The Requirement of B-cell Lymphoma Maloney-Murine Leukemia Virus Insertion Region-1 Homolog for the Proliferative Effects of Glucagon-like Peptide-2 in the Murine Small Intestine

by

Bradley Smither

A thesis submitted in conformity with the requirements for the degree of Masters of Science

Physiology
University of Toronto

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Abstract

Glucagon-like peptide-2 (GLP-2) stimulates growth of the intestinal epithelium upon nutrient ingestion. The goal of the present study was to determine which crypt cell population GLP-2 activates to induce its proliferative effect. It was hypothesized that GLP-2-induced proliferation requires a full complement of B-cell specific Mo-MLV insertion region-1 homolog (Bmi-1) in the small intestine, assessed using the Bmi-1<sup>enhanced Green Fluorescent Protein (eGFP)/+</sup> mouse model. The acute (1d) and chronic (11d) proliferative response to GLP-2 in the transit-amplifying zone was impaired in Bmi-1<sup>eGFP/+</sup> crypts compared to Bmi-1<sup>+/+</sup> crypts. Chronic GLP-2-treated Bmi-1<sup>eGFP/+</sup> mice had an adaptive growth response to GLP-2 in which the epithelial cell migration rate was increased. After a lethal dose of ionizing-radiation, the Bmi-1<sup>eGFP/+</sup> intestine also had an impaired proliferative response to chronic (18-19d) GLP-2 treatment. Therefore, a full genetic complement of Bmi-1 is required for GLP-2’s proliferative effects in the physiological and pathological settings.
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time I put into this project worried them and others around me. I love them all and this thesis is dedicated to them.
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<thead>
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<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>+4</td>
<td>position – 4</td>
</tr>
<tr>
<td>b.i.d</td>
<td>bis in die – twice a day</td>
</tr>
<tr>
<td>Bmi-1</td>
<td>b-cell specific MoMLV-insertion region-1 homolog</td>
</tr>
<tr>
<td>Bmi-1 HET</td>
<td>bmi-1^GFP/+</td>
</tr>
<tr>
<td>Bmi-1 WT</td>
<td>bmi-1^+/+</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>BrdU</td>
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</tr>
<tr>
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<tr>
<td>CBC</td>
<td>crypt-base columnar</td>
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<td>complementary deoxyribonucleic acid</td>
</tr>
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<td>centimeter</td>
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<td>cellular myelocytomatosis</td>
</tr>
<tr>
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<td>cage-side observation</td>
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<tr>
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<td>cre recombinase – estrogen receptor T2</td>
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<tr>
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<tr>
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<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------------</td>
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<td>GFP</td>
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<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>EphB</td>
<td>ephrin type-B receptor</td>
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<td>hour</td>
</tr>
<tr>
<td>H2A</td>
<td>histone 2A</td>
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</table>
$^3$H-thymidine  tritiated thymidine
H&E  hematoxylin and eosin
HCl  hydrochloric acid
Hes1  hairy enhancer of split – 1
h(Gly$^2$)GLP-2  human-(glycine$^2$)glucagon-like peptide-2; also known as teduglutide
hopx  HOP homeobox
IE-IGF-1R  intestinal epithelial-insulin-like growth factor-1 receptor
IF  immunofluorescence
IGF-1  insulin-like growth factor-1
IHC  immunohistochemistry
IP-2  intervening peptide-2
IR  ionizing radiation
IRES  internal ribosomal entry site
ISC  intestinal stem cell
kbp  kilobase pair
kg  kilogram
KO  knockout
LacZ  β-galactosidase
Lgr5  leucine-rich repeat G-protein coupled receptor
LRC  label-retaining cell
µg  microgram
µL  microliter
µm  micrometer
M   molar
Math1 murine atonal homolog – 1
mAU milli-activating units
mg  milligram
min minutes
mM  millimolar
MoMLV moloney murine leukemia virus
Msi-1 musashi-1
n   number of biological replicates
N   normal
%   percent
PBS phosphate buffered saline
PC  prohormone convertase
PCR polymerase chain reaction
PGDP proglucagon-derived peptide
pH  \(-\log_{10}[H^+]\)
.pmol/L picomoles per liter
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>PRC</td>
<td>polycomb repressive complex</td>
</tr>
<tr>
<td>q.d.</td>
<td>quaque die – once a day</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>Rac1</td>
<td>ras-related C3 botulinum toxin substrate 1</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>R-spondin</td>
<td>Rspo</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>S</td>
<td>Svedberg units</td>
</tr>
<tr>
<td>SI wt</td>
<td>small intestinal weight</td>
</tr>
<tr>
<td>Sox9</td>
<td>SRY-box 9</td>
</tr>
<tr>
<td>Tert</td>
<td>telomerase reverse transcriptase</td>
</tr>
<tr>
<td>TPN</td>
<td>total parenteral nutrition</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling</td>
</tr>
<tr>
<td>vs</td>
<td>versus</td>
</tr>
<tr>
<td>wk</td>
<td>week</td>
</tr>
<tr>
<td>wnt</td>
<td>wingless type mouse mammary tumour virus integration site</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
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</table>
Chapter 1

1 Introduction

1.1 Rationale

The beneficial intestinal effects of glucagon-like peptide-2 (GLP-2) in patients with short bowel syndrome (SBS) are well-documented (1-4). Thus, degradation-resistant GLP-2, teduglutide, improved enteral nutrition and measures of intestinal growth and function in phase II and III clinical trials (2-4). This pharmacological GLP-2 analogue has therefore been approved for use in patients with SBS as the drug GATTEX® (Shire Plc, Lexington, MA, USA), a direct result of its success in these clinical trials. The improved gut growth is attributable to several of the known biological activities of GLP-2, most importantly its trophic effect on the intestinal epithelium (5,6). This profound GLP-2 action is mediated by indirect signalling mechanisms to promote crypt cell proliferation (7-9). However, the proliferative crypt cell population GLP-2 acts on to induce intestinal epithelial growth is not known. Since intestinal stem cells (ISCs) constantly divide to generate the entire epithelium, these cells are potentially stimulated by GLP-2 (10,11). The transit-amplifying (TA) cells, which divide multiple times and more quickly, also drive the proliferative output of the crypt and could therefore be affected by the actions of GLP-2 (12). The goal of the present study was therefore to uncover what crypt cell population GLP-2 activates for its proliferative effect and the essential mechanisms that underlie this stimulation.

1.2 GLP-2

1.2.1 Synthesis

1.2.1.1 Proglucagon Gene

GLP-2 is a 33 amino-acid long peptide hormone that is derived from the proglucagon gene within chromosome 2, in humans (13). The proglucagon gene is expressed in the α-cells of the endocrine pancreas, the L-cells of the distal intestine, and in neurons of the hypothalamus and brainstem (14,15). The human proglucagon
gene codes for the pre-proglucagon signal peptide, glucagon, glucagon-like peptide-1 (GLP-1), and GLP-2, each within separate exons (16). On the other hand, glicentin-related pancreatic peptide and the intervening peptides-1 and -2 (IP-2) are each spread over two consecutive exons (16). The human proglucagon gene likely encodes exons for the 5’ and 3’ untranslated regions, as they are present in the hamster and rat (16,17).

1.2.1.2 Proglucagon Processing

Translation of the human preproglucagon transcript produces the 159 amino-acid long proglucagon, after the signal sequence is lost (16). The proglucagon-derived peptides (PGDPs) that then become liberated depend on the endocrine cell in which proglucagon is synthesized, due to the presence of prohormone convertases (PCs) which cleave specific pairs of basic amino acids within proglucagon. In the α-cell, PC2 produces glicentin-related pancreatic polypeptide, glucagon, intervening peptide-1, and the major proglucagon fragment (18). In the intestinal L-cell, PC1/3 liberates glicentin, oxyntomodulin, GLP-1, IP-2, and GLP-2 from proglucagon (Figure 1-1) (19,20).
Figure 1-1 Proglucagon processing in the enteroendocrine L-cell. (Yellow lightning bolt) Prohormone convertase-1 (PC1) cleaves at the C-terminal side of several (black line) pairs of basic amino acids along proglucagon and its derivatives, as well as at (orange line) a single basic amino acid within the sequence of GLP-1. Carboxypeptidase E cleaves the C-terminal arginine of proglucagon (red lightning bolt), as well as residual basic residues at PC1/3 cleavage sites. The proglucagon derived peptides liberated are: glicentin, oxyntomodulin, GLP-11-37, GLP-17-37, GLP-17-36NH₂, IP-2, and GLP-21-33. The C-terminus of GLP-1 can become (red triangle) amidated at the final amino acid.

1.2.2 Secretion

The L-cell secretes GLP-2 along with the rest of the intestinal PGDPs upon nutrient ingestion (19,21). Together, they are secreted in a biphasic pattern. Initially, there is a rapid response to nutrients entering the duodenum, followed by a later peak in secretion due to the direct contact of luminal nutrients with the L-cell (22,23). Upon
lipid and carbohydrate entry into the duodenum, K-cells release glucose-dependent insulinotropic peptide which activates the vagus nerve to stimulate L-cell secretion via gastrin-releasing peptide and acetylcholine (22-25). In the late peak of GLP-2 secretion, lipids and carbohydrates in the ileum contact L-cells directly to stimulate secretion (23,24). Once released into the circulation, physiological plasma concentrations of GLP-2<sub>1-33</sub> range from 10-20pmol/L in the fasted state to 60-80pmol/L in the fed state (23,26).

1.2.3 Biological Activities

1.2.3.1 GLP-2 Receptor

Circulating GLP-2<sub>1-33</sub> travels to and binds the GLP-2 receptor (GLP-2R), located in the stomach, small and large intestine, hypothalamus, brain stem, lungs, cervix, and vagus (27-30). This 7-transmembrane G-protein coupled receptor is expressed throughout the small intestine, but is highest in the jejunum (27,31). More specifically within the gut, enteroendocrine cells, intestinal sub-epithelial myofibroblasts (ISEMFs), and enteric neurons express the GLP-2R (7,27,32). The first two N-terminal amino acids of GLP-2, histidine<sup>1</sup> and alanine<sup>2</sup>, are critical for receptor activation, while the remainder of the peptide is important for binding (33,34). Upon binding of GLP-2<sub>1-33</sub>, the GLP-2R increases cyclic-adenosine monophosphate, likely via G<sub>αs</sub> protein-mediated activation of adenylyl cyclase, to transduce the many effects of GLP-2 (27,31,35,36).

1.2.3.2 Degradation/Excretion

In the circulation, dipeptidylpeptidase-IV (DPP-IV) cleaves the first two N-terminal amino acids of the biologically active GLP-2<sub>1-33</sub> to produce GLP-2<sub>3-33</sub> (37). This degradation product acts as a weak partial agonist of the GLP-2R, increasing cAMP levels and small intestinal weight, at very high doses (38). However, at lower levels of truncated GLP-2, it competitively inhibits the active full-length hormone from binding its receptor and reduces intestinal growth (34,38). Therefore, the biological activity of GLP-2<sub>1-33</sub> is short-lived because DPP-IV degrades it to GLP-2<sub>3-33</sub> approximately 7 minutes after its release from the L-cell (39,40). The accumulation
of GLP-2\textsuperscript{1-33} in bi-laterally nephrectomized rats shows that the biological activity of circulating levels of GLP-2\textsuperscript{1-33} is also limited by renal clearance (40).

The lack of an equally strong enterotrophic GLP-2 effect in rat, as compared to murine models, prompted the study and development of degradation resistant GLP-2 (5,37). When the alanine at the second position of GLP-2 is replaced with glycine, this blocks the degradation of the peptide by DPP-IV (33,37). Not only does glycine\textsuperscript{2}-GLP-2 remain in circulation longer, it still maintains the ability to bind and activate the receptor (31,33,37,40).

1.2.3.3 Intestinal and Extra-intestinal Effects

The primary biological action of GLP-2 is to stimulate intestinal growth and function. Initially, its proliferative effect on gut growth was identified when nude mice, injected subcutaneously with PGDP over-expressing tumours, were found to have increased mucosal thickness of the jejunum and ileum (5). Then after synthesis and separate injections of glicentin, GLP-1, IP-2, and GLP-2 into CD1 mice, only GLP-2 was able to increase small intestinal weight, villus height, and the epithelial proliferative index (5). The endogenous role of GLP-2 is one of adaptive re-growth (34). It mediates the nutrient regulation of intestinal re-growth in fed mice after a prolonged period of fasting (34). This enterotrophic effect is a result of the stimulation of proliferation as well as a reduction of apoptosis (5,34,41). Furthermore, this role is consistent with the GLP-2R knock-out model where the intestinal adaptation to a fasting re-feeding challenge is lost (42). Also, endogenous GLP-2 signalling protects the small intestine from mucosal damage as a result of inflammation (43).

The administration of GLP-2 not only increases epithelial growth, but also intestinal function (5,6,41). Treatment with GLP-2 increases the activity of duodenal maltase, sucrase, lactase, glutamyl transpeptidase, and DPP-IV (6). These brush border enzymes are important for the degradation of luminal carbohydrates and protein by absorptive enterocytes (6). In addition to improved nutrient breakdown, GLP-2 increases nutrient absorption of glucose, leucine, and triolein via sodium dependent glucose transporter – 1, glucose transporter – 2, and cluster
of differentiation – 36 (6,44-46). Another GLP-2 effect is increased barrier function through decreased paracellular and transcellular permeability (47,48). The increased presence of tight junctional complexes, such as claudin-3 and -7, improves the paracellular epithelial barrier (48,49). Other improvements in gut function as a result of GLP-2 administration include increased mesenteric blood flow via increased expression and activation of endothelial nitric oxide synthase (eNOS), and reduced gut motility by inhibiting cholinergic neurons (50-52).

