previously shown that iron is necessary for Ggly biological activity and that hypoxia increases the gastrin expression *in vitro* and the iron upload *in vivo*.

The aim was to explore the interaction between gastrin, iron homeostasis and hypoxia in colorectal cancers. The overexpression of progastrin affects the connection between iron homeostasis and oxygen availability and allows mice to cope better under hypoxic conditions. Gastrin promoter activity was stimulated by both Co^{2+} and Zn^{2+} ions *in vitro* and *in vivo*.

Metal ion-gastrin complexes (indium, ruthenium ou gallium) do not bind to the CCK2 receptor *in vitro* and could therefore act as inhibitors of gastrins. Bi^{3+} treatment led to a significant decrease of intestinal tumors larger than 3mm in male $APC^{\Delta 14/+}$ mice. These findings confirm a correlation between gastrins, hypoxia and iron homeostasis in colorectal cancers.