INTRODUCTION & REVIEW OF LITERATURE

CHAPTER 1

Chapter 1. Introduction & Review of Literature

1. The Pancreas

The human body runs on a tightly regulated network of various hormones and neuropeptides which ultimately maintain the homeostasis of the body. The pancreas holds a prominent position in this regulating network. It is a gland that is located connecting to the duodenum behind the stomach in humans. The pancreas makes a dynamic part of both the digestive system and endocrine system of the vertebrates. Being an important player in controlling energy metabolism, it plays a key role in maintaining the glucose metabolism of the body. Regulated and controlled pancreatic functions are integral to systematic bodily functions which are fruitfully achieved by the functional and morphological dual nature of the pancreas: endocrine and exocrine (Fig 1.1).

Human Pancreas, structurally and functionally contains two compartments

A.Exocrine Pancreas – The exocrine compartment is a majority part that secretes digestive enzymes, which play a role in digestion, like amylase, pancreatic lipase, trypsinogen, etc., transported through acinar cells in a network of main and subsidiary pancreatic ducts (Röder, Wu et al. 2016).

B.Endocrine Pancreas – The endocrine compartment secretes various hormones in response to external signals like nutrient intake or stress which regulates the metabolism of the body, which is carried out by highly vascularized cell clusters which are island-like structures within the exocrine pancreatic tissue called “The Islets of Langerhans” (Chandra and Liddle 2009).

The islet of Langerhans is the mixed population of endocrine cells of the pancreas which majorly performs hormonal regulation and are distributed in the pancreatic parenchyma. It was discovered by German anatomist Paul Langerhans in 1969 (Morrison 1937).

The total islet count in a human pancreas is found to be approximately between 3.2 and 14.8 million which can be circular or irregular in shape (Hellman 1959) (Da Silva Xavier 2018). The interspecies difference is found in the pancreatic islets in terms of cellular composition and architecture. Human islets consist of 30% α-cells which produces glucagon, 60% insulin-producing β-cells and 10% δ-cells (somatostatin-producing), γ- or PP cells (pancreatic polypeptide-producing), and ε-cells (ghrelin-producing) (Brissova, Fowler et al. 2005) (Da Silva Xavier 2018). Rodent islets have a different architecture with a core of β-cells which is surrounded by the other endocrine cell (Steiner, Kim et al. 2010). Regulation of glucose metabolism is

orchestrated by harmonised action of hormones insulin and glucagon. Increasing levels of blood glucose stimulate insulin release which directs glucose towards metabolism or storage. On the other hand, Glucagon has a contrasting function to insulin and helps in glucose release or gluconeogenesis from glycogen (Prentki, Joly et al. 2002).

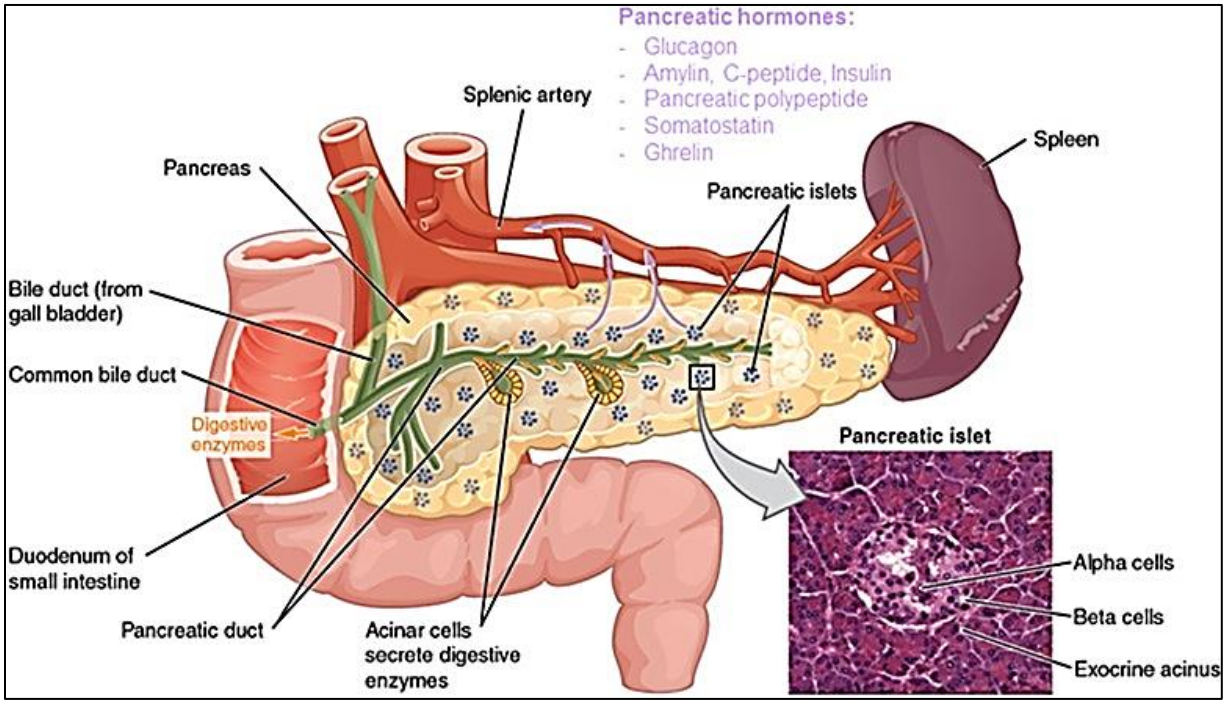

Figure 1.1 Anatomical organization of the pancreas depicting exocrine and endocrine sections with various cell types (Marieb and Hoehn 2007)

1.1.Types of Endocrine Cells

Human islets are comprised of at least five different endocrine cell types which are as follows:

i) α-cells

Alpha cells constitute about 20% of endocrine cells in the islets of the pancreas. It synthesizes the peptide hormone glucagon. Glucagon is the counter hormone to insulin, with hyperglycemic action, which elevates the glucose levels in the blood and is characteristic of the fasted state. The primary role of the counter-regulatory response is the prevention of hypoglycemia, a response that is weakened in diabetes (Da Silva Xavier 2018). Some other derived products are GLP-1, GLP-2, and glicentin (Orskov, Holst et al. 1986). Glucagon activates glycogen phosphorylase, inside the

hepatocyte to hydrolyze glycogen to glucose to achieve glycogenolysis (Adeva-Andany, González-Lucán et al. 2016).

ii) β-Cells

Human β-cells are widely studied and considered for key therapeutic interventions (Rutter, Pullen et al. 2015) (Chen, Cohrs et al. 2017). β cells are a type of endocrine cells that forms the majority of the pancreatic endocrine cell mass which is ideally between 50 and 80% and can make and release insulin in response to glucose (Dolenšek, Rupnik et al. 2015).

Insulin is an anti-hyperglycemic hormone that antagonizes glucagon, growth hormone, glucocorticosteroids, epinephrine, and other hyperglycemic hormones, to maintain circulating glucose concentrations within a narrow physiologic range (Marchetti, Bugliani et al. 2017). Insulin is proteolytically derived from proinsulin. This biologically inactive precursor is cleaved into A and a B chain forming the biologically active insulin molecule, and a C chain, which is released along with insulin. Insulin hormone is necessary for glucose uptake inside the insulin-dependent cells and thus is important for the survival of the organism. Insulin is packaged into cytoplasmic secretory vesicles (Orci 1986). Within the granule, insulin is complexed to zinc, forming insulin–zinc hexamers and crystalline granule cores. Insulin secretion is released by high glucose concentration and neuropeptides which are stimulated by food and boosted by the presence of incretin hormones (Liu, Weiss et al. 2018). Insulin secretion is inhibited in presence of some hormones like somatostatin (Ensinck, Laschansky et al. 1989), epinephrine (Lacey, Berrow et al. 1990), leptin, galanin (Renström, Ding et al. 1996), ghrelin (Wierup, Sundler et al. 2014), and zinc ions (Ferrer, Soria et al. 1984). Islet Associated Polypeptide (IAPP, also called amylin), related to calcitonin gene-related peptide (CGRP) is also co secreted by β-cell (Akter, Cao et al. 2016).

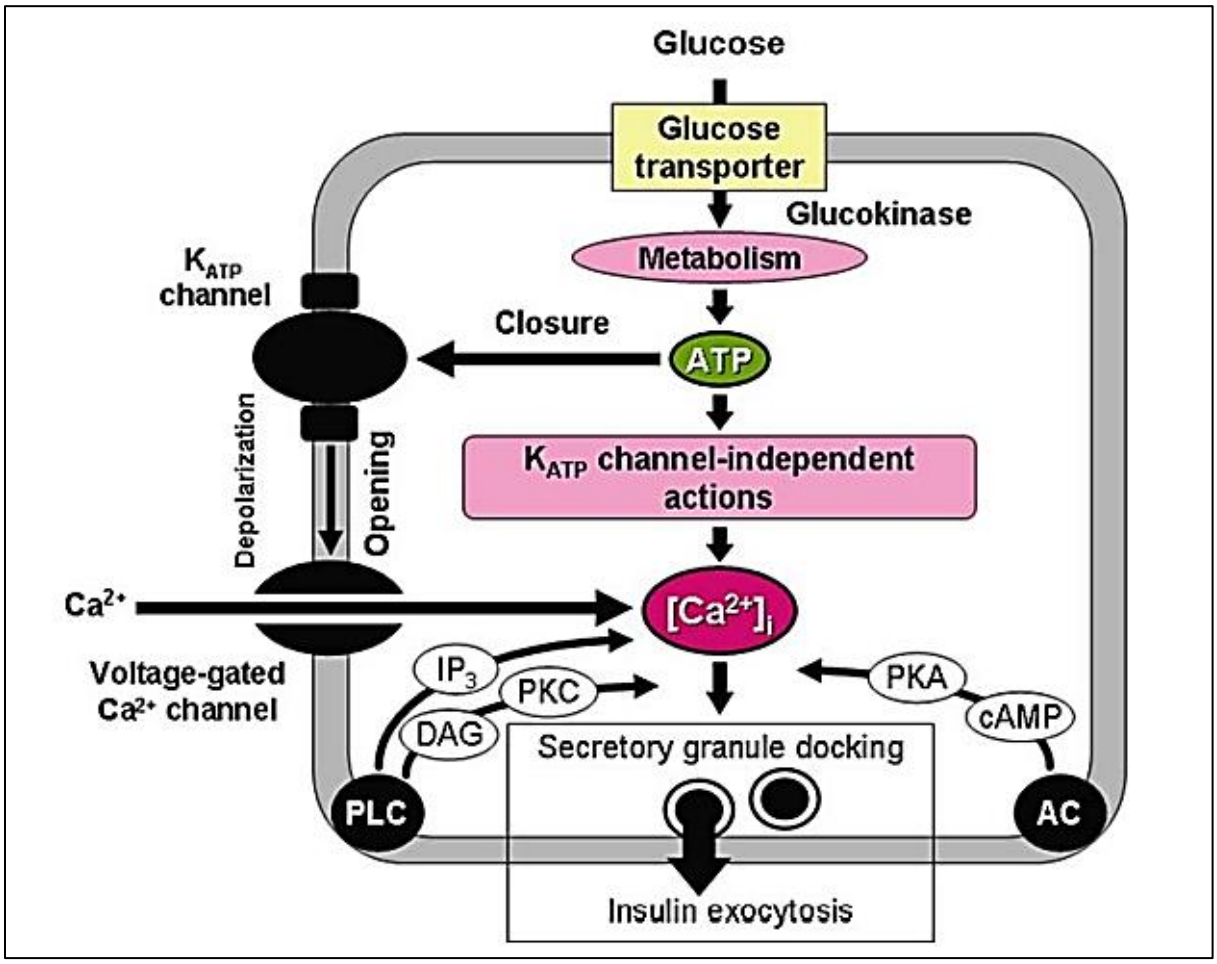

Figure 1.2 Mechanism of glucose-stimulated insulin secretion in pancreatic islet β cells

(Newsholme, Gaudel et al. 2010)

Glucose metabolism in the β-cell occurs in the presence of low affinity, high transport capacity glucose transporter(s) (GLUT1 and 2), and the low-affinity hexokinase-glucokinase (Thorens 2015) (Doliba, Qin et al. 2012). Glucose apart from stimulating insulin secretion, also increases proinsulin biosynthesis. Glucose-stimulated insulin secretion (GSIS) is the increase in insulin secretion over basal release in response to increased extracellular, and ultimately intracellular glucose (Fu, Gilbert et al. 2013).

Post meal increase in the blood glucose (>5mM), uptake of glucose occurs by the β cell, this glucose then gets phosphorylated by the enzyme glucokinase, which has a high Km for glucose to give glucose-6-phosphate (G6P). Glucokinase, control the rate of glucose utilization by the β-cell over a range of physiological glucose levels (3–20 mM) and the combination of transport and

phosphorylation regulates metabolic flux through glycolysis. Glycolysis ends with the production of pyruvate that enters the mitochondria to be oxidated. Activation of this cycle leads to the generation of NADH and FADH2, and these reducing equivalents are employed in the production of ATP in the electron transport chain. The augmented cytosolic ATP levels determine the closure of the KATP channels, reduction of K+ conductance, and plasma membrane depolarization. ATP production leads to the closure of the ATP-sensitive K+ channel (KATP) channel on the cell surface. In turn, depolarization of the plasma membrane induces the opening of L-type voltage-dependent Ca2+ channels. Ca2+ influx into the β cell cytoplasm mobilizes the insulin granules, and also activates insulin gene expression via the Calcium Responsive Element Binding Protein or CREB. In mature adult islets, insulin secretion happens very quickly after glucose administration with biphasic kinetics. This biphasic pattern of insulin secretion includes a KATP channel-dependent pathway and another KATP channel-independent pathway. Both are critically Ca2+ influx dependent affecting the different pools of insulin-secretory granules. KATP-dependent pathway accomplishes exocytosis of an "immediately releasable pool" of granules that marks the first phase (10 min) response, the KATP channel-independent pathway, working in cooperation with the KATP-dependent pathway is responsible for the second phase (60 min) response (Fig 1.2) (Straub and Sharp 2002).

In addition to (pro)insulin, C-peptide, zinc, IAPP,and proteolytic enzymes, the secretory granule contains calcium, biogenic amines, adenine nucleotides and a series of additional peptide (pro)hormones including chromogranin A and β granin (Hutton, Peshavaria et al. 1988) (Eiden 1987).

In patients with type I or type II diabetes, decreased β -cell mass and function leads to inadequate insulin secretion and hyperglycemia (Gepts 1965) (Westermark, Wilander et al. 1987). Several changes occur in type 2 diabetic β cells. Increased oscillations in the intracellular Ca2+ concentration is related with GSIS which can stimulate mitochondrial ROS generation. Reactive oxygen species (ROS) acutely stimulates but chronically induces inhibitory effects on β-cell metabolic pathways.It promotes KATP channel opening with resulting inhibitory effects on insulin secretion. Pancreatic β-cells have inherently relatively low levels of free radical detoxifying and redox-regulating enzymes, such as glutathione reductase, glutathione peroxidase, catalase, and thioredoxin, rendering them vulnerable to damage and destruction. This mechanism is chiefly

involved in β-cell dysfunction during the development of diabetes (Nakazaki, Kakei et al. 1995) (Morgan, Rebelato et al. 2009).

iii) Delta-Cells

D-cells form 5–10% of islet volume. The D (or δ) cells release somatostatin first isolated from in the hypothalamus and also found in the central nervous system, peripheral neurons and the gastrointestinal tract (Hökfelt, Efendić et al. 1975). This peptide hormone is a potent inhibitor of glucagon and insulin (Hauge-Evans, King et al. 2009).

iv) PP Cells

Pancreatic polypeptide-containing cells, also called PP cells or F-cells, make up 1–2% of the islet cell population (Ekblad and Sundler 2002). PP cells are more concentrated in the head of the pancreas (Rahier, Wallon et al. 1983). Post-prandial pancreatic polypeptide release is responsive to arginine but not glucose stimulation (Weir, Samols et al. 1979). The pancreatic polypeptide is an inhibitor of glucagon release at low glucose (Aragón, Karaca et al. 2015). The major function of PP appears to be a satiety hormone (Tan and Bloom 2013).

v) Epsilon Cells

Epsilon or Ghrelin cell releases the hormone ghrelin. Adult islets contain less than 1% epsilon cells (Wierup, Svensson et al. 2002). The hormone is thought to be of importance in growth hormone release, metabolic regulation, and energy balance. Ghrelin-positive cells are mainly found in the gut. It is increased in fasting; plasma ghrelin content has a reciprocal relationship with plasma insulin content and is an inhibitor of insulin secretion in humans and rodents. Ghrelin may also be a regulator of glucagon, PP, and somatostatin release (Egido, Rodriguez-Gallardo et al. 2002).

