The Role and Regulation of Insulin-Like Growth Factor Binding Protein-4 in the Rodent Intestinal Mucosa

by

Kaori Austin

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Graduate Department of Physiology

University of Toronto

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Abstract

Insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF) and glucagon-like peptide-2 (GLP-2) are all hormones that induce intestinal mucosal proliferation and growth. They are also interdependent as GLP-2 relies on IGF-1 and EGF to act as intermediary factors, which in turn act synergistically on the intestinal epithelium to stimulate proliferation. IGF binding protein-4 (IGFBP-4) regulates the activity of IGF-1 and is expressed highly in the intestine. IGFBP-4 can inhibit IGF-1 activity, but also promotes IGF-1-induced growth in a protease-dependent manner in whole body and bone. However, the role of IGFBP-4 within the intestine is not well defined. Thus, I hypothesized that IGFBP-4 is required for the growth promoting effects of GLP-2, which require the actions of IGF-1, but inhibits basal intestinal epithelial proliferation. Because the intestinal growth factors stimulate Sox9 expression, a transcriptional promoter for IGFBP-4, I also hypothesized that mucosal IGFBP-4 expression is stimulated by GLP-2, IGF-1, and EGF. GLP-2-treated control mice displayed increased intestinal weight per body weight, crypt-villus height and epithelial proliferation while the IGFBP-4 knockout (KO) mice had impaired
increases in all of these parameters. In contrast, vehicle-treated IGFBP-4 KO mice had elevated
intestinal weight per body weight, crypt-villus height, and epithelial proliferation compared to
vehicle-treated control mice. Consistently, IGFBP-4 dose-dependently decreased IEC-6 cell
proliferation in an IGF-1 receptor (IGF-1R)- and MEK1/2-dependent manner. Finally, chronic
GLP-2 treatment in vivo increased mucosal IGFBP-4 mRNA expression. However, chronic
inhibition of EGFR and/or intestinal epithelial IGF-1R KO in vivo did not alter mucosal IGFBP-4
eexpression. Altogether, the results demonstrate that IGFBP-4 is integral for promoting GLP-2-
induced mucosal growth and inhibiting growth under basal conditions and that mucosal IGFBP-4
expression is regulated in a GLP-2-dependent but not IGF-1/EGF-dependent manner.
Acknowledgments

First, I am most grateful to my supervisor, Dr. Patricia Brubaker for giving me the opportunity to pursue a PhD in her laboratory, and for her mentorship and supervision of my project throughout the five years. Pat has been a strong female role model for me and her commitment to excellence in leadership, teaching, and mentorship has undoubtedly been an integral part in my personal and academic growth during graduate school.

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<th>Description</th>
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<tbody>
<tr>
<td>ALS</td>
<td>Acid-labile subunit</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>B6-Igf1&lt;sup&gt;tm1.1Mhz&lt;/sup&gt;</td>
<td>C57BL/6-insulin-like growth factor I receptor; targeted mutation 1.1, Martin Holzenberger</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Bmi-1</td>
<td>B-cell lymphoma Moloney murine leukemia virus insertion region-1 homolog</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2’-deoxyuridine</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxyl-terminal</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>Cre</td>
<td>cyclization recombinase</td>
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<tr>
<td>CK1</td>
<td>casein kinase 1</td>
</tr>
<tr>
<td>c-Myc</td>
<td>MYC proto-oncogene, bHLH transcription factor</td>
</tr>
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<td>CON</td>
<td>control</td>
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<tr>
<td>Ct</td>
<td>cycle threshold</td>
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<td>Dcamkl</td>
<td>doublecortin-like and CAM kinase-like 1</td>
</tr>
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<td>DLL1</td>
<td>Delta-like 1</td>
</tr>
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<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>DPP-4</td>
<td>dipeptidyl peptidase-4</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EdU</td>
<td>5-ethynyl-2’-deoxyuridine</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Mitogen-activated protein kinase 3/1</td>
</tr>
<tr>
<td>FAC</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>flox</td>
<td>Flanked by locus of X-over of P1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GLP</td>
<td>Glucagon-like peptide</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>Het</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Heparin-binding EGF-like growth factor</td>
</tr>
<tr>
<td>Hopx</td>
<td>HOP homeobox</td>
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<tr>
<td>ISEMF</td>
<td>Intestinal subepithelial myofibroblast</td>
</tr>
<tr>
<td>IE</td>
<td>Intestinal epithelial</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IGFBP-7</td>
<td>Insulin like growth factor binding protein-7; Mac25, TAF, or PSF</td>
</tr>
<tr>
<td>IGFBP-rPs</td>
<td>Insulin-like growth factor binding protein-related proteins</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LEF</td>
<td>Lymphoid enhancer factor</td>
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<tr>
<td>Lgr5</td>
<td>Leucine-rich repeat-containing G-protein coupled receptor 5</td>
</tr>
<tr>
<td>Lrig1</td>
<td>Leucine-rich repeats and immunoglobulin-like domains 1</td>
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<tr>
<td>MEK1/2</td>
<td>Mitogen-activated protein kinase kinase 1/2</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>Msi-1</td>
<td>Musashi RNA binding protein 1</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino terminal</td>
</tr>
<tr>
<td>p</td>
<td>Phospho</td>
</tr>
<tr>
<td>PAPP-A</td>
<td>Pregnancy-associated plasma protein-A</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositide 3-kinases</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginylglycylaspartic acid</td>
</tr>
<tr>
<td>R</td>
<td>Receptor</td>
</tr>
<tr>
<td>Rosa26</td>
<td>Gene trap ROSA 26, Philippe Soriano</td>
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<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SI</td>
<td>Small intestine</td>
</tr>
<tr>
<td>Sox9</td>
<td>SRY-box 9</td>
</tr>
<tr>
<td>t</td>
<td>Total</td>
</tr>
<tr>
<td>TA</td>
<td>Transit-amplifying</td>
</tr>
<tr>
<td>TCF</td>
<td>T-cell factor</td>
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<tr>
<td>Tg(Vil-cre/ERT2)23Syr</td>
<td>Transgene insertion 23, Sylvie Robine</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor-α</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-related integration site</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
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<td>^3H</td>
<td>Tritium</td>
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**Unit Prefixes**

<table>
<thead>
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<tr>
<td>k</td>
<td>kilo</td>
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<tr>
<td>c</td>
<td>centi</td>
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<tr>
<td>m</td>
<td>milli</td>
</tr>
<tr>
<td>µ</td>
<td>micro</td>
</tr>
<tr>
<td>n</td>
<td>nano</td>
</tr>
<tr>
<td>p</td>
<td>pico</td>
</tr>
</tbody>
</table>
Units
M  molar
Da daltons
m meter
g grams
L litre
U units
°C degree Celcius

Nucleotide Abbreviations
A adenine
T thymine
G guanine
C cytosine

Amino Acid Abbreviations
Ser serine
Gly glycine
Trp tryptophan
Tyr tyrosine

Other Symbols
p statistical p-value
n statistical n-value
~ approximately
% percent
± plus or minus
X times
& and
Δ delta
Chapter 1

Introduction

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Author Contributions:

Austin K. produced all text and figures presented in this chapter
1 Introduction

1.1 Rationale

The intestinal mucosa is a key site of nutrient absorption and barrier function against pathogens. The epithelial layer within the mucosa undergoes constant shedding and is renewed by the intestinal stem cells and progenitor cells to facilitate these functions. The mesenchymal cells, which underlie the epithelial layer within the mucosa, play a key role in secreting growth factors to support the growth and proliferation of the epithelium. Key growth factors that play a role in the growth and maintenance of the intestinal mucosa include glucagon-like peptide-2 (GLP-2), insulin-like growth factor-1 (IGF-1), and epidermal growth factor (EGF). However, how the activities of these peptides work together to regulate the growth of the intestinal mucosa remain incompletely understood.

Understanding the fundamental biology that regulates mucosal growth and proliferation is essential for understanding how mucosal growth can be altered under diseased states as well as how therapeutics could improve mucosal function. For example, the epithelium can display aberrant growth such as the case in colorectal cancer or undergo increased apoptosis in response to radiation therapy which targets proliferating cells. In the case of intestinal insufficiency, such as short bowel syndrome, the intestinal capacity to absorb and digest nutrients is insufficient to support life and patients must rely on parenteral nutrition. Teduglutide, a long acting form of the peptide hormone, GLP-2, can promote mucosal growth and improve function of the remaining intestine in patients with short bowel syndrome (1). However, GLP-2 relies on the presence of intermediary growth factors to exert its effects (2). One of the intermediary growth factors is IGF-1 whose activity is known to be regulated by Insulin-like Growth Factor Binding Proteins
(IGFBPs) (3). Within the intestine, IGFBP-4 has been implicated to be playing a role in regulating IGF-1 activity. Specifically, this work aims to elucidate how IGFBP-4 regulates the activity of IGF-1 within the intestinal mucosa, its impact on the GLP-2-induced intestinal growth, and its overall contribution to the regulation of intestinal mucosal growth.

1.2 The Small Intestinal Mucosa

1.2.1 Structure and Function

The small intestinal mucosa is the inner most layer of the small intestine where luminal nutrient digestion and absorption takes place. The mucosa is surrounded by the submucosa, muscularis externa, and finally by the serosa (Figure 1.1). The serosa is made up of connective tissue. It surrounds the muscularis externa which contain the circular and longitudinal smooth muscles, and is innervated by the submucosal plexus, which allows for the slow mixing and moving of the luminal contents within the small intestine. The muscularis externa surrounds the submucosa which is made up of more connective tissue, blood, and lymph vessels and surrounds the mucosa. To maximize the surface area available for nutrient absorption, the small intestinal wall forms folds called plicae. However, to increase the surface area further, the mucosa also forms protrusions and invaginations known as the villi and crypts, respectively. The mucosa is composed of the intestinal epithelium, the lamina propria and the muscularis mucosa (Figure 1.1). The muscularis mucosa is the outermost layer of the mucosa composed of smooth muscle cells. The lamina propria is a layer of connective tissue that underlies the epithelium. It contains a number of different tissues including the intestinal subepithelial myofibroblasts, intestinal immune cells, blood vessels, and lacteals. The intestinal subepithelial myofibroblasts exist as a syncytium and secrete growth factors to support epithelial proliferation, restitution, and
inflammatory responses (4). The intestinal immune cells, which includes lymphoid cells and macrophages, protect against pathogens and support gut and intestinal epithelial homeostasis (5). The blood vessels and lacteals, or lymph vessels, support cell metabolism of the gut, and carry nutrients that are transported across the epithelial layer out of the intestine. The intestinal epithelium is the layer directly in contact with the intestinal chyme, and is shed into the lumen as it is continuously regenerated.

The intestinal epithelium is made up of many different types of differentiated cells including enterocytes, goblet cells, enteroendocrine cells, tuft cells, and M cells that reside in the intestinal villi, and Paneth cells that reside within the intestinal crypt (6). These cells are derived from the intestinal stem cells that are situated at the bottom of the intestinal crypt, intercalated with the Paneth cells and continuously divide to provide progenitors, called transit amplifying cells (Figure 1.2). The enterocytes make up the majority of the population and are responsible for facilitating nutrient absorption. The mucosa plays an integral role in digesting food through enzymatic and mechanical digestion, absorbing food as well as forming a protective barrier against pathogens (7). Goblet cells secrete mucins which form a protective layer of mucus within the small and large intestine (8). A number of different enteroendocrine cells also exist and function to secrete hormones in response to meal consumption such as cholecystokinin, glucose-dependent insulinotropic polypeptide, GLP-2, and glucagon-like peptides-1 (GLP-1) to regulate various aspects of digestion and metabolism including gut motility, insulin secretion, satiety, and intestinal mucosal growth (9). Tuft and M cells both play a role in mucosal immune responses (10). Last but not least, Paneth cells secrete antimicrobial enzymes such as lysozyme and defensin (6).
The innermost lining of the intestine is the mucosa which facilitates nutrient digestion and absorption. The mucosa is surrounded by submucosa, followed by muscularis externa and then the serosa. The intestinal mucosa is made up of three layers. The innermost layer is the epithelium which forms invaginations and protrusions, called crypts and villi respectively. The lamina propria, which underlies the epithelium, contains a number of different cells including subepithelial myofibroblasts, immune cells, and blood vessels. The muscularis mucosa layer contains smooth muscle cells.
The intestinal crypt houses the stem cells responsible for providing progenitors that eventually differentiate into one of the epithelial cell types present in the intestine. Active stem cells, also known as crypt base columnar (CBC) cells, reside at the very bottom of the crypt, intercalated between Paneth cells. These cells proliferate approximately every 24 hours. Progenitors are termed transit-amplifying (TA) cells, which rapidly proliferate as they travel up, differentiate and move out of the crypt and into the villi where they are eventually shed into the lumen. Some of the progenitor cells move down and differentiate into Paneth cells. Quiescent stem cells reside in
approximately +4 cell position in the crypt. Interconversion between the quiescent stem cells and active stem cells is possible; however, quiescent stem cells are mainly thought to convert to an active stem cell phenotype under conditions of radiation damage or following ablation of the active stem cells.
In addition to the plicae, crypt, and villi, the surface area of the intestinal epithelium is further increased by the presence of brush-border, or microvilli, which form finger-like projections on the cell membrane on the apical side of the epithelium (11). Microvilli express enzymes such as disaccharides and peptidases for digestion and absorption of luminal contents. Finally, while the intestinal microbiome serves an important purpose of aiding the digestion of the luminal contents, the intestine maintains a barrier against the invasion of pathogens by forming tight junctions that prevent paracellular transport of bacteria and macromolecules (12). Compromised intestinal barriers are associated with intestinal inflammation and inflammatory bowel disease (12).

1.2.2 Small Intestinal Growth

Intestinal epithelial growth is facilitated by proliferation of stem cells and progenitor cells. Two populations of stem cells have been identified – the active stem cells, also known as the crypt-base columnar cells, and the quiescent stem cells located at the position +4 location of the crypt (Figure 1.2). Various factors supplied from the surrounding cells, such as the wingless-related integration site (Wnt) ligands, delta-like 1 (DLL1), and EGF, have been identified as essential for creating a ‘niche’ that supports the survival of the stem cells (7). Progenitors derived from these stem cells continue to proliferate as ‘transit amplifying cells’ and are pushed out from the crypts and to the villi where they eventually differentiate into goblet, enterocyte, enteroendocrine or tuft cells (7). Some also migrate down towards into the crypt as Paneth cells which play a critical role in secreting factors such as Wnt ligands, EGF, and Notch ligands, thereby creating the niche for the stem cells (7,13). The differentiated intestinal epithelial cells are eventually shed from the top of the villi and are replaced by new cells. Thus, the epithelial cells are renewed every 3-5 days with the exception of Paneth cells which are replaced at a slower rate (7).
While interconversion between the quiescent and actively proliferating stem cells has been established (14), various theories exist regarding the role and relationship between the intestinal stem cell types present within the intestinal crypts (7). The actively proliferating stem cell is often identified and isolated by the expression of leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5), which is a Wnt target gene. Lgr5 itself also functions as a regulator of Wnt signaling in the intestine (15), potentially through direct interaction with the Wnt receptor Frizzled and co-receptor LRP5/6 (16). Its role as an intestinal stem cell marker was demonstrated using lineage tracing techniques using Lgr5-Cre/Rosa26-LacZ mice which allows for the visualization of lacz+ epithelial cells derived from stem cells that expressed Lgr5 at the time of induction (17). Whole-body, or total Lgr5 knockout (KO) mice are neonatal lethal (18), demonstrating the critical role Lgr5+ cells play in sustaining life. Similarly, the quiescent stem cell at position 4, has also been characterized as expressing B-cell lymphoma Moloney murine leukemia virus insertion region-1 homolog (Bmi-1) (19). Similar to the Lgr5 KO mice, stem cells that express Bmi-1 have been demonstrated through lineage tracing to produce progenitors that populate the epithelium (19). Bmi-1 is part of a Polycomb repressor complex and is thought to regulate stem cell proliferation downstream of Notch signaling (20). Similarly, Hopx (HOP homeobox) has also been demonstrated through lineage tracing to label the quiescent stem cell pool (14). Other markers such as Dcamkl (doublecortin-like and CAM kinase-like 1) and Lrig1 (Leucine-rich repeats and immunoglobulin-like domains 1), an EGF receptor inhibitor, are also expressed by a slowly cycling stem cell population of the crypt (21–23). In conditions such as radiation which ablate only the actively proliferating stem cells, quiescent stem cells convert into active stem cells and allow for the repopulation of the intestinal epithelium as seen through lineage tracing studies in vivo and single cell organoid studies in vitro (24,25). However, recent studies show that Bmi-1 as well as Hopx and Lrig1 expression are not restricted to the +4
position of the intestinal crypt and are expressed by Lgr5+ stem cells as well (26,27). Thus, other methods of characterizing the stem cells may be more useful in studying stem cell dynamics. One such proposed alternative is using relative expression of Sox9 to differentiate between quiescent and actively proliferating stem cells. Mice that express enhanced green fluorescent protein (EGFP) under the Sox9 promoter (Sox9EGFP mice) show variable, relative expression levels of EGFP within the intestinal crypt depending on cell type (27,28). The quiescent, intestinal stem cells at the position +4 location have ‘high’ levels of EGFP, while the active stem cells that are intercalated among the Paneth cells are sensitive to radiation treatment and have ‘low’ levels of EGFP (27,28). Irradiation studies using Sox9EGFP mice show increased proliferation of both the ‘high’ and ‘low’ Sox9EGFP expressing cells, as well as increased expression of genes related to DNA replication and repair, expressed in the ‘high’ Sox9EGFP which are characterized as being similar to the quiescent stem cells (29). Overall, the identities and characterizations of the two stem cell populations remains controversial; however, various means of studying these cells are available and this remains a rapidly changing field of study.

A number of signaling pathways have been identified as crucial for the maintenance of the progenitor and stem cells and have been linked to stimulating proliferation and growth within the intestine. The canonical Wnt signaling pathways is one such pathway. Under non-stimulated conditions, β-catenin, the downstream target of canonical Wnt signaling, is phosphorylated for ubiquitination and degradation by the destruction complex which includes: axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3), and casein kinase 1 (CK1), among other proteins (30). Binding of the Wnt ligand, secreted from the Paneth and surrounding mesenchymal cells (13,31,32), to Frizzled results in the recruitment LRP5/6 (30). Together, the ternary complex activates Disheveled and results in the recruitment of the destruction complex to
the receptors and deactivation of the complex. This allows β-catenin to translocate into the
nucleus to induce transcription of the Wnt target genes which includes Sox9 and c-Myc, through
its interaction with the DNA bound T-cell factor/lymphoid enhancer factor proteins (TCF/LEFs)
(33,34). In the intestine, stimulation of the Wnt signaling pathway by administration of the Wnt
signaling agonist, R-spondin1, results in increased small intestinal weight (35). Similarly, hepatic
infection with an R-spondin1-encoding adenovirus also leads to crypt hyperplasia (24). Aberrant
Wnt signaling is often linked to epithelial hyperplasia and colon cancer. Indeed mice with a
mutation of the APC gene, such as in APC<sup>min/+</sup> mice which express a truncated version of APC
are widely used as a model of human familial adenomatous polyposis (36).

