Development of a novel Pck-1:eGFP reporter zebrafish line for the
discovery and evaluation of potential anti-diabetic drugs

By

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Development of a novel \textit{Pck-1:eGFP} reporter zebrafish line for the discovery of potential anti-diabetic drugs

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Abstract

Overexpression of Phosphoenolpyruvate carboxykinase - cytosolic (PEPCK, encoded by \textit{Pck-1} gene) has been found to be associated with the prevalence of hyperglycemia in Type 2 Diabetes Mellitus (T2DM) patients. The \textit{Pck-1} enzyme catalyzes the rate limiting step in endogenous glucose production. The aims of this study are to develop a \textit{Pck-1:eGFP} reporter zebrafish and validate it as a potential tool for the screening of novel anti-diabetic compounds. 3.6 kb zebrafish \textit{Pck-1} promoter fragment was cloned and a \textit{Pck-1:eGFP} expression vector was constructed. After DNA microinjection, we generated \textit{Pck-1:eGFP} reporter zebrafish with strong eGFP expression in developing liver. Validation studies confirmed that \textit{Pck-1:eGFP} zebrafish embryos responded to treatment of glucose, cAMP and dexamethasone, metformin and rosiglitazone similarly to that of humans. This novel \textit{Pck-1:eGFP} reporter fish line can serve as a tool for the screening and development of novel anti-diabetic drugs that may have potential in the treatment of T2DM.
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<th>Description</th>
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<tbody>
<tr>
<td>Ac/Ds</td>
<td>Activator/Dissociation transposon system</td>
</tr>
<tr>
<td>ADME</td>
<td>Administration, distribution, metabolism, excretion</td>
</tr>
<tr>
<td>AF-1</td>
<td>Accessory Factor-1</td>
</tr>
<tr>
<td>Akt/PKB</td>
<td>Akt/Protein Kinase B</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>aPKC</td>
<td>Atypical protein kinase C</td>
</tr>
<tr>
<td>AT-Ra</td>
<td>All trans retinoic acid</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CAAT/enhancer binding protein-α</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP regulatory element binding protein</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Dax-1</td>
<td>Dosage sensitive sex reversal adrenal hypoplasia congenital critical region on X chromosome gene 1</td>
</tr>
<tr>
<td>DEX</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
</tr>
<tr>
<td>Dpf</td>
<td>Days post fertilization</td>
</tr>
<tr>
<td>DPP-4</td>
<td>Dipeptidyl peptidase-4</td>
</tr>
<tr>
<td>DSP</td>
<td>Distal symmetrical polyneuropathy</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>FOXO1</td>
<td>Forkhead box 1</td>
</tr>
<tr>
<td>Fructose 1, 6-bis-P, F1,6P</td>
<td>Fructose 1,6-bisphosphate</td>
</tr>
<tr>
<td>GLIP</td>
<td>Glipizide</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon like peptide-1</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid regulatory element</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase-3β</td>
</tr>
<tr>
<td>G6P</td>
<td>Glucose 6-P’ase: Glucose-6-phosphatase</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter type 4</td>
</tr>
<tr>
<td>HNF-1</td>
<td>Hepatocyte nuclear factor 1</td>
</tr>
<tr>
<td>Hpf</td>
<td>Hours post fertilization</td>
</tr>
<tr>
<td>HTS</td>
<td>High throughput screening</td>
</tr>
<tr>
<td>IPF-1</td>
<td>Insulin promoting factor-1</td>
</tr>
<tr>
<td>MET</td>
<td>Metformin</td>
</tr>
</tbody>
</table>
OAA: Oxaloacetate

Pck-1: Phosphoenolpyruvate Carboxykinase-1 (gene)

Pdx1: Pancreatic and duodenal homeobox 1

PEP: Phosphoenolpyruvate

PEPCK: Phosphoenolpyruvate carboxykinase – cytosolic isozyme

PEPCK-M: Phosphoenolpyruvate carboxykinase – mitochondrial isozyme

PGC-1: Peroxisome proliferator-activated receptor-γ coactivator 1

PP: Pancreatic polypeptide

PI3K: Phosphoinositide-3-kinase

PPARγ: Peroxisome proliferator-activated receptor-γ

ROSI: Rosiglitazone

RXR: Retinoic x receptor

SCORE: Specimen in a corrected optical rotational enclosure

SIRT1: Sirtuin 1

SNP: Single nucleotide polymorphism

STZ: Streptozotocin

TBDs: Transcription binding domains

TORC2: Target of rapamycin complex 2

T1DM: Type 1 Diabetes Mellitus

T2DM: Type 2 Diabetes Mellitus
Chapter 1: Introduction

1.1 Diabetes Mellitus

Diabetes Mellitus (DM) is a prevalent and current medical problem for the global society. It is predicted that by 2030, more than 438 million people will be affected by DM [1]. The prevalence continues to increase in westernized countries and it was projected that in the year 2000, every one of 3 individuals would have had a likelihood of being diagnosed with DM [1]. In Canada, there are 3 million people living with DM and it is expected that in 2020, more than 3.7 million people will have DM [1]. Globally, 285 million people are affected by DM and by 2030, the number of people with DM is projected to double [1]. Financially, the cost of DM to the health care system in Canada will be $16.9 billion per year [1]. DM is mainly divided into 2 categories, Type 1 and 2, which are the most common forms of DM [2]. DM is characterized by hyperglycemia as well as the dysregulation of lipid, protein and carbohydrate metabolism in the body [2]. This is due to impaired insulin action and/or insulin secretion. In patients, DM causes thirst, blurry vision, weight loss, and polyuria [2]. Often, hyperglycaemia leads to pathological and functional changes in the body such as blindness, kidney failure, foot ulcers[2]. DM patients have an increased risk of vascular specific diseases. In severe forms of DM and in the absence of treatment, ketoacidosis may develop resulting in coma and death [2]. Approximately 80% DM patients will die as a result of vascular related disease [1].
1.1.1 Type 1 Diabetes Mellitus (T1DM)

T1DM accounts for 5-10% of DM patients worldwide [3]. It is an autoimmune disease that causes pancreatic β-cell destruction that typically develops in younger individuals [2]. β-cell destruction is caused by autoimmune processes, with individuals being asymptomatic for years until the amount of destruction is significant enough to cause insufficient insulin secretion to regulate glucose homeostasis [3]. These individuals typically require insulin supplementation for survival.

1.1.2 Type 2 Diabetes Mellitus

Type 2 DM (T2DM) is a metabolic disorder of multiple aetiologies. This metabolic disorder is characterized by insulin resistance with relative defects in insulin secretion not due to the destruction of β-cells [2, 4]. Compared to T1DM, it is more common that the onset of T2DM occurs at later stages of life [4] and is preceded by a period of mild hyperglycaemia and insulin resistance [4]. An acute consequence of DM is hyperglycaemia leading to glycosuria, osmotic diuresis and decreased organ function [2]. Another consequence is the increase of lipolysis and amino acid breakdown, resulting in ketoacidosis and muscle wasting [2]. Retinopathy, the most common specific complication of T2DM is the leading cause of blindness within the US population [5]. Nephropathy leading to diabetic kidney disease [6] and neuropathy with the clinical trademark of distal symmetrical polyneuropathy (DSP)[7], are also common in T2DM patients. Long term complications of DM also include increased risk of atherosclerosis, i.e. coronary [8], peripheral disease [9] and cerebrovascular disease [8]. The elevation of fasting blood glucose seen in T2DM patients is related to increased endogenous hepatic glucose production [10].
1.1.3 Current and emerging anti-diabetic drugs

Drugs that are currently used to treat T2DM include sulfonylureas, metformin (biguanides), α-glucosidase inhibitors, thiazolidinediones (also known as glitazones), and glucagon like peptide-1 (GLP-1) receptor agonists. The sulfonylureas, such as Glipizide (GLIP), work through stimulating insulin secretion from the pancreatic islet β-cells. A limitation of these compounds is that they are only suitable for treating patients who still retain adequate pancreatic β-cell function. This is due to the fact that the drug’s ability to increase insulin secretion depends on the viability of the pancreatic β-cells, which decreases over time in diabetic patients. Furthermore, the sulfonylurea use has been associated with weight gain, making it an unfavourable choice for individuals who are obese. In addition, sulfonylureas are known to cause hypoglycaemic episodes and are contraindicated in patients with renal and hepatic impairments as well in elderly individuals [11]. Moreover, it has been reported that prolonged use of sulfonylureas, such as glibenclamide, may lead to β-cell toxicity.

Biguanides such as Metformin (MET) cause a decrease in hepatic glucose production and increase insulin sensitivity. MET is largely used as the initial treatment of diabetes. It is not associated with an increase in body weight and thus is suitable for obese patients. Also, Metformin treatment does not cause hypoglycaemic episodes in patients. However, MET should be avoided in patients with renal and hepatic impairment. In addition, it should be avoided in patients with congestive heart failure [11].
α-glucosidase inhibitors such as acarbose reduce the post-prandial hyperglycaemia levels in diabetic patients. Their effects are due to the inhibition of α-glucosidase enzymes in the small intestine. As a result, the rate of polysaccharide digestion at the proximal small intestine is reduced. Unfortunately, the increase in levels of undigested polysaccharides in the small intestine also leads to adverse side effects such as abdominal discomfort and diarrhea [11].

Thiazolidinediones such as rosiglitazone (ROSI) exhibit anti-diabetic effects by enhancing the insulin sensitivity in adipose tissue, muscle, and to a lesser degree at the liver. These effects are through the activation of nuclear peroxisome proliferator-activated receptor (PPAR-γ). Also, drugs belonging in this class decrease the rate of hepatic glucose production, adipocyte lipolysis, and reduce blood pressure [11, 12]. These drugs increase absorption of sodium at the distal nephron, causing increased fluid retention and peripheral edema in diabetic patients. Thus, the use of thiazolidinediones leads to an increase risk of heart failure and should be avoided in patients who have a history of cardiovascular disease and heart failure [12, 13]. Some thiazolidinediones such as rosiglitazone have been reported to increase the risk of myocardial ischemia [13].

Also, there are emerging treatments such as GLP-1 receptor agonists (incretin mimetics) and dipeptidyl peptidase-4 (DPP-4) inhibitors. GLP-1 is a hormone produced and secreted by the entero-endocrine L-cells at the ileum of small intestine[14]. Within minutes of oral food consumption, GLP-1 will enter the bloodstream to cause glucose dependent insulin secretion from the pancreas. In addition, GLP-1 decreases glucagon
secretion, decreases gastric emptying and suppresses appetite [14]. The result is a decrease in blood glucose levels. Incretin mimetics (GLP-1 receptor agonists) such as Exanatide and Liraglutide act on the GLP-1 pathway to cause glucose-dependent insulin secretion. The duration of action of GLP-1 is relatively short because the hormone is subjected to peptide degradation via the serine protease DPP-4. The advantage of incretin mimetics over GLP-1 is their prolonged duration of activity since they are more resistant to degradation via DPP-4 compared to GLP-1 [15]. A disadvantage is that these drugs require administration via subcutaneous injection [11]. Exanatide and Liraglutide are widely tolerated with mild side effects such as GI discomfort, nausea, and vomiting [16, 17]. These drugs may lead to an increased risk of hypoglycaemia when used in combination with sulphonylureas [16, 17]. It is suspected that GLP-1 therapy may increase the risk of acute pancreatitis although this assertion has not been fully confirmed [17].