1.2 Pancreatic Islet Cell Development

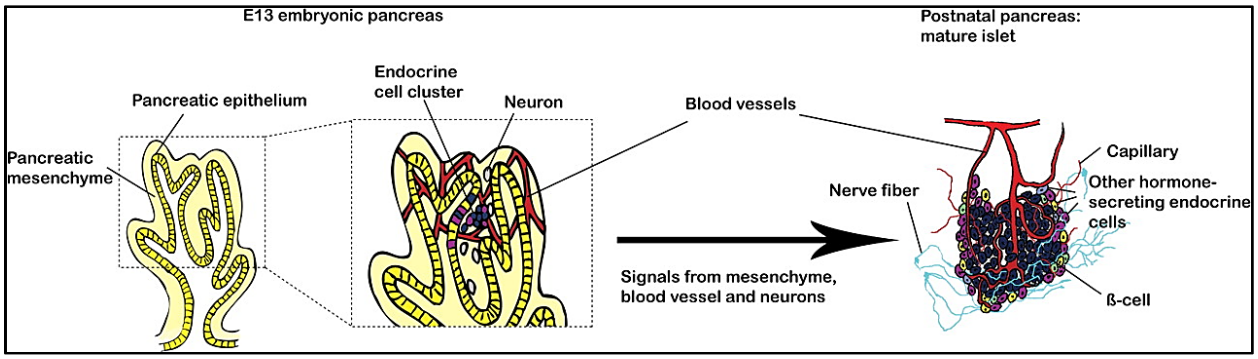

Figure 1.3 Pancreatic organogenesis demonstrating islet cell development
(Guo and Hebrok 2009)

After gastrulation, the three germ **layers**, ectoderm, mesoderm, and endoderm, are created. The pancreas is an endoderm-derived glandular organ that comprises endocrine and exocrine compartments. The commitment to a pancreatic fate arises through a progressive interaction with neighboring tissues with the endoderm. Signals coming from the notochord, mesenchyme, and endothelium encourage pancreatic buds via the formation and proliferation of multipotent progenitor cells. Dorsal and ventral endoderm gives rise to the dorsal pancreatic bud and ventral pancreas respectively (Kim, Hebrok et al. 1997) (Lammert, Cleaver et al. 2001). Even though forming similar mature tissues, differences in the genetic program between the dorsal and the ventral pancreatic buds are also emancipated in later development. Around the embryonic day (E) 9.5 in the mouse embryo, epithelial buds invade the neighboring mesenchyme by successive branching morphogenesis. Tips of the branching network occupy the multipotent progenitors which give rise to all the pancreatic cell types. Some of the glucagon-positive cells appear at this stage of development. But most of the hormone-expressing cells are seen around E13.5, a period known as the second transition. Around this time, the gut tube rotates to bring both buds into juxtaposition. As embryogenesis progresses, the organ differentiates and grows while the exocrine acinar cell clusters that empty into the ductal cells and the endocrine cells establish into islet clusters. The main pancreatic duct further buds into secondary ducts forming the smaller ducts. Here, the endodermal cells implant, leading to the formation of pancreatic lobules. A small group of cells organized as strands remains separated from the pancreatic ducts. Dense capillaries and

connective tissues then surround these strands directing them to the formation of the future islet of Langerhans (Fig 1.3).

The development of various cells in the pancreas takes place by numerous cell signaling pathways and the interplay of several transcriptional factors (Zhou, Law et al. 2007).

1.3 Transcriptional regulation of islet development in the pancreas

Cell differentiation is accomplished by the initiation and maintenance of well-regulated gene expression patterns monitored by a specific spatial and temporal combination of transcription factors. These transcription factors play a very crucial role in pancreatic progenitors, islet progenitors, and β-cells differentiation and maturation and maintenance (Fig 1.4) (Rojas, Khoo et al. 2010).

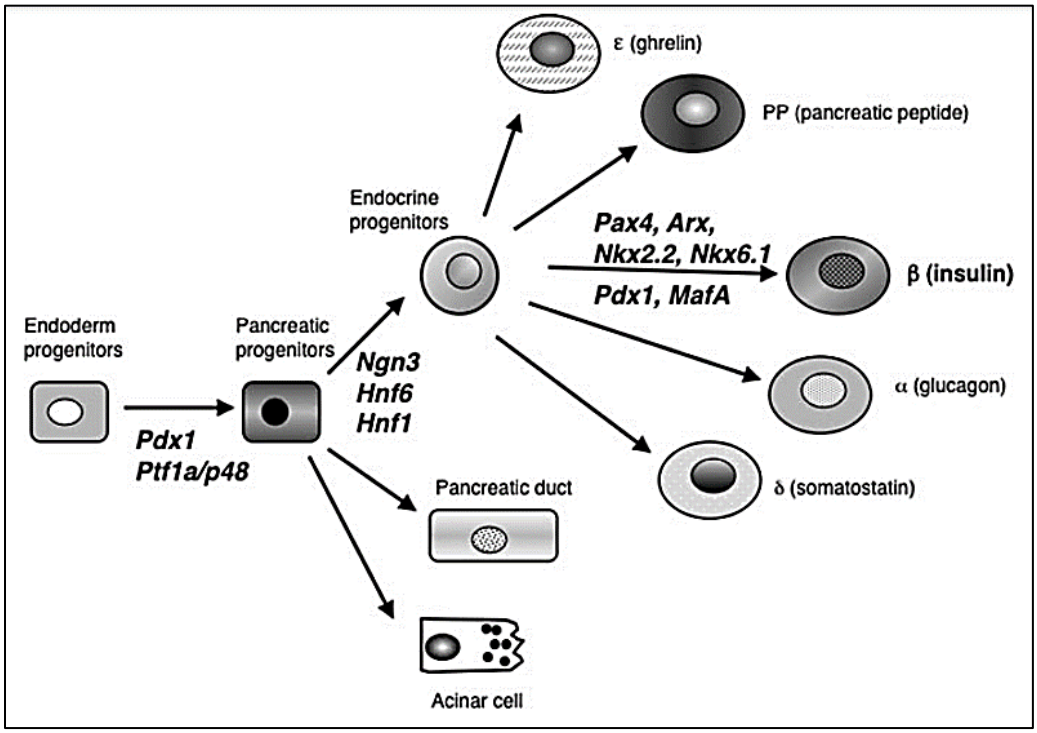

Figure 1.4 Key transcription factors involved in different steps of Pancreatic islet β-cell formation (Rojas, Khoo et al. 2010)

A) HNF-3B

HNF-3B or Foxa2 is redundant in the formation of a primed enhancer state and chromatin architecture through developmental lineage specification. FOXA2 knockout hPSCs formed reduced numbers of pancreatic progenitors (Lee, Cho et al. 2019). FOXA2 lacking mice are severely hypoglycemic with a 90% reduction in glucagon expression and a decrease in α-cells. (Lee, Sund et al. 2005). FOXA2 is required for the normal function of β-cells and the expression of the two subunits of the K_{ATP} channel (Sund, Vatamaniuk et al. 2001) (Lantz, Vatamaniuk et al. 2004)

B) Pdx1

Pancreatic duodenal homeobox 1 (Pdx1) is a transcription factor that plays a fundamental role in pancreatic β -cell function and survival. It is among the earliest transcription factors expressed in pancreatic progenitors (Offield, Jetton et al. 1996, Fujimoto and Polonsky 2009). It regulates insulin gene expression and is important for the normal development of the pancreas. Pdx1 is also critical for the function of the mature β-cell and is thus mainly found in insulin-producing cells (Brissova, Shiota et al. 2002). Complete deficiency of Pdx1 function results in pancreatic agenesis, and partial deficiency leads to severe β-cell dysfunction and increases β-cell death and diabetes both in rodents and humans (Fujimoto and Polonsky 2009). In Pdx1 knockout mice, the initial buds of the pancreas form but subsequent branching and morphogenesis of these buds is arrested which suggests that Pdx1 expression might depend on various sets of transcriptional cues controlling each pancreatic bud (Guz, Montminy et al. 1995). Pdx1 is a major player in the maintenance of healthy β-cells in adults (Rojas, Khoo et al. 2010). Thus in addition to its role in early pancreas development, Pdx1 is also a key regulator of β-cell.

C) NESTIN

The cellular role of nestin in islet cell development and differentiation is of utmost importance (Hunziker and Stein 2000). Nestin localization has been established in the pancreatic islets and ducts and nestin expression has been observed during embryogenesis to precede the appearance of β cells. In human neonatal pancreatic sections, an increased number of islet-associated positive nestin cells as compared with adult islets has been observed, pointing to a role for Nestin in early pancreatic development (Wang, Li et al. 2005) (Lechner, Leech et al. 2002).

D) Ngn3

Ngn3 identified as specific for endocrine development is a member of the basic helix–loop–helix transcription factor family. Expression of Ngn3 is first observed in the dorsal pancreatic epithelium at E9 in the mouse, increasing between E9.5 and E15.5, corresponding to the endocrine differentiation wave (Apelqvist, Li et al. 1999) (Schwitzgebel, Scheel et al. 2000).Ngn3-expressing cells (Ngn3+) function as endocrine precursor cells which are critical for pancreatic endocrine fates give rise to all hormone-secreting pancreatic cells (Gu, Dubauskaite et al. 2002). Ngn3 knockout mice lack all endocrine cells types and neonates die postnatally from diabetes In contrast, overexpression of Ngn3 in the pancreatic progenitors in mouse embryos leads to premature endocrine differentiation and blocks exocrine development (Gradwohl, Dierich et al. 2000) (Apelqvist, Li et al. 1999). Thus the important place of Ngn3 in establishing endocrine cell fate in a coordinated cascade of transcription factors activation and inactivation in cells is very important in the understanding of endocrine differentiation.

E) Pax4

Pax4 is selectively expressed in the developing pancreas. As development proceeds, the expression pattern is restricted to mature endocrine cells especially to β- and δ-cells (Sosa-Pineda 2004) (Dohrmann, Gruss et al. 2000). Pax4 favors β- and δ-cell fates at the expense of α-cell destiny. Pax4-deficient mice develop severe diabetes at birth as inactivation of Pax4 leads to islets form of α- and δ-cells and characterized by a deficiency of β and δ cells Pax4 deletion leads endocrine progenitors to adopt an alternative cell-subtype fate (Sosa-Pineda, Chowdhury et al. 1997). Thus, in the hierarchical network in endocrine lineage specification, Pax4 acts downstream Ngn3 and determine the final proportions of the different endocrine cell types (Collombat, Mansouri et al. 2003).

F) Nkx6.1

NKX6.1 is expressed at primary stages of pancreatic development as well as in adult β cells, where it is involved in several functions. Nkx6.1 is redundantly required for endocrine α and β cell development. It is broadly expressed until E10.5 but is expressed entirely in β-cells by the end of gestation (Zhou, Law et al. 2007) (Schaffer, Freude et al. 2010). The expression can be observed

in both multipotent pancreatic progenitor cells and functional β cells. Mice that lack Nkx6.1 exhibits a discerning reduction of β-cells with a usual complement of other endocrine cell types (Sander, Sussel et al. 2000) (Nelson, Schaffer et al. 2007)

G) NeuroD

When the cells within the islet are differentiated into the hormone-expressing cells, they need to maintain their identity. This is accomplished by the persistent expression of transcription factors required for the maturation and development of each cell type.

NeuroD also known as B2 is a bHLH transcription factor expressed from E9.5 in scattered pancreatic cells and E14.5 is expressed in Ngn3+ cells. After birth, its expression becomes restricted to mature β-cells (Chae, Stein et al. 2004). In NeuroD-null mice decrease in islet, cells number is seen, especially in β-cells by undergoing apoptosis and die of severe diabetes shortly after birth (Naya, Huang et al. 1997). NeuroD is a strong inductor of Insulin transcription (Sharma, Moore et al. 1999). In humans, mutations in NeuroD can incline individuals to develop maturity-onset diabetes of the young (MODY6) (Gu, Stein et al. 2010).

H) MafA

MafA is a β cell-specific transcription factor that binds to insulin promoter. It is a positive indicator of β cell formation and functionality (Hang and Stein 2011). MafA expression starts at E13.5 in the first insulin-producing cells, and its expression remains in the β-cells to adulthood. Inactivation of MafA in the mouse embryo leads to β cell mass decrease and β-cell apoptosis (Zhang, Moriguchi et al. 2005). MafA interacts with Pdx1 and NeuroD to initiate insulin transcription (Olbrot, Rud et al. 2002).

I) GLUT2

GLUT2 is the major glucose transporter in pancreatic β cells which plays an important role in insulin secretion (Wu, Fritz et al. 1998). Impaired Glucose stimulated insulin secretion (GSIS) is linked to reduced expression of GLUT2 in diabetic mice and rats (Thorens, Weir et al. 1990) (Orci, Unger et al. 1990). Transplantation of islets from control animals to diabetic mice normalized GLUT2 levels and reestablished GSIS (Thorens, Wu et al. 1992) (Ogawa, Noma et al. 1995).

Any disturbances in the hormonal regulation by the pancreatic islets lead to aberrant actions on glucose metabolism which ultimately results in metabolic diseases like Diabetes Mellitus.

2. Diabetes Mellitus

Diabetes mellitus (DM) is a chronic heterogeneous metabolic disorder characterized by differences in carbohydrate, fat, and protein metabolism culminating in severe disturbances of energy metabolism. It is marked by chronic high blood glucose levels (hyperglycemia) that require frequent monitoring and proper control. The primary cause for this is defects in insulin- either secretion or action or both. Long-term damage, dysfunction, and failure of some organs like eyes, kidney, and microvasculature are seen due to continuous hyperglycemia (American Diabetes 2009).

Pancreatic β cells produce the hormone insulin which facilitates the absorption of glucose into the cells to provide energy and is also involved in a variety of other functions. Insulin is secreted from the pancreas on getting triggered by high blood glucose levels and effectively maintains blood-glucose homeostasis. It stimulates the cells for glucose uptake via its signaling pathway. But in diabetes either the pancreas is not producing insulin at appropriate levels or the cells are unable to utilize glucose although insulin is produced at an appropriate level (Fu, Gilbert et al. 2013) (Röder, Wu et al. 2016).

2.1 Epidemiology

In current times, 537 million adults (20-79 years) are living with diabetes worldwide. This number is predicted to rise to 643 million by 2030 and 783 million by 2045. Diabetes is responsible for 6.7 million deaths in 2021. 541 million adults have Impaired Glucose Tolerance (IGT), placing them at high risk of type 2 diabetes. In South East Asia, 90 million have diabetes. The number of adults with diabetes is expected to reach 113 million by 2030 and 151 million by 2045. 747 lakh deaths are caused by diabetes in 2021 (IDF 2021). In India, 9.6% of the population (20-79 years) is suffering from diabetes currently. By the year 2045, this will rise to 10.8%. Presently 53.1% of people are with undiagnosed diabetes. People suffering from microvascular complications of diabetes are Nephropathy: 5.9%, Retinopathy: 0.8%, and Neuropathy: 10.6%. People suffering from macrovascular complications of diabetes are Coronary artery disease: 2.5%, Cerebrovascular disease: 0.3%, Peripheral artery disease: 0.0% and Heart failure: 0.2% (IDF 2021). Indian Council

of Medical Research–INdia DIABetes (ICMR–INDIAB) population-based cross-sectional study was conducted in various states of India which reported 7.1 % reported cases of diabetes in Gujarat population (Anjana, Deepa et al. 2017).

2.2 Classification of Diabetes

Type 1 Diabetes Mellitus

Type 1 Diabetes Mellitus (T1DM) is an autoimmune disease with the genetic aberration of the immune system. It is also referred to as insulin dependent diabetes or juvenile-onset diabetes. Only 5–10% of those with diabetes account for T1DM. It is perceived as a chronic immune disease with selective loss of insulin-producing β-cells in the pancreatic islets (American Diabetes 2009).

There are different forms of the disease, the commonest being occurring in preadolescent growth. This form of the disease progresses slowly with the environmental stimulus involved several years before the autodestruction of the β cell reduces its mass adequately to develop the symptoms. Higher frequencies of other autoimmune diseases are often found in these children like Graves' disease, hypothyroidism, Hashimoto's thyroiditis, Addison's disease, vitiligo, celiac sprue, autoimmune hepatitis, myasthenia gravis, pernicious anemia, etc. (Juneja and Palmer 1999) (Guthrie and Guthrie 2004) (ADA 2014).