Growth factors that influence the stem cell and progenitor cell proliferation and thereby growth,
can be used to recapitulate and mimic the stem cell niche in vitro. Landmark in vitro studies by
the Clevers group established the three key factors necessary for intestinal stem cell survival and
proliferation in vitro as EGF, R-spondin1, and the bone morphogenic protein signaling inhibitor,
noggin (37). Isolation of intestinal crypts or of single, Lgr5 positive stem cells and plating them
with media supplemented by these growth factors and embedded in Matrigel for structural
support allows the intestinal stem cells to proliferate and form three dimensional structures called
intestinal organoids (Figure 1.3) (37). Such organoids have been characterized as having
protruding buds where the stem cells localize and that effectively act as the intestinal crypt. The
stem cells within the organoid provide progenitors which migrate in towards the center of the
structure and differentiate into all of the differentiated cell types that are typically found within
the intestinal epithelium (37). Organoids are able to continuously propagate with consistent
media change and weekly splitting (38), and are thought to be a more representative model of the
intestinal epithelium compared to cell lines because of their heterogeneity and ability to create all
of the different types of cells present in vivo, and their structural representation of the epithelium in vivo.
Isolation of intestinal crypts or intestinal stem cells by FAC sorting for Lgr5-EGFP+ cells and culturing in Matrigel domes with media supplemented with EGF, R-spondin-1, and noggin as per an established protocol (37) results in three dimensional structures known as organoids. These structures recapitulate the epithelial configuration with the lumen on the inside and crypts that protrude outward and contain actively proliferating stem cells. Organoids grown under these conditions have been shown to make all of the differentiated epithelial cell types in vitro including enterocytes, Paneth cells, goblet cells, and enteroendocrine cells.

**Figure 1.3 Intestinal Organoids**
In recent years, both actively cycling and quiescent intestinal stem cells have been demonstrated to be capable of forming organoids. The actively cycling stem cells, typically isolated through FAC sorting for expression of Lgr5-GFP, only require the regimen described above although isolation as a doublet attached to a Paneth cell greatly increases their survival, likely due to the Wnt and Notch ligands that the Paneth cells express to support stem cell proliferation (39). The ability of the quiescent stem cells to form organoids has been assessed using a number of different markers including Bmi-1 and Sox9\textsuperscript{EGFP} expression. Single, FAC sorted Bmi-1-YFP expressing intestinal epithelial cells isolated from Bmi1-CreER; Rosa26-YFP mice were demonstrated to give rise to Lgr5+ cells, and were multipotent, generating all of the differentiated epithelial cell types in vitro (24). Using the Sox9\textsuperscript{EGFP} mice characterized by the Magness group, and described earlier (27,28), the Lund group demonstrated that the quiescent stem cell-like cells, isolated by FAC sorting for cells that have high expression levels of Sox9\textsuperscript{EGFP}, can also form organoids in vitro if isolated after mice were subjected to an activating stimulus such as abdominal irradiation (29).

The intestine is very sensitive to alterations in circulating growth factor levels such as EGF, IGF-1 and GLP-2. In vivo, administration of EGF chronically and acutely to mice has been demonstrated to increase stem cell and progenitor cell proliferation as well as villus height and small intestinal weight (40). EGF, as described above, plays a critical role in maintaining the crypt niche, and facilitating growth and development of the intestine (37,41). EGF is a 53 amino acid peptide that was discovered in 1962 as a factor that stimulates the eye-lid opening of newborn mice (42). Since then, EGF has been shown to regulate proliferation, differentiation, migration, and apoptosis in many cell types (43). EGF exerts effects through the EGF receptor (EGFR, also known as ErbB-1) which belong to the ErbB receptor family, containing ErbB-1
through ErbB-4 (43). ErbB1 is localized to the cell membrane and upon ligand binding forms a homodimer or a heterodimer with another ErbB receptor, (43). EGF belongs to a group of EGF-like ligands, or ErbB-ligands. These include EGF, TGF-α (transforming growth factor-α), HB-EGF (heparin-binding EGF-like growth factor), amphiregulin, betacellulin, epigen, and epiregulin, which have high affinity for ErbB1, and neuregulin-1 through -4 which have higher affinity for ErbB-3 and ErbB-4 (44). Whole-body EGFR (ErbB1) KO mice do not survive postnatally, demonstrating the importance of the ErbB system in prenatal and postnatal development (45–47). Additionally, overexpression of the ErbB receptor is often associated with hyperplasia and cancer (43). Whole-body EGF KO mice are more susceptible to cysteamine-induced duodenal ulceration but the knockout is not fatal, likely due to compensation by redundant signaling pathways (48). However, the EGF, Amphiregulin, and TGF-α triple KO mice display significant growth retardation, have smaller intestinal weight/length ratios, and display duodenal lesions, thinner muscularis externa, as well as reduced ileal villus height and crypt proliferation (48). Activation of ErbB1 is associated with direct activation of the MEK pathway and STAT5, as well as indirect activation of the PI3K/Akt pathway (43). Overall, the ErbB ligand-receptor plays a critical role not only in intestinal physiology but also in whole body growth and development.

Similarly, systemic administration of IGF-1 in rats subjected to total parenteral nutrition has been demonstrated to prevent total parenteral nutrition-induced intestinal atrophy (49). IGF-1 will be discussed in more detail in subsequent sections in this chapter. Both EGF and IGF-1 are also established intermediary factors of GLP-2, a potent intestinotrophic factor (35,40). GLP-2 is a 33 amino acid peptide that is released from the intestinal enteroendocrine L-cell in response to nutrient ingestion in rodents and in humans (50,51). GLP-2 receptors are expressed in the
subepithelial myofibroblasts, as well as some enteroendocrine and enteric neurons, but not the epithelium of the intestine (2,52–55). As such, the proliferative and growth effects of GLP-2 rely on the actions of intermediary growth factors such as IGF-1 and EGF to act on the intestinal epithelium (35,56,57). GLP-2 treatment is associated with increased crypt cell survival in normal and radiation-induced intestinal damage mouse models (58,59) as well as increasing the proportion of cells expressing Msi-1 (musashi RNA binding protein 1), a stem and progenitor cell marker (35) and the number of proliferating cells within the crypt cells (35,40). In addition to stimulating intestinal growth, GLP-2 has a number of other beneficial effects in the bowel including improving the barrier function of the epithelium and reducing inflammation (54,60,61). Physiologically produced GLP-2 is subject to degradation by dipeptidyl peptidase-4 (DPP-4), a peptidase that cleaves GLP-2 at alanine on position 2 (51). Thus, a DPP-4 degradation resistant isoforms of GLP-2, teduglutide, is now an approved drug for treatment of patients with short bowel syndrome (1).

Physiological changes such as fasting followed by refeeding alter circulating levels of several of the growth factors described above that are important in maintenance and stimulation of intestinal proliferation and growth. Reductions in these growth factors due to fasting are associated with reduced bowel mass as well as crypt to villus height. Most notably, a fasting-refeeding paradigm is associated with a decrease (62), followed by an increase in circulating IGF-1 (62,63). Similar trends are also observed with circulating GLP-2 (64). The effects of fasting and refeeding on intestinal growth are mediated, at least in part, by GLP-2 as administration of the GLP-2 receptor antagonist (GLP-23-33) prevents or reduces the adaptive changes in small intestinal weight, crypt-villus height, and proportion of proliferating cells (65). These changes are also dependent on the presence of both the intestinal epithelial IGF-1 receptor
(IE-IGF-1R) and the GLP-2R, as both the inducible IE-IGF-1R KO mice and the GLP-2R KO mice display impaired adaptive increases in intestinal proliferation and crypt-villus height in response to refeeding after fasting (56,64).

While crypt proliferation and crypt-villus height increases are used commonly as a measure for intestinal growth, other parameters such as inhibition of apoptosis and crypt fission, as well as intestinal smooth muscle growth also contribute to intestinal growth (66,67). Recovery and adaption from intestinal resection in mice is associated with increased intestinal crypt proliferation, as well as a significant increase in crypt fission, observed through bifurcating crypts sharing a common, proliferating wall (68). Consistently, GLP-2 treatment in mouse models of intestinal resection results in increased crypt fission and is associated with increased crypt density (69). Similarly, systemic treatment with growth factors such as GLP-2 and IGF-1 in rodents is also associated with decreased epithelial apoptosis (2,59,66). Collectively, IGF-1 alone, or in response to GLP-2 plays a crucial role in maintaining gut homeostasis and stimulating mucosal growth.
1.3 Insulin-Like Growth Factor-1 (IGF-1)

1.3.1 Structure, Expression, and Secretion

IGF-1 is a 70 amino acid peptide hormone whose sequence was identified in 1978 (70) and the cDNA sequence for its precursor in 1983 (71). In humans, IGF-1 is expressed on chromosome 12, whereas the related peptides, insulin and insulin-like growth factor-2 (IGF-2) are expressed on chromosome 11 (72).

Structurally, IGF-1 contains an A and B domain connected together with disulfide chains, as well as a C and D domain and has a 43% sequence homology to insulin and a 62% homology to IGF-2 (73). IGF-1 and IGF-2 are expressed by many different tissues but circulating IGF-1 and IGF-2 are derived predominantly from the liver (73,74). IGF-1 was first identified as a sulfation factor that was intermediary to the actions of growth hormone, hence its alternative name, somatomedin (73,75). However, it was later established to also have insulin-like effects as well as growth-promoting effects which are carried out in an endocrine, as well as paracrine and autocrine manner (76). In contrast to insulin, which is secreted from the β-cells of the pancreas in response to increases in blood glucose levels, IGF-1 release from the liver and is stimulated by growth hormone, or somatotropin, which in turn is secreted from the anterior pituitary in response to growth hormone releasing hormone, as well as nutrient intake (66). Serum IGF-2 is also predominantly produced by from the liver (73). However, its release from the liver is less responsive to growth hormone compared to IGF-1 as patients with acromegaly, caused by elevated levels of growth hormone, demonstrate excessive levels of circulating IGF-1 but not IGF-2 (73). Normal circulating levels of IGF-1 in the serum range between 9-35 nM (77,78). IGF-1 levels are also dependent on age, with serum concentrations rising during puberty and
slowly decreasing with age (77,79). IGF-2 levels are much higher than those of IGF-1, ranging between 80-130 nM. Both serum IGF-1 and IGF-2 concentrations are higher than serum insulin concentrations, which for a normal non-diabetic patient under fasting conditions is ~36.2±19.7 pM (3,78,80,81). The structure, expression, and secretion of IGF-1 lead to its unique biological action, as discussed below.

1.3.2 Biological Activity

IGF-1 plays an integral role in prenatal and postnatal growth and development, as whole-body IGF-1 KO mice are severely growth restricted with less than 60% of the wild-type (WT) body weight at birth (82), and retarded longitudinal bone growth postnatally (83). IGF-1 stimulates osteoblast expansion (84) and growth and maturation in a variety of compartments such as muscle and adipose tissue. In humans, lack of IGF-1 production or growth hormone insensitivity results in dwarfism, resulting in smaller birth height, and slower growth trajectories postnatally, that can be reversed with IGF-1 treatment (76). In contrast, IGF-2 plays a more central role in prenatal development compared to postnatal growth (85) and the anabolic actions of IGF-2 are less pronounced as compared to IGF-1 in adult humans (73). In addition to its anabolic effects, IGF-1 also stimulates metabolic responses. A bolus injection of free, unbound IGF-1 in healthy adult humans results in an insulin-like response wherein blood glucose levels are transiently decreased (86). However, in the circulation, 99% of the IGF proteins are bound to IGF Binding Proteins (IGFBPs), that prolong their half-lives and regulate their biological activity (3). A bolus injection of free, unbound IGF-1 results in a half-life of 20 minutes, whereby some of the IGF-1 is taken up by circulating IGFBP, resulting in an extended increase in the duration of total IGF-1 present in the circulation (86). The unbound IGF is eventually cleared by the kidney (87). Specifics of the IGFBPs will be discussed later in this chapter.
As compared to the acute, insulin-like, metabolic effects of IGF-1, the growth effects by IGF-1 are slower. These are evident in vitro, through observations of increased proliferation using H3-thymidine incorporation assays (73,88), as well as in long-term IGF-1 infusion studies in rats in vivo where continuous subcutaneous infusion of IGF-1 for 6 days results in increased tibial epiphysis width and increased thymidine incorporation in costal cartilage in hypophysectomized rats (89). Postnatal growth and development has been attributed to both paracrine actions of local expression of IGF-1 as well as endocrine actions of circulating IGF-1 secreted from the liver. Mice that have liver-specific deletion of IGF-1 due to transgenic expression of cre recombinase under the albumin promoter acting on an IGF-1R flox/flox gene, display a dramatic reduction in the circulating IGF-1 levels. They also have a surprisingly normal liver, kidney, and heart wet weight, as well as normal body weight and femur length (90). These results suggest that local IGF-1 plays a key anabolic role in growth and development. However, liver-specific IGF-1 knock-in mouse on a IGF-1 null background as compared to IGF-1 null mice showed normal circulating IGF-1 levels and a significant increase in mouse body weight compared to IGF-1 null mice, demonstrating that the endocrine actions of circulating IGF-1 also contribute to postnatal growth and development and can overcome developmental deficiencies when local expression of IGF-1 is abrogated (91,92).

To stimulate anabolic effects, IGF-1 binds and activates its receptor, IGF-1R, a heterotrimeric membrane-spanning tyrosine kinase receptor, composed of two α-subunits and two β-subunits linked by disulfide bridges (93–95). Activation of IGF-1R by IGF-1 is associated with many pathways that regulate cell proliferation, differentiation and cell survival (96–98). Ligand binding occurs in the cysteine rich domain of the α-subunits and results in a conformational change in the β-subunit to induce autophosphorylation of tyrosine residues (95), including
positions 950, 1131, 1135, and 1136 (96). Downstream, IGF-1R activates multiple Insulin Receptor Substrates (IRS) (98,99), but exerts its growth promoting/antiapoptotic effects predominantly through IRS-1 and IRS-2, whereas IRS-3 and IRS-4 have been suggested to act as negative regulators of IRS-1 and IRS-2 (99–101). Activation of the IGF-1R-IRS pathway is associated with multiple downstream signaling pathways including the Akt/PI3K and the MEK/ERK signaling pathways (98).

The underlying mechanisms of IGF-1 activated signaling pathways are further complicated by the fact that IGF-1 can also bind to the structurally similar insulin receptor (IR), IGF-2 receptor (IGF-2R), and the IGF-1R/IR heterodimer (78,102). While many of the signaling pathways activated by each of the receptors overlap with one another, the receptors are not functionally redundant as homozygous IR KO mice and IGF-1R KO mice both die shortly after birth but are phenotypically distinct. The former dies from diabetic ketoacidosis with minimal growth retardation (103,104) while the latter displays severe growth restriction (45% of WT) and dies from respiratory failure (105). In terms of structural isoforms, the IGF-1R does not have any splice variants. However, the IR has two forms, IR-A and IR-B, where exon 11 of the IR transcript encodes for a 12 amino acid domain is excluded in IR-A (78). The IR-B isoform homodimer only binds insulin while the IR-A homodimer can bind both insulin and IGF-2 with equal affinity (106). The IR-A homodimer can also bind IGF-1 but its affinity is lower by ten-fold (106,107). The IR-B is associated with the major metabolic effects of insulin and is expressed in a higher ratio in tissues such as adipose, muscle, and liver (108). In contrast, IR-A is associated with the mitogenic effects of insulin (108,109). The classical IGF-1R homodimer, and the IGF-1/IR-A as well as the IGF-1/IR-B heterodimer can bind both IGF-1 and IGF-2 (78). The
IGF-2R, in contrast, has no intrinsic kinase activity but binds IGF-2 and targets it for degradation (78,110).

Finally, activation of the IGF-1R can be influenced via transactivation, independent of ligand binding, or by mechanisms regulating IGF-1R gene expression. In a mouse fibroblast cell line that overexpresses the EGFR and does not express IGF-1R, transfection with the IGF-1R gene showed that EGFR activation results in tyrosyl phosphorylation of IRS-1 downstream of the IGF-1R and increases survival of the cells. This effect is also observed in cells that are transfected with a mutant IGF-1R that cannot bind IGF-1 but not with a mutant IGF-1R that cannot bind to ATP. As this effect was not attributable to the effects of EGF alone, the study demonstrates the ability of EGF to transactivate the IGF-1R (111). However, whether this transactivation occurs within the intestine is unknown.

1.3.3 IGF-1 in the Intestine

IGF-1 is a potent growth factor that plays a key role in intestinal growth (66). Downstream of its receptor, IGF-1 exerts its proliferative and antiapoptotic effects on the intestinal epithelium predominantly through an IRS-1 dependent manner (112). Systemic administration of IGF-1 to rats mitigates total parenteral nutrition-induced reductions in bowel weight and crypt-villus height (49). IGF-1 treatment also prevents radiation-induced apoptosis of intestinal epithelial stem cells (113,114). In intestinal resection studies in rats, a short bowel syndrome model, IGF-1 enhances bowel adaptation through increased jejunal protein and DNA content, and contributes to significantly increased body weight compared to rats that do not receive IGF-1 treatment (115). Although the intestines of the IGF-1 KO mice are responsive to systemic administration of Wnt signaling agonist, Rspondin-1, they fail to respond to the growth promoting effects of GLP-
2 (35), demonstrating the importance of IGF-1 as a mediator of other intestinal growth stimulating hormones. Interestingly, IGF-2 is also thought to contribute to the GLP-2-induced intestinal growth, with IGF-2 KO mice displaying abrogated proliferative responses to systemic GLP-2 administration. However, the role of IGF-2 is not as prominent as IGF-1 as GLP-2 still induces increases in crypt villus height and mucosal cross sectional area, as well as in intestinal weight in IGF-2 KO mice (35). In the inducible, intestinal epithelial specific IGF-1R KO (IE-IGF-1R KO) model, which expresses cre recombinase under the villin promoter, mice display normal intestinal morphology, with normal intestinal weight, intestine to body weight ratio, crypt-villus height and crypt proliferation after tamoxifen induction (56). However, in response to fasting-refeeding paradigms, the intestine of the IE-IGF-1R KO mice display an impaired ability to recover, with a crypt-villus height that remains reduced after refeeding. Similarly, intestinal epithelial proliferation in IE-IGF-1R KO mice does not increase in response to systemic administration of IGF-1 or GLP-2 (56), demonstrating the critical role that the epithelial IGF-1R plays in facilitating intestinal growth responses to IGF-1 and GLP-2.

In the intestine, the proliferative effects of IGF-1 are regulated, in part, through changes in the expression of IGF-1R. IGF-1 has been shown in vitro to increase intestinal epithelial proliferation in the rat intestinal epithelial cell line (IEC-6) in a synergistic manner with EGF as measured by $^3$H-thymidine incorporation (88). In the same study, IGF-1 was reported to decrease IGF-1R mRNA expression in the IEC-6 cells and this decrease was prevented by co-administration of EGF (88). The synergistic effects of EGF and IGF-1 on IEC-6 cell proliferation has been attributed to maintenance of IGF-1R expression by EGF in the presence of IGF-1 (88). EGF and the intestinal epithelial EGFR (IE-EGFR) have also been demonstrated to be required for the growth and proliferative effects of GLP-2 in vivo (40,57), suggesting that the synergistic
effects of EGF and IGF-1 may also occur in vivo. The control mechanism that regulates intestinal IGF-1R gene expression in vivo is more nuanced, however. In total parenteral nutrition studies, systemic IGF-1 treatment results in decreased intestinal mucosal atrophy compared to rats that only receive vehicle treatment, but this also decreased IGF-1R binding to IGF-1 in whole jejunum sections (49). However, in normal ad libidum fed mice, systemic IGF-1 administration, for 7 days (twice a day) results in increased IGF-1R expression in whole jejunum, likely contributing to the intestinotrophic effects of IGF-1 (116).

