ROLE OF THE INTESTINAL EPITHELIAL INSULIN-LIKE GROWTH FACTOR-1 RECEPTOR IN GLUCAGON-LIKE PEPTIDE-2-MEDIATED SMALL INTESTINAL GROWTH RESPONSES

By

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Role of the intestinal epithelial insulin-like growth factor-1 receptor in glucagon-like peptide-2-mediated small intestinal growth responses

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ABSTRACT

The gut hormone glucagon-like peptide-2 (GLP-2) has numerous beneficial effects on the intestinal epithelium, including increased mucosal growth and proliferation. GLP-2 is also necessary for the adaptive intestinal re-growth that occurs upon re-feeding after fasting. Although insulin-like growth factor (IGF)-1 and the IGF-1 receptor are known to be required for GLP-2-induced crypt-cell proliferation, the precise cellular localization of the IGF-1 receptor through which the intestinotrophic actions of GLP-2 are mediated remains unknown. I hypothesized that small intestinal growth responses to GLP-2 occur through an intestinal epithelial IGF-1 receptor-dependent pathway, through the use of an inducible, intestinal epithelial-specific IGF-1 receptor knockout (IE-igf1rKO) mouse. Intestinal growth and proliferative responses were examined in IE-igf1rKO and control mice following treatment with GLP-2, as well as in animals that were fasted and re-fed to induce GLP-2-dependent adaptation. In Chapter 3, it was demonstrated that IE-igf1rKO mice, as compared to control littermates, had normal small intestinal weight, morphometric parameters, proliferative index and differentiated epithelial cell lineage distribution. Administration of GLP-2 for 30 minutes increased nuclear translocation of β-catenin in non-Paneth crypt-cells, and stimulated the

crypt-cell proliferative marker c-Myc 90 minutes following GLP-2 treatment, in control littermates but not in IE-igf1rKO mice. In Chapter 4, adaptive re-growth was studied by fasting IE-igf1rKO and control animals for 24 hours, or by fasting and then re-feeding mice for 24 hours. Small intestinal weight, crypt depth, villus height and crypt-cell proliferation were decreased in both control and IE-igf1rKO mice after 24 hour fasting. While re-feeding in control mice restored all of these parameters, re-fed IE-igf1rKO mice displayed abrogated adaptive re-growth of the crypt-villus axis as well as reduced crypt-cell proliferation. In Chapter 5, control mice responded to chronic GLP-2 with increased small intestinal weight, mucosal cross-sectional area, crypt depth, villus height and crypt-cell proliferation. However, the GLP-2-induced increase in crypt-cell proliferation was absent in IE-igf1rKO mice, in association with impaired growth of the crypt-villus axis. Taken together, these results indicate that the proliferative responses of the intestinal epithelium to exogenous GLP-2 administration and during conditions of GLP-2-dependent adaptive re-growth are dependent on the intestinal epithelial IGF-1 receptor.

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When asked what was one thing that he would change regarding his humanitarianism and accomplishments, Canadian politician Steven Lewis stated that he would have directed more efforts towards the environment because without it, none of the most important global initiatives even matter. From this idea, I would like to point out that mindfulness of sustainability and the environment should be highly revered because without the world that we live in, advances in science and humanity will be worthless. We all must adapt to this changing environment by making sustainability a priority.

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LIST OF ABBREVIATIONS

α-SMA	α-Smooth muscle actin
ALS	Acid-labile subunit
ANOVA	analysis of variance
APC	Adenopolyposis Coli
Apo	Apolipoprotein
AUC	Area under the curve
BAD	bcl2-associated death promoter protein
BMP	Bone morphogenetic protein
bp	base pair
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CK1	Casein-kinase-1
CNS	Central nervous system
CR	Cysteine-rich
Cre	Cyclization recombination
CREB	cAMP response element-binding
C(t)	threshold cycle
cWnt	Canonical Wnt
DAPI	4'-6-diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
DPPIV	Dipeptidyl peptidase IV
DSH	Dishevelled
e	exon boudary
EGF	Epidermal growth factor
ER	Estrogen receptor
eNOS	Endothelial nitric oxide synthase
EPH	Chicken ephrin type
ERK	Extracellular Signal-Regulated Kinase
FGF	Fibroblast growth factor
FRZ	Frizzled
GH	Growth hormone
GI	Gastrointestinal
GLP-2	Glucagon-like peptide-2
GLP-2R	Glucagon-like peptide-2 receptor
GPC	G-protein coupled
GPR49	G-Protein-coupled Receptor 49
GSK3	Glycogen synthase kinase 3
H&E	Hematoxylin & eosin
HB	Heparin binding
HGF	Hepatocyte growth factor
HES	mammalian hairy and enhancer of split homolog
IBD	Inflammatory bowel disease
IE	Intestinal epithelial
IFN	Interferon
IGF	Insulin-like growth factor

IGF-1R	Insulin-like growth factor-1 receptor
IGF-2	Insulin-like growth factor-2
IGF-2R	Insulin-like growth factor-2 receptor
IGFBP	Insulin-like growth factor binding protein
IHC	Immunohistochemistry
IL	Interleukin
IP	Intervening peptide
IR	Insulin receptor
IRS	Insulin receptor substrate
ISEMF	Intestinal subepithelial myofibroblast
KGF	Keratinocyte growth factor
KO	Knockout
L1	Leucine-rich repeat domain 1
L2	Leucine-rich repeat domain 2
LCM	Laser capture microdissection
LGR5	Leucine-rich repeat-containing G-protein-coupled receptor 5
LI	Large intestine
LR^3IGF-1	Long-$Arginine^3$-insulin-like growth factor-1
LRP	Lipoprotein receptor-related protein
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
mRNA	Messenger ribonucleic acid
miRNA	micro RNA
Msi-1	Musashi-1
Myc	Myelocytomatosis oncogene
P	Phosphorylated
PBS	Phosphate-buffered saline
PC	Prohormone convertase
PCR	Polymerase chain reaction
PDK-1	3-Phosphoinositide dependent protein kinase-1
PI3K	Phosphoinositide-3-kinase
$PI(3,4,5)P_3$	Phosphatidylinositol-3,4,5-phosphate
PKA	Protein kinase A
PTEN	Phosphatase and tensin homolog
qRT-PCR	Quantitative real time polymerase chain reaction
Rspo	Roof-plate spondin
R	Receptor
SBS	Short bowel syndrome
SI	Small intestine
Sox9	Sry-type high-mobility-group box 9
TBST	Tris-buffered saline with Tween
TCF	T-cell factor
TNFα	Tumor necrosis factor α
TPN	Total parenteral nutrition
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
VEGF	Vascular endothelial cell growth factor
VIP	Vasoactive intestinal polypeptide

Wnt	Wg (wingless) + Int1 (chromosomal integration site of mouse mammary tumor virus on mouse chromosome 15)
Z/EG	lacZ/EGFP

Methodological Abbreviations

~	approximately
%	percent
º C	degrees Celsius
g	gram
hr	hour(s)
l	litres
m	metre
M	molar (moles/l)
min	minute(s)
mol	moles
p	statistical p-value
s	second(s)
SEM	standard error of the mean
wk	week

Prefixes

k	kilo- (x 10^{3})
c	centi- (x 10^{-2})
m	milli- (x 10^{-3})
µ	micro- (x 10^{-6})
n	nano- (x 10^{-9})
p	pico- (x 10^{-12})

Nucleic Acid Abbreviations

A	adenosine or deoxyadenosine
C	cytidine or deoxycytidine
G	guanosine or deoxyguanosine
T	thymidine or deoxythymidine

Amino Acid Abbreviations

Ala	A	alanine
Arg	R	arginine
Asn	N	asparagine
Asp	D	aspartic acid
Cys	C	cysteine
Gln	Q	glutamine
Glu	E	glutamic acid

Gly	G	glycine
His	H	histidine
Ile	I	isoleucine
Leu	L	leucine
Lys	K	lysine
Met	M	methionine
Phe	F	phenylalanine
Pro	P	proline
Ser	S	serine
Thr	T	threonine
Trp	W	tryptophan
Tyr	Y	tyrosine
Val	V	valine

CHAPTER 1

INTRODUCTION

Some of the text of this chapter is reproduced from:

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2. Rowland, K.J. and P.L. Brubaker, *The 'cryptic' mechanism of glucagon-like peptide-2.* Am J Physiol Gastrointest Liver Physiol, 2011. **301**(2): G1-8.

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1 Rationale

Glucagon-like peptide-2 (GLP-2) is an intestinotrophic peptide that has specific actions on the gut, including increased mucosal surface area, nutrient absorption, digestion and barrier function [1-4]. The beneficial effects of GLP-2 have propelled this peptide towards clinical status resulting in a long-acting GLP-2 analog that is in clinical trials (FDA approved) for the treatment of short bowel syndrome (SBS), Phase 2 for Crohn's disease, and preclinical development for gastrointestinal mucositis and pediatric indications (www.npsp.com). Despite the depth of knowledge that exists on the biological effects of GLP-2 on the intestinal epithelium, the underlying mechanism of action is not fully understood. Studies examining the mechanism of action of GLP-2 revealed that the cells that respond to GLP-2, the crypt and villus epithelial cells, do not express the GLP-2 receptor (R) [5-9]. The GLP-2R has, instead, been localized to several other intestinal cell types. Therefore, it was hypothesized that GLP-2 acts indirectly, possibly via other intestinal growth factors [5]. Hence, there are very limited *in vitro* models to study this complex mechanism of action. Recently, reports have indicated that the intestinotrophic actions of GLP-2 require the insulin-like growth factor (IGF)s, among other growth factors; although mechanistic details surrounding the reliance of GLP-2 on these growth factors are limited. Studies using global IGF-1 and IGF-2 knockout (KO) mice determined that IGF-1 and, to a lesser extent, IGF-2, is required for GLP-2's trophic effects on the small (SI) and large intestine (LI) [10]. The dramatic lack of response seen in the IGF-1 null mice in response to GLP-2 administration solidifies the importance of further investigation into GLP-2's dependence on the IGF-1 signalling pathway in mediating its biological effects. However, due to the global-nature of IGF-1 ablation and complications involving potential developmental alterations, it is beneficial to

use an inducible, intestinal-specific mouse model to follow up with these studies. Therefore we developed an inducible intestinal epithelial (IE)-specific IGF-1R KO mouse model (IE-igf1rKO) to investigate the role of IE-IGF-1R signalling in the beneficial effects of GLP-2 on the SI.

1.1 Small intestinal structure and growth

1.1.i Structure and organization of the small intestine

The overall health of an organism is strongly dependent on the epithelium that lines the gastrointestinal tract. This intricate cell layer not only serves as a physical boundary between the external and internal milieu, but also functions to digest and absorb nutrients, as well as to secrete ions, mucus and anti-microbial peptides; many of these activities are regulated by the epithelial cells, through secretion of a plethora of peptide hormones and growth factors [11]. The intestine's elaborate function is clearly reflected in its structural complexity, with 4 main cell types; absorptive, goblet, Paneth and endocrine. The importance of these cells to normal physiology is illustrated by the pathophysiology that results from loss or damage to the epithelial cell layer, including maldigestion, malabsorption and inflammation, as well as systemic disorders that result from poor nutrient absorption, such as growth impairment [12]. Furthermore, as the epithelial layer exists in a dynamic equilibrium between cellular proliferation and apoptosis, abnormalities in either of these parameters may also result in abnormal growth and/or intestinal cancer [13].

The apical aspect of the epithelial layer is exposed to the luminal contents, whereas the basolateral side is supported by the mesenchyme. Together, these layers form a series of folds in the SI resulting in villi that project into the lumen, and crypts which surround the

base of each villus and penetrate into the mesenchymal region. Each villus is supplied by approximately 8-11 crypts in the mouse, the function of which are to produce and deliver mature cells into the villus through a sequential process of proliferation, migration and differentiation [14, 15]. Absorptive enterocytes, goblet and enteroendocrine cells migrate from the depths of the crypt to the villus tip, a voyage that ends after 3 - 7 days when they are removed by either apoptosis or anoikis [16]. Conversely, the anti-microbial Paneth cells descend to the base of the crypt, where they are phagocytosed by neighboring cells after ~20 days [17]. Sitting immediately above the crypt base is the proliferative region, which contains both a slowly-dividing stem cell zone immediately above and amongst the Paneth cells, and a more proximal, hierarchical layer of early progenitor cells (Figure 1.1). It has been theorized that each crypt contains 1 - 6 ancestral stem cells, which divide approximately once per day in the rodent [18, 19]. The progenitor cells then undergo 3 - 6 cell divisions before reaching the crypt-villus junction, where they differentiate and acquire a phenotype of one of the mature villus cell lineages [13, 18, 20]. As all of the differentiated cell types of the epithelium are believed to derive from a single stem cell [21, 22], the key to unlocking intestinal cell dynamics therefore revolves around the stem cell, a multipotent, undifferentiated progenitor that maintains its numbers while enabling crypt replacement every ~2 days.

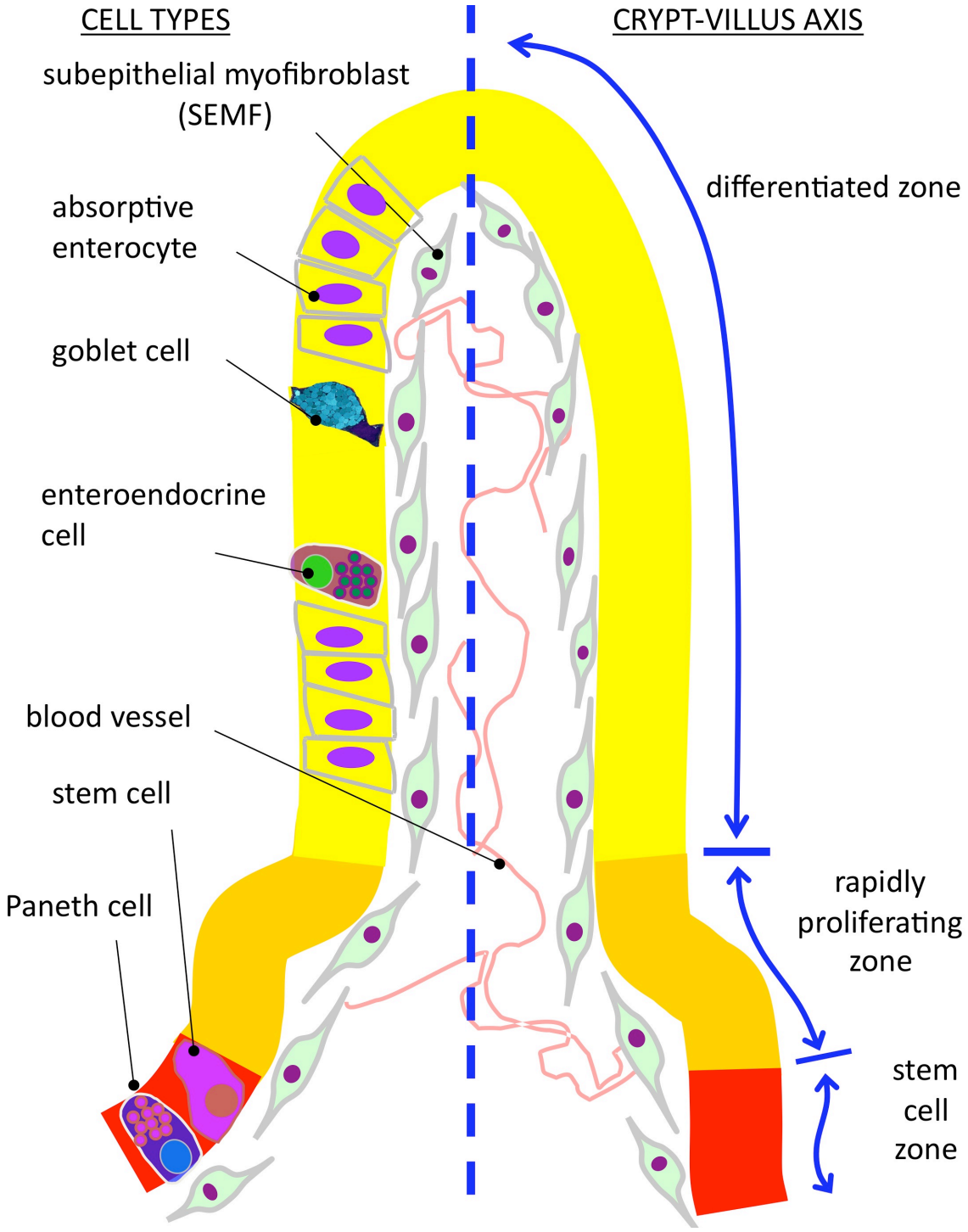


Figure 1.1 *Epithelial cell types and functional zones of the small intestine*. The villus contains differentiated epithelial cells including enteroendocrine cells, goblet and absorptive enterocytes, while the antimicrobial Paneth cells are localized to the crypt base. Subtending

the epithelium is a syncytium of intestinal subepithelial myofibroblasts (ISEMFs). The crypt-villus axis is functionally divided into three main regions, comprised of the stem cell zone at the base of the crypt, the rapidly proliferating zone that contains the progenitor cells, and the differentiated zone that extends to the villus tip.

Intestinal stem cell biology has been a fascinating and controversial topic over the last few decades due to the potential of such cells in tissue regeneration. Furthermore, the slowly-dividing nature of these cells makes detection by immunohistochemistry (IHC) for proliferative markers quite difficult (e.g. only 2 - 4% of putative stem cells label for Ki-67) [21]. Nonetheless, initial studies using tritiated-thymidine labeling demonstrated a population of slowly-dividing cells deep in the crypt that ultimately generate the 4 epithelial cell lineages [22]. Furthermore, irradiation has been shown to destroy the rapidly proliferating progenitor zone but does not prevent re-population of the epithelial layer, again establishing the existence of a very slowly-dividing stem cell [23]. Studies on mouse chimeras using chemical mutagenesis or gene induction have also shown that crypts contain short-lived and long-lived progenitor cells of enterocytic or goblet cell lineages, as well as long-lived multipotent stem cells [24, 25]. Finally, stochastic growth of stem cells is believed to follow one of several paths: asymmetric division to produce one differentiated daughter cell and one stem cell; symmetric division producing two daughter cells; or symmetric division producing two stem cells. Ultimately, the crypt cell population is maintained although, if cell numbers exceed or fall below capacity, the crypt may undergo fission, a process in which new crypts are generated with its own stem cell population [15, 25].

Putative stem cell markers have now been identified in several rapidly renewing tissues, such as the epidermis and both the nervous and hematopoietic systems. These markers include nestin, an intermediate filament protein [26], and Musashi-1 (Msi-1), an RNA-binding protein involved in asymmetric cell division [27]. Although nestin is expressed in the muscularis layer of the intestine, it has not been detected in the epithelial layer [28]. In contrast, Msi-1 is enriched in the intestinal crypt and was previously suggested to be a marker for intestinal stem cells [29]. However, Msi-1 also appears to be expressed by

descendent cells in the progenitor zone, thereby limiting its use for detection of the true stem cell population [10, 21]. Similarly, a number of genes with known roles in intestinal crypt cell proliferation and/or lineage determination (e.g. sry-type high-mobility-group box (Sox)9, phosphorylated (P)-Akt, P-phosphatase and tensin homolog (PTEN), chicken ephrin type (Eph)B2, EphB3, telomerase, Noggin, T-cell factor (Tcf)4, 14-3-3ζ, cluster of differentiation (CD)44 and mammalian hairy and enhancer of split homolog (Hes)1) also exhibit a decreasing gradient of expression from the deep crypt towards the villus and are therefore not likely to be exclusive to the stem cell [27, 30-35]. Nonetheless, Dekaney *at el.* have recently isolated a population of IE cells that are enriched for Msi-1, β1-integrin, and cytokeratin, but that are negative for CD45, suggesting enrichment of non-hematopoietic stem cells [36]. However, as these cells did not proliferate in vitro, their identity as stem cells remains unclear [37]. Recently, other strategies have associated cellular markers with their localization at the base of the crypt. Serine/threonine protein kinase DCAMKL1 is a microtubule-associated Ca^{2+}/calmodulin-dependent protein kinase located in post-mitotic neurons and was proposed to be an intestinal stem cell marker. Through radiation studies, these DCAMKL1-positive cells are considered to be a subgroup of quiescent stem cells. However, none of these markers have been shown to demonstrate lineage labeling and therefore cannot be used to delineate stem cell characteristics [38, 39]. Leucine-rich repeat containing G-protein coupled receptor-5 (Lgr5, Gpr49), has also been identified as a putative stem cell marker that can give rise to a clonal population. Inducible Lgr5-Cre knockin-lacZ mice demonstrated that Lgr5 expression is restricted to the base of the intestinal crypt. These Lgr5-positive cells can generate all epithelial lineages, thereby being an indication of multipotency in vivo and in vitro [40]. Other putative stem cell markers have also been explored, such as olfactomedin 4 [41]

which demonstrates similar localization as Lgr5, and polycomb complex protein, BMI-1 [42, 43], which was also identified to have stem cell potential. However, the debate remains as to the exact identity of the intestinal stem cells, as well as to when they lose their 'stemness' and become early progenitor cells committed to differentiation, with a lack of clarity of defined markers continuing to be a major limitation to the field.

1.1.ii Intrinsic and extrinsic signals regulating crypt cell dynamics

Intestinal crypt cell maintenance and proliferation is tightly regulated by a number of cellular signals, of which two major pathways have garnered particular attention; canonical (c) Wnt and bone morphogenic protein (BMP). Current evidence suggests that cWnt signalling is a critical regulator of stem cell behaviour. Signaling is initiated when Wnt ligands engage a complex consisting of a frizzled (Frz) receptor and a low-density lipid receptor (LRP5 or LRP6) (Figure 1.2). Another key molecule in this pathway is cytoplasmic β-catenin, the stability of which is regulated by a destruction complex comprising of adenomatous polyposis coli (APC), axin, casein kinase I (CKI) and glycogen synthase kinase 3-β (GSK3-β). In the absence of the Wnt ligand, β-catenin is phosphorylated by the destruction complex and targeted for ubiquitin-mediated proteosomal degradation [44]. However, in the presence of Wnt ligand, activation of disheveled (Dsh) inhibits the activity of this complex. As a result, β-catenin accumulates, translocates to the nucleus and binds to nuclear DNA binding proteins of the Tcf/Lef family to affect expression of cWnt target genes. Although β-catenin is constitutively expressed in the nucleus of Paneth cells [30, 45], two reports indicated that cWnt signaling is involved in the regulation of intestinal stem cell behaviour by the demonstration of nuclear β-catenin in non-Paneth crypt cells [30, 46]. Consistent with a requirement for active

cWnt signaling in the stem cell zone, over-expression of Dickkopf-1, an inhibitor of Frz, leads to loss of proliferative crypts [47]. Furthermore, there is a total absence of the proliferative compartment in $Tcf4^{-/-}$ neonatal mice, indicating that cWnt signaling is required for maintenance of the intestinal stem cell zone [48]. Deletion of the Tcf4 target gene, c-myelocytomatosis (Myc) also results in a marked reduction in crypt cell proliferation, due in large part to an increased cell cycle time [49]. Conversely, mutations that activate the cWnt/β-catenin pathway lead to adenomatous polyp formation in the mouse intestine [50-52] and colorectal cancer in humans [53-55]. Taken together, therefore, the cWnt signaling pathway is believed to play an essential role in maintaining intestinal crypt cell proliferation.

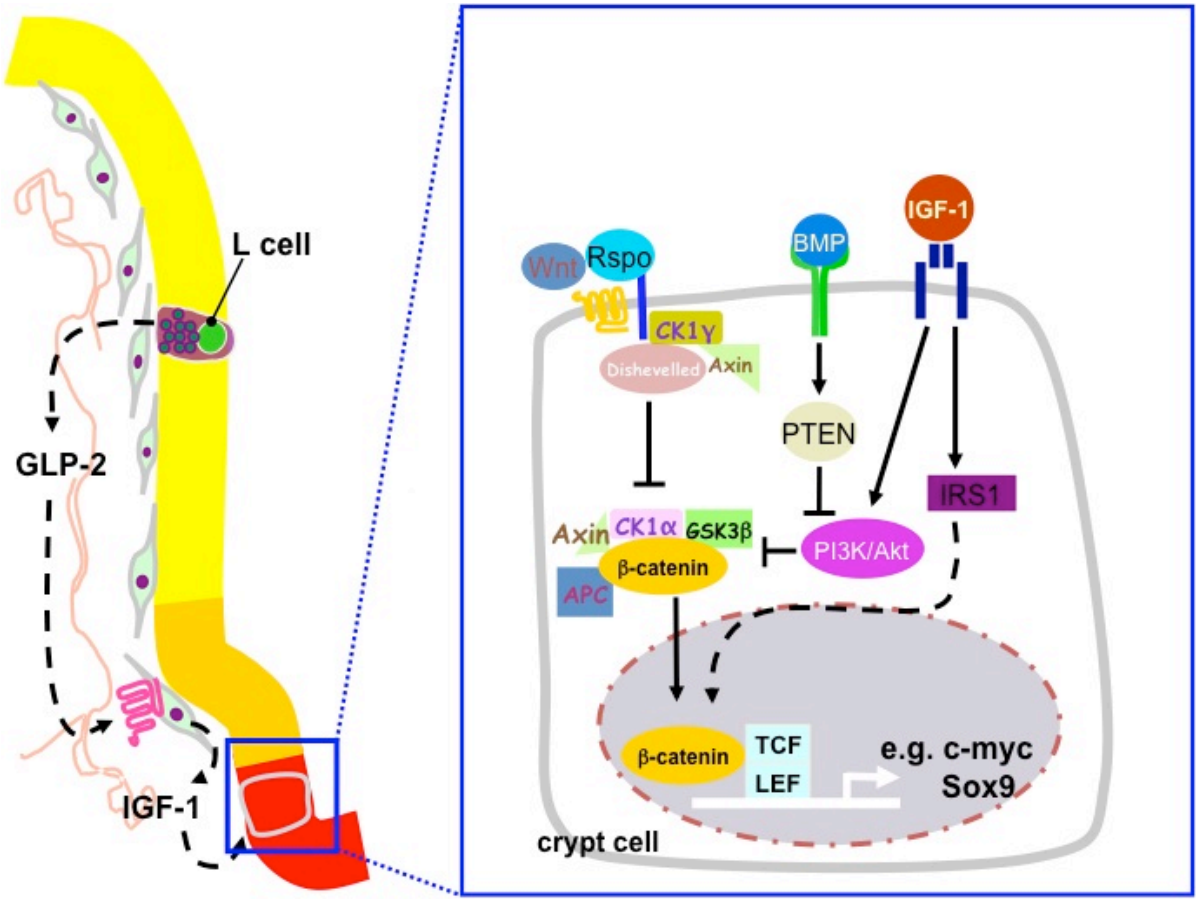


Figure 1.2 *Schematic of the extrinsic and intrinsic (inset) signaling pathways proposed to link glucagon-like peptide-2 (GLP-2) to crypt stem cell proliferation.* Release of GLP-2 from the intestinal L cell stimulates insulin-like growth factor-1 (IGF-1) secretion from intestinal subepithelial myofibroblasts (ISEMFs) expressing the G protein-coupled GLP-2 receptor. IGF-1, in turn, activates intestinal crypt cells expressing the tyrosine kinase IGF-1 receptor. (Inset) Wnt proteins, R-Spondin1 (Rspo), bone morphogenic protein (BMP) and IGF-1 elicit individual signaling pathways in the intestinal stem and proliferating cells that are integrated to regulate proliferation and differentiation. Solid lines indicate known interactions in crypt cells, while the dotted line represents a proposed pathway.

IE homeostasis is also tightly regulated by the BMP/Smad4 signalling pathway. Signalling is initiated when BMPs activate their receptors and transduce a signal to the nucleus via Smad transcription factors. Although most mutations in the BMP signaling pathway are embryonically lethal in mice, inhibition of BMP signaling in the villus via transgenic expression of the secreted inhibitor, noggin, resulted in a juvenile polyposis syndrome-like phenotype, including the late development of adenomatous polyps [56]. Interestingly, a similar phenotype is observed in conditionally-inactivated Bmpr1a mutant mice, in which loss of BMP signalling increases stem cell proliferation by activation of cWnt signalling, suggesting that BMP signalling serves as a molecular brake to cWnt-mediated stem cell renewal [32, 56] (Figure 1.2). Mechanistically, it has been suggested that the interactions between BMP and the cWnt pathway are mediated through the tumor suppressor PTEN, an inhibitor of phosphoinositide-3 kinase (PI3K)/Akt signalling [32]. Consistent with this notion, deletion of PTEN results in enhanced nuclear localization of β-catenin in Msi-1-positive proliferating crypt cells [21].

