Investigation of the effect of acute radiation on enteroendocrine L-cell health and function

by

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Abstract

Radiation enteropathy is a commonly observed syndrome induced by abdominal and pelvic radiation therapy characterized by acute and/or chronic pain and loss of proper gut function. Glucagon-like peptide-2 (GLP-2), an important peptide hormone in the enhancement of proper intestinal function, proliferation of intestinal tissue and inhibition of enterocyte apoptosis, is a known treatment option of malabsorption disorders of the intestines. Recent developments have demonstrated that exogenous GLP-2 treatments attenuate and facilitate recovery of radiation-induced gut injury. This study aims to elucidate the effects of radiation therapy on endogenous GLP-2 secretion from intestinal L-cells as this is poorly understood. In mice, L-cell counts remained unchanged 48 hours post-irradiation but total circulating GLP-2 was increased. Irradiation of *in-vitro* L-cell models demonstrated a trend of increased hormone secretion at intermediate doses and significant increase in cell viability and mitochondrial activity. The stimulatory effect of radiation on L-cell should be investigated in future studies.

Keywords

Glucagon-like peptide-2, gastrointestinal, enteroendocrine, ionizing radiation, L-cells

Abbreviations

5-FU: 5-fluorouracil

BSA: Bovine serum albumin

cAMP: Cyclic adenosine monophosphate

EECs: Enteroendocrine cells

FFAR : Free fatty acid receptor

GI: Gastrointestinal

GIP: Glucose-dependent insulinotropic polypeptide

GIT: Gastrointestinal tract

GLP-1: Glucagon-like peptide-1

GLP-2: Glucagon-like peptide-2

GLP-2R: Glucagon-like peptide-2 receptor

GPBAR1: G protein-coupled bile acid receptor 1

GRPP: Glicentin-related pancreatic peptide

GLUT2: Glucose Transporter 2

IP-1: Intervening peptide 1

IP-2: Intervening peptide 2

ISC: Intestinal stem cells

LCFA: Long chain fatty acid

MCFA: Medium chain fatty acid

MPGF: Major proglucagon fragment

SBS: Short bowel syndrome

SCFA: Short chain fatty acid

SGLT1: Sodium-glucose transporter 1

PC1/3: Proprotein convertase 1/3

PC2: Proprotein convertase 2

RPL13a: Ribosomal protein L13a

Co-Authorship Statement

The submitted review paper provided in section 1.2 was written and completed by myself with the guidance of, and reviewed by, Dr. Jeffrey Gagnon.

For the research project provided in section 2 of the document, the general outline of the project, training in experimental techniques, methods and analysis as well as counsel during the manuscript writing process were provided by Dr. Gagnon. The animal work, including maintenance, blood collections and harvesting of tissue was completed along with members of Dr. Sujeenthar Tharmalingam and Dr. Christopher Thome's radiation biology research group, under their established animal protocol. All cell culture work, protein and RNA measurements were completed by myself. Emily Cooke provided some immunohistochemistry data on the effects of ionizing radiation on enteroendocrine L-cell distribution and localization in mouse ileum.

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1 Introduction

This chapter will begin with a submitted review article outlining the therapeutic potential of glucagon-like peptide-2 (GLP-2) in attenuating injuries to the gastrointestinal tract (GIT) resulting from treatments of cancer. Further remarks regarding enteroendocrine L-cells, GLP-2 and the effects of ionizing radiation on the health of the GIT will follow this review

1.1 Therapeutic Potential of Glucagon-like Peptide-2 in Attenuating Cancer-Therapy Induced Gut Injuries: A Review

1.1.1 Abstract

Glucagon-like peptide-2 (GLP-2) is a potent intestinotrophic proglucagon-derived peptide secreted from intestinal enteroendocrine L-cells. GLP-2 signaling increases proliferative and absorptive pathways within the gut, whilst simultaneously suppressing apoptotic and inflammatory processes. The trophic and cytoprotective effects of GLP-2 have been utilized to treat malabsorptive disorders such as short bowel syndrome (SBS) and inflammatory bowel disease (IBD). Exogenous GLP-2 administration may be a candidate to facilitate healing of injuries to the gastrointestinal tract such as those resulting from treatment of neoplasms. However, due to its potent effects on cell proliferation, there is concern that GLP-2 may induce or facilitate development of intestinal dysplasia. This review provides an up-to-date summary of the available research on (1) GLP-2's potential therapeutic benefits in treating cancer treatment-induced gut injuries, and (2) the current understanding on the potential links between GLP-2 and the development of neoplasms.

1.1.2 Introduction

Cancer is a leading cause of disease burden (1) and mortality worldwide, accounting for nearly 10 million deaths annually (2). As recently as 2015, the Global Burden of Disease Study (GBD) estimated that approximately 90 million individuals globally are currently diagnosed with a form of cancer (3), with a projected increase of 47% in new cancer cases annually by 2040 (2). This trend is reflected both by the ageing and growing population as well as the significant advances in the treatment of infectious and neonatal diseases in developing countries (1,2). Correspondingly, considerable research is dedicated to preventing, diagnosing, and treating these diseases, leading to substantial increases in age-adjusted cancer survival rates. Nevertheless, many of the individuals afflicted by these diseases experience a decreased quality of life (QoL) resulting from their course of treatment (4).

Studies concerning the causes of decreased quality of life in response to cancer treatment have identified gastrointestinal (GI) complications as a primary source of discomfort and hospitalization (4,5). These complications primarily present as inflammation and ulceration of the GI epithelium in upwards of 50% of all chemotherapy and radiotherapy patients (6) with a significant increase to 90% prevalence for head and neck cancer patients (HNC) (7). These injuries to the intestinal epithelium, referred to as GI mucositis or enteropathy, promote NF-κB mediated inflammatory cytokine storm (8,9) and apoptosis of the intestinal crypt cells resulting in loss of efficient proliferation of the intestinal epithelium (10). This induces atrophy of the absorptive microvilli and the formation of ulcers or polyps (10). This generally presents through acute and/or chronic abdominal pain, decreased appetite, nausea, constipation, and diarrhea (11).

Ulceration of the epithelial membrane may also lead to increased susceptibility to infection from pathogenic agents (10,12). This is a primary concern in immunosuppressed cancer patients, including those who have leukemia or those treated with chemotherapeutic agents. In these patients there is a 4-fold increase in the incidence of sepsis compared to the general population (12). Importantly, septic cancer patients have an observed increase in mortality of 43% compared to non-oncological related cases of sepsis (12,13).

To date, treatments of mucositis and enteropathy of the GI tract have been limited to symptomatic treatments such as administration of statins and angiotensin-converting enzyme (ACE) inhibitor (14,15) or by supplementation of amino acids and vitamins (15). The chemoprotective agent amifostine has shown promising results in early-stage clinical trials as a potential adjuvant treatment option of enteropathy (11,16), but its use in clinical practice is limited due to potentially severe adverse effects (17). Glucagon-like peptide-2 (GLP-2) (18), an enteric peptide hormone, has recently been shown to alleviate the impairment and damage of the GI tract sustained during cancer treatments (19). This literature review will present these recent findings and their potential limitations in the hope of highlighting the potential clinical benefits of GLP-2 as an adjuvant therapy to cancer treatments.

1.1.3 Glucagon-like Peptide-2

GLP-2, a 33-amino acid peptide, is a potent intestinotrophic hormone involved in the post-prandial proliferation of the intestinal epithelium, increasing the absorptive surface, blood flow, and nutrient uptake by the intestinal epithelium (Figure 1) (20). GLP-2 derives from the processing of the proglucagon peptide by proprotein convertase 1/3 (20) in the intestinal L cells, primarily

localized in the ileum and proximal colon (21). Proglucagon-processing additionally induces production of the incretin glucagon-like peptide-1 (GLP-1), oxyntomodulin as well as several other peptides with poorly defined functions (22). Interaction of GLP-2 with the G-protein coupled GLP-2 receptor (GLP-2R) expressed in a variety of cells throughout the intestinal tract (23) promotes downstream secretion of growth factors such as insulin-like growth factor-1 (IGF-1) (24) and epidermal growth factor receptor ligands (ErbB-1) to increase proliferation of intestinal stem cells (ISCs) (Figure 2) (25). This hyperproliferative activity induces an increase in microvilli height, crypt depth and total intestinal length. GLP-2R signaling additionally enhances barrier function through increased expression of tight junction (TJ) proteins (26), protecting against potential infections and septicemia (27,28). In addition, GLP-2R signaling protects against apoptotic and inflammatory processes in the gastrointestinal tract (GIT) (29,30) by inhibiting NF-κB, ERK1/2 and JNK1/2 pro-inflammatory pathways (31).