More recently, systemic administration of IGF-1 has been suggested to induce the proliferation of the reserve stem cell pool within the intestinal epithelium. This was identified by the use of the Sox9EGFP mice, described earlier (27–29). The quiescent stem cell-like cells were identified by the relatively ‘high’ Sox9EGFP expression within the intestinal crypt and the proportion of these cells that were proliferating increased in response to systemic IGF-1 administration (117). Concurrently, the number of cells that had ‘low’ expression of Sox9EGFP, that had been previously characterized as active stem-like cells (27,28) increased, suggesting that the proliferation of the quiescent stem cell-like cells gives rise to more actively proliferating cells (117). Consistently, derivation of intestinal organoids was enhanced for the cells having ‘high’ expression of Sox9EGFP (ie. quiescent stem cell-like) following supplementation of the established organoid media with IGF-1 (100ng/ml) (117).

Finally, in the intestine, the IGF-1R expression has been localized on the epithelium to both the apical and basolateral side (49). However, as discussed earlier, IGF-1 can bind to multiple receptors including the IR and the IGF/insulin heterodimer receptors. Interestingly, within the intestinal epithelium, the IR isoforms IR-A and IR-B demonstrate different gradients of expression (118). IR-A, associated with mitogenic activities, is expressed in higher proportions
in actively proliferating stem cells, and in progenitor cells from Sox9<sup>EGFP</sup> mice, identified by ‘low’ and ‘sublow’ expression of Sox9<sup>EGFP</sup> (27,28), while the differentiated, postmitotic cells that do not express Sox9<sup>EGFP</sup> express a higher proportion of IR-B (118). These findings were consistent in vitro, wherein the human, colon cancer cell line, Caco-2 cells, express more IR-B compared to IR-A as they become more confluent and differentiated (118).

1.4 Insulin-Like Growth Factor Binding Protein (IGFBP)

1.4.1 Structure, Expression, and Secretion

99% of IGF-1 present within the circulation is bound to an IGFBP which acts as a reservoir for IGF-1 and extends its half-life (3). There are six main IGFBPs (IGFBP-1 through IGFBP-6) with other related binding proteins within the IGFBP super-family showing lower affinity for the IGFs (119). IGFBP-1 was first isolated from human amniotic fluid in 1984 (120). Subsequently, five other IGFBP isoforms were identified each with different cDNA and protein sequences (121). The IGFBPs have 35% sequence homology amongst themselves and 80% homology between species, and have highly conserved structures with three domains, ranging from 216-289 amino acids and with molecular mass between 22.8 and 31.3kDa (3,122,123). The N-terminal and C-terminal domains are relatively conserved amongst the IGFBP isoforms.

However, the central linker domain varies amongst the IGFBP isoforms and contains various sequences such as glycosylation sites in IGFBP-3 and -4, and phosphorylation sites in IGFBP-1, -3 and -5 (122,124). All IGFBPs genes have four exons except IGFBP-3 which has an extra, untranslated exon (119).

The distribution of IGFBP isoforms differs between fluid compartments. In human serum, IGFBP-3 is present with the highest concentration, followed by IGFBP-4, -5, -2, -6, and -1, with
a 50% excess of IGFBP isoforms present compared to IGF proteins (3). In the interstitial fluid of the skin, IGFBP-1 through -4 have been identified (125). In the cerebral spinal fluid, IGFBP-2 and -6 are the major isoforms while in amniotic fluid, IGFBP-1 is the major IGFBP isoform (3,120). The presence of IGFBP isoforms has also been detected in a number of other fluids, including vitreous and aqueous humour, and synovial fluids (3). The concentration of IGFBP isoforms in these fluid compartments can also differ in response to various physiological states and stimuli including: diurnal states, nutrition, pregnancy, development, and aging (3). Various disease states are also known to affect IGFBP level in fluids including diabetes, rheumatoid arthritis, acromegaly, and renal failure (3). The following paragraphs will give a brief overview of the six IGFBP isoforms, their structures, expression, and secretion patterns. A summary of the IGFBP isoform structures and characteristics can also be found in the following table (Table 1.1).
<table>
<thead>
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<td>Major isoform in amniotic fluid</td>
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<tr>
<td>IGFBP-2</td>
<td>RGD sequence</td>
<td>Major isoform in cerebral spinal fluid &amp; lymph</td>
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| IGFBP-3      | Three glycosylation sites | Major isoform in serum  
Forms ternary complex with IGF-1 and Acid-Labile Subunit (ALS) | (3,126) |
| IGFBP-4      | Glycosylation site | Can interact with Frizzled 5/6, and inhibit Wnt signaling | (127–129) |
| IGFBP-5      | Glycosylation site | Interacts with extracellular matrix  
Potentiates IGF-1 activity  
Forms ternary complex with IGF-1 and ALS  
High affinity for hydroxyapatite in bone | (130–132) |
| IGFBP-6      | Two less cysteines than other isoforms  
One glycosylation site | Higher affinity for IGF-2, inhibit IGF-2 activity | (133–135) |

Table 1.1 Summary of Unique IGFBP Isoform Characteristics (3,122).
IGFBP-1 is the major IGFBP isoform present in the amniotic fluid (3,136), but is secreted from the liver, kidneys, and decidua and has been identified in various fluids including serum and interstitial fluid (125,136). Structurally, it is 234 amino acids long with a molecular weight of 28-34kDa, with three phosphorylation sites at Ser101, Ser119, and Ser169. It is expressed on chromosome 7 in humans (119,121,137). Circulating IGFBP-1 displays diurnal variation and fluctuates in response to food intake (3). Its serum levels are lowest at night, highest in the morning, and are especially high in the morning after an overnight fast (3). Clinically, subjects with type-1 diabetes have higher levels circulating of IGFBP-1 (138,139). In addition, intrauterine growth restriction is associated with elevated levels of IGFBP-1 in fetal cord serum (140) and this has been suggested to directly inhibit fetal growth in transgenic overexpression mouse models (141).

IGFBP-2, at 36kDa, was first isolated from rat liver cells in 1986 (142). Structurally, it is 289 amino acids long, its molecular weight is 31kDa, and it is expressed on chromosome 2 in humans (119). It is the predominant IGFBP isoform found in seminal fluid (136) but is also the one of the dominant IGFBP isoforms in cerebral spinal fluid and milk (3). In contrast to IGFBP-1, serum IGFBP-2 levels do not fluctuate significantly. However, levels have been found to be diminished in humans in response to protein restriction, and in patients suffering from anorexia (3). Its secretion is decreased in neuroblastoma cells in response to retinoic acid (143).

IGFBP-3 was discovered in 1986 in human plasma (144). Structurally, it is 264 amino acids long, and is expressed on chromosome 7 in humans (119). It has three glycosylation sites (145) and thus, the molecular weight ranges between 30-44 kDa (123). In the circulation, IGFBP-3 is the most abundant isoform with a 3200µg/L mean concentration in adult serum, about ten-fold higher than the other isoforms (3,146,147), and its serum levels show strong association with
growth hormone levels, similar to IGF-1 (126). Thus, serum levels of IGFBP-3, like IGF-1, differ by age- increasing through puberty and decreasing throughout adult life (126). Consistently, in patients with acromegaly who have high levels of circulating IGF-1 due to high levels of growth hormone, IGFBP-3 levels are also elevated, whereas growth hormone deficient patients have low levels of IGF-1 and IGFBP-3 (126). Interestingly, circulating IGFBP-3 levels are also found to be elevated in those with liver failure and in pregnant women in their third trimester (126). IGFBP-3 expression and secretion has been demonstrated in various non-hepatic tissues as well in rats and humans including intestine, skin fibroblasts and bones (121,136).

Of particular interest for this work, IGFBP-4 was first isolated from human osteosarcoma TE89 cells and rat serum in 1989 (127,128). Rat and human IGFBP-4 cDNA were first cloned in 1990 with a predicted amino acid length of 233 amino acids for rats and 237 for humans (148). Mouse IGFBP-4 was subsequently cloned in 1993 and was found to be highly homologous to rat IGFBP-4 with differences in only 3 amino acids, whereas the peptide differs from human IGFBP-4 by 24 amino acids (149). In humans, IGFBP-4 gene is located on chromosome 17 and is 24-36 kDa depending on reducing conditions and glycosylation states (123). The C-terminal and N-terminal domains of IGFBP-4 are highly conserved compared to other IGFBP isoforms but the mid-protein, linker domain differs with a N-glycosylation site (119,124) as well as two extra cysteine residues (3,150). Whether the cysteine residues contribute to the unique biological actions of IGFBP-4 is not well defined (151). The glycosylation state of IGFBP-4 has been shown to not affect its affinity for IGF in the rat cell model (151). The known biological actions of IGFBP-4 will be discussed further in the next section. IGFBP-4 has no known polymorphism with an altered physiological activity to date. The C-terminal does not contain an arginylglycylaspartic acid (RGD) sequence that other isoforms have to bind to integrins for
interaction with cell membrane and the extracellular matrix (119). Although lower than IGFBP-3, IGFBP-4 is the second most abundant binding protein isoform in the serum (3).

Within different tissues, the mechanisms that influence IGFBP-4 expression are variable by cell-type and are not well understood. In mouse osteoblasts, IGFBP-4 expression and secretion is increased in response to 1,25-dihydroxyvitamin D3 treatment in vitro (152) and results in increased serum levels in vivo (153). 1,25-dihydroxyvitamin D3 is thought to inhibit osteoblast proliferation in part through inhibition of IGF-1 activity by enhanced IGFBP-4 secretion (127,152). IGFBP-4 expression levels have also been reported to increase in rat osteoblasts in response to treatment with basic fibroblast growth factor, platelet-derived growth factor, TGF-α, and EGF (154). In rat granulosa cells in vitro, IGFBP-4 is increased transiently by follicle stimulating hormone and IGF-1 (155). In neuroblastoma cells, retinoic acid has been shown to decrease IGFBP-4 secretion (143). There is also evidence that, in mouse Leydig cells, IGF-1 stimulates while IGF-2 reduces the secretion of IGFBP-4 (156). Finally, in colorectal cancer cells, IGFBP-4 expression is stimulated by Sox9, a downstream target of the canonical Wnt signaling pathway, such that Sox9 directly binds to the promoter region of IGFBP-4 to increase its expression and secretion and, thereby, inhibiting epithelial cell proliferation in vitro (157). Overall, therefore, expression and secretion control mechanisms of IGFBP-4 are variable and both cell- and environment-dependent.

IGFBP-5 is the third most abundant binding protein the serum, discovered in 1991 in rat serum (130). Structurally, human IGFBP-5 has 252 amino acids and is expressed in chromosome 2 (119). It has one glycosylation site (119), resulting in molecular weight between 29-32 kDa. In general, IGFBP-5 expression is stimulated in response to growth stimulating factors. In human smooth muscle cells, expression was found to decrease with increasing confluency and in
response to endogenously produced transforming growth factor-β (131). In rat osteoblast cells in vitro, endogenous and exogenous IGF-1 were found to stimulate IGFBP-5 secretion (158).

Retinoic acid in human osteoblast cells also increases IGFBP-5 mRNA expression (159).

IGFBP-6 was discovered in human cerebral spinal fluid in 1989 (134). It has 216 amino acids and, in the human, is expressed on chromosome 12 (119,160). It differs from the other IGFBPs because it lacks the N-terminal domain six disulfide bonds found in all isoforms (122). Similar to the other isoforms, IGFBP-6 is expressed in a variety of cells including fibroblasts, intestine, and osteoblasts and is regulated by a number of stimuli. For example, in human osteoblast cells in vitro, IGFBP-6 mRNA expression is increased by retinoic acid treatment (159).

A number of other IGFBP-related proteins (IGFBP-rPs) also exist within the IGFBP superfamily. These include IGFBP-rP1 through IGFBP-rP9, which differ from IGFBP-1 through -6 due to their lower affinity for the IGF proteins (119). For example, IGFBP-rP1 (also known as Mac25, IGFBP-7, TAF, or PSF) was independently isolated by three different groups from human meningioma, fibroblast, and bladder carcinoma cell lines, respectively (161–163).

Structurally, IGFBP-rPs have 40-57% sequence similarities to the IGFBP family proteins (119). Similar to IGFBP-3, the IGFBP-rP genes have five exons, with the fifth exon being an untranslated region (119). The secretion of IGFBP-rPs is stimulated by a number of stimuli including parathyroid hormone, cortisol, and glucocorticoids in osteoblasts, and transforming growth factor-β in breast cancer cell lines (119).

1.4.2 Biological Activity

Binding of IGFs to IGFBPs in the circulation extends the half-life of IGF-1 and promote the mitogenic activity of IGF-1. Figure 1.4 depicts the role of IGFBPs in the circulation. The
following will briefly cover various factors that regulate the biological activity of IGFBP isoforms, with a predominant focus on IGFBP-3, IGFBP-4, and IGFBP-5 which are the three most common isoforms in the circulation, as well as in the intestine (3,164).
Figure 1.4 Role of IGFBP Isoforms in the Circulation

IGFBP-3 is the predominant isoform present in the circulation, with IGFBP-4 and IGFBP-5 being the next two most common isoforms. Binding of IGFs to IGFBPs extends their half-life in the circulation. In addition, because the majority of IGFs present in the circulation are bound to IGFBPs, IGFBPs prevent the acute metabolic effects of IGFs through the insulin receptor and promote the chronic mitogenic effects of IGF by maintaining a reservoir of IGF. IGFBP-3 and IGFBP-5 can also associate with Acid-Labile Subunit (ALS) which further extends the half-life of the IGFs.
As a modulating protein, IGFBPs can inhibit or stimulate IGF-activity. This occurs by either preventing IGFs from binding to their receptor or potentiating IGF action by interacting with the extra-cellular matrix or the cellular membrane in certain tissues, sequestering IGFs, and bringing the IGF closer to its receptor. Both activities exist in the literature depending on the IGFBP isoform and system being studied. Differences in the regulation of IGF activities by the IGFBP isoforms also occur through differences in IGF affinity, increased expression in specific tissues, ability to interact with cellular membranes and extracellular matrices in certain tissues, and through susceptibility to various IGFBP proteases. Some of the IGFBPs also have IGF- and IGF-1R-independent effects through interaction with other receptors and associated stimulation of downstream signaling pathways. IGFBP bioactivity is complex with many regulatory mechanisms in place. Figure 1.5 depicts the various biological activity that IGFBP may have in the extravascular space.
IGFBPs exist in the circulation and extravascular state. IGFBPs can potentiate the growth/proliferation-promoting effects of IGF proteins by bringing them in close contact with the IGF-1R. This may occur through IGFBP binding to the cellular membrane or the extracellular matrix, or its ability to cross the vascular endothelial barrier from the circulation into a specific extravascular space. IGFBPs can also inhibit the growth/proliferative actions of IGFs by inhibiting them from binding to the IGF-1R. Finally, IGFBPs can have IGF-independent effects by binding to their own receptor and promoting cellular proliferation and differentiation process in this way.

Figure 1.5 Various Biological Actions of IGFBP In the Extravascular Space
Binding affinity of the IGFBPs for IGF-1 and IGF-2 differs depending on the isoform. IGFBP-1, -3, -4 have comparable affinity for IGF-1 and IGF-2, whereas IGFBP-2, -5, and -6 have higher affinity for IGF-2 (3). Affinity for the IGFs can also be modulated. In the case of IGFBP-1, phosphorylation of Ser101 increases its affinity for IGF-1 (137). Compared to the IGFBP isoforms, the IGFBP-rPs have about 100-fold lower affinity to IGF-1 and IGF-2 (119).

Some IGFBP isoforms also have their own receptors, or exert IGF-1R-independent effects. For example, in intestinal smooth muscle cells, IGFBP-5 induces cell proliferation and IGF-1 secretion through its own receptor, independent of IGF-1/IGF-1R and in a MEK/ERK1/2-dependent manner (131,165). Since IGFBP-5 also augments the actions of IGF-1 (131), this creates a positive feedback loop that facilitates smooth muscle proliferation in the intestine. In the bone, IGFBP-5 has similarly been suggested to act as a growth factor, independently of IGF-1, increasing bone alkaline phosphatase activity, an indicator of osteoblast activity, in the absence of IGF-1 administration and in IGF-1 KO mice in vivo (166). IGFBP-3 also has been observed to induce IGF-1- and IGF-1R-independent inhibition of cell proliferation (167), as well as mediating apoptosis in breast cancer and prostate cancer cell lines, likely through cell surface interaction with another receptor (168,169). IGFBP-4 can also directly interact with the Wnt receptor, Frizzled 5 and 6, to inhibit the canonical Wnt signaling pathways in cardiomyocytes, independent of IGF-1 and IGF-1R to stimulate cardiomyocyte differentiation (129).

IGFBPs have proteases that specifically target their degradation as well as non-specific proteases, providing an additional regulation of IGFBP activity. Of particular interest to this work, pregnancy-associated plasma protein-A (PAPP-A) was originally identified as a circulating factor that was elevated in pregnant women (170,171) and was later established as an IGFBP-4 and IGFBP-5 protease in in vitro studies using human fibroblast cells (172). PAPP-A is
in the pappalysin class of the metzincin superfamily, which is a large group of proteases that share structural similarities and includes proteases such as matrix metalloproteases (MMPs), adamlynsins, and serralysins (170). The PAPP-A protein is composed of five domains: the N-terminal, C-terminal, proteolytic domain, an uncharacterized mid sequence domain, and a complement control protein module which allows for PAPP-A to adhere to cell surfaces to cleave IGFBP-4/-5 and release IGF-1 in specific local environments (170). PAPP-A is expressed in a number of different cells including the placenta, granulosa cells, osteoblasts, and vascular smooth muscles (170). It is not reported to be expressed in the human adult small or large intestine (173). Overall, PAPP-A has been implicated in regulating the bioactivity of IGFBP-4 and thus, IGF-1, in a variety of physiological settings including folliculogenesis, wound healing, vascular remodeling, bone morphogenesis, and fetal growth (170). Interestingly, PAPP-A proteolytic activity is regulated by the conformation of IGFBP-4. Binding of IGF proteins to IGFBP-4 induces a conformational change in the protein which renders IGFBP-4 more susceptible to proteolytic cleavage by enzymes such as PAPP-A (172). Thus, cleavage by proteases provides an additional layer of control that regulates the bioactivity IGFBP-4, and in turn, that of IGF-1.

Other notable IGFBP protease proteins include PAPP-A2, which is also in the metzincin superfamily and is structurally and functionally related to PAPP-A but has a higher affinity for IGFBP-5 (170). However, unlike PAPP-A, PAPPA-A2 does not display affinity for IGF-bound IGFBP-5 over unbound IGFBP-5. Finally, in the serum, there are proteases which can degrade multiple IGFBP isoforms such as serine proteases and plasmin (3).