Inhibitors of DPP-4 such as Sitagliptin and Alogliptin have also been developed, which prolong the activity of GLP-1 within the body and can be administered orally. These drugs are beginning to be widely used and are well tolerated but information on the side effects is still limited. The use of Sitagliptin in maternal patients is not recommended as it is a category B agent and the effects on nursing infants are currently unknown [18, 19].

Although current anti-diabetic drugs are effective in the treatment of DM, they are unquestionably associated with a multitude of adverse side effects (vomiting, nausea, hypoglycaemia), contraindications (pregnancy and heart failure) and drug-drug interactions. Furthermore, it is common practice for physicians to prescribe more than one class of drugs to
treat diabetic patients, which may increase the likelihood of side effects. These shortcomings justify further development of novel anti-diabetic drugs with improved efficacy and lowered toxicity [11]. The development of novel drugs are usually done with High throughput screening (HTS) on in vitro models (protein or cell based screens), which may indicate many of the side effects as seen in humans. Thus, it would be more translatable to use in vivo models for the screen. To use mice in HTS for drug development is impractical due to physical and financial infeasibilities. Instead, zebrafish can be used as a robust in vivo HTS model. In this study, we provide a zebrafish model that can be used as viable tool in the HTS of novel anti-diabetic drugs.

1.2 Process of drug discovery

Thousands of compounds and millions of dollars are usually invested for the development of one FDA approved drug that would make it to the market. There are 4 stages of in this process: drug discovery, pre-clinical, clinical, post-marketing surveillance[20]. More than 90% of ‘hit’ HTS screened compounds at the beginning of the drug development pipeline do not make it past the clinical trials phases [21]. In addition to this, many drugs that do make it to the market are removed from the shelves due to post-marketing reports of adverse effects seen in the population that were not observed during clinical trials [21]. Novel tools must be developed in order to increase success rate and better evaluate compound/drug specificity to avoid toxicity or non-target effects. This study offers a potential tool in the drug discovery and preclinical phases of the drug development pipeline that may minimize the failure rate ‘hit’ compounds.
1.2.1 Traditional high throughput screening (HTS) method

The traditional HTS screening method that many pharmaceutical companies use comes with several shortcomings [22]. The screening process requires that there is an identifiable, screenable target [22, 23]. Although in vitro models provide high throughput of compounds tested, the results may not be translatable to what is seen in whole animals [22, 24]. This is due to the fact that in vitro models do not have the physiological complexity seen in in vivo models. Drugs that are later tested on mammalian models show problematic administration, distribution, metabolism, excretion (ADME) issues[24]. For instance, ‘hit’ compounds found to be effective in in vitro models may turn out to have poor dose-limiting solubility, poor absorption, or undesirable metabolic conversions on the drug of interest [24]. As a result, many drugs do not pass the pre-clinical stages of drug development [24]. The use of whole organisms for high throughput screening would address these problems[25]. In addition to many advantages of zebrafish over other in vivo model in HTS experiments. The use of zebrafish would make many HTS drug screens to be cost effective relative to in vivo models such as mice [22]. (Figure 1) illustrates the traditional HTS method modified from Giacomotto et al. 2010 [22], and the alterations that come with the use of zebrafish for drug screening. In the traditional HTS method, an identifiable drug target is drug screened with the use of in vitro models and is further validated in mice before entering preclinical and clinical trials[22].
Figure 1. Traditional HTS method. 1, black lines display the traditional HTS drug discovery workflow. 2, blue lines display that in vivo models such as zebrafish can allow HTS screening of compound libraries which are further validated via mammalian models such as rodents. 3, the use of in vivo models such as a zebrafish may allow the identification of novel mechanisms and drug targets, which can be further studied through the traditional HTS drug discovery workflow (shown with red lines). 4, the use of zebrafish may act as a bridge between traditional HTS drug discovery workflow and drug validation studies with the use of rodent models.

The use of zebrafish in chemical screens would allow for new compounds to be identified, which are more likely to succeed further into the drug discovery process [25]. Also, the screening of an in vivo model would pave the way for the identification of targets and pathways that were not previously suspected in the development of disease [25] – these can be used in the traditional HTS screening method. In addition, the use of zebrafish as an in vivo model for high throughput drug screening can alleviate the shortcomings of HTS on in vitro models such as identifying the efficacy of drug metabolites, and act as a preliminary validation screen before ‘hit’ compounds are further validated in mammalian models.
1.3 The zebrafish model

Although rodent models are the gold standard, the use of zebrafish in biomedical research is increasing every year. There are close physiological and genetic similarities between zebrafish and mammalian species. As an experimental tool, zebrafish have an array of advantages that other species including mice do not share such as optical transparency, high fecundity, and quick, external development. Changes to morphology and modulations in gene and protein expression can be easily assayed through the use of fluorescent proteins as markers or reporters [26]. The relatively small physical size allows for multiple zebrafish to fit into a small 96 or 384 well plate, making the scaling of experiments an easy transition [26]. Also, the relatively cheaper costs associated with fish husbandry, coupled with the frequency of progeny that zebrafish can achieve, are other reasons that make this organism an attractive tool for biomedical researchers. HTS of compound libraries in an in vivo model that is both genetically and physiologically similar to mammalian species at very economical costs can be achieved through the use of zebrafish.

Zebrafish, with their fully sequenced and well-characterized genome, are genetically and physiologically similar to humans and other mammalian species in terms of their glucose metabolism. Many genes that partake in glucose metabolism have been noted to have similar roles in mammalian species. Previous studies on the glucose metabolism of zebrafish suggest that physiologically, both zebrafish and mammalian species respond similarly in order to maintain glucose homeostasis [27-30]. At 2-3 dpf, the pancreas is both fully formed and functional [30]. By 3-4dpf, the liver is also fully formed and functional [31]. During this time, the yolk sac, containing carbohydrate nutrients is depleted, causing the zebrafish to be in a
developmental period where the blood glucose level is at a plateau [27]. The genetic and physiological similarities between zebrafish and mammalian species make it an excellent model in the study of DM.

1.3.1 Development of transgenic lines via transposon technology (Ac/Ds)

The Activator (Ac) and Dissociation (Ds) elements that make up the Ac/Ds transposon system was first discovered by Barbara McClintock [32]. These elements are part of the hAT family of transposons[33]. The Ac element is the autonomous part of the transposon system of a 4.565kbp in length whereas the nonautonomous element, Ds, is of various lengths and internal sequences[33]. Both elements share a 11bp terminal inverted repeats (TIRs) [34]. As well, the system requires that the termini of the sequences must consists of multiple copies of hexameric motifs of at least 200-250bp in length [35]. The Ac carries the Ac transposase gene, which codes for 102kD (807 amino acid protein) transposase enzyme [36]. The transposase enzyme is required to excise sequences enclosed by the Ds elements (nonautonomous) [37] by recognizing the hexameric motifs within the flanking subterminal sequences [36]. Although there are other transposon systems available such as tol2 and sleeping beauty (SB) [38], the use of the Ac/Ds transposon system has several advantages over other systems: it requires a relatively small size of flanking sequences, a large sequence construct can be carried as an insert by this system, and it has relatively high frequency of transposition in comparison to other transposon systems [39]. The Ac/Ds transposon system has been shown to be an effective tool for the development of transgenic lines in zebrafish [39].
1.3.2 Overview of organ development: specific focus on pancreas and liver

1.3.2.1 Pancreas

There are many similarities when comparing the development and physiological role of the pancreas in zebrafish with other vertebrates such as rodents and humans [25, 30]. Many important genes such as Pancreatic and duodenal homeobox 1 (pdx1) (referred to in mammals as insulin promoting factor-1, IPF-1) and developmental pathways such as retinoic acid signalling [40-42] that play a role in the development of the mature pancreas and islet formation is conserved in zebrafish and other vertebrates[43-45].

Similar to other vertebrate organisms, the zebrafish pancreas contains both the exocrine and endocrine system [45]. The zebrafish pancreas exocrine component contain acinar and ductal cells which are involved in digestion processes [30]. The acinar cells are responsible for the secretion of digestive enzymes secretion, which are transported via ductal cells.

In the endocrine component of the zebrafish pancreas are similar islet cells found in other vertebrates: β cells, α cells, δ cells, and ε cells[30]. Similar to mammals, insulin is secreted from β cells, glucagon from α cells, somatostatin from δ cells, and pancreatic polypeptide (PP) from ε cells. One particular difference between zebrafish and mammals is the localization of the cells, specifically β cells are in the central location of the islet core, surrounded by the other islet cells [46]. β cells compose the majority of the endocrine pancreas. Similar to mammalian models, insulin from β cells is secreted in response to the increase in blood glucose levels. Glucagon is secreted from α cells in response to reduced blood glucose levels [30, 45, 47] to break down glycogen to become glucose[30].
Somatostatin and PP inhibit insulin and glucagon secretion, respectively [30]. The organization of the different islet cells within the pancreas is further illustrated in the following diagram (Figure 2) Error! Reference source not found.[48].

Figure 2. Islet cell organization in the zebrafish pancreas, 4dpf.

Zebrafish have Zebrafish pancreas is located dorsally of the liver and anteriorly of the intestine. Pancreas contains 4 islet cell types as seen in mammalian species. Organization of islet cell types is similar to mammalian species.

At 24hpf, the dorsal bud of the budding pancreas forms a single primary islet cell within the zebrafish endocrine pancreas containing all 4 islet cells. In the ventral bud, the exocrine pancreas is formed at 32hpf. Both ventral and dorsal buds fused together to form a singular pancreas at 50hpf.
By 72-96hpf, more β cells are formed in the ventral bud and these contribute to the primary endocrine islet. Acinar cells of the exocrine pancreas, responsible for the secretion of digestive enzymes, are functional at 72hpf [49]. Compared to mammals, where various organs from the endodermal layer are formed by budding out from the gut tube, zebrafish organs begin initially in the bilateral sections of the body and migrate towards the anatomical midline[30]. The mature pancreas is spatially located to the right of the midsagittal line[50].

Many of the genes that are involved in the development of the pancreas are conserved among zebrafish and mammals [51]. The human IPF-1 transcription factor is known as pdx-1 in zebrafish and mice. Homozygosity for an inactivating exon mutation in IPF-1 in humans is linked to early onset of T2DM (Maturity-onset diabetes of the young 4, MODY4). Pdx-1 is essential for mammalian islet development [52]. The use of pdx1 inhibitor showed a reduction in islet cells [28]. Zebrafish administered with a pdx1 inhibitor showed hyperglycemia [28]. Also, knock-down of pdx-1 in zebrafish is reported to show a reduction of the pancreas [53].

Zebrafish has shown to be a valid model for the etiology of pancreatic regeneration. In one particular study, β-cells in a insa:GFP zebrafish line were destroyed via streptozotocin or surgical removal recovered after 2 weeks with normal blood glucose levels and do not require insulin injections[54]. Although this is not similar to other mammals where β-cell destruction would result in a permanent T1DM phenotype, zebrafish can provide a model in which to dissect the important signalling pathways that are required in the regeneration of the pancreas and the proliferation and differentiation of β cell progenitors[54]. Another transgenic line using the insulin promoter has been used in the screening of compounds that promote β-cell regeneration [55]. For instance, a double transgenic line that allows for conditional ablation of
the β-cells was used to screen compound libraries for potential drugs that promote β-cell regeneration [55].

