UTS UNIVERSITY OF TECHNOLOGY SYDNEY

# Investigating the impact of the parasite derived peptide FhHDM-1 on β-cell survival and function

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Thesis submitted in fulfilment of the requirements for the degree of

**Doctor of Philosophy**

under the supervision of
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## Certificate of Original Authorship

I, Inah Camaya, declare that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Life Sciences, Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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## Abbreviations

| Abbreviation | Term |
|---|---|
| AKR1B7 | Aldo-keto reductase family 1 member B7 |
| AMPK | AMP-activated protein kinase |
| Arg1 | Arginase-1 |
| Bad | Bcl-2 associated death promoter |
| CCL2 | C-C ligand 2 |
| CCL5 | C-C ligand 5 |
| CCR2 | C-C chemokine receptor 2 |
| CM | Cytokine mix |
| CRAMP | Cathelicidin-related antimicrobial peptide |
| CSF | Colony-stimulating factor |
| CXCL10 | C-X-C motif chemokine ligand 10 |
| DAMP | Damage associated molecular pattern |
| DAVID | Database for Annotation, Visualization and Integrated Discovery |
| DE | Differentially expressed |
| DPP-4 | Dipeptidyl peptidase-4 |
| DTT | Dithioethreitol |
| EC50 | Half maximal effective concentration |
| EGF | Epidermal growth factor |
| ER | Endoplasmic reticulum |
| ERK | Extracellular-related kinase |
| ES | Excretory/secretory |
| FBP1 | Fructose biphosphatase 1 |
| FC | Fold change |
| FDR | False discovery rate |
| FFA | Free fatty acids |
| FhHDM-1 | *Fasciola hepatica* helminth defence molecule-1 |
| Fizz1 | Found in inflammatory zone |
| FOXO | Forkhead box O |
| GAD | Gamma-aminobutyric acid |
| GIP | Gastric inhibitory polypeptide/glucose-depedent insulinotropic polypeptide |
| GLP-1 | Glucagon like peptide-1 |
| GO | Gene ontology |
| GSF | Wine grape seed flour |
| GSIS | Glucose stimulated insulin secretion |
| GSK3 | Glycogen synthase kinase 3 |
| h | hours |
| hESC | Human embryonic stem cells |
| HFD | High fat diet |
| HIF | Hypoxia-inducible factor |
| HIP | Hybrid insulin peptides |

| | |
|---|---|
| HMGB1 | High-mobility group box 1 |
| HNF1A | Hepatocyte nuclear transcription factor 1 alpha |
| IAPP | Islet amyloid polypeptide |
| IFNγ | Interferon gamma |
| IGF-1 | Insulin growth factor 1 |
| IGF1R | Insulin growth factor 1 receptor |
| IGF-2 | Insulin growth factor 2 |
| IL | Interleukin |
| IL1β | Interleukin 1 beta |
| iNOS | Inducible nitric oxide synthase |
| i.p. | Intraperitoneal |
| IR | Insulin receptor |
| IRS | Insulin receptor substrate |
| JNK | Jun N-terminal kinase |
| JTXK | Jiang Tang Xiao Ke granule |
| kDA | Kilodalton |
| LBP | Lycium barbarum |
| Lins | Linsitinib |
| LNFPIII | Lacto-N-fucopentaose III |
| LPS | Lipopolysaccharide |
| MAPK | Mitogen-activated protein kinase |
| MCP1 | Monotype chemoattractant protein 1 |
| MEK | Mitogen-activated protein kinase |
| MHC | Major histocompatibility complex |
| min | Minutes |
| miRNA | MicroRNA |
| mRNA | Messenger mRNA |
| ml | Mililitre |
| mTORC | Mammalian target of rapamycin |
| MyD88 | Myeloid differentiation primary response |
| NAD | Nicotinamide adenine dinucleotide |
| NAV1 | Neuron navigator 1 |
| NF-κB | Nuclear factor kappa ligand |
| ng | Nanogram |
| NLRP3 | NLR family pyrin containing domain 3 |
| nm | Nanometre |
| NO | Nitric oxide |
| NOD | Non-obese diabetic |
| NRF2 | Nuclear factor erythroid-2 related factor-2 |
| OD | Optical density |
| O/N | Overnight |
| PAMP | Pathogen associated molecular pattern |
| PARP | Poly-ADP ribose polymerase |

| | |
|---|---|
| PBS | Phosphate buffered saline |
| PDGF | Platelet-derived growth factor |
| PD-L1 | Programmed death-ligand 1 |
| PI3K | Phosphoinositide 3-kinase |
| PICK | Protein interacting with C-kinase |
| PK | Prolink |
| PPARγ | Peroxisome proliferator-activated receptor gamma |
| PTEN | Phosphatase and tensin homolog |
| PTM | Post-translational modification |
| RELM | Resistin-like molecules |
| ROI | Reactive oxygen intermediates |
| ROS | Reactive oxygen species |
| RT | Room temperature |
| RT | Iodoacetamide |
| SCFA | Short chain fatty acid |
| SEA | Soluble egg antigen |
| sec | Seconds |
| SEM | Standard error of means |
| SGK | Serum/glucocorticoid regulated kinase |
| Shc | Src homology 2 (SH2)-containing protein |
| SOCS-1 | Suppressor of cytokine signalling-1 |
| STZ | Streptozotocin |
| T1D | Type 1 diabetes |
| T2D | Type 2 diabetes |
| TGF | Transforming growth factor |
| THBS1 | Thrombospondin 1 |
| TLR | Toll-like receptor |
| TNF | Tumour necrosis factor |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labelling |
| Tyr | Tyrosine |
| Un | Untreated |
| VC | Vehicle control |
| VEGF | Vascular endothelial growth factor |
| Ym1 | Chitinase-like protein 3 |
| β-cells | Beta-cells |
| ω | Omega |
| µg | Microgram |
| µm | Micrometre |
| µM | Micromolar |
| µl | Microlitre |
| °C | Degrees celsius |

## Abstract

The prevalence of type 1 diabetes (T1D) has been increasing worldwide in recent decades, affecting 8.4 million people in 2021, with this figure predicted to increase to 17.4 million by 2040. Pathogenesis is driven by the irreversible immune-mediated destruction of the insulin producing β-cells within the pancreatic islets. This results in dependence on exogenous insulin for survival. However, insulin is not a cure and cannot mimic the minute-to-minute glucose responsiveness of β-cells, and so patients experience repeated episodes of hypo- and hyperglycaemia, which increase morbidity and mortality. Given its autoimmune pathogenesis, modulation of immune cells, and/or their effectors, has been widely explored to cure T1D. However, immune suppression strategies have had limited clinical success to date. It is now evident that the β-cells themselves play central roles in the pathogenic immune dialogue, and actively contribute to their own destruction, thereby initiating and perpetuating autoimmunity (reviewed in Chapter 1). Therefore, therapeutic strategies that directly impact β-cells to preserve function and/or survival, will ultimately cure T1D.

*Fasciola hepatica* helminth defence molecule-1 (FhHDM-1), was previously identified as a parasite-derived peptide that permanently prevented T1D development in non-obese diabetic (NOD) mice. Only six intraperitoneal injections administered on alternate days coincident with the initiation of autoimmunity was efficacious in preventing T1D. Disease prevention was associated with increased insulin content and decreased insulitis within the islets. The aim of this project was to determine whether this beneficial effect of FhHDM-1 was mediated by alterations to the survival and/or function of pancreatic β-cells. Initial analysis (Chapter 2) revealed that FhHDM-1 localised to the pancreas *in vivo* and directly interacted with β-cells *in vitro* to promote survival/function and prevent apoptosis in the presence of pro-inflammatory cytokines, without inducing proliferation. These positive effects were associated with the activation of PI3K/Akt signaling.

Investigation of the underlying transcriptome and proteome changes induced by FhHDM-1 that led to activation of PI3K/Akt signaling identified insulin growth factor 1 receptor (IGF1R) as the probable initiating factor (Chapter 3). However, despite an increase in expression and protein abundance of this receptor in FhHDM-1 treated β-cells, no direct interaction between FhHDM-1 and IGF1R occurred. Instead, FhHDM-1 treatment enhanced the secretion of IGF-2, in turn increasing the total abundance and phosphorylation of IGF1R at the tyrosine residue 1316. This phosphorylation site is associated with the recruitment of

PI3K to IGF1R, and subsequent activation of the PI3K/Akt signaling cascade, in turn leading to enhanced β-cell survival and function.

Since IGF-2 is not stored in the intracellular vesicles of β-cells, the data indicated that FhHDM-1 was activating the transcription of IGF-2 to mediate its positive downstream effects (Chapter 2). This was confirmed by analysis of the miRNA-gene regulation within FhHDM-1 treated β-cells, which identified several miRNAs that correlated with IGF-2/PI3K/Akt modulation. The FhHDM-1 mediated upregulation of miR-483-3p, and downregulation of miR-466i-5p, and miR-7689-3p was linked to an increase in IGF-2 secretion. In addition, the FhHDM-1 induced upregulation of miR-124-3p, and inhibition of miR-30d-5p, miR-3470a, miR-677-5p, and miR-7033-5p correlated with the promotion of PI3K/Akt signaling. Despite the alteration to a broad range of miRNAs and their corresponding gene targets in FhHDM-1 treated β-cells, the biological pathways they impact paralleled each other and corroborate the positive cellular outcomes previously described, that is, the preservation of β-cell mass/function, and ultimately prevention of T1D.

In addition to its beneficial effects on the β-cells, FhHDM-1 has also been previously demonstrated to regulate the activation of macrophages by inhibiting the pro-inflammatory M1-like phenotype. This multimodal effect of FhHDM-1 on both cell types would potentially impact the immune dialogue between macrophages and β-cells, whose crosstalk has emerged as a key determinant of β-cell fate: be it immune tolerance and preservation of β-cell/mass, or initiation/perpetuation of inflammation and β-cell destruction (reviewed in Chapters 1 and 5).

The collective outcomes of this research have revealed new insights into the effects of FhHDM-1 within β-cells, which has been previously unexplored, and has contributed to novel perspectives for diabetes treatment in which targeting of β-cells is central. FhHDM-1 offers a unique therapeutic approach that would be efficacious across multiple pathogeneses in which preservation of functional β-cell mass is required such as in individuals who are at-risk of T1D, in individuals that have undergone seroconversion, in recently diagnosed T1D patients with residual β-cell mass, in patients undergoing islet transplantation, and putatively in T2D, in which inflammation compromises β-cell function. Accordingly, FhHDM-1 represents a potential cure for a compilation of diseases that are the fastest growing and most chronic conditions worldwide. Insulin was the first peptide therapeutic in man – and now, over 100 years later, FhHDM-1 may be an alternative naturally occurring peptide to take its place.

## Hypothesis and Aims

The novel peptide, FhHDM-1, secreted by the parasitic worm *Fasciola hepatica*, has been previously shown to prevent the onset of T1D in NOD mice. Remarkably, only six intraperitoneal injections of FhHDM-1 administered on alternate days coincident with initiation of autoimmunity (4 weeks of age) was sufficient to prevent disease development, with protected mice remaining normoglycaemic up to 30 weeks of age (experimental endpoint). Examination of the pancreas of treated, non-diabetic mice revealed a significant reduction in insulitis and increased insulin within the islets, suggesting the preservation of β-cell mass/function against an autoimmune background characteristic of T1D.

Accordingly, this thesis hypothesized that FhHDM-1 may positively enhance β-cell survival and function as part of the protective mechanism associated with T1D prevention. The following experimental aims were investigated:

i) Determine whether FhHDM-1 promotes β-cell survival/function, and if these beneficial effects are exerted by directly interacting with the β-cells and via activation of specific biological pathways (Chapter 2)

ii) Determine the biological pathway(s) which activates the PI3K/Akt pathway in FhHDM-1 treated β-cells, including putative binding partner(s) (Chapter 3)

iii) Investigate FhHDM-1 induced changes to β-cell miRNAs associated with pathways of survival and function (Chapter 4)

# Chapter 1: Helminth parasites modulate macrophage and β-cell crosstalk to prevent diabetes

## 1.1 Introductory Statement

The work presented in this chapter has been accepted as a review article, for publication in Frontiers in Endocrinology.

Inah Camaya, Bronwyn O'Brien, Sheila Donnelly. How do parasitic worms prevent diabetes? An exploration of their influence on macrophage and β-cell crosstalk.

The text presented here is the accepted version of the manuscript with the numbering of sections, tables and figures altered to align with the formatting of the thesis.

**Author Contributions**

IC conceived and researched the topic and drafted the manuscript; BOB and SD provided conceptual advice and revised the manuscript.

Inah Camaya (graduate research student) Production Note: Signature removed prior to publication.

Bronwyn O'Brien (PhD Principal supervisor) Production Note: Signature removed prior to publication.

Sheila Donnelly (PhD Co-Supervisor) Production Note: Signature removed prior to publication.

## 1.2 Abstract

Diabetes is the fastest growing chronic disease globally, with prevalence increasing at a faster rate than heart disease and cancer. While the disease presents clinically as chronic hyperglycaemia, two distinct subtypes have been recognised. Type 1 diabetes (T1D) is characterised as an autoimmune disease in which the insulin-producing pancreatic β-cells are destroyed, and type 2 diabetes (T2D) arises due to metabolic insufficiency, in which inadequate amounts of insulin are produced, and/or the actions of insulin are diminished. It is now apparent that pro-inflammatory responses cause a loss of functional β-cell mass, and this is the common underlying mechanism of both T1D and T2D. Macrophages are the central immune cells in the pathogenesis of both diseases and play a major role in the initiation and perpetuation of the proinflammatory responses that compromise β-cell function. Furthermore, it is the crosstalk between macrophages and β-cells that orchestrates the inflammatory response and ensuing β-cell dysfunction/destruction. Conversely, this crosstalk can induce immune tolerance and preservation of β-cell mass and function. Thus, specifically targeting the intercellular communication between macrophages and β-cells offers a unique strategy to prevent/halt the islet inflammatory events underpinning T1D and T2D. Due to their potent ability to regulate mammalian immune responses, parasitic worms (helminths), and their excretory/secretory products, have been examined for their potential as therapeutic agents for both T1D and T2D. This research has yielded positive results in disease prevention, both clinically and in animal models. However, the focus of research has been on the modulation of immune cells and their effectors. This approach has ignored the direct effects of helminths and their products on β-cells, and the modulation of signal exchange between macrophages and β-cells. This review explores how the alterations to macrophages induced by helminths, and their products, influence the crosstalk with β-cells to promote their function and survival. In addition, the evidence that parasite-derived products interact directly with endocrine cells to influence their communication with macrophages to prevent β-cell death and enhance function is discussed. This new paradigm of two-way metabolic conversations between endocrine cells and macrophages opens new avenues for the treatment of immune-mediated metabolic disease.

## 1.3 Introduction

While the term 'diabetes' is defined as an individual's inability to regulate blood glucose concentrations with resultant chronic hyperglycaemia, traditionally two major clinically distinct subtypes have been characterised. Type 1 diabetes (T1D) results from the complete autoimmune mediated destruction of the insulin producing beta (β) cells within the pancreatic islets (1). In contrast, type 2 diabetes (T2D) arises because the β-cell population cannot satisfy insulin demand and/or peripheral tissues are resistant to the actions of insulin (2). Although T1D and T2D have been clinically classified as separate disease entities with distinct pathogeneses, there is now increasing evidence that they share disease sequalae. Loss of β-cell function and mass is the common underlying mechanism driving the progression of both conditions, and β-cell death/dysfunction is caused by pro-inflammatory responses largely initiated and perpetuated by macrophages (3).

The incidence of T1D and T2D have been exponentially increasing in recent decades, with the global prevalence predicted to reach almost 600 million cases by 2035 (4). Such rapid increases in disease prevalence cannot be attributable to genetic modifications, and instead suggest the removal of a protective environmental factor or introduction of a predisposing agent (5, 6). Initially, epidemiological studies established a robust inverse relationship between the incidence of multiple autoimmune/inflammatory diseases, and the prevalence of endemic helminth infections (7). Subsequently, compelling results from several human and animal studies have corroborated a protective effect of helminth infection (or their excretory/secretory [ES] molecules) against the development of both TID and T2D (8). It has been broadly proposed that this positive impact on disease outcome is mediated by the potent ability of helminths to regulate pro-inflammatory host immune responses.

Interestingly, macrophages have been identified as the key players in both the modulation of host responses during helminth infections (9), and the initiation and perpetuation of pro-inflammatory responses during diabetes development. Over the years, the central role of macrophages in β-cell differentiation and homeostasis has been well demonstrated (10). However, more recently macrophages have emerged as central players in the initiation of autoimmune insulitis (immune cell infiltration of the islets) in T1D, and as the dominant immune cell population causing intra-islet inflammation in T2D (11, 12). Macrophages are highly dynamic and adopt distinct phenotypes and functions in response to cues received from adjacent cells and the surrounding microenvironment (13, 14). Further,

macrophages are among the first immune cells to traffic to the islets during the development of T1D and T2D (11, 12). Thus, it is not surprising that macrophages can, and do, communicate intimately with β-cells, and *vice versa*. This intercell crosstalk determines if the islet micro-environment becomes pro- or anti-inflammatory, thereby promoting or mitigating the development of diabetes, respectively (15, 16).

This review discusses the evidence suggesting that modulation of the host immune responses by helminths alters the interplay between macrophages and β-cells to prevent β-cell dysfunction and death, and therefore prevent the development of both T1D and T2D. Helminth infection has also been shown to alter host metabolism (17), suggesting an additional effect on cells with endocrine function, such as β-cells. Therefore, this review also explores the biological changes mediated by helminths, and their ES products, to determine if they may directly alter the communication between β-cells and macrophages, to enhance β-cell survival and function, thereby preventing the development of T1D and T2D.

## 1.4 β-cell dysfunction and death underpin T1D and T2D

In T1D, β-cells are lost due to a sustained process of autoimmune destruction driven by pro-inflammatory immune cells. This destructive process progresses over several years such that at diagnosis approximately only 10-20% of β-cell mass remains (1). Within the autoimmune islet environment, dying β-cells are phagocytosed by antigen presenting cells (macrophages and dendritic cells), and autoantigens are processed and presented to autoreactive Th1 and Th17 $CD4^+$ cells. Subsequently, autoreactive cytotoxic $CD8^+$ T cells undergo activation and clonal expansion, and traffic to the pancreas where they infiltrate the islets and destroy β-cells (18, 19). During insulitis, infiltrating immune cells, such as macrophages and T cells, secrete pro-inflammatory cytokines (notably IL-1β, TNF, and IFNγ), which further promotes β-cell apoptosis (20, 21) (Figure 1). These pro-inflammatory macrophages are pivotal to T1D development as their deletion attenuates disease development (22).

While the events initiating β-cell loss are different, T2D is also ultimately an inflammatory syndrome of the islets. Indeed, conditions of overnutrition trigger inflammation and insulin resistance, along with elevated levels of inflammatory factors, such as circulating glucose and free fatty acids (FFA) (23). This induces β-cell endoplasmic reticulum (ER) stress and β-cell secretion of pro-inflammatory cytokines/chemokines (such as IL-1β, TNF, and IFNγ) as well as islet amyloid polypeptide (IAPP). In turn, this pro-inflammatory milieu

leads to the recruitment of macrophages to the islet, akin to insulitis development in T1D, in addition to the activation of islet resident macrophages. These macrophage populations exhibit a pro-inflammatory M1-like phenotype and release the same inflammatory cytokines/chemokines characteristic of T1D development (24, 25). This inflammatory sequalae is exacerbated by stressed and/or necrotic adipocytes, which similarly cause the recruitment of pro-inflammatory macrophages, and reductions in anti-inflammatory macrophage and regulatory T cell populations within the adipose tissue. This, in turn, triggers inflammation in organs targeted by insulin and initiates insulin resistance. To compensate for these adverse metabolic events, β-cells produce and secrete increased amounts of insulin, resulting in β-cell hyperplasia, stress, exhaustion, and ultimately death (26-28). Collectively, these processes lead to the perpetuation and maintenance of a pro-inflammatory environment within the islet, ultimately culminating in β-cell dysfunction, death and T2D (2) (Figure 1.1).

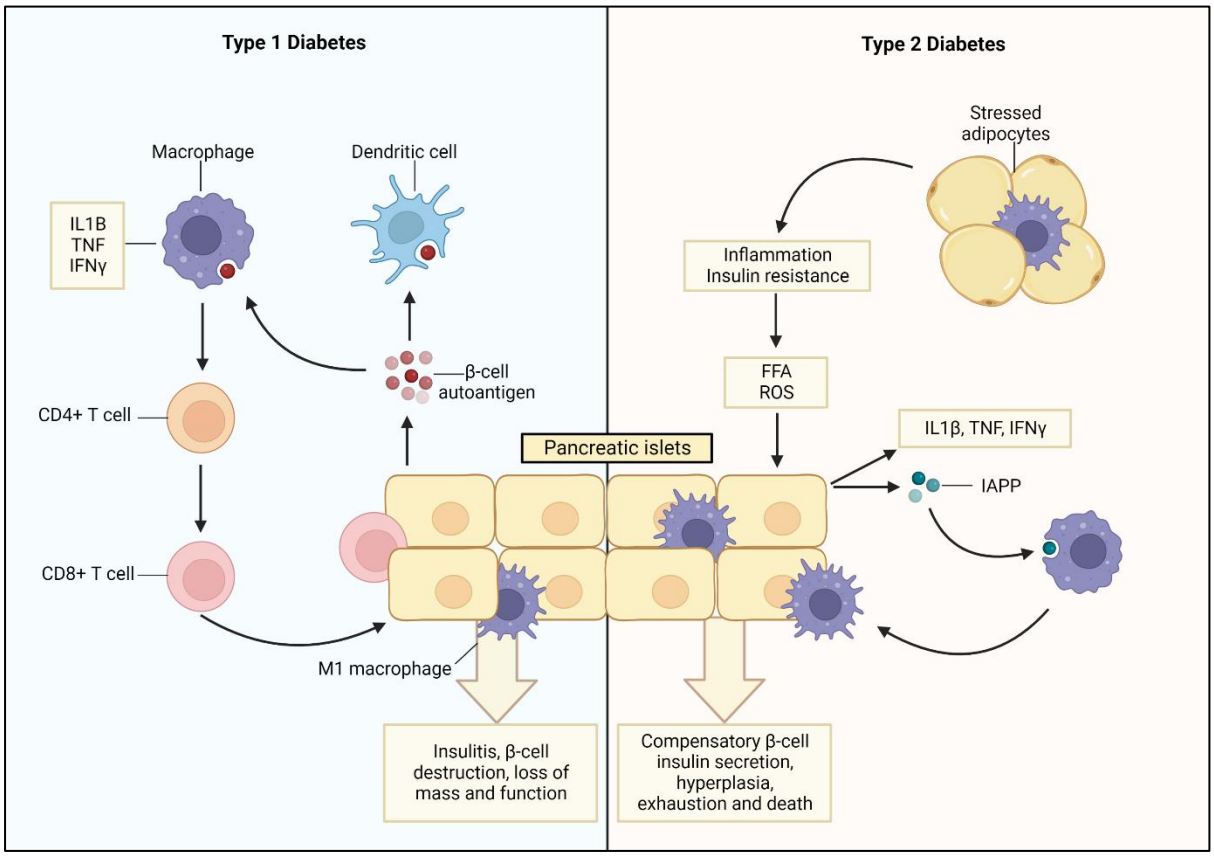


**Figure 1.1 Pancreatic islet inflammation and β-cell death underpin type 1 and type 2 diabetes.** In type 1 diabetes (T1D), β-cell autoantigens are phagocytosed by antigen presenting cells, namely macrophages and dendritic cells. These autoantigens are then presented to autoreactive Th1 and Th17 $CD4^+$ cells, which, in turn, cause activation and clonal expansion of autoreactive cytotoxic $CD8^+$ T cells that infiltrate the islets (termed insulitis) and destroy the β-cells. During insulitis, infiltrating macrophages and T cells secrete pro-inflammatory cytokines, such as IL1β, TNF and IFNγ, which exacerbates β-cell apoptosis. In type 2 diabetes (T2D), overnutrition causes inflammation and insulin resistance, along with hyperglycaemia and increased levels of free fatty acids (FFAs). This induces β-cell endoplasmic reticulum (ER) stress and secretion of pro-inflammatory cytokines, akin to those observed in T1D, as well as islet amyloid polypeptide (IAPP). In turn, this leads to recruitment of macrophages into the islet and activation of islet resident macrophages, which both exhibit pro-inflammatory features. This process is exacerbated by stressed adipocytes that trigger inflammation in peripheral organs and initiate insulin resistance. Consequently, β-cells undergo compensatory hyperplasia and increased insulin secretion, which ultimately leads to β-cell exhaustion, death and T2D. Created with BioRender.com.

## 1.5 β-cells are active participants in their own destruction

The destructive consequences of insulitis on β-cells led to the long-held paradigm that β-cells were passive victims of the detrimental pro-inflammatory islet environments characteristic of T1D and T2D. However, this notion has been challenged by more recent evidence in the context of T1D, that β-cells are active participants in their own demise (1, 29).

The neonatal phase of pancreatic remodelling is characterized by waves of β-cell proliferation, apoptosis and neogenesis. While this is intended as a physiological phenomenon, the process can lead to the generation of β-cell autoantigens, and the stimulation of autoreactive T-cells, which play a major role in the β-cell destruction that leads to diabetes progression. However, the frequencies of these autoreactive T-cell populations in the peripheral blood of T1D patients is comparable to those observed in healthy individuals. This suggests that the activation of insulitis, which underpins the initiation and progression of diabetes, requires the immune tolerance to β-cell autoantigens to be broken (1, 30).

Analyses of human insulitic lesions have suggested that signals released from stressed β-cells precedes, and putatively initiates, the development of insulitis (31). This sequence of events is recapitulated in a humanized mouse model of diabetes, in which the presence of autoreactive T-cells alone was insufficient to induce disease, which was triggered only when β-cells were stressed by the addition of the diabetogenic agent, streptozotocin (STZ) (32). Furthermore, immunosuppressive therapies that solely target T-cells fail to provide long-term protection against T1D as they do not typically address the underlying loss of β-cell immune tolerance (33). Thus, it has now been proposed that activation of the cellular stress response in β-cells due to their metabolic activities promotes cell death pathways, and participates in the initiation and amplification of inflammation and the active destruction of β-cells in T1D (34). Likewise, while T2D has different pathogenesis as compared to T1D, β-cells similarly undergo metabolic stress due to overnutrition and inflammation that causes β-cell compensatory insulin secretion, followed by exhaustion and ultimately death (2).

In T1D, β-cells rapidly respond to the minute-to-minute fluctuations in blood glucose levels by tightly regulating insulin secretion. Such high metabolic demand to produce and secrete insulin can render the β-cells susceptible to exceeding ER protein folding capacity, in turn leading to accumulation of misfolded proteins and ER stress (35). This can dysregulate several processes, such as inhibition of β-cell function, induction of apoptosis and activation of cytosolic post-translational modification (PTM) enzymes (36), which can generate a group

of neoantigens called hybrid insulin peptides (HIPs) by covalently linking insulin peptides to β-cell granule peptides, such as insulin c-peptide and IAPP. These HIPs contribute to autoimmune responses and ultimately β-cell destruction, as they are recognized by autoreactive $CD4^+$ T cells in both mouse models and human patients of T1D (37, 38). Beta cells from diabetic patients also express increased levels of MHC-I and MHC-II molecules (HLA-I and HLA-II, respectively, in humans), the latter being conventionally expressed only by antigen presenting cells. This enables β-cells to present peptides (notably autoantigens) to $CD8^+$ and $CD4^+$ T cells, respectively (39, 40). Moreover, immune cells have direct access to islets through the dense network of islet vasculature, which can be particularly detrimental in the presence of activated pro-inflammatory immune cells and cytokines that induce β-cell stress and apoptosis (34, 41).

These β-cell characteristics make them vulnerable to destruction, and this is exacerbated in the pathogenic conditions of T1D and T2D, in which there are increased levels of pro-inflammatory cytokines, reactive oxygen species (ROS) and nitric oxide (NO), which are especially detrimental since β-cells have limited antioxidant defence capabilities (42, 43), and are highly sensitive to cytokine mediated damage. The central pro-inflammatory cytokines, IL-1β, TNF and IFNγ, have been shown to inhibit β-cell function and activate apoptotic pathways in β-cell lines, human islets, and rodent models (34, 44). In turn, this inflammatory milieu stimulates the β-cells themselves to secrete pro-inflammatory cytokines and chemokines, thereby actively contributing to their own destruction (45). Indeed, β-cells of diabetic animals and within the islets of T2D patients were observed to produce IL-1β (46). This is corroborated by *in vitro* studies, wherein cultured human islets exposed to high glucose were observed to secrete IL-1β, with production increasing due to autocrine feedback (47).

Furthermore, β-cells produce several chemokines: (i) C-C ligand 5 (CCL5), also named RANTES, (ii) C-X-C motif chemokine ligand 10 (CXCL10), also called IP-10, and (iii) C-C ligand 2 (CCL2), also termed monotype chemoattractant protein or MCP1 (34). CXCL10 and CCL5 are secreted by murine islets, β-cell lines, and cultured human islets after exposure to IL-1β, TNF, and IFNγ. Both chemokines attract activated immune cells to the islets (48, 49), and CXCL10 has been shown to exert direct toxicity on the β-cells (50). Similarly, CCL2 is produced by human and murine islets in response to IL-1β and can be induced *in vivo* by environmental triggers (such as viral infections), causing inflammation and macrophage recruitment (51-53).

Collectively, these β-cell vulnerabilities, along with the signals they release under conditions of stress within the islet microenvironment, can create a self-perpetuating cycle of β-cell destruction, which is exacerbated in conjunction with pro-inflammatory immune cells (Figure 1.2).

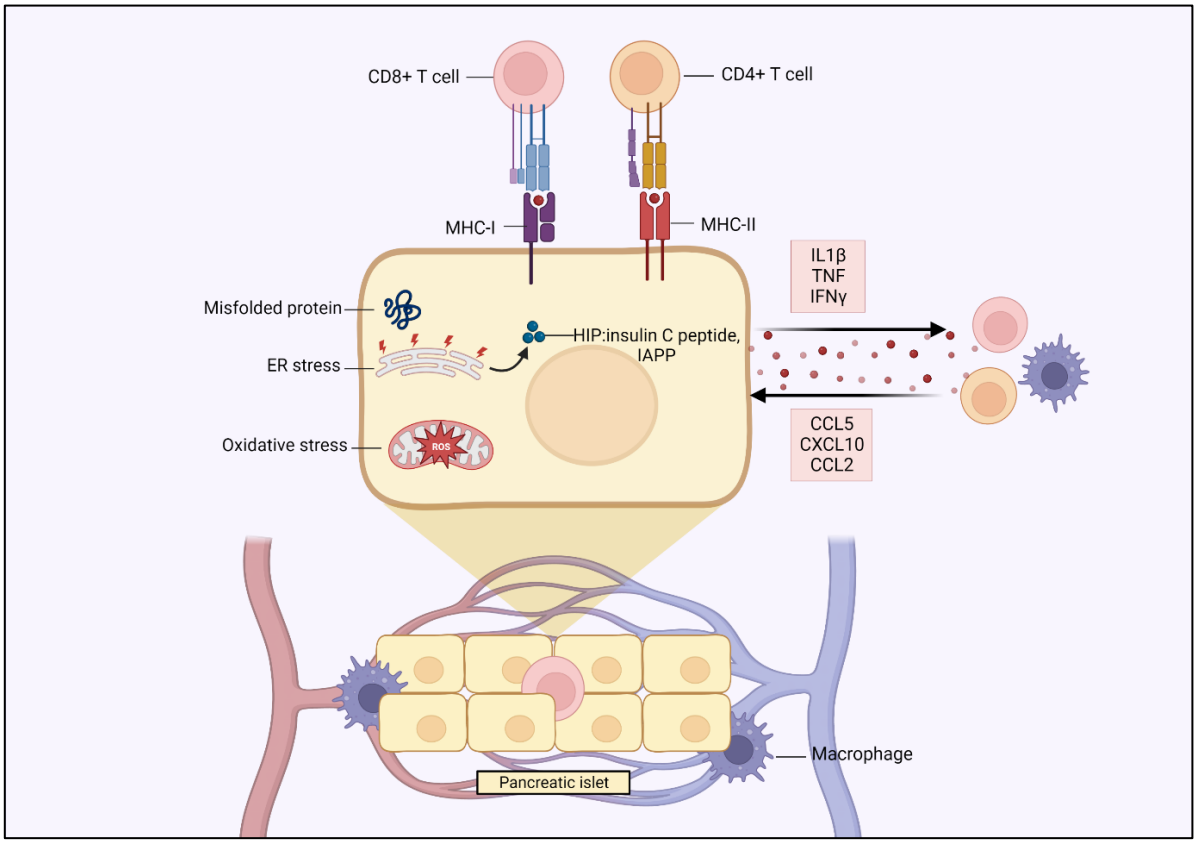


**Figure 1.2 Pancreatic β-cells play an active role in their own destruction.** The high metabolic demand of insulin production/secretion can render β-cells vulnerable to exceeding endoplasmic reticulum (ER) protein folding capacity, which can lead to accumulation of misfolded proteins and ER stress. This results in dysregulation of β-cell function, induction of apoptosis and generation of hybrid insulin peptides (HIP), such as insulin c-peptide and islet amyloid polypeptide (IAPP), which can be recognized by autoreactive immune cells, thereby contributing to β-cell destruction. Beta-cells can also express MHC-I/II, the latter conventionally only expressed by antigen presenting cells to present peptides to $CD8^+$ and $CD4^+$ T cells, respectively. The dense network of islet vasculature also allows immune cells direct access to the islets, which can be especially detrimental under diabetic conditions wherein immune cells generally exhibit pro-inflammatory activity. In a diabetic environment, there are elevated levels of reactive oxygen species (ROS) and pro-inflammatory cytokines, to which β-cells have suboptimal defense mechanisms due to their limited production of antioxidant enzymes and sensitivity to cytokine damage. In response to this inflammation, β-cells themselves secrete pro-inflammatory cytokines (IL1β, TNF, IFNγ) and chemokines (CCL5, CXCL10, CCL2), which contribute to β-cell destruction through self-toxicity or activation/recruitment of immune cells such as macrophages. Created with BioRender.com.

## 1.6 Crosstalk between macrophages and β-cells predetermines the fate of β-cells

Macrophages constitute a heterogeneous and dynamic immune cell population whose phenotype and function are highly dependent on the tissue microenvironment. Macrophages can derive from embryonic hematopoietic precursors and inhabit specific tissues, in which they actively participate in maintaining homeostasis under physiological conditions. Following pathogen infection, tissue damage, release of inflammatory mediators, or metabolic cues, circulating monocytes are recruited in different tissues where they differentiate into resident macrophages. Here, stimuli in the surrounding micro-environment induce macrophages to adopt pro-inflammatory or anti-inflammatory phenotypes and functions, thereby mediating inflammatory/autoimmune or homeostatic/reparative responses, respectively (22, 54).

Macrophages are among the first cells to traffic to the pancreas under both physiological and diabetogenic conditions. This is because macrophages play central roles during neonatal islet remodeling, which is characterised by waves of β-cell differentiation, proliferation, and apoptosis, due to their potent phagocytic abilities. After taking up residence in the islets, macrophages remain in intimate contact with the β-cells, where they act as exquisite sensors of the function and viability of β-cells (15). Through a two-way exchange of signals, such as cytokines and chemokines, hormones and growth factors, insulin-containing vesicles and exosomes, and metabolites, macrophages are highly responsive to β-cell activities, and the composition of their secretions is modulated accordingly to promote β-cell survival and metabolic activity (under physiological cues). On the other hand, macrophage/β-cell crosstalk can drive β-cell dysfunction and apoptosis when physiological equilibrium is disturbed by the inflammatory signals present during the development of T1D and T2D (16) (Figure 1.3).

In the context of T1D, the populations of resident islet macrophages in both animal models and humans display a pro-inflammatory phenotype, characterised by the secretion of cytokines (such as IL-1β and TNF), upregulated expression of MHC-II and increased antigen presentation capacity, and decreased phagocytosis of apoptotic β-cells (55-57). Decreased uptake of dying β-cells by macrophages and/or increased rates of β-cell apoptosis leads to an accumulation of dying β-cells, and their progression to necrosis, during the intended physiological process of neonatal islet remodeling. Also, after the phagocytosis of apoptotic β-cells, macrophages secrete increased levels of pro-inflammatory IL-12 and decreased levels

of IL-10, which contradicts the expected anti-inflammatory response (58-60). Aside from this cytokine profile, intra-islet macrophages secrete NO and chemokines, and express chemokine receptors (notably CCR5, CXCR3 and CCR8) which further recruits and activates other pro-inflammatory immune cells (61, 62). These phenomena modulate the crosstalk between β-cells and macrophages to trigger the initiation of disease.

Furthermore, monocyte-derived macrophages are among the first, and most abundant, immune cells to traffic into the islet in both murine models and human patients (11, 63), and it is hypothesized that this initial influx is induced by increased levels of CCL2. Indeed, CCL2 overexpression in murine β-cells promoted monocyte recruitment to islets, infiltration, and β-cell destruction (53). Moreover, macrophages express C-C chemokine receptor 2 (CCR2) to which CCL2 can bind, thereby inducing macrophage secretion of the pro-inflammatory mediators, IL-1β, TNF, IL-12, and CXCL10. These islet infiltrating macrophages are predominantly characterized as a pro-inflammatory phenotype and release potent inflammatory mediators (such as IL-1β and CCL2) that promote β-cell apoptosis (64). Simultaneously, signals from apoptotic β-cells (IL-1β, CCL5, CXCL10, CCL2) amplify/maintain this pro-inflammatory phenotype (65).

This inflammatory environment can be exacerbated by priming factors that mediate crosstalk between macrophages and β-cells. For instance, β-cells and islet resident macrophages express toll-like receptors (TLRs), which are activated by pathogen activated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) released due to viral infection and other tissue damaging agents. This stimulates the secretion of pro-inflammatory cytokines (such as IL-1β and TNF) that can reinforce and amplify a pro-inflammatory function/phenotype of islet resident macrophages and activate apoptotic pathways within β-cells (66). As aforementioned, stressed and/or apoptotic β-cells release pro-inflammatory signals, which are endocytosed by macrophages, thereby perpetuating the cycle of inflammation. Furthermore, insulin vesicles secreted by β-cells contain immunogenic peptides, which can also be taken up by resident M1-like macrophages for processing and presentation to autoreactive T cells. Aside from the uptake of secreted vesicles, intracellular cargo from apoptotic β-cells can be transferred to macrophages during phagocytosis (64). Additionally, under the pro-inflammatory conditions β-cells begin to express MHC-II, which enables them to present autoantigens (notably insulin neoantigens) to antigen presenting cells (40). Ultimately, the activities of β-cells and macrophages culminate

in the activation and clonal expansion of autoreactive T cells, which drive the accelerated and irreversible loss of β-cell mass leading to T1D.

Similarly, under T2D conditions the crosstalk between macrophages and β-cells plays a major initiating role in establishing β-cell dysfunction, and a pro-inflammatory macrophage phenotype. In T2D, overnutrition and transient hyperglycaemia induce β-cell hyperactivation and hyperplasia to satisfy the increased insulin demand (23). Aside from increased insulin secretory rates, β-cells undergo multiple functional changes induced by the nutrient rich environment and increased metabolic rates, including the production of pro-inflammatory mediators, such as NO and ROS (67). As islet macrophages constantly probe their microenvironment for signals, they sense these fluctuations in β-cell activity, via uptake of insulin-containing vesicles or through overactivation of their purinergic receptors by ATP. This can lead to desensitisation and downregulation of homeostatic cues, as supported by reports of decreased purinergic receptor gene expression in T2D macrophages (68). Islet resident macrophages also contribute to islet vascular remodelling that supports compensatory hyperinsulinemia (69). While this may be advantageous in the early phases of compensatory prediabetes, this advantage is nullified by the chronic background of inflammation in which β-cell hyperactivation and dysfunction are amplified, ultimately driving β-cell exhaustion and loss.

Moreover, in T2D patients, β-cells secrete IAPP along with insulin (70). The former can aggregate to form plaques and fibrils, which skew islet macrophages to a pro-inflammatory phenotype and induce IL-1β secretion in circulating monocyte-derived macrophages to facilitate their recruitment into the islet (71). Additionally, this inflammatory dialogue is amplified by increased levels of FFAs (i.e. overnutrition) and pro-inflammatory cytokines (IL-1β, TNF, IL-6, and IL-8) (72, 73). Collectively, this environment induces β-cell dysfunction and eventual death (due to the actions of inflammatory mediators and metabolic exhaustion) (15). In addition, pro-inflammatory M1-like macrophages in T2D mice were found to secrete exosomes containing the microRNA, miR-212-5p, that can be taken up by β-cells, in turn inhibiting SIRT2-mediated Akt activation and subsequently impairing glucose stimulated insulin secretion (74).

Importantly, islet macrophages do not exclusively display an M1-like phenotype, expressing both pro-inflammatory (CD68) and anti-inflammatory (CD163) markers (75). Furthermore, it has been observed that in animal models of T2D, islet macrophages undergo

local expansion and specific transcriptional changes, with two distinct subsets identified: intra-islet ($CD11c^+$) and peri-islet ($CD11c^-$) macrophages. The former was found to inhibit β-cell function putatively through increased uptake of β-cell secreted insulin vesicles in a cell-to-cell contact dependent manner. On the other hand, both macrophage populations promoted compensatory β-cell proliferation (76). It has also been reported that in response to increased β-cell death in murine models of induced diabetes (STZ alone or in combination with a high fat diet) and in a genetic mouse model of T2D, islet macrophages exhibited increased expression levels of insulin like growth factor 1 (IGF-1), concomitant with decreased expression of IL-6 and TNF, a profile that is associated with a reparative phenotype (77). This functional activity is supported by a multitude of studies demonstrating that anti-inflammatory M2-like macrophages are induced in various models of pancreatic injury and inflammation, and operate to promote β-cell survival, proliferation, and maintenance of functional β-cell mass, through the release of protective factors, such as IGF-1, epidermal growth factor (EGF), transforming growth factor (TGF), platelet-derived growth factor (PDGF), and Wnt3a (78).

Collectively, these observations demonstrate that macrophages exhibit distinct expression profiles and associated functional activities that are highly specialized in response to the islet microenvironment. As a result, the crosstalk between β-cells and macrophages plays a central role in both the maintenance of islet homeostasis and the initiation/perpetuation of inflammation. Accordingly, the macrophage / β-cell crosstalk can dictate autoimmunity and β-cell destruction, or conversely, immune tolerance and preservation of β-cell mass and function. Manipulating this metabolic conversation offers a unique and new strategy to directly affect β-cell survival and preservation of β-cell mass, and thus prevent the islet inflammatory events underpinning T1D and T2D.

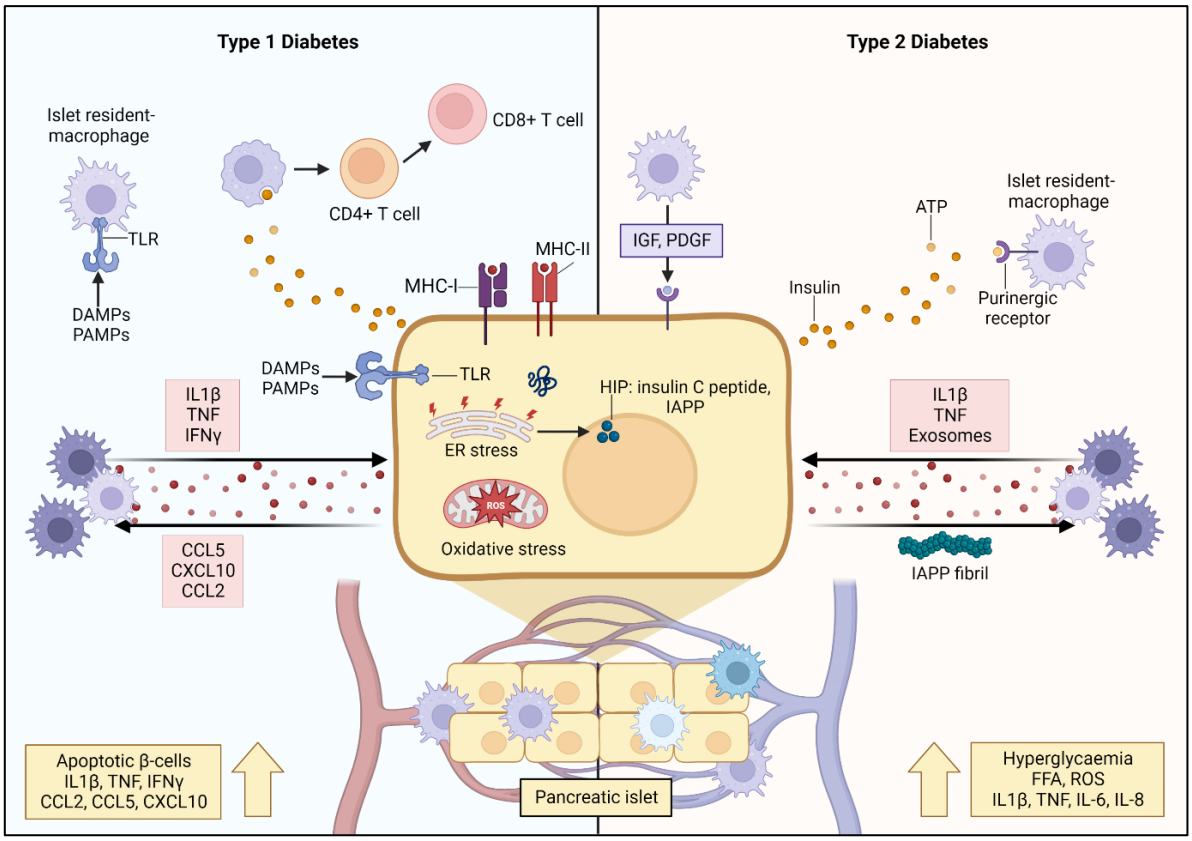


**Figure 1.3 Crosstalk between macrophages and β-cells orchestrates inflammation and β-cell destruction in type 1 and type 2 diabetes.** When physiological homeostasis is disrupted by inflammatory signals, communication between β-cells and macrophages drive β-cell dysfunction and apoptosis. In the initiating stages of T1D, resident islet macrophages exhibit a pro-inflammatory M1-like phenotype characterized by secretion of pro-inflammatory cytokines, upregulated MHC-II expression and increased antigen presentation capacity. The β-cells also secrete pro-inflammatory cytokines and chemokines that recruit monocyte-derived macrophages with an M1-like phenotype into the islet. Furthermore, β-cells also secrete insulin vesicles containing immunogenic peptides that are taken up by macrophages for presentation to autoreactive T cells, and express MHC-II, allowing them to present autoantigens as well. Together, these interactions reinforce and amplify β-cell apoptosis and destruction underlying the irreversible loss of β-cell mass leading to T1D. During T2D, hyperglycaemia and overnutrition causes β-cell hyperactivation and hyperplasia leading to functional changes and increased metabolic rates (ER stress, oxidative stress) within the β-cells. These changes are sensed by resident islet macrophages via uptake of insulin-containing vesicles, or through overactivation of purinergic receptors by ATP. Furthermore, β-cells secrete islet amyloid polypeptide (IAPP) which can aggregate to form plaques and fibrils that skew macrophages to a destructive pro-inflammatory M1-like phenotype, Islet macrophages do not purely exhibit M1 features and can also have anti-inflammatory M2-like activity, wherein macrophages exhibit increased expression reparative factors such as IGF-1 that promote β-cell survival and proliferation. Created with BioRender.com

## 1.7 Using the immune regulatory mechanisms of helminths to positively modulate macrophage and β-cell crosstalk for diabetes prevention

The inverse correlation between the prevalence of infection with helminths and the incidence of immune-mediated metabolic disease has been explained by the 'old friends hypothesis'. This proposes that the coexistence of helminths and their human hosts over millennia has enabled them to potently regulate the mammalian immune system and skew responses to be anti-inflammatory/tolerogenic/reparative (79-81). This phenomenon affords mutual benefit by promoting longevity and tissue integrity for helminth and host, respectively.

Consequently, the elimination of helminths from human populations, due to enhanced sanitation practices, has also removed their regulatory influence on host immune responses. The result of this is the aberrant activation of inflammatory pathways, thereby increasing the incidence of immune-mediated diseases, such as T1D and T2D. Given these observations, live helminth infection, or the administration of their ES products, are being actively investigated for therapeutic potential (82-84).

### 1.7.1 Helminths are potent modulators of macrophage phenotype and function

To have sufficient time to mature and reproduce, thereby completing their lifecycle, helminths must establish chronic infections within their mammalian hosts. Accordingly, helminths have developed elegant mechanisms to educate the host's immune system to tolerate their presence, and therefore support their prolonged survival. Thus, all helminth parasites typically drive the immune response of their hosts towards a predominantly anti-inflammatory phenotype and suppress the development of a pro-inflammatory immune response (85). Reflecting this bias in immune cell activation, helminth infection is commonly associated with an increase in Th2 cytokines, such as IL-4 and IL-13, and a reduction in Th1 cytokines, such as IL-12 and IFNγ (85-87).