IGFBP-3 and IGFBP-4 can be N-glycosylated and IGFBP-5 and IGFBP-6 can be O-glycosylated (122). Whether glycosylation of IGFBP affects their affinity to IGF proteins is not known.
However, the glycosylation and binding status of the IGFBP may delay clearance, at least for IGFBP-6 (174). In addition, perfusion studies of rat hearts show preferential localization of glycosylated IGFBP-4 to the connective tissues while unglycosylated IGFBP-4 localizes to the muscle layer (175).

Many location-specific factors regulate the activity of the IGFBPs. Consequently, IGFBP isoforms have been described to have opposing roles, potentiating the effects of IGF in certain situations while inhibiting IGF actions in others. For example, IGFBP-3 and IGFBP-5 form a ternary complex with IGF-1 and the glycoprotein, Acid-Labile Subunit (ALS) to extend the half-life of circulating IGF-1 (3,122,176,177). However, this complex cannot cross the endothelial barrier from the circulation to the extracellular fluid, thus restricting the actions of IGF-1 on tissues. Consequently, the ternary structure created by IGFBP-3 and IGFBP-5 functions as an IGF-1 reservoir in the circulation (3). Thus, in response to a bolus injection of IGF-1, IGFBP-3 and IGFBP-5 inhibit the metabolic actions of IGF-1 while potentiating the mitogenic actions of IGF-1 by extending its half-life in the circulation (3) (Figure 1.4).
Consistently, triple KO mice of all of the three major IGFBP isoforms in the circulation, IGFBP-3, IGFBP-4, and IGFBP-5, results in impaired postnatal growth, largely attributed to a significant reduction in circulating IGF-1 levels (178). However, in specific tissues and cell types, these IGFBPs have been found to play different roles. In human intestinal smooth muscle cells, IGFBP-3 inhibits IGF-1-mediated proliferation in vitro (131). Conversely, in bones and intestinal smooth muscle, IGFBP-5 has a more distinct IGF-potentiating role. IGFBP-5 has an RGD sequence, known to interact with integrins expressed on cell membranes (119) and, when administered in vitro, IGFBP-5 potentiates IGF action in a mouse clonal osteoblastic cell line, and human fetal fibroblast cell line (179,180). IGFBP-5 also has a high affinity for hydroxyapatite found in bones and is speculated to stimulate IGF-mediated bone remodeling and proliferation as well as IGF-1-independent proliferation (132,166). Consistently, systemic administration of IGFBP-5 in vivo stimulates bone formation observed through increased bone alkaline phosphatase activity and serum osteocalcin levels, both of which increase in association with increased osteoblast activity (181). IGFBP-6 in contrast, has been reported to largely inhibit the actions of IGF-2, specifically due to its higher affinity for IGF-2 over IGF-1 (122,133–135).

While the role of IGFBP-4 in vivo has been described as growth-promoting as well as growth-inhibiting, many studies suggest that IGFBP-4 is inhibitory (3,179). IGFBP-4 does not directly interact with the cellular membrane or extracellular matrix, which is one way in which IGFBP potentiates the actions of IGFs. However, perfusion studies using rat hearts have demonstrated the ability of IGFBP-4 to cross the endothelial barrier and preferentially localize in the connective and muscle tissue depending on the IGFBP-4 glycosylation states (175). In addition, systemic administration IGFBP-4 results in enhanced bone formation which occurs in a protease dependent manner, suggesting an inhibitory role of IGFBP-4 at the same time as enhancing IGF-
delivery to the bone (182,183). Interestingly, whole-body, or total IGFBP-4 KO mice are growth restricted, suggesting that IGFBP-4 can play a growth stimulatory role (184). Specifically however, IGFBP-4 seems to facilitate IGF-1/IGF-2-stimulated prenatal growth and IGF-1-stimulated postnatal growth due to the actions of IGFBP-4 protease, with the IGFBP-4 KO mice having smaller birth weight by 10-15% compared to the wild-mice, a deficit that is maintained throughout postnatal growth and into adulthood (170,184). Collectively, these studies establish a role for circulating IGFBP-4 in promoting growth and development; however, whether the growth promoting effects of IGFBP-4 always requires degradation or actions of proteases to liberate IGF proteins is not well established.

Many studies support the notion that IGFBP-4 requires the presence of proteases that cleave and release IGF proteins to stimulate growth. For example, the enhancement of bone formation by systemic administration of IGFBP-4 has been demonstrated to be protease dependent as mutant, protease resistant IGFBP-4 does not stimulate bone growth in the same way in vivo (182). In addition, the whole-body, or total PAPP-A KO mice are severely growth restricted at birth (~60% WT body weight), in addition to the accumulation of serum IGFBP-4 levels, attributed to lack of enzymatic cleavage of IGFBP-4 (184). The growth restricted phenotype of the PAPP-A KO mice, which is similar to IGF-2 KO mice, suggests that in this model, because IGFBP-4 remains uncleaved, IGFBP-4 inhibits the actions of IGF-2 prenatally (184,185). Consistently, overexpression of PAPP-A results in enhanced growth due to increased IGFBP-4 cleavage and release of IGFs in the localized bone and skeletal muscle growth (186,187). However, regardless of the mechanism through which IGFBP-4 regulates IGF-induced growth, what remains clear is that both circulating and locally expressed IGFBP-4 contribute to the regulation of IGF activity. Overall, these studies demonstrate the complexity of IGFBP activity and its proteases in growth
and development and indicate that the role of systemic and locally expressed IGFBP-4 in regulating the bioactivity of IGF-1 is context-dependent. Finally, the IGFBP-4 KO mice do not display any compensatory changes in serum levels of other IGFBP isoforms (178,184), and are thus an important loss-of-function tool in studying the role of IGFBP-4 in specific tissues in this work. Conversely, as PAPP-A KO mice are established to have an accumulation of circulating IGFBP-4 (184) and because PAPP-A has been reported to not be expressed in the small intestine or the colon (173), the PAPP-A KO mice were used in this work as a gain-of-function model to study the role of circulating IGFBP-4 in intestinal growth. Please refer to Figure 1.6 for a summary of what is known about IGFBP-4 and IGFBP-4 bioactivity to date.
Figure 1.6 Known Biological Actions of IGFBP-4

IGFBP-4 is secreted into the circulation from the liver and is also expressed in various tissues including intestine, bone, and granulosa cells. It is cleaved by the IGFBP-4-specific protease PAPP-A as well as by non-specific proteases which liberates the bound IGF proteins. It has been shown to be largely inhibitory to IGF-mediated actions except for instances where it plays a role in sequestering IGF in specific tissues and indirectly potentiates the actions of IGFs. It is also known to inhibit the canonical Wnt signaling pathway by directly interacting with the Wnt receptor, Frizzled 5/6.
1.4.3 IGFBP-3/4/5 in the Intestine

Within the intestine, IGFBP-3, -4, and -5 are most highly expressed (164), with expression that gradually decreases postnatally and into adulthood in rodents (188). Rats 19 days postnatally, appear to express IGFBP-3 through IGFBP-6 largely within the lamina propria and IGFBP-5 in the muscularis externa layer (188). Within the intestine, the expression of IGFBP-3 and IGFBP-5 in rats with total parenteral nutrition is highest in the lamina propria and the muscularis externa layer, respectively (189). In parenteral nutrition models, systemic administration of IGF-1 has also been associated with enhanced intestinal IGFBP-3 and IGFBP-5 expression (189,190). Combined with the fact that IGFBP-5 can associate with the extracellular matrix to potentiate IGF-1 action (180), as well as with findings that IGFBP-3 can enhance the effects of IGF-1 in breast cancer cells in vitro (154), it was proposed that IGFBP-3 and IGFBP-5 play potentiating roles in the intestinotrophic effects of IGF-1 (116). IGFBP-5 KO mice retain their proliferative response to systemic IGF-1 treatment, potentially due to compensatory increases in jejunal IGFBP-3 expression (191). However, whole-body IGFBP-3 and IGFBP-5 double KO mice have increased intestinal weight under basal conditions and an abrogated increase in intestinal weight, crypt mitotic index, and crypt-villus height in response to systemic IGF-1 treatment for 7 days (116). This suggests that IGFBP-3 and IGFBP-5 play an inhibitory role in intestinal growth, but play a potentiating effect in response to systemic administration of IGF-1. Interestingly, in response to systemic GLP-2 administration, IGFBP-3 and IGFBP-5 double KO mice display a sustained growth response with normal increases in jejunal weight, crypt mitotic index, and crypt-villus height (116). This was unexpected as the IGF-1-IE-IGF-1R system is an established intermediary pathway that is required for the growth effects of GLP-2 to take place (35,56).
In contrast to IGFBP-3 and -5, not as much is known about changes in the expression of IGFBP-4 in response to IGF-1 or GLP-2 treatment, as well as regarding its role within the intestine in response to these treatments. A role for IGFBP-4 within the intestine was first suggested when its expression was found to increase in response to intestinal resection (63). Within the intestinal mucosa, IGFBP-4 is localized to the intestinal epithelial crypt in adult mice (157) where increased proliferation plays a critical role in stimulating intestinal growth. IGFBP-4 expression has also been reported in the intestinal subepithelial myofibroblasts with evidence of increased IGFBP-4 cleavage in response to GLP-2 treatment, in vitro (192). In addition, unlike IGFBP-3 and IGFBP-5 which complex with ALS with IGFs in the circulation, IGFBP-4 does not form this ternary complex (122). The IGF-1+IGFBP isoform complex is thought to be able to cross the vascular endothelial layer into local tissue/intracellular fluid while the ternary complexes are essentially confined to the circulation (122). Thus, if IGFBP-4 plays a role in intestinal mucosal growth, both the circulating and the locally expressed IGFBP-4 may contribute.

In vitro ChIP assays using the Caco-2 and DLD-1 colonic cell lines, transfected with an IGFBP-4 plasmid and a Sox9-pcDNA vector established that Sox9 has multiple binding sites close to the IGFBP-4 promoter region (157). In addition, transfection of these cells with the Sox9-pcDNA vector results in a significant decrease in the proliferation rate, and this is reversed in the presence of an anti-IGFBP-4 antibody in the cell media. Consistently, siRNA knockdown of Sox9 results in increased proliferation (157). Therefore, with human colonic epithelial models in vitro, IGFBP-4 appears to be inhibitory to epithelial growth, and Sox9 appears to be an important regulator of IGFBP-4 expression and function. Given that both IGFBP-4 and Sox9 expression has been localized to the intestinal crypt (27,28,157), it becomes increasingly paramount to
investigate the role and mechanism through which IGFBP-4 exerts its effects on epithelial proliferation and the role that Sox9 plays in regulating IGFBP-4 expression in vivo.

Finally, both GLP-2 and IGF-1 are established to stimulate the intestinal epithelial Wnt signaling pathway through increased β-catenin translocation to the nucleus and enhanced Wnt target expression including c-Myc and Sox9 (193). IGF-1 has been shown to stabilize β-catenin through inhibition of the GSK-3 substrate of the β-catenin destruction complex via PI3K/Akt pathway in vitro (193–195). In vitro studies using the mouse fibroblast cells have also suggested that IGF-1-mediated IRS-1 activation induces its interaction with the β-catenin complex to stimulate β-catenin translocation into the nucleus (196). In addition, overexpression of IRS-1, the mediator of the proliferative/anti-apoptotic effects of IGF-1, in the rat intestinal epithelial cell line, IEC-6, results in an increase in Sox9 expression (197). Finally, EGFR (ErbB1), has also been demonstrated to directly interact with β-catenin (198,199). Thus, investigation of the role of GLP-2, and its intermediaries IGF-1 and EGF in regulating IGFBP-4 expression in the intestine is warranted.
1.5 Rationale and Hypotheses

Intestinal mucosal growth is regulated by multiple factors and mechanisms. Both IGF-1 and GLP-2 play important roles in intestinal physiology under homeostatic conditions, and in disease states. In addition, IGFBP-3 and IGFBP-5 have been demonstrated to play an important role in regulating intestinal physiology both under basal conditions and in response to systemic administration of IGF-1 (116). IGFBP-4 is another IGFBP isoform that is highly expressed within the intestine that likely regulates the bioactivity of IGF-1 in this tissue (164). The aim of this work, thus, is to define the role and regulation of IGFBP-4 specifically in the context of intestinal mucosal growth and proliferation by testing the following hypotheses, using both in vivo and in vitro models:

1. IGFBP-4 is required for the intestinal growth effects of GLP-2;

2. IGFBP-4 inhibits basal intestinal epithelial proliferation; and

3. Intestinal IGFBP-4 expression is stimulated by GLP-2 and its intermediate factors IGF-1 and EGF.
Chapter 2 Materials and Methods

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2 Materials and Methods

2.1 In Vivo Studies

All animals were housed at the University of Toronto, Division of Comparative Medicine with ad libitum feeding under a 14-hour light and 10-hour dark cycle. All animal protocols were approved by the University of Toronto Animal Care Committee.

2.1.1 IGFBP-4 KO Mice

IGFBP-4 KO mice and the WT mice on a C57BL/6 background were developed and provided by Dr. John Pintar (Rutgers University, New Jersey) (184). Heterozygous breeding pairs were used to breed mice for experiments and litters were genotyped with primers outlined in Table 2.1 to yield the following products by PCR: 222bp IGFBP-4 gene and 493bp neomycin resistance cassette. PCR was conducted with a denaturation temperature of 94°C, annealing temperature of 62°C and extension temperature of 72°C for 35 cycles.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>IGFBP-4 Forward</td>
<td>5’-GGTTGCGAGGAGTGGTG-3’</td>
</tr>
<tr>
<td>IGFBP-4 Reverse</td>
<td>5’-AGAGGCTGTGCAGGCTTTTCTT-3</td>
</tr>
<tr>
<td>Neomycin Forward</td>
<td>5’-AGGATCTCCTGTCATCTCACCTTGCTCCTG-3’</td>
</tr>
<tr>
<td>Neomycin Reverse</td>
<td>5’-AAGAATGCAAGAAGACGATAGAAGGC-3’</td>
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</table>

Table 2.1 Primers Used To Genotype IGFBP-4 KO Mice
2.1.2 PAPP-A KO Mice

PAPP-A KO and WT mice on a C57BL/6,129 background were a kind gift from Dr. Cheryl A. Conover (Mayo Clinic, Minnesota) (185). Mice were genotyped according to primers outlined in Table 2.2 to yield the following products by PCR: 306bp pappalysin-1 gene and 481bp neomycin resistance cassette. PCR was conducted with a denaturation temperature of 94°C, annealing temperature of 60°C and extension temperature of 72°C for 35 cycles.

<table>
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<tr>
<td>Pappalysin-1 Forward</td>
<td>5'-ATGATTTCATGAGATTGGGCATAG-3'</td>
</tr>
<tr>
<td>Pappalysin-1 Reverse</td>
<td>5'-TGTTGTAAGGAGTGTTGAAGAAGC-3'</td>
</tr>
<tr>
<td>Neomycin Forward</td>
<td>5'-AGGATCTCCTGTCATCTCACCTTGCTCCTG-3'</td>
</tr>
<tr>
<td>Neomycin Reverse</td>
<td>5'-AAGAACTCGTCAAGAAGGCATAGAAGGC-3'</td>
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Table 2.2 Primers Used To Genotype PAPP-A KO Mice

2.1.3 IE-IGF-1R KO Mice

The IE-IGF-1R KO mice (B6-Igf1tm1.1Mhz; Tg(Vil-cre/ERT2)23Syr) were made as previously described (56,60). Tg(Vil-cre/ERT2)23Syr mice, previously known as the Villin-CreERT₂+/0 mice were originally from Dr. Sylvie Robine (Institut Curie-CNRS, Paris) (200). The B6-Igf1tm1.1Mhz mice, also known as the IGF-1R^{flox/flox} mice, were originally from Dr. Martin Holzenberger (Université Pierre et Marie Curie, Paris) (201,202). The mice were maintained in the animal facility and crossed by Melanie Markovic, a fellow graduate student in the laboratory. Mice were genotyped with primers as outlined previously (203), and in Table 2.3 by PCR: 390bp
Cre gene and 327bp floxed IGF-1R gene or the 271bp WT IGF-1R gene. PCR was conducted with a denaturation temperature of 94°C, annealing temperature of 60°C and extension temperature of 72°C for 39 cycles. KO was induced by tamoxifen injection interperitoneally (1mg per mouse, per day) for 5 days to excise the floxed exon 3 of the IGF-1R gene. KO was confirmed by semi-quantitative RT-PCR for IGF-1R expression in the intestinal mucosa (refer to section 2.1.3 Gene Expression).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Cre Forward</td>
<td>5’-CCTGGAAAAATGCTTCTGTCCG-3’</td>
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<tr>
<td>Cre Reverse</td>
<td>5’-CAGGGTGTATAAGCAATCCCC-3’</td>
</tr>
<tr>
<td>IGF-1 flox/flox Forward</td>
<td>5’-ATCTTGGAGTGGGTGCTTGTTT-3’</td>
</tr>
<tr>
<td>IGF-1 flox/flox Reverse</td>
<td>5’-ATGAATGCTGAGGTGGTGTCTTT-3</td>
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</tbody>
</table>

**Table 2.3 Primers Used To Genotype IE-IGF-1R KO Mice (B6-Igf1rtm1.1Mhz; Tg(Vil-cre/ERT2)23Syr)**

**2.1.4 Acute and Chronic Treatment Protocols**

Female and male mice between the age of 8-12 weeks were age, sex and weight matched for experiments.

For acute inhibitor treatment studies, mice were given wortmannin (1.5µg/kg, Sigma) in 4% methanol or vehicle by oral gavage 6.5 and 3.5 hours before sacrifice by isofluorane overdose.

For chronic GLP-2 treatment studies, the human degradation-resistant GLP-2 analog, h(Gly²)-GLP-2 (American Peptide Company, Inc) was used as previously described (35,56,60). Briefly,
mice were given subcutaneous injections of either vehicle (phosphate buffered saline (PBS)), or a pharmacological (0.1µg/g) or suprapharmacological (10µg/g) dose of h(Gly²)-GLP-2, daily for 10 days with a final injection 3 hours before euthanasia.

For chronic EGF receptor inhibition studies, mice were administered CI-1033 (20mg/kg) in H₂O by oral gavage for 10 days, which has been previously reported to be efficacious and well tolerated (204,205).

For all in vivo experiments, mice were weighed, intestines were collected, and whole intestinal lengths were measured vertically under constant tension. The intestines were then flushed with PBS, blotted, and weighed. Two 2-cm segments of jejunum were collected from each sample, flash-frozen, and stored at -80°C for RNA extraction from mucosal scrapes or whole thickness. One 2-cm segment of jejunum was collected from each sample, fixed in 10% formalin, and paraffin embedded for immunohistochemical and morphometric analyses.

2.2 In Vitro Studies

2.2.1 Fetal Rat Intestinal Culture Cells

As described and validated previously (35,206), fetal rat intestinal cells (FRICs) were collected by enzymatic digestion of whole fetal rat intestines (mixed sex) from pregnant female Wistar rats at gestational day 19-20 (Charles River) and cultured overnight in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) containing glucose (4.5g/L), 5% fetal bovine serum (FBS, Gibco), penicillin (40 U/mL), and streptomycin (40µg/ml) in 10-cm plates. Cells were then treated with h(Gly²)-GLP-2 (10⁻⁸ M) or vehicle in DMEM containing glucose (1g/L) with 0.5% FBS for 0.5–24 hours.
2.2.2 IEC-6 cells

Rat IEC-6 cells were purchased from ATCC or kindly provided by Dr. P. Kay Lund (UNC-Chapel Hill, North Carolina), originally developed by Dr. Andrea Quaroni (207). As previously reported (88,208), these cells were grown subconfluently (20-25%) and passaged weekly and grown in complete growth medium containing: high-glucose DMEM (4.5g/L glucose), with 10% heat-deactivated FBS, pencillin (50U/ml), streptomycin (50µg/ml), insulin (5µg/ml), transferrin (5µg/ml), and sodium selenite (5ng/ml) (ITS; Roche). For experiments, cells were cultured in serum free media which contains DMEM supplemented with glucose (4.5g/L), 0.1% bovine serum albumin (BSA, Sigma), pencillin (50U/ml), streptomycin (50µg/ml), and transferrin (5µg/ml). Cells between passages 17-23 were used for experiments.