1.3.2.2 Liver

The liver plays an important role in the regulation of glucose levels in the body. This organ is largely responsible for endogenous glucose production [56]. During a starving state, endogenous glucose production increases, whereas during post-prandial periods the liver uptakes glucose and carbohydrates and to glycogen [57]. The liver is responsible for maintaining the fine balance of endogenous glucose production and uptake of glucose in order to achieve homeostatic blood glucose levels in the body [56]. In T2DM, the glucose metabolism in the liver is perturbed, resulting in overproduction of glucose and fatty acids as well as decreased glucose uptake from exogenous sources (food intake). In addition to this, the increased levels of blood glucose causes increased secretion of insulin from β cells, leading to increased peripheral insulin resistance [58].

In zebrafish, the liver consists of 3 lobes that lie adjacent to the intestinal gut tract [59]. Similar to other vertebrates such as mice and humans, the liver in the zebrafish also plays a large role in maintaining homeostatic blood glucose levels in addition to other duties such as detoxification and production of serum proteins such as albumin [60]. There are notable differences between zebrafish and mammals. Compared to mammals which have portal triads and distinct hepatocytes, the portal triads and hepatocytes in zebrafish livers are not clearly defined [59, 60]. As well, zebrafish liver does not have Kuppfer cells [59]. The following (Figure 3) [61] illustrates the location and orientation of the zebrafish liver.
Figure 3. Location of gut related organs in zebrafish. 5dpf zebrafish A) Dorsal view, B) right later view, C) right later view.

Pharynx (Ph), esophagus (E), liver (L), pancreas (P) with islet (Pi), gallbladder (G), swimbladder (SB), and intestine (I). Broken and solid lines represent liver, pancreas and gallbladder ducts.
1.4 The use of zebrafsh as an organism for drug discovery

1.4.1 Overview of pharmacological and toxicological studies in zebrafish

Previous genetic and chemical screens performed in zebrafish show toxicological and pharmacological results that highly suggest that zebrafish can be a representative model for other mammalian organisms, including humans [26, 62]. As well, many developmental genes found in zebrafish were further validated in rodent models and humans [26, 62]. In previous studies, the screening of compound libraries show that the effects seen in zebrafish are similar to the effects seen in humans [26, 63]. Toxicological studies on various organs and organ systems on zebrafish have been previously investigated: teratogenicity, cardiototoxicity, hepatotoxicity, nephrotoxicity, neurotoxicity, GI toxicity [26]. Drugs assays on embryonic zebrafish have been used in order to investigate teratogenicity [64, 65]. Also, embryonic zebrafish have been used in order to investigate whether tested drugs may cause cardiovascular problems such as arrhythmias (torsades de pointes) and other heart contractility dysfunction [26]. Many drugs that elicit cardiovascular defects in zebrafish also exhibit similar effects physiological effects in humans [66, 67]. There are several known drugs causing hepatotoxicity in mammals that also cause similar toxicological effects in zebrafish liver. Further investigation will be required in order to use zebrafish as a screening tool for hepatotoxicity. Recent studies suggest that many drugs found to be hepatotoxic in mammals are also found to have similar effects in zebrafish [68, 69]. Nephrotoxicity effects of drugs have also been studied in zebrafish. Drug toxicity to the kidney structures decrease the effectiveness of many drugs such as antibiotics [70]. Drugs such as cisplatin, a drug used for the treatment of
cancer, that is widely known to be clinically toxic to the kidneys, is also found to be true in zebrafish [70].

Many drugs exhibit neurological effects and as such, the screening of drugs displaying neurological toxicity is highly beneficial [26]. Currently, there are several research groups that carry out drug assays on zebrafish for neurological toxicities such as learning [71], sleep [72], and addiction[73]. Many drugs on the market have side effects that affect the GI tract. It is clear that GI tract irritation and motility issues lead to non-compliance in clinical patients, thus it is important to screen potential drugs for GI toxicity. In zebrafish, gut motility is present as early as 4-5 dpf, and drugs can be screened beginning at 3 dpf [74]. High throughput assays have been developed, but still require optimization in order to be applicable for GI toxicological investigations [26].

1.4.2 Zebrafish models of disease

Although there are few models of diabetes in zebrafish [75], zebrafish have been used extensively to elucidate the pathogenesis of various human diseases. There are numerous biological similarities in both anatomy and physiology to mammals and humans [76]. As such, previous studies have demonstrated that zebrafish can be used a model for many diseases and pathologies, which include cancer [76], cardio-vascular disorders [25], infection [77], immunity[78], and diabetic complications [79].

1.4.2.1 Diabetic complications

Zebrafish have very similar glucose metabolism and regulation in comparison to other mammalian organisms. As discussed previously, zebrafish have vital organs such as pancreas
[45], liver[56] as well as related genes relevant to glucose regulation such as insulin (insa) [28] and gluconeogenesis genes (G6P, Pck-1) [29].

In addition to this, glucoregulation and metabolism in zebrafish are found to be similar to other mammalian organisms during the introduction of exogenous glucose [28] and known anti-diabetic drugs [29]. A previous study demonstrates a model of T1DM in zebrafish via the IP injection of streptozocin leading to β-cell destruction [75]. This causes diabetic complications such as signs of retinopathy, nephropathy, increased glycated serum proteins in the blood, and impaired wound healing[75].

1.4.3 The zebrafish model in HTS and drug discovery

The use of zebrafish would act as a bridge between in vitro and in vivo studies[26] due to the organism’s feasibility for HTS in combination with it’s similarities with higher order mammals such as humans[26]. Many genes identified in drug screens on zebrafish have also been validated in mammals such as rodents and humans [80, 81]. The zebrafish model allows for the investigation in integrative biology via the use of tools such as fluorescent gene markers and morpholino-mediated gene knock-down technology. To date, there have been several zebrafish reporter lines that fluorescence specific genes or cell types, allowing for ease of molecular changes from exposure to drugs [27, 82, 83]. As well, there are many disease models that have been developed in zebrafish [26, 84, 85].

More recently, a zebrafish reporter line of the natriuretic peptide has been used as a screening system to identify compounds that modulate signals largely responsible in
Some groups have previously reported to have used zebrafish to screen compounds that modulate development and suppress pathogenesis of disease [26, 63, 80]. In addition to this, the screening of drugs on an whole organism allow for higher “hit” rates compared to traditional drug discovery methods as in vivo screening would allow the integration of multiple targets and pathways as oppose to a singular target and pathway [26]. Compounds that were identified with the use of zebrafish screens are progressing toward structure-activity studies and clinical studies [26]. ADME has yet to be fully validated. Previous studies that describe evidence of many metabolic enzymes (P450 cytochrome family of proteins) suggest similar ADME between zebrafish and humans [87]. Furthermore, studies on prodrugs metabolism is more efficient and less biased in a whole organism as opposed to in vitro/specific tissue testing approaches [88]. Structure-activity-relationship studies are more promising in zebrafish compared to in vitro testing, given that in vivo testing of structurally modified drugs would detect detrimental ADME as well as changes in drug potency and toxicity effects [88].

The zebrafish can be a novel model for the screening of anti-diabetic compounds [28, 29] and there are many advantages in the use of zebrafish for HTS for novel anti-diabetic drugs. The zebrafish model allows for easy monitoring of genes marked by fluorescent proteins, which is advantageous in HTS.

1.5 **Pck-1: A potential target for anti-diabetic treatment**

*Pck-1* plays a large role in the glucose homeostasis of vertebrates. Of the two isoymes, PEPCK-1 cytosolic (PEPCK) is considered to be more important in the regulation of gluconeogenesis and diabetes research in comparison to the mitochondrial counterpart.
In mammals, 95% of the enzymatic activity of PEPCK is by PEPCK-C [90]. The isozymes are encoded by separate genes. In humans, PEPCK-C is encoded by \textit{Pck-1}, in chromosome 20q13.31, whereas PEPCK-M is encoded by \textit{Pck-2} at 14q11.2. PEPCK-C is made up of 622 amino acids, whereas PEPCK-M is composed of 640 amino acids[89]. In zebrafish, \textit{Pck-1} is located at chromosome 6:59719200-59726056 and \textit{Pck-2} is located at chromosome 24:12824153-12842687.

Increased gluconeogenesis may contribute to hyperglycaemia seen in T1DM and T2DM [91, 92]. Unidirectional enzymes such as fructose-1,6 bisphosphatase (F1,6P), glucose-6-phophatase (G6P) and \textit{Pck-1} are largely involved in glucose regulation [93]. The zebrafish \textit{Pck-1} (6.857kbp long and found on chromosome 6, position: 59719200-59726056) is a rate-limiting step in the gluconeogenic pathway within the liver. One of the early steps of gluconeogenesis is the conversion of oxaloacetate (OAA) to phospholenolpyruvate (PEP) via PEPCK [94], and is further converted to glucose, leading to an increase in endogenous glucose levels [95]. Excessive hepatic glucose production resulting in hyperglycemia is found in T1DM and T2DM [96].

\textit{Pck-1} overexpression has been found in hyperglycaemic patients [97]. \textit{Pck-1} expression levels and regulation have been shown to be a sensitive marker for blood glucose levels [29, 94, 98]. In \textit{db/db} mice, silencing of the \textit{Pck-1} in the liver has been shown to improve glycaemic control. In a glucose tolerance test, mice with a knockdown of liver specific \textit{Pck-1} via shRNA showed a lower blood glucose levels, suggesting improved glucose clearance [99]. In both fed
and fasting state, *Pck-1* silenced db/db mice showed lower plasma insulin levels, suggesting improved insulin sensitivity [99].

In contrast, another study showed that liver specific knockout of *Pck-1* in mice display viability with impaired hepatic glucose production, abnormal lipid metabolism, and fasting mild hyperglycemia [100]. It is suspected that extrahepatic organs such as kidney and intestine may play a role in the expression of other gluconeogenic genes [100]. As well in compensation, the overall energy metabolism of these mice is dramatically altered as they display an increase in tricarboxylic acid cycle activity. Also, glycogen synthesis in tissues such as muscle and liver is impaired [100].

1.5.1 *Pck-1* sequence conservation among vertebrate species

*In silico* analysis comparing the rat *Pck-1* sequence to other vertebrate species such as humans and zebrafish showed that the gene is highly conserved among vertebrate species [101] (Figure 4). *In silico* analysis of the rat *Pck-1* promoter suggested that several TBDs (CRE, HNF-1, C/EBP, HNF4α/AP-1, GR1, PPARγ) are highly conserved among vertebrate species [101].
Pck-1 is highly conserved among rat and human, mice, zebrafish. Green spikes represent high conservation of gene sequences within species. Blue spikes show that there is high conservation of gene exons between the analysed vertebrate species.

1.5.2 The Regulation of the Pck-1

Pck-1 is primarily expressed in the liver and secondarily in the white/brown adipose tissue and proximal tubule of the kidney. Currently, the mechanisms that regulate the expression of the Pck-1 in specific tissue are not fully known but key transcription factors are suspected to play important roles. For instance, the deletion of the C/EBP gene in the mice results in the lack of Pck-1 liver expression, leading to lethal hypoglycaemia. Also, the interaction of transcription factors on the HNF-1 regulatory binding regions of the gene promoter is required for Pck-1 expression in the proximal tubule of the kidney [101].