Within the immune response to helminth infection, macrophages have emerged as the dominant innate immune cells playing a central role in controlling the development of the adaptive immune response, and the pathological outcomes of infection. During parasite infection, macrophages primarily display an anti-inflammatory M2-like phenotype, as characterised by the expression of specific effector molecules, such as arginase-1 (Arg1), Ym1 and resistin-like molecules (RELM). The primary functional role for these cells is the mediation of tissue repair mechanisms (9). Arg1 metabolises L-arginine into polyamines,

urea and L-orthinine, which contributes to collagen synthesis, fibrosis, and wound healing (88, 89). This consumption of L-arginine can also inhibit NO synthesis, as it is the same substrate required by inducible nitric oxide synthase (iNOS) (90), thereby regulating inflammatory activity. Similarly, Ym1 and RELMα are both associated with wound healing, with the former also directly regulating tissue repair (91-93), and the latter mediating collagen deposition and vascular stability (94, 95). In addition to these characteristic markers, helminth induced M2-like macrophages secrete growth factors, such as IGF-1, vascular endothelial growth factor (VEGF) and PDGF, which orchestrate collagen deposition, angiogenesis and the recruitment, activation, and proliferation of reparative cells, such as fibroblasts and endothelial cells (96, 97). Besides this functional activity, the M2-like macrophages also regulate excessive inflammation by dampening pro-inflammatory macrophages (9). Thus, as a combined effect, the modulation of macrophage phenotypes during helminth infections acts to minimize/prevent excessive inflammatory immune responses within the host and to repair tissue damage caused by the migratory and feeding activities of the parasite (85).

The modulation of macrophage activities must be mediated by the molecules that are actively secreted by the parasites, and generally referred to as ES products. The ES products from all parasites are heterogenous mixes of proteins, glycoproteins and lipids, which mimic the modulation of immune responses induced by parasite infection *per se*, thus supporting the notion that these ES molecules hold considerable immune modulatory power (98). Accordingly, the ES products of several parasites have been mined to characterise the individual constituent molecules with a capacity to interact with, and modulate, the phenotype/function of host macrophages (Table 1.1)

**Table 1.1 Helminth-derived products and molecules that modulate macrophage phenotype and function.**

| Helminth | Helminth-derived product | Macrophage cell type | Biological effect on macrophage | Reference |
|---|---|---|---|---|
| *Acanthocheilonema viteae* | Excretory/secretory products (ES)-62 purified from adult fluke | Ex vivo murine (BALB/c and 129) peritoneal macrophages | Suppressed IFNγ/LPS-induced production of IL-12, IL-6, and TNF via interaction with TLR-4 | (99, 100) |
| | Cysteine protease inhibitor (AvCystatin); *E. coli* recombinant | Murine (C57BL6 and BALB/c) peritoneal macrophages | Induced regulatory/M2-like phenotype and increased production of IL-10, via activation of MAPK signalling pathways | (101, 102) |
| *Ancylostoma ceylanicum* | *A. ceylanicum* metalloprotease 2 (Ace-MTP-2); *E. coli* and *P. pastoris* recombinant | Human monocyte cell line (THP-1) and THP-1 differentiated macrophages | Enhanced the secretion of TNF and induced the release IFNγ in LPS-exposed macrophages | (103) |
| *Ascaris lumbricoides* | Lumbricoides protein with cysteine protease inhibitor activity (AI-CPI); *E. coli* recombinant | Murine macrophage cell line (RAW 264.7) | Inhibited macrophage secretion of IL1β, TNF, IFNγ and IL-6 following LPS exposure. Enhanced production of IL-10 and TGFβ, suggesting polarisation to an anti-inflammatory M2 phenotype | (104) |
| *Ascaris suum* | Adult body fluid (ABF) | Human monocyte-derived macrophages (from peripheral blood mononuclear cells) | Inhibited secretion of LPS-induced TNF and IL-6 | (105) |
| *Brugia malayi* | Macrophage migration inhibitory factor (MIF); *E. coli* recombinant | Murine (C57BL6) bone marrow derived macrophages, murine (BALB/c) peritoneal macrophages | Synergized with IL-4 to induce M2-like macrophages expressing key markers (Arg1, RELMα, Ym1 and mannose receptor) | (106) |
| | | Murine (BALBc) peritoneal macrophages | Increased macrophage expression of Ym1 | (107) |
| | Abundant larval transcript (ALT); *E. coli* recombinant | Murine (C57BL6 and CBA) bone marrow derived macrophages, murine macrophage cell line (J774) | Increased expression of SOCS-1 and GATA-3 which are both associated with polarization to an anti-inflammatory phenotype and/or inhibition of pro-inflammatory macrophages | (108) |

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| *Clonorchis sinensis* | Type 1 cystatin (CsStefin-1); *E. coli* recombinant | Murine (C57BL6) spleen and mesenteric lymph node derived macrophages | Induced IL-10 secreting macrophages in the spleen and mesenteric lymph nodes, which were associated with reduction in intestinal inflammation | (109) |
| | Host defence molecule (CsMF6p/HDM); *E. coli* recombinant | Murine macrophage cell line (RAW 264.7) | Induced pro-inflammatory response associated with M1-like phenotype, such as increased expression of TNF and IL-6 | (110) |
| *Echinococcus granulosus* | Cyst fluid (EgCF) | Murine peritoneal macrophages and murine macrophage cell line (RAW 264.7) | Suppressed LPS-induced TNF, IL-12 and IL-6, and increased IL-10 | (111) |
| *Echinococcus multilocularis* | *E. multilocularis* miR-71 (emu-miR-71) | Murine macrophage cell line (RAW 264.7) | Inhibited nitric oxide release from macrophages | (112) |
| *Fasciola hepatica* | Peroxiredoxin (Prx/Trx); *E. coli* recombinant | Murine (BALB/c) peritoneal macrophages, murine macrophage (RAW 264.7) cell line | Induced markers (Arg1, Ym1, Fizz1) associated with an M2-like phenotype and promoted secretion of IL-10 | (113) |
| | Native fatty acid binding protein (Fh12) purified from adult fluke extract | Human monocyte derived macrophages | Induced markers (Arg1, Ym1) associated with an M2-like phenotype, promoted secretion of IL-10 and downregulated production of NO, TNF, IL1β and IL-12. Effects are likely mediated via TLR4 | (114) |
| | | Murine (C57BL6) bone marrow derived macrophages | Suppressed LPS-induced production of TNF, IL1β, IL-12 and IL-6 and inhibited TLR4 activation | (115) |
| | Fatty acid binding protein (Fh15); *E. coli* recombinant | Murine (C57BL6) bone marrow derived macrophages | Suppressed LPS-induced production of TNF and IL1β, and inhibited TLR4 activation | (116) |
| | Native glutathione S-transferase (nFhGST) isolated from adult fluke soluble extract | Murine (C57BL6) bone marrow derived macrophages | Suppressed LPS-induced NF-κB-dependent production of TNF and IL1β | (117) |
| | Cathepsin-L1 (FheCL1); *P. pastoris* recombinant | Ex vivo murine (BALB/c) peritoneal macrophages | Suppressed TLR3-dependent cytokine production (IL-6, IL-12 and TNF) induced by LPS, via cleavage of TRIF | (118) |

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| | Transforming growth factor-like molecule (FhTLM); *E. coli* recombinant | Bovine blood derived macrophages | Induced regulatory phenotype expressing increased levels of IL-10, Arg1, PD-L1 and mannose receptor, along with decreased levels of IL-12 and NO | (119) |
| | Helminth defence molecule-1 (FhHDM-1); synthetic molecule | Murine (C57BL6) bone marrow derived macrophages, ex vivo non-obese diabetic mice peritoneal macrophages | Suppressed LPS-induced production of TNF, prevented activation of NLRP3 inflammasome via inhibition of lysosomal vATPase, thereby suppressing production of IL1β | (120-122) |
| | Novel omega-class glutathione transferase (GSTO2); *E. coli* recombinant | Murine macrophage cell line (RAW 264.7) | Decreased expression of IL-6, IL1β, IFNγ and TNF in LPS-exposed macrophages and increased expression of IL-10 and TGFβ | (123) |
| *Heligmosomoides polygyrus* | *H. polygyrus* derived extracellular vesicles | Murine (C57BL6 and BALB/c) bone marrow derived macrophages, murine macrophage cell line (RAW 264.7) | Suppressed activation of both pro- and anti-inflammatory macrophages, leading to decreased levels of IL-6, IL-12, TNF and CD206, Ym1 and RELMα, respectively | (124) |
| *Hymenolepis dimimnuta* | *H. dimimnuta* antigen (HdAg) | Murine (BALB/c) bone marrow derived macrophages | Suppressed LPS-induced release of TNF and IL1β by promoting IL-10 signalling | (125) |
| *Nippostrongylus brasilliensis* | Acetylcholinesterase (AChE); expressed and delivered via *T. musculi* | Murine (BALB/c) peritoneal macrophages | Promoted M1-like macrophages with increased NO and lowered arginase activity, suggesting inhibition of an M2-like phenotype | (126) |
| *Schistosoma mansoni* | Soluble egg antigen (SEA) | Human macrophages differentiated from monocytes (isolated from human volunteers) | Induced a mix of pro- and anti-inflammatory characteristics; increased expression of IL-10, TNF, IL-12 and TGFβ | (127) |
| | Schistosomal-derived lypophosphatidylcholine (LPC) | Murine (C57BL6) bone marrow derived and peritoneal macrophages | Induced M2-like macrophages as evidenced by increased Arg1, TGFβ and IL-10 | (128) |
| | Omega 1 (ω1) derived from SEA | Ex vivo murine (C57BL6) peritoneal macrophages | Induced IL1β secretion in peritoneal macrophages stimulated with toll-like receptor 2 ligand | (129) |

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| | Omega 1 (ω1); recombinant, purified from human embryonic kidney 293 cells | Murine (C57BL6) macrophages derived from epididymal white adipose tissue | Promoted IL-33 secretion, leading to polarisation to an anti-inflammatory phenotype | (130) |
| | Immunomodulatory molecule (Sm16/SPO-1/SmSLP); *P. pastoris* recombinant and synthetic molecule | Murine (BALB/c) bone marrow derived macrophages and human THP-1 differentiated macrophages | Decreased IL-6 and TNF induced by LPS exposure, but increased the same pro-inflammatory cytokines when administered alone | (131) |
| *Schistosoma japonicum* | CP1412 protein; *E. coli* recombinant | Murine macrophage cell line (RAW 264.7) | Increased expression of CD206, Arg1 and IL-10 associated with polarization to an M2-like phenotype | (132) |
| | Sj16 protein; *E. coli* recombinant | Murine (BALB/c) peritoneal macrophages | Downregulated LPS-induced TNF expression and upregulated IL-10, associated with polarization to an anti-inflammatory phenotype | (133) |
| | *S. japonicum* derived extracellular vesicles | Murine macrophage cell line (RAW 264.7) | Promoted polarization to M1-like phenotype, with increased expression of CD16/32, iNOS and TNF | (134) |
| *Taenia crassiceps* | Excretory/secretory products (TcES) | Murine (BALB/c) bone marrow derived macrophages | Decreased IL-6, IL-12 and TNF, and increased IL-10 in LPS exposed macrophages | (135) |
| *Taenia pisiformis* | Isolated exosome-like vesicles derived from ES | Murine macrophage cell line (RAW 264.7) | Induced production of IL-4, IL-6, IL-10, IL-13 and Arg1, and decreased expression of IL-12, IFNγ and iNOS | (136) |
| *Toxocara canis* | Excretory/secretory products (ES) | Murine (C57BL6) peritoneal macrophages | Promoted TNF secretion, decreased IL1β and inhibited IL-6 initially followed by continuous increase over time. Induced expression of inflammatory NFκB | (137) |
| *Trichinella spiralis* | *T. spiralis* excretory/secretory antigens | Murine macrophage cell line (RAW 264.7) | Decreased IL-12 and TNF following LPS exposure, and promoted IL-10 secretion | (138) |
| | | Murine macrophage cell line (J774A.1) | Inhibited TNF, IL1β, IL-6 and IL-12 production following LPS exposure, and promoted IL-10, TGFβ and Arg1 | (139) |
| | | Murine (C57BL6) peritoneal macrophages and murine macrophage cell line (RAW 264.7) | Attenuated colitis by promoting M2-like macrophage polarization, as evidenced by increased CD206 and Arg1 | (140) |

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| | *T. spiralis*-specific 53 kDA glycoprotein (rTsP53); *E. coli* recombinant | Murine (BALB/c) colon macrophages | Inhibited colitis by promoting polarization to an anti-inflammatory phenotype, as evidenced by increased Arg1 and Fizz1 markers. Increased IL-10 and TGFβ, and decreased IL-6 and TNF | (141) |
| | | Murine (BALB/c) bone marrow derived and peritoneal macrophages | Attenuated sepsis through promotion of M2-like macrophage polarization, associated with increased Arg1 and Fizz1, and reduced iNOS | (142) |
| | *T. spiralis* novel statin (rTsCstN); *E. coli* recombinant | Murine (BALB/c) bone marrow derived macrophages | Decreased pro-inflammatory IL1β, IFNγ, TNF and iNOS following LPS exposure and inhibits macrophage antigen presentation | (143) |
| | *T. spiralis* cystatin (Ts-Cys); *E. coli* recombinant | Murine (BALB/c) bone marrow derived macrophages | Promoted macrophage polarization from pro- to anti-inflammatory phenotype by inhibiting TLR2/MyD88 signal pathway, TNF, IL-6, IL-1β, and increasing mannose receptor expression and TGFβ | (144) |
| | *T. spiralis* cathepsin B-like protein (rTsCPB); *E. coli* recombinant | Murine (BALB/c) intestinal tissue macrophages | Ameliorated ischemia/reperfusion injury by induction of M2-like macrophages, as evidenced by decrease in M1-like markers and increase in M2-like markers | (145) |

Abbreviations: Arg1, arginase 1; Ym1, chitinase-like protein 3; Fizz1, Found in inflammatory zone; IL; interleukin, NO; nitric oxide, TNFα; tumour necrosis factor alpha, IL1β, interleukin 1 beta; TLR, toll like receptor; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa ligand; TRIF, TIR-domain-containing adapter-inducing interferon beta; PD-L1, programmed death-ligand 1; NLRP3, NLR family pyrin domain containing 3; RELMα, resistin-like molecule alpha; SOCS-1, suppressor of cytokine signalling-1; MAPK, mitogen-activated protein kinases; CCL, chemokine ligand; iNOS, inducible nitric oxide synthase; MyD88, myeloid differentiation primary response

### 1.7.2 Regulation of macrophage phenotypes by helminths prevents the development of disease in animal models of T1D and T2D

The possibility that macrophages mediate the beneficial effect of helminths in diabetes emerged from experimental studies using mouse models in which the depletion of T-cells, or the genetic ablation of T-cell signalling molecules, failed to impact the protection from disease elicited by infection with *Litomosoides sigmodontis, Heligmosomoides polygyrus,* or *Schistosoma mansoni* (146, 147). Further analysis of immune cell populations within the infected non-diabetic animals showed an increase in the expression levels of M2 macrophage markers in the pancreatic lymph nodes. This finding indicated a central role for M2 macrophages in disease protection (148). Subsequent studies using the ES products of *Fasciola hepatica* and *Taenia crassiceps* confirmed the association between helminth-mediated protection and the recruitment of M2-like macrophages (121, 149). A functional role for macrophages was confirmed by depletion studies in mice, in which the removal of macrophage populations, by the administration of clodronate-liposomes reversed the protective effect of the *T. crassiceps* ES products (149). Albeit, such macrophage ablation studies must be interpreted with caution, as in a recent study, the anti-inflammatory effects of clodronate liposomes in arthritis models were attributed to the modulation of neutrophil effector functions after phagocytosis of liposomes (150).

Analysis of the individual constituents of the *F. hepatica* ES products identified a single protein (FhHDM-1), which mimicked the protective effect of the ES products in preventing both insulitis and hyperglycaemia in the non-obese diabetic (NOD) mouse model of T1D (151). While the reduction in disease progression was correlated to the modulation of macrophage activity, in contrast to the administration of the ES products, FhHDM-1-mediated protection was associated with the reduced ability of macrophages to respond to pro-inflammatory ligands, suggesting the regulation of an M1-like phenotype, and indicating multiple mechanisms mediating an overall switch from a predominance of pro-inflammatory to anti-inflammatory macrophages (151).

Helminth-mediated macrophage modulation is also advantageous in the prevention of T2D. Indeed, infection with various helminths, such as *S. mansoni* (152), *Strongyloides venezuelensis*, *L. sigmodontis (153)*, *Nippostrongylus brasiliensis* (154), *Trichinella spiralis* (155), and *H. polygyrus* (156, 157), have been shown to exert beneficial effects (such as improvement of glucose tolerance and insulin sensitivity) in various T2D mouse models (Table 1.2). The mechanism of action has been putatively attributed to the

activation/recruitment of M2-like macrophage populations within the adipose tissue. These M2-like populations oppose the obesity-induced elevation of pro-inflammatory macrophages and promote an anti-inflammatory environment akin to that observed in lean adipose tissue. Of these helminths, a wide array of molecules derived from *S. mansoni* (130, 152, 158, 159) and *L. sigmodontis* (153) similarly provided protection from T2D through the same mechanism (Table 1.2). This helminth-mediated reduction of inflammation in adipocyte tissue and subsequent improvements in glucose homeostasis could also have positive effects on the β-cells, by reducing FFAs levels and hyperglycaemia that contribute to β-cell stress and exhaustion (8).

**Table 1.2 Helminth infection and helminth-derived products exert protective effects against obesity and type 2 diabetes.**

| Helminth/product | Infection/dose | T2D model | Macrophage activation | Therapeutic effect | References |
|---|---|---|---|---|---|
| *Schistosoma mansoni* infection | Percutaneous infection with 36 larvae, chronic/12wk infection | HFD-induced obese C57BL/6 mice | Promoted M2-like macrophages in white adipose tissue | Reduced weight and fat mass gain, improved glucose tolerance and insulin sensitivity | (152) |
| *S. mansoni* SEA | i.p. injection with 50µg SEA once every 3d for 4wks | | | | |
| *S. mansoni*-derived ω1 | Recombinant, i.p. injection with 25µg ω1 on 0, 2 and 4d | HFD-induced obese C57BL/6 mice[a] | Promoted M2-like macrophages in adipose tissue dependent on IL-33 release | Reduced weight gain and promoted glucose homeostasis in an IL-33 dependent manner | (130) |
| *S. mansoni*-derived ω1 | Recombinant, 50µg i.p. injection every 3d for 4wks | HFD-induced obese C57BL/6 mice | Promoted M2-like macrophages in white adipose tissue | Reduced weight gain and improved glucose tolerance and insulin sensitivity | (159) |
| LNFPIII | Synthetic glycan, 25µg i.p. injection twice per wk., 4-6wks after HFD-induced obesity onset | HFD-induced obese C57BL/6 mice | Promoted M2-like macrophages in metabolic tissue | Improved glucose tolerance and insulin sensitivity, partly mediated through M2 macrophage-derived IL-10 | (158) |
| *Litomosoides sigmodontis* infection | Larvae transmitted with blood meal of infected *Ornithonyssus bacoti* mites at 8-10wks of age (1-2wks after HFD onset) | HFD-induced obese BALB/c mice | Promoted M2-like macrophages in epididymal adipose tissue | Improved glucose tolerance, LsAg reduced inflammatory immune response and enhanced insulin signalling | (153) |
| *L. sigmodontis* antigen (LsAg) | LsAg derived from adult worms, 2µg i.p. injection daily at 8-10wks or 12-14wks of HFD | HFD-induced obese C57BL/6 mice | | | |
| *Nippostrongylus brasiliensis* infection | Subcutaneous infection with 500 larvae once every 4wks for total of 3 infections for HFD mice, at 10 and 17wks of age for RIP2 Opa1KO mice | HFD-induced obese RIP2-Opa1KO mice or C57BL/6 mice | Promoted M2-like macrophages in epididymal adipose tissue | Reduced weight gain and improved glucose tolerance | (154) |

Cont'd

| | | | | | |
|---|---|---|---|---|---|
| *Trichinella spiralis* infection | Oral infection with 400 larvae, after 4wks on HFD | Ob/ob mice and HFD-induced obese C57BL/6 mice | Promoted M2-like macrophages in adipose tissue | Improved glucose tolerance and insulin sensitivity | (155) |
| *Heligmosomoides polygyrus* infection | Oral infection with 200 larvae after ~4wks on HFD | HFD-induced obese C57BL/6 mice | Promoted M2-like macrophages in gonadal fat tissue | Reduced weight gain, improved glucose tolerance and increased browning of white adipose tissue. Adoptive transfer of *H. polygyrus* induced M2 macrophages similarly attenuated HFD-induced obesity | (156) |
| *Heligmosomoides polygyrus* infection | Oral infection with 200 larvae at 10-12wks of age | HFD-induced obese C57BL/6 mice | Promoted M2 -like macrophages in adipose tissue | Reduced weight gain and improved glucose tolerance | (157) |

Abbreviations: wk, week; SEA, soluble egg antigen; IP, intraperitoneal; HFD, high fat diet; ω1, omega-1; LNFPIII, Lacto-N-fucopentaose III; LsAg, *Litomosoides sigmodontis* antigen; $Lepr^{db/db}$, diabetes (db) mutation of the leptin receptor, [a]Various mouse models were crossed to C57BL/6 background for mechanistic studies, including T1/ST2-deficient mice, IL-33 citrine reporter mice, and IL-33 deficient mice. $CD206^{-/-}$ and $Rora^{sg/sg}$ were also used.

### 1.7.3 Helminth-mediated changes to macrophages may alter the crosstalk with β-cells

While the aforementioned studies clearly demonstrate a critical role for macrophages in the protective mechanisms of helminths against diabetes development, most reports simply concluded that the beneficial effects were attributable to a switch in the predominant inflammatory phenotype of macrophage. However, we propose that the changes to macrophages elicited by helminths, and their ES products, are also exerting a protective effect through communication with β-cells. Indeed, the M2-like macrophages induced by helminth infection exhibited increased expression levels of IGF-1 (160). Additionally, ablation of IGF-1 signalling in macrophages has been shown to disrupt endocrine IGF1-mediated signalling, resulting in a significant increase in insulin resistance in mice (160). Although a specific link to β-cell function was not explored in these studies, it has been reported that macrophages are the primary source of IGF-1 in pancreatic islets, and that these cells function to enhance insulin secretion from β-cells (77). Furthermore, other growth factors (such as TGFβ1 and EGF) secreted by M2-like macrophages have been shown to enhance the survival and proliferation of β-cells through the increased expression levels of SMAD7 (161).

Supporting the beneficial effect of M2-anti-inflammatory macrophages in diabetes prevention, is the helminth-mediated regulation of pro-inflammatory macrophages. Under diabetic conditions, phagocytosis of apoptotic β-cells by macrophages generates ROS, leading to inflammasome activation, and the secretion of pro-inflammatory cytokines (notably IL-1 β), all hallmarks of an M1-like phenotype (60). As mentioned above, these macrophages have deleterious effects on β-cells by impairing glucose-stimulated insulin secretion, inducing apoptosis, and causing β-cell dedifferentiation. Further crosstalk between pro-inflammatory macrophages and pathogenic β-cells work in concert to drive inflammation and disease. Indeed, macrophages within the islets of NOD mice have been found to exhibit M1-like features (10), and depletion of these macrophages prevented T1D onset (162). Furthermore, typically, M1-like macrophages exhibit decreased phagocytic ability, as observed in diabetes-prone NOD mice (163) and humans (164), which could initiate and exacerbate inflammation because apoptotic β-cells are not efficiently cleared. Helminths can hinder this deleterious activity of pro-inflammatory macrophages (through inhibition of the inflammasome and/or secretion of pro-inflammatory cytokines (122) ) or skew macrophages towards an anti-inflammatory M2-like phenotype. Consequently, the destructive pathway of macrophage/β-cell communication would be inhibited, the perpetuation of β-cell destruction

prevented, and anti-inflammatory pathways conducive with preservation of β-cell function and mass, and disease prevention, would prevail.

## 1.8 Helminths modulate whole body metabolism independently of immune regulation

In recent years, it has become evident that infection with helminth parasites triggers a remodelling of the systemic metabolism of the host, which is demonstrated by altered production of pancreatic hormones, namely incretins and adipokines (165). Additionally, there is a shift in metabolic pathways in both infected tissue and in organs beyond the location of the parasite (17). The current theoretical framework suggests that this metabolic shift is required to direct the host immune status, and eventually impacts cells and organs that are not directly involved in the immune response (17, 166). This rewiring of the host's metabolism commonly manifests as a significant increase in insulin sensitivity in non-diabetic helminth infected humans and mice (160, 167).

### 1.8.1 Helminth derived molecules interact with endocrine cells

We have recently shown that the *F. hepatica* FhHDM-1, which was previously shown to prevent T1D via modulation of macrophage activity, can also interact directly with pancreatic β-cells to modulate their activities (168, 169). After interaction through a currently unknown binding partner, FhHDM-1 activates the PI3K/Akt signalling pathway in β-cells. This consequently promotes β-cell survival and function, without enhancing proliferation, and inhibits pro-inflammatory cytokine induced apoptosis in both a NOD-derived (diabetogenic) β-cell line and in human islets (Chapter 2) (169).

Due to the focus on the contributory role for immune modulation in the protective effect mediated by helminths, there are limited additional studies specifically investigating the interactions of helminths, and their ES products, with β-cells. However, there is evidence supporting this premise. As mentioned previously, pancreatic β-cells express TLRs, which can modulate β-cell viability, influence insulin homeostasis (170), and contribute to T1D initiation upon interaction with high-mobility group box 1 (HMGB1) (171). Since the molecules secreted by helminths can interact with TLRs, these receptors may allow direct helminth regulation of β-cells (172). Furthermore, given the diversity of helminth molecules (including unique glycoproteins) it is plausible that helminth-derived molecules can directly

modulate metabolic processes through glycan activation of metabolic cell receptors and C-type lectins (173). For example, the glycan, lacto-N-fucopentaose III (LNFPIII), which is the predominant polylactosamine sugar found on secreted egg antigens of *S. mansoni* exerted a similar dual effect as FhHDM-1. Under pre-T2D conditions (obesity), LNFPIII improved glucose tolerance and insulin sensitivity, by modulating pro-inflammatory responses and by directly inhibiting lipogenesis in hepatocytes, thereby ameliorating hepatic steatosis (158). Similarly, the T2 RNase ω1 secreted from the same helminth, promoted metabolic homeostasis in a murine model of diet induced T2D. In this case, ω1 was found to enhance adipocyte expression and secretion of IL-33, likely through interaction with the mannose receptor (130). Several other studies have reported similar direct helminth modulation of non-immune, metabolically active cells (8, 154, 174) (Table 1.3), predominantly adipocytes and hepatocytes, which communicate with, and influence the function of, β-cells. Indeed, adipocytes secrete several adipokines, such as leptin, adipsin and adiponectin, that contribute to regulation of β-cell insulin secretion and proliferation (175). Furthermore, extracellular vesicles (EVs) secreted by healthy adipocytes have been shown to promote normal β-cell physiology and provided protection from palmitate or pro-inflammatory cytokine induced death. Conversely, EVs derived from obese adipocytes led to β-cell dysfunction and death. Similar crosstalk has been observed between β-cells and hepatocytes (176). Hepatocytes secrete EVs and proliferative factors, such as IGF-1, that contribute to compensatory β-cell hyperplasia in conditions of obesity/T2D. Moreover, under this inflammatory environment, pancreatic β-cell dysfunction leads to impaired insulin secretion that normally modulates glucagon release, thus leading to elevated glucagon levels. In turn, this exacerbates the increased glucose output from the liver associated with insulin resistance and induces production of kisspeptin1 in hepatocytes that can further impair β-cell insulin secretion (175). Therefore, helminth-mediated changes to adipocytes and hepatocytes would be expected to have significant knock-on effects to the β-cells.

**Table 1.3 Helminths and helminth derived products directly affect endocrine cells**

| Helminth/product | Interaction with non-immune cells | Reference |
|---|---|---|
| *Fasciola hepatica*; FhHDM-1 | Promoted β-cell survival and function without enhancing proliferation, and inhibited pro-inflammatory cytokine-induced apoptosis, via activation of PI3K/Akt signalling pathway | (169) |
| *Schistosoma mansoni;* LNFPIII | Suppressed lipogenesis in hepatocytes and protected against hepatic steatosis by upregulating bile acid sensing nuclear receptor *Fxr-α* signalling | (158) |
| *Schistosoma mansoni;* ω1 | Enhanced adipocyte expression and secretion of IL-33, likely through interaction with the mannose receptor | (130) |
| *Nippostrongylus brasiliensis* infection | Suppressed lipogenesis in hepatocytes and ameliorated hepatic steatosis, and decreased expression of key glucose transporters in intestinal cells, which could normalise glucose homeostasis under conditions of obesity | (154) |
| *Schistosoma japonicum* SEA | Impaired NF-κB activation in hepatic stellate cells, thereby inhibiting TNF-induced pro-fibrotic IL-34 and progression of hepatic fibrosis | (174) |
| *Litomosoides sigmodontis* infection | Downregulated adipogenesis related genes such as PPARγ and C/EBPα in epididymal adipose tissue | (8) |
| *Litomosoides sigmodontis* antigen | Reduced differentiation of pre-adipocyte cell line (3T3-L1) into mature adipocytes, suggesting suppression of adipogenesis | (8) |

FhHDM-1, *Fasciola hepatica* helminth defence molecule-1; LNFPIII, lacto-N-fucopentaose III; ω1, omega-1; SEA, soluble egg antigen; PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPα, CCAAT/enhancer binding protein alpha

### 1.8.2 Helminths alter the gut-islet axis

Extensive analyses of the human microbiome have revealed that the gut microbiota is deeply interconnected with diabetes, with alterations to the populations of species in the gut impacting several metabolic effects and immune response processes (177, 178). It is widely acknowledged that gastrointestinal factors, such as glucagon like peptide (GLP)-1 and gastric inhibitory polypeptide (GIP), interact directly with β-cells to induce proliferation, and enhance resistance to apoptosis, thereby increasing/maintaining β-cell mass (179, 180). In addition, short chain fatty acids (SCFA) released by gut microbiota modulate the function and survival of β-cells (175). Thus, any changes to the bacteria populating the gut will subsequently alter the metabolic activity of β-cells within the pancreas.

As the gut mucosa hosts the largest population of macrophages in the human body (181), it is not surprising that SCFA released by gut microbiota also influences their functional phenotypes. For example, it has been reported that an increased abundance in *Bacteroides fragilis*, *Lactobacillus* spp. *and Clostridia* class induce the polarisation of anti-inflammatory M2-like macrophages (182, 183). In contrast, the presence of *Enterococcus faecalis* polarises colonic macrophages towards a pro-inflammatory phenotype (184). However, these effects are not confined to the intestinal microenvironment, as it has been shown that butyrate produced by gut microbiota induces the expression of mouse β-defensin 14 in pancreatic islets, which in turn drives the activation of regulatory macrophages and prevents the development of T1D in a mouse model (185).

Several studies of human and murine helminth infection have demonstrated that the presence of the parasites alters both the abundance and diversity of gut microbiomes (186, 187). More recently it has been demonstrated that these changes mediate a beneficial effect in models of T2D diabetes. Infection of mice with either *S. venezuelensis* (188) or *Nippostrongylus brasiliensis* (189) resulted in significant compositional changes in gut microbiota, most notably by increasing *Lactobacillus* spp., a bacterial species which has been assessed as a beneficial probiotic for diabetes (190). In both cases, these changes in gut microbiota resulted in improved insulin signalling and sensitivity. Although a specific effect on β-cells was not assessed in these studies, increased expression of M2 macrophage markers were found in adipose tissue, liver, and gut.

## 1.9 Concluding remarks

T1D and T2D are characterised by a progressive dysfunction and loss of the insulin-producing β-cells in the pancreatic islets leading to insulin deficiency. Despite this knowledge, none of the currently prescribed anti-diabetic agents effectively target the maintenance of functional β-cell mass. In T1D, the focus remains on blocking autoreactive immune cells and their mediators. However, diabetes reversal is not achieved, protection of residual β-cell mass is short-term, and global immune suppression is induced (191-193). Due to the scarcity of organ donors, significant β-cell loss during islet preparation, and allogenic and recurrent autoimmune reactions, which mediate β-cell death post-transplant, islet transplantation is not a realistic cure for T1D. In T2D, therapeutic options, such as dipeptidyl peptidase-4 (DPP-4) inhibitors, incretin-based drugs, and GLP-1 analogues, target β-cell function, but not all patients are responsive and approximately 50% ultimately require daily insulin injections due to β-cell exhaustion and death (194). Clearly, alternate, more effective strategies, are urgently required. By regulating the activities of islet macrophages and β-cells (and other endocrine cells), helminth parasites shape their crosstalk (Figure 1.4). This offers a unique opportunity to exploit helminths' mechanisms for survival in their mammalian hosts to establish an environment that preserves β-cell mass and function and thus offers the potential as a cure for both T1D and T2D.

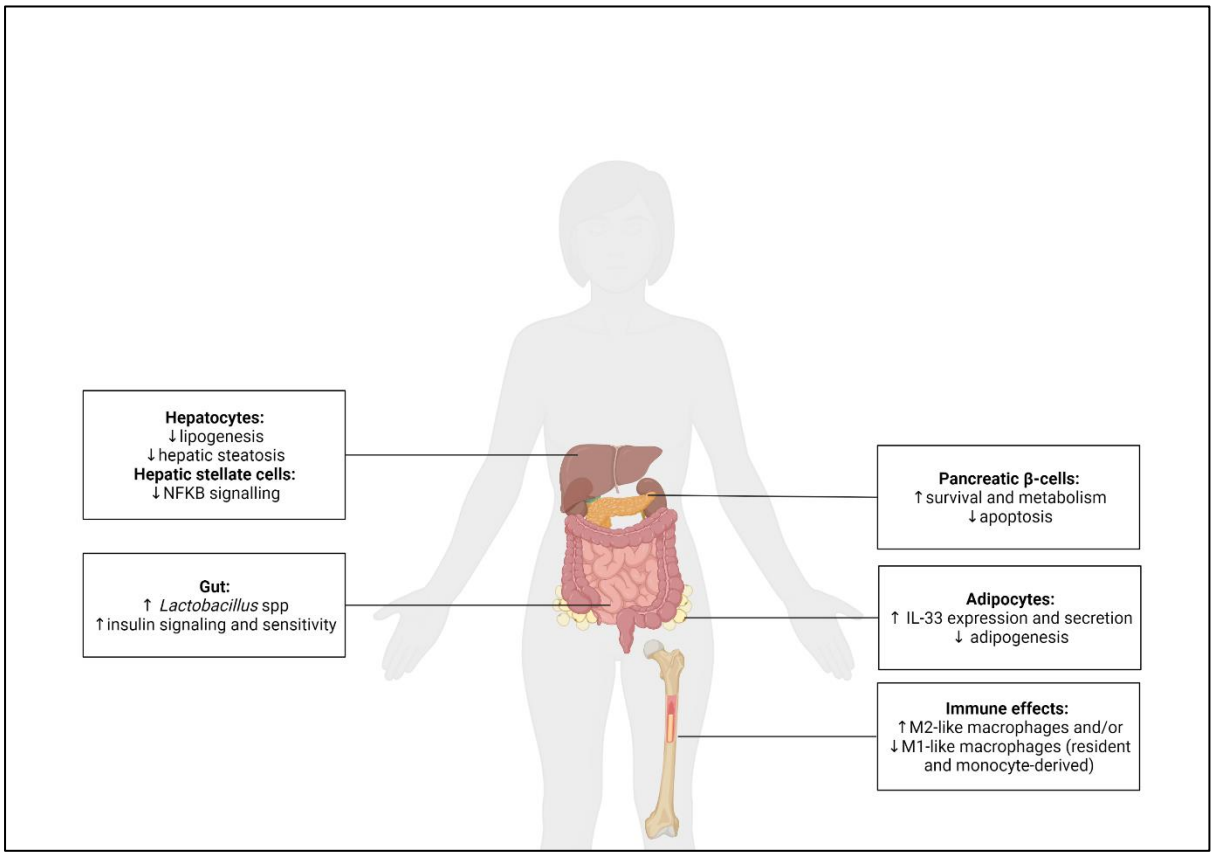


**Figure 1.4 The systemic effect of helminths and their secreted molecules on the function of metabolic and immune cells.** Helminths are recognised as potent immune modulators, typically promoting the polarisation of macrophages (resident and monocyte-derived) to an anti-inflammatory/regulatory M2-like phenotype and/or inhibiting a pro-inflammatory M1-like phenotype. This immune regulation has been shown to be beneficial for type 1 and type 2 diabetes. Helminths also positively impact endocrine cells and have been demonstrated to (i) enhance pancreatic β-cell survival and metabolism, (ii) promote adipocyte expression of anti-inflammatory IL-33 and inhibit adipogenesis, (iii) reduce lipogenesis and prevent steatosis within hepatocytes, and decrease pro-inflammatory nuclear factor kappa (κ) B signalling in hepatic stellate cells, and (iv) increase the presence of probiotic *Lactobacillus* spp. in the gut, which promotes insulin signalling/sensitivity. Created with BioRender.com.

# Chapter 2: The parasite-derived peptide FhHDM-1 activates the PI3K/Akt pathway to prevent cytokine-induced apoptosis of β-cells

## 2.1 Introductory Statement

The chapter has been published as an original research paper in the **Journal of Molecular Medicine**:

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### Author Contributions

IC designed, performed, analysed and interpreted the experimental work throughout. She also drafted the initial manuscript and was actively involved in the editing process. TYM provided technical assistance in the measurement of glucose secretion. Previous research performed by ML led to the foundation of the hypothesis for this project. NB performed mass spectrometry on β-cells and JT, MWR and JS performed the mass spectrometry on pancreatic protein samples. BOB and SD provided conceptual advice and revised the manuscript.

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### Copyright Declaration

## 2.2 Abstract

Type 1 diabetes (T1D) is an autoimmune disease characterised by the destruction of the insulin-producing beta (β)-cells within the pancreatic islets. A parasite-derived molecule, termed *Fasciola hepatica* helminth defence molecule 1 (FhHDM-1), was previously identified as a novel peptide that prevented T1D development in non-obese diabetic (NOD) mice. In this study, proteomic analyses of pancreas tissue from NOD mice suggested that FhHDM-1 activated the PI3K/Akt signalling pathway, which is associated with β-cell metabolism, survival, and proliferation. Consistent with this finding, FhHDM-1 preserved β-cell mass in NOD mice. Examination of the biodistribution of FhHDM-1 after intraperitoneal administration in NOD mice revealed that the parasite peptide localised to the pancreas, suggesting that it exerted a direct effect on the survival/function of β-cells. This was confirmed *in vitro*, as the interaction of FhHDM-1 with the NOD-derived β-cell line, NIT-1, resulted in increased levels of phosphorylated Akt, increased NADH and NADPH, and reduced activity of the NAD-dependent DNA nick sensor, poly(ADP-ribose) polymerase (PARP-1). As a consequence, β-cell survival was enhanced, and apoptosis was prevented in the presence of the pro-inflammatory cytokines that destroy β-cells during T1D pathogenesis. Similarly, FhHDM-1 protected primary human islets from cytokine-induced apoptosis. Importantly, while FhHDM-1 promoted β-cell survival it did not induce proliferation. Collectively these data indicate that FhHDM-1 has significant therapeutic applications to promote β-cell survival, which is required for T1D and T2D prevention and islet transplantation.

## 2.3 Introduction

Type 1 diabetes (T1D) is an autoimmune disease in which the insulin-producing beta (β)-cells within the pancreatic islets are destroyed. The immune responses directed against β-cell autoantigens precede disease development by several years, such that at the time of diagnosis β-cell mass is significantly reduced (1). Although this residual β-cell mass can allow the patient to revert to normoglycaemia for a transient period of time, it is then ultimately destroyed, and the patient is irreversibly dependent on exogenous insulin for survival. However, the delivery of bolus insulin doses cannot mimic the minute-to-minute glucose responsiveness of β-cells, and so the patient suffers episodes of hypo- and hyper-glycaemia and is predisposed to several complications, which increase morbidity and mortality (195).

Initiation of β-cell directed autoimmunity likely occurs during a physiological phase of neonatal pancreatic remodelling, which is characterised by waves of β-cell neogenesis, proliferation and apoptosis. Dysregulation in the relative rates of β-cell neogenesis and proliferation versus apoptosis can lead to the generation of β-cell autoantigens, resulting in the activation and expansion of autoreactive T cell populations, which traffic to the pancreas (insulitis) to destroy β-cells (18, 19). This insulitis leads to increased concentrations of pro-inflammatory cytokines, chemokines, reactive oxygen intermediates (ROI), and other pro-apoptotic stimuli (perforin/granzyme and Fas/FasL system) (196-199). Additionally, β-cells play an active role in their own destruction by secreting pro-inflammatory cytokines, thereby perpetuating a cycle of cytotoxicity (200, 201). The induction of apoptotic pathways in β-cells converge upon the activation of caspase-3, which, in turn, cleaves its downstream substrates (notably poly-ADP ribose polymerase [PARP-1]), ultimately leading to DNA fragmentation, apoptosome formation and apoptotic cell death.

Given its immune-mediated pathology, one of the most widely studied approaches to prevent/treat T1D is immunotherapy. These strategies aim to suppress the activities of specific autoreactive effector immune cells and/or effector molecules, and/or re-establish immune tolerance to β-cells by vaccination with β-cell antigens, thereby allowing recovery/preservation of β-cell function/mass (202). The most widely explored immunotherapy for T1D is anti-CD3 antibody. Recently multiple small studies have demonstrated that Teplizumab (modified CD3 antibodies) are efficacious in the delay of T1D onset when administered prior to hyperglycaemia development. Despite some level of success (203-207) these immunotherapeutic strategies primarily rely upon the functional

modulation of immune cell populations, and do not directly target β-cells. Testament to these shortcomings, diabetes reversal is not achieved, protection of residual β-cell mass is short-term, global immune suppression is induced, and several severe side effects are experienced. The optimal strategy for T1D prevention and treatment may well be a therapy that can act directly on β-cells to enhance their survival/function to render resistance to immune-induced apoptosis, and perhaps also exert a regulatory effect on the autoreactive immune response. Indeed, an alternative viewpoint in which β-cells are the key contributors to T1D because of their susceptibility to biosynthetic stress with limited abilities to mitigate this stress is rapidly emerging. Therefore, immunotherapy, while necessary, might be insufficient to cure T1D. Ultimately, strategies that target β-cell stress and islet autoimmunity will create the greatest clinical gains (1).

A novel peptide (termed FhHDM-1) secreted by the parasitic worm *Fasciola hepatica*, was previously identified as possessing the unique ability to regulate mammalian immune responses (120, 208). Administration of FhHDM-1 to non-obese diabetic (NOD) mice, the most widely studied animal model of T1D, prevented the onset of disease with protected mice remaining normoglycaemic up to 30 weeks of age (experimental endpoint). Consistent with the immune-regulatory activities of FhHDM-1, examination of the pancreas of the treated mice showed a significant reduction in insulitis, as compared to the control diabetic animals (151).

The aim of the current study was to identify the biological signals within the pancreas that were altered in response to FhHDM-1 treatment to decrease insulitis and prevent hyperglycaemia. The data revealed a direct interaction of FhHDM-1 with β-cells in the pancreas, resulting in the activation of the PI3K/Akt signalling pathway, which operated to prevent pro-inflammatory cytokine induced apoptosis. Combined with our previous observations (151), this would suggest that FhHDM-1 is capable of both regulating the autoimmune response, and exerting direct effects on the target cell to enhance β-cell survival.

## 2.4 Materials and Methods

### 2.4.1 FhHDM-1 treatment of NOD mice

Female NOD mice at 3 weeks of age were purchased from the Animal Resources Centre (Western Australia), and, upon arrival, were allowed to acclimatise for one week before being used in experiments. At 4 weeks of age, mice were randomly divided into two treatment groups, each with 10 mice. Each mouse received either FhHDM-1 (10µg; chemically synthesised by GenScript, USA) or phosphate buffered saline (PBS) by a total of six intraperitoneal injections (i.p.) administered on alternate days. At 10 weeks of age, the pancreas tissues were harvested and snap frozen in liquid $N_2$(l) for analysis of β-cell mass.

A second cohort of mice (n=5 per treatment group) was aged and the development of diabetes was determined by weekly blood glucose measurements using Accu-check Advantage blood glucose strips (Roche). PBS-treated animals were sacrificed at diabetes onset (defined by two consecutive blood glucose concentrations above 14 mM), and aged matched non-diabetic FhHDM-1 treated mice were euthanised at the same time. The pancreas tissues from these mice were analysed by mass spectrometry.

To examine the biodistribution of FhHDM-1 in NOD mice *in vivo*, FhHDM-1 was labelled with the fluorescence dye CF® 800 succinimidyl ester (CF 800, 0.25µmol, Biotium, 92127) (797/816 nm) prior to the injection in mice. Four-week-old female NOD mice were administered a single i.p. injection of the labelled FhHDM-1 or an equivalent amount of the CF800 dye only or an equivalent volume of sterile saline. After 20mins, the fluorescent signal (excitation: 745nm, emission: 800nm) *in vivo* was captured using an IVIS Spectrum imaging system (IVIS Spectrum, Perkin Elmer). Organs of euthanised mice were also screened for fluorescence using the same system.

All animal work was conducted in accordance with the approved guidelines of the University of Technology Sydney Animal Care and Ethics Committee (protocols ETH17-1226 and ETH19-4082).

### 2.4.2 Preparation of protein extracts of pancreas tissue from NOD mice

Whole pancreata stored in $N_2$(l) were thawed on ice and homogenised in 1 ml extraction buffer, containing 7M urea/2M thiourea, using a Stuart homogeniser with a 5 mm stainless steel probe. Triton-X100 was then added to a final concentration of 0.1 % (v/v) and the

samples were rotated for 1.5 h at room temperature (RT; 18-21°C). Samples were then centrifuged (14,000 rpm for 10 min at 4°C), and proteins in the supernatant were precipitated with 5 volumes of acetone for 4 h at 4°C to remove detergent prior to electrophoresis. The suspension was centrifuged (14,000 rpm for 10 min at 4°C) and the resulting protein pellet was washed in 5 ml of ice-cold acetone before the final precipitate was resuspended in 7M urea/2M thiourea by gentle pipetting and vortexing.

### 2.4.3 Gel electrophoresis and mass spectrometry

Whole pancreas protein extracts (in biological triplicate) were separated on reducing NuPage 4-12 % Bis-Tris gels (Thermo Scientific), which were then lightly stained with Colloidal Coomassie Blue G250 (Sigma). Gel lanes were cut into 5 sections for analysis by mass spectrometry. Briefly, the gel sections were cut into smaller pieces (approximately 1 $mm^2$) and reduced and alkylated with 5 mM tributylphosphine (Sigma) and 20 mM acrylamide (Sigma) in 100 mM $NH_4HCO_3$ (Sigma) for 90 mins. The excised sections were then in-gel digested with 100ng/µl sequencing grade trypsin (Sigma) and the peptides solubilised with 2% formic acid (Sigma) prior to analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Tempo nanoLC system (Applied Biosystems) with a C18 column (Vydac) coupled to a QSTAR Elite QqTOF mass spectrometer running in IDA mode (Applied Biosystems).

### 2.4.4 Database searching and criteria for protein identification

Tandem mass spectra were extracted by Protein Pilot v1.0 software (Applied Biosystems) using default parameters. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analysed using Mascot (Matrix Science, London, UK; version 2.4.1). Mascot was set up to search the contaminants_all_10062014 database (selected for *Mus musculus*, 113222 entries) assuming the digestion enzyme semiTrypsin. Mascot was searched with a fragment ion mass tolerance of 0.20 Da and a parent ion tolerance of 100 PPM. Deamidation of asparagine and glutamine, oxidation of methionine and propionamide of cysteine were specified in Mascot as variable modifications.

Scaffold (version Scaffold_4.4.5, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet

algorithm (209) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet (210). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters (Supplementary Table 2.1).

### 2.4.5 Systems bioinformatic analysis

The initial data interrogation utilised the mass spectrometry data collected from either PBS or FhHDM-1 treated mice completed in biological replicates (n=3). The biological replicates were merged so as to not artificially bias or cut unique proteins. Complete lists of proteins were then utilised to construct interaction networks in Cytoscape (version 3.7.2) (211) and for KEGG pathway analysis.

### 2.4.6 Immunohistochemical staining of pancreas and quantification of insulin staining

Frozen pancreata were embedded into optimal cutting temperature compound (Proscitech, IA018) and sectioned at 5µm thickness. For each sample, 6-8 representative sections that were at least 100µm apart from each other were collected. Sections were fixed and permeabilised in acetone for 10mins prior to addition of blocking buffer (PBS, BSA 1% (w/v), $NaN_3$ (0.05% w/v), foetal calf serum (2% v/v)) and staining with the following antibodies; goat anti-insulin (1/100) (Santa Cruz, SC7839) and donkey anti-goat Alexa Fluor 647 (1/1000) (Abcam, ab6566). DAPI (1µg/ml) (Sigma, D9524) was used as the nuclear counterstain. Whole pancreata were imaged using the DeltaVision Elite Microscope or the Axio Scan Zeiss Slide Scanner, and analysed using Image J software, to calculate the area positive for insulin staining relative to the total area of the tissue section.

### 2.4.7 Culture of β-cells

NIT-1 β-cells (ATCC, CRL2055; Passage 4) were cultured in Ham's F12K media with 2mM L-glutamine, adjusted to contain 1.5g/L sodium bicarbonate (ATCC, ATC302004), supplemented with 10% (v/v) heat-inactivated dialyzed foetal bovine serum (FBS). MIN-6 β-cells (ATCC, CRL-11506, passage 22) were cultured in Dulbecco's Modified Eagle's

medium (DMEM), supplemented with high glucose (25mM), 15% (v/v) heat-inactivated FBS and 70μM 2-mercaptoethanol. Primary human pancreatic islets (Celprogen, 3500204) were cultured in Pancreatic Islets of Langerhans Cell Culture Complete Growth Media with Serum (Celprogen, M35002-045). All cells were maintained at 37˚C in a humidified chamber (5% $CO_2$, 95% air atmosphere).