The second form of the disease is mainly observed in younger patients which descends more quickly than the first form and is often preceded by viral infection. Other autoimmune diseases are not usually associated. There is a quick loss of almost all the β cells and not often have much of a remission period. Ketoacidosis is the first indicating symptom, particularly in children and adolescents.

The third form of the disease occurs mainly in adults and is called latent autoimmune diabetes mellitus (Juneja and Palmer 1999). This is similar to the first form but the onset is observed in late adulthood while pancreatic autoantibodies are present beforehand. The critical differentiating marker of T1DM from T2DM is the existence of pancreatic autoantibodies especially glutamic acid decarboxylase (GAD) which also serves as a predictor of the onset of T1DM. The specific characteristic of type 1 diabetes is autoantibody generated towards the islet. In all the mentioned forms, the mutually common factor is the destruction of the β cells of the pancreas by T lymphocytes of the immune system, with subsequent loss of insulin production. Islet cell

autoantibodies, autoantibodies to insulin, and autoantibodies to the tyrosine phosphatases IA-2 and IA-2β are other markers for β cell destruction (ADA 2014) (Vermeulen, Weets et al. 2011, Bonifacio 2015). These antibodies are often observed in 85–90% of individuals when fasting hyperglycemia is first detected. Some develop modest fasting hyperglycemia that quickly transforms to severe hyperglycemia in existence with infection and various stresses. Some adults may retain residual-cell functioning which may delay serious symptoms but eventually become insulin-dependent (Taylor, Accili et al. 1994).

The insulin deficiency thus produced is lifelong and complete. This absolute insulin deficiency ultimately fails in the anabolic process. Hyperglycemia persists in blood since the entry of glucose is prohibited inside the cells. Dehydration and electrolyte deficiency occurs when fluid and food intake outrun the losses. Free fatty acids (FFAs) are released from fat cells as a result of fat breakdown. FFAs start getting accumulated in the liver and products like acetone, acetoacetic acid, and β hydroxybutyric acid or ketone bodies are formed. Acidosis is more pronounced as a result of the increased formation of ketone bodies. If this condition remains untreated, it results in coma and eventually death. The lifelong treatment option that remains is insulin (Guthrie and Guthrie 2004) (Dabelea, Rewers et al. 2014).

A) Symptoms

Classic symptoms of the disease are excessive urination (polyuria), unquenched thirst (polydipsia), excess hunger (polyphagia) is observed due to fuel and energy-deficient cells. Cell death is commonly observed with marked weight loss despite polyphagia (ADA 2014) (Craig, Hattersley et al. 2009) (Galtier 2010).

B) Diagnosis

Diabetes mellitus can be diagnosed by blood glucose measurements. In the presence of characteristic clinical symptoms, diabetes is diagnosed based on a fasting plasma glucose (FPG) of ≥126 mg/dl, a random blood glucose (RBG) of ≥ 11·1 mmol/L (200 mg/dL) or 2-hour plasma glucose during a glucose tolerance test of ≥200mg/dl (Committee 2022). Haemoglobin A1c (HbA1c) is a diagnostic and prognostic marker used for chronic glycemia

that measures the non-enzymatic glycation of hemoglobin and estimates an average blood glucose levels over a 2-3 month period of time (Sherwani, Khan et al. 2016).

C) Etiology of T1DM

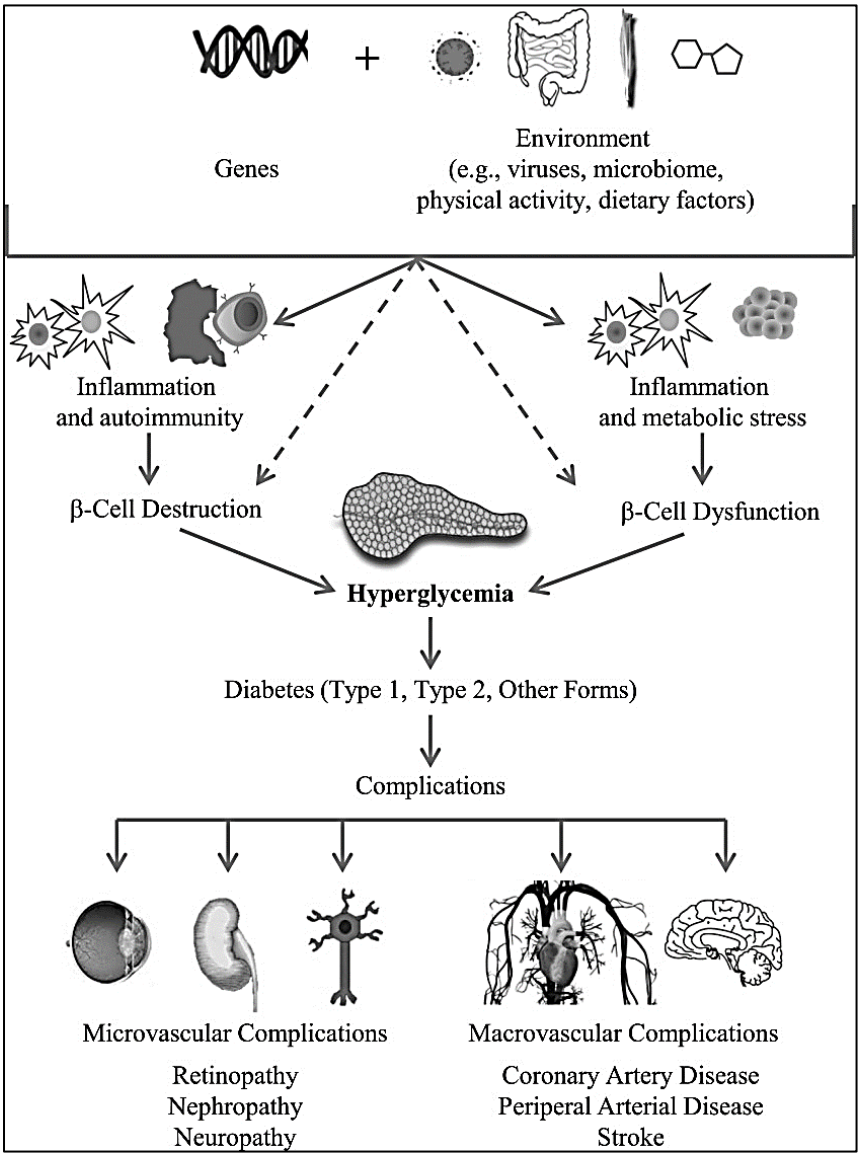

Figure 1.5 Risk Factors associated with the pathogenesis of Diabetes Mellitus (Skyler, Bakris et al. 2017)

There are several risk factors associated with T1DM (Fig 1.5)

i. Genetic

In T1DM, there is a development of auto-immunity (Fig 1.6). Type 1 Diabetes is of two kinds, type 1A is usually characterized by the presence of anti-insulin, anti-GAD, anti-islet cell antibodies and in type 1B there is a lack of auto-immune antibodies but the symptoms of the two are the same and the levels of insulin in the blood are similar. These patients generally require insulin treatment. Immune responses are mediated through both T-cell mediated inflammatory response and humoral (B cell) response. During inflammation, β – cells increase the number of MHC II and thereby present antigen to diabetogenic CD4 T-cells. Also, multiple chemokines produced in β – cells infiltrate islets of Langerhans and recruit immune cells to the pancreas via chemokines receptors. Autoimmune type 1 diabetes has strong HLA associations, with linkage to DR and DQ genes and development of the first islet autoantibody (Skyler, Bakris et al. 2017) (Pociot and Lernmark 2016).

Islet cell antibodies (ICA) are directed against cytoplasmic components of the islet cells. ICA presence may precede the development of type 1 DM. HLA-DR/DQ alleles can be either predisposing or protective. When TIDM develops in adults it is clinically known as Latent autoimmune diabetes of adults (LADA). It has a slower onset when compared to the same condition in children (Tuomi, Groop et al. 1993).

Indian population is also associated positively with a high frequency of pancreatic autoantibodies. But the haplotypes most frequented in Indian population are A26-B8-DRB1*03 and Ax-B50-DRB1*03 (found in 25% each of patients), a study conducted in North Indian children. Haplotype A1-B8-DRB1*03 was found in a minority (7.2%) of this Asian Indian population which is a classic haplotype favouring autoimmunity in white individuals. This clearly suggests HLA associations with T1DM differ in Asian Indians compared with those described for white populations (Kanga, Vaidyanathan et al. 2004) (Unnikrishnan, Anjana et al. 2016).

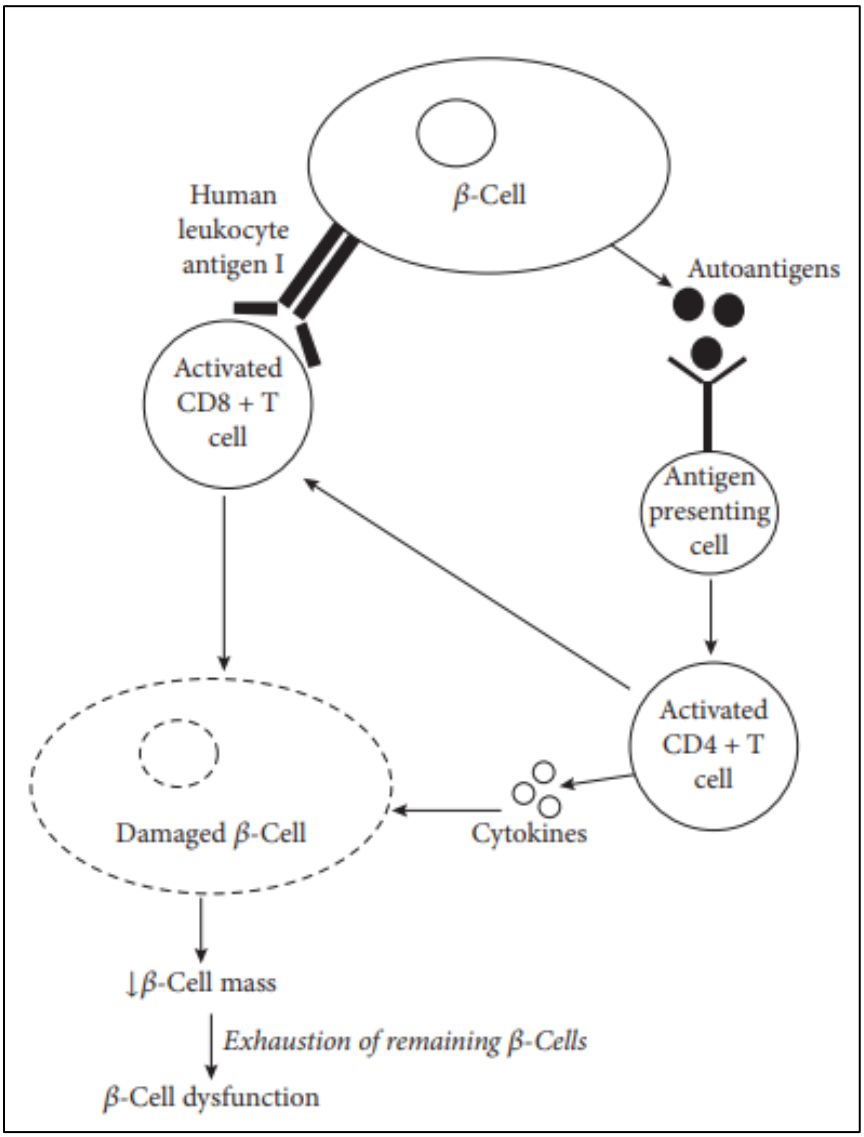

Figure 1.6 Mechanism of β cell destruction in T1DM (Wickramasinghe, Kalansuriya et al. 2021)

ii. Environmental

Though genetic predisposition is of major importance in type 1 diabetes, several environmental factors also contributes to the etiology of the disease. Pancreatic β cells are also targeted and sequentially destroyed by various environmental stimuli eventually causing insulin deficiency and resulting in diabetes (Roep 2003). Coxsackieviruses have been identified in some patients (Andréoletti, Hober et al. 1997). Type 1 DM is caused by an interaction between environmental

factors and an inherited genetic predisposition. Numerous viruses attack the pancreatic β-cell either directly through a cytolytic effect or by triggering an autoimmune attack against the β-cell. In addition, patients newly presenting with type 1 DM may exhibit serologic evidence of viral infection. Higher immunoreactivity to enteroviral VP1 protein is also found in the β cells of children with type 1 diabetes (Richardson, Leete et al. 2013). Other environmental factors contributors for type 1 diabetes are cesarean delivery, early childhood diet, and use of antibiotics and also interlinked with the human microbiome (Wen, Ley et al. 2008) (Maslowski and Mackay 2011).

iii Chemicals and Drugs

Toxins in food or water might activate autoimmune mechanisms in genetically susceptible individuals which results in pancreatic islet cell death (Benson, Vanleeuwen et al. 2010). Nivolumab and pembrolizumab are monoclonal antibodies used for the treatment of cancer have been reported to occasionally induce autoimmune diabetes (de Filette, Andreescu et al. 2019). Nitrosamines may act as diabetogenic (Moltchanova, Rytkönen et al. 2004) Many chemicals are known to be toxic to pancreatic β-cells like alloxan, streptozotocin (Radenković, Stojanović et al. 2016), and the rat poison Vacor (Thayer, Heindel et al. 2012).

D) Type 1 Diabetes Management

Diabetes management aims at restoring carbohydrate metabolism with effective control of blood glucose Strategies to manage T1D are rapidly growing to avoid long-term diabetic complications. With various lifestyle management and proper medication type 1 diabetes is manageable.

i. Diabetic diet and lifestyle modification

People with type 1 diabetes are advised to follow a diet which can controlglycemic variability (Seckold, Fisher et al. 2019) (Kasuga 2006). Poorly controlled diabetes also leads to high blood pressure and other long-term complications. Lower carbohydrate intake and lower dietary carbohydrate-to-fat ratio provides lower variability in the blood glucose concentrations, lower blood pressure, and higher HDL-cholesterol concentration (Hu, Mills et al. 2012). The benefits of exercise and physical activity in patients with type 1 diabetes are favorable and recommended (Chetty, Shetty et al. 2019).

ii. Insulin therapy

Patients with type 1 diabetes mellitus are in a need of lifelong insulin therapy. Most require 2 or more injections of insulin daily, with doses adjusted based on self-monitoring of blood glucose levels. Administration of insulin through injections, insulin pumps, or insulin pens has proved to be effective in maintaining glucose homeostasis in T1D patients (McCall and Farhy 2013).

Insulin discovery was one of the greatest medical achievements in history (Bliss 1982). The primary aim of treatment of type 1 diabetes mellitus is the external replacement of the functions of β cells to achieve near normoglycemia as close to the normal range as possible (McCall and Farhy 2013). Efficient tools for managing T1D have improved over time since insulin was discovered. Initially, insulin preparation was bovine or porcine pancreata origin but production techniques became more efficient in terms of purity and ease of isolation (Hirsch, Juneja et al. 2020).

Types of Insulin

Selecting the appropriate insulin depends mainly on the anticipated time of action of insulin. Regular insulin is structurally the same as physiological insulin produced by β cells which consist of six monomers of insulin, each of which consists of an A chain and a B chain. It results in a delayed onset of action of 30 to 60 minutes, a peak of 2 to 3 hours, and an effective duration of 6 to 8 hours (Donner and Sarkar 2000). Rapid-acting insulin are insulin analogs with a rapid onset in 15-30 minutes, peaking in 30-90 minutes, and reach an effective duration of 4 to 5 hours when it is injected subcutaneously. Rapid-acting: e.g., insulin lispro (Lalli, Ciofetta et al. 1999), insulin aspart, and insulin glulisine (Quianzon and Cheikh 2012). Intermediate-acting insulin are a combination of recombinant human insulin with protamine which results in crystal formation results in Neutral protamine Hagedorn (NPH) insulin. Its onset of action occurs 2 to 4 hours from the time of injection, with a peak effect lasting 6 to 10 hours, and an effective duration of 10 to 16 hours (Hagedorn, Jensen et al. 1984) (Hirsch, Juneja et al. 2020). Long-acting insulin analogs are with enhanced pharmacokinetics and pharmacodynamics without a peak effect, it maintains a longer duration of action. Insulin glargine, insulin detemir are Long-acting insulin analogs (Poon and King 2010) (Donner and Sarkar 2000). Ultralong-acting insulin analogs are Insulin degludec is an ultra-long acting basal insulin that has a deletion of the threonine amino acid residue at B30

and the addition of a fatty acid to the lysine at B29, the rest amino acid sequence is the same as human insulin (Heise, Hermanski et al. 2012) (Tambascia and Eliaschewitz 2015).

iii. Cell replacement therapies

Cell replacement therapy is a striking approach to treat diabetes and has acquired a foremost position in the field of diabetes research. Several substantial strategies are being employed to obtain a renewable source of β-cells for transplantation purposes. Replacement of a pancreatic β-cell to accomplish a more physiological means for normalizing glucose homeostasis is therapeutically remarkable. There are various strategies employed for β-cell replacement which are majorly being employed in diabetes therapeutics (Kin 2010).