The biological activity of GLP-2 is however transient as it only has an active half-life of approximately 7 minutes in circulation (19). This results from filtration of the blood by the kidneys and the circulation of the peptide-cleaving enzyme dipeptidyl peptidase-4 (DPP-IV) (19,32). The GLP-2 analogue teduglutide [(Gly2)-GLP-2], which is resistant to degradation from DPP-IV, is used in the clinical setting for the treatment of malabsorption disorders such as

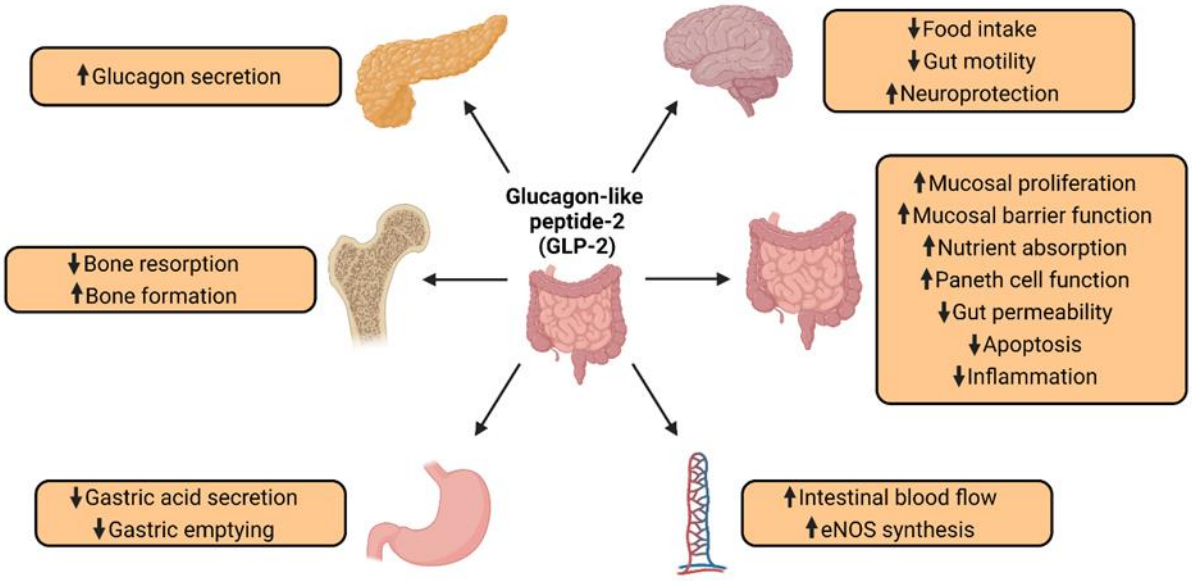

Figure 1. Physiological effects of GLP-2 signaling via GLP-2R expressing cells in the GIT.

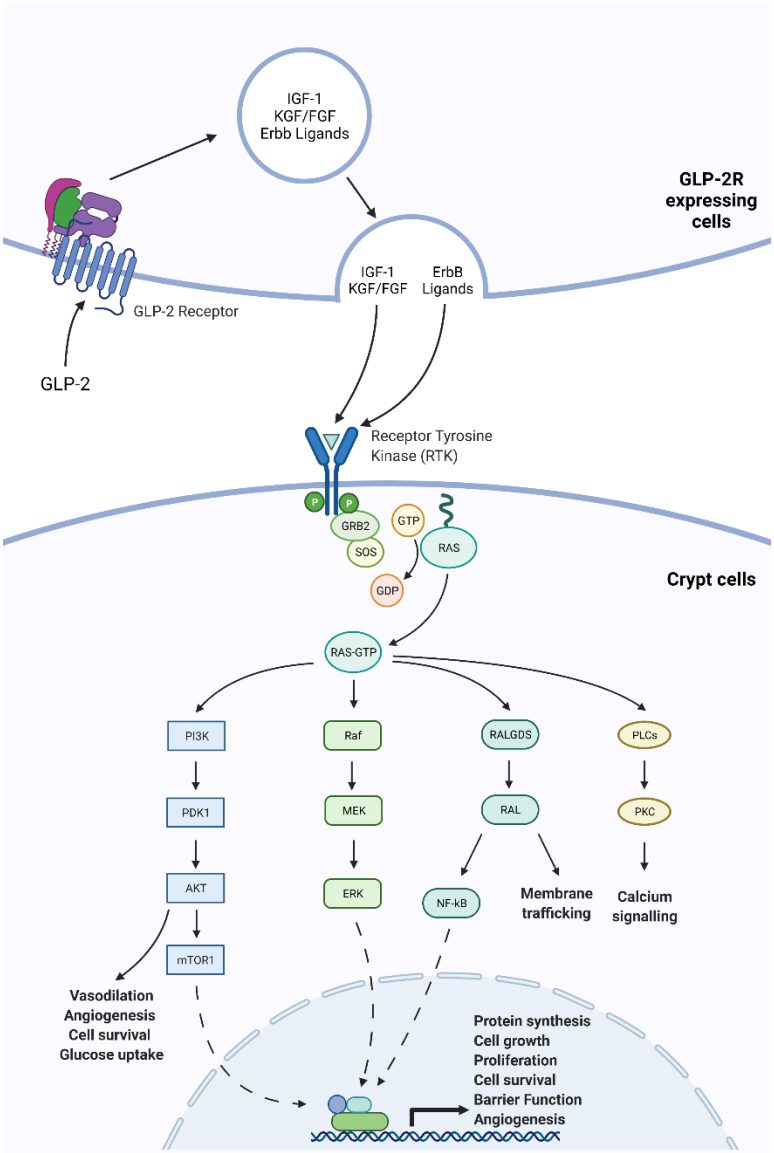

Figure 2. GLP-2R signaling causes secretion of various growth factors and ErbB ligands inducing activation of intestinotrophic pathways in intestinal crypt cells. Adapted from "Ras Pathway", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

short bowel syndrome (SBS) and Crohn's disease (33,34). Due to the increased half-life and biological activity of teduglutide, treatments significantly improve overall gut length, nutrient absorption and decrease expression of pro-inflammatory cytokines in patients (19,30,33,34). Recent studies have shown that administration of GLP-2 analogues as adjuvant therapies to cancer treatments such as chemo- and radiotherapy may significantly improve gut function and limit adverse effects. However, a better understanding of the physiological effects of these treatments in treating cancer-related gut injuries is needed.

1.1.4 Surgical Resection of Cancerous Tissue

Current treatment regimens of malignancies within the GI tract, regardless of disease progression, primarily employ open or laparoscopic surgical resection of the cancerous tissues (35,36). Early-stage colon and rectal cancer patients typically rely solely on local tumor excision or removal of portions of the colon and rectum without adjuvant chemotherapy or radiotherapy (RT) (84% and 62%, respectively) (36). Individuals who have undergone colectomies generally have their bowel reformed through end-to-end anastomosis of the remaining sections of the GIT or the formation of a colostomy (36). Consequentially colorectal patients present with severe bowel dysfunction and severe diarrhea resulting from loss of colonic absorptive surface and loss of GLP-2 secreting L-cells (37,38). Hospitalization and mortality rates following end-to-end anastomosis of the GIT post-resection are also elevated due to blockage and leakage resulting from improper anastomotic wound healing (37,38). Current research on adjuvant therapies for facilitating anastomotic wound healing is limited, and there is a need to fill this gap in both the literature and clinical practice.

Subcutaneous administration of GLP-2 and GLP-2R agonists has been shown to facilitate the healing of colonic anastomoses by increasing crypt cell proliferation and growth factor production. This is primarily observed by increased insulin-like growth factor-1 (IGF-1) (24,39), which increases expression of both the type I (COL1A1) and type III (COL3A1) collagen subtypes necessary to form the intestinal extracellular matrix (40,41). Administration of a long-acting GLP-2 analog, ZP1849, in Wistar albino rat models, increased Ki67 expression compared to non-injured and vehicle controls at both the 3- and 5-day post-anastomosis (40). Elevated expression of IGF-1, COL1A1 and COL3A1 were observed during the healing stage compared to all controls, which reflected the observed increase in anastomotic breaking strength in ZP1849 treated rats (40). Teduglutide, the most commonly administered GLP-2 analogue, has similarly been shown to promote mucosal re-epithelialization and increase submucosal expression of type III collagen in response to ileal anastomosis in rat models (42). This study however indicated that submucosal type I collagen content was decreased 7-days following anastomosis in response to teduglutide treatment, contradicting similar studies (42). This study also varied from the other related studies in that collagen content was observed in the ileum and was visualized with Gordon and Sweet's silver staining technique and assigned a qualitative score (42) rather than assess the expression of COL1A1/COL1A3 and of circulating collagen (40,41).

Some studies also suggest that administration of GLP-2 may decrease postprandial bone resorption in patients who have undergone colectomies (43). A study comparing individuals with SBS, both with and without functional colons, determined that individuals with SBS without a functional colon presented with lower baseline and post-prandial GLP-2 levels than healthy and SBS with colon controls. These individuals lacking a colon showed no postprandial changes in bone resorption, indicating a potential role of the colon in maintaining bone health (44). Although

research regarding the application of GLP-2 as an adjuvant treatment to non-GI-related cancers is limited, rat models have shown that exogenous GLP-2 administration limits bone resorption and increases bone formation following ovariectomy, potentially offering therapeutic benefit to the treatment of cancer-treatment induced osteoporosis (45). Research studying the effects of GLP-2 following anastomosis is limited to those presented in this article, and application of GLP-2 analogues as a clinical adjuvant to recission of cancerous tissue needs to be further investigated.

Contrary to colorectal cancer, rare cancers of the small bowel such as small bowel adenocarcinomas (SBAs) are generally poorly differentiated and in later stages of disease at the time of diagnosis (46,47). Correspondingly, removal of cancerous tissue is typically not applicable in treating small bowel malignancies, and intense chemotherapy and/or RT treatment patterns are applied directly (47). Exogenous GLP-2 has however shown similar effects following resection and anastomosis of the small bowel, limiting the symptomatology of SBS in both animal models (48–50) and human trials (51). Loss of jejunal tissue within the small bowel generally leads to SBS development, partly resulting from loss of absorptive surface (52) and GLP-2R signaling, which is believed to be primarily located within the jejunum (23). Elevated levels of exogenously administered GLP-2 in animals with simulated SBS models lead to prolonged gastric emptying, intestinal adaptation and lengthening (33,48,49).