### 2.4.8 Binding of FhHDM-1 to β-cells

FhHDM-1 peptide was labelled with Alexa Fluor 488 (Life Technologies, Mulgrave, VIC, Australia), according to the manufacturer's instructions. NIT-1 cells ($1x10^6$ cells) or MIN6 cells ($1x10^6$ cells) were incubated with the fluorescently labelled FhHDM-1 (10 μg/ml) for 30 min at 4°C and binding of the peptide was assessed by flow cytometry.

### 2.4.9 Differential expression of genes in NIT-1 β-cells

To identify changes in the expression levels of genes stimulated by FhHDM-1 treatment of β-cells, NIT-1 cells ($2x10^6$) were plated overnight (37°C/5% $CO_2$) to allow adhesion. Cells were then either left untreated or treated with FhHDM-1 (10μM) for a further 24h. After harvesting the cells, the pellets were washed with sterile PBS and total RNA was extracted using the Isolate II RNA mini kit (Bioline, BIO52073), according to the manufacturer's instructions.

Sequencing of the transcriptome was performed on 18 paired-ends samples by Macrogen (Seoul) using a TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, part #15031047) and a NovaSeq 6000 S4 reagent kit (Illumina) to create an mRNA library. To ensure the validity of the results, the quality control of the sequenced raw reads was analysed. Then low-quality reads, adaptor sequence, contaminant DNA sequences, and PCR duplicates were removed to minimise biases in the analysis. After that, adapter sequences were removed by Trimmomatic (Version 0.38) and the cleaned reads were mapped to the mouse genome (mm10) using StringTie (Version 2.1.3b).

The raw read counts of genes for each sample were analysed for differential expression using the edgeR package within Rstudio (Rstudio Team, Boston, MA, USA, Version 1.3.1073). Firstly, only genes that had at least a minimum read count across all samples were retained. Genes were then normalised using the trimmed mean of M (TMM)-

values method. The comparison between groups was performed by deducting the baseline readings (untreated cells) from the FhHDM-1 group readings. To extract the most differentially expressed genes, an additional threshold of logarithmic fold change > 1 and < -1 and a false discovery rate < 0.05 were applied. The resulting p-value and fold change of each differentially expressed gene were calculated by the software and utilised for visualisation and functional annotation analysis. The enrichment of Gene Ontology (GO) categories and KEGG pathways for differentially expressed genes were determined using the DAVID (v6.8) functional annotation analysis tool (https://david.ncifcrf.gov) (212).

### 2.4.10 Western analyses

NIT-1 cells ($2x10^6$) were either untreated or treated as with FhHDM-1 (10μM, 24h or 48h) or vehicle control (water) diluted in cell culture media under basal conditions. Under apoptotic conditions, cells were incubated with FhHDM-1 (10μM, 24h) or vehicle control (water), in combination with the pro-inflammatory cytokine mix (CM) (10ng/ml IL1β, 50ng/ml TNF, 50ng/ml IFNγ; BD Pharmingen). Cells were lysed in RIPA buffer (Thermo Fisher, 89900) containing 1x protease (Thermo Fisher, 1862209) and phosphatase inhibitors (Thermo Fisher, 78420). Protein content was measured by the Braford assay (Thermo Fisher, 23225) and protein lysates (14μg) were separated on 4-12% Bis-Tris gels (Life Technologies, NP0321), then transferred to polyvinylidene difluoride membranes (Life Technologies, IB24001), which were then probed with primary antibodies for pAkt (Cell Signalling Technologies, 9271S) and total Akt (Cell Signalling Technologies, 9272S), followed by horseradish peroxidase (HRP)-conjugated anti-rabbit as the secondary antibody (Cell Signalling Technologies, 7074S) The blots were probed for actin (Abcam, ab20272) as a loading control. Enhanced chemiluminescence (ECL) HRP substrate (Thermo Fisher, 34076) was then added, and protein bands were visualised using the Amersham Imager 600.

### 2.4.11 Cell proliferation assays

Proliferation of NIT-1 cells ($1x10^5$) after incubation with FhHDM-1 (10μM) or vehicle control (water) diluted in cell culture media was measured using the BrdU Cell Proliferation Elisa Kit (Abcam, ab126556) or CyQuant Cell Proliferation Assay Kit (Invitrogen, C7026), according to manufacturers' instructions.

### 2.4.12 Cell viability assay

An XTT cell viability assay kit (Roche, 11465015001) was used to measure cell survival. NIT-1 cells ($1x10^5$) were incubated with FhHDM-1 (10µM) or vehicle control (water) for 48h. An additional group of FhHDM-1 treated cells was pre-treated with a wortmannin, a PI3K inhibitor, (100nM, 15mins), protected from light. Four hours prior to the end of incubation, XTT mixture was added to each sample and the production of formazan was measured (450/650nm).

### 2.4.13 NADH and NADPH measurement

NIT-1 cells ($1x10^6$) seeded overnight were left untreated or incubated with FhHDM-1 (10µM) for 24h, after which the cells were collected and resuspended in ice-cold 80% (v/v) mass spectrometry grade methanol. The intracellular levels of NADH/NADPH were measured using liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS). Briefly, 100 µL of cell lysate was added to 20 µL of labelled standards in 5% (v/v) formic acid. Samples were then mixed with 300 µL of acetonitrile, vortexed for 15 sec, and then centrifuged, and dried via speed vacuum overnight at 37 °C. Samples were then reconstituted in 100µL of 10 mM ammonium acetate with 0.1% formic acid. Metabolites were separated on a Phenomenex $NH_2$ column (150 mm x 2 mm x 3µm; Phenomenex, Torrance, CA).

### 2.4.14 Measurement of glucose-induced insulin production

To measure the levels of insulin produced in response to glucose, MIN-6 or NIT-1 cells ($1x10^5$ cells/well) were incubated with FhHDM-1 (10µM) or vehicle control (water) for 48h. Cells were then incubated in low glucose basal media (0.14M NaCL, 2.7mM KCl, 1.5mM $KH_2PO_4$, 10mM $Na_2HPO_4$, 0.9mM $CaCl_2$, 0.5mM $MgCl_2$, 27mM $NaHCO_3$, 20mM HEPES, 2g/L BSA, 1.5mM glucose) for 1h. The supernatants were collected and replaced with the same media containing a higher concentration of glucose (33mM) and the cells were cultured for 1h. After this time, the supernatants were collected, and the cells cultured for a further 1h in the low glucose basal media. To quantify the levels of insulin in the supernatants, an ultra-sensitive mouse insulin ELISA kit (Crystal Chem Inc., 90080) was used, according to the manufacturer's instructions.

### 2.4.15 Caspase activity assay

NIT-1 cells ($1x10^5$) or primary human islets ($1x10^5$) seeded overnight were left untreated or treated with FhHDM-1 (10μM) or vehicle control (water) for 1h. Apoptosis was induced by incubation with a pro-inflammatory cytokine cocktail (final concentration murine cytokine cocktail: 10ng/ml IL1β, 50ng/ml TNF, 50ng/ml IFNγ; BD Pharmingen, human cytokine cocktail: 10ng/ml TNF, 5ng/ml IL-1β and 10ng/ml IFN-γ) for 10h. A fluorometric activity assay kit (Abcam, ab219915) was then used to quantify caspase-3 activity within the cells according to the manufacturer's instructions.

### 2.4.16 Quantification of PARP activity

PARP activity was measured using a new operational protocol relying on the chemical quantification of $NAD^+$ modified from Putt et al. (213). Briefly, NIT-1 cells ($0.5x10^6$ cells) were seeded overnight then left untreated or incubated with FhHDM-1 (10μM) or vehicle control (water) for 1h. Apoptosis was then induced by incubation with a pro-inflammatory cytokine cocktail (final concentration: 10ng/ml IL1β, 50ng/ml TNF, 50ng/ml IFNγ; BD Pharmingen) for 24h. Plated cells are washed twice with Dulbecco's phosphate buffered saline (DPBS) and another 500 μl was added per well. DPBS solution was then aspired and PARP lysing buffer (200 μl) was added to the cell plate. The buffer solution contained $MgCl_2$ (10 mM), Triton X-100 (1%), and $NAD^+$ (20 μM) in Tris buffer (50mM, pH 8.1). The plate was then incubated for 1 h and the amount of $NAD^+$ consumed was measured by the NAD(H) microcycling assay using the Fluorostar Optima microplate reader (BD Biosciences).

### 2.4.17 TUNEL assay

NIT-1 cells ($2x10^6$) were seeded overnight and then left untreated or treated with FhHDM-1 (10μM) or vehicle control (water) for 1h. Additional cells pre-treated with wortmannin (100nM, 15mins) were also incubated with FhHDM-1. Apoptosis was then induced by the addition of a pro-inflammatory cytokine cocktail (final concentration: 10ng/ml IL1β, 50ng/ml TNF, 50ng/ml IFNγ; BD Pharmingen) for 18h. Cells were collected and fixed with 1% (w/v) paraformaldehyde for 15mins, washed twice with PBS, and resuspended in cold 70% (v/v) ethanol. The presence of DNA strand breaks induced during apoptosis of NIT-1 cells was then measured using the terminal deoxynucleotidyl transferase dUTP Nick End Labelling

(TUNEL) assay kit (Abcam, ab66108), according to the manufacturer's instructions. Quantification of apoptosis was performed using a BD LSRII flow cytometer. Data was analysed using FCS Express software. Results were expressed as the percentage of apoptotic cells, which was proportional to the number of cells positive for TUNEL staining.

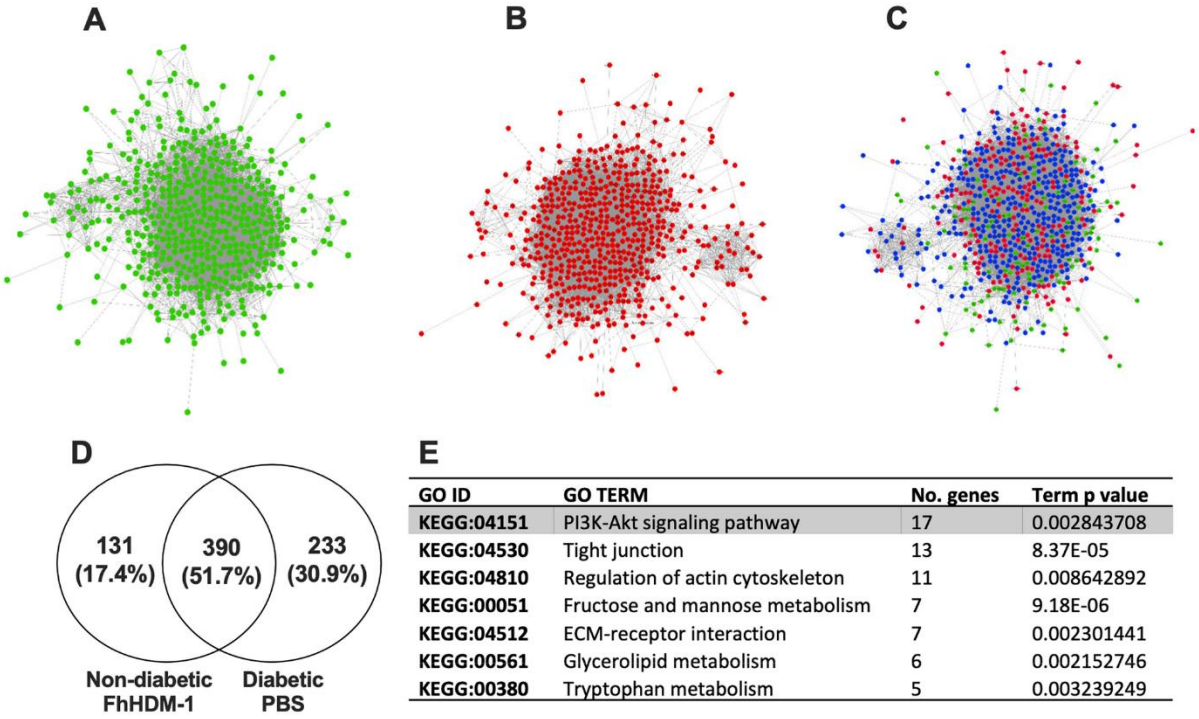
### 2.4.18 Data Analyses and Statistical Tests

All values were expressed as means ± standard error of means (SEMs). Data were statistically analysed using an unpaired two-tailed t-test or one-way ANOVA with Turkey's multiple comparisons *post hoc* test with GraphPad Prism, and differences were considered significant if $p \geq 0.05$.

## 2.5 Results

### 2.5.1 Treatment with FhHDM-1 activates the PI3K/Akt pathway in the pancreas of NOD mice

Although FhHDM-1 was previously shown to significantly reduce the incidence of T1D in NOD mice, associated with decreased insulitic scores (151), the mechanism underlying this protective effect had not been fully elucidated. Therefore, a tissue-level proteomic approach was used to identify the biological pathways within the pancreas that were modulated by FhHDM-1. Thus, the proteomic profiles of pancreas isolated from aged-matched diabetic (PBS-treated) and non-diabetic (FhHDM-1-treated) mice were compared (Supplementary Table 2.2). A reductive topological interaction network analysis of proteins within the pancreas revealed that the majority of proteins (n=390) were identified as common to both PBS diabetic and FhHDM-1 nondiabetic mice. (Fig.2.1A-D). However, this approach also revealed that 131 proteins were uniquely expressed in the FhHDM-1 nondiabetic mice. Using the KEGG pathway analysis resulted in 30 of these proteins being mapped to 7 functional biological processes. Of these, the most highly represented, with 17 contributing proteins, was the phosphoinositide 3-kinase (PI3K)/Akt signalling pathway (Fig. 2.1E). This finding was of particular interest as PI3K/Akt signalling is known to play an important role in regulating the proliferation, survival, and metabolic activity of β-cells (214-219). This result suggested that FhHDM-1 activated the PI3K/Akt pathway to preserve β-cell mass, (and possibly functionality), even on a background of β-cell directed autoimmunity, and thus prevented the development of T1D. Supporting this hypothesis, interrogation of the protein network using relevant search terms (such as ‘apoptosis’, ‘proliferation’, ‘insulin synthesis and secretion’) identified a group of 11 uniquely expressed proteins in the diabetes protected, FhHDM-1 treated, mice with annotated roles in the regulation of apoptosis (Supplementary Table 2.3). While the majority of these proteins were scattered throughout the network, there was an interlinked contiguous quartet of interacting proteins made up of Cdh1, Agt, Rps6kb1, and Bmi1, which formed part of a single pathway (Supplementary Figure 2.1). The biological activity of three of these proteins has also been intrinsically linked to the activation of the PI3K/Akt pathway (220-222).




E

| GO ID | GO TERM | No. genes | Term p value |
|---|---|---|---|
| KEGG:04151 | PI3K-Akt signaling pathway | 17 | 0.002843708 |
| KEGG:04530 | Tight junction | 13 | 8.37E-05 |
| KEGG:04810 | Regulation of actin cytoskeleton | 11 | 0.008642892 |
| KEGG:00051 | Fructose and mannose metabolism | 7 | 9.18E-06 |
| KEGG:04512 | ECM-receptor interaction | 7 | 0.002301441 |
| KEGG:00561 | Glycerolipid metabolism | 6 | 0.002152746 |
| KEGG:00380 | Tryptophan metabolism | 5 | 0.003239249 |

**Figure 2.1 Treatment with FhHDM-1 activates the PI3K/Akt pathway in the pancreas of NOD mice. (A)** Network analysis of the protein lysates isolated from the pancreas of non-diabetic mice that had been treated with FhHDM-1 shows 495 proteins (nodes) and 8286 interactions (edges) within this sample; **(B)** Network analysis of the proteins within the pancreas of diabetic NOD mice shows 592 proteins (nodes) and 12307 interactions (edges) within this sample; **(C)** Merging the diabetic and non-diabetic (ND) pancreatic networks shows 709 proteins (nodes) and 14280 interactions. Green nodes unique to ND FhHDM-1, Red nodes unique to diabetic mice and blue nodes shared between the two treatments. **(D)** Venn diagram breakdown of proteins unique and shared in the merged network shows 131 proteins unique to ND FhHDM1, 233 proteins unique to D PBS and 390 shared proteins between networks. **(E)** Differentially enriched biological pathways in FhHDM-1-treated non-diabetic mice as compared to age-matched PBS-treated diabetic mice.

### 2.5.2 FhHDM-1 treatment of NOD mice preserves β-cell mass

To more specifically test the possibility that FhHDM-1 was regulating biological signals to promote β-cell survival, the β-cell mass of mice treated with FhHDM-1 was compared to that of mice that received PBS. Analyses were performed on pancreas tissue harvested from NOD mice at 10 weeks of age, a time point juxtapositioned after the initiation of autoimmunity (i.e. commencement of insulitis) and coincident with the perpetuation phases of autoimmunity (i.e. amplification of insulitis, and hence β-cell destruction). Sections of pancreas were stained by immunofluorescence for insulin to identify β-cells, and DAPI for nuclei (Fig. 2.2A-F), and the areas occupied by β-cells were quantified using Image J software analysis. These studies revealed that FhHDM-1 preserved β-cell mass in NOD mice, as evidenced by a significantly greater ($p=0.003$, Fig. 2.2G) area of insulin positivity relative to the total pancreas tissue area analysed, as compared to the PBS control group. This observation supported the hypothesis suggested by the proteomic analysis that the administration of FhHDM-1 promoted the survival of β-cells and provides a putative mechanism of action for FhHDM-1 in preventing T1D.

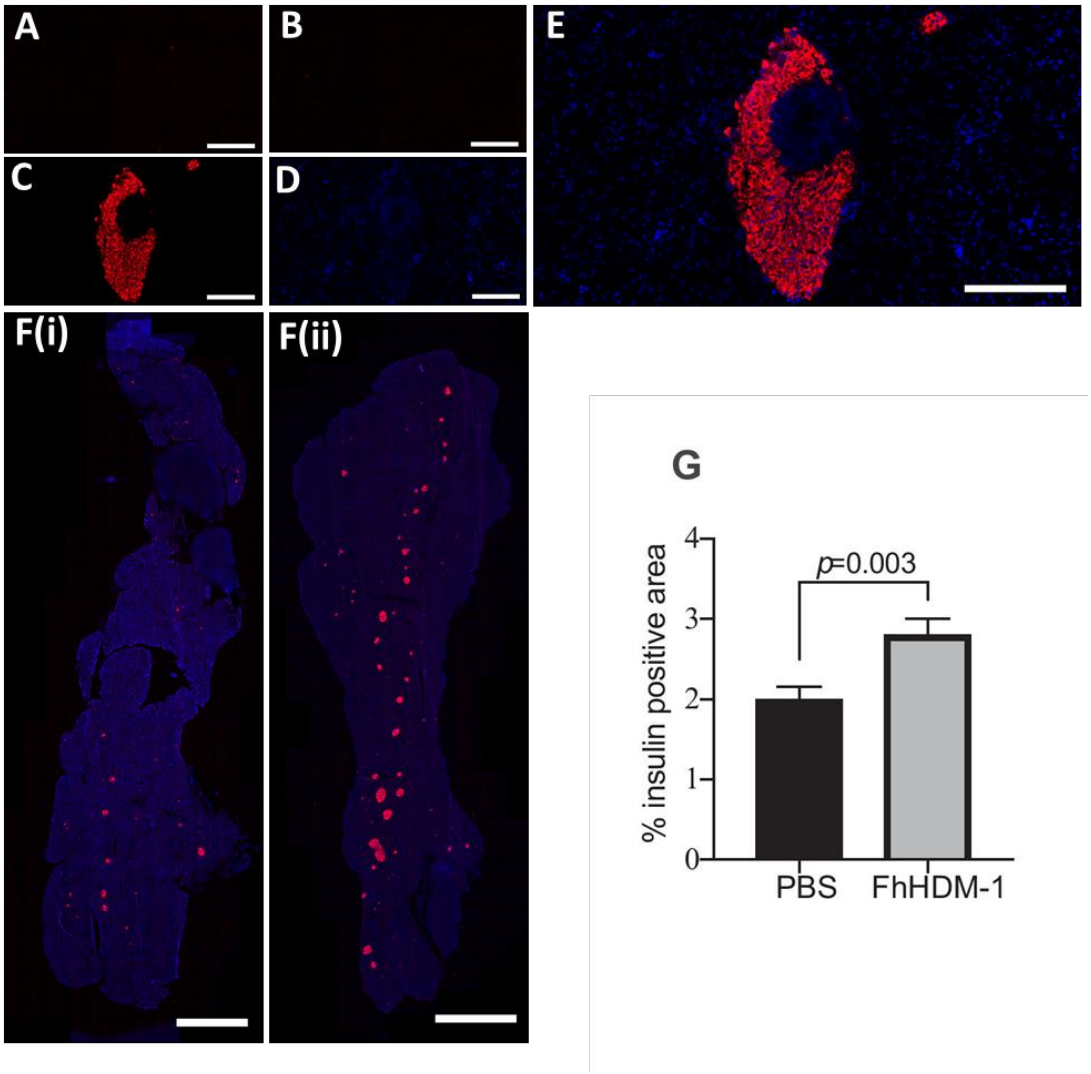



**Figure 2.2 FhHDM-1 treatment of NOD mice preserves β-cell mass.** Pancreatic cryosections (5µm) were fixed/permeabilised with acetone before immunofluorescence labelling of insulin (red) and counterstaining of nuclei with DAPI (blue). **(A)** Autofluorescence control to which no antibodies were added. **(B)** Secondary antibody only control, in which the primary antibody was omitted. **(C)** Pancreatic islet positive for insulin staining was visualised via the far-red filter (645/665nm). **(D)** DAPI-counterstained nuclei of the same islet were visualised via the blue filter (435/455). **(E)** Merged image C and D. All images were taken using the Delta Vision Elite microscope or the Zeiss Axio Scan Slide Scanner. **(F)** Representative whole pancreatic tissue sections from (i) PBS and (ii) FhHDM-1 treated mice. **(G)** Quantification of insulin positivity using ImageJ software. A total of nine mice pancreata from each treatment group (PBS or FhHDM-1) were analysed. Within each pancreas, six to eight representative tissue sections that were at least 100µm apart were observed, and percentages of insulin positive regions relative to area of the whole section were calculated. Scale bar represents 200µM (A-E), or 2000µm (F).

### 2.5.3 FhHDM-1 localises to the pancreas after intraperitoneal administration and interacts directly with β-cells

The finding that FhHDM-1 treatment of NOD mice led to both the induction of the PI3K/Akt pathway and an increase in β-cell mass *in vivo* suggested a direct functional link between the ability of FhHDM-1 to prevent T1D by PI3K/Akt-induced preservation of β-cell mass. To determine whether the parasite peptide was mediating these changes directly, through interaction with β-cells, the distribution of FhHDM-1 *in vivo* was traced. FhHDM-1 was labelled with the fluorescent dye, CF800, and administered to NOD mice by intraperitoneal i.p. injection. Twenty minutes after injection, fluorescence was predominantly concentrated in the lower abdomen of FhHDM-1 treated mice (Fig. 2.3A). By comparison, in mice injected with CF800 dye only, fluorescence was more expansively spread across the anterior body. Within the peritoneal cavity of mice treated with FhHDM-1, fluorescent signal was specifically localised to the pancreas and kidneys (Fig. 2.3A). This distribution of fluorescence suggested that the presence of fluorescent signal in the pancreas and kidney was specifically attributable to the presence of the FhHDM-1 peptide, as opposed to disassociated dye alone. While it is not unexpected for peptides to be excreted through the kidney after i.p. administration (223), the presence of FhHDM-1 in the pancreas suggested a targeted localisation, and supported the direct interaction of FhHDM-1 with β-cells. To ascertain whether this was the case, MIN-6 (non-diabetogenic mouse-derived) and NIT-1 (NOD/diabetogenic mouse-derived) β-cells were incubated *in vitro* with the fluorescently labelled FhHDM-1 for 30mins on ice. Measurement of fluorescence using flow cytometry supported the *in vivo* imaging data and revealed that FhHDM-1 bound to both β-cell lines (Fig. 2.3B and 2.3C).

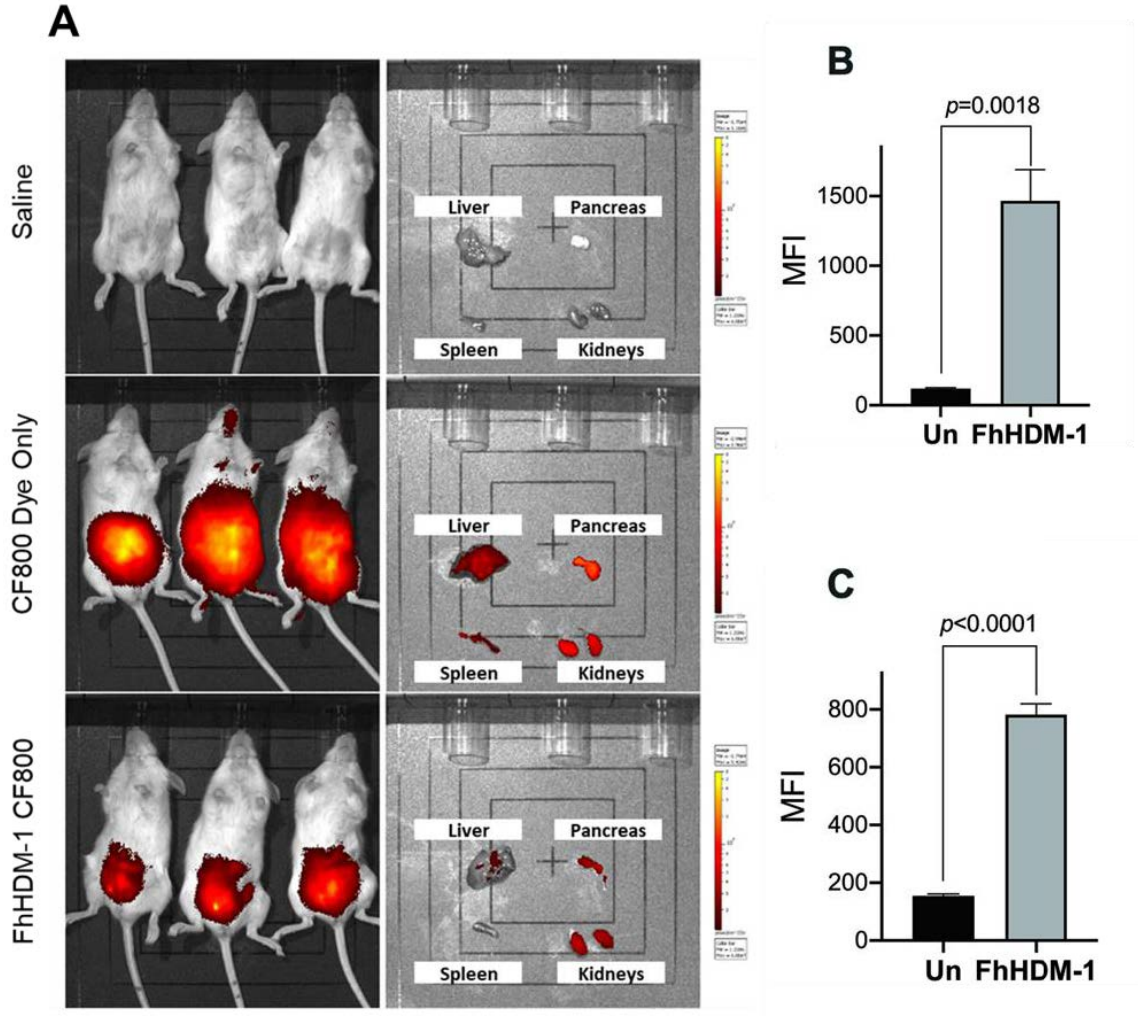
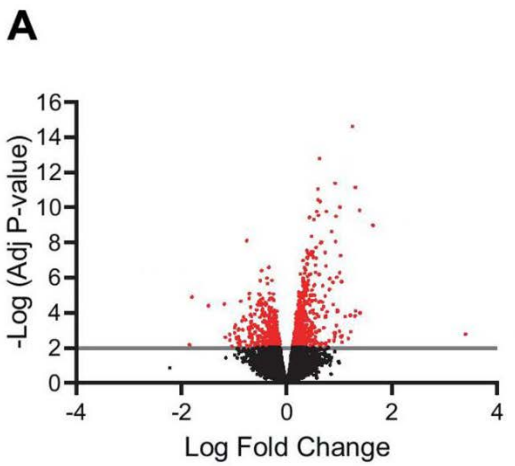
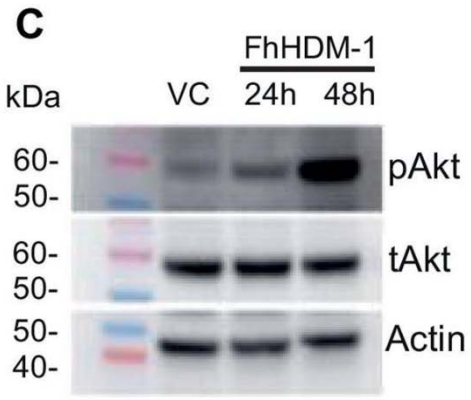



**Figure 2.3 FhHDM-1 localises to the pancreas after intraperitoneal administration to NOD mice and binds to β-cells. (A)** Female/Lt NOD mice (n=3) at 4 weeks of age were injected i.p. with FhHDM-1 labelled with fluorescent CF800 dye (100ug/ml in 100ul), CF800 dye only or sterile saline. Whole-body scans of mice were acquired 20mins post-injection using the IVIS Lumina II imaging system. Organs were then harvested for ex vivo imaging and representative images are shown. Fluorescence images are displayed as photons, with the colour scale normalised across all images and represented logarithmically. **(B)** NIT-1 β-cells derived from NOD mice and **(C)** MIN6 cells derived from Balb/C mice were left unstained (Un) or incubated with FhHDM-1 (10uM) labelled with AlexaFluor488 dye for 10mins at 4˚C, then analysed via flow cytometry to observe binding. Data shown represent the means ± SEMs of two independent experiments and statistical significance was determined using an unpaired t-test.

### 2.5.4 FhHDM-1 activates the PI3K/Akt signalling pathway in β-cells *in vitro*

To understand the impact of the cellular interaction between FhHDM-1 and β-cells, the transcriptome of FhHDM-1 treated NIT-1 cells was sequenced. Initial assessment of this data showed that FhHDM-1 induced an increase in the expression levels of 7345 genes and a reduction in the expression levels of 7066 genes (Fig. 2.4A). Applying a threshold of differential expression of a logarithmic fold change of >1 or <1, and a false discovery rate <0.05, resulted in the identification of only 17 upregulated and 4 downregulated genes (Supplementary Table 2.4). Remarkably, and in support of the proteomic analysis of the whole pancreas, application of the KEGG pathway analysis to the genes that exhibited increased expression levels revealed their predominant involvement in the PI3K/Akt signalling pathway (Fig. 2.4B). This outcome was further validated by western analyses, which showed increased levels of phosphorylated Akt (pAkt) in NIT-1 β-cells treated with FhHDM-1, as compared to untreated cells (Fig. 2.4C).




**B**

| GO ID | GO TERM | Attributed genes | p value |
|---|---|---|---|
| **KEGG:04151** | PI3K-Akt signaling pathway | SCK1, THBS1, IGF1R | 0.0485 |
| **KEGG:00051** | Fructose and mannose metabolism | FBP1, AKR1B7 | 0.0348 |

**Figure 2.4 FhHDM-1 induced differential expression of genes associated with the PI3K-Akt pathway in NIT-1 cells after 24h under basal conditions. (A)** NIT-1 cells ($2x10^6$ cells/well) were untreated or incubated with FhHDM-1 for 24h, prior to cell collection and RNA extraction. Gene expression read counts of samples were provided by Macrogen, which were further used for comparison between baseline and FhHDM-1 treated samples to identify differentially expressed genes using the edgeR package in Rstudio. **(B)** Genes that satisfied the high threshold of differential expression (logarithmic fold change >1 or <1 and false discovery rate <0.05) were mapped to KEGG pathways. P-value was calculated using Fisher's Exact test as provided by DAVID Bioinformatics Resources. **(C)** The phosphorylation of Akt (pAkt) was determined by western blot which analysed protein lysates from NIT-1 cells ($2x10^6$/well) cultured in the presence of FhHDM-1 for 24h or 48h. The presence of total Akt (tAkt) and actin served to normalise the levels of protein in each sample. The image shown is representative of three independent experiments.

### 2.5.5 The induction of the PI3K/Akt pathway by FhHDM-1 increases the metabolic activity of β-cells

To determine whether activation of the PI3K/Akt pathway was increasing the proliferation of β-cells to increase β-cell number/mass, three distinct assays were utilised; (i) BrdU incorporation (Fig. 2.5A), (ii) CyQuant assay (Fig. 2.5B), and (iii) XTT assay (Fig. 2.5C). Of these, only the XTT assay showed a significant increase ($p$=0.0004) in viability of NIT-1 cells treated with FhHDM-1, as compared to vehicle control (water) cells (Fig. 2.5C). Importantly, in the presence of the PI3K/Akt inhibitor, wortmannin, this increase in β-cell viability was abolished (p=0.0218), confirming once again that FhHDM-1 activated the PI3K/Akt pathway (Fig. 2.5C).

Furthermore, the application of these different biological measures of cell number indicated a potential mechanism by which FhHDM-1 was mediating its protective effect on β-cells. BrdU is a thymidine analog that is incorporated into the DNA during cell proliferation, with high rates of BrdU incorporation correlating with high proliferative rates. Similarly, the CyQuant assay utilises a dye that fluorescently labels nucleic acids, thus allowing the quantification of total cellular DNA content, an increase in which reflects cell division. While the XTT assay is commonly used as a measure of cell viability, it quantifies cellular metabolic activity as an indicator of cell viability, proliferation, or cytotoxicity, as opposed to changes in cell numbers *per se*. More specifically, the biochemical reaction of the XTT assay depends on the presence of NADH/NADPH as products of glycolytic activity in cells. An increase in both of these molecules within β-cells treated with FhHDM-1 (Fig. 2.5D and 2.5E) corroborated the outcomes from the XTT assay.

A specific characteristic of the β-cell is not only the strong association between glycolysis and mitochondrial oxidative metabolism, but also rapid glucose stimulated insulin secretion. After glucose equilibrates across the plasma membrane of the β-cell it is phosphorylated by glucokinase, and this determines the rate of glycolysis. The high rates of glycolysis required in the presence of increased glucose concentrations is maintained through the activity of mitochondrial shuttles, which allow the reoxidation of cytosolic NADH. The importance of additional shuttles generating cytosolic NADPH has also been emphasised. However, despite the evidence of increased glycolysis, the treatment of either MIN-6 or NIT-1 β-cells with FhHDM-1 did not enhance the production of insulin in response to glucose (Fig. 2.5F and data not shown). This finding may have been attributable to the decreased

glucose stimulated insulin secretion that characterises β-cell lines (224), and the possibility that the β-cells may have reached maximum metabolic activity under conditions of high glucose (33mM) stimulation and so no increased insulin secretion was observed.

Importantly, while NADH/NADPH are central to metabolic activity, their depletion has also been reported to be a very early event in apoptosis, notably in β-cells (225). Pancreatic β-cells are highly susceptible to the ROI released in the insulitic lesions and by the actions of pro-inflammatory cytokines. Strand breaks in DNA and the activation of PARP-1 and the concomitant depletion of NAD(P)H in β-cells are predominant early consequences of ROI action early in the pathogenesis of T1D. Collectively, these data suggest that the activation of the PI3K/Akt pathway by FhHDM-1 alters the metabolic activity of β-cells to enhance their survival.

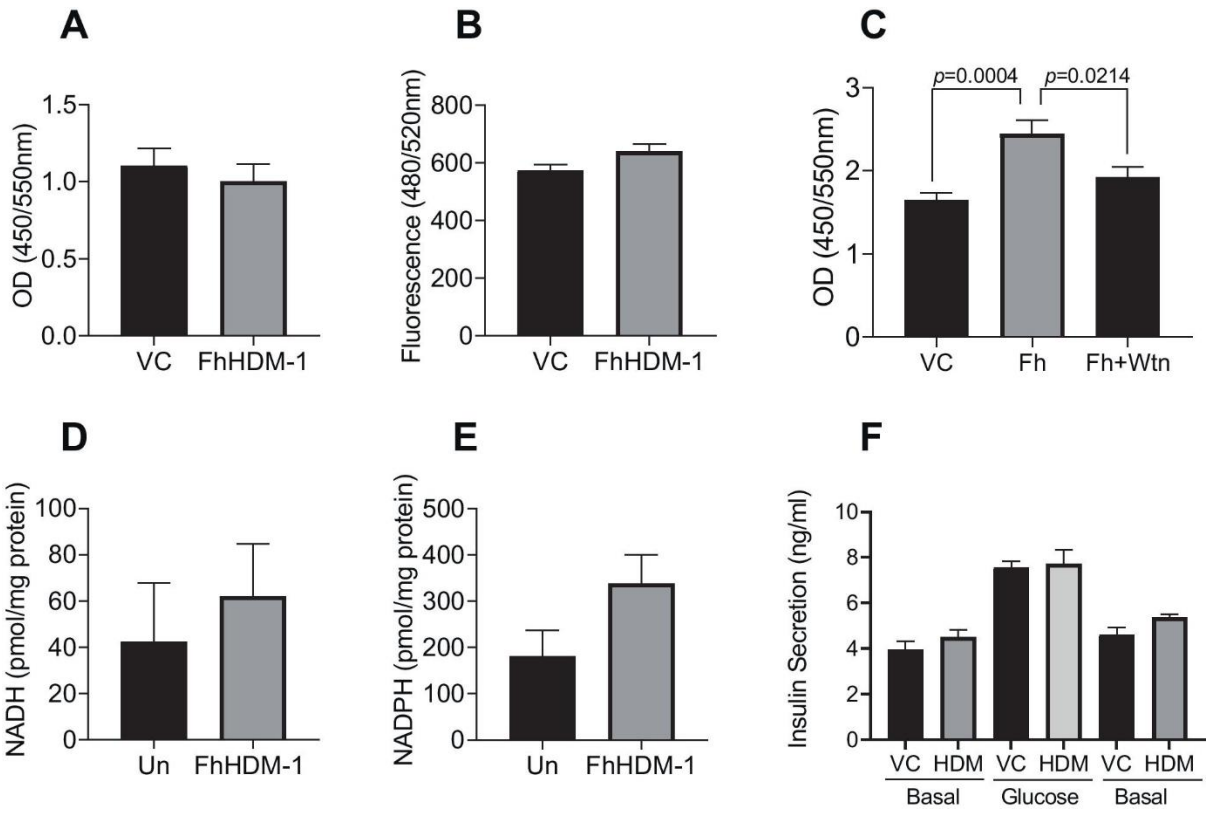



**Figure 2.5 Activation of the PI3K/Akt pathway by FhHDM-1 promotes β-cell survival without inducing proliferation. (A)** NIT-1 cells ($1x10^5$/well) were incubated with FhHDM-1 or vehicle control (VC) (water) diluted in cell culture media for 48h before proliferation was quantified using BrdU incorporation or **(B)** CyQuant dye labelling. Data is presented as means ± SEMs of five of three independent experiments, respectively. **(C)** NIT-1 cells ($1x10^5$/well) were treated with FhHDM-1 (10μM) or vehicle control (water) and incubated for 48h. Additional cells were pre-treated with wortmannin (PI3K/Akt inhibitor; 100nM, 15mins) before incubation with FhHDM-1. Cell viability was quantified using the XTT assay and presented as optical density (OD). Data is presented as means ± SEMs of six biological replicates and is representative of two independent experiments. **(D)** Levels of NADH and **(E)** NADPH in lysates of NIT-1 cells ($1x10^6$) left untreated (Un) or treated with FhHDM-1 (10μM) for 24 h were quantified using liquid chromatography mass spectrometry (LC-MS). Data shown is the means ± SEMs of three biological replicates. (**F**) MIN-6 cells ($1x10^5$cells/well) were incubated with FhHDM-1 (10μM) or vehicle control (water) for 48h. Cells were then washed three times and starved in basal media (1.5mM glucose) for 1h. Cells were then stimulated with high glucose (33mM) for 1h. Finally, cells were incubated once again in basal media for 1h. All collected media was analysed for insulin secretion by ELISA. Data is presented as means ± SEMs of triplicate samples and is representative of three independent experiments. Statistical significance was determined using either an unpaired two-tailed t-test or one-way ANOVA with Turkey's multiple comparisons *post hoc* test.

### 2.5.6 FhHDM-1 stimulates the PI3K/Akt pathway to prevent pro-inflammatory cytokine stimulated apoptosis of murine and human β-cells

To test the premise that FhHDM-1 enhanced β-cell survival, NIT-1 cells were treated with FhHDM-1 (or vehicle control), and then exposed to a mixture of pro-inflammatory cytokines (TNF, IFNγ and IL-1β) to simulate, in part, the environment of the diabetic pancreas after the initiation of insulitis. Treatment of both NIT-1 cells (Fig. 2.6A), and primary human β-cells (Fig. 2.6B) with FhHDM-1, significantly inhibited the activation of caspase-3, which is the downstream mediator of cytokine stimulated apoptotic β-cell death. Furthermore, PARP-1 activity was also significantly reduced in β-cells treated with FhHDM-1 as compared to untreated cells (Fig. 2.6C). Finally, using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL; which measures the apoptotic endpoint of DNA cleavage subsequent to caspase 3 activation), as a definitive morphological marker of cell death, confirmed that FhHDM-1 significantly reduced the incidence of β-cell apoptosis after pro-inflammatory cytokine exposure, as compared to vehicle control ($p=0.0016$; Fig. 2.6D). In fact, treatment of NIT-1 cells with pro-inflammatory cytokines in the presence of FhHDM-1 reduced the percentage of apoptotic β-cells to levels equivalent to those observed in untreated cells. This indicates the ability of FhHDM-1 to completely reverse the cytotoxic effects of pro-inflammatory cytokines known to play a major role in β-cell death. This enhanced level of survival of FhHDM-1 treated β-cells correlated with increased levels of phosphorylated of Akt (Fig. 2.6E). Further, inhibition of the PI3K/Akt pathway using wortmannin reversed the protective effect of FhHDM-1 on β-cell survival (Fig. 2.6D). These observations corroborate the overarching hypothesis that activation of the PI3K/Akt signalling pathway is the mechanism by which FhHDM-1 prevents β-cell death, to prevent T1D.

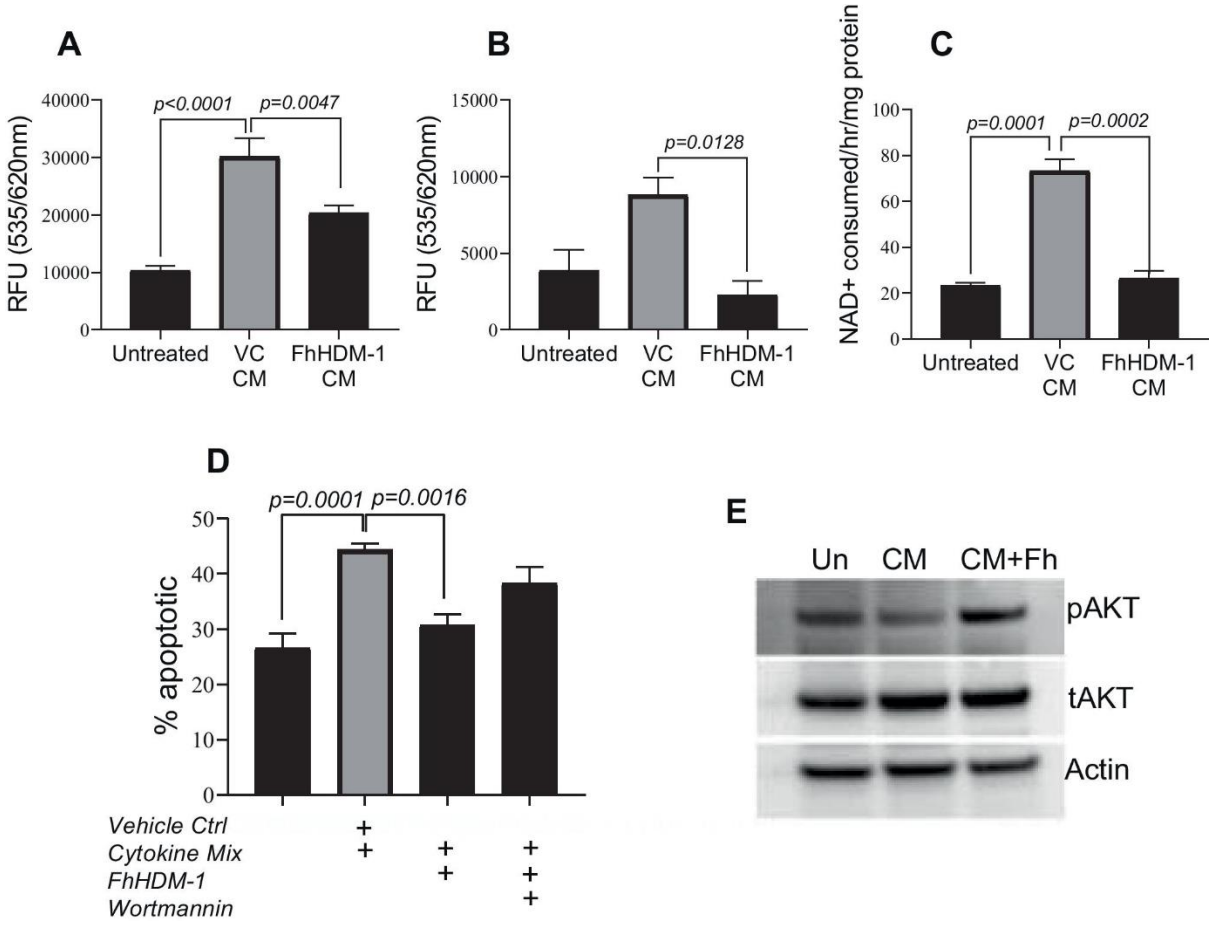



**Figure 2.6 FhHDM-1 prevents β-cell apoptosis induced by pro-inflammatory cytokines.** **(A)** NIT-1 β-cells ($1x10^5$/well) or **(B)** primary human islets ($1x10^5$/well) were left untreated (Un) or treated with vehicle control (VC) (water), or FhHDM-1, in the presence of pro-inflammatory cytokines (murine CM; 50ng/ml TNF, 10ng/ml IL-1β and 50ng/ml IFN-γ, human CM; 10ng/ml TNF, 5ng/ml IL-1β and 10ng/ml IFN-γ) for 10h. Levels of activated caspase-3 were measured by relative fluorescence units (RFU 535/620nm) of a fluorogenic indicator released upon caspase-3 cleavage. **(C)** PARP-1 activity was measured in the cells ($0.5x10^6$cells/ml) using a spectrophotometric assay relying on the chemical quantification of NAD+ consumed. **(D)** Apoptotic cells ($2x10^6$ cells/well) were identified by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining and quantified by flow cytometry. Data for all graphs is presented as means ± SEMs of triplicate samples and is representative of three independent experiments. Statistical significance was determined by one-way ANOVA with Turkey's multiple comparisons *post hoc* test **(E)**. The phosphorylation of Akt (pAkt) in protein lysates from the treated cells ($2x10^6$ cells/well) was assessed by western blot. The presence of total Akt (tAkt) and actin served to normalise the levels of protein in each sample. The image shown is representative of three independent experiments.

## 2.6 Discussion

FhHDM-1 has previously been identified as a novel parasite-derived molecule which prevents T1D development in NOD mice when delivered over only a short time course, coincident with the initiation of autoimmunity (151). In the current study, the application of global proteomic analyses followed by *in vivo* and *in vitro* assays has revealed that FhHDM-1 localises to the pancreas, interacts directly with β-cells, and activates the PI3K/Akt signalling pathway. As a consequence, β-cell survival is enhanced and apoptosis is prevented, even in the presence of the pro-inflammatory cytokines that destroy β-cells during T1D pathogenesis.

While there are a number of signalling cascades triggered by the activation of PI3K, Akt, a serine/threonine protein kinase, has been identified as the predominant downstream effector that promotes cell survival. Overexpression of activated Akt rescues cells from apoptosis induced by various stressors, whereas the inactivation of Akt decreases the pro-survival effect of growth factors (226, 227). Specific to the development of diabetes, and in agreement with the observations presented in this current study, activated Akt has been shown to prevent apoptosis in both primary murine islets and β-cell lines *in vitro* (228, 229). In contrast, increased β-cell apoptosis was induced in insulinoma cells where the phosphorylation of Akt was negatively regulated by c-43 Jun N-terminal kinase (JNK) in response to a number of environmental stimuli, such as pro-inflammatory cytokines and oxidative stress (230). Relating this effect to the development of disease, constitutively active Akt was reported to protect mice against streptozotocin (STZ)-induced diabetes, which causes β-cell apoptosis due to NADH depletion and PARP-1 activation, and protection was associated with a significant increase in β-cell mass (215, 231).

Mechanistically, Akt signalling serves to promote cell survival, in part, by modulating cellular metabolism. An Akt-dependent switch to glycolysis has been coupled to the inhibition of early apoptotic events (232-234), and the depletion of NAD(P)H, a product of glycolysis, is an early event in apoptotic cell death (225). In the current study, FhHDM-1 treatment of β-cells was associated with enhanced levels of NAD(P)H, suggesting a contribution to the preservation of β-cells. Akt phosphorylates a number of transcription factors that control the expression levels of genes that encode metabolic enzymes. Two of these, forkhead box O (FOXO) and hypoxia-inducible factor (HIF)-1, contribute to the regulation of glucose uptake and glycolysis (235). However, neither of these were observed in the list of differentially expressed genes within the transcriptome of FhHDM-1-treated β-

cells, which may explain why these cells did not show an enhanced insulin secretion in response to glucose. In contrast, the expression of thrombospondin 1 (THBS1) was significantly increased in the FhHDM-1-treated β-cells. The expression of this glycoprotein is reportedly upregulated by activation of the PI3K/Akt pathway (236), and, in turn, has been shown to activate nuclear factor erythroid-2 related factor-2 (NRF2) in β-cells (237). NRF2 is a transcription factor that regulates the expression of a range of genes involved in the antioxidant response, including enzymes involved in the regeneration of NAD(P)H (238). Furthermore, the phosphorylation of Akt leads to the stabilisation and increased abundance of NRF2 (235). Based on this evidence, it is proposed that the activation of PI3K/Akt in β-cells by FhHDM-1 provides the necessary reducing power [NAD(P)H] for antioxidant responses, which regulate intracellular oxidative stress to prevent apoptosis.