2.2.3 Organoids

Jejunal mouse organoids were cultured under standard organoid culture conditions (37). Briefly, jejunal crypts were isolated by mucosal scrape and PBS wash, followed by EDTA digestion (5mM, 4°C, 30min). Crypts were then centrifuged and embedded in Matrigel domes (Corning) in 24 well plates (Grenier) with media (500µl/well) containing Advanced DMEM/F12 (Gibco) supplemented with the following: Glutamax (1x, Gibco), Hapes (10mM, Gibco), Pencillin, Streptomyycin (100U/ml,100µg/ml, Gibco), B27(1x, Gibco), N2 (1x, Gibco), n-acetylcysteine (1mM, Sigma), Noggin (100ng/ml, PeproTech), EGF (50ng/ml, Gibco), and 10% R-spondin1 conditioned medium. The media was also supplemented with Rock inhibitor, Y-2763 (10mM, Sigma) when the organoids were first derived and when they were passaged. Media was changed every 3-4 days. Organoid surface area was measured as a measure of growth (209) using AxioVision Version 4.9.1 Software (Carl Zeiss).
For R-spondin1 conditioned medium, HEK293T-HA-RSP-FC- Z cells, a kind gift from Dr. Catherine O’Brien (University of Toronto) were used. Cells were grown in Advanced DMEM/F12 supplemented with pencillin/streptomycin (100U/ml,100µg/ml), Hepes (10mM), Glutamax (1x), and Zeocyn (300µg/ml, Invitrogen). Cells were split weekly. Upon reaching confluency, the media was changed to Zeocyn-free media and collected one week after. Media was centrifuged, sterile filtered, and stored as frozen aliquots for organoid media.

2.2.4 Proliferation Assays

Proliferation assays were adapted from previously reported studies (88,208). Briefly, IEC-6 cells were plated in 24 well plates (1x10^4 cells per well) and incubated in complete growth medium for 16 hours before 24 hour serum-starvation in serum-free DMEM. Medium was then aspirated and cells were washed with Hank’s balanced salt solution; cells were then cultured for 24 hours in serum-free medium with or without rat IGF-1 (10-1000ng/ml, Abcam) and/or rat IGFBP-4 (6.25-25nM, GroPep). For EGF pre-treatment studies, cells were cultured in serum-free medium with and without mouse EGF (5ng/ml; Life Technologies) for 6 hours after the 24 hour serum-starvation, then treated with IGF-1 and/or IGFBP-4 for 18 hours. Both pre-treatment and treatment medium contained [methyl-^3H]-thymidine (1µCi/ml). Cells were then washed in 4°C PBS twice and treated with 4°C 5% trichloroacetic acid, and the precipitated DNA was collected in 0.1 M NaOH. DNA synthesis was assessed by quantifying radioactivity of the samples by scintillation counting.

For inhibitor experiments, cells were co-incubated with NVP-AEW541 (0.1-1 µM, Caymen Chemicals), wortmannin (0.5 µM, Sigma), LY294002 (5µM, Sigma), PD98059 (10µM, Tocris), or U0126 (5µM, Tocris).
2.3 Gene Expression

Total RNA from whole intestine, mucosal scrapes, or cells was extracted using the QIAshredder (QIAGEN) and RNeasy (QIAGEN) kits. For semi-quantitative RT-PCR, RNA samples were reverse-transcribed with 5x All-in-One RT Mastermix (Applied Biological Materials Inc), and PCR was conducted using probes outlined in Table 2.4 (Applied Biosystems). Expression of the gene of interest was calculated using the ΔΔ Ct method using 18S or histone as the internal control. For RT-PCR, the QIAGEN OneStep RT-PCR kit was used to assess transcript expression using GAPDH as loading control. Primer sequences, product size and the annealing temperatures are as listed in Table 2.5. Products were run on 1% agarose gel and visualized by SYBR Safe (Invitrogen). Tissues from Wistar rats (Charles River) were used as positive controls.
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<tr>
<td>Mouse IGFBP-1</td>
<td>Mm00833447_m1</td>
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Table 2.4 Primers Used For Semi-Quantitative RT-PCR
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<td>5’-CTGCGAGGAACGACACTGCTG-3’</td>
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Table 2.5 Primer Sequences For RT-PCR Used for Mouse and Rat Tissues/Cells.

2.4 Western Blot

Cells were collected in radioimmunoprecipitation assay buffer supplemented with protease inhibitor (complete mini EDTA-free protease inhibitor, Roche). Protein concentration was assessed by the Pierce Bicinchoninic Acid (BCA) assay (Thermo Scientific). For analysis of secreted proteins, 1ml of cell media was collected and frozen. Media samples were then lyophilized overnight and reconstituted in 200 µl of radioimmunoprecipitation assay buffer supplemented with protease inhibitor, described above. Samples were run through a polyacrylamide gel and transferred onto PVDF membranes. Proteins of interests were probed using rabbit antibodies for IGFBP-4 (1:1000, Santa Cruz, sc-13092), phospho(Ser473)-Akt (pAkt, 1:1000, Cell Signaling, 9271), total Akt (1:1000, Cell Signaling, 9272), phospho(Trp202/Tyr204)-ERK1/2 (pERK1/2,1:1000, Cell Signaling, 4377), total ERK1/2 (1:1000, Cell Signaling, 9102), or β-actin (1:5000, Sigma A2066) with overnight incubation at 4°C. Following, membranes were probed with horseradish peroxidase-linked goat anti-rabbit IgG (1:8000, Cell Signaling, 7074). The membrane was imaged using the Kodak Image Station 4000MM pro (Carestream). Protein densitometry was conducted by Carestream Molecular Imaging software (Carestream). Relative expression was normalized per membrane and β-actin expression was used as loading control. For assessment of secretion assay, protein secretion was normalized to cell protein content.
2.5 Microscopy

For morphometric analysis and immunohistochemistry, formalin-fixed, paraffin-embedded jejunal cross-sections were used. For morphometric analysis, sections were stained with hematoxylin and eosin and imaged with a Zeiss microscope and analyzed using AxioVision Version 4.9.1 software (Carl Zeiss). Crypt depth was measured from the crypt base to the villus junction and villus height was measured from the tip of the villus to the villus junction. Crypt-to-villus height was calculated as the sum of the mean crypt depth and mean villus height for each mouse. At least 20 well-oriented crypts and villi were measured per mouse for the IGFBP-4 KO/WT mice. However, the PAPP-A KO mouse intestines were found to be very fragile upon section cutting, and a minimum of 10 crypts and villi were therefore measured for each mouse. All measurements were done in a blinded manner.

Immunohistochemistry for the proliferative marker Ki-67 was conducted using rabbit antihuman Ki-67 antibody (1:100, Abcam, ab16667). In brief, antigen retrieval was performed in citrate buffer and nonspecific binding was blocked using 5% normal goat serum. Endogenous peroxidase activity was blocked using 3% H₂O₂, and slides were incubated with the primary antibody overnight at 4˚C, followed by goat antirabbit secondary antibody (1:200, Vector Laboratories, Inc, BA-1000) for 30 minutes at room temperature. Negative controls were performed without the primary antibody. Visualization was conducted using Ultra Streptavidin-Horseradish Peroxidase Complex: Level 2 labeling reagent (Signet) and Sigma Fast-3,3’ Diaminobenzidine (Sigma-Aldrich, Inc). For positional analysis, the percentage of Ki-67-positive cells was determined by recording Ki-67 positive and negative cells at each cell position, counting up 1 side of the crypt to cell position 22, for at least 20 crypts per mouse. All analyses were conducted in a blinded manner. Apoptosis was similarly assessed for the intestinal
villi using the Apoptag Peroxidase In Situ Apoptosis Detection kit (EMD Millipore). Cells undergoing apoptosis were quantified per intestinal section, 4 sections per mouse.

2.6 Statistical Analysis

All data are presented as mean ±SEM. Data were analyzed using Student’s t-test, or one- or two-way ANOVA followed by appropriate post-hoc analysis. Significance was set at p<0.05.
Chapter 3

IGFBP-4 is Required for Glucagon-Like Peptide-2-Induced Intestinal Epithelial Proliferation

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Author contributions:

Imam NA was a 4th year undergraduate student working under my supervision. In this chapter, Imam NA helped conduct in vivo experiments whose data are presented in Figures 3.2, 3.3D, and 3.3. Imam NA also conducted immunohistological analysis presented in Figure 3.3 A, B, C. Pintar JE supplied the original breeding pairs for the IGFBP-4 KO mice
3 IGFBP-4 is Required for Glucagon-Like Peptide-2-induced Intestinal Epithelial Proliferation

3.1 IGFBP-4 Knockout Mice Have Impaired Growth Responses to GLP-2 Treatment

To determine the role of IGFBP-4 in GLP-2-stimulated intestinal growth, control and whole-body IGFBP-4 KO mice were genotyped (Figure 3.1) and subjected to chronic treatment with either 0.1μg/g h(Gly²)-GLP-2 or vehicle alone. Changes in mucosal mRNA expression of IGFBP-4 as a result of h(Gly²)-GLP-2 treatment in control mice, as well as potential compensatory mRNA expression changes in other IGFBP isoforms by treatment or genotype were investigated and is reported in Chapter 5. Vehicle-treated IGFBP-4 KO mice were not significantly different from control animals with respect to body weight (Figure 3.2A) or small intestinal weight (Figure 3.2B). In order to account for the sex differences in body weight and associated differences in intestinal size, small intestinal weight was also normalized to body weight (Figure 3.2C). The small intestinal weight per body weight of vehicle-treated IGFBP-4 KO mice was found to be increased, by 30.6%±11.5% (p<0.05), compared to control mice; this change was independent of intestinal length, which was not different between the groups of animals when normalized to body weight (Figure 3.2C,D). The inhibitory role of IGFBP-4 on basal intestinal growth is explored in more detail in Chapter 4.

The intestinal weight of control mice treated with GLP-2 was increased by 28.0±8.7% vs. vehicle-treated animals (p<0.05, Figure 3.2B), as expected (35,56,213). However, this growth response to GLP-2 was impaired in IGFBP-4 KO mice (p>0.05). Consistent with the absolute
small intestinal weight data, GLP-2-treated control mice also demonstrated a 24.4%±8.6% (p<0.05) and 23.4%±6.9% (p<0.05) increase in small intestine weight compared to vehicle-treated control mice when normalized to body weight or intestinal length, respectively, whereas the growth response to GLP-2 was again found to be impaired in KO mice (p>0.05, Figure 3.2C,E).

Finally, in order to assess whether or not the lack of response to GLP-2 treatment was due to the intestine of the IGFBP-4 KO mice already having reached the maximum capacity to increase intestinal growth, a preliminary study was conducted using the GLP-2 dose that is 100 times higher than is used pharmacologically (10µg/g). Consistent with the previous study, mice were given daily injections of this supra-pharmacological dose for 10 days. For both the control and IGFBP-4 KO mice, GLP-2 treatment resulted in increased intestinal weight overall by 60%–70% (Figure 3.2F). This was greater than that seen using the lower dose of GLP-2 and indicates that the lack of response to the lower dose of GLP-2 in the IGFBP-4 KO mice was not due to any limitation in intestinal growth.
Figure 3.1 Genotype of IGFBP-4 Control and KO mice.

The genotype of the mice was confirmed by the PCR. IGFBP-4 KO mice were identified through the presence of the neomycin resistance cassette and absence of the IGFBP-4 gene. WT mice were identified through the presence of the IGFBP-4 gene and the absence of the neomycin resistance cassette. Heterozygous mice were identified through the presence of both genes. Representative gel with respective genotype of each sample is shown.
Figure 3.2 IGFBP-4 Knockout Mice Have Impaired Intestinal Growth Responses to GLP-2.

IGFBP-4 KO (KO) and control (CON) mice were treated with 0.1 µg/g h(Gly²)-GLP-2 (GLP-2) or vehicle (PBS) for 10 days. (A) Body weight, (B) small intestinal weight, (C) small intestinal weight normalized to body weight, (D) small intestinal length normalized to body weight, and (E) small intestinal weight normalized to small intestinal length; n=11-12 per group. (F) Preliminary study with small intestinal weight of KO and CON mice treated with 10µg/g h(Gly²)-GLP-2 (GLP-2) or vehicle (PBS) for 10 days. *p<0.05
3.2 IGFBP-4 Knockout Mice Have Impaired Intestinal Proliferative Responses to GLP-2 Treatment

To determine whether the absence of IGFBP-4 impaired the known intestinal proliferative effect of GLP-2 (35,56), jejunal sections of mice were stained for the proliferative marker, Ki-67, and subjected to positional analysis. Control mice displayed a significant increase in the percentage of Ki-67 positive cells at cell positions 12-14 (p<0.05-0.001, Figure 3.3A) with GLP-2 treatment, whereas IGFBP-4 KO mice demonstrated no change in the percentage of Ki-67 positive cells at any position within the jejunal crypt (p>0.05). To compare between genotypes, the area under the curve (AUC) from cell position 12-17 was calculated for each group. Consistent with the intestinal weight and proliferative data, the proliferative-AUC increased by 96.3±27.3% (p<0.01) in GLP-2-treated control mice as compared to vehicle-controls as expected (35,56). However, despite an increase in the basal proliferative-AUC in vehicle-treated IGFBP-4 KO mice (by 81.9±35.8%, p<0.05) as compared to vehicle-treated control mice, the KO mice displayed an abrogated proliferative-AUC response to GLP-2 treatment (p>0.05, Figure 3.3C). Preliminary studies staining for apoptotic cells using TUNEL assay suggested that IGFBP-4 KO mice had fewer apoptotic cells per intestinal section compared to control mice. As expected, GLP-2 appeared to reduce the number of apoptotic cells in control mice; however, in the IGFBP-4 KO mice, GLP-2 did not seem to decrease the number of apoptotic cells compared to the vehicle-treated IGFBP-4 KO mice (Figure 3.3E). Finally, to determine whether the proliferative differences translated to morphometric changes in the intestinal epithelium, crypt depth and villus height were measured. As expected based on the gravimetric and proliferative responses, the crypt-to-villus height of control mice increased by 28.4±7.4% (p<0.05) with GLP-2 treatment, while this response was impaired in the IGFBP-4 KO animals (p>0.05 Figure 3.3D).
Interestingly, and consistent with the increased basal proliferation observed in the vehicle-treated IGFBP-4 KO mice, the crypt depth of the vehicle-treated IGFBP-4 KO mice was found to be increased by 22.1±5.6% compared to vehicle-treated control mice (p<0.05 Figure 3.3D).
Figure 3.3 IGFBP-4 Knockout Mice Have Impaired Intestinal Proliferative Responses to GLP-2.

IGFBP-4 KO (KO) and control (CON) mice were treated with 0.1 µg/g h(Gly²)-GLP-2 (GLP-2) or vehicle (PBS) for 10 days. (A-B) Percentage of Ki-67-positive cells within the jejunal crypt for each cell position from the base to cell position 22 for (A) CON mice and (B) KO mice; n=7-8 per group. (C) Area under the curve between cell positions 12-17 for CON and KO mice shown in A and B; n=7-8 per group. (D) Crypt depth and villus height measurements for CON
and KO mice; n=4-6 per group. (E) Preliminary studies assessing apoptotic cells per jejunal section for CON and KO mice; n=2 per group. *p<0.05, **p<0.01, ***p<0.001 as indicated.
3.3 Pregnancy Associated Plasma Protein-A Knockout Mice Have Preserved Growth Responses to GLP-2 Treatment

IGFBP-4 is known to be able to cross the endothelial barrier of the vasculature (169). Thus, to investigate the role of circulating IGFBP-4 in GLP-2-induced intestinal growth, a chronic study using PAPP-A KO model was conducted. PAPP-A is not expressed in the intestine (173), and the PAPP-A KO mice have elevated levels of circulating IGFBP-4 (184). Thus these mice were used as a gain-of-function model for circulating levels of IGFBP-4 (185). Mouse genotypes were confirmed using PCR (Figure 3.4). However, as previously reported (184,185), PAPP-A KO mice were smaller by 36.7±3.9% (p<0.01) compared to WT animals (Figure 3.5A). In addition to impaired overall growth, the small intestinal weight of PAPP-A KO mice was reduced by 32.4±3.5% (p<0.001, Figure 3.5B). As a consequence, the small intestinal weight per body weight of vehicle-treated KO mice was not different from that of the vehicle-treated WT animals (Figure 3.5C). However, normalized intestinal length was unexpectedly increased by 42.4±6.9% in the vehicle-treated KO mice (p<0.05, Figure 3.5D), resulting in a decrease in small intestinal weight per unit intestinal length, by 25.1±2.9% (p<0.001, Figure 3.5E). This is likely an artifact of the measurement method used, due to the PAPP-A KO intestine being fragile and more easily stretched.

As expected (35,56,213), GLP-2 stimulated intestinal growth in WT mice, with increases of 20.8%±5.1% (p<0.01) and 17.9%±3.2% (p<0.01) when intestinal weight was normalized to body weight and intestinal length, respectively (Figure 3.5C,E). Similar increases of 21.6±4.5% (p<0.01) and 18.4±3.9% (p<0.01) respectively, were observed in the KO animals (Figure 3.5C,E). Consistent with these findings, GLP-2 also stimulated an increase in the crypt-to-villus
height, by 29.4%±5.4% and 11.5%±5.7% in WT and PAPP-A KO mice respectively (p<0.01 and p<0.05 Figure 3.5F). IGFBP-4 mRNA expression within the whole intestine of PAPP-A KO was comparable with that of WT mice (1.1±0.3-fold of control; p>0.05, Figure 3.5G).

**Figure 3.4 Genotype of PAPP-A WT and KO mice.**
The genotype of the mice was confirmed through the PCR. PAPP-A KO mice were identified through the presence of the neomycin resistance cassette and absence of the pappalysin-1 gene. WT mice were identified through the presence of the pappalysin-1 gene and the absence of the neomycin resistance cassette. Heterozygous (Het) mice were identified through the presence of both gene. Representative gel with respective genotype of each sample is shown.
Figure 3.5 PAPP-A Knockout Mice Have Preserved Intestinal Growth Responses to GLP-2.

