Abstract

Type 2 diabetes (T2D), a chronic metabolic disorder characterized by hyperglycemia, is growing in prevalence in both young and old people worldwide. One of the current treatments for T2D involves the use of oral hypoglycemic medications that stimulate insulin secretion from pancreatic β-cells. However, these drugs risk inducing hypoglycemia and, as β-cell function declines over time, lose their effectiveness. An attractive alternative for the treatment of T2D is glucagon-like peptide-1 (GLP-1), a gut hormone with a variety of anti-diabetic properties including stimulation of glucose-dependent insulin secretion and enhancement of β-cell mass. However, the clinical use of GLP-1 is limited due to its rapid degradation, primarily by dipeptidyl peptidase IV (DPIV). As a result, continuous administration of GLP-1 is necessary to maintain its therapeutic effect. Gene therapy may represent a promising alternative approach for achieving long-term automatic release of GLP-1 from cells in the body. In this thesis, the efficacy of in vitro and in vivo GLP-1 gene transfer to the liver using an adenoviral vector expressing GLP-1 and its effects on glucose homeostasis in diabetic db/db mice was examined. A replication-deficient adenoviral vector was constructed to express DPIV-resistant GLP-1 under the regulation of a liver-specific, glucose-responsive L-type pyruvate kinase (LPK) promoter (AdLPK-GLP-1). In vitro transduction of AdLPK-GLP-1 in hepatocytes and non-hepatocytes induced immunoreactive and bioactive GLP-1 production in a liver-specific and dose-dependent manner. Furthermore, when these transduced hepatocytes were transplanted into the peritoneal cavity or under the kidney capsule of normal mice, an ~2-fold increase in fasting plasma GLP-1 levels was observed. However, this approach was not effective in improving glucose homeostasis in severely diabetic db/db mice. Moreover, intravenous delivery of AdLPK-GLP-1 in normal mice and rats did not result in an increase in fasting plasma GLP-1 levels. Thus while a virus was generated that is capable of inducing hepatocytes to produce GLP-1, additional studies will be required to demonstrate a therapeutic effect with this agent.
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<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>3 Letter Code</th>
<th>1 Letter Code</th>
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<tbody>
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<td>Alanine</td>
<td>Ala</td>
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<td>Arginine</td>
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<tr>
<td>Asparagine</td>
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<td>N</td>
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<td>Aspartate</td>
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<td>D</td>
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<td>Glutamine</td>
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<td>Q</td>
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<td>Glu</td>
<td>E</td>
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<tr>
<td>Glycine</td>
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<tr>
<td>Histidine</td>
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<td>Isoleucine</td>
<td>Ile</td>
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<td>Leucine</td>
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<tr>
<td>Lysine</td>
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<tr>
<td>Methionine</td>
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<td>Phenylalanine</td>
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<tr>
<td>Proline</td>
<td>Pro</td>
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<tr>
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<tr>
<td>Threonine</td>
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<tr>
<td>Tryptophan</td>
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<td>Tyrosine</td>
<td>Tyr</td>
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<tr>
<td>Valine</td>
<td>Val</td>
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**Abbreviations**

- **[Gly8]**-GLP-1<sub>7-37</sub>: GLP-1 containing a glycine substitution at the 8<sup>th</sup> amino acid position
- **[Ser8]**-GLP-1<sub>7-36 amide</sub>: GLP-1 containing a serine substitution at the 8<sup>th</sup> amino acid position
- **Ad5**: Recombinant type 5 adenovirus
- **AdCMV-βGal**: Adenoviral vector expressing β-galactosidase under the control of the CMV promoter
- **AdLPK-GLP-1**: Adenoviral vector expressing GLP-1 under the control of the LPK promoter
- **BSA**: Bovine serum albumin
- **CaCl<sub>2</sub>**: Calcium chloride
- **cAMP**: Adenosine-3', 5'-cyclic monophosphate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CPM</td>
<td>Counts per minute</td>
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<tr>
<td>CRE</td>
<td>cAMP response element</td>
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<tr>
<td>DPIV</td>
<td>Dipeptidyl peptidase IV</td>
</tr>
<tr>
<td>E1 to E4</td>
<td>Early regions 1 to 4</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GIP</td>
<td>Glucose-dependent insulinotropic polypeptide</td>
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<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>GLP-2</td>
<td>Glucagon-like peptide-2</td>
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<tr>
<td>GLUT2</td>
<td>Glucose transporter 2</td>
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<tr>
<td>GRPP</td>
<td>Glicentin-related pancreatic polypeptide</td>
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<tr>
<td>HG-DMEM</td>
<td>High glucose Dulbecco's modified Eagle medium</td>
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<tr>
<td>HNF</td>
<td>Hepatocyte nuclear factor</td>
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<tr>
<td>IP</td>
<td>Intervening peptide</td>
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<tr>
<td>IR-GLP-1</td>
<td>Immunoreactive GLP-1</td>
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<tr>
<td>ISE</td>
<td>Intestinal specific element</td>
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<tr>
<td>LPK</td>
<td>Liver-type pyruvate kinase</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
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<tr>
<td>MOPS</td>
<td>3-[N-Morpholino]propanesulphonic acid</td>
</tr>
<tr>
<td>NEP 24.11</td>
<td>Neutral endopeptidase 24.11</td>
</tr>
<tr>
<td>PACAP</td>
<td>Pituitary adenylate cyclase activating polypeptide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS&lt;sup&gt;+&lt;/sup&gt;</td>
<td>PBS without CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PC</td>
<td>Prohormone convertase</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming units</td>
</tr>
<tr>
<td>RCF</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light units</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>SV</td>
<td>Simian virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside</td>
</tr>
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ACKNOWLEDGEMENTS

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INTRODUCTION

Type 2 Diabetes Mellitus

Diabetes mellitus is a chronic disease that is growing in epidemic proportions worldwide with a projected growth from 171 million in 2000 to 366 million diagnosed cases by 2030 [1]. According to the World Health Organization, an estimated 2 million Canadians have diabetes in 2000 and this number is expected to increase to 3.5 million in 2030 [2]. The majority of this increase is attributed to urbanization in developing countries, population growth, aging, physical inactivity, unhealthy diets and obesity [1-4]. Given the chronic nature and epidemic rise of diabetes, an increasingly huge burden is placed on the world health care systems with respect to health care needs, resource utilization and costs for prevention and treatment of this disease. In Canada, 13.2 billion dollars are spent annually by the health care system for treating diabetes and its complications and this is expected to increase to 19.2 billion per year in 2020 [5]. Undoubtedly, diabetes has become a major health concern of the 21st century worldwide. As a result, there is a great impetus towards research into a better understanding of this disease in order to find a cure.

Diabetes is a heterogeneous group of disorders characterized by elevated blood glucose levels due to a defect in the ability to adequately produce and/or use insulin, a glucose-lowering hormone produced by the pancreatic β-cells [6-9]. There are 2 main types of diabetes. Type 1 diabetes (T1D) represents approximately 5-10% of diagnosed cases of diabetes. This disease typically strikes children and adolescents and is characterized by absolute insulin deficiency due to autoimmune destruction of the β-cells. As a result, people with T1D rely on daily exogenous insulin injections for survival [6-9]. The more prevalent type of diabetes, attributing to more than 90% of the diagnosed cases, is Type 2 diabetes (T2D). T2D is typically associated with adults although in recent years an alarming number of children and adolescents are being diagnosed with this disease [10]. An increasing concern is that this type of diabetes will become the predominant form of diabetes in childhood. This issue is already evident in Japan where T2D accounts for approximately 80% of
childhood diabetes [11]. T2D is characterized by insulin resistance and/or reduced insulin secretion. Currently, it is proposed that T2D begins as insulin resistance. As the need for insulin increases, β-cell mass and insulin production initially increase, but subsequently decline leading to elevated blood glucose levels. If left untreated, this sustained elevation in blood glucose leads to long-term complications, including blindness, kidney damage and lower limb amputations, responsible for the shortened life expectancy of people with diabetes [6-9, 12]. Therefore, the goal of diabetes therapy is to maintain blood glucose levels as close to normal as possible. The importance of blood glucose control in diabetes was demonstrated by findings from the UK Prospective Study Group and Diabetes Control and Complications Trial. These studies demonstrated that intensive glycemic control substantially decreases the risk of complications in people with T2D [13, 14].

**Current Treatments for Type 2 Diabetes**

Current treatments for T2D involve proper diet, exercise and oral medications (biguanides, sulphonylureas, and thiazolidinediones) that help improve insulin sensitivity or enhance insulin secretion. If these treatments are no longer effective in maintaining glucose homeostasis, daily exogenous insulin injections are used [6, 12, 15-17]. Although current drugs significantly improve blood glucose control, these drugs fail to effectively restore glucose homeostasis in the long-term because they do not adequately target the metabolic abnormalities that underlie T2D. These include the inability of the drugs to (i) prevent the complications associated with chronic elevated blood glucose levels, (ii) reverse insulin resistance and (iii) stop the progressive loss of β-cell function and mass. In addition, these drugs are often associated with hypoglycemia and weight gain, a risk factor for T2D, and often lose their effectiveness upon loss of β-cell function [6, 12, 15-17]. Therefore, a major focus in the coming years is to develop new therapeutic approaches that effectively target one or more of the metabolic defects associated with T2D. Novel therapeutic agents will aim to (i) enhance glucose uptake by muscle and fat, (ii) decrease hepatic glucose production, (iii) increase growth and proliferation of β-cells and (iv) increase glucose-dependent insulin secretion. One
potential candidate that can mediate these effects and is emerging as a promising new treatment for T2D is the gastrointestinal incretin hormone, glucagon-like peptide-1 (GLP-1).

The Incretin Effect

In 1932, LaBarre [18] introduced the term “incretins” to describe gut-derived extracts that were shown to be capable of lowering blood glucose levels [18-21]. Even more interesting was the observation that oral intake of glucose resulted in a greater rise in plasma insulin than intravenous administration of glucose at the same concentration – an observation that was subsequently termed the “incretin effect” [21-23]. Consequently, this led to the search for the identity of the incretins responsible for up to 50-60% of postprandial insulin secretion. Two hormones were discovered that mediate this incretin effect – glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). GIP is a 42 amino acid peptide released from K-cells of the duodenum. GLP-1, consisting of 36 or 37 amino acids, is secreted from the L-cells located in the distal jejunum, ileum, colon and rectum. Both hormones are released in response to nutrient ingestion to act on specific GIP and GLP-1 receptors expressed on islet cells and various other tissues to exert their glucose-dependent insulinotropic effects [24, 25]. The importance of these hormones in normal glucose homeostasis is demonstrated by the presence of glucose intolerance in mouse models lacking GIP or GLP-1 receptors [26].

In addition to insulin resistance and reduced insulin secretion, T2D patients also have a reduced incretin effect [25, 27-30]. The majority of studies have reported that GIP secretion is normal or elevated in people with T2D than in healthy subjects [30-33]. Conflicting results have been obtained for GLP-1 secretion in T2D. Both a decrease and increase in secretion has been observed [34, 35]. However, more recent studies using a larger number of subjects have demonstrated a general reduction in GLP-1 secretion [32, 36]. Interestingly, while the insulinotropic effect of GIP is lost in T2D patients, GLP-1 retains its potent insulinotropic activity [35]. This makes GLP-1 an attractive anti-diabetic agent since it has been shown in several studies that exogenous administration of GLP-1
results in normalization of blood glucose levels in subjects with T2D [37-44]. Therefore, several programs are underway to develop GLP-1-based therapies, with the first compound, Byetta™ recently approved by U.S. Food and Drug Administration [45].

**Biosynthesis of GLP-1**

The two bioactive forms of GLP, GLP-1 and GLP-2, belong to the glucagon superfamily of peptide hormones due to their high degree of sequence homology with glucagon [46-49]. Other peptides that belong to this family include GIP, secretin, pituitary adenylate cyclase activating polypeptide (PACAP), PACAP-related peptide, growth hormone releasing factor and vasoactive intestinal polypeptide. These peptides have diverse functions and are produced in various organs including gut, pancreas, central nervous system (CNS) and peripheral nervous system [24].

Sequence analysis revealed that glucagon and GLPs are encoded from the preproglucagon gene consisting of 6 exons and 5 introns. The exons encode a 2-kb transcript consisting of a 5'-untranslated region, a protein-coding region and a 3'-untranslated region. Specifically, the protein-coding region is comprised of the N-terminal signal sequence, the proglucagon gene consisting of the glicentin-related pancreatic polypeptide (GRPP) and sequences that encode glucagon, GLP-1 and GLP-2 (Fig. 1) [24, 46, 50].

The proglucagon gene is expressed in (i) the α-cells of the pancreatic islets, (ii) the L-cells located in the distal ileum, colon and rectum and (iii) the nucleus tractus solitarius of the CNS [49, 51-53]. Expression of the proglucagon gene is regulated by the proglucagon gene promoter. Studies in glucagon-expressing cell lines revealed that the promoter consists of 5 important transcriptional DNA control elements: G1, G2, G3, adenonsine-3',5'-cyclic monophosphate (cAMP) response element (CRE) and intestinal specific element (ISE). G1 is involved in directing pancreatic α-cell specific expression while G2 and G3 are involved in enhancing islet cell expression. A CRE confers cAMP responsiveness to transcription of proglucagon gene and an ISE is important for determining transcriptional expression of the gene in intestinal L-cells [24]. In mammals, cell-specific processing
Figure 1. Schematic diagram of the major bioactive hormones derived from tissue-specific post-translational processing of proglucagon by prohormone convertases (PCs). In the pancreas, proglucagon is cleaved by PC2 to produce glucagon, glicentin-related pancreatic polypeptide (GRPP) and major proglucagon fragment. In the intestine and brain, proglucagon is cleaved by PC1/3 to produce glucagon-like peptide (GLP)-1, GLP-2, oxyntomodulin and glicentin. IP-1 and IP-2, intervening peptides.
of proglucagon by prohormone convertases (PCs) gives rise to different peptide hormone products. In the α-cells, proglucagon is cleaved by PC2 to form glucagon, GRPP, and major proglucagon fragment (Fig. 1). In the L-cells and the neurons of the brain, the main products derived from proglucagon by the actions of PC1/3 are GLP-1, GLP-2, glicentin and oxyntomodulin (Fig. 1) [24, 54, 55]. The mammalian GLP-1 sequence, from rodents to humans, is completely conserved and indicates the importance of the biological processes regulated by this hormone [24]. Prior to its release from L-cells, N-terminal truncation of the full-length GLP-1_1-37 or GLP-1_1-36 amide gives rise to GLP-1_7-37 and GLP-1_7-36 amide, respectively, with the latter found in greater abundance in the circulation [24, 25].

**Secretion of GLP-1**

Like insulin, the release of GLP-1 following a meal is biphasic consisting of an immediate peak (15 to 30 minutes) followed by a second peak approximately an hour later [24]. Previous studies in humans have demonstrated that carbohydrates and fat are the most important nutrient stimulus for GLP-1 secretion whereas protein alone does not increase GLP-1 release [56-58]. However, since the majority of glucose is absorbed before reaching the L-cells in the ileum, fat is believed to be the more important nutrient in inducing GLP-1 secretion. In particular, long-chain monosaturated fatty acids induce greater GLP-1 secretion than short-chain polyunsaturated or saturated fatty acids [59, 60]. Interestingly, when fat is placed in the duodenum, GLP-1 secretion also occurs [61]. This observation combined with the fact that the initial peak of GLP-1 release occurs before nutrient contact with L-cells suggests that there are other stimuli involved in regulating GLP-1 secretion.

Several studies have provided evidence for endocrine and neural control in the rapid release of GLP-1 following a meal. To date, the only endocrine hormone shown to stimulate GLP-1 secretion is GIP. Studies conducted *in vivo* in the rat and *in vitro* in rat intestinal cells and isolated canine L-cells have demonstrated that GIP is a potent stimulant of GLP-1 synthesis and release [62-67]. Based on these findings, it has been proposed that a proximal-distal loop exists whereby nutrients in the upper
intestine stimulate the release of GIP that, in turn, circulates to the L-cells to stimulate GLP-1 release. However, this appears to be an unlikely mechanism in humans since GIP infusion in fasted subjects did not increase GLP-1 secretion [58, 68, 69]. In addition to hormones, the CNS has been shown to be important in regulating GLP-1 release. The importance of vagal cholinergic innervation in mediating GLP-1 secretion has been demonstrated in studies in which muscarinic agonists have been shown to stimulate GLP-1 secretion in both rat and human L-cell lines [70, 71]. Furthermore, fat and glucose-induced GLP-1 secretion is reduced by atropine, a non-specific muscarinic blocker, in rats and humans, respectively [71, 72]. GLP-1 secretion is also influenced by the sympathetic nervous system. Epinephrine and isoproterenol, a β-adrenergic agonist, have been shown to stimulate GLP-1 secretion from perfused rat intestine [67, 73, 74]. Finally, non-adrenergic non-cholinergic stimulation of GLP-1 secretion has been observed with gastrin-releasing peptide (GRP). Both in vitro and in vivo studies have shown that GRP is a potent stimulant of GLP-1 secretion [64, 66, 74]. Together, these results demonstrate the complexity of the regulation of GLP-1 secretion involving interactions between nutrient, endocrine and neural stimuli.

**Metabolism of GLP-1**

Once released into circulation, GLP-1 has a plasma half-life of approximately 1-2 minutes [75, 76]. This extremely short biological half-life of GLP-1 is due to renal clearance, hepatic clearance and degradation by proteases such as dipeptidyl peptidase IV (DPIV) or neutral endopeptidase 24.11 (NEP 24.11) [24, 77]. The majority of GLP-1 degradation in the circulation is mediated by DPIV, a ubiquitous serine protease that cleaves GLP-1 at the N-terminus (His^7^-Ala^8^) to produce GLP-1\_9\_37 or GLP-1\_9\_36 amide [78]. These non-insulinotropic fragments have been reported to be biologically inactive and even may act as competitive antagonists at the GLP-1 receptor [79-82]. Another enzyme involved in mediating GLP-1 degradation is NEP 24.11. This membrane-bound zinc metallopeptidase is widely distributed in tissues and specifically cleaves at the N-terminal side of GLP-1 that contains aromatic or hydrophobic amino acids [83]. While the kidney is less important
for GLP-1 degradation, it is a major site for metabolite clearance of GLP-1 since patients with renal impairments have relatively high levels of GLP-1 [76, 84, 85]. Specifically, both glomerular filtration and tubular catabolism are believed to be involved in removal of GLP-1 metabolites by the kidney [86]. The liver is the first organ where endogenous GLP-1 must pass through prior to entering the circulation. This has important significance since inactivation of GLP-1 has been observed in the liver due to the presence of high concentrations of DPIV in the hepatocytes [87, 88].

**Physiological Actions of GLP-1**

GLP-1 elicits its biological effects through binding to the GLP-1 receptor, a member of the seven transmembrane G protein-coupled family of receptors [89]. Activation of GLP-1 receptor leads to the stimulation of different signal transduction pathways including the (i) cAMP and protein kinase A-dependent and -independent pathways, (ii) phospholipase C pathway and (iii) phosphatidylinositol 3-kinase pathway. In β-cells, these signalling pathways are implicated in the regulation of ion channels, intracellular calcium stores and transcription factors that are important for mediating glucose-dependent insulin secretion, gene transcription and proliferation [90]. The GLP-1 receptor is widely distributed in the body and is expressed in the pancreatic islets, stomach, brain, heart, lung, kidney, small intestine and colon of mice and rats [89]. This distribution of GLP-1 receptor reflects the wide range of physiological actions mediated by GLP-1.

The hallmark action of GLP-1 is stimulation of glucose-dependent insulin secretion from β-cells [91, 92]. Administration of GLP-1 at concentrations similar to those observed in plasma after meals enhances insulin secretion in humans with elevated blood glucose levels. Furthermore, GLP-1 has been shown to stimulate insulin gene transcription and biosynthesis [93], a feature that is not found in current oral anti-diabetic drugs. Notably, the insulinotropic action of GLP-1 is attenuated at low blood glucose levels (~ 4.5 mM) therefore preventing the risk of hypoglycemia [92].

An exciting aspect of GLP-1 is its role in β-cell growth and development. Recent studies have demonstrated that GLP-1 can increase β-cell mass by enhancing β-cell neogenesis and proliferation.
and by reducing β-cell apoptosis [94-96]. The progressive loss of β-cell function and mass in T2D are the key factors leading to reduced insulin secretion [97, 98]. Currently, there are no treatments that can prevent this reduction in islet function and mass. Therefore, GLP-1 could represent a novel therapy that could delay or even reverse the natural progression of T2D.

In addition to β-cells, GLP-1 regulates hormone secretions from other pancreatic islet cells. In the δ-cells, GLP-1 stimulates secretion of somatostatin, a hormone involved in inhibiting insulin and glucagon secretion [99]. In the α-cells, GLP-1 has been reported to inhibit secretion of glucagon, a hormone that increases blood glucose levels [100, 101]. The ability to inhibit glucagon release has been shown to play a role in the normalization of blood glucose levels in T2D subjects treated with exogenous GLP-1 [38]. Furthermore, T1D patients with no residual β-cells exhibited a decrease in blood glucose levels after GLP-1 administration despite undetectable C-peptide levels perhaps, in part, as a result of the ability of GLP-1 to lower glucagon levels [102]. Interestingly, when GLP-1 was tested in vitro, stimulation of glucagon secretion was observed [103]. This suggests that GLP-1 exerts its inhibitory effects on glucagon secretion via an indirect paracrine pathway mediated by glucose and hormones such as insulin and somatostatin.