Independently to a role in cellular metabolism, activation of the PI3K/Akt signalling pathway, by insulin, insulin-like growth factor (IGF), glucose, glucagon-like peptide-1 (GLP-1), or glucose-dependent insulinotropic polypeptide (GIP), results in the prevention of cell death via the phosphorylation and inhibition of a family of pro-apoptotic factors (FOXO) (229, 239). The phosphorylation of specific amino acid sequences results in the translocation of FOXO proteins from the nucleus to cytoplasm leading to their transcriptional inactivation (240). Of relevance to this pathway, β-cells treated with FhHDM-1 showed a significant increase in the expression levels of the receptor for IGF-1 (IGF1R). Similarly, GLP-1 strongly enhanced the expression of IGF-1R expression in β-cells, which was required for GLP-1 activation of PI3K/Akt and subsequent phosphorylation of FOXO1 to protect against pro-inflammatory cytokine induced apoptosis (241). The transcriptome and proteome data presented here corroborates these outcomes due to activation of the PI3K/Akt pathway in β-cells by FhHDM-1.

The expression levels of the serum/glucocorticoid regulated kinase, SGK1, which operates downstream of PI3K, were also significantly increased in FhHDM-1 treated β-cells. This kinase is highly homologous to Akt and thus, like Akt, SGK1 promotes cell survival, in part, by phosphorylating and inactivating FOXO3. Although ultimately mediating the same biological effect, SGK and Akt display different preferences for phosphorylation sites within the sequence of FOXO3 (242). This observation has led to the proposition that both function independently to facilitate the ability of PI3K activation to mediate cell survival. While not specific to the pancreas, increased expression levels of FOXO3 have been reported in

peripheral blood mononuclear cells from patients with new onset T1D, and a role for FOXO3 in the pathogenesis of T1D has been suggested (243).

Besides the activation of the PI3K/Akt pathway, the fructose and mannose metabolism pathways were also identified (by both proteomics and transcriptome analyses) as activated in the pancreas of NOD mice treated with FhHDM-1 and in treated NIT-1 cells. While glucose and mannose stimulate the synthesis and secretion of insulin, the contribution for these metabolic pathways in the pathogenesis of diabetes has largely focused on type 2. The two genes whose expression levels were upregulated in NIT-1 cells in response to FhHDM-1 treatment were fructose bisphosphatase 1 (FBP1) and aldo-keto reductase family 1 member B7 (AKR1B7). The former functions to increase glycolytic flux and regulate glucose sensing and insulin secretion in β-cells, while downregulation enhances glucose stimulated insulin secretion (244). Increased expression levels of FBP1 in the transcriptome of FhHDM-1-treated β-cells may explain, in part, why β-cells did not show an enhanced insulin secretion in response to glucose. Enhanced glycolytic flux induced by FBP1 may operate synergistically with the Akt-dependent switch to glycolysis to inhibit early apoptotic events. Conversely, a role for FBP1 in β-cell dysfunction and apoptosis via the promotion of glycogen storage/production and caspase 3 activation has been reported (245). While AKR1B7 is not expressed at significant levels in β-cells it has been ascribed anti-diabetogenic roles (246). While it is yet to be elucidated if modulation of fructose or mannose metabolism is one of the mechanism(s) of action of FhHDM-1 in β-cells, it is noteworthy that modulation of this metabolic pathway(s) occurred in both NOD mice and NIT-1 β-cells, and there is considerable crosstalk between the fructose metabolism, glucose metabolism and the PI3K/Akt pathway.

It is now of interest to determine how the interaction of FhHDM-1 with β-cells results in the activation of the PI3K pathway. Some insight into this mechanism may be inferred from the biological activity of the mammalian cathelicidin-related antimicrobial peptide (CRAMP) (247). This peptide is constitutively expressed in mice and shares structural similarity with FhHDM-1 as both are predominantly comprised of an alpha helix with an amphipathic C-terminal region. Moreover, like FhHDM-1, CRAMP has been reported to prevent the development of T1D in NOD mice (248), and to promote β-cell viability and afford protection against β-cell apoptosis induced by pro-inflammatory cytokines (249). While the stimulatory effect of CRAMP on β-cell viability was originally shown to be dependent on the epidermal growth factor receptor (EGFR), more recent studies have

revealed that CRAMP has the capacity to directly bind IGRF1 and subsequently activate the PI3K/AKT pathway (250). It is relevant to note that the structure of GLP-1 is also comprised primarily of an amphipathic alpha helix (251). While there is little sequence similarity between GLP-1 and FhHDM-1 or CRAMP, molecular modelling suggests that the agonist potency of GLP-1 and its synthetic derivatives correlates with predicted propensity to form alpha helices (252). Similarly, it has been shown that the immune modulating activity of FhHDM-1 is dependent on the presence of an intact amphipathic alpha helix (208). It will be of interest to determine whether the parasite-derived peptide shares the same binding partner on β-cells as GLP-1 and CRAMP, to activate the PI3K/Akt pathway.

It has long been acknowledged that exogenous insulin delivery is a treatment and not a cure for T1D. The vast majority of treatments designed to cure T1D involve immunotherapy, which aims to suppress the activities of specific autoreactive effector immune cells and/or effector molecules or re-establish immune tolerance to β-cells by vaccination with β-cell antigens, thereby allowing recovery/preservation of β-cell function/mass. Amongst the putative therapies tested for efficacy are the secreted proteins of parasitic worms. During parasite infection, these proteins modulate the immune response of their mammalian hosts to prevent expulsion and to drive tissue repair (253, 254). This results in the regulation of inflammation and thus has been proposed to protect against autoimmune diabetes, as such an outcome would limit the pathogenic Th1/Th17-driven inflammation associated with insulitis and the development of T1D. This study serves as one of the first forays into the effect of helminth-derived molecules on the pancreatic β-cells themselves. Together, the outcomes of this study have revealed potentially novel mechanisms that warrant further investigation for applicability to human T1D. The data provides compelling evidence that a single parasite-derived peptide, FhHDM-1, affects pancreatic β-cells, both human and murine, and the mechanism may be via activation of the PI3K/Akt pathway to maintain redox balance and prevention of pro-inflammatory cytokine-induced apoptosis. The ultimate result of FhHDM-1 exposure being the preservation of β-cell mass, and prevention of T1D. It will be both interesting and crucial to now demonstrate the mechanism of the peptide *in vivo* and to identify its cognate receptor.

Additionally, adopting a multi-drug approach of FhHDM-1 treatment and immunotherapy strategies that target β-cell stress and islet autoimmunity, respectively, may provide the long-sought cure for T1D (1). Considering the efficacy of FhHDM-1 to permanently prevent TID development in NOD mice, when administered at a time point

coincident with pancreatic remodelling and the initiation of autoimmunity, it is proposed that the reduced β-cell death at this critical timepoint serves to decrease the autoantigen load, which in turn, enables the balance of the immune response to be tipped from autoimmunity to tolerance. However, given the critical role for macrophages in the initiation of autoimmunity at this same timepoint (255), the proven ability of FhHDM-1 to regulate the pro-inflammatory response of macrophages may assist in the switch towards reduced autoimmunity and thus contribute to the overall reduction in pathogenesis (122). These findings warrant the further investigation of FhHDM-1 as a candidate to preserve residual β-cell mass in recent onset T1D patients. Likewise, it may have significant applications as a therapeutic for other conditions mediated by β-cell death and inflammation such as T2D and islet transplantation.

# Chapter 3: FhHDM-1 enhances IGF-2 production in β-cells and IGF1R expression, suggesting activation of the IGF-2/IGF1R autocrine loop

## 3.1 Introduction

Having discovered that FhHDM-1 promoted the preservation of β-cell mass and inhibition of pro-inflammatory cytokine-induced apoptosis via activation of PI3K/Akt pathway signalling, (Chapter 2), the mechanisms mediating this effect were next investigated.

The activation of PI3K/Akt signalling can occur through an array of upstream pathways coupled to different cell surface receptors. Generally, these include receptor tyrosine kinases (RTK), cytokine receptors, B cell and T cell receptors, integrins, and G-protein-coupled receptors. However, of these cellular receptors, the insulin-like growth factor 1 receptor (IGF1R), was of particular interest as its expression levels were significantly increased in the transcriptome of β-cells treated with FhHDM-1 (Chapter 2).

IGF1R is classified as an RTK (Figure 3.1A) and is classically activated by insulin-like growth factor-1 (IGF-1), IGF-2 and insulin. Upon ligand binding, IGF1R undergoes autophosphorylation, thereby promoting intrinsic kinase activity. This activated IGF1R can recruit and phosphorylate intracellular substrates, diverting into two major pathways (Figure 3.1B). The first pathway occurs through activated insulin receptor substrate (IRS), which recruits and phosphorylates phosphatidylinositol 3-kinase (PI3K). In turn, PI3K activates Akt, which then phosphorylates downstream targets (such as Bcl-2 associated death promoter [Bad], mammalian target of rapamycin [mTORC]1 and glycogen synthase kinase 3 beta [GSK3β]) to regulate cell survival and metabolism. The second pathway is through activated src homology 2 (SH2)-containing-protein (Shc), which recruits Ras to the membrane and activates it, in turn inducing a chain of phosphorylation mediated activations: Raf followed by mitogen-activated protein kinase (MEK) 1/2 and finally extracellular-related kinase (ERK) 1/2, which then phosphorylates an array of substrates associated with cell growth, proliferation and differentiation (256).

Within the pancreatic β-cells, IGF1R is essential in maintaining normal β-cell function and its deletion results in defective glucose stimulated insulin secretion (GSIS) (257, 258). Its ligand, IGF-1, contributes to insulin secretion and is essential for β-cell survival, and IGF-1 overexpression within β-cells confers protection against diabetes induced by streptozotocin (STZ) (259) and pro-inflammatory cytokines (260-262) in animal models. IGF-2 plays a similar role within β-cells, predominantly early in life, during which the growth factor contributes to β-cell development and function (263). Later in life, IGF-2 is synthesised and secreted by adult β-cells and contributes to the regulation of β-cell mass and

function, particularly in response to metabolic stress (264). In the context of diabetes, IGF-2 expression has also been shown to ameliorate disease in genetic mouse models (265, 266), and inhibit β-cell apoptosis induced by pro-inflammatory cytokines (267, 268). However, overexpression of IGF-2 within β-cells can also lead to endoplasmic reticulum (ER) stress, islet dysfunction and subsequently diabetes onset, if unregulated (269).

Alpha helix peptides that are structurally similar to FhHDM-1, such as murine cathelicidin like antimicrobial peptide (CRAMP) and the human homologue, LL37, as well as the hormone, glucagon-like peptide-1 (GLP-1), have all been shown to interact with IGF1R. CRAMP has been shown to bind directly to IGF1R, thereby activating PI3K/Akt signalling in cardiomyocytes to attenuate pathological cardiac hypertrophy (270). Likewise, LL37 interacts with IGF1R, although this peptide has been implicated as a biased agonist due to the preferential activation of the ERK1/2 arm of the signalling pathway, and an inability to promote recruitment of IRS1 to IGF1R. Consequently, cellular proliferation but not protein synthesis is enhanced (271). In contrast, GLP-1 has been demonstrated to indirectly activate IGF1R by inducing the IGF-2/IGF1R autocrine loop in which IGF-2, secreted by the β-cells themselves, increases the expression levels and thus enhances the activation of IGF1R on their cell membranes. By promoting IGF-2 secretion and IGF1R expression on the β-cells, GLP-1 conferred protection against pro-inflammatory cytokine induced apoptosis (272).

Considering these beneficial effects, GLP-1 analogues, such as exenatide and liraglutide, have been used as therapeutics for T2D, and they have been demonstrated to enhance glucose-dependent insulin secretion (273). Similarly, GLP-1 analogues have also been tested for efficacy in T1D, with several studies demonstrating beneficial effects on weight, reduction in total daily insulin requirements, and modest improvements in glucose control (274). Although GLP-1 lacks stability *in vivo* (275), these studies provide proof-of-principle that targeting the IGF-2/IGF1R autocrine loop has beneficial effects in diabetes. Therefore, the hypothesis that IGF1R was the binding partner for FhHDM-1 on β-cells, with resultant activation of PI3K/Akt signalling and enhancement of viability, was investigated.

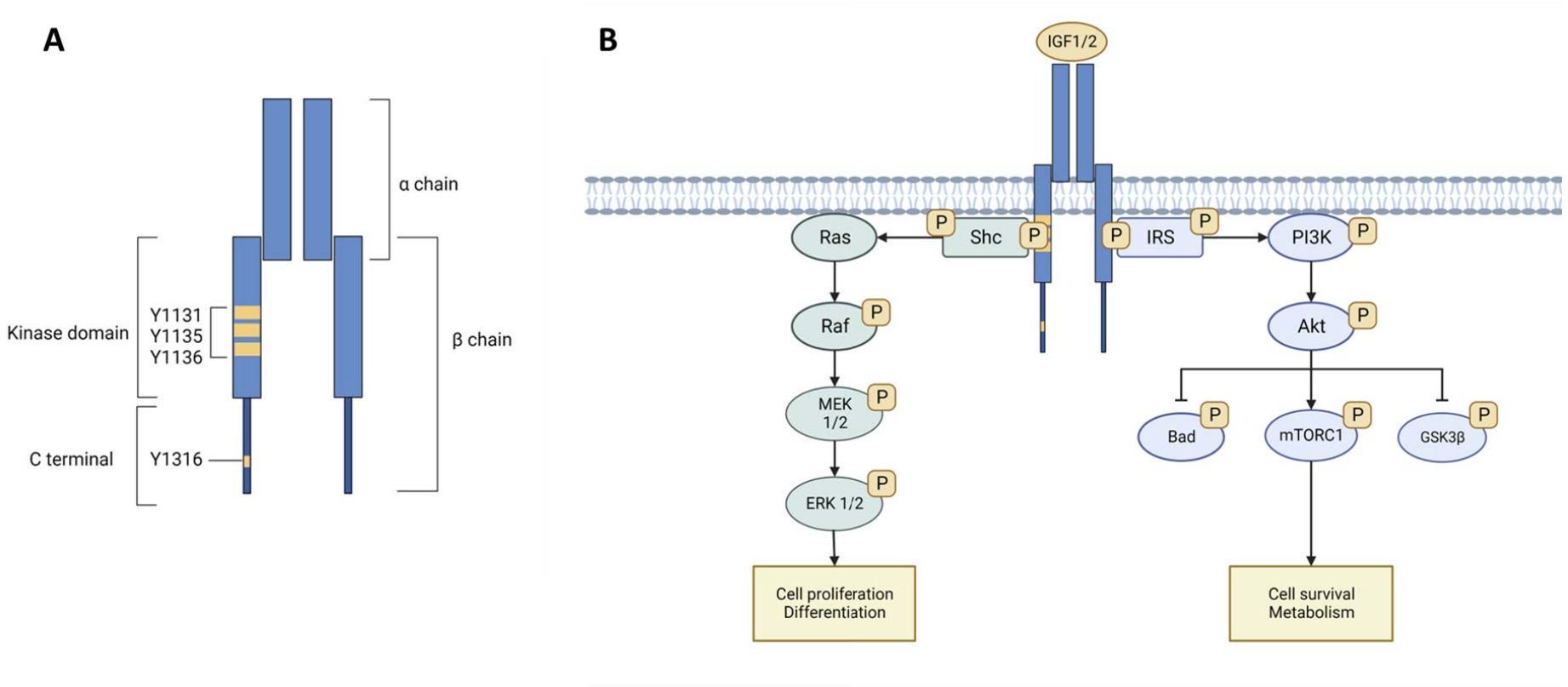



**Figure 3.1 Overview of the IGF1R and its downstream pathways. (A)** Insulin growth factor 1 receptor (IGF1R) is comprised of an alpha (α) chain and beta (β) chain. Within the intracellular domain of the β-chain is the kinase domain containing the three major phosphorylation sites of IGF1R, Tyr1131, Tyr1135 and Tyr1136. Another phosphorylation site, Tyr1316, is found within the C terminal region of IGF1R. **(B)** Once activated by its ligands, IGF1R phosphorylates its intracellular substrates, diverting into two main pathways: phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular-related kinase (ERK). IGF1R phosphorylates the insulin receptor substrate (IRS), which recruits and phosphorylates PI3K. Subsequently, PI3K activates Akt, which then phosphorylates its downstream targets (such as Bcl-2 associated death promoter [Bad], mammalian target of rapamycin [mTORC1] and glycogen synthase kinase 3 beta [GSK3β]) to promote cell survival and metabolism. IGF1R activated src homology 2 (SH2)-containing-protein (Shc) recruits and activates Ras, which subsequently phosphorylates Raf. Activated Raf then phosphorylates mitogen-activated protein kinase (MEK) 1/2, which, in turn, activates extracellular-related kinase (ERK) 1/2 that affects targets associated with cell growth, proliferation, and differentiation. Created with BioRender.com and adapted from (276).

## 3.2 Methods

### 3.2.1 Protein extraction and proteolysis

NIT-1 β-cells ($6x10^6$) were untreated (Un) or incubated with FhHDM-1 (10μM) for 24h. Cells were washed four times with PBS, then cell pellets were collected and stored at -80°C until proteomic analyses, which were conducted by the Australian Proteome Analysis Facility (APAF). Cells were suspended in S-Trap lysis buffer (10% SDS, 100mM triethylammonium bicarbonate, TEAB, pH 8.55) and lysed by probe sonication. Protein disulphide bridges were reduced with 10mM dithiothreitol (DTT) at 56°C then alkylated with 20mM iodoacetamide (IAA) at room temperature (RT) in the dark. Subsequently, protein samples were processed using S-trap spin columns (Protifi, USA), as per manufacturer's instructions. Briefly, proteins were acidified using phosphoric acid and diluted with S-Trap binding buffer (90% (v/v) methanol, 100mM triethylammonium bicarbonate, pH 7.1). The samples were then transferred into S-Trap Micro spin columns, centrifuged, and washed twice with S-Trap binding buffer. Proteolysis was carried out with 20μg of trypsin (in 50mM TEAB) for 3h at 47°C. Peptides were eluted sequentially using (i) 80μL TEAB 50 mM, (ii) 80μL 0.2% (v/v) formic acid (HCOOH) in $H_2O$, and (iii) 80μL 50% (v/v) acetonitrile ($CH_3CN$) and 0.2 % (v/v) formic acid. Eluted peptides were pooled and lyophilized in a vacuum centrifuge.

### 3.2.2 TMT labelling of peptides

Peptides from each biological replicate were labelled with the respective 10-plex TMT reagents (Thermo Fisher, USA), as per manufacturer's instructions. Briefly, labelling reactions were carried out at RT for 1h, after which 5% (v/v) fresh hydroxylamine was added to fully quench unbound TMT labels. Portions of each sample were then combined, desalted using StageTip (https://pubs.acs.org/doi/10.1021/ac026117i), and analysed by LC-MS, and total intensities of TMT labels calculated in each TMT and normalisation factors calculated. Peptides from each sample were combined at 1:1 ratio using these normalisation factors, dried completely using a vacuum centrifuge, and desalted using Sep-Pak C18 cartridges (Waters, USA), following manufacturer's instructions.

### 3.2.3 Offline high pH reversed phase (HpH) fractionation of TMT labelled peptides

The following proteomic analysis was performed by APAF. Briefly, the peptide mixture was subjected to phospho-peptide enrichment using a Phosphopeptide Enrichment Kit (Thermo Scientific, USA), as per manufacturer's instructions. The unbound flowthrough fraction of non-phosphorylated peptides was dried using a vacuum centrifuge. The flowthrough peptide mixture was resuspended in loading buffer (5 mM ammonia solution, pH 10.5), then separated into a total of 96 fractions using an Agilent 1260 HPLC system equipped with a fraction collector. The 96 fractions were consolidated into 20 individual fractions, dried by vacuum centrifugation, then reconstituted in 0.1 % (v/v) formic acid for LC-MS/MS analysis.

### 3.2.4 LC-MS/MS analysis of TMT labelled peptides

Samples were analysed on a Q-Exactive Orbitrap-HFX mass spectrometer (Thermo Fisher Scientific, USA) coupled to an UltiMate 3000 HPLC system (Thermo Fisher Scientific, USA). Samples were first loaded on a reverse phase peptide trap column, washed with 0.1 % (v/v) formic acid, then loaded onto a reversed-phase nano-LC column (Solidcore Halo-C18, 160 Å, 2.7µM, 100µM x 30cm, Advanced Materials Technology, USA). Peptides were separated using a linear gradient of 95:5 mobile phase A (0.1% (v/v) formic acid): mobile phase B (99.9% (v/v) acetonitrile/0.1% (v/v) formic acid) to 70:30 mobile phase A: mobile phase B over 110 min. The mass spectrometer was operated in data-dependent mode. A survey scan over 350 to 1850*m/z* for was acquired at a resolution of 60,000 with an automated gain control (AGC) target value of $3 \times 10^6$ ions and a maximum injection time of 45ms. The ten most abundant ions with charge state +2 to +5 were selected for higher-energy collisional dissociation (HCD) fragmentation at a normalized collision energy of 33%. The maximum injection time for target ions selected for MS/MS was set to 85ms. Spectra were acquired in a resolution of 45,000 with an AGC target value of $1 \times 10^5$ ions.

### 3.2.5 Protein identification and quantification

Peak lists derived from LC-MS/MS data files were searched against *Mus musculus* protein sequences from the Uniprot database (55,485 protein sequences downloaded 14th January 2021) using MaxQuant (Version_1.6.10.43) employing the Andromeda search engine (https://pubs.acs.org/doi/10.1021/pr101065j). The MS and MS/MS tolerances were each set to ± 20 ppm, and trypsin digestion was specified with two missed cleavages. Oxidation of methionine and oxidation of protein N-termini were set as variable modifications.

Carbamidomethylation of cysteine was set as a static modification. Protein, peptide and PSM false discovery rates of < 1% were employed. Protein abundances were normalized to total abundance of all the proteins in the respective channel. Relative quantitation of proteins was achieved by comparison of TMT reporter ion intensities using the TMTPrepPro data analysis suite (https://pubmed.ncbi.nlm.nih.gov/27975283/). For each treatment versus control, the statistical significance of differentially expressed proteins (DEPs) was evaluated using comparisons by student's t-test. DEPs with protein abundance fold change of ± 1.2 and t-test P value < 0.05 were subjected to downstream evaluation.

### 3.2.6 PathHunter IGF1R Bioassay

The PathHunter IGF1R Bioassay kit (Eurofins Discover X, 93-0505Y1-00069) was used to determine activation of IGF1R kinase activity (via detection of receptor phosphorylation) by FhHDM-1, as compared to IGF1. Briefly, PathHunter HEK 293 IGF1R Bioassay cells ($1x10^4$) were incubated with serial dilutions of recombinant human IGF1 ligand or FhHDM-1 for 16-18h at 37°C and 5% $CO_2$. The cells were then incubated with Bioassay detection reagents 1 and 2, for 15mins and 1h, respectively, at RT protected from light, prior to measurement of luminescence signal from which dose curves were generated. To allow comparison of doses between the two treatment groups, the $EC_{50}$ of IGF-1 was converted based on its molecular weight of 7.649kDA, resulting in an $EC_{50}$ of 0.002μM.

### 3.2.7 Binding of FhHDM-1 to β-cells

FhHDM-1 peptide was labelled with Alexa Fluor 488 NHS Ester (Thermo Fisher Scientific, A20000), according to manufacturer's instructions. Mouse fibroblast clones ($1x10^6$ cells) overexpressing IGF1R (P6) or having little/no expression of IGF1R (R-) were kindly provided by Prof. Girnita (Karolinska Institute, Sweden). Cells were incubated with fluorescently labelled FhHDM-1 for 30mins at 4°C prior to flow cytometric analyses to assess binding to each clone.

### 3.2.8 Cell viability assay

A CyQUANT XTT cell viability assay kit (Thermo Fisher Scientific, X12223) was used to measure cell survival. NIT-1 β-cells ($1x10^5$) were plated in serum-free media, then left

untreated (Un) or incubated with DMSO control, IGF1 (1µg/ml, which is equivalent to 0.13 µM when converted based on IGF1 molecular weight of 7.649kDA), or FhHDM-1 (10µM) for 24h. An additional group of IGF1 and FhHDM-1 treated cells were pre-treated with linsitinib (Novusbio), an IGF1R inhibitor (10µM, 1h). Four hours prior to the end of incubation, XTT mixture was added to each sample and the production of formazan by viable cells was measured (450/650nm).

### 3.2.9 Measurement of IGF1 and IGF-2 secretion

NIT-1 β -cells ($2x10^6$) were serum-deprived for 18h then left untreated or incubated with IGF1 (1µg/ml or 0.13µM) or FhHDM-1 (10µM) for 6h or 24h. Following incubation, cell supernatants were collected and assayed for IGF-1/2 production using an IGF-1 (Thermo Fisher Scientific, EMIGF1) or IGF-2 ELISA kit (Thermo Fisher Scientific, EMIGF2).

### 3.2.10 Western blot analyses

NIT-1 β -cells ($2x10^6$) were serum-deprived for 18h then left untreated or incubated with IGF1 (1µg/ml or 0.13µM) or FhHDM-1 (10µM) for 6h or 24h. Following incubation, cells were lysed in RIPA buffer (Thermo Fisher Scientific, 89900) containing 1x protease (Thermo Fisher Scientific, 1862209) and phosphatase inhibitors (Thermo Fisher Scientific 78420). Protein content was measured using the Bradford assay (Thermo Fisher Scientific, 23225), and protein lysates (7µg) were separated on 4 – 12% Bis-Tris gels (Thermo Fisher Scientific, NP3021), and then transferred to polyvinylidene difluoride membranes (Thermo Fisher Scientific, IB24001), followed by probing with primary antibodies for pIGF1R Tyr1161/Tyr1165/Tyr1166 (also known as Tyr1131, 1135, 1136) (Merck, ABE332), pIGF1R Ty1316 (Cell Signalling Technologies, 28897S), and total IGF1R (Cell Signalling Technologies, 9750S), followed by horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody (Cell Signalling Technologies, 7074S). The blots were probed for actin (Abcam, ab20272) as a loading control. Membranes were then incubated with enhanced chemiluminescence (ECL) HRP substrate (Thermo Fisher Scientific, 34076) prior to visualisation of protein bands using the Amersham Imager 600. Band intensities were determined using ImageJ software (277).

## 3.3 Results

### 3.3.1 IGF1R may be required for the FhHDM-1 mediated increase in β-cell viability

To initially investigate whether IGF1R was required by FhHDM-1 to exert its beneficial effects, β-cell viability was measured following treatment with FhHDM-1 with or without the addition of a potent inhibitor of IGF1R kinase activation (Linsitinib; Lins). As a comparative control, NIT-1 β-cells were treated with IGF-1, the natural ligand for IGF1R. This approach confirmed that treatment of cells with IGF-1 and FhHDM-1 significantly enhanced the viability of β-cells, as compared to cells treated with vehicle control (DMSO) (Figure 3.2). Furthermore, the addition of Lins significantly reversed the effect of both IGF1 and FhHDM-1 ($p<0.0001$), suggesting IGF1R may be required by FhHDM-1 to exert its positive effects on β-cell viability. However, this effect was also observed in cells treated with Lins alone.

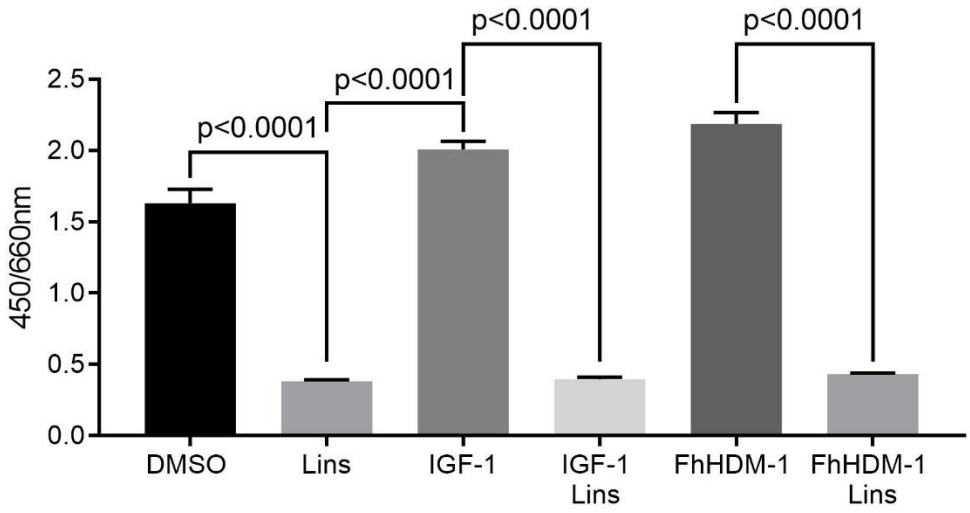



**Figure 3.2 Inhibition of IGF1R prevented FhHDM-1 mediated increase in β-cell viability.** NIT-1 β-cells ($1x10^5$) were plated in serum-free media, incubated with DMSO (vehicle control), Linsitinib (Lins; 10μM, 1h), IGF1 (1μg/ml or 0.13μM), or FhHDM-1 (10μM) for 24h. An additional group of IGF1 and FhHDM-1 treated cells were pre-treated with Lins. Four hours prior to the end of incubation, XTT mixture was added to each sample and the production of formazan by viable cells was measured (450/650nm). Data is presented as the means ± SEMs and representative of three independent experiments, each with at least three replicates per treatment group. Statistical significance was determined using a one-way ANOVA with Turkey's multiple comparisons *post hoc* test.

### 3.3.2 FhHDM-1 increased the abundance of IGF1R in β-cells

It could not be definitively determined whether FhHDM-1 was exerting its beneficial effects on the β-cells through IGF1R. To address this uncertainty, a subsequent investigation was conducted to determine the abundance of IGF1R in FhHDM-1 treated β-cells, as this can provide an indication of whether FhHDM-1 mediates its effect by driving increased abundance of the receptor, as suggested by the previous transcriptome data (Chapter 2). A global proteomic analysis was performed to examine whether there were also complementary alterations in the abundance of downstream components of the PI3K/Akt pathway, enabling further dissection of the intracellular signals activated by FhHDM-1 treatment of β-cells. Thus, in replicating the previous transcriptome analysis (Chapter 2), NIT-1 β-cells were left untreated (Un) or treated with FhHDM-1 (10μM) for 24h prior to protein extraction and mass spectrometry analysis.

The results showed that only 96 proteins showed significant ($p<0.05$) changes in abundance. Of these, 24 showed an increase in abundance in FhHDM-1 treated β-cells, and 74 were decreased in abundance (Supplementary Table 3.1, Figure 3.3). While collectively, no biological pathways were identified when KEGG pathway analysis was applied to this protein list, it was of interest that one of the most abundant proteins in FhHDM-1 treated β-cells was IGF1R, with a log fold increase of 1.24 ($p=0.0031$). This corroborates previous transcriptome data (Chapter 2) in which IGF1R was identified as one of the most upregulated genes in FhHDM-1 treated β-cells.

This outcome was further validated by western blot analyses. NIT-1 β-cells were left untreated (Un) or treated with IGF-1 (1μg/ml) or FhHDM-1 (10μM) for 6h or 24h, prior to protein extraction. The presence of total IGF1R in cell lysates was determined using a specific antibody, and the protein abundance quantified by densitometry and normalisation to the presence of actin (Figures 3.4A and B, 3.5A and B). This confirmed all the previous data sets, and once again showed that FhHDM-1 increased the abundance of IGF1R in comparison to untreated cells, although this difference was greater at 24h (Figure 3.5A, B). Furthermore, this effect contrasted with the outcome following treatment with IGF1 which, at both time points, resulted in a reduction in the quantity of IGF1R that was detected on β-cells, as compared to untreated cells (Figure 3.4 and 3.5).

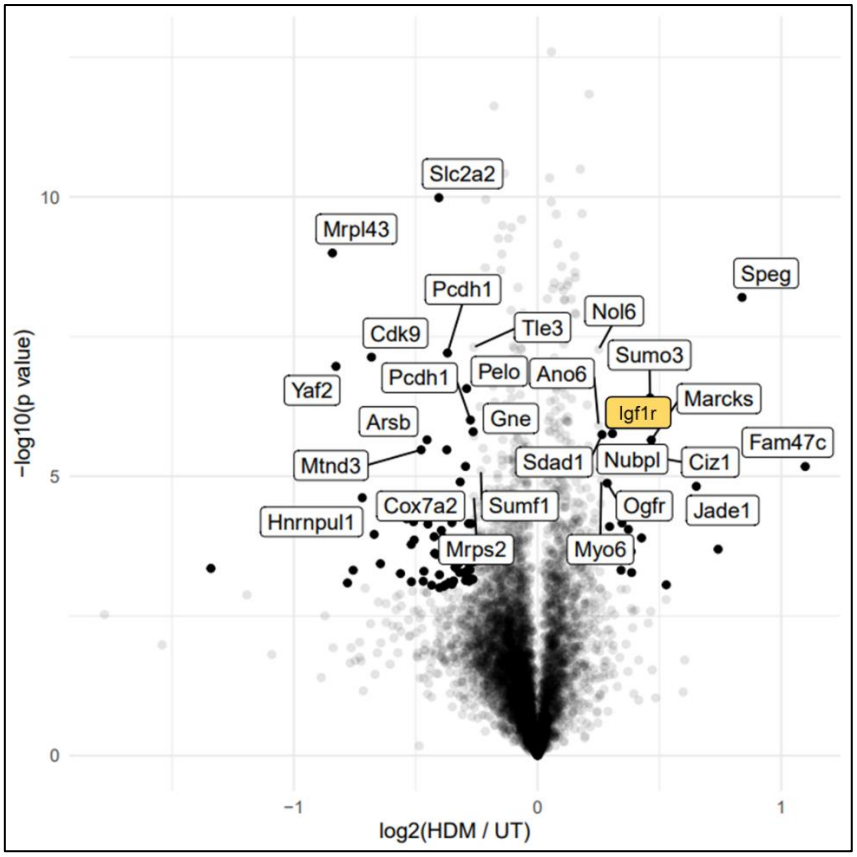



**Figure 3.3 IGF1R is upregulated in FhHDM-1 treated β-cells.** NIT-1 β-cells ($6x10^6$, n=5) were left untreated or incubated with FhHDM-1 (10µM) for 24h prior to protein extraction and mass spectrometry analysis to determine total protein abundance. Volcano plot represents protein fold change expression in FhHDM-1 treated β-cells as compared to untreated cells, versus the corresponding p-value. The darker dots represent proteins that were differentially expressed, i.e., with a fold change beyond the threshold of ±1.2 and p-value <0.05.

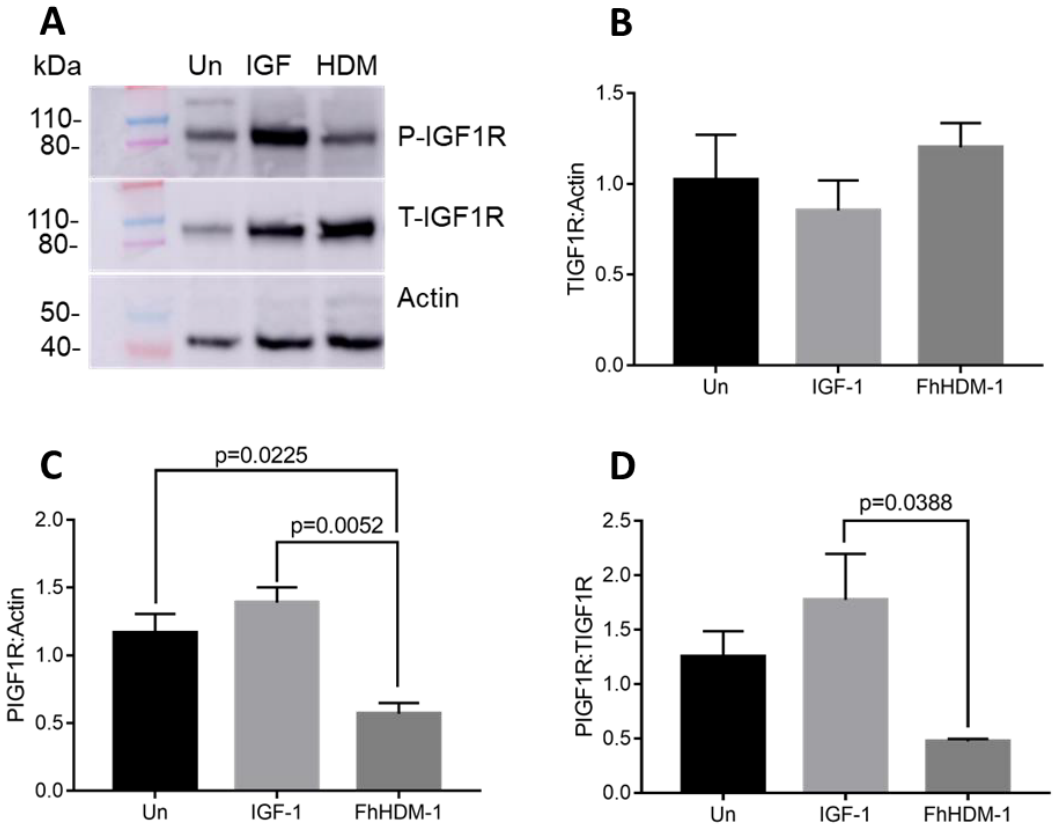



**Figure 3.4 FhHDM-1 treated NIT-1 β-cells (6h) showed increased levels of total IGF1R, but not phosphorylated IGF1R (Tyr1131/1135/1136). (A)** NIT-1 β-cells ($2x10^6$) were serum-deprived for 18h, then left untreated or incubated with IGF1 (1µg/ml or 0.13µM) or FhHDM-1 (10µM) for 6h. Following incubation, protein lysates were collected for western blot analysis to determine phosphorylation of IGF1R (P-IGF1R) at Ty1131/Tyr1135/Ty1136 (P-IGF1R). The presence of total IGF1R (T-IGF1R) and actin served as controls. Densitometry was then performed using ImageJ to compare protein abundance between samples, with **(B)** T-IGF1R and **(C)** P-IGF1R normalised to actin. The ratio between the normalised bands were then calculated as a measure of receptor activation **(D)**. Data is presented as means ± SEMs and representative of two independent experiments, each with three replicates per treatment group. Statistical significance was determined using a one-way ANOVA with Turkey's multiple comparisons *post hoc* test.

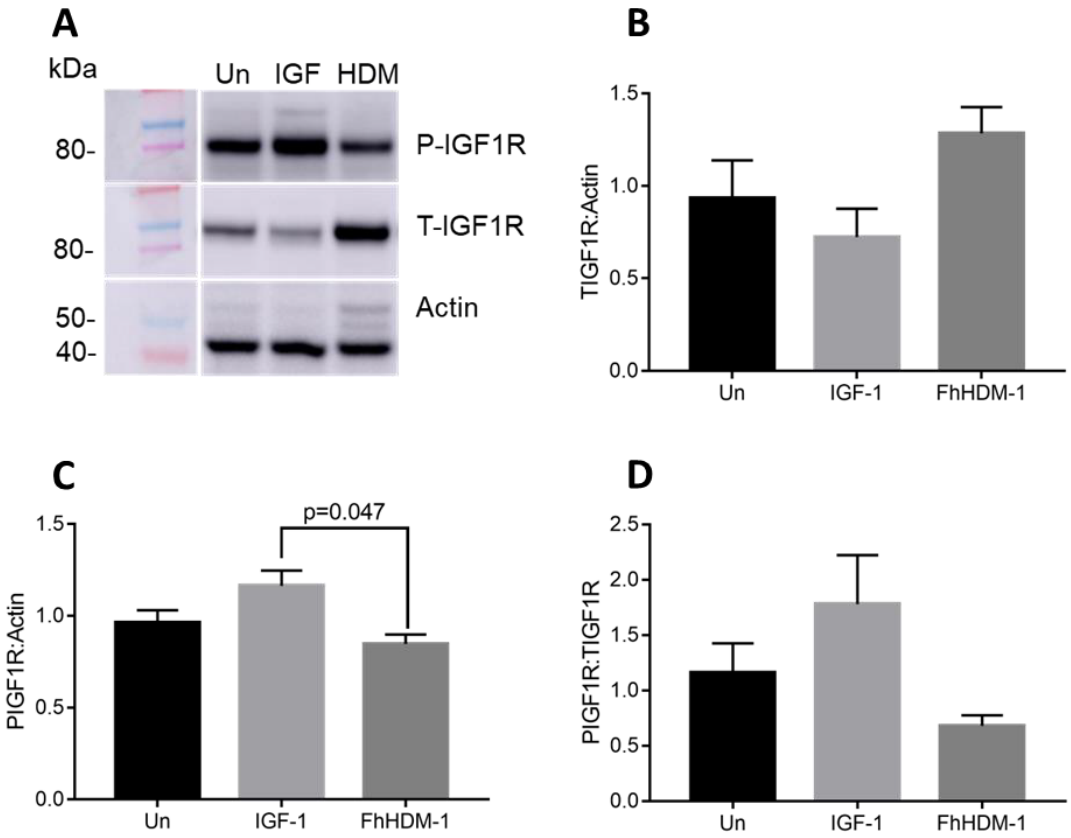



**Figure 3.5 FhHDM-1 treated β-cells (24h) showed increased levels of total IGF1R, but not phosphorylated IGF1R (Tyr1131/1135/1136). (A)** NIT-1 β-cells ($2x10^6$) were serum-deprived for 18h, then left untreated or incubated with IGF1 (1µg/ml or 0.13µM) or FhHDM-1 (10µM) for 24h. Following incubation, protein lysates were collected for western blot analysis to determine phosphorylation of IGF1R (P-IGF1R) at Ty1131/Tyr1135/Ty1136. The presence of total IGF1R (TIGF1R) and actin served as controls. Densitometry was then performed using ImageJ to compare protein abundance between samples, with **(B)** T-IGF1R and **(C)** P-IGF1R and normalised to actin. The ratio between the normalised bands were then calculated as a measure of receptor activation **(D)**. Data is presented as means ± SEMs and representative of two independent experiments, each with three replicates per treatment group. Statistical significance was determined using a one-way ANOVA with Turkey's multiple comparisons *post hoc* test.

### 3.3.3 FhHDM-1 increases the phosphorylation of IGF1R at Tyr1316, but not at Tyr1131-1136

To determine whether the increased abundance of IGF1R in FhHDM-1 treated β-cells reflected an increase in receptor activation, the phosphorylation status of selected tyrosine (Tyr) residues within the IGF1R was examined. Initially, the phosphorylation of the three Tyr residues within the kinase domain (Tyr1131, Ty1135 and Tyr1136), which is regarded as the primary autophosphorylation site, was measured. To compare protein abundance between samples, densitometry of total protein (T-IGF1R) and phosphorylated protein (P-IGF1R) were normalised to actin. The ratios between the normalised bands were then calculated as a measure of receptor activation, with a greater ratio indicating true increased phosphorylation/activation of the receptor.

As expected, treatment of β-cells with IGF-1 enhanced the phosphorylation of these Tyr residues, as compared to untreated cells. However, despite the increased abundance of IGF1R, in FhHDM-1 treated cells, there was no change in the levels of phosphorylation of the receptor at either 6h (Figure 3.4 C, D) or 24h (Figure 3.5C, D), as compared to the untreated cells.

IGF1R phosphorylation can also occur outside the kinase domain and forms docking sites for downstream signal transduction molecules (Figure 3.1A). Of interest, the phosphorylation of Tyr1316, has been reported to induce direct recruitment of PI3K to the cytoplasmic region of the receptor and subsequently activate the PI3K/Akt pathway. Thus, the lysates from β-cells that had been treated with either IGF1 or FhHDM-1 for 24h were next examined for phosphorylation at Tyr1316 (Figure 3.6). Remarkably, FhHDM-1 induced significantly greater phosphorylation, as compared to untreated cells ($p=0.0017$), paralleling the level of phosphorylation induced by IGF-1 treatment ($p=0.0025$).

Together, these analyses revealed that FhHDM-1 increased both total receptor abundance and phosphorylation of IGF1R at Tyr1316, independent of the three major phosphorylation sites (Tyr1131, Ty1135 and Tyr1136) within the kinase domain.

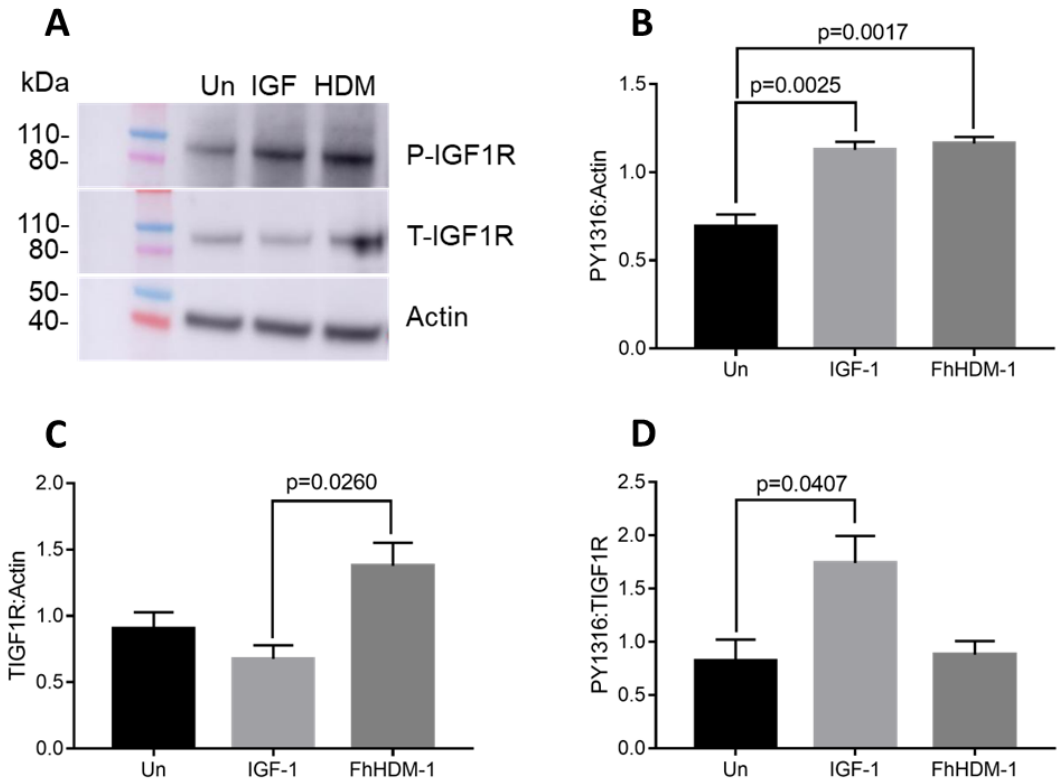



**Figure 3.6 FhHDM-1 treated β-cells (24h) showed increased levels of both phosphorylated IGF1R (Tyr1316) and total IGF1R. (A)** NIT-1 β-cells ($2x10^6$) were serum-deprived for 18h, then left untreated or incubated with IGF1 (1µg/ml or 0.13µM) or FhHDM-1 (10µM) for 24h. Following incubation, protein lysates were collected for western blot analyses to determine phosphorylation of IGF1R (P-IGF1R) at Tyr1316 (PY1316). The presence of total IGF1R (T-IGF1R) and actin served as controls. Densitometry was then performed using ImageJ to compare protein abundance between samples, with **(B)** P-IGF1R and **(C)** T-IGF1R normalised to actin. The ratios between the normalised bands were then calculated as a measure of receptor activation **(D)**. Data is presented as means ± SEMs and representative of one experiment, with three replicates per treatment group. Statistical significance was determined using a one-way ANOVA with Turkey's multiple comparisons *post hoc* test.

### 3.3.4 FhHDM-1 does not directly enhance IGF1R kinase activity in β-cells

To complement the western blot analyses and further explore the activation of IGF1R by FhHDM-1 in β-cells, a bioassay to assess ligand-based activation of IGF1R kinase activity was employed. Briefly, the assay utilises enzyme fragment complementation technology in which the β-galactosidase (β-gal) enzyme is divided into two fragments. One fragment (Prolink; PK) is fused to the c-terminus of the IGF1R receptor, which is overexpressed in the HEK293 human cell line. The second fragment is fused to a phosphotyrosine SH2 domain containing protein (termed EA). When the IGF1R tyrosine kinase is activated by interaction with a ligand, the SH2 is recruited and binds to the c-terminus of the IGF1R receptor. This interaction forces complementation of PK and EA to form an active β-gal enzyme, the activity of which is measured using a chemiluminescent substrate. Thus, this assay was used to compare the activation of IGF1R by FhHDM-1, as compared to IGF-1.

As expected, the native ligand, IGF-1, potently activated IGF1R, and this provided the arbitrary maximal response from a half maximal effector concentration ($EC_{50}$) of 0.002μM. By comparison, FhHDM-1 showed a maximal response of 37% and an $EC_{50}$ of 2μM (Figure 3.7), suggesting that FhHDM-1 did not strongly induce activation of IGF1R. This outcome was not entirely unexpected given that FhHDM-1 did not induce phosphorylation of IGF1R at the major autophosphorylation sites required for kinase activity, namely Tyr1131, Ty1135 and Tyr1136. Instead, FhHDM-1 induced IGF1R phosphorylation at Tyr1316, which is located outside the kinase domain at the C-terminus of the receptor, and to which PI3K can be directly recruited.