PAPP-A KO (KO) and wild-type (WT) mice were treated with 0.1 µg/g h(Gly²)-GLP-2 (GLP-2) or vehicle (PBS) for 10 days. (A) Body weight, (B) small intestinal weight, (C) intestinal weight normalized to body weight, (D) intestinal length normalized to body weight, and (E) intestinal...
weight normalized to intestinal length; n=8-9 per group. (F) Crypt depth and villus height; n=5-8 per group. (G) Whole intestinal IGFBP-4 expression; N=3-4 per group. *p<0.05, **p<0.01, ***p<0.001 as indicated.
Chapter 4

IGFBP-4 Inhibits Basal Intestinal Proliferation in an IGF-1- and IGF-1R-Dependent Manner
4 IGFBP-4 Inhibits Basal Intestinal Proliferation in an IGF-1- and IGF-1R-Dependent Manner

4.1 Rat Intestinal Epithelial Cells Express IGFBP-4 and IGF-1 Receptor mRNA

The enlarged intestinal weight of vehicle-treated IGFBP-4 KO mice compared to control mice, found in Chapter 3, suggested that IGFBP-4 inhibits basal intestinal growth. Therefore, to determine the mechanism underlying this effect of IGFBP-4 on epithelial growth, in vitro studies were conducted. Thus, to characterize the rat intestinal epithelial cell line (IEC-6), RT-PCR was conducted to assess the expression of IGFBP isoforms 1 through 7, IGF-1, IGF-1R, PAPP-A, GLP-2R, and GAPDH as loading control (Figure 4.1A, B,) with whole jejunum, liver and fetal rat tail as positive controls (Figure 4.1C). IEC-6 cells from Dr. Kay Lund (UNC-Chapel Hill, North Carolina) were found to express IGF-1R, IGFBP-2, -4, -5, -6, -7 and PAPP-A but not IGF-1, IGFBP-1, -3 or GLP-2R (Figure 4.1A). However, IGF-1 was detectable in IEC-6 cells from Dr. Kay Lund by qRT-PCR with Ct values ranging from 33 to 36. IEC-6 cells from American Type Culture Collection (ATCC) were found to express the same genes as well as IGF-1 and GLP-2R (Figure 4.1B). However, IEC-6 cells purchased from American Type Culture Collection also displayed reduced responsiveness to IGF-1 (Appendix Figure 1); thus, for all subsequent experiments, the IEC-6 cells from Dr. Kay Lund were used.
Figure 4.1 Rat Intestinal Epithelial Cell Lines Express IGFBP-2, -4, -6 and -7 and decreased or no expression of IGFBP-1 or IGFBP-3 mRNA.

RT-PCR was conducted to assess IGFBP mRNA isoform expression in (A) IEC-6 cells from Dr. P. Kay Lund (UNC-Chapel Hill) with rat liver as positive control for IGF-1 mRNA, (B) IEC-6 cell lines American Type Culture Collection (ATCC) and (C) positive controls, using rat liver, jejunum, and fetal rat tail samples. Product size is as indicated below each band. Two bands (417&365bp) resulted for the IGF-1 product due to alternative splicing.
4.2 IGFBP-4 Dose Dependently Decreases IGF-1 Activity in Vitro

To test the responsiveness to IGF-1 and EGF in the IEC-6 cells, the cells were treated with IGF-1 (0-1000ng/ml) for 18 hours following a vehicle or EGF (5ng/ml) pre-treatment. Changes in cell proliferation were assessed by thymidine incorporation assay. As previously reported (88), pre-treatment with EGF treatment alone significantly increased subsequent IEC-6 cell proliferation by 81.8±20.3% compared to vehicle treated cells (p<0.01, Figure 4.2A inset). Also, as expected (88), EGF pre-treatment enhanced the IEC-6 cell responsiveness to IGF-1 treatment whereby, for the same 10, 100 and 1000 ng/ml of IGF-1 treatment, EGF pre-treatment increased cell proliferation by 52.0±15.4%, 87.2±25.7%, and 105.3±27.9%, respectively, after subtracting the effects of EGF alone (p<0.01, Figure 4.2A) (88).

To assess the effects of IGFBP-4 on proliferation, IEC-6 cells were treated with increasing concentrations of IGFBP-4 (0-25nM) with and without EGF pre-treatment (5ng/ml) and/or IGF-1 treatment (100ng/ml). Increasing concentrations of IGFBP-4 alone resulted in a trend of decreased IEC-6 proliferation, although this was not statistically significant. Similarly, increasing concentrations of IGFBP-4 in the presence of IGF-1 also resulted in a trend of decreased proliferation but again, this was not statistically significant. As expected (88), IEC-6 cells given EGF pre-treatment with IGF-1 treatment displayed a significant increase in proliferation by 301.8±43.9% compared to cells given vehicle only (p<0.001, Figure 4.2B). More importantly, IGFBP-4 decreased EGF+IGF-1-induced proliferation in a dose-dependent manner wherein 6.25nM IGFBP-4 treatment decreased proliferation by 21.8±8.8% (p<0.05)
compared to 0nM IGFBP-4 and a 25nM IGFBP-4 treatment resulted in further reduction by 27.0±6.5\% relative to cells given a 6.25nM IGFBP-4 treatment (p<0.05, Figure 4.2B).
Figure 4.2 IGFBP-4 Decreases the Intestinal Proliferative Bioactivity of IGF-1 in vitro.

$^3$H-thymidine incorporation was measured to assess IEC-6 proliferation in response to the following treatments: (A) EGF or vehicle pre-treatment (5ng/ml; 6 hours), followed by various doses of IGF-1 treatment for 18 hours (0-1000ng/ml; data were normalized to their own control, with inset showing EGF effect alone; n=10-12 per group), and (B) vehicle pre-treatment with IGFBP-4 treatment (0-25nM), vehicle pre-treatment with IGF-1 (100ng/ml) and IGFBP-4, or
EGF pre-treatment (5ng/ml; 6 hours) with IGF-1 and IGFBP-4 treatment; n=9-12 per group. 

*p<0.05, **p<0.01, ***p<0.001 as indicated and, ###p<0.001 compared to IGFBP-4 only within group.
4.3 IGFBP-4 Decreases Intestinal Proliferation in an IGF-1R Dependent Manner

To determine the role of the IGF-1R in IGFBP-4-induced inhibition of cell proliferation, IEC-6 cells were treated with and without the IGF-1R inhibitor NVP-AEW541 (0.1&1µM), and treated with and without IGFBP-4 (25nM). NVP-AEW541 treatment at 0.1 and 1µM significantly decreased IEC-6 proliferation by 42.2±6.7% (p<0.01) and 76.2±4.5% (p<0.001) respectively, compared to vehicle control (Figure 4.3A). The addition of IGFBP-4 (25nM) did not result in a further decrease in cell proliferation (p>0.05, Figure 4.3A). This experiment was repeated following 6 hours of EGF pre-treatment, followed by 18 hour treatment with NVP-AEW541 (0.1&1µM) and with or without IGFBP-4 (25nM). NVP-AEW541 treatment at 0.1 and 1µM significantly decreased IEC-6 proliferation by 54.0±9.5% (p<0.001) and 88.2±0.7% (p<0.001) respectively, compared to vehicle control (Figure 4.3B). Consistent with the previous experiment, even after EGF pre-treatment, IEC-6 cells treated with IGFBP-4 and NVP-AEW541 did not differ significantly from those treated with only NVP-AEW541 (p>0.05, Figure 4.3B).
Figure 4.3 IGFBP-4 Decreases Intestinal Proliferation in an IGF-1R Dependent Manner.

$^{3}$H-thymidine incorporation was measured to assess IEC-6 proliferation in response to the following treatments: (A) IGFBP-4 (25nM) and/or NVP-AEW541 (0.1 or 1 µM) vs vehicle controls for 24 hours; n=4-8 per group and (B) EGF pre-treatment (5ng/ml) for 6 hours, followed by IGFBP-4 (25nM) and/or NVP-AEW541 (0.1 or 1 µM) vs vehicle controls for 18 hours. (Data were normalized to cells given only EGF pre-treatment; inset shows EGF effect); n=4 per group. *p<0.05 compared to vehicle, **p<0.01, ***p<0.001 compared to without inhibitor, ##p<0.01 compared to without IGFBP-4.
4.4 IGFBP-4 Decreases Intestinal Proliferation in an MEK1/2 Dependent Manner

To investigate the downstream signaling pathway involved in IGFBP-4-induced inhibition of IEC-6 proliferation, pharmacological inhibition of the PI3K/Akt and the MEK/ERK pathway was conducted. To test the efficacy of the PI3K inhibitors and the MEK1/2 inhibitors, immunoblot was conducted for pAkt, pERK1/2, as well as total Akt, total ERK1/2 and β-actin as loading control, using dosages shown in previous studies to be effective in intestinal subepithelial myofibroblasts (214) or IEC-6 cells (215,216). As expected, the PI3K inhibitors, wortmannin (0.5µM) and LY294002 (5µM), decreased expression of pAkt relative to total Akt. Similarly, the MEK1/2 inhibitors, PD98059 (10µM) and U0126 (5µM), decreased expression of pERK1/2 relative to total ERK1/2 (Figure 4.4A). Next, cell proliferation was assessed by thymidine incorporation assay after the cells were treated with the PI3K or MEK1/2 inhibitors and IGFBP-4 (25nM) vs vehicle controls. IGFBP-4 treatment alone significantly decreased IEC-6 cell proliferation by 64.3±5.4% (p<0.0001). In addition, inhibition of PI3K by LY2904002 resulted in a 63.9±3.2% (p<0.0001) reduction of IEC-6 proliferation with no reduction by wortmannin (p>0.05 Figure 4.4B). Inhibition of MEK1/2 by PD98059 and U0126 both decreased IEC-6 proliferation by 76.4±7.8% (p<0.0001) and 83.6±7.37% (p<0.0001) respectively (Figure 4.4B). IGFBP-4 treatment reduced IEC-6 proliferation in the presence of the PI3K inhibitors, wortmannin and LY294002 by 37±3.6% (p<0.0001) and 49.3±7.6% (p<0.05) respectively (Figure 4.4B). In contrast, IGFBP-4 did not further decrease IEC-6 cell proliferation in the presence of the MEK1/2 inhibitors, PD98059 and U0126 (p>0.05 Figure 4.4B).
Figure 4.4 IGFBP-4 Decreases the Intestinal Proliferation in a MEK1/2 Dependent Manner.

(A) IEC-6 cells were treated with IGFBP-4 (25nM) and/or wortmannin (0.5µM), LY294002 (5µM), PD98059 (10µM) or UO126 (5µM), with respective vehicle control for 24 hours.

Western blot was performed to probe for the expression of pAkt, pERK1/2, as well as total Akt (tAkt), total ERK1/2 (tERK1/2) and β-actin as loading control, representative of two blots. (B) ³H-thymidine incorporation study was measured to assess IEC-6 proliferation in response to the same inhibitors or vehicle, with or without IGFBP-4 (25nM) for 24 hours; n=3-4 per group.

***p<0.001 compared to without inhibitor, #p<0.05, ###p<0.001 compared to without IGFBP-4
Chapter 5

Mucosal IGFBP-4 Expression is Increased by GLP-2 But Not by IGF-1 and/or EGF

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Author contributions:

Imam NA was a 4th year undergraduate student working under my supervision. In this chapter, Imam NA helped conduct in vivo experiments for data presented in Figure 5.1A and 5.2. Pintar JE contributed the IGFBP-4 KO mice.
5 Mucosal IGFBP-4 Expression is Increased by GLP-2
But Not by IGF-1 and/or EGF

5.1 GLP-2 Treatment Enhances Mucosal Expression of IGFBP-4
In Vivo and In Vitro

GLP-2 is known to induce the expression of intestinal Sox9 mRNA (193), a known transcriptional activator of IGFBP-4 (157). Thus, to establish whether jejunal IGFBP-4 mRNA expression is regulated in vivo by GLP-2, mucosal scrapes from control animals subjected to chronic treatment with GLP-2 were examined for changes in IGFBP-4 mRNA expression. GLP-2-treated animals demonstrated a 518±22% increase (p<0.05) in mucosal IGFBP-4 mRNA levels as compared to vehicle-treated controls (Figure 5.1A). Because the mucosal expression of IGFBP-4 transcripts was found to be very low relative to that of the whole intestine (Figure 5.1A), FRIC cultures were used as an in vitro model of the entire intestine. FRIC cultures have previously been established to express a functional GLP-2R that displays a cAMP response, as well as enhanced IGF-1 mRNA expression and IGF-1 secretion in response to GLP-2 treatment (35). When incubated with h(Gly²)-GLP-2 (10⁻⁸M) for 2 hours, IGFBP-4 mRNA expression in the FRIC cultures was also found to be increased, by 40.8±15.2% (p<0.05) compared to vehicle-treated cells (Figure 5.1B). This effect was transient and was not observed at any other time point tested.
Figure 5.1 Intestinal IGFBP-4 mRNA Expression is Increased by GLP-2.

(A) Relative expression of IGFBP-4 mRNA in mucosal scrapes extracted from control mice treated with 0.1 µg/g h(Gly²)-GLP-2 (GLP-2) or vehicle (PBS) for 10 days; n=9-12. The adjacent bar graph indicates relative small intestinal (SI) mRNA expression collected from whole jejunum of control mice; n=4. (B) Relative expression of IGFBP-4 mRNA extracted from FRIC cultures treated with $10^{-8}$ M h(Gly²)-GLP-2 (GLP-2) or vehicle (PBS) for 0.5-24 hours; n=4-12 per group. *p<0.05 vs. paired control.
5.2 GLP-2 Treatment Does Not Alter Mucosal Expression of IGFBP-3, -5, -6 and -7 In Vivo

Altered IGFBP-4 gene expression due to chronic GLP-2 treatment may lead to alterations in expression of other IGFBP isoforms within the mucosa. Furthermore, IGFBP-4 KO mice, investigated in Chapter 3, may have altered IGFBP isoform expression due to compensation. Thus, the expression of IGFBP1-3 and 5-7 was determined in jejunal mucosal scrapes of control and IGFBP-4 KO mice treated with vehicle or GLP-2, by semi-quantitative RT-PCR. IGFBP-1 and -2 mRNA expression was not detectable in the mucosa, and levels were extremely low overall in whole jejunum. Furthermore, although mRNA for IGFBP-3 and 5-7 was readily detected in whole jejunum, their relative expression in the mucosa was much lower (Figure 5.2). Notwithstanding, there were no significant differences in the expression of any of these IGFBPs between control and IGFBP-4 KO mice, with and without GLP-2 treatment. Similarly, mucosal expression of the IGFBP-4 cleaving enzyme, PAPP-A, was also extremely low with Ct values ranging between 35-37 to undetectable.
Figure 5.2 Intestinal IGFBP isoform -3, -5, -6, or -7 Expression is Not Affected by GLP-2.

IGFBP-4 KO (KO) and control (CON) mice were treated with 0.1 µg/g h(Gly²)-GLP-2 (GLP-2) or vehicle (PBS) for 10 days. mRNA expression of IGFBP isoform (A) -3, (B) -5, (C) -6, and (D) -7, as assessed by semi-quantitative PCR from mucosal scrapes; n=3-5 per group. Adjacent bar graph in each panel indicates relative small intestinal (SI) mRNA expression collected from whole jejunum of control mice; n=4 per group.
5.3 Chronic Loss of Epithelial IGF-1R and EGFR Signaling Does Not Alter Mucosal IGFBP-4 Expression In Vivo

IGF-1 and EGF are established mediators of the proliferative effects of GLP-2 (35,40,56). Additionally, IGF-1 is known to induce intestinal expression of Sox9 mRNA(193), and EGF stimulates Wnt signaling, in the intestine (57), whose target gene includes Sox9. Thus, to determine whether these intermediate growth factors play a role in the increased mRNA expression of mucosal IGFBP-4 mRNA in response to GLP-2 as above, the inducible IE-IGF-1R KO mice were examined either alone (vehicle) or following administration of the EGFR inhibitor CI-1033 (20mg/kg) orally for 10 days. Genotypes of the mice were determined through PCR (Figure 5.3). Successful knockout of the IGF-1R by tamoxifen injection was confirmed in the IE-IGF-1R KO mice with significantly reduced mucosal IGF-1R mRNA expression (p<0.001, Figure 5.4A). The WT and IE-IGF-1R KO mice treated with and without the CI-1033 displayed no differences in small intestinal weight (Figure 5.4B,C). In addition, mucosal expression of IGF-1, Sox9, and IGFBP-4 mRNA was assessed. CI-1033 treatment resulted in a significant increase in IGF-1 mRNA expression in IE-IGF-1R KO mice by 3.5±1.8 fold (p<0.05, Figure 5.4 D). In contrast, IE-IGF-1R KO mice did not exhibit any difference in Sox9 expression, a known transcriptional regulator of IGFBP-4 (157) or in IGFBP-4 mRNA with and without CI-1033 treatment (p>0.05 Figure 5.4E,F).
Figure 5.3 Genotype of IGF-1R<sup>flox/flox</sup>, Villin-CreER<sup>T2+/0</sup>, and Villin-CreER<sup>T2+/0</sup>/IGF-1R<sup>flox/flox</sup> mice.

The genotype of the mice was confirmed through PCR. Villin-CreER<sup>T2+/0</sup>/IGF-1R<sup>flox/flox</sup> mice were identified through the presence of the Cre gene and the floxed IGF-1R gene. IGF-1R<sup>flox/flox</sup> mice were identified through the presence of the floxed IGF-1R gene and the absence of the Cre gene. Villin-CreER<sup>T2+/0</sup> mice were identified through the presence of the Cre gene and the WT IGF-1R gene, and the absence of the floxed IGF-1R gene. Representative gel with respective genotype of each sample is shown.
Figure 5.4 Chronic Loss of Epithelial IGF-1R and EGFR Signaling Does Not Alter IGFBP-4 Expression.

Inducible IE-IGF-1R KO (KO) and control (CON; Cre-, flox/flox- with/without tamoxifen, Cre-flox/flox mice without tamoxifen) mice were given ErbB Inhibitor, CI-1033, or vehicle orally for 11 days. (A) Mucosal expression of IGF-1R mRNA; n= 6-10 per group. (B) Small intestinal weight and (C) small intestinal weight normalized to body weight; n=7-10 per group. Mucosal expression of (D) IGF-1 mRNA, (E) Sox9 mRNA, and (F) IGFBP-4 mRNA; n= 5-10 per group.
5.4 Acute PI3K Inhibition Does Not Alter Expression of Mucosal IGFBP-4 In Vivo

IGF-1 is thought to induce intestinal Wnt signaling via GSK3β inhibition in a PI3K/pAkt dependent manner (193). Thus, to determine whether mucosal IGFBP-4 mRNA expression was altered in an acute time frame by PI3K/Akt signaling in vivo, normal C57/BL6 mice were treated with wortmannin (1.5 µg/g) or vehicle by oral gavage 6.5 and 3.5 hours before sacrifice. Inhibition of PI3K by wortmannin was confirmed as wortmannin reduced mucosal pAkt significantly (p<0.001 Figure 5.5A) with no changes in pERK1/2 expression (p>0.05 Figure 5.5 B). Mucosal IGFBP-4 mRNA expression was not significantly different in response to altered PI3K signaling (p>0.05 Figure 5.5C).
Figure 5.5 Acute PI3K Inhibition Does Not Alter Expression Of Mucosal IGFBP-4.