High blood glucose levels leads to the secretion of insulin from pancreatic β-cells in order to maintain glucose homeostasis. Insulin activates the insulin receptor (a tyrosine kinase) leading to recruitment and activation of Phosphoinositide-3-kinase (PI3-K). PI3-K converts PI-bisphosphate to the secondary messenger, PI3 [102]. PI3 leads to the activation of Akt/PKB [102]. Akt/PKB phosphorylates Forkhead box 1 (FOXO1), leading to the inactivation and nuclear...
exclusion [102]. It is suggested that FOXO1, which would upregulate *Pck-1* expression if bound to the promoter’s AF2 or distal AF2 (dAF2) TBDs, is the primary mediator of insulin action on the regulation of *Pck-1* [103]. Thus, FOXO1 inactivation and nuclear exclusion result in the downregulation of *Pck-1* expression [102]. In mice, the inactivation of the FOXO1 encoding gene in the liver resulted in a decrease in *Pck-1* expression and lower blood glucose levels [103]. Also, mice that are knock-out for the FOXO1 gene and insulin receptor deficiency showed increased survival and decreased *Pck-1* expression [103]. The Akt/PKB also phosphorylates and inactivates glycogen synthase kinase-3 (GSK-3), promoting glycogen synthesis [102]. Glycogen synthase kinase 3-β (GSK3-β) activates CREB to upregulate *Pck-1* expression [104].

Insulin also downregulates *Pck-1* expression through Akt/PKB, which promotes the activity of salt-inducible kinase 2 (SIK2) to phosphorylate target of rapamycin complex 2 (TORC2), also referred to as CREB regulated transcription factor 2 (CRTC2), leading to CRTC2 inactivation and nuclear exclusion [105]. CRTC2 is part of the CREB/CREB binding protein (CBP)/CRTC2 complex that acts on the CRE binding domains within the *Pck-1* promoter, resulting in the upregulation of *Pck-1* expression [106]. Insulin may promote the CBP phosphorylation, leading to the dissociation of CBP from CRTC2 and the degradation of CRTC2 [105]. CBP phosphorylation also happens through the activation of atypical protein kinase C (aPKC) [105]. Insulin and metformin activate aPKC, resulting in the decrease of *Pck-1* expression [105]. In addition, in high fat diet induced insulin resistance in mice metformin treatment can promote CBP phosphorylation where insulin could not, suggesting that metformin may promote CBP phosphorylation through an alternative pathway not shared with
insulin. MET may downregulate Pck-1 expression by activating aPKC via AMP activated protein kinase (AMPK) [107].

In contrast to the action of insulin, glucagon promotes the increase in endogenous glucose production, leading to an increase in blood glucose levels. In a fasting state, glucagon is secreted from pancreatic α-cells. Glucagon activates the glucagon receptor (a G-protein coupled receptor), activating protein kinase A (PKA), and leading to the increase in cAMP levels [108].

Increased levels of cAMP activates calcium release channels, inositol 1, 4, 5-trisphosphate receptor 1 (InsP3R1), leading to an increase in intracellular calcium from the endoplasmic reticulum calcium stores [108]. Increased intracellular calcium levels cause an increase in activity of serine/threonine phosphatase calcineurin (Calna), leading to the dephosphorylation CRTC2 [108]. Dephosphorylated CRTC2 may enter the nucleus to bind with CREB, promoting the CREB/CBP/CRTC2 complex and the upregulation of Pck-1 [108].

Glucocorticoids bind to the glucocorticoid receptor (GR), which interacts with the glucocorticoid regulatory element (GRE) to stimulate PEPCK expression [102]. Glucocorticoids may promote the binding of factors C/EBPα, C/EBPβ and PPARα onto the AF2 and dAF2 sites of the Pck-1 promoter, leading to upregulation of Pck-1 [102]. Also, glucocorticoids promote the binding of HNF4-α to the dAF1 site of the Pck-1 promoter, promoting the upregulation of Pck-1 [102]. In addition, peroxisome proliferator-activated receptor-γ coactivator (PGC-1) co-activate GR and HNF-4α, promoting the upregulation of Pck-1 [109]. Also, CREB can activate PGC-1 to stimulate the glucocorticoid pathway for Pck-1 expression [109].
There is also regulation of gene expression via DNA methylation, chromatin modulations and histone methylation. It has been shown in rats that tissues with high Pck-1 expression such as the liver and kidney contain under methylation of the gene. In contrast, in tissues not expressing Pck-1, such as the heart and spleen, Pck-1 is heavily methylated. Currently, a total of 18 methylation sites have been identified on Pck-1. The administration of insulin in 19-day old rats led to rapid decrease in transcription of the Pck-1 chromatin and decrease in methylation of the H3 histone[101]. Currently, it is postulated that the demethylation of the H3 histone is one of insulin’s mechanism of action to downregulate Pck-1 expression [101]. In addition, there have been studies reporting several DNase-1 hypersensitive gene sites on the rat Pck-1. Past studies on transgenic mice suggest that DNase-1 hypersensitive sites on the Pck-1 will interact with specific regulatory binding elements within the gene promoter to obtain tissue specific expression of the gene. For instance, the DNase-1 hypersensitive site at +1900 within Pck-1 interact with the HNF-1 regulatory element on the gene promoter to cause kidney specific expression [101]. In the regulation of glucose metabolism, Pck-1 is highly regulated at the transcription level, with many hormones and related transcriptions factors that play important regulatory roles.
1.5.3 *Pck-1* protein regulation

*Pck-1* protein uses the high-energy bond from the phosphate of a GTP to convert OAA to PEP (along with CO₂) (Figure 5) [110].

\[
\text{OAA} + \text{GTP} \rightarrow \text{PEP} + \text{CO}_2 + \text{GDP}
\]

*Figure 5. OAA is converted to PEP by PEPCK-C through the use of GTP.*

It has been shown that the binding of GTP to the *Pck-1* enzyme is accomplished through the interaction of several aromatic residues (PHe517, Phe525, Phe530) (Figure 6)[111]. The guanine base (purine ring) of the GTP is found to be placed between the Phe517 and Phe530 residues of the enzyme.

*Figure 6. Binding of GTP and xanthine inhibitor to PEPCK-C. (a) Guanosine unit of GTP bound to PEPCK. (b) Competitive inhibitor, Xanthine 1, bound to PEPCK. Guanosine unit of GTP in red and light green bonds structure represent PEPCK; orange lines represent hydrogen bonds, blue represents water, black represents xanthine 1. Backbone (bb) and side chain (sc) groups of PEPCK.*
Interestingly, Phe517 has been found to be conserved among the \textit{Pck-1}-GTP utilizing protein family [110]. Xanthine inhibitors (compound I and II) have been developed that competitively inhibit the \textit{Pck-1} enzyme through this GTP binding pocket. Both xanthine inhibitors take advantage of the interaction between GTP and \textit{Pck-1} enzyme through Phe525 via a phenyl ring in both compounds [110]. The phenyl ring interacts with Phe530 on the \textit{Pck-1} protein [110].

Also, the purine ring in compounds I and II are also found to be placed between the Phe517 and Phe530 residues. In addition, the allyl group of the xanthine inhibitor, compound I, act as a hydrogen bonding acceptor of the Asn533 amide group [110].

1.5.4 \textit{Pck-1} protein post translation regulation

In a particular study, it has been found that acetylation of the \textit{Pck-1} protein is one mechanism through which the \textit{Pck-1} is regulated at the protein level [112]. The acetylation at lys-19 and 514 has been found to increase enzyme activity in yeast and hepatoma cells [113].

The acetylation of \textit{Pck-1} protein is mediated by SIRT2, stabilization PEPCK where P300 acetyltransferase destabilizes PEPCK, leading to protein degradation [112] via the ubiquitylation by HECT E2 ligase [114].

1.5.5 Past clinical studies on \textit{Pck-1}

Although there have been many reports of \textit{Pck-1} polymorphisms among DM patients [115] there have not been many studies on DM patients that specifically investigate the associations of the \textit{Pck-1} and DM. It is worth noting that in a particular study, a polymorphism (-232C->G) in the PEPCK promoter is found among T2DM patients [116]. Further \textit{in vitro} studies
of this single nucleotide polymorphism (SNP) suggest that the polymorphism leads to increased basal gene activity and a reduction in the effect that insulin has on PEPCK gene activity [116]. The findings of another study from the UK further corroborates this assertion about SNP [117]. In addition, many SNPs previously reported [115] to be associated with T2DM patients are screened [118], with the above mentioned SNP is found to be associated with T2DM [118].

1.5.6 *Pck-1* as a potential therapeutic target for anti-diabetic compounds

The various means of the regulation of *Pck-1* in glucose metabolism suggest the potential of this gene as a target for compounds that modulate gene expression, with the therapeutic potential to treat the symptoms of DM such as high blood glucose levels. Past studies have explored the *Pck-1* as a drug target. Researchers from a study fused a *Pck-1* promoter construct to a luciferase gene in order to measure gene expression in rat hepatocytes [119]. The expression of *Pck-1* was increased with the treatment of lipid extracts to zucker fatty, lean, and wistar rats. *Pck-1* expression was decreased upon treatment of hepatocytes with insulin [119]. Polyphenolic compounds extracted from plants have been found to decrease *Pck-1* expression and the rate of gluconeogenesis in streptozotocin (STZ) [120] induced diabetic rats and in the hepatocyte cell line (H4IIE) [121]. Synthesized compounds (6-demethoxycapillarisin and 2’,4’-dihydroxy-4-methoxydihydrochalcone) of these mentioned polyphenolic compounds regulate *Pck-1* through the typical insulin mediated or AMPK mediated pathway [121].

A previous study identified an oral novel inhibitor of the FOXO1, 5-amino-7-(cyclohexylamino)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (AS1842856)
In diabetic (db/db) mice, the oral administration of this FOXO1 inhibitor caused a decrease in fasting blood glucose levels. Also, the novel FOXO1 inhibitor showed a decrease in hepatic glucose production in db/db mice. In contrast, the administration of the FOXO1 inhibitor in normal mice did not induce any significant reduction in fasting blood glucose levels [122].

Another compound, 20-hydroxyecdysone, extracted from plants such as spinach (Spinacia oleracea) decreased gene expression in diabetic mice that were overexpressing Pck-1 in the liver, resulting in reduced weight and hyperglycemia [123]. The study show that the mechanisms of action for these compounds may be through both the typical and atypical insulin mediated pathways [123].