Interestingly, the effects of GLP-2 are not limited to the intestine. It reduces gastric motility by vasoactive intestinal peptide (VIP)-mediated relaxation of the murine fundus in vitro (53). Also, it promotes satiety by stimulating the action of pro-opiomelanocortin-expressing neurons in the hypothalamus to reduce gastric emptying and feeding behaviour (54). In contrast, murine models of exogenous GLP-2 administration and GLP-2R antagonism show no effect of GLP-2 on appetite (34,41,54). Thus, the effects of GLP-2 as a satiety factor remain to be conclusively established. In the murine lungs, GLP-2 protects them from tissue damage and inflammation (55). Additionally, it increases bone formation by reducing bone resorption in healthy post-menopausal women (56,57).

As GLP-2 promotes normal gut growth, it is not surprising that the actions of administered GLP-2 also protect the intestine from patho-physiological challenges. Human-**(glycine)^2** glucagon-like peptide-2 (h**(Gly)^2**)-GLP-2 reduces mortality in murine models of gut inflammation by increasing proliferation and reducing apoptosis, preventing bacterial infiltration in the circulation, improving glucose transporter-2 expression, and reducing intestinal cytokines (58-61). Another insult GLP-2 safeguards the intestine from is radiation. Chronic pre-treatment with h**(Gly)^2**-GLP-2 increases clonogenic crypt survival after exposure to a lethal dose of ionizing radiation (IR) (62). Additionally, in the setting of neonatal total-parenteral nutrition (TPN), administered GLP-2 prevents mucosal atrophy by reducing apoptosis (63,64). As well, long-lasting GLP-2 improves digestion of carbohydrates, absorption of carbohydrates and amino-acids, and intestinal blood flow during TPN (50,65).
These beneficial intestinal effects of long-lasting GLP-2 administration translate in SBS patients with impaired intestinal function. Chronic treatment with native GLP-2 improves nutrient and water absorption, reduces gastric emptying, and increases urine output and mucosal growth in SBS patients (1). Furthermore, the pharmacological equivalent of h(Gly\(^2\))GLP-2, teduglutide, significantly improves wet weight absorption and reduces fecal water content in association with increased villus height, crypt depth, and mitotic index (2,3). Since SBS patients treated with this drug have reduced oral fluid intake and increased urine output, less parenteral support is required (3). Furthermore, long-term teduglutide treatment, with aggressive guidelines for reduced parenteral support, still safely improves the enteral nutrition of responders with SBS (4). Importantly, no serious adverse events were linked to teduglutide action in SBS patients, but it did cause manageable adverse events such as enlargement of the jejunal stoma and lower limb edema (2-4). As a result of its success in these clinical trials to improve oral nutrition in SBS patients, teduglutide has been cleared by the United States Food and Drug Administration for human use as the drug, GATTEX® (Shire Plc).

Since GLP-2 is a growth factor, there is the potential for it to contribute to the development of intestinal cancer. Human(Gly\(^2\))-GLP-2 promotes the formation of aberrant crypt foci in the jejunum and mucin-deficient foci and tumours in the colon, in an azoxymethane-induced model of colon carcinogenesis (66). Furthermore, in two rodent models that combine a carcinogen with intestinal inflammation, rats fed a high-fat diet combined with 2-Amino-1-methyl-6-phenylimidazo[4,5,b]-pyridine and azoxymethane-injected mice with dextran sodium sulfate-induced colitis, h(Gly\(^2\))-GLP-2 increases aberrant crypt growth (67). However, endogenous and exogenous GLP-2 did not increase the growth or survival of colon cancer cells, in vitro or when transplanted into nude mice, and they did not promote tumour formation in an adenomatous polyposis coli – multiple intestinal neoplasia murine model (68). Taken together, GLP-2 can promote the formation of adenomas depending on the context of cancer initiation, but has yet to be shown to initiate de novo cancer development (66-68).
1.2.3.4 Downstream Signalling Mediators

Despite the numerous effects GLP-2 imparts on the intestinal epithelium, puzzlingly the GLP-2R is not found within it except for sparse enteroendocrine cells (27). Therefore, GLP-2 requires downstream signalling pathways to mediate its enterotrophic and other enteric effects. One important pathway is the insulin-like growth factor-1 (IGF-1) – intestinal epithelial-IGF-1 receptor (IE-IGF-1R) pathway. IGF-1 expression and secretion is increased by degradation resistant GLP-2 in vitro and IGF-1 is required for the intestinal growth and proliferative effects of chronic GLP-2 treatment in vivo (69). The source of IGF-1 is believed to be the syncytium of ISEMFs below the epithelium (70). IGF-1 is expressed by these GLP-2R-expressing cells, and this is stimulated upon GLP-2 administration (7,70). IGF-1 binds to the IE-IGF-1R, which is also required for the enterotrophic effects of GLP-2 (9). Secreted IGF-1 is thought to be bound by intestinal IGF-1 binding protein-4 to facilitate IGF-1 binding to the IE-IGF1R (71). Furthermore, IGF-1 and the IE-IGF-1R are required in vivo for the crypt cell nuclear translocation of β-catenin and the upregulation of the proliferation transcription factor, cellular myelocytomatosis (c-myc), as well as the stem cell gene, SRY-box 9 (Sox9), with acute GLP-2 treatment (9,72).

An alternative GLP-2 signalling pathway includes a group of intestinal growth factors, the ErbB receptor ligands. The gene expression of epidermal growth factor (EGF), amphiregulin, epiregulin, and heparin-binding – EGF is up-regulated in response to acute GLP-2 treatment and this is recapitulated with EGF treatment alone (8). These ligands can then bind to the ErbB family of receptors, such as the ErbB1 receptor, present in the intestinal epithelium (73). In vivo, inhibition of the ErbB family of receptors causes impaired proliferation, with acute GLP-2 treatment (8). During the physiological challenge of fasting re-feeding, ErbB ligand signalling is required for the increased proliferation and adaptive regrowth of the intestinal epithelium (42). How these observations fit with the IGF-1 – IE-IGF-1R findings remains to be determined.
Keratinocyte growth factor (KGF) also mediates the GLP-2 growth effects in the gut. Primarily, it is important for colonic mucosal growth because increases in colonic weight, mucosal area, and crypt depths are abrogated after immunoneutralization of KGF combined with GLP-2 administration (7). Even though chronic KGF administration increases small bowel mass, length, and villus height, immunoneutralization of KGF does not prevent the growth effects of chronic GLP-2 treatment in the small intestine (7,8). Therefore, KGF mediates the enterotrophic effects of GLP-2 in the colon and not the small intestine (7). The source of KGF is also thought to be the ISEMFs that lie below the epithelium which have KGF co-localized with the GLP-2R (7).

In addition to intestinal growth, several mediators are also required for other enteric effects of GLP-2. The increased epithelial barrier effect requires IGF-1 signalling because conditional knock-out of the IE-IGF-1R impairs barrier permeability and reduces proteins present in the tight junctional complexes (48). The production of nitric oxide by eNOS is critical for the GLP-2-induced increase in intestinal blood flow as well as glucose and amino acid uptake, during TPN (50). Nitric oxide is produced from the eNOS- and VIP-expressing enteric neurons which are stimulated by GLP-2 (51). In models of ileitis and colitis, GLP-2’s anti-inflammatory actions are mediated by VIP produced from enteric neurons of the submucosal ganglia (61). Hence, the GLP-2-induced improvements in the inflammation scores, body weight loss, production of cytokines, and proliferation are lost by pharmacological inhibition of VIP (61). However, GLP-2 does not protect VIP−/− or VIP+/+ mice from dextran sulfate-induced colitis, which could be due to the difference in genetic background with the mice from the previous study (61,74). Since this VIP knock-out (KO) study may still suggest GLP-2 does not require VIP for its anti-inflammatory effects, the role of VIP needs to be further evaluated (74).

1.3 Intestinal Stem Cells

1.3.1 The Intestinal Epithelium

Eventually, the signalling mediators of GLP-2 converge onto the intestinal epithelium to put many of GLP-2’s effects into motion. The morphology of this
simple columnar epithelium increases the individual functional surface area, where it folds into finger-like projections, known as villi, and invaginations, known as the crypts of Lieberkühn. There are four main differentiated cell-types that are found within the intestinal epithelium (Figure 1-2), as well as two that are less common. Most of the villi are made up of absorptive enterocytes (75). These cells have apical membrane enzymes that digest luminal nutrients, which are then absorbed into circulation via apical and basolateral transporters (6,76,77). Absorptive enterocytes have tiny finger-like projections, microvilli, located on the apical membrane that further increase the surface area for nutrient digestion and absorption (77-79). A second cell type of the intestinal epithelium is the goblet cell (80,81). These secretory cells release mucus into the lumen to protect the epithelium from physical damage as well as from pathogens (80,81). Another group of secretory cells are the enteroendocrine cells (82). Upon nutrient ingestion these cells release many hormones such as GLP-1, GLP-2, glucose-dependent insulinotropic peptide, and cholecystokinin into circulation to travel to their target tissue for specific actions (19,22,83). The fourth main epithelial cell-type is the Paneth cell, which exclusively resides at the base of the crypt between the ISCs (84,85). These secretory cells release lysozyme and other anti-microbial factors to defend the body from harmful pathogens (86). As well, Paneth cells provide niche signals required to maintain the ISC population (87). One of the rarer cell types is the tuft cell, whose function is not well understood (88-90). This unique cell has long microvilli and a specifically glycosylated membrane (88). Lastly, another rare population of epithelial cells is the micro-fold cell that is predominantly found in the ileum and does not have microvilli (91). These are important for transporting antigens from the lumen to the submucosal Payer’s patches that can mount an immune response if necessary (91).

Overall, the intestinal epithelium is a highly dynamic tissue, which almost completely regenerates every 6 days (92). To maintain this high cellular turnover rate, ISCs present at the bottom of the crypt proliferate approximately once a day and eventually produce all the differentiated cell types of the epithelium (Figure 1-2) (10). The ISCs immediately give rise to a highly proliferative
progenitor cell population, called TA cells, which both express Musashi-1 (Msi-1) (93,94). These cells reside just above the ISC niche along the rest of the crypt (Figure 1-2) (95). They become committed towards either an absorptive or secretory lineage as they divide and migrate up the crypt, and they are fully differentiated once they reach the villus (10,95). This migration of cells along the crypt-villus axis progresses until the differentiated cells reach the tip of the villus where they undergo anoikis, specialized apoptosis where cells are shed from the epithelium and die (75,96,97). Paneth cells are the exception to this conveyer belt progression towards the villus tip because instead they migrate towards the base of the crypt and, when they die, are phagocytosed by neighbouring stem cells (84,98).

![Figure 1-2](image-url)

**Figure 1-2** The intestinal epithelium and the niche signals of the crypt. The four main differentiated cell types are absorptive enterocytes (green), enteroendocrine cells (pink), goblet cells (orange with light blue mucin), and Paneth cells (blue with black dots). Within the crypt, there are TA cells (light purple) and stem cells (orange or dark green). Also, soluble factors are secreted or cell surface receptors are expressed in gradients along the crypt axis to promote proliferation only in the...
crypt and differentiation in the villus. As the gradient increases moving towards the base of the crypt, wingless type mouse mammary tumour virus integration site (Wnt), R-spondin (Rspo)-1 and -2, noggin, ephrin type-B receptor (EphB) 2 and 3, and Notch, promote proliferation (red). On the other hand, EphrinB2 and bone morphogenetic protein (BMP) are found in a gradient that increases moving towards the top of the crypt, which promote differentiation.

1.3.2 ISC Pools

1.3.2.1 The Intestinal Stem Cell

The ISCs are adult stem cells (12). Thus, by definition, they are undifferentiated cells that have the ability to undergo self-renewing divisions over the lifespan of an organism and give rise to progeny that can then differentiate into the multiple cell types of the somatic tissue in which it resides (99). Self-renewal of a dividing cell is achieved by the production of at least one daughter cell that remains undifferentiated, with equal potency to generate differentiated cells. Thus, over the course of many subsequent divisions, the stem cell is never lost. To identify crypt cells with self-renewal capability and multipotency, radiation- and transgenic reporter-based techniques are used to specifically label a cell over a long period of time (10,11,99,100). If the crypt retains the label over several months and the label co-localizes with multiple differentiated intestinal cell-types, then the targeted cell is capable of self-renewal and is multipotent (10,11). Additionally, the ability of a singly sorted crypt cell to generate in vitro intestinal organoids, long term, can also be used to assess these qualities (101,102). There are three ISCs identified in the crypt, crypt-base columnar (CBC) stem cells, reserve position – 4 (+4) ISCs, and the label-retaining cell (LRC) (10,11,100).