Maintenance of glucose homeostasis does not rely solely on the effects of GLP-1 on the pancreas. Extrapancreatic effects of GLP-1 have also been shown to play an important role in this aspect. In the stomach, GLP-1 has been shown to be involved in the “ileal brake”, a mechanism by which the presence of nutrients in the distal small intestine can cause inhibition of upper gastrointestinal motor and secretory activity [104, 105]. Studies have shown that GLP-1 inhibits gastrointestinal secretion and motility which can reduce nutrient absorption and the subsequent rise in blood glucose levels [42, 106, 107]. In the brain, GLP-1 and its receptors are expressed in high concentrations in the hypothalamic regions involved in food and water intake. A role in satiety and body weight regulation was demonstrated by the reduction in food intake of rats given intracerebroventricular administration of GLP-1. This effect was reversed upon co-administration of exendin9-39, a GLP-1 receptor
antagonist [108]. Finally, GLP-1 has been suggested to be important in increasing glucose uptake and insulin sensitivity in liver, skeletal muscle and adipose tissues [109-111], although this remains controversial [112-114].

Collectively, these studies demonstrate the wide range of blood glucose-lowering effects mediated by GLP-1. As a result, GLP-1 is increasingly being viewed as an attractive candidate for the treatment of T2D.

**GLP-1 as an Anti-Diabetic Agent**

The first study that demonstrated the clinical potential of GLP-1 was conducted by Gutniak *et al.* [109] where continuous GLP-1 infusion reduced the amount of exogenous insulin required to maintain postprandial blood glucose levels in both T1D and T2D patients. The effects of GLP-1 under more physiological settings, such as during ingestion of regular meals, were demonstrated by Rachman *et al.* [40] who continuously infused GLP-1 into T2D patients resulting in normalization of their blood glucose levels. Several clinical studies have reported that increased insulin secretion, reduced glucagon secretion, inhibition of gastric emptying and enhanced insulin sensitivity all contribute to the glucose-lowering effects of GLP-1 in T2D subjects [38, 42, 109, 115-117].

Diabetes is a chronic disease and requires long-term treatment. Many clinical studies have demonstrated the efficacy of GLP-1 in lowering blood glucose concentrations in acute situations but few have investigated the effects of long-term treatments with exogenous GLP-1. Not only is this more relevant for development of novel diabetes therapy, it is important to examine whether long-term administration of GLP-1 might lead to undesirable side effects or desensitization of the GLP-1 receptor thus reducing its efficacy in the long run. A 1 week treatment of subcutaneous GLP-1 injections in T2D patients demonstrated that the anti-diabetic effect of GLP-1 was maintained for the duration of the study [118]. Similarly, Zander *et al.* [119] demonstrated that 6 weeks of continuous subcutaneous infusion of GLP-1 significantly improved fasting and 8 hour mean plasma glucose levels as well as decreased hemoglobin A1C (HbA1C) by 1.3%. Notably, no significant side effects
were observed. Recently, a 12 week study was conducted in elderly T2D patients given either an oral anti-diabetic medication (sulphonylurea) or continuous subcutaneous administration of GLP-1. This treatment was well tolerated and while both the sulphonylurea-treated and GLP-1 treated groups achieved similar HbA1c levels, there were significantly less episodes of hypoglycemia in the GLP-1 treated group (1 compared to 87 episodes) [120]. Therefore, these findings indicate that long-term exposure to GLP-1 for up to 12 weeks does not cause significant side effects, and more importantly, does not diminish its anti-diabetic effects in vivo.

Despite these impressive effects of GLP-1 in numerous clinical studies, its short biological half-life and the requirement for continuous administration has necessitated the development of other strategies to optimize the use of GLP-1. Some of these new approaches include stimulating endogenous GLP-1 release, producing longer-acting GLP-1 analogues and investigating alternative administration routes. To stimulate endogenous GLP-1 release, the use of α-glucosidase inhibitors and prostaglandin E2 agonists have been proposed due to their ability to increase nutrient contact with the L-cells by delaying gastric emptying or propelling nutrients to the distal gut respectively [121, 122]. In addition, studies in rats have shown that monosaturated fatty acids and fibre consumption increase endogenous GLP-1 secretion [60,123]. Generation of longer-acting GLP-1 analogues has been accomplished through modifications of the GLP-1 structure to make them resistant to DPIV degradation. These modifications include substitution of N-terminal sequences, acylation to fatty acid chain or conjugation to reactive chemistry groups that bind to albumin in vivo [124, 125].

Recently, the success of long-acting GLP-1 analogues has been realized with the introduction of Byetta™ into the U.S. market for prescription as an adjunctive therapy for T2D (Amylin Pharmaceuticals, Inc./Eli Lilly & Co, San Diego, California and Indianapolis, Indiana, USA; [45]). Byetta™ is the trade name for Exenatide, a synthetic form of naturally derived exendin-4 from the salivary glands of the lizard Glia monster (Heloderma suspectum) [45]. Exendin-4 is a 39 amino acid peptide that shares ~52% sequence homology with native GLP-1 [125,126]. Furthermore, it is resistant to DPIV degradation due to the presence of a penultimate N-terminal glycine instead of
alanine found in native GLP-1 [125, 126]. While the introduction of Byetta™ is a big step towards bringing GLP-1 to the forefront as an adjunct treatment for T2D, it requires daily subcutaneous administration for a therapeutic effect [45]. This reflects one of the biggest challenges of using GLP-1 as a new drug for diabetes therapy. Since GLP-1 is a peptide, it is not orally available and has to be injected into the body [124, 125]. Therefore, alternative administration routes that can simplify GLP-1 delivery into the body and, most importantly, reduce the requirement for daily treatment will be important in increasing the appeal of GLP-1 therapy for diabetes. Amongst the proposed approaches, such as buccal, pulmonary, nasal, transdermal and gene therapy routes [15, 17, 124, 125], gene therapy may offer the most potential in reducing the strict daily regimen of current diabetes treatment.

**Gene Therapy Strategies**

The most important goal of diabetes therapy is to maintain normal blood glucose levels by linking blood glucose-sensing mechanisms with the rapid release of sufficient amounts of glucose-lowering factors. Unfortunately, current drug treatments, such as insulin injections or oral anti-diabetic medications, fall short of meeting this goal. However, the rapid development in molecular genetics has led to the birth of gene therapy, a promising technology that may address the shortcomings of current diabetes treatments.

The attractive feature of gene therapy is its ability to achieve automatic endogenous release of therapeutic proteins from genetically engineered cells within the body. Gene therapy involves targeting specific cells in the body with a therapeutic gene that can alter gene expression or replace defective genes [127-130]. Currently, *ex vivo* and *in vivo* methods are used for introducing vectors containing the gene of interest into the body. For both approaches, a vector containing a desired gene is delivered into a surrogate cell such that it can express, store and produce the resulting therapeutic protein in a regulated manner.
Ex vivo approaches involve isolating the target cells from the patient. These cells are subsequently cultured, transduced with a therapeutic gene and transplanted into the patient [127-130]. Several strategies have been employed for transplanting cells into the body. One strategy involves encapsulating cells prior to transplantation into the peritoneal cavity. Cell encapsulation involves the use of semipermeable microcapsules to protect transplanted cells from the body’s immune system by acting as a barrier to immune cells and antibodies [131]. However, at the same time, the microcapsules must permit passage of nutrients, oxygen, cellular products and wastes between the transplanted cells and the surrounding environment [131]. Another strategy involves transplanting cells under the kidney capsule. The kidney capsule is a thin layer covering the outside surface of the kidney, a highly vascularized organ [132]. The presence of good vasculature has been reported to be important for survival of transplanted cells [133]. Furthermore, the close proximity of the transplanted cells to the circulation has been shown to permit more effective transport of secreted products from the cells into the bloodstream [134, 135]. Despite the invasive procedures involved with ex vivo gene delivery, this approach permits targeting of specific tissues with the therapeutic gene thereby avoiding systemic toxicity associated with widespread dissemination of vectors that could occur with in vivo administration [136].

In vivo gene delivery involves administering vectors directly into the circulation through various routes, such as intravenous, subcutaneous or intrabronchial injections, or into target tissues by local injections [127-130]. Given the simplicity and convenience of this method, the in vivo approach is an attractive method of delivery for gene therapy. However, as mentioned earlier, a limiting factor with this approach is the concern with systemic toxicity and immune reaction against the administered vector [136].

Both ex vivo and in vivo gene delivery rely on a vector to deliver the gene of interest into the body. There are two main types of vectors used in gene therapy research: non-viral and viral vectors. Non-viral vectors utilize a recombinant DNA plasmid that can be delivered alone by mechanical administration or in combination with chemical carriers such as liposomes and biodegradable
polymers. An important feature of non-viral system is its association with low toxicity due to a reduced immunogenic profile. However, non-viral vectors are limited by low transfection efficiency, transient gene expression and the ability to deliver only small nucleotide sequences [128, 130, 137]. Despite its higher immunogenicity compared to non-viral vectors, viral vectors are the most commonly used delivery system in gene therapy due to the ability to deliver genes with high efficiency. In order to generate viral vectors, viruses are genetically modified to become replication-deficient but still retain the ability to infect cells. To achieve this, parts of the coding regions from the viral genome are deleted and replaced with the gene of interest. The deleted regions of the viral genome are involved in replication or generation of viral capsid and/or envelope proteins. In order to produce the recombinant virus, so-called packaging cells containing the deleted viral genes in trans are transfected with the recombinant viral genome. The resulting viral vectors are harvested from the infected cells and purified. There are 4 main classes of viral vectors that have been used extensively for gene therapy research, including retroviral, adeno-associated viral, herpes simplex viral and adenoviral vectors. Of these different viral vectors, adenoviral vectors have been the most widely used for many proof-of-concept experiments [138, 139].

Adenoviral Vectors

Adenoviruses are non-enveloped viruses consisting of a linear, double-stranded DNA genome (36 kb) enclosed in an icosahedral-shaped capsid. The capsid is made up of proteins (hexon, penton base and fiber) important for providing viral structure and mediating virus-cell interactions. To date, 51 different serotypes of adenoviruses have been identified. Many studies focused on adenovirus serotypes 2 and 5 for generating adenoviral vectors for gene delivery because they have not been associated with severe diseases in humans [138, 140, 141]. Depending on the portion of gene region that is deleted from the viral genome, there are three main types of replication-deficient adenoviral vectors: (i) first generation, (ii) second generation and (iii) gutless adenoviral vectors. First generation vectors involve the deletion of the early region 1 (E1) and/or E3 genes. The E1 genes are
important for initiating genome replication whereas E3 genes are believed to be involved in providing protection of the vector or transduced cells from the immune system. Second generation vectors involve additional deletion of the E2 and/or E4 genes that are required, in combination with E1 genes, for viral genome replication. Gutless adenoviral vectors are completely devoid of all viral regions involved in encoding adenoviral proteins [138, 140].

Adenoviral vectors are an attractive vector for gene therapy due to a high transduction efficiency into both quiescent and proliferating cells [128, 130, 137, 140]. This results in a rapid and high level of transgene expression in different cell types. Numerous animal studies have demonstrated effective gene transfer by adenoviral vectors in many tissues including liver, lung, muscle, pancreas, arteries, joints, brain and tumors [138, 142]. Other attractive features of the adenoviral vector include the ability to generate these vectors in high titers (10^{11} to 10^{12} pfu/ml) and the ability of these vectors to accommodate transgenes up to 30 kb in size [128, 130, 137, 140]. In 1995, the first clinical application of adenoviral vector was carried out in patients with cystic fibrosis [143]. To date, there are approximately 260 clinical studies involving adenoviral vectors of which 5 are in phase III for cancer applications [144].

One of the drawbacks of adenoviral vectors for widespread applications is the lack of stable transgene expression due to the inability of these vectors to integrate into the host genome. The duration of transgene expression is often further attenuated by a strong immune and inflammatory response. As a result, the transduced cells are destroyed leading to loss of transgene expression. Furthermore, the production of antibodies against the adenoviral vector may prevent its re-administration into the body [130, 140, 145]. The activation of the immune system can also lead to serious consequences as demonstrated by a 1999 clinical gene therapy trial for ornithine transcarbamylase deficiency [139]. In this study, a high dose of adenoviral vector induced a massive immune response leading to the death of a patient.

There are other viral vectors which are integrative and less immunogenic, such as retroviral and adeno-associated viral vectors, that may be more suitable for treating a chronic disease such as
diabetes. However, for proof-of-concept experiments, adenoviral vectors are ideal due to the ability to generate high titers, induce high levels of gene expression and infect different cell types. Furthermore, the safety and efficacy of these vectors can be improved by targeting adenoviral vectors to deliver and express genes in specific tissues [141].

Liver as a Target for Gene Therapy in Diabetes

The liver has been regarded as an attractive target for gene therapy in diabetes for several reasons. First, the liver is highly vascularized and therefore easily accessible after in vivo delivery of viral vectors. Second, the liver has the ability to rapidly regenerate to its normal mass following removal of its cells – an ideal characteristic for ex vivo gene delivery [146]. Finally, the liver contains glucose-responsive genes such as glucose transporter 2 (GLUT2), glucokinase and liver-type pyruvate kinase (LPK) [147, 148]. Therefore, many investigators have examined the feasibility of genetically engineering the liver as a surrogate organ for insulin production. However, one of the main limitations of using the liver for insulin production is the absence of secretory granules. As a result, insulin secretion from the liver is not regulated to the degree that occurs in β-cells. In an attempt to address this problem, hepatocyte promoters involved in regulating expression of glucose-responsive genes have been exploited for driving insulin expression.

One liver-specific promoter that has been extensively used in studies involving regulated insulin gene expression in the liver is the LPK promoter. The LPK promoter is involved in stimulating the expression of the LPK gene in response to glucose and insulin but inhibiting expression in response to glucagon. Specifically, a 183 bp region of the LPK promoter (-183 to +11 bp relative to the LPK gene cap site) has been shown to contain the regulatory DNA elements involved in conferring liver specificity and glucose sensitivity [149]. However, the LPK promoter is relatively weak in driving gene expression since transgenic mice expressing hepatic proinsulin driven off the LPK promoter were not protected from streptozotocin (STZ)-induced diabetes [147]. When a simian virus (SV) 40 enhancer was coupled with the LPK promoter to enhance expression, the resulting hepatic insulin
expression was sufficient to achieve remission of STZ-induced diabetes in rats [150]. A limitation in using transcriptional regulation of hormone secretion is the lag time in turning secretion on and off in response to glucose as compared to the rapid response of the regulated secretory pathway in the β-cells. For example, an increase in insulin after an oral glucose challenge was observed in normal rats after 30 minutes compared to 2 hours in STZ-induced diabetic rats expressing hepatic insulin under the control of the LPK promoter [150]. This type of insulin release kinetics from hepatocytes is undesirable for clinical use due to the lack of tight coupling between glucose stimulation and insulin secretion and the potential risk of hypoglycemia. However, this type of release kinetics may be suitable for GLP-1 secretion due to its dependence on glucose for exerting its insulinotropic actions.

**Prospect of GLP-1 Gene Therapy for Diabetes**

In healthy individuals, the coordinated release of regulatory hormones allows blood glucose to be maintained within a narrow range throughout the day. This tight regulation of blood glucose levels is absent in people with T2D due to an inability to effectively dispose of glucose absorbed by the gut during meals. Therefore, an ideal gene therapy for diabetes would achieve basal secretion of a glucose-lowering factor that changes in response to glucose levels during meals. Due to its dependence on elevated glucose levels for biological effects, GLP-1 is an ideal peptide to deliver by gene therapy in T2D. Previous studies have shown that sustained production of GLP-1 may be possible by *ex vivo* gene transfer of this peptide into surrogate cells. For example, Burcelin *et al.* [151] genetically engineered C2C12 murine myoblasts to produce GLP-1 that were subsequently encapsulated in semi-permeable hollow fibers. When these encapsulated cells were implanted into mice given a high fat diet, improved glucose tolerance was observed. However, this approach has limited clinical applications due to the potential risk of uncontrolled cell growth and the absence of regulated GLP-1 secretion in response to glycemic requirements. In another study, *in vivo* delivery of GLP-1 using a plasmid-polyethyleneimine polymer complex was successful in decreasing blood glucose levels in a rat model of T2D. However, similar to the Burcelin study, GLP-1 production was
not glucose-dependent [152]. The objective of this thesis was to explore the possibility of using ex \textit{vivo} and \textit{in vivo} gene delivery methods to engineer liver cells with adenoviral vectors to produce therapeutic levels of GLP-1 within the body for the treatment of T2D.
THESIS INVESTIGATION

Approximately 90% of people diagnosed with diabetes have T2D and its prevalence continues to increase at a rapid rate worldwide. Given that current treatments do not adequately address the underlying metabolic abnormalities of T2D, there is a great impetus towards a search for better therapeutic options. One potential candidate is GLP-1, a gut hormone released from the L-cells of the distal intestine following nutrient ingestion. Studies have shown that exogenous administration of GLP-1 to patients with T2D results in normalization of blood glucose levels. However, a drawback that limits the clinical use of GLP-1 is its short biological half-life due to, in part, its degradation by DPIV. Therefore, the goal of this thesis is to examine the possibility of using gene therapy as an approach for achieving long-term endogenous production of GLP-1.

The hypothesis of this research is that GLP-1 production can be induced in liver by ex vivo and in vivo gene delivery of adenoviral vectors expressing a GLP-1 gene to treat diabetes. To address this hypothesis, 4 specific aims were examined in this thesis. First, experiments were conducted to determine the effectiveness of an adenoviral vector expressing a DPIV resistant GLP-1 gene in transducing hepatocytes to produce GLP-1 in vitro. Second, a GLP-1 bioassay was developed and characterized to measure biologically active GLP-1 released from genetically engineered hepatocytes. Third, the effectiveness of ex vivo and in vivo AdLPK-GLP-1 gene delivery in increasing plasma GLP-1 levels in normal rodents was examined. Finally, the effect of ex vivo AdLPK-GLP-1 gene delivery on glucose homeostasis in a mouse model of T2D was evaluated.
MATERIALS AND METHODS

IN VITRO STUDIES

Cell lines

Cell lines described herein were grown in media (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin G (Invitrogen) and 100 μg/mL streptomycin (Invitrogen) at 37°C in a 5% CO₂ atmosphere. Any additional growth media supplements will be outlined below for the specific cell line. Unless otherwise specified, all cell lines were obtained from American Type Culture Collection with the corresponding catalogue number indicated in brackets (Manassas, Virginia, USA).

Construction of a Recombinant Adenovirus Expressing GLP-1

GLP-1 transgene

In order to confer resistance to DPIV degradation, a human GLP-1_{7,37} oligonucleotide was synthesized to contain a substitution at the 8th amino acid sequence (A → G) ([Gly8]-GLP-1; Table I). This GLP-1 sequence was also synthesized to contain an upstream human GIP signal peptide sequence (21 amino acid; Table I; Fig 2). The resulting 192 bp fragment was amplified by PCR (forward primer: AGG CGC GCA AGC TTG GAA GAT and reverse primer: ACG TAC GTG GGC CCT TAT CCT) and cloned into pGeneB’/V5-His vector (Invitrogen) at HindIII and Apal sites producing pGeneB’/GLP-1 (Fig. 2). To allow GLP-1 secretion from the cells, a rat albumin secretory signal peptide (Table I) was inserted upstream to GLP-1. Furin is a ubiquitous endoprotease that functions in the constitutive secretory pathway. Therefore, a furin cleavage site (Table I) was added between the signal sequence and the first residue of GLP-1 so that the signal peptide could be removed prior to secretion of GLP-1. Oligonucleotides for the signal peptide and furin cleavage site
Figure 2. Cloning strategy for constructing the pGeneB'/FurGLP-1 plasmid. A dipeptidyl resistant glucagon-like peptide-1 sequence ([Gly8]-GLP-1) and a glucose-dependent insulinotropic peptide signal sequence (GIPSS) were ligated into pGeneB'/V5-His vector to produce pGeneB'/GLP-1. The GIPSS was replaced with a sequence containing a rat albumin secretory signal peptide (Alb) and a furin cleavage site (F) to produce pGeneB'/FurGLP-1. All plasmids confer resistance to both ampicillin and zeocin. Texts in *italics* are restriction enzymes used in this cloning strategy.
were synthesized and inserted into the *HindIII*/*PpuMI*-digested fragment of pGeneB'/GLP-1 to generate pGeneB'/FurGLP-1 (Fig. 2).