Similarly, a clinical study of teduglutide's effectiveness in treating SBS further indicated that individuals with SBS treated with GLP-2 presented with decreased dependence on parenteral nutrition (PN) and intravenous fluid (IVF) administration compared to controls (53). Current clinical applications of GLP-2 analogues for SBS treatment should be considered in the rare occurrence of resection of short bowel malignancies. Still, they should be mindful of the potential

implications due to the lack of available literature studying GLP-2 administration following resection of SBAs.

1.1.5 Chemotherapy

Chemotherapy treatments generally function by inhibiting replication of cancerous tissues through various drug classes, including alkylating agents and antimetabolites (54,55). Alkylating agents such as cisplatin can alkylate proteins and nucleic acids, causing DNA strand breaks leading to apoptosis of the afflicted cells (55). Contrarily, antimetabolites can incorporate themselves into typical cellular processes such as DNA replication and transcription due to structural similarities to essential compounds. Two of these antimetabolites, methotrexate and 5-fluorouracil (5-FU), are structurally similar to folic acid and uracil, respectively and bind to associated enzymes, both inhibiting thymidine production as well as other mitotic processes (54–56). These compounds impact malignancies due to the uncontrolled and rapid nature of their replication. However, as these drugs are not specific to tumor cells, healthy tissues with increased stem cell counts and elevated rates of proliferation, such as the GI epithelium, are at high risk of being affected by chemotherapeutic agents. In humans, this is especially true in the intestines, where complete turnover of the intestinal epithelium occurs in a period of 3-7 days (57). This results in ulceration and inflammation of the oral cavity and the GIT, commonly referred to as mucositis (58), in up to 40% of patients treated with chemotherapeutic drugs [7]. Incidence of mucositis increases to 80% in patients receiving high-dose radiation therapy and hematopoietic stem cell transplantation (HSCT) (58). Those afflicted present with increased risk of infection and septicemia as well as GI

dysfunction, dehydration and poor nutrient uptake due to damage to the GIT and the debilitating pain caused by the ulceration of the epithelial tissues, notably in the mouth (12,13).

GLP-2 analogues have shown promising results in the treatment of chemotherapy induced GI mucositis. Subcutaneous injections of teduglutide in mouse models caused increased overall survival rates and significantly decreased incidence of bacteremia in healthy tissues and circulation in mice treated with either 5-FU or the topoisomerase I inhibitor irinotecan (59). GLP-2 treatment also attenuated epithelial injury and inhibited apoptosis in the intestinal crypts compared to controls (59). Importantly, tumor-bearing mice treated with irinotecan with/without subcutaneous injections of GLP-2 displayed similar tumor suppression levels, indicating that the teduglutide treatment did not impact the effectiveness of the chemotherapeutic agents (59).

A previous study examining the influence of teduglutide on 5-FU induced mucositis in rat models observed comparable findings, where administration of GLP-2 following injection led to increased intestinal weight, villus height and decreased crypt cell death compared to 5-FU control animals (56). This study demonstrated that daily injections of teduglutide in the week before injecting the chemotherapeutic agent did not significantly increase the jejunal weight and villus height compared to control animals and posited that pre-treatment of GLP-2 impaired attenuation of injury (56). This conclusion has seen criticism in the literature as total intestinal weight was not measured and due to potentially outdated investigative parameters (56,60). Further evidence from a 2012 study indicated that pre-treatment of a GLP-2 analogue in rats two days before 5-FU injection mitigated intestinal weight and microvillus height loss while decreasing MPO-positive cell localization within the lamina propria (60). Additional experimental and clinical studies need to be conducted to clarify physiological responses to pre-treatments of GLP-2 preceding injections

of chemotherapeutic agents to delineate appropriate time frames for future clinical administrations of GLP-2 analogues.

GLP-2R localization is not limited to the GIT as it has also been shown to be expressed in enteric neurons and myofibroblast where it exerts neuroprotective functions (20,61). Recent studies have shown that adjuvant treatments with teduglutide may also be partially neuroprotective against chronic administration of cisplatin (61,62). Female C57BL/6 mice were treated with 4mg/kg injections of cisplatin with or without teduglutide (62). At the time of termination, the animals treated with the adjuvant teduglutide presented decreased inflammation and reduce injuries to the GIT. Chronic treatment with cisplatin led to neuronal death in colonic tissue, notably ChAT- and nNOS-immunoreactive neurons (62). However, adjuvant teduglutide treatment significantly attenuated neuronal death, principally through the protection of nNOS-IR neurons and SOX-10-R expressing glial cells (61,62). Future studies should aim to study the cellular mechanisms through which GLP-2R signaling protects the enteric nervous system (ENS).

Recent findings indicate that the role of GLP-2R signaling in mitigating symptomology of GI mucositis results from facilitating the healing of the GIT rather than mitigating the acute response to injury (63). Wild-type (WT) mice treated with a single dose of 5-FU to induce injury of the intestinal tract responded displayed the expected natural healing response to injury through increased intestinal weight, microvilli height and crypt depth in comparison to the acute inflammatory stage of injury (63). However, this natural response to healing was absent in GLP-2R knockout (GLP-2R(-/-)) littermates, while the acute response to injury remained statistically identical to WT mice, except for slightly increased inflammation of the jejunum (63). However, due to limited time points in this study, additional work will be needed to understand how GLP-2R can mitigate early stage apoptosis and injury (63). GLP-2R(-/-) mice did not display lowered

replication nor increased apoptosis both 3- and 5- days post-injury, but this may be attributed to selected time-points and the use of cleaved caspase-3 as the sole indicator of apoptosis (63). As previous literature seems to indicate that GLP-2R signaling plays a pivotal role in crypt cell proliferation (29,56) and inhibition of cleaved caspase-8 activation (59), further research should be conducted to examine the impact of chemotherapy-induced gut injuries in the early stages of gut injury to clarify the mechanism through which GLP-2R signaling mitigates injury.

1.1.6 Radiation Therapy

Current estimates predict that ionizing radiation is used in treating approximately 50% of all cancer patients (11). This is principally observed in the treatment of later-stage malignancies localized within abdominopelvic organs such as the prostate, bladder, uterine corpus (64) and GIT (11,36,65). Controlled exposure to ionizing radiation damages the DNA of cancerous cells, inducing genetic abnormalities, inhibited cell replication and increased rates of apoptosis (6,11). Although the accuracy of current radiotherapy regimens has increased significantly over the past decades, stray radiation continues to interact with the healthy tissues surrounding tumors inducing toxicity in at-risk tissues such as the rapidly dividing epithelium of the GIT. This toxicity damages the DNA of enterocytes and intestinal stem cells (ISCs) within the intestinal crypts inducing secretion of pro-inflammatory cytokines and apoptosis of ISCs) (6,65). This results in both acute and chronic irritation of the bowel, impacting feeding habits, nutrient and fluid uptake, as well as a severe risk of contracting infections leading to septicemia (7,11).

A 2007 study by Torres et al. indicated that treatment with a GLP-2 analogue significantly improved GIT integrity following irradiation of murine ileum isolated by laparotomy (66). Wistar

rats treated with GLP-2 presented with decreased ulceration and significantly healthier intestinal walls due to lowered rates of scarring of afflicted tissues (66). Subsequent analyses of the chronic effects of radiation indicated that 15 weeks after irradiation, rats treated with GLP-2 no longer presented with ulceration (66). The overall length of the intestine also remained identical to sham controls (66). Lack of change in total intestinal length was likely induced by the 2-fold increase in proliferative crypt cells observed in GLP-2 treated rats compared to sham-irradiated mice (25,66). GLP-2 treatment also significantly decreased the incidence of fibrosis while ensuring protection of the circular layer of smooth muscle of the muscularis externa (66).

Recent studies applying novel analogues of GLP-2 to combat the elevated prices and the comparatively short half-lives of analogues of GLP-2 currently in clinical use have shown similar findings in protecting villus height and intestinal integrity (19,67–71). These studies further confirmed the ability of GLP-2 treatments to improve healing of intestinal injuries by decreasing expression of NF-kB, MPO, TNF-α, IL-6 and IL-1β, which are the primary mediators of inflammation and fibrosis in radiation-induced injuries to the GIT (19,68,69). Interestingly, in one of these studies conducted by Gu et al., pre- and post-treatment of mice with teduglutide and their prepared GLP-2 degradation-resistant dimer increased survival following administration of a lethal dose of radiation by 2- and 2.7-fold, respectively compared to sham-mice (19).