The intestine demonstrates enormous plasticity in that it can adapt physiologically to nutrient status, such as during periods of fasting and re-feeding. Fasting causes intestinal atrophy due to increased apoptosis and decreased proliferation of IE cells. As a result, there are structural changes, including decreased cellularity and shortening of the villus as well as reduction of bowel mass [57-59]. However, these effects are reversed by re-feeding, therefore suggesting that a period of fasting does not impair rapid functional restoration. This re-growth can be attributed to the fast rate of epithelial cellular renewal. Furthermore, there is a large body of evidence that strongly suggests that nutrients are the principle stimulus of intestinal growth. For instance, infusion of dextrose intragastrically causes intestinal hyperplasia, but this effect is abolished when hydrolysis of disaccharides is prevented [60, 61].

Other dietary components, such as amino acids, also stimulate mucosal growth and it appears that, from individual amino acid studies, histidine has a more potent trophic effect than valine or glycine [62, 63]. Interestingly, glutamine has garnered significant attention since removal of this amino acid from cultured intestinal mucosal cells inhibits cell proliferation, while supplementation in rats significantly increases villus height [64]. Moreover, glutamine is also necessary for the maintenance of intestinal mucosal integrity, since deprivation of this amino acid is associated with the breakdown of epithelial junctions [65]. Dietary lipids also enhance adaptive response to partial SI resection in rats, where long-chain free fatty acids have a greater effect than long-chain triglycerides. Conversely, deficiency of dietary fatty acids attenuates adaptive response of the SI mucosa following mucosal injury in rats [66, 67].

In addition to direct effects on crypt cell dynamics, luminal nutrients also stimulate intestinal growth through a process involving the release of growth factors. Although a number of intestinal growth factors have been described in the literature, their potential interactions with cWnt- and/or BMP- signalling in the crypt have not been intensively studied. However, IGF-1 has recently been shown to increase nuclear β-catenin localization and proliferation in crypt cells [68]. Cytoplasmic accumulation of β-catenin is also enhanced in the crypt by fibroblast growth factor (FGF) [69]. In contrast, epidermal growth factor (EGF) appears to stimulate crypt cell proliferation through a cWnt-independent pathway [70]. Finally, epimorphin, a mesenchymal protein that may play a role in the secretion of intestinal growth factors, negatively regulates intestinal proliferation through BMP-mediated down-regulation of cWnt signalling [71]. Recent studies have now identified another intestinal growth factor, GLP-2, as a mediator of cWnt signalling in the intestinal crypt [68]. As previous studies have also demonstrated that GLP-2 is a potent activator of intestinal proliferation under both fed

conditions and in re-growth following fasting, as well as enhancing Msi-1, β-catenin target genes (c-Myc and Sox9) expression in the crypt [10], these findings implicate GLP-2 as an extrinsic regulator of cWnt-dependent intestinal crypt cell behaviour.

1.2 Glucagon-like peptide-2

1.2.i Proglucagon and the proglucagon-derived peptides

The 160 amino acid precursor protein, proglucagon, is processed into the glucagon-like peptides, GLP-1 and GLP-2, as well as oxyntomodulin, glicentin, and intervening peptide-2 in the enteroendocrine L cell (Figure 1.3), while in the pancreatic α-cell, glucagon, intervening peptide-1, glicentin-related pancreatic peptide and the major proglucagon fragment which contains both GLP-1 and GLP-2 are liberated [72-74]. This differential processing of proglucagon is achieved through the distinct actions of prohormone convertase (PC)1/3, which is expressed in intestinal L cells, and PC2 in the pancreatic α-cells [72-75].

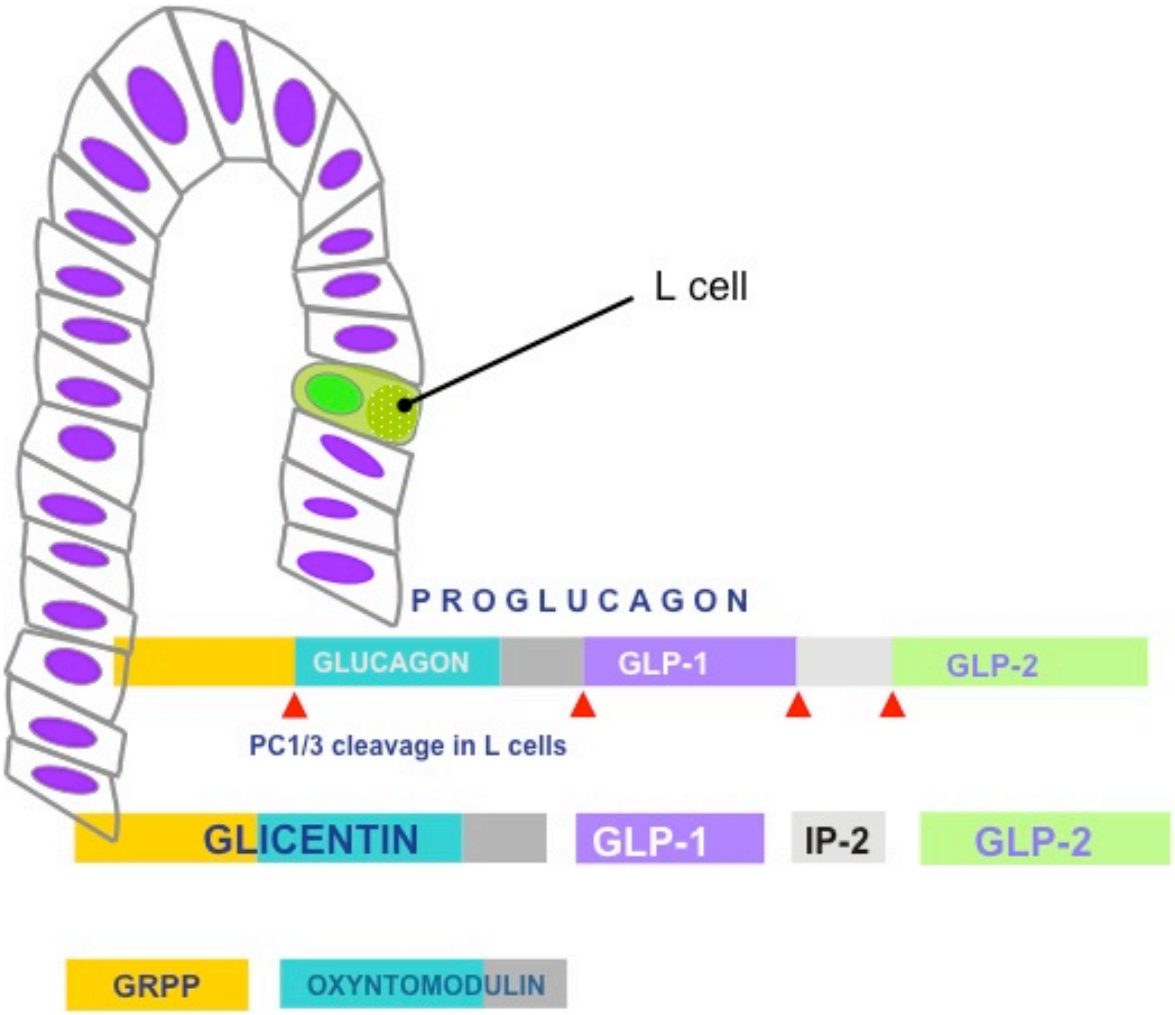


Figure 1.3 *Synthesis of the proglucagon-derived peptides by the intestinal L cell.* The 160 amino acid precursor protein, proglucagon, is cleaved by prohormone convertase (PC; red arrowheads) 1/3 in the intestinal L cell to yield either glicentin or glicentin-related pancreatic peptide (GRPP) plus oxyntomodulin, as well as glucagon-like peptide-1 (GLP-1), intervening peptide-2 (IP-2) and glucagon-like peptide-2 (GLP-2). The pancreatic hormone glucagon, which is also contained within the sequence of proglucagon, is not liberated in the intestinal L cell.

GLP-2 and GLP-1 are co-secreted from the L cell upon nutrient ingestion via both indirect and direct mechanisms [76-78]. In brief, the indirect mechanism involves activation of the vagus nerve by nutrients in the duodenum, resulting in muscarinic receptor-dependent stimulation of the ileal and colonic L cells [79, 80]. In contrast, the direct mechanism is mediated by contact of the luminal nutrients with the distal L cells [77, 78, 81]. Together, these pathways contribute to a biphasic secretory pattern of GLP-2 in humans, with a first peak at 30-60 minutes after a meal, and a second at 90-120 minutes post-prandially [77, 82].

1.2.ii Metabolism and degradation of GLP-2

Upon secretion, GLP-2 has a short serum half-life (~ 7 minutes in rats and humans), owing both to renal clearance and inactivation via N-terminal cleavage at Ala^2 by the protease dipeptidylpeptidase IV (DPP IV). DPP IV is a cell surface protein expressed in epithelial cells of the liver, intestine and kidney, but also exists as a soluble protein in the circulation. Cleavage by DPP IV produces the metabolite GLP-2^{3-33} which depending on concentration, has antagonistic effects on the GLP-2R as well as being a partial agonist [82-85]. Thus, most studies on the actions of GLP-2 in vivo have been conducted using DPP IV-resistant, long-acting analogues of GLP-2, such as Gly^2-GLP-2 and teduglutide (Gattex®); both of these peptides demonstrate an extended half-life and improved efficacy due to substitution of Ala^2 with Gly^2 [84, 86]. Moreover, differences in DPP IV activity is seen between species; rat have higher DPP IV activity compared to mice; therefore, there is significantly reduced biological response to exogenous GLP-2 in these rodents unless given through continuous intravenous infusion [87].

Both full-length and cleaved GLP-2 are subject to renal clearance at the same rate as glomerular filtration, as confirmed by inulin clearance in rats [88]. Although there are increased

levels of circulating GLP-2 in nephrectomised rats [84], GLP-2 continues to be cleared at a steady level, thereby implicating other sites of GLP-2 clearance, such as the splanchnic bed and peripheral tissues (e.g. hind limb), but not the liver [89].

1.2.iii Bioactivities of GLP-2

1.2.iii.a. Actions of exogenous GLP-2 on intestinal growth

Intestinotrophic properties were first associated with an unknown product of proglucagon processing in patients with proglucagon-expressing tumors who demonstrated enhanced crypt-villus growth [90, 91]. However, it was not until 1996 when studies of exogenous peptide administration in rodents showed that GLP-2 was the proglucagon-derived peptide with specific intestinotrophic properties [92-94]. Normal mice or rats treated with GLP-2 demonstrate increased SI weight in association with enhanced mucosal thickness, mucosal surface area, crypt-depth, villus height and crypt cell proliferation, as well as reduced crypt and villus apoptosis [10, 86, 92-96]. These effects are not limited to the SI, as the colon also responds to GLP-2 with increased crypt depth and proliferation, albeit to a lesser extent than the SI [10, 97, 98]. Importantly, GLP-2-induced growth appears to be relatively specific for the intestinal epithelium, as there have been only limited reports of growth in the intestinal muscularis [99] and no indication of growth in any other organs [92]. Furthermore, GLP-2 enhances intestinal function in parallel with this growth, such that nutrient digestion and absorption, as well as barrier function are all increased in normal rodents following chronic administration of GLP-2 [100-103].

1.2.iii.b. Actions of endogenous GLP-2 on intestinal growth

Interestingly, while exogenously-administered GLP-2 produces a robust effect on gut growth and function, the trophic actions of the endogenously-produced peptide appear to be

relatively more modest. The importance of endogenous GLP-2 has largely been elucidated through the use of a GLP-2R antagonist (e.g. the GLP-2 metabolite, GLP-2^{3-33}), the GLP-2R KO mouse model or immunoneutralization techniques. Of note, while the GLP-2 metabolite, GLP-2^{3-33} acts as an antagonist at lower concentrations, it also functions as a partial agonist at increased doses, with significant trophic effects observed in both the small and large bowel following administration of higher amounts of GLP-2^{3-33} [104]. Hence, caution must be taken in interpreting the results of studies using the GLP-2R antagonist, and appropriate controls must always be included. Nonetheless, administration of GLP-2^{3-33} for either 24 hr or 4 wk demonstrated a physiological role for GLP-2 in basal intestinal growth, reducing SI weight and decreasing crypt-villus height; these changes occurred in the absence of any detectable changes in proliferation but, at least in the 24 hr model, were associated with increased IE cell apoptosis [85, 105]. Although these findings stand in contrast to the report of normal intestinal weight, crypt-villus height and proliferative index in the GLP-2R null mouse [106, 107], it is possible that chronic adaptation occurs in these animals in order to maintain basal intestinal growth. Endogenous GLP-2 is also essential for the adaptive intestinal growth that occurs in mice and rats in response to oral re-feeding after a period of nutrient deprivation, as demonstrated using both the GLP-2 antagonist and GLP-2R null mice [85, 107, 108]. Finally, immunoneutralization of GLP-2 reduces the adaptive intestinal growth that is associated with experimental type 1 diabetes in rats [109], although these findings were not recapitulated in streptozotocin-diabetic GLP-2R KO mice [106]. Hence, the trophic effects of endogenous GLP-2 appear to be related to adaptation of the intestine in response to varying nutrient intake, although the relative importance of this role appears to vary with the species and/or the model utilized. Furthermore, the cellular mechanism of action of GLP-2 to increase intestinal growth remains a subject of intense interest, as discussed in more detail below.

1.2.iii.c. Other intestinal activities of GLP-2

In addition to the growth-promoting properties of GLP-2, several recent studies have begun to address the pathways by which GLP-2 increases intestinal function. Long known to enhance triolein absorption [100], treatment of mice and hamsters with GLP-2 has now been linked to increased plasma triglyceride and cholesterol levels, through a CD36-dependent mechanism that results in stimulation of intestinal apolipoprotein (apo)B48 secretion following an oral fat load [110]. Studies in pigs and humans also demonstrate that GLP-2 increases mesenteric blood flow [9, 111, 112], thus providing another mechanism to facilitate digestion and absorption of nutrients. Finally, the effects of GLP-2 to increase barrier function [113, 114] have been confirmed in murine models of both type 1 and type 2 diabetes (the non-obese diabetic and *ob/ob* mouse, respectively) [115, 116], and the mechanism whereby these effects are transduced has begun to be elucidated. Thus, administration of a prebiotic to *ob/ob* mice not only promotes GLP-2 synthesis, but also results in GLP-2-dependent up-regulation of the tight junction proteins, ZO-1 and occludin [115].

1.2.iv Therapeutic potential of GLP-2

Unlike other mitogenic factors, the apparent intestinal specificity of GLP-2 renders this peptide attractive for use in conditions of intestinal dysfunction, indeed these notable intestinal actions of GLP-2 have also been demonstrated in humans. Hence, chronic administration of either GLP-2 for 6 wk or of teduglutide for 3 wk (0.03 – 0.15 mg/kg/d) to subjects with SBS increases crypt-villus height and mitotic index, and reduces fecal output in association with increased absorption of enteral nutrients [117, 118]. Similarly, chronic treatment with teduglutide (for 20-24 weeks, 0.05 mg/kg/d) reduces the need for total parenteral nutrition (TPN) by 20% or more in 63% of SBS patients [119]. Interestingly, statistical

reductions were not observed in this study with a higher dose of teduglutide (0.1 mg/kg/d), although the decrease in parenteral volume was equal between the two groups. A pilot study investigating the effects of teduglutide (0.05 – 0.2 mg/kg/d) in patients with Crohn's disease is also suggestive of beneficial effects to increase intestinal mucosal mass and/or mucosal healing, as assessed through measurements of plasma citrulline concentration [120]. However, although a trend towards increased clinical responses and/or remission were observed in these subjects, there were no differences in their Crohn's Disease Activity Index. This was suggested to be due to a lack of power in the study; however, it also remains possible that there is a difference in the response to GLP-2 between humans with Crohn's disease and animal models of intestinal inflammation. Furthermore, the findings suggest that both the low (0.05 mg/kg/d) and the high (0.2 mg/kg/d) dose of teduglutide are more effective than the intermediate dose (0.1 mg/kg/d). Although the reasons for the apparent discrepancies in teduglutide effectiveness are not clear, previous studies in normal mice indicated that the intestinotrophic effects of GLP-2 are dose-dependent [93]. Hence, further studies in humans to determine the exact relationship between the dose of teduglutide and clinical effectiveness are required. Finally, the growth-promoting effects of GLP-2 in mice, and those of teduglutide on nutrient absorption in humans with SBS are reversed upon withdrawal of treatment [93, 118]. Thus, teduglutide (Gattex®) has recently completed clinical trials (phase 3) for the treatment of SBS and is currently under investigation for Crohn's disease (phase 2); teduglutide is also in preclinical development for gastrointestinal mucositis and pediatric indications (http://www.npsp.com; [118-120]). However, teduglutide will likely require chronic administration, and potential long-term adverse effects cannot be discounted, as discussed in more detail below. Nonetheless, the therapeutic advantage of the intestinal-specific actions of GLP-2 make this hormone an attractive candidate over other gut growth factors that also

exhibit extra-intestinal trophic actions, such as IGF-1. However, as with any growth factor, the potential for carcinogenic effects of chronic GLP-2 treatment must not be overlooked. Due to our incomplete knowledge of the mechanism of action of GLP-2 on the intestine, current studies are focusing on the cellular mechanism of GLP-2.

Several studies have examined the effects of GLP-2 treatment on cancer initiation and/or progression in rodents. In the first of such studies, methylating carcinogen (dimethylhydrazine)-induced colonic tumors were increased in mice treated with long-acting Gly^2-GLP-2 compared to untreated control groups [121]. Interesting, Gly^2-GLP-2 specifically increased the number of small, medium and large polyps in these animals, with medium and large polyps characterized as pedunculated non-malignant adenomas, whereas native GLP-2 only increased the number of small polyps, none of which were malignant. The potential carcinogenic effect of GLP-2 has also been studied in mice that were pre-treated with the carcinogen azoxymethane [105]. Azoxymethane is a rare dietary carcinogen that is established for use as a model of 'sporadic' colon cancer; however, the exact relevance of this model to colon cancer induction in humans remains uncertain. Nonetheless, this study demonstrated not only a significant increase in the number of colonic aberrant crypt foci (ACF), but also of the more dysplastic, mucin-depleted foci. Further, the number of ACF was reduced by treatment with GLP-2^{3-33}, implicating GLP-2 in the development of colonic dysplasia in this model. Together, these findings suggest that GLP-2 increases tumor initiation as well as progression in the dimethylhydrazine- and azoxymethane-models of sporadic murine colon cancer. Although the mechanism of this action has not been established, one possibility is the regulatory relationship between the cWnt and PI3K/Akt signalling pathways, both of which have been implicated in the pathogenesis of colorectal cancer [122]. However, in contrast to these studies, other findings indicate that GLP-2 does not modulate tumor growth in vitro or

in vivo. For instance, treatment of stable colon cancer cell lines transfected with the GLP-2R did not affect cell proliferation or survival [123]. Furthermore, injection of these cells into nude mice followed by treatment with GLP-2 did not induce tumor growth. Finally, GLP-2 did not modulate tumor growth in $APC^{min+/-}$ (adenomatous polyposis coli; multiple intestinal neoplasia) mice, which have an activating mutation in APC causing increased cWnt signaling and are, thus, a model of familial colon cancer [123]. Thus, despite the beneficial effects of GLP-2 and its long-acting derivative, teduglutide, in the treatment of gastrointestinal insufficiency and disease, the potential for GLP-2 to induce carcinogenesis, albeit controversial, is an important issue that warrants further long-term investigation.

1.2.v The GLP-2 receptor

1.2.v.a. Structure

The bioactivities of GLP-2 are transduced through a G protein-coupled (GPC) receptor (R) that displays a typical 7-transmembrane topology. The GLP-2R belongs to the glucagon-secretin class B receptor family [124]. Polymerase chain reaction (PCR) and hybridization screening of conserved motifs between GLP-1R and glucagon R led to the cloning of the GLP-2R from rat hypothalamus and duodenum/jejunum. There is 81.6% sequence homology between human and rat GLP-2R, whereby the human GLP-2R encodes 553 amino acids and the rat GLP-2R encodes 550 amino acids [124]. To date, there is nothing known about possible GLP-2R or GLP-2 mutations in humans.

1.2.v.b. Location

Distribution of the GLP-2R appears to be largely restricted to the intestinal tract, although limited expression has been also detected in the lung and hypothalamus [5, 124]. However, despite a broad knowledge of the intestinal actions of GLP-2, mechanistic studies have been

limited due to the fact that the GLP-2R is not localized to the epithelial cells, the major site of the proliferative and cytoprotective actions of GLP-2 [5, 6, 8, 9]. Rather, the GLP-2R is expressed in the intestinal subepithelial myofibroblast (ISEMF)s that underlie the epithelium, dispersed enteroendocrine cells and the enteric nervous system. It was therefore proposed that GLP-2 exerts its intestinotrophic actions indirectly, via downstream mediators derived from GLP-2R-expressing cells [5].

1.2.v.c. Intracellular signalling

Although there have been numerous advances recently in the understanding of downstream mediators of GLP-2 action using in vivo models, these studies do not provide information regarding intracellular signalling mechanisms of the GLP-2R. Thus, a number of different in vitro models have been used to examine GLP-2R signalling. Similar to other class B GPCRs, ligand binding in heterologous cells expressing the transfected GLP-2R results in a dose-dependent increase in 3'-5' cyclic adenosine monophosphate (cAMP) and activation of protein kinase A (PKA), cAMP response element-binding protein (CREB) and AP-1 [124, 125]. Furthermore, GLP-2 has a small, PKA-independent proliferative effect on cells transfected with the GLP-2R, and decreases apoptosis through inhibition of GSK3-β and bcl-2-associated death promoter protein (BAD) [126, 127]. However, the results of studies using heterologous cell models are not entirely consistent with data from cells expressing the GLP-2R endogenously. Thus, although primary rat mucosal cells, intestinal muscle strips and fetal rat intestinal cells do respond to GLP-2 treatment with an increase in cAMP levels [10, 85, 99, 128], a recent study with primary ISEMF cells, which also naturally express the GLP-2R, demonstrated no effect of GLP-2 on the cAMP-PKA-CREB pathway [129]. A similar lack of effect of GLP-2 on cAMP levels was also reported using HeLa cells, a model cell line that also expresses the endogenous GLP-2R [130]. However, unexpectedly, the stimulatory effect of

GLP-2 on IGF-1 mRNA transcript levels in the ISEMF cells was found to be dependent upon PI3K/Akt, a pathway that had not previously been linked to GLP-2R activation in vitro[129]. Indeed, although GLP-2 does activate PI3K/Akt signalling in IE cells in vivo [68, 101, 131], this is presumed to be downstream of the cellular mediators, rather than due to direct signalling by the GLP-2R. This hypothesis is supported by findings that both the IGF-1R and ErbB receptors stimulate PI3K/Akt signalling in IE cells [132, 133]. Similarly, although the cWnt/β-catenin signalling pathway, an integral system for cell cycle regulation, has recently been implicated in the effects of GLP-2 on the crypt cell in vivo [68], activation of this pathway must be mediated indirectly, rather than through direct linkage to the GLP-2R. This notion is also supported by the finding that PI3K/Akt signalling activates β-catenin in intestinal stem and progenitor cells via phosphorylation at Ser[552] [21, 134], through a mechanism involving Ras activation [135] and GSK3-β phosphorylation [136]. Therefore, although treatment with GLP-2 in vivo activates a number of signalling pathways in the IE cells, there is currently only limited information about GLP-2R signalling in the actual target cells of this hormone in the intestine, namely the ISEMFs, with nothing reported to date on the signalling pathways activated by GLP-2 in either intestinal neurons or enteroendocrine cells. Nonetheless, although there currently exist few in vitro cell models that allow for the study of such a complex system, numerous in vivo studies have now demonstrated that GLP-2 indeed elicits a variety of its intestinal effects indirectly, through several paracrine mediators.

1.2.vi Downstream mediators of GLP-2 action in the intestine

Several studies originally described keratinocyte growth factor (KGF) and endothelial nitric oxide synthase (eNOS) as mediators involved in GLP-2-induced colonic growth and

intestinal blood flow, respectively [8, 9, 112]. However, more recent studies have focused on the IGFs [10, 68, 108, 129, 137], the ErbB network [107, 138] and vasoactive intestinal polypeptide (VIP) [139] as key players in the trophic actions of GLP-2 (Table 1.1).

Table 1.1 *Indirect mediators of GLP-2 intestinal action*

Indirect mediator	Intestinal site of action	References
ErbB ligands	jejunal mucosa	[138]
insulin-like growth factor-1	jejunal, ileal, colonic mucosa	[10, 68]
insulin-like growth factor-2	jejunal, colonic mucosa	[10]
keratinocyte growth factor	colonic mucosa	[8]
nitric oxide	mucosal blood vessels	[9, 112]
vasoactive intestinal polypeptide	ileal, colonic mucosa	[139]

The IGFs, IGF-1 and IGF-2, are mitogenic peptides with functions encompassing cellular proliferation, survival and differentiation [140]. Importantly, the IGFs and GLP-2 share many similar biological actions in the gut (Table 1.2) [10, 96, 141, 142]. The rationale for the involvement of IGF-1 in the intestinotrophic effects of GLP-2 has been previously reviewed [143]. Briefly, studies performed in IGF-1- and IGF-2 KO mice determined that IGF-1 and, to a lesser extent, IGF-2, is required for the trophic effects of GLP-2 on both the SI and LI [10]. Hence, there was a dramatic lack of response to GLP-2 administration in IGF-1 global KO mice, in terms of crypt cell proliferation, crypt-villus length, and intestinal weight. In contrast, the role of IGF-2 in the intestinotrophic effects of GLP-2 was more modest, and appears to be restricted to mucosal surface area. Additionally, use of an IGF-1R inhibitor, NVP-AEW541 prevented various proliferative responses of GLP-2 in SI crypt cells. In further support of a requirement for IGF-1 in the actions of GLP-2 are findings that GLP-2 increases IGF-1 mRNA transcript levels in murine and rat intestine, as well as in cultures of murine ISEMF cells, and induces IGF-1 secretion by fetal rat intestinal cells in vitro [10, 129, 137]. Nelson *et al.* have also demonstrated that mucosal growth upon re-introduction of luminal nutrients in the rat is not only associated with elevated jejunal IGF-1 mRNA levels, but is also partially blocked by administration of GLP-2$^{3\text{-}33}$ [108]. Nonetheless, it is important to recognize that IGF-1 is produced in many tissues in addition to the intestine, and also circulates in the blood stream [144]. Hence, more studies are required to determine whether the intestine and if so, which cell type, is the source of the IGF-1 required for the trophic actions of GLP-2.

Table 1.2 *Comparison of the SI actions of GLP-2 and IGF-1 in healthy animal models*

Parameter	GLP-2 action	Reference	IGF-1 action	Reference
Weight	↑	86, 87, 92-100, 103, 145, 146	↑	96, 142, 147-155
Length	↑ or no change	87, 95, 96, 98, 156	↑ or no change	96, 142, 147, 149, 157
Protein content	↑	94, 102, 146	↑ or no change	142, 147-150, 153, 155, 158-160
DNA content	↑	94, 102, 146	↑	142, 148, 150, 153, 155, 158, 159
Mucosal weight	↑	103, 146	↑	147, 148, 150, 153, 158, 159, 161
Mucosal area	↑	87, 98, 103, 162	n.r.	
Muscularis growth	↑ or no change	95, 99	↑	96, 147, 149, 153, 155, 157
Villus height	↑	7, 86, 87, 93-95, 98, 99, 103, 146, 156, 163	↑	96, 149, 150, 153, 158
Crypt depth	↑	7, 86, 87, 93, 95, 98, 99, 145, 146, 163	↑	96, 149-151, 153, 155, 158
IE cell proliferation	↑	95, 99, 145, 163-167	↑	149, 151, 158, 159, 168-170
IE cell apoptosis	↓	95	↓	158, 159, 170
Barrier function	↑	103	↑	150, 169
Digestion	↑	100, 146, 164	↑	96, 155, 161
Nutrient transporters	↑	100-102, 146, 171-173	↑	174-176
β-catenin signalling	↑	68	↑	68

In addition to the IGFs, ErbB ligands have recently generated interest as downstream mediators of GLP-2-induced intestinotrophic actions. The ErbB network is a potent proliferative system that contributes to the maintenance of intestinal mucosal growth and function [177]. The ErbB ligands, epiregulin and neuregulin are upregulated in the murine small intestine one and 4 hr after GLP-2 treatment [138]. Similarly, administration of GLP-2 or EGF increased mRNA expression of the ErbB ligands, amphiregulin, epiregulin and HB-EGF, as well of the immediate early genes, c-fos, egr-1 and phlda-1 within intestinal sections. However, of some note, these sections were full-thickness, including all layers of the intestinal wall, which precluded definitive localization of the cell type in which the effects were exerted. Furthermore, the ErbB receptors were required for the chronic intestinal proliferative and growth effects of GLP-2, as determined through the use of the pan ErbB inhibitor, CI-1033. More recently, EGF administration was also found to rescue the lack of adaptive re-growth in the re-fed GLP-2R KO mouse [107]. In contrast, using the ErbB receptor inhibitor, gefitinib, others have reported that the positive effects of GLP-2 on intestinal weight and crypt-villus length are independent of ErbB signalling [178]. However, gefitinib is not a pan ErbB inhibitor and has limitations compared to CI-1033. Nonetheless, collectively, the data suggest the involvement of an ErbB ligand – ErB signalling pathway in the proliferative actions of GLP-2.