**Table I: Amino acid sequences used in constructing pGeneB'/FurGLP-1.**

<table>
<thead>
<tr>
<th>Peptides or Cleavage Site</th>
<th>Amino Acid Sequences</th>
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<tr>
<td><strong>GLP-1</strong>7,37</td>
<td>HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG</td>
</tr>
<tr>
<td>[Gly8]-GLP-1</td>
<td>HGEFTFTSDVSSYLEGQAAKEFIAWLVKGRG</td>
</tr>
<tr>
<td>GIP signal peptide</td>
<td>MVATKTFALLLSLFLAVGLG</td>
</tr>
<tr>
<td>Albumin secretory signal peptide</td>
<td>MKWVTFLLLLFSGSFSAFS</td>
</tr>
<tr>
<td>Furin cleavage site</td>
<td>RARYKR</td>
</tr>
</tbody>
</table>

**GLP-1**7,37 was not used in constructing pGeneB'/V5-HisFurGLP-1. It only serves as a comparison for [Gly8]-GLP-1.

A 183 bp fragment spanning the -183 to +11 nucleotide relative to the cap site of the rat LPK promoter was obtained by *SalI*/*XhoI* digest of 183PK/CAT (generously provided by Dr. Bruno Doiron; Fig. 3; [153]). The resulting fragment (183LPK) contains the L1/L3 and L4 sequences which have been shown to confer liver specificity and glucose sensitivity, respectively (Fig. 3; [149, 154, 155]). This fragment was subsequently inserted into *SalI*/*XhoI*-digested fragment of pSSBS, a pBluescriptII plasmid in which the *XbaI* site was converted to a second *SalI* site (generously provided by Dr. Paul Orban, Biomedical Research Centre, Vancouver, BC, Canada), to produce pSSBS-LPK (Fig. 3). To remove the *NotI* site from pSSBS-LPK, the *NotI* site was digested, filled in and re-ligated to produce pSSBS-LPK-Δ*NotI*. To increase mRNA stability of the construct, a SV40 splice site and poly A signal was added downstream to the LPK promoter. This was accomplished by digesting a pCMVΔβ vector (Clontech, Mississauga, Ontario, Canada) with *SalI*/*XhoI* and ligating the fragment containing the SV40 splice site and poly A signal into *XhoI*/dephosphorylated-digested fragment of pSSBS-LPK-Δ*NotI* to produce pSSBS-LPK-SV (Fig. 3). In order to enhance expression levels by several fold, a SV40 enhancer was introduced upstream to the LPK promoter by blunt ligation of *EcoRV*-digested pSSBS-LPK-SV to *PvuII*/NcoI-digested fragment of pRL-SV40 vector (Promega, Madison, Michigan, USA) to produce pSSBS-LPK-SVE (Fig. 3).
Figure 3. Cloning strategy for constructing the pSSBS-LPK-SVE plasmid. A 183 bp liver-type pyruvate kinase promoter (183LPK) from 183PK/CAT plasmid was ligated into pSSBS plasmid to produce pSSBS-LPK. A simian virus 40 (SV40) poly A (SV40PolyA) and SV40 splice site (SV40SS) from pCMVΔβ vector was ligated into pSSBS-LPK plasmid to produce pSSBS-LPK-SV. A SV40 enhancer (SVenh) from pRL-SV40 plasmid was blunt ligated into pSSBS-LPK-SV plasmid to produce pSSBS-LPK-SVE. All plasmids confer resistance to ampicillin. Texts in italics indicate restriction enzymes used in this cloning strategy.
To construct the complete GLP-1 transgene, a NotI-digested fragment of the LPK promoter construct (pSSBS-LPK-SVE) was blunt ligated to the HindIII/Pmel-digested fragment of the GLP-1 construct (pGeneB'/FurGLP-1) to produce pSSBS-LPK-SVE-FurGLP-1 (Fig. 4).

Recombinant adenoviral vector expressing GLP-1

A recombinant type 5 adenoviral vector (Ad5) expressing GLP-1 was constructed using the Adeno-X™ Expression System (Clontech) according to the manufacturer’s instruction. Briefly, pSSBS-LPK-SVE-FurGLP-1 (GLP-1 transgene) was digested with SpeI/PvuII and blunt ligated into pShuttle (SpeI/Pmel) to produce pShuttle-LPK-SVE-FurGLP-1 (Fig. 5). The GLP-1 transgene was removed from the pShuttle-LPK-SVE-FurGLP-1 with I-Ceu-I/PI-Scel (Fig. 5). This linearized fragment was subsequently ligated into I-Ceu-I/PI-Scel-digested pAdeno-X™ Viral DNA and the resulting recombinant adenoviral DNA was transformed into STBL2 E. coli cells (Stratagene, La Jolla, California, USA). Ampicillin-resistant transformants were selected to obtain AdLPK-GLP-1 plasmid (pAdLPK-GLP-1) (Fig. 5).

HEK 293, a human embryonic kidney cell line transformed to stably express Ad5 E1 genes (CRL-1573), was cultured in minimum essential medium (MEM) and used for packaging and propagating recombinant adenoviruses. Recombinant adenoviruses were produced by transfecting pAdLPK-GLP-1 into HEK 293 cells using standard methods for Lipofectamine™ and DNA Plus Reagents™ (Invitrogen). After transfection, cells were overlaid with MEM supplemented with 10% FBS and 1.25% SeaPlaque agarose (Mandel Scientific, Guelph, Ontario, Canada). Plaques were picked at approximately 10 days after transfection and propagated successively from 12-well tissue culture plates to T75 tissue culture flasks containing HEK 293 cells.

Propagation and purification of recombinant adenoviruses

To propagate AdLPK-GLP-1, HEK 293 cells were grown in T75 flasks and infected with the recombinant adenovirus. Once complete cytopathic effect was observed (3 to 4 days after infection), cells were harvested followed by 3 freeze/thaw cycles to lyse the cells and release the recombinant
Figure 4. Cloning strategy for constructing the pSSBS-LPK-SVE-FurGLP-1 plasmid containing the GLP-1 transgene. The GLP-1 transgene consists of a dipeptidyl peptidase resistant glucagon-like peptide-1 ([Gly8]-GLP-1) placed downstream of a rat albumin secretory signal peptide (Alb). A cleavage site for furin (F), a ubiquitous protease, was placed in between the secretory signal peptide and GLP-1 to permit removal of the signal peptide prior to GLP-1 secretion. To regulate GLP-1 expression, a 183 bp liver-type pyruvate kinase promoter (183LPK) and a simian virus 40 (SV40) enhancer (SVenh) was placed upstream to GLP-1. SV40 splice site (SV40SS) and poly A signal (SV40PolyA) was added to enhance mRNA stability. All plasmids confer resistance to ampicillin and/or zeocin. Texts in italics indicate restriction enzymes used in this cloning strategy.
Figure 5. Cloning strategy for constructing the pAdLPK-GLP-1 plasmid. The GLP-1 transgene was cloned into the pShuttle vector and the resulting plasmid (pShuttle-LPK-SVE-FurGLP-1) was amplified in *E. coli* under kanamycin selection. After amplification, GLP-1 transgene was removed and ligated into Adeno-X Viral DNA, a replication incompetent human adenoviral type 5 (Ad5) genome, to produce pAdLPK-GLP-1. The pAdLPK-GLP-1 plasmid confers resistance to ampicillin. Texts in *italics* are restriction enzymes used in this cloning strategy.
adenovirus. The resulting cell suspension was centrifuged at 2000 rcf (relative centrifugal force) for 5 minutes and cell lysates were purified using Adenopure Adenovirus Purification Kit (Puresyn, Malvern, Pennsylvania) according to the manufacturer's instructions. The resulting purified AdLPK-GLP-1 was sent to ViraQuest Inc. for generation of high-titer stocks.

**In Vitro Transduction of Cell Lines With AdLPK-GLP-1**

Six cell lines were used for in vitro AdLPK-GLP-1 transduction experiments: (i) WRL 68, a fetal human hepatic cell line (CL-48), (ii) Huh7, a human hepatoma cell line (generously provided by Dr. Chris Richardson, University of Toronto, Canada), (iii) HEPA 1-6, a mouse (C57/L) hepatoma cell line (CRL-1830), (iv) IEC-6, a rat intestinal cell line, (v) INS-1, a rat insulin-secreting cell line (generously provided by Dr. Claes Wolheim, University of Geneva, Switzerland) and (vi) 3T3-L1, an embryonic mouse fibroblast cell line (CL-173). WRL 68 cells were grown in MEM while INS-1 cells were grown in RPMI 1640 supplemented with 50 μM β-mercaptoethanol (Sigma-Aldrich Canada Inc., Oakville, Ontario, Canada). Huh7, HEPA 1-6, 3T3-L1 and IEC-6 cells were grown in high glucose (25 mM) Dulbecco's modified Eagle medium (HG-DMEM). Human insulin (0.1 U/mL; Novolin, Novo Nordisk Canada Inc., Mississauga, Ontario, Canada) was added into the growth media of IEC-6 cells.

Cells were seeded into 6-well (5x10⁵ cells/well) tissue culture plates and placed in a 37°C incubator. After 2 hours, cells were left uninfected or were infected with virus (AdLPK-GLP-1 or AdCMV-βGal, a recombinant Ad5 expressing β-galactosidase (βGal) under the control of the cytomegalovirus (CMV) promoter [kindly provided by Dr. Jay K. Kolls, Louisiana State University Medical Center, New Orleans, Los Angeles, USA] [156]) for 2 hours at 37°C. Each condition was performed in triplicate and the average number of virus particles to cell ratio was expressed as a multiplicity of infection (MOI). Infection was performed by replacing culture medium with 300 μL of infection medium, consisting of the necessary volume of virus and MEM supplemented with 2% FBS. For uninfected cells, culture medium was replaced with 300 μL of MEM supplemented with
2% FBS. Following the infection period, medium was aspirated and replaced with 2 mL fresh growth medium. To examine the duration of GLP-1 production, culture medium was removed from each well at different days post infection. To ensure that the harvested medium represented an accumulation of protein over a 24 hour period, culture medium was replaced with fresh media every day. For all other experiments, medium was removed on the third day after infection. The harvested medium samples were transferred to microcentrifuge tubes and centrifuged at 21,000 rcf for 1 minute. The resulting supernatant was transferred into microcentrifuge tubes and stored at -80°C for subsequent GLP-1 assay.

To investigate the effects of glucose on GLP-1 production from transduced cells, 100 mm diameter tissue culture plates were seeded with $1.5 \times 10^6$ cells and placed in a 37°C incubator. Some cell lines are normally cultured in high glucose medium and acute exposure to low glucose medium could lead to reduced cell growth. As a result, cells were first equilibrated to culture in low glucose (5.5 mM) growth medium for approximately 1 week prior to experimentation. This was to ascertain that changes in GLP-1 production were more likely due to differences in glucose concentration in the medium and not due to changes in cell growth. Infection was performed as described above with the exception that 900 µL of infection medium was used to replace the culture medium. On the following day, cells were trypsinized and transferred into 6-well tissue culture plates in low glucose (2 mM) or high glucose (20 mM) growth medium for each condition. At three days post infection, culture medium was removed from each well and stored in -80°C for subsequent GLP-1 assay.

The medium samples from uninfected and infected cells were assayed for total immunoreactive GLP-1 using a GLP-1 (total) radioimmunoassay (RIA) kit (GLP1T-36HK; sensitivity ~3 pM, 100% cross-reactivity with GLP-17-36 amide, GLP-17-37 and GLP-19-36 amide; Linco Research Inc., St. Charles, MO, USA) according to the manufacturer’s instructions.
**β-galactosidase staining of liver cells**

To examine the susceptibility of different liver cell lines to adenovirus infection, β-galactosidase staining for AdCMV-βGal infected cells was performed. Cells were seeded into 4-well chamber slides (1.25x10⁵ cells/well, Lab-Tek® ChamberSlide™ System, Nalge Nunc International, Rochester, New York, USA) and placed in a 37°C incubator. After 6 hours, cells were infected with AdCMV-βGal (MOI 50). Three days after infection, cells were fixed using 0.2% glutaraldehyde (Sigma-Aldrich Canada Inc.) in phosphate buffered saline (PBS; Invitrogen) without CaCl₂ (PBS⁻) for 5 minutes at room temperature. After fixing, cells were washed with PBS⁻ and incubated in 5-bromo-4-chloro-3-indoly1-beta-D-galactopyranoside (X-Gal) solution (1 mg/mL X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂; all from Sigma-Aldrich Canada Inc.) for 1 to 2 hours at 37°C. Once the desired stain intensity was obtained, cells were washed with PBS⁻, counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, California, USA) and mounted with coverslips. Images of the cells were captured under 100x magnification using an Axiovert 200 inverted microscope (Carl Zeiss Canada Ltd., Port Moody, British Columbia, Canada) connected to a camera (Retiga 2000R, Qimaging, Burnaby, British Columbia, Canada) with a colour monitor and imaging software (Openlab, Improvision Include, Lexington, Massachusetts, USA).

**Development of a GLP-1 Bioassay**

The disadvantage of using the Linco GLP-1 (total) RIA for measuring GLP-1 from transduced cells is that it is unable to distinguish between biologically active and inactive forms of GLP-1. While a GLP-1 enzyme linked immunosorbent assay (ELISA) that detects active forms of GLP-1 is commercially available, it is important to recognize that immunoreactivity to an active form of GLP-1 does not necessarily indicate bioactivity. As a result, a GLP-1 bioassay was developed for determining the levels of bioactive GLP-1. To develop a GLP-1 bioassay, the inherent ability of the activated GLP-1 receptor to, in turn, activate the cAMP response element (CRE) was exploited. HEK 293 cells stably expressing the human GLP-1 receptor (HEK-hGLP1R; generously provided by Dr. [Name] at [Institution]) were transfected with a reporter plasmid containing a CRE-luciferase construct. The cells were then incubated with increasing concentrations of GLP-1 and the luciferase activity was measured. A dose-response curve was generated to determine the EC₅₀ of GLP-1. This bioassay provides a more accurate measure of bioactive GLP-1 levels compared to the RIA methods.
Jesper Gromada, Novo Nordisk A/S, Denmark) were stably transfected with a plasmid expressing the luciferase gene (Luc) driven by the CRE, which is activated as a result of signalling cascades downstream of GLP-1 receptor activation. In these stably transfected cells, CRE activation would result in the expression of the luciferase gene that, in turn, could be quantified by measuring luminescence emitted from the cells. Therefore, the amount of luminescence would be proportional to the concentration of biologically active GLP-1 in the sample.

**Determination of N-terminally modified forms of bioactive GLP-1 using Linco GLP-1 ELISA**

The Linco GLP-1 (active) ELISA kit was designed to detect all biologically active forms of GLP-1 (EGLP-35K; sensitivity ~2 pM, 100% cross-reactivity with GLP-17-36 amide and GLP-17-37). Since the GLP-1 sequence in AdLPK-GLP-1 was modified by a N-terminal substitution with glycine in the penultimate amino acid position, the suitability of Linco ELISA for detecting GLP-1 produced from AdLPK-GLP-1 transduced cells was examined.

To perform this experiment, medium samples from transduced WRL 68 and HEPA 1-6 cells were assayed according to the manufacturer’s instructions. To further examine the ability of GLP-1 ELISA in measuring bioactive GLP-1 from AdLPK-GLP-1 transduced cells, a parallel experiment was performed using different concentrations (0.975 to 500 pM) of two synthetic GLP-1 peptides containing either a glycine substitution ([Gly8]-GLP-17-36; generous gift from Dr. Bernard Thorens, Institute of Pharmacology and Toxicology, Lausanne, Switzerland) or a serine substitution ([Ser8]-GLP-17-36 amide; American Peptide Company Inc., Sunnyvale, California, USA). As a positive control, the same concentrations of GLP-17-36 amide (American Peptide Company Inc.) were also used in the ELISA. To prepare the different concentrations of different forms of bioactive GLP-1s, the peptides were reconstituted in sterile saline (100 µM) and subsequently diluted in HG-DMEM supplemented with 1% bovine serum albumin (BSA; Invitrogen).
Transformation and purification of pHTS-CRE plasmid

A pHTS-CRE plasmid (Biomyx, San Diego, California, USA) containing the luciferase gene driven by a CRE was amplified by transformation into DH5α chemically competent *E. coli* cells (Invitrogen) according to the manufacturer’s instructions (Fig. 6). The resulting transformation mixture was spread onto agar plates containing ampicillin (Sigma-Aldrich Canada Inc.) and incubated at 37°C. The following day, ampicillin-resistant colonies were selected and transferred into polypropylene tubes containing 3 mL LB (Luria Bertani; Sigma-Aldrich Canada Inc.) medium supplemented with 50 µg/mL ampicillin for incubation at 37°C overnight. Initial isolation and purification of the pHTS-CRE plasmid from the bacterial culture was performed using the QIAprep Spin Miniprep Kit (QIAGEN, Mississauga, Ontario, Canada). After confirmation of the correct plasmid by restriction enzyme digest (*HindIII/EcoR*V) and gel electrophoresis, large-scale purification of the pHTS-CRE plasmid was performed using the EndoFree Plasmid Maxi Kit (QIAGEN) according to the manufacturer’s instructions (Fig. 6). The DNA concentration for the purified pHTS-CRE plasmid was 1830 µg/mL.

Transfection and selection of HEK-hGLP1R cells with pHTS-CRE plasmid

HEK-hGLP1R cells were seeded at 2x10⁶ cells into 100 mm diameter tissue culture plates and incubated at 37°C. The next day, cells were transfected with pHTS-CRE plasmid (16 µg/mL) using Lipofectamine™ 2000 Transfection Reagent (Invitrogen) according to the manufacturer’s instructions (Fig. 6). Twenty-four hours after transfection, the cells were trypsinized and seeded into 100 mm diameter tissue culture plates at different dilutions varying from 1:10 to 1:100. Culture medium was replaced on the following day with selection medium containing 800 µg/mL each of hygromycin and G418 to select for HEK 293 cells stably expressing human GLP-1 receptor and luciferase gene (HEK-hGLP1R-Luc). After 1 month of selection, HEK-hGLP1R-Luc clones were selected using cloning discs (Scienceware, Pequannock, New Jersey, USA) and transferred to 24-well tissue culture plates (Fig. 6). HEK-hGLP1R-Luc clones were successively expanded in selection medium until the cells
Figure 6. Schematic representation of the steps performed to generate stably transfected HEK-hGLP1R cells expressing the luciferase gene (HEK-hGLP1R-Luc). A plasmid containing the luciferase gene driven by the cAMP response element (pHTS-CRE) was transformed into E. coli cells. After selection and purification, the purified pHTS-CRE was transfected into HEK 293 cells expressing GLP-1 receptor (HEK-hGLP1R). HEK-hGLP1R-Luc cells were subsequently selected from cells grown in selection medium for approximately 1 month.
were transferred into 100 mm diameter tissue culture plates after which they were screened for use in the GLP-1 bioassay.

**GLP-1 receptor binding assay**

To confirm GLP-1 binding in the stably transfected HEK-hGLP1R-Luc clones, a GLP-1 receptor binding assay was performed as described previously [157]. Briefly, 1x10^6 cells were resuspended in 800 μL binding buffer (138 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl₂, 2.6 mM CaCl₂, 10 mM HEPES, 1% protease-free BSA, 10 mM glucose [all from Sigma-Aldrich Canada Inc.] and 1% Trasylol [Bayer Inc., Toronto, Ontario, Canada]) and transferred into microcentrifuge tubes. Cells were incubated with 100 μL of [125I]GLP-1₇₋₃₆ amide (~30,000 counts per minute [cpm]; Linco) in the presence or absence of 100 μL of 100 μM GLP-1₇₋₃₆ amide for 30 minutes at room temperature. Following the 30 minute incubation, cells were centrifuged at maximum speed for 2 minutes and the supernatant was aspirated. After washing the cells twice in ice-cold binding buffer, the cell pellet was solubilized in 0.1 M NaOH (Sigma Aldrich Canada Inc.) and transferred to borosilicate tubes for measuring [125I] radioactivity using a 1277 Wallac GammaMaster gamma counter (LKB Wallac, Uppsala, Sweden).

**GLP-1 bioassay**

HEK-hGLP1R-Luc cells were seeded into a 96-well (5x10^4 cells/well) white opaque tissue culture plate and placed in a 37°C incubator. The next day, medium was aspirated and replaced with 100 μL of GLP-1 standards or medium samples from transduced cells. For the initial screening experiment, 100 μM forskolin was added to some cells as a positive control. To make up GLP-1 standards, GLP-1₇₋₃₆ amide was reconstituted in sterile saline to a concentration of 100 μM. This concentrated stock was subsequently diluted in medium to generate GLP-1 standards ranging in concentration from 0.975 to 500 pM. To assess the cross-reactivity of the GLP-1 bioassay, 100 μL of 500 pM glucagon or GIP was incubated with HEK-hGLP1R-Luc cells. After incubation of standards and samples for 5 hours at 37°C, luciferase assay was performed using the Bright-Glo Luciferase Assay Kit (Promega) and, unless otherwise specified, according to the manufacturer’s instructions. Briefly, cells were
equilibrated at room temperature for 15 minutes prior to the addition of 100 μL of Bright-Glo Luciferase Reagent to each well. After a 5 minute incubation at room temperature, the luminescence (expressed as relative light units [RLU]) was measured using LMax II Microplate Reader (Molecular Devices, Sunnyvale, California, USA).

For optimization experiments, modifications to 4 different parameters of the protocol were examined including (i) different incubation times with GLP-1 (3, 5 or 7 hours), (ii) GLP-1 standards made up in HG-DMEM supplemented with either 10% FBS or 1% BSA, (iii) different number of cells seeded per well (2.5\times10^4, 5\times10^4 or 1\times10^5 cells/well) and (iv) GLP-1 standards made up using freshly or previously reconstituted GLP-1\textsubscript{7-36} amide.

To compare the effects of phenol red and different medium types on the sensitivity of the GLP-1 bioassay, the 96-well plate was inverted after the 5 hour incubation period in order to remove medium from the wells. To each well, 100 μL of PBS or medium and 100 μL of Bright-Glo Luciferase Reagent was added and the resulting luminescence was measured.

To examine the effects of plasma on GLP-1 bioassay, undiluted or diluted charcoal extracted plasma samples in HG-DMEM supplemented with 1% BSA were spiked with different concentrations of GLP-1. Luciferase assay was subsequently performed as outlined above.