Beyond acting as a potent cytoprotective agent against radiation-induced injury, GLP-2 has also shown promise in protecting ISCs. Pre-treatment of teduglutide was shown to be protective of murine crypt stem cells from gamma-radiation released by a cesium 137 gamma irradiator (71). Various treatment regimens of teduglutide administered at a dose of 0.2mg/kg/day increased the quantity of surviving stem cells following irradiation by 1.3- to 1.5-fold compared to the survival rates observed in irradiated control mice (71). Recent findings have elucidated that teduglutide

treatment altered subpopulation ratios of ISCs following surgical resection of ileal tissue (72). ISCs can be characterized into two separate subpopulations dependent on the presence or absence of the leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) and the B-cell-specific Moloney murine leukemia virus insertion site 1 (Bmi1) (72,73). Administration of teduglutide in rat models increased the proportion of Lgr^{+}/Bmi^{-} crypt base columnar ISCs compared to position +4 Lgr^{-}/Bmi^{+} ISCs (72). Protection of actively replicating $Lgr5^{+}$ ISCs intercalated between Paneth crypt cells is necessary to heal radiation-induced trauma to the intestinal epithelium by increasing crypt viability (72). Evidence suggests that Lgr^{+} ISCs may be partially resistant against radiation-induced damage (73,74). This may be attributable to non-mitotically active heterogeneous $Lgr+/Bmi1^{+}$ subpopulations, but the necessary validation is lacking (73). However, this may be critical in the surgical resection of malignant tissues of the intestinal tract with adjuvant radiotherapy. Administration of a GLP-2 analogue may be capable of increasing the proportion of Lgr^{+}/Bmi^{-}-expressing ISCs following anastomosis of the GIT to protect ISC-mediated wound healing following radiation treatment. Additional studies to understand the ability of teduglutide to be radioprotective following joint application of surgical resection and irradiation of malignant tissue are required.

1.1.7 Limitations of Clinical Administration of GLP-2 in Response to Cancer-Treatment Induced Gut Injuries

An important question associated with the use of GLP-2 analogues in the attenuation of cancer treatment-induced injuries to the gut epithelium is the potential link between GLP-2 and the development of neoplasia. Due to the potent hypertrophic and anti-apoptotic roles of GLP-2 in gut

preservation, evidence suggests that increasing circulating GLP-2 may promote the proliferation of cancerous cells (75–79). A 2004 study by Thulesen et al. demonstrated that GLP-2 treatment of mice with induced colonic tumors increased the incidence of chronic small, medium and large colonic polyps compared to irradiated controls (75).

These findings were however refuted by Koehler et al. (2006) (80). DLD-1 and SW480 human colon cancer cell lines were transfected with the GLP-2R to examine the response to GLP-2 (80). Cells placed in GLP-2 media did not present with more rapid doubling times than those cultured in serum-free media (80). Follow-up experiments were conducted to induce colon cancer xenografts using the GLP-2R-transfected cells (80). Observed results contradicted previous observations as GLP-2 treatments led to significantly smaller tumors in mice injected with SW480: hGLP-2R than PBS controls (80). Similar trends were noted in the DLD-1: hGLP-2R xenografts but were however not significant (80). Polyp burden was also shown to not be dependent on GLP-2R signaling in $Apc^{Min/+}$ mice as incidence and sizes of polyps remained identical regardless of GLP-2R genotype ($GLP2R^{+/+}$, $GLP2R^{+/-}$ and $GLP2R^{-/-}$) (80).

Long-term studies of teduglutide-mediated treatment of patients with SBS did not present development of dysplasia (81–85). In two studies, individuals were treated with varying doses of teduglutide with examination of the GIT conducted by colonoscopy with adjuvant biopsy collection after 24 weeks of treatment (82,83). After 24 weeks, no individuals in either study presented any malignancies or dysplasia of the intestinal tract following GLP-2 treatments (82,83). In the study by Schwartz et al. associated with the Study of Teduglutide Effectiveness in Parenteral Nutrition-Dependent Short Bowel Syndrome Subjects (STEPS), follow-ups conducted 24-30 months post-initiation of teduglutide treatments did not find any evidence of dysplasia or malignancies of the intestinal tract (82). One individual presented with metastatic cancer of the

liver, but the origin of the malignancy was unknown (82). However, further STEPS studies indicated that the incidence of colon polyps rose to 18% after 24-36 months of treatment from the baseline value of 12% (86). It is not out of the question that the trophic effects of degradation-resistant GLP-2 agonists may promote tumor cell growth due to the lack of consistent observations in the literature. This is very much the key question that needs to be answered to fully elucidate the safety concerns associated with long-term administration of GLP-2 analogues in treating cancer-treatment-induced gut injuries.

1.1.8 Conclusion

It has been shown that exogenous administration of GLP-2 and its various available analogues significantly improve the healing of various injuries induced during treatments of malignancies, offering a potential adjuvant therapy to increase QoL of those afflicted. However, a significant question remains to be answered regarding the long-term effects of GLP-2 treatments regarding potentially substantial adverse effects in patients. The most common GLP-2 analogue, teduglutide, has been available for clinical treatment of various malabsorption diseases of the intestine, such as SBS, for approximately a decade, and the long-term effects may be clarified in the near future. Although this review has focused on the ability of exogenous GLP-2 to promote healing of cancer-treatment-induced gut injuries, current research is seeking to apply probiotics and modulation of the microbiome to increase native GLP-2 secretion in the hopes of protecting the gut. Even though many significant questions remain to be answered before we may take advantage of GLP-2R signaling in treating injuries to the GIT, recent discoveries are promising, and our knowledge of the role of GLP-2 signaling in protecting our gut should be clarified in the near future.

1.2 Enteroendocrine cells

In addition to being comprised of absorptive enterocytes, the GIT epithelium is also comprised of specialized cells responsible for the secretion of gastrointestinal hormones. These enteroendocrine cells (EECs), which comprise approximately 1% of the epithelial lining of GIT, combine to form the body's largest endocrine organ (87,88). Although some EECs demonstrate increased expression in a fasting state, the secretory function of EECs is typically stimulated by the direct detection of luminal contents such as nutrients and microbial byproducts, notably short-chain fatty acids resulting from microbial fermentation (87,89). This secretion via exocytosis of peptides/hormones from hormone-containing granules localized within EECS can interact by paracrine or endocrine interactions to regulate digestion, gut motility, enterocyte replenishment, nutrient absorption, and appetite (87,89). EECs are typically classified by the contents of their secretory granules, but recent studies have indicated that some EECs are capable of secreting hormones that have historically been used to differentiate different cell types within the literature (90,91). These include the Gastrin-positive gastric G-cells, the glucose-dependent insulinotropic polypeptide (GIP)-positive intestinal K-cells and the proglucagon-positive intestinal L-cells (87).

Enteroendocrine L-cells, which are primarily localized within the distal ileum and proximal colon, produce proglucagon peptide capable of being differentially processed by proprotein convertase 1/3 to produce several distinct hormones such as glucagon-like peptide-1 (GLP-1), glicentin, GLP-2, and oxyntomodulin (Figure 3) (21,92). Unlike pancreatic α-cells, which predominately process proglucagon into glucagon during fasting to increase blood glucose levels, L-cells are primarily active postprandially (87,89). The apical surface of L-cells contains

microvilli capable of interacting with luminal nutrients via expression of surface receptors and transporters. Notable receptors found on the apical surface of L-cells include short chain fatty acid (SCFA) receptors such as the free fatty acid receptors (FFARs) 2 and 3, medium chain fatty acid (MCFA) and long chain fatty acid (MCFA) receptors FFAR1 and 4, the secondary bile acid receptor CPBAR1 and carbohydrate-sensing receptors glucose transporter 2 (GLUT2) and sodium-glucose transporter 1 (SGLT1)(89,93,94). These surface receptors increase cytoplasmic Ca^{2+} and cyclic adenosine monophosphate (cAMP) levels to induce exocytosis of these hormones into the lamina propria via granule-membrane fusion on the cell's basal membrane (95,96).

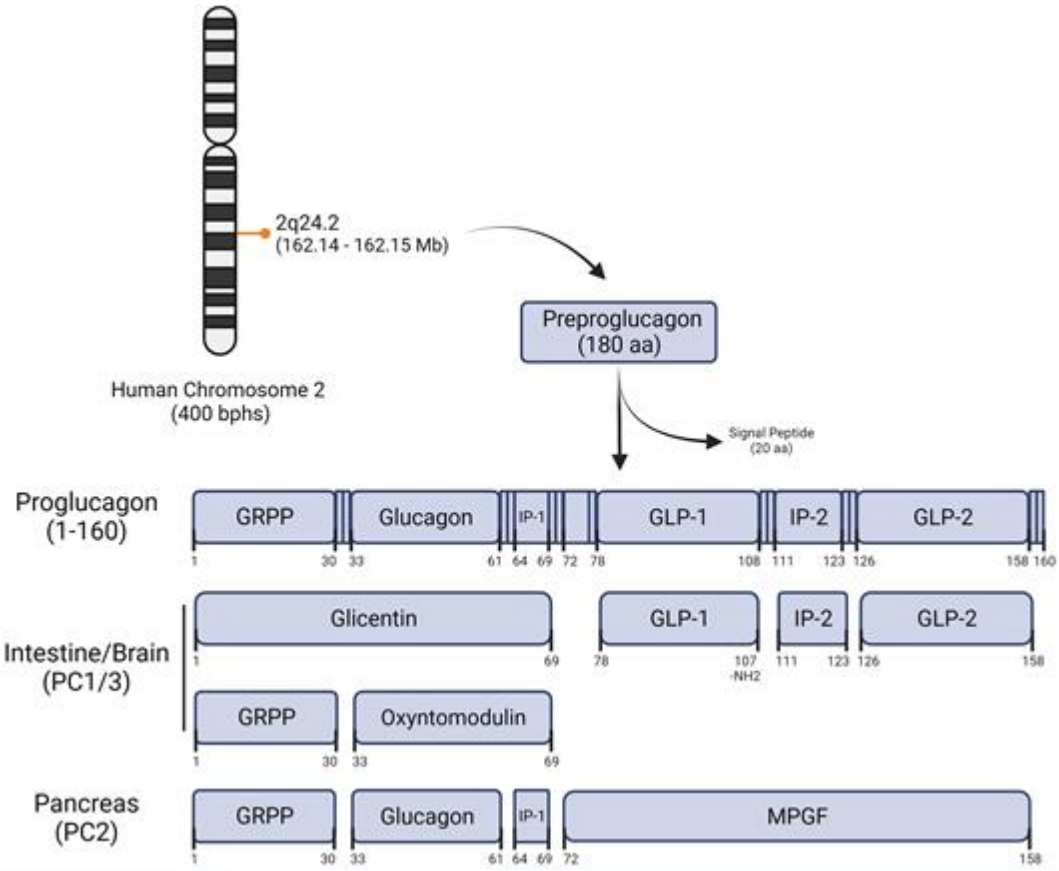

Figure 3. Gene location and post-translational modification of proglucagon.