C57/Bl6 mice were treated with wortmannin (1.5 ug/g) or vehicle by oral gavage 6.5 and 3.5 hrs before sacrifice. (A) pAkt levels normalized to actin; n=9-10, representative blots shown above. (B) pERK 1/2 levels normalized to total ERK 1/2; n=9-10, representative blot shown above. (C) Mucosal IGFBP-4 mRNA expression; n=10. ***p<0.001
5.5 EGF and IGF-1 Do Not Alter Expression or Secretion of Epithelial IGFBP-4 Expression In Vitro

Finally, to determine whether epithelial expression of IGFBP-4 is regulated by IGF-1 or EGF in vitro, the IEC-6 cells were used. Cells treated with EGF and IGF-1 together for 24 hours displayed a 39.1±5.8% reduction in Sox9 mRNA expression (p<0.01, Figure 5.6A). This did not correlate with any alterations in IGFBP-4 mRNA expression (p>0.05, Figure 5.6B). Consistently, protein expression of IGFBP-4 did not change significantly after 48 hours of IGF-1, EGF, or IGF-1+EGF treatment compared to vehicle treated cells (p>0.05, Figure 5.6C). Secretion of IGFBP-4 was also not altered compared to vehicle treated cells (p>0.05, Figure 5.6C). As expected, pAkt was significantly increased by 48 hour IGF-1 treatment alone and with EGF by 599.3±195.1% and 521.1±177.3%, respectively (p<0.05, Figure 5.6D). IGF-1 treatment alone also resulted in increased pERK1 expression by 54.9±23.3% relative to vehicle treated control (p<0.05, Figure 5.6D), with similar trends in pERK2 expression, while no significant changes were observed with EGF treatment alone and with IGF-1 together.
**Figure 5.6 EGF and IGF-1 Do Not Stimulate IGFBP-4 Expression or Secretion.**

IEC-6 cells were treated with EGF (5ng/ml) and/or IGF-1 (100ng/ml) for 24 hours. mRNA expression of (A) Sox-9 and (B) IGFBP-4; n=6-8. IEC-6 cells were then treated with EGF (5ng/ml) and/or IGF-1 (100ng/ml) for 48 hours. (C) Cellular IGFBP-4 protein expression normalized to actin (n=12), and IGFBP-4 protein secretion normalized to cell content (n=3-4 per group). (D) pAkt expression normalized to total Akt, pERK1 normalized to total ERK1, and pERK2 normalized to total ERK2; n=6-8. *p<0.05,**p<0.01 compared to vehicle
Chapter 6
Discussion

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6 Discussion

6.1 Summary

The physiology of the intestinal mucosa is regulated by multiple, interacting signaling pathways that are stimulated by growth factors such as GLP-2, IGF-1 and EGF. The mechanisms through which these growth factors are regulated and how these growth factors interact and affect the activity of each other are critical in understanding intestinal physiology. As IGF-1 and the IGF-1R are expressed by many different tissues, the therapeutic benefits of this growth factor for patients with bowel disease such as inflammatory bowel disease or short bowel syndrome is limited, considering the numerous off-target growth effects of IGF-1 (66). EGF and EGFR are also expressed in many target tissues (43). In contrast, teduglutide, a long-acting GLP-2, is an approved drug in Canada for patients with short bowel syndrome (1). Interestingly, the GLP-2R is not expressed on the intestinal epithelium. As such, GLP-2 is thought to act through the receptors expressed on the intestinal subepithelial myofibroblasts, enteroendocrine cells, and enteric neurons (52–55). The proliferative effects of GLP-2 is known to rely on IGF-1 and EGF to act as secondary factors that act on their receptors on the intestinal epithelium to stimulate proliferation and growth (56,57). In addition, the activity of IGF-1 is enhanced in a synergistic manner by EGF (88). Within this context, this work identifies that IGF-1 activity is also regulated by the binding protein, IGFBP-4, which thereby regulates intestinal epithelial growth and proliferation under GLP-2-treated, as well as under basal conditions. Potential signaling pathways through which expression of mucosal IGFBP-4 is regulated are also identified. In Chapter 3, lack of IGFBP-4 was found to impair the epithelial proliferative effects of GLP-2 using whole-body IGFBP-4 KO mice as a loss-of-function model and whole-body PAPP-A KO
mice as a gain-of-function model. However, given that the IGFBP-4 KO mice had significantly enlarged bowel weight and crypt depth under control conditions, the role of IGFBP-4 in basal epithelial proliferation was investigated in Chapter 4. Interestingly, in contrast to being required for growth and proliferation under GLP-2-stimulated conditions, IGFBP-4 played an inhibitory role intestinal epithelial proliferation under basal states. In Chapter 5, GLP-2, a known stimulator of intestinal Wnt-signaling and Sox9 expression, was established to increase the expression of mucosal IGFBP-4. Recent studies have suggested that intestinal epithelial IGFBP-4 expression is enhanced by binding of Sox9, a down-stream target of the Wnt-signaling pathway, to its promoter region (157). Interestingly, the established mediators of GLP-2, IGF-1 and EGF, were found to not regulate the expression of IGFBP-4 either in vitro or in vivo.

6.2 Overall Discussion

6.2.1 Requirement of IGFBP-4 for the Growth Effects of GLP-2

The complex mechanisms that regulate GLP-2 activity are not well understood. GLP-2 is known to be released from the intestinal epithelial enteroendocrine L-cell in response to nutrient ingestion (2). It acts on the intestine through its receptor which is expressed by intestinal subepithelial myofibroblasts that underlie the epithelium (52,53), enteroendocrine cells (2,55), and the enteric nervous system (54). Interestingly, the intestinal epithelium where the growth and proliferative effects are observed does not express the GLP-2R (2). As such, GLP-2 requires the actions of secondary growth factors, such as IGF-1 and EGF, to be released and to activate the IE-IGF-1R and the IE-EGFR, respectively, for its growth and proliferative effects (35,40,56,57). However, how the actions of IGF-1 in particular are regulated within the intestine are not well understood.
IGFBPs are known to promote or inhibit the activity of IGF proteins, with IGFBP-3, -4, and -5 being the most highly expressed isoforms in the intestinal mucosa (164). While IGFBP-3 and -5 have been demonstrated to not contribute to the growth effects of GLP-2 (116), IGFBP-4 has not been studied as extensively in the context of intestinal growth. As IGFBP-4 has been demonstrated in vivo in other models to facilitate growth/proliferation (183,184), I hypothesized that IGFBP-4 would potentiate the IGF-1-dependent actions of GLP-2. To test this hypothesis, whole-body IGFBP-4 KO mice and control mice were treated with h(Gly²)-GLP-2 for 11 days. Control mice displayed normal responses to chronic GLP-2 treatment with increased intestinal weight relative to body weight, increased crypt-villus height, and increased epithelial proliferation, as expected (35,56). In previous studies, differences in cell positions with increased proportion of proliferating crypt cells in response to GLP-2 treatment have been found due to the use of different proliferation markers, mouse strains, and duration of treatment. For example, CD-1 mice treated with GLP-2 for 11 days had an increased proportion of proliferating cells at positions ~11-17 with the Ki-67 marker (35), whereas with mice on a C57B/6 background expressing either villin-Cre or IGF-1R<sup>flox/flox</sup>, increased proportion or proliferation was observed at positions ~16-20 (56). In contrast, with the BrdU marker, proliferation was observed to be increased at positions 3-10 in CD1 mice treated acutely (6 hours) with GLP-2 (40). In this study, the increase in the proportion of proliferating cells was observed at cell positions 12-14, with the trend extending to cell position 17, following 11 days of treatment with GLP-2. Regardless of the exact cell position that increases in proliferation in response to GLP-2 treatment, the present analyses were conducted in a blinded manner and thus, were not subject to observer bias. In addition, increases in the proliferative index have consistently been found within the crypt but above cell position 4, which corresponds to the region where transit-amplifying cells reside (6). While increases in the proliferation of stem cell region by GLP-2 have been reported with BrdU
as a marker (40), Ki-67 may not be sensitive enough to measure changes in proliferation in this area.

Importantly, and consistent with my hypothesis, IGFBP-4 KO mice displayed impaired intestinal growth and proliferation in response to GLP-2 treatment with impaired increases in intestinal weight, crypt-villus height, and proportion of cells proliferating in the crypt. This is in contrast to chronic GLP-2 studies conducted with IGFBP-3/IGFBP-5 double knockout where the mice remained responsive to the growth stimulatory effects of GLP-2 with preserved responses including increased intestinal weight, crypt-villus height and crypt cell proliferation (116). GLP-2 is thought to contribute to increased intestinal crypt-villus height and intestinal weight through enhanced epithelial stem cell and transit-amplifying cells and reduced apoptosis. The impaired responses to GLP-2 in these parameters in IGFBP-4 KO mice suggests that IGFBP-4 aids in the growth promoting actions of GLP-2.

It must be noted that, while the growth and proliferative responses to GLP-2 in IGFBP-4 KO mice were impaired, they were not completely abrogated, with a trend of increased growth and proliferative parameters that were not statistically significant but still present. This is likely due to the fact that there are multiple growth factors involved in facilitating the effects of GLP-2, with IGFBP-4 being only one of many regulatory proteins present within the intestine. The impaired response is also not due to a ceiling effect as preliminary studies conducted using a supra-pharmacological dosage demonstrated the ability of the mouse intestine to increase in both genotypes. With increased dosage of GLP-2, the intermediary factors, EGF and IGF-1, may therefore be able to overcome the deficiency of IGFBP-4 and stimulate growth, although alternative pathways that may also be activated cannot be excluded. In addition, preliminary studies demonstrated a reduction in apoptotic cells in response to GLP-2 treatment in control.
mice, as expected (2). Conversely, IGFBP-4 KO mice treated with GLP-2 had an impairment in the reduction of apoptosis. This may also contribute to the impaired increase in crypt-villus height and, in turn, to the impaired increase in intestinal weight in response to GLP-2 seen in these animals. However, the contribution of apoptosis to the impaired response to GLP-2 needs to be investigated further.

In previous studies, IGF-2 KO mice have also been demonstrated to have impaired proliferative responses to GLP-2, with the suggestion of reduced increases in crypt fission (35). Importantly, IGFBP-4 is known to bind to IGF-1 as well as IGF-2, both of which are able to bind and activate the IGF-1R (78). While IGF-2 does not contribute to GLP-2-induced proliferation to the same extent to that of IGF-1 (35), the potential for impaired potentiation of IGF-2 activity in the IGFBP-4 KO mice, contributing to altered epithelial proliferation and crypt fission cannot be discounted.

Finally, this study investigated the role of local/paracrine actions of IGFBP-4 and whether serum IGFBP-4 contributes to the mucosal growth effects of GLP-2, using PAPP-A KO mice in a gain-of-function study. A previous study had reported a 40% increase in the levels of serum IGFBP-4 in PAPP-A KO mice (184) and, since IGFBP-4 does not form a ternary complex with ALS like IGFBP-3 and -5, and is thus able to cross the endothelial barrier from the vascular space (122), I hypothesized that IGFBP-4 could cross into the extracellular space of the mucosa. However, since the intestinal growth response to GLP-2 was preserved in the PAPP-A KO mice, the results suggest that the requirement of IGFBP-4 for GLP-2-induced growth is due to locally expressed IGFBP-4 within the intestine that acts in a paracrine/autocrine fashion to contribute to the growth/proliferative response, by supporting the bioactivity of IGF-1. Whether this occurs
through increased retention of the IGF proteins leading to inhibition of IGF degradation, or increased release of bioactive IGF proteins following IGFBP-4 degradation was not determined.

A limitation of these studies is that intestinal epithelial IGF-1R activation was not assessed in response to GLP-2. If IGFBP-4 improved the delivery of IGF-1 to the IGF-1R, we would expect to see increased phosphorylation and, thereby, activation of the IGF-1R in response to GLP-2 treatment, and a reduction in this response in the IGFBP-4 KO mice. Consistently, with the PAPP-A KO mice, since the GLP-2-induced growth effects were preserved, we would expect to see a preserved response in IGF-1R phosphorylation in response to GLP-2 treatment. Similarly, degradation of IGFBP-4 in response to GLP-2 treatment was not assessed in vivo. If there is increased degradation of IGFBP-4 in response to GLP-2, as has been shown in vitro (192), it would suggest that the degradation of IGFBP-4 increases the activity of IGF proteins, and allows the growth/proliferative responses of GLP-2.

Last but not least, both the IGFBP-4 KO and PAPP-A KO mice are whole-body knockout models. The mucosal expression of the other isoforms was found to be not different between the IGFBP-4 KO and control mice in Chapter 5, and circulating serum IGFBP-4 levels were found to be also not different in previous studies (184). Serum IGF-1 levels and embryonic expression of IGF-2 are also reported to be not different between PAPP-A KO and WT mice (185), and intestinal IGFBP-4 expression levels were found to be not different between the PAPP-A KO and WT mice. However, PAPP-A KO mice lack PAPP-A expression in the whole body and display severe growth restriction in utero and postnatally (185). In addition, expression and activity of other proteases that can cleave IGFBP isoforms, as well as changes in the expression and activation of IGF-1R within the intestine were not investigated. Considering all of these factors together, compensatory changes in these models in terms of IGFBP degradation,
sensitivity of the mucosa to IGF-1, and expression of other growth factors within the intestine and the circulation may confound the results in both models.

6.2.2 Requirement of IGFBP-4 for Inhibiting Basal Intestinal Growth and Proliferation

Analysis of the vehicle treated IGFBP-4 KO mice in the chronic GLP-2 treatment study showed that IGFBP-4 KO mice had overall larger intestinal weight per body weight and deeper crypts compared to control mice. Consistent with the morphometric differences, the proportion of proliferating cells within the crypts was also elevated in the IGFBP-4 KO animals. All of these results suggest that under basal conditions in vivo, ie. under conditions where mice are fed ad libitum and not treated with exogenous GLP-2, IGFBP-4 plays an inhibitory role in basal intestinal epithelial proliferation.

Small intestinal weight normalized to body weight was not significantly different between the gain-of-function model, PAPP-A KO mice and the WT mice. While IGFBP-4, unlike IGFBP-3 and -5 does not form ternary complexes with ALS in the circulation and, thus, is able to cross the vascular endothelial barrier into interstitial fluids (122), this result suggests that locally-expressed IGFBP-4 contributes to the inhibition of IGF-induced-basal intestinal growth and proliferation, but that circulating IGFBP-4 does not. Interestingly, PAPP-A KO small intestines were significantly smaller than found in the WT mice with respect to small intestinal weight per unit length because the intestinal length was found to be longer relative to body weight in the PAPP-A KO mice. This is likely due to increased stretch that occurred during length measurement under constant tension as the PAPP-A KO intestinal tissue was notably more fragile upon handling and likely susceptible to more stretch. However, because the PAPP-A KO mice are severely growth restricted, the contribution of systemic IGFBP-4 to basal intestinal
proliferation could be better determined by systemic administration of IGFBP-4 to normal mice, followed by morphometric and immunometric measures of intestinal growth.

In order to study this inhibitory action of IGFBP-4, in vitro experiments using the rat epithelial IEC-6 cells were conducted. Consistent with the in vivo findings, IGFBP-4 treatment in vitro inhibited IGF-1-induced proliferation in IEC-6 cells in a dose-dependent manner. However, this dose-dependency was not proportional to the molar increase; in other words, 2x the molar concentration of IGFBP-4 did not lead to inhibition of IGF-1 activity by 2x. This may be because the synergistic activity of EGF pretreatment on IGF-1 actions prevents the proportional inhibition of IGF-1 activity, that endogenously produced IGF-1 and IGF-2 present in the media mitigates the inhibitory effects of IGFBP-4, or that IGFBP-4 proteases are present in the media that inhibit the actions of the administered IGFBP-4.

The in vitro experiments assessing changes in IEC-6 cell proliferation by IGFBP-4 in the presence of IGF-1R-, PI3K-, and MEK1/2- inhibitors suggested that inhibition of IGF-1-induced epithelial proliferation by IGFBP-4 occurs in an IGF-1R and MEK1/2-dependent pathway. A limitation to the in vitro studies using the IGF-1R inhibitor NVP-AEW541 is that this drug also inhibits the activity of the insulin receptor albeit, to a lesser extent (217). However, to assess the role of signaling pathways downstream of IGF-1R, two different inhibitors were used to inhibit PI3K and MEK1/2 pathways respectively, to account for non-specific drug effects (218). The fact that basal proliferation did not decrease in response to wortmannin by itself was unexpected. This may be due to differences in non-specific targets of wortmannin or LY294002 (218,219). Nonetheless, western blot for pAkt expression demonstrated reduced levels in IEC-6 cells following both wortmannin and LY294002 treatment at the same dose used in the proliferation assay. The finding that IGFBP-4 still decreased IEC-6 proliferation in the presence of the PI3K
inhibitors, therefore suggests that IGFBP-4 exert its inhibitory effect through another signaling pathways, most likely, MEK1/2 and ERK1/2.

A number of limitations exist with the use of IEC-6 cells as a model of the intestinal epithelium in vitro. While IEC-6 cells are rat primary cultures that have an epithelioid morphology with sparse microvilli and can persist in culture as many as 40-50 passages, they do not express markers of differentiated cells (207). Thus, they do not fully represent the stem cells or the differentiated cell types that are normally present within the epithelium in vivo. In addition, with increased passages and highly dense culture conditions, IEC-6 cells lose responsiveness to IGF-1 treatment (Drs. Simmons and Lund, via communication). Consistently, IEC-6 cells purchased from American Type Culture Collection display reduced responsiveness to IGF-1 (Appendix Figure 1). All 3H-thymidine incorporation assays were conducted with the ‘IGF-1-responsive’ IEC-6 cells from Dr. P. Kay Lund, originally received from Dr. A. Quaroni (207), with at least two different passage numbers between 17- and 23 to account for reduction in responsiveness. However, whether there are other differences within these cells with increasing passages is unknown. While IGFBP-4 treatment of IEC-6 cells by itself reduced cell proliferation, IEC-6 cells received from Dr. Kay Lund express substantially lower IGF-1 mRNA transcripts than IEC-6 cells purchased from ATCC. As such, the role of IGF-2 in promoting basal IEC-6 proliferation from Dr. Kay Lund also cannot be discounted. In the proliferation studies where signaling pathway inhibitors were used, the treatment of MEK1/2 inhibitors resulted in a dramatic reduction of proliferation. It is possible that IEC-6 cells were already at the lowest possible rate of proliferation, such that addition of IGFBP-4 could not have reduced proliferation regardless of the signaling pathway that was responsible for IGFBP-4-induced reduction of proliferation. A dose dependent study with the MEK1/2 inhibitors at various dosages with and without IGFBP-4
would be beneficial to further study the role of MEK1/2 signaling in response to IGFBP-4 treatment.

Lastly, while this study focused on intestinal crypt cell proliferation in vivo and IEC-6 proliferation in vitro, whether IGFBP-4 plays a role in regulating epithelial cell differentiation is not known. Previous studies in cardiomyocytes have shown that IGFBP-4 can inhibit Wnt signaling by physically interacting with the Frizzled 5/6 receptor (129). This inhibition occurs independently of the IGF-1-IGF-1R pathway, and results in enhanced differentiation of the cardiomyocytes. Whether IGFBP-4 contributes to the regulation of intestinal transit amplifying cell differentiation is not known and, in this study, expression of differentiated epithelial cell markers in the IGFBP-4 KO mice was not assessed. Further studies will be needed to address whether IGFBP-4 induces alterations in the epithelial differentiation and cell cycle.