To examine the ability of the GLP-1 bioassay to detect N-terminally modified forms of bioactive GLP-1, different concentrations (0.975 to 500 pM) of [Gly8]-GLP-1\textsubscript{7-37} and [Ser8]-GLP-1\textsubscript{7-36} amide were incubated with HEK-hGLP1R-Luc cells. As a comparison, the same concentrations of GLP-1\textsubscript{7-36} amide were also incubated with HEK-hGLP1R-Luc cells. The GLP-1 samples were prepared as described previously for the Linco GLP-1 ELISA. After 5 hour incubation at 37°C, the cells were assayed for luciferase activity according to the manufacturer’s instructions.
**IN VIVO STUDIES**

**Animals**

Three different strains of mice and one strain of rat were used for the following studies. Animals were housed (4 to 6 mice per cage or 1 rat per cage) in a 12 hour light/dark cycle and given standard rodent chow and water *ad libitum*. For the encapsulation studies, male CD-1 mice (*Study 3.1.1*: 8-9 weeks of age; *Study 3.1.2*: 10-11 weeks of age; *Study 3.1.3*: 16-17 weeks of age) and male Lepr<sup>db/db</sup> (BSK.Cg-m<sup>+</sup>/+Lepr<sup>db</sup>/J; abbreviated as *db/db*) mice (15 weeks of age) were used. Male C57Bl/6 mice (10 weeks of age) were used for kidney subcapsular transplantation study while male CD-1 mice (10 weeks of age) and male Sprague Dawley (SD) rats (~ 250 g) were used for *in vivo* AdLPK-GLP-1 delivery studies. CD-1 mice and SD rats were obtained from the Animal Care Centre at the University of British Columbia (UBC, Vancouver, British Columbia, Canada). C57Bl/6 (stock no. 000664) and *db/db* (stock no. 000642) mice were obtained from Jackson Laboratories (Bar Harbour, Maine, USA). All animal research protocols were approved by the Animal Care Committee of the University of British Columbia in compliance with guidelines of the Canadian Council for Animal Care.

Body weight and 4 hour fasting blood glucose (8 am to 12 pm) were monitored three times per week. Twice a week, blood samples were obtained via the saphenous vein (mice: ~ 65 µL) or tail vein (rats: ~ 200 µL) for measurements of blood glucose or plasma GLP-1. Blood glucose was measured via the tail vein using a portable glucometer (OneTouch, Lifescan Canada, Burnaby, British Columbia, Canada). For the *db/db* mouse study, blood glucose was also determined using a commercially available Glucose (Trinder) Assay kit (Diagnostic Chemicals Ltd. Charlottetown, PE, Canada). The assay was performed on 96-well plates and read spectrophotometrically at 490 nm using a microplate reader (Dynatech MRX, Chantilly, Virginia, USA). All animals were matched by blood glucose and body weight prior to the onset of treatment in each experiment.
At the end of these studies, a maximum volume blood sample was obtained from each animal via cardiac puncture using a 25G $\frac{5}{8}$ needle and 3 mL syringe. The blood sample was transferred into microcentrifuge tubes containing heparin sodium solution (final concentration: 5 U/mL, Fisher Scientific, Fair Lawn, New Jersey, USA) and immediately placed on ice. Plasma was separated by centrifugation (7000 revolutions per minute [rpm] for 9 minutes) and stored at –20°C within one hour of collection. Samples were analyzed for total immunoreactive GLP-1 using the Linco GLP-1 (total) RIA kit.

Transplantation of Encapsulated GLP-1 Producing Hepatocytes in Mice

Preparation of cells for encapsulation

WRL 68 (Study 3.1.1 and 3.1.2) and HEPA 1-6 (Study 3.1.3 and 4) cells were left uninfected or were infected with AdLPK-GLP-1 (MOI 500) as described for in vitro AdLPK-GLP-1 transduction experiments. The only modification to the protocol is that the infection was performed in 100 mm diameter tissue culture plates (1.5x10$^6$ cells/plate). At 1 to 2 days after infection, cells were trypsinized and washed twice in PBS*. One plate each of uninfected and infected cells was left untouched in order to assess GLP-1 production from unencapsulated cells. The trypsinized cells were resuspended in MOPS (3-[N-Morpholino]propanesulphonic acid) solution (10 mM MOPS and 0.85% NaCl) and 1.5% alginate (InoTech, Rockville, Maryland, USA) at a 1:5 ratio. The cell-alginate mixture was aspirated into 20 mL syringes using a blunt-ended 14G stainless steel needle (Popper and Sons Inc., New Hyde Park, New York, USA) and encapsulated using the Encapsulator® IE-50 R (InoTech) according to the manufacturer’s instructions. The settings used for the speed, frequency, and electrostatic voltage of the Encapsulator® were 500, 1100 Hz and 1.04 kV, respectively.

Encapsulated cells were resuspended in serum-free medium, transferred into a 250 mL bead collection Erlenmeyer flask (InoTech) and allowed to settle by gravity. After removing excess medium, the encapsulated cells were drawn up into 10 mL syringes using a blunt-ended stainless steel 14G needle until 3 mL of encapsulated cells were obtained. This process was repeated for each
animal and the remaining microcapsules were transferred into 60 mm diameter tissue culture plates containing growth medium and placed in a 37°C incubator. Images of capsules were captured at 100x magnification using an Axiovert 200 inverted microscope connected to a camera with a colour monitor and imaging software.

**Transplantation of encapsulated cells into mice**

Animals were anesthetized with isoflurane (Baxter Co., Mississauga, Ontario, Canada) and a 1 cm midline incision through the linea alba was made in the abdomen. Encapsulated cells (Study 3.1.1: not determined; Study 3.1.2: ~2x10⁶ cell/mouse; Study 3.1.3: ~2x10⁶ cells/mouse; Study 4: ~3x10⁶ cells/mouse) were transplanted into the peritoneal cavity of the animals using an 18G catheter attached to a syringe containing the encapsulated cells. In the db/db mouse study, a sham surgery group was also included as a control. After suturing the muscle layer and skin, animals were placed on a heating pad until full recovery from the anesthetic was observed.

Two days after transplantation, animals were euthanized for terminal blood sampling and capsule retrieval. Animals were anesthetized with isoflurane and a midline incision was made in the abdomen. The peritoneal cavity was exposed and washed with PBS to remove the capsules. The retrieved capsules from each animal were transferred into 60 mm diameter tissue culture plates containing growth medium and placed in a 37°C incubator.

**Medium harvest from unencapsulated and encapsulated engineered cells**

On different days after infection, media was removed from unencapsulated and encapsulated cells and transferred to microcentrifuge tubes. Medium samples were centrifuged at 21,000 rcf for 1 minute and the resulting supernatant was transferred to new microcentrifuge tubes. After each medium harvest, fresh growth media was added to each plate in order to replace the removed volume of media. Samples were stored at -80°C for subsequent analysis of total GLP-1 using the Linco GLP-1 (total) RIA kit.
Kidney Subcapsular Transplantation of GLP-1 Producing Hepatocytes

HEPA 1-6 cells were left uninfected or were infected with AdLPK-GLP-1 as described for the encapsulation studies. The following day, cells were trypsinized and transferred in serum-free medium to microcentrifuge tubes (~2x10^6 cells/tube). Cells were centrifuged at 200 rcf for 20 seconds and the cell pellet was aspirated into polyethylene 50 (PE50) tubing (Intramedic®, BD Biosciences, Mississauga, Ontario, Canada) using a micrometer (Mitutoyo, Mississauga, Ontario, Canada). One end of the PE50 tubing was sealed with ligaclips (Ethicon Endo-Surgery Inc., Cincinnati, Ohio, USA) and the cells were spun down to the bottom of the tubing using a mechanical cell spinner (Heidolph RZR1, Rose Scientific Ltd., Edmonton, Alberta, Canada).

Animals were anesthetized with isoflurane and a 2 cm incision was made on the lower left quadrant of the dorsal side. The kidney was externalized and a small pouch was made in the kidney capsule using a 30G 1/2 needle and a handmade glass probe. After removing the ligaclips, the PE50 tubing containing the cell pellet was gently inserted into the kidney capsule pouch and the cells were slowly expelled using a micrometer. Once all the cells were expelled, the hole in the kidney capsule was cauterized. After suturing the muscle layer and skin, animals were placed on a heating pad until full recovery from the anesthetic was observed. Two days after transplantation, animals were euthanized for a terminal blood sample by cardiac puncture.

In Vivo Delivery of AdLPK-GLP-1 in Rodents

Animals were fasted for 4 hours prior to intravenous injection of AdLPK-GLP-1. Following induction of anesthesia by isoflurane, animals were injected with 100 μL virus (Ad5-βGal: 1x10^9 plaque forming units [pfu]/animal or AdLPK-GLP-1: 1 x10^9 or 5x10^9 pfu/animal) by tail vein. Body weight and blood glucose were monitored for 1 week (CD-1 mice) or 3 weeks (SD rats) after virus injection.
Liver Harvest and Terminal Blood Sample

At 1 week (mice: n = 12; AdCMVβ-Gal-infected rats: n = 2) or 3 weeks (rats: n = 12) after virus injection, animals were anesthetized and the liver was exposed via a ventral midline incision. Following excision, a 1 cm³ liver section was rinsed briefly in cold PBS, blotted dry, embedded in a cryomold containing Optimal Cutting Temperature compound (Tissue Tek, Sakura Finetek USA Inc., Torrance, California, USA) and immersed slowly in cold isopentane for subsequent cryosection. Frozen tissues were cut into 10 µm sections (2 sections per slide) at the UBC Department of Pathology and Laboratory Medicine (Vancouver, British Columbia, Canada). The slides were stored in -80°C until β-galactosidase staining and immunohistochemistry could be performed on the sections. Following liver harvest, a maximum volume blood sample was obtained via cardiac puncture as previously described.

β-galactosidase staining of liver sections

To determine the level of adenoviral transduction in the liver, β-galactosidase staining was performed. Liver sections were fixed using 0.2% glutaraldehyde in PBS for 5 minutes at room temperature. After fixing, sections were washed with PBS and incubated in X-Gal solution for 20 hours at 37°C. Once the desired stain intensity was obtained, sections were washed with PBS and mounted with coverslips.

Quantification of liver transduction

Quantification of liver transduction was performed on two 10 µm sections per animal. The percentage of liver area transduced with virus was calculated by dividing the area of β-galactosidase positive cells by the total area of the section. The area of each section was determined with an Axiovert 200 inverted microscope connected to a camera with a colour monitor and imaging software.
**Immunohistochemistry of liver sections**

To confirm the expression of GLP-1 in the liver of rats given AdLPK-GLP-1, GLP-1 immunostaining was performed. Liver sections were fixed in acetone for 10 minutes at -20°C. After fixing, the slides were rinsed in cold (4°C) PBS for 5 minutes. To block endogenous peroxidase activity, liver sections were incubated with 0.3% hydrogen peroxide in methanol for 10 minutes at room temperature. Following hydration of slides, the sections were incubated with serum-free protein block (DakoCytomation, Mississauga, Ontario, Canada) for 10 minutes. Primary anti-GLP-1 antibody (C-terminal specific, goat polyclonal antibody; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) was incubated at 1:100 dilution with the liver sections at 4°C overnight. The following day, liver sections were washed with PBS and incubated with secondary antibody (biotinylated anti-goat IgG, 1:400 dilution, Santa Cruz Biotechnology Inc.) for 1 hour at room temperature. To stain the liver sections, 3-3'-diaminobenzidine (DAB, Vector Laboratories) was used as a substrate chromagen. After the desired stain intensity was obtained, liver sections were washed with tap water, counterstained with hematoxylin (Gill’s Formula, Vector Laboratories) and mounted with coverslips. Images of liver sections were captured at 100x magnification using an Axiovert 200 inverted microscope connected to a camera with a colour monitor and imaging software.

**DATA ANALYSIS**

Data are presented as mean ± standard error of the mean (SEM) with the number of experiments or replicates shown in the figure legends. Data were assessed using Student’s t-test and was considered statistically significant when $P < 0.05$. Data analysis was performed using the Prism software package (Version 4.0b, GraphPad, San Diego, California, USA).
RESULTS

STUDY 1

In Vitro Characterization of an Adenoviral Vector Expressing Glucagon-like Peptide-1

1.1 Evaluation of GLP-1 production from hepatocytes transduced with AdLPK-GLP-1

Given that AdLPK-GLP-1 was constructed to express GLP-1 under the direction of a liver specific promoter, an investigation was initiated to examine the effectiveness of AdLPK-GLP-1 in transducing hepatocytes to produce GLP-1. Pilot studies were performed to determine whether GLP-1 production was increased in hepatocytes transduced with AdLPK-GLP-1 and, if so, whether this effect was due to the specific transgene expressed by the recombinant adenovirus. To address this question, the level of GLP-1 production was examined in WRL 68 cells under three different conditions: (i) uninfected, (ii) infected with AdCMV-βGal or (iii) infected with AdLPK-GLP-1 (Fig. 7). The pilot studies discussed herein are shown as separate graphs due to the different conditions used for carrying out the experiments. For both studies, infection with AdCMV-βGal at various MOIs (1, 10, and 100) did not have an effect on the level of immunoreactive GLP-1 production compared to the uninfected controls. A small increase in GLP-1 production was observed in cells infected at the lowest MOI of AdLPK-GLP-1 in both studies (MOI 1: infected: 11.1 ± 0.4 pM vs. uninfected: 9.2 ± 2.1 pM [Fig. 7A]; MOI 10: infected: 10.6 ± 2.3 pM vs. uninfected: 6.4 ± 0.5 pM [Fig. 7B]). However, this increase was not statistically significant. Finally, when the highest MOI of AdLPK-GLP-1 (MOI 10 and 100) was used to infect WRL 68 cells in each study, a greater increase in immunoreactive GLP-1 production relative to uninfected cells was observed. Specifically, a 3.4-fold increase in the levels of GLP-1 production was observed (31.4 ± 3.5 pM vs. 9.2 ± 2.1 pM) in the first study (Fig. 7A) whereas a 7.6-fold increase was observed (48.4 ± 27.3 pM vs. 6.4 ± 0.5 pM) in the second study (Fig. 7B). However, due to the large variability in GLP-1 production levels in the second study, the increase in GLP-1 was not considered statistically significant.
Figure 7. Effect of AdCMV-βGal and AdLPK-GLP-1 on immunoreactive GLP-1 production from transduced WRL 68 cells. WRL 68 cells were seeded at (A) 5x10^5 cells/well or (B) 1x10^5 cells/well into 6-well plates. After 5-6 hours at 37°C, cells were infected with AdCMV-βGal and AdLPK-GLP-1 at a MOI of (A) 1 and 10 or (B) 10 and 100. Three days after infection, medium samples were harvested from the cells and immunoreactive GLP-1 was assayed using a total GLP-1 RIA kit (Linco). (A) Mean of 3 replicates; (B) Mean of 6 replicates; *P < 0.05 (compared to uninfected cells).
1.2 Generation and selection of AdLPK-GLP-1 clones

In light of the observation that AdLPK-GLP-1 was capable of transducing WRL 68 cells to produce GLP-1, additional AdLPK-GLP-1 clones were generated and screened. The reason for generating new clones of AdLPK-GLP-1 was to obtain a homogenous population of virus. Six clones (A to F) were screened for their ability to infect WRL 68 cells by comparing the levels of GLP-1 production. This screening process was performed since differences in infection efficiency of the AdLPK-GLP-1 clones could affect the level of GLP-1 production from the infected cells. While all of the AdLPK-GLP-1 clones induced a significant increase in immunoreactive GLP-1 production from WRL 68 cells compared to the uninfected cells (fold increase for clone A: 4.9, B: 6.8, C: 9.4, D: 7.7, E: 7.1, F: 4.4), AdLPK-GLP-1 clone C induced the highest level of GLP-1 production (Fig. 8). Given that the clones infected cells at a similar titer (~ 1 x10¹¹ optical particle unit [opu]/mL as determined by measuring the optical density of the virus at 260 nm), clone C was selected for further purification by ViraQuest Inc. to generate high-titer stocks. Unless otherwise stated, purified AdLPK-GLP-1 clone C was used in all subsequent in vitro and in vivo experiments.

1.3 Optimization of GLP-1 production from hepatocytes transduced with AdLPK-GLP-1

All preliminary in vitro experiments were performed in WRL 68 cells, a fetal human hepatic cell line. To examine whether AdLPK-GLP-1 can transduce other hepatocyte cell lines to produce GLP-1, immunoreactive GLP-1 production from a human hepatoma cell line, Huh7, was examined (Fig. 9A). While AdLPK-GLP-1 effectively transduced both WRL 68 and Huh7 cell lines to produce immunoreactive GLP-1 compared to uninfected controls, the increase in GLP-1 levels was statistically significant only for WRL 68 cells (infected: 65.5 ± 5.1 pM vs. uninfected: 4.3 ± 1.6 pM), and not for Huh7 cells (infected: 28.4 ± 19.6 pM vs. uninfected: 5.6 ± 2.0 pM). Furthermore, the level of GLP-1 production from WRL 68 cells was 2.3-fold greater than Huh7 cells.

The observation that infected Huh7 cells produced lower levels of GLP-1 compared to WRL 68 cells prompted an examination of the effects of cell numbers and incubation time on the amount of
Figure 8. Comparison of immunoreactive GLP-1 production from WRL 68 cells transduced with different AdLPK-GLP-1 clones. WRL 68 cells were seeded at 5x10^5 cells/well into 6-well plates. After 6 hours at 37°C, cells were infected with 200 µl of AdLPK-GLP-1 (Clones A to F). Three days after infection, medium samples were harvested from the cells and immunoreactive GLP-1 was assayed using a total GLP-1 RIA kit (Linco). Mean of 3 replicates; *P < 0.05, **P < 0.01 (compared to uninfected cells).
Figure 9. Optimization of AdLPK-GLP-1 transduction in (A) WRL 68 and Huh7 cells by comparing immunoreactive GLP-1 production due to (B) different number of cells/well and (C) different incubation time prior to infection. Cells were seeded at (A and C) 5x10^5 or (B) the indicated concentration of cells per well into 6-well plates. After (A and B) 5-6 hours or (C) the allotted period of time at 37°C, cells were infected with 200 μl AdLPK-GLP-1 (A: Clone B; B: WRL68 Clone E, Huh7 Clone F; C: Clone A). Three days after infection, medium samples were harvested from the cells and immunoreactive GLP-1 was assayed using a total GLP-1 RIA kit (Linco). Mean of 3 replicates; *P < 0.05, **P < 0.01, ***P < 0.0001 (compared to uninfected cells.)
GLP-1 released from the transduced cells. For WRL 68 and Huh7 cells, an increase in the number of cells seeded per well resulted in a corresponding decrease in GLP-1 production from the transduced cells compared to uninfected cells (Fig. 9B). Similarly, an increase in incubation time prior to infection decreased the level of GLP-1 produced by both cell lines (Fig. 9C). However, there were two caveats in these experiments that must be taken into account when interpreting these results. The first caveat was that different AdLPK-GLP-1 clones (A to F) were used for each experiment; therefore, results from different experiments cannot be compared. However, a general trend obtained from each individual experiment could be drawn. The second caveat was that a constant volume of virus (200 μl), rather than a specific MOI (virus particle to cell ratio), was used for infecting the cells under different conditions. Therefore, the decrease in GLP-1 production observed with increasing cell number or longer incubation time could be associated with the presence of less infective virus particles per cell. Despite these caveats, all subsequent in vitro AdLPK-GLP-1 transduction experiments were performed using 5x10⁵ cells/well that were incubated for 2 hours at 37°C prior to infection.

1.4 Liver-specific GLP-1 production mediated by AdLPK-GLP-1

The AdLPK-GLP-1 vector was designed such that GLP-1 expression is driven by the liver-specific rat LPK promoter [149, 153]. To examine the liver specificity of AdLPK-GLP-1, several hepatocyte (WRL 68, Huh7 and HEPA 1-6) and non-hepatocyte (IEC-6, INS-1 and 3T3-L1) cell lines were transduced with AdLPK-GLP-1. As shown in Figure 10, AdLPK-GLP-1 was capable of transducing different hepatocyte cell lines to produce immunoreactive GLP-1 in a dose-dependent manner. Furthermore, consistent with study 1.3 (Fig. 9A), the level of immunoreactive GLP-1 production was different between hepatocyte cell lines. At a MOI of 500, mouse HEPA 1-6 cells produced 2483 ± 436.3 pM GLP-1 (527-fold increase vs. uninfected), human WRL 68 cells produced 333.7 ± 10.0 pM GLP-1 (58-fold increase vs. uninfected) and human Huh7 cells produced 97.8 ± 16.6 pM GLP-1 (23-fold increase vs. uninfected). However, at lower MOIs, the difference in GLP-1
Figure 10. Comparison of immunoreactive GLP-1 production from different hepatocyte cell lines transduced with AdLPK-GLP-1. (A) WRL 68, (B) Huh7 and (C) HEPA 1-6 cells were seeded at 5x10^5 cells/well. After 2 hours at 37°C, cells were infected with AdLPK-GLP-1 at a MOI of 5, 50, and 500. Three days after infection, medium samples were harvested from the cells and immunoreactive GLP-1 was assayed using a total GLP-1 RIA kit (Linco). n = 3 (in triplicate); *P < 0.05, **P < 0.01, ***P < 0.0001 (compared to uninfected cells).
production between different hepatocyte cell lines was not significant. These results were also
important in demonstrating the ability of AdLPK-GLP-1 to transduce a mouse hepatocyte cell line
(HEPA 1-6) since subsequent in vivo studies were to be performed in rodents. Three other rodent
hepatocyte cell lines (FAO, Clone 9 and H4-II-E-C3) were also infected with AdLPK-GLP-1 (MOI 5,
50, and 500) but did not demonstrate an increase in GLP-1 production (data not shown).