The Pck-1 is highly regulated and past studies suggest the potential of modulating Pck-1 expression to treat DM in rodent models. Thus, the Pck-1 promoter may be a potential target to be used for the screening of novel anti-diabetic compounds [124]. Combining the potential of Pck-1 as a therapeutic target with the advantages of using zebrafish as a HTS model, the development of a Pck-1 reporter transgenic zebrafish line would be beneficial in the HTS screening of potential anti-diabetic drugs.
Chapter 2: Rationale, Hypothesis, and Objectives

2.1 Rationale:

Dysregulation of gluconeogenesis plays an important role in the prevalence of hyperglycaemia in DM [91, 92, 116, 117]. Phosphoenolpyruvate carboxykinase gene (Pck-1) is a potential target for the screening of anti-diabetic compounds as it produces a rate limiting gluconeogenic enzyme, thus playing a very important role in endogenous glucose production and glucose metabolism [116]. Pck-1 is highly conserved among humans, mice and zebrafish [125]. Dysregulation of Pck-1 has been found to be related to hyperglycaemia in T2DM patients [116, 117]. Pck-1 promoter single nucleotide polymorphisms at the CRE region has been found to be associated with the development of T2DM in humans [116]. In zebrafish, Pck-1 expression have been reported to indicate the efficacy of anti-diabetic drugs [29]. In diabetic mice, partial Pck-1 silencing decreased Pck-1 expression and improved glycaemia [99]. Also, Pck-1 is regulated by compounds which target the gene promoter, such as a novel inhibitor of Foxo1 (an activator of PEPCK)[13]. In addition, known anti-diabetic drugs such as MET [105] can regulate Pck-1 expression in diabetic mice, which further suggest the potential of the promoter as a target for anti-diabetic drug discovery.

Traditional high throughput screening for anti-diabetic drugs is largely performed with in vitro models [126]. On the other hand, mice are the predominant in vivo mammalian model for diabetes research [127] but slow embryonic development, low throughput and high cost make
it unfavourable to be used for large scale drug screens [22]. The shortcomings of current in vitro and in vivo models for HTS contribute to current low success rate in the development of new anti-diabetic drugs. As an emerging model organism, zebrafish allows for easy, cost-effective and large-scale chemical genetic screen. As this screen is performed in vivo, drug toxicity can be evaluated in parallel to assess drug efficacy, allowing for a higher success rate compared to the traditional in vitro drug screen on cultured cells [22, 128, 129]. The generation of eGFP reporter fish strains allow for easy assessment of drug efficacy in vivo. It is clear that the development of zebrafish screening models for anti-diabetic drugs is a very attractive and novel strategy in diabetic research as it will bridge the gap between in vitro models and mammalian in vivo models, thus accelerating the current drug discovery method.
2.2 Hypothesis:

The Pck-1 promoter:eGFP reporter zebrafish transgenic line will be a valid in vivo model for the screening of drugs that may have anti-diabetic efficacy resulting from the down regulation of Pck-1 expression.

2.3 Objectives:

The objectives of this study include:

1) Clone the zebrafish Pck-1 promoter

2) Create and characterize zebrafish Pck-1:eGFP reporter lines

3) Validate the Pck-1:eGFP reporter line as a tool for the screening of novel anti-diabetic drugs
Chapter 3: Materials and Methods

3.1 Fish maintenance and breeding

3.1.1 Fish husbandry

Zebrafish (AB strain and Leo strains) were maintained in an Aquaneering water recirculation fish system at a constant water temperature of 28°C. The fish were kept at a 14 hours light:10 hours dark photoperiod day cycle. The larval fish were fed with fry food (First Bites, Hikari Tropical) from 5 dpf to 14dpf. At 14dpf, fish were fed with brine shrimp. Embryonic fish water (E2 water) was used as media for larval fish.

3.1.2 Fish generations

F₀ founder fish are outcrossed with wild-type fish in order to obtain F₁ generation fish. F₁ generation fish are outcrossed with wild-type fish in order to obtain F₂ generation fish embryos.

3.2 Gene promoter PCR amplification

The Pck-1 promoter of 3.87kbp upstream of the 5’ UTR was amplified via nested PCR. The following PCR primer pair were used (F1: 5’AGTATTAATAGAATAAACATGCAC3’, R1: 5’AAACAGGAGCGAATAAATCTGCCTTC3’) followed by a second round of PCR with nested primer pair (F2: 5’GGCGAGGCATGCACAAATAGCTATAG3’, R2: 5’GGCGAGGCATGCACAAATAGCTATAG3’) in order to substitute SphI and Smal restriction enzyme sites at the 5’ and 3’ ends respectively of the expected PCR amplicons.
PCR product is cloned into TA cloning vector (pCR-XL-TOPO cloning vector, Invitrogen) and then subcloned into the Ac/Ds transposon expression vectors. Genomic DNA template of PCR experiments were extracted from adult wildtype zebrafish by following the genomic DNA extraction protocol listed in the Zebrafish Book: Guide to Laboratory Use of Zebrafish [130].

3.3 Cloning

3.3.1 Transformation

100ul of chemically competent DH5-α E.coli cells (stored at -80°C) are thawed on ice. 100ng of the plasmid of interest is mixed with the E.coli cells and incubated on ice for 30 minutes. Cells are subjected to heat shock at 42°C for 90 seconds followed immediately by 2 minutes of incubation on ice. 150-250µl of LB is added to the cells and shaken at 140rpm in a 37°C water bath for at least 1 hour. The transformation mix is then plated on a LB agar plate containing 100µg/ml ampicillin and incubated at 37°C for 16-18 hours.

3.3.2 Amplification of zebrafish Pck-1 promoter

3.87kb of the Pck-1 promoter was amplified via nested PCR. The “outer” 1st set of primers are designed to amplify a 4kb Pck-1 promoter segment, where the 2nd set of primers are designed to substitute bases close to the 5’ and the 3’ ends to be compatible with SmaI and SphI restriction enzyme digest sites, respectively. Amplified PCR products were analyzed via gel electrophoresis with a 1kb DNA ladder (Fermentas) in a 1% agarose gel solution containing either ethidium bromide or RedSafe DNA staining solution (Frogger Bio) (Figure 7).
Figure 7. Amplification and cloning of a 4kb zebrafish Pck-1 promoter.  
A) Amplification of 3.87kb Pck-1 promoter via nested PCR. Outer F1,R1 PCR primers followed by inside primers F2, R2 to make Sphl and Smal restriction sites. B) Cloning of Pck-1 promoter in pCR-XL-TOPO cloning vector with relevant restriction enzyme sites. C) Gel electrophoresis, pcr product with amplified 3.87kb Pck-1 promoter noted with arrow. D) 1, undigested Pck-1 promoter-TOPO cloning vector. 2, Digest with EcoRI and Smal results in bands of 4kb, 2 and 1.5kb. E) 1, undigested Pck-1 promoter-TOPO cloning vector. 2, digest with Smal results in a band between 5-6kb and a band at ~2kb. 3, digest with Smal+Sphl results in bands of 3kb, 2kb, 1.5kb, 0.8kb.
3.3.3 TA cloning and expression vector subcloning

The Pck-1 promoter was inserted into a PCR-XL-TOPO (Invitrogen) cloning vector and sequenced with a T7 promoter. Two lengths of the promoter were made via restriction enzyme digest. A 3.6kb fragment was made by restriction enzyme digest with Sall and SmaI. A 0.8 kb was made by restriction enzyme digest with SphI and SmaI. These promoter constructs were then subcloned into a Ac/Ds transposon system expression vector (pMDs-EGFP vector, Emelyanov & Parinov, Temasek Life Sciences Laboratory, Singapore University) to be used for microinjection.

Bands that were at the expected molecular weight were isolated and purified via a gel purification kit (Qiagen). Purified isolated 3.87kbp PCR products were then used in ligation reactions with pCR-XL TOPO vectors (3.5kbp). Ligation reaction protocols included in the TOPO ligation kit were followed. The ligation reaction was used for transformation in chemically competent DH5-α E. coli and transferred to a 100µg/ml ampicillin LB agarose plate [131]. The plates were then incubated overnight for 16-18 hours. Single colonies were isolated and incubated in 100µg/ml ampicillin LB media for 16-18 hours. Cultures were mini prep (Qiagen) and inserts were checked by restriction enzyme digest with EcoRI, SmaI and SphI restriction enzymes. Digested plasmids were analyzed via gel electrophoresis on a 1% agarose gel (Figure 7). Correct plasmids are used for subcloning into Ac/Ds transposon expression vectors for microinjection experiments. Plasmids are sequenced via T7 promoter.
The 3.87 kbp Pck-1 promoter in the TA vector is digested with a combination of Smal, Sphl or Smal, Sall in order to obtain promoter constructs of 0.8kb and 3.6kb. These constructs are then subcloned into a pMDS-eGFP Ac/Ds transposon expression vectors of 4.632 kbp in size (Figure 8).

Figure 8. Expression vectors with PEPCK promoter constructs. A) Top: TOPO cloning vector containing cloned Pck-1 promoter is digested with either Smal, Sphl or Smal, Sall to obtain promoter constructs of 0.8kb and 3.6kb. Bottom: An empty Ac/Ds transposon system expression vector with restriction enzyme sites upstream of the eGFP gene. B) Top: expression vectors containing the 0.8 kbp Pck-1 promoter. Bottom: expression vector containing the 3.6 kbp promoter construct. C) Gel electrophoresis of expression vectors containing either the 0.8kb or the 3.6kb Pck-1 promoter constructs. Expression vector containing the 3.6kb Pck-1 promoter construct, (1) undigested, (2) Smal digest, band at 8.2kb, (3) Sall digest, band at 8.2kb (4) Smal + Sall digest, bands at 4.6kb and 3.6kb. (5) 1kb DNA ladder (Fermentas). Expression vector containing the 0.8kb Pck-1 promoter construct, (6) undigested, (7) Smal digest, band at 5.4kb (8) Sphl digest, band at 5.4kb (9) Smal + Sphl digest, bands at 4.6kb and 0.8kb.
3.4 Generating reporter zebrafish lines

3.4.1 Ac transposase in vitro transcription

Ac transposase vector (Parinov labs, Temasek Life Sciences Laboratory, Singapore University) containing the 2.2kb cDNA sequence in a 3.626kb p64T backbone vector was linearized with BamHI restriction enzyme digest to be used as a template for SP6 in vitro transcription reaction and RNA extraction and purification (Ambiogen), following the in vitro transcription kit protocol.

3.4.2 Microinjection

A solution of the following final concentrations was used for microinjection: 40ng/µl transposase mRNA, 25ng/µl Pck-1 (3.6kb):eGFP Ac/Ds transposon system expression vector DNA. The fish embryos were collected at 5-10 minutes post-fertilization (prior to the 1-cell developmental stage) for the microinjection experiments. Embryos were lined up on a 2% agarose gel mount and a microinjector device (Eppendorf) was used for microinjection. Microinjection needles were made by pulling borosicillate Pasteur glass pipettes with a glass pipette tip puller.
3.4.3 Generating the Pck-1:eGFP reporter line – workflow

(Figure 9) summarizes the workflow used in this study for the generation of Pck-1 reporter fish lines. Wild type zebrafish embryos prior to the 1-cell developmental stage are microinjected Ac transposase mRNA (Figure 10) in combination with the expression vector containing the 3.6kb Pck-1 promoter construct, generating chimeric transgenic fish.

Figure 9. Workflow of generating the Pck-1 promoter:eGFP reporter line. Embryos prior to the 1-cell stage are microinjected with Pck-1:eGFP vector and transposase mRNA to produce chimeric fish (F0). F0 fish are outcrossed with WT fish to produce F1 fish. F1 fish with the correct fluorescence expression pattern as endogenous Pck-1 expression are used for validation studies. These F1 fish that expression fluorescence at the liver are outcrossed with WT fish to produce F2 for validation studies.
Figure 10. In vitro transcription of Ac transposase mRNA. A) 1, pAC-SP6 vector linearized with BamHI to be used as template for in vitro transcription of Ac transposase mRNA. 2, undigested pAC-SP6 vector. B) Ac transposase mRNA via SP6 in vitro transcription.