1. Pancreas Transplantation

Pancreas transplantation is a highly effective and successful treatment for type 1 diabetes. It was established due to the advancement of technology as to surgical techniques and the preservation of organs. But it is associated with surgical complications and immunosuppression (Meirelles Júnior, Salvalaggio et al. 2015). The first vascularized pancreas transplant was performed simultaneously with a renal graft to treat a type 1 diabetes patient with uremia at the Hospital of the University of Minnesota, USA, in 1966 by William Kelly and Richard Lillehei. Improved life quality and survival were observed after the pancreas transplant. This treatment normalizes glucose levels and serum levels of glycosylated hemoglobin in type 1 diabetes patients (Kelly, Lillehei et al. 1967).

2. Islet Transplantation

Besides the daily administration of exogenous insulin or pancreas transplantation, islet transplantation is the treatment of choice for type 1 diabetic patients. It has many advantages and low morbidity. So recent advances in islet isolation technology have accelerated the successful development of new clinical islet transplantation programs around the world (Kin 2010). It is a preferred therapeutic option for T1DM patients with severe hypoglycaemic episodes. A decrease in the frequency and severity of hypoglycemia along with ideal graft function and optimum levels of C-peptide is seen in most of the patients post-transplantation (Ang, Meyer et al. 2014).

In the Edmonton protocol in the year 2000, seven T1DM patients attained insulin independence with normalized glycosylated hemoglobin (HbA1c) levels by using freshly isolated sufficient islet

mass from multiple donors and corticosteroid-free immunosuppressive anti-rejection therapy (Shapiro, Lakey et al. 2000) (Ryan, Lakey et al. 2001). This protocol has been adapted worldwide for islet transplantation with successful outcomes with reproducibility of the Edmonton results in 21 of 36 patients (58%) who attained posttransplant insulin independence in a subsequent international trial at nine centers (Markmann, Deng et al. 2003) (Hering, Kandaswamy et al. 2004) (Warnock, Meloche et al. 2005). However, most of the islet transplant patients went back to insulin injections after a five-year follow-up in the Edmonton center. Only ∼10% of 65 patients ensued insulin independence while around ∼80% continued to be C-peptide positive (Ryan, Paty et al. 2005) (Rekittke, Ang et al. 2016).

Despite the several advancements in islet cell transplantation, its transition from the stage of clinical research to routine clinical practice is faced by multiple complications like graft rejection, recurrent autoimmunity, and some technical aspects like successful isolation of high-quality sufficient number of viable islets which survive and functional for a longer time and show improved long-term clinical outcome (Kin 2010). The gradual allograft abrasion is also accredited to some nonimmunological factors as well (Oberholzer, Triponez et al. 1999). Although a majority of patients achieve insulin independence following transplantation which is 82% of patients at 1 year after islet allotransplantation it is short terms and most of them experience an increase in graft dysfunction over time, with a success rate of only 10% at 5 years post-transplantation (Shapiro, Ricordi et al. 2006) (Ryan, Paty et al. 2005).

So as we saw the exogenous cell replenishment strategies, we will now examine endogenous replenishment of pancreatic β-cells which is the major interest of this thesis.

3. Islet neogenesis

The capacity of an organism to sustain its β-cell mass in an intricate developmental and physiological complex during adulthood is crucial for maintaining glucose homeostasis and preventing diabetes. It depends upon progressive and sequential hierarchical layers of cellular organizations (Khadra and Schnell 2015). The β-cell mass is regulated by β-cell neogenesis, β-cell proliferation or replication, β-cell hypertrophy or hypotrophy, and β-cell death (Ackermann and Gannon 2007). An important strategy for a lasting cure for type 1 and type 2 diabetes is to stimulate the regeneration in the pancreas *in vivo*, which can increase new β-cells while providing enough

β-cells to reverse diabetes (Bonner-Weir 2001) (Bonner-Weir and Sharma 2002) (Lee and Nielsen 2009) (Bonner-Weir, Guo et al. 2012).

Islet neogenesis can arise from stem or progenitor cells during embryonic or postnatal growth. Stem or progenitor cells can ascend from various locations such as pancreatic ducts, islets, and bone marrow. The process can also include trans-differentiation of pancreatic acinar and liver cells, epithelial-mesenchymal transition, differentiation of intra-islet precursors or splenocytes, and induced genetic reprogramming of adult exocrine cells to functional β-cells (Gershengorn, Hardikar et al. 2004) (Lipsett and Finegood 2002) (Sapir, Shternhall et al. 2005).

- **Sources of islet neogenesis**

There are several sources for islet neogenesis (Fig 1.7)

A) Embryonic stem cells (ESC)

Human Embryonic stem cells (hESCs) are the gold standard of all the stem cells They are telomerase-positive, immortal, capable of both self-renewal and can differentiate into any cell type of the body. Their enormous replicative potential makes them an alluring alternate source for providing an unlimited supply of β cells for transplantation into diabetic patients (Thomson, Itskovitz-Eldor et al. 1998). D'Amour et al have generated pancreatic endocrine precursors, definitive endoderm, primitive gut tube-like cells, and endocrine cells from hESC using a differentiation protocol that recapitulates the signaling cascades of β-cell differentiation in the embryo (D'Amour, Agulnick et al. 2005) (D'Amour, Bang et al. 2006).

The use and application of hESC are hindered by ethical concerns, but recapitulating the steps involved in the differentiation process adds valuable information to the islet neogenic research (Zulewski 2008).

B) Induced pluripotent stem cells (iPSC)

Induced pluripotent stem cells (iPSC) are obtained by reprogramming differentiated cells. Pluripotency is supported by a complex system of signaling molecules and a gene network that is specific for pluripotent cells.

Somatic cells are reprogrammed into iPSC by introducing several transcription factors combination of Oct3/4, Sox2, Klf4, and c-Myc11 giving rise to induced pluripotent (Okita and Yamanaka 2011). Human iPSC has been differentiated into insulin-secreting islet-like cells that have proven therapeutic for T1DM therapy. Differentiation is induced by recapitulating pancreas development and reprogramming to a pluripotent stage with various growth factors and chemical compounds, in the order of definitive endoderm, primitive gut tube, posterior foregut tube, pancreatic progenitor cells, endocrine progenitor, and pancreatic islet-like cells: α cells, δ cells, and β cells. But compared to ESC and iPSC, adult stem cell-like, mesenchymal stem cells happen to be a better choice for generating differentiated cells for therapy in terms of clinical applications (Medvedev, Shevchenko et al. 2010) (Choi, Shinohara et al. 2021).

C) Adult stem cells

Mesenchymal Stem Cells are immunomodulatory which help to control the auto-immune response and thus prevent immune injury of newly proliferating cells (Claiborn and Stoffers 2008). They are highly plastic stem cells that can be isolated from the bone marrow, blood, fat, skin, oral cavity, urine, umbilical cord, amniotic fluid, and placenta (Hass, Kasper et al. 2011). MSC is similar to pluripotent stem cells in terms of the differentiation abilities to generate germ layer cells (Grove, Bruscia et al. 2004). Especially, bone marrow-derived MSC is superior and well explored with generating differentiated cells for diabetic therapy (Domínguez-Bendala, Lanzoni et al. 2012).

MSCs can differentiate into functional pancreatic islet-like cells *in vitro*. It was demonstrated that the transplanted mesenchymal cells differentiated into insulin-secreting cells and increased glucagon-stimulated C-peptide. They also did not generate an immune reaction in the subjects, and therefore articulates themselves as promising candidates for diabetes therapy (Bhansali, Asokumar et al. 2014). If MSCs from diabetes patients themselves can be isolated, proliferated, differentiated into functional pancreatic islet-like cells, and transplanted back into their donor, their high proliferation potency and rejection avoidance will prove to be highly therapeutic for diabetic patients (Pavathuparambil Abdul Manaph, Sivanathan et al. 2019).

Our lab has also used the MSC cell model for regenerative therapy approaches for T1DM therapeutics. mouse Bone Marrow Stem Cell was assessed for *in vitro* islet differentiation and formation of neo islets. A novel pharmacological *in vivo* lineage tracing approach was used for

stimulating direct migration and homing of therapeutic mBMSC in STZ-induced diabetic mice (Dadheech, Srivastava et al. 2020). We have also demonstrated that human Bone Marrow Stem Cells have tremendous *in vitro* expansion potential and can be differentiated into highly efficient stepwise multistep lineage, including definitive endocrine, pancreatic progenitor, endocrine progenitor, and mature pancreatic islets, by evaluating temporal gene and protein expression. Thus, recapitulating *in vivo* pancreatic islet development (Mitul Vakani Ph.D. Thesis 2020) (Vakani 2020).

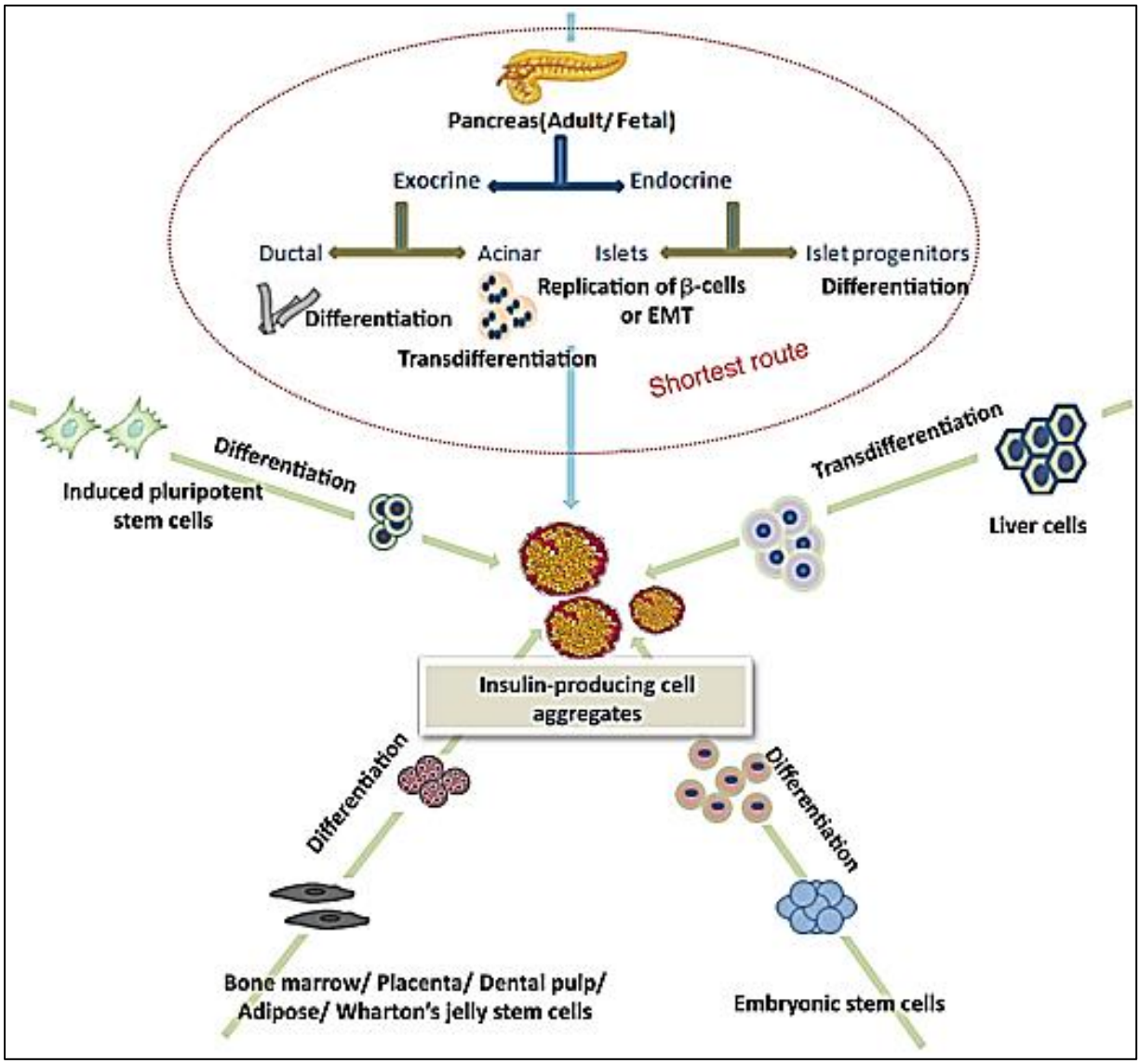

Figure 1.7 Different cell sources for islet neogenesis (Venkatesan, Gopurapilly et al. 2011)

D) Pancreatic progenitors cells

Neogenesis or the formation of new islet cells from pancreatic progenitor/stem cells is widely accepted as being responsible for the initial embryonic formation of the endocrine pancreas. Pancreatic progenitors are the cells residing within the pancreas and are capable of differentiating into insulin-producing cells (Venkatesan, Gopurapilly et al. 2011). There are ductal epithelial cells that form a promising approach for β-cell mass expansion (Liao, Verchere et al. 2007) (Katdare, Bhonde et al. 2004). Then Nestin positive cells (NPC) can differentiate into islet endocrine cells which correlates NPC with islet precursors (Zulewski, Abraham et al. 2001). Stellate cells could form a new source for islet neogenesis (Docherty 2009). More importantly, the role of intra islet precursor cells in generating β-cell mass has been an attractive approach. Banerjee and Bhonde et al. demonstrated the generation of *in vitro* neoislets from the expansion of intra islet precursor cells (Banerjee and Bhonde 2003).

Our lab has also demonstrated differentiation of pancreatic resident endocrine progenitors (PREPs) for their profound characteristics and unique commitment to generate islet like cell clusters (ILCC) exploring the shortest route of islet differentiation. We differentiated Panc-1 (Dadheech, Srivastava et al. 2015) and NIH3T3 (Sarita, Dadheech et al. 2010) (Dadheech, Soni et al. 2013), pancreatic cell lines into functional islet clusters establishing islet neogenesis and functionally and morphologically characterizing newly formed islets. Primary cultured mouse intra-islet progenitor cells (mIPC) were induced for islet neogenesis by monitoring key transcription factors temporally (Dadheech, Srivastava et al. 2015). Pancreatic progenitors were triggered for islet differentiation by up-regulating key transcription factors necessary for replenishment of lost β cells and thus recovering impaired pancreatic endocrine function (Srivastava, Dadheech et al. 2018). PREPs were isolated, purified and characterized from BALB/c mice. PREPs were transplanted intravenously into streptozotocin (STZ) diabetic mice while monitoring their robust homing and differentiation toward the damaged pancreas, leading to amelioration in the diabetic condition (Srivastava, Dadheech et al. 2019). (Srivastava, Dadheech et al. 2018)

- **Induction of differentiation of Stem/Progenitors cells into islets by chemical compounds**