In addition, these results suggested that FhHDM-1 was not acting as a specific agonist ligand for IGF1R. To further confirm this, the binding of a fluorescently tagged FhHDM-1 to IGF1R was examined using mouse fibroblast clones that overexpress (P6) or had little/no expression (R-) of IGF1R. Flow cytometry analysis of these cells following incubation with FhHDM-1 showed no difference in the mean fluorescence intensity. This data confirmed that FhHDM-1 did not interact with IGF1R to enhance its abundance or phosphorylation at Tyr1316 (Figure 3.8).

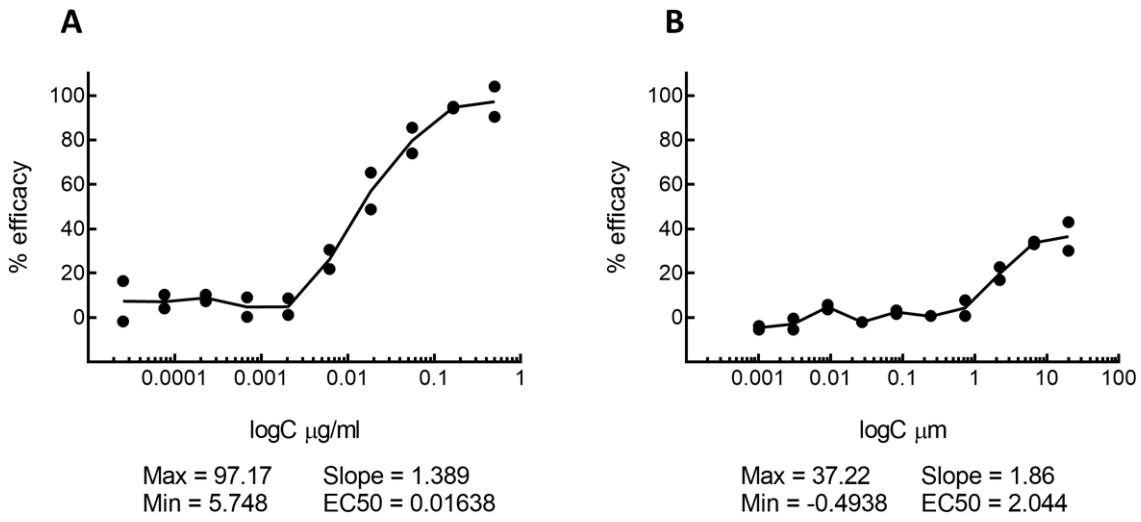



**Figure 3.7 FhHDM-1 did not directly enhance IGF1R kinase activity.** PathHunter HEK 293 IGF1R Bioassay cells ($1x10^4$) were incubated with serial dilutions of recombinant human **(A)** IGF-1 ligand or **(B)** FhHDM-1 for 16-18h prior to addition of bioassay detection reagent and reading of luminescence signal to generate dose curves. When converted to the same units as FhHDM-1, the $EC_{50}$ of IGF-1 is equivalent to 0.002μM. Data represents one independent experiment.

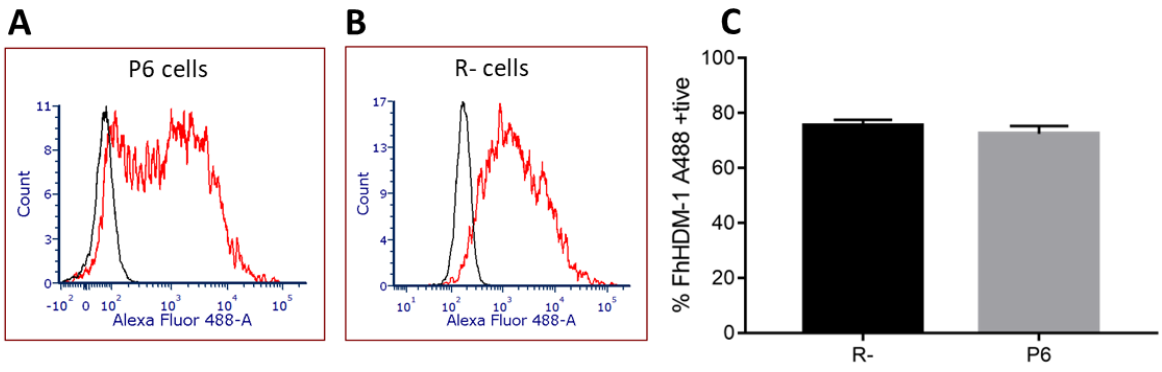



**Figure 3.8 FhHDM-1 did not bind directly to IGF1R.** Mouse fibroblast clones ($1x10^6$ cells) **(A)** overexpressing IGF1R (P6) or **(B)** having little/no expression of IGF1R (R-) were left unstained (depicted by black lines) or incubated with fluorescently labelled FhHDM-1 for 30mins at 4°C (depicted by red lines) prior to flow cytometric analyses. **(C)** The percentage of FhHDM-1 AlexaFluor488 (A488) positive cells for each clone were quantified and compared. Data is presented as means ± SEMs and representative of two independent experiments, each with three replicates per treatment group. Statistical significance was determined using an unpaired t-test.

### 3.3.5 FhHDM-1 promotes IGF-2 secretion within β-cells

The lack of interaction with the receptor and the absence of kinase activation reliant on a specific interaction with IGF1R, suggested that FhHDM-1 must be indirectly inducing phosphorylation at IGF1R Tyr1316, and enhancing total IGF1R expression in β-cells. As mentioned previously, indirect receptor activation can occur through the IGF1R autocrine loop, which is activated by both IGF-1 and IGF-2. Thus, the secretion of IGF-1 and IGF-2 by FhHDM-1 treated β-cells was measured via ELISA. In contrast to IGF-1 treated cells, FhHDM-1 did not enhance secretion of IGF-1, with levels observed similar to those of untreated cells at both 6h (Figure 3.9A) and 24h (Figure 3.9B) time points. However, it is important to note that the assay cannot differentiate between exogenous IGF-1 versus IGF-1 secreted by the β-cells. Accordingly, the increased IGF-1 levels detected in IGF-1 treated β-cells may be exogenous, which would indicate IGF-1 was not detected across all treatment groups. Nevertheless, FhHDM-1 significantly increased the secretion of IGF-2, as compared to both untreated and IGF-1 treated cells, at both 6h (Figure 3.9C) and 24h (Figure 3.9D). These data indicate that FhHDM-1 promotes IGF-2 secretion and IGF1R expression within the β-cells, suggestive of an IGF-2/IGF1R autocrine loop, consequently activating the PI3K/Akt pathway and enhancing β-cell viability.

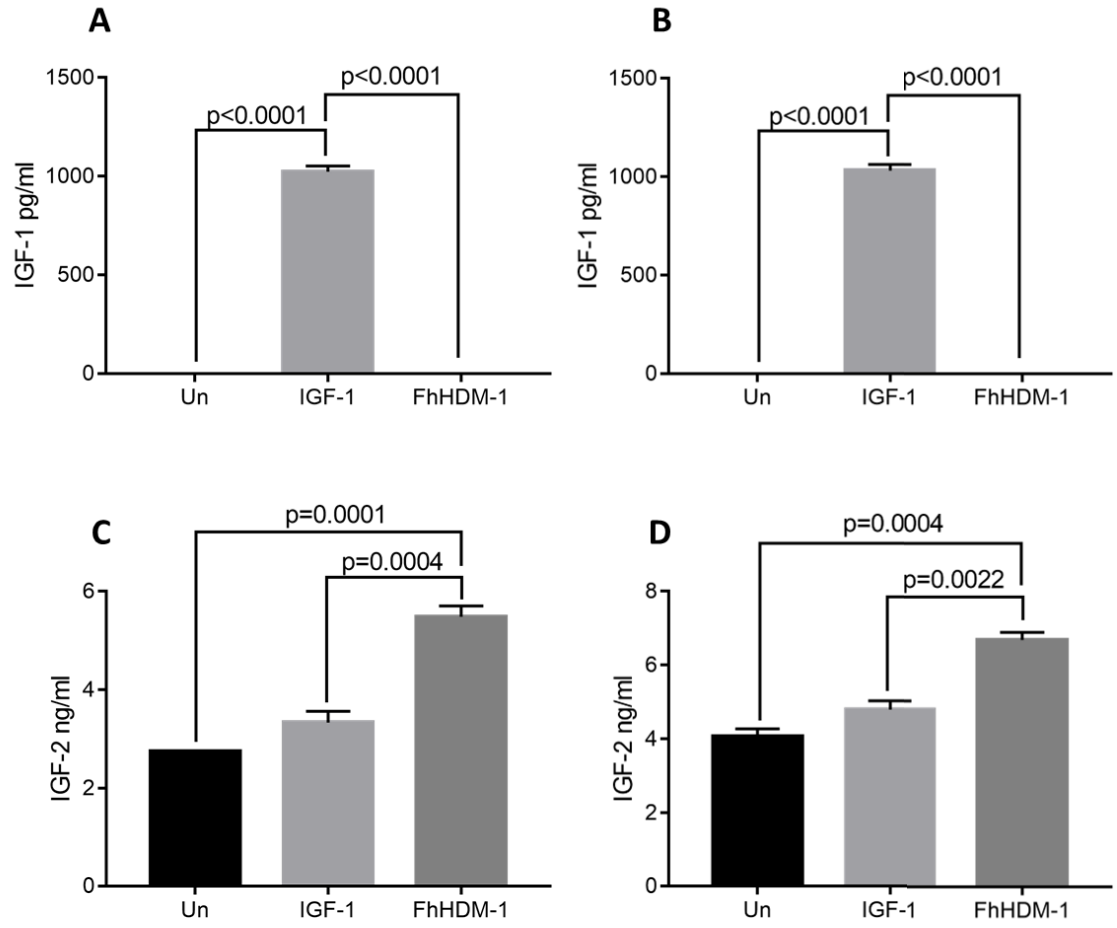



**Figure 3.9 FhHDM-1 treated β-cells did not secrete IGF-1.** NIT-1 β -cells ($2x10^6$ cells) were serum deprived for 18h, then left untreated (Un) or incubated with IGF-1 (1µg/ml or 0.13µM) or FhHDM-1 (10µM) for 6h or 24h. Following incubation, cell supernatants were assayed for IGF-1 (**A**:6h, **B**:24h) and IGF-2 (**C**:6h, **D**:24h) secretion. Data is presented as the means ± SEMs and representative of two independent experiments, each with three replicates per treatment group. Statistical significance was determined using a one-way ANOVA.

## 3.4 Discussion

The data presented in this chapter builds on the findings presented in Chapter 2 and supports a central role for IGF1R in the enhanced viability of β-cells mediated by FhHDM-1. More specifically, the data revealed that FhHDM-1 increased both the abundance and the phosphorylation of IGF1R. Surprisingly, this phosphorylation occurred at a Tyr residue that is outside of the predominant kinase activation domain of the receptor, but at a site specifically associated with the recruitment of PI3K. These downstream effects were not a consequence of a specific interaction between FhHDM-1 and IGF1R but correlated with a significant increase in the production of IGF2. Together, these data suggest that FhHDM-1 enhances the activity of an IGF-2/IGF1R autocrine loop within β-cells, which drives the phosphorylation at Tyr1316, leading to the direct recruitment of PI3K to IGF1R, and subsequent activation of PI3K/Akt signalling. The culmination of these events being an enhancement of β-cell function and survival.

The increased abundance of IGF1R in FhHDM-1 treated β-cells was maintained up to 24h after treatment, in contrast to IGF-1 treated β-cells, which exhibited reduced levels IGF1R akin to those observed for untreated cells. The prolonged expression of IGF1R induced by FhHDM-1 may be explained, in part, by the biological activity of FhHDM-1. It has been shown that after FhHDM-1 is internalised by cells, it localises to the endolysosome where it inhibits vacuolar(v)ATPase activity, and thus prevents the acidification of the lysosome (122). Generally, following ligand-mediated activation and phosphorylation of IGF1R, receptor expression levels are downregulated via receptor internalisation, thereby enabling cells to return to basal state. This can occur through clathrin- or caveolin-mediated internalisation wherein the receptor progresses from early endosomes to late endosomes, during which the fate of the receptor is determined, whether that be recycling or degradation. If it is the latter, the receptor progresses to the hydrolase-containing lysosomal compartments that are highly acidic (256). Given its ability to prevent lysosomal acidification, the presence of FhHDM-1 would prevent the degradation of IGF1R. Furthermore, internalisation of a receptor does not necessarily terminate its signalling capacity immediately. Indeed, signalling through several receptors, such as epidermal growth factor receptor (EGFR) and colony-stimulating factor-1 receptor (CSF1R), can be maintained in endosomal compartments that determine the longevity of signalling (278, 279). Following endocytosis, IGF1R has also been shown to traffic and signal through various subcellular locations, such as the nucleus, Golgi and mitochondria, though endosomal compartments are not well-characterised (280).

Furthermore, redirection of IGF1R to the nucleus can influence the transcription of genes, by forming transcription complexes, modulating the activity of chromatin remodelling proteins, and contributing to DNA damage tolerance mechanisms (281). Thus, the effects FhHDM-1 could allow preservation of IGF1R and maintain signalling even after internalisation, thereby driving the IGF1R autocrine loop. To determine the mechanism by which this is achieved, the location of IGF1R can be tracked following FhHDM-1 treatment. This can be achieved via fluorescent labelling of IGF1R in FhHDM-1 treated β-cells or using a cell line with labelled IGF1R.

In the classical paradigm of IGF1R activation, interaction with a ligand induces phosphorylation at three tyrosine residues 1131, 1135 and 1136 within the kinase domain, a central process for receptor autophosphorylation and subsequent downstream signalling. However, phosphorylation has also been reported to occur outside the kinase domain, such as on docking sites within the C-terminus, which contains residues that regulate IGF1R function (Figure 3.1A). Of these, Tyr1316 has been established as a direct binding site of PI3K. Indeed, prolonged (>9h) activation of IGF1R by IGF-1 induced recruitment of PI3K to the phosphorylated Tyr1316 site (282). Given the parallel with the sustained activation of IGF1R by FhHDM-1 (up to 24h), this could represent a mechanism by which FhHDM-1 activates PI3K/Akt signalling, via direct recruitment of PI3K to IGF1R following Tyr1316 phosphorylation. To confirm this mechanism, future studies could include FhHDM-1 treatment of cells expressing mutant IGF1R with inactivated Tyr1316 to determine if phosphorylation of PI3K is maintained. Moreover, western blot analyses of the membrane proteins isolated from FhHDM-1 treated β-cells would reveal if PI3K is recruited to the receptor. On the other hand, it is important to note that the phosphorylation of IGF1R was only examined within longer time points (≥6h). To definitively confirm the lack of classic IGF1R phosphorylation at Tyr1131, 1135 and 1136, which has been shown to occur rapidly (283), FhHDM-1 treated β-cells need to be analysed at earlier time points, such as at 5, 10 and 30mins.

While the IGF1R autocrine loop can be activated by both IGF-1 and IGF-2 (160), it was found that FhHDM-1 only enhanced the secretion of IGF-2 in NIT-1 β-cells. This is not necessarily unexpected given that primary islets, non-diabetogenic MIN6 β-cells, and primary β-cells have all been shown to express extremely low levels of IGF-1 (284). Conversely, IGF-2 is highly expressed in primary islets and in purified β-cells, and is secreted in response to glucose, which corroborates the detection of IGF-2 within insulin granules (285, 286). The

anti-apoptotic effects associated with induction of autocrine IGF-2 is supported by previous studies showing the critical role of IGF-2 in the regulation β-cell function early in life. Indeed, impaired β-cell function in adult rodents has been linked to decreased IGF-2 expression levels within the pancreas during embryonic development (287). Moreover, the neonatal phase of pancreatic remodelling in which waves of β-cell apoptosis occur coincides with reduced expression levels of IGF-2 within the islets (268). Conversely, apoptosis can be prevented by increased circulating levels of IGF-2, as shown in studies in which transgenic overexpression of IGF-2 in peripheral tissues was used (288). In adult mice, transgenic expression of IGF-2 within β-cells results in increased β-cell mass (289). Further, β-cell injury triggers the re-expression of IGF-2, which is crucial for subsequent β-cell regeneration (290). Thus, it is evident that autocrine IGF-2 is an important regulator of β-cell mass in both foetal and adult life. Furthermore, IGF dysregulation has been shown to precede and follow T1D development in humans (291). Unsurprisingly, both IGFs have also demonstrated the ability to ameliorate disease in various models of diabetes (259-262, 265, 266).

This activity of FhHDM-1 is comparable to the outcome of treatment of β-cells with GLP-1. While there is little similarity in sequence between FhHDM-1 and GLP-1, both form alpha helices, which may correlate with the activation of the IGF-2/IGF1R/PI3K signalling axis. Indeed, GLP-1 exerted anti-apoptotic effects by activation of the IGF-2/IGF1R autocrine loop and subsequent PI3K/Akt signalling (272). Protection of primary murine β-cells from pro-inflammatory cytokine induced apoptosis was associated with stimulation of IGF1R expression, with peak expression observed at 18h, and subsequent Akt phosphorylation, which was dependent on active secretion of IGF-2 as protection was abolished by reduction in levels of IGF1R or IGF-2 expression or secretion. Although it is evident that FhHDM-1 promotes secretion of IGF-2, a demonstration that this subsequently induces the autocrine loop will need to be validated by blocking IGF-2 expression and/or neutralising secreted IGF-2 to determine if the beneficial effects of FhHDM-1 are abolished.

The beneficial effects of FhHDM-1 on β-cell viability were impaired in the presence of the selective IGF1R inhibitor, linsitinib. However, it is important to note that linsitinib also inhibits the insulin receptor (IR) due to the high homology between IR and IGF1R, albeit linsitinib inhibits the former with slightly reduced potency; with the half-maximal inhibitory concentration of linsitinib for IR at 75nM, as compared to IGF1R at 35nM (292). The anti-apoptotic effects induced by GLP-1 was inhibited by knocking down IGF1R but not the insulin IR, thus identifying IGF1R as the primary receptor upstream of phosphorylated Akt.

While the data presented here supports a similar pathway of activation induced by FhHDM-1, it will be important to confirm IGF1R as the primary receptor. This can be achieved in future experiments by using IR deficient cells to determine if the effects of FhHDM-1 are maintained, including the activation of PI3K/Akt and enhancement of β-cell survival. Additionally, the requirement for the alpha helical structure in mediating the peptide's biological effects could be tested by using truncated/mutated versions of FhHDM-1 in which the alpha helix is disrupted.

As aforementioned, GLP-1 analogues have been tested and used as therapeutics for both T1D and T2D. However, they lack stability *in vivo* due to degradation by dipeptidyl peptidase-4-enzyme (DPP-4), giving them short half-lives of ~2min (275). FhHDM-1 represents a preferable peptide as it is highly stable and not susceptible to protease cleavage (151). Furthermore, due to the increase in cell proliferation following treatment with GLP-1 and GLP-1 analogues there have been concerns raised surrounding their carcinogenic potential. This risk is not a concern for the application of FhHDM-1 as a therapeutic peptide as it has been shown (Chapter 2) to promote β-cell viability without stimulating proliferation, putatively by activating the PI3K/Akt pathway downstream of IGF1R, but not ERK (Figure 3.1). This possibility of biased agonists is supported by observations in studies using LL37, which preferentially activates ERK, but not PI3K/Akt, signalling (271). This hypothesis could be tested by investigating the phosphorylation of proteins within the ERK pathway, in FhHDM-1 treated β-cells.

In summary, this chapter indicates that FhHDM-1 promotes the secretion of IGF-2 within β-cells, which, in turn, enhances/sustains expression and activation of IGF1R. Consequently, PI3K is recruited to IGF1R at Tyr1316, leading to activation of PI3K/Akt signalling (Figure 3.10A). As detailed above, further studies are required to address some gaps within this proposed mechanism, and to clarify the putative mechanisms by which FhHDM-1 promotes IGF1R expression (Figure 3.10 B-C). Nonetheless, the data presented here validates a central role for the activation of IGF1R signalling in mediating the beneficial effect of FhHDM-1 to beta-cells and identifies IGF2 as the likely initiating factor.

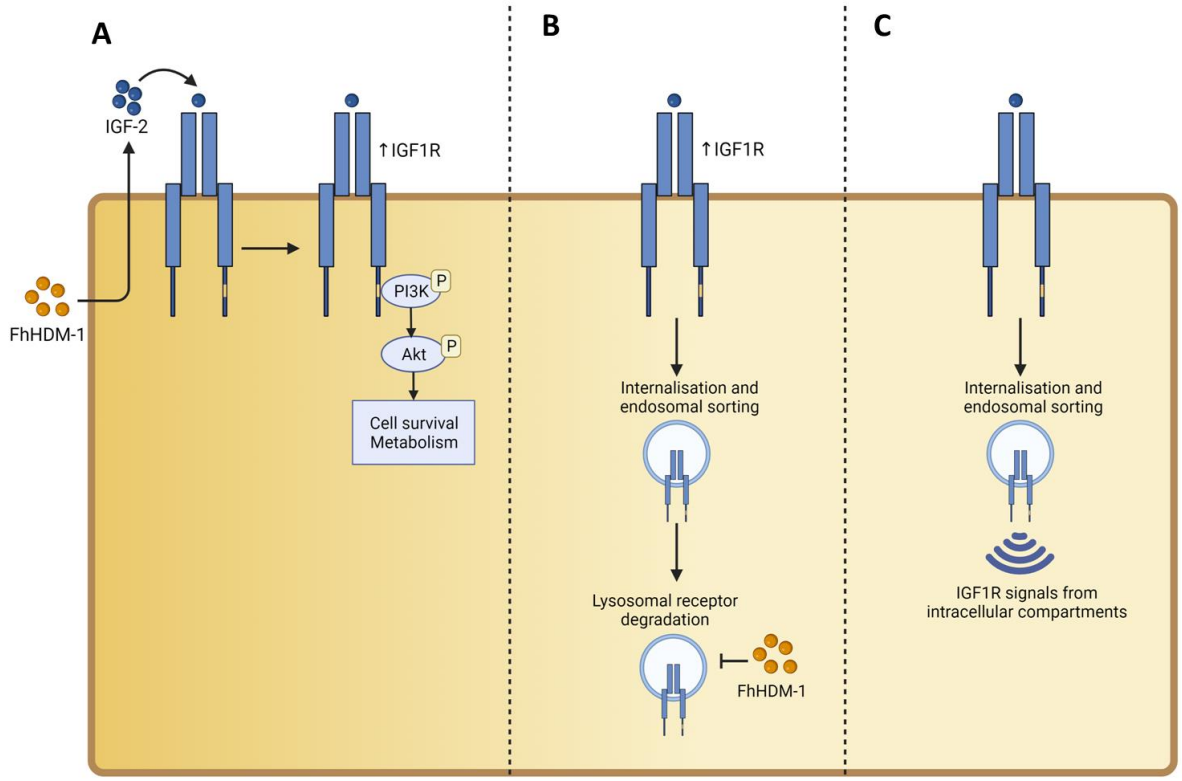



**Figure 3.10 (A) Confirmed mechanisms by which FhHDM-1 affects IGF1R signalling.** FhHDM-1 enhances the secretion of insulin growth factor-2 (IGF-2) within the β-cells. Consequently, IGF-2 binds to insulin growth factor receptor (IGF1R) and activates the receptor, leading to the recruitment of PI3K to IGF1R at Tyr1316, thereby causing phosphorylation/activation of phosphatidylinositol 3-kinase (PI3K)/Akt and its downstream signalling components associated with cell survival and metabolism. Furthermore, the binding of FhHDM-1 induced IGF-2 to IGF1R results in enhancement and/or maintenance of IGF1R expression. **(B-C) Putative mechanisms by which FhHDM-1 promotes IGF1R expression. (B)** FhHDM-1 prevents lysosomal acidification, which in turn can inhibit IGF1R degradation following receptor internalisation and endosomal sorting. **(C)** Despite internalisation, IGF1R may continue to signal from various intracellular compartments. Created with BioRender.com

# Chapter 4: FhHDM-1 induces changes in β-cell miRNA expression associated with positive effects on β-cell biology via PI3K/Akt signalling

## 4.1 Introductory Statement

The analysis of differentially expressed miRNAs and their gene targets in β-cells treated with pro-inflammatory cytokines with and without FhHDM-1 (as described in sections 4.2, 4.3, 4.4.3, 4.4.4, 4.5) has been extracted from this chapter, and is under review as a research paper at the Journal of Molecular Endocrinology:

Camaya I, O'Brien B, Donnelly S. The parasite-derived peptide FhHDM-1 induces the differential expression of MiRNAs to regulate gene networks that mediate β-cell survival against pro-inflammatory cytokine challenge.

### Author Contributions

IC designed, performed, analysed, and interpreted the experimental work throughout. She also drafted the initial manuscript and was actively involved in the editing process. BOB and SD provided conceptual advice and revised the manuscript.

Inah Camaya (PhD graduate student) Production Note: Signature removed prior to publication.

Bronwyn O'Brien Production Note: Signature removed prior to publication.

Sheila Donnelly Production Note: Signature removed prior to publication.

## 4.2 Introduction

The data presented thus far (Chapters 2 and 3) indicated that FhHDM-1 induced the activation of the PI3K/Akt pathway and enhanced the expression levels (mRNA and protein) of IGF1R. While Chapter 3 suggests that an increase in IGF-2 may be mediating this effect, the underlying mechanism has yet to be determined. Given the emerging role of miRNA in the activation and regulation of the PI3K/Akt pathway (293), this was next investigated.

MicroRNAs are short non-coding RNAs that regulate gene expression at the posttranscriptional level, by binding to specific target messenger RNA (mRNA). Subsequently, the target mRNA is silenced through repression of translation or induction of mRNA degradation. The central role of miRNAs in the regulation of cellular responses to both physiological and disease conditions has been well established (294), an in more recent years, a characterization of the biological function of miRNAs in the context of the pancreatic β-cells is emerging. Genetic loss- and gain-of-function studies have revealed an extensive network of miRNAs that are evolutionary conserved and highly expressed, and function as regulators of stress signals by preserving β-cell function, and promoting proliferation, and survival (295, 296).

Some of the most highly enriched miRNAs within the pancreas include miR-375, miR-200 and miR-7, all of which play crucial roles in β-cell biology. Specifically, miR-375 is involved in the regulation of β-cell proliferation and insulin secretion, and its inactivation leads to overt diabetes due to decreased β-cell mass (297). Conversely, an abundance of miR-200 is correlated with β-cell dysfunction and apoptosis, and its deletion has a protective effect against β-cell injury, such as that induced by STZ (298). The miR-7 family displays a more dynamic regulation in response to metabolic stress, suggesting involvement in the functional adaptation of β-cells during diabetes pathogenesis, especially during compensatory diabetes (299). These observations combined with additional similar outcomes for several other miRNAs (293), provides solid evidence that miRNAs play critical roles in the functional responses of β-cells.

This regulation of β-cell biology by miRNAs creates an opportunity to protect β-cells during pathological conditions of T1D and T2D, and consequently, several miRNAs have been identified as potential therapeutics to preserve β-cell mass and promote β-cell function. Of relevance to this research project, some of these miRNAs directly or indirectly target the PI3K/Akt pathway (described in Chapter 2 and (293)). Notable miRNAs include miR-132,

the increased expression of which is associated with increased β-cell proliferation and survival, via inhibition of PTEN and consequent upregulation of PI3K/Akt signaling (300). Furthermore, the presence of miR-17-92 has been associated with β-cell survival and repair, as its deletion within β-cells promoted impaired glucose tolerance and diabetes development induced by STZ, which were attributable to reduced β-cell mass and increased apoptosis. These deleterious effects were associated with increased PTEN expression levels, which suppressed PI3K/Akt signaling (301).

Conversely, inhibition of specific miRNAs can promote PI3K/Akt signaling, and thus β-cell survival. For instance, in response pro-inflammatory cytokines, there is increased β-cell apoptosis and dysfunction associated with increased miR-18 that inhibits neuron navigator 1 (NAV1), a constituent of the PI3K/Akt pathway, thereby downregulating phosphorylated Akt and PI3K expression levels (302). Likewise, overexpression of miR-139-5p suppresses protein interacting with C-kinase 1 (PICK1) expression levels, which normally confers β-cell protection via PI3K/Akt signaling (303). Although their corresponding gene targets have not been investigated, inhibition of miR-122 (304) or let-7 (305) induces PI3K/Akt signaling within β-cells, which mediates protection from STZ-induced destruction. Therefore, upregulation of PI3K/Akt signaling through crosstalk with various miRNA can exert positive effects on β-cell function and survival. Given the promotion of PI3K/Akt signaling by FhHDM-1 at the transcriptome level, it was hypothesized that this effect may be regulated by modulation of the β-cell miRNome.

## 4.3 Methods

### 4.3.1 Sample preparation and RNA extraction

NIT-1 cells ($2x10^6$) were allowed to adhere overnight (37°C/5% $CO_2$) before being left untreated or treated with FhHDM-1 (10µm) for a further 24h. In addition to basal conditions, another set of cells were pre-treated with vehicle control or FhHDM-1(10µm) for 1h prior to exposure to pro-inflammatory cytokines (10ng/ml IL1β, 50ng/ml TNF, 50ng/ml IFNγ; BD Pharmingen) for a further 24h. Cells were then washed twice with sterile PBS and total RNA was isolated using the Isolate II RNA mini kit (Bioline, BIO52073) according to the manufacturer's instructions.

### 4.3.2 Sequencing

Sequencing library preparation of miRNA from samples was performed by Macrogen (Seoul) using a TrueSeq Small RNA Library Sample Prep Kit and sequenced using Illumina NextSeq 500. The extracted RNA from the three biological replicates were pooled to obtain enough for sequencing. From fastq sequencing files, adaptor sequences were excised and filtered for low quality (<20 phred score) sequences and low length sequences (<18 nt) using bioinformatic tool CutAdapt (v3.4). Sequencing of the transcriptome of NIT-1 cells treated with pro-inflammatory cytokines and FhHDM-1 was performed on 18 paired-ends samples by Macrogen (Seoul) using a TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, part #15031047) and a NovaSeq 6000 S4 reagent kit (Illumina) to create an mRNA library. To ensure the validity of the results, the quality control of the sequenced raw reads was analysed. Then low-quality reads, adaptor sequence, contaminant DNA sequences, and PCR duplicates were removed to minimise biases in the analysis. After that, adapter sequences were removed by Trimmomatic (Version 0.38), and the cleaned reads were mapped to the mouse genome (mm10) using StringTie (Version 2.1.3b).

### 4.3.3 Expression Analysis

The raw read counts of miRNAs for each sample were analysed for differential expression using the DESeq2 package (v1.38.3) in Rstudio (Rstudio Team, v1.3.1073) to identify differentially expressed miRNAs in (i) FhHDM-1 treated cells versus untreated (Un) cells, (ii) FhHDM-1 treated cells exposed to pro-inflammatory cytokines (FhHDM-1 CM) versus

cells exposed to cytokines only (CM), and (iii) Un cells versus CM cells. The miRNAs with a Hochberg adjusted p-value <0.1 were considered as differentially expressed (DE) miRNAs. All DE miRNAs were plotted using RPM values on a heatmap, and using log adjusted p-value versus log fold change on volcano plots. A log fold change threshold of ±1 was then applied to the resulting list of DE miRNAs for consideration of potential role in diabetes or β-cell biology. The DE analysis of the transcriptome was conducted as previously described (Chapter 2, Section 2.4.9).

### 4.3.4 Gene target prediction and transcriptome correlation

To determine the putative gene targets of all DE miRNAs (without log fold change cut-offs), the following online target prediction tools were used: miRDB, DIANA and TargetScan. For miRDB (https://mirdb.org/), only genes with a target score of >80 were included, while miRNAs with more than 5000 predicted targets were excluded. For DIANA (http://diana.imis.athena-innovation.gr/DianaTools/index.php), the microT-CDS v5.0 database was used with the default threshold of 0.7. Lastly, a custom prediction with a murine background using the TargetScan database (http://www.targetscan.org) was completed. Only genes that were identified by all three tools were considered for further analysis. The combination of multiple predictive tools is considered one of the most effective approaches in providing a subset of high-quality predictions with minimal false positives and provides the greatest effect in supporting validation of gene targets (306). The resulting list of common predicted gene targets was then analysed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov/home.jsp) functional annotation analysis tool to determine the enrichment of Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways. Where there were insufficient genes to attribute to pathways, gene ontology enrichment for molecular function and biological process was completed using Panther DB (http://www.pantherdb.org/). For the final analysis, the common predicted gene targets were correlated to previously identified DE genes from the same treatment groups within the NIT-1 β-cell transcriptome. The transcriptome-matched genes were similarly mapped to KEGG pathways. Cytoscape 3.9.1 was used to visualise miRNA-gene target interactions.

## 4.4 Results

### 4.4.1 FhHDM-1 treatment of β-cells induced changes in the expression of miRNAs expression

To analyse the alterations in miRNA expression that were associated with FhHDM-1 activated PI3K/Akt signalling, the same treatment conditions were used that previously led to an increase in β-cell viability (Chapter 2). Thus, NIT-1 β-cells were left untreated or treated with FhHDM-1 (10μm) for 24h prior to in-depth small RNA sequencing. This analysis identified 11 miRNAs that were differentially expressed (DE) as a result of FhHDM-1 treatment, and of these, 5 were upregulated and 6 were downregulated. Further, only 4 of the DE miRNAs displayed a log fold change in expression >1, as compared to the untreated cells, and all of these were reduced in their expression. Consistent with their change in expression, 9 of the DE miRNAs have previously been reported to play important roles role in diabetes and/or β-cell biology (Table 4.1). Specifically, miR-5100 (log fold change [FC] of 0.81, p=1.18E-04) and miR-7033-5p (log FC of -2.11, p=1.86E-26), have reduced expression levels in mice exposed to a high fat diet (HFD) and in HFD mice given anti-diabetic treatment, respectively. This finding corroborates the hypothesis that FhHDM-1 may be modulating the expression levels of genes via the regulation of miRNA expression to ultimately prevent diabetes. Further, this potentially occurs through the activation of pathways that enhance β-cell viability. While this is an attractive premise, it is difficult to make a definitive conclusion because published studies of the impact of β-cell derived miRNAs are limited. Additionally, several other miRNAs exhibit an opposing effect, which is testament to the complexities of miRNA expression and regulation of gene targets. Therefore, further analyses to better determine the biological processes mediated by the DE miRNAs induced by FhHDM-1 were conducted.

**Table 4.1. Differentially expressed miRNAs in FhHDM-1 treated β-cells (24h) compared to untreated (Un) controls**

| miRNA Name | Log Fold Change (FhHDM-1 vs Un) | P-value | Known role in diabetes or β-cell biology | Reference |
|---|---|---|---|---|
| mmu-miR-1931 | 0.91 | 1.07E-03 | Dysregulated in mitochondria of genetic T2D mouse model | (307) |
| mmu-miR-5100 | 0.81 | 1.18E-04 | Downregulated in HFD mice, upregulated in MIN6 β-cell exosomes and parental cells, suggesting putative association with differentiation of cells into β-like cells | (308, 309) |
| mmu-miR-3968 | 0.73 | 2.87E-04 | Upregulated by maternal pregestational diabetes in embryonic heart, upregulated in MIN6 β-cell exosomes and parental cells (as above) | (309, 310) |
| mmu-miR-483-3p | 0.62 | 2.08E-04 | Upregulated in MIN6 β-cell exosomes and parental cells (as above) | (309) |
| mmu-miR-1249-3p | 0.60 | 1.41E-03 | Upregulated in pancreatic tissue of STZ-induced diabetic mice and human homologue is increased in plasma of pre-diabetic individuals | (311, 312) |
| mmu-miR-30d-5p | -0.36 | 8.32E-04 | Upregulated in STZ and pro-inflammatory cytokine treated mouse islets | (313) |
| mmu-miR-3470a | -0.70 | 7.35E-04 | Upregulated in MIN6 β-cell exosomes and parental cells (as above) | (309) |
| mmu-miR-677-5p | -1.42 | 5.59E-04 | Not determined | |
| mmu-miR-7033-5p | -2.11 | 1.86E-26 | Downregulated by anti-diabetic GSF in HFD mice | (314) |
| mmu-miR-7037-5p | -3.81 | 1.41E-04 | Not determined | |
| mmu-miR-7068-5p | -3.41 | 9.60E-04 | Upregulated by anti-diabetic GSF in HFD mice | (314) |

GSF, wine grape seed flour; HFD, high fat diet; STZ, streptozotocin

### 4.4.2 The predicted gene targets for differentially expressed miRNAs in FhHDM-1 treated β-cells were associated with positive modulation of β-cell activity and PI3K/Akt signaling

To elucidate the functional roles of the FhHDM-1 induced DE miRNAs in regulating the biological activity of β-cells, gene targets for each miRNA were predicted from the 3'UTR regions of genes within the murine genome using the software tools miRDB, Diana tools and TargetScan (Supplementary Table 4.1). To obtain high-quality predictions, limit false positives, and identify the most authentic miRNA:mRNA relationships, only those gene targets that were identified by *all* three tools were selected for further analysis. With these parameters, a total of 847 predicted gene targets were identified in FhHDM-1 treated β-cells. Of these, 63 were attributed to the five upregulated miRNA sequences, and 784 were attributed to the six downregulated miRNA sequences (Supplementary Table 4.2). Of the five upregulated miRNAs, two (miR-5100 and miR-1249-3p) had no predicted gene targets. Of the remaining three miRNAs, 30 gene targets were attributed to miR-1931, 15 to miR-3968, and 18 to miR-483-3p (Figure 4.1A). For the downregulated miRNA sequences, approximately 60% of the total gene targets (482 out of 784) were attributed to miR-30d-5p, followed by 99 to miR-7033-5p, 88 to miR-3470a, 87 to miR-677-5p, 18 to miR-3037-5p, and 10 to miR-7068-5p (Figure 4.1B).

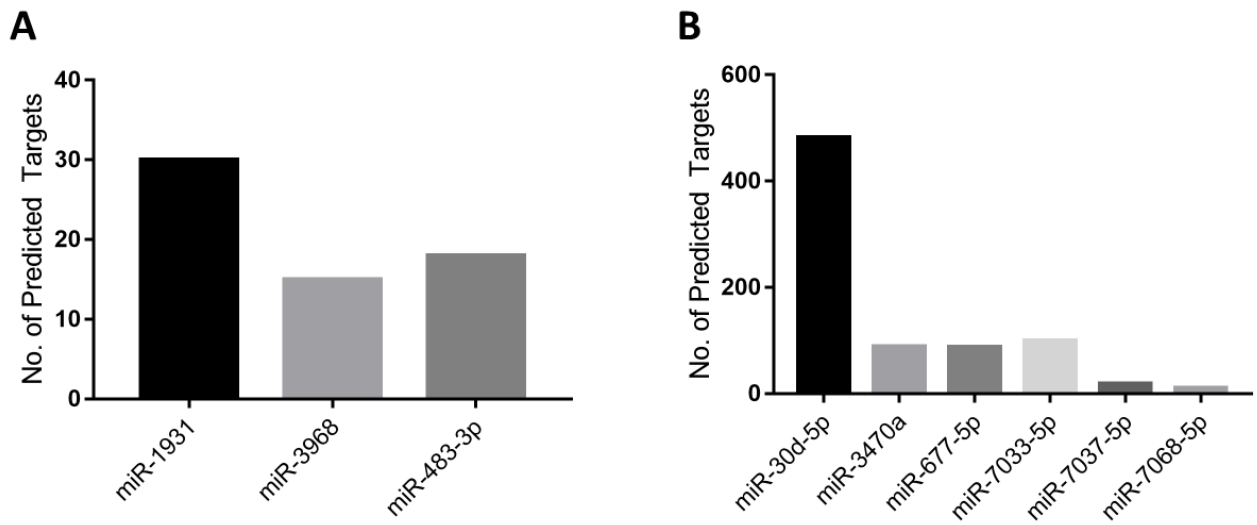



**Figure 4.1 The number predicted gene targets for each of the (A) upregulated and (B) downregulated miRNAs in FhHDM-1 treated β-cells.** Within the 63 upregulated miRNAs, 30 were attributed to miR-1931, 15 to miR-3968 and 18 to miR-483-3p. Within the 784 downregulated miRNAs, 482 were attributed to miR-30d-5p, 99 to miR-7033-5p, 88 to miR-3470a, 87 to miR-677-5p, 18 to miR-3037-5p and 10 to miR-7068-5p.

These predicted gene targets were then mapped to biological pathways using KEGG pathway analysis. Assuming an inverse relationship between the expression of a given miRNA and its gene target(s), it was presumed that the gene targets for the miRNA sequences that were reduced in expression levels by FhHDM-1 treatment of β-cells would exhibit increased expression levels, and this would contribute to the increased survival of β-cells mediated by FhHDM-1, and *vice versa*. While there were an insufficient number of predicted gene targets for the upregulated miRNAs to assign to pathways, an analysis of the molecular functions revealed a predominance of binding and catalytic activities (Figure 4.2, Supplementary Table 4.3A). For these gene targets, the top three biological processes were identified as cellular process, metabolic process, and biological regulation. Within these gene ontologies, a substantial number of genes were shared (including Sos1, Sema4a, Sema6a, Spam1, Fmr1, Dsel, Nbr1, Itga1, Tle1, Cnot61, Phf20l1, and Ahcyl1). This is likely due to the dependency of software algorithms learning on published literature, leaving genes that have not been extensively studied unaccounted for.

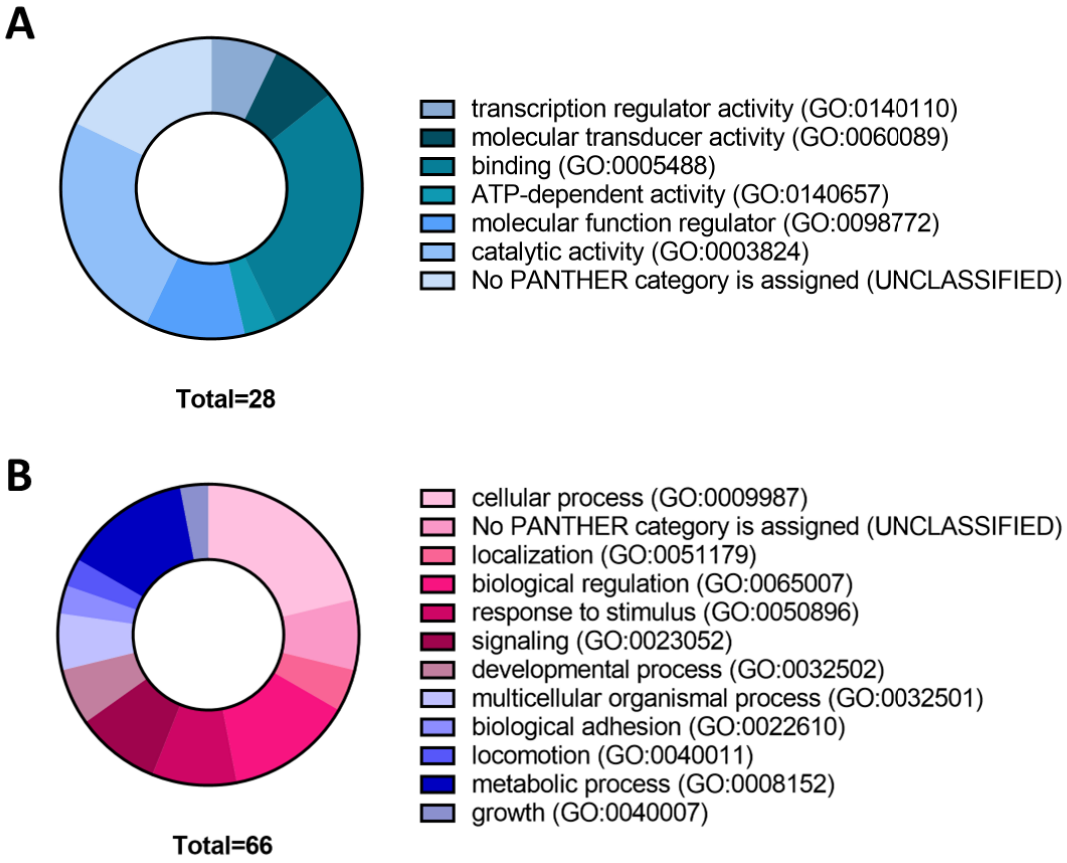



**Figure 4.2 Molecular function (A) and biological processes (B) associated with predicted gene targets of upregulated miRNA in FhHDM-1 treated β-cells.** Gene ontology enrichment for molecular function and biological process of the predicted gene targets of miRNAs that were upregulated in FhHDM-1 treated β-cells was completed using Panther DB (http://www.pantherdb.org/), with the percentage of genes in each category represented by the colour-coded donut charts. The genes within each category are listed in Supplementary Table 4.3A and the total number of genes is noted at the bottom of each chart.

The genes identified as targets of the downregulated miRNA sequences were predictively mapped to 43 biological pathways (Supplementary Table 4.3B). Of these, ten were intricately linked to the biological activity of β-cells (Figure 4.3), the most notable being the PI3K/Akt pathway that was previously shown to be activated by FhHDM-1 to positively modulate β-cell function/survival (Chapter 2). Further exploration of the specific predicted genes targets identified within the PI3K/Akt pathway (Figure 4.4), revealed several genes of interest, including IGF and PI3K, amongst others that ultimately lead to pro-survival and anti-apoptotic functional outcomes. These genes were regulated by miR-30d-5p, miR-3470a, miR-677-5p and miR-7033-5p, which were predicted to govern the expression of 15, 4, 3 and 1 gene(s), respectively (Table 4.2).

The genes within PI3K/Akt signalling were also shared with other pro-survival pathways such as MAPK, FoxO, Wnt, and mTOR signalling pathway. Therefore, it is perhaps not surprising that "pathways in cancer" was found to be the most highly attributed pathway, given its association with cell survival and proliferation. However, importantly FhHDM-1 has been shown to enhance β-cell viability and prevent β-cell death without inducing proliferation, and this is the mechanism by which T1D is prevented (Chapter 2). This bioinformatic approach also identified other biological pathways that are clearly crucial for β-cell function, including insulin signalling, suggesting additional positive effects that may be mediated by FhHDM-1 treatment.

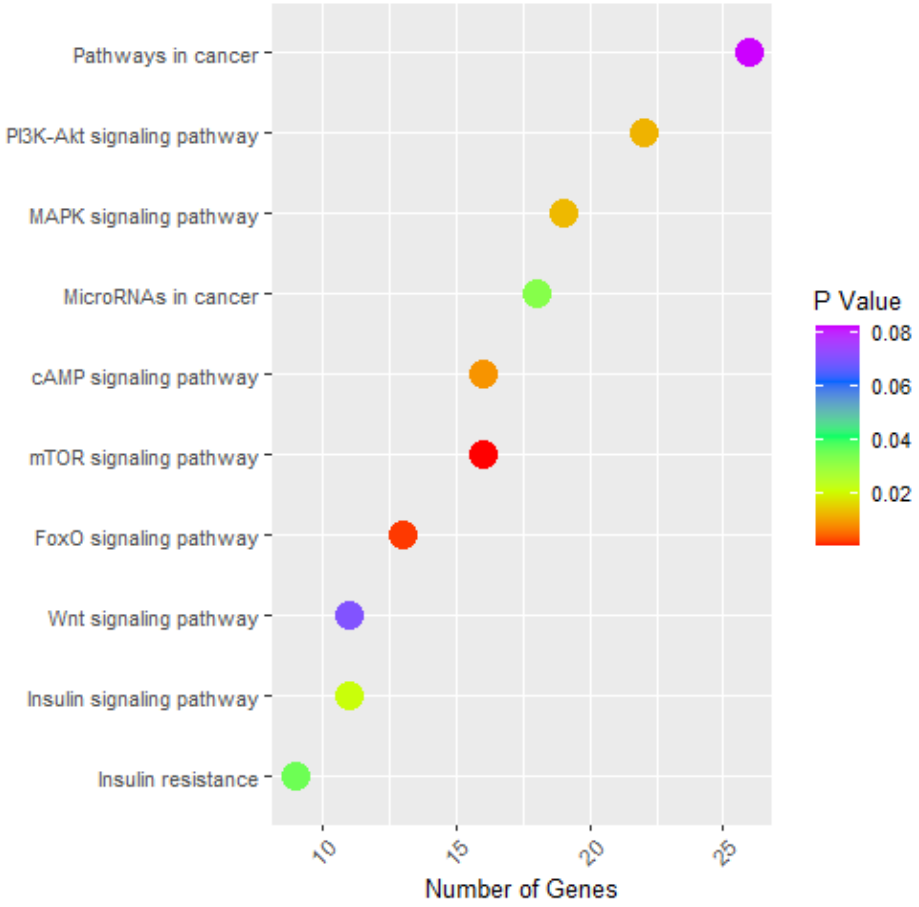



**Figure 4.3 KEGG pathway analysis of predicted gene targets from downregulated miRNAs in FhHDM-1 treated β-cells show several are associated with β-cell biology.** Dot plot showing the top 10 pathways most relevant to β-cells from the predicted gene targets of downregulated miRNAs in FhHDM-1 treated β-cells. The genes within each pathway are found in Supplementary Table 4.3B. Dot colour indicates p-value and dot placement on the x-axis indicates the number of genes in that pathway.