How the findings on the ErbB axis can be reconciled with data on the IGF-1 – IE– IGF-1R pathway remains unclear. Recent data have indicated that GLP-2 treatment increases IGF-1, but not ErbB ligand mRNA transcript levels in ISEMF cultures [129], suggesting that the ErbB system lies downstream of the IGF-1 network. Conversely, exogenous EGF, but not IGF-1, rescues the growth deficit in re-fed GLP-2R null mice [107]. While these findings suggest that IGF-1 lies downstream of EGF/ErbB signalling, it is important to recognize that

the IGF-1 used in these studies was not proven to be functional in control animals, which may have important implications for the conclusion. Nonetheless, it has been well-established that the IGF-1R can transactivate ErbB receptors, and vice versa, demonstrating the existence of cross-talk between these two pathways [179-182]. Studies in other cell models have also indicated that the two pathways demonstrate co-dependence [183]. Additional studies utilizing cell-specific models are clearly required to delineate the exact relationship between the IGF-1R and the ErbB receptors in the proliferative response to GLP-2.

Finally, in association with expression of the GLP-2R on submucosal enteric neurons, recent evidence indicates that GLP-2 reduces intestinal inflammation and damage via activation of VIP-producing neurons [139]. In a variety of rat and mouse models of inflammatory bowel disease (IBD), GLP-2 treatment reduced the levels of inflammatory cytokines (i.e. IFN-γ, TNF-α, IL-1β), and increased the production of the anti-inflammatory cytokine, IL-10, in association with increased numbers of VIP-expressing neurons in the submucosal plexus of the SI. However, in contrast to the well-established mitogenic effects of GLP-2 in the normal bowel, Sigalet *et al.* observed a GLP-2-mediated reduction in epithelial proliferation rates in these inflammatory models, with a reduction in inflammation-induced proliferation towards normal levels [139]. Nonetheless, treatment with either GLP-2 or VIP improved weight loss and reduced intestinal damage in these animals, and antagonism of the actions of VIP prevented the anti-inflammatory actions of GLP-2. Finally, despite the initial findings on IL-10, studies in the IL-10 null model of colitis demonstrated that GLP-2 mediated decreases in mucosal inflammation and crypt cell proliferation occur through an IL-10-independent mechanism [139]. Rather, the anti-inflammatory actions of GLP-2 in this model involved activation of suppressor of cytokine signalling-3 signalling in IE cells, and an IL-6-mediated increase in signal transducer and activator of transcription-3 in colonic mucosal

scrapings [184]. Hence, although VIP is an essential regulator and downstream mediator of GLP-2 in rodent models of IBD, the exact pathway by which these effects are modulated remains unclear. Collectively, therefore, multiple studies now indicate that the actions of GLP-2 on the intestine are complex, involving multiple mediators (e.g. eNOS, ErbB ligands, IGF-1, IGF-2, KGF and VIP), the roles of which are both cell-type specific and dependent upon the physiological/pathophysiological state of the organism.

1.3 Insulin-like growth factors

1.3.i The IGF peptides

The IGFs or somatomedins are peptide hormones that have main functions in growth and development with important roles in cell migration, proliferation, differentiation and cell survival. The IGF system is comprised of the ligands: IGF-1 and IGF-2 (70% amino acid sequence homology), the receptors: IGF-1R and IGF-2R (also known as the mannose-6-phosphate R), as well as six IGF binding proteins (IGFBP-1 to -6). The IGFs were first identified as mediators of growth hormone (GH; aka somatotrophin)-induced skeletal growth [185] and were recognized to be structurally homologous to insulin [140, 186], which functions largely in metabolism and nutrient homeostasis. In contrast, the IGFs are known to serve more of a mitogenic role under normal physiology in all vertebrates [187]. IGF-1 and IGF-2 both structurally resemble proinsulin in that they consist of four evolutionarily-conserved domains, A, B, C and D, with A- and B-domains connected by disulfide bonds that are homologous to those of insulin. However, IGF-1 (70 amino acids) and IGF-2 (67 amino acids) do not undergo PC1/3- or PC2-mediated proteolytic cleavage, as is the case with proinsulin, to produce the mature insulin bi-peptide with A- and B-chains. Instead, mature

IGF-1 and IGF-2 remain intact with a C-domain and an additional D-domain, which is not found on proinsulin [188].

Due to differential exon splicing, IGF-1 exists as different isoforms that have different biological effects and may initiate varied signalling pathways [189]. Variants of IGF-1 (Figure 1.4) that form starting from exon 1 are classified as C1 or Class 1 isoforms, while isoforms that initiate from exon 2 are grouped as Class 2 (C2) isoforms. Overexpression of Class 1 IGF-1 (IGF-1:C1) in dystrophic *mdx* mice demonstrate that this isoform causes skeletal muscle hypertrophy, decreases the progression of myofiber atrophy following denervation, and slows the onset of skeletal muscle necrosis. Similarly, previous studies show that over-expression of Class 2 IGF-1 (IGF-1:C2) isoform leads to a 5-times greater increase in total muscle IGF-1 levels and concomitantly increased muscle mass [189].

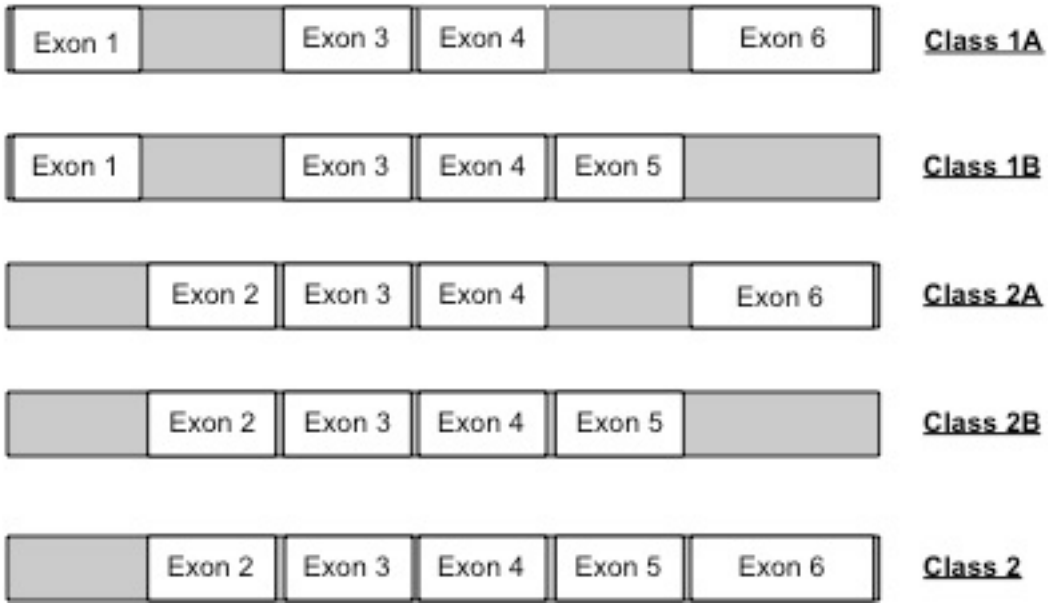


Figure 1.4 *Human IGF-1 mRNA splicing.* IGF-1 isoforms include Class 1, which contain coding regions starting from exon 1 and Class 2 that initiate from exon 2. Grey intron and white exon regions are representative and, therefore, not to scale.

IGF-1 is expressed and secreted by many somatic tissues and the site of secretion reflects its actions [190]. The majority of circulating IGF-1 is produced from the liver via control by the hypothalamic-pituitary-liver axis and its respective endocrine hormones: growth hormone releasing-hormone, GH and IGF-1; both post-natal and pubertal growth is regulated through this axis. In contrast, IGF-2 is released from the liver into the circulation in a constitutive manner. Normal fetal growth is regulated by both IGF-1 and IGF-2, despite low levels of GH and GH receptors in the liver of the fetus. Initially, IGF-2 predominates in the fetus with highest levels early in gestation, followed by rising IGF-1 levels closer to full gestation. IGF-1 and IGF-2 are also expressed and secreted in numerous organs and tissues outside of the liver including, but not limited to intestine, bone and ovary – in all of these tissues, autocrine and paracrine mechanisms of actions are important [191, 192].

Several pathways integrate to regulate the expression and production of the IGFs. Unlike insulin, IGF-1 is not affected by serum glucose levels and, in fact, GH is the principle stimulus of IGF-1 release from hepatocytes along with lesser effects of insulin, cortisol and triiodothyronine, as well as inhibitory actions of glucagon and amino acids [140, 193-196]. In most tissues, IGF-2 and IGF-2R mRNA expression is regulated via genomic imprinting, causing expression of only the paternal allele [197-199]. The maternal IGF-2 allele is transcriptionally repressed due, in part, to non-methylation and to the transcriptional CCCTC-binding factor, which binds enhancer elements upstream of the H19 gene and, hence, restricts transcription of the adjacent Igf2 gene. The paternal allele is methylated, thus preventing transcriptional CCCTC-binding factor binding, (and in turn this region is free to associate with cis enhancer elements of Igf2 gene to drive its transcription). In addition, posttranscriptional regulation of IGFs occurs through the activity of a number of microRNAs and this has been studied in different model systems. One study demonstrated that the microRNAs, miR-1 and miR-206,

target the 3'UTR of the IGF-1 mRNA and repress its translation. Similarly, IGF-1 down-regulation was observed due to the microRNA, miR-32, in myocardial microvascular endothelial cells in a diabetic rat model. IGF-2 mRNA expression levels are also down-regulated by the IGF-2 mRNA-binding proteins (IMP1, IMP2, IMP3). Furthermore, the RNA-binding protein, Lin-28, has also been shown to bind IGF-2 mRNA and activate translation in both embryonic and adult tissues [200]. Collectively, therefore the IGFs represent a rather complex system involving multiple forms and differential regulation pre- and post-transcriptionally.

1.3.ii Metabolism and clearance of the IGFs

Circulating IGF-1 and IGF-2 are bound to the high-affinity IGFBPs that not only modulate the availability of the IGFs to enter freely into tissues and bind to their receptors, but also prolong their plasma half-lives and potency. In addition, a series of low-affinity IGFBPs exists, including IGFBP-7 and -8 [201]. IGFBPs are primarily localized within the extracellular matrix, circulation and extravascular fluids [202], and mainly function as a reservoir for IGFs, sequestering them for controlled release and delivery. Of the six binding proteins, IGFBP-3 is the most prominent and, hence, has the most binding capacity (80% in humans). However, IGFBP-3, -4 and -5 predominate in the rodent and human postnatal intestine . The IGF-binding protein complex exists in a 1:1 molar ratio along with an 85-kDa acid-labile subunit (ALS) to form a large 150kDa complex [140]. Although the role of the ALS is often dismissed, the IGFBPs are known to have both IGF-inhibitory and potentiating properties, in addition to sequestering the IGFs. Hence, inhibitory actions have been demonstrated for IGFBP-1, -2, -3, -4 and -6, while IGFBP-1, -3 and -5 have potentiating actions through their close association with the extracellular matrix or cell surface [140]. Futhermore, the affinity of the IGFBPs to the

IGFs is at least equal to or greater than that of IGF-1R, depending on the cell-type. For example, a study utilizing bovine aortic endothelial cells demonstrated that there were twice as many binding sites associated with IGFBPs as IGF-1Rs per cell [203]. Both IGFBP and receptor interactions inhibit transport of IGF peptides; however, only IGF-1R binding leads to peptide degradation. Proteases such as caspases and prostate-specific antigen, in turn, can limit IGFBP activity by digestion and therefore increase IGF availability [202]. Finally, increasing evidence suggests that the IGFBPs also have actions independent of their association with the IGFs, through IGFBP receptors, although the physiological role is not known [204-206].

In addition to regulated metabolism of IGFs by IGFBPs, activity of the IGFs is also limited by degradation, which occurs through the actions of the proteolytic insulin-degrading enzyme [207, 208], as well as via rapid renal clearance when IGF-1 or IGF-2 is not complexed with IGFBPs. The degradation of IGF-1 in the rat duodenum and ileum was found to be rapid, with a half-life of 2 min as determined via radiolabelled tracer methodology [209].

1.3.iii Bioactivities of IGFs

The IGFs and their receptors are indispensible components of embryonic and postnatal growth as evidenced by gene mutagenesis studies [210]. The majority of IGFs are secreted from the liver with IGF-1 being the dominant form that is secreted in adult life. Circulating IGF-1 released by the liver in response to GH acts in an endocrine manner, while also providing negative feedback on GH release [188]. It has been long known that over-expression of GH [211] and IGF-1 [212] in transgenic mice elevates the levels of these peptides, resulting in enhanced body growth. Similarily, global knockout of the Igf1 [210, 213-215], Igf2 [215-218] and Igf1r [215, 218] genes causes severe embryonic and postnatal growth retardation, resulting in high neonatal

lethality and birth weights that are 60% and 40% of normal, respectively. Nevertheless, mutations in GH or the IGF system do not appear to be deleterious in adulthood and are not associated with morbidity.

In addition to its endocrine role, IGF-1 is also secreted in a tissue-specific manner, therefore acting as an autocrine or paracrine growth factor for many tissues [219]. While the exact roles of these two sources of IGF-1 have not been fully elucidated, it is clear that they play different roles as exemplified by the liver-specific Igf1 gene-deleted mouse model [191, 220]. Hence, studies in these mice indicated that liver-derived IGF-1 is essential for periosteal bone growth and for negative feedback on GH secretion, but not for linear growth. These studies also demonstrated that liver-derived IGF-1 constitutes 75% of IGF-1 found in the circulation and that this fraction of circulating IGF-1 is not necessary for postnatal tissue growth. Thus, peripheral sources of IGF-1 and IGF-2 are sufficient to transduce mitogenic cues. This conclusion was further exemplified through the use of the α-smooth muscle actin (αSMA) promoter-driven IGF-1 transgenic mouse model, in which IGF-1 is overexpressed in mesenchymal cells [147, 157, 221]. The αSMA-IGF-1 transgenic mouse displays normal serum IGF-1 levels but has enhanced growth of peripheral αSMA-positive tissues. For example, these mice exhibit increased growth of the intestinal muscularis, since cells within the lamina propria and smooth muscle layer are major sites of IGF-1 expression in the bowel [222, 223]. While it is difficult to ascertain the endogenous effects of locally-expressed tissue IGF-1 using IGF-1 over-expression methodologies, tissue-specific deletion of the IGF-1R provides a powerful tool in order to dissect locally-expressed IGF-mediated actions in peripheral tissues (Table 1.3). Nevertheless, this tissue-specific approach also presents its own limitation, as it does not differentiate between local and circulating IGF-1. Therefore, to

delineate the actions of IGF-1 both methodologies must be taken together to form an integrative conclusion.

Table 1.3 *Tissue-specific IGF-1R KO models and their phenotypes*

KO location	Igf1r$^{flox/flox}$	Cre recombinase	Phenotype	Reference
Prostate epithelium	√	Arr2PBi-Cre	Cell autonomous proliferation and hyperplasia ↑ p53-regulated apoptosis ↑ rate of prostate cancer with compromised p53 activity	[224]
Brain (CNS)	√	Nestin-Cre	↓ lifespan Altered development of somatotrophic axis	[225]
Sertoli cell	√	Cre lentiviral construct	↓ proliferation ↑ apoptosis ↓ lactate and transferrin secretions	[226]
Skin/organo-typic model	√	Cre adenoviral construct	↓ proliferation (keratinocyte) ↑ differentiation (keratinocyte) IRS2 overexpression	[227]
Hepatocyte/ cholangiocyte	√	α-fetoprotein enhancer-Cre	↓ proliferation in males (hepatocyte) ↓ ERK1/2 Downregulation IRS1	[228]
Adipose tissue	√	Meu-Cre	↓ adipose tissue mass ↑ adipose tissue lipid content	[229]
Osteoblast	√	Cre adenoviral construct	↓ cancellous bone volume ↓ connectivity ↓ rate of mineralization of osteoid	[230]
Vascular endothelium	√	Tie-2-Cre	↓ retinal neovascularization during hypoxia ↓ VEGF during hypoxia ↓ eNOS during hypoxia	[231]
Venous smooth muscle cell	√	Cytomegalovirus-Cre	↓ in mechanical stretch-induced proliferation ↓ neointima formation	[232]
β-cell	√	Pdx-Cre, RIP-Cre	↓ acute phase insulin release No change in β-cell apoptosis during development No change in β-cell growth during early development	[233]
CNS	√	CamKIIα-Cre	↓ remyelination ↓ proliferation and survival of oligodendrocyte progenitors	[234]

In order to examine the role of the IGFs in bowel growth, it is important to first consider the location of locally-expressed IGF-1 and IGF-2. Microarray analysis of isolated epithelial and mesenchymal fractions confirmed that IGF-1 is most highly enriched within the mesenchymal region of the intestine [235] and, more specifically, in the ISEMFs and external smooth muscle layer [222, 223]. The ISEMF layer underlies the epithelial cells of the crypt-villus unit and is known to have an important role in controlling enterocyte proliferation and differentiation [236-238]. ISEMFs engage in reciprocal cross-talk with adjacent epithelial cells by releasing various signalling molecules and growth factors, including the IGFs, EbrB ligands, KGF, b-fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) [239-241]. It is not entirely clear what mechanisms regulate IGF-1 expression in ISEMFs, although IGF-1 mRNA levels increase in a time- and dose-dependent manner in primary ISEMF cell cultures following treatment with GLP-2 [129]. Furthermore, factors associated with intestinal disease, intestinal resection and altered nutrient intake may regulate IGF-1 secretion from these cells [223, 242, 243]. Further studies that examine the functional response of epithelial cells to ISEMF IGF-1 release through the use of a cell co-culture system could prove beneficial in this regard. In addition, IGF-1 is also expressed in muscularis smooth muscle and lamina propria macrophages where their paracrine actions influence neighboring cell growth [184, 244, 245].

In normal physiology and in multiple animal models of disease, IGF-1 administration has trophic effects, including most somatic tissues as well as, most importantly to this discussion, the SI and LI [96, 141, 246]. Consistent with these findings, the proliferation and survival of IE cells increases in response to transgenic over-expression or administration of IGF-1 [142, 148, 149]. Specifically, both GH and IGF-1 transgenic mice have been reported to demonstrate increased intestinal weight and length, while IGF-1 transgenic animals also

exhibit increased crypt cell proliferation. Moreover, it was shown that IGF-1 can mediate the same trophic actions as GH but does not require the presence of GH, with the intestine being particularly responsive to IGF-1 excess. Trophic effects of exogenously-administered IGF-1 have also been studied in a rat model of TPN-induced mucosal atrophy [160]; these mice demonstrate increased protein synthesis, DNA and crypt depth in the jejunal mucosa and increased protein synthesis in the muscularis, therefore displaying an IGF-1-induced reversal of TPN-associated mucosal atrophy. Additionally, IGF-1 administration reverses intestinal atrophy in rodent models of intestinal damage, such as radiation enteritis and severe burn injury, while IGF-1 transgenic mice exhibit reduced spontaneous apoptosis and irradiation-induced apoptosis of IE cells [247-250]. Interestingly, in numerous instances, IGF-1 administration mimics the intestinotrophic and functional effects observed with exogenously-administered GLP-2; specifically, GLP-2 and IGF-1 appear to overlap in their roles in intestinal adaptation following loss of mucosal function due to disease, bowel injury or resection.

Endogenous release of intestinal IGFs may be altered in disease states in which there is adaptive growth of the SI and LI. Adaptive hyperplasia is stimulated by luminal nutrients, circulating hormones, such as GLP-2 (as discussed above) and locally-produced growth factors, including the IGFs. Thus, intestinally-derived IGFs have been reported to act on the IE-IGF-1R in response to various forms of intestinal insult, including intestinal adaptation to fasting and re-feeding, resection, mucositis and IBD [201]. Several studies have further examined the association between fasting/re-feeding and endogenous IGF-1 and GLP-2 production [108, 223]. The data demonstrate that fasting and re-feeding in rats results in increased jejunal IGF-1 mRNA levels and this effect is temporally associated with restored intestinal mass and functional capacity. Furthermore, re-feeding-induced jejunal IGF-1 mRNA levels

are reduced when the GLP-2R antagonist, GLP-2^{3-33} is administered during the period of re-feeding [108]. A temporal association also exists between the GLP-2-induced expansion of putative intestinal stem cells and increased jejunal IGF-1 mRNA levels following ileo-cecal resection [242]. Furthermore, endogenous IGF-2 may increase intestinal adaptation following resection by stimulating crypt fission [251].

1.3.iv IGF-1 receptor signalling

Both IGF-1 and IGF-2 are ligands for the type 1 or IGF-1R, which is similar in structure and signalling to the insulin receptor (IR). The IGF-1R is a cell-surface, transmembrane receptor belonging to the growth factor family of receptors with intrinsic tyrosine kinase activity (Figure 1.5) [252, 253]. IGF-1R and IR are $\alpha_2\beta_2$ heterotetrameric stuctures composed of extracellular α-subunits, involved in ligand binding, and membrane-spanning β-subunits, with intrinsic tyrosine kinase activity. Encoded on chromosome 15, the IGF-1R is comprised of 21 exons, with exons 1-10 encoding the α-subunit and exons 12-21 the β-subunit [252, 253]. The IGF-1R precursor molecule is a 1367 amino acid protein that undergoes proteolytic cleavage resulting in separate α- and β-subunits linked by four disulfide bonds. Due to the highly homologous nature of the IR and IGF-1R α- and β-subunits, as well as the similar location of the disulfide bonds between individual αβ dimers, mature receptors in this family can present as hybrid receptors. Hence, cells that express both the IGF-1R and IR can produce hybrid receptors comprising the IGF-1R αβ dimer bound to the IR αβ dimer (including either the fetal/cancer IR-A isoform or the adult IR-B isoform). Importantly, IGF-1 can bind all of these hybrid receptors, whereas IGF-2 can only bind the IGF-1R/IR-A hybrid, and insulin has insignificant affinity for any of the hybrid receptors [254]. Moreover,

IGF-2 can also interact with the monomeric transmembrane mannose-6-phosphate receptor. The IGF-2R is believed to function mainly as a clearance receptor for IGF-2. However, reports have suggested that IGF-2 binding to IGF-2R can indirectly activate extracellular signal-regulated kinase (Erk) signalling through transactivation of sphingosine-1 phosphate receptors [255].

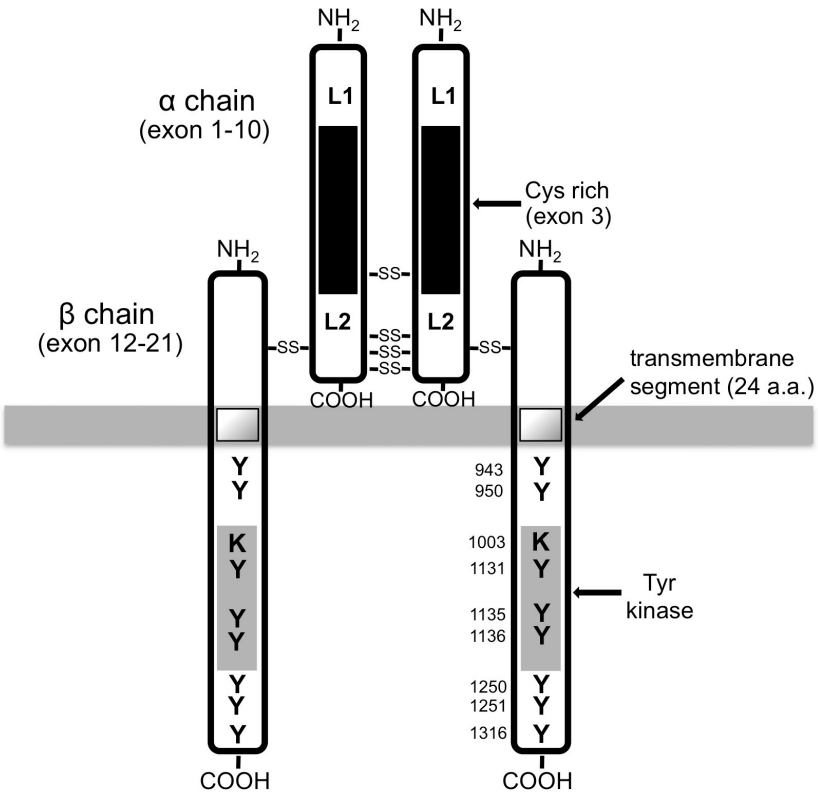


Figure 1.5 *Structure of human insulin-like growth factor-1 receptor (IGF-1R).* A member of the growth factor family of receptors with intrinsic tyrosine kinase activity, IGF-1R is a heterotetramer composed of two extracellular α-chains and two membrane-spanning β-chains. α-chains contain 2 large leucine-rich repeat domains (L1 and L2) that are separated by a cystine-rich domain, which is important for ligand binding and contains, including but not limited to, exon 3. β-chains contain the tyrosine kinase domain that is the site of autocatalytic phosphorylation. α- and β-chains are linked by 4 disulfide bonds. β-chain amino acid sequence numbers are indicated as well as the 24 amino acid transmembrane domain (gradient).

IGF receptors have been localized throughout the body and, more specifically, throughout the SI and LI, with receptor expression in both the muscularis and mucosa [201, 256]. Within the mucosa, the IGF-1R is found on both the apical and basolateral region of crypt and villus epithelial cells with increasing abundance in the proliferative crypt cells, and the highest density on the basolateral membrane of these IE cells [148, 257].

The ligand binding region of the IGF-1R is composed of three domains, the large leucine-rich repeat domain (L1), the cysteine-rich domain (CR) and the leucine-rich repeat domain (L2) [252, 253]. Upon ligand binding, tyrosine residues in the β-subunits of the IGF-1R undergo autocatalytic phosphorylation (e.g. Y^{1131}, Y^{1135}, Y^{1136}) [258, 259]. This results in further tyrosine kinase activity that phosphorylates tyrosine residues in the juxtamembrane Y^{943} and Y^{950}, and C-terminal Y^{1316} regions, thereby opening docking sites for signing molecules that contain a phosphotyrosine binding domain, including insulin receptor substrate (IRS)-1 to IRS4 and the Src-homology adaptor protein [260-264]. The possibility of heterodimerization of IGF-1R and IR adds further complexity and specificity to this signalling pathway by permitting/increasing association with alternate intracellular proteins. Nevertheless, IRS proteins are the major IR and IGF-1 signalling route, wherein they are phosphorylated by the receptor tyrosine kinase, leading to further availability of docking sites for downstream molecules such as PI3K and Grb2 [265-267]. Interestingly, various IRS isoforms have relatively important but different roles in growth and metabolism. For example, IRS1 KO mice have severe growth retardation with mild insulin resistance [268]; however, IRS2 null animals have severe metabolic disorder including insulin resistance and beta cell dysfunction leading to diabetes [269, 270]. Collectively, these studies reveal that IRS1 appears to be preferentially required for growth, whereas IRS2 mediates the metabolic actions of IGF-1 and IR signalling. In contrast, IRS3 KO does not confer any changes in phenotype, while IRS4 KO

results in only modest growth retardation and glucose intolerance [271, 272]. However, this does not negate their importance, as IRS proteins can play overlapping and complementary roles. For instance, double KO of IRS1 and IRS3 produces a synergistic effect resulting in severe lipoatrophy and diabetes [273].