At sexual maturity, chimeric fish are outcrossed with wild type zebrafish to generate F_1 generation reporter line fish. Only the reporter fish lines that are similar to the endogenous gene expression of *Pck-1* in zebrafish seen in situ hybridization of past studies [27, 28] are further used in the validation studies with various physiologically related and known anti-diabetic compounds, as illustrated with a red dashed outline box.

3.4.4  **F₀ chimeric fish of 3.6kb *Pck-1* promoter construct**

At 48hpf and 96hpf, brightfield and fluorescent microscopy images of chimeric F₀ generation fish microinjected with the expression vector containing the 3.6 kb *Pck-1* promoter construct. Chimeric fish that display fluorescence in the body are isolated and maintained into
adulthood. In 3.6kb Pck-1 promoter:eGFP chimeric fish, fluorescence in present in the yolk sac, head, and liver.

3.5 Transgenic line: model validation

The Pck-1 promoter:eGFP transgenic fish were tested with glucose and anti-diabetic drugs in order to validate that the Pck-1 promoter would respond as expected and similarly to other mammals such as rodents and humans. Glucose and anti-diabetic drugs concentrations from previous study [29] were used.

3.5.1 Drug compound assays on reporter fish line

Reporter line fish at 3 dpf were placed in a 6 well cell assay plate, 25 fish per well and treated with glucose (GLU: 100mM; Sigma) for 72 hours at 28°C. Also, Pck-1 promoter:eGFP reporter fish were also tested with the following compounds: MET (10µM, Sigma), ROSI (1µM; Sigma), cAMP (100µM; AbCam) and DEX (0.5µM, 1µM; Sigma). 0.1% DMSO was used for compound solubility in E2 water.

Specimen in a corrected optical rotational enclosure (SCORE) imaging was used to obtain fluorescence and bright-field images. ImageJ software (version 1.46r, NIH) was used to analysis the level of fluorescence intensity at the liver in F2 generation Pck-1:eGFP reporter line fish.
3.5.2 RT qPCR

RNA samples were extracted with the following protocol. Larval fish from drug compound assays were placed in Trizol solution (Invitrogen) and stored at -80°C before RNA extraction. Chloroform is added and the samples were incubated for 2-3 minutes and spun down at max rpm at 4°C for 15 minutes. Isopropanol was then added to the tubes containing the supernatant and incubated at room temperature for 10 minutes. Tubes are spun down at max rpm at 4°C for 10 minutes. RNA pellets were washed with 75% ethanol and spun down at 9000rpm at 4°C for 5 minutes. Ethanol was removed and pellets were allowed to dry at room temperature for 5-10 minutes. RNA pellets were resuspended in DEpc H2O and stored at -80°C until further use.

RT qPCR was performed through reverse transcription of the extracted RNA samples using SuperScript III Reverse Transcriptase (Invitrogen) kit protocol and RT2 SYBR Green qPCR Mastermix (Qiagen) kit protocol. The following Pck-1 primers are used for RT qPCR: PEPCK1 qPCR Fwd, 5’AGTGGGACAAAGCCATGAAC; PEPCK1 qPCR Rev, 5’TATCTTGGAGAGCGGAGAGC3’.

GAPDH is used as the house keeping gene. The following GAPDH primers are used: GAPDH Fwd, 5’ GATACACGGAGCACCAGGTT3’, GAPDH Rev, 5’GCCATCAGGTCACATACACG3’.

3.6 SCORE imaging

A modified version of SCORE imaging protocol by Petzold et al. 2010 [132] was used in the imaging of Pck-1 promoter:eGFP reporter line fish at various developmental stages and validation studies via drug compound assays. Larval fish specimens are anaesthetised with 100ppm clove oil solution and are placed in a 2.5% methylcellulose solution. 50ml solution:
1.25g of methyl cellulose (USP/FCC, 400 centipoises), 49.5ml nanopure water, 25µL of clove oil (diluted 1:10 in ethanol). Fish specimens are sucked into a borate glass tube with a p200 micropipette and pipette tip. Glass tube is mounted on a 3cm dish and water is filled to the point where the glass tube is submerged in water. The specimen can then be rotated to the desired orientation for imaging purposes.

### 3.7 In silico analysis

A 4kb sequence of the zebrafish *Pck-1* (cytosolic) upstream of the transcription start site (UCSC genome browser)[133] was analysed with the animal Ghosh transcription binding domains database (NSITE, Softberry)[134].

### 3.8 Statistical tests

2-tailed student's t-test was used to determine statistical significance. P-values that were found to be less than 0.05 were considered statistically significant. Error bars within the bar graphs are expressed in mean +/- SEM.
Chapter 4: Results

4.1 Cloning and characterization of zebrafish *Pck-1* promoter

4.1.1 *In silico* analysis of zebrafish *Pck-1* promoter

Past literature suggests that the gene sequences of *Pck-1* is largely conserved among vertebrate species [101]. But the similarities between the gene promoter of vertebrate species and zebrafish have yet to be compared. An *in silico* analysis of a zebrafish 4kb *Pck-1* promoter sequence with a vertebrate regulatory motifs database suggests that several transcription regulatory motifs within the zebrafish *Pck-1* promoter corresponds to the binding of transcription factors found to be important in the regulation of the *Pck-1* in rodent species (Figure 11).

![Diagram of transcription factors binding to the *Pck-1* promoter](image)

**Figure 11.** In silico analysis of Pck-1 zebrafish promoter of similar regulatory motifs with mammalian promoters.

There are many conserved TBDs that were found in silico in zebrafish Pck-1 promoter that bind to transcription factors: CREB, accessory factor 1 (AF1), PPARγ, and RXR, hepatocyte nuclear factor (HNF-1), hepatocyte HNF-4α, CAAT/enhancer binding protein-α (C/EBP), GR, nuclear factor κβ (NFκβ), and specificity protein 1 (SP1). In rodent species, CREB binding in rodent species is required for the upregulation of Pck-1 expression via the upstream action of glucagon [102]. AF1 binding domain is required for glucocorticoid response on the Pck-1 promoter [135]. PPARγ is required for the regulation of Pck-1 expression in adipose tissue in rodent models [102] and RXR is required for the up regulation of Pck-1 expression via retinoic acids in rodents and humans [136, 137]. HNF-1 is required for the transcription of Pck-1 in the kidney [102]. Also, HNF-4α binding to the Pck-1 promoter leads to the down regulation of Pck-1 [125]. cAMP activity leads to C/EBP binding to CRE, promoting the up regulation of Pck-1 expression [102]. Glucocorticoids promote the upregulation of Pkc-1 with GR bind to the GRE on the Pck-1 promoter [102]. SP1 may promote upregulation of Pck-1 expression, which competes with SREBP-1c to bind to Sterol regulatory element (SRE) [102]. Insulin leads to the competitive inhibition of SP1 by SREBP-1c [102].

4.1.2 Cloning of the Pck-1 promoter regions and construction of eGFP expression vectors

In rodent models, many of the transcription factors are less than 1kb upstream of the transcriptional start site [138]. In silico analysis of the Pck-1 promoter sequence, 4kb upstream, suggest that many TBDs in mammalian species are present in the zebrafish Pck-1 promoter within 1kb. In order to observe whether the regulation of Pck-1 may be different between a shorter or longer promoter sequence, Pck-1 promoter sequences of 0.8kb and 3.6kb were
amplified and cloned into cloning vectors. The 0.8kb and 3.6kb Pck-1 promoter sequences were subcloned into Ac/Ds transposon system-eGFP expression vectors. Pck-1-eGFP expression vectors were transfected into HepG2 cells via lipofectamine (Invitrogen) and incubated for 48hrs. At 48hrs, transfected cells are lysed and whole cell lysates are used to measure fluorescence intensities between the cells transfected with 0.8kb or 3.6k Pck-1 promoter constructs (Figure 12). Cells transfected with the longer Pck-1 promoter (3.6kb) displayed lower level of eGFP fluorescence. In comparison, cells transfected with the shorter promoter displayed eGFP fluorescence that was much higher than what is observed even in cells transfected with a CMV:eGFP control vector. This may suggest possible TBDs that are required for normal gene regulation of Pck-1 in the longer promoter are not present in the shorter Pck-1 promoter. Further studies will be required to validate this finding in zebrafish. In order to ensure that most TBDs (many of which may potentially be vital for normal Pck-1 regulation) would not be omitted in the development of the Pck-1:eGFP reporter fish, the longer Pck-1 promoter construct of 3.6kb was used in the creation of the transgenic line.
4.2 Generation of the Pck-1: eGFP reporter lines

Microinjection of the 3.6kb Pck-1:eGFP expression vector with Ac transposase was performed 6 times, each time with the injection of 100-250 embryos. 70 F₀ generation fish were screened between 1-6 dpf for fluorescence pattern that is similar to Pck-1 expression seen in wild-type fish.

4.2.1 F₂ generation fish of the Pck-1:eGFP reporter line

Chimeric F₀ fish are outcrossed with wild-type zebrafish. Fluorescence at the liver is not detected until 4-5 dpf. F₁ fish that display fluorescence at the liver at 4-5 dpf are raised into adulthood. Adult F₁ fish containing the 3.6kb Pck-1 promoter were outcrossed with wild type zebrafish in order to obtain F₂ generation fish embryos which can be further used for validation studies. In this study, reporter lines 1 and 2 are observed but only reporter line 1 is used for
further validation experiments. In both lines, fluorescence prior to 3dpf is not visible. At 4 dpf, fluorescence is visible in the liver and yolk sac in reporter line 1. The fluorescence pattern is similar in reporter line 2, but fluorescence in the liver is not as visible compared to reporter line 1 until 6dpf. At 5dpf, the fluorescence is seen at the liver and faintly in the intestinal tract in reporter line 1.

In comparison, reporter line 2 shows fluorescence in the intestinal tract and faintly visible in the liver. By 6dpf, the Pck-1:eGFP reporter lines 1 and 2 only display fluorescence in the liver. The fluorescence expression pattern of 2-7 day old embryos of reporter lines 1 is shown in (Figure 13) and line 2 is shown in (Figure 14).
Figure 13. Pck-1:eGFP reporter line 1 fish from 2-7dpf.

A-D) 2 dpf, E-H) 3 dpf, I-L) 4 dpf, M-P) 5 dpf, fluorescence is observed at the liver and faintly in the intestinal tract. Q-T) 6 dpf, fluorescence is only observed at the liver. U-X) 7 dpf. Magnifications: 27.4x, 101x.
Figure 14. Pck-1:eGFP reporter line 2 fish from 2-7 dpf.

A-D) 2 dpf. E-H) 3 dpf. I-L) 4 dpf. M-P) 5 dpf, Fluorescence is observed at the liver, intestinal tract and pronephros tissue. Q-T) 6 dpf, fluorescence is only observed at the liver. U-X) 7 dpf. Magnifications: 27.4x, 101x.
4.3 Validation of Pck-1:eGFP reporter line for anti-diabetic drug evaluation

To validate the reporter line for anti-diabetic drug discovery, the line is tested with various physiologically relevant compounds as well as known anti-diabetic drugs. F2 generation reporter zebrafish at 3dpf were treated with the following compounds for 72 hours with previously tested concentrations[29]: cAMP + DEX, glucose, MET, and ROSI.