1.3.2.2 CBC Stem Cells

The intestinal stem cell niche contains actively proliferating CBC stem cells that reside in-between Paneth cells (Figure 1-3) (75,85,92). This was first discovered by Cheng and Leblond, who injected tritiated (H)-thymidine to follow the lineage
progression of cells with vacuoles containing phagocytosed, radio-labelled CBC cell components (92). CBC cells initially incorporate the $^3$H-thymidine during deoxyribonucleic acid (DNA) replication, but some die as a result of the radiation and are phagocytosed by, surviving, neighbouring CBC cells (92). The unique vacuole, containing radioactive CBC stem cell components was used as a marker for lineage tracing (92). The label first appears in CBC stem cells and is followed much later by the appearance in the differentiated cell types (92). As well, electron microscopy revealed that, later on, there are radio-labelled-crypt cells that contain both characteristics of CBC cells and of each of the individual differentiated cells, in addition to the presence of intermediate secretory cells that have different types of secretory granules (92). Furthermore, extra-epithelial lymphocytes do not contribute to the origin of the different epithelial cells because radioactively labelled bone-marrow cells do not produce progeny that incorporate into the intestinal epithelium (92). Together, this evidence showed that the CBC cell is a multipotent stem cell that gives rise to the four main differentiated cell types of the epithelium, constituting the main principle of ‘The Unitarian Theory of the Origin of Epithelial Cells of the Gastrointestinal Tract’ (92).
**Figure 1-3** The two-stem cell model of the intestinal crypt. There are two pools of ISCs at the bottom of the crypt, the reserve +4 ISCs (orange) and active CBC ISCs (green). At the base of the crypt, Paneth cells (blue) separate the CBC ISCs. Eventually the ISCs divide and produce rapidly dividing TA cells that populate the rest of the crypt above the reserve +4 ISCs.

The findings of Cheng et al. are consistent with studies in a more recently developed leucine-rich repeat G-protein coupled receptor (Lgr5)-enhanced green fluorescent protein (eGFP)-internal ribosomal entry site (IRES)-Cre recombinase estrogen receptor T2 (Cre-ERT2); Rosa26-β-galactosidase (LacZ) transgenic mouse model (Figure 1-4A and B) (10). The CBC stem cells divide approximately once a day, express high levels of the Lgr5 gene, and the in situ hybridization pattern for Lgr5 at the crypt base does not overlap with the patterns of other crypt cell populations, such as Paneth cells and TA cells (10). After 60d-lineage tracing,
Lgr5\(^+\) cells with LacZ reporter expression produce a continuous line of cells along the crypt-villus axis (10). Over this time frame, the Lgr5\(^+\) CBC stem cells recapitulate a complete and functional intestinal epithelium, with their progeny demonstrating LacZ co-localization with periodic-acid-Schiff staining that labels goblet and Paneth cells, as well as with synaptophysin labeling of enteroendocrine cells (10). Also, labelled crypts have equal proportions of the four differentiated cell types compared to those without lineage tracing, in the same tissue (10). Moreover, a single Lgr5\(^+\) cell can grow and maintain an organoid that recapitulates the normal architecture and composition of the gut, for over a year (101).

**Figure 1-4** Reporter transgenic constructs for ISC gene expression. (A) The Lgr5-eGFP-IRES-CreERT2 construct expresses both eGFP and CreERT2 at the Lgr5 locus. (B) The Rosa26 reporter construct that, upon CreERT2-mediated excision of the STOP cassette, flanked by LoxP sites, expresses LacZ or YFP depending on the model used. (C) The Bmi-1-CreERT2 only expresses CreERT2, which is inserted into (yellow) the 3’ untranslated region. (D) The Hopx construct expresses just
CreERT2, which is found in the 3' untranslated region. (E) The Bmi-1-eGFP construct only expresses eGFP which replaces exons 2-10 at the Bmi-1 locus.

1.3.2.3 Reserve +4 ISCs and the Gene Marker Bmi-1

In addition to the CBC cells, there is a more quiescent, reserve, +4 ISC that was first discovered by Sangiorgi and Capecchi. The transgenic B-cell lymphoma MoMLV-insertion region-1 homolog (Bmi-1)-Cre-ERT2; Rosa26-LacZ or - yellow fluorescent protein (YFP) transgenic mouse labels a cell at the fourth position from the base of the crypt, on average (Figure 1-3, 1-4B and C) (11). After 5d of tracking their progeny, some crypts only have as few as two YFP+ cells, and after 7d, only 1.7% of YFP+ cells proliferate (11,102). Therefore, these Bmi-1-expressing +4 cells are more quiescent in nature than CBC stem cells (11,102). However, these cells generate similar lineage stripes that span the entire crypt-villus axis one month after labelling is initiated (11). Within these traces, there are cells with the co-immunofluorescence (IF) of YFP and either lysozyme for Paneth cells, dolichos biflorus agglutinin for goblet cells, or chromogranin A for enteroendocrine cells (11). Taken together, Bmi-1-expressing +4 crypt cells therefore divide to give rise to all the differentiated cells of the epithelium (11). Further demonstrating their role as an ISC, these cells have the ability to self-renew because completely LacZ+ crypts are found 12 months after induction of lineage tracing (11).

The Bmi-1 gene, expressed by reserve +4 ISCs, encodes a chromatin-remodeling complex protein that is responsible for the transcriptional silencing of many genes to promote proliferation and self-renewal in stem cells (103-105). The polycomb repressive complex (PRC) 1 is made up of several proteins that are held together by Bmi-1 (103). Although Bmi-1 does not contain the E3 ubiquitin ligase activity required to modify chromatin, it promotes optimal ubiquitination (103). The methylation of lysine 27 in histone 3 by PRC2 at Bmi-1 target genes, recruits PRC1 to ubiquitinate histone 2A (H2A) at lysine 119, leading to transcriptional silencing (103).
An important group of Bmi-1 transcriptional targets in several non-intestinal cell types are cell cycle genes, such as p16 and p19 at the Ink4a locus (104,106). In the absence of Bmi-1, these two cell cycle regulators prevent the proliferation and self-renewal of stem and progenitor cells in the nervous and hematopoietic system in the absence of Bmi-1 (104-106). Cerebellar granule neural progenitor cells also require Bmi-1 to proliferate in response to sonic hedgehog in order to populate the layers of the cerebellum (104). Without Bmi-1 expression, cultured neural stem cells from the subventricular zone, also do not grow as large or survive as many passages as Bmi-1-expressing controls (104). Finally, in a lethally irradiated host, transplanted hematopoietic stem cells require Bmi-1 to recapitulate the ablated white blood cell population, while hematopoietic cells in bone marrow, the spleen, and thymus need Bmi-1 to maintain the lymphocyte population and to proliferate in response to growth factors (105,107).

In addition to regulating cell cycle genes, Bmi-1 extends the lifespan of stem cells by up-regulating the expression of telomerase reverse transcriptase (Tert) (108). Up-regulation of Tert leads to the stable maintenance of telomeres at 3-4kbp so that stem cells do not reach replicative senescence (108). It is hypothesized that the increased transcriptional activation is most likely due to the de-repression of a negative regulator of Tert by Bmi-1 (108). Another way Bmi-1 contributes to the longevity of highly proliferative cells is by facilitating the recruitment of DNA repair mechanisms to sites of DNA damage (109). Bmi-1 brings the PRC1 to ubiquitinate H2A lysine 119 at double stranded breaks in DNA, which is dependent on phosphorylated H2A.X (109). Normal homologous recombination at these sites to repair DNA damage requires Bmi-1 so that cells can pass through the G2/M checkpoint and continue to progress through the cell cycle (109).

Finally, the expression of Bmi-1 is important for the proliferation and self-renewal of stem cells in the intestine (110). Low level induction of diphtheria toxin to ablate Bmi-1⁺ cells in transgenic mice causes altered epithelial morphology, with patches of missing jejunal and duodenal crypts (11). However, higher induction of Bmi-1⁺ ablation causes lethality 3d-post insult, and accumulation of dead intestinal cells (11). Furthermore, whole-body Bmi-1 KO mice have thin intestines that are
significantly shorter than in controls (110). The whole-body and intestine-specific loss of Bmi-1 reduces ISC proliferation in the small intestine and underlie the impaired intestinal growth phenotype (110). This proliferative defect begins during the development of the intestinal epithelium at embryonic day 15-16 (110). Additionally, the expression of Bmi-1 is important for ISC self-renewal because organoids generated from Bmi-1 null ISCs do not grow and survive as many passages as when Bmi-1 is fully expressed (110).

Several downstream mechanisms for the impairments in ISC function with Bmi-1 loss are due to Bmi-1’s role in regulating cell cycle gene expression, Tert, and DNA repair (104-106,108,109). Bmi-1 deficiency leads to the increased crypt cell expression of p16 and p19, cell cycle genes that halt progression through the cell cycle (110). The expression of Tert is reduced in the whole-body Bmi-1 KO model (108,110). As well, after exposure to whole-body IR, more intestinal crypt cells accumulate unrepaired DNA damage, as a result of Bmi-1 deficiency (110).

In addition to Bmi-1, a few other genes functionally identify the reserve +4 ISC, including HOP homeobox (Hopx), mouse telomerase reverse transcriptase (Tert), leucine-rich repeats and immunoglobulin-like domains-1, and doublecortin and calmodulin kinase-like – 1 (DCAMKL-1) (111-113). These all mark quiescent cells that are able to maintain themselves long-term and can recapitulate the differentiated cell types of the intestinal epithelium (111-114).

1.3.2.4 Label Retaining Stem Cells

Prior to the characterization of the Bmi-1⁺, +4 reserve ISC, Potten found another +4 ISC within this niche (100). This ISC is a slowly proliferating LRC which incorporates ³H-thymidine that is retained long term despite numerous cell divisions, reflecting the high self-renewal capacity of this cell (100,115). The LRC maintains the radioactive label because it selectively segregates its DNA to retain the original template strand and give the newly synthesized strand to the daughter cell (100). As well, the LRC is highly sensitive to radiation exposure as it undergoes apoptosis a few hours after low level, 1Gy, IR (100). These unique characteristics of the LRC are believed to preserve the integrity of the genome
within this ISC, as retaining the template strand prevents errors in DNA replication, while hypersensitivity to radiation-induced DNA damage suggests that normal DNA repair mechanisms are absent to prevent errors arising during repair (100).

1.3.2.5 Secretory Progenitors

Although not found within the ISC niche, there are two kinds of secretory progenitor cells that have been shown to have ISC behaviour after an injury-induced stimuli, much like the reserve +4 ISCs. Identified by its retention of histone 2B-green fluorescent protein (GFP), there is a LRC that expresses high levels of ISC genes such as Lgr5 and genes specific for different secretory cell types (116). This distinct population of LRCs is a secretory progenitor cell because it produces Paneth and enteroendocrine cells in the physiological setting (116). However, when the intestinal epithelium is subjected to 6Gy IR or inhibitors of DNA replication, these secretory LRCs can regenerate the epithelium and produce the four main differentiated cell types (116). When they are isolated in vitro, they can also produce organoids efficiently, recapitulate the intestinal architecture, and produce all the differentiated cell types (116).

The other secretory progenitor cell that displays reserve ISC behaviour resides above the niche at position – 5 (117). This secretory cell is identified by its expression of the Notch ligand, delta-like (DII) 1 (117). As well, it expresses low levels of Lgr5 and other ISC genes, high levels of early secretory-lineage genes, high levels of murine atonal homolog – 1 (Math1) and low levels of Notch receptor genes (117). Normally, this Dll1\(^+\) secretory precursor proliferates and produces goblet, Paneth, enteroendocrine, and tuft cells, but it is not capable of long-term self-maintenance (117). However, since this progenitor cell is almost an immediate daughter cell of the CBC ISC, under the right conditions it has the ability to revert back to its parent cell and act as a CBC stem cell (117). Thus isolated Dll1\(^+\) secretory progenitor cells, in the presence of wingless type mouse mammary tumour virus integration site (Wnt)3a, can generate organoids in vitro and, in vivo, 6Gy of IR activates these progenitors to regenerate the intestinal epithelium (117).
1.3.2.6 Current ISC Model

Under normal physiological conditions (Figure 1-3), the workhorse ISC pool that generates most of the epithelial cells is the CBC stem cells, whereas the reserve ISCs contribute significantly fewer cells over the same time period (102). However, reserve ISCs have the ability to produce the entire intestinal epithelium, when the system is stressed, for example, by abolition of the CBC ISCs using diphtheria toxin targeting of Lgr5 expressing cells or exposure to lethal doses of IR (102,118). To recapitulate the mucosa, the reserve ISCs increase their proliferative output by as much as 10-fold, to produce new CBC stem cells or bypass CBC stem cell production to directly produce the differentiated intestinal cell types (102,111,118). Interestingly, even though the reserve ISC pool can generate the active ISC pool, the reverse is also true, as long-term lineage tracing shows overlap of CBC progeny with the labelled +4 ISC (111).

Despite the ability of both ISC pools to interconvert and the overlapping expression of Bmi-1 and Hopx in Lgr5-expressing cells, they are still two molecularly and functionally distinct cell populations (119,120). The reserve +4 ISC is most reliably labeled with a Hopx-CreERT2 construct, whereas the CBC ISC is most reliably labeled by an Lgr5-eGFP-CreERT2 construct (Figure 1-4A and D) (119). Reporter constructs can be combined with these transgenes to isolate and analyze the expression patterns of each ISC pool (119). The gene signature of reserve +4 ISCs consists of high Bmi-1 and Hopx expression relative to Lgr5, low expression of Lgr5 and other Wnt signalling genes, and high expression of p21 and the vitamin B12 receptor (119). In contrast, the CBC ISC gene signature is characterized by high levels of Wnt signalling and target genes including Lgr5 and Sox9, as well as Notch target genes like olfactomedin – 4 and hairy enhancer of split – 1 (Hes1) (119). However, the expression of Bmi-1, Hopx, Lrig, and Tert is more highly expressed in the CBC ISCs than in the reserve ISCs, the stem cell pool they are intended to label (119). Therefore, the mRNA expression of those genes cannot be used to specifically label the reserve ISCs (119). Despite this, the reserve and active ISCs express a unique array of genes that characterize them as distinct stem cell pools (119). Additionally, the reserve ISC population does not express the same
genes as the Dll1-expressing secretory precursors, which have high expression of Dll1 and low expression of Notch signalling and target genes, and so the two cell types also represent discrete cell populations within the crypt (117,119).