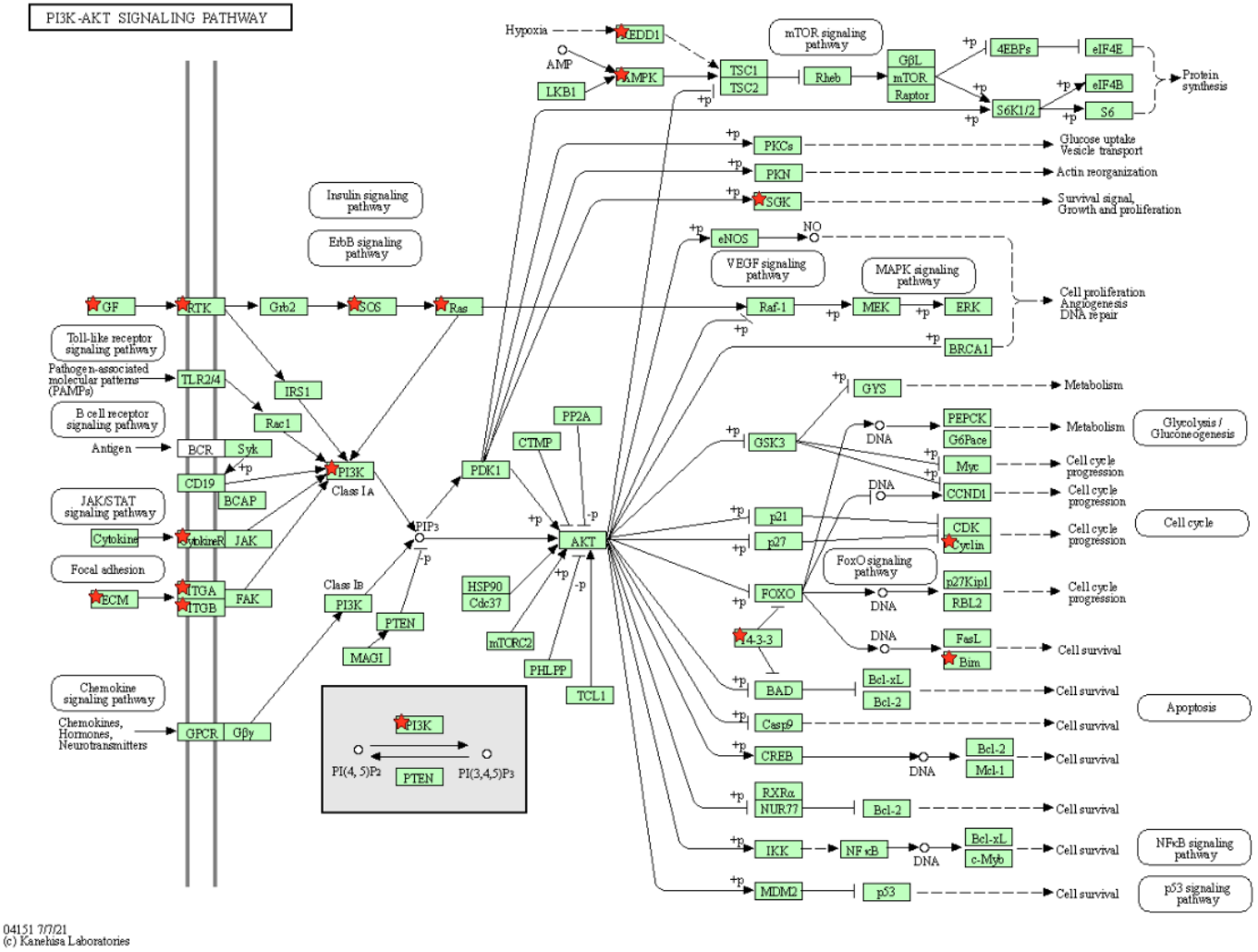



**Figure 4.4 The (upregulated) gene targets of downregulated miRNA impacted within the PI3K/Akt pathway as shown through DAVID (v6.8) functional annotation analysis tool.** Expression changes observed within FhHDM-1 treated β-cells compared to untreated controls. Activated genes are indicated by the red stars.

**Table 4.2 Upregulated genes with their corresponding regulatory miRNA within the PI3K/Akt pathway.**

| miRNA Name | Gene Symbol | Gene Name |
|---|---|---|
| mmu-miR-30d-5p | Bcl2l11 | BCL2-like 11 (apoptosis facilitator) |
| mmu-miR-30d-5p | Ccne2 | cyclin E2 |
| mmu-miR-30d-5p | Col9a3 | collagen, type IX, alpha 3 |
| mmu-miR-30d-5p | Ddit4 | DNA-damage-inducible transcript 4 |
| mmu-miR-30d-5p | Efna3 | ephrin A3 |
| mmu-miR-30d-5p | Itga8 | integrin alpha 8 |
| mmu-miR-30d-5p | Itgb3 | integrin beta 3 |
| mmu-miR-677-5p | Itgb6 | integrin beta 6 |
| mmu-miR-30d-5p | Kras | Kirsten rat sarcoma viral oncogene homolog |
| mmu-miR-677-5p | Lama4 | laminin, alpha 4 |
| mmu-miR-3470a | Lamc2 | laminin, gamma 2 |
| mmu-miR-3470a | Ntrk2 | neurotrophic tyrosine kinase, receptor, type 2 |
| mmu-miR-7033-5p | Pgf | placental growth factor |
| mmu-miR-30d-5p | Pik3cd | phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta |
| mmu-miR-677-5p | Prkaa1 | protein kinase, AMP-activated, alpha 1 catalytic subunit |
| mmu-miR-30d-5p | Prkaa2 | protein kinase, AMP-activated, alpha 2 catalytic subunit |
| mmu-miR-30d-5p | Prlr | prolactin receptor |
| mmu-miR-30d-5p | Sgk3 | serum/glucocorticoid regulated kinase 3 |
| mmu-miR-30d-5p | Sos1 | SOS Ras/Rac guanine nucleotide exchange factor 1 |
| mmu-miR-3470a | Sos1 | SOS Ras/Rac guanine nucleotide exchange factor 2 |
| mmu-miR-3470a | Sos2 | SOS Ras/Rho guanine nucleotide exchange factor 2 |
| mmu-miR-30d-5p | Tnxb | tenascin XB |
| mmu-miR-30d-5p | Ywhaz | tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide |

To further refine this analysis, and more accurately determine the differential expression of the miRNA gene targets in FhHDM-1 treated β-cells, the common predicted targets of miRNAs were next matched to changes in gene expression levels, previously characterised from the transcriptome of FhHDM-1 treated β-cells (Chapter 2). Using this approach, 19 of the genes that were predicted as targets of the downregulated miRNAs (Table 4.3), displayed an increase in transcriptional levels in FhHDM-1 treated β-cells. From this, an interaction network was generated (Figure 4.5), which showed that miR-30d-5p exerted the most influence, regulating a total of 11 miRNAs, followed by miR-3470a and miR-677-5p.

This number of genes was too low to map to any biological pathways using the DAVID (v6.8) functional annotation analysis tool (https://david.ncifcrf.gov). However, gene ontology analysis reflected the same biological functions as previously predicted from non-matched downregulated genes (Figure 4.2), with the top three molecular functions identified as catalytic, binding, and transcription regulator activities. Similarly, the analysis of biological processes revealed a predominance of cellular process, metabolic process, and biological regulation (Figure 4.6, Supplementary Table 4.4). In addition, an exploration of the literature revealed that 9 of the identified gene targets were associated with the PI3K/Akt pathway. Of these, 7 (Nap1l5, Stk35, Ncam1, Calu, Rap1b, Rgs17, and Galnt2) were attributed to miR-30d-5p (Table 4.3; Figure 4.5), 1 (Pde4d) to miR-3470a, and 1 to miR-677-5p. Unfortunately, none of the downregulated genes in the transcriptome matched the predicted gene targets of upregulated miRNA in FhHDM-1 treated β-cells.

**Table 4.3 Upregulated transcriptome-matched genes with their corresponding regulatory miRNA in FhHDM-1 treated β-cells compared to untreated controls.**

| miRNA Name | Gene Symbol | Gene Name | Log 2 Fold Change | P-value |
|---|---|---|---|---|
| mmu-miR-30d-5p | Cth | Cystathionase | 0.39 | 1.84E-06 |
| mmu-miR-30d-5p | Nap1l5 | Nucleosome Assembly Protein 1-Like 5 | 0.24 | 1.33E-03 |
| mmu-miR-30d-5p | Stk35 | Serine/Threonine Kinase 35 | 0.34 | 1.51E-08 |
| mmu-miR-30d-5p | Ncam1 | Neural Cell Adhesion Molecule 1 | 0.15 | 8.30E-05 |
| mmu-miR-30d-5p | Calu | Calumenin | 0.14 | 1.21E-03 |
| mmu-miR-30d-5p | Rap1b | Ras Related Protein 1B | 0.19 | 2.91E-03 |
| mmu-miR-30d-5p | Ell2 | Elongation Factor For Rna Polymerase Ii 2 | 0.15 | 7.41E-03 |
| mmu-miR-30d-5p | Rgs17 | Regulator Of G-Protein Signaling 17 | 0.39 | 3.73E-03 |
| mmu-miR-30d-5p | Fosl2 | Fos-Like Antigen 2 | 0.41 | 1.52E-08 |
| mmu-miR-30d-5p | Mboat1 | Membrane Bound O-Acyltransferase Domain Containing 1 | 0.29 | 3.69E-06 |
| mmu-miR-30d-5p | Galnt2 | Polypeptide N-Acetylgalactosaminyltransferase 2 | 0.16 | 1.01E-03 |
| mmu-miR-3470a | Scpep1 | Serine Carboxypeptidase 1 | 0.22 | 1.33E-06 |
| mmu-miR-3470a | Ell2 | Elongation Factor For Rna Polymerase Ii 2 | 0.15 | 7.41E-03 |
| mmu-miR-3470a | Pde4d | Phosphodiesterase 4D, Camp Specific | 0.33 | 6.78E-05 |
| mmu-miR-677-5p | Canx | Calnexin | 0.14 | 5.54E-03 |
| mmu-miR-677-5p | Emc7 | Er Membrane Protein Complex Subunit 7 | 0.21 | 8.61E-04 |
| mmu-miR-677-5p | Zeb2 | Zinc Finger E-Box Binding Homeobox 2 | 0.33 | 7.92E-03 |
| mmu-miR-7033-5p | Cdr2l | Cerebellar Degeneration-Related Protein 2-Like | 0.21 | 1.18E-06 |
| mmu-miR-7068-5p | Crot | Carnitine O-Octanoyltransferase | 0.43 | 3.34E-14 |

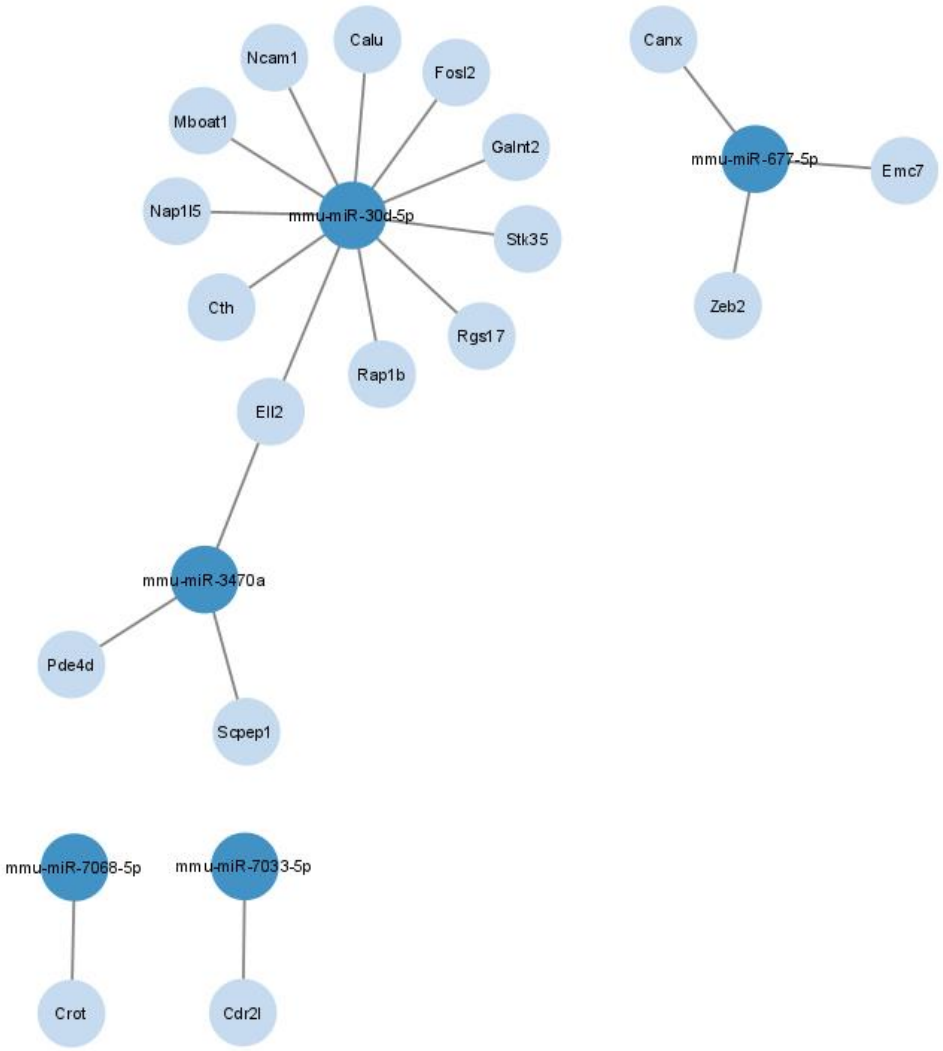



**Figure 4.5 Interaction network of matched genes upregulated in the transcriptome with their corresponding regulatory miRNA downregulated in FhHDM-1 treated β-cells.** Each dark blue node represents the miRNAs and the connections to light blue nodes represent their corresponding matched gene target(s). Created using Cytoscape 3.9.1.

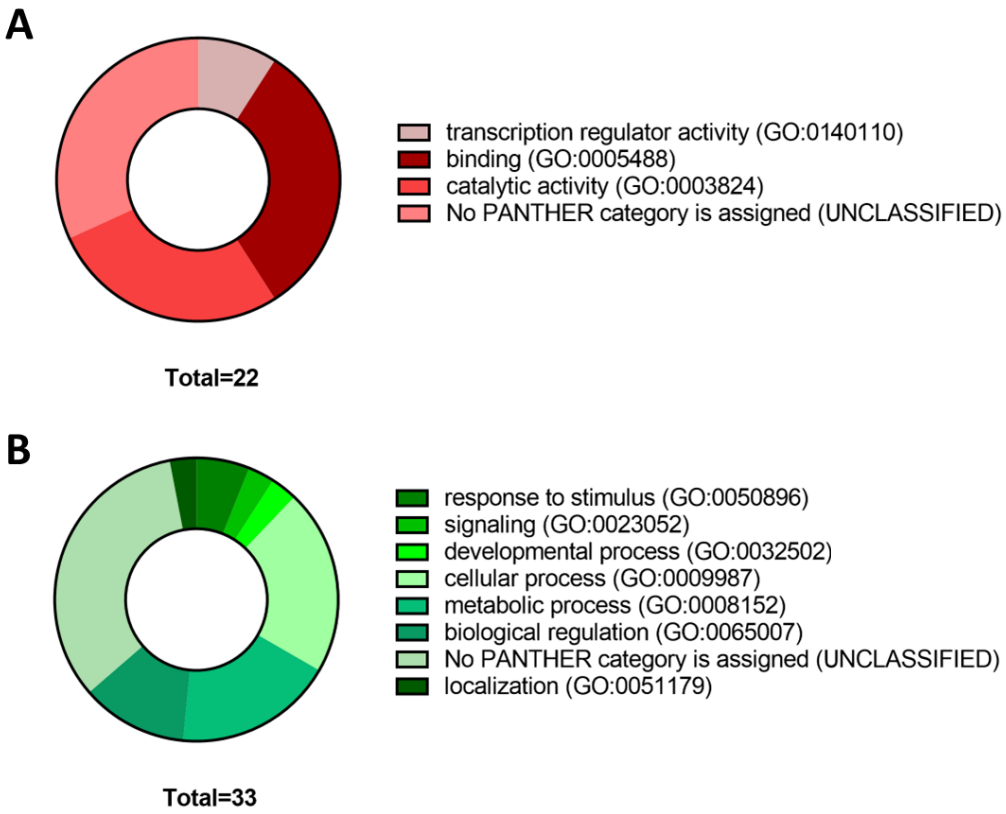



**Figure 4.6 (A) Molecular function and (B) biological processes associated with matched gene targets of the downregulated miRNA in FhHDM-1 treated β-cells.** Gene ontology enrichment for molecular function and biological process of the predicted gene targets of miRNAs upregulated in FhHDM-1 treated β-cells were completed using Panther DB (http://www.pantherdb.org/), with the percentage of genes in each category represented by the colour-coded donut charts. The genes within each category are listed in Supplementary Table 4.4 and the total number of genes is noted at the bottom of each chart.

### 4.4.3 FhHDM-1 treatment of apoptotic β-cells induces changes in miRNA expression levels

To next investigate the changes underlying FhHDM-1 mediated prevention of β-cell apoptosis driven by pro-inflammatory cytokines, the experimental conditions previously used in chapter 2 were again mirrored: NIT-1 β-cells were pre-treated with vehicle control or FhHDM-1 (10μm) for 1h prior to exposure to pro-inflammatory cytokines (10ng/ml IL1β, 50ng/ml TNF, 50ng/ml IFNγ) for a further 24h, before RNA samples were isolated. From these samples, in-depth small RNA sequencing identified that 291 miRNAs were differentially expressed in β-cells exposed to pro-inflammatory cytokines (IL1β, TNF, IFNγ) for 24h compared to untreated controls (Fig 4.7A), validating the expected significant change in the miRNA landscape from addition of cytokines alone (315).

Compared to β-cells treated with cytokines, in the presence of both pro-inflammatory cytokines and FhHDM-1, 188 miRNAs were differentially expressed (Fig 4.7A, Supplementary Table 4.5). Of these, a total of 56 miRNAs (Fig 4.7B) reached beyond a log fold change threshold of ±1, with 37 upregulated (Table 4.4) and 19 downregulated (Table 4.5). Within the upregulated miRNA list, several were associated with diabetes and β-cell biology, such as miR-100-5p which was found to be downregulated in the serum of T1D patients. Other miRNAs of interest include miR-369-5p, which is associated with regulation of glucose stimulated insulin secretion, let-7f-5p, and miR-26b-5p, which were both downregulated in pancreatic tissue of streptozotocin (STZ)-induced diabetic mice, and miR-132-5p, which promotes β-cell regeneration via downregulation of PTEN and consequent upregulation of Akt.

A notable miRNA within the downregulated list included miR-193b-3p, the human homologue of which is associated with pre-diabetes/insulin resistance, and miR-3102-5p, which is normally upregulated in pancreatic tissue of STZ diabetic mice. Further, miR-7033-5p, which was also downregulated in FhHDM-1 treated β-cells under basal conditions, was found to be downregulated by anti-diabetic grape seed flour treatment in high fat diet (HFD) mice. Additionally, miR-345-5p was found to be elevated in the sera of children with recent-onset T1D. While generally the reported functions of these miRNAs support the premise that FhHDM-1 exerts positive effects on the β-cells, analysis of the predicted, and matching, gene targets was performed to gain a more specific insight into the biological processes impacted by the alteration in miRNAs expression induced in β-cells by FhHDM-1.

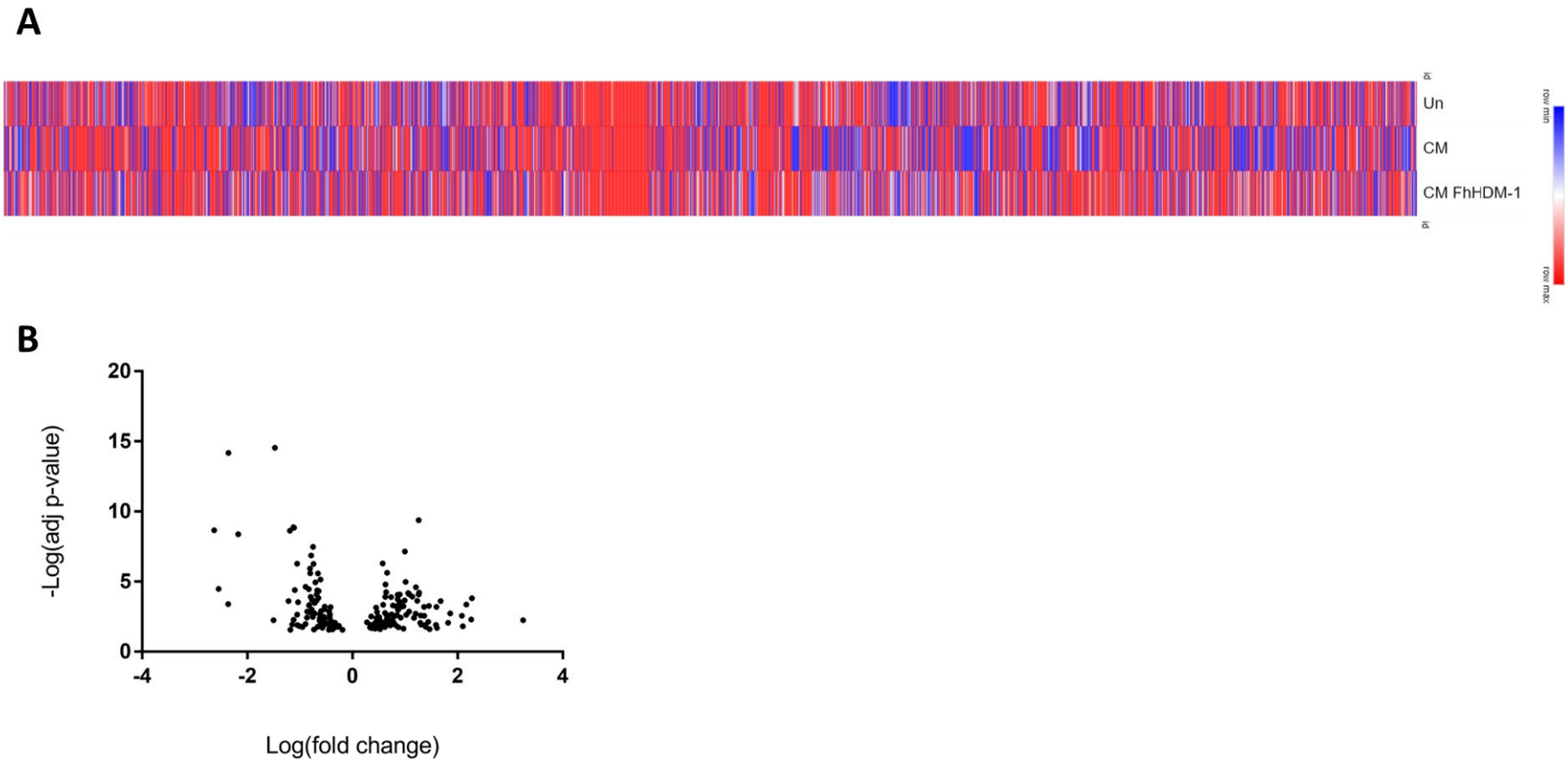



**Figure 4.7 Exposure of β-cells to pro-inflammatory cytokines induced changes in miRNA expression levels, which were altered by treatment with FhHDM-1.** NIT1 β-cells ($2x10^6$) were left untreated (Un) or treated with FhHDM-1 (10µm, 1h) prior to exposure to pro-inflammatory cytokines (CM: IL-1β, TNF, IFNγ) for 24h, followed by RNA extraction and sequencing**. (A)** Heat map of relative abundance of miRNAs from β-cells (reads per million). **(B)** Volcano plot of differentially expressed miRNAs in FhHDM-1 treated β-cells compared to β-cells exposed to CM alone.

**Table 4.4 Differentially expressed miRNAs with a log fold change >±1 in FhHDM-1 treated β-cells exposed to pro-inflammatory cytokines compared to cytokines alone (CM)**

| miRNA Name | Log Fold Change (FhHDM-1 vs CM) | P value | Known role in diabetes or β-cell biology | Reference |
|---|---|---|---|---|
| mmu-miR-802-3p | 3.25 | 5.67E-03 | Downregulated in genetic T2D mouse model | (316) |
| mmu-miR-758-3p | 2.27 | 1.57E-04 | Not determined | (317) |
| mmu-miR-301a-5p | 2.26 | 5.07E-03 | Not determined | |
| mmu-miR-181a-1-3p | 2.17 | 4.31E-04 | Not determined | |
| mmu-miR-376a-5p | 2.10 | 1.53E-02 | Not determined | |
| mmu-miR-134-3p | 2.08 | 2.81E-03 | Not determined | |
| mmu-miR-218-5p | 1.86 | 1.88E-03 | Not determined | |
| mmu-miR-152-5p | 1.82 | 8.86E-03 | Upregulated in plasma of individuals with T2D and T1D | (318) |
| mmu-miR-1193-5p | 1.68 | 2.47E-04 | Not determined | |
| mmu-miR-3962 | 1.61 | 2.02E-02 | Downregulated in developing embryonic heart of diabetic mice | (310) |
| mmu-miR-130a-3p | 1.60 | 6.54E-04 | Elevated in INS1 β-cells with dysregulated GSIS, islets of hyperglycaemic individuals and T2D rats. Downregulated in HFD and high sucrose mice. | (319, 320) |
| mmu-miR-100-5p | 1.59 | 1.23E-02 | Downregulated in plasma of individuals with T1D | (321) |
| mmu-miR-3061-5p | 1.47 | 2.49E-02 | Not determined | |
| mmu-miR-411-5p | 1.46 | 5.48E-04 | Downregulated in islets of individuals with T2D | (322) |
| mmu-miR-3068-5p | 1.44 | 7.56E-03 | Downregulated in HFD mice | (323) |
| mmu-miR-411-3p | 1.40 | 1.66E-02 | Downregulated in HFD mice | (323) |
| mmu-miR-410-3p | 1.39 | 1.28E-02 | Enhanced hESC-derived pancreatic endoderm transplant which alleviated gestational diabetes | (324) |
| mmu-miR-138-2-3p | 1.37 | 2.74E-03 | Downregulated in insulin resistant mice | (325) |
| mmu-miR-126a-3p | 1.36 | 6.43E-04 | Downregulated in plasma of individuals with T2D | (326) |
| mmu-miR-369-5p | 1.31 | 1.20E-02 | Downregulated in MIN6 β-cells with GSIS dysfunction | (327) |
| mmu-let-7f-5p | 1.30 | 2.68E-03 | Downregulated in pancreatic tissue of STZ diabetic mice | (311) |
| mmu-miR-148b-5p | 1.29 | 8.76E-03 | Not determined | |
| mmu-miR-26b-5p | 1.27 | 6.08E-05 | Downregulated in STZ diabetic mice | (326) |

Cont'd

| | | | | |
|---|---|---|---|---|
| mmu-miR-132-5p | 1.26 | 8.43E-05 | Promoted β-cell regeneration after partial pancreatectomy via PI3K signalling, downregulation reduced proliferation and enhanced apoptosis in MIN6 β-cells | (328) |
| mmu-miR-3968 | 1.26 | 4.18E-10 | Upregulated in developing embryonic heart of diabetic mice | (310) |
| mmu-miR-203-3p | 1.23 | 2.40E-04 | Downregulated in islets of diabetic mice, associated with anti-apoptotic function | (329) |
| mmu-miR-27b-5p | 1.21 | 2.55E-05 | Upregulated in HFD and high sucrose fed rats | (320) |
| mmu-miR-130b-3p | 1.20 | 1.93E-03 | Elevated in INS1 β-cells with dysregulated GSIS, islets of hyperglycaemic individuals and T2D rats | (319) |
| mmu-miR-9-5p | 1.17 | 3.88E-03 | Upregulated in AMPK double knock out mice with impaired insulin secretion | (330) |
| mmu-miR-29a-3p | 1.14 | 1.18E-04 | Expression induces inflammation and diabetes in β-cells due to defective insulin secretion | (331) |
| mmu-miR-101c | 1.08 | 1.44E-03 | Downregulated in pancreatic tissue of STZ diabetic mice | (311) |
| mmu-miR-191-3p | 1.08 | 8.05E-05 | Downregulated in plasma of children with low grade obesity | (332) |
| mmu-miR-101a-3p | 1.08 | 1.37E-03 | Downregulated in pancreatic tissue of STZ diabetic mice | (311) |
| mmu-miR-1839-5p | 1.08 | 7.47E-05 | Downregulated in HNF1A silencing-induced diabetes in MIN6 β-cells | (333) |
| mmu-miR-743a-3p | 1.06 | 6.60E-05 | Downregulated in cutaneous wounds of STZ diabetic mice | (334) |
| mmu-miR-30e-3p | 1.03 | 2.34E-03 | Negatively correlated with islet autoantibody GAD titers | (335) |
| mmu-miR-185-5p | 1.01 | 1.07E-05 | Targeted SOCS3 to inhibit β-cell dysfunction in diabetes | (336) |

HNF1A, hepatocyte nuclear transcription factor 1 alpha; GSIS, glucose stimulated insulin secretion; T1D, type 1 diabetes; HFD, high fat diet; hESC, human embryonic stem cells; STZ, streptozotocin; NOD, non-obese diabetic mice; GAD, gamma-aminobutyric acid; SOCS3, suppressor of cytokine signalling-3

**Table 4.5 Differentially expressed miRNAs with a log fold change <±1 in FhHDM-1 treated β-cells exposed to pro-inflammatory cytokines compared to cytokines alone (CM)**

| miRNA Name | Log Fold Change (FhHDM-1 vs CM) | P value | Known role in diabetes or β-cell biology | Reference |
|---|---|---|---|---|
| mmu-miR-677-3p | -2.63 | 2.17E-09 | Not determined | |
| mmu-miR-7068-5p | -2.54 | 3.37E-05 | Upregulated by anti-diabetic GSF in HFD mice | (314) |
| mmu-miR-193b-3p | -2.36 | 4.06E-04 | Human homologue is linked with obesity, insulin resistance and pre-diabetes | (337) |
| mmu-miR-2137 | -2.36 | 6.60E-15 | Downregulated in total visceral fat of obese mice | (338) |
| mmu-miR-677-5p | -2.17 | 4.22E-09 | Not determined | |
| mmu-miR-698-5p | -1.50 | 5.81E-03 | Not determined | |
| mmu-miR-7033-5p | -1.47 | 2.87E-15 | Downregulated by anti-diabetic GSF in HFD mice | (314) |
| mmu-miR-3473a | -1.21 | 2.43E-04 | Associated with MIN6 β-cell differentiation | (309) |
| mmu-miR-3470a | -1.19 | 2.41E-09 | Associated with MIN6 β-cell differentiation | (309) |
| mmu-miR-3102-5p | -1.18 | 2.82E-02 | Upregulated in pancreatic tissue of STZ diabetic mice | (311) |
| mmu-miR-8097 | -1.14 | 1.13E-02 | Upregulated in HFD mice and downregulated after treatment with anti-diabetic LBP | (339) |
| mmu-miR-700-5p | -1.12 | 1.33E-09 | Upregulated in pancreatic tissue of STZ diabetic mice | (311) |
| mmu-miR-6970-5p | -1.12 | 5.27E-03 | Not determined | |
| mmu-miR-871-3p | -1.11 | 1.45E-09 | Upregulated in HNF1A silencing-induced diabetes in MIN6 β-cells | (333) |
| mmu-miR-690 | -1.10 | 4.00E-05 | Downregulated in MIN6 β-cells chronically exposed to high glucose | (340) |
| mmu-miR-345-5p | -1.05 | 5.26E-07 | Elevated in sera of children with recent-onset T1D | (341) |
| mmu-miR-1934-3p | -1.05 | 2.27E-03 | Associated with MIN6 β-cell differentiation, upregulated in pancreas of mice treated with anti-diabetic JTXK | (309, 323) |
| mmu-miR-3572-3p | -1.05 | 1.29E-02 | Not determined | |
| mmu-miR-7679-3p | -1.03 | 3.03E-04 | Upregulated in HNF1A silencing-induced diabetes in MIN6 β-cells | (333) |

GSF, wine grape seed flour; HFD, high fat diet; STZ, streptozotocin; LBP, lycium barbarum; HNF1A, hepatocyte nuclear transcription factor 1 alpha; T1D, type 1 diabetes; JTXK, Jiang Tang Xiao Ke granule

### 4.4.4 Predicted gene targets for differentially expressed miRNAs in FhHDM-1 treated β-cells exposed to pro-inflammatory cytokines mapped to metabolic pathways and PI3K/Akt signaling

The functional role for the miRNAs in regulating the biological activity of β-cells was once again investigated through gene target prediction from the 3'UTR regions of genes within the murine genome using miRDB, Diana tools and TargetScan (Supplementary Table 4.6). The common gene targets identified by all three tools totalled 324,884 predicted gene targets, of which 321,788 and 3096 were attributed to upregulated or downregulated miRNAs in FhHDM-1 treated β-cells in the presence of pro-inflammatory cytokines, respectively (Supplementary Table 4.7).

The frequency distribution of the number of targets per miRNA (Figure 4.8) showed that within the upregulated miRNA list, gene targets were evenly distributed across miRNAs, with most having 3000-4500 gene targets. The miRNAs with the most predicted gene targets included miR-128-3p with 4788, miR-494-3p with 4820, miR-329-3p with 4892, miR-27b-3p with 5029, and miR-124-3p with 5056. In contrast, most downregulated miRNAs had between 1 to 80 predicted gene targets, apart from 6 miRNAs that had greater than 100 targets. These included miR-330-3p with 105 gene targets, miR-129-5p with 117, miR-350-5p with 140, miR-493-5p with 185, miR-181a-5p with 250, and miR-466i-4p with 394.

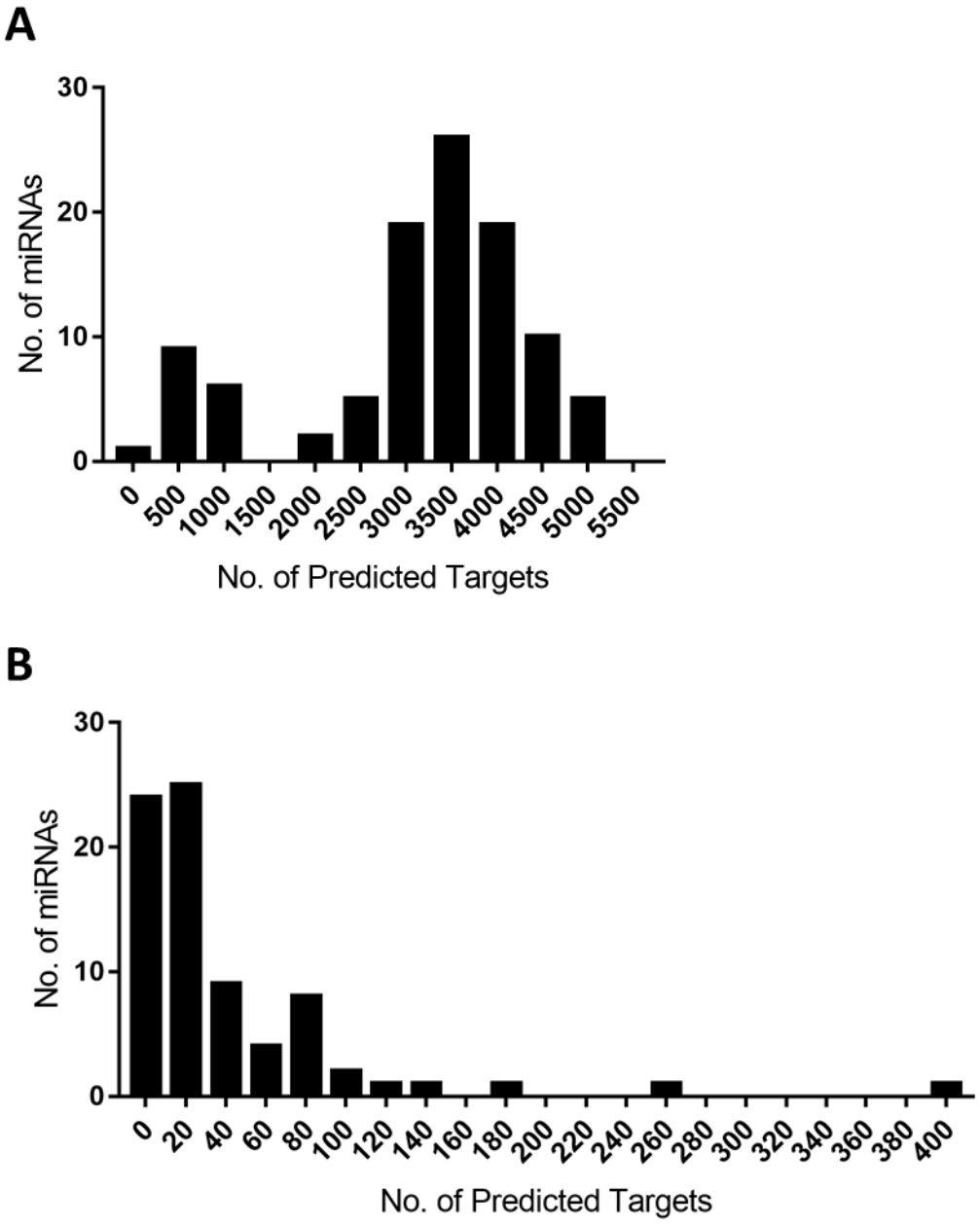



**Figure 4.8 Frequency distribution histogram of (A) upregulated and (B) downregulated miRNAs in FhHDM-1 treated β-cells under pro-inflammatory cytokine conditions, with their total number of predicted gene targets common across all three prediction tools.**

These predicted gene targets were then mapped to biological pathways using KEGG pathway analysis, resulting in the identification of 269 (Supplementary Table 4.8) and 142 (Supplementary Table 4.9) biological pathways, for the increased and decreased miRNAs, respectively. Paralleling the KEGG analysis of FhHDM-1 treated β-cells under basal conditions, the predicted gene targets of miRNAs from FhHDM-1 treated β-cells exposed to pro-inflammatory cytokines mapped to similar pathways associated with β-cell biology, with the top 10 most relevant presented in Figure 4.9. Shared between both the upregulated (Figure 4.9A) and downregulated (Figure 4.9B) miRNA gene targets were the following signaling pathways: PI3K/Akt, MAPK, Ras, Wnt, FoxO, AMPK, insulin signaling, and pathways in cancer. Unique pathways to which upregulated miRNA gene targets were attributed included metabolic pathways and cAMP signaling. On the other hand, unique pathways to which downregulated miRNA gene targets mapped included insulin resistance, mTOR and TGF-β signaling.

Since PI3K is shared between upregulated and downregulated miRNA gene targets, it is difficult to delineate the bonafide effect(s) of FhHDM-1 by these analyses. Therefore, the specific genes predicted by the DAVID (v6.8) functional annotation analysis tool (https://david.ncifcrf.gov) to be involved in the PI3K/Akt pathway were examined further. For the gene targets predicted to be reduced in expression (Figure 4.10), most (if not all) genes were affected, predicting that the PI3K pathway is broadly impacted by FhHDM-1, though no singular effect can be ascertained. The regulation of these genes within the PI3K pathway was evenly distributed across 6354 different miRNAs, though the topmost functional were miR-128-3p, miR-124-3p, miR-107-3p, which govern 104, 101 and 100 genes, respectively. Analysis of the gene targets that were predicted to be enhanced in expression through downregulation of the corresponding regulator miRNA(s) (Figure 4.11), identified genes such as IGF and its downstream targets such as IRS1, Ras and ERK. Importantly, while PI3K was predictively activated, its inhibitor PTEN was not. Of the 94 genes within the pathway predicted to be increased in expression, regulation was mostly attributed to the top three miRNA, miR-466i-5p, miR-3473a, miR-350-5p, which govern 10, 8 and 7 genes, respectively. The enhanced expression of these gene targets would ultimately lead to pro-survival effects, which corroborates the outcomes observed in FhHDM-1 treated β-cells, in which β-cell survival is enhanced, apoptosis is inhibited (Chapter 2), and IGF-2 is increased (Chapter 3).

Interestingly, various members of the IGF1 family were well-represented within the lists of both downregulated and upregulated gene targets, reflecting the paradoxical attribution of both downregulated and upregulated gene targets to the PI3K/Akt pathway (Figure 4.12). Indeed, miR-466i-5p and miR-3473a predictively upregulated Igf2 and Igf1, while concurrently the same genes are predicted to be downregulated by let-7i-5p and miR-185-5p. Additionally, miR-411-3p and

miR-152-3p downregulate Igf1r and Pigf. The balance between these miRNAs and the regulation of their gene targets could explain the outcomes in Chapter 3, in which there was increased production of IGF-2 but not IGF-1, enhanced expression of total IGF1R and distinct phosphorylation of the receptor.

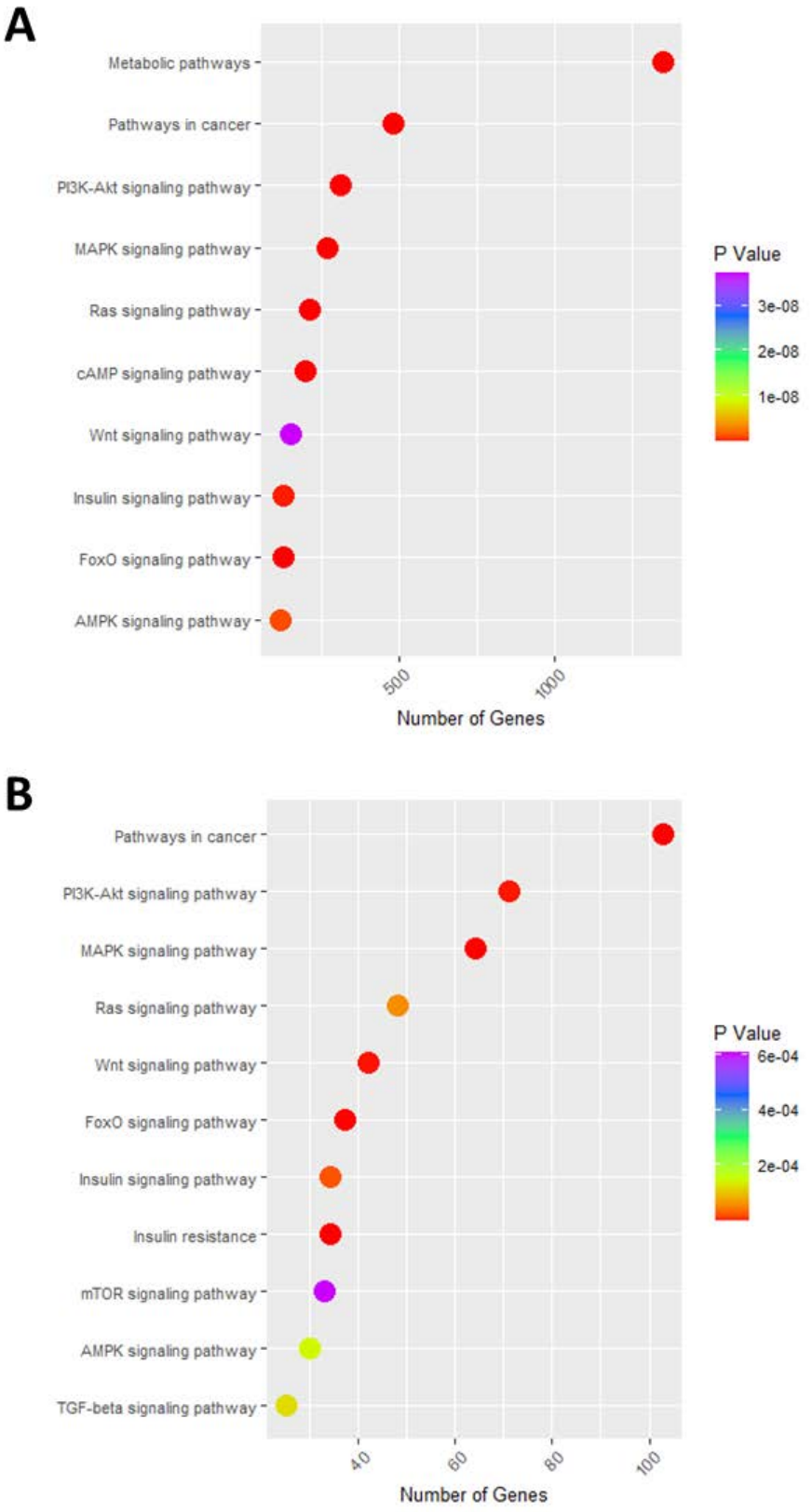



**Figure 4.9 KEGG pathway analysis of predicted gene targets from (A) upregulated and (B) downregulated miRNAs in FhHDM-1 treated β-cells revealed highly represented pathways associated with β-cell biology.** Dot plot showing the top 10 pathways most relevant to β-cells. The genes in each pathway are found in Supplementary Table 4.8 and 4.9. Dot colour indicates p-value and dot placement on the x-axis indicates the number of genes in that pathway.

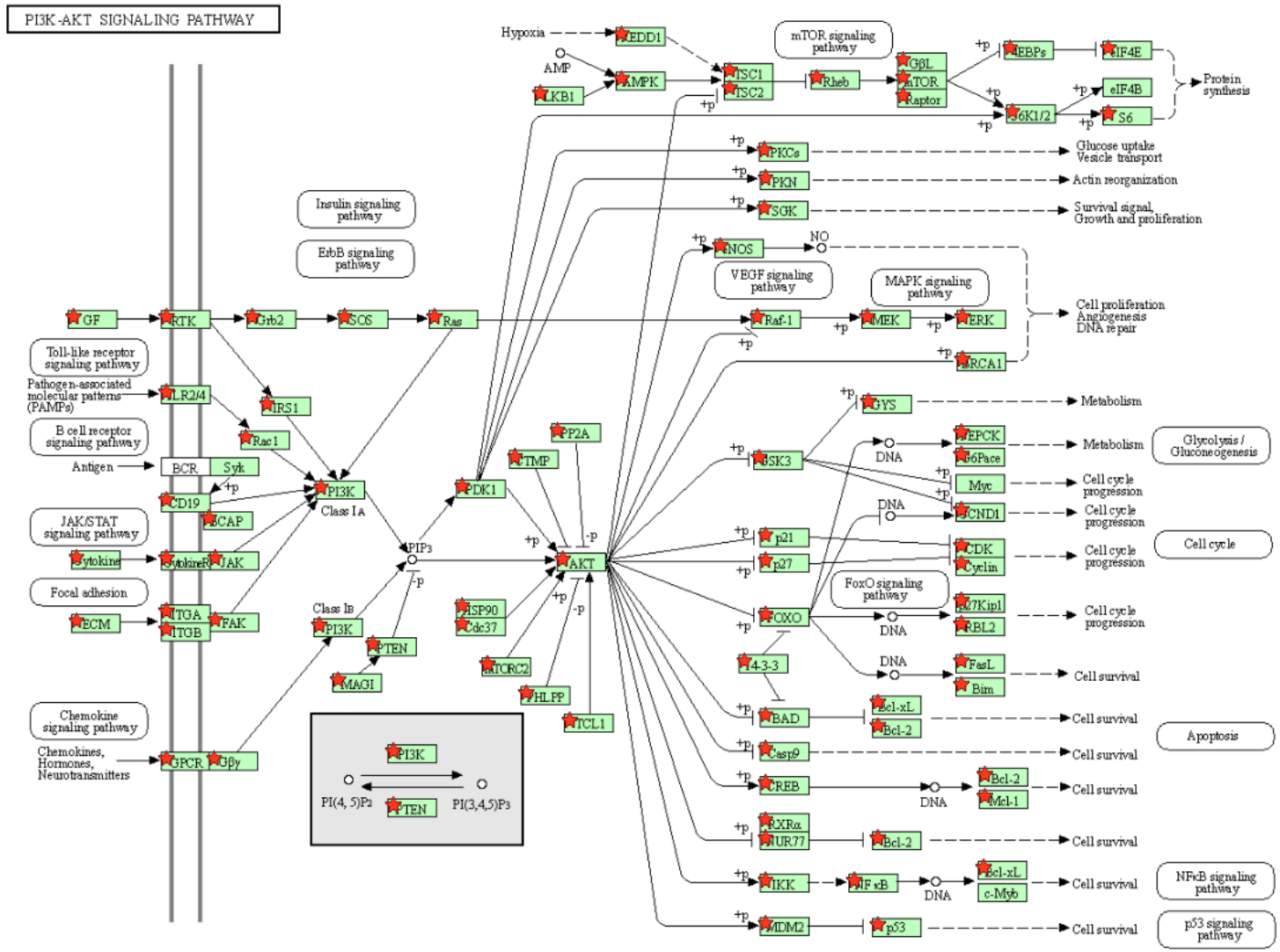



**Figure 4.10 The gene targets of miRNA that were increased in expression, identified within the PI3K/Akt pathway as determined by DAVID (v6.8) functional annotation analysis tool.** Differential expression of miRNAs observed within FhHDM-1 treated β-cells under inflammatory conditions compared to controls treated with pro-inflammatory cytokines only, were predictively mapped to gene targets, which were then attributed to the PI3K/Akt pathway. Targeted genes, assumed to be decreased in expression, are indicated by the red stars.