4.3.1 cAMP + DEX upregulate Pck-1 expression in the Pck-1:eGFP zebrafish

Physiologically, transcription factors that are activated in the presence of glucagon act through the CRE domain of the Pck-1 promoter through the activation of cAMP, resulting in the up regulation of the Pck-1 [29]. The presence of a synthetic glucocorticoid, DEX, would activate downstream transcription factors such as GRs, as well as co-activation of Pck-1 through cAMP/CRE activation pathway.

In order to validate that the eGFP expression in the Pck-1 reporter zebrafish line may be modulated through glucocorticoids and glucagon activation related pathways, the treatment of cAMP + DEX is used. Past studies suggest that the treatment of cAMP + DEX may up regulate Pck-1 expression, similar to what is observed in rodent models and humans.

The reporter line responds to the treatment of cAMP and DEX with a dose dependent increase in fluorescence intensity (Figure 15). With the treatment of cAMP + DEX (0.5mM), there was an increase in fluorescence intensity (p=0.058). With the treatment of cAMP and a higher dose of DEX (1mM), the increase in fluorescence intensity was significantly higher than that seen with the lower treatment of cAMP + DEX (p=0.024).
Figure 15. Pck-1 promoter reporter line fish treated with cAMP + DEX.

A, control. B, cAMP(100µM) + DEX(0.5µM). C, cAMP(100µM) + DEX(1µM). D, mean fluorescence intensity of control and treatment groups. Student T test, control vs cAMP+1µM DEX (*p=0.024), control vs cAMP+0.5µM DEX (+p=0.058). E, mean mRNA fold change of control and treatment groups. Student’s t-test, control vs cAMP+DEX (0.5µM)(**p=0.003), control vs cAMP+DEX(1µM)(p=0.12).
In order to further verify that the response to cAMP + DEX in the reporter fish seen with fluorescence intensity is similar to the modulation in endogenous Pck-1 expression, RT qPCR was performed. With the treatment of cAMP + DEX (0.5mM), there is an increase in Pck-1 expression, similar to what is seen via the modulation of fluorescence intensity with the treatment of cAMP + DEX (0.5mM) (Figure 16E). These results suggest that glucagon signalling messenger, cAMP, and glucocorticoid, DEX, may modulate Pck-1 expression in the zebrafish reporter line by acting on the Pck-1 promoter.

4.3.2 Glucose downregulates Pck-1 expression in the reporter line

Past studies with rodent and zebrafish models have shown that glucose cause a decrease of Pck-1 expression [27, 29]. Treatment with glucose would cause the secretion of insulin, leading to the down regulation of gluconeogenic enzymes such as Pck-1. In this study, the reporter line fish was tested with glucose in order to validate that the effect seen in other mammalian models is also observed in our zebrafish model. The Pck-1:eGFP reporter line responds to glucose with a decrease in fluorescence intensity at a dose dependent manner (Figure 16). With a lower dose of glucose, there was a decrease in the fluorescence intensity (p=0.13). At a higher dose of glucose, fluorescence intensity was seen to be significantly decreased (p<0.01) in comparison with control fish. The decrease in fluorescence intensity between the lower and higher dose of glucose treatment groups was also significant (p<0.01). Similar to the cAMP + DEX compound assay, RT qPCR was performed in order to compare the modulation of Pck-1 expression with that seen via fluorescence in the reporter line fish.
Figure 16. Pck-1:eGFP reporter line fish treated with glucose.

A, control. B, glucose (50mM). C, glucose (100mM). D, Mean fluorescence intensity of control and treatment groups. Student’s t-test, control vs 50mM glucose (p=0.13), control vs 100mM glucose (**p>0.01). E, Mean mRNA fold change of control and treatment groups. Student’s t-test, control vs 50mM (++p=0.002), control vs 100mM (**p=0.01).
Similar to what is seen via fluorescence, treatment of glucose resulted in a significant decrease in endogenous *Pck-1* expression. These results suggest that mammals and zebrafish share the same *Pck-1* regulatory pathway responding to higher glucose intake. With the expected effects of cAMP + DEX and glucose observed in the reporter line, the transgenic fish line was then tested with anti-diabetic drugs, MET and ROSI.

4.3.3 Anti-diabetic drugs downregulate *Pck-1* expression in the reporter line

To further validate the reporter line, several known anti-diabetic drugs were tested. Reporter *Pck-1:eGFP* zebrafish embryos treated with MET displayed a significant decrease in *Pck-1* expression, *p*=0.025 (Figure 17). Reporter zebrafish embryos were also treated with ROSI, an insulin sensitizer, also showed a significant decrease in fluorescence intensity (Figure 18).
Figure 17. Pck-1 promoter reporter line fish treated with MET.

Fluorescent microscopy images of A) control B) MET treated fish. C) Average fluorescence intensity of MET treated reporter fish is significantly lower compared to the control. Student’s t-test, *P<0.05.
Figure 18. Pck-1 promoter reporter line fish treated with ROSI.

Fluorescent microscopy images of A) control B) ROSI treated fish. C) Average fluorescence intensity of ROSI treated reporter fish is significantly lower compared to the control. Student's t-test, *P<0.05.
Chapter 5: Discussion, Conclusions, and Future Directions

5.1 Discussion

Zebrafish have conserved physiological and genetic similarities with mammalian models [25, 26]. Past studies have shown that zebrafish have similar physiological glucose regulation [28]. In this study, a fluorescent Pck-1 reporter line was created and validated to be potentially used for the screening of novel anti-diabetic compounds. Pck-1 is highly involved in the regulation of gluconeogenesis within the body. Similar to other mammals, zebrafish also contain two isozymes of Pck-1, namely a cytosolic and mitochondrial isozyme [124]. Past literature suggest that the former is regarded as more relevant for the regulation of glucose metabolism and the study of T2DM[124]. In many vertebrates such as mice and zebrafish, the enzyme activity of Pck-1 is largely due to the cytosolic isozyme (95:5). In humans however, the activity of Pck-1 may be due to an equivalent activity of both the cytosolic and the mitochondrial isozymes. Although the subject of which isozyme is more relevant in the study of glucose metabolism in mammalian models remains to be controversial, it is clear that cytosolic Pck-1 plays a very important role in glucose regulation and the production of endogenous glucose in mammalian species[124], which are important in the treatment of T2DM.

Previous studies suggest that Pck-1 play an important role in glucoregulation in zebrafish [27, 29]. Zebrafish Pck-1 regulation respond similarly to that observed in mammalian species when treated with compounds relevant to glucose metabolism such as glucose (promotes
insulin signaling), cAMP (signalling messenger of glucagon), and DEX (synthetic glucocorticoid). In this study, *in silico analysis* of the *Pck-1* promoter suggest that many TBDs present on the *Pck-1* promoter in mammalian species [101, 102] may also be present on the zebrafish *Pck-1* promoter. The presence of many of these TBDs also found on the zebrafish *Pck-1* promoter support why the observed responses in zebrafish due to the above mentioned compounds are similar to that seen in rodent models. Further investigation would be required to validate the presence and role of these TBDs on the *Pck-1* promoter in zebrafish.

Transfection of a smaller and longer promoter constructs of *Pck-1* suggest that there may be TBDs upstream of 0.8kb from the transcriptional start site that are required for normal gene regulation. The longer promoter construct was used to create transgenic reporter lines that express eGFP under the control of a 3.6kb *Pck-1* promoter construct.

Although there are alternative transposon systems that could have been used in the development of our *Pck-1:eGFP* reporter fish line [38], the use of an Ac/Ds transposon system was utilized for a number of reasons. Firstly, the rate of successful transposition of the desired sequence construct in zebrafish is quite high in Ac/DS transposon systems in comparison with other transposon systems [39]. Secondly, previous studies suggest that the transposition of larger sequence constructs in zebrafish can be done with greater ease using an Ac/Ds transposon system in comparison instead of other available strategies [39]. Outcrossing of our generated reporter lines consistently showed that 50% of the progeny have fluorescence, suggesting that the eGFP gene was transmitted in a Mendelian fashion where there is a single insertion in the established
lines. In addition, the “cut and paste” mechanism that is seen in Ac/Ds transposon systems make it more plausible that only one insert was introduced into the established zebrafish lines[139].

To validate that the reporter lines would respond to glucoregulation related compounds such as glucose and cAMP + DEX similarly to mammalian species, the transgenic line was tested with these compounds. In addition to this, the reporter line fish is tested with known anti-diabetic drugs, MET and ROSI, in order to validate that reporter line respond similarly to that observed in mammalian species. To show that modulations in fluorescence observed in the reporter fish in response to drug treatments is similar to the modulation in $Pck$-1 expression, RT qPCR was performed.

5.1.1 $Pck$-1 promoter length and reporter gene expression

Many developmental pathways such as the insulin regulatory pathway are conserved between zebrafish and higher vertebrates such as mice [28]. Prior to the completion of hepatogenesis, $Pck$-1 is expressed in multiple pre-hepatic cell types in mouse and zebrafish embryos [28, 140]. Past literature state that $Pck$-1 is expressed in the zebrafish liver by 72-96 hpf [28, 140].

A previous study also suggest that the endogenous expression of $Pck$-1 by 3.5 dpf is found predominantly within the liver, yolk sac, intestinal cells, and possibly pronephrono tissue [27]. Similar to what is observed in a previous study ISH [27], the reporter line in this study displays fluorescence in the liver at 4dpf and onwards. Similarly to what is observed in a previous study [27], our reporter fish line displayed fluorescence within the yolk sac, intestinal
cells, with slightly stronger fluorescence within liver at approximately the same developmental stages. In a previous study by Gut et al. (2012), the Pck-1 reporter line fish displayed a markedly high level of fluorescence not only in the liver, but also in the pronephronos tissue at 6-7dpf [27]. In contrast, our Pck-1:eGFP transgenic reporter line fish at similar developmental time points do show fluorescence in the liver but lack fluorescence within the pronephronos tissue by 7dpf. Further experiments would be required to further confirm these observations but the differences may be due to changes in transcriptional regulation of the Pck-1 promoter as the previous study developed a reporter line with 2.8kb Pck-1 promoter construct [27] compared to our reporter line which contains a 3.6kb gene promoter sequence. This may suggest that sequences 2.8kb upstream of the Pck-1 transcriptional start site may play a role in the regulation of Pck-1 expression in zebrafish pronephronos. Thus, it is plausible that there may be differences observed in response to drug treatments such as glucose, cAMP + DEX, and anti-diabetic drugs.

5.1.2 Comparison of our established Pck-1:eGFP reporter line with another published Pck-1 reporter line

The reporter line embryos were treated at 3 dpf for a number of reasons. Firstly, by 3dpf, most organ systems within zebrafish are both fully formed and functional, which include organs that play important roles in glucoregulation: pancreas, kidney, and liver. Secondly, in order to validate the reporter fish line with glucose and other drug compounds under fasting state conditions, these embryos should be tested at a developmental stage where both endogenous and exogenous sources of nutrients would not be confounding factors. By 3-4dpf, the yolk sac of the zebrafish is almost depleted if not fully depleted and thus, drug compound
treatment on the reporter line fish during this time period would greatly reduce confounding results caused by endogenous sources of nutrients. A previous study by Elo et al. (2007) also considered 3dpf as an appropriate developmental stage to assume that the zebrafish is in a near fasting state [27]. To remove the confounding results due to exogenous sources of nutrients, reporter line fish that would be tested with glucose or other drug compounds are not fed at all. Similarly, other studies have done this in order to avoid confounding results in their experiments [27, 29]. Thus, our validation studies were tested with the assumption that the reporter line are in a fasting state.