The human proglucagon gene is located at the chromosomal locus 2q24.2 and is transcribed/translated into preproglucagon. Differential post-translational modification of proglucagon occurs with regards to organ of expression. Ideogram of human chromosome 2 presented at a resolution of 400 bands per haploid set. GRPP: Glicentin-related pancreatic peptide, IP-1: Intervening peptide-1, GLP-1: Glucagon-like peptide-1, IP-2: Intervening peptide-2, GLP-2: Glucagon-like peptide-2, PC1/3: Proprotein convertase 1, PC2: Proprotein convertase 2, MPGF: Major proglucagon fragment.

1.3 Glucagon-like peptide-2

As previously stated, GLP-2 has been shown to regulate multiple important bodily functions such as nutrient absorption, enteric blood flow, intestinal proliferation, and barrier function (20). This occurs through downstream mediators secreted following interaction with its associated receptor GLP-2R. GLP-2R, a 7-transmembrane G-protein coupled-receptor, is primarily expressed by intestinal subepithelial myofibroblast, certain EECs and enteric neuronal cells (97–99). Although expression of GLP-2R positive cells is observable throughout the intestinal tract with restricted expression in other organs such as the lungs and central nervous system, the receptor is predominantly expressed within the jejunum (23). Activation of GLP-2R in jejunal myofibroblasts induces expression and secretion of IGF-1, interacting with receptors on the basal membrane of ISCs to promote proliferation of the GI epithelium and expression of tight junction proteins for nutrient absorption purposes (39,100). Knockout of intestinal epithelium IGF-1 receptor resulted in significant loss of microvilli length and an inability for exogenous administration of GLP-2R agonist to stimulate proliferation of the intestinal epithelium (24,100). Consequently, the intestinotrophic effects of GLP-2R activation are dependent on IGF-1/IGF-1R signaling in ISCs, and loss of ISC due to injury would result in loss of proliferation and differentiation of intestinal epithelial cells (24,72,100).

1.4 Radiation enteropathy

As previously outlined, radiation therapy as part of cancer treatment may often cause both acute and chronic issues in patients due to damage to the GIT, known as radiation enteropathy or

intestinal radiation toxicity. Irradiation of the intestinal tissue leads to gut dysbiosis and cell death of the enterocytes and other cells of the epithelium, such as ISCs and enteroendocrine cells (11,101,102). This cell loss is typically further associated with inflammatory cell infiltration, remodeling of the tissue, loss of vascularization, fibrosis of the intestinal wall and enteric neuron damage, which may induce permanent modification of bowel habits and long-term issues such as nutrient malabsorption (11,103). However, research has demonstrated possible benefits to exposure to low-dose radiation treatment. Exposure to low-dose radiation has shown to have certain beneficial effects such as immune modulation and increased anti-inflammatory effects, with some studies showing no significant loss of intestinal crypt cells (104–106). This may be of benefit as a primer to abdominal radiation exposure where increased inflammation may lead to long term complications in patients. Certain studies have demonstrated increased expression of enteroendocrine hormones in certain intestinal injury models as a potential compensatory mechanism (107,108). The effects of intestinal radiation toxicity on enteroendocrine hormone secretion, notably GLP-2, are poorly understood and filling in the gap in the literature that may be beneficial to understanding and treating the disease state.

1.5 Rational and Hypothesis

As GLP-2 is a potent intestinotrophic hormone with strong evidence indicating cytoprotective properties against injuries to the GIT, there is a potential link between impaired endogenous GLP-2 secretion and GIT injury pathogenesis. We hypothesize that ionizing radiation will enhance GLP-2 production from the L-cell. We predict that radiation dosage will determine the degree of GLP-2 levels in both animals and L-cells.

2 Investigation of the effect of acute radiation on enteroendocrine L-cell health and function

2.1 Materials and Methods

2.1.1 Animals

Male and female C57BL/6 mice (7-12 weeks old) were purchased from Charles River Laboratories (St. Constant, QC, CA). Animals were housed in pairs in standard cages on a 12-hour light/dark cycle in the Paul Field Animal Care Facility at Laurentian University. Experimental protocols were conducted as outlined by the animal use protocol approved by the Laurentian University Animal Care Committee, adhering to guidelines outlined by the Canadian Council for Animal Care.

2.1.2 Animal Irradiation Protocol

Animals were randomly assigned to one of five treatment groups upon arrival in the animal care facility. Upon reaching 16 weeks of age, animals were transported from the facility to the Northern Ontario School of Medicine (NOSM) University in transfer cages equipped with HEPA filters and transported in environmentally controlled conditions. The animals were placed in pie cages and irradiated by X-ray radiation via an X-RAD 320 irradiator before being rehoused in the animal care facility. The animals were euthanized via anesthesia by isoflurane followed by cervical dislocation 48 hours post-irradiation, and blood was collected via cardiac puncture. Roughly 1cm sections of duodenum, jejunum, ileum and proximal colon were collected via snap-freezing in liquid nitrogen before being stored at -80°C until analysis. Sections of the ileum were

collected in paraformaldehyde (PFA) for fixation before being embedded in paraffin blocks by the University Health Network.

2.1.3 Fluorescent Immunohistochemical Quantification of L-cells

Paraffin-embedded ileal tissue blocks were sectioned into 4 µm thick ribbons via microtome and allowed to float in a 37°C water bath. The ribbons were then sectioned into individual slices and collected onto positively-charged slides before being dried in a 37°C oven for 2-3 minutes. Slides were deparaffinized via Xylene/ethanol and washed with serially decreasing dilutions of ethanol before being rehydrated in 1X TBS for 15 minutes. Samples were blocked with a 5% v/v Normal Goat Serum blocking solution, obtained from Abcam (ab2532166, Cambridge, MA, USA), for 20 minutes in a humidity chamber. Blocking serum was removed from the slide, and a 1:250 Mouse-anti-GLP-1 antibody from Abcam (ab26278, Cambridge, MA, USA) was introduced and left to incubate in a humidity chamber at 4°C for 16-20 hours. The primary antibody had previously been validated in-lab for use in murine ileal tissue and no primary antibody controls were conducted to evaluate for non-specific binding or false positives (109). Following the incubation, the samples were rinsed and incubated on a shaker at 80 rpm for 5 minutes with TBS three times before being incubated with a 1:150 Goat-anti-mouse 488 secondary antibody (ab150113, Cambridge, MA, USA) on a plate shaker for 45 minutes. Samples were washed with TBS as previously indicated, mounted with DAPI mounting media (ab104139, Cambridge, MA, USA), and covered with a cover slip sealed using clear nail polish. Slides were visualized via Zeiss fluorescence microscope and Axiovision Rel 4.5 software, and L-cell and villi counts were taken by manual counting. Surface area of the absorptive surface of the ileal sections was determined via the Axiovision Rel 4.5 software.

2.1.4 Gene Expression by Real-Time PCR

Snap-frozen sections of ileal tissue were homogenized via Tissue-Tearor in Lysis Buffer, with β-mercaptoethanol, and RNA was purified and extracted as indicated in the illustra RNAspin Mini RNA Isolation Kit obtained from GE Healthcare Life Sciences, now Cytiva (25-0500-71, Marlborough, MA, USA). cDNA was synthesized from the extracted RNA via SensiFAST cDNA Synthesis Kit obtained from Meridian Bioscience Inc. (BIO-65053, Cincinnati, OH, USA) and stored at -20°C until analysis. PCR primers were designed via the NCBI's Primer-BLAST tool and obtained from Integrated DNA Technologies (Coralville, IA, USA). The primer set and their appropriate annealing temperatures were validated in lab via staining of the gel electrophoresis of gradient PCR products using SYBR Safe DNA Gel Stain from Invitrogen (S33102, Waltham, MA, USA). Genes of interest were quantified via real-time PCR using SensiFAST SYBR No-ROX Kit obtained from Meridian Bioscience (BIO-98020, Cincinnati, OH, USA). Gene expression was quantified via absolute quantification by comparing the samples against a standard curve prepared by diluting pure PCR product of the gene of interest.

Table 1. Primer sets used for real-time PCR analysis

RPL13α	5'- GAAGCAGATCTTGAGGTTACGGA-3' (forward) 5'- AGGCATGAGGCAAACAGTCT-3' (reverse)
GCG	5'-TTGAGAGGCATGCTGAAGGG-3' (forward) 5'-TCTTCTGGGAAGTCTCGCCT-3' (reverse)

2.1.5 Quantification of GLP-2 in Murine Samples

Snap-frozen sections of ileal tissue were homogenized via Tissue-Tearor in a 2X dilution of 10X Cell Lysis Buffer (obtained from Cell Signaling Technology, #9803, Danvers, MA, USA) with

cOmplete™ EDTA-free Protease Inhibitor Cocktail (obtained from Roche, 04693132001, Basel, Switzerland). Homogenized samples were centrifuged at 14,000 x g at 4°C for 10 minutes, and the supernatant was collected for analysis. Total GLP-2 content within the collected plasma and protein extraction was measured via Mouse GLP-2 enzyme-linked immunosorbent assay (ELISA) from Crystal Chem (obtained from Catalog# 81514, Elk Grove Village, IL, USA). Ileal GLP-2 content was normalized to total protein measured by Bradford Reagent protein assay (obtained from MilliporeSigma, St. Louis, MO, USA).