The PI3K pathway involves the recruitment of the p85 regulatory subunit to IRS proteins, resulting in activation of the p110 catalytic subunit of the PI3K molecule [274]. As a result, PI3K converts phosphatidylinositol-(4,5)-bisphosphate to phosphatidylinositol-(3,4,5)-triphosphate ($PI(3,4,5)P_3$). Recruitment by $PI(3,4,5)P_3$ of pleckstrin homology domain-containing proteins, such as serine/threonine kinase Akt/protein kinase B (PKB), to the membrane then allows for the activation of this pleiotrophic growth and survival enzyme [275]. Hence, binding of $PI(3,4,5)P_3$ to Akt leads to its activation by 3-phosphoinositide-dependent kinase-1 (PDK-1)-mediated phosphorylation at T^{308}, as well as phosphorylation by an unknown enzyme at S^{473} [275].

Another integral component of IGF-1R signalling is the mitogen-activated protein kinase (MAPK)/ ERK pathway, which is synonymous with proliferation, transcription and survival. The MAPK signalling cascade is initiated by recruitment of Grb2 adapter protein and the GTP-exchange factor Sos by IRS or by Shc association with the IGF-1R [276]. Subsequent signalling involves sequential activation of the G protein Ras, Raf, MEK1/2, and then p44/p42 MAPK (ERK1/2). The serine/threonine kinases, p44/p42 MAPK, are activated through phosphorylation at T^{202} and Y^{204}, and are known to signal both transcriptional regulation and proliferation responses [277]. Thus, IGF-1R signalling, as induced by IGF-1 and/or -2, results in stimulation of cell growth pathways, such as differentiation, proliferation and survival.

1.3.v Clinical relevance of GH/IGF-1 axis mutations

The impact of the GH/IGF-1 axis on body growth is best emphasized through the study of GH/IGF mutations. First reported in 1966, *Laron* syndrome or growth hormone insensitivity syndrome (GHIS) presents as a result of GH receptor mutations, which cause postnatal growth defects that are partially reversible by treatment with IGF-1 [190]. Interestingly, IGF-2 deficiency results in delayed lung development associated with mal-organized alveoli and thicker alveolar septae [278]. However, generally, mutations to either IGF-1 or the IGF-1R cause pre- and postnatal growth restriction leading to low birth weight, no pubertal growth, deafness and craniofacial development defects; notwithstanding, there are no reported gastrointestinal (GI) tract abnormalities in such individuals [279].

Unlike the IR, which has many described mutations that lead to altered biological actions, there are only a few functional analyses of IGF-1R mutations [280, 281]. While IGF-1R point mutations are known to cause growth retardation in humans, studies using animal models of IGF-1R mutations also allow for examination of the role of this receptor in embryogenesis. For example, mice with Igf1r gene ablation display severe prenatal growth delay and high levels of mortality at birth due to respiratory failure [215]. However, heterozygous $Igf1r^{+/-}$ do not die at birth but have growth retardation, metabolic disorders and, interestingly, enhanced longevity [282]. Clinical observances of IGF-1R point mutations have been associated with significant reductions in IGF-1R phosphorylation and downstream signalling [253, 280, 281]. Consistent with this finding, a familial IGF-1R mutation involving a substitution of Arg for Gln at position 481 results in a structural alteration of the IGF-1R near the α-subunit disulphide bond and, subsequently, impaired activation of the intrinsic tyrosine kinase of the IGF-1R, thereby causing growth retardation [253]. Analysis of IGF-1/IGF-1R mutations in humans has thus furthered our understanding of the IGF signalling pathways

and their role in longitudinal growth. However, no study has thoroughly investigated the intestinal phenotype consequent to these mutations, with a resultant lack of implications for intestinal morphology and function.

1.4 Rationale and hypothesis

The insulin-like growth factors, IGF-1 and IGF-2, are related peptides that regulate growth in a wide variety of tissues through actions on the shared IGF-1R [140]. IGF-1 is expressed within the GI tract, primarily in ISEMFs and smooth muscle cells [141-144]. Furthermore, the IGF-1R is expressed on the apical and basolateral membranes of the SI epithelial cells [145]. Importantly, it has been established that: 1) GLP-2 and IGF-1 share many similar biological effects in the small intestine [81]; 2) treatment of fetal rat intestinal cells in culture with GLP-2 increases IGF-1 secretion and IGF-1 mRNA transcript levels, while administration of GLP-2 to mice increases the expression of IGF-1, but not of IGF-2, in the small intestine [58], and treatment of ISEMFs with GLP-2 induces a dose- and time-dependent increase in IGF-1R mRNA levels [10]; 3) while wild-type mice respond to GLP-2 treatment with increased intestinal weights and crypt cell proliferation, *Igf1*$^{-/-}$ mice are almost completely unresponsive to the intestinotrophic effects of GLP-2; in contrast, the proliferative actions of GLP-2 are not completely lost in *Igf2*$^{-/-}$ mice [95]; and 4) GLP-2 and IGF-1 both induce β-catenin translocation in crypt IE cells, and the effects of GLP-2 are prevented by prior administration of an IGF-1R blocker (NVP-AEW541) and are lost in *Igf1*$^{-/-}$ mice, while both GLP-2 and IGF-1 induce expression of the proliferative cWnt marker, c-Myc, and of the de-differentiation protein, Sox9, in the murine SI [10]. These observations identify both IGF-1 and the IGF-1R as essential mediators of GLP-2-induced SI proliferation, growth and cWnt

signaling**.** However, it is unknown whether the proliferative effects of GLP-2 in the SI are due to the direct action of IGF-1 on the IE-IGF-1R or if there are other paracrine and/or autocrine pathways involved.

Thus, the current thesis focuses on the role of the IE-IGF-1R in the intestinotrophic actions of GLP-2. However, the IGF-1R$^{-/-}$ mouse is perinatally lethal, which precludes such analyses in adult animals [54]. Furthermore, although use of global KO mice as an experimental model has advantages, such as relative ease of development, it also has disadvantages, including the inability to determine the role of the IGF-1R in specific cell types or organs. Thus, to surmount these problems, a novel, tissue-specific, inducible IE-IGF-1R KO mouse model was developed, in order to determine the requirement for the IE-IGF-1R in the intestinal growth effects of GLP-2. The hypothesis of these studies was, therefore, that ***small intestinal growth responses to GLP-2 occur through an IE-IGF-1R-dependent pathway***. The specific aims of this thesis were thus to determine:

i. the role of the IE-IGF-1R in the ***acute*** effects of GLP-2 on β-catenin signalling (Chapter 3);
ii. the role of the IE-IGF-1R in GLP-2's actions on intestinal growth in ***fasting and re-feeding*** (Chapter 4); and
iii. the role of the IE-IGF-1R in the ***chronic*** effects of GLP-2 on intestinal growth (Chapter 5).

CHAPTER 2

MATERIALS AND METHODS

Some of the text of this chapter has been submitted for publication:

1. K.J. Rowland, S. Trivedi, D. Lee, K. Wan, R.N. Kulkarni, M. Holzenberger and P.L. Brubaker, *Loss of glucagon-like peptide-2-induced proliferation following intestinal epithelial insulin-like growth factor-1 receptor deletion.* Gastroenterology, 2011. **Accepted, in press**.

Author contributions:

K.J. Rowland produced all text and tables within this chapter.

2.1 Animals

All animals were bred at the University of Toronto or purchased from Charles River Canada (CD1 and C57BL/6; St. Constant, PQ, Canada). IE-igf1rKO mice were generated by crossing *villin-CreER*$^{T2+/0}$ and *Igf1r*$^{flox/flox}$ mice [225, 228, 283], both on a C57BL/6 background; the resultant *villin-CreER*$^{T2+/0}$ x *Igf1r*$^{flox/+}$ offspring were then mated to *Igf1r*$^{flox/flox}$ mice to generate the *villin-CreER*$^{T2+/0}$; *Igf1r*$^{flox/flox}$ (IE-igf1rKO) animals (Figure 2.1a). Care was taken throughout to avoid female *villin-Cre-ER*^{T2} breeders in the F1 and F2 generations due to potential Cre excision from the maternal genome [284]. Age- and sex-matched littermate *villin-Cre-ER*$^{T2+/0}$;*Igf1r*$^{flox/flox}$ and *Igf1r*$^{flox/flox}$ (control) mice were used in all experiments, and *villin-CreER*$^{T2+/0}$ were also used in chronic studies. Female and male mice were combined in all experiments due to breeding times and limited N values for individual experiments. To localize Cre recombinase expression, LacZ/EGFP reporter mice (*Z/EG*, a kind gift from A. Nagy, University of Toronto [285]) were crossed with *villin-Cre-ER*$^{T2+/0}$ animals. Mice were genotyped as previously described [228, 283, 285], and were housed in a 12-hour light/dark cycle animal facility at the University of Toronto. All animal studies were approved by the University of Toronto Animal Care Committee.

Nuclear translocation of Cre recombinase was induced in mice aged 8-11 wk by daily intraperitoneal injections of tamoxifen (100 μL, 10 mg/mL suspended in ethanol and solved in sunflower oil; MP Biomedicals, Solon, OH) for 5 days [283]. Thirty-two hr after tamoxifen injection (at 8:30-9:30 am), some mice were fasted overnight in clean, individual cages before receiving an intraperitoneal injection of vehicle (phosphate-buffered saline (PBS)), Long-Arg^3-IGF-1 (LR^{3}IGF-1; 1 μg/g; media-grade human long-acting IGF-1 analog; GroPep, Adelaide, Australia) or h(Gly^2)GLP-2 (0.05 μg/g; a long-acting DPP-IV resistant

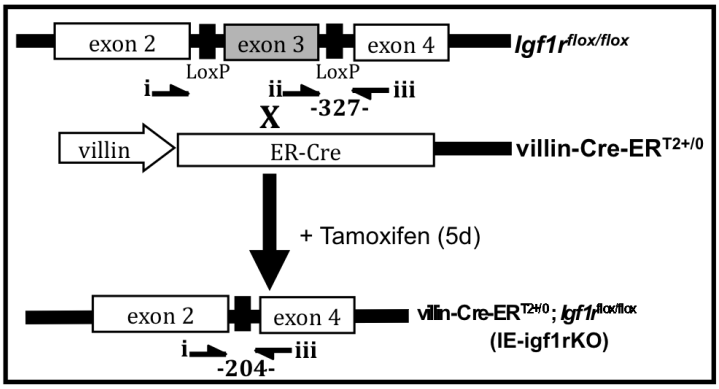


Figure 2.1 *Inducible tissue-specific deletion of IGF-1R in murine IE cells.* (a) Two rounds of breeding of *villin-Cre-ER*$^{T2+/0}$ and *igf1R*$^{flox/flox}$ mice generated *villin-Cre-ER*$^{T2+/0}$; *Igf1R*$^{flox/flox}$ (IE-igf1rKO) mice. Primers (i, ii, iii) were used to detect floxed and recombined *Igf1r* alleles in a triplex PCR reaction.

GLP-2 analog [84]; American Peptide Company; Sunnyvale, CA) at t = 0 min, followed by euthanasia at t = 30 – 90 min ('acute' studies). For 'fasting-re-feeding studies', animals were fasted by restricting access to standard chow for 24 hr. 'Fasted' mice were then sacrificed, while 're-fed' mice were subsequently allowed access to chow for 24 hr prior to euthanasia. 'Fed' mice were also individually housed but had free access to chow for 48 hr after the final tamoxifen injection and were then sacrificed. For 'chronic' studies, mice were subcutaneously injected with PBS (500 μL; q24hr), LR^3IGF-1 (4 μg/g; q12hr) or h(Gly^2)GLP-2 (0.1 μg/g; q24hr) for 10 days, with the final injection being 3 hr prior to sacrifice.

Mice were weighed prior to sacrifice, at which point a 1 cm^2 section of liver was collected and/or the luminal contents of the SI and LI were gently removed. The SI and LI were weighed, and 2 cm tissue segments from the jejunum and ileum and 1 cm from the colon were collected, immediately frozen and stored at -80°C for water, protein or RNA extraction, while separate tissue segments for morphometry and cell staining were immersed in 10% neutral buffered formalin and fixed for 24 hr before paraffin embedding and sectioning. A 1 cm section of jejunum was also immersed in O.C.T. Compound (Sakura Finetek, Torrance, CA), frozen in Cytocool II (Thermo Fisher Scientific, Nepean, ON) on dry ice, and stored at -80°C.

2.2 Laser capture microdissection (LCM) and PCR

Fresh-frozen jejunal sections were cut at 9 μm thickness and fixed in 70% ethanol, stained with HistoGene Staining solution (Arcturus Bioscience, Mt. View, CA), dehydrated in xylene and a graded-ethanol series, and then left to dry completely. Approximately 3,500 spots (7.5

μm diameter) of villus and crypt epithelium were collected by laser capture onto LCM caps (CapSure Macro LCM Caps, Arcturus Bioscience) using an Arcturus PixCell lle LCM system. Special care was taken to microdissect cells from well-oriented crypt-villus units only, as well as to exclude sub-epithelium from the burn spots. DNA was isolated from the captured cells using the PicoPure DNA Extraction Kit (Arcturus Bioscience) and PCR was performed as previously described, using the following primers to detect floxed and recombined *Igf1r* alleles in a triplex PCR reaction: 5'-CCATGGGTGTTAAATGTAATGGC-3'; 5'-ATGAATGCTGGTGAGGGTTGTCTT-3'; and 5'-ATCTTGGAGTGGTTGGGTCTGTTT-3' [71].

2.3 RNA isolation and real-time qRT-PCR

Tissues used for RNA extraction were liver, whole jejunum, and jejunal, ileal or colonic mucosa. Mucosal isolation was conducted by allowing frozen intestine to thaw briefly on ice, followed by longitudinal opening of the tissue and collection of the mucosal fraction by gentle scraping with a glass slide. Samples were lysed and total RNA was isolated using the RNeasy kit according to the manufacturer's instructions (Qiagen Inc., Mississauga, ON, Canada). RNA was quantified by spectrophotometry and then converted to cDNA by reverse transcription using Superscript II Reverse Transcriptase, according to the manufacturer's instructions (Invitrogen Life Technologies Inc., Burlington, ON, Canada). First-strand cDNAs were treated with ribonuclease H to remove RNA, and semi-quantitative RT-PCR (qRT-PCR) was performed using Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and monitored with MJ Opticon Monitor Analysis Software V 3.1 on the Chromo4 Continuous Fluorescence Detector (Bio-Rad Laboratories, Mississauga, ON, Canada). The TaqMan Gene Expression Assays (Applied Biosystems) used are listed in

Table 2.1. Relative mRNA expression levels were calculated using the ΔΔC(t) method [10] and all reactions were performed in duplicate. 18S ribosomal RNA was used as the endogenous control for all quantitative analyses [10].

Table 2.1 *TaqMan gene expression assays for real-time qRT-PCR*

Target	**TaqMan Assay**
18S	Hs99999901_s1
Amphiregulin	Mm00437583_m1
c-myc	Mm00487803_m1
Epiregulin	Mm00514794_m1
Erbb1	Mm01187858_m1
Erbb2	Mm00658541_m1
GLP-2R	Mm01329473_m1
HB-EGF	Mm00439307_m1
IGF-1	Mm00439559_m1
IGF-1R exon 2-3	Mm00802837_m1
IGF-1R exon 7-8	Mm00802841_m1
IGF-2	Mm00439564_m1
IRS1	Mm01278327_m1
Proglucagon	Mm00801712_m1
Sox9	Mm00448840_m1

2.4 Morphometry and cell staining

Crypt and villus height were measured on hematoxylin and eosin (H&E)-stained 4-μm jejunal cross-sections. An average of 39 well-oriented villi and 43 intact crypts from 2-4 cross-sections per mouse were scored in a blinded manner. Average mucosal cross-sectional area was determined by measuring the area enclosed by crypt-villi in 3 cross-sections per tissue segment.

IHC for the proliferative marker, Ki67, was performed using a rat anti-mouse Ki67 antiserum (Dako, Glostrup, Denmark) with a biotinylated mouse anti-rat secondary antiserum (Vector Laboratories, Burlington, ON, Canada). Negative staining in the absence of primary antiserum was confirmed (Appendix). For each mouse an average of 38 intact half-crypts from 2-3 cross-sections were analyzed. Cell positions 1-20 were counted per half-crypt with the base of the crypt designated as position 1. The incidence of Ki67-positive cells ('proliferative index') was determined by analyzing the relative number of labeled cells to total cells for each cell position.

TUNEL assay for apoptosis was performed following the manufacturer's protocol (Roche Diagnostics Corp., Indianapolis, IN). The total number of TUNEL-positive cells per half-villus was counted [10, 85]. Negative staining in the absence of enzyme solution was confirmed. For each mouse an average of 33 intact half-crypt+villi from 2-3 cross-sections were analyzed (Figure 2.2).

Synaptophysin and lysozyme immunofluorescence was conducted using a rabbit anti-human synaptophysin antiserum (1: 150 dilution, Dako), and a rabbit anti-lysozyme antiserum (1: 300 dilution; Dako), respectively, followed by a Cy3-linked donkey anti-rabbit IgG for synaptophysin and an Alexa488-linked goat anti-rabbit IgG antiserum for lysozyme.

The number of stained cells was counted in at least 20 half-villi per mouse. Negative staining in the absence of primary antiserum was confirmed (Appendix).

Mucin histochemistry with the Rapid Mucin Stain kit (Polysciences, Warrington, PA) was performed according to the manufacturer's instructions. The number of stained cells was counted in at least 20 half-villi per mouse.

Immunofluorescence analysis for nuclear localization of β-catenin in non-Paneth crypt cells was performed, as previously described [68]. In brief, mouse anti-β-catenin (1: 200 dilution; BD Biosciences, Mississauga, ON, Canada) and rabbit anti-lysozyme (Dako) antisera were used, with a biotinylated goat anti-mouse secondary antiserum coupled to streptavidin-horseradish peroxidase (1: 200 dilution; Vector Laboratories, Burlington, ON, Canada) with tyramide-Cy3 signal amplification (PerkinElmer, Waltham, MA), as well as an Alexa488-conjugated goat anti-rabbit secondary antiserum (1: 300 dilution; Invitrogen). Nuclei were visualized by staining for DNA with 4'-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Negative staining in the absence of primary antiserum was confirmed (Appendix). The quantification of nuclear localization of β-catenin was performed by counting the number of crypt cells with co-localization of β-catenin and DAPI, but excluding cells demonstrating the presence of the Paneth cell marker, lysozyme. For each mouse, the cells in an average of 32 intact half-crypts from 2-3 cross-sections were scored.

A Zeiss AxioPlan epifluorescence microscope was used to capture all digital images (Carl Zeiss, Canada, Don Mills, ON, Canada).

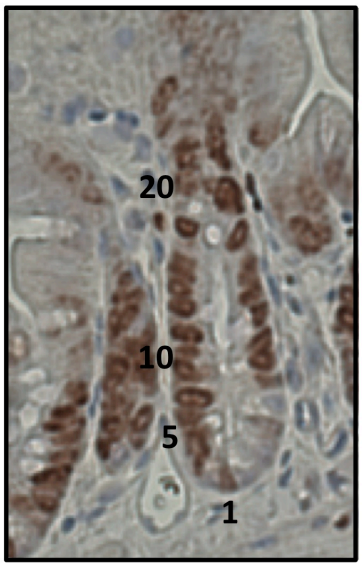


Figure 2.2. *Cell positional analysis of Ki67-positive cells in the SI crypt.* Positively stained cells are counted starting at the base of the crypt, which is cell position 1.

2.5 Immunoblotting

Jejunal mucosal scrapes were homogenized in ice-cold RIPA lysis buffer (50 mM ®-glycerol-phosphate, 10 mM Hepes pH 7.4, 1% Triton X-100 (v/v), 70 mM sodium chloride, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride and 1 complete mini EDTA-free protease inhibitor tablet (Roche Diagnostics)). The protein concentration of the supernatant was determined by the Bradford method (Bio-Rad). For western blot analysis to detect Ser^{473} phosphorylation of Akt, 75 µg of protein from each sample was added to 4X SDS-PAGE loading buffer (0.2 M Tris-HCl, 8% SDS w/v, 40% glycerol, 200 mM dithiothreitol, 0.01% bromophenol blue) and RIPA lysis buffer, boiled for 5 minutes at 95°C and run on an 8% SDS-PAGE gel. Proteins were then transferred to polyvinylidene difluoride membranes (Bio-Rad) at 4°C overnight. Following transfer, membranes were washed in Tris-buffered saline/0.1% Tween 20 (v/v) (TBST). The membranes were blocked in 5% non-fat dry milk (w/v) in TBST for 1 hr at room temperature, followed by a brief wash and overnight incubation at 4°C with primary antibodies in 0.1% TBST. Membranes were subsequently washed and incubated with appropriate horseradish peroxidase-linked secondary antibodies. Protein bands were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech). When appropriate, membranes were stripped by incubation in stripping buffer (62.5 mM Tris-HCl pH 6.7, 2% sodium dodecyl sulfate w/v, 100 mM β-mecaptoethanol) for 20 min at 50°C and re-probed. Primary antisera used were rabbit anti-phospho-Ser^{473}Akt (1: 1000 dilution, pAkt; Cell Signaling Technology, Danvers, MA) and rabbit anti-actin (1: 1000 dilution, Sigma-Aldrich). The secondary antibody was a horseradish peroxidase-linked anti-rabbit IgG (1: 2000 dilution, Cell Signaling).

2.6 Statistical analysis

All data are expressed as mean ± standard error. Some experimental data were expressed as log10 to normalize variance for statistical analysis. Results were analyzed by Student *t* test, or by one- or two-way analysis of variance (ANOVA), followed by Bonferroni *post hoc* analysis, as appropriate. *P* values of < 0.05 were considered to be statistically significant.

CHAPTER 3:

The IE-IGF-1R is required for acute GLP-2-induced small intestinal β-catenin and proliferative signaling

Some of the text of this chapter has been submitted for publication:

1. K.J. Rowland, S. Trivedi, D. Lee, K. Wan, R.N. Kulkarni, M. Holzenberger and P.L. Brubaker, *Loss of glucagon-like peptide-2-induced proliferation following intestinal epithelial insulin-like growth factor-1 receptor deletion.* Gastroenterology, 2011. **Accepted, in press**.

Author contributions:

S. Trivedi, D. Lee and K. Wan were undergraduate project students under the direct supervision of K.J. Rowland. R.N. Kulkarni and M. Holzenberger provided original mouse breeding pairs. All other work, data analysis and writing were complete by K.J. Rowland.

3.1 IGF-1 receptor deletion in IE-igf1rKO mice

To investigate the interactions between GLP-2 and IGF-1R signalling in a site-specific manner, an inducible IE cell-specific KO of exon 3 of the IGF-1R was generated. PCR analysis of IE cells collected by LCM demonstrated the presence of the excised gene fragment (204 bp) only in cells from IE-igf1rKO mice as compared to control (*Igf1r*$^{flox/flox}$) animals (Figure 3.1a). Densitometric analysis of the PCR products from 2 mice indicated ~85% recombination in IE cells from the IE-igf1rKO mice. Moreover, qRT-PCR for IGF-1R mRNA (exon boundaries (e) 2-3 and 7-8) in jejunal mucosa showed an 88.4 ± 9.0% decrease in IGF-1R (e2-3) transcripts in the KO mice relative to controls ($p<0.05$; Figure 3.1b). A trend towards a similar reduction in IGF-1R (e7-8) mRNA levels was also found in control and KO animals. Additionally, recombination was achieved in *villin-Cre-ER*$^{T2+/0}$*;Z/EG* reporter mice, as determined by the appearance of fluorescence in IE cells but not surrounding tissues (Figure 3.1c). Therefore, recombination in IE cells of the SI was found to be highly efficient in IE-igf1rKO mice following Cre induction.

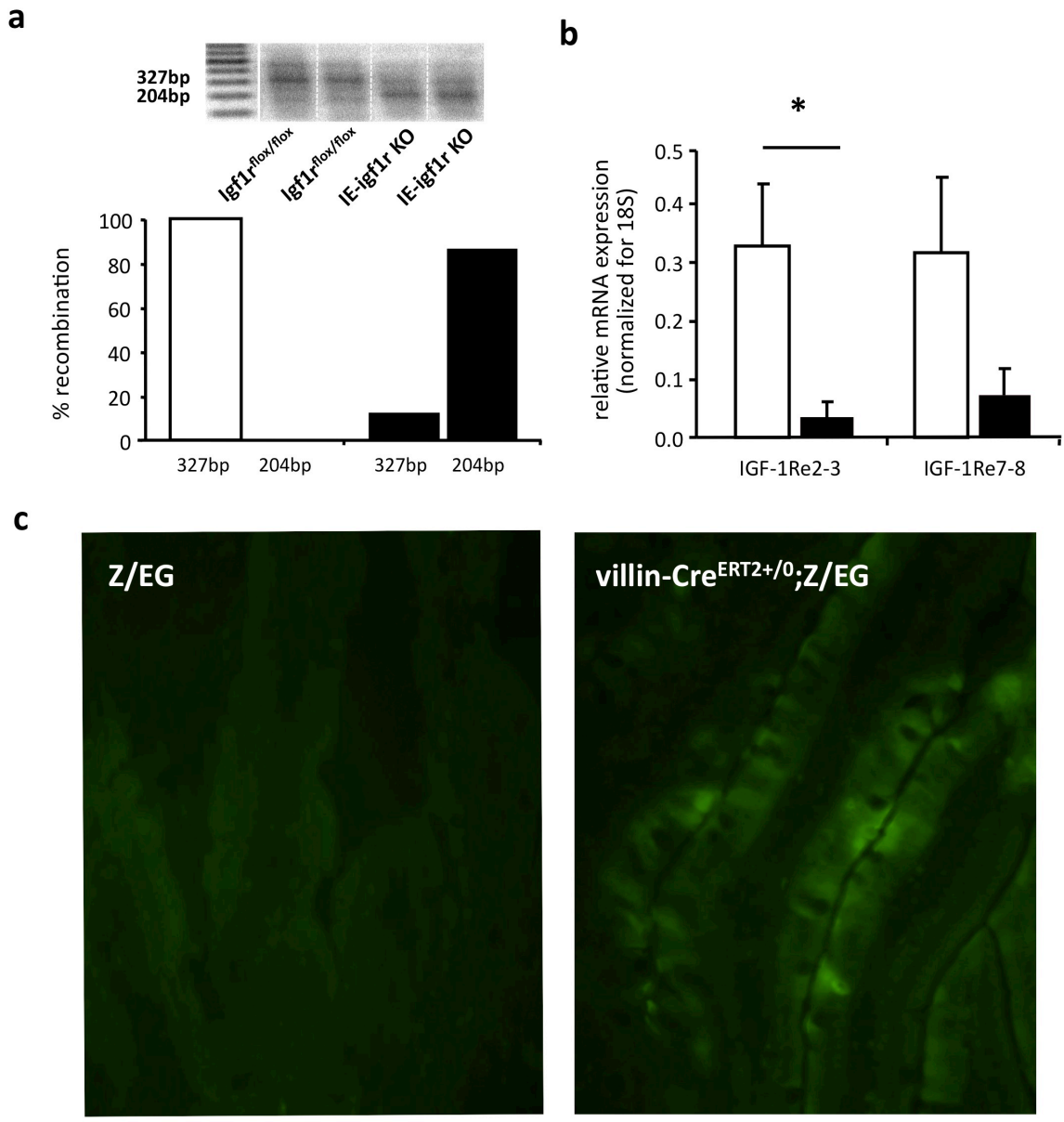
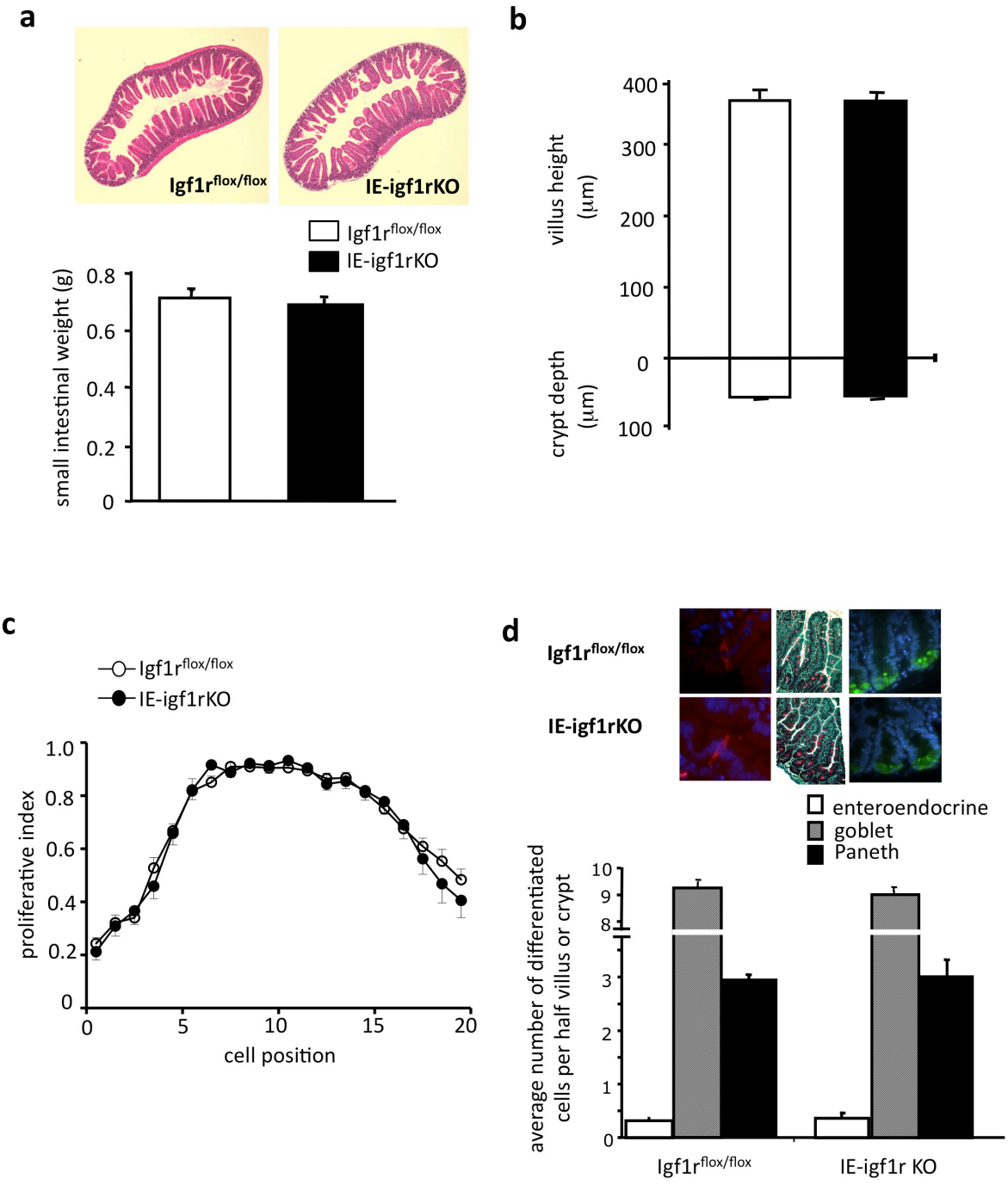


Figure 3.1 *Inducible tissue-specific deletion of IGF-1R in murine IE cells.* (a) Recombination percentage as determined by relative band intensities measured from ethidium bromide-stained gel. Samples were obtained from laser-captured jejunal IE cells of $Igf1r^{flox/flox}$ (control, open bars; n=2) and IE-igf1rKO (closed bars; n=2) mice and were subjected to PCR analysis using genotype-specific primers. (b) Relative qRT-PCR quantification of IGF-1R mRNA transcripts (exon boundaries 2-3 and 7-8) from jejunal mucosa of $Igf1r^{flox/flox}$ (open

bars; n=7) and IE-igf1rKO (closed bars; n=9) mice. (c) Representative sections of jejunum from tamoxifen-treated Z/EG, and villin-Cre$^{ERT2+/0}$;Z/EG mice. * $p<0.05$.