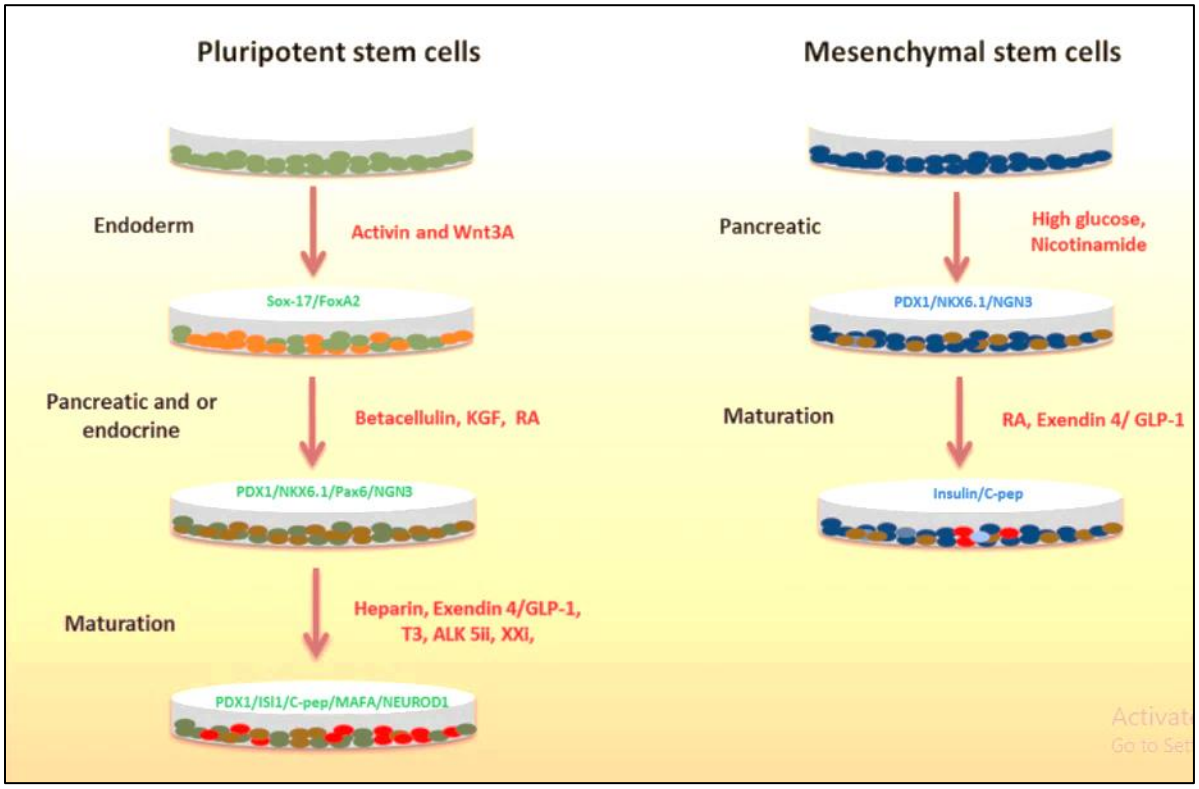

Figure 1.8 Small molecules induced MSC differentiation to β cells (Pavathuparambil Abdul Manaph, Sivanathan et al. 2019)

Small molecules efficiently promote cell differentiation by regulating signal transduction pathways and metabolism (Fig 1.8). Their use has made it easier to mimic *in vivo* environments by the activation/inhibition of cell-specific signaling. There are several molecules demonstrating islet differentiation capabilities (Thakur, Lee et al. 2020). Pancreatic progenitor differentiation can be achieved by using nicotinamide with or without growth factors (Sun, Roh et al. 2007). Chemicals like L-taurine and sodium butyrate also amplified the endocrine differentiation of MSC (Zhou, Li et al. 2010). The final maturation to β -like cells can be achieved by nicotinamide combined with exendin-4 (Millman, Xie et al. 2016) or glucagon-like peptide-1 (Chandra, G et al. 2009). Activin A combined with β cellulin helps to maintain PDX1 expression during pancreatic differentiation and maturation in ESC (Paz, Salton et al. 2011). KGF is also known for the

induction of differentiation (Millman, Xie et al. 2016). KGF promotes β -cell regeneration by stimulating duct cell proliferation *in vivo* leading to β -cell neogenesis (Uzan, Figeac et al. 2009). Activin-A promotes islet differentiation via ACT--MEPK-TKK pathway mediated through activin receptors which facilitate islet generation (Kojima and Umezawa 2006).

Our lab has deployed the islet neogenic potential of Keratinocyte growth factor (KGF) as a positive control while differentiating two model cell lines PANC-1 and NIH3T3 generating islet like cellular aggregates which were positive for islet hormones (Sarita, Dadheech et al. 2010) and Activin-a was used as an islet differentiation inducer agent with positive results using NIH3T3 cell line (Dadheech, Soni et al. 2013). Activin-a was also used as islet neogenic agent while differentiating human pancreatic progenitor PANC-1 and Mouse intra-islet progenitor cells into newly formed islet clusters (Dadheech, Srivastava et al. 2015). We also demonstrated role of Activin-a potentiation in migration, homing, and β-cell differentiation of transplanted BMSCs, whereby significant migration of BMSC (~ 6%) into diabetic pancreas with Activin-a treatment was observed. This is accredited to key endocrine transcriptional reprogramming initiation with Activin-a treatment (Dadheech, Srivastava et al. 2020).

- **Induction of islet neogenesis by medicinal plants**

Plant extracts have been attractive therapeutic candidates and their potential use in the treatment of diabetes. Various plant extracts are known to have beneficial effects on pancreatic β cell function. Dietary intake of these extracts may serve as a promising strategy for diabetes prevention (Oh 2015) (Abhay Srivastava 2021). Medicinal plant *Citrullus colocynthis* has regenerative effect on β cells and affects intra-islet vasculature (Amin, Tahir et al. 2017). *Tinospora cordifolia* also has β cell regenerative properties (Rajalakshmi and Anita 2016). *Moringa oleifera* is known for regeneration of damaged pancreatic cells by employing its antioxidant properties (Abd El Latif, El Bialy et al. 2014). *Oreocnide integrifolia* has *in vivo* pancreatic regeneration ability of 70% as seen in pancreatectomized BALB/c mice (Bharucha, Umarani et al. 2012).

➢ Enicostemma littorale: Anti diabetic effects and islet neogenesis

Our Lab has long been active in the principal area of research related to the systematic elucidation of various properties of plant-derived biomolecule in diabetes healthcare. The study of islet biology and the fate of pancreatic regeneration and the development of endocrine pancreatic islet cells from adult stem/ precursors has been a key component of the core research area in the lab. Current therapeutic avenues available for diabetes treatment are confined to managing diabetes transiently. As synthetic drugs have their side effects, alternative and complementary approaches as therapeutics are fast. Natural plant compounds have long been in traditional knowledge as anti-diabetic medications which have provided greater insight into them being safe and cost-effective (Abhay Srivastava 2021).

One such medicinal plant is *Enicostemma littorale (EL)* whose antidiabetic activity has been well documented by our lab (Fig 1.19). *Enicostemma littorale* (family: Gentianaceae) is a glabrous perennial herb. It grows throughout India up to 1.5 feet high. It is called Chota-kirayat or Chota chirayata in Hindi, Mamejavo in Gujarati, Nagajivha in Bengal, and Vellarugu or Vallari in Tamil. As for the high nutritional value of EL, 2 g of EL fresh leaves is daily recommended in diabetes (Upadhyay and Goyal 2004) (Sathishkumar, Lakshmi et al. 2009).

Traditionally aqueous extract and dried powder of this herb have been used for the treatment of malaria and diabetes. Our Lab showed that a long-term 30 days treatment of aqueous extract significantly reduced blood sugar levels in alloxan-induced diabetic rats (Vijayvargia, Kumar et al. 2000). We then demonstrated that a single dose of aqueous extract of EL (15 g dry plant equivalent extract per kg) had shown a significant increase in the serum insulin levels in alloxan-induced diabetic rats along with insulin secretagogue action in isolated rat pancreatic islets (Maroo, Vasu et al. 2002). The dose-dependent blood-glucose-lowering effect of aqueous extract of E. littorale Blume in alloxan-induced diabetic rats was also demonstrated (Maroo J., Vasu TV. et al. 2003). Apart from animal studies, we have also reported the antidiabetic effect of aqueous extract EL in newly diagnosed Non-insulin-dependent diabetes mellitus (NIDDM) patients showing hypoglycemic, antioxidant, and hypolipidemic actions. A decrease in glycosylated hemoglobin and an increase in serum insulin levels (Vihas T Vasu, C Ahsvinikumar et al. 2003). Aqueous extract of E. littorale (1.5 g/100g body weight/day) has also been shown by our lab having

hypolipidaemic and antioxidant effects within 6 weeks in hypercholesterolemia-induced rats (Vasu, Modi et al. 2005). The protective effect of EL was observed with 45 days of treatment to alloxan-induced diabetic neuropathy in rats with significant reduction in nociception (Niraj Mukundray Bhatt 2009). The protective effect of EL methanolic extract on gentamicin-induced nephrotoxicity in rats was observed (Bhatt, Chauhan et al. 2011). Cardioprotective and antihypertensive effects of EL in HF-fed rats were observed (Bhatt, Chavda et al. 2012). Our group has also suggested that the methanolic extract of Enicostemma littorale imparts cytoprotective and anti-apoptotic effects to the islet of Langerhans against oxidative stress (Srivastava, Bhatt et al. 2016).

EL extract was used to differentiate two model stem cell lines PANC-1 and NIH3T3 into functional insulin-producing islet-like clusters, which showed tremendous islet neogenic potential and significant islet yield. Morphological and molecular characterization of newly generated islet-like cellular aggregates proved them differentiated and positive for islet hormones (Gupta S, Dadheech N et al. 2010).

Our Lab has been granted patent for "Islet neogenic potential of methanolic extract of *Enicostemma Littorale* herb". Patent filed to Department of Biotechnology, Government of India. (Patent number- #2425/del/2009)

- **Induction of islet neogenesis by bioactive molecules**

Natural bioactive compounds are a source of novel pharmaceuticals enabling phytochemical-based therapies because of their diversity, complex structures, and biological potency which render them as safe, cost-effective, and therapeutically efficacious treatment of diabetes (Dias, Urban et al. 2012, Abhay Srivastava 2021).

Geniposide promotes β cell regeneration and survival in isolated mouse islets & mouse pancreatic cell line (MIN6) (Yao, Yang et al. 2015). Improved integrity of islets of Langerhans was observed in the Kinsenoside treated rats, indicating pancreatic β-cell regeneration (Zhang, Cai et al. 2007). Silymarin recovers the normal morphology and endocrine function of damaged pancreatic tissue in alloxan-treated diabetic rats (Soto, Raya et al. 2014). Genistein boosts insulin secretion & prevents pancreatic β-cell apoptosis (Gilbert and Liu 2013). Conophylline addition to pancreatic

rudiments and AR42J cells (a model of pancreatic progenitors) increased the number of insulin, and Pdx-1-positive cells. These indicates differentiation of pancreatic progenitors' cells (Kojima and Umezawa 2006) (Ogata, Li et al. 2004). The researcher suggests pancreatic islets regeneration in diabetic rats when treated with Curcumin. Anti-inflammatory and antioxidant effects of curcumin generate a favorable environment to promote islet neogenesis (Abdul-Hamid and Moustafa 2013) (Aziz, El-Asmar et al. 2013) (Chuengsamarn, Rattanamongkolgul et al. 2012).

Our lab has significant contribution in carrying out several research concerning bioactive in the field of healthcare biology with various advanced techniques in isolation, characterization, lab scale production, and mechanistic elucidation of biomolecule in diabetes therapeutics. Swertisin, one of the active components of EL which has been systematically explored in our lab for its potent islet neogenic and anti-hyperglycemic properties using various stem cells/progenitors (Panc-1 and NIH3T3) (Gupta S, Dadheech N et al. 2010) (Dadheech, Soni et al. 2013) and differentiating them into functional islet-like clusters. Investigation on the molecular mechanism was done for swertisin for generating and promoting differentiation of pancreatic progenitors into islet cells. Panc-1 and primary cultured mouse intra-islet progenitor cells (mIPC) were used for Swertisin induced islet neogenesis mechanism. It was established that swertisin follows MEPK-TKK pathway for islet neogenesis involving the role of p38 MAPK via activating Neurogenin-3 (Ngn-3) and Smad Proteins cascade. The mechanism was explored *in vivo* in the partial pancreatectomised (PPx) mice model, where swertisin exerted a potential increase in insulin transcript levels with persistent down-regulation of progenitor markers within three days post PPx (Dadheech, Srivastava et al. 2015). We also reported that Pancreatic Resident Endocrine Progenitors were differentiated into mature islet clusters by swertisin and functionally characterized. Furthermore, PREPs transplanted in STZ diabetic mice migrated and localized within the injured pancreas (Srivastava, Dadheech et al. 2018). Swertisin's role in triggering resident pancreatic progenitors for islet neogenesis in streptozotocin-diabetic mice was discovered. Swertisin was administered to STZ diabetic mice and normoglycemia with an elevation of fasting serum insulin levels was observed. This highpoint pancreatic innate competence to regenerate and restore using its resident progenitors upon appropriate stimulus, which acts for effective diabetic therapy (Srivastava, Dadheech et al. 2018). We have examined differentiation of mouse Bone Marrow Stem Cells into islet like cell clusters by differentiating agent swertisin (Nidheesh Dadheech Ph.D. Thesis). We have also demonstrated

that human Bone Marrow Stem Cells recapitulates *in vivo* pancreatic islet development by differentiating into mature pancreatic islets by swertisin containing biomolecule cocktail (Mitul Vakani Ph.D. Thesis).Our Lab has been granted a patent by The Patent Office, Government of India for ‘’Swertisin as a potent and novel molecule for islet differentiation from human bone marrow derived mesenchymal stem cells’’ (Patent no. 391796 (2016))

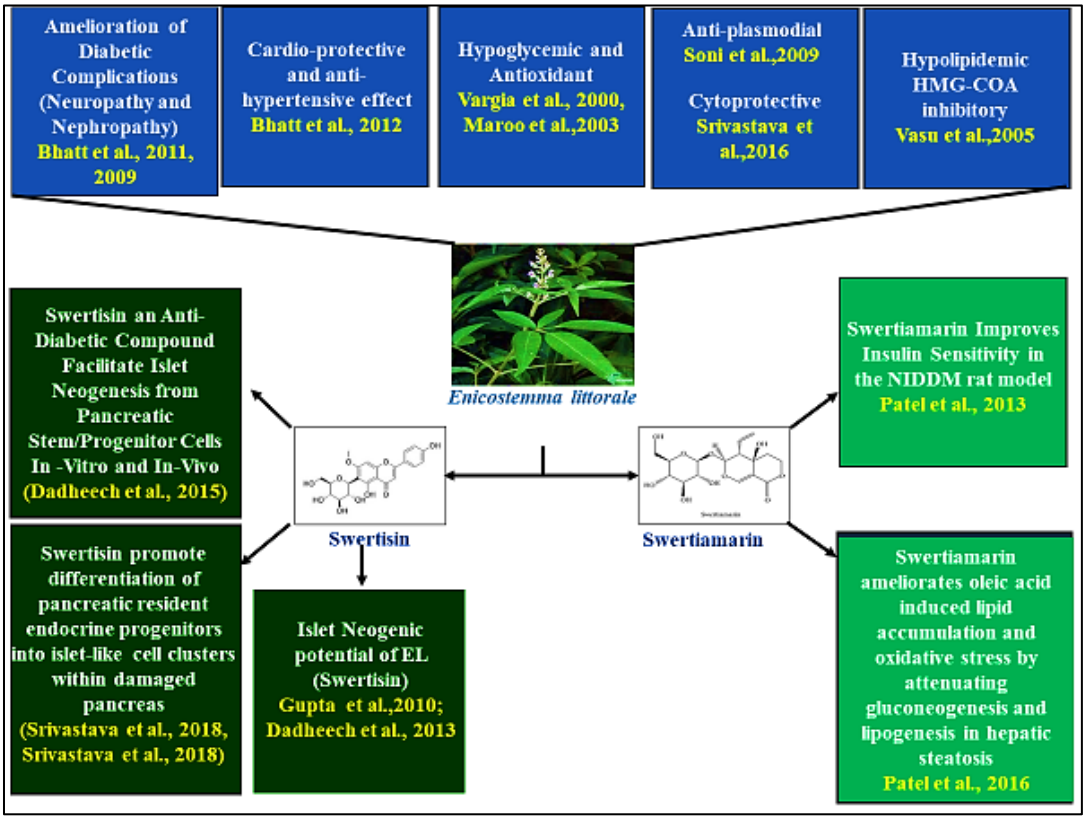

Figure 1.9 Properties of Enicostemma Littorale, Swertisin and swertiamarin (Our Lab Reports)

Type 2 Diabetes Mellitus

Type 2 Diabetes Mellitus (T2DM) also referred to as non-insulin-dependent diabetes (NIDDM) or adult-onset diabetes, is one of the most common metabolic disorders worldwide which comprises 90–95% of all diabetes (Committee 2022). It is a chronic disease associated with several macrovascular and microvascular complications. Its development is characterized by a combination of two main factors: relative insulin deficiency due to defective insulin secretion by pancreatic β cells and peripheral insulin resistance which is the inability of insulin-sensitive tissues to respond to insulin (Roden and Shulman 2019) (Galicia-Garcia, Benito-Vicente et al. 2020). There is an inadequate compensatory insulin secretory response when β cells fail to secrete sufficient insulin to keep up with demand to maintain glucose homeostasis. Thus, the progression of the disease results in hyperglycemia (Zheng, Ley et al. 2018) (Skyler, Bakris et al. 2017).