In addition, the two stem cell pools are functionally distinct. The population of reserve ISCs slowly divide to produce progeny mostly consisting of themselves or the CBC ISCs, and negligible numbers of TA cells (119). In comparison, the CBC ISCs’ rapid divisions lead to more CBC stem cells or TA cells, and minimal numbers of reserve ISCs (119). Furthermore, although the reserve ISCs are quiescent compared to the CBC ISC, they still are more active than any LRC, thus excluding both the +4 LRCs and secretory LRCs as the reserve +4 ISC pool (100,116,119). Lastly, the ability of individual ISCs from each pool to self-renew long-term is equal (101,102). However, at the population level, reserve ISCs have a greater capacity to self-renew long-term than the CBC ISCs (119). Importantly, this difference and the unequal inter-conversion between these two ISCs gives the reserve ISC the greatest ‘stemness’ of the two pools (119).

Together, the CBC and reserve ISC pools dynamically populate the crypt in a way that is best modeled by neutral competition, resulting in population asymmetry (85,121). In the ISC niche, there are approximately 14 equipotential CBC stem cells per crypt (85). These ISCs divide symmetrically within the niche (85). However, even though individual CBC stem cells have the same potential to populate an entire crypt with its progeny, they all do not have the same likelihood of accomplishing this end (85,121). This is because there is a spatial divide amongst these ISCs, as a result of the confined space where the niche provides its resources (121). Thus, the CBC stem cells located at the very bottom of the niche are more likely to drive a crypt towards monoclonality than those at the border of the niche (121). Thus, the closer a CBC stem cell is to the base of the crypt, the less likely it is to be forced out of the niche after a cell division, lose access to critical niche signals, and become a TA cell (121). Therefore, the stable maintenance of the niche, and the crypt in which it resides, is a result of neutral drift dynamics where the random ISC divisions are balanced by the loss of expelled ISCs that become TA cells (85,121). The evidence for this is illustrated by long-term lineage tracing of
individual ISCs in the same niche, with separately coloured fluorescent labels, drifts towards a crypt of only one colour (85).

Most of the contributions to crypt cell growth are by the active ISCs because they are more numerous and proliferate more frequently under physiological conditions than the reserve ISCs (102). However, the predicted end result of this model, increasing monoclonal crypts as time progresses, is observed after long-term lineage tracing of reserve ISCs, as well as of active ISCs, under homeostasis (10,11,111). Interestingly, the regeneration dynamics of the niche can be predicted after depletion of all Lgr5+ ISCs, when the likelihood of individual border cells to migrate to the crypt base is increased (121). This leaves room, in this stochastic model of ISC dynamics, for reserve +4 ISCs that recapitulate the ISC niche after a pathophysiological challenge (102).

1.3.3 Niche signals

The proliferation of ISCs and TA cells of the crypt and differentiation of cells moving towards the villus tip is driven by soluble factors as well as the surface receptors present on the cells (Figure 1-2). Paneth and ISEMFs provide many of these niche signals (87,122,123). The soluble factor, Wnt3a, is one of these secreted factors and is responsible for stimulating proliferation through the canonical Wnt signalling pathway (87). It binds to its receptor, Frizzled-7, expressed by proliferative crypt cells, preventing the degradation of β-catenin, and then up-regulating T-cell-specific transcription factor-mediated transcription of genes, such as c-myc and Lgr5 (95,124). The expression of c-myc promotes cell-cycle progression and Lgr5 potentiates Wnt signalling in ISCs by binding Rspo, which is an enhancer of Wnt signalling, to further promote growth (87,95,101,123). Another proliferative cue is mediated by ephrin-EphB signalling (98). The membrane-bound ephrin receptors, EphB2 and EphB3, are expressed in a gradient that is highest at the base of the crypt (98). These receptors are responsible for approximately 50% of crypt cell proliferation (98). Also, the counter gradient of its membrane bound ligand, ephrin, causes a repulsive interaction between the two, pushing EphB3 expressing Paneth cells into the base of the crypt and keeping ephrin expressing
cells towards the top of the crypt (95,98). The expression of Notch is also important for maintaining proliferation in ISCs and promoting differentiation of TA cells (125). The membrane-bound Notch ligand, Dll4, binds to the Notch receptor causing its intracellular domain to be released by enzymatic cleavage, so that it can translocate to the nucleus and up-regulate recombination signal sequence-binding protein-J – mediated transcription of Hes1 (95,125). The expression of this gene in TA progenitor cells, and its suppression of the secretory fate gene Math1, leads to the specification of the cell towards an absorptive enterocyte fate (95,126). However, in the progenitor cells that express Dll4, the absence of Notch signalling to prevent Hes1 expression, and thus permit Math1 gene expression, results in the specification of this cell towards a secretory cell fate (95,125-127). As well, the presence of noggin in the ISC niche inhibits the differentiation-promoting bone morphogenetic protein signalling pathway, to promote self-renewal in the ISCs (95,128). Many of these niche signals are fundamental to epithelial growth because crypts isolated in vitro and treated with just Rspo-1, EGF, and noggin, can grow into organoids (101). Adding a Rho kinase inhibitor and a Notch agonist to this niche factor cocktail creates an environment conducive to the development of a long-lived organoid from even a single ISC (101). In both cases, the organoids survive long-term and recapitulate the structure and function of the intestinal epithelium (101).

1.4 GLP-2 and ISCs

Although the proliferative crypt cell that is targeted for GLP-2’s enterotrophic effects is not known, there is evidence that implicates a specific effect on a population of crypt cells within the ISC niche. First, GLP-2 administration increases crypt cell proliferation (5,41). This is true with the administration of h(Gly\(^2\))GLP-2 in both murine models and patients with SBS (2,9,69,71). However, GLP-2 could be acting on any or all of the three proliferative populations: the CBC stem cells, the reserve +4 ISCs, or the TA cells. Furthermore, in a specific progenitor assay of somatic mutation, administration of this enterotrophic hormone increases the clonogenicity of ISCs and absorptive progenitors, but not of mucous progenitors (32). This would suggest that GLP-2 signalling is directed towards the
TA cell compartment (32). Furthermore, acute and chronic h(Gly²)GLP-2 treatment in murine models increase proliferation in both the base of the crypt and in the TA cell compartment (8,9,69).

However, other studies suggest GLP-2 primarily stimulates the reserve +4 ISCs. Chronic teduglutide treatment increases clonogenic crypt survival in mice exposed to 12-16Gy of whole-body lethal IR, 4d post-IR (62). Lethal doses of IR induce DNA damage in the proliferative crypt cell compartment and result in crypt loss (62,110,129). This large pathophysiological stress causes the intestinal epithelium to atrophy, translating to maximal damage around 4d-post IR (129). The crypts that avoid obliteration by the IR are able to regenerate the epithelium and this is evident 6d-post IR (62,129). In order for a crypt to survive radiation damage, it must have an ISC that endures the IR (129). Importantly, the reserve +4 ISC is resistant to and activated by lethal doses of radiation whereas the CBC stem cells are known to be more radiation-sensitive (11,102). Therefore, GLP-2 could be stimulating the reserve +4 ISCs, activating them in the permissive setting of radiation damage, and promoting the survival of crypts in this pathophysiological setting. Additionally, GLP-2 increases Msi-1 labelling, a gene expressed by both ISCs and TA cells, in the ISC compartment, including position – 4 of the crypt, as well as in the TA cell compartment (69).

Further demonstrating GLP-2’s stimulation of ISCs is a downstream signalling mediator of its enterotrophic effects, IGF-1 (130). In the physiological setting, IGF-1 stimulates the reserve +4 ISCs and increases the number of CBC stem cells (130). After 5d-post lethal IR, IGF-1 increases the proliferation of both pools of ISCs and increases the number of CBC stem cells (130). In both settings, the reserve +4 ISCs proliferate to produce CBC ISCs, which increases the population of the latter (102,119,130). As well, sorted Sox9-eGFP<sup>high</sup> cells that contain the reserve +4 ISCs only grow intestinal organoids in the presence of IGF-1, whereas Sox9-eGFP<sup>low</sup> cells, which are CBC stem cells, do not require IGF-1 to grow intestinal organoids nor do they have a growth response to IGF-1 (130). All of this suggests the actions of GLP-2, as mediated by IGF-1, may be effected at the reserve +4 ISCs.
To determine whether the reserve +4 ISCs are the target of GLP-2-induced proliferation in the small intestine, the studies presented in this thesis utilized the Bmi-1eGFP/+ murine model to label Bmi-1-expressing cells with eGFP (Figure 1-4E) (131). This transgenic model properly labels hematopoietic stem cells and was initially intended to mark Bmi-1 expression with fluorescence, specific to the reserve +4 ISCs (11,102,131). Although labeling of this ISC pool has been functionally demonstrated in the Bmi-1-CreERT2 mouse, this now does not appear to be true of the Bmi-1eGFP/+ murine model, as Bmi-1 is expressed in both pools of ISCs (Figure 1-4B,C, and E) (119). This means the Bmi-1eGFP/+ murine model cannot be used to exclusively label the reserve +4 ISC pool. These mice were therefore used as a model of Bmi-1 haploinsufficiency in the context of both the acute and chronic proliferative effects of GLP-2, as well as chronically in the pathophysiological setting of lethal IR. The hypothesis of these studies is that a full complement of Bmi-1 is required for the proliferative effects of GLP-2 in the murine small intestine.
Chapter 2

2 Methods

2.1 Animal Studies

2.1.1 Transgenic Murine Models

Bmi-1<sup>eGFP/+</sup> mice have eGFP knocked-in to exon 2 of the Bmi-1 locus in mice on a C57Bl/6/Ka-Thy1.1, CD45.2 background (131). Since these mice also have specific isoforms of hematopoietic cell surface markers knocked-in, Thy1.1 leukocyte alloantigen (Thy1<sup>a</sup>) and CD45.2 (Ptprc<sup>b</sup>), Bmi-1<sup>+/+</sup> mice on a C57Bl/6/Ka-Thy1.1, CD45.2 background were used as controls (131). These mice grow and survive equally well and have similar hematopoietic parameters (107,131).

To determine the genotype of the Bmi-1 locus, murine ear tissue samples were collected and lysed with 1% sodium dodecyl sulfate in a 50mM Tris, 20mM sodium chloride, and 1mM ethylenediaminetetraacetic acid tail clip buffer and a greater than 600mAU/mL proteinase K (Qiagen Inc., Toronto, ON, CA) to liberate DNA from cells. The isolated DNA was amplified by polymerase chain reaction (PCR) using DNA primers for the Bmi-1<sup>+/+</sup> (WT) allele and Bmi-1<sup>eGFP/+</sup> (HET) allele expressing eGFP (Integrated DNA Technologies® San Jose, CA, USA; Table 2-1A), and the products were separated by electrophoresis in a 1.5% agarose gel and stained with SYBR Safe (Thermo Fisher Scientific Inc., Grand Island, NY, USA) to label DNA. The resulting DNA bands in Figure 2-1 were from Bmi-1<sup>+/+</sup> and Bmi-1<sup>eGFP/+</sup> mice, where the predicted size of the WT DNA band was 220bp and the MT DNA band was 330bp. In all experiments, both Bmi-1<sup>+/+</sup> and Bmi-1<sup>eGFP/+</sup> mice were sex-, age-, and litter-matched. At the end of each study, they were weighed, heavily anaesthetized with isoflurane and then euthanized by cardiac puncture. The Animal Care Committee at the University of Toronto approved all animal experiments.
A

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>Sequence</th>
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<tr>
<td>Bmi-1 WT/HET forward</td>
<td>5’-GAG AAT CCA GCT GTC CAG TGT-3’</td>
</tr>
<tr>
<td>Bmi-1 WT reverse</td>
<td>5’-TAC CCT CCA CAC AGG ACA CA-3’</td>
</tr>
<tr>
<td>Bmi-1 HET reverse</td>
<td>5’-GAA CTT CAG GGT CAG CTT GC-3’</td>
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B

<table>
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<tr>
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<tr>
<td>Bmi-1</td>
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</tr>
<tr>
<td>DCAMKL-1</td>
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<td>eGFP</td>
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</tr>
<tr>
<td>18S</td>
<td>Hs99999901_s1</td>
</tr>
</tbody>
</table>

Table 2-1

List of primers for (A) genotyping and (B) qPCR.
**Figure 2-1** Genotyping for Bmi-1 WT and Bmi-1 HET mice. DNA was isolated from ear samples and then amplified by PCR with Bmi-1 common forward, Bmi-1 WT reverse, and Bmi-1 HET reverse primers. DNA was separated by electrophoresis in a 1.5% agarose gel stained with SYBR Safe. A 100bp DNA ladder (Thermo Scientific) was used to determine size of DNA bands. The predicted Bmi-1 WT band was 220bp and the Bmi-1 HET band was 330bp.

In preliminary proliferation studies, IGF-1R$^{\text{Flox/Flox}}$ mice were administered 100mg/kg body weight (BW) of 5-bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich Co. LLC., Oakville, ON, CA) intraperitoneally, one or three hours prior to sacrifice, to determine the optimal time for subsequent proliferative analyses in Bmi-1$^{\text{eGFP/+}}$ mice. These mice were heavily anaesthetized with isofluorane and then euthanized by cardiac puncture.