3.2 IE-igf1rKO mice display normal small intestinal characteristics

IE-igf1rKO mice were phenotypically normal, with no difference in body weight ($Igf1r^{flox/flox}$: 19.1 ± 0.89 g *vs.* IE-igf1rKO: 19.4 ± 0.73 g). Histological analysis revealed no gross alterations in crypt-villus architecture after IGF-1R knockout and no significant differences in SI weight, crypt depth or villus height, compared to littermate control mice (Figure 3.2a-b). The incidence of Ki67-positive cells, a marker of proliferation, did not differ between IE-igf1rKO and control mice, and was highest between cell positions 5-15 in both groups of animals (Figure 3.2c). We next determined whether loss of the IE-IGF-1R leads to altered IE cell lineages using specific markers of the differentiated cell types. The number of synaptophysin-positive enteroendocrine cells, mucin-positive goblet cells and lysozyme-positive Paneth cells was found to be comparable in KO and control animals (Figure 3.2d). qRT-PCR analysis was also carried out to assess baseline mRNA expression of several key factors known to be involved in GLP-2-induced SI proliferation. No significant differences were observed in transcript levels for IGF-1, GLP-2R, the ErbB ligands epiregulin, amphiregulin and HB-EGF, and the ErbB receptor ErbB1, in jejunal (Figure 3.2e), ileal (Figure 3.2f) and colonic (Figure 3.2g) mucosa of control and IE-igf1rKO mice. Reduced mRNA levels of proglucagon and ErbB2 were seen in ileal ($p < 0.05$), but not in jejunal and colonic mucosa of KO animals. Significant reductions in IGF-1R mRNA (e2-3 and e7-8) were found in jejunal, ileal and colonic mucosa. However, consistent with the specificity of the villin promoter for IE cells [283], no change in IGF-1R (e2-3) mRNA transcript levels were observed in the liver of IE-igf1rKO mice, nor were IGF-1 mRNA levels altered, compared to control littermates (Figure 3.2h). Therefore, the basal architecture of and growth factor expression in the SI of the IE-igf1rKO mice appeared to be normal.


a
Igf1r^flox/flox
IE-igf1rKO
Igf1r^flox/flox
IE-igf1rKO
small intestinal weight (g)
0.8
0.6
0.4
0.2
0
b
villus height (μm)
400
300
200
100
0
crypt depth (μm)
100
c
Igf1r^flox/flox
IE-igf1rKO
proliferative index
1.0
0.8
0.6
0.4
0.2
0
0
5
10
15
20
cell position
d
Igf1r^flox/flox
IE-igf1rKO
enteroendocrine
goblet
Paneth
average number of differentiated cells per half villus or crypt
10
9
8
3
2
1
0
Igf1r^flox/flox
IE-igf1r KO

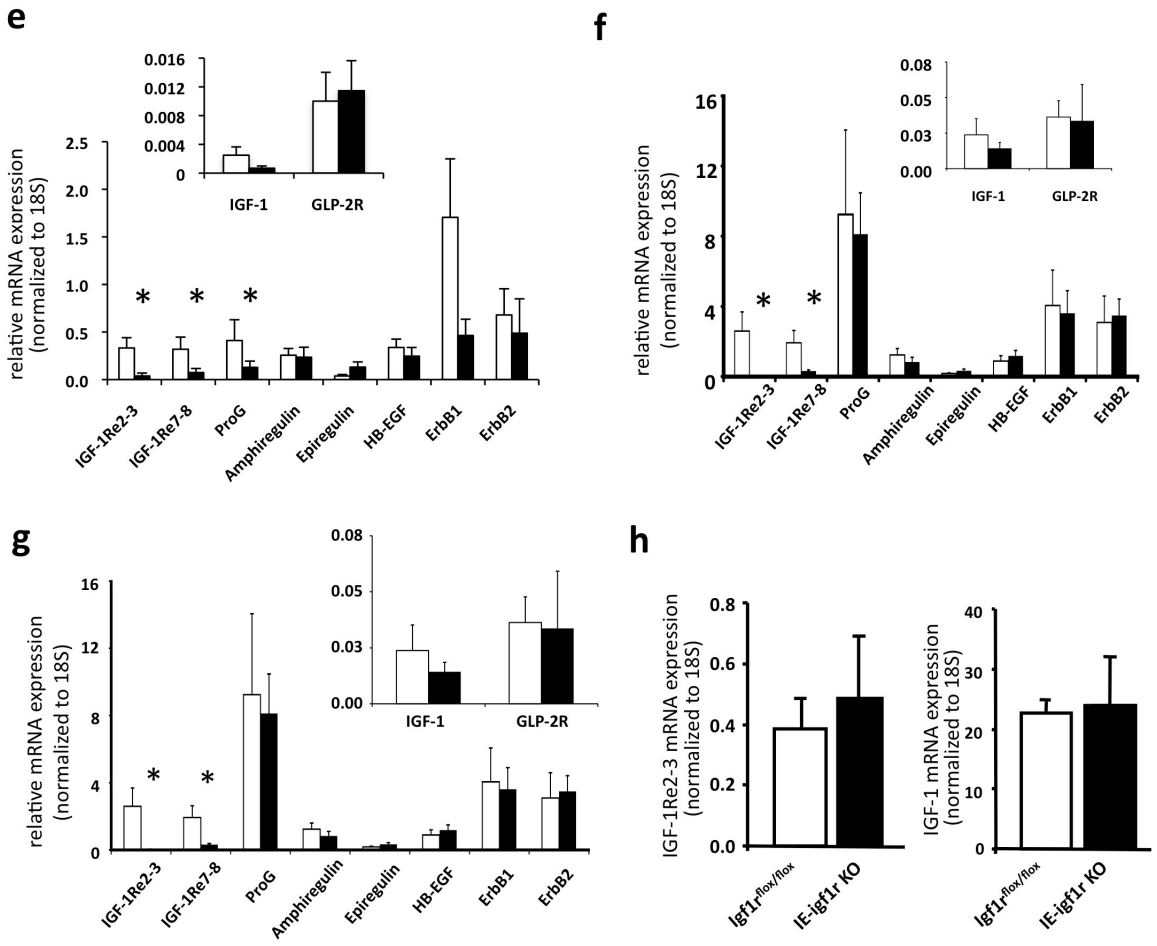


Figure 3.2 *Characterization of IE-igf1rKO mice under basal conditions.* Igf1r$^{flox/flox}$ (control) and IE-igf1rKO mice were fasted overnight, followed by examination of SI (a) weight (n=9, controls; n=15 KO), and (b) analysis of Ki67-labelled jejunal crypt IE cells from control (n=5) and IE-igf1rKO (n=10) mice. Position 1 designates the cell at the base of the crypt. (d) Quantification of differentiated IE cell types in control and IE-igf1rKO jejunum through analysis of synaptophysin- (open bars, n=4-6), mucin- (hatched bars, n=5), and lysozyme- (black bars, n=5-6) positive cells. (e-h) Expression of mRNA transcripts in control (open bars) and IE-igf1rKO (closed bars) mice was determined by qRT-PCR for IGF-1, proglucagon, GLP-2R, ErbB ligands (amphiregulin, epiregulin, HB-EGF) and ErbB receptors (ErbB1, ErbB2) in jejunal (e), ileal (f), and colonic (g) mucosa (n=6-9); and (h) IGF-1 and

IGF-1R at exon boundary 2-3 in liver (n=6). Expression was normalized for expression of 18S RNA.

3.3 Strain-specific differences between C57BL/6 and CD1 mice in response to acute administration of IGF-1

Preliminary studies indicated that commercially-available C57BL/6 mice did not respond to a dose of IGF-1 (e.g. 0.5 μg/g) that we had previously reported to increase IE cell P-Akt in CD1 animals [68]. Therefore, to determine a dose at which C57BL/6 mice do respond to IGF-1 treatment, fasted CD1 mice were treated for 30 min with either 0.75 μg/g IGF-1 or vehicle, while fasted C57BL/6 mice were treated for 30 min with either 0.75 μg/g IGF-1, 1.0 μg/g IGF-1 or vehicle. Acute signalling responses to IGF-1 treatment were then analyzed by examining activation of the cWnt signalling pathway via IHC for nuclear localization of β-catenin, and of the PI3K/Akt pathway via immunoblotting for phospho-Akt. CD1 mice treated with 0.75 μg/g IGF-1 displayed a slight but not significant activation of β-catenin signalling within the jejunal IE cells (Figure 3.3a) but significantly increased P-Akt (Ser473) relative to vehicle-treated controls (Figure 3.3b; $p<0.05$). However, C57BL/6 mice treated with the high (1.0 μg/g), but not the lower (0.75 μg/g) IGF-1 dose demonstrated significant increases in both nuclear localization of β-catenin and P-Akt in the jejunal mucosa ($p<0.05$). These findings demonstrated that a dose of 1.0 μg/g IGF-1 was sufficient to induce IE cell signalling in C57BL/6 mice.

3.4 Absence of the P-Akt response to IGF-1 treatment in IE-igf1rKO and control mice

To determine whether the acute signalling effects of IGF-1 treatment are lost in absence of the IE-IGF-1R, control and IE-igf1rKO mice were treated with 5 daily injections of tamoxifen (100 μL, 10 mg/mL), fasted overnight on the fifth day, and then treated with vehicle or 1.0 μg/g IGF-1 for 30 minutes. Unexpectedly, both control and KO mice

demonstrated no significant change in the phosphorylation (Ser^{473}) of Akt in response to IGF-1 treatment relative to vehicle-treated controls (Figure 3.3c). Further analyses of Akt signalling were therefore not conducted in the present study.

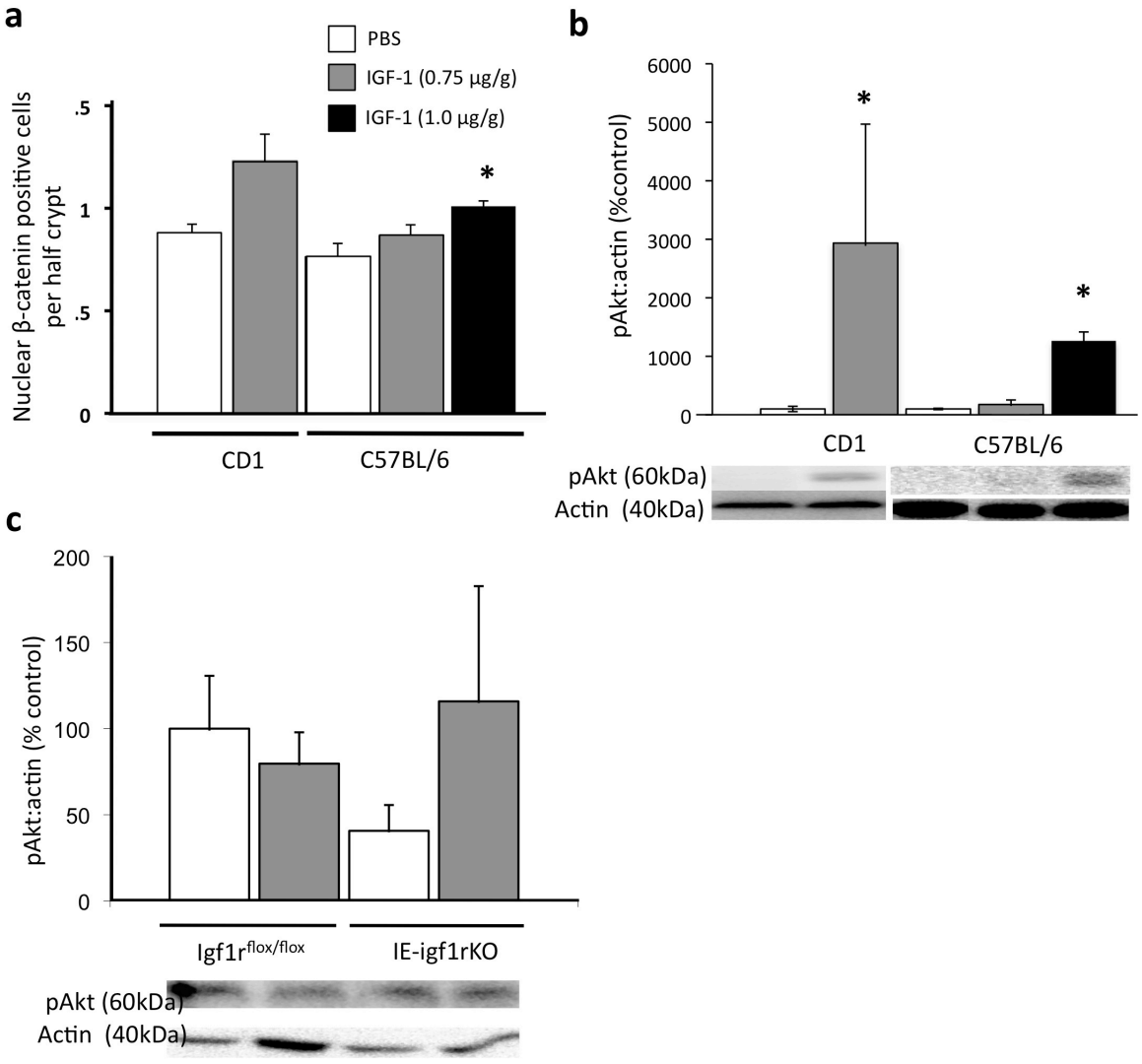


Figure 3.3 *β-catenin and PI3K/Akt signalling in response to IGF-1 in CD1, C57BL/6, control and IE-igf1rKO mice.* (a) Immunofluorescent staining for nulear localization of β-catenin in non-Paneth crypt cells in mouse jejunal sections of CD1 and C57BL/6 mice treated with vehicle (open bars) or IGF-1 at low (0.75 ug/g; gray bars) and high (1.0 ug/g; black bars) (n=4 each). (b and c) P-Akt protein levels of (b) normal CD1 and C57BL/6 (c) control and IE-igf1rKO mice, from jejunal mucosa treated with vehicle (open bars) or IGF-1 (closed bars; n=4-8). * $p<0.05$ as indicated

3.5 cWnt signalling is reduced in IE-igf1rKO mice following acute GLP-2 administration

We have previously shown that GLP-2 acutely activates β-catenin signalling in the SI crypt, using global, acute IGF-1R inhibition to demonstrate that this response requires IGF-1R signalling [68]. To examine the specific role of the IE-IGF-1R in GLP-2-induced β-catenin activation in intestinal crypt cells, IE-igf1rKO mice and controls were pre-treated with tamoxifen (as above), and then treated with h(Gly^2)GLP-2 (herein referred to as GLP-2) for 30 min, followed by analysis of nuclear localization of β-catenin in non-Paneth crypt cells (Figure 3.4a). Administration of GLP-2 to control mice significantly increased nuclear β-catenin in non-Paneth crypt cells, to 191.9 ± 28.7% of vehicle ($p<0.05$), consistent with previous reports [68]. Following deletion of the IGF-1R in IE cells, the ability of GLP-2 to induce β-catenin activation was abolished (Figure 3.4a). Analysis of β-catenin target gene induction by qRT-PCR similarly revealed that both c-Myc ($p< 0.05$) (Figure 3.4b) and Sox9 (Figure 3.4c) transcript levels were increased in control mice 90 min following GLP-2 administration. In contrast, basal c-Myc and Sox9 ($p<0.05$) mRNA levels were both upregulated in vehicle-treated IE-igf1rKO mice, but no further increase was observed in response to treatment with GLP-2.

Taken together, the results presented in this chapter are consistent with a role for the IE-IGF-1R in the actions of GLP-2 to induce cWnt signalling in SI crypt epithelial cells.

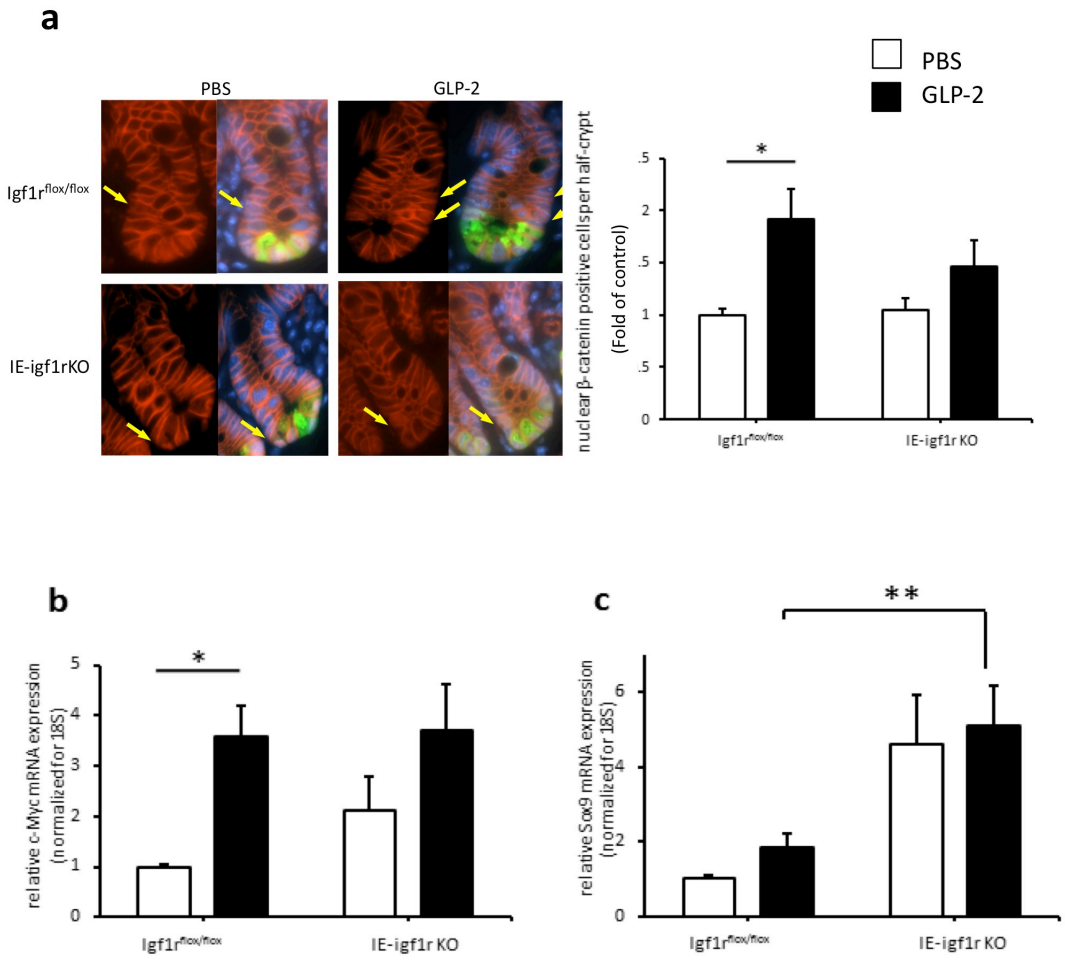


Figure 3.4 *β-catenin signalling in response to GLP-2 in IE-igf1rKO mice.* (a) Immunofluorescent staining for β-catenin (red) and lysozyme (green) in mouse jejunal tissue; DNA was stained with DAPI (blue). Quantification of nuclear β-catenin-positivity in non-Paneth crypt cells (indicated with the arrows in the representative photomicrograph) of control and IE-igf1rKO mice treated with vehicle (open bars) or GLP-2 (closed bars; n=6-8 each). (b and c) mRNA transcript levels of (b) c-Myc and (c) Sox9, normalized to 18S, in jejunal extracts of control and IE-igf1rKO mice treated with vehicle (open bars) or GLP-2 (closed bars; n=5-10 each)* $p<0.05$, ** $p<0.01$ as indicated.

CHAPTER 4:

IE-IGF-1R IS REQUIRED FOR FASTING AND RE-FEEDING-INDUCED SMALL INTESTINAL RE-GROWTH

Some of the text of this chapter has been submitted for publication:

1. K.J. Rowland, S. Trivedi, D. Lee, K. Wan, R.N. Kulkarni, M. Holzenberger and P.L. Brubaker, *Loss of glucagon-like peptide-2-induced proliferation following intestinal epithelial insulin-like growth factor-1 receptor deletion.* Gastroenterology, 2011. **Accepted, in press**.

Author contributions:

S. Trivedi, D. Lee and K. Wan were undergraduate project students under the direct supervision of K.J. Rowland. R.N. Kulkarni and M. Holzenberger provided original mouse breeding pairs. All other work, data analysis and writing were complete by K.J. Rowland.

4.1 IE-IGF-1R signalling is required for SI re-growth in the re-fed mouse

To determine if IE-IGF-1R signalling is required for GLP-2-dependent intestinal adaptation and re-growth during fasting and re-feeding, respectively, control and IE-igf1rKO mice were pre-treated with tamoxifen, and then either normally fed for 48 hr, fasted for 24 hr, or fasted for 24 hr followed by 24 hr of re-feeding. No significant differences were detected between the body weights (Figure 4.1a) and SI weights (Figure 4.1b) of control and IE-igf1rKO mice within any of the treatment groups. However, mice of both genotypes demonstrated a decrease in SI weight with fasting ($p<0.05$ for IE-igf1rKO animals), while re-feeding for 24 hr increased SI weight as compared to fasted mice of the same genotype ($p<0.05$-0.01). Similar trends were observed in mucosal cross-sectional area (Figure 4.1c). Jejunal and ileal dry weight was not different from control values within any of the treatment groups (Figure 4.1d, Figure 4.1e). Villus heights and crypt depths also did not differ between fed control and fed IE-igf1rKO mice, while fasting decreased crypt-villus height in mice of both genotypes ($p<0.05$-0.001; Figure 4.1f). In contrast, while re-fed control mice displayed an increase in villus height to 115.8 ± 4.7% relative of fasted control mice ($p<0.05$), re-feeding was not associated with increased villus height in IE-igf1rKO mice. Re-feeding did not affect crypt depths in mice of either genotype.