Insulin and its target organs

Insulin is a small protein of molecular weight of 5.8 kDa. It is produced by the β cells of the islets of Langerhans of the pancreas, in response to blood glucose levels and is deposited into the intracellular space (Fu, Gilbert et al. 2013). It then passes into the bloodstream and proceeds through the circulation to the cells of the body and stimulates glucose use differently in various tissues. Insulin has target organs in which it determines various metabolic changes. Insulin-dependent organs comprise skeletal muscle, liver, and adipose tissue. After food consumption, it is digested and absorbed from the intestinal tract into the bloodstream. After meeting the immediate energy needs of the body, most of the assimilated food is stored for later use. Carbohydrate is stored in the form of glycogen in skeletal muscle and liver more importantly as a fuel for the brain which is completely insulin-dependent. Fat is stored in adipocytes in adipose tissue for future use. Insulin also inhibits glycogenolysis in myocytes, decreases hepatic gluconeogenesis, and inhibits lipolysis of fat in adipose tissue. All of these cellular uptakes as well as the storage of glucose from the blood into the tissue are mediated by the action of insulin. Therefore, insulin is very critical for glucose transportation. Thus, a deficit of insulin results in a reversal of these processes and leads to a state of starvation. All of the insulin action promotes glucose uptake by cells and reduces circulating levels of glucose in the blood. Insulin exhibits

several functions on different cells. It is also involved in protein metabolism, cell division, and growth through its mitogenic effects. (Guthrie and Guthrie 2004).

Insulin signaling

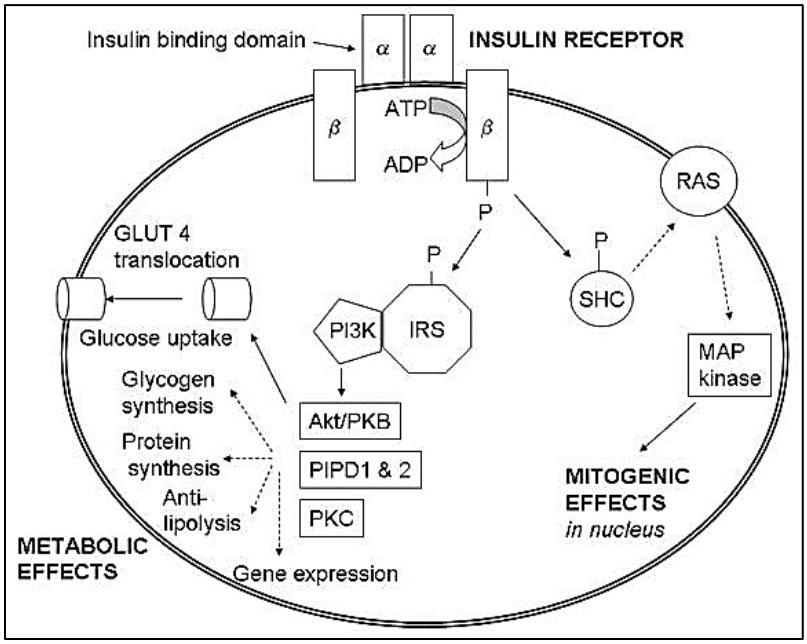

Figure 1.10 Insulin signaling pathway (Wilcox 2005)

Physiological insulin signaling follows the binding of insulin to the insulin receptor, a ligand-activated tyrosine kinase. Insulin binds to its transmembrane receptor and promotes its autophosphorylation at tyrosine residues (p-IR). The insulin receptor was first characterized in 1971. It consists of a heterotetramer consisting of 2 α and 2 β glycoprotein subunits which are linked by disulfide bonds. Insulin binds to the extracellular α subunit, resulting in conformational change enabling ATP to bind to the intracellular component of the β subunit. ATP binding, in turn, triggers phosphorylation of the β subunit conferring tyrosine kinase activity. Activated p-IR recruits IR substrate (IRS) and enhances its activation by mediating its phosphorylation (p-IRS). Phosphorylated IRS proteins bind specific src-homology-2 domain proteins (SH2). p-IRS subsequently binds to p85, the regulatory subunit of phosphoinositide-3 kinase (PI3K), and elevates the activation of its catalytic subunit p110, which then activates phosphoinositide-dependent kinase (PDK). PI 3-kinase acts via serine and threonine kinases such as Akt/protein kinase B (PKB), protein kinase C (PKC), and PI dependent protein kinases1& 2 (PIPD 1&2). As

the upstream kinase of Akt, PDK promotes the phosphorylation of Akt (p-Akt) at Thr308 and Ser473. Activated Akt regulates glucose metabolism in two pathways. One is promoting glucose transporter GLUT translocation from the cytoplasm to the membrane, which mediates glucose uptake; another one is repressing the function of glycogen synthesis kinase 3 (GSK3) by enhancing its phosphorylation at Ser9, and then enhancing the activation of GS and promoting glycogen synthesis (Fig 1.10) (Copps and White 2012) (Wilcox 2005) (Fu, Gilbert et al. 2013).

Insulin Resistance

The study of insulin resistance and its associated clinical manifestations remain at the forefront of medical research, at the center of healthcare biology. Insulin resistance is defined where a normal or elevated insulin level produces an attenuated biological response; classically this refers to impaired sensitivity to insulin-mediated glucose metabolism. Physiologically, insulin actions are influenced by the action of other hormones. Insulin acts in coordiantion with growth hormone and IGF1. Other counter-regulatory hormones include glucagon, glucocorticoids, and catecholamines. These hormones drive metabolic processes in the fasting state. In addition to insulin resistance, the increased demand for insulin could not be met by the pancreatic β cells due to defects in the function of these cells. On the contrary, insulin secretion decreases with the increased demand for insulin over time due to the gradual destruction of β cells that could transform some type 2 diabetes patients from being independent to becoming dependent on insulin. As insulin resistance develops, the β cells increase insulin production to compensate and maintain the blood glucose level in the narrow range needed for normal bodily function. If insulin resistance persists or increases over time (usually years), the β cells will begin to fail. When insulin resistance persists but insulin secretion decreases and blood glucose levels begin to rise, true diabetes has developed. Insulin resistance is probably the first defect in T2DM. Insulin resistance in type 2 diabetes patients increases the demand for insulin in insulin-target tissues and begins many years before the onset of symptoms or developing a blood glucose level high enough to make the diagnosis (Wilcox 2005) (Galicia-Garcia, Benito-Vicente et al. 2020) (Kharroubi and Darwish 2015) (Guthrie and Guthrie 2004).

Manifestation sites for insulin resistance

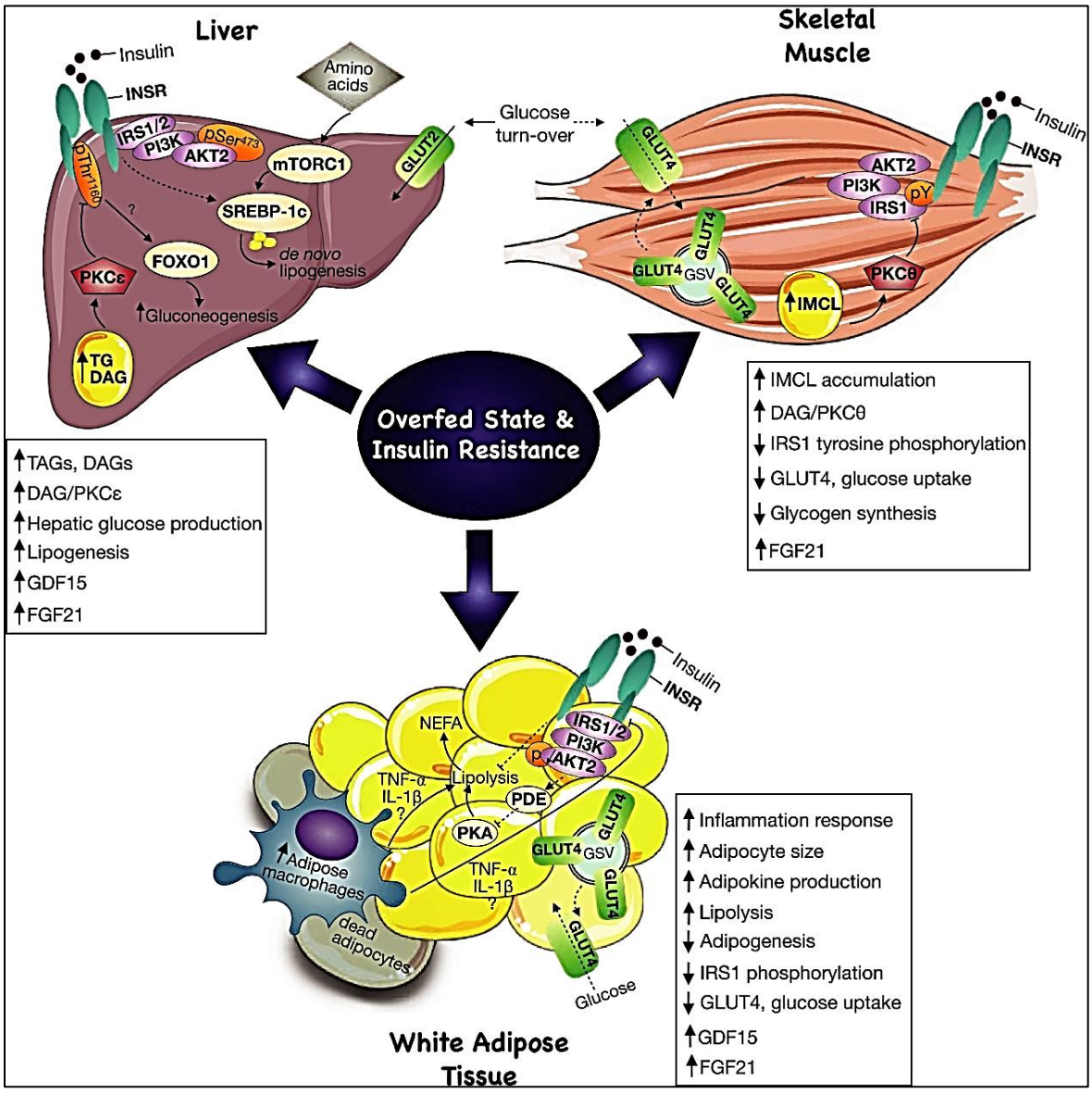

Figure 1.11 Physiological insulin resistance and insulin dependent tissues (da Silva Rosa, Nayak et al. 2020)

A) Skeletal muscle

The skeletal muscle accounts for nearly 80% of the postprandial site for disposal of ingested glucose in lean healthy normal glucose tolerance (NGT) individuals. Approximately one-third of ingested glucose is taken up by the liver, post-meal, and the remaining by peripheral tissues, chiefly by insulin-dependent skeletal muscle. The postprandial hyperglycemia stimulates pancreatic β

cells to release insulin which then stimulates glucose uptake in skeletal muscle. In the skeletal muscle, glucose is transported into the myocyte by the GLUT4 transporter and immediately phosphorylated by hexokinase. Phosphorylated glucose either is stored as glycogen, or enters the glycolytic pathway for oxidation which is 75% of all glucose disposal (Thiebaud, Jacot et al. 1982). Skeletal muscle utilizes both glucose and free fatty acid (FFA) as fuel sources for energy production (Groop, Bonadonna et al. 1989). During the fasting state, muscle glucose uptake is low and FFA serves as the main fuel source for energy production, while the brain exclusively depends on glucose. In the fed state, increasing glucose levels and resultant hyperinsulinemia suppresses lipolysis which leads to a decline in plasma FFA concentration, and simultaneously insulin stimulates glucose uptake in skeletal muscle, activating key enzymes in glucose metabolism, enabling muscle glucose oxidation (DeFronzo 1988) (Defronzo 2009). The ability of the skeletal muscle to switch from fat oxidation during the fasting state to glucose oxidation during the postprandial state has been referred to as metabolic flexibility (Abdul-Ghani and DeFronzo 2010) (da Silva Rosa, Nayak et al. 2020).

Skeletal muscle, by its mass and high rate of insulin-stimulated glucose transport, represents an important tissue in the development of insulin resistance which represents a major defect in the maintenance of normoglycemia and results in metabolic abnormalities like T2DM (Galicia-Garcia, Benito-Vicente et al. 2020).

i. Molecular mechanism of insulin resistance

Insulin binding to the α-subunit of the Insulin receptor causes phosphorylation of the β-subunit on multiple tyrosine residues and activates insulin-mediated signaling. Thus, mutations in any of the main phosphorylation sites can affect INSR tyrosine kinase activity, thus impairing insulin action on skeletal muscle. In insulin-resistant conditions, serine phosphorylation of IRS (Insulin Receptor substrate) protein is promoted and mutations in IRS-1 and IRS-2 impairs insulin action on the muscle and progress into diabetes. In T2DM subjects, reduced IRS-1 tyrosine phosphorylation is observed which is related to their increased serine/threonine phosphorylation (Bjornholm, Kawano et al. 1997). As a result, Insulin-stimulated protein kinases like serine/threonine kinase AKT and PI3K levels are reduced (Kim, Nikoulina et al. 1999) (Kim, Kotani et al. 2003). Disruption in the AKT and PKC kinases is central to the development of diabetes and is associated with all major

features of the disease including hyperinsulinemia, dyslipidemia, and insulin resistance. Apart from defects in intermediate steps in the insulin signaling cascade, defects in GLUT4 translocation and trafficking are responsible for an insulin-resistant state in skeletal muscle. Thus, mutations that reduce the expression of insulin receptor or GLUT4 activity/translocation, as well as any defect in either upstream or downstream signaling pathway would reduce glucose uptake into the muscle resulting in a hyperglycaemic state leading to diabetes (Abdul-Ghani and DeFronzo 2010) (Garvey, Maianu et al. 1998).

B) Adipose tissue

Adipose tissue is an insulin-responsive tissue that accounts for about 10% of insulin-stimulated whole-body glucose uptake. It is critically important in influencing both glucose and lipid metabolism by releasing adipokines, proinflammatory cytokines, and free fatty acids (FFAs). Here, insulin promotes the storage of triglycerides by stimulating the differentiation of preadipocytes to adipocytes, inhibiting lipolysis, and increasing the uptake of fatty acids and glucose (Jung and Choi 2014). Similar to the mechanisms in muscle, insulin exerts its biological effects via the IRS-PI3K-Akt2-GLUT4 signaling pathways (Chadt, Immisch et al. 2015). A major role of insulin in adipose tissue is to promote the suppression of lipolysis where lipid triglycerides are hydrolyzed into glycerol and fatty acids and used to provide stored energy during fasting or exercise but in the presence of an obesogenic environment, this leads to the deleterious effect of ectopic lipid accumulation. Excess energy storage leads to the hypertrophy of adipocytes which affects the secretion of many adipokines specifically, proinflammatory adipokines. Therefore, a reduction in anti-inflammatory adipokine secretion, concurrent with an increase in inflammatory cytokine secretion plays an important role in the onset of adipose tissue insulin resistance. Certainly, adipose tissue is an essential regulator of overall health. Therefore, impaired adipose tissue function may lead to a series of complications such as insulin resistance, T2D, among other metabolic diseases (Fig 1.11) (da Silva Rosa, Nayak et al. 2020).

i. Adipose derived stem cells

Adipose tissue is a very dynamic, metabolically active organ involved in multiple physiological processes. It is composed of adipocytes and a stromal vascular fraction (SVF) consisting of pericytes, monocytes, endothelial cells, macrophages, and Adipose derived Stem Cells (ADSC).

Stem cell therapy has great potential for the treatment of a wide variety of diseases. They can expand and differentiate into a variety of cell types, to repopulate and revitalize the damaged cells correcting tissue defects, improving their performance. They also expand and differentiate to different cell types like adipocytes, chondrocytes, myocytes, osteoblasts, and neurocytes among other cell lineages (Coelho, Oliveira et al. 2013). They have an enormous use in tissue repair and regeneration owing to their developmental plasticity and therapeutic potential. Compared with other sources, the resources of adipose tissue are much less expensive with the minimum invasive operation. Therefore, adipose tissue represents an abundant, practical, and attractive source of donor tissue for autologous cell replacement (Baer and Geiger 2012). ADSCs secrete several cytokines, growth factors, and antioxidant factors into their microenvironment regulating intracellular signaling pathways in neighboring cells. Thus, ADSC therapy has demonstrated beneficial effects, suggesting that secreted factors protect correcting tissue defects (Miana and González 2018) (Rochette, Mazini et al. 2020). However, a harsh microchemical environment especially in terms of high glucose levels like in diabetic conditions affects the potential of ADSC. Glucotoxicity impairs β-cell function and induces apoptosis. Not only β-cells, stem cells obtained from high glucose conditions induced cellular senescence, while reduction of glucose enhanced proliferation, decreased apoptosis. Thus, the deleterious effect is seen on the stem cells which is a result of aggravation of oxidative stress triggered by high glucose (Saki, Jalalifar et al. 2013).