When non-hepatocyte cell lines were infected with AdLPK-GLP-1 at a MOI of 500, no
significant increase in immunoreactive GLP-1 production was observed (Fig. 11). Interestingly,
transduced INS-1 cells produced significantly less GLP-1 compared to the uninfected controls (11.33
± 0.06 pM vs. 28.9 ± 1.5 pM; Fig. 11B). However, it is important to note that the cell confluency was
different between uninfected and infected INS-1 cells (uninfected: 40-50% vs. infected: 5-10%).

1.5 Comparison of adenoviral vector transduction in hepatocytes

To examine whether differences in the level of GLP-1 production among the hepatocyte cell lines
were due to the difference in susceptibility to adenovirus infection, β-galactosidase staining was
examined in hepatocytes infected with AdCMV-βGal (Fig. 12). All of the transduced cell lines
stained strongly for β-galactosidase as indicated by the presence of blue staining in the cytoplasm of
the cells, which was absent in cells that were not infected. Specifically, the intensity of β-
galactosidase staining was similar for Huh7 and HEPA 1-6 cells whereas a stronger β-galactosidase
staining was observed for WRL 68 cells. Furthermore, the percentage of cells that were β-
galactosidase positive was similar amongst the cell lines (WRL 68: 81% [Fig. 12B], Huh7: 90% [Fig.
12D], and HEPA 1-6: 93% [Fig. 12F]).

Cell confluency was different between cell lines at the time of staining with ~80-90% confluency
observed in WRL 68 cells and ~95-100% confluency in both Huh7 and HEPA 1-6 cell lines. In
addition, the cell morphology appeared slightly altered after adenovirus infection; cells appeared to
develop a larger and more rounded shape compared to the smaller and more elongated shape observed
in the uninfected cells (Fig 12).
Figure 11. Effect of AdLPK-GLP-1 on immunoreactive GLP-1 production from non-hepatocyte cell lines transduced with AdLPK-GLP-1. (A) IEC-6, (B) INS-1 and (C) 3T3-L1 cells were seeded at 5x10^5 cells/well. After 2 hours at 37°C, cells were infected with AdLPK-GLP-1 at a MOI of 500. Three days after infection, medium samples were harvested from the cells and immunoreactive GLP-1 was assayed using a total GLP-1 RIA kit (Linco). (A and B) Mean of 6 replicates; (C) Mean of 3 replicates; **P < 0.01 (compared to uninfected cells).
Figure 12. β-galactosidase staining of hepatocyte cell lines transduced with AdCMV-βGal. (A and B) WRL 68, (C and D) Huh7 and (E and F) HEPA 1-6 cells were seeded at 1.25×10^5 cells/well in a 4-chamber well slide. After 6 hours at 37°C, cells were infected with (B, D and F) AdCMV-βGal at a MOI of 50. Three days after infection, cells were stained for β-galactosidase. Bar = 95 μm at 100x magnification.
1.6 Regulation of GLP-1 production by glucose from engineered hepatocytes

In addition to conferring liver specificity, the LPK promoter in AdLPK-GP-1 has also been shown to confer glucose sensitivity [149, 154, 155]. Therefore, glucose regulation of GLP-1 production from AdLPK-GLP-1 infected hepatocytes was examined. A pilot study using AdLPK-GLP-1 clone C demonstrated a 1.3-fold increase in GLP-1 production in infected Huh7 cells cultured in high glucose medium compared to those in low glucose medium (Huh7: high glucose: $24.9 \pm 1.5$ pM vs. low glucose: $19.7 \pm 1.9$ pM; Fig. 13A). Despite taking into account the high basal level of GLP-1 produced from uninfected WRL 68 cells in low glucose medium, there was no statistically significant difference in GLP-1 production from infected WRL 68 cells cultured in low or high glucose medium (low glucose $15.2 \pm 3.0$ pM vs. high glucose $21.5 \pm 0.1$ pM; Fig 13A).

Based on these initial results, the effects of glucose on GLP-1 production from engineered Huh7 and HEPA 1-6 cells were investigated using purified AdLPK-GLP-1. In agreement with the trend observed in the previous pilot study, a 3.8-fold increase in GLP-1 production was observed in transduced Huh7 cells cultured in high glucose medium compared to those cultured in low glucose medium (high glucose: $78.8 \pm 13.6$ pM vs. low glucose: $20.7 \pm 6.5$ pM; Fig. 13B). In contrast, HEPA 1-6 cells did not display a change in GLP-1 production when the cells were cultured in low or high glucose media (Fig. 13C).

1.7 Time course of GLP-1 production from engineered hepatocytes

Since adenoviral vectors do not integrate into the host DNA upon infection of cells, expression of the desired genes in the vector is typically a few weeks [140, 158]. To determine the duration of the GLP-1 production by the engineered hepatocytes, GLP-1 levels in the medium were measured at different days post infection. Since WRL 68 and HEPA 1-6 cells produced the highest levels of GLP-1 amongst the hepatocyte cell lines examined, the duration of GLP-1 production was determined in these cell lines. For WRL 68 and HEPA 1-6 cells, peak GLP-1 production was observed at 3 days post infection (WRL 68: $65.4 \pm 4.7$ pM; HEPA 1-6: $4113 \pm 296$ pM) and this production declined to
Figure 13. Effect of low and high glucose on immunoreactive GLP-1 production from hepatocytes transduced with AdLPK-GLP-1. (A) Pilot experiment was performed in WRL 68 and Huh7 cells using 1.8 ml AdLPK-GLP-1 clone C. Subsequent experiments were conducted in (B) Huh7 and (C) HEPA 1-6 cells using AdLPK-GLP-1 at a MOI of 500. Cells were seeded at 1.5x10^6 cells/well into 100 mm diameter plates. After 2 hours at 37°C, cells were infected with AdLPK-GLP-1 at the indicated (A) volume or (B and C) MOI. Twenty-four hours after infection, cells were transferred into 6-well plates in medium containing 2 or 20 mM glucose. Medium samples were harvested at 3 days post infection and immunoreactive GLP-1 was assayed using a total GLP-1 RIA kit (Linco). (A) Mean of 3 replicates, (B and C) n = 3 (in triplicate); *P < 0.05 (compared to infected cells cultured in 2 mM glucose).
negligible levels by 13 to 14 days post infection, respectively (Fig. 14A and 14B). To address whether the decline in GLP-1 production was due to cells reaching confluency, a parallel experiment was performed in transduced HEPA 1-6 cells that were continuously passaged for 2 weeks after infection. Since levels of GLP-1 production might reflect the number of cells present, the level of GLP-1 production in passaged cells was normalized to the same cell confluency as the non-passaged cells. The rationale for this is that at day 2 post infection, passaged cells were approximately 40-50% confluent and subsequently decreased to 25% confluence from days 5 to 11 post infection, followed by an increase to 40-50% confluency from day 12 post infection. In contrast, non-passaged cells remained at approximately 90-100% confluent from day 1 post infection onwards (data not shown). While passaging cells increased the level of GLP-1 produced from days 3 to 11 post infection, it did not appear to prolong the duration of GLP-1 production since insignificant GLP-1 levels were observed by day 14 post infection (Fig. 14C).
Figure 14. Time course of immunoreactive GLP-1 production from hepatocytes transduced with AdLPK-GLP-1. (A) WRL 68 and (B and C) HEPA 1-6 cells were seeded at 5x10^5 cells/well into 6-well plates. After 2 hours at 37°C, cells were infected with AdLPK-GLP-1 at a MOI of (A) 100 or (B and C) 500. HEPA 1-6 cells were (B) unpassaged or (C) passaged for 2 weeks. Medium was harvested at the indicated days post infection and immunoreactive GLP-1 was assayed using a total GLP-1 RIA kit (Linco). (C) Immunoreactive GLP-1 levels were normalized to the same cell confluency as (B) unpassaged HEPA 1-6 cells. Mean of 3 replicates; *P < 0.05, **P < 0.01, ***P < 0.0001 (compared to uninfected cells).
STUDY 2

Development of a GLP-1 Bioassay

2.1 Evaluation of a commercial GLP-1 (active) ELISA for measuring biologically active GLP-1 released from engineered cells

Experiments from study 1 demonstrated that AdLPK-GLP-1 could transduce hepatocytes to produce immunoreactive GLP-1 (Fig. 7-10, 13 and 14). Therefore, the next question was whether the immunoreactive GLP-1 produced by these hepatocytes was biologically active since this would have important implications for this construct’s utility in vivo. While the Linco GLP-1 (total) RIA provides a sensitive method for detecting picomolar concentrations (range of linearity: 3 to 333 pM) of all the major circulating forms of GLP-1 (GLP-17-36 amide, GLP-17-37, GLP-19-36 amide, GLP-19-37, GLP-11-36 amide and GLP-11-37), it does not provide information on the specific levels of biologically active GLP-1 (GLP-17-36 amide and GLP-17-37). The Linco GLP-1 (active) ELISA kit was designed to detect the active forms of GLP-1 by using a monoclonal antibody that recognizes the N-terminus of GLP-17-36 amide and GLP-17-37. Therefore, the goal of this study was to examine the utility of the Linco GLP-1 (active) ELISA kit for detecting biologically active GLP-1 produced by hepatocytes transduced with AdLPK-GLP-1.

Given that the N-terminal region of GLP-1 sequence in AdLPK-GLP-1 was modified at the 8th amino acid by substitution with glycine, a potential limitation of the Linco GLP-1 ELISA is that its antibody cannot recognize the GLP-1 produced by AdLPK-GLP-1 infected hepatocytes. Indeed, Linco had confirmed that the GLP-1 ELISA was unable to detect a modified GLP-1 analogue containing a substitution at the 8th amino acid (Sonali Nayak, personal communication, November 11, 2002). However, unlike the modified GLP-1 in AdLPK-GLP-1, the GLP-1 analogue used by Linco did not contain a glycine substitution. To test whether the Linco GLP-1 ELISA could detect the modified GLP-1 in AdLPK-GLP-1, the assay was used to measure bioactive GLP-1 in medium samples harvested from engineered WRL 68 cells.
As shown in Figure 15A, a dose-dependent increase in immunoreactive GLP-1 from WRL 68 cells was observed using the Linco GLP-1 (total) RIA kit (black bar). In contrast, no dose-dependent increase in bioactive GLP-1 was observed using the Linco GLP-1 (active) ELISA (white bar). To further validate this result, the ability of the GLP-1 ELISA to detect synthetic GLP-1 peptides containing a substitution at the 8th amino acid position was examined. When GLP-1 \(7.36\)amide was measured in the GLP-1 ELISA, an ~40-fold increase in bioactive GLP-1 levels relative to background was observed (Fig. 15B). In contrast, the same concentrations of \([\text{Ser8}]\)-GLP-1 \(7.36\)amide and \([\text{Gly8}]\)-GLP-1 \(7.37\) did not produce a significant increase in bioactive GLP-1 levels (Fig. 15B). A similar trend was observed at different concentrations of GLP-1s (data not shown). This further confirms that the Linco GLP-1 ELISA cannot be used for measuring N-terminally modified forms of bioactive GLP-1.

In the absence of other commercially available kits for assaying bioactive GLP-1 levels, these results necessitated the development of a bioassay that would allow measurements of bioactive GLP-1 produced by AdLPK-GLP-1 transduced cells.

2.2 Validation of HEK-hGLP1R-Luc clones for use in GLP-1 bioassay

To develop the GLP-1 bioassay, a HEK 293 cell line expressing human GLP-1 receptor (HEK-hGLP1R) was used. This cell line was transfected with a pHTS-CRE plasmid containing a luciferase gene (HEK-hGLP1R-Luc) that is coupled to the activation of the GLP-1 receptor signalling pathway. Prior to using HEK-hGLP1R-Luc clones for the bioassay, the ability of these cells to bind to GLP-1 was tested using a GLP-1 receptor binding assay. This assay involves incubating the cells in the presence of a radiolabeled GLP-1 tracer and measuring changes in radioactivity (expressed cpm) in the absence or presence of a competing unlabeled GLP-1 peptide. The principle behind this assay is that cells expressing the GLP-1 receptor will bind to the tracer and, upon exposure to the competing GLP-1 peptide, the tracer will be displaced resulting in a decrease in radioactivity. As shown in Figure 16A, all HEK-hGLP1R-Luc clones displayed similar maximal binding of labeled GLP-1 as the parental cell line (HEK-hGLP1R). This was represented by an increase in radioactivity to
Figure 15. Evaluation of the ability of Linco GLP-1 (active) ELISA in detecting N-terminally modified forms of biologically active GLP-1. (A) Comparison of the level of immunoreactive GLP-1 measured from transduced WRL 68 cells using a GLP-1 (active) ELISA kit (white bar) or GLP-1 (total) RIA kit (black bar). WRL 68 cells were seeded at 5x10^5 cells/well into 6-well plates. After 2 hours, cells were infected with AdLPK-GLP-1 at a MOI of 5, 50, and 500. Three days after infection, medium was harvested from the cells and immunoreactive GLP-1 was assayed using a GLP-1 (active) ELISA and a GLP (total) RIA kit. n = 3 (in triplicate). (B) Comparison of bioactive GLP-1 levels of three different forms of synthetic biologically active GLP-1 (GLP-1_7-36 amide, [Ser8]-GLP-1_7-36 amide and [Gly8]-GLP-1_7-37) using the Linco GLP-1 (active) ELISA. Mean of duplicates. **P < 0.01 compared to 0 pM GLP-1.
Figure 16. Screening HEK-hGLP1R-Luc clones for GLP-1 bioassay. (A) GLP-1 receptor binding assay was performed to confirm binding to GLP-1 by HEK-hGLP1R-Luc clones. Cells (1x10^6 cells/800 μl) were incubated with 0 or 10 μM GLP-1_7-37 in the presence of ^125^I-GLP-1_7-37 tracer for 30 minutes at room temperature. Cells were centrifuged and radioactivity was measured from the resulting cell pellet. (B) GLP-1 bioassay was performed to compare the level of luciferase activity between HEK-hGLP1R-Luc clones. Cells were seeded at 5x10^4 cells/well into 96-well plates and allowed to incubate at 37°C overnight. The next day, cells were stimulated with 0 or 10 μM GLP-1 or 10 μM forskolin for 5 hours at 37°C. Luciferase activity was expressed as relative light units (RLU). Mean of 3 replicates.
~ 20,000 cpm in the absence of a competing GLP-1 peptide compared to a cell line lacking the human GLP-1 receptor (HEK 293). Furthermore, addition of 10 μM GLP-1 peptide in HEK-hGLPIR and HEK-hGLPIR-Luc cells resulted in the displacement of GLP-1 tracer from the receptor as indicated by the decrease in radioactivity to the levels of HEK 293 cells (~125 cpm).

Due to differences in transfection efficiency, some of the HEK-hGLPIR-Luc clones may contain more pHTS-CRE plasmids resulting in higher luciferase gene expression upon GLP-1 stimulation. Since luciferase gene expression results in an increase in luminescent signals from the cells, the amount of luminescence produced by the HEK-hGLPIR-Luc clones in response to GLP-1 was compared. Out of the five clones tested, clones 9 and 11 produced the highest luminescence when exposed to 10 μM GLP-1 (clone 9: 19.3 ± 1.8 RLU and clone 11: 19.8 ± 2.5 RLU; Fig. 16B). Since clone 9 had less background compared to clone 11 as indicated by a lower luminescent signal in the absence of GLP-1 stimulation (clone 9: 2.5 ± 0.1 RLU vs. clone 11: 5.4 ± 0.1 RLU), it was selected for use in subsequent GLP-1 bioassays. As a positive control, cells were incubated with forskolin, a stimulator of cAMP. The rationale is that expression of the luciferase gene in HEK-hGLPIR-Luc clones is dependent on the activation of cAMP. As expected, increased luminescence was observed in all clones stimulated with forskolin. Furthermore, the amount of luminescence produced in forskolin-stimulated clones was equivalent to that observed in cells that were incubated with GLP-1 (Fig. 16B). Luminescence was also measured in HEK 293 and HEK-hGLPIR cells to serve as negative controls. Since these cells do not contain the luciferase gene, no luminescent signal was detected in these cells (data not shown).

2.3 Optimization of GLP-1 bioassay protocol

The standard protocol for the GLP-1 bioassay involved seeding 5x10⁴ cells/well in 96-well plates. After overnight incubation at 37°C, cells were incubated with freshly reconstituted GLP-1₇₋₃₇ peptide made up in HG-DMEM supplemented with 10% FBS. After a 5 hour incubation at 37°C, luciferase activity was measured. To enhance the sensitivity of the GLP-1 bioassay, modifications to four
parameters of the protocol were examined: (i) incubation time with GLP-1, (ii) presence of serum in the medium, (iii) number of cells per well and (iv) freshly and previously reconstituted GLP-1 peptide.

When cells were incubated with GLP-1 for different lengths of time, a significant increase in luminescence was observed from 3 to 5 hours (11.5 ± 0.5 to 16.5 ± 0.7 RLU; Fig. 17A). However, no further increase in luminescence was observed from 5 to 7 hours (16.5 ± 0.7 to 15.9 ± 0.1 RLU).

GLP-1 is known to rapidly degrade in human serum due to the presence of enzymes such as DPIV and NEP 24.11 [77]. Since FBS has been shown to contain DPIV activity [159], one of the concerns with reconstituting GLP-1 in medium containing 10% FBS is the potential for GLP-1 degradation. In order to address this issue, GLP-1 was prepared in medium containing either 10% FBS or 1% protease-free BSA. Incubation of cells with GLP-1 prepared in medium containing 1% BSA resulted in a significant increase in luminescence compared to GLP-1 prepared in medium containing 10% FBS (FBS: 16.5 ± 1.1 vs. BSA: 20.0 ± 0.8 RLU; Fig. 17B).

Changes in the level of luminescence were also examined by testing different cell concentrations. Cells seeded at different densities (2.5x10^4, 5x10^5 or 1x10^5 cells/well) were incubated with concentrations of GLP-1 ranging from 1.95 to 125 pM. As shown in Figure 17C, increasing the number of cells per well corresponded to an increase in luminescence with a significant increase observed between 2.5x10^4 to 5x10^4 cells/well from 3.9 to 125 pM GLP-1. From 5x10^4 to 1x10^5 cells/well, a significant increase in luminescence was only observed at higher GLP-1 concentrations (31.25 and 125 pM). Furthermore, at lower GLP-1 concentrations (1.95 to 7.8 pM), the slope for 5x10^4 cells/well (0.6 ± 0.2 RLU/pM) was greater than the slope for 1x10^5 cells/well (0.3 ± 0.1 RLU/pM).

The GLP-1 peptide used for preparing GLP-1 standards is initially in a lyophilized form and is reconstituted in sterile saline prior to dilution in medium for the GLP-1 bioassay. In general, the lyophilized form is more stable than the reconstituted form due to the susceptibility of peptides to degradation. Therefore, to determine if the sensitivity of the GLP-1 bioassay was significantly
Figure 17. Optimization of GLP-1 bioassay by examining the effects of (A) incubation time, (B) serum in media, (C) number of cells seeded per well and (D) freshly or previously reconstituted GLP-1 on luciferase activity. Cells were seeded at 5x10^4 cells/well into 96-well plates and allowed to incubate at 37°C overnight. The next day, cells were stimulated with (A and B) 100 pM or (C and D) at the indicated GLP-1 concentrations for (A) the indicated hours or (B to D) 5 hours at 37°C. Luciferase activity was expressed as relative light units (RLU). Mean of 3 replicates; (A) **P < 0.01 (compared to cells incubated with 100 pM GLP-1 for 3 hours); (B) **P < 0.01 (compared to cells incubated with 100 pM GLP-1 made up in media containing 10% FBS); (C) *P < 0.05, **P < 0.01, ***P < 0.0001 (compared to 5x10^4 cells/well).
affected by the reconstitution state of GLP-1 (freshly prepared or previously prepared, frozen and
thawed), cells were incubated with GLP-1 standards prepared from freshly or previously reconstituted
peptide. When freshly reconstituted GLP-1 peptide was used for preparing GLP-1 standards, a
steeper standard curve was observed compared to GLP-1 standards made up using previously
reconstituted peptide (1.3 RLU/pM vs. 0.7 RLU/pM; Fig. 17D).

Based on these results, all subsequent experiments were performed using 5x10^4 cells/well that
were incubated with GLP-1 standards and samples for 5 hours at 37°C. Furthermore, standards were
made up using freshly reconstituted GLP-1 diluted in HG-DMEM supplemented with 1% BSA.

2.4 Effects of phenol red on the sensitivity of the GLP-1 bioassay

Since luciferase gene expression is measured based on luminescence, the presence of phenol red,
present in cell culture media, might interfere with the luminescence emitted by the cells. To examine
whether this factor could affect the sensitivity of the bioassay, luminescence was measured in the
presence and absence of phenol red. To perform this experiment, phenol red-containing media
samples were removed from the wells by inverting the 96-well plate after the 5 hour GLP-1
incubation. Wells then received either phenol red-containing media or PBS and the cells were lysed
with Bright-Glo Luciferase Reagent for measurements of luciferase gene expression. As expected,
the presence of phenol red lowered the luminescence by ~2.5-fold across different GLP-1
concentrations (Fig. 18A). However, when the luminescence was normalized as a percent increase,
the GLP-1 standard curves generated in the presence or absence of phenol red completely overlapped
each other (Fig. 18B).