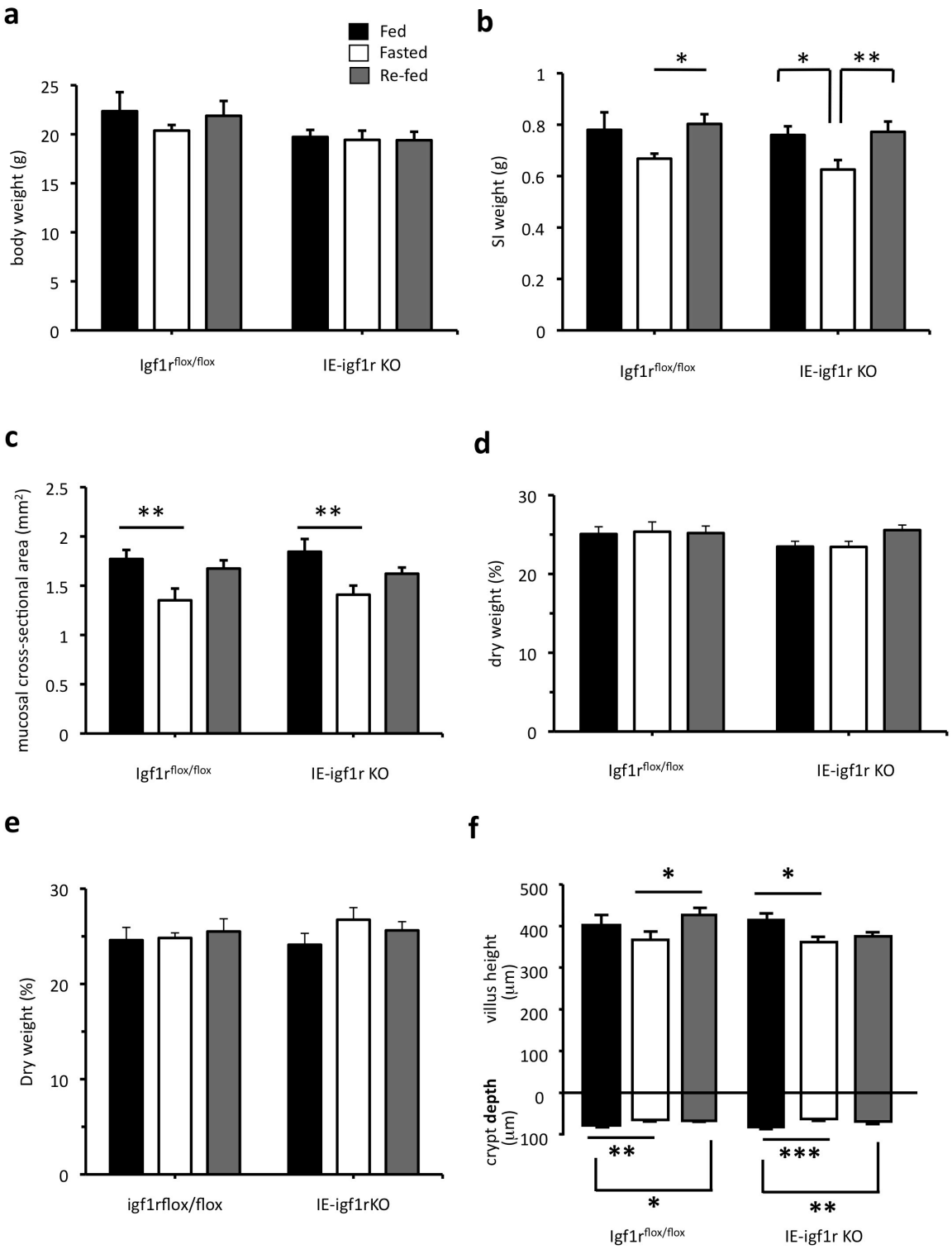
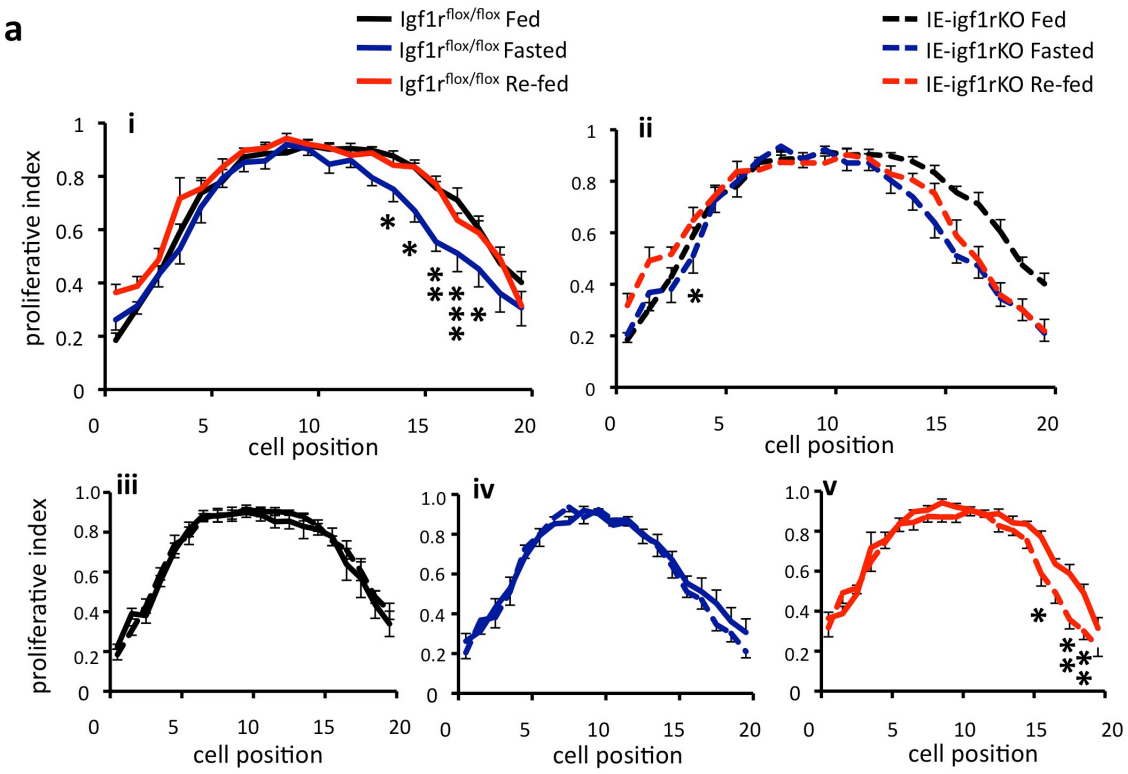
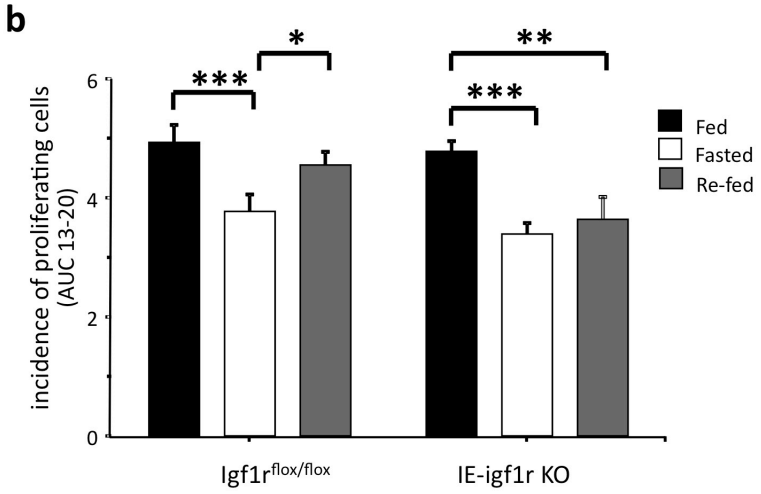

a
Fed
Fasted
Re-fed
body weight (g)
25
20
15
10
5
0
Igf1r flox/flox
IE-igf1r KO
b
SI weight (g)
1
0.8
0.6
0.4
0.2
0
*
*
**
Igf1r flox/flox
IE-igf1r KO
c
mucosal cross-sectional area (mm²)
2.5
2
1.5
1
0.5
0
**
**
Igf1r flox/flox
IE-igf1r KO
d
dry weight (%)
30
20
10
0
Igf1r flox/flox
IE-igf1r KO
e
Dry weight (%)
30
20
10
0
igf1rflox/flox
IE-igf1rKO
f
villus height (µm)
crypt depth (µm)
500
400
300
200
100
0
-100
*
*
**
***
*
**
Igf1r flox/flox
IE-igf1r KO

Figure 4.1. *Weights and morphometric analyses of fed, fasted and re-fed IE-igf1rKO mice.* Control and IE-igf1rKO mice were normally fed for 24 hr (closed bars; n=5-9), fasted for 24 hr (open bars; n=6-8) or fasted for 24 hr and then re-fed for 24 hr (gray bars; n=6-11), followed by determination of (a) body weight, (b) SI wet weight, (c) jejunal mucosal surface-area, (d) jejunal dry weight**,** (e) ileal dry weight, and (f) crypt depth and villus height, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ as indicated.

4.2 IE-IGF-1R signalling is required for SI proliferation in the re-fed mouse

To ascertain whether the failure to increase jejunal villus height during re-feeding in IE-igf1rKO mice was due to abnormal crypt cell proliferation, Ki67-positive jejunal crypt cells were quantified. In control mice, there was a small but non-significant decrease in proliferation in response to fasting, consistent with previous findings [85]. However, as reported [85], re-feeding was associated with an increase in Ki67-positive cells at crypt cell positions 13-17 ($p<0.05$-0.001; Figure 4.2a-i). This resulted in a significant increase in overall proliferation (Figure 4.2b; AUC for cell positions 13-20; $p<0.05$). In contrast, although fasted IE-igf1rKO mice demonstrated a similar trend to decreased proliferation with fasting, re-fed IE-igf1rKO mice exhibited an increase in proliferation only at cell position 4 ($p<0.05$; Figure 4.2a-ii), with no increase in overall proliferation (Figure 4.2b). Thus, although no differences in the distribution or number of proliferating cells were observed between fed IE-igf1rKO mice and fed littermate controls (Figure 4.2a-iii) or between fasting KO and control mice (Figure 4.2a-iv), only the control animals were found to demonstrate an adaptive proliferative response to re-feeding ($p<0.05$-0.01; Figure 4.2a-v). Furthermore, there was no difference in apoptotic TUNEL-positive cells in the intestinal epithelium between IE-igf1rKO and control mice in any of the feeding groups (Figure 4.2c). Collectively, therefore, these results indicate that IE-igf1rKO mice fail to demonstrate adaptive re-growth of the crypt-villus unit upon re-feeding after a fast and that this occurs in association with a dysregulation of crypt cell proliferation.


a
Igf1r flox/flox Fed
Igf1r flox/flox Fasted
Igf1r flox/flox Re-fed
IE-igf1rKO Fed
IE-igf1rKO Fasted
IE-igf1rKO Re-fed
i
ii
iii
iv
v
proliferative index
cell position


b
incidence of proliferating cells (AUC 13-20)
Fed
Fasted
Re-fed
Igf1r flox/flox
IE-igf1r KO

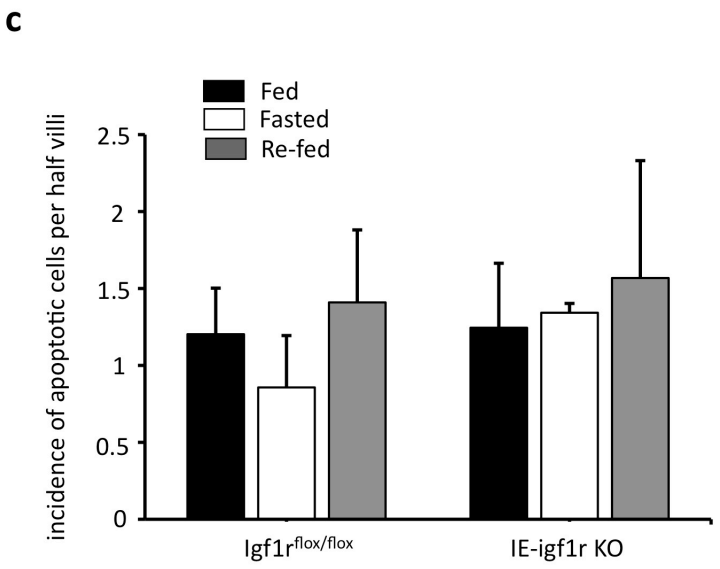


Figure 4.2. *Jejunal crypt cell proliferation and apoptosis in fed, fasted and re-fed IE-igf1rKO mice.* (a) Positional analysis of Ki67-labelled jejunal crypt IE cells from control (solid lines) and IE-igf1rKO (dashed lines) mice. Position 1 designates the cell at the base of the crypt. Mice were normally fed (black; n=7-9), fasted for 24 hr (n=7-8; blue) or fasted for 24 hr followed by 24 hr of re-feeding (n=7-11; red). The data shown in panels (i) and (ii) are reproduced in panels (iii-v) to allow direct comparisons between control and IE-igf1rKO mice. For clarity, significance is only reported for fasted vs. re-fed groups in panels (i) and (ii) (b) Total incidence of proliferating cells between cell positions 13-20 (AUC) for the data shown in (a). (c) The total number of TUNEL-positive apoptotic cells per half-villi in fed, 24 hr fasted, or 24 hr fasted followed by 24 hr re-fed mice (n=3). * $p<0.05$, ** $p<0.01$, *** $p<0.001$

CHAPTER 5:

THE IE-IGF-1R IS REQUIRED FOR CHRONIC GLP-2-INDUCED SI PROLIFERATION AND GROWTH OF THE CRYPT-VILLUS AXIS

Some of the text of this chapter has been submitted for publication:

1. K.J. Rowland, S. Trivedi, D. Lee, K. Wan, R.N. Kulkarni, M. Holzenberger and P.L. Brubaker, *Loss of glucagon-like peptide-2-induced proliferation following intestinal epithelial insulin-like growth factor-1 receptor deletion.* Gastroenterology, 2011. **Accepted, in press**.

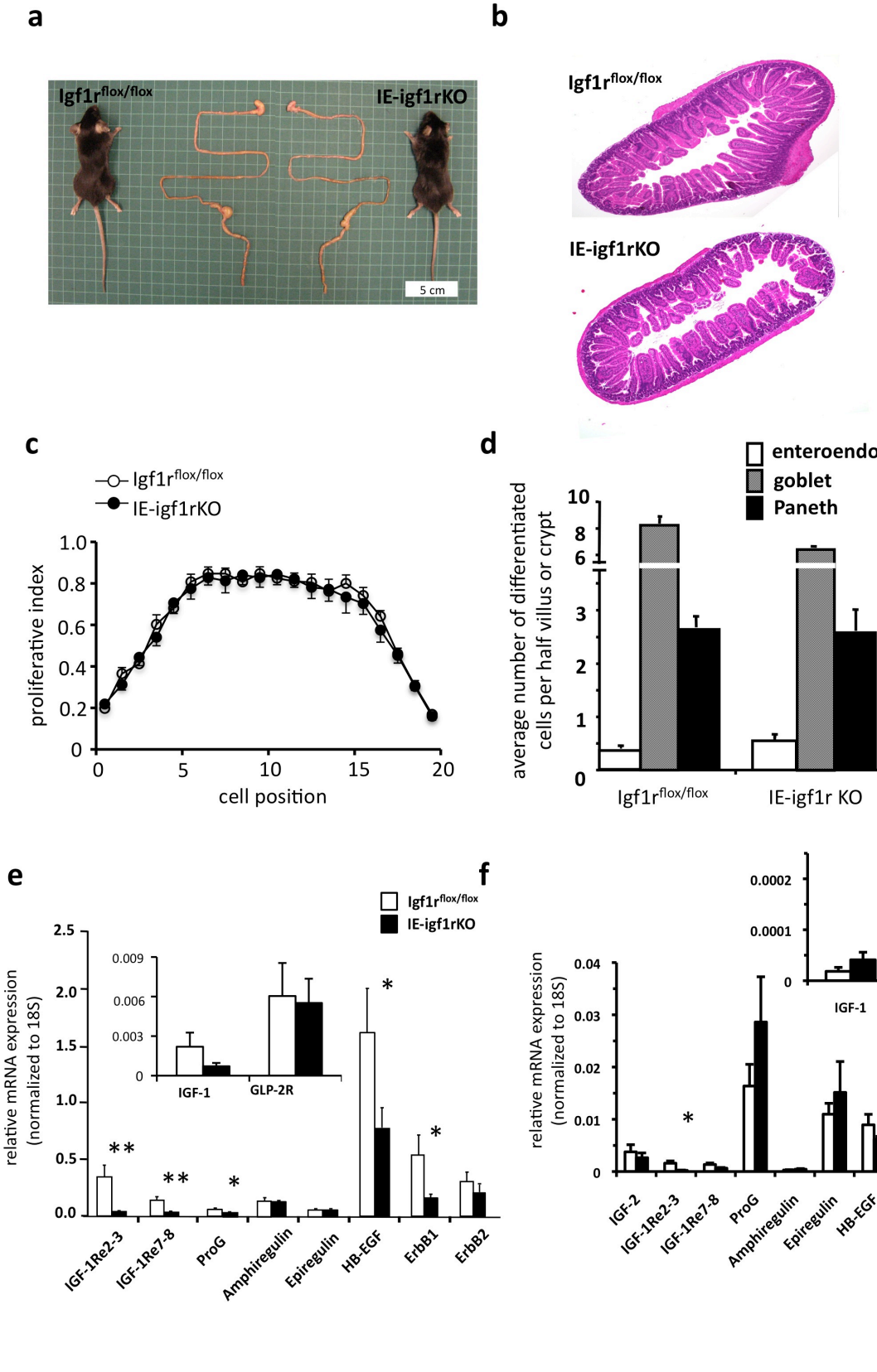
Author contributions:

S. Trivedi, D. Lee and K. Wan were undergraduate project students under the supervision of K.J. Rowland. R.N. Kulkarni and M. Holzenberger provided original mouse breeding pairs. All other work, data analysis and writing were complete by K.J. Rowland.

5.1 IE-igf1rKO mice display normal SI characteristics 10 d after tamoxifen induction

Ten days after pre-treatment with tamoxifen, IE-igf1rKO mice were phenotypically normal, with no difference in body weight ($Igf1r^{flox/flox}$: 17.6 ± 0.96 g *vs.* IE-igf1rKO: 18.8 ± 0.83 g). Histological analysis revealed no gross alterations in body, intestine and crypt-villus architecture after IE-IGF-1R KO, as compared to littermate control mice (Figure 5.1a-b). The incidence of Ki67-positive cells, a marker of proliferation, did not differ between IE-igf1rKO and control mice, and was highest between cell positions 5-15 in both groups of animals (Figure 5.1c). We next determined whether loss of the IE-IGF-1R leads to altered IE cell lineages using specific markers of the differentiated cell types. The number of synaptophysin-positive enteroendocrine cells, mucin-positive goblet cells and lysozyme-positive Paneth cells was found to be comparable in KO and control animals (Figure 5.1d). qRT-PCR analysis was also carried out to assess baseline mRNA expression of several key factors known to be involved in GLP-2-induced SI proliferation. No significant differences were observed in transcript levels for IGF-1, GLP-2R, the ErbB ligands epiregulin and amphiregulin, and the ErbB receptor ErbB2, in jejunal (Figure 5.1e) and ileal (Figure 5.1f) mucosa of control and IE-igf1rKO mice. Reduced mRNA expression of proglucagon, HB-EGF, and ErbB1 was observed in jejunal mucosa of IE-igf1rKO mice, as compared to controls, with no significant differences of these transcripts observed in the ileal mucosa between control and KO mice. However, as expected based upon the results of the acute studies on these animals (Chapter 3), significant decreases e2-3 and e7-8 of the IGF-1R transcripts were found in jejunal mucosa, as well as for e7-8 in ileal mucosa. Furthermore, the incidence of Ki67-positive proliferating crypt cells was increased at crypt cell positions 19 and 20 in IGF-1-treated control mice, as compared to IGF-1-treated IE-igf1rKO littermates ($p<0.05$; Figure 5.1g). Therefore, the basal architecture and differentiated cell

distribution of the SI appeared to be normal in the IE-igf1rKO mice. However, these mice demonstrated basal downregulation of proglucagon, HB-EGF, and ErbB1 mRNA expression in the jejunal mucosa. Furthermore, IE cell proliferative activity in response to IGF-1 treatment was reduced in IE-igf1rKO mice, as compared to control littermates.


a
Igf1r flox/flox
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c
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goblet
Paneth
average number of differentiated cells per half villus or crypt
Igf1r flox/flox
IE-igf1r KO
e
Igf1r flox/flox
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IGF-1Re2-3
IGF-1Re7-8
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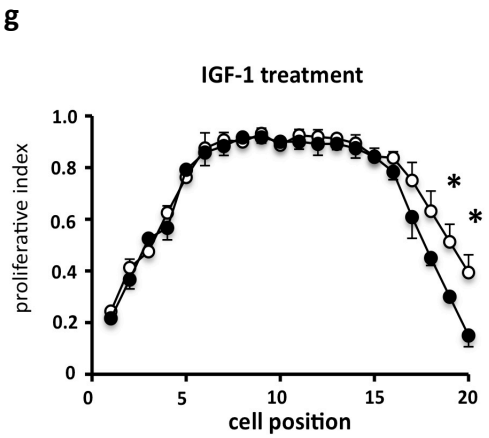


Figure 5.1 *Characterization of IE-igf1rKO mice 10 d after tamoxifen induction.* Igf1r$^{flox/flox}$ (control) and IE-igf1rKO mice (representative photomicrographs; a) were fasted overnight, followed by examination of SI (b) morphology (representative photomicrographs are shown), and (c) positional analysis of Ki67-labelled jejunal crypt IE cells from control (n=9) and IE-igf1rKO (n=8) mice. Position 1 designates the cell at the base of the crypt. (d) Quantification of differentiated IE cell types in control and IE-igf1rKO jejunum through analysis of synaptophysin- (open bars, n=4), mucin- (hatched bars, n=4), and lysozyme- (black bars, n=4) positive cells. (e and f) Expression of mRNA transcripts in control (open bars) and IE-igf1rKO (closed bars) mice was determined by qRT-PCR for IGF-1 (inset), IGF-2, proglucagon (ProG), GLP-2R (inset), ErbB ligands (amphiregulin, epiregulin, HB-EGF) and ErbB receptors (ErbB1, ErbB2) in jejunal (e) and ileal (f) mucosa (n=6-9). Expression was normalized for expression of 18S RNA. (g) Positional analysis of Ki67-positive jejunal crypt cells from control (n=4) and IE-igf1rKO (n=3) mice following chronic treatment with IGF-1. Cell position 1 designates the IE cell at the base of the crypt. * $p<0.05$, ** $p<0.01$ as indicated

5.2 Tamoxifen administration or Cre recombinase transgene insertion does not alter small intestinal growth responses to GLP-2

To determine if tamoxifen pretreatment and/or insertion of the villin-Cre-ER$^{T2+/0}$ transgene confers an altered response to GLP-2 in mice, villin-Cre-ER$^{T2+/0}$ (control) mice were pretreated with tamoxifen or vehicle followed by 10 d of treatment with GLP-2 or PBS. Compared to PBS controls, chronic administration of GLP-2 significantly increased jejunal weight ($p < 0.01$; Figure 5.2a), crypt depth and villus height ($p < 0.01$; Figure 5.2b), as well as increasing crypt cell proliferation (cell positions 16-20; $p < 0.05$; Figure 5.2c) in both the tamoxifen- and vehicle-pretreated mice. Furthermore, no significant differences were detected in the responses to GLP-2 between tamoxifen- and vehicle-pretreated mice. Hence, tamoxifen pretreatment did not significantly interfere with the effects of GLP-2 in the small intestine, and the *villin-Cre-ER$^{T2+/0}$* mice display appropriate intestinotrophic responses to GLP-2 treatment.

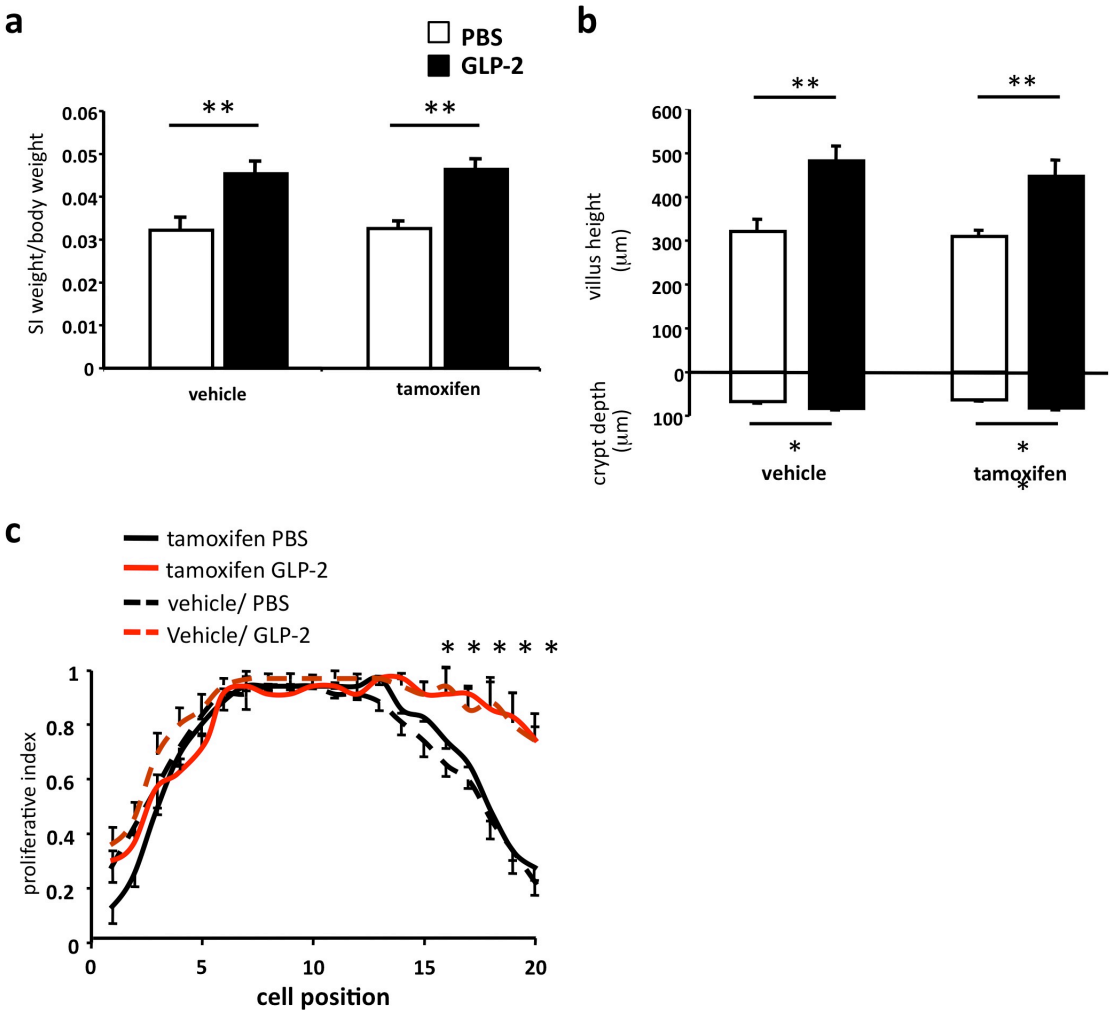


Figure 5.2 *Weights and morphometric analyses of villin-Cre-ER$^{T2+/0}$ mice.*

villin-Cre-ER$^{T2+/0}$ (control) mice were pretreated with tamoxifen or vehicle (oil/ethanol) for 5 d followed by treatment with 0.1 μg/g GLP-2 or PBS q24h for 10 d. Chronic administration of GLP-2 (closed bars or red lines) or PBS (empty bars or black lines) to vehicle-pretreated (as indicated or dashed lines) or tamoxifen-pretreated (as indicated or solid lines) villin-Cre-ER$^{T2+/0}$ mice was performed followed by analyses of (a) small intestinal weight normalized for body weight (n=4), (b) crypt depth and villus height (n=4), and (c) Ki67-labelling index of crypt cell proliferation (n=4). * $p<0.05$, ** $p<0.01$ as indicated.

5.3 Chronic GLP-2 administration does not change IGF-1 and IGF-2 mRNA expression levels in both control and IE-igf1rKO mice

qRT-PCR analysis was carried out to assess if KO of the IE-IGF-1R leads to altered IGF-1 and/or IGF-2 mRNA levels in the jejunal mucosa of mice treated with vehicle or GLP-2. Chronic GLP-2 treatment of control and IE-igf1rKO mice did not result in alterations of IGF-1 mRNA expression levels in jejunal mucosa, compared to vehicle-treated control mice (Figure 5.3a). No significant differences in basal IGF-1 or IRS1 mRNA levels were observed between control and KO mice. However, a significant effect of GLP-2 treatment was observed, such that IGF-1 expression levels were increased between treated control and IE-igf1rKO mice ($p<0.05$). No alterations in IGF-2 mRNA transcript expression were detected in mice of any treatment group or genotype (Figure 5.3b).