Although there are many similarities between our reporter line and what has been developed by Gut et al. (2012), there are also many differences. The approach used by Gut et al. (2012) in order to quantify the responses of compounds used in the validation and drug library screens is similar to a standard luciferase assay. In their study, Gut et al. (2012) developed a reporter line where a luciferase gene is driven by a 2.8kb zebrafish Pck-1 promoter. Reporter line fish at 3dpf are treated for 2 days (6dpf). Fish are then lysed followed by quantification via a plate reader. In contrast to our study, Gut et al. (2012) treated their reporter line fish at 4 dpf compared to 3 dpf. Their study justified treating the reporter line fish at 4dpf as it was deemed to the appropriate developmental period (4-6dpf) where glucose levels are at a plateau before calorie deficit lead to net depletion of glucose in the fish. We treated our reporter line fish at 3dpf, the fish are treated for 3 days. This is to overcome the eGFP half-life of 24 hours, which would require a period of 2 to 3 days in order to quantitate treatment effects on the reporter line. Similar to Gut et al. (2012), drug responses during our validation studies are also quantified at 6dpf. Thus, our reporter line fish are also tested during
the appropriate developmental period. In addition to this, most organs are functional by 3dpf. Thus, developmental issues would not play a role in the differences seen between our reporter line fish and the one developed by Gut et al. (2012). Another difference between reporter line fish is that we quantified drug responses via fluorescence microscopy compared to a luciferase assay. In future studies, this approach would allow HTS confocal imaging of drug treated reporter line fish, simultaneously allowing for both quantitation of drug responses and qualitative observation. This allows for quantification of fluorescence only at the liver. In comparison, Gut et al. (2012) used whole body lysate in order to quantify drug responses. Also, their reporter line does not only express their Pck-1 construct in liver. Thus, it may be plausible that the quantified drug responses may be confounded. In comparison, our reporter line only expresses our longer Pck-1 construct at the liver, and only fluorescence at the liver is quantified.

5.1.3 Glucose effect and potential regulatory pathway on Pck-1 expression in zebrafish

In mammalian species, the administration of glucose will decrease Pck-1 expression and endogenous glucose production. Glucose would cause increased insulin secretion in the body, affecting gluconeogenic genes such as Pck-1. In a previous study, glucose was tested on zebrafish in order to show that blood glucose levels as well as the effect on gluconeogenic genes were similar to other mammalian species [29]. In this study, the reporter fish tested with glucose displayed a similar response, with a significant decrease in eGFP fluorescence within the liver. Similarly, endogenous Pck-1 expression is also reduced with the treatment of glucose. This result suggests that pathways that cause a down regulation in response to glucose seen in mammalian species are also present in zebrafish.
It is plausible that similar signaling pathways through the secretion of insulin and the activation of downstream messengers may also be present in zebrafish. Further experiments where the administration of insulin may validate that Pck-1 is down regulated through an insulin activated pathway.

5.1.4 Potential presence of glucagon and glucocorticoid activated regulation of Pck-1 expression in zebrafish

The administration of cAMP and DEX, activators of Pck-1 expression, cause an increase in PEPCK gene expression in zebrafish [27, 29]. Similar to other studies, the treatment of cAMP and DEX are to mimic a similar signalling pathways and physiological response due to glucagon [29]. Another study [27] where a Pck-1 reporter line was also used, the administration of isoprenaline, a non-selective agonist of several adrenergic receptors (α1, α2, α3, β1, β2, β3) in order to stimulate the increase in blood glucose in the body [27]. Isoprenaline would increase the expression gluconeogenic genes such as Pck-1 in order to increase blood glucose levels in the body[27], through increased cAMP levels[141]. Thus, the effect of isoprenaline administration is similar to the administration of glucagon in the body. In either treatments [27, 29], the response was an increase in blood glucose levels and an increased gene expression of Pck-1. Similarly, our reporter fish line were treated with cAMP and DEX and showed a dose dependent increase in eGFP fluorescence in the liver, suggesting a similar response in PEPCK gene expression. Similarly, there is an increase in gene expression of Pck-1 in larval fish treated with cAMP + DEX. Although this study suggest that the treatment of a downstream glucagon related signaling messenger and a glucocorticoid causes the down regulation of Pck-1, treatment of these compounds individually may delineate a better understanding of the regulation of Pck-1 in zebrafish. In addition, although cAMP is a downstream signaling
messenger of glucagon which leads to the regulation of \( Pck-1 \), the treatment of glucagon to our reporter line may further validate the presence of a glucagon activated pathway.

### 5.1.5 Potential presence of insulin activated and atypical pathways that regulate \( Pck-1 \) expression in zebrafish

MET is commonly used in the clinical setting and is known to a “front line” drug in the treatment of T2DM [105]. MET falls within the biguanide class of anti-diabetic drugs that cause an inhibition to endogenous glucose production. The suggested mechanism of action of metformin is through a similar signalling pathway of insulin. It has been suggested that in T2DM patients, metformin treatment causes a decrease in endogenous glucose production through the down regulation of gluconeogenic genes such as \( Pck-1 \), resulting in a decrease in blood glucose levels, through another pathway. It has been suggested that in T2DM patients, insulin insensitivity leads to a decrease in the inhibition of gluconeogenic genes, which can lead to an increase in blood glucose levels [105]. MET treatment causes the downregulation of \( Pck-1 \) and other gluconeogenic genes through an atypical signalling cascade of PI3K and aPKC, leading to the nuclear exclusion of CBP. Nuclear exclusion of CBP causes the dissociation of the CBP/TORC2/CRTC2 binding complex, which is stimulates the up regulation of gluconeogenic genes such as \( Pck-1 \) [105]. In zebrafish, the administration of MET in larval fish at 4dpf over a 24 to 48 hour period caused a decrease in the mRNA expression of \( Pck-1 \) [29].

Similarly, exposure of metformin at a previously proposed concentration [29] to larval reporter line fish lead to a decrease in the fluorescence intensity of the liver, which highly suggestive of a decrease in \( Pck-1 \) expression. This further supports the notion that \( Pck-1 \) may be regulated by an insulin activated pathway in zebrafish.
5.1.6 Potential presence of PPARγ/RXR activated pathway that regulate Pck-1 expression in the reporter line

In addition to validating the Pck-1:eGFP reporter line with MET, we have also tested the reporter line using another anti-diabetic drug, ROSI. ROSI is a thiazolidinedione class anti-diabetic drug, which are insulin sensitizing compounds. Currently, the mechanism of action of thiazolidinedione drugs is not well understood. It is suggested that ROSI treatment leads to the activation of PPARγ and RXR, resulting in the upregulation of insulin-responsive genes such as lipoprotein lipase, fatty acid transporter protein and Glucose transporter type 4 (GLUT4) glucose transporter [142]. This leads to an increase in the utilization of glucose and increased fatty acid uptake, ultimately to a decrease in blood glucose levels and hyperlipidemia [142]. Targets of action of thiazolidinedione drugs are adipocytes, skeletal muscle cells and hepatocytes [142]. A previous study, the treatment of ROSI on larval zebrafish at 4dpf led to the decrease in mRNA expression of Pck-1 [29]. Similarly with our Pck-1:eGFP reporter zebrafish line, the treatment of ROSI on 3dpf larval fish led to a decrease in fluorescence intensity in the liver, which suggest that there is down regulation of Pck-1 expression. This result suggests that pathways involving transcription factor, PPARγ, are present in order for ROSI to regulate the expression of Pck-1 in zebrafish.

5.1.7 Validation studies - conclusions

To validate the established Pck-1:eGFP reporter line for potential ant-diabetic drug screens, the treatment of glucose, cAMP + DEX, and known anti-diabetic drug compounds were used to determine whether the reporter line would respond to the selected compounds similarly to what has been described in previous publications [27, 29]. These results suggest
that regulatory pathways activated through insulin, glucagon and glucocorticoids in mammalian species are also conserved in zebrafish.

Our findings suggest that the *Pck-1:eGFP* reporter line respond similarly to what has been previously seen, validating the line to be used a potential tool in the screening of compounds that affect glucose metabolism through the modulation of the *Pck-1* expression.

5.2 Conclusion

Due to its close physiological and genetic similarities to mammals in combination with the advantages in HTS methods, the zebrafish has emerged as a powerful *in vivo* tool for the discovery of novel drugs, along with delineating disease mechanisms. In this study, a *Pck-1:eGFP* reporter zebrafish line is developed as a novel drug screening tool to identify potential anti-diabetic compounds.

This fluorescent reporter shows similar expression pattern as endogenous *Pck-1* and a number of *Pck-1* expression modulators and anti-diabetic drugs, such as glucose, cAMP + DEX, MET, and ROSI, have been validated to regulate fluorescence in the liver of developing *Pck-1:eGFP* zebrafish embryos. Although further validation of our *Pck-1:eGFP* reporter fish line with other known anti-diabetic drugs such as GLIP would be required in future studies, the results in this study strongly suggest the potential of using this novel fluorescent reporter zebrafish line for the identification of novel anti-diabetic drugs.
5.3 Future directions

Although this reporter line has been tested against several anti-diabetic drugs and known modulators of Pck-1 expression, additional experiments with compounds such as GLIP and all trans retinoic acid (AT-Ra) is required to further validate this transgenic line as AT-Ra has been tested in mammalian models to upregulate the gene expression of Pck-1. Also further validation of the Pck-1:eGFP reporter line can be done with RT qPCR studies on reporter line fish treated with MET, ROSI, and GLIP in order to compare mRNA levels of Pck-1 and the observed eGFP fluorescence intensity. Comparison between the fluorescence intensity observed in the reporter fish line and the mRNA expression of Pck-1 would provide insight in whether the changes in fluorescence intensities seen in the transgenic fish are similar to the changes in mRNA levels.

Preliminary results of transfected promoter constructs of different sequence lengths in HepG2 cells suggest that changes in gene regulation may be due to the presence of essential TBDs in the longer promoter construct (3.6kb) that may not be present within the shorter promoter construct (0.8kb). Further in vitro studies of the Pck-1 promoters would elucidate the details of transcriptional gene regulation. In future studies, transfection of Pck-1 promoters of various lengths fused with luciferase reporter gene into HepG2 cells should be done to measure the differences in gene expression.

These promoter constructs of various lengths could be transfected into HepG2 cells and tested upon addition of glucose, cAMP + DEX, and MET, in order to observe the effects of these
compounds on Pck-1 expression. These cell lines may serve as in vitro models to study drug mechanisms related to transcriptional regulation of Pck-1.

Ultimately, the goal of this study is to create and validate a tool and model system for HTS of anti-diabetic compounds in vivo. In the future, this reporter fish line should be optimized to be used in 96 - 394 well assay plates and imaged with confocal microscope of the Zebrafish HTS Platform at St. Michael’s Hospital. This would accelerate the screening of compound libraries in order to discover novel anti-diabetic compounds for the treatment of DM.
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