2.5 Effects of different media types on GLP-1 bioassay

Although removal of media from the wells did not affect the relative luminescence produced in
the presence or absence of phenol red (Fig. 18B), a study was conducted to examine whether this
modified protocol could serve as a way to measure and compare samples from cell cultures in
different media using one standard curve. When GLP-1 standards were prepared in either
Figure 18. Effect of phenol red on luciferase activity of HEK-hGLP1R-Luc clones stimulated with varying concentrations of GLP-1. Cells were seeded at $5 \times 10^4$ cells/well into 96-well plates and allowed to incubate at 37°C overnight. The next day, cells were stimulated at the indicated GLP-1 concentrations for 5 hours at 37°C. After incubation, the plate was inverted to remove medium samples from the wells. Each well was replaced with either 100 µl medium or PBS and 100 µl Bright-Glo Reagent. Luciferase activity was expressed as (A) relative light units (RLU) or (B) percent increase in luciferase activity. Mean of 3 replicates.
HG-DMEM + 1% BSA or Hams-F10 + 0.5% BSA, a significant decrease in luminescence (~1.5-fold) was observed with standards made up in HG-DMEM + 1% BSA (Fig. 19).

2.6 Effects of plasma on GLP-1 bioassay

To examine the feasibility of the GLP-1 bioassay in measuring GLP-1 levels in plasma samples, luminescence was measured from GLP-1 standards that were prepared in undiluted or diluted charcoal extracted plasma samples. The charcoal extraction procedure was performed in order to remove endogenous peptides, such as GLP-1, from plasma. As shown in Figure 20A, higher luminescent signal was detected from HEK-hGLP1R-Luc cells incubated with plasma alone or with plasma containing low concentrations of GLP-1 (0.975 or 1.95 pM) compared to cells incubated with medium under the same conditions. Furthermore, increasing dilutions of plasma in medium (50% to 10% plasma in medium) were associated with decreasing luminescence production from the cells. More importantly, the relative luminescence of the GLP-1 bioassay was significantly reduced for undiluted and diluted plasma samples compared to medium samples (Fig. 20B). Although diluting the plasma in medium produced a slight increase in relative luminescence, the increase was still not comparable to the relative luminescence measured from GLP-1 standards prepared in medium (Fig. 20B).

2.7 Characteristics of GLP-1 bioassay

Several aspects of the GLP-1 bioassay were determined from studies 2.1 to 2.6. Based on results from 15 experiments, the lowest level of GLP-1 that was detectable using the bioassay was 3.4 ± 0.8 pM when a 100 μl sample size was used. Furthermore, the EC_{50} of this GLP-1 bioassay is 17.8 ± 2.5 pM. To determine the precision of this assay, the intra- and inter-assay variability was determined by calculating the coefficient of variation (CV: 100 • [standard deviation/mean value of set]). Based on 10 replicates from 3 different experiments, the intra-assay CV is 5%. The inter-assay CV is 40% based on results from 10 different experiments. This high inter-assay CV may be dependent on how often the HEK-hGLP1R-Luc cells were passaged prior to its use for the bioassay. It was observed
Figure 19. Effect of different types of media on luciferase activity of HEK-hGLP1R-Luc clones stimulated with varying concentrations of GLP-1. Cells were seeded at 5x10^4 cells/well into 96-well plates and allowed to incubate at 37°C overnight. The next day, cells were stimulated at the indicated GLP-1 concentrations made up in HG-DMEM + 1% BSA or Hams-F10 + 0.5% BSA for 5 hours at 37°C. After incubation, the plate was inverted to remove medium from the wells. Each well was replaced with 100 μl PBS and 100 μl Bright-Glo Reagent. Luciferase activity was expressed as relative light units (RLU). Mean of 3 replicates.
Figure 20. Effect of plasma on luciferase activity of HEK-hGLP1R-Luc clones stimulated with varying concentrations of GLP-1. Cells were seeded at 5x10^4 cells/well into 96-well plates and allowed to incubate at 37°C overnight. The next day, cells were stimulated at the indicated GLP-1 standards prepared in HG-DMEM + 1% BSA, 100% charcoal extracted plasma or 10% and 50% charcoal extracted plasma diluted in HG-DMEM + 1% BSA for 5 hours at 37°C. After incubation, the plate was inverted to remove medium from the wells. Each well was replaced with 100 µl PBS and 100 µl Bright-Glo Reagent. Luciferase activity was expressed as (A) relative light units (RLU) or (B) percent increase in luciferase activity. Mean of 3 replicates.
that the cells become less responsive to GLP-1 stimulation with greater number of passages in culture. The GLP-1 bioassay was found to be quite specific for GLP-1, with less than 0.1% cross-reactivity for GIP and 3.2% cross-reactivity for glucagon.

2.8 Measurement of biologically active GLP-1 in ADLPK-GLP-1 transduced hepatocytes

Prior to measuring medium samples of AdLPK-GLP-1 transduced hepatocytes for biologically active GLP-1, the ability of the GLP-1 bioassay to detect biologically active forms of GLP-1 containing a N-terminal penultimate substitution was examined. To do this, GLP-1 standard curves generated from three different synthetic GLP-1 peptides: GLP-17-36 amide, [Ser8]-GLP-17-36 amide and [Gly8]-GLP-17-37 were compared. As shown in Figure 21A, a rightward shift in the standard curve was observed with both N-terminally modified GLP-1s compared to the GLP-17-36 amide. This rightward shift is reflected in the EC50 values with 8.3 pM, 18.2 pM and 28.4 pM corresponding to GLP-17-36 amide, [Ser8]-GLP-17-36 amide and [Gly8]-GLP-17-37, respectively.

Since the mouse liver cell line, HEPA 1-6, produced the highest level of immunoreactive GLP-1 upon transduction with AdLPK-GLP-1 (Fig. 10), the objective of this study was to determine whether the GLP-1 produced by these cells was biologically active. When media samples from uninfected and infected HEPA 1-6 cells were subjected to GLP-1 bioassay, transduced HEPA 1-6 cells produced significant levels of bioactive GLP-1 (MOI 50: 3.1 ± 1.0 pM and MOI 500: 170.8 ± 24.1 pM vs. uninfected: 0.07 ± 0.01 pM; Fig. 21B, black bar). This bioactive level of GLP-1 represents ~10 to 15% of total immunoreactive GLP-1 as measured by RIA. A similar trend was observed with engineered WRL 68 cells (data not shown).
Figure 21. Assessing the ability of GLP-1 bioassay in detecting N-terminally modified GLP-1. (A) Comparison of standard curves generated in the GLP-1 bioassay using three different GLP-1 peptides (GLP-1_{7-36 amide}, [Ser8]-GLP-1_{7-36 amide} and [Gly8]-GLP-1_{7-37}). Mean of 3 replicates. (B) Comparison of bioactive and immunoreactive GLP-1 levels in AdLPK-GLP-1 infected HEPA 1-6 cells. Cells were seeded at 5x10^5 cells/well into 6-well plates. After 2 hours at 37°C, cells were infected with AdLPK-GLP-1 at a MOI of 5, 50, and 500. Three days after infection, medium was harvested from the cells and immunoreactive GLP-1 was assayed using the GLP-1 bioassay (black bar) or total GLP-1 RIA kit (Linco; gray bar). n = 3 (in triplicate); *P < 0.05, **P < 0.01 (compared to uninfected samples measured using GLP-1 bioassay).
STUDY 3

Ex Vivo and In Vivo Delivery of AdLPK-GLP-1 in Normal Rodents

3.1 Transplantation of encapsulated GLP-1 producing hepatocytes in normal male mice

The greatest limitation in transplantation of cells in the body is immune rejection. To circumvent this problem, the technique of cell encapsulation, using a semipermeable polymer membrane that prevents direct interaction of immune cells with encapsulated cells, was developed as a way to provide immunoprotection for the transplanted cells. Successful implementation of this technique has been demonstrated in animal studies. For example, transplantation of encapsulated insulin-producing cells (βTC6-F7 or INS-1) or xenograft islet cells provided long-term restoration of normoglycemia in mouse and rat models of diabetes [160-162].

Earlier studies demonstrated that AdLPK-GLP-1 transduced hepatocytes produce immunoreactive and bioactive GLP-1 in vitro (Fig. 10 and 21B). Therefore, the next objective was to examine the effects of encapsulation and subsequent transplantation of these engineered hepatocytes on plasma GLP-1 levels in mice and rats.

3.1.1 Transplantation of encapsulated GLP-1 producing WRL 68 cells in normal male CD-1 mice (8-9 weeks old)

Since WRL 68 cells produced relatively high levels of GLP-1 (~300 pM) when infected with AdLPK-GLP-1 at a MOI of 500 (Fig. 10A), this cell line was selected for transplantation studies. Two days after AdLPK-GLP-1 infection, WRL 68 cells were encapsulated and subsequently transplanted into the peritoneal cavity of mice. Uninfected WRL 68 cells were used as a negative control. Plasma GLP-1 levels were measured, using a GLP-1 (total) RIA, at two days after transplantation in order to examine the effects of transplantation of encapsulated engineered WRL 68 cells.
To confirm that the transduced WRL 68 cells were producing immunoreactive GLP-1, medium samples from uninfected and AdLPK-GLP-1 infected cells were harvested prior to encapsulation and assayed by a GLP-1 (total) RIA. As shown in Figure 22A, infected WRL 68 cells produced ~68-fold higher levels of immunoreactive GLP-1 compared to uninfected cells (infected: 231.6 ± 10.6 pM vs. uninfected: 3.4 ± 0.3 pM). However, when plasma GLP-1 levels were examined from recipient animals that were fasted overnight and then fed ad libitum for 7.5 hours, no significant difference was observed between the two treatment groups (infected: 19.0 ± 8.1 pM vs. uninfected: 14.1 ± 4.4 pM; Fig. 22B).

3.1.2 Transplantation of encapsulated GLP-1 producing WRL 68 cells in normal male CD-1 mice (10-11 weeks old) with increased number of cells per capsule

In study 3.1.1, there was no difference in plasma GLP-1 levels between animals transplanted with encapsulated uninfected or infected hepatocytes despite the fact that the infected cells were producing significantly high levels of immunoreactive GLP-1 in vitro (Fig. 22). Therefore, the goal of this study was to repeat the same protocol with increased number of cells per capsule prior to transplantation into the animals.

Similar to the previous study, an ~94-fold increase in GLP-1 production by engineered WRL 68 cells was observed prior to encapsulation and transplantation (infected: 217.3 ± 5.5 pM vs. uninfected: 2.3 ± 0.4 pM; Fig. 23A). However, no difference in 4 hour fasted plasma GLP-1 levels was observed at two days post transplantation (infected: 5.6 ± 1.8 pM vs. uninfected: 6.7 ± 3.7 pM; Fig. 23B).

3.1.3 Transplantation of encapsulated GLP-1 producing HEPA 1-6 cells in normal male CD-1 mice (16-17 weeks old)

Despite increasing the number of cells per capsule, an increase in plasma GLP-1 was not observed in animals transplanted with capsules containing WRL 68 cells engineered to produce GLP-1 (Fig. 23). Therefore, the goal of this study was to encapsulate a different hepatocyte cell line (HEPA 1-6),
Figure 22. Transplantation of encapsulated engineered WRL 68 cells in normal male CD-1 mice (8-9 weeks of age). Immunoreactive GLP-1 levels were determined from (A) transduced cells prior to encapsulation at 2 days post infection and (B) fed plasma samples from mice at 2 days post transplantation (4 days post infection) using GLP-1 (total) RIA kit (Linco). n = 2 for each treatment group; ***P < 0.0001 (compared to uninfected cells).
Figure 23. Transplantation of encapsulated engineered WRL 68 cells in normal male CD-1 mice (10-11 weeks of age) with increased number of cells per capsule. Mice were transplanted with ~2x10^6 cells and fasted plasma samples were obtained at 2 days post transplantation (4 days post infection). Immunoreactive GLP-1 levels were determined from (A) transduced cells prior to encapsulation at 2 days post infection and (B) plasma samples using GLP-1 (total) RIA kit (Linco). n = 2 for each treatment group; ***P < 0.0001 (compared to uninfected cells).
which has been shown to produce approximately 10-fold higher levels of GLP-1 compared to WRL 68 cells upon infection by AdLPK-GLP-1 at a MOI of 500 (Fig. 10C). Furthermore, the protocol was modified such that the transduced cells were encapsulated and transplanted at 1 day rather than 2 days after AdLPK-GLP-1 infection. The rationale for this modification is such that the time of blood harvest from the animals (2 days post transplantation) would correspond to the peak GLP-1 production of HEPA 1-6 cells (3 days post infection) observed in earlier studies (Fig. 14).

To confirm immunoreactive GLP-1 production from the engineered hepatocytes during this study, cells were kept in culture at different stages of the encapsulation process: (i) unencapsulated cells, (ii) untransplanted encapsulated cells and (iii) transplanted encapsulated cells retrieved from animals 2 days after transplantation (Fig. 24A to C). It is important to note that the levels of immunoreactive GLP-1 produced at the different stages of the encapsulation process cannot be compared since the number of cells or capsules at each stage were not the same. However, it can be concluded that at all stages of the study, the engineered hepatocytes produced significant amounts of GLP-1 compared to uninfected cells starting at 2 days post infection (Fig. 24A to C). Furthermore, the level of GLP-1 production declined beyond 3 days post infection. Compared to previous encapsulation studies with WRL 68 cells (Fig. 22A and 23A), HEPA 1-6 cells produced ~15 times more immunoreactive GLP-1 at 2 days post AdLPK-GLP-1 infection (HEPA 1-6 3704 ± 683 pM; Fig. 24A). To examine whether this increase in immunoreactive GLP-1 production from transduced HEPA 1-6 cells was sufficient to change plasma GLP-1 levels in mice, 4-hour fasted plasma samples were obtained 2 days post transplantation. Indeed, a 2-fold increase in plasma GLP-1 levels was observed in animals transplanted with encapsulated AdLPK-GLP-1 infected HEPA 1-6 compared to those transplanted with encapsulated uninfected cells (infected 38.8 ± 6.0 pM vs. uninfected 18.9 ± 3.9 pM; Fig. 24D).

Encapsulated cells were examined prior to transplantation and after retrieval from transplanted animals at 2 days post transplantation (Fig. 25). All capsules were fairly uniform in size, varying from 700 to 800 μm in diameter. Furthermore, all capsules retained their spherical shape throughout the study. Although similar cell density per capsule was observed for the two different conditions
Figure 24. Effect of transplantation of encapsulated engineered HEPA 1-6 cells on plasma GLP-1 levels in normal male CD-1 mice (16-17 weeks of age). Mice were transplanted with ~2x10^6 cells and immunoreactive GLP-1 was determined from (D) 4-hour fasted plasma samples obtained at 2 days post transplantation (3 days post infection). At various days post infection, immunoreactive GLP-1 levels were measured from (A) unencapsulated engineered cells and encapsulated engineered cells that were (B) not transplanted or (C) transplanted and subsequently retrieved from animals. All samples were assayed using GLP-1 (total) RIA kit. n = 2 for each treatment group; *P < 0.05, **P < 0.01 ***P < 0.0001 (compared to uninfected control on the same day post infection).
(uninfected and AdLPK-GLP-1 infected), the size of the AdLPK-GLP-1 infected cells appears to be larger than the uninfected cells prior to transplantation into the animals (Fig. 25A and B). There appears to be a slight increase in the number of cells per capsule 2 days post transplantation. In addition, adherent cells were present at the bottom of the tissue culture plates (Fig. 25C and D). These cells may come from capsules that have ruptured during transplantation or capsule retrieval from the animals.

3.2 Kidney subcapsular transplantation of GLP-1 producing hepatocytes in normal male C57Bl/6 mice (10 weeks old)

While transplantation of encapsulated engineered HEPA 1-6 cells into the peritoneal cavity increased the basal levels of plasma GLP-1 by approximately 2-fold (Fig. 24D), a potential limitation of this transplantation strategy is the absence of direct contact of the GLP-1 producing cells with blood vessels. As a result, it was reasoned that perhaps only some of the GLP-1 produced by the engineered cells was being effectively taken up into the circulation.

To address this issue, the kidney capsule was considered as another potential transplantation site for cells due to its close proximity to the circulation [133]. Therefore, the objective of this study was to examine whether transplanting engineered hepatocytes under the kidney capsule would further increase plasma GLP-1 levels of the transplanted animals. To minimize immune attack against the transplanted HEPA 1-6 cells, normal male C57Bl/6 mice were used for this study since the HEPA 1-6 cells were derived from this strain.

To examine the effects of kidney subcapsular transplantation of AdLPK-GLP-1 infected HEPA 1-6 cells in mice, cells were transplanted into the animals 1 day post infection. To allow for comparison to encapsulation study 3.1.3, the same number of cells was transplanted under the kidney capsule. Similar to the results obtained in the previous encapsulation study, a 2.1-fold increase in plasma GLP-1 levels was observed (infected $10.2 \pm 1.7$ pM vs. uninfected $4.7 \pm 1.7$ pM; Fig. 26).
Figure 25. Comparison of capsules containing (A and C) uninfected or (B and D) AdLPK-GLP-1 infected HEPA 1-6 cells. Capsules obtained (A and B) after encapsulation or (C and D) after retrieval from transplanted male CD-1 mice were examined under 100x magnification with an Axiovert 200 inverted microscope (Carl Zeiss). Bar = 95 μm.
Figure 26. Evaluation of plasma GLP-1 levels after transplantation of uninfected or AdLPK-GLP-1 infected HEPA 1-6 cells under the kidney capsule of C57Bl/6 mice (10 weeks of age). Animals were monitored for ~ 2 weeks prior to transplantation of 2x10^6 cells per animal. Two days after transplantation, 4-hour fasted plasma samples were obtained and immunoreactive GLP-1 levels were measured using GLP-1 (total) RIA kit. n = 4 for each treatment group; *P < 0.05 (compared to uninfected controls).
3.3 *In vivo* delivery of AdLPK-GLP-1 in normal rodents

Given that *ex vivo* delivery of AdLPK-GLP-1 was effective in increasing plasma GLP-1 levels in normal mice (Fig. 24D and 26), the effect of direct viral vector administration into the circulation was examined. *In vivo* delivery of an adenoviral vector in mice and rats has been shown to induce effective expression of transgene in the liver in which ~80 to 90% of hepatocytes were transduced [163, 164]. Therefore, the goal of this study was to determine if intravenous delivery of AdLPK-GLP-1 could engineer the cells in the liver to produce GLP-1 and thereby increase basal plasma GLP-1 levels in normal rats and mice.

3.3.1 *In vivo* delivery of AdLPK-GLP-1 in normal male Sprague Dawley rats (~250 g)

To examine the effects of *in vivo* delivery of AdLPK-GLP-1 in normal male Sprague Dawley rats, body weight and blood glucose levels were monitored for 2 weeks before and 3 weeks after injection with AdLPK-GLP-1 (1 x 10^9 or 5 x 10^9 pfu) or as a control, AdCMV-βGal (1 x 10^9 pfu; Fig. 27A and B). In addition, plasma GLP-1 levels were examined 7 and 11 days after viral injection (Fig. 27C). Body weight of all animals continued to increase throughout the study from 92.8 ± 1.3 g at the start of the study to 401.7 ± 13.7 g at the end of the study (Fig. 27A). Although the body weight of the animals in the AdCMV-βGal group was consistently lower than those in the AdLPK-GLP-1 treatment groups after virus injection, this difference in body weight was not statistically significant (Fig. 27A). Similarly, fasted blood glucose levels for all treatment groups remained at approximately 6.7 mM throughout the study, with no significant differences observed after viral injection (Fig. 27B). Finally, no significant difference in fasted plasma GLP-1 levels was observed 7 and 11 days after infection (Table II; Fig. 27C).
Figure 27. Effects of intravenous delivery of AdLPK-GLP-1 on body weight, blood glucose and plasma GLP-1 levels in normal male Sprague Dawley rats (~250 g). (A) Body weight and (B) blood glucose levels were monitored 3 times a week. Rats were monitored for 2 weeks and placed into one of three treatment groups: (i) $1 \times 10^9$ pfu AdCMV-βGal, (ii) $1 \times 10^9$ pfu AdLPK-GLP-1 or (iii) $5 \times 10^9$ pfu AdLPK-GLP-1. At 7 and 11 days after virus injection, 4 hour fasted plasma GLP-1 levels were determined using GLP-1 (total) RIA kit. AdCMV-βGal group: $n = 6$ (Days -14 to 7 post virus injection), $n = 4$ (Days 7 to 21 post virus injection); AdLPK-GLP-1 group: $n = 4$. 

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Table II: Plasma GLP-1 levels in normal Sprague Dawley rats at different days post virus injection

<table>
<thead>
<tr>
<th>Days</th>
<th>AdCMV-βGal</th>
<th>1x10⁹ pfu AdLPK-GLP-1</th>
<th>5x10⁹ pfu AdLPK-GLP-1</th>
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<td>7</td>
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<td>11</td>
<td>7.7 ± 2.1 pM</td>
<td>8.6 ± 3.1 pM</td>
<td>6.3 ± 2.2 pM</td>
</tr>
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</table>

To examine the efficacy of intravenous virus delivery in rats, β-galactosidase staining was performed on liver sections obtained from animals (n = 2) at 1 week after injection of Ad5CMV-βGal. Positive β-galactosidase staining is indicated by the presence of dark blue staining in the cells. As shown in Figure 28, β-galactosidase staining was not observed in liver sections from either animal.