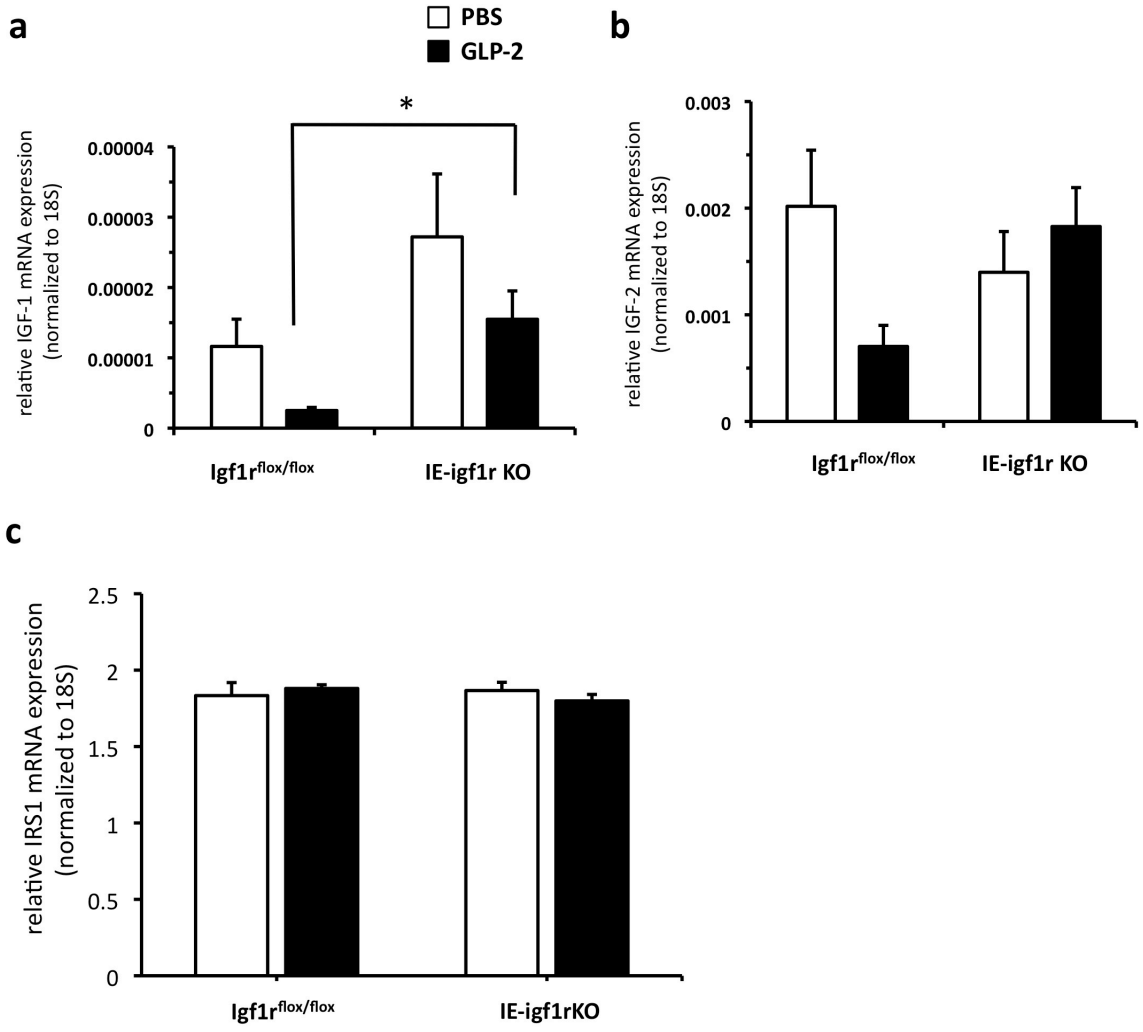


Figure 5.3 *IGF-1, IGF-2 and IRS1 mRNA expression in response to GLP-2 in control and IE-igf1rKO mice.* mRNA transcript levels of (a) IGF-1, (b) IGF-2 and (c) IRS1 normalized to 18S, in jejunal mucosa extracts of control and IE-igf1rKO mice treated with vehicle (open bars) or GLP-2 (closed bars; n=7-8 each) * $p<0.05$

5.4 IE-IGF-1R signalling is required for SI growth and proliferation in response to GLP-2

To assess whether IE-IGF-1R KO affected intestinal growth responses to GLP-2, adult, age-matched control and IE-igf1rKO mice were pre-treated with tamoxifen and then treated with vehicle or GLP-2 for 10 days. Administration of GLP-2 to control and IE-igf1rKO mice caused no significant changes in body weight (Figure 5.4a), but did result in a 21 ± 7 % (control mice) and a 16 ± 4 % (IE-igf1rKO mice) increase in SI weight (Figure 5.4b; $p < 0.05$), as compared to vehicle-treated control and KO mice, respectively. Mucosal cross-sectional area also increased in response to GLP-2 administration in both control and IE-igf1rKO mice, by 36 ± 10 % and 29 ± 4 %, respectively (Figure 5.4d; $p<0.01$). However, there were no changes in SI length in mice of any treatment group or genotype (Figure 5.4c). To further characterize the role of the IE-IGF-1R in the intestinotrophic effects in response to chronic GLP-2 administration, crypt depths and villus heights were measured (Figure 5.4e). Treatment with GLP-2 caused increases in crypt depths ($p < 0.05$) and villus heights ($p < 0.05$) in both control and IE-igf1rKO mice; however, the increase in both villus height and crypt depth was significantly reduced in GLP-2-treated IE-igf1rKO mice, as compared to GLP-2-treated control littermates ($p < 0.05$).

To determine whether the reduced villus height and crypt depth following GLP-2 treatment in IE-igf1rKO mice was associated with abnormal crypt cell proliferation, Ki67-positive jejunal crypt cells were quantified. Control mice treated with GLP-2 exhibited an increased incidence of proliferating cells at cell positions 18-20 in the proximal jejunum (Figure 5.5a; $p < 0.05 – 0.001$). Importantly, indicative of a requirement for the IE-IGF-1R in proliferative responses to GLP-2, Ki67 labeling indices in jejuna of IE-igf1rKO mice were

not increased by GLP-2 at any crypt cell position (Figure 5.5b). The results observed in the cell positional analysis are further demonstrated by analysis of the AUC for the proliferative responses, such that there was an 25 ± 8 % increase in overall proliferation (AUC cell position 15-20) following administration of GLP-2 in control mice, whereas there was no increase in total proliferation in GLP-2-treated IE-igf1rKO animals (Figure 5.5c; $p<0.05$). Collectively, these results demonstrate that IE-igf1rKO mice exhibit reduced growth of the crypt-villus unit following chronic administration of GLP-2 and that this occurs in association with a dysregulation of crypt cell proliferation.

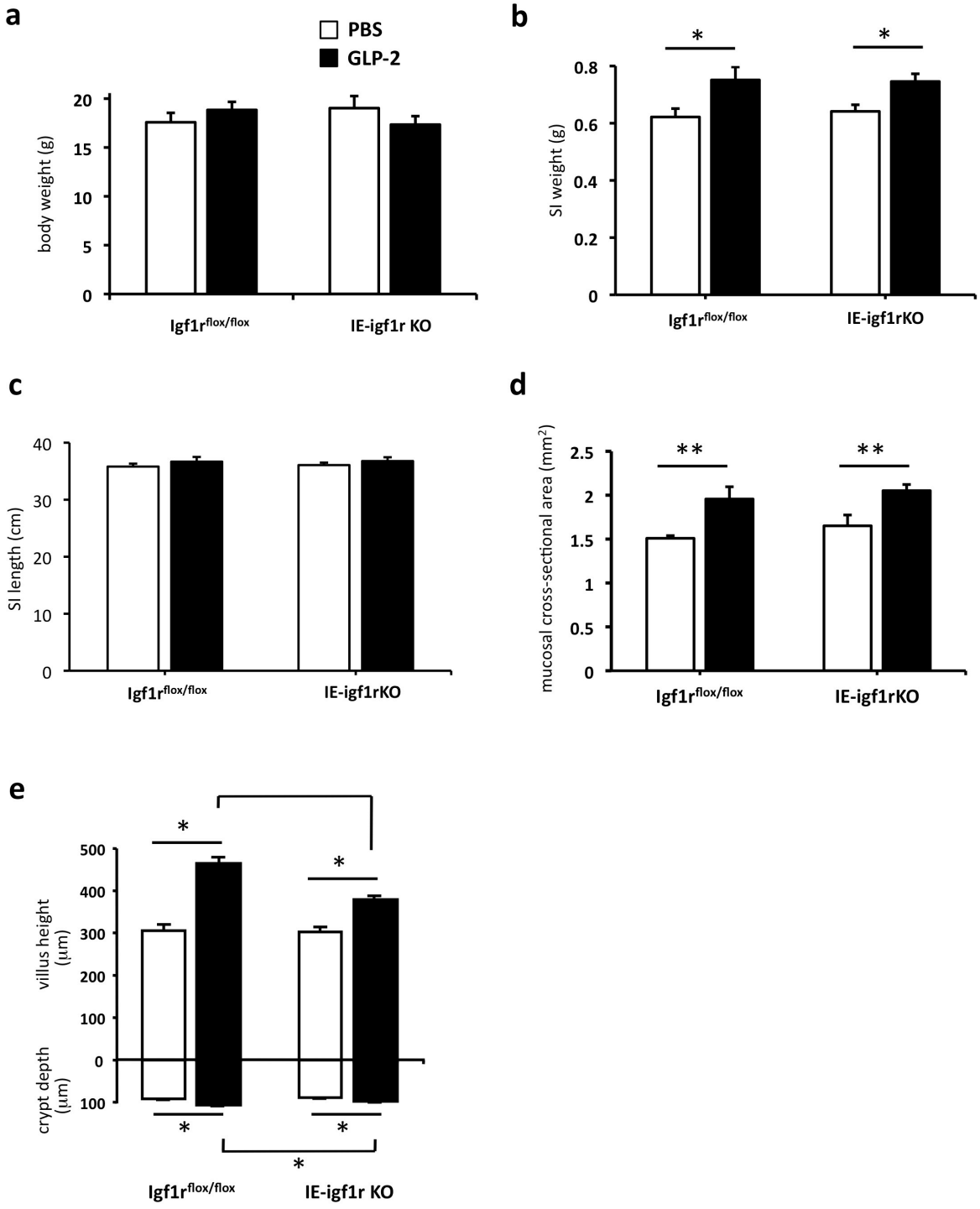


Figure 5.4 *Weights and morphometric analyses of Igf1r$^{flox/flox}$ and IE-igf1rKO mice.* Control and IE-igf1rKO mice were treated with a 0.1 μg/g dosage of GLP-2 (closed bars) or vehicle (open bars) q24h for 10 d, followed by determination of (a) body weight (n=8-10), (b) SI

weight (n=8-10), (c) SI length (n=7-9), (d) jejunal mucosal cross-sectional area (n=7-9), (e) crypt depth and villus height (n=7-10), * $p<0.05$, ** $p<0.01$ as indicated

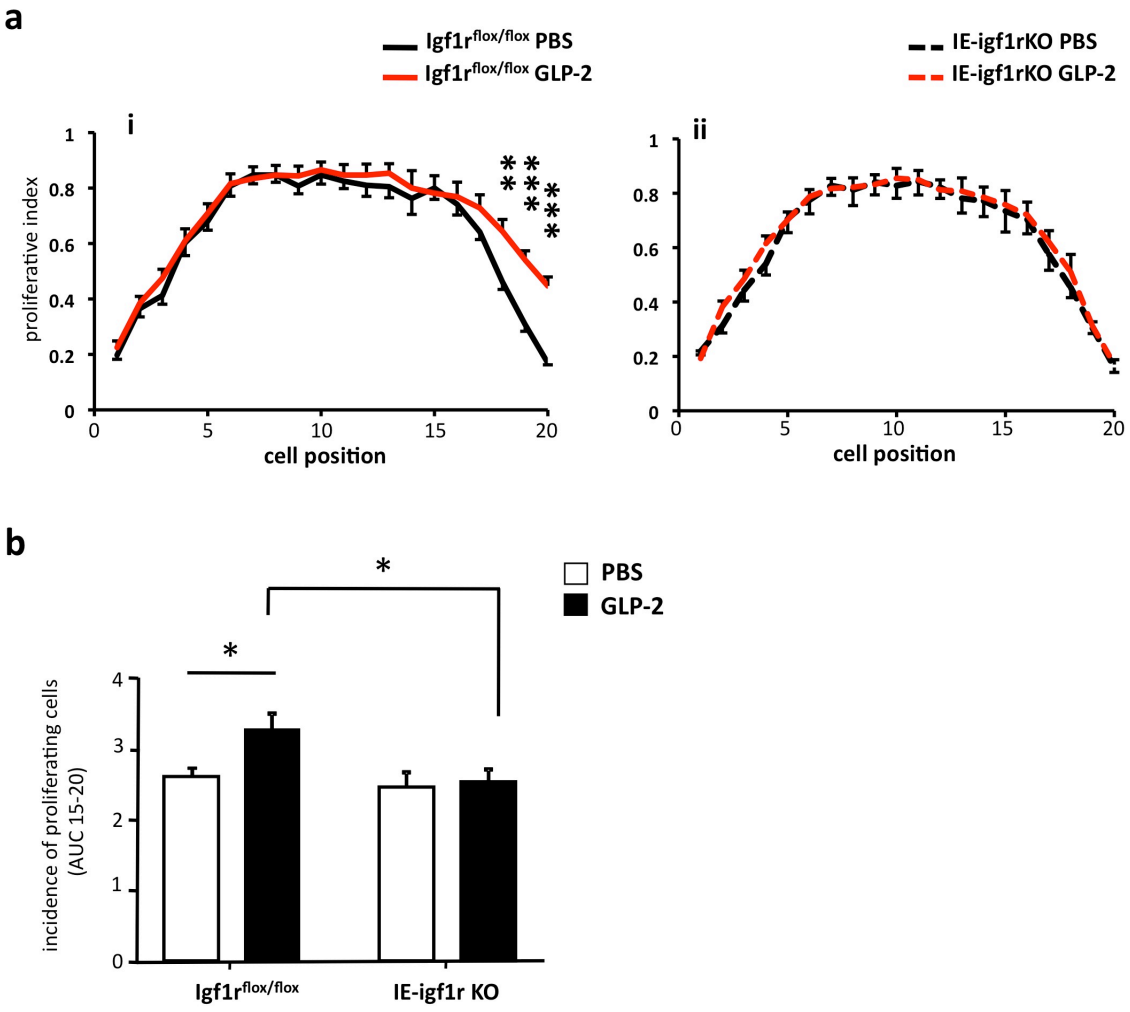


Figure 5.5 *Jejunal crypt cell proliferation in control and IE-igf1rKO mice.* (a) Positional analysis of Ki67-labelled jejunal crypt IECs from control (solid lines) and IE-igf1rKO (dashed lines) mice. Position 1 designates the cell at the base of the crypt. Mice were administered with vehicle (black; n=8-9) or GLP-2 (0.1 μg/g; red; n=8-10) for 10 d following tamoxifen induction. The data shown in panel (i) represents control animals alone and (ii) displays IE-igf1rKO mice of both vehicle- and GLP-2-treatment groups. (b) Total incidence of proliferating cells between cell positions 15-20 (AUC) for the data shown in (a). * $p<0.05$, ** $p<0.01$, *** $p<0.001$

CHAPTER 6

DISCUSSION

Some of the text of this chapter is reproduced from:

1. Rowland, K.J. and P.L. Brubaker, *Life in the crypt: a role for glucagon-like peptide-2?* Mol Cell Endocrinol, 2008. **288**(1-2): p. 63-70.
2. Rowland, K.J. and P.L. Brubaker, *The 'cryptic' mechanism of glucagon-like peptide-2.* Am J Physiol Gastrointest Liver Physiol, 2011. **301**(2): G1-8.
3. K.J. Rowland, S. Trivedi, D. Lee, K. Wan, R.N. Kulkarni, M. Holzenberger and P.L. Brubaker, *Loss of glucagon-like peptide-2-induced proliferation following intestinal epithelial insulin-like growth factor-1 receptor deletion.* Gastroenterology, 2011. **Accepted, in press**.

Author contributions:

K.J. Rowland produced all text and figures within this chapter.

6.1 General summary

One of the prominent features that makes GLP-2 an attractive growth-promoting agent is its highly intestinal-specific effects. The trophic actions of GLP-2 predominate in the mid SI and are less pronounced in the colon due to decreased expression of the GLP-2R in the distal intestine [5, 124]. Recent advances in clinical trials involving GLP-2 necessitate elucidation of the exact signalling pathways by which GLP-2 acts. It is now well established that GLP-2 is linked to a complex network of indirect mediators that induce diverse signalling pathways. Not only does this raise the question as to what and where are the indirect mediators that GLP-2 acts through in terms of its intestinotrophic effects, but what specific cell types are these downstream mediators, such as IGF-1, acting on to elicit GLP-2-mediated intestinal effects. Hence, our understanding of the downstream mechanistic pathway(s) underlying GLP- 2's intestinotrophic actions remains incomplete. Therefore, it is important to delineate the downstream mediators and signalling pathways of GLP-2, both in a temporal and spatial manner.

In this thesis, I have addressed the role of IE-IGF-1R signalling in the acute, the adaptive and the chronic growth responses to GLP-2. In Chapter 3, the important role of the cWnt pathway, β-catenin and IGF-1 in IE cell proliferation was addressed, demonstrating a novel relationship between GLP-2 and the IE-IGF-1R. In addition to the acute proliferative responses to exogenously-administered GLP-2, the endogenous actions of GLP-2 and the role of IE-IGF-1R signaling were explored in Chapter 4 through the use of a fasting and re-feeding adaptive gut model. Furthermore, Chapter 5 was directed towards the chronic intestinotrophic effects of GLP-2, as we sought to examine how the IE-igf1rKO mouse responds within this chronic setting. Specifically, the results indicate that the acute, adaptive and chronic intestinotrophic effects of GLP-2 are dependent upon the IE-IGF-1R, such that

crypt cell proliferation and the resultant increase in crypt-villus height were not induced by GLP-2 in the IE-igf1rKO mouse. Cumulatively, these studies demonstrate for the first time an association between the SI crypt cell proliferative responses to GLP-2 and the IE-IGF-1R signalling pathway.

6.2 Utilizing an inducible, tissue-specific KO mouse model to study the mechanism of action of GLP-2

The localization of the intestinal GLP-2R to enteroendocrine cells, enteric neurons and ISEMFs reflects the indirect mechanism(s) that underlie the actions of this hormone on the IE cells [5-9]. Our proposed model of a role for the IE-IGF-1R in GLP-2-mediated SI proliferation is similarly supported by the localization of the components of the IGF system in the intestine. Hence, IGF-1R protein expression has been demonstrated on the apical and, most importantly, basolateral membranes of crypt and villus IE cells [148, 286]. In addition, IGF-1 has been localized to the ISEMFs that underlie the IE cells [148, 196, 243, 287, 288]. Moreover, we have recently shown that GLP-2 not only stimulates IGF-1 secretion from fetal rat intestinal cell cultures, which presumably include ISEMF but also increases IGF-1 mRNA transcript levels in both these cells and ISEMFs in vitro [10, 129]. Finally, consistent with our hypothesis, the Igf1 null mouse is unresponsive to the trophic effects of GLP-2 [10], while whole-body administration of an IGF-1R antagonist prevents the acute effects of GLP-2 on IE cell β-catenin signalling [68]. However, the interpretation of results using global KO mice is limited when examining the role of a mediator such as IGF-1 that is produced both systemically (e.g. by the liver) and locally, preventing identification of the origin of the IGFs. Moreover, long-term adaptation can potentially occur in animals with genetic deletion from conception. Similarly, as the IGF-1R is expressed in multiple tissues, identification of the specific cell

types that are responsible for the altered phenotype is precluded in studies involving administration of a receptor antagonist to the whole animal. I therefore developed the IE-igf1rKO mouse as a novel, tissue-specific inducible model to circumvent these limitations [289].

6.3 Validation of the IE-igf1rKO mouse

Several approaches were taken to prove tissue-specific inducible KO of the IE-IGF-1R. First, successful excision of the Igf1r-exon 3 gene fragment was confirmed in laser capture microdissected IE cells, demonstrating 80% recombination efficiency at the genomic level. Additionally, the IGF-1Re2-3 and IGF-1Re7-8 primers were used to compare mRNA transcript levels for the excised part of the gene against a part of the gene that was not manipulated, respectively. It was shown that IGF-1Re2-3 mRNA transcript levels were significantly lower in the IE-igf1rKO mice than in control littermates, with only ~20% of control mRNA expression found in the jejunal mucosa of acutely-induced animals (Chapter 3), and a further decrease to ~10% of the levels detected in the jejunal mucosa of chronically-treated mice (Chapter 5). Of note, while IGF-1Re7-8 mRNA levels in jejunal mucosa were not significantly reduced in the IE-igf1rKO mice in the acute setting ($p=0.08$), they did show a marked decrease. Furthermore, in the chronic studies, this decrease became statistically significant, suggesting that the creation of a non-functional mRNA transcript following the excision of exon 3, possibly due to degradation of the interrupted transcript by mRNA surveillance pathways [290]. Moreover, other studies using the $Igf1r^{flox/flox}$ mice crossed to achieve gene KO have also confirmed that altered exon 3 transcripts do not contribute to an mRNA signal. Finally, of some note, the excision of the Igr1r appeared to be cell-specific, based upon previous reports of the specificity of the villin promoter [283] , the finding of GFP

only in the IE cells of the villin-Cre x Z/EG animals, and the observation that the reduction in exon 3 levels was not as great when cells in addition to the IE were included in the laser capture.

Given the strong decrease in mRNA levels, in principle there should also be a significant decrease in IGF-1R expression at the protein level. Attempts to visualize the IE-IGF-1R via IHC resulted in very low signal strength in some tissue sections and no anti-IGF-1R-positive staining in other tissue sections (Appendix). Previous localization of the IGF-1R protein through IHC was performed successfully on SI sections of rats following prolonged fasting [148] or massive small bowel resection [291], suggesting that such approaches are necessary to induce protein expression for visualization. Nonetheless, a functional confirmation of diminished proliferative activity in IE-igf1rKO mice following IGF-1 administration was observed. Analysis of the incidence of Ki67-positive proliferating crypt cells indicated increases at cell positions 19 and 20 in IGF-1-treated control mice, compared to IGF-1-treated IE-igf1rKO littermates.

6.4 IE-igf1rKO mice appear phenotypically normal

Given the known importance of IGF-1 and the IGF-1R to whole-body growth [215, 292], it was somewhat surprising that no abnormalities were detected in the basal growth parameters of the SI in IE-igf1rKO animals. However, this observation is consistent with similar findings of normal or even enhanced SI weight (relative to body weight), crypt-villus height and proliferative index in mice lacking IGF-1 or IGF-2, the known IGF-1R ligands [10]. Similarly, the SI of the *Glp2r* knockout mouse demonstrates normal weight and architecture under basal conditions [107]. Finally, no major intestinal abnormalities have been reported in humans with inactivating mutations in either IGF-1 or the IGF-1R [279]. Relevant to the current villin-Cre

model, is the finding that villin is also expressed in cells of the proximal metanephric and proximal tubules of the kidney, as well as ductuli efferentes, paradidymis and epoophoron of the gonads [293], where IGF-1R is present. Although the IE-igf1rKO mice did not appear to have gross kidney or gonadal alterations, these tissues were not morphologically or functionally analyzed. Collectively, these findings indicate that neither GLP-2 nor the IGF-1/IE-IGF-1R signalling pathway are essential for normal intestinal epithelial homeostasis.

6.5 Tamoxifen and interactions with systemic IGF system

In order for the induction of gene KO to occur, IE-igf1rKO and control mice were treated with tamoxifen (100 μL; 10 mg/mL) daily for 5 d prior to experimental protocols. Tamoxifen is an anticancer drug, widely used for treatment of ER-positive breast cancers through partial antagonistic actions on the ER. Inducibility of the villin-Cre-ER$^{T2+/0}$ mice is mediated via the fusion of the coding sequence for Cre recombinase to that of the ligand binding domain of a mutant ER that specifically recognizes tamoxifen. Binding of tamoxifen to the Cre-ER fusion protein results in translocation of the tamoxifen/Cre-ER complex from the cytosol into the nucleus. Although any potential interactions between tamoxifen and proglucagon or GLP-2 has not been reported, several studies have reported interactions between tamoxifen and members of the IGF system, using various models. The administration of tamoxifen in vivo was inhibited IGF-1 mRNA expression in liver and lung, and inhibited GH output, as a consequence of a pituitary-independent effect of tamoxifen. In addition, rats displayed a 25% decline in serum IGF-1 levels following administration of 5 mg tamoxifen for two d [294, 295]. IGFBP-4 and -6 serum levels were also found to increase following tamoxifen treatment (20 mg/day for 4 months) of breast cancer patients [296].

In the current study, the experiments in Chapter 3 were conducted in an acute setting,

where mice were sacrificed 24-26 h following the final tamoxifen injection. Furthermore, in Chapter 4, experiments were performed 24-48 h after the final tamoxifen injection, such that there may still be interactions with the IGF-1-IGF-1R axis in the gut and, perhaps, with circulating IGF-1 levels. There has been recent attention given to potential toxicity of Cre expression and tamoxifen administration associated with the Cre/lox methodology [297]. Interestingly, one report demonstrated that tamoxifen-induced Cre genotoxicity is present in gastric epithelial cells, but not IE cells [298]. Furthermore, Cre expression in the pancreatic β-cell has been reported to alter cell function [299, 300]. Despite having also injected control animals with tamoxifen, this short duration between the final tamoxifen injection and the initiation of acute experiments in Chapters 3 and 4 may present underlying effects on the endogenous IGF system and, hence be a limitation in these studies. Notwithstanding, the findings of Chapter 5, which were conducted 10 d after the final tamoxifen injection, were consistent with those of Chapters 3 and 4, suggesting that tamoxifen and/or Cre toxicity may not have been an issue in the present studies.

6.6 Strain-specific differences in the intestinal responses to IGF-1 administration

It is well known that the two widely-used experimental mouse strains CD1 and C57BL/6 vary in coat colour; furthermore, CD1 mice are out-bred, while C57BL/6 mice are inbred. More generally, there are common defects that are observed in pure backgrounds, as compared to mice of hybrid backgrounds. For example, C57BL/6 mice have greater lean body mass, as well as higher susceptibility to diet induced obesity and lower susceptibility to tumor formation and progression [301-303]. Hence, to compare studies performed in the C57BL/6 IE-igf1rKO mice with existing data in the literature, any differences in the relevant signalling pathways between the CD1 and C57BL/6 mouse strains must be known. Hence,

for the studies in Chapters 3 and 5, it was necessary to determine the dose of IGF-1 to which normal CD1 and C57BL/6 mice exhibit acute responses in the SI epithelium. I found that neither CD1 nor C57BL/6 mice showed a significant increase in crypt cell nuclear localization of β-catenin with a 0.75 μg/g dose of IGF-1. In contrast, IGF-1 null mice on a CD1 background required only 0.5 μg/g IGF-1 to activate this pathway, whereas a dose of 1.0 μg/g IGF-1 was necessary in the present study for C57BL/6 mice. Similarly, analysis of Ser^{473} Akt phosphorylation revealed that while a 0.75 μg/g IGF-1 was sufficient to increase P-Akt levels in the SI mucosa of CD1 mice, a higher, 1.0 μg/g dose of IGF-1 was required in C57BL/6 mice. Thus, it was determined that a 1.0 μg/g dose of IGF-1 would be utilized for further studies in mice on a C57BL/6 background.

Therefore, IE-igf1frKO and control ($Igf1r^{flox/flox}$) mice, both on a C57BL/6 background, were treated with 1.0 μg/g IGF-1 and the acute signalling responses to IGF-1 administration were determined. Surprizingly, although control mice responded to IGF-1 treatment with appropriate changes in β-catenin nuclear localization, these same mice failed to respond to this dose of IGF-1 with an increase in P-Akt in the jejunal mucosa. Further study is required to determine if the activity of other downstream signalling molecules are changed in these mice. Nonetheless, as C57BL/6 mice are a widely-used strain, the information acquired in this study may be important for future studies involving IGF-1 treatment of genetically-engineered C57BL/6 mice.

6.7 Sexual dimorphism-related alterations in body weight of IE-igf1rKO mice

Given the key role of IGF signalling in regulating tissue and organ growth during postnatal development, several results in this study bring to light interesting correlations between altered body weight and deletion of various components of the IGF system in female mice. It

is well known that the somatotrophic axis is sexually dimorphic in mammals [292]. While no significant changes were seen in body weight between control and IE-igf1rKO mice of any treatment group, including normally fed, 24 h fasted and 24 h re-fed animals (Chapter 4), or mice treated chronically with vehicle or GLP-2 (Chapter 5), this was likely due to similar numbers of males and females within groups. In chronic GLP-2 studies, female IE-igf1rKO mice weighed on average 16.3 g, whereas in male IE-igf1rKO mice, the average body weight was 20.4 g. However, the substantial differences in body weight between males and females was not observed in terms of SI weight, therefore indicating a slightly larger SI weight relative to whole body weight in the females. Hence, SI weight was not expressed in the present study as a percentage of body weight. Conversely, villin-Cre-ER$^{T2+/0}$ control mice did not present with striking differences in body weight between genders and, as such, SI weight was expressed per body weight in Figure 5.2a, as is normally done for studies of this nature [10, 107, 138]. Studies examining postnatal growth responses in IRS1-deficient mice also reported that absolute or partial IRS1-deficiency mediated body overgrowth to a lesser extent in male than female mice [152]. Similarly, male liver-specific IGF-1 KO mice and control mice did not have any alterations in body weight, whereas female liver-specific IGF-1KO mice demonstrated a non-significant decrease in body weight, as compared to female control mice [191]. It is interesting to note that induction of this liver-specific IGF-1 KO was mediated through interferon treatment and is thus unrelated to exogenous estradiols, such as tamoxifen. Furthermore, male, but not female liver-specific IGF-1R KO mice had defective liver regeneration, and control female Igf1r$^{flox/flox}$ mice had considerably fewer Ki67-positive hepatocytes than males [228]. Testosterone has also been implicated in increasing GH receptor mRNA levels in the epiphyseal growth plate, another rapidly proliferating tissue [304]. Exactly how these findings of sexual dimorphism may transcend to other mammals and, more

specifically, to the interactions of GLP-2 with the IGF-1-IGF1R pathway, remain to be determined.