Our Lab has also worked on the metabolism, regulation, stemness and therapeutic potentials of adipose derived stem cells from Indian population. hADSC of obese Indians are highly vulnerable to detrimental effects at a very low BMI compared to those of Caucasian population. We were the first to explain that obesity mitigates insulin signaling of human ADSC (hADSC) and promotes insulin resistance with diminished metabolism of hADSC owing to high inflammation with concomitant reduced pluripotent markers. A cautious application of hADSC from Indian obese in regenerative therapy is suggested which can be modulated for their potential applications in stem cell therapy (Rawal, Patel et al. 2020) (Komal Rawal Ph.D Thesis 2019) (Rawal, Patel et al. 2020, Rawal, Purohit et al. 2021)

Insulin resistance at the systemic level

A complex range of factors and cellular disturbances in glucose and lipid metabolism in various tissues contribute to the development of insulin resistance. Obesity is critically the most predominant risk factor leading to insulin insensitivity and diabetes. Obesity-induced insulin resistance is directly linked to increased nutrient influx and energy accumulation in tissues that directly affect cell responsiveness to insulin. Increased immune cell infiltration and secretion of proinflammatory molecules in intramyocellular and perimuscular adipose tissue lead to skeletal muscle inflammation. This leads to myocyte inflammation, impaired myocyte metabolism, and contributes to Insulin Resistance. When the capacity of visceral and subcutaneous adipose tissue is overwhelmed in obesity, circulating levels of fatty acids are markedly increased and lipid deposition in other tissues like liver, muscle etc. is observed. Thus, lipotoxicity culminates in IR in muscles, liver and pancreas. Apart from mutations or defective epigenetic regulation, environmental factors play an important role in glucose uptake by muscle. Reduced mitochondrial function has been considered as an important contributor to 'toxic' lipid metabolite accumulation, reduced insulin sensitivity and insulin resistance (Wu and Ballantyne 2017) (Petersen and Shulman 2002) (Samuel and Shulman 2012) (Guilherme, Virbasius et al. 2008).

Interorgan crosstalk and its impact in normal and diabetic condition

The major focus of the present thesis is on interorgan crosstalk of insulin sensitive tissues with pancreatic islets

i. Skeletal muscle as a secretory organ and its effect on pancreas/islets

Apart from being a locomotory organ, skeletal muscle has been identified as secretory in nature which orchestrates various roles in the body (Fig 1.12). Far from being an inert organ, skeletal muscle actively takes part in interorgan communication by various secretory products which constitutes the secretome of the skeletal muscle (Goldstein 1961) (Rai and Demontis 2016) (Jalabert, Vial et al. 2016) (Pedersen and Febbraio 2012). Various comprehensive quantitative analysis reports of skeletal muscle secretome point towards a wide range of secretory products. With being identified as an endocrine organ, the cytokines which were released from the skeletal muscle were termed as myokines (Whitham and Febbraio 2016). These myokines exert autocrine, paracrine, or endocrine functions and are part of a complex network that mediates communication

between different organs like a muscle, the liver, adipose tissue, the brain, and other organs. Myokines regulated by muscle contraction plays role in mediating the health-promoting effects of regular physical activity and in the prevention of insulin resistance, inflammation and metabolic dysfunction.

Studies in human muscle indicate that the synthesis and secretion of myokines are regulated by several conditions like differentiation (Le Bihan, Bigot et al. 2012), exercise (Norheim, Raastad et al. 2011), *in vitro* electrical stimulation (Scheler, Irmler et al. 2013), and insulin resistance (Bouzakri, Plomgaard et al. 2011). Thus, the secretome of muscle gets altered with different stimulus conditions. The aberrant secretory function of skeletal muscle in T2DM impacts multiple tissues which are strikingly detrimental than a normal healthy muscle secretome (Ciaraldi, Ryan et al. 2016).

The islet pathophysiology gets detrimentally affected by secretome from IR myotube secretome. It is observed that the secretome profile of skeletal muscle changes with the given stimuli, so considerable change was observed in insulin-resistant conditions where a different panel of myokines is observed which has unfavorable effects on other tissues of the body including pancreatic islet β cells (Bouzakri, Plomgaard et al. 2011) (Ciaraldi, Ryan et al. 2016).

IL-6 is the first identified and most studied myokine. The basal plasma IL-6 concentration increases up to 100-fold after aerobic exercise which elicits a systemic cytokine response. IL-6 enhances glucose uptake and increases intramyocellular or whole-body fatty acid oxidation by activation of AMPK in skeletal muscle (Carey, Steinberg et al. 2006). Muscular IL-6 has a role in metabolism rather than in inflammation. Upon muscle contraction, IL-6 is released into the circulation the amount of which is dependent on the intensity and duration of exercise and the energy status of a muscle (Steensberg, van Hall et al. 2000). Insulin sensitivity depends on the duration of exposure to IL6 whether it is acute or chronic. Acute IL-6 treatment increased glucose uptake, while chronic exposure resulted in insulin resistance due to activation of JNK and impairment of insulin signaling by the aberrant effect on IRS-1 (Eckardt, Görgens et al. 2014). It is demonstrated that myotubes from type 2 diabetic patients are resistant to the acute effect of IL-6 on glucose metabolism (Jiang, Duque-Guimaraes et al. 2013). IL-6 increases the proliferation of both alpha and β cells in islets of the pancreas and prevents apoptosis of alpha cells which resulted

from metabolic stress. Importantly, IL-6 promotes glucagon-like peptide-1 (GLP-1) secretion and production in intestinal L cells and pancreatic alpha cells, thereby leading to improved β cell insulin secretion and glucose tolerance. The increase in GLP-1 plasma levels during exercise is mediated by skeletal muscle-derived IL-6. An interaction between GLP-1 and IL-6 in the brain indicates the role of central IL-6 in mediating the anorexic and body weight loss effects of GLP-1 receptor activation (Ellingsgaard, Hauselmann et al. 2011). Moreover, IL-6 released by contracting skeletal muscle enhances the production of anti-inflammatory cytokines like IL-10, IL-1, IL-1RA, etc.(Pedersen and Febbraio 2008) Of which IL-10 is known to inhibit the production of proinflammatory factors like IL-1α, IL-1β, TNFα, IL-8, and MIPα (Peterson and Pizza 2009).

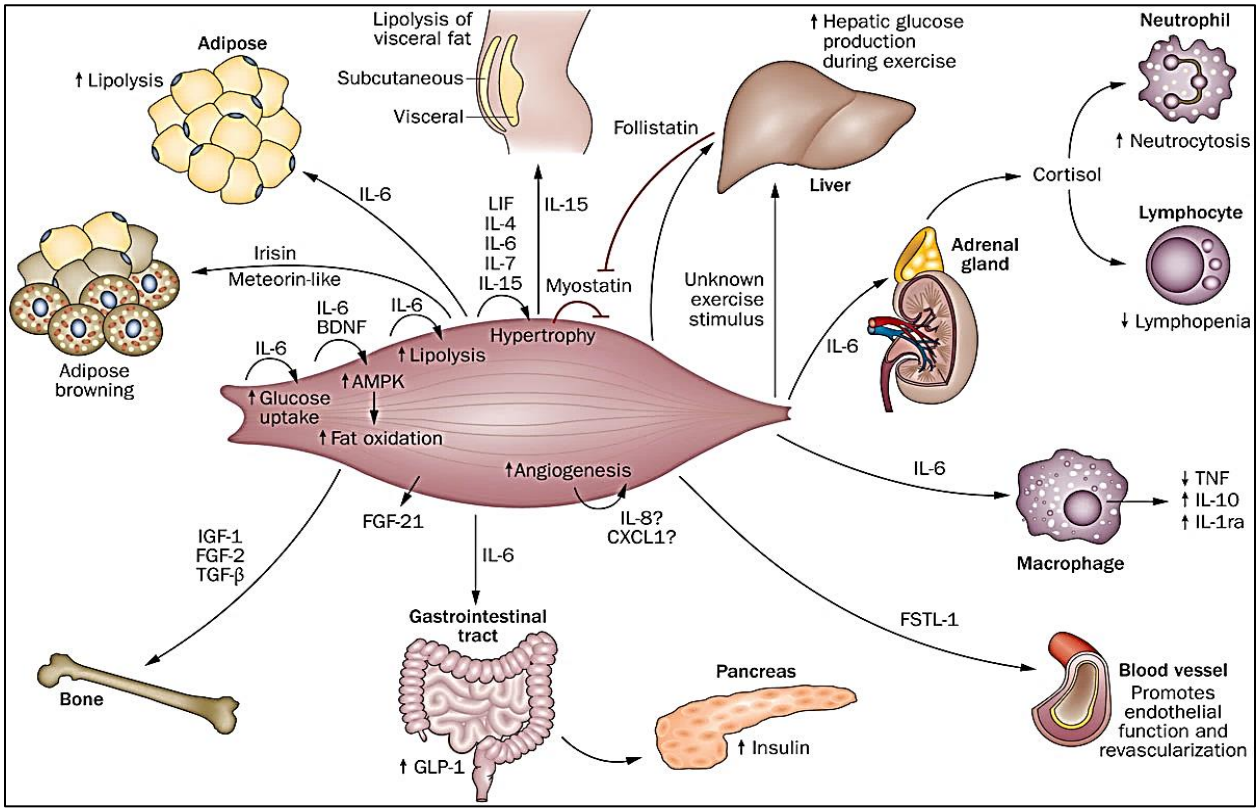

Figure 1.12 Effects of various myokines on different organs (Benatti and Pedersen 2015)

IL-13 myokine is released by human myotubes. It increases glucose uptake and oxidation and also increases glycogen synthesis in skeletal muscle while decreasing glucose production in the liver. Individuals with T2DM have significantly reduced serum levels of IL-13 which is 75% less IL-13 than myotubes from controls (Jiang, Franck et al. 2013) (Stanya, Jacobi et al. 2013).

IL-15 is expressed and released by skeletal muscle and belongs to IL-2 superfamily (Peterson and Pizza 2009). It has anabolic effects on skeletal muscle and plays a potential role in lipid metabolism by decreasing lipid deposition in preadipocytes and the mass of white adipose tissue and increasing fatty acid oxidation by induction of PPARδ expression. Lower levels of IL-15 have been reported in T2DM patients (Al-Shukaili, Al-Ghafri et al. 2013)and obese individuals (Barra, Reid et al. 2010). Thus, IL-15 plays a role in muscle–fat interaction and skeletal muscle fiber growth-regulating metabolic diseases like obesity and diabetes (Almendro, Busquets et al. 2006).

Fractalkine (also known as CX3CL1) plays a role in crosstalk to the pancreas and is associated with obesity, insulin resistance, and T2DM (Henningsen, Rigbolt et al. 2010) (Shah, Hinkle et al. 2011). Treatment of islets with CX3CL1 increases insulin secretion in both mouse and human islets. It is observed that human islets express and secrete CX3CL1 and have a protective effect on islets and decrease basal apoptosis of human β-cells (Rutti, Arous et al. 2014). Another muscle-derived myokine CXCL1 reduces diet-induced obesity through the improvement of fatty acid oxidation and oxidative capacity in skeletal muscle tissue (Pedersen, Olsen et al. 2012).

FGF21 is a member of the fibroblast growth factor superfamily which is involved in cell proliferation, growth, and differentiation. FGF21 is an Akt-regulated myokine (Izumiya, Bina et al. 2008). FGF21 acts on several tissues by affecting carbohydrate and lipid metabolism, enhancing insulin sensitivity, decreasing triacylglycerol concentrations, and causing weight loss. FGF21 promotes a white to the brown shift of WAT as demonstrated by increased oxygen consumption in WAT (Hojman, Pedersen et al. 2009). Serum levels of FGF21 are significantly higher in type 2 diabetic patients than in healthy controls (Cheng, Zhu et al. 2011).

ii. ADSC Secretome and its effect on pancreas/islets

Stem cell therapy has gained wider recognition for its remarkable ability to support cell survival and homing capacity to the damaged tissue. One such remarkable element is the regenerative capacity of secreted factors from adipose tissue-derived stem cells (ADSCs). ADSC secretome is known to orchestrate various functions like wound healing, angiogenesis, anti-inflammation, immunomodulatory, anti apoptosis etc. Proangiogenic properties of the ADSC secretome have been demonstrated using rodent limb ischemia and acute myocardial infarction models (White, Acton et al. 2014). ADSCs have a greater anti-inflammatory effect on preparations of monocyte

derived dendritic cells thus exhibiting immunomodulatory effect (Ivanova-Todorova, Bochev et al. 2009). Pro-inflammatory stimuli have commonly been used to study the ASC secretome which explored wound healing properties in a rodent model (Heo, Jeon et al. 2011). Conditioned media (CM) from stem cells have garnered much attention for regenerative potential without the involvement of the cell itself. It has also proved to have a beneficial effect on islet functionality. Various reports suggest direct or indirect co-culture of ADSC with islet have shown anti apoptotic effect and increased insulin secretion.

Indirect and direct effects of human ADSC (hADSCs) on rat islets viability and functionality have been studied. The secreted amount of insulin from islets was improved and restored when associated with hADSCs or/and FGF2 under normoxia and hypoxia respectively. hADSC-conditioned medium contained various anti-apoptotic factors which improve islets viability and decrease apoptosis (Bhang, Jung et al. 2013). hADSCs improve the viability of porcine islet cells in indirect co-culture by paracrine signaling of trophic factors, particularly VEGF, which enhanced the survival and function of islets (Yamada, Shimada et al. 2014). Encapsulation of rat islets with ADSC was also done within collagen-alginate composite microfibers and implanted in diabetic mice which resulted in lower average blood glucose and high survival rate of co-cultured islets-ADSC before being compromised by the host immune system after a long term study (Jun, Kang et al. 2014). Pancreas examination of diabetic mice revealed increased expression of insulin which received ADSC injections which protected pancreatic β-cell from damage induced by STZ (Li, Liu et al. 2012). In a T1DM patient study, it was demonstrated that co-infusion of insulin-making cells, undifferentiated ADSCs and BMSC in conjunction with live donor renal transplant is a safe and effective therapy for patients with end-stage renal disease (Dave, Vanikar et al. 2013).

Type 2 Diabetes Mellitus Management

A prominent concern for a therapeutic approach in diabetes Mellitus is Hyperglycemia which wreaks havoc on overall glucose homeostasis. Thus, Anti-hyperglycemic agents play a key role in maintaining normoglycemia. Most of the drugs used in diabetes treat diabetes mellitus by lowering glucose levels in the blood (Chaudhury, Duvoor et al. 2017). T2DM is characterized by insulin resistance whose treatment includes (1) drugs increasing insulin production from pancreatic β cells (2) drugs that lower glucose absorption from the gastrointestinal tract (3) drugs that increase the

sensitivity of target organs to insulin. (Ergun-Longmire B et al 2014). Current treatment available for treating insulin resistance includes the use of Insulin Sensitizers like Biguanides, Thiazoidinediones, Insulin Secretogogues like sulfonylureas. Non insulin medications such as incretin mimetics and amylin analogs ,Dipeptidyl peptidase-4 (DPP-4) inhibitors Alpha-glucosidase inhibitors (Fig 1.13) (Wilcox 2005). Another class of emerging medication is SGLT(Sodium glucose co transporters) inhibitors which is the major study of the present thesis.