6.2.3 Regulation of IGFBP-4 Expression in the Intestinal Mucosa

Chronic in vivo treatment of control mice with GLP-2 resulted in a significant increase in mucosal IGFBP-4 expression. As discussed above, other mucosal IGFBP isoforms were not altered in response to GLP-2 treatment in either control mice or IGFBP-4 KO mice, suggesting that this was an IGFBP-4-specific response to GLP-2. Nevertheless, the expression of intestinal IGFBP isoforms was found to be consistently higher in the muscularis externa than within the mucosa of control mice. Whether expression of the other IGFBP isoforms in the muscle layer is altered in the IGFBP-4 KO mice compared to the controls, as well as in response to chronic GLP-2 treatment was not assessed, and is a confounding factor. Consistent with the in vivo results that showed increased mucosal expression of IGFBP-4 mRNA after chronic GLP-2 treatment in normal mice, in vitro studies using FRIC cultures treated with GLP-2, demonstrated a transient increase in IGFBP-4 gene expression. This finding is in keeping with the finding of
another group who reported that IGFBP-4 expression is increased in human, colon tumour myofibroblasts in response to GLP-2 treatment (192). However, because FRIC cultures are heterogeneous, likely containing fibroblasts, epithelial cells, endocrine, and muscle cells, the cell type in which IGFBP-4 gene upregulation occurred was not assessed.

In vivo treatment with GLP-2 and IGF-1 increases mucosal expression of Sox9 (193), a known transcriptional stimulator of IGFBP-4 (157). In total parenteral nutrition models, EGF treatment has also been associated with increased crypt cell nuclear β-catenin levels, an indicator of enhanced Wnt signaling, whose downstream target includes Sox9 (57). As EGF and IGF-1 are established intermediate growth factors of GLP-2 (35), it was expected that in vitro treatment of IEC-6 cells with IGF-1 with or without EGF pre-treatment, would increase the expression of IGFBP-4, and that in vivo inhibition of the IGF-1R and EGFR activity would lead to a reduction in IGFBP-4 gene expression. The results were unexpected as in vitro, EGF and IGF-1 treatment of IEC-6 cells reduced Sox9 mRNA expression. In addition, neither IGF-1 nor EGF treatment, together or separately, altered expression of IGFBP-4 under the conditions tested. In vivo, IGF-1R KO in combination with the EGFR inhibitor CI-1033 did not affect mucosal Sox9 or IGFBP-4 gene expression. CI-1033 administration in vivo however, resulted in a compensatory increase in mucosal IGF-1 mRNA expression in the IE-IGF-1R KO mice; thus, unlike the in vitro experiments, there may have been too many compensatory responses within the mucosa in response to knocking out the IE-IGF-1R and to EGFR inhibition to study the regulation of mucosal IGFBP-4 mRNA expression in vivo. However, because the in vitro results were also not significant, IGFBP-4 gene expression may be induced by GLP-2 within the mucosa by other intermediary factors induced by GLP-2 (54,220,221) and not by Sox9 via IGF-1 and EGF. In fact, this finding is consistent with a recent study that found that, in a total parenteral nutrition
model, intestinal epithelial PI3K, which was originally proposed to enhance GLP-2-induced Wnt signaling (193), was not required for the GLP-2-stimulated nuclear translocation of β-catenin (57). An alternate reason why IGFBP-4 gene expression did not change in response to IGF-1 and EGF treatment may be because basal expression of IGFBP-4 is not dependent on Sox9 expression and thus the reduction of Sox9 did not alter the expression of IEC-6 IGFBP-4 expression. Gain-of-function studies in vitro and in vivo would be needed to be conducted to better study the relationship between IGF-1, EGF, and IGFBP-4.

6.2.4 Reconciling the Apparent Contradictory Role of IGFBP-4 in the Intestinal Mucosa: Proposed Role of IGFBP-4

In chapter 3, IGFBP-4 was shown to be required for intestinal proliferation under GLP-2-treated conditions while in chapter 4, IGFBP-4 was established to inhibit proliferation under basal conditions. The dual roles of IGFBP-4 that is required for and at the same time, inhibits proliferation under different circumstances is similar to IGFBP-3/IGFBP-5 within the intestine. As described previously, whole-body IGFBP-3 and IGFBP-5 double KO mice have increased intestinal weight under basal conditions and an abrogated increase in intestinal weight, crypt mitotic index, and crypt-villus height in response to systemic IGF-1 treatment for 7 days (116). This suggests that IGFBP-3 and IGFBP-5 play an inhibitory role in intestinal growth, but play a potentiating effect in response to systemic administration of IGF-1.

IGFBP-4 has been demonstrated to enhance growth in several models including fetal and postnatal development, as well as bone growth in a protease-dependent manner (182–184). The ability of IGFBP-4 to promote growth effects of IGF-1 is observed through the reduced growth trajectories of IGFBP-4 KO mice (184). In contrast, application of exogenous IGFBP-4 in vitro in the IEC-6 proliferation experiments, inhibited the proliferative actions of IGF-1. Although
IGFBP-4 degradation was not assessed in this study, as mentioned previously, new studies demonstrate increased IGFBP-4 degradation by MMP secreted by the intestinal subepithelial myofibroblasts in response to GLP-2 (192). Thus, while GLP-2 also increases mucosal expression of IGFBP-4, the major mechanism of action through which locally expressed IGFBP-4 stimulates intestinal proliferation may be through its degradation. The lack GLP-2-induced proliferation in the IGFBP-4 KO mice may thus be explained due to a lack of GLP-2-induced IGFBP-4 degradation, thereby, leading to lack of increased IGF-1 bioavailability. Interestingly, in vivo, acute administration of the MMP inhibitor (GM6001) has been shown to preserve the acute GLP-2-induced upregulation of ErbB ligands. However, whether GLP-2-induced proliferation and long-term growth effects are preserved with the MMP inhibitor have not been investigated (40). Thus, the roles of IGFBP-4 degradation by MMP under GLP-2 stimulated conditions leading to IGF-induced growth and proliferation remains to be determined. In addition, whether increased expression/secretion of IGFBP-4 in response to GLP-2 contribute to altered IGFBP-4 degradation and IGF-1 bioactivity remains to be determined but may be addressed through assessments of total IGFBP-4 protein expression in the mucosa under chronic GLP-2 treatment. Overall, the results support the notion that under basal conditions where less IGFBP-4 degradation is taking place, IGFBP-4 inhibits the action of IGF proteins, thereby inhibiting basal proliferation. The proposed mechanisms of action of IGFBP-4 and a summary of finding to date is illustrated in Figure 6.1.
Figure 6.1 Schematic Representing Proposed Role of IGFBP-4 Within the Intestinal Mucosa.

GLP-2, once released into the circulation from the intestinal L-cells, binds to GLP-2 receptors expressed by intestinal subepithelial myofibroblast (ISEMF) cells, a key source of IGF-1 within the mucosa, some of which is bound to locally-expressed IGFBP-4 from the epithelium and the ISEMf. Increased degradation, potentially by MMP, of IGFBP-4 in response to GLP-2, releases IGF-1 leading to increased IGF-1R activation and consequent stimulation of IEC proliferation within the intestinal crypt niche. Conversely, under basal unstimulated conditions, IGFBP-4 plays an inhibitory role in basal intestinal growth by binding to IGF-1 and preventing its interaction with the IE-IGF-1R. Lack of the IGFBP-4 protease, PAPP-A, leads to increased circulating IGFBP-4 levels; however, circulating IGFBP-4 does not influence mucosal growth and proliferation under basal conditions or in response to GLP-2 treatment.
6.3 Future Directions

IGFBP-4 was found to have contradictory effects depending on whether or not the intestine was stimulated with GLP-2. It would be of particular interest to further delineate the integral mechanisms that govern the growth and proliferation within the mucosa and reconcile the seemingly contradictory roles of IGFBP-4.

In chapter 3, where a key role of intestinal IGFBP-4 in facilitating GLP-2-induced proliferation was identified, the next step is to investigate whether IGFBP-4 cleavage occurs more frequently in response to GLP-2 in vivo. IGFBP-4 fragment expression in mucosal scrapes isolated from control mice treated chronically with GLP-2 or vehicle can be determined by western blot analysis (192). Increased IGFBP-4 fragmentation in control mice treated with GLP-2 would suggest that IGFBP-4 degradation increases IGF-1 bioavailability and would help reconcile the contradictory roles of IGFBP-4. As well, in order to determine the role of MMP in GLP-2-induced proliferation, as there are many MMP isoforms, a broad spectrum MMP inhibitor (GM6001) or vehicle can be administered to normal mice intraperitoneally (40). The chronic proliferative effects of GLP-2 would then be assessed via Ki-67 incorporation as previously established (35,56). As well, to quantify the IGFBP-4 degradation, western blot of mucosal scrapes collect from the mice can again be conducted to determine changes in IGFBP-4 degradation by GLP-2 or vehicle, with and without GM6001 administration.

In chapter 4, where IGFBP-4 was demonstrated to inhibit basal intestinal epithelial proliferation in an IGF-1R and MEK1/2 dependent manner, further pharmacological studies are required to assess the role of MEK1/2 in the regulating epithelial proliferation. Namely, a second proliferation study using reduced MEK1/2 inhibitor dosages followed by IGFBP-4
administration would further characterize the effectiveness of IGFBP-4 in inhibiting IEC-6 proliferation.

In addition, studies could be conducted using the intestinal organoid model system of primary intestinal epithelial cells. Preliminary studies on murine organoid cultures have already been used to study the function of IGFBP-4, using change in surface area as a proxy for growth and proliferation. Phenotypically, IGFBP-4 KO organoids developed larger crypts (Figure 6.2A) as compared to control organoids and required more frequent splitting. IGFBP-4 KO in the organoids was confirmed by semi-quantitative RT-PCR (Figure 6.2B). Interestingly, IGFBP-4 KO organoids were found to express significantly higher Lgr5 mRNA by 40.0±20.7% as compared to control organoids (p<0.05, Figure 6.2C). Established organoids were also split and plated, and treated with exogenous IGF-1 (100ng/ml) for 24 hours. Organoids with crypt structures and of similar starting size were assessed for change in surface area in response to the treatment. Organoids in all experimental groups displayed increased surface area over 24 hours. Control organoids, displayed no significant difference in the % of surface area increase after 24 hours of IGF-1 treatment (p>0.05, Figure 6.2A,D). In contrast, IGFBP-4 KO organoids treated with IGF-1 displayed a significant increase in the % change in surface area which was 1.7±0.3 fold that of the vehicle treated IGFBP-4 KO organoids (p<0.05, Figure 6.2D).
Figure 6.2  IGFBP-4 KO Organoids Increase Surface Area In Response to IGF-1.

Jejunal organoids were grown from crypts under standard culture conditions. (A) Control and IGFBP-4 KO (KO) organoids were treated with IGF-1 (100 ng/ml) or vehicle for 24 hours, 3 days after plating. Representative of n= 20-35 organoids, 2 mice per group. (B) IGFBP-4 mRNA expression and (C) Lgr5 mRNA expression of the organoids; n=3-5. (D) Surface area of organoids with crypt formations before treatment and (E) % Change in surface area was quantified as a measure of epithelial proliferation following 24hr treatment of IGF-1 (100ng/ml); n= 20-35 organoids, 2 mice per group. *p<0.05, ***p<0.001
Because the intestinal organoid model contains a variety of cells, including stem cells and fully differentiated cells, this model is arguably more representative of the small intestinal epithelium in vivo. Consistent with the larger crypt depth observed in IGFBP-4 KO mice in vivo, the morphology of the IGFBP-4 KO organoids suggests that the buds that protrude outward are larger than in control organoids. Furthermore, the finding of increased expression of Lgr5 mRNA in IGFBP-4 KO organoids suggests increased stemness of the IGFBP-4 KO organoids. However, whether the proportions of stem cells and differentiated cells, as well as proliferating cells are different between control and IGFBP-4 KO organoids have not been determined. Interestingly, when change in surface area was used as a measure of growth, the intestinal IGFBP-4 KO organoids were more responsive to IGF-1 treatment than control organoids. This suggests that, consistent with the hypothesis, IGFBP-4 secreted from intestinal organoids inhibits the growth promoting effects of IGF-1 under basal conditions. However, whether the activation of IGF-1R and its downstream signaling pathways by IGF-1 is different between the control and IGFBP-4 KO organoids was not determined. In addition, whether Wnt signaling, known to be directly inhibited by IGFBP-4 (129), is altered between the two groups is also undetermined. The in vivo, systemic administration of IGF-1 to Sox9EGFP mice resulted in increased proliferation of cells classified as quiescent stem cell-like, which also only formed spheroids in vitro with IGF-1 supplementation (117). Since only the IGFBP-4 KO organoids responded to IGF-1, it is also possible that IGFBP-4 KO organoids contain altered proportions of active and quiescent stem cell populations compared to those in the control organoids. Although analysis of Ki-67 positive cells were also assessed by immunocytochemistry in the organoids, this marker was found to be expressed in most of the crypt cells and was not specific enough to delineate changes in proliferation rates between control and IGFBP-4 KO organoids,
as well as IGF-1 and vehicle-treated organoids. Thus, the next step is to assess proliferation by EdU incorporation to quantify the proportion of proliferating cells per organoid in response to IGF-1 treatment in control and IGFBP-4 KO organoids. In addition, whether IGFBP-4 alters cell differentiation is not known. Thus in vivo, the next step is to characterize the mucosal epithelium population in IGFBP-4 KO mice compared to the WT mice and in vitro, in IGFBP-4 KO and WT organoids, using cell markers such as alkaline phosphatase inhibitor, mucin, chromogranin A, and villin. Finally, for the proliferation assay where IGFBP-4 treatment decreased IEC-6 cell proliferation, the contribution of IGF-2 needs to be assessed. IEC-6 cells could therefore undergo siRNA knockdown of IGF-2 or IGF-1 and undergo the same proliferation studies with the IGF-1R, PI3K and MEK1/2 inhibitors to assess whether IGFBP-4 inhibits the action of IGF-1, IGF-2, or both to inhibit basal intestinal epithelial proliferation.

In chapter 5, IGFBP-4 expression was demonstrated to increase in response to GLP-2 but not IGF-1 or EGF treatment in vivo and in vitro. Myofibroblasts generated from colon tumors have been reported to increase secretion and degradation of IGFBP-4 in response to GLP-2 treatment (192). While human colon myofibroblast cell lines such as CCD-18Co have been reported to increase in proliferation in response to GLP-2 (192), I did not observe an increase in the expression/secretion of IGFBP-4 in this cell line or in mouse myofibroblasts in vitro in response to GLP-2 treatment (please see Appendix for further data). Furthermore, degradation of IGFBP-4 by the CCD-18Co cells and mouse myofibroblasts in response to GLP-2 treatment would need to be assessed as this would also help establish the mechanism by which IGFBP-4 aids in the growth and proliferative effects of GLP-2.

In addition, gain-of-function studies in vitro and in vivo are necessary to confirm the findings of the loss-of-function studies that demonstrate that IGFBP-4 gene expression is not regulated by
IGF-1 and EGF. Thus, using organoids in vitro, change in IGFBP-4 expression can be assessed in response to IGF-1 treatment using immunostaining or in situ hybridization as it is possible that only certain cell types within the intestinal epithelium alters their expression and release of IGFBP-4. Transfection of a Sox9 vector or activation of the Wnt signaling pathway by treatment of IEC-6 cells with Rspondin-1 may also be a useful gain-of-function model to assess whether IGFBP-4 gene expression increases in response to increased expression of Sox9 as was found in colon epithelial cell lines (157). In vivo, systemic co-administration of EGF and IGF-1 is another way to assess regulation of IGFBP-4 gene expression. However, because subcutaneous IGF-1 injections (2µg/g every 12 hours) (35) results in insulin-like effects as well as intestinal growth effects, the dose would have to be carefully calibrated or administered by osmotic-mini-pumps to deliver IGF-1 slowly (117). EGF co-administration with GLP-2 has already been studied in neonatal piglets (222); however, whether EGF with IGF-1 administration in vivo alter the expression of IGFBP-4 would be a beneficial complement to the loss-of-function study conducted using IE-IGF-1R KO mice treated with and without CI-1033. Additionally, GLP-2 is also known to act through vasoactive intestinal polypeptide (54,221), and nitric oxide (220). Thus, the role of these intermediary factors in inducing mucosal IGFBP-4 expression in response to GLP-2 treatment also need to be investigated.

### 6.4 Overall Conclusions and Significance

Overall, this study establishes a critical role of locally-expressed IGFBP-4 as a key mediator of mucosal growth and proliferation within the intestine through, first, its requirement for intestinal proliferation under GLP-2-stimulated conditions and, second, its role in inhibiting basal intestinal crypt cell growth. Finally, while GLP-2 stimulates mucosal IGFBP-4 expression, the known GLP-2-intermediary growth factors, IGF-1 and EGF do not contribute to its expression.
Intestinal mucosal growth and proliferation are regulated by multiple pathways which interact with one another. IGF-1 and EGF are both integral mediators of the growth effects of GLP-2, used as treatment therapy for patients with short bowel syndrome (1). Thus, investigating the mechanisms by which these growth factors interact with one another and how the activity of IGF-1 may be regulated by IGF binding proteins within the mucosa is crucial both for understanding intestinal physiology and how GLP-2 exerts its beneficial effects in clinical applications.
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Appendix
Appendix Figure 1 IEC-6 Cells From ATCC Demonstrate Poor IGF-1 Responsivity

A) DNA synthesis in IEC-6 cells from ATCC treated with IGF-1 doses ranging from 10 ng/ml to 1000 ng/ml; serum-containing medium was used as a positive control. B) DNA synthesis in IEC-6 cells from Dr. P. Kay Lund treated with IGF-1 doses ranging from 10 ng/ml to 1000 ng/ml; serum-containing medium was used as a positive control. (N=4 per group; cell from two different splits in duplicates) *p<0.05, **p<0.01, ***p<0.001
Appendix Figure 2 CCD-18Co Cell IGFBP-4 mRNA Expression and Protein Secretion Do Not Change in Response to GLP-2 Treatment

Relative IGFBP-4 mRNA expression in CCD-18Co cells given hGly^2-GLP-2 treatment for 2 hours at varying doses (A; 10^{-9}-10^{-6} M, N=7-8 per group) or for varying time periods (B; 10^{-8} M 2-24 hours, N=4 per group). Relative IGFBP-4 protein secretion from CCD18-Co cells given hGly^2-GLP-2 treatment (10^{-8} M) for 12 or 24 hours (C; N=5-6 per group). *p<0.05. Open bars indicate vehicle treatment and closed bars indicate hGly^2-GLP-2 treatment.
Appendix Figure 3 Mouse ISEM F Cells Are Not a Good Model for Assessing Intracellular Effects of GLP-2

A) IGF-1 mRNA expression of ISEM F cells were treated with hGly2-GLP-2 at $10^{-8}$ M for 2 hours. (n=10) B) Cells under serum-starvation conditions were treated with $10^{-8}$ M of GLP-2 for 30 minutes and compared to control (n =5). Representative blot shown for control and GLP-2
treated cells. C) GLP-2R expression was compared between ISEMFs at different passages (n=3-5 per group). D) IGFBP-4 mRNA expression does not have a dose dependent response to GLP-2 treatment. ISEMFs were treated with hGly2-GLP-2 at doses ranging from 10^{-12} to 10^{-6} M for 4 hours (n=7). E) IGFBP-4 mRNA does not have a time dependent response to GLP-2 treatment. ISEMFs were treated with hGly2-GLP-2 at a dose of 10^{-8} M for a time period ranging from 0.5 to 24 hours (n=4-7 per group).
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