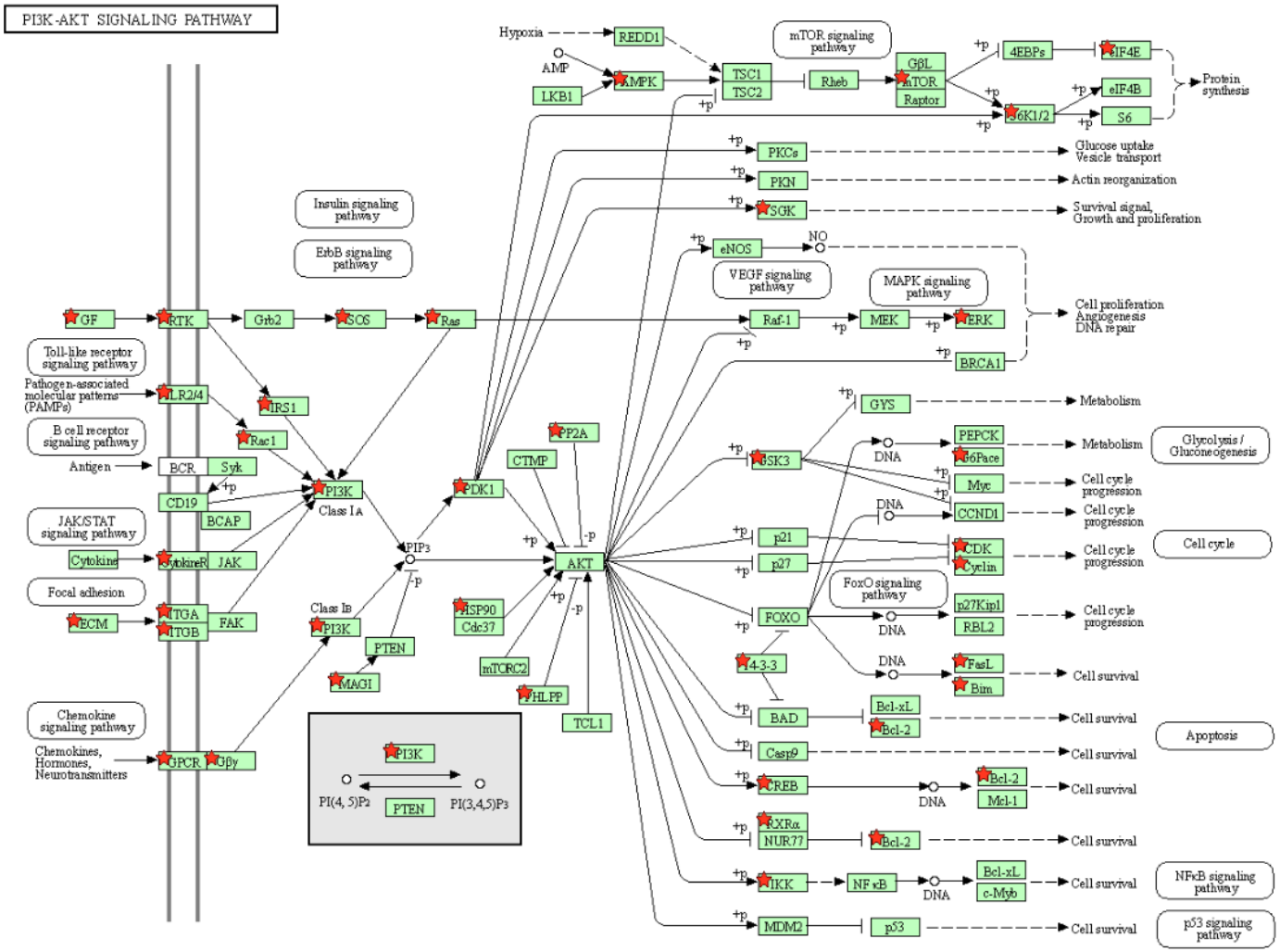



**Figure 4.11 The gene targets of miRNAs that were decreased in expression, identified within the PI3K/Akt pathway as determined by DAVID (v6.8) functional annotation analysis tool.** Differential expression of miRNAs observed within FhHDM-1 treated β-cells under inflammatory conditions compared to controls treated with pro-inflammatory cytokines only, were predictively mapped to gene targets, which were then attributed to the PI3K/Akt pathway. Targeted genes, assumed to be increased in expression, are indicated by the red stars.

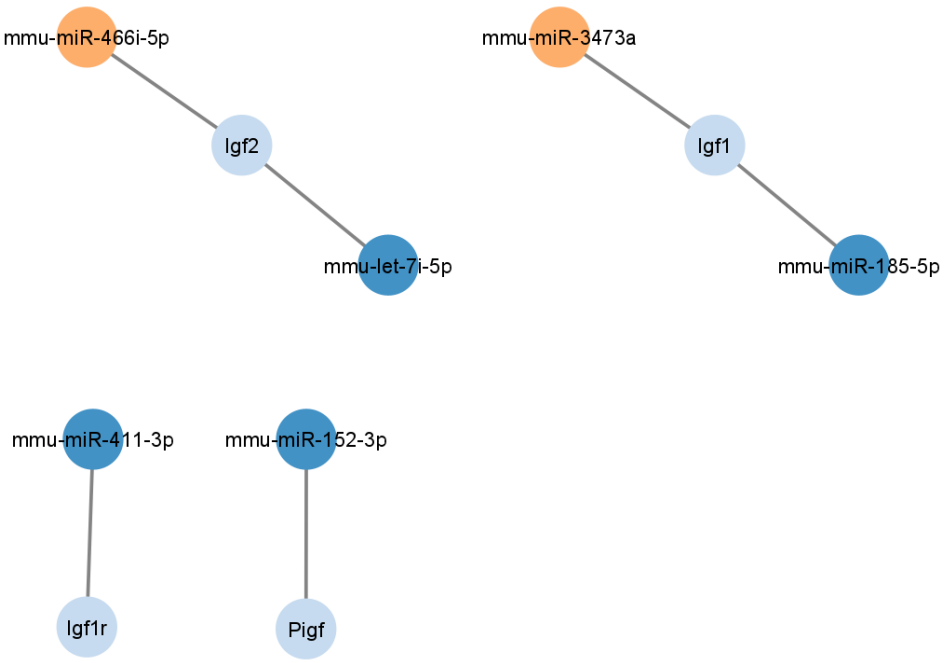



**Figure 4.12 Regulation of genes within the IGF family by upregulated and downregulated regulatory miRNA.** Each dark blue node represents upregulated miRNAs, orange nodes represent downregulated miRNAs, and the connections to light blue nodes represent their corresponding matched gene target(s). Created using Cytoscape 3.9.1.

To more precisely determine the relationship between the DE miRNAs and gene expression in FhHDM-1 treated β-cells under pro-inflammatory conditions, the common predicted gene targets of miRNAs (Supplementary Table 4.6) were matched to gene expression changes identified from the transcriptome of CM + FhHDM-1 treated β-cells (Supplementary Table 4.10). The resulting genes (Supplementary Table 4.11) were then mapped to KEGG pathways, with 37 (Supplementary Table 4.12) and 14 (Supplementary Table 4.13) pathways associated with the downregulated and upregulated genes, respectively.

Of the 37 pathways associated with downregulated genes, the most relevant pathways (Figure 4.13A) included MAPK signaling, apoptosis, TNF signaling, NFKβ signaling, insulin resistance, and pancreatic cancer. This suggests that FhHDM-1 downregulates apoptosis and pathways (TNF, insulin resistance) associated with inflammation. Furthermore, downregulation of pancreatic cancer pathways corroborates previous observations (Chapter 2) that FhHDM-1 selectively promotes β-cell survival but not proliferation. Interestingly, MAPK signaling was downregulated, which could contribute to elucidation of the downstream pathways activated by FhHDM-1 as described in Chapter 3, in which investigation of MAPK/ERK activation was proposed as a future study.

On the other hand, the upregulated genes were highly attributed to metabolic pathways and PI3K/Akt signaling, with 26 and 9 genes, respectively (Figure 4.13B). The metabolic pathways fell into the general categories of energy, carbohydrate, lipid, amino acid, and nucleotide metabolism, and featured pathways such as the citrate cycle, glycolysis/gluconeogenesis, fatty acid biosynthesis/degradation, fructose/mannose metabolism, phosphonate/phosphinate metabolism, and nicotinate/nicotinamide metabolism (Figure 4.14). Within PI3K/Akt signaling, the attributed genes include Igf2, thus corroborating the finding that FhHDM-1 induces IGF-2 secretion within the β-cells, thereby activating PI3K/Akt signaling and exerting anti-apoptotic effects.

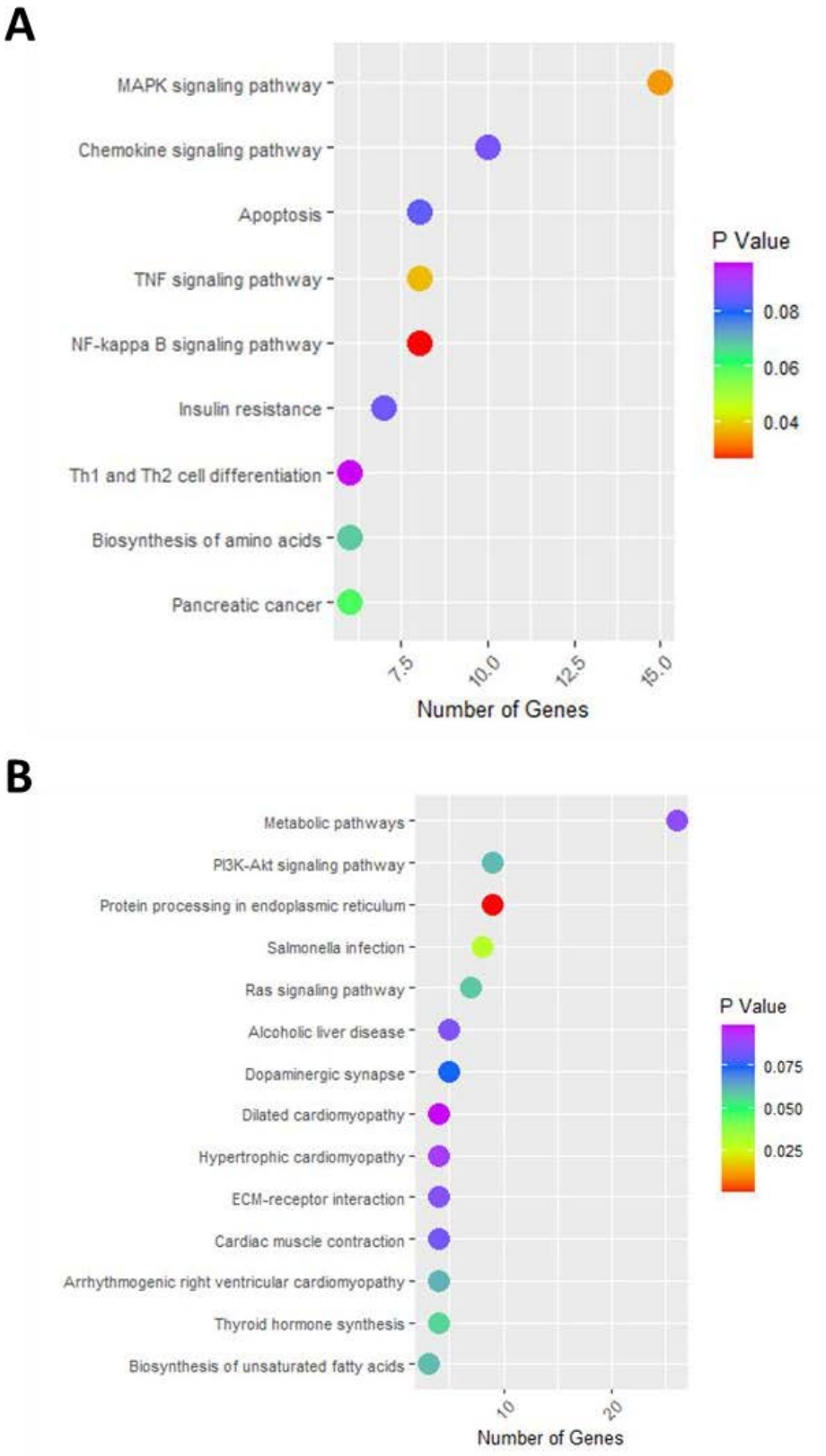



**Figure 4.13 KEGG pathway analysis of matched gene targets from (A) upregulated or (B) downregulated miRNA in FhHDM-1 treated β-cells show pathways relevant to β-cell biology.** Dot plot showing all pathways identified from KEGG pathway analysis. The genes in each pathway are found in Supplementary Table 4.12 and 4.13. Dot colour indicates p-value and dot placement on the x-axis indicates the number of genes in that pathway.

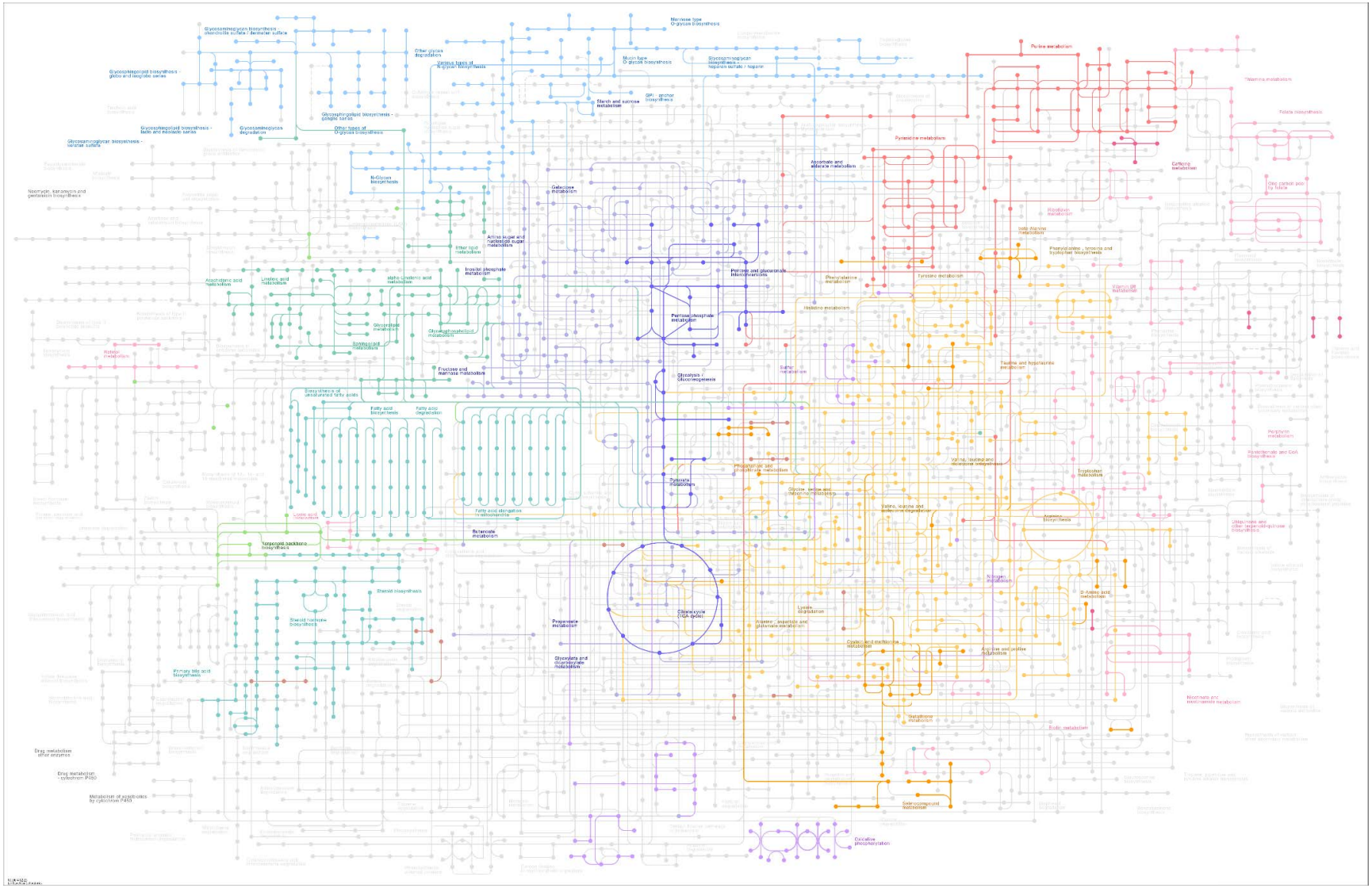


**Figure 4.14 KEGG metabolic pathways of matched gene targets of downregulated miRNAs in FhHDM-1 treated β-cells.** Of the matched gene targets from downregulated miRNA that were mapped to KEGG pathways, 26 genes were attributed to metabolic pathways (Supplementary Table 4.13), as denoted by the different coloured networks. Notable pathways include carbohydrate (dark purple), lipid (teal), amino acid (yellow and orange), and nucleotide (red) metabolism. Image generated using DAVID (v6.8) functional annotation analysis tool (https://david.ncifcrf.gov).

Interaction networks were then built from the transcriptome-matched genes and their corresponding regulatory miRNAs to identify significant miRNAs that could be candidates as central players in the biological effects mediated by FhHDM-1. The downregulated genes (predicted due to correspondingly upregulated miRNAs) were clustered discretely (Figure 4.15), with each miRNA regulating unique sets of gene targets without any overlap. The genes attributed to significant KEGG pathways, including apoptosis, insulin resistance, TNF, NFKB, pancreatic cancer, and MAPK, were distributed among different miRNAs. Consequently, no individual miRNA displayed a broad regulatory effect which could be singularly attributed to the enhanced viability mediated by FhHDM-1. However, based on the number of gene targets per miRNA, let-7e-5p, miR-124-3p, and miR-101a-3p, were the most impactful, regulating 51,47, and 43 gene targets, respectively. These three miRNAs regulate genes associated with pancreatic cancer (Bcra2), insulin resistance (Pck2, Trib3) and shared significant pathways (Rela, Fos).

Among the upregulated genes (as correlated to downregulated miRNA levels), Igf2 was of particular interest given its association with the PI3K pathway, and because it has been previously implicated in exerting the positive effect of FhHDM-1 in β-cells (Chapter 3). From the interaction network (Figure 4.16), Igf2 was found to be regulated by miR-466i-5p and miR-7689-3p, both of which also regulate other genes associated with PI3K signaling and metabolic pathways. Moreover, miR-466i-4p regulated the greatest number of genes at 36, thus establishing itself as the most prominent miRNA in these studies.

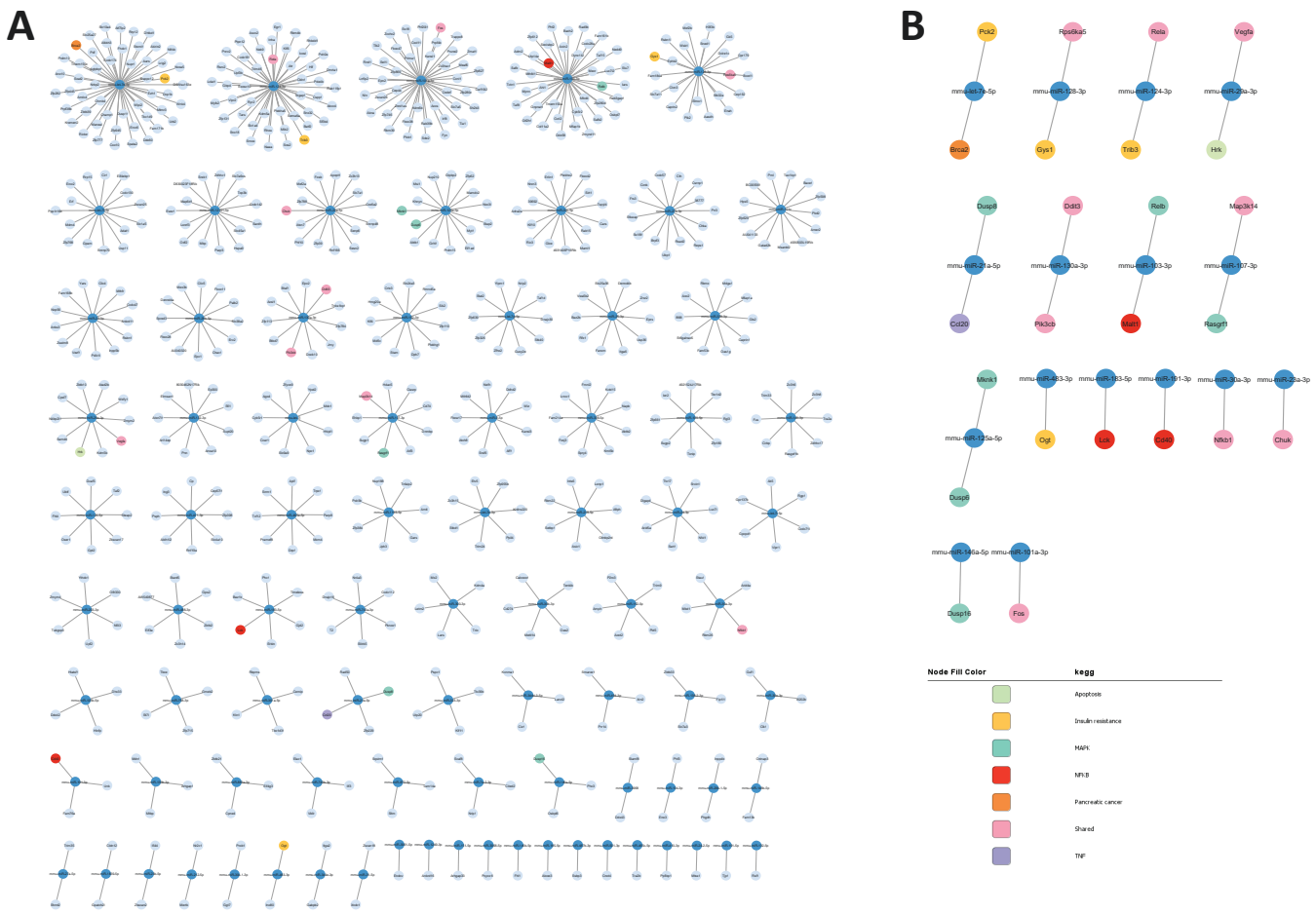



**Figure 4.15 Interaction network of transcriptome-matched genes downregulated in the transcriptome with their corresponding regulatory miRNA upregulated in FhHDM-1 treated β-cells under inflammatory conditions.** The transcriptome-matched genes were attributed to KEGG pathways, after which the genes within the topmost relevant biological pathways were matched back to their corresponding regulatory miRNA to generate interaction networks displaying **(A)** all interactions or **(B)** only the interactions within the relevant pathways. Each dark blue node represents the miRNAs and connections to light blue nodes represent the corresponding matched gene target(s), apart from gene nodes attributed to relevant biological pathways, namely apoptosis, insulin resistance, MAPK, metabolic pathways, NFKB, PI3K, pancreatic cancer and TNF, which are colour coded according to the legend. Genes that appear across multiple pathways are coloured pink. Created using Cytoscape 3.9.1.

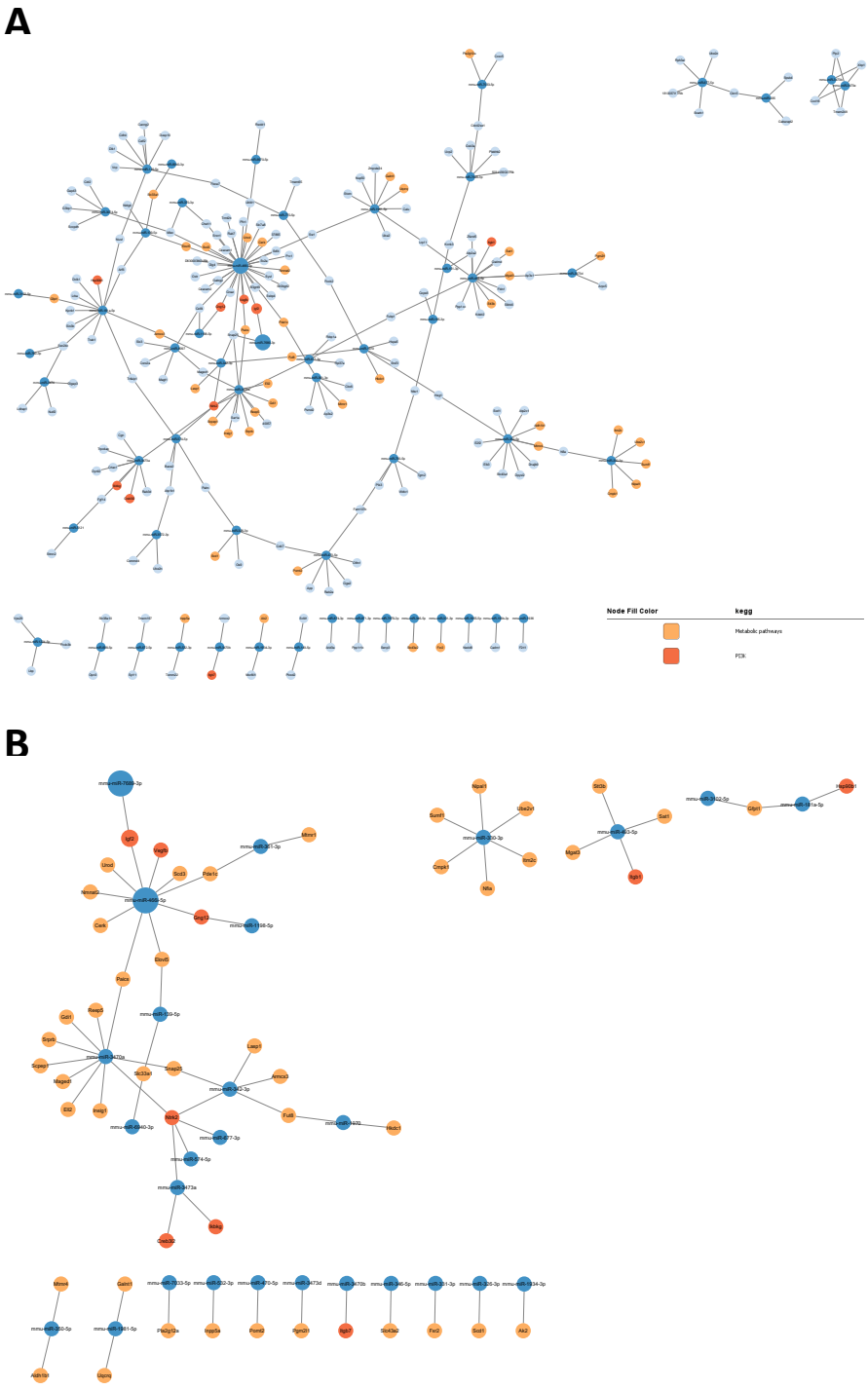



**Figure 4.16 Interaction network of matched genes upregulated in the transcriptome with their corresponding regulatory miRNA downregulated in FhHDM-1 treated β-cells under inflammatory conditions.** The transcriptome-matched genes were attributed to KEGG pathways, after which the genes within the topmost relevant biological pathways were matched back to their corresponding regulatory miRNA to generate interaction networks displaying **(A)** all interactions or **(B)** only the interactions within the relevant pathways. Each dark blue node represents the miRNAs and connections to light blue nodes represent the corresponding matched gene target(s), apart from gene nodes attributed to metabolic pathways and PI3K, which are colour coded according to the legend. Created using Cytoscape 3.9.1.

## 4.5 Discussion

This study has established that FhHDM-1 exerts substantial changes to the miRNA profile within β-cells. To distinguish the most impactful miRNAs that could act as candidates for validation, notable miRNAs from (1) common predicted gene targets attributed to PI3K/Akt pathways were extracted and (2) transcriptome-matched gene targets associated with genes attributed to the biological effects of FhHDM-1 such as IGF1R, Igf2 and components of the PI3K/Akt pathway were identified. If no significant associations were determined, then the miRNAs regulating the greatest number of genes were considered most influential. Using this approach, under basal conditions, the common predicted gene targets of downregulated miRNA were highly attributed to the PI3K pathway, in which genes were regulated by miR-30d-5p, miR-3470a, miR-677-5p and miR-7033-5p. This parallels the final analysis of transcriptome-matched genes, in which the same miRNAs were identified as most impactful based on the number of genes they regulated.

Under apoptotic conditions induced by pro-inflammatory cytokines, notable upregulated miRNAs that governed common predicted gene targets within the PI3K pathway included miR-128-3p, miR-124-3p and miR-107-3p. In addition, the analysis of transcriptome-matched gene targets of the upregulated miRNAs led to identification of let-7e-5p, miR-124-3p, and miR-101a-3p as likely candidates of interest. For the downregulated miRNAs, the top miRNAs that regulated common predicted gene targets within the PI3K pathway included miR-466i-5p, miR-3473a, and miR-350-5p, whereas the most notable miRNAs from the final analysis of transcriptome-matched genes miR-466i-5p and miR-7689-3p.

While different miRNAs and gene targets were identified under basal versus inflammatory conditions, the biological pathways impacted were remarkably similar and reflected the functional outcomes induced by FhHDM-1 as previously shown in Chapter 2 and 3, in which IGF-2 production and IGF1R expression were enhanced, thus activating PI3K/Akt signaling to promote β-cell survival and function, but not proliferation. Indeed, the upregulated gene targets (of downregulated miRNAs) under both basal and inflammatory conditions mapped to significant biological pathways of PI3K/Akt signaling and other associated pro-survival pathways such as MAPK, Wnt, FoxO and mTOR. Furthermore, detailed examination of the targeted genes within the PI3K pathway revealed that the expression of PI3K and IGF were specifically altered, suggesting that FhHDM-1 treatment

induced the activation of these genes via downregulation of their corresponding regulatory miRNA.

In addition, the downregulated gene targets (of upregulated miRNA) under inflammatory conditions mapped to biological pathways of apoptosis, insulin resistance, TNF, and pancreatic cancer. This suggests that FhHDM-1 inhibits these pathways, thereby exerting anti-apoptotic and anti-inflammatory effects, which once again corroborates the inhibition of apoptosis shown in previous cellular studies (Chapter 2). Moreover, it is attractive to speculate that the downregulation of genes associated with pancreatic cancer pathways indicates that the miRNAs modulated by FhHDM-1 play regulatory roles in preventing proliferative effects that could lead to cancer. This agrees with data described in Chapter 2 showing that FhHDM-1 promoted β-cell viability without inducing proliferation.

This study utilised a robust and inclusive approach in which miRNAs were analysed with multiple gene target prediction tools, from which only the common gene targets were assessed. Furthermore, these gene targets were integrated with the mRNA transcriptome of the same β-cells to more accurately identify genes regulated by the miRNAs. However, there are caveats that must be considered, some of which are revealed when examining the basal data. Firstly, the common predicted gene targets of upregulated miRNAs could not be mapped to biological pathways due to insufficient numbers of genes, and upon investigation of gene ontologies (molecular function and biological process), most genes were the same throughout the analysis. This reflects the shortcomings of currently available prediction tools, which rely on published literature, thereby leaving unidentified gene targets, and thus, pathways, omitted from the analysis. Secondly, none of the predicted gene targets of upregulated miRNA could be matched to the downregulated genes in the transcriptome. As both the miRNA and mRNA samples were harvested at the same time point following treatment, this may reflect the diverse and complex kinetic relationships between miRNA expression and regulation of gene expression. Accordingly, miRNA-gene regulation may extend beyond the equivalent time points analysed, potentially overlooking miRNA-gene relationships that have varied temporal expression. Thirdly, throughout the study, there is the standard assumption of a negative association between the expression of a given miRNA and the expression of its gene targets. This reflects the predominant biological outcomes that have been characterised to date. However, there is also the possibility that expression of certain miRNAs and their gene targets are positively correlated. Of interest in this study is miR-483-3p, which was found to be upregulated in FhHDM-1 treated β-cells under basal conditions

and has been reported to be co-expressed with IGF-2. Furthermore, a positive feedback loop has been observed whereby miR-483-5p amplified IGF-2 expression (342). This suggests that increased expression levels of miR-483-3p induced by FhHDM-1 treatment could be associated with an increased expression of IGF-2, as supported by the data in Chapter 3. Therefore, consideration of this unconventional positive miRNA-gene relationship presents opportunities for further analysis of the miRNA sequencing data.

While this analysis has produced a detailed and comprehensive view of the miRNA-mRNA relationships that are instructed by the treatment of β-cells by FhHDM-1 and has identified several key miRNAs as central regulators of the positive effect of FhHDM-1 on β-cell survival, experimental validation of these findings is required. The accepted standard approach would be to knock in/knock out the miRNAs of interest in β-cells, followed by determination of key cellular outcomes, such as cell survival, apoptosis, and expression of IGF-2 and IGF1R, to determine if the effect of FhHDM-1 is paralleled. This can be combined with an *in vitro* reporter system which would measure the regulation of the expression of specific gene targets by a single miRNA sequence to functionally validate the predicted miRNA/mRNA relationship.

Despite these limitations, this study has provided a novel insight into the molecular regulation of enhanced β-cell survival by the parasite derived peptide FhHDM-1. Significantly, the identification of specific gene targets associated to the induction and sustained activation of the PI3K/Akt pathway corroborated the previous biological activity of FhHDM-1, which validates the bioinformatic approach that was employed here, and strongly supports the regulatory role for the miRNA candidates that were identified. The findings presented here sets the foundation for future targeted analysis to fully determine the critical nature of the contribution that the identified miRNAs and corresponding gene targets make towards the positive effect of FhHDM-1 on the function and survival of β-cells.

# Chapter 5: General Discussion

The complete and irreversible immune mediated destruction of β-cells underpins T1D (1). As the delivery of exogenous insulin cannot mimic the minute-to-minute glucose responsiveness of β-cells, morbidity and mortality for the patient is significantly increased (195). Additionally, the incidence of T1D has been increasing appreciably in recent decades (4). It has been over 100 years since insulin was first discovered. Since that time, exogenous insulin delivery has remained the mainstay of treatment for T1D (195). However, "insulin is not a cure for diabetes; it is a treatment" (Frederick Banting). Given its autoimmune pathogenesis, immune suppression long been investigated as a cure for T1D, with disappointingly limited success to date (196, 202, 343). It is now recognised that β-cells are active participants in the immune dialogue, which instigates their own destruction and initiates autoimmunity (1, 29). Accordingly, treatment strategies that directly target β-cells to preserve function and/or survival will ultimately cure T1D.

By characterising the protein component of the excretory/secretory products of the liver fluke, *Fasciola hepatica*, a single peptide (FhHDM-1) that permanently prevented T1D in NOD mice was previously identified. Remarkably, only six intraperitoneal injections of FhHDM-1 (10μg/mouse) administered over 12 days was sufficient to prevent T1D when delivered coincident with the initiation of autoimmunity. Disease prevention was associated with increased insulin content and decreased insulitis within islets (151). However, the putative impacts of FhHDM-1 on β-cells were yet to be elucidated. Here it has been demonstrated that treatment of β-cells with FhHDM-1 enhanced their viability and function, but importantly did not increase proliferation. Improved β-cell survival/function was maintained against a background of inflammation, and other cellular stress stimuli, in both human and murine β-cells. Accordingly, FhHDM-1 has potential applications for various inflammatory conditions in which β-cell survival is compromised, such as to support exhausted β-cells in T2D, and to inhibit β-cell apoptosis during islet isolation and post-transplantation. To date, no other parasite peptide offers a therapeutic strategy which can be applied to the different pathogeneses affecting β-cells. This study investigated the new paradigm of shifting the focus to the β-cell and determined the impact of FhHDM-1 on β-cells and the mechanisms underlying these effects.

First, a global proteomic approach was employed to identify putative biological pathways within the pancreas that were modulated by FhHDM-1. These analyses revealed that proteins involved in the activation of the PI3K/Akt pathway were highly abundant. Given that this pathway plays a fundamental role in regulating β-cell proliferation, survival, and

metabolic activity (293), it was hypothesised that FhHDM-1 enhanced PI3K/Akt signalling to preserve β-cell mass, and thus prevent T1D development. Exploring this hypothesis (Chapter 2), it was established that FhHDM-1 localised to the pancreas *in vivo*, and directly interacted with the β-cells *in vitro* to promote survival/function. These beneficial effects correlated with the activation of PI3K/Akt signalling, as evidenced by increased levels of phosphorylated Akt, and NADH and NADPH, and reduced activity of the NAD-dependent DNA nick sensor, PARP-1. These intracellular changes induced by FhHDM-1 would explain why β-cells were protected from apoptosis in the presence of the pro-inflammatory cytokines, which are responsible for the destruction during T1D pathogenesis. Importantly, while FhHDM-1 promoted β-cell survival and function, it did not induce proliferation.

Having identified the ability of FhHDM-1 to enhance β-cell survival and function, the mechanisms exerting these effects were investigated. Activation of the PI3K/Akt signalling cascade can occur through various upstream pathways coupled to different cell surface receptors. Of these cellular receptors, IGF1R was of particular interest as its expression levels were significantly increased within the transcriptome of FhHDM-1 treated β-cells (Chapter 2). IGF1R and its' major ligands, IGF-1, and IGF-2, are essential to regulation of β-cell survival, mass, and function. Moreover, alpha helix peptides, such as CRAMP (270), LL37 (271) and GLP-1 (272), which are structurally similar to FhHDM-1, have all been demonstrated to interact with IGF1R and subsequently directly/indirectly activate PI3K/Akt signalling (270-272). However, despite these similarities in structure and activity, the data here suggests that IGF1R is not the binding partner/activating receptor for FhHDM-1 (Chapter 3).

Nonetheless, the treatment of β-cells with FhHDM-1 enhanced the production of IGF-2. Consequently, there was both increased abundance and phosphorylation of IGF1R in FhHDM-1 treated β-cells. Interestingly, this phosphorylation occurred at a tyrosine residue outside the major kinase activation domain of the receptor, at the Tyr1316 site, which is specifically associated with the recruitment of PI3K. Together, this suggests that FhHDM-1 likely induced the IGF-2/IGF1-R autocrine loop within the β-cells, thus initiating direct recruitment of PI3K to IGF1R, and subsequent activation of PI3K/Akt signalling, ultimately leading to promotion of β-cell survival and function. Importantly IGF-2 is not stored in intracellular vesicles within β-cells, which implied that FhHDM-1 was activating the transcription of IGF-2 to mediate its positive downstream effects. Therefore, the

transcriptional profile of FhHDM-1 treated β-cells, and more specifically, the post-transcriptional regulation governed by miRNAs, was investigated.

Analysis of miRNA gene regulation (Chapter 4) identified several miRNAs induced/inhibited by FhHDM-1 treatment that are associated with IGF-2/PI3K/Akt modulation. Correlated with the increase in IGF-2 secretion was the FhHDM-1 mediated upregulation of miR-483-3p and downregulation of miR-466i-5p, and miR-7689-3p. Associated with promotion of PI3K/Akt signalling was the FhHDM-1 induced enhancement of miR-124-3p, and inhibition of miR-30d-5p, miR-3470a, miR-677-5p, and miR-7033-5p. While these miRNAs and their respective gene targets are diverse, the biological pathways they impact parallel each other, and corroborate the positive cellular outcomes induced by FhHDM-1 previously described in Chapters 2 and 3.

Collectively, this study has provided new insights into the mechanisms by which FhHDM-1 putatively prevents T1D, namely by exerting direct effects on β-cells (Figure 5.1). Notwithstanding the additional experimental validation that has been detailed in the discussion section of each results chapter, a putative cascade of events has emerged from the data obtained in this PhD project. The interaction of FhHDM-1 with β-cells induces notable changes in the miRNA profile, correlating with increased expression, and secretion of IGF-2. It can then be assumed that this ligand binds to IGF1R, leading to an increased and prolonged abundance of IGF1R. This results in the phosphorylation of IGF1R at Tyr1316, thereby inducing the recruitment of PI3K. Subsequently, the PI3K/Akt signalling cascade is activated, culminating in the promotion of β-cell survival and metabolism, without induction of proliferation. As a result, the β-cells are protected from pro-inflammatory cytokine induced apoptosis, leading to preservation of β-cell mass/function, thus representing a putative mechanism by which T1D is prevented.

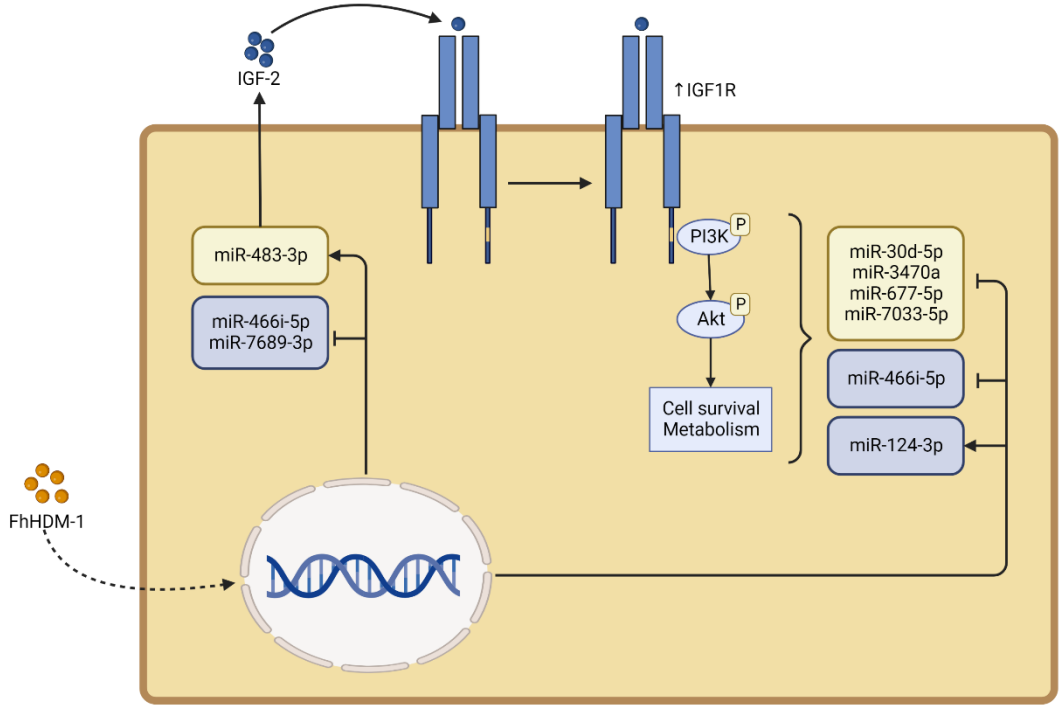



**Figure 5.1 Putative mechanism by which FhHDM-1 exerts beneficial effects within pancreatic β-cells.** FhHDM-1 is internalised into the β-cells via a mechanism yet to be elucidated, after which it impacts the expression of various miRNAs, of which the most significant are presented. FhHDM-1 induces the upregulation of miR-483-3p and downregulation of miR-466i-5p, and miR-7689-3p. Modulation of these miRNAs are associated with increased production of IGF-2. Consequently, IGF-2 binds to IGF1R, leading to (1) enhanced expression of IGF1R on the cell surface, and (2) phosphorylation of IGF1R at the Tyr1316, causing the recruitment and activation of PI3K, and subsequently Akt. Activated Akt then phosphorylates various downstream substrates, ultimately culminating in increased cell survival and metabolism, without induction of proliferation. Additionally, FhHDM-1 also positively modulates miR-124-3p, and downregulates miR-30d-5p, miR3470a, miR-677-5p, and miR-7033-5p, which are associated with activation of PI3K/Akt signalling. Note that miRNAs within yellow text boxes were identified from FhHDM-1 treated β-cells under basal conditions while those within blue text boxes were identified under apoptotic conditions induced by pro-inflammatory cytokines.

In addition to the maintenance of insulin secretion, the prevention of β-cell apoptosis by FhHDM-1 may putatively positively impact the initiation of the pro-inflammatory innate immune responses that drive the onset of T1D. Specifically, intra-islet macrophages are in intimate contact with β-cells, where there is communication between the two via the release of signals (such as cytokines, chemokines, hormones, growth factors, and exosomes) and through responses to cues from the microenvironment (Chapter 1). This intercellular crosstalk has emerged as a key determinant of the fate of β-cells, be it normal development and preservation of β-cell mass/function, or development of a pro-inflammatory environment leading to the initiation of autoimmunity, and the development of diabetes. The enhanced β-cell survival mediated by FhHDM-1 as described here has the potential to alter the communication with macrophages such that the differentiation of pro-inflammatory/M1-like macrophages would not occur. Apart from this effect, it has been previously reported that FhHDM-1 also directly regulates the activation of macrophages, reducing their ability to respond to pro-inflammatory ligands, indicating an inhibition in the predominance of pro-inflammatory M1-like characteristics (151). This multimodal effect of FhHDM-1 could beneficially modulate the conversation between macrophages and β-cells, offering a unique strategy to positively modulate β-cell survival while simultaneously inhibiting pro-inflammatory macrophage responses, and thus promoting preservation of β-cell mass and prevent/ameliorate diabetes.

Further studies can validate this exciting possibility. Pancreatic β-cells can be treated with FhHDM-1, then exposed to various diabetogenic agents, such as pro-inflammatory cytokines, high glucose, and streptozotocin. The supernatants from these β-cells can then be analysed by proteomic array, and used to stimulate macrophages, after which cytokine secretion and gene expression can be analysed, to determine if M1-like characteristics are inhibited. Conversely, supernatants from macrophages treated with FhHDM-1 and diabetogenic agents can be analysed then used to stimulate β-cells, after which β-cell viability and glucose responsiveness can be assessed, to determine if β-cell survival is enhanced. Lastly, to determine whether cell contact is required for intercell crosstalk, β-cells can be co-cultured with macrophages along with FhHDM-1 and diabetogenic stimuli, followed by measurement of β-cell viability and quantification of insulin uptake by macrophages.

In recent years, a mechanistic framework has emerged suggesting that the regulation of the host immune status by helminths is mediated by a shift in metabolic pathways within both the infected tissue, and in organs beyond the location of the parasite (17). This

'reprogramming' of the host's metabolism not only drives immune regulation, but also impacts non-immune cells; specifically altering the production of pancreatic hormones, which manifests as a significant increase in insulin sensitivity in helminth infected humans and mice (160, 165). This has led to the proposition that helminths represent a new class of biologics to alleviate inflammatory diseases and metabolic disorders. However, the application of live parasite infection as a treatment strategy is problematic for many reasons. For instance, delivery of an accurate dose is challenging as there is no reliable way to predict the number of eggs that will hatch into viable worms within the intestine of the recipient. Live helminth infection can cause pathology due to the migratory and feeding activities of the parasitic worms. As it is necessary to harvest helminths from another mammalian host, there is also a risk of inadvertent contamination with other pathogens. Additionally, administration of worms is distasteful and so patient compliance is often poor. Therefore, research has shifted to identification of the individual molecules produced by the parasites that mediate these positive effects. FhHDM-1 is the first parasite peptide shown to have the dual biological activities of interest, interacting with both immune cells (macrophages) and endocrine cells, specifically the pancreatic β-cells, thus establishing it as a first in class peptide therapeutic.

It would now be of interest to determine if this dual activity is specific only to FhHDM-1, or if it is present in other members of the parasite-derived HDM family. The HDM peptides are uniquely expressed by trematodes (flatworms) with no equivalent sequences found within the genomes of other helminths (nematodes or cestode) or mammals (120), suggesting highly specific biological activity. Phylogenetic analysis has revealed the existence of two distinct branches, one classified as Fasciola-like HDMs, and the second, which comprises peptides of a larger molecular weight and exclusively produced by the Schistosomatoidea (131). The *Fasciola*-like HDM branch currently contains HDMs from *Fasciola hepatica*, *Echinostoma caproni*, *Clonorchis sinensis,* and *Opisthorchis vivverrini*, which cluster together. It also contains HDMs from various species of the Schistosome family, although these are found on a separate extended branch (131). All the HDM peptides display the same structural components; an alpha helix and an amphipathic C-terminal, both of which are required for the immune-modulatory activity (344). As this structure is also common to peptides previously shown to interact with β-cells and enhance survival (as mentioned in Chapter 3), there is a clear relationship between structure and function, which suggests a common biological activity across the HDM family. Biochemical investigation of

this structure/function association of the HDM family peptides, through the production and functional analyses of mutants and/or truncated peptides, would provide insight into whether a specific amino acid sequence or structure are fundamental to the multimodal biological effect. These analyses would also determine if the biological activity of FhHDM-1 is analogous in macrophages and β-cells.

In addition, a better understanding of the structure/function relationships would assist in characterising the cellular interaction of the HDM peptides. The data presented in this project suggested that IGF1R is not the binding partner for FhHDM-1, an unexpected result given that the activation of the receptor's downstream pathways in the presence of FhHDM-1. However, this finding corroborates a previous study indicating that FhHDM-1 may not interact with a protein receptor, as removal of proteins from the cell membrane by trypsin cleavage did not significantly reduce FhHDM-1 binding (344). FhHDM-1 binds specifically within the lipid rafts of mammalian cell membranes (344). This is of interest as lipid rafts are a specialised microdomain in cell membranes that play a central role in facilitating signal transduction from membrane receptors, specifically enabling the interaction between cellular receptors and downstream signalling components, such as IGF-1/IGF1R (345, 346) and PI3K/Akt (346, 347). The lipid composition of the membrane rafts appears to be the controlling factor, as alterations in the abundance/availability of cholesterol influences the signal transduction from IGF1R (348-350). *In vitro* biochemical assays showed that FhHDM-1 had the capacity to bind to cholesterol, which suggests that this interaction could mediate enhanced activation of IGF1R signalling in β-cells. This proposal will need to be further investigated.

The work presented here, in combination with previous studies, indicates that FhHDM-1 warrants further investigation as a candidate in the enduring search for a T1D cure. FhHDM-1 is a naturally occurring peptide (not unlike insulin) that has evolved over millennia of co-evolution with mammalian hosts to be well tolerated by humans. Potentially, FhHDM-1 could be administered at multiple stages in the pathogenic process of T1D. In individuals who carry susceptibility alleles, FhHDM-1 could be delivered to prevent the initiation of autoimmunity to preserve β-cell mass and function prior to any β-cell loss. In individuals who have seroconverted, the significant, residual β-cell mass could be maintained. At diagnosis when patients enter the honeymoon period and the remaining β-cells can transiently maintain normoglycaemia, FhHDM-1 could be employed to extend β-cell survival. Finally, for TID patients undergoing islet transplantation, FhHDM-1 would

promote β-cell survival and function during islet preparation and post-transplant. Putatively, FhHDM-1, or derivatives thereof, would have therapeutic benefit for T2D in which inflammation compromises β-cell function.