3.3.2 In vivo delivery of AdLPK-GLP-1 in normal male CD-1 mice (10 weeks of age)

In contrast to the effect of transplantation of AdLPK-GLP-1 induced hepatocytes in mice (Fig. 24D and 26), no increase in plasma GLP-1 levels was observed in rats after in vivo delivery of the same adenoviral vector (Fig. 27C). This observation prompted an investigation of in vivo gene delivery in normal male mice since the majority of gene delivery studies have been conducted in these animals.

Similar to the rat study, the effects of in vivo AdLPK-GLP-1 delivery in CD-1 mice were examined by monitoring body weight, blood glucose and GLP-1 levels (Fig. 29). As shown in Figure 29A, CD-1 mice were beginning to reach a plateau in their body weight (38.5 ± 0.3 g) by 10 weeks of age (day -14). After intravenous delivery AdCMV-βGal (1x10⁹ pfu) or AdLPK-GLP-1 (1 x10⁹ or 5x10⁹ pfu), no significant difference in body weight or blood glucose was observed between mice of the different treatment groups (Fig. 29A and B). In addition, there was no effect on plasma GLP-1 levels in mice given the highest dose of AdLPK-GLP-1 compared to the mice given AdCMV-βGal (AdLPK-GLP-1: 27.9 ± 7.1 pM vs. AdCMV-βGal: 29.4 ± 2.6 pM; Fig. 29C). Surprisingly, a 1.6-fold
Figure 28. β-galactosidase staining of liver sections from male Sprague Dawley rats (~250 g). After a 4 hour fast, rats were given an intravenous injection of AdCMV-βGal. One week after virus injection, liver tissues were harvested from (A and B) two rats and frozen for cryosection. Tissue sections (10 μm) were examined under 100x magnification with an Axiovert 200 inverted microscope (Carl Zeiss). Bar = 95 μm.
Figure 29. Effects of intravenous delivery of AdLPK-GLP-1 or AdCMV-βGal on body weight, blood glucose and plasma GLP-1 levels in male CD-1 mice (10 weeks of age). (A) Body weight and (B) blood glucose levels were monitored 2 to 3 times a week. Mice were monitored for 2 weeks and placed into one of three treatment groups: (i) $1 \times 10^9$ pfu AdCMV-βGal, (ii) $1 \times 10^9$ pfu AdLPK-GLP-1 or (iii) $5 \times 10^9$ pfu AdLPK-GLP-1. One week after virus injection, (C) 4-hour fasted plasma GLP-1 levels were determined using GLP-1 (total) RIA kit. $n = 4$ for each treatment group; $^*P < 0.05$ (compared to uninfected controls).
decrease in plasma GLP-1 levels was observed with the lowest dose of AdLPK-GLP-1 (AdLPK-GLP-1: 18.2 ± 1.6 pM vs. AdCMV-βGal: 29.4 ± 2.6 pM; Fig 29C).

Since plasma GLP-1 levels did not increase in mice following AdLPK-GLP-1 injection, β-galactosidase staining was performed on liver sections obtained from mice (n = 4) 1 week after injection of Ad5CMV-βGal in order to determine whether the virus was effectively delivered to the liver (Fig. 30). Approximately 90% of liver cells were β-galactosidase positive in 3 out of 4 animals (Fig. 30B, D and E). The staining was homogenous across the whole liver section with greater staining intensity observed in cells located near the blood vessels. The remaining liver section contained approximately 5% of stained liver cells (Fig. 30C). A liver section from a mouse given AdLPK-GLP-1 was stained for β-galactosidase as a negative control (Fig. 30A). As expected, no staining was observed in this section. Although GLP-1 immunostaining was performed on liver sections from mice that received AdLPK-GLP-1, no convincing staining was observed (data not shown). One concern is that the GLP-1 antibody used may have been unable to detect the modified form of GLP-1 produced from AdLPK-GLP-1 transduced hepatocytes. Therefore, at this stage, no conclusions could be drawn from the GLP-1 staining until more suitable antibodies are found.
Figure 30. β-galactosidase staining of liver sections from male CD-1 mice (10 weeks of age). After a 4 hour fast, mice were given either an intravenous injection of (A) AdLPK-GLP-1 or (B to E) AdCMV-βGal. One week after virus injection, liver tissues were harvested from the mice and frozen for cryosection. Tissue sections (10 μm) were examined under 100x magnification with an Axiovert 200 inverted microscope (Carl Zeiss). $Bar = 95 \mu m$. 
STUDY 4

Evaluation of the Effect of Ex Vivo GLP-1 Gene Therapy on Glucose Homeostasis in a Rodent Model of Diabetes

Transplantation of encapsulated GLP-1 producing HEPA 1-6 cells in male db/db mice

Given that transplantation of engineered HEPA 1-6 cells by both encapsulation (Fig. 24D) and kidney subcapsular (Fig. 26) techniques resulted in an increase in fasted plasma GLP-1 levels in normal male mice, the next goal was to examine whether this increase could improve glucose homeostasis in male db/db mice. This strain of mice is characterized by a spontaneous mutation in the gene encoding the leptin receptor leading to the development of obesity and diabetes by 4 to 8 weeks of age [165-170]. Due to this phenotype, the db/db mice are used in many studies as rodent model for T2D.

Body weight, blood glucose, and circulating plasma GLP-1 levels

To carry out this study, male db/db mice were given a sham surgery or transplanted with encapsulated HEPA 1-6 cells that were uninfected or infected with AdLPK-GLP-1. The encapsulation technique was selected because it is a less invasive procedure and can accommodate more cells compared to kidney subcapsular transplantation. In line with their phenotype, the db/db mice gained weight at a rapid rate, from 27.8 ± 0.1 g at 5 weeks of age (day 0) to a plateau weight of 47.2 ± 0.3 g at 11 weeks of age (day 42; Fig. 31A). In addition, these animals developed hyperglycemia (blood glucose levels > 15 mM) by 6 weeks of age (day 7). Within a month (day 28), their blood glucose levels had increased to values greater than or equal to 33.3 mM (Fig. 31B).

At the time of transplantation (day 65), db/db mice had reached their plateau weight (47.9 ± 0.3 g) and their blood glucose levels were ~ 35 mM (Fig. 31C). After transplantation, no significant difference in body weight was observed between the three treatment groups although a slight decline in body weight was observed in all animals. As a result, the post-transplantation body weight of the mice (day 67 to 91; 46.1 ± 0.4 g) remained below their pre-transplantation body weight (day 42 to 66;
Figure 31. Effects of transplantation of encapsulated engineered HEPA 1-6 cells in male \( db/db \) mice (15 weeks of age) on body weight, blood glucose and plasma GLP-1 levels. (A) Body weight and (B and C) blood glucose levels were monitored 2 to 3 times a week. After ~7 weeks of monitoring, animals were placed in one of three treatment groups: (i) sham surgery, (ii) transplantation with \( \sim 3 \times 10^6 \) encapsulated uninfected cells or (iii) transplantation with \( \sim 3 \times 10^6 \) encapsulated AdLPK-GLP-1 infected cells. Blood glucose levels were determined with either a (B) glucometer or (C) Glucose (Trinder) Assay kit. At 3 weeks after transplantation, (D) 4-hour fasted plasma GLP-1 levels were determined using a GLP-1 (total) RIA kit. \( n = 4 \) for each treatment group; \(*P < 0.05 \) (compared to uninfected controls).
48.1 ± 0.1 g) for the duration of the study. A limitation with the glucometer used in this study is that it is unable to accurately measure blood glucose levels greater than 33.3 mM. Since the blood glucose levels of db/db mice were greater than or equal to 33.3 mM, it was difficult to assess whether the transplantation had any effect on their blood glucose using the glucometer. To address this problem, plasma samples from db/db mice 1 week before and 2 weeks after transplantation (day 56 to 74) were assayed using a glucose assay that permitted accurate measurements of blood glucose levels greater than 33.3 mM. Results from the glucose assay indicated that there was no significant difference in blood glucose levels between the treatment groups after transplantation (Fig. 31C).

When the plasma GLP-1 levels were examined at ~3.5 weeks after transplantation, a 1.5-fold increase was observed in animals transplanted with capsules containing AdLPK-GLP-1 infected cells compared to those transplanted with capsules containing uninfected cells (infected: 51.5 ± 8.5 pM vs. uninfected: 34.9 ± 4.5 pM; Fig. 30D). Animals receiving sham surgery or encapsulated uninfected cells had similar plasma GLP-1 levels (sham: 35.6 ± 8.5 vs. uninfected: 34.9 ± 4.5 pM; Fig. 31D).

**Imaging of capsules containing uninfected or AdLPK-GLP-1 infected HEPA 1-6 cells**

Encapsulated cells were examined prior to transplantation and after retrieval from transplanted animals (~3.5 weeks after transplantation) to examine changes in cell growth and capsule structure (Fig. 32). Prior to transplantation, similar cell density per capsule was observed for the two treatment groups (uninfected and AdLPK-GLP-1 infected). Furthermore, the capsules were fairly uniform in size (700 to 800 μm in diameter) and were spherical in shape (Fig. 32A and B). After ~3.5 weeks in the transplanted animals, large aggregates of cells were noticeable in the capsules (Fig. 32C and D). While the majority of the capsules remained intact, the presence of cells at the bottom of the tissue culture plates indicated that some of the capsules might have ruptured during the transplantation or capsule recovery process (Fig. 32C and D). It is also possible that these cells are not the transplanted hepatocyte cells but are cells that originated from the animal. Furthermore, fibrosis developed on the outside surface of some of the capsules making it difficult to examine its contents.
Figure 32. Comparison of capsules containing (A and C) uninfected or (B and D) AdLPK-GLP-1 infected HEPA 1-6 cells. Capsules obtained (A and B) after encapsulation or (C and D) after retrieval from transplanted male \(db/db\) mice were examined under 100x magnification with an Axiovert 200 inverted microscope (Carl Zeiss). \(Bar = 95 \mu m\).
In Vitro GLP-1 Production from Hepatocytes Transduced with an Adenoviral Vector Expressing GLP-1

GLP-1 is a potent glucose-dependent insulinotropic hormone released from L-cells of the distal intestine after a meal. In addition to its insulinotropic effects, GLP-1 enhances β-cell mass, suppresses glucagon secretion, inhibits gastric emptying and acid secretion, promotes satiety and possibly stimulates insulin-independent glucose disposal in peripheral tissues [24]. Continuous infusion of GLP-1 in subjects with T2D resulted in the normalization of their blood glucose levels [40]. However, clinical applications of GLP-1 are limited due to its short biological half-life. Gene therapy may be an attractive mode of GLP-1 delivery whereby endogenous release of GLP-1 from cells within the body can be achieved [130]. In order to deliver the desired gene into the body, both non-viral and viral vectors have been used in gene therapy research. Amongst these vectors, the adenoviral vector is the most commonly used in preclinical and clinical studies due to its ability to effectively transduce a wide range of cells [140, 142]. In this study, an adenoviral vector expressing DPIV resistant GLP-1 under the control of a LPK promoter (AdLPK-GLP-1) was constructed using standard molecular cloning techniques (Fig. 2 to 5). Initial studies demonstrated that AdLPK-GLP-1 could effectively transduce a fetal human hepatic cell line, WRL 68, to produce immunoreactive GLP-1 (Fig. 7). Furthermore, this effect was due to the specific transgene expressed by the AdLPK-GLP-1 since no increase in immunoreactive GLP-1 was observed with cells infected with AdCMV-βGal (Fig. 7).

The LPK promoter is normally involved in regulating the expression of LPK, an important enzyme involved in the glycolytic process in the liver [171]. The LPK promoter used for driving GLP-1 expression in AdLPK-GLP-1 is a 183 bp fragment spanning from -183 to +11 nucleotide relative to the LPK cap site [153]. The DNA elements found in this portion of the LPK promoter, in the 3'-5' direction upstream of the TATA box, include L1, L2, L3, and L4 [147]. Previous studies
have demonstrated that the L1 and L3 elements are important for conferring liver-specificity [149, 153, 172, 173]. Consistent with these studies, AdLPK-GLP-1 was able to transduce cells in a dose-dependent and, more importantly, liver-specific manner. This latter effect was demonstrated by the presence of GLP-1 production in hepatocytes (WRL 68, Huh7, and HEPA 1-6) but not in non-hepatocytes (IEC-6, INS-1, and 3T3-L1) (Fig. 10 and 11). Interestingly, uninfected INS-1 cells produced high basal levels of GLP-1 and upon infection with AdLPK-GLP-1, the amount of GLP-1 produced decreased (Fig. 11B). This observation can be explained by the fact that this cell line expresses the proglucagon gene and PC3 [174]. The decrease in GLP-1 production is attributed to the lower number of infected cells compared to uninfected cells (10% vs. 50% cell confluency). Given that E1-deleted vectors have been shown to cause cytopathic effects leading to death of transduced cells [175], the decrease in the number of INS-1 cells may be a result of cell death due to infection by AdLPK-GLP-1, an E1/E3-deleted vector.

Two liver transcriptional factors, hepatocyte nuclear factor (HNF) 1 and HNF4, are important for activating the LPK promoter by binding to L1 and L3 elements, respectively [176]. Therefore, the absence of increased GLP-1 production from non-hepatocytes transduced with AdLPK-GLP-1 could be explained by the fact that these cells lack HNF1 and HNF4 protein required for activating the LPK promoter. Indeed, both HNF1 and HNF4 mRNA levels are low in most non-liver cells [177, 178]. Furthermore, Park et al. [179] were able to induce LPK promoter activity in a kidney cell line (HEK 293) following introduction of HNF1α cDNA [179]. Similarly, co-transfection of fibroblast cells (NIH 3T6) with HNF4 expression vector induced activation of the LPK promoter [180]. Surprisingly, not all the hepatocyte cell lines (FAO, H4-II-E-C3 and Clone 9) examined in this study produced GLP-1 upon infection with AdLPK-GLP-1 (data not shown). It is possible that HNF1 and HNF4 are expressed at insufficient levels in these cell lines and therefore, similar to non-hepatocyte cell lines, are unable to activate the LPK promoter. A correlation between HNFα mRNA levels and LPK activity has been previously reported [181]. Variable expression of these transcription factors could also explain why the other liver cell lines express differing amounts of GLP-1 (Fig. 10).
Indeed, several studies have demonstrated that the level of HNF1 and/or HNF4 mRNA levels are variable between different liver cell lines [177, 181, 182]. Therefore, it is possible that differing levels of HNF1 and HNF4 between WRL 68, Huh7 and HEPA 1-6 cells activate the LPK to different degrees resulting in the variable GLP-1 expression. Given the similar expression levels of β-galactosidase in these cell lines following transduction with AdCMV-βGal (Fig. 12), differing transduction efficiencies by adenovirus do not appear to account for the differences.

An attractive feature of the LPK promoter is its ability to confer glucose responsiveness in vivo and in primary hepatocytes [154, 155, 173]. Specifically, this glucose sensitivity is regulated by a glucose/insulin response element, L4, present within the 183 bp 5'-flanking region of the LPK promoter [153, 154, 180, 183, 184]. This characteristic is ideal for GLP-1 gene delivery in diabetes whereby it may be possible to achieve maximal production of GLP-1 under hyperglycemic conditions (e.g. diabetes) and minimal during hypoglycemia from engineered hepatocytes. Therefore, the ability of transduced hepatocytes to produce different levels of GLP-1 in response to different concentrations of glucose was examined. Of the 3 hepatocyte cell lines examined, transduced Huh7 cells produced GLP-1 in a glucose-dependent manner (Fig. 13). However, this is not surprising since it has been shown that glucose control of the LPK promoter is lost in some well-differentiated rat hepatoma cell lines [185]. Meienhofer et al. [185] reasoned that this effect was due to changes that occurred in the cells during the establishment of these hepatoma lines resulting in the loss of regulatory systems normally found in the liver. Consequently, Antoine et al. [186] demonstrated that GLUT2, a glucose transporter normally found in the liver, is important for the glucose regulation of LPK promoter in hepatoma cell lines. In their study, hepatoma cells expressing GLUT2 were able to induce LPK expression in a glucose-dependent manner. In contrast, LPK expression from hepatoma cells lacking GLUT2 is not regulated by glucose and upon transfection with a GLUT2 expression vector, glucose-responsiveness was observed. Huh7 cells have been shown to express GLUT2 mRNA [187]. Therefore, this might explain the glucose-dependent GLP-1 production from Huh7 cells transduced
with AdLPK-GLP-1. Conversely, the absence of glucose-dependent GLP-1 production in transduced WRL 68 and HEPA 1-6 cells may be due to the absence of GLUT2.

Most in vivo studies using first-generation adenoviral vectors resulted in transgene expression that typically lasted only a few weeks [188-191]. Consistent with these findings, GLP-1 production from transduced WRL 68 and HEPA 1-6 cells peaked at 3 days post infection followed by a rapid decline to undetectable GLP-1 levels by 2 weeks (Fig. 14A and B). Furthermore, passaging HEPA 1-6 cells did not prolong the duration of GLP-1 production (Fig. 14C). These data suggest that the decline in GLP-1 production is not due to a decrease in cell activity as a result of cells reaching confluency. Given that first-generation adenoviral vectors have been shown to express low levels of viral proteins that are responsible for inducing death of transduced cells [175, 190, 192], it is possible that part of the decline in GLP-1 production is due to cell death. A more important contribution to the transient GLP-1 production may be due to the episomal nature of adenoviral vectors. Since adenoviral vectors do not integrate their genome into the host chromosome, the viral genome is more susceptible to degradation by nucleases over time [193].

**Determination of Biologically Active GLP-1 using a GLP-1 Bioassay**

Numerous in vivo studies have reported that a glycine substitution at the 8th amino acid position of GLP-1\textsubscript{7.36 amide} and GLP-1\textsubscript{7.37} yields an analogue with increased resistance to DPIV degradation and improved biological activity compared to native GLP-1 [159, 194, 195]. As a result of these characteristics, a glycine-substituted GLP-1\textsubscript{7.37} was incorporated in AdLPK-GLP-1. Although studies demonstrated that AdLPK-GLP-1 transduced hepatocytes can produce immunoreactive GLP-1 (Fig. 10), its biological activity could not be confirmed with the commercially available GLP-1 ELISA kit from Linco (Fig. 15A) as the antibody used in this ELISA is unable to recognize the N-terminally modified GLP-1 (Fig. 15B). Therefore, this result necessitates the development of an assay for detecting bioactive GLP-1.
Binding of biologically active GLP-1 to its receptor, a member of the G-protein-coupled receptor family, leads to activation of the cAMP pathway resulting in gene transcription driven by CRE [24]. Therefore, one of the ways for assessing biological activity of a protein is its ability to activate intracellular signalling pathways upon receptor binding. Luciferase reporter genes coupled to G-protein-linked receptors are commonly used for detecting the activation of intracellular signalling pathways because it encodes a functional enzyme immediately upon translation [196]. The luciferase enzyme is involved in a catalytic reaction that results in the emission of light, or luminescence, that is easily quantified using a luminometer [196]. Given this knowledge, a GLP-1 bioassay was developed utilizing a HEK 293 cell line stably expressing human GLP-1 receptor and a luciferase gene whose expression is driven by CRE (HEK-hGLP1R-Luc). As a result, the level of luminescence signal measured is proportional to the amount of biologically active GLP-1 exposed to the cells.

Since the luminescence signal can be affected by assay conditions, the effects of phenol red and different culture media on the GLP-1 bioassay were examined prior to measuring medium samples of transduced hepatocytes for bioactive GLP-1. When measuring GLP-1 standards in the presence or absence of phenol red-containing medium, an ~2.5-fold increase in luminescence was observed in the absence of phenol red (Fig. 18A). This is in agreement with studies conducted by Promega that observed a 2.5-fold increase in luminescence upon removal of phenol red [197]. Surprisingly, the GLP-1 standard curves generated under the two different conditions (phenol red vs. PBS) were superimposable when the luminescence was normalized (Fig. 18B). Therefore, these results suggest that the presence of phenol red does not affect the sensitivity of the bioassay.

The possibility of using one standard curve in the GLP-1 bioassay for measuring samples obtained from cells cultured in different media would greatly enhance the simplicity and convenience of this assay for future use. However, a significant difference in luminescence was observed when the standard curves generated from GLP-1 standards were prepared in either HG-DMEM + 1% BSA or Hams-F10 + 0.5% BSA (Fig. 19). Specifically, HEK-hGLP1R-Luc cells incubated with GLP-1 standards prepared in Hams-F10 produced higher luminescence than those incubated with standards...
prepared in HG-DMEM. This would be an expected observation if the medium samples were left in
the wells during luminescence measurements because the phenol red concentration in Hams-F10 is
less than HG-DMEM (1.2 mg/L vs. 15 mg/L phenol red). However, in this study, luminescence was
measured after the medium was removed from the wells by inverting the 96-well plate. Therefore, it
was anticipated that there would be no difference in luminescence between GLP-1 standards prepared
in Hams-F10 and HG-DMEM. The fact that a difference was observed suggests that the presence of
even a small amount of medium left in the well was sufficient to induce significant changes in
luminescence. Based on these results, it is important that samples are compared to GLP-1 standards
made up in the same medium.

Since future studies with AdLPK-GLP-1 will be conducted in animals, it was important to
examine the potential of using the bioassay for measuring GLP-1 levels in plasma samples.
GLP-1 standards prepared in undiluted or medium-diluted plasma produced significantly lower
relative luminescence compared to standards prepared in medium (Fig. 20B) suggesting a decrease in
sensitivity of the GLP-1 bioassay. Incubation of GLP-1 with plasma has been shown to lead to
degradation of the peptide due to the presence of DPIV in the plasma [198]. Therefore, the decrease
in sensitivity of the GLP-1 bioassay in measuring GLP-1 standards prepared in plasma can be
attributed to the degradation of GLP-1 by DPIV. This reasoning is strengthened by the fact that an
increase in relative luminescence was observed when plasma was diluted in protease-free medium
(Fig. 20B). Therefore, these findings demonstrate that the GLP-1 bioassay is able to measure
bioactive GLP-1 from plasma samples, albeit with a narrower range of concentrations and this can be
improved by diluting the plasma.