### 2.1.2 Experimental Protocol

#### 2.1.2.1 Acute Study

To determine the acute proliferative effects of GLP-2, 0.2µg/g BW of h(Gly$\beta$)GLP-2 (American Peptide Company Inc., Sunnyvale, CA, USA) or the equivalent
volume of phosphate buffered saline (PBS) was subcutaneously administered 6h and then 3h prior to sacrifice in 11-14.5wk Bmi-1+/+ and Bmi-1cGFP/+ mice (N=6) (8). To label proliferating crypt cells, 100mg/kg BW of EdU (Thermo Fisher Scientific Inc.) was injected intraperitoneally 1h before sacrifice (8).

2.1.2.2 Physiological Chronic Study

To determine the chronic proliferative effects of GLP-2, 0.1µg/g BW of h(Gly²)GLP-2 or the equivalent volume of PBS was subcutaneously administered q.d. for 11d in 8-18wk Bmi-1+/+ and Bmi-1cGFP/+ mice (n=6-8) (9,48,69,71). On the final day, h(Gly²)GLP-2 or PBS was administered 3h prior to sacrifice and then 100mg/kg BW of BrdU was injected intraperitoneally 1h before sacrifice. In a separate experiment, to assess cell migration, 8-11wk old mice were similarly administered h(Gly²)GLP-2 or PBS q.d. for 11d (n=6) but were injected with 100mg/kg BW of BrdU 24h and 100mg/kg BW of EdU 1h prior to sacrifice (132).

2.1.2.3 Pathological Chronic Study

To determine the chronic proliferative effect of GLP-2 in combination with the pathological challenge of high-dose radiation, 0.2µg/g BW of h(Gly²)GLP-2 or the equivalent volume of PBS was subcutaneously administered b.i.d. for 18d or 19d in 5.5-12.5wk old Bmi-1+/+ and Bmi-1cGFP/+ mice (n=7). This study was modified from that which was used by the Potten group (62). Initially, to establish the appropriate dose of radiation used in a Gammacell® 40 Exactor (MDS Nordion, Ottawa, ON, CA), two Bmi-1cGFP/+ mice were exposed to 120Gy radiation, without prior h(Gly²)GLP-2 or PBS treatment. However by the fourth morning post-irradiation, these mice were moribund and euthanized. Thus, on the 15th day of the pathological chronic study, mice were exposed to 100Gy whole-body IR. h(Gly²)GLP-2 and PBS injections were then adjusted each day to the rapidly declining body weight of each mouse. Intestinal tissues were collected 4d- or 5d-post IR, to assess the effects of GLP-2 at the stage of the most epithelial damage and at the regenerative phase, respectively (129).
The health status of these mice was monitored b.i.d. by recording body weight and using a cage-side observation (CO) score calculated from the sum of three different categories: posture, eye appearance, and activity level (133). For each category, a mouse was designated a score from 0-3, in which the decline in health for a particular category was given a higher score. Once a mouse reached a total CO score of 7 or its BW dropped 30% post-irradiation, it was deemed moribund, euthanized, and removed from the rest of the experiment (133). Lastly, the percent change in BW post-irradiation was converted to a score from 0-3, where a change of 0-10% was a score of 0, 11-20% was a score of 1, 21-30% was a score of 2, and a change greater than 30% was a score of 3. Then this BW score was combined with the CO score.

2.2 Analyses

2.2.1 Gravimetric Analyses

The small intestine was harvested from the pyloric sphincter to the ileo-cecal junction. Then the intestine was flushed with cool PBS, to remove chyme within the lumen. After gently squeezing out and dabbing excess PBS onto a paper towel, the intestine was weighed. In the pathological chronic study before the mice were irradiated, 3-5 wet stool samples were collected and weighed. Afterwards, they were freeze-dried in a Speed Vac Concentrator (Thermo Fisher Scientific Inc.) using a refrigerated condensation trap (Thermo Fisher Scientific Inc.) and weighed again to measure dry weight. As well, post-irradiation, both lungs were harvested, padded down on wax paper to remove excess blood, and weighed.

2.2.2 Morphometric Analyses

A 2cm segment of jejunal tissue was mounted on Whatman paper in a plastic cassette, and then fixed in 10% buffered formalin at room temperature overnight. Afterwards, the sample was stored in 70% EtOH at 4°C until the formalin-fixed tissues were paraffin-embedded and sectioned into 4µm slices by University Health Network’s Histological Services. Histological sections were stained with hematoxylin and eosin (H&E) for visualization of the intestinal epithelial crypt-
villus axis. On each slide, one piece of jejunal tissue was separated into four cross-sections from four equally-spaced locations along the length of the jejunal segment. From each slide, a minimum of 12 villus heights (or 7 in the IR studies) and 26 crypt depths (or 20 sub-villus depths in the IR studies) were measured using an Axioplan 2 Imaging Microscope with AxioVision Rel. 4.8 software (Carl Zeiss, Don Mills, ON, CA). The sub-villus depth acted as a surrogate marker for crypt depths in the pathological chronic study due to the complete absence of intestinal crypts. Sub-villus depths measured the distance below the villus but did not include the muscle layer.

2.2.3 Molecular Analyses

RNA was extracted from ~30mg segments of flash-frozen jejunal tissue using an RNeasy® Plus Mini Kit with QiaShredder (Qiagen Inc.). The extracted RNA was then quantified with a spectrophotometer for quality of nucleic acid content and for nucleic acid concentration. The RNA samples were reverse transcribed, with 5x All-In-One Reverse Transcriptase MasterMix (Applied Biological Materials Inc. Richmond, BC, CA). Primers that spanned an exon-intron-exon region for Bmi-1, Lgr5, Msi-1, DCAMKL-1, and eGFP, with 18S rRNA as a housekeeping transcript were then used to amplify the cDNA (Table 2-1B). Dilution curves for these primers were carried out to determine the linear range of amplification (Figure 2-2A-F). Relative changes in gene expression were calculated using the ΔΔC(t) method (134).
Figure 2-2 Dilution curves for each primer used for qPCR. Primers for (A) Bmi-1, (B) Lgr5, (C) Msi-1, (D) DCAMKL-1, (E) eGFP, and (F) 18S were added to amplify their respective genes by qPCR in 4 different sample dilutions. Due to the high expression of 18S, small volumes of cDNA were used and, thus, the –log₁₀ volume cDNA was plotted vs average C(t) for 18S. The sample volume that was in
the middle of the linear portion of the curve was chosen for future qPCR experiments. \( C(t) = \) cycle threshold.

### 2.2.4 Immunometric Analyses

For immunohistochemical staining, 2cm jejunal segments were fixed in 10% buffered formalin at room temperature (RT) overnight, then stored in 70% EtOH at 4\(^{\circ}\)C. The fixed samples were paraffin-embedded and sectioned into 4µm slices by University Health Network’s Histological Services. For immunofluorescent staining, the segments were fixed in 4% paraformaldehyde at 4\(^{\circ}\)C overnight, in the dark to prevent photobleaching of eGFP. The next day the samples were transferred into 30% sucrose for cryo-protection and were kept in the dark. The tissue was then cut into four equal pieces that were oriented vertically in a plastic mold filled with Tissue Tek\textsuperscript{®} Optimum Cutting Temperature\textsuperscript{™} Compound (Sakura Finetek USA Co. Ltd., Torrance, CA, USA). The tissue was then flash-frozen in isopentane at -80\(^{\circ}\)C, mounted onto a Leica CM1510S CryoStat (Leica Biosystems Nussloch GmbH\textsuperscript{©}, Nussloch, Germany), and 4µm cross-sections were sliced onto Superfrost\textsuperscript{®} Plus microslides (VWR International LLC., Mississauga, ON, CA) and stored at -80\(^{\circ}\)C. All slides from either preparation were visualized and photographed using an Axioplan 2 Imaging Microscope and examined using AxioVision Rel. 4.8.

To analyze the position of proliferating cells, in the preliminary proliferative labeling experiment or the physiological chronic study, jejunal cross-sections were stained for BrdU by 3,3’-diaminobenzidine (DAB) immunohistochemistry (IHC). Formalin-fixed, paraffin-embedded tissue sections were de-paraffinized in xylene and rehydrated through an ethanol gradient. Then, endogenous peroxidase activity was quenched with 3% hydrogen peroxide at 37\(^{\circ}\)C for 30min. This was followed by treatment with 2M hydrochloric acid (HCl) and then 10mM HCl with 0.4% pepsin, both at 37\(^{\circ}\)C for 30min. Tissues were neutralized with 100mM sodium borate pH 8.5 at room temperature (RT) for 10min. After slides were washed in PBS, they were blocked with 5% normal donkey serum in PBS pH 7.4, for 30min at RT, and then incubated overnight at 4\(^{\circ}\)C with a mouse anti-BrdU anti-body (1:100, Thermo
Fisher Scientific Inc.) in PBS with 1% bovine serum albumin (BSA) and 0.05% Tween20. After a wash in tris buffered saline with 0.05% Tween20, tissues were incubated with biotinylated goat anti-mouse anti-body (1:200, Vector Laboratories Inc., Burlingame, CA, USA) in tris buffered saline pH 7.6, at RT for 30min, followed by UltraStreptAvidin-Horseradish Peroxidase (Cedarlane®, Burlington, ON, CA), for 30min at RT, and lastly Sigmafast™ DAB tablets (Sigma-Aldrich Co. LLC.), dissolved in 5mL distilled de-ionized water, for 20min at RT, followed by a 20s rinse in tap water. To label nuclei, Mayer’s Hematoxylin Solution (Sigma-Aldrich) was applied to tissue for 5min and slides were then placed into distilled de-ionized water for 30s to 3.5min, and then rinsed for 1min under tap water. Tissues were dehydrated in 100% ethanol and xylene, mounted in Vectashield Mounting Medium (Vector Laboratories), and coverslipped. Positional analysis was conducted in a blinded fashion. The number of BrdU positive cells was counted in a minimum of 20 right-half crypts, from the base of the crypt at the +1-position up to the +20-position.

To analyze the position of proliferating cells in the acute study or proliferation in the pathological chronic study, frozen-fixed jejunal sections were stained for EdU by IF. Slides were thawed, washed in PBS with 3% BSA, and then permeabilized in 0.05% TritonX-100 in PBS for 20min. Next, the EdU Click-iT® reaction cocktail (Thermo Fisher Scientific Inc.) was added for 30min in the dark. After washing the tissues in PBS with 3% BSA, Vectashield Mounting Medium with 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) or Prolong® Antifade Diamond Mountant with DAPI (Thermo Fisher Scientific Inc.) was added and the slides were coverslipped and stored at 4°C in the dark. In slides generated from the acute study, positional analysis of immunofluorescent EdU+ cells and the natural fluorescence of eGFP+ cells were quantified in a minimum of 20 right-half crypts, from the base of the crypt at the +1-position up to the +20-position. In the pathological chronic study, the number of EdU+ cells per intestinal cross-section was quantified due to the absence of intestinal crypts. The circumference of each cross-section was measured to ensure that all four sections were consistent within each slide and were
comparable between slides. In both analyses, the data were collected in a blinded fashion.

To analyze the cell migration rate in the physiological chronic study, frozen-fixed jejunal sections were co-stained for BrdU and EdU IF. Sections were thawed, washed in PBS, and then submerged in 10mM citrate buffer pH6 for 40mins at approximately 98°C, for antigen retrieval. Histones were then denatured with 2N HCl for 30min at 37°C and slides were neutralized with 100mM sodium borate pH 8.5 at RT for 10min. Then, the tissues were blocked in PBS with 1% BSA for 1h, followed by incubation with mouse anti-BrdU anti-body (1:100, Thermo Fisher Scientific Inc.) overnight at 4°C. Jejunal sections were then washed in PBS, incubated in goat anti-mouse Alexa-Fluor® 488 secondary anti-body (1:1000, Thermo Fisher Scientific Inc.) at RT for 1h, in the dark. After washing in PBS, tissues were permeabilized in PBS with 0.5% TritonX-100 for 20min and then an EdU Click-iT® reaction cocktail (Thermo Fisher Scientific Inc.) was added for 30min, in the dark. Once washed in PBS, they were coverslipped with Prolong® Antifade Diamond Mountant with DAPI (Thermo Fisher Scientific Inc.), stored at +4°C, and light-protected. In a blinded fashion, cell migration rate was quantified in more than 8 right-half crypt - left-half villus units. The cell migration rate was calculated as the distance between the length of the individual BrdU and EdU labels, over their 23h administration interval.

2.3 Statistics

Data are presented as mean ± SE. Two-way analysis of variance was performed using GraphPad Prism® Version 6.0c (GraphPad Software Inc., La Jolla, CA, USA). Some data were log10-transformed to normalize variance. If significance was found for variation in the treatment, the genotype, or the interaction of both, a two-tailed Student’s t-test was used to determine significant changes in the data due to the appropriate independent variable.
Chapter 3

3 Results

3.1 Acute Study

3.1.1 Intestinal Growth and Proliferation

To study the acute effects of GLP-2 on proliferation in the Bmi-1eGFP/+ mice, h(Gly\(^2\))GLP-2 or PBS was administered 6h and then 3h prior to sacrifice (8). The mice within this experiment did not differ in BW (Figure 3-1A). In a pilot study on Bmi-1eGFP/+ mice, due to the short time course of this experiment, there was no apparent GLP-2 effect on small intestinal weight normalized to body weight (SI wt/BW) and, therefore, intestinal weight was not measured for the rest of the mice in the acute study (Figure 3-1B).