2.1.6 Cell Culture

The murine GLUTag cell line, obtained from Dr. Drucker of the Lunenfeld-Tanenbaum Research Institute, were chosen for this study as a model of the murine enteroendocrine L-cell secretagogue. The GLUTag cells were maintained in 5mM glucose containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (P/S) (obtained from MilliporeSigma, St. Louis, MO, USA) and were incubated in a humidified incubator at 5% CO_2. Cells were passaged once 75-85% plate confluency was achieved. All experiments were conducted after 10-25 passages. GLUTag cells were seeded in 6-well plates at a density of 1,000,000 cells per well 12 hours prior to irradiation using low glucose DMEM supplemented with 10% FBS and 1% P/S. The cells were irradiated by X-ray via the X-RAD 320 Biological Irradiator located at NOSM University, with a sham plate serving as a control. The irradiated cells were allowed to proliferate for 48 hours before experiments were conducted.

2.1.7 *In-vitro* Secretion Assays

After 48 hours of proliferation in a humidified incubator at 5% CO2, the cells were washed. The growth media was replaced with secretion media (Low glucose DMEM with 0.5% FBS and 1% P/S) containing either no additional treatment or 10 µM forskolin. Following 2 hours of incubation at 37°C and 5% CO_2, secretion media was collected and acidified using trifluoroacetic acid (TFA) at a final concentration of 0.1% and stored at -80°C until analysis. Total GLP-2 content within the secretion media was quantified using a multi-species GLP-2 ELISA from ABCam (ab222863, Cambridge, MA, USA) as indicated within manufacturer-provided protocols. Total GLP-1 content within the secretion media was quantified using a multi-species GLP-1 ELISA from Invitrogen (BMS2194, Waltham, MA, USA) as indicated within manufacturer-provided protocols.

2.1.8 Resazurin Reduction Assay

A 10 mg/mL stock solution of Resazurin (Biotium, Inc. Fermont, CA, USA) was diluted to 0.15 mg/mL using secretion media. Following 48 hours of proliferation in a humidified incubator at 5% CO_2, the cells were washed, and the growth media was replaced with 1:5 Resazurin to treatment secretion media. Treatment media used to create the 1:5 Resazurin to treatment mixture given included no additional treatment, 10 µM forskolin or a 0.3% H_2O_2 positive control. Cells were allowed to incubate at 37°C, 5% CO_2 following media replacement, and fluorescence was measured at the 1- and 2-hour time points following treatment (Ex/Em 530nm/590nm). Correction for background was conducted by subtracting the positive controls' fluorescence values from the treatment wells' measured fluorescence values.

2.1.9 Statistical Analysis

All of the data presented is expressed as the mean ± SEM. Studies with multiple doses of the same treatment were analyzed by one-way ANOVA, followed by a Bonferroni post hoc test, where applicable. Immunohistological studies were analyzed by non-parametric Kruskal-Wallis tests. Studies with two independent variables were analyzed by two-way ANOVA, followed by a Bonferroni post hoc test, where applicable. P value of <0.05 was considered to be statistically significant.

2.2 Results

2.2.1 Acute irradiation did not impact short-term L-cell distribution within mouse ileum

To examine if exposure to acute irradiation impacted short-term L-cell distribution within the ileum, ileal tissue collected 48 hours after irradiation was fixed, sectioned, and subjected to immunohistological staining to visualize L-cells (Figure 4A). Manual counting of GLP-1-stained cells did not indicate any significant change in L-cell counts between irradiation groups 48 hours following irradiation protocol (Figure 4B). Normalization of L-cell counts to the surface area of the absorptive surface of the ileal mucosa similarly did not demonstrate any significant difference in relative L-cell counts between treatment groups (Figure 4C). These results indicate that acute irradiation did not impact L-cell counts within the ileum in the short-term following treatment.

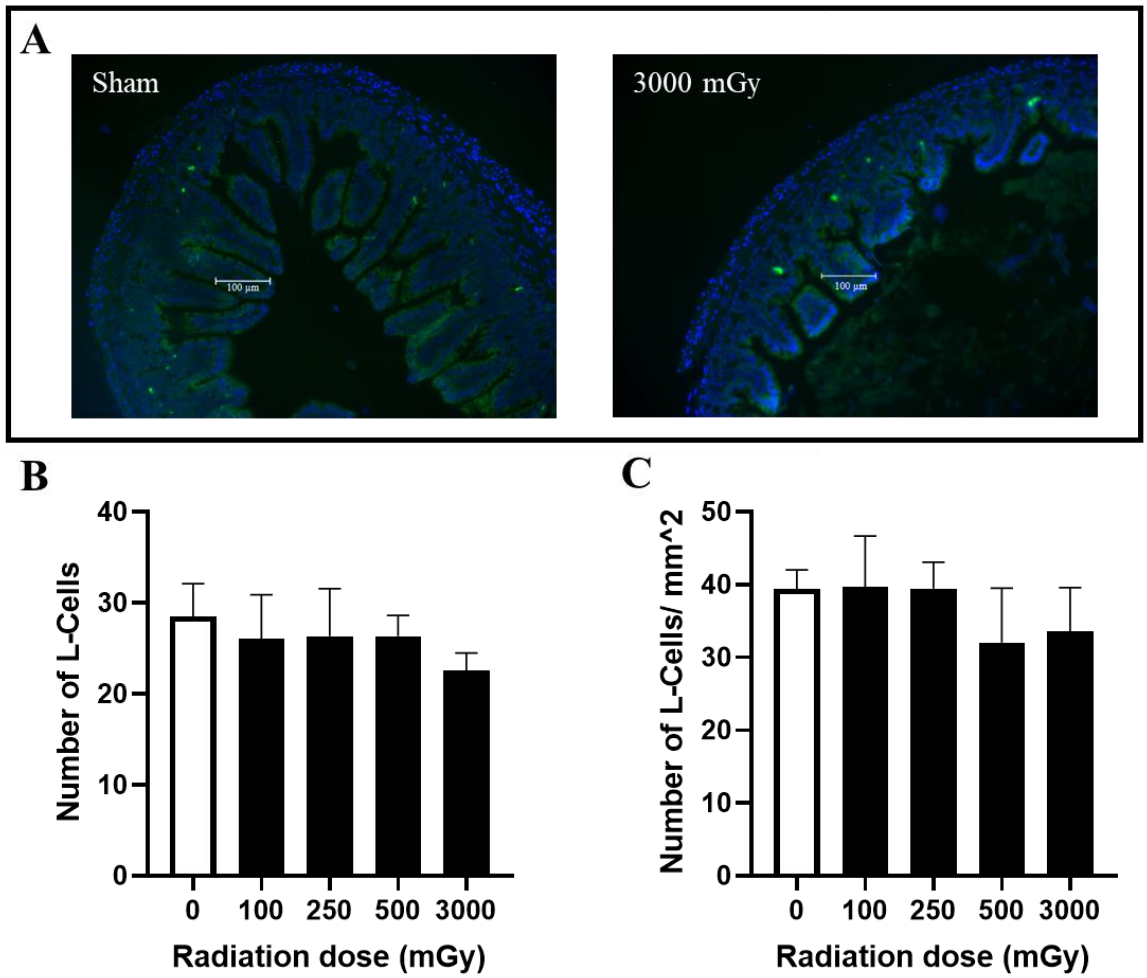

Figure 4. Acute irradiation does not impact short-term L-cell distribution within the ileum of mice.

L-cell distribution was visualized via immunohistochemistry in sections of mouse ileum collected 48 hours post-acute irradiation. Tissue samples were stained with anti-GLP-1 antibodies (green) and DAPI mounting media (blue) as shown in stained images of Sham and 3000 mGy samples (A). Green-fluorescent cells were manually counted (B) and normalized to the surface area of intestinal epithelium (C). n=6. Data is shown as mean ± SEM and was analyzed using a non-parametric Kruskal-Wallis test.

2.2.2 Acute irradiation increased GLP-2 levels in mice

In order to examine the short-term effect of acute irradiation on GLP-2 production in mice, RT-PCR analysis was used to quantify proglucagon RNA within tissue lysate collected 48 hours post-acute irradiation. The results demonstrated a trend of increased proglucagon expression within the tissue following irradiation but were not significant due to high variability within groups (Figure 5).

Following proglucagon gene expression analyses, GLP-2 levels were measured by ELISA to investigate the effects of acute irradiation on GLP-2 production and secretion by analyzing collected plasma and ileal tissue. The results demonstrated that acute irradiation significantly increased circulating GLP-2 levels compared to sham treatments ($F(2,20)=4.486$, $p<0.05$) (Figure 6A). Post-hoc analyses revealed that mice irradiated with an acute dose of 500 mGy of X-ray irradiation had significantly higher levels of total GLP-2 in circulation compared to sham models (1.0685±0.02025 ng/mL for 500 mGy treated mice compared to 0.8151±0.09042 ng/mL for sham mice, $p<0.05$, Fig 6A). Similarly to the trend observed in gene expression assays, GLP-2 levels within tissue lysate demonstrated high variance in the 500 mGy treatment group, but significant change was observed in tissue GLP-2 levels as a result of irradiation (Figure 6B). These results indicate that acute exposure to ionizing radiation increases baseline circulating GLP-2 levels 48 hours following treatment but that production of GLP-2 within the ileum was not a driving force of this change.