Never has a single, small, naturally occurring peptide shown efficacy across multiple scenarios in which preservation of β-cell function and mass is required. Importantly, FhHDM-1 exerts positive effects on β-cell survival and metabolism without ultimately inducing β-cell exhaustion and death, and it does not induce β-cell proliferation. Testament to these unique qualities, FhHDM-1 permanently prevents T1D in a murine model and preserves β-cell survival and function of human islets. Lastly, being a small protein, FhHDM-1 would be amenable to optimisation, and recombinant or synthetic production. Altogether, FhHDM-1 represents a potential cure to a compilation of diseases (T1D and T2D) that are the fastest growing and most chronic conditions worldwide. Insulin was the first peptide therapeutic in man – and now, over 100 years later, we may have another naturally occurring peptide to take its place

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# Appendix

# Targeting the PI3K/Akt signalling pathway in pancreatic β-cells to enhance their survival and function; an emerging therapeutic strategy for type 1 diabetes

**Running Title: Targeting PI3K/Akt signalling in β-cells**

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**Abstract**

Type 1 diabetes (T1D) is an autoimmune disease caused by the destruction of the insulin-producing beta (β)-cells within the pancreas. Islet transplantation represents one cure, however during islet preparation and post-transplantation significant amounts of β-cell death occur. Therefore, prevention and cure of T1D is dependent upon the preservation of β-cell function and the prevention of β-cell death. PI3K/Akt signalling represents a promising therapeutic target for T1D due to its pronounced effects on cellular survival, proliferation, and metabolism. A growing amount of evidence indicates that PI3K/Akt signalling is a critical determinant of β-cell mass and function. Modulation of the PI3K/Akt pathway, directly (via the use of highly specific protein and peptide-based biologics, excretory/secretory products of parasitic worms, and complex constituents of plant extracts) or indirectly (through miRNA interactions) can regulate the β-cell processes to ultimately determine the fate of β-cell mass. An important consideration is the identification of the specific PI3K/Akt pathway modulators that enhance β-cell function and prevent β-cell death without inducing excessive β-cell proliferation, which may carry carcinogenic side effects. Among potential PI3K/Akt pathway agonists, we have identified a novel parasite derived protein, termed FhHDM-1, which efficiently stimulates the PI3K/Akt pathway in β-cells to enhance function and prevent death without concomitantly inducing proliferation unlike several other identified stimulators of PI3K/Akt signalling. As such FhHDM-1 will inform the design of biologics aimed at targeting the PI3K/Akt pathway to prevent/ameliorate not only T1D, but also T2D, which is now widely recognised as an inflammatory disease characterised by β-cell dysfunction and death. This review will explore the modulation of the PI3K/Akt signalling pathway as a novel strategy to enhance β-cell function and survival.

**KEYWORDS: Type 1 diabetes, β-cell, PI3K/Akt, FhHDM-1**

**Highlights**

PI3K/Akt signalling is an emerging promising therapeutic target for diabetes. Modulation of this pathway, via protein and peptide-based biologics, parasite-derived molecules, plant extracts, or through miRNA interactions, will regulate the processes that determine the function of β-cells and the fate of β-cell mass. In doing so, such new therapeutic approaches will have broad-reaching applications for the prevention of Type 1 diabetes, the preservation of islets pre- and post-transplantation, and the treatment of Type 2 diabetes.

## INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease caused by the destruction of the insulin-producing beta (β)-cells within the pancreatic islets of Langerhans.[1] Multiple immune cell subsets contribute to the elimination of β-cells. Neutrophils, macrophages and T helper (Th)1 and Th17 $CD4^+$ T cells produce pro-inflammatory cytokines and other cytotoxic mediators, which can directly destroy β-cells and/or prime autoreactive $CD8^+$ cytotoxic T cells. The initiation and amplification of immune responses against β-cell autoantigens occurs during an extensive, yet silent, preclinical period, which precedes the onset of clinical symptoms (notably hyperglycaemia) by months to years. Initiation of the destructive autoimmune processes likely begins early in life, concurrent with waves of β-cell neogenesis (formation of β-cells from non-β-cell precursors), proliferation and apoptosis, which occur during a physiological phase of neonatal pancreatic remodelling. Perturbations in the balance between these proliferative and apoptotic processes within β-cells can have pathogenic consequences. Specifically, increased rates of β-cell apoptosis, (perhaps coupled with impaired clearance of dying cells by macrophages), can lead to the generation of autoantigens,[2,3] which activate autoreactive pro-inflammatory Th1 and Th17 cells along with cytotoxic T cells. Following the destruction of 80-90% of the β-cell population, the patient becomes irreversibly hyperglycaemic and must rely on exogenous insulin to survive. However, insulin injections cannot mimic the minute-to-minute glucose responsiveness of β-cells. Therefore, the patient is subject to episodes of hypoglycaemia and hyperglycaemia, which increase morbidity and mortality.[1] Preservation of the survival and metabolic activities of β-cells is required to prevent and cure T1D.

## TARGETING β-CELLS TO TREAT TYPE 1 DIABETES

The progressive loss and dysfunction of pancreatic β-cells is the key pathogenic process of T1D. Testament to this, for several months up to a year after diagnosis over half of T1D patients experience a ‘honeymoon period’ in which the remaining β-cell mass produces sufficient insulin to maintain normoglycaemia. However, this residual β-cell population is ultimately destroyed by autoreactive immune cells and their mediators. Similarly, after islet transplantation, recurrent autoimmunity eliminates the allograft in the absence of chronic immunosuppression, which carries multiple adverse side effects, including β-cell death. Additionally, significant amounts of β-cell destruction occur during the preparation of islets for transplantation. This demands the availability of 2-3 cadaver pancreata to revert a single patient to normoglycaemia, and reversion is often transient due to subsequent β-cell

destruction. Therefore, prevention and cure of T1D is dependent upon the preservation of β-cell function and the prevention of β-cell death.

Despite the knowledge that β-cell death underpins T1D, none of the currently used anti-diabetic agents directly target the maintenance of endogenous β-cell mass. This is because the therapeutic focus has been on blocking the pro-inflammatory autoimmune responses to halt the β-cell destructive autoimmune sequelae in T1D patients, and those predisposed to T1D. These strategies rely upon modulation of immune cell populations, and do not directly impact β-cells to maintain function and/or prevent apoptosis. Testament to these shortcomings, diabetes reversal is not achieved, protection of residual β-cell mass is short-term, global immune suppression is induced, and multiple adverse side effects are experienced.[4-6] With an understanding that it is the dysregulation in the rates of β-cell proliferation versus apoptosis early in life that serve as pivotal initiators of autoimmunity, and that T1D development proceeds as autoreactive immune cells perpetuate the destructive responses,[7-9] regulation of these processes in β-cells, could effectively maintain sufficient β-cell mass to halt T1D development. Given the limitations of current T1D therapies, it is crucial to investigate more direct strategies to preserve β-cell mass and/or promote β-cell function.

One pathway that is emerging as a central process to the development, and therefore prevention, of T1D is the PI3K/Akt signalling pathway. PI3K/Akt signalling has long been recognised as a major regulator of important cellular processes, such as survival, proliferation, lipid metabolism, protein synthesis, glucose homeostasis, and apoptosis. It is now apparent that this holds true for the target cells destroyed in T1D, the pancreatic β-cells. Indeed, a growing body of evidence indicates that PI3K/Akt signalling is a critical regulator of β-cell processes that determine the fate of β-cell mass, including proliferation, survival, metabolism, and apoptosis. This review will explore the modulation of the PI3K/Akt signalling pathway as a novel strategy to preserve β-cell function and avoid β-cell death to prevent/ameliorate T1D.

## THE PI3K/Akt SIGNALLING PATHWAY

Phosphoinositide-3-kinase (PI3K) belongs to a family of kinases that catalyse the phosphorylation of inositol lipids. There are three PI3K classes (denoted I, II and II), which are differentiated by structure and substrate specificity. Class I is further subdivided into Class IA PI3K and Class IB PI3K. The former are dimers of a p110 catalytic subunit (isoforms: p110α, p110β, p110δ) and a p85 regulatory subunit (isoforms: p85α, p85β, p55α,

p55γ and p50α), while the latter are dimers of p100γ catalytic subunit, with a p101 or p87 regulatory subunit.[10,11] In the context of β-cell survival and function in T1D, Class I PI3Ks are the most relevant and will herein be referred to as "PI3K".

Generally, PI3K is activated by the binding of extracellular growth ligands to their cognate receptors, including receptor tyrosine kinases (RTKs), cytokine receptors, B cell and T cell receptors, integrins, and G-protein-coupled receptors. After ligand/receptor interaction, PI3K binds to the receptor, either directly (via its regulatory subunit), or indirectly (through adapter molecules, such as the insulin receptor substrate [IRS] proteins), thereby causing PI3K activation (Figure 1). Additionally, direct or indirect activation of PI3K can be mediated by the catalytic subunit (p110) in conjunction with small GTPases, notably Ras and Ras-related protein Rab-5A.[12,13]

Once activated through its catalytic subunit, PI3K converts phosphatidylinositol (3,4)-bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-trisphosphate (PIP3), to which Akt (also known as protein kinase B [PKB]) then binds. This allows phosphoinositide dependent protein kinase 1 (PDK1) to phosphorylate Akt at its Thr308 site. This event causes partial activation of Akt. Subsequently, the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) becomes activated and interacts with multiple downstream substrates. For full activation, Akt must be phosphorylated at the Ser473 site, which is catalysed by mTOR complex 2 (mTORC2) or DNA-dependent protein kinase (DNA-PK). Once fully activated, Akt can induce further substrate-specific phosphorylation events in both the cytoplasm and nucleus.[11,14]

The precise function of Akt is determined by the specific context of its phosphorylation, including factors such as isoforms and different phosphorylation sites. There are three conserved isoforms of Akt: Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ. Akt1 and Akt2 are expressed ubiquitously, while Akt3 is highly expressed in nervous tissue. Evidence from mouse models suggests that these isoforms are associated with distinct functions in various cells.[11,15] Furthermore, Akt activation is modulated by phosphorylation at different sites, aside from the major sites Thr308 and Ser473. Indeed, isoforms Akt1, Akt2 and Akt3 have 31, 22 and 18 known potential phosphorylation sites, respectively.[16] It is the large number of potential phosphorylation sites in Akt, and its ability to catalyse the phosphorylation of multiple substrates, that facilitates the involvement of fully activated Akt in multiple and varied cellular events.

Activated Akt can have an inhibitory or stimulatory effect, depending on the target substrate and the consequent phosphorylation events, thereby resulting in the modulation of

several cellular processes, such as glucose metabolism, apoptosis, proliferation, gene transcription, and migration.[11,14] For instance, Akt exerts an inhibitory effect on forkhead box protein O (FOXO) to prevent its pro-apoptotic and catabolic activities.[11,17] Akt also represses glycogen synthase kinase 3 (GSK3β), leading to inhibition of GSK3β-mediated apoptosis and glycogen synthase.[18] Akt can also directly inhibit the activity of the pro-apoptotic proteins, caspase-9 and Bcl-2-associated death promoter (Bad), thereby promoting cell survival. Collectively, activated Akt modulates the functions of its substrates to promote cell proliferation, survival and/or metabolism, while simultaneously inhibiting apoptosis.[11,19]

This pro-survival pathway is tightly controlled by key negative regulators, such as the phosphatase and tensin homolog (PTEN), which antagonises Akt signalling by dephosphorylating the products of PI3K activity (i.e. reverting PIP3 to PIP2). Protein phosphatase 2 (PP2A) and PH-domain leucine-rich-repeat-containing protein phosphatases (PHLPP1/2) also suppress Akt activity by dephosphorylating Akt at the Thr308 or Ser473 sites, respectively.[11,14] The tight regulation of PI3K activities enables the optimal balance between pro-survival/proliferative/metabolic and apoptotic events to be achieved.

## TRANSGENIC MOUSE MODELS PROVIDE EVIDENCE THAT PI3K/AKT SIGNALLING MODULATES β-CELL MASS AND FUNCTION

Experiments using transgenic murine models, in which specific components of the PI3K/Akt pathway have been overexpressed in β-cells, have provided direct evidence that PI3K/Akt signalling is a critical determinant of β-cell mass and function. Overexpression of the constitutively active form of Akt1 (CA-Akt) in β-cells *in vivo* induced a significant increase in β-cell size and total islet mass, with resultant improved glucose tolerance, which protected mice against streptozotocin (STZ)-induced diabetes (multiple low doses of 40mg/kg body weight for 5 consecutive days, which causes autoimmune diabetes).[20] Given that the rate of β-cell proliferation remained unchanged, it was concluded that disease protection was associated with an Akt-mediated preservation of β-cell mass and increased metabolic activity, specifically the anabolic processes that drive cell growth. Similar maintenance of islet mass and glucose tolerance were observed in an equivalent CA-Akt mouse model, which also exhibited resistance to β-cell death ordinarily rapidly induced by higher doses of STZ.[21] Interestingly, in contrast to the original CA-Akt model, protection against diabetes was attributed to enhanced β-cell proliferation (and putatively β-cell neogenesis) resulting in increased islet mass, which counteracted the rates of β-cell destruction.[21] Thus, modulation of

the PI3K/Akt signalling, via increased expression levels of CA-Akt, positively regulated β-cell mass and function to prevent diabetes. The enhancement of β-cell proliferation, while effective in the short term to negate β-cell destruction, must be monitored to ensure that pancreatic carcinogenesis does not ensue.

Likewise, expression of constitutively active epidermal growth factor receptor (CA-EGFR, a RTK that preferentially stimulates PI3K/Akt signalling), at a time coincident with neonatal pancreatic remodelling, increased rates of β-cell proliferation in mice. This positive effect on β-cell growth was not observed in adult mice, suggesting that the modulation of β-cell mass dynamics, and prevention of T1D development, is most effectively achieved early in life.[22] This is an interesting observation, given that the physiological wave of neonatal islet remodelling is juxta-positioned with the initiation of autoimmunity in susceptible animal models, such as nonobese diabetic (NOD) mice, with concomitant increased rates of β-cell apoptosis and trafficking/infiltration of autoreactive immune cell populations to the islets (insulitis).[2,3,7] Nevertheless, in adult mice, CA-EGFR expression improved glucose tolerance and significantly inhibited β-cell apoptosis following either a single high dose (200mg/kg body weight) or multiple low doses (50mg/kg body weight daily for 5 consecutive days) of STZ, thereby conferring partial protection against diabetes development. Further, islets isolated from diabetes-resistant (CA-EGFR overexpression) animals were resistant to the cytotoxic effects of the pro-inflammatory cytokines (IL1β, TNF and IFNγ) that are primarily responsible for β-cell destruction in T1D. The protective mechanism was likely attributable to PI3K/Akt-mediated inhibition of the pro-apoptotic protein, Bcl-2-interacting mediator of cell death (BIM), which facilitates cytokine-induced β-cell apoptosis during T1D development.[22]

Indirect modulation of the PI3K/Akt pathway has also been achieved by expression of the caspase-3-generated RasGAP N-terminal fragment (fragment N), a molecule that exerts an anti-apoptotic effect via activation of Ras-PI3K-Akt signalling in various cell types,[23] including insulin secreting cells. This is supported by *in vivo* studies utilising a transgenic NOD mouse model in which fragment N was expressed specifically within the β-cells. These mice exhibited slower progression to hyperglycaemia and overt diabetes, as compared to non-transgenic NOD controls. Further *in situ* studies of islets isolated from these transgenic animals at 16 weeks of age (when significant rates of β-cell apoptosis would normally be occurring) showed that fragment N expression was associated with reduced numbers of apoptotic β-cells, as compared to controls. This anti-apoptotic effect was attributable to the activity of Ras, which, in turn, activated PI3K/Akt signalling.[24]

Consistent with these observations, deletion of the key negative regulator of the PI3K/Akt pathway, PTEN, in β-cells has also been shown to provide protection against STZ-induced diabetes.[25] *In vitro* studies using the murine β-cell line, β-TC-6, showed that silencing of PTEN conferred resistance to cytokine-induced apoptosis and partially reversed Akt inhibition.[26] Collectively, these observations provide compelling evidence that activation of the PI3K pathway in β-cells has the potential to positively modulate β-cell function to prevent diabetes development.

## MODULATORS OF PI3K/Akt SIGNALLING ARE POTENTIAL THERAPEUTICS

### Proteins and peptide-based biologics

The use of protein or peptide-based agents are now being extensively studied for their ability to stimulate β-cell proliferation/function and/or inhibit β-cell apoptosis, specifically through modulation of PI3K/Akt signalling (Table 1). Treatment of NOD-derived NIT-1 β-cells with erythropoietin (EPO) suppressed pro-inflammatory cytokine-induced apoptosis, and improved insulin secretion, in a PI3K/Akt dependent manner.[27] Similarly, the protein Wnt3a was found to exert an equivalent effect in NIT-1 β-cells. In this case, activation of the Wnt/β-catenin pathway resulted in cross-talk with the PI3K/Akt pathway,[28] to promote β-cell proliferation and to suppress β-cell apoptosis, resulting in increased islet mass and improved β-cell function.[29,30] Although these outcomes are encouraging, it should be noted that the assessments of these proteins were performed *in vitro* with no evidence of efficacy *in vivo*. It is also important to consider that neither EPO nor Wnt3a specifically interact with β-cells, with receptors for both proteins expressed by multiple cell types.[31-33] The administration of either of these proteins may therefore have non-specific off-target effects. This risk is already evident for Wnt3a, as the activation of Wnt/β-catenin signalling initiates and maintains several cancer states,[32,34] which would likely preclude its value as a treatment for diabetes.

Circumventing the issue of cell specificity, is the exploitation of the gut-derived incretin, glucagon-like-peptide-1 (GLP-1), which is naturally produced in response to food ingestion to enhance insulin secretion, through interaction with its receptor which is expressed only on β-cells (and neurons). Specifically, GLP-1 has been reported to promote proliferation and inhibit apoptosis of β-cells, *in vivo* and *in vitro*, and to attenuate apoptosis induced by several pro-apoptotic agents (such as STZ and reactive oxygen species [ROS]).[35,36] Chang et al (2016) showed that GLP-1 suppressed methylglyoxal (MG)-induced apoptosis in a rat insulinoma cell line (RINm5F), by improving mitochondrial function and activating PKA and PI3K/Akt signalling, thereby causing Akt phosphorylation and

subsequent inhibition of the cleavage (i.e. activation) of pro-apoptotic caspase-3. These effects were corroborated by experiments using MIN6 and INS-1 β-cells.[37] In addition to enhancing proliferation and survival, GLP-1 has been recently shown to putatively promote β-cell neogenesis in a severe insulin deficient diabetic rat model induced by administration of a single high dose of STZ. Indeed, the consequent increase in β-cell number following GLP-1 treatment was not attributable to β-cell replication, but rather to alpha (α) cell dedifferentiation and subsequent transdifferentiation into glucose responsive insulin secreting β-cells. This was associated with the regulation of the GLP-1 receptor and its downstream transcription factor pathway, PI3K/Akt/FOXO1.[35]

While these beneficial effects of GLP-1 strongly support its use as a therapeutic agent, it is rapidly degraded *in vivo* ($t_{1/2}$ ~ 2min) by the endogenous enzyme, dipeptidyl-peptidase-IV (DPP-4).[38] To extend bioavailability to improve clinical efficacy, several synthetic GLP-1 receptor agonists have been developed. For instance, the native molecule exendin-4 has been shown to enhance β-cell proliferation and inhibit apoptosis, both *in vitro* and *in vivo*, thereby increasing β-cell number and mass, respectively. These effects were abolished in the presence of the PI3K inhibitor, LY294002, establishing that the pro-survival/proliferation effect was mediated by PI3K/Akt signalling in β-cells.[39] Likewise, the human GLP-1 analogue, liraglutide, has been shown to promote β-cell proliferation and inhibit apoptosis, both *in vitro* and *in vivo*. Indeed, in BTC6 β-cells, liraglutide suppressed apoptosis induced by serum withdrawal through PI3K/Akt phosphorylation, leading to the inhibition of caspase-3 activity, via a mechanism like that of GLP-1. The downstream Akt targets, pro-apoptotic Bad and FOXO1 transcription factor, also underwent inhibitory phosphorylation. These observations were corroborated in an animal model of overt diabetes where liraglutide restored islet size, ameliorated β-cell apoptosis, and increased expression levels of nephrin,[40] a key protein involved in β-cell survival signalling.[41] Despite the positive effects on β-cell survival *in vivo*, there are some concerns regarding the safety profile of these GLP-1 analogues due to their ability to enhance the proliferation of cells. While a consensus has not been reached, evidence from some clinical studies have shown an expansion of exocrine and endocrine pancreatic cells with a possible association to pancreatic cancer.[42]

As an alternative biologic, γ-aminobutyric acid (GABA), may be a candidate therapeutic agent. It is endogenously produced in β-cells and has been found to stimulate β-cell proliferation in mouse and human islets through the PI3K/mTORC1 pathway. Co-treatment of mice with both GABA and Ly49, a novel GABA type A receptor positive

allosteric modulator, amplified these positive effects. Furthermore, co-treatment with GABA and Ly49 increased β-cell area and proliferation, as compared to mice treated with GABA alone, and similar observations were made using human islets.[43] Importantly, a number of $GABA_A$ receptor agonists are currently in clinical use as a treatment for epilepsy,[44] which suggests that they are considered safe for use in humans and so could potentially be tested in clinical trials as a treatment for T1D.

**The secreted products of parasitic worms**

Numerous epidemiological studies and experimental investigations have now clearly demonstrated an inverse relationship between infection with parasitic worms (helminths) and the incidence of autoimmune diseases, such as T1D.[45,46] The beneficial effect of a parasite infection in preventing autoimmunity has been explained by the "old friends' hypothesis".[47] This postulates that because the normal protective immune response mounted in response to pathogens is largely inefficient against large multicellular worms, humans have evolved, over millennia of co-existence, to tolerate the presence of helminths. This outcome is supported by the potent modulation of human immune responses by the parasites. The net result is the suppression of anti-microbial pro-inflammatory responses, which are of the same phenotype associated with the development of T1D (i.e. pronounced Th1 and Th17 immune responses).[48] Testament to this, the development of T1D may indeed be driven by some forms of bacteria.[49] Accordingly, the removal of helminth exposure, due to improved hygiene and medical practices, has been associated with an increased risk of inappropriate immune responsiveness to autoantigens, and the development of autoimmunity.[48] Therefore, controlled helminth parasite infection, or exposure to their excretory/secretory products, has been investigated as a therapeutic strategy to prevent and/or ameliorate immune-mediated diseases.[50,51]

With the assumption that the capacity for helminths to modulate host immune responses is the primary mechanism by which these parasites are preventing disease, helminth secretions have been specifically mined for immune-modulatory molecules to be used as therapeutic modalities. While several agents have been identified,[51] we have recently discovered that the helminth defence molecule 1 secreted by the liver fluke *Fasciola hepatica* (FhHDM-1) has a unique capacity to directly target β-cells. This presents an opportunity for the development of a novel therapeutic for treating diabetes that has been pharmacologically optimised through the co-evolution of a parasitic worm and its human hosts.

Administration of FhHDM-1 via intraperitoneal injection prevented the onset of T1D in NOD mice.[52,53] Importantly, only a short-course of FhHDM-1 treatment was required, with mice presenting as non-diabetic up to 26 weeks after the final injection (experimental endpoint). While the half-life of FhHDM-1 has not been determined, this outcome would suggest that the biological effect is long-lasting, which provides a positive pharmacological comparison to GLP and its analogues. Disease protection in FhHDM-1 treated mice was associated with decreased insulitis (likely indicative of decreased β-cell apoptotic rates, and hence auto-antigen generation) and increased levels of pancreatic insulin (suggesting that FhHDM-1 preserved β-cell mass against an aggressive autoimmune background), as compared to vehicle controls. Further, biodistribution studies showed that FhHDM-1 localised to the pancreas, and *in vitro* FhHDM-1 directly interacted with β-cells, of both murine and human origin, to enhance viability and prevent apoptosis without inducing β-cell proliferation. These positive effects on the β-cells were associated with the activation of PI3K/Akt signalling, as revealed by proteomics and RNAseq analysis. The inhibition of the PI3K/Akt pathway reversed the protective effects of FhHDM-1 on β-cells, confirming a functional role for this pathway in mediating the positive effect of the peptide.[54]

Interrogation of numerous parasite and mammalian genomes has established that the HDM peptide family is unique to flatworms[55,56], suggesting an adaptation for a specific biological function while they reside within their mammalian hosts. Testament to this host-parasite relationship, FhHDM-1 is not cytotoxic to mammalian cells[57], and it does not induce any adverse activity in a broad range of clinically relevant pharmacology assays (unpublished data). Therefore, FhHDM-1 is highly selective and efficacious in its ability to preserve β-cell mass, without inducing proliferation, while simultaneously being safe and well tolerated. Collectively, these properties make FhHDM-1 an innovative candidate for maintaining β-cell survival and function against the inflammatory backgrounds underlying T1D.

**Plant extracts**

The healing properties of medicinal herbs has been applied for therapeutic intervention since the dawn of human history. Today, hundreds of plants have been reported to exert positive effects for the treatment of diabetes, by reducing blood glucose levels and decreasing the occurrence of the complications caused by hyperglycaemia.[58-60] However, there remains a lack of detailed information regarding the specific bioactive component(s) and the exact mechanism(s) of action through which many of these plants exert their therapeutic effect. Of the plant extracts that have been identified and tested specifically in models of T1D to date,

seven have been shown to exert their therapeutic effects via PI3K/Akt-mediated modulation of β-cell function (Table 1).[61-64] All these compounds commonly protected β-cells from cytotoxic stimuli (*in vitro;* pro-inflammatory cytokines[61,62] or high glucose,[63] and *in vivo;* pro-inflammatory cytokines[61] or ROS[64]). In all cases, apoptosis was actively inhibited, β-cells retained viability, and glucose-stimulated insulin secretion was restored.

**Modulation of miRNA expression**

MicroRNAs (miRNAs) are short, non-coding RNAs that are recognised as major regulators of gene expression through post-transcriptional mechanisms, thereby indirectly modulating diverse physiological and pathological processes.[65] Although miRNA therapeutics have not yet translated as approved therapies, many are in clinical trials, and the first small-interfering RNA was recently granted FDA approval, which signals the emergence of this therapeutic approach.[66] There is evidence of clinical application for both miRNA mimics (to target gene expression), as well as miRNA inhibitors (to enhance gene expression).

An exploration of the literature unsurprisingly reveals several miRNAs that directly or indirectly modulate PI3K/Akt signalling to exert positive effects on β-cell survival and function under conditions akin to T1D development (Table 1). Enhanced β-cell proliferation, induced by partial pancreatectomy in mice, was associated with an upregulation of miR-132 expression, which, in turn, indirectly activated PI3K/Akt signalling through inhibition of PTEN. Conversely, deletion of miR-132 *in vivo* inhibited β-cell proliferation. These findings were corroborated *in vitro* using MIN6 β-cells, wherein downregulation of miR-132 suppressed proliferation while concomitantly increasing levels of cleaved (apoptotic) caspase 9. On the other hand, overexpression of miR-132 produced the opposite effect through inhibition of PTEN, subsequent upregulation of phosphorylated Akt levels, and, thus, downregulation of its pro-apoptotic substrate, FOXO3. Therefore, targeting the miRNA mediated activation of PTEN/Akt signalling may serve as a therapeutic avenue to positively modulate β-cell mass.[67] This notion is further supported by the observation that β-cell specific deletion of miR-17-92 *in vivo* promoted the development of experimental autoimmune diabetes induced by multiple low doses of STZ, characterised by elevated fasting blood glucose levels and impaired glucose tolerance. These outcomes were attributable to reduced β-cell number and mass, and an increased incidence of β-cell apoptosis. In turn, these deleterious effects were associated with higher levels of PTEN expression, which supressed PI3K/Akt signalling.[68]

Conversely, the inhibition of specific miRNAs can enhance PI3K/Akt signalling to confer β-cell survival. For instance, under pro-inflammatory conditions induced by IL-1β treatment, MIN6 β-cells showed significant upregulation of miR-18 expression levels, which was associated with an increased incidence of apoptosis, and dysregulated insulin production and secretion in response to glucose. These deleterious effects on β-cell survival and function were associated with miR-18-induced repression of neuron navigator 1 (NAV1), a constituent of the PI3K/Akt pathway, resulting in the downregulation of pAkt and PI3K expression levels. Importantly, the knockdown of miR-18 generated the opposite outcomes, suggesting that miR-18 can be inhibited to promote PI3K/Akt signalling, and consequently ameliorate β-cell dysfunction and apoptosis.[69] Similarly, overexpression of miR-139-5p negatively regulated the expression levels of protein interacting with C-kinase 1 (PICK1), which normally provides functional protection of β-cells through PI3K/Akt activation.[70] Likewise, although their specific gene targets were not investigated, the inhibition of miR-122[71] or let-7 miRNA[72] resulted in the activation of PI3K/Akt signalling in β-cells, which mediated their protection from STZ-induced destruction *in vitro*[71,72] and *in vivo*.[72]

Collectively, the aforementioned studies indicate that upregulation of PI3K/Akt signalling through dialogue with various miRNAs can exert positive effects on β-cell function and survival and, as such, these miRNAs represent viable therapeutics/targets for the treatment of T1D.

## MODULATION OF PI3K/Akt IN β-CELLS HAS APPLICATIONS FOR TREATMENT OF T2D AND ISLET TRANSPLANTATION

While their etiopathologies are different, T1D and T2D are both pro-inflammatory disorders characterised by an ultimate decline in β-cell mass and function. In T1D, β-cell loss is mediated by a sustained process of autoimmune destruction that occurs over several months/years, such that at diagnosis β-cell mass is reduced by 80-90%. In T2D, β-cells undergo a compensatory expansion in response to insulin resistance, thereby causing β-cell exhaustion and death. Furthermore, genome-wide association studies of T2D show that most genes are modulators of β-cell mass and/or function. In addition, preservation of β-cell mass during islet preparation and after islet transplantation is critical and represents a major clinical hurdle to widespread application for patients. Therefore, approaches to preserve functional β-cell mass offers therapeutic potential for both T1D and T2D (Figure 2).[73]

Many of the therapeutic strategies targeting the PI3K/Akt pathway in β-cells that are described above for the treatment of T1D, have also been tested, and in some instances utilised, as T2D therapeutics because they modulate β-cell function to enhance glucose responsiveness (Table 2). While they are efficacious and generally well-tolerated, these treatments are limited by several shortcomings. As mentioned, GLP-1 analogues are hindered by a very short half-life,[38] adverse effects (the most common being gastrointestinal and injection site reactions),[74] and potential associations with pancreatic cancer due to the induction of cellular proliferation.[42] Metformin, is an alternatively prescribed treatment for T2D, which, has been shown to inhibit endoplasmic reticulum stress, dysfunction and apoptosis in NIT-1 cells via PI3K/Akt signalling.[75] However, metformin is contraindicated in patients with risk factors for lactic acidosis and has common side effects, such as gastrointestinal intolerance, although they are usually transient.[76] Thus, there is a continued clinical need for alternative therapeutic strategies. Like the reported efficacy in T1D, several plant extracts, proteins/peptides, and miRNAs have been identified with potential anti-diabetic effects in T2D. These agents have been shown to protect β-cells from injury and apoptosis *in vivo*, such as in high fat diet (HFD)[77] and HFD/STZ[78-80] murine models and i*n vitro*, under pro-inflammatory conditions following exposure to STZ[81] or inducers of oxidative[82,83] and endoplasmic reticulum stress.[75] They also exert beneficial β-cell effects under basal conditions by enhancing glucose stimulated insulin secretion.[84] All these positive effects were mediated by regulation of PI3K/Akt signalling and its associated pathways.

## CONCLUSION

PI3K/Akt signalling represents a promising therapeutic target for diabetes. Modulation of the pathway, singularly or through miRNA interactions, will aid in regulating the β-cell processes that determine the fate of β-cell mass. For instance, upregulation of PI3K/Akt signalling can promote β-cell function, survival, and/or proliferation, thereby counteracting the disproportionately large waves of apoptosis during neonatal pancreatic remodelling, at a time concurrent with the initiation of autoimmunity. Likewise, β-cell mass lost from autoimmune destruction may be restored or maintained by PI3K/Akt-mediated stimulation of β-cell survival, metabolic, and anti-apoptotic pathways. All these activities are crucial not only in T1D, but also in the context of T2D, which is now recognised as an inflammatory disease in which β-cell mass and function need to be preserved. Indeed, modulation of PI3K/Akt signalling can support exhausted β-cells to alleviate hyperglycaemia and insulin resistance. Therapeutic applications of enhancing PI3K/Akt signalling in β-cells could also be

extended to islet transplantation, to counteract the large amount of β-cell death that occurs throughout the process of isolation and delivery, and then post-transplant against allogeneic and autoimmune destruction.

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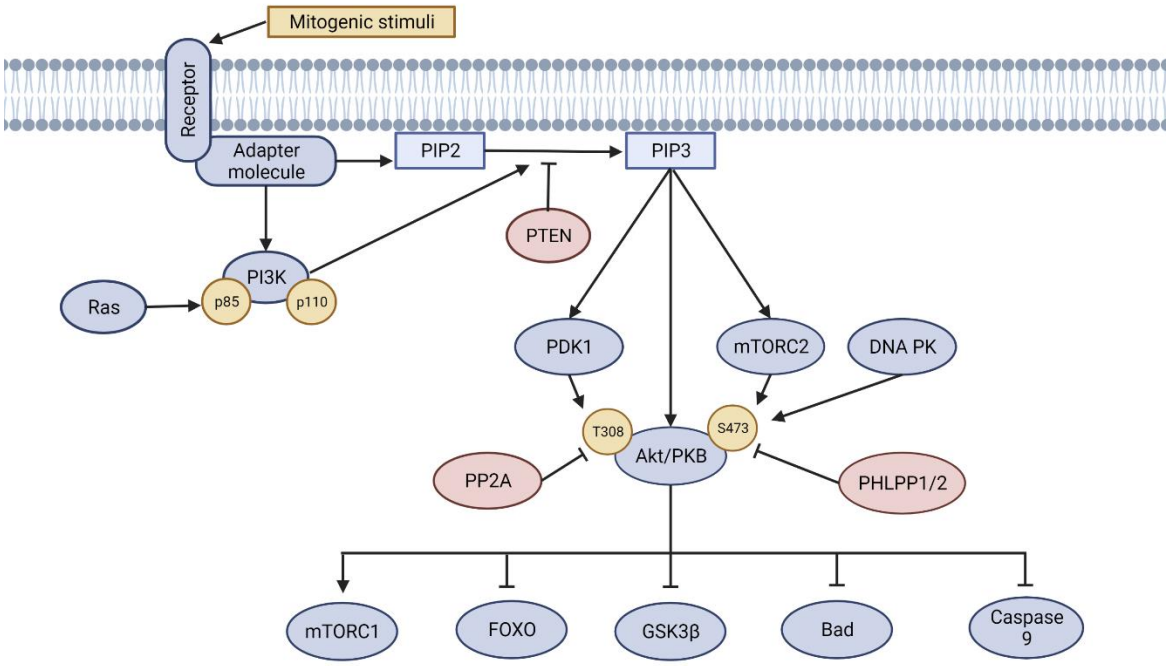


***Figure 1. Overview of PI3K/Akt activation and signalling.*** The PI3K/Akt cascade begins with extracellular mitogenic cues that stimulate various receptors, such as receptor tyrosine kinases, cytokine receptors, B cell and T cell receptors, integrins, and G-protein-coupled receptors. PI3K then binds to the receptor directly, or indirectly via adapter molecules (such as the insulin receptor substrate proteins), thus activating PI3K. Membrane-bound Ras can also activate PI3K. Subsequently, activated PI3K converts phosphatidylinositol (3,4)-bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Akt then binds to PIP3 at the plasma membrane, which allows phosphoinositide-dependent protein kinase 1 (PDK1) to phosphorylate Akt at the Thr308 site, thus causing partial Akt activation. This is sufficient to activate mammalian target of rapamycin (mTOR) complex 1 (mTORC1) becomes activated. The mTOR complex 2 (mTORC2) or DNA-dependent protein kinase (DNA-PK) must phosphorylate Akt at the Ser473 site for full activation. Activated Akt mediates the inhibitory or stimulatory phosphorylation of its various downstream targets, such as forkhead box protein O (FOXO), glycogen synthase kinase 3 (GSK3β), caspase-9 and Bcl-2-associated death promoter (Bad). Important negative regulators control this signalling cascade. Specifically, phosphatase and tensin homolog (PTEN), which dephosphorylates PIP3 into PIP2, and PH-domain leucine-rich-repeat-containing protein phosphatases (PHLPP1/2) that dephosphorylates Akt at the Thr308 or Ser473 sites, respectively. Created with BioRender.com.

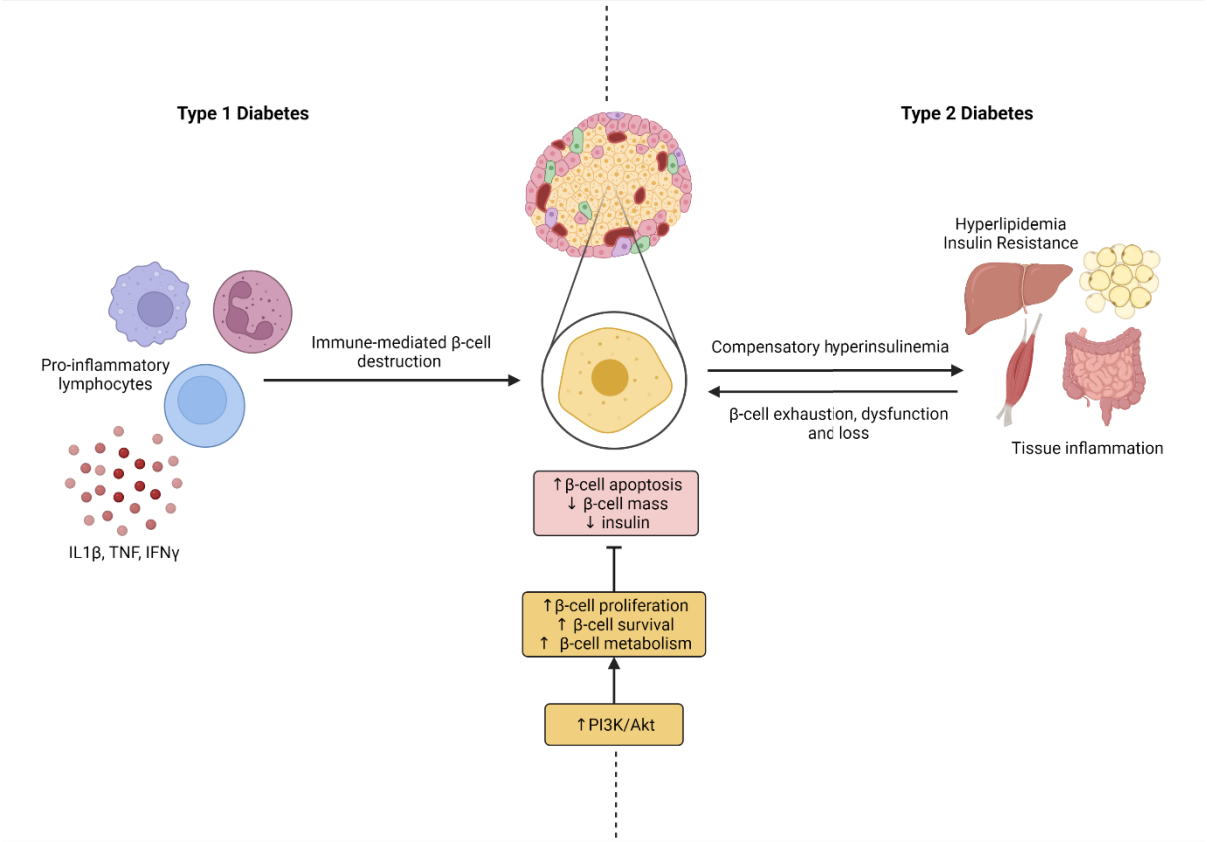


***Figure 2. The pathogenesis of type 1 (T1D) and type 2 diabetes (T2D) both ultimately lead to the decline of β-cell mass and function. Modulation of PI3K/Akt signalling can act as a therapeutic strategy to counteract β-cell loss.*** In T1D, autoreactive lymphocytes and pro-inflammatory cytokines (such as IL1β, TNF and IFNγ) drive β-cell destruction. In T2D, β-cells undergo compensatory expansion and increased insulin secretion in response to hyperlipidaemia and insulin resistance in the tissues, thus causing β-cell exhaustion and death. In both conditions, there is a decrease in β-cell mass and insulin secretion. This decline can be counteracted by activation of the PI3K/Akt pathway, which has been shown to promote β-cell proliferation, survival and metabolism. Created with Biorender.com

***Table 1. Modulators of PI3K/Akt signalling as putative therapeutics for Type 1 diabetes***

| Modulator | β-cell effect[†] | Reference |
|---|---|---|
| **Protein and peptide-based biologics** | | |
| **rEPO** | *In vitro*: Suppressed cytokine-induced[‡] apoptosis and improved insulin secretion through PI3K/Akt signalling in NIT-1 β-cells. Increased expression of downstream Akt target, anti-apoptotic Bcl2. | [27] |
| **Wnt3a** | *In vitro*: Enhanced β-cell proliferation, improved insulin secretion and inhibited cytokine-induced apoptosis via PI3K/Akt and Wnt signalling, in NIT-1 β-cells. | [29] |
| **GLP-1** | *In vitro*: Inhibited reactive oxygen species (hydrogen peroxide) induced apoptosis and enhanced cell survival in MIN6 cells, mediated by cAMP- and PI3K-dependent signalling pathway. | [36] |
| | *In vitro*: Suppressed methylglyoxal-induced toxicity, improved mitochondrial function and inhibited pro-apoptotic caspase-3 via PKA and PI3K/Akt signalling in RINm5F, MIN6 and INS-1 β-cells. | [37] |
| | *In vivo:* Increased β-cell number in a STZ-induced diabetic rat model, by promoting β-cell neogenesis through α-cell transdifferentiation into β-cells, via regulation of GLP-1 receptor and downstream transcription factor pathway, PI3K/Akt/FOXO1. | [35] |
| **Exendin-4** | *In vivo* and *in vitro*: Enhanced β-cell proliferation in C75BL/6 mice and isolated islets, leading to increased β-cell mass and number, respectively, in a PI3K/Akt dependent manner. | [39] |
| **Liraglutide** | *In vivo:* Restored islet size, prevented apoptosis and improved nephrin expression (involved in β-cell survival) in diabetic mice.<br>*In vitro*: Enhanced β-cell proliferation, inhibited serum withdrawal induced-apoptosis, suppressed caspase-3, and downregulated pro-apoptotic Bcl-2-associated death promoter (Bad) and forkhead box protein O1 (FOXO1) in BTC-6 cells through PI3K/Akt signalling. | [40] |
| **Nephrin** | *In vitro*: Stimulated recruitment of PI3K and activated PI3K/Akt signalling, in turn inhibiting downstream Akt substrates; pro-apoptotic Bad and FOXO, in mouse islets and BTC-6 cells. | [41] |

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| **GABA** | *In vivo* and *in vitro*: Enhanced β-cell proliferation in mouse and human islets through PI3K/mTORC1 pathway. This regenerative effect was amplified by co-treatment with Ly49, a GABA type A receptor positive allosteric modulator, which enhanced β-cell area and proliferation. | [43] |
| **FhHDM-1** | *In vivo:* Prevented the onset of T1D in NOD mice and increased the levels of pancreatic insulin, indicating preservation of β-cell mass.<br>*In vitro:* directly interacted with β-cells to enhance viability and prevent cytokine-induced apoptosis without inducing proliferation, through activation of PI3K/Akt pathway. | [54] |
| **Plant extracts** | | |
| **Puerarin** | *In vivo*: Suppressed STZ-induced diabetes and preserved β-cell mass, inhibited apoptosis and reversed hyperglycaemia in established diabetes.<br>*In vitro*: Conserved β-cell viability and insulin secretion following cobalt chloride-induced apoptosis, mediated by PI3K/Akt signalling. Increased expression of anti-apoptotic Bcl2 in MIN6 β-cells and primary islets. | [61] |
| **BAI** | *In vitro:* Suppressed TNF-induced apoptosis, enhanced insulin production and increased expression of anti-apoptotic Bcl2 and Bcl2-associated X protein in MIN6 β-cells, in a PI3K/Akt dependent manner. | [62] |
| **Saponins** | *In vitro*: Improved INS1 cell morphology, viability, and insulin secretion under conditions of glucotoxicity. These effects were associated with increased phosphorylation of Akt and decreased levels of FOXO1. | [63] |
| **C3G** | *In vivo*: Restored normoglycaemia after transplantation of C3G-treated neonatal porcine islets in diabetic mice.<br>*In vitro*: Inhibited reactive oxygen species toxicity via PI3K/Akt and extracellular signal-regulated kinase 1/2 signalling in neonatal porcine islets. | [64] |
| **miRNAs** | | |
| **miR-132** | *In vivo*: Increased β-cell proliferation and survival induced by partial pancreatectomy in mice was associated with enhanced miR-132 expression, which in turn indirectly activated PI3K signalling via PTEN inhibition.<br>*In vitro*: Downregulation inhibited MIN6 β-cell proliferation and increased cleaved caspase 9. Overexpression generated the opposite effects; it enhanced levels of phosphorylated Akt | [67] |

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| | and downregulated pro-apoptotic FOXO3. | |
| **miR17-92** | *In vivo*: Deletion in mice promoted the development of diabetes induced by multiple low doses of STZ, decreased β-cell number and mass, and increased apoptosis. These effects were associated with suppressed PI3K/Akt signalling due to increased PTEN expression. | [68] |
| **miR-18** | *In vitro*: Upregulated in MIN6 β-cells after exposure to pro-inflammatory cytokine stress and was associated with increased apoptosis and dysregulated insulin secretion. These effects were attributed to miR-18 induced repression of a component within the PI3K/Akt pathway (neuron navigator 1), thus downregulating pAkt and PI3K. Knockdown of miR-18 produced the opposite results. | [69] |
| **miR139-5p** | *In vivo*: Overexpression of miR139-5p in mice downregulated expression of protein interacting with C-kinase 1(PICK1), which normally protects β-cells via PI3K/Akt activation.<br>*In vitro*: Negative regulation by miR139-5p of PICK1 repressed PICK1-mediated activation of PI3K/Akt signalling. Overexpression of PICK1 protected β-cells from glucotoxicity via PI3K/Akt activation. | [70] |
| **miR-122** | *In vitro*: Inhibition of miR-122 suppressed oxidative stress and apoptosis induced by STZ in INS-1 β-cells, via activation of PI3K/Akt signalling. | [71] |
| **Let-7** | *In vivo and in vitro*: Biogenesis of let-7 miRNA (PI3K/Akt suppressor) was reduced by protein Lin28a. Reduction of let-7 biogenesis via Lin28a overexpression resulted in activation of PI3K/Akt and protected β-cells from STZ induced destruction in mice and MIN6 cells. | [72] |

†Various β-cell lines have been investigated in the different studies, including murine BTC6, NIT-1, MIN6, and INS-1, and the rat β-cell line, RINm5F. ‡The most commonly used cytokine mix to induce β-cell apoptosis combines interleukin-1 β, tumour necrosis factor and interferon γ, all of which have been shown to act synergistically to induce β-cell apoptosis. rhEPO; recombinant human erythropoietin, Wnt3a protein; promotes the activation of PI3K/Akt signalling, with crosstalk with Wnt signalling, GLP-1; glucagon-like peptide-1, STZ; streptozotocin, GABA; γ-aminobutyric acid, FhHDM-1; *Fasciola hepatica* helminth defence molecule-1, Puerarin; extracted from *Radix puerariae*, BAI; baicalin derived from *Scutellaria baicalensis*, Saponins; derived from *Momordica* charantia, C3G; cyanidin-3-O-glucoside derived from anthocyanin.

*Table 2. Modulators of PI3K/Akt signalling as putative therapeutics for Type 2 diabetes*

| Modulator | β-cell effect | Reference |
|---|---|---|
| **Plant extracts** | | |
| **Hydroxysafflor yellow A** | *In vivo*: Protected β-cells from inflammatory damage and apoptosis, through activation of PI3K/Akt signalling, in T2D rats induced by HFD and low dose STZ. | [78] |
| **Jiaogulan tea and white tea** | *In vivo*: Ameliorated T2D in C57BL/6 mice exposed to HFD/STZ and protected β-cells against oxidative and inflammatory damage, mediated through the AMPK/PI3K pathway. | [79] |
| **Vin-C01 and Vin-F03** | *In vitro*: Promoted β-cell survival and inhibited STZ-induced apoptosis in INS-1 cells via regulation of IRS2/PI3K/Akt signalling pathway. | [81] |
| **Methyl caffeate** | *In vitro*: Enhanced glucose-stimulated insulin secretion and activation of IRS2, PI3K, and Akt proteins in INS-1 cells. | [84] |
| **Banxia xiexin** | *In vitro*: Suppressed tert-butyl hydroperoxide-induced apoptosis and improved insulin secretion through regulation of PI3K/Akt signalling and FOXO1 in MIN6 cells. | [82] |
| **Proteins** | | |
| **Irisin** | *In vivo and in vitro*: Attenuated lipotoxicity-induced β-cell insulin resistance and inflammatory response in HFD C57BL/6J mice and MIN6 cells, via activation of PI3K/Akt/FOXO1 signalling. | [77] |
| **Sericin** | *In vivo*: Ameliorated HFD/STZ induced islet damage and improved β-cell function through enhanced PI3K/Akt signalling. | [80] |
| **Metformin** | *In vitro*: Inhibited endoplasmic reticulum stress, dysfunction and apoptosis in NIT-1 cells via AMPK and PI3K/Akt signalling. | [75] |
| **miRNA** | | |

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| **miR-126** | *In vitro*: Resveratol-induced upregulation of miR-126 alleviated uric acid-induced injury and apoptosis in MIN6 cells, through the activation of PI3K/Akt signalling. | [83] |

T2D; type 2 diabetes, HFD; high fat diet, STZ; streptozotocin, IRS2; insulin receptor substrate 2, AMPK; AMP activated protein kinase