![Figure 3-1](image)

**Figure 3-1** No growth effects of acute (t = -6 and then -3h) GLP-2 treatment. Bmi-1\(^{+/+}\) and Bmi-1eGFP/+ (A) end body weight was measured for all mice (n=6). The (B) weight of the entire small intestine was normalized to BW (SI wt/BW) for some of the Bmi-1eGFP/+ mice in this study (n=3).

To mark proliferating cells entering S-phase of the cell cycle EdU was administered 1h prior to sacrifice (8) (Figure 3-2A). Proliferating eGFP\(^+\) and eGFP\(^-\) cells were
counted along the length of the crypt, but apparent eGFP+ cells were surprisingly observed in Bmi-1+/+ crypts, presumably due to non-specific fluorescence (Figure 3-2A). Cell counting also showed no clear difference in apparent eGFP labeling between Bmi-1+/+ and Bmi-1eGFP/+ crypts and, therefore, eGFP was not used further (Figure 3-3).

The proliferative index demonstrated significant increases in proliferation with GLP-2 treatment in Bmi-1+/+ mice at positions 10-13, stimulated by 1.5-, 1.3-, 1.5- and 1.8-fold at each position, respectively (p<0.05 to 0.01; Figure 3-2B). In contrast, only positions 6 and 8 were stimulated by GLP-2 in the Bmi-1eGFP/+ mice, where both increased 1.3-fold (p<0.05; Figure 3-2C). Also, there was significantly higher proliferation in Bmi-1+/+ crypts as compared to Bmi-1eGFP/+ crypts at positions 10, 13, and 17, which were increased 1.3-, 1.5-, and 3.1-fold (p<0.05 to 0.01). To determine any compartment-specific effects of GLP-2 on proliferation, the area under the curve was calculated for proliferation in the three different zones of the crypt: the active stem cell zone, composed primarily of CBC ISCs from cell positions 1-3; the reserve stem cell zone, containing the quiescent +4 position stem cell from cell positions 4-6; and the TA zone, where rapidly dividing TA cells reside from cell positions 7-16 (10,11,92,135). In the active and reserve stem cell zones, GLP-2 had no effect on proliferation regardless of genotype (Figure 3-4A,B). However, in the TA zone (Figure 3-4C), there was an impaired proliferative response to acute GLP-2 treatment in the Bmi-1eGFP/+ crypts compared to the Bmi-1+/+ crypts (p<0.001). Thus, the Bmi-1+/+ mice had a 44% increase in proliferation within the TA zone due to acute GLP-2 treatment (p<0.01), while there was no significant effect in the Bmi-1eGFP/+ mice. Also, the GLP-2 – treated Bmi-1+/+ mice had 18% more proliferation as compared to the Bmi-1eGFP/+ mice, in the TA zone (p<0.05).
Figure 3-2 Differential acute (t = -6 and then -3h) GLP-2 proliferative effects in Bmi-1+/+ and Bmi-1eGFP/+ intestinal crypts. The proliferative index was quantified using EdU Click-iT® with jejunal sections from mice injected with EdU 1h before sacrifice. (A) Representative immunofluorescent section (100µm scale bar). (B-C) Quantitative analysis of EdU fluorescence in sections from (B) Bmi-1+/+ and (C) Bmi-1eGFP/+ mice (*, p<0.05; **, p<0.01; n=6).
Figure 3-3 No clear difference in apparent eGFP labelling between acutely PBS-treated Bmi-1+/+ and Bmi-1 eGFP+/+ mice. The percentage of eGFP+ cells was quantified by counting green fluorescent crypt cells in jejunal sections from Bmi-1+/+ and Bmi-1 eGFP+/+ mice. Also, the percentage of EdU+ cells was quantified by
counting EdU-IF in cells of the same crypts. The error bars were omitted for clarity (n=4).

Figure 3-4 Potential impaired acute (t = -6 and then -3h) GLP-2 proliferative responses within the TA zone of Bmi-1 eGFP/+ mice. The intestinal crypt was divided into three different zones by cell position: (A) the active stem cell zone from position 1-3, (B) the reserve stem cell zone from position 4-6, and (C) the TA zone from position 7-16. The area under the curve of proliferation was calculated from the proliferative indices of acute study Bmi-1+/+ and Bmi-1 eGFP/+ crypts (*, p<0.05; **, p<0.01; n=6).
3.2 Physiological Chronic Study

3.2.1 Intestinal Growth and Proliferation

To determine whether this potentially impaired proliferative response to acute GLP-2 treatment could translate into an impaired epithelial growth response to chronic GLP-2 administration in the Bmi-1^{eGFP/+} mice, h(Gly^2)GLP-2 was chronically administered for 11d (9,48,69,71). To assess the appropriate time to label proliferating cells in this setting, a preliminary chronic GLP-2 experiment with control IE-IGF-1R^{fl/fl} mice was conducted in which BrdU was administered 1h or 3h prior to sacrifice. Positional analysis of proliferation showed no effect of GLP-2 on proliferation when BrdU was injected 3 hours prior to sacrifice (Figure 3-5A). In contrast, when BrdU was injected 1h prior to euthanasia, GLP-2 appeared to effect proliferation from cell positions 6-9 (Figure 3-5B). Therefore, in the chronic study with Bmi-1^{+/+} and Bmi-1^{eGFP/+} mice, BrdU was injected one hour before sacrifice.

![Figure 3-5 A and B](image)

**Figure 3-5** A BrdU injection one hour before sacrifice was appropriate to study an effect of chronic (11d) GLP-2 on proliferation. In this preliminary study, positional analysis of proliferation was conducted in IGF-1R^{fl/fl} mice administered BrdU (A) 3h prior (n=3) or (B) 1h prior to sacrifice (n=2).

Chronic GLP-2 did not increase BW in either Bmi-1^{+/+} or Bmi-1^{eGFP/+} mice (Figure 3-6A), but increased SI wt/BW in these animals by 27% and 28%, respectively (p<0.001) (Figure 3-6B). In a direct assessment of epithelial growth,
jejunal crypt depths and villus heights were measured in H&E-stained slides (Figure 3-6G). Chronic GLP-2 treatment increased villus height by 30% and crypt depth by 15% (both p<0.001) in Bmi-1\(^{+/+}\) mice and increased villus height by 28% and crypt depth by 11%, in Bmi-1\(^{GFP/+}\) mice (p<0.001 and p<0.05, respectively). Proliferation was then studied as the potential mechanism underlying this mucosal growth response to chronic GLP-2.
Figure 3-6 Different intestinal growth response to physiological chronic (11d) GLP-2 treatment in Bmi-1^{+/+} and Bmi-1^{eGFP/+} mice. (A) BW and (B) SI wt/BW for Bmi-1^{+/+} and Bmi-1^{eGFP/+} mice. (C-F) Representative intestinal morphology visualized by H&E staining of a Bmi-1^{+/+} mouse chronically administered (C) PBS or (D) h(Gly$_2$)GLP-2 and of a Bmi-1^{eGFP/+} mouse chronically administered (E) PBS.
or (F) h(Gly²)GLP-2 (100µm scale bar; inset shows intestinal crypts). (G) Crypt depths and villus heights. (*, p<0.05; ***, p<0.001; n=12-14).

Proliferation was assessed in a sub-group of mice by administration of BrdU 1h before sacrifice. Positional analysis revealed that GLP-2 increased proliferation (p<0.05; Figure 3-7B) in Bmi-1+/+ mice 3.7- and 4.5-fold at positions 14 and 15, respectively, but there was no significant change in the Bmi-1eGFP/+ mice at these positions or throughout the rest of the crypt (Figure 3-7C). To determine the proliferative effect of GLP-2 in different crypt compartments, the crypt was divided into the same three zones as in the acute study (Figure 3-7A), and the area under the curve for the proliferation index was analyzed for each zone. There was no effect of GLP-2 on proliferation in either the active and reserve stem cell zones, regardless of genotype (Figure 3-8A,B). However, within the TA zone, there was an impaired proliferative response to GLP-2 in the Bmi-1eGFP/+ crypts as compared to the Bmi-1+/+ crypts (Figure 3-8C, p<0.05).
Figure 3-7 Diminished chronic (11d) GLP-2 proliferative effect in Bmi-1 eGFP/+ intestinal crypts. Bmi-1+/+ and Bmi-1 eGFP/+ mice administered BrdU 1h before sacrifice. (A) Representative BrdU DAB immunohistochemical section (100µm scale bar). (B-C) The proliferative index of intestinal crypts from (B) Bmi-1+/+ and (C) Bmi-1 eGFP/+ mice (*, p<0.05; n=5-8).
Figure 3-8 Impaired chronic (11d) GLP-2 proliferative responses within the TA zone of Bmi-1\(^{eGFP/+}\) mice. The intestinal crypt was divided into three different zones by cell position: (A) the active stem cell zone from position 1-3, (B) the reserve stem cell zone from position 4-6, and (C) the TA zone from position 7-16. The area under the curve of proliferation was calculated from the proliferative indices of Bmi-1\(^{+/+}\) and Bmi-1\(^{eGFP/+}\) intestinal crypts. (Δ = GLP-2 response; *, p<0.05; n=5-8).

3.2.2 Cell Migration

To uncover a possible mechanism driving intestinal growth in the absence of a proliferative effect of GLP-2, h(Gly\(^2\))GLP-2 was administered for 11d but, this time, BrdU was injected into a sub-group of mice 24h prior to sacrifice and EdU was injected 1h prior to sacrifice to track migration of intestinal epithelial cells.
undergoing DNA replication (132). Interestingly, chronic GLP-2 administration increased cell migration rate 1.6-fold (p<0.05) in the Bmi-1<sup>eGFP/+</sup> mice, but did not alter it in the Bmi-1<sup>+/+</sup> mice (Figure 3-9B).

**Figure 3-9** Chronic (11d) GLP-2 increased cell migration rate only in Bmi-1<sup>eGFP/+</sup> mice. Bmi-1<sup>+/+</sup> and Bmi-1<sup>eGFP/+</sup> mice were administered BrdU and EdU, 24h and 1h prior to sacrifice, respectively. (A) Representative BrdU/EdU co-immunofluorescent image (100µm scale bar). (B) The cell migration rate was quantified from the difference between the lengths of each proliferative label over the 23h time interval (**, p<0.01; n=5-6).

### 3.2.3 Gene Expression

To explore the ISC gene signature in response to chronic GLP-2 treatment, the expression of the stem and TA cell genes Bmi-1 (11) and Msi-1 (94,136), the intestinal stem cell-specific gene Lgr5 (10), the tuft cell marker and potential intestinal stem cell marker DCAMKL-1 (114,136,137), and the reporter gene eGFP were studied by semi-quantitative RT-PCR relative to expression of the housekeeping gene, 18S, in a sub-group of mice (Figures 3-10A-E). Although there appeared to be a chronic GLP-2 effect on Bmi-1 mRNA levels in the Bmi-1<sup>+/+</sup> mice, this did not reach significance (Figure 3-10A, p=0.051). However, in the Bmi-1<sup>eGFP/+</sup> mice, GLP-2 treatment significantly reduced Bmi-1 gene expression.
(Figure 3-10A, p<0.05). Furthermore, the GLP-2-treated Bmi-1eGFP/+ mice had significantly lower Bmi-1 mRNA levels than GLP-2-treated Bmi-1+/+ mice (Figure 3-10A, p<0.05). Unexpectedly, GLP-2 decreased Msi-1 in the Bmi-1+/+ intestine (Figure 3-10B p<0.05). No eGFP expression was detected in the Bmi-1+/+ mice (Figure 3-10C). Lastly, the gene expression of Lgr5, DCAMKL-1, and eGFP was not altered with GLP-2 treatment in either genotype (Figure 3-10C-E).
Figure 3-10 Impaired Bmi-1 gene expression after chronic (11d) GLP-2 treatment in Bmi-1eGFP/+ mice. Semi-quantitative RT-PCR of relative changes in gene expression of (A) Bmi-1, (B) Msi-1, (C) eGFP, (D) Lgr5, and (E) DCAMKL-1 normalized to the expression of 18S rRNA (Δ = GLP-2 response; *, p<0.05; n=5-7).
3.3 Pathological Chronic Study

3.3.1 Health Status

Finally, to study the effects of GLP-2 on the reserve stem cell pool, h(Gly²)GLP-2 was chronically administered for 18d or 19d in combination with the pathological challenge of lethal 100Gy whole-body irradiation, on day 15 (62). Over the 4-5d post-IR, the health of the mice declined regardless of genotype or treatment (Figure 3-11A-C). There was no effect of genotype or treatment on the percent BW change. At 1d-post IR, there was a decline in the health of the Bmi-1⁺/⁺ mice as compared to the Bmi-1eGFP/⁺ mice, regardless of treatment (Figure 3-11B; p<0.05, n=14-16). On day 4- or 5-post IR, CO score was not affected by GLP-2 treatment or genotype, with the exception of a small decrease in the CO score for, PBS-treated, Bmi-1⁺/⁺ mice 4d-post IR (Figure 3-11B; p<0.05, n=14-15). Similarly, when the percent change in BW was converted into a score that was combined with the CO score (Figure 3-11C), the combined score did not reveal an effect of genotype or treatment on murine health status except for Bmi-1⁺/⁺ mice which had a worse combined score than Bmi-1eGFP/⁺ mice on day 1, regardless of treatment, and day 4, with PBS treatment (p<0.05, n=14-16). Since radiation syndrome can lead to edema in the lungs, this could increase the BW of the mice in this study (138,139). To clarify this potential limitation of measuring BW as a health indicator, the lungs were collected 4d- and 5d-post IR. There was no effect of treatment or the day post-IR on lung wet weight in the Bmi-1⁺/⁺ lungs (Figure 3-11D, n=4-5). Due to the very few lungs collected from Bmi-1eGFP/⁺ mice (n=0-2), effects on lung weight could not be determined. Finally, as the experiment was ongoing, it appeared that, prior to irradiation, the Bmi-1⁺/⁺ mice had larger stools than the Bmi-1eGFP/⁺. As an indirect assessment of gut function and metabolism, 2-5 stool samples were collected per mouse (n=3-6). Despite the initial visual observation, there was no effect of genotype or treatment on the total or dry stool weight and water content of the stool collected (Figure 3-12A,B). As a result, this parameter was not determined post-IR.
Figure 3-11 Health status declined post-100Gy IR. Both Bmi-1<sup>+/+</sup> and Bmi-1<sup>eGFP/+</sup> mice were chronically administrated (14d) h(Gly<sup>2</sup>)GLP-2, exposed to 100Gy IR on the 15<sup>th</sup> day, and then h(Gly<sup>2</sup>)GLP-2 was administered for another 4-5d post-IR.
(A) BW, (B) CO Score, and (C) Combined BW change and CO Scores were determined up to 4d- or 5d-post IR (significant genotype effect in PBS treated mice *, p<0.05; significant genotype effect in GLP-2 treated mice #, p<0.05; n=7-16).