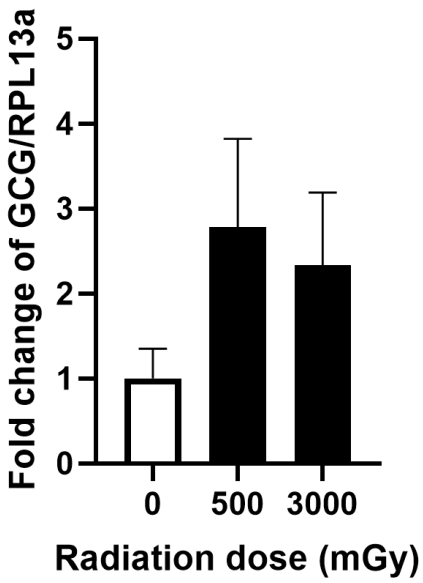

Figure 5. Acute irradiation does not significantly impact short-term intestinal proglucagon expression.

Proglucagon (GCG) gene expression was measured via RT-PCR in protein extracted from murine ileum collected 48 hours post-acute irradiation. GCG expression was normalized to RPL13α expression in corresponding samples. Sham cohort n=8, 500 mGy cohort n=6, 3000 mGy cohort, n=8. Data is shown as mean ± SEM and was analyzed using a one-way ANOVA with Bonferroni post hoc test.

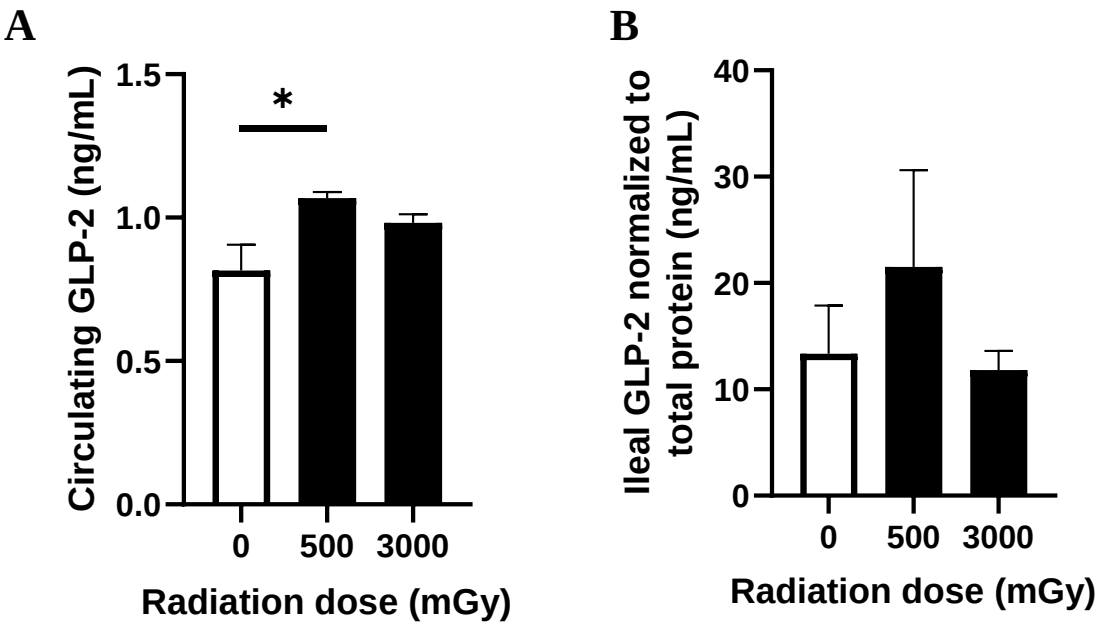

Figure 6. Low-dose radiation increases circulating and intestinal GLP-2 levels in male mice.

GLP-2 concentration was measured via GLP-2 ELISA in mouse (A) plasma and (B) ileum collected 48 hours post-acute irradiation. GLP-2 concentration measured from ileal tissue lysate was normalized to total protein. Sham cohort n=8, 500 mGy cohort n=6, 3000 mGy cohort n=9. Data is shown as mean ± SEM and was analyzed using a one-way ANOVA with Bonferroni post hoc test. *, $p<0.05$ vs Sham.

2.2.3 L-cell secretions remained unchanged following ionizing radiation exposure in GLUTag L-cell model

To investigate the effects of acute irradiation on L-cell secretion, the GLUTag L-cell models were irradiated, and GLP-1/GLP-2 levels were measured via ELISAs following 2-hour secretion experiments both in the presence and absence of the GLUTag secretagogue forskolin. Irradiation of GLUTag cells did not induce a significant change in GLP-1 secretion by GLUTag cells in any individual treatment group compared to sham, but a bell-shaped trend was observed in regards to the effect of acute irradiation on GLP-1 secretion ($F(4,50)=1.992$, $p=0.11$ for radiation dose) (Figure 7A). Two-way ANOVA analyses of the GLP-1 secretion results did not demonstrate any significant interaction between radiation and forskolin ($F(4,50)=1.222$, $p=0.3135$ for interaction), whilst the effect of forskolin supplementation remained significant ($F(1,50)=101.5$, $p<0.0001$ for forskolin) (Figure 7A). GLP-2 secretion assays demonstrated a similar non-significant but more pronounced bell-shaped trend in relation to radiation dose ($F(4,45)=2.436$, $p=0.0608$ for radiation dose) (Figure 7B). The stimulatory function of forskolin on GLP-2 secretion was not significantly impacted by radiation dose as no interaction was observed between the two factors ($F(4,45)=1.052$, $p=0.3910$ for interaction, $F(1,45)=39.15$, $p<0.0001$ for forskolin) (Figure 7B). These results indicate that exposure to ionizing radiation did not suppress the secretion of enteroendocrine hormones from GLUTag L-cell models, nor the stimulatory function of its secretagogue forskolin.

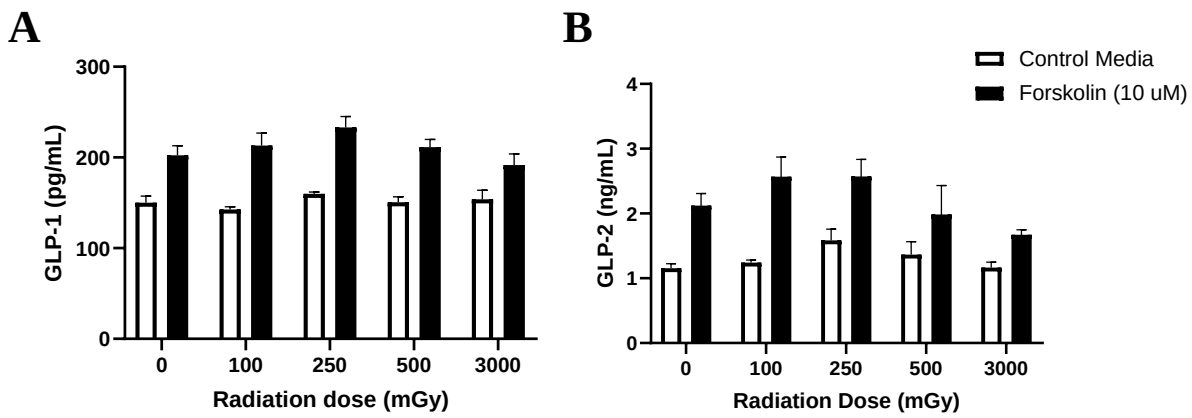

Figure 7. L-cells have moderately enhanced secretion 48 hours post-acute irradiation.

GLUTag secretions were measured 48 hours post-acute irradiation via 2-hour secretion assays, with and without stimulation via forskolin. Total GLP-1 (A) and total GLP-2 (B) levels were measured via GLP-1 and GLP-2 ELISAs, respectively. n=6 for GLP-1 assay, n=6 for all GLP-2 control media, Sham Forskolin and 100 mGy Forskolin groups, n=5 for 250 mGy Forskolin and 500 mGy Forskolin groups, n=3 for 3000 mGy Forskolin group. Data is shown as mean ± SEM and was analyzed using a two-way ANOVA with Bonferroni post hoc test.

2.2.4 Mitochondrial activity was increased 48 hours post-acute irradiation in GLUTag L-cell model

As acute ionizing radiation is known to impair mitochondrial function by damaging both mitochondrial DNA and membranes, a resazurin reduction assay was conducted to assess mitochondrial metabolic activity of GLUTag cells 48 hours following irradiation. Irradiation of GLUTag cells increased total resazurin reduction (F (4,25)=3.389, p=0.0240) with significant increases for the 250 mGy and 3000 mGy treatment groups (14.1±2.7% increase for the 250 mGy group, $p<0.01$, and 11.3±2.4% increase for the 3000 mGy group compared to the sham group, $p<0.05$) (Figure 8). This outcome indicates that ionizing radiation increased total mitochondrial activity of GLUTag cultures.