Unlike the Linco GLP-1 ELISA, this GLP-1 bioassay is able to detect N-terminally modified
GLP-1 (Fig. 21A). However, it is important to note that a rightward shift in the GLP-1 standard
curves was observed in this bioassay when standards were prepared using [Gly8]-GLP-17-36 amide or [Ser8]-
GLP-17-36 amide compared to GLP-17-36 amide (Fig. 21A). Therefore, this shift suggests that the N-
terminally modified GLP-1 analogues have reduced biological activity compared to the native
peptide. This is a reflection of reduced receptor binding and activation of the GLP-1 receptor.

Indeed, several studies have demonstrated, in certain cases, slightly lower binding affinity of these analogues to GLP-1 receptor [194, 195]. However, this reduced activity in vitro might be outweighed by increased bioactivity in vivo as a result of increased stability in plasma. Indeed, Xiao et al. [195] demonstrated that glycine-substituted GLP-1 have improved biological activity in vivo despite a relatively lower receptor binding and cAMP activation in vitro.

This novel bioassay confirmed that biologically active GLP-1 was produced from AdLPK-GLP-1 transduced hepatocytes (Fig. 21B). In addition, this level of bioactive GLP-1 reflects ~10 to 15% of total immunoreactive GLP-1 as assessed by GLP-1 RIA. The lower percentage of bioactive GLP-1 compared to total GLP-1 is not surprising given that degradation of GLP-1 can occur. Although the GLP-1 sequence in AdLPK-GLP-1 was constructed to have DPIV resistance, the possibility that GLP-1 degradation by other enzymes cannot be ruled out. In addition to DPIV, NEP 24.11 is another enzyme found in the body that has been shown to be involved in GLP-1 degradation [77]. Since the transduced cells are cultured in media supplemented with FBS, it is possible that this enzyme is also present to degrade GLP-1. Furthermore, bioactive GLP-1 was measured from medium samples that were left incubating with transduced cells for 3 days before being collected for assay thereby increasing the likelihood of GLP-1 degradation.

**Ex Vivo Delivery of AdLPK-GLP-1**

**In normal mice: effects of AdLPK-GLP-1 on plasma GLP-1 levels**

*Ex vivo* gene delivery is achieved by transducing isolated cells with a gene of interest and subsequently transplanting the engineered cells into the body. A major limitation with this method of delivery is the risk of immune response against the transplanted cells. To address this problem, Chang [199] developed the technology of cell microencapsulation. This technology involves enveloping cells in a semipermeable membrane in order to form a mechanical barrier separating the host immune cells and antibodies from the cells yet allow the diffusion of cellular products, nutrients
and wastes [131, 133]. Encapsulated insulin-producing cells retained function and were protected from immune destruction for up to 3 months when transplanted into diabetic rodents [160, 161]. Therefore, these studies demonstrate the feasibility of using encapsulated cells in animals.

WRL 68 and HEPA 1-6 cells produced greater than 100 pM GLP-1 upon infection with AdLPK-GLP-1 at a MOI of 500 (Fig. 10) and were thus selected for transplant. Initial studies did not increase plasma GLP-1 levels in CD-1 mice transplanted with encapsulated engineered WRL 68 cells despite the fact that these cells were producing ~250 pM GLP-1 at 2 days post infection (Fig. 22A and 23A). This result was attributed to the fact that these cells were not producing sufficient levels of GLP-1 to increase plasma GLP-1 levels. To address this problem, HEPA 1-6 cells were next used for encapsulation since these cell lines were found to produce ~10 times more GLP-1 than WRL 68 cells when transduced with AdLPK-GLP-1 at a MOI of 500 (Fig. 10). Furthermore, cells were encapsulated and transplanted into animals at 1 rather than 2 days post infection. This was to ensure that the day of plasma collection (2 days post transplantation) coincided with the day HEPA 1-6 cells produce the maximum amount of GLP-1 (3 days post infection; Fig. 14B and 24A). When CD-1 mice were transplanted with encapsulated engineered HEPA 1-6 cells, a 2-fold increase in plasma GLP-1 levels was observed 2 days post transplantation (Fig. 24D).

Vos et al. [133] have reported that alginate-based capsules, such as the ones used in this study, can invoke an immune response leading to fibrotic overgrowth of capsules and subsequent death of the encapsulated cells. Therefore to determine whether this could also be a contributing factor to the relatively small increase in plasma GLP-1 levels, the capsules retrieved from the transplanted animals were examined. Since the majority of the retrieved capsules remained intact and free of fibrosis with clear signs of cell growth, immune response to the capsules is not the reason for the relatively small increase in plasma GLP-1 levels (Fig. 25C and D).

The transplantation site has been shown to be an important factor on the kinetics of hormone transport into the bloodstream. Several studies mimicked the release of insulin from encapsulated islets transplanted into the peritoneal cavity by intraperitoneal infusion of insulin in rats. In these
studies, Vos et al. [134, 135] observed a small and delayed increase in plasma insulin levels compared to intraportal infusion of insulin. This was attributed to the lack of proximity of the peritoneal site to the bloodstream resulting in the limited diffusion of insulin from the peritoneal cavity to the bloodstream. Unlike the peritoneal cavity, the kidney capsule is a highly vascularized site. Okugawa et al. [200] reported that islet transplantation under the kidney capsule of rats was more effective in normalizing blood glucose levels than transplantation into the peritoneal cavity. Therefore, to address whether improved proximity of transplantation site to the bloodstream could increase plasma GLP-1 levels, engineered HEPA 1-6 cells were transplanted under the kidney capsule of C57Bl/6 mice. Similar to the encapsulation study, a 2.1-fold increase in plasma GLP-1 levels was observed (Fig. 26). Since the same number of cells was transplanted into the peritoneal cavity and kidney capsule, this observation suggests that the proximity of the cells to the circulation does not limit GLP-1 transport to the circulation under the current experimental conditions. Although a 2-fold increase in plasma GLP-1 levels observed in this study appears relatively small, previous studies have demonstrated that a 2 to 4-fold increase in total GLP-1 levels was sufficient for a therapeutic effect in T2D subjects [37, 201].

In db/db mice: effects of AdLPK-GLP-1 on glucose homeostasis

The db/db mouse lacks a functional leptin receptor and, as a result, develops obesity and diabetes. Therefore, it has been widely used as a rodent model of T2D. Treatment of pre-diabetic and diabetic db/db mice with exogenous GLP-1 or GLP-1 analogues has been shown to delay the onset of diabetes and ameliorate hyperglycemia [166-168, 170, 202]. Furthermore, Burcelin et al. [151] have demonstrated improvement in glucose tolerance in high fat fed mice transplanted with encapsulated myoblast cells secreting the same GLP-1 analogue used in this study. Given the success of the ex vivo AdLPK-GLP-1 delivery in increasing plasma GLP-1 levels in normal mice, the effects of transplanting encapsulated hepatocytes engineered to produce GLP-1 on glucose homeostasis in db/db mice were examined.
The *db/db* mice exhibited a rapid increase in body weight and fasting blood glucose over the span of 6 to 9 weeks of age; at which point the blood glucose levels were equal to or greater than 33.3 mM (Fig. 30A and B). Similar to the results obtained in normal mice, transplantation of encapsulated engineered hepatocytes into *db/db* mice at ~15 weeks of age resulted in a 1.5-fold increase in plasma GLP-1 levels. Even more interesting is that this increase was observed at 3.5 weeks post transplantation suggesting that the encapsulated cells were still capable of secreting GLP-1 *in vivo* at that time. Moreover, the GLP-1 levels may have been even higher at earlier time points. When capsules were retrieved from transplanted animals, the majority remained intact with some capsules exhibiting fibrotic overgrowth (Fig. 31). Fibrotic overgrowth has been reported to contribute to islet cell death due to limited passage of nutrients to the cells. However, in this study, it appears that the presence of fibrotic overgrowth in some capsules has not prevented an increase in plasma GLP-1 levels at 3.5 weeks post transplantation of transduced AdLPK-GLP-1 hepatocytes.

Although studies in rats and human subjects with diabetes have demonstrated a reduction in body weight after treatment with GLP-1 receptor agonists [119, 203], no significant reduction in body weight was observed in *db/db* mice transplanted with encapsulated AdLPK-GLP-1 transduced cells compared to control animals (Fig. 30A). Other studies in these animals have also noted no significant reduction of body weight after treatment with GLP-1 receptor agonists [166, 169, 202]. Therefore, the effect of GLP-1 on body weight reduction may depend on specific characteristics of the rodent models or human subjects used. As mentioned earlier, *db/db* mice have a mutated leptin receptor. Leptin is a hormone released from adipose tissue and acts on the leptin receptor to reduce food intake and body weight [204]. It is possible that the inability to activate the leptin receptors in *db/db* mice results in an increased tendency to eat excessively and conserve energy thereby counteracting the GLP-1 effects on reducing body weight.

No reduction in blood glucose levels was observed in *db/db* mice transplanted with encapsulated transduced cells (Fig. 30C). A factor that could contribute to the lack of efficacy of GLP-1 on improving blood glucose levels in diabetic *db/db* mice is the severity of diabetes at the onset of
treatment. Most studies examining effects of GLP-1 on glucose homeostasis of \( db/db \) mice were carried out in animals that were moderately diabetic, with fasting blood glucose levels at \(~12\) to \(15\) mM. In this study, all animals had blood glucose levels at \(~35\) mM on the day of transplantation. Furthermore, studies have shown that treatment with GLP-1 analogues in \( db/db \) mice were less effective in normalizing blood glucose levels in older and more diabetic animals \([166, 169]\). Similarly, a 3-fold increase in plasma active GLP-1 in \( db/db \) mice at 23 weeks of age after inhibition of DPIV did not improve glucose tolerance whereas an improvement was observed when these animals were 6 weeks of age \([165]\). Thus the lack of efficacy in these studies is likely attributable to severe insulin resistance and reduced function of the \(\beta\)-cells in severely diabetic animals. The lack of efficacy of GLP-1 in reducing body weight and blood glucose levels may also be attributed to insufficient plasma levels of bioactive GLP-1.

### In Vivo Delivery of AdLPK-GLP-1 in Normal Male Rats and Mice

Several studies have demonstrated that \textit{in vivo} delivery of adenoviral vectors, at a dose of \(0.1\) to \(8 \times 10^{10}\) pfu, has been shown to be effective in inducing transgene expression from hepatocytes in rodents \([191, 205-207]\). Numerous methods have been described for liver-directed gene delivery in rodents including direct administration of viral vectors into the liver, portal vein, or tail vein \([164, 205-209]\). Systemic administration via the tail vein is attractive because it is a simple and non-invasive technique. Furthermore, a recent comparison of portal and tail vein delivery of an adenoviral vector encoding green fluorescent protein (GFP) in rats demonstrated that a stronger and more homogenous distribution of GFP was achieved with the tail vein method \([164]\). Recently, Oh \textit{et al.} \([152]\) demonstrated the effectiveness of GLP-1 gene therapy using a GLP-1 plasmid-polyethyleneimine polymer complex. In this study, they observed a decrease in blood glucose levels in a rat model of T2D that was associated with an increase in both plasma GLP-1 and insulin levels. Given the effectiveness of \textit{ex vivo} delivery of AdLPK-GLP-1 in increasing plasma GLP-1 levels in
normal rodents, the effects of administering AdLPK-GLP-1 via the tail vein at a dose of 1 or 5x10^9 pfu in normal rats and mice were examined in this study.

In normal rodents, peripheral administration of GLP-1 receptor agonists have been associated with, in certain cases, modest weight loss [202, 203, 210]. Furthermore, the glucose lowering effect of GLP-1 has been shown to be glucose-dependent [168]. Consistent with this finding, other studies have reported only slight reduction or no change in blood glucose levels in normal rodents treated with GLP-1 [169, 202, 203]. Therefore, in addition to examining the effects of in vivo delivery of AdLPK-GLP-1 in plasma GLP-1 levels, body weight and blood glucose levels of normal mice and rats were monitored for 1 to 2 week following virus injection.

In contrast to the ex vivo study (Fig. 24D and 26), no increase in plasma GLP-1 levels in rats or mice given an intravenous administration of AdLPK-GLP-1 compared to those given AdCMV-βGal were observed (Fig. 27C and 29C). Given this observation, it was not surprising that there was no significant difference in body weight or blood glucose between treatment groups (Fig. 27A,B and 29A,B). To determine whether the absence of increase in plasma GLP-1 levels was associated with decreased efficacy of viral delivery and transduction, β-galactosidase staining in the liver of animals given AdCMV-βGal was performed. For the rat study, no β-galactosidase positive cells were observed in the liver sections (Fig. 28). These data suggest that the lack of effect on plasma GLP-1 levels in AdLPK-GLP-1 treated rats may be associated with ineffective tail vein delivery of the adenoviral vector into the liver. Problems with viral delivery could have occurred if the virus was injected subcutaneously rather than directly into the tail vein. Injection of virus into the surrounding tissues rather than the vein is often indicated by the formation of a bleb or whitening of the surrounding tissue. Given that this was observed in some of the rats during tail vein injection, it is possible that problems associated with tail vein administration is one of the reasons for the absence of viral delivery into the liver. In addition, Wang et al. [211] demonstrated that administration of AdCMV-βGal vector at 7x10^10 pfu/animal resulted in only 40% of the liver cells being intensely
stained for β-galactosidase. Since the dose of AdCMV-βGal used in this study was 70 times lower, the lack of β-galactosidase staining could also be associated with low viral dose. Indeed, intravenous administration of low doses of adenoviral vectors (1 to 3x10⁹ viral particles) have been shown to be ineffective in inducing transgene expression from the liver [212, 213]. Furthermore, Worgall et al. [214] demonstrated that 90% of the viral genome was eliminated from the liver within 24 hours following intravenous administration of adenoviral vectors in mice. These effects were shown to be due to uptake and phagocytosis of adenoviral vectors by Kupffer cells, a type of tissue macrophage located in the liver sinusoids [212, 213, 215]. Studies reported that depletion of Kupffer cells prior to adenoviral vector administration resulted in increased transgene expression [212, 215]. Therefore, the combination of poor viral delivery and low viral dose may have contributed to the lack of increase in plasma GLP-1 levels following intravenous delivery of AdLPK-GLP-1 in rats.

In contrast to the rat study, β-galactosidase staining was observed in all liver sections of mice given AdCMV-βGal (Fig. 30). Specifically, ~80-90% of cells were β-galactosidase positive in 3 of 4 mice. This is in agreement with numerous studies that observed 80-90% β-galactosidase transduction of liver sections using adenoviral vector dose of 1 to 5x10⁹ pfu [163, 191]. Therefore, the lack of increase in plasma GLP-1 levels of AdLPK-GLP-1 treated mice compared to AdCMV-βGal treated mice was not due to inefficient viral delivery and transduction of the liver cells. Furthermore, it is unlikely that the doses of AdLPK-GLP-1 (1 and 5x10⁹ pfu) used were too low given that a high percentage of hepatocytes were transduced with AdCMV-βGal at 1x10⁹ pfu (Fig. 30).

First-generation (E1 or E1/E3-deleted) viral vectors have been shown in several animal studies to mediate only transient protein expression lasting anywhere from 7 to 21 days [188-190, 205]. This effect is believed to be due to activation of both innate and adaptive immune responses against the adenoviral vectors leading to the elimination of adenoviral vectors and transduced cells [216]. The innate immune response, the first line of host defense, is activated rapidly following viral entry and typically lasts 4 days [216]. This response is mediated by neutrophils, macrophages and natural killer
cells. The contribution of the innate immune response in the clearance of adenoviral vectors has been demonstrated by the rapid elimination of viral genome from immunodeficient mice and by increased production of inflammatory cytokines [214, 217, 218]. The adaptive immune response occurs several days after the initiation of the innate immune response and consists of both cellular and humoral components [216]. Of these two components, only the cellular immune response affects the persistence of transgene expression. Several studies have reported that low expression of viral genes is sufficient to activate cytotoxic T-cells against the adenoviral vector resulting in the elimination of adenoviral vector transduced cells [163, 192, 219]. Since AdLPK-GLP-1 is a first generation adenoviral vector, it is possible that there was an activation of the immune response leading to the destruction of AdLPK-GLP-1 and transduced hepatocytes in the mice. This may have resulted in transient expression of GLP-1 such that an increase in plasma GLP-1 levels was no longer present in mice at 7 days following AdLPK-GLP-1 administration when blood samples were analyzed.

Many adenoviral vectors contain a deletion in the E3 region because it is not essential for viral replication and allows for incorporation of a larger transgene [216]. However, there is increasing evidence that the presence of E3 region is important for modulating the host immune response. For example, Ilan et al. [220] reported that administration of an adenoviral vector containing the E3 region resulted in the inhibition of cellular and humoral immune response against the transduced cells. Therefore, the absence of E3 region in AdLPK-GLP-1 may increase its susceptibility to immune attack thereby reduce the duration of GLP-1 expression.

An unresolved issue in the mouse study is the reason for a significant decrease in plasma GLP-1 levels of mice given $1 \times 10^9$ pfu AdLPK-GLP-1 compared to the control mice (Fig. 29C). It is possible that the difference in plasma GLP-1 levels is a reflection of the amount of endogenous GLP-1 produced by these animals. Therefore, one potential reason for the lower levels of GLP-1 detected is the duration of fasting period in these animals. This is relevant since endogenous GLP-1 secretion is stimulated by the presence of nutrients. All animals were fasted at the same time but due to the lengthy procedures involved in tissue harvest and cardiac puncture, some of the animals were fasted...
for significantly longer times than others. Since plasma samples from the majority of animals in the
AdCMV-βGal group were obtained earlier than animals in the 1x10⁹ pfu AdLPK-GLP-1 group, it is
possible that they had higher endogenous GLP-1 present in their plasma.
CONCLUSIONS

Since the initiation of this thesis investigation, the majority of gene transfer studies for diabetes have focused on delivering insulin by adenoviral vectors to achieve endogenous hepatic insulin secretion. Although there have been studies that examined the potential of gene transfer of GLP-1 for treatment of diabetes, there have not been studies that were directed to adenovirally mediated GLP-1 production in the liver. The results from these current studies provide further support of the effectiveness of using adenoviral vectors to transfer therapeutic genes into cells. Specifically, an adenoviral vector expressing DPIV resistant GLP-1$_{7,37}$ under the control of a liver-specific promoter (AdLPK-GLP-1) can transduce liver, but not several non-liver cell lines to produce GLP-1 in vitro.

In addition, GLP-1 production from the engineered hepatocytes could, depending on the characteristics of the cell line, be regulated by glucose. Furthermore, GLP-1 production from hepatocytes was transient in vitro. Due to the N-terminal penultimate substitution in the GLP-1 sequence of AdLPK-GLP-1, a novel GLP-1 bioassay that was capable of detecting N-terminally modified GLP-1 was developed. From this bioassay, it was established that the engineered hepatocytes were producing biologically active GLP-1. Furthermore, transplantation of GLP-1 producing hepatocytes resulted in an increase in plasma GLP-1 levels in both normal and db/db mice. Despite the increase in plasma GLP-1 levels, no effect on blood glucose or body weight in db/db mice was observed. These results are in agreement with previous studies that found little or no improvement in glucose homeostasis in severely diabetic db/db mice compared to those that were only moderately diabetic. Therefore, at this stage, it cannot be concluded whether GLP-1 gene therapy would be a feasible option for treating diabetes in these animals. Finally, while these studies revealed that intravenous administration of AdLPK-GLP-1 could not effectively increase plasma GLP-1 levels in normal rodents, the success of the ex vivo gene therapy approach provides a basis for further investigation into optimizing AdLPK-GLP-1 for in vivo gene delivery.
FUTURE STUDIES

Several future studies based on this thesis are warranted in order to evaluate the feasibility of a GLP-1 gene therapy strategy for the treatment of diabetes. First, the structure of the biologically active GLP-1 produced from AdLPK-GLP-1 transduced cells should be established. To determine the structure of the peptide, reverse-phased high performance liquid chromatography (RP-HPLC) and mass spectroscopy on medium samples from transduced cells could be performed. RP-HPLC can detect small changes in the GLP-1 sequence such as that occurs with N-terminal degradation by DPIV. Mass spectroscopy permits the determination of the molecular mass of the degraded products separated by RP-HPLC.

Second, in addition to the low levels of biologically active GLP-1, the severe diabetic state of the db/db mice used in this study could contribute to the lack of therapeutic effect on blood glucose levels of the AdLPK-GLP-1. Therefore, to address this issue, younger db/db mice should be used for subsequent transplantation experiments using AdLPK-GLP-1 transduced cells.

Finally, it should be determined whether the lack of effect on plasma GLP-1 levels following in vivo AdLPK-GLP-1 delivery was due to the transient nature or the dose of viral vector. This issue could be addressed by examining plasma GLP-1 levels at earlier days post viral injection and using a broader range of AdLPK-GLP-1 dose. Furthermore, studies should be carried out to examine whether the immune response plays an important role in limiting GLP-1 expression from the transduced hepatocytes. Evaluation of immune response activation could be accomplished by measuring cytokine levels involved in mediating both innate and adaptive immune responses.
REFERENCES


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