(D) Lung weight in Bmi-1 +/- mice 4d- and 5d-post IR (n=4-5).

**Figure 3-12** No differences in stool dry weight and water content after 11-13d of GLP-2 treatment. (A) Dry stool weights and (B) water content of stool (n=3-6).

### 3.3.2 Intestinal Growth and Proliferation

To assess the enterotrophic and proliferative effects of GLP-2 at the time of highest intestinal damage and during recovery, these parameters were assessed 4d- and 5d-post IR. GLP-2 treatment increased SI wt/BW in Bmi-1 +/- mice 4d-post IR by 41% (p<0.01), an effect that was lost at 5d-post IR (Figure 3-13A and B). Furthermore, in Bmi-1eGFP/+ mice GLP-2 increased SI wt/BW by 33% 4d-post IR (p<0.01) and by 36% (p<0.01) 5d-post IR (Figure 3-13A and B). Strikingly, crypts were completely absent from the jejunum regardless of genotype, treatment or the day post-IR (Figure 3-13C-F). Therefore, sub-villus depth replaced crypt depth as a surrogate measure of the space below the villus, not including the muscle layer. Despite no effect of GLP-2 treatment on sub-villus depth 4d or 5d-post IR, there was a genotype effect in PBS-treated mice 5d post-IR (Figure 3-13H). Bmi-1 +/- sub-villus depth was 30% deeper than in the Bmi-1eGFP/+ mice (p<0.05). There was also a GLP-2 effect on villus height, 4d-post IR (Figure 3-13G), a 35% increase in the
Bmi-1+/+ intestine (p<0.01) and a 45% increase in the Bmi-1eGFP/+ intestine (p<0.01). A two-way analysis of variance revealed an effect of treatment on villus height 5d-post IR (p<0.05), but a Student’s t-test revealed no difference between mice administered PBS vs GLP-2 regardless of genotype (p>0.05).
**Figure 3-13** Intestinal growth increase with chronic (18-19d) GLP-2 and 100Gy IR at day 15 in Bmi-1+/+ and Bmi-1eGFP/+ mice. SI wt/BW at (A) 4d- or (B) 5d- post-100Gy IR in Bmi-1+/+ and Bmi-1eGFP/+. (C-F) Representative H&E stained sections from jejunal tissues 4d-post IR (C) PBS-treated and (D) h(Gly²)GLP-2-treated Bmi-1+/+ mice and (E) PBS-treated and (F) h(Gly²)GLP-2-treated Bmi-1eGFP/+ mice (100µm
scale bar; inset shows lack of intestinal crypts). Sub-villus depth and villus height were measured (G) 4d-post IR and (H) 5d-post IR (Significant treatment effect *, p<0.05; **, p<0.01; significant genotype effect #, p<0.05; n=7).

Finally, to understand the mechanism underlying the GLP-2-induced growth responses, EdU was administered 1h prior to sacrifice 4d-post IR and 5d-post IR. Strikingly, there were no EdU+ cells within the sub-villus region, consistent with the complete absence of crypts seen in the jejunum (Figure 3-13C-F). However, there were some EdU+ cells sparsely located within the epithelium between and at the base of the villi (Figure 3-14A). Interestingly, at 4d-post IR (Figure 3-14B), proliferation increased by 96% with GLP-2 treatment in the Bmi-1+/+ intestine (p<0.01), but not in the Bmi-1eGFP+/+ intestine.

**Figure 3-14** Impaired proliferative response to GLP-2 in Bmi-1eGFP+/+ mice, 4d-post IR. (A) Representative EdU immunofluorescent section (100µm scale bar). Proliferation was quantified from (B) the number of EdU+ cells per jejunal cross-section. (**, p<0.01; n=7).
Chapter 4

4 Discussion

4.1 Rationale

One missing end of the enterotrophic GLP-2 pathway is the identity of the proliferative crypt cells it stimulates. Since proliferation within the crypt increases with GLP-2 administration, its possible targets are the TA cells, the active ISCs, or the reserve ISCs and there is evidence for each (5). Hence, chronic GLP-2 stimulates absorptive progenitor cells and increases the proliferation within the TA cell compartment (32,69). As well, teduglutide promotes crypt survival after lethal IR and increases Msi-1 labeling at +4, suggesting that GLP-2 stimulates the reserve ISC pool (62,69). Also IGF-1, a GLP-2 signal transducer (9,48,69), can stimulate proliferation in one or both of the ISC pools, depending on the physiological context (130). Importantly, impaired intestinal growth due to abnormal proliferation in the crypt can be caused by the KO of the Bmi-1 gene (110). However, whole-body KO of Bmi-1 leads to hematopoietic and neural abnormalities that cause death within a few months of birth (107,110). Therefore, a more viable Bmi-1 haploinsufficient model was used to determine if the proliferative effects of GLP-2 on the dividing cells of the crypt required the full gene expression of this transcriptional repressor.

4.2 Major Findings

In the acute setting, proliferation was stimulated with GLP-2 treatment in the TA zone of Bmi-1+/+ crypts, but not in the Bmi-1eGFP/+ crypts. Normally, the short-term administration of GLP-2 increases the cell division of TA cells (8). Furthermore, the TA zone of Bmi-1eGFP/+ mice also had an impaired jejunal proliferative response to chronic GLP-2 as compared to their Bmi-1+/+ controls. The impairment was specific to this crypt compartment, as the reserve and active ISC zones did not have a proliferative response to chronic GLP-2, regardless of genotype.
It is known that Bmi-1 is important for regulating basal intestinal proliferation because whole-body Bmi-1 KO mice have reduced crypt cell proliferation and a specific reduction in ISC proliferation (110). Furthermore, intestinal epithelial-specific Bmi-1−/− mice have reduced numbers of Ki67+ cells in the crypt (110). Consistent with respect to the reported normal survival and hematopoietic parameters (107), in this study there was no difference between PBS-treated Bmi-1eGFP/+ mice and Bmi-1+/+ mice in terms of proliferation throughout the three crypt zones. This means that normal crypt proliferation is maintained in homeostasis despite the haploinsufficiency of Bmi-1. One compensatory mechanism for having just a single copy of the gene is that transcription of Bmi-1 could be increased in the Bmi-1eGFP/+ intestine to maintain similar basal gene expression levels as in the Bmi-1+/+ intestine. However, importantly, when the crypt is driven to increase the number of cell divisions with GLP-2 treatment, the high proliferative demand would require an increase in Bmi-1 to repress the expression of p16 and p19 (104,106,110). However, after GLP-2 administration in the Bmi-1eGFP/+ mice, Bmi-1 gene expression was reduced and was also significantly less than in the GLP-2-treated Bmi-1+/+ intestine. Hence, while a compensatory increase in Bmi-1 expression may prevent altered basal proliferation of the epithelium, this mechanism failed in response to chronic GLP-2 treatment. Therefore, a full complement of Bmi-1 is required for the acute and chronic proliferative effects of GLP-2 in the TA zone.

Puzzlingly, the Bmi-1eGFP/+ mice had an intestinal growth response to chronic GLP-2 treatment despite the impaired proliferative response. It was therefore hypothesized that intestinal epithelial cell migration could help explain this growth. In fact, GLP-2 treatment increased the cell migration rate in the Bmi-1eGFP/+ but not the Bmi-1+/+ mice. Analogous to the TA zone proliferative response to GLP-2, basal cell migration rate was comparable between both genotypes and possibly due to compensation in Bmi-1 expression. When this was lost with GLP-2 treatment, the lack of a normal proliferative response in the TA zone of Bmi-1eGFP/+ mice may therefore have allowed for a new unidentified driving force that increased the cell migration rate. A proliferation-independent increase in cell migration rate could be
due to faster movement of differentiated cells out of the crypt and into the villus. Fundamentally, this mechanism alone cannot account for increased growth because it would only cause cells of the epithelium to reach the villus tip faster to undergo anoikis. Also, the enterotrophic effects of GLP-2 include reducing apoptosis (41). Therefore, in the chronic study, a proliferation-independent adaptive response to increase cell migration rate could have led to increased intestinal growth, possibly, in combination with a GLP-2-induced reduction in apoptosis at the villus tip.

Interestingly, suppressor of zeste 12 prevents differentiation of TA cells by transcriptionally repressing genes expressed by the absorptive enterocyte lineage, such as sucrase isomaltase and DPP-IV (140). Since suppressor of zeste 12 is an integral part of the polycomb repression complex 2, methylation of its target genes recruits PRC1 to further repress their expression (103,140). As an important PRC1 protein, Bmi-1 should also be able to control and prevent differentiation in the TA cell compartment (103). Since Bmi-1 is found throughout the crypt, this lends further support to the present findings that its haploinsufficiency affects TA cells and this could lead to more differentiation at the expense of proliferation (110,120,136).

Even though the epithelium of Bmi-1^{GFP/+} mice is less responsive to GLP-2’s proliferative effects, GLP-2 can still drive cell migration in these animals. Independent of proliferation, GLP-2 promotes ‘wound’ healing in the intestinal epithelial cell-18 cells, a rat ileal cell line, mediated by vascular endothelial growth factor – A and transforming growth factor – β (141). Furthermore, the intestinal epithelium heals over jejunal wounds induced by the application of HCl within 3h of GLP-2 treatment, a time-scale too fast to be driven by increased proliferation (142). The combination of Bmi-1 haploinsufficiency to increase differentiated enterocytes in the TA zone, at the expense of proliferation, and GLP-2 to drive proliferation-independent movement towards the villus may, therefore, increase the epithelial cell migration rate.

Finally, it is possible that there could be a proliferation-dependent mechanism underlying the increased epithelial cell migration rate. Although GLP-2 typically
increases the number of dividing cells (8,9,69), its administration combined with Bmi-1 haploinsufficiency could have altered cell-cycle dynamics to increase the frequency of proliferation within the same number of mitotic cells. This mechanism could account for epithelial growth on its own because a faster proliferation rate per cell would produce more progeny along the crypt-villus axis. Still, a reduction in apoptosis by GLP-2 could contribute to mucosal growth, but would not be necessary if the cell migration rate was proliferation-dependent.

To elucidate the role of Bmi-1 in mediating the proliferative effects of GLP-2 on the reserve ISCs, Bmi-1^{eGFP/+} mice were exposed to, lethal, 100Gy IR in combination with long-term GLP-2 treatment. The SIwt/BW increased comparably at 4d-post IR for Bmi-1^{+/+} and Bmi-1^{eGFP/+} mice. Furthermore, this coincided with mucosal growth as demonstrated by an increase in villus height for both genotypes. In the Bmi-1^{+/+} gut, the intestinal epithelial growth can be attributed to an increase in proliferation. Since +4 reserve ISCs are known to be resistant to lethal doses of radiation, as opposed to the CBC stem cells, GLP-2 should mostly be acting on this population to stimulate proliferation and regenerate the damaged epithelium (11,102). In agreement with this, a mediator of GLP-2’s enterotrophic effects, IGF-1, stimulates the reserve population of ISCs to enter S-phase of the cell-cycle after 14Gy IR (130). However, GLP-2 was unable to stimulate proliferation in the Bmi-1^{eGFP/+} intestine 4d-post IR. As was the case in the chronic physiological study, GLP-2 treatment created a proliferative demand that could not be met with half of the normal complement of the Bmi-1 gene following 100Gy IR. However, as increased mucosal growth was again found, despite the impaired proliferative response, it is highly likely that the increased villus height was due to an adaptive GLP-2 response that increases cell migration rate possibly combined with a reduction in apoptosis.