6.8 GLP-2-induced β-catenin signalling is reduced in IE-igf1rKO animals

Induction of cWnt/β-catenin signalling in the intestinal crypt epithelium causes proliferation and differentiation through the activation of various transcription factors [47]. Previously, exogenous GLP-2 administration has been shown to activate cWnt signalling in the mouse SI, as evidenced by increased β-catenin nuclear localization in non-Paneth crypt IE cells and enhanced mRNA expression of the cWnt target genes, c-Myc and Sox9 [68]. Furthermore, the actions of GLP-2 on β-catenin nuclear localization were found to be IGF-1R-dependent, as they were abrogated by acute administration of a global IGF-1R inhibitor [68]. The results of the present study confirm and extend these findings by the demonstration that GLP-2 induces a two-fold increase in nuclear β-catenin localization in non-Paneth crypt cells of control animals, and this effect is lost in IE-igf1rKO mice. Furthermore, GLP-2 treatment was unable to increase c-Myc or Sox9 expression in IE- igf1rKO animals. Interestingly, a trend to higher basal levels of c-Myc and Sox9 was also noted in the mutant mice. Although the change was not statistically significant, this observation suggests the possibility of a compensatory response to the loss of the IGF-1R in IE cells. Consistent with this notion, basal levels of the IGF-1R docking protein, IRS1, are increased in hepatocytes from liver-specific Igf1r null animals [228], and IRS1 has been reported to enhance c-Myc gene expression, at least in mouse embryonic fibroblasts [305]. However, real-time PCR confirmed that jejunal mucosal IRS1 mRNA levels were not different between control and IE-igf1rKO mice under basal conditions. Moreover, recent studies have demonstrated that cross-talk occurs in the intestinal

epithelium between cWnt/β-catenin and Notch signalling [306]. The cWnt and Notch pathways control IE cell fate decisions, particularly proliferation and differentiation at both the tissue and cellular level. The unexpected upregulation of c-Myc and Sox9 in IE-igf1rKO could be due to compensation of other molecular signalling pathways, such as Notch, since it is known that Sox9, mouse atonal homolog1 (Math1) and recombining binding protein suppressor of hairless (RBP)-J all have roles in IE cell fate decisions and are regulated by Notch signalling [307-309]. An additional explanation for this unexpected finding could be related to decreased expression of select members of the ErbB network in IE-igf1rKO mice, which could be inhibitory or stimulatory on other downstream signalling molecules. Indeed, microarray analysis has revealed that IGF-1R activation results in up-regulation of the mitogen, HB-EGF in 3T3-L1 adipocytes [310], which corresponds to the chronic ileal mucosa data presented in Figure 5.1d. Further studies are needed to understand how IE cells may compensate following KO of the IE-IGF-1R, through signalling pathways, such as Notch and ErbB.

6.9 IGF-1R — ErbB interactions

In Chapter 3, acute administration of GLP-2 to IE-igf1rKO mice demonstrated that there is a partial dependence between GLP-2 and the IE-IGF-1R, as there was a slight but non-significant increase in β-catenin/cWnt signalling in response to treatment with GLP-2. This finding provides a key mechanistic understanding on the role of the IE-IGF-1R in GLP-2-induced proliferative responses; however, it also suggests that other proliferative pathways may be involved in the acute mechanism of action of GLP-2. In light of recent data by Yusta *et al.*[139] and Bahrami *et al.*[108] that ErbB ligand – ErbB signalling pathway is also involved in

the acute proliferative actions of GLP-2, this strongly suggests the possibility of receptor-receptor interactions [107, 138].

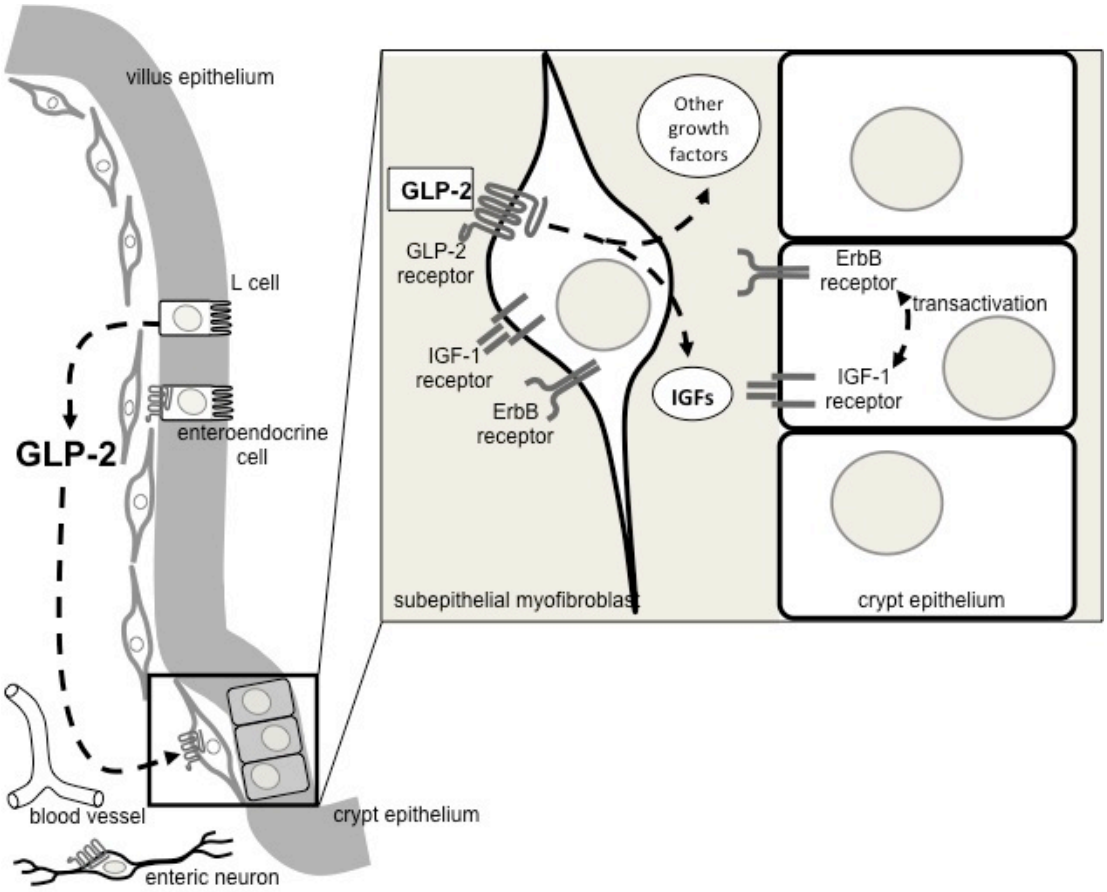


Figure 6.1 *Model for the proposed mechanism of action of glucagon-like peptide-2 (GLP-2) on intestinal crypt cells.* The GLP-2 receptor (R) is expressed by intestinal subepithelial myofibroblasts (ISEMF), enteroendocrine cells including the L-type endocrine cell, and enteric neurons. Following nutrient-induced release into the circulation, GLP-2 acts via GLP-2R binding on ISEMF cells, leading to the release of ISEMF-derived insulin-like growth factor-1 (IGF-1) and, likely, other growth factors, such as ErbB ligands. IGF-1 then binds to the IGF-1R located on crypt epithelial cells, where it may transactivate the ErbB receptor, leading to cell proliferative responses including phosphatidylinositol-3-kinase/Akt and β-catenin signalling. The role of the IGF-1R and/or ErbB receptor expressed by the ISEMF cells has not been delineated but may mediate autocrine effects of ISEMF-derived factors, leading to the release of other growth factors and/or support factors for the lamina propria.

How the findings of the Drucker laboratory on the ErbB axis can be reconciled with those of our laboratory on the IGF-1–IE–IGF-1R pathway remains unclear (Figure 6.1). Recent data have indicated that GLP-2 treatment increases IGF-1, but not ErbB ligand, mRNA transcript levels in ISEMF cultures [129], suggesting that the ErbB system lies downstream of the IGF-1 network. Conversely, exogenous EGF, but not IGF-1, rescues the growth deficit in re-fed GLP-2R null mice [107], suggesting that IGF-1 lies downstream of EGF/ErbB signalling. Nonetheless, it has been well-established that the IGF-1R can transactivate ErbB receptors, and vice versa, demonstrating the existence of cross-talk between these two pathways [179-182] . Several studies provide evidence to support the proposed cross-talk mechanism. For instance, in COS-7 cells, both IGF-1R and EGFR are activated following IGF-1 treatment. Interestingly, the EGFR kinase inhibitor, AG1478, prevents IGF-1 activation of the EGFR in COS-7 cells, while treatment with the EGFR inhibitor, ZD1839 prevents IGF-1-induced ERK1/2 activation. These results suggest that IGF-1R signalling partially requires the EGFR. Furthermore, it was found that the cross-talk between IGF-1R and EGFR is dependent on matrix-metalloprotease-mediated release of HB-EGF in COS-7 and HEK-293 cells. However, the matrix metalloprotease inhibitor, GM6001, as well as the HB-EGF inhibitor, CRM-197, had no effect on IGF-1-induced ERK activation. Therefore, transactivation of EGFR by IGF-1 is independent of matrix metalloprotease activity. Cross-talk between IGF-1R and EGFR can also occur through heterodimerization of these receptors. In mammary fibroblasts, the IGF-1R co-immunoprecipitates with EGFR. Regardless of the mechanism, cross-talk between IGF-1R and EGFR represents an additional mechanism that may mediate the intestinotrophic effects of GLP-2. This is supported by studies using the IGF-1R inhibitor, NVP-AEW541, and the ErbB inhibitor, CI-1033, both of which resulted in abolishment of GLP-2-induced intestinal growth in mice. Furthermore, the

effects of GLP-2 were independent of metalloproteinase activity consistent with the findings on transactivation in other models. Finally, studies in other cell models also indicate that the two pathways demonstrate co-dependence [183]. Additional studies utilizing cell-specific models are required to delineate the exact relationship between the IGF-1R and the ErbB receptor in mediating the crypt cell proliferative response to GLP-2.

As an alternative possibility, GLP-2 may cause the release of IGF-1 from ISEMF cells, which binds in an autocrine fashion to the IGF-1R on ISEMFs, leading to the release of other growth factors that bind to receptors located on IE cells. This explanation would reconcile the findings from Chapter 3, wherein GLP-2-induced increases in nuclear β-catenin localization and cMyc expression were not completely abolished in IE-igf1rKO mice, with the results from studies using the IGF-1R global inhibitor, NVP-AEW541 [10]. Mice treated with NVP-AEW541 did not display a GLP-2-dependent increase in β-catenin activation in the SI crypt. This hypothesis could be addressed through the use of ISEMF-Cre recombinase mice (α-smooth muscle actin-creER) crossed with IGF-1R$^{flox/flox}$ mice. Similarly, dependence of GLP-2 and/or IGF-1 on ErbB signalling could be examined using ISEMF-Cre recombinase mice crossed with Egfr$^{flox/flox}$ mice, which also could be bred to villin-Cre-ER$^{T2+/0}$ mice, to delete the EGFR in both the ISEMF and IE cells. Further consideration of the molecular cross-talk and downstream signalling pathways mediating the intestinotropic effects of GLP-2 is warranted.

6.10 Involvement of the GLP-2 — IGF-1R axis in fasting and re-feeding

The model of 24 hr fasting followed by 24 hr re-feeding represents an adaptive gut growth response in which fasting-induced mucosal atrophy due to increased epithelial apoptosis, is

reversed by nutrient availability, leading to crypt cell proliferation and restoration of the mucosal architecture [85, 311]. Previous studies utilizing both a GLP-2R antagonist and GLP-2R null mice demonstrated that the adaptive re-growth phase is strictly dependent on the presence of endogenous GLP-2 and associated GLP-2R signaling [85, 107]. The results of the present study demonstrate that, although the IE-IGF-1R does not modulate the SI atrophy associated with fasting in mice, it is an essential component of the proliferative response to re-feeding after a fast. Hence, IE-igf1rKO mice demonstrated a defect in re-feeding-associated lengthening of the crypt-villus axis, as well as loss of the proliferative response in the crypt cells in positions 13 to 17, which are found in the transit amplifying zone. Previous studies implicate IGF-1 as an endogenous mediator of intestinal mucosal growth in the transition from fasting to re-feeding where the parallel effects of GLP-2 and IGF-1 were examined. In one study, re-fed rats exhibited increased levels of circulating IGF-1 and increased jejunal IGF-1 mRNA expression, and these responses were also associated with increased SI growth [223]. In another study, rats fasted for 48 hrs followed by ad libitum re-feeding for 2 d displayed increased circulating levels of IGF-1 in association with increased small intestinal mass, DNA and protein content - administration of the GLP-2 antagonist, GLP-2^{3-33}, prevented these responses [108]. Thus, changes in re-feeding-induced jejunal mass in rats correlate with changes in serum IGF-1 [108, 223]. A recent study in mice indicates that exogenous administration of IGF-1 to fasted animals does not restore SI growth [107], although these findings may be related to the relatively low dose of IGF-1 utilized in these C57BL/6 mice (0.5 μg/g) [85, 107]. Nonetheless, together, these in vivo findings strongly implicate the GLP-2–GLP-2R–IGF-1–IGF-1R axis in the SI proliferative response to re-feeding. However, a recent report that SI proliferation can be restored in fasting $Glp2r^{-/-}$ mice by exogenous administration of the ErbB ligand [107], suggests that intestinal adaptive re-growth requires

numerous mediators that interact in a highly complex fashion, as discussed above.

6.11 IE-igf1rKO mice demonstrate selective loss of GLP-2-induced proliferation but retain normal changes in SI weight and mucosal cross-sectional area

Interestingly, despite the inability of IE-igf1rKO mice to increase proliferation and villus height in response to re-feeding, SI wet and dry weight, as well as mucosal cross-sectional area, were restored in a normal fashion. Similar findings were made in chronic GLP-2-treated mice (Chapter 5), such that only the proliferative response and growth in villus height were lost in the IE-igf1rKO animals. Although these findings appear contradictory, adaptive changes to the murine SI are known to occur through proportional shrinkage and restoration of all intestinal tissue components [311]. Hence, IGF-1R deletion in the epithelial compartment would not be expected to alter either IGF-1- or re-feeding-induced growth of the lamina propria or muscularis layers. Of note, examination of potential changes in SI length did not demonstrate any differences between mice of either genotype. Collectively, these findings indicate that the loss of the IE-IGF-1R confers a highly-specific defect to the proliferative cells of the epithelial crypt under conditions of nutrient excess following a period of restriction or after GLP-2 administration.

6.12 Future directions

In Chapter 3, I identified a role for the IE-IGF-1R in β-catenin signalling following acute GLP-2 administration. Specifically, my findings demonstrated that acute GLP-2 administration leads to increases in nuclear β-catenin in the crypt compartment and induction of the cWnt targets, c-myc and Sox9, in control mice, and that these responses are reduced in

IE-igf1rKO animals. An alternate indicator of cWnt signalling pathway activation is the cytoplasmic regulation of β-catenin phosphorylation. Recently, a link has been found between PI3K/Akt signalling and activation of β-catenin in intestinal stem and progenitor cells via phosphorylation at Ser^{552} (P-β-$catenin^{552}$). As such, IGF-1 increases P-β-$catenin^{552}$, through a mechanism involving Ras activation and the regulation of GSK3β phosphorylation, as well as via inhibition of PI3K signalling through PTEN and BMP. Therefore, a mechanistic basis exists for GLP-2- IGF-1 signalling leading to activation of the cWnt pathway in the crypt epithelium, thereby increasing the number of proliferating cells. I was unable to examine Akt signaling in the present mouse model; therefore, in order to address this finding and others indicating that GLP-2-mediated IE cell trophic actions converge at P-Akt in the crypt cell, I would test the actions of GLP-2 in the inducible IE-specific PI3K KO mouse model, in which the class IA subunit of PI3K (functional domain) is conditionally deleted [134].

As outlined in Chapter 4, my study is the first to demonstrate that adaptive re-growth of the SI during the transition of fasting to re-feeding requires the IE-IGF-1R to increase IE cell proliferation. Differences in gene expression of a number of growth factor- and nutrient-regulated molecules could be assessed in IE-igf1rKO mice through microarray studies. Endogenous GLP-2 also plays a role in intestinal adaptation in rodent models of resection, as both Dahly *et al.* and Martin *et al.* demonstrated that intestinal resection is followed by a marked increase in intestinal proliferation [312, 313]. Furthermore, Garrison *et al.* demonstrated that GLP-2 administration immediately after ileo-cecal resection in mice transiently increases P-β-$catenin^{552}$-positive putative intestinal stem cells, and this is temporally correlated with increased jejunal IGF-1 mRNA expression [242]. Therefore, future studies aimed at examining

P-β-catenin^{552}-positive putative intestinal stem cells during fasting and re-feeding-induced intestinal re-growth in IE-igf1rKO mice will help clarify the importance of GLP-2 and IGF-1R signalling in this model of adaptation.

In Chapter 5, GLP-2 was administered to IE-igf1rKO mice over a 10 d time-course and it was determined that the IE-IGF-1R is responsible for chronic GLP-2-induced proliferative effects and growth of the crypt-villus unit. Although the experiments in Chapter 5 were directed at the proliferative effects of GLP-2, and I did not analyze the inhibitory effect of chronic GLP-2 on apoptosis (e.g. through positional analysis of caspase 3- or TUNEL-positive apoptotic cells); these are interesting studies that could be employed in the future. Furthermore, even though the most potent intestinotrophic actions of GLP-2 are directed towards the SI, there has been shown to be IGF-1-dependent effects of GLP-2 on the LI. Thus, future efforts should be directed towards examining the role of the IE-IGF-1R in the colon. Additionally, studies by Yusta *et al.* and Bahrami *et al.* implicating ErbB in the intestinotrophic effects of GLP-2 through the use of the GLP-2R null mice [106, 138], address the need for future studies that integrate IE-IGF-1R KO with a diminished SI ErbB system, possibly through the use of an ErbB inhibitor or genetic deletion models.

Finally, GLP-2 is known to exert a number of additional effects on the SI epithelium, including enhanced digestion, absorption and barrier function. Whether any of these effects also require the IE-IGF-1R is not known, although IGF-1 treatment exhibits similar biological actions to those of GLP-2 for all of these actions [96, 100-103, 146, 150, 155, 161, 164, 169, 171-176, 314, 315].

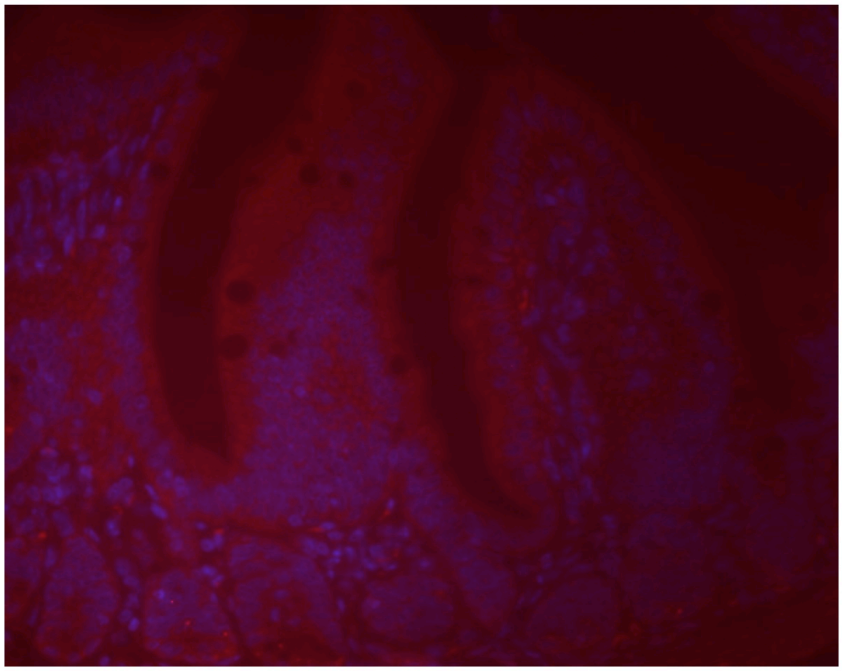
6.13 Conclusions

In summary, I have established for the first time, a genetic model of inducible IE cell IGF-1R deficiency, demonstrating a prominent, cell-specific role for this receptor in GLP-2-induced

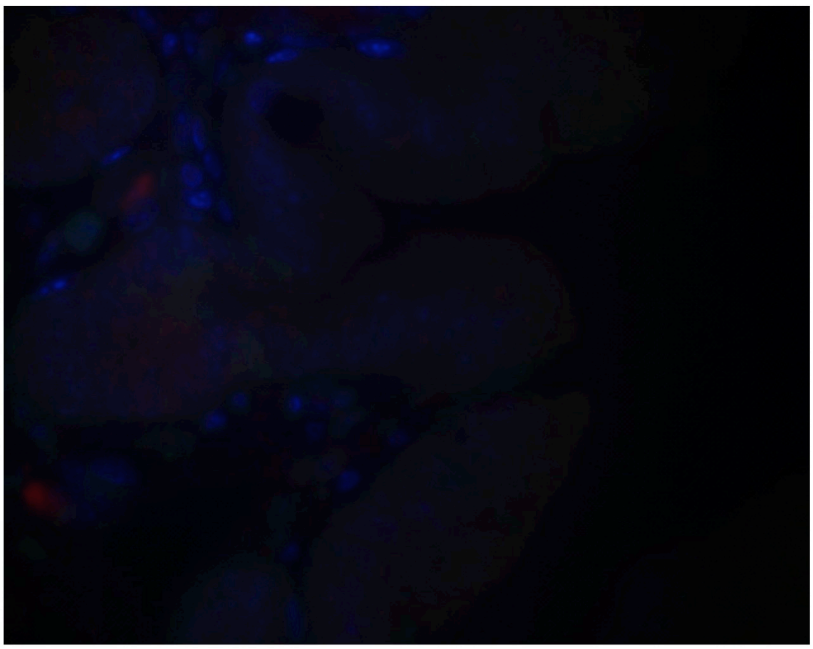
crypt cell proliferation. These findings extend our knowledge of the mechanism of action of both exogenous and endogenous GLP-2, as well as of the IGFs, to stimulate SI proliferative responses. The progression of GLP-2 in clinical trials for Crohn's disease emphasizes the importance of these mechanistic studies, especially since endogenous IGF-1 and IGF-1R expression is increased in patients with IBD. As recent data suggest that other factors (e.g. ErbB ligands) also act in concert with GLP-2 to promote intestinal growth, further studies are required to develop an integrative picture of the complex interplay between all of these factors in the regulation of intestinal homeostasis.

APPENDIX A

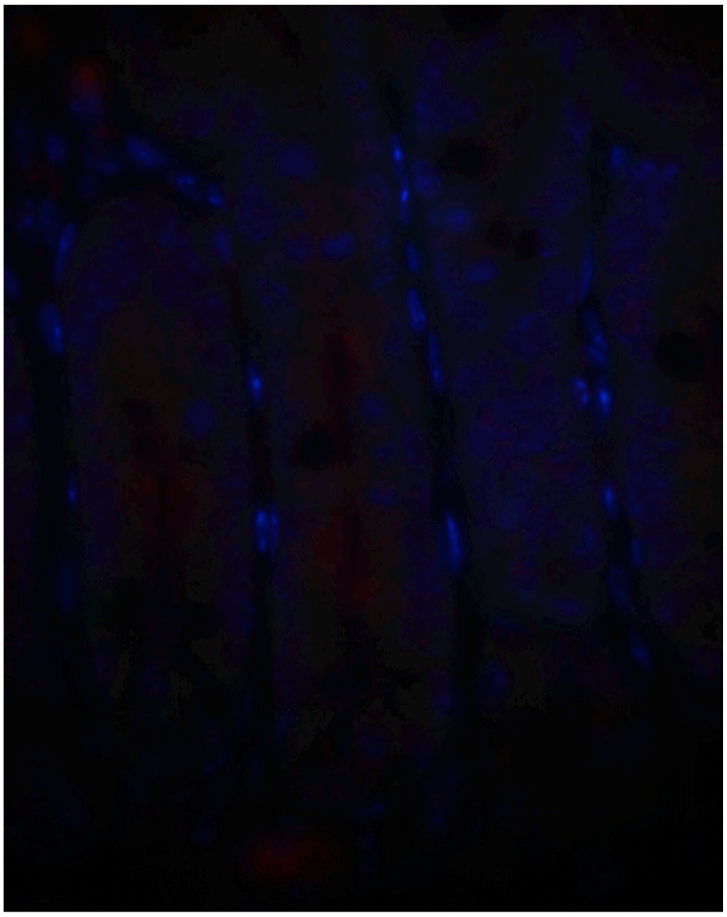
IMMUNOFLUORESCENT STAINING



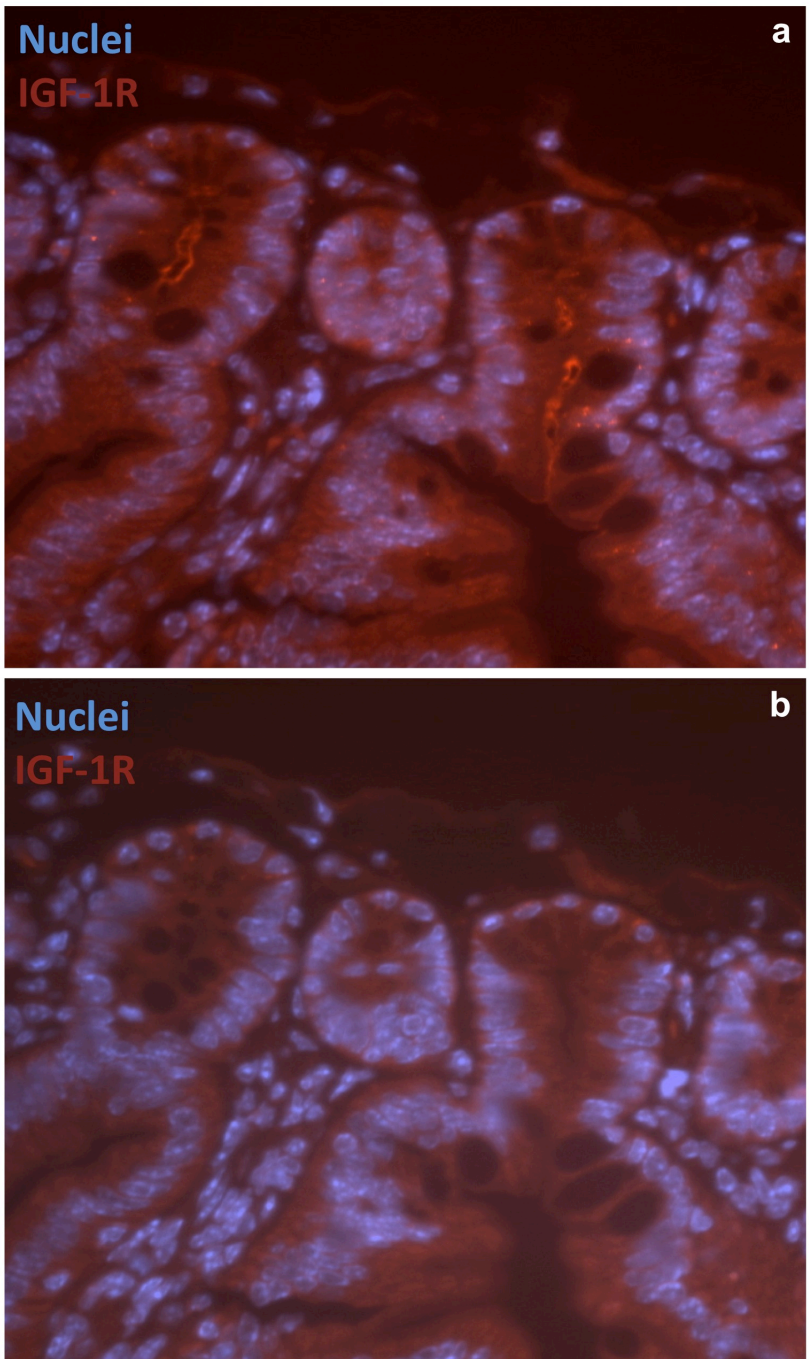
Negative staining in the absence of synaptophysin primary antiserum.



Negative staining in the absence of lysozyme primary antiserum.



Negative staining in the absence of beta-catenin and lysozyme primary antisera.



(a) Immunofluorescent staining for IGF-1 receptor (R) in C57Bl/6 mice. (b) Negative staining in the absence of IGF-1R primary antiserum.

APPENDIX B

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