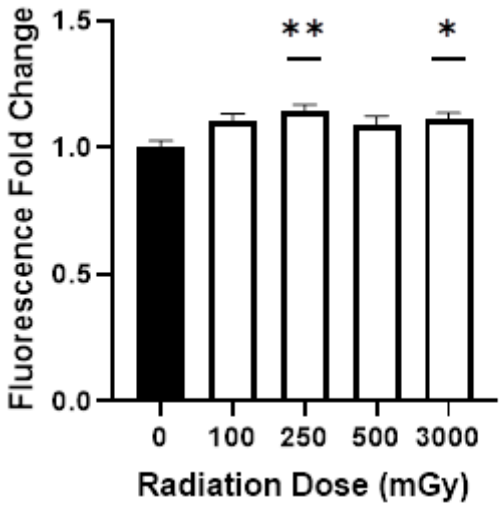

Figure 8. Radiation increases L-cell Mitochondrial activity.

Mitochondrial activity of GLUTag cells was measured via Resazurin Reduction assay. GLUTag cells were incubated with Resazurin 48 hours post-irradiation and incubated for an hour. n=6 Data is shown as mean ± SEM and was analyzed using a one-way ANOVA with Bonferroni post hoc test. .*, $p<0.05$, **, $p<0.01$ vs Sham.

2.3 Discussion

Firstly, visualization of L-cells within murine ileal tissue did not reveal any significant change in relative counts throughout the intestinal epithelium between irradiation treatments. As total regeneration of the ileal epithelium in mice typically occurs after approximately 3-5 days (110,111), it is possible that the full effects of the death of proliferating crypt epithelial cells are not observable in histological assays on ileal tissue collected 48 hours following irradiation. It should be noted that initials signs of radiation-induced apoptosis of the crypt cells typically presents within 3-6 hours following treatment as a result of the radiosensitivity of these cells (111,112). However, this rapid turnover of crypt cells as a result of apoptosis is typically observed for treatment doses of 1 Gy and above (111), and it is thus possible that minimal observation of apoptosis would have been noted in animals treated with low-dose radiation comparatively to the 3 Gy treatment. Quantifying apoptosis markers such as cleaved caspase 3 3-6 hours following irradiation may have provided better insight into the health of the intestinal wall. Treatment regimens may have also not been suitable for modelling of long-term effects of radiation exposure on L-cell health and distribution due to the acute whole-body nature of the irradiation. Typical treatment of abdominal and pelvic cancers by radiotherapy employs multiple lower-dose fractions of irradiation to avoid damaging healthy cells, limiting tissue and organ level damage that could induce severe side-effects (6,11). These fractionated doses of low-doses radiation may have demonstrated increased L-cell distribution or activity via direct or indirect methods such as accelerated immune senescence, minimizing damage to the intestinal epithelium and crypts.

To explore the effects of ionizing radiation exposure on L-cell endocrine function in mice, GCG expression and GLP-2 levels were measured 48 hours post-irradiation. No change was observed in GCG gene expression or protein expression within ileal tissue. However, exposure to 500 mGy of ionizing radiation increased circulatory GLP-2 levels compared to the sham treatment. This suggests that the enhancement of circulatory GLP-2 is not a result of enhanced glucagon gene expression but rather a result of differential post-translational processing of the peptide. GLP-2 is first produced as an inactive proprotein in L-cells and must be cleaved by PC1/3 before secretion (20,107). Enhanced expression of PC1/3 has been observed following injury to the intestinal walls, namely in colitis models (108). This indicates a potential link to increased circulatory GLP-2 as increased PC1/3 expression has been linked to increased processing of GLPs in L-cells (22). It should be noted that the ELISA used on these tissue samples may have also detected the uncleaved Proglucagon peptide. Future work should explore the impact of radiation exposure on the expression and activity of PC1/3 in L-cells.

Alternatively, the amplified levels of circulating GLP-2 may also have resulted from reduced GLP-2 turnover by the peptidase DPP-IV. DPP-IV is widely expressed in animals, including cells of the immune system (113). As low-dose irradiation has been shown to reduce immune cell numbers in mice (105), it is possible this reduction led to the increased GLP-2 found in our animals. Future studies could resolve this by assaying DPP-IV activity in the plasma.

Assay timing may have also been altered to better understand the effects of radiation exposure during the acute injury phase. As previously mentioned, peak apoptosis in intestinal cells following radiation exposure occurs in the first 3-6 hours post irradiation (111,112). Examination of total GLP-2 levels in circulation within this timespan may have demonstrated significant changes that may have shown more insight as to the L-cells response to gut injury. Furthermore,

GLP-2 levels may have been measured after a longer timespan to examine the impact of radiation exposure on enteroendocrine secretion to better understand the impact of treatment following injury recovery.

Finally, it should be noted that the plasma analysis of GLP-2 was carried out in non-fasted mice. This would have led to variability in GLP-2 levels, as food consumption is a potent secretion signal for this hormone (20). Future studies will ensure that all animals were under the same fed or fasted conditions.

To explore the direct effects of radiation on L-cells, GLUTag cells were treated with the same doses used in the animal study for the same duration. GLUTag secretion was then examined over a 2-hour timeframe in both cAMP-stimulated (Forskolin) and non-stimulated conditions. A trend towards a bell-shaped dose-response curve was observed in both secreted GLP-1 and GLP-2 levels, with peak response being observed at 500 mGy. Although this finding did not reach significance, it does suggest that a potential dose-response relationship may be present in regards to L-cell response to ionizing radiation exposure, but that this trend is eventually lost. Due to the large gap between the peak-response dose of 500 mGy and the uppermost dose of 3000 mGy, a trend may have been observed had intermediary doses been studied for their impact on GLUTag secretion. It should be noted that no statistically significant loss in cell viability was observed following radiation treatment with 3000 mGy. Unexpectedly, irradiation of GLUTag cells led to increased mitochondrial activity, as observed via Resazurin assay. This contradiction could be explained by the mechanism of action of the resazurin assay, which measures the activity of the electron transport chain via reduction of resazurin to resofurine. Some studies have demonstrated that exposure to low-dose ionizing radiation has been linked to increased mitochondrial fusion and increased expression of specific electron transport chain proteins in hippocampal neurons

(114). Treatment of these neurons by low-dose radiation similarly demonstrated increased response in a similar mitochondrial activity-based cell viability assay (114). As these hippocampal neurons are post-mitotic and can no longer undergo mitosis, this would be indicative of the fact that radiation treatment did not increase cell viability but rather mitochondrial activity (114). However, other studies have demonstrated that low-dose radiation can induce signaling cell pathways associated with proliferation and survival through both hormesis and adaptive responses in certain cell lines (104). Unlike the previously outlined study, hormetic responses induced by low-dose radiation are a result of epigenetic modulation that may temporarily increase mitochondrial respiration due to the increased rate of proliferation (104). To examine whether the increased reduction of resazurin observed was a result of increased mitochondrial activity or cell proliferation, a non-metabolic cell viability assay such as neutral red uptake or Annexin V/Propidium Iodide apoptosis assays can be used.

Overall, it should be noted that further *in-vivo* work would be beneficial to the understanding of the impact of radiation therapy on enteroendocrine function. Use of both extended timelines and of fractionated treatment may help elucidate the long-term effects of radiation therapy observed in patients undergoing radiation therapy. Use of radiation in cancer treatment is typically applied in multiple fractionated doses to allow repair of surrounding healthy tissue and application of a similar dose regimen may be more representative of radiation therapy. This future study should also capitalize on the use of focused abdominopelvic radiation. Animals were irradiated whilst being contained in pie cages that did not limit radiation exposure to the abdominal area which may have caused radiation-induced injuries and changes to other organ systems that may be detrimental to the survival of the animal and that may have had unaccounted for impacts on enteroendocrine function and differentiation. Animals in this study were limited to male mice

which is not representative of the true impact of radiation therapy on organ-system responses that may have impacted enteroendocrine response and use of both male and female animals should be applied in future studies to account for potential sex-based physiological responses.

This future *in-vivo* study design would further benefit from use of other assays to expand both on the information gleaned into during this study and to expand on certain aspects that were not addressed. As previously mentioned, use of PC1/3 and DPP-IV ELISAs may explain the changes in circulatory GLP-2 that was not observed during gene expression assays. However, in a longer-term study, stem cell differentiation may be examined to assess any changes in enteroendocrine cell populations. This may be examined by measuring transcription factors associated with enteroendocrine cell differentiation such as chromogranin A, neurogenin3 and neurod1 (115). Use of these transcription factors in immunohistology may have also further demonstrated any potential change in both enteroendocrine cell counts and distribution within the intestinal tract. Additionally, markers of immune and inflammatory response or cell death such as interleukin-1, interleukin-6 and cleaved caspase 3 can be measured to demonstrate any potential increased cell death following radiation exposure, either via quantitative ELISA or by visualization by immunohistochemistry (116).

Finally, as certain luminal nutrients such as SCFAs stimulate L-cell secretion, an *in-vivo* study examining the impact of adjuvant L-cell secretagogues during radiation treatment may be used to further our understanding of the role of L-cells during the acute phase of injury. This may be accomplished by measuring daily changes in bodyweight both during and after treatment as well as by measuring any potential changes in crypt/villi measurements throughout the intestinal tract, namely in the duodenum where GLP-2R is most highly expressed.

2.4 Conclusion

In conclusion, low-dose radiation treatment seems to increase GLP-2 secretion 48 hours post-treatment. GLUTag cells demonstrated a similar trend of increased secretion following treatment, as well as increased cell viability via increased mitochondrial activity. This study indicates the potential for future work exploring the effects of ionizing radiation treatment on the potential adaptive response of enteroendocrine cells to better understand the role of GLP-2 in radiation enteropathy and other gut injuries.

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