In summary, the results of all three studies presented in this thesis indicate that a full complement of Bmi-1 is required for the proliferative effects of GLP-2 treatment in both the physiological and pathological settings.
4.3 Limitations

Although the Bmi-1^eGFP/+ mouse model was used to elucidate the importance of a complete Bmi-1 gene complement, it could not be used for fluorescently labeling the reserve ISC pool. The +4 reserve ISC is functionally identified by the expression of Bmi-1 using a Bmi-1-CreERT2 transgene (11). However, even though the Bmi-1^eGFP/+ model properly labels hematopoietic stem cells, it does not mark solely the +4 reserve ISCs (119,131), as indicated by eGFP fluorescence throughout the crypt and in lone cells of the villus (present study) (119). One possible reason, for this, is that eGFP could be present long after Bmi-1 normally is expressed in a +4 reserve ISC and could remain present in its non-reserve ISC progeny (119). As well, eGFP is knocked-in to exon 2 of the Bmi-1 gene, knocking-out Bmi-1 expression in one allele, while CreERT2 is inserted in the 3’ untranslated region of the Bmi-1 gene, maintaining full Bmi-1 expression in mice expressing the Bmi-1 CreERT2 construct (11,119,131). This could cause adaptive mechanisms to maintain basal Bmi-1 expression in the Bmi-1^eGFP/+ mice leading to a non-Bmi-1-like eGFP expression pattern. Lastly, the high background fluorescence of these intestines observed in this and another study, further explain the broader labeling within the crypt (119). Therefore, cells that exhibit eGFP fluorescence in this model cannot be specifically identified as reserve ISCs.

Additionally, this murine model is limited in molecularly identifying the +4 reserve ISCs because expression of Bmi-1 itself cannot label this pool of ISCs (119). The expression of Bmi-1 is found throughout the crypt base, overlapping with the CBC stem cell pool (119,120). Furthermore, the highest levels of Bmi-1 expression are found in the CBC stem cells that highly express Lgr5 (119). Therefore, in this model and others, a crypt cell that expresses Bmi-1 cannot be classified as a reserve ISC.

Also, the impaired proliferative response to chronic GLP-2 in the TA zone of Bmi-1^eGFP/+ mice was not compared to a positive control, as there is still the possibility that the Bmi-1^eGFP/+ mice are unresponsive to mitogens, in general. Since administration of Rspo-1 increases proliferation in the crypt in a manner that is at
least partially independent of GLP-2 signaling (69,143), proliferation should have been assessed in a separate group of Bmi-1\textsuperscript{eGFP/+} mice chronically treated with R\textsuperscript{spo}-1.

It is also important to consider how the proliferative index assessed proliferation. The positional analysis of proliferation quantified the number of dividing cells at each position of the crypt. However, this does not account for the proliferative rate of these crypt cells because only a single division was labelled. Since a single BrdU or EdU injection was administered, multiple divisions could not be observed. Furthermore, the label was given just an hour prior to sacrifice, a time frame too short to allow for a second division to take place. Therefore, the proliferative response to chronic GLP-2 may not be completely impaired, as a population of cells could have undergone faster cell divisions.

Another important limitation of this study was that the appropriate dose of radiation was not used in the pathological study. Normally, the intestinal crypts in C57Bl/6 mice undergo apoptosis after exposure to 10-12Gy IR because their highly proliferative cells are susceptible to DNA damage (129). At 4d-post IR, the epithelium reflects the consequences of radiation damage with shrunken villi and minimal, but intact crypts (129). Still, the intestine regrows back to its normal morphology, 6d-post IR, because of a regenerative period driven by surviving clonogenic crypts (100,129). In comparison, at 100Gy IR, both Bmi-1\textsuperscript{+/+} and Bmi-1\textsuperscript{eGFP/+} had intestines with completely absent crypts. Furthermore, this was also observed 5d-post 100Gy IR. In pilot studies, Bmi-1\textsuperscript{eGFP/+} mice were taken to 6d-post 100Gy IR or 4d-post 120Gy IR, where they either died or were deemed moribund by the CO score and euthanized. This reflected a much greater impact of radiation damage than what is observed in WT, C57Bl/6 mice exposed to a 10-fold lower dose of IR (129). Although one would expect the manipulation of an important proliferation-related gene in the crypt could predispose the intestinal epithelium to greater damage, the same effect was seen in the Bmi-1\textsuperscript{+/+} mice. Taken together, this suggests that the radiation dose was too high to properly determine the proliferative effects of GLP-2 in the pathological setting.
Although the Bmi-1^eGFP/+ mice have only one copy of the Bmi-1 gene, there was no significant difference in mRNA expression in the PBS-treated Bmi-1^eGFP/+ intestine as compared to the PBS-treated Bmi-1^+/+ intestine. It was expected that a single copy of Bmi-1 would lead to half the amount of Bmi-1 expressed in the intestine. What this means, is that there was an adaptive response to Bmi-1 haploinsufficiency to maintain normal Bmi-1 expression, at least at the mRNA level. However, this was not confirmed at the protein level, which is important because mRNA levels do not necessarily correspond to the same abundance of protein. Although attempts were made to observe Bmi-1 protein by IF, and indirectly with eGFP, the available antibodies were not found to be specific; thus, western blots have not yet been attempted. If the adaptive Bmi-1 mRNA expression pattern was also true at the protein level, then the basal effects of this haploinsufficient model could be masked.

Lastly, to assess proliferation throughout the different studies, BrdU or EdU incorporation within the cells of the crypt was quantified. In cells that are in S-phase of the cell cycle, these thymidine analogues become incorporated into the DNA during replication. Usually, the cell can then progress through the G2 and M-phases of the cell cycle to divide into two daughter cells. However, a cell that is in S-phase does not always successfully move forward through the cell cycle and undergo mitosis. For example, in the chronic pathological study, a cell exposed to 100Gy IR could initiate the process by replicating its DNA, incorporating EdU, but then undergo apoptosis before dividing due to the radiation damage it incurred. Also, radiation-induced DNA damage could initiate DNA repair mechanisms that incorporate EdU into the cell’s DNA. Therefore, EdU and BrdU labelled cells are not exclusively proliferating cells.

4.4 Future Directions

A promising message from the pathological chronic study is that GLP-2 caused mucosal growth in the Bmi-1^+/+ mice after a lethal-dose of IR, suggesting that it stimulated the reserve ISC pool, and that the impaired proliferative response in the Bmi-1 haploinsufficient intestine could be a result of reduced proliferation in the
reserve ISC zone. This could then provide the opportunity for compensation via faster cell migration and, potentially, a reduction in apoptosis to maintain mucosal growth, as in the physiological setting. However, this compensatory growth mechanism and which population of crypt cells have defective proliferation could not be pinpointed in the Bmi-1^{GFP+} mice, as was done in the acute and chronic studies. This was because the 100Gy IR was too strong a dose and ablated all intestinal crypts by 4d- and 5d-post IR, leaving a few, stranded, proliferative cells between villi. To overcome this limitation, one mouse was exposed to 10Gy IR, in a pilot study. The mouse was taken to 4d-post IR, when the epithelium exhibits the most damage, and its jejunal morphology was assessed by H&E histochemistry. This dose of IR showed the presence of enlarged crypts, as opposed to the complete absence of crypts at 100Gy, and so this appears to be an appropriate dose of radiation that could cause enough damage to activate the reserve ISCs, but still allow for the epithelium to regenerate. This dose was therefore chosen for an ongoing chronic pathological study in which gravimetric, morphometric, and proliferative analyses will be performed. This time, due to the presence of crypts, positional analysis of proliferation will be quantified. Each of the three crypt zones will be compared to see which population of cells has impaired proliferation due to Bmi-1 haploinsufficiency. Also, these mice will not only be taken to 4d-post IR, but also to 6d-post IR, to determine if Bmi-1-dependent proliferation in response to GLP-2 is required for the regeneration and recovery of the intestinal epithelium. Furthermore, as was done in the chronic study, cell migration rate could be assessed as a potential proliferation-independent mechanism of epithelial growth.

Interestingly, chronic GLP-2 was found to increase cell migration rate, possibly independent of an increase in proliferating cells. This effect in the intestine is not fully understood. It has been shown in vitro that this is mediated by vascular endothelial growth factor – A and transforming growth factor – β (141). Whether and how they contribute to this phenotype in the intestinal epithelium is not known. Fortunately, analogous intestinal epithelial movements are studied in the restitution phase of ‘wound’ healing (141,144,145). Cell migration rate is elevated to rapidly cover the gap in the epithelium to maintain its barrier integrity and function, before
proliferation can contribute to the healing process (141,144,145). From these studies there are a few possible downstream effectors that could be involved in the adaptive response to chronic GLP-2 treatment. One option is Ras-related C3 botulinum toxin substrate 1 (Rac1), which is important for the migration of the endoderm of the embryo and mouse embryonic fibroblasts in vitro (146,147). Rac1 is important for the formation of lamellipodia, focal adhesions, and actin cytoskeletal rearrangements that promote the movement of epithelial cells (146,147). Using similar mechanisms, mammalian target of rapamycin is known to promote EGF-stimulated cell migration (148). Its actions can also be stimulated by IGF-1 in combination with high levels of amino acids (144). Another possibility is that enterocytes express different integrin sub-units, such as α3, α6, β1, and β4, that could promote interactions with extra-cellular matrix proteins, such as laminin, to favour migration (145). To assess the possibility that increased cell migration may coincide with increased gene expression of any and all of these factors, qRT-PCR could be carried out in jejunal samples from the chronic GLP-2 study. This could be followed up with western blot to confirm any increased mRNA expression at the protein level.

Importantly, apoptosis would need to be assessed in the chronic study to confirm the enterotrophic effect of GLP-2 is in part due to its anti-apoptotic action (41). Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling (TUNEL) could be done to quantify apoptosis at the villus tip. Since TUNEL marks fragmented DNA that indicates cell apoptosis, cleaved caspase-3 immunohistochemistry could also be done to examine programmed cell death because it is an important apoptotic signalling mediator.

What still needs to be explored is the possibility that the chronic GLP-2-induced increase in epithelial cell migration rate of Bmi-1GFP/+ intestines could be proliferation-dependent. In vitro overexpression of IGF-1, a GLP-2 signal mediator, increases the number of retinal pigment epithelial cells that reach S-phase and G2 of the cell-cycle, in 27h, as compared to synchronized control cells (149). Thus, GLP-2 could be shortening the cell-cycle of proliferating cells via IGF-1, to increase cell migration rate without increasing the total number of proliferating
cells. To determine the cell-cycle kinetics in the crypt, positional analysis of double-labelled cells from the chronic study, with BrdU/EdU co-labelling over a 23h interval, could elucidate faster cell-cycle dynamics in reserve ISCs. Since reserve ISCs take more than a day to divide, an increased number of BrdU+/EdU+ cells around position +4 of the crypt would suggest they are cycling faster. However, the cell migration rate was increased 1.6-fold and to detect an equivalent increase in the proliferative rate of active ISCs, which normally divide once a day (10), a 16h interval between the BrdU and EdU labels could be done in a separate chronic study. Also, to detect the same increase in cell-cycle time of the TA cells, a 7.5h interval could be used in another chronic study because they normally cycle every 12h (12,94).

Lastly, what remains to be definitively answered is why a full complement of Bmi-1 is required for the acute and both physiological and pathological chronic proliferative effects of GLP-2. The various actions of Bmi-1 could contribute to this role, such as its repression of p19 and p16 expression, the promotion of Tert expression, the activation of DNA repair mechanisms, or simply the repression of differentiation, which all need to be explored (103,104,106,108,109,140). To assess repression of transcription and translation of p16 and p19, these cell-cycle regulators could be analyzed in all three GLP-2 studies by qRT-PCR and western blot. Additionally, Tert expression could be examined in the exact same way to determine if GLP-2 requires Bmi-1 to increase its expression, thus delaying the replicative senescence in the proliferative cells of the intestine. The role of driving DNA repair may be more prominent in the chronic pathological study due to the lethal dose of IR. So in this study, phosphorylated gamma-H2A.X – IF could be quantified in jejunal sections as an indicator of Bmi-1-mediated DNA repair (110). To support this, the number of cells that have DNA damage but are not apoptotic could be quantified from the co-immunofluorescent staining of TUNEL and cleaved caspase-3. If the incidence of non-apoptotic cells with DNA damage is higher in GLP-2-treated Bmi-1 haploinsufficient mice as compared to the Bmi-1+/+ mice, then it will suggest that Bmi-1 is important for maintaining proliferation by promoting DNA repair. Lastly, quantification of the four main differentiated cell
types of the crypt-villus unit will show if there is more overall differentiation, as well as if there is a particular cell type that is favoured, in the Bmi-1 haploinsufficient intestine. This could be characterized in the chronic studies, where jejunal sections could be stained for lysozyme (Paneth cells), mucin (goblet cells), sucrase-isomaltase (absorptive enterocytes), and chromogranin A (enteroendocrine cells). De-repression of differentiation due to less Bmi-1 expression could inherently reduce the amount of proliferation in the TA zone of the crypt. Potentially, a GLP-2-dependent increase in differentiation overall or of a specific cell type in the Bmi-1^{eGFP/+} intestine could explain the increased cell migration rate, as these cells may have unique mechanisms to equip themselves for efficient transit towards the villus tip.

4.5 Conclusions

In conclusion, Bmi-1 is important for the proliferative actions of GLP-2 in the intestinal epithelium. In the physiological setting, a full gene complement of this chromatin-remodeling complex protein is required for the acute and chronic proliferative response to GLP-2 in TA cells. Specifically, in the chronic setting, Bmi-1 haploinsufficiency leads to an adaptive growth response to GLP-2 administration that increases the epithelial cell migration rate, despite impaired proliferation. When the intestine is challenged with a lethal dose of IR, both copies of the Bmi-1 gene are also required for the proliferative effects of chronic GLP-2 treatment. These findings could be attributable to the downstream epigenetic effects of Bmi-1 and/or effects of GLP-2 to induce proliferation-independent or -dependent cell migration and/or cell survival. These possibilities will be explored in future studies to completely elucidate how this vital pathway facilitates the enterotrophic actions of GLP-2 on the intestinal epithelium.
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