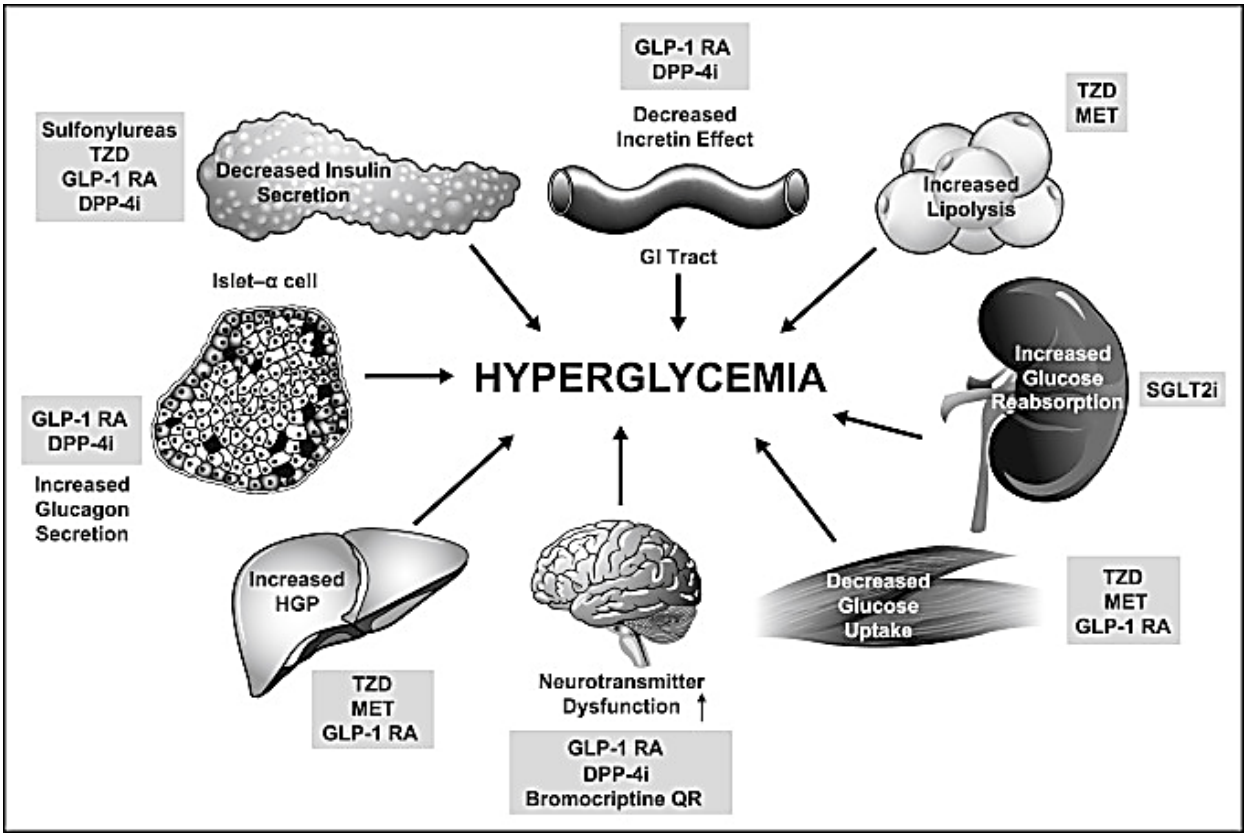

Figure 1.13 Site of action for glucose-lowering medications (Thrasher 2017)

i. Insulin sensitizers

A) Biguanides

Reduction of glucose production by the liver and decreasing the absorption of glucose from the intestine is involved by the biguanides mechanistic approach in improving the body's response to natural insulin. By decreasing gluconeogenesis and stimulating glycolysis, it reduces the glucose output of the liver. They act by augmenting the insulin receptor activity and thereby increasing

insulin signaling (Quillen, Samraj et al. 1999). Metformin, Phenformin, and Buformin are included in this class. Metformin, a biguanide derivative, is one of the most widely and commonly used drug to treat type 2 diabetes (T2D) which is often the foremost primary choice of treatment due its outstanding ability to reduce blood glucose (Bailey 2017).

B) Thiazolidinediones

The insulin sensitizers are also known as Peroxisome Proliferator Activated Receptor agonists (PPARs). PPARs are the regulators of protein and carbohydrate metabolism and maintain glucose homeostasis. PPARγ agonists are generally thiazolidinedione called as "glitazones". Glitazones increase the sensitivity of cells to insulin. The first-generation molecules under this category are Pioglitazone, Rosiglitazone, and Ciglitazone.

Our Lab has also worked on insulin sensitizer bioactive swertiamarin, a bitter secoiridoid glycoside, isolated from the medicinal plant *Enicostemma Littorale.* Swertiamarin enhances insulin sensitivity resulting in restoration of altered gene expression of glucose metabolism in liver in NA-STZ diabetic rats. In dyslipidemic conditions, swertiamarin plays an important role in lowering high cholesterol levels by inhibiting HMG-CoA reductase activity (Patel, Soni et al. 2013). It has lipid lowering action which ameliorates insulin resistance in T2DM. Antioxidant and hypolipidemic activity of swertiamarin was studied in ameliorating NAFLD caused due to hepatic lipid accumulation, inflammation, and insulin resistance. It reduces lipogenesis and TG accumulation. It targets potential metabolic regulators AMPK and PPAR-α, through which it regulates hepatic glycemic burden, insulin resistance, fat accumulation, and ROS generation in hepatic steatosis (Patel, Rawal et al. 2016).

ii. Insulin secretagogues

A) Sulfonylureas

The drugs falling under this class mechanistically act by increasing the secretion of insulin from the pancreas. They bind to the sulfonylurea receptor (SUR) of ATP sensitive potassium channel on pancreatic β cells (Seino, Sugawara et al. 2017). They exert their effect by closing the ATP sensitive potassium channel (Sola, Rossi et al. 2015). Tolbutamide, Chlorpropamide, Tolazamide, Acetohexamide are 1st generation sulfonylurea and 2nd generation sulfonylurea includes

Glibenclamide, Glipizide, Glimepiride (Kalra, Bahendeka et al. 2018). Repaglinide and Nateglinide are other molecules used in this category (Hemmingsen, Sonne et al. 2016). Meglitinide is the prototype molecule that is a derivative of benzoic acid of the non-sulfonylurea moiety of Glibenclamide (Sola, Rossi et al. 2015).

iii. Alpha-glucosidase inhibitors

These are oral antidiabetic drugs. They delay the entry of glucose, when it enters the bloodstream after a meal, by slowing the digestion of carbohydrates in the small intestine. Alpha-glucosidase inhibitors have structural similarities to disaccharides or oligosaccharides. Because of this reason they bind strongly to the enzyme Alpha -Glucosidases, located in the brush border of the small intestine. This interaction competitively inhibits the carbohydrate–glucosidase complexes and hence carbohydrates cannot be absorbed in the intestine and reduces the rate of digestion of carbohydrates. Acarbose, miglitol, voglibose are included in this class (Liu and Ma 2017) (Gökçay Canpolat and Şahin 2021).

iv. Incretin mimetics

Incretins are insulin secretagogues. Glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (glucose-dependent insulinotropic peptide, GIP) are the major incretin hormones in humans. Both GLP-1 and GIP are secreted from the gastrointestinal tract. K-cells located in the duodenum produce GIP and the L-cells chiefly found in the ileum and colon produce GLP-1. They can also be found throughout the whole intestine. GLP-1 Receptors are present in the central nervous system which have a role in the gut-brain axis and regulates energy homeostasis by affecting appetite and body weight. Glucagon-like peptide (GLP) agonists bind to a membrane GLP receptor and insulin release from the pancreatic β cells is increased (Nauck, Homberger et al. 1986) (Kim and Egan 2008). Exenatide or Exendin-4 is the first GLP-1 agonist approved for the treatment of type 2 diabetes which is an agonist rather than analog (Briones and Bajaj 2006) (Gallwitz 2006). Other analogs include Liraglutide, Lixisenatide, Dulaglutide, etc. Common side effect includes a decrease in gastric motility, nausea, and weight loss (Raccah 2017).

v. Dipeptidyl peptidase-4 inhibitor

DPP-4 inhibitors are oral hypoglycemic drugs that block the enzyme dipeptidyl peptidase-4 (DPP-4). Both GLP-1 and GIP are quickly inactivated by the enzyme dipeptidyl peptidase-4 (DPP-4). So by inhibiting the action of DPP-4, the level of incretin hormones is increased. This results in an increase in insulin and decrease in glucagon levels. This decreases gastric emptying, and decreases blood glucose levels. Sitagliptin, saxagliptin, linagliptin, alogliptin, and vildagliptin drugs are included in this class (Röhrborn, Wronkowitz et al. 2015) (Amori, Lau et al. 2007) (Gökçay Canpolat and Şahin 2021)

vi. Glycosurics

The major focus of the present thesis is on SGLT2 inhibitors which fall under the class of glycosuric.

Both glucose ingested through diet and synthesized within the body need to be transported from the blood circulation into the target cells. Therefore, glucose uptake is mediated to facilitate transportation across the plasma membranes, which occurs through important transport proteins. There are two major types of glucose transporters which are GLUTs which transports through facilitated diffusion and SGLTs which use active transport. GLUTs are used by the cells to bring glucose from the extracellular fluid into the cells. Depending on the concentration gradient, they can transport molecules in either direction across the cell membrane. SGLTs can move the substrate into the cell by following the Na+K+ gradient. Therefore, are found only on transporting epithelial cells that use this active transport gradient to bring glucose into the body from the extracellular fluid (Poulsen, Fenton et al. 2015) (Abdul-Ghani and DeFronzo 2008) (Bays 2013).

A) Classification of SGLT

Various isoforms of SGLT are distributed throughout different tissues of the body. SGLTs belong to a large family of sodium glucose cotransporter SLC5. All SGLT proteins have 14 transmembrane helices in topology. Their expression and activity increase by raised plasma glucose concentration but is unrelated to renal gluconeogenesis, which may be seen increased in diabetes. SGLT1 and SGLT2 function as glucose/galactose transporter across the membrane. Human SGLT1 functions as a water channel. In humans, SGLT3 is thought to be a glucose sensor

that functions as a glucose-sensitive sodium channel expressed in the plasma membrane of cholinergic neurons of the enteric nervous system and muscle. The functions of SGLT4, SGLT5, and SGLT6 are not known. SGLT1 has limited tissue expression and is found essentially on the apical membranes of the small-intestinal absorptive cells (enterocytes) and renal proximal straight tubules (S3 cells) and in the myocardium. SGLT2 has a low affinity and is expressed mainly on the apical membrane of renal convoluted proximal tubules (S1 and S2 cells). The affinity of SGLT1 for D-glucose is 10 times higher than SGLT2. The NaK/glucose coupling ratio is two for SGLT1 and one for SGLT2. Selectivity for glucose transport renders major differentiation between SGLT1 and SGLT2 (Gallo, Wright et al. 2015) (Uldry and Thorens 2004) (Wright and Turk 2004).

B) Mechanism of SGLT

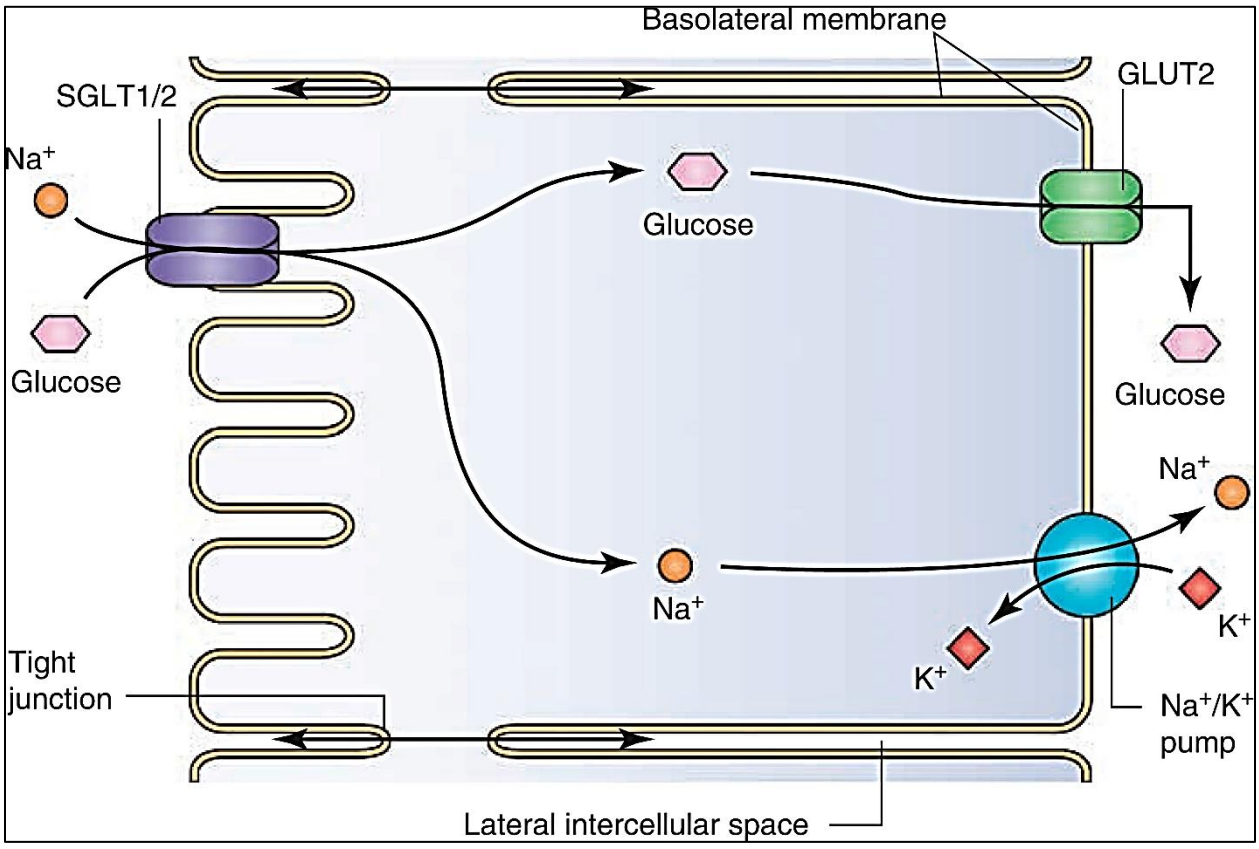

Figure 1.14 Glucose reabsorption by SGLT1/2 through a proximal tubule epithelial cell in nephron (Bakris, Fonseca et al. 2009)

The kidney plays an important role in regulating normoglycemia in blood. It regulates glucose homeostasis through gluconeogenesis, glomerular filtration, and reabsorption of glucose in the proximal convoluted tubules. In healthy individuals, blood glucose concentration under normal conditions is nearly 5.5 mmol/l. Nearly all of the glucose filtered by the glomeruli (>99%) is reabsorbed and returned to the circulation. At plasma glucose concentrations beyond the resorptive threshold (~180 g/d), glucose begins to appear in the urine, assuming that the glomerular filtration rate is unaffected.

Glucose initially gets collected within the epithelium by SGLTs in the brush-border membrane and is then transported out of the cell across the basolateral membrane by GLUTs. Both the active and facilitative glucose transporters have distinct distribution profiles along the proximal tubule related to their kinetic characteristics. The majority (~90%) of re-absorbance of all of the glomerular filtered glucose is done through high capacity SGLT2 in the early proximal tubule and the low capacity SGLT1 in more distal regions of the tubule reabsorb the remainder. These co-transporters are secondary active as they depend on Na+K+ATPase activity in the basolateral membrane for the active removal of sodium. GLUT2 and GLUT1 respectively facilitate glucose transport across the basolateral membrane in the early and more distal regions of the proximal tubule. The filtered glucose load is the product of the plasma glucose concentration and the glomerular filtration rate (GFR). The filtered glucose load increases in a linear manner as the plasma glucose concentration increases. When the reabsorption capacity of the proximal tubule is exceeded, as occurs during hyperglycemia, glucose appears in the urine (Fig 1.14). This maximum reabsorption capacity is called ‘the maximum transport rate (Tm)’. In healthy individuals without diabetes, Tm for glucose is reached at blood glucose concentrations of approximately 200 mg/dL (Wright and Turk 2004, Abdul-Ghani and DeFronzo 2008) (Wright, Hirayama et al. 2007).

C) The action of SGLT inhibitors

Under diabetic conditions, proximal tubule glucose reabsorption is higher resulting from increased expression of SGLT2, which contributes to overt hyperglycemia. The increased proximal tubule sodium reabsorption reduces the downstream sodium and chloride concentration at the macula densa, thus activating the tubuloglomerular feedback mechanism. This causes early diabetic hyperfiltration. In poorly controlled diabetic individuals, maximal transport rate (Tmax) of kidney

is 20% higher i.e. 352 mg/ min (19.5mmol/l/min) to 419mg/min (23.3mmol/l/ min) than healthy ones which is around 300mg/min. Increased expression of SGLTs in diabetes is a physiological response to increased glucose delivery to the nephrons that is ultimately maladaptive.

Thus, antagonizing SGLT2 transporters with SGLT2 inhibitors provides an effective insulin-independent mechanism that efficiently manages hyperglycemia and serves as a newer class of anti-diabetic medications. Inhibition of SGLT2 in the proximal convoluted tubules and SGLT1 in the intestine leads to decreased absorption of glucose and thereby increased exertion of glucose into the urine. So SGLT inhibitors help to achieve better glycemic control and reduction in the amount of already ingested glucose (Fig 1.15) (DeFronzo, Davidson et al. 2012) (Mogensen 1971) (Bakris, Fonseca et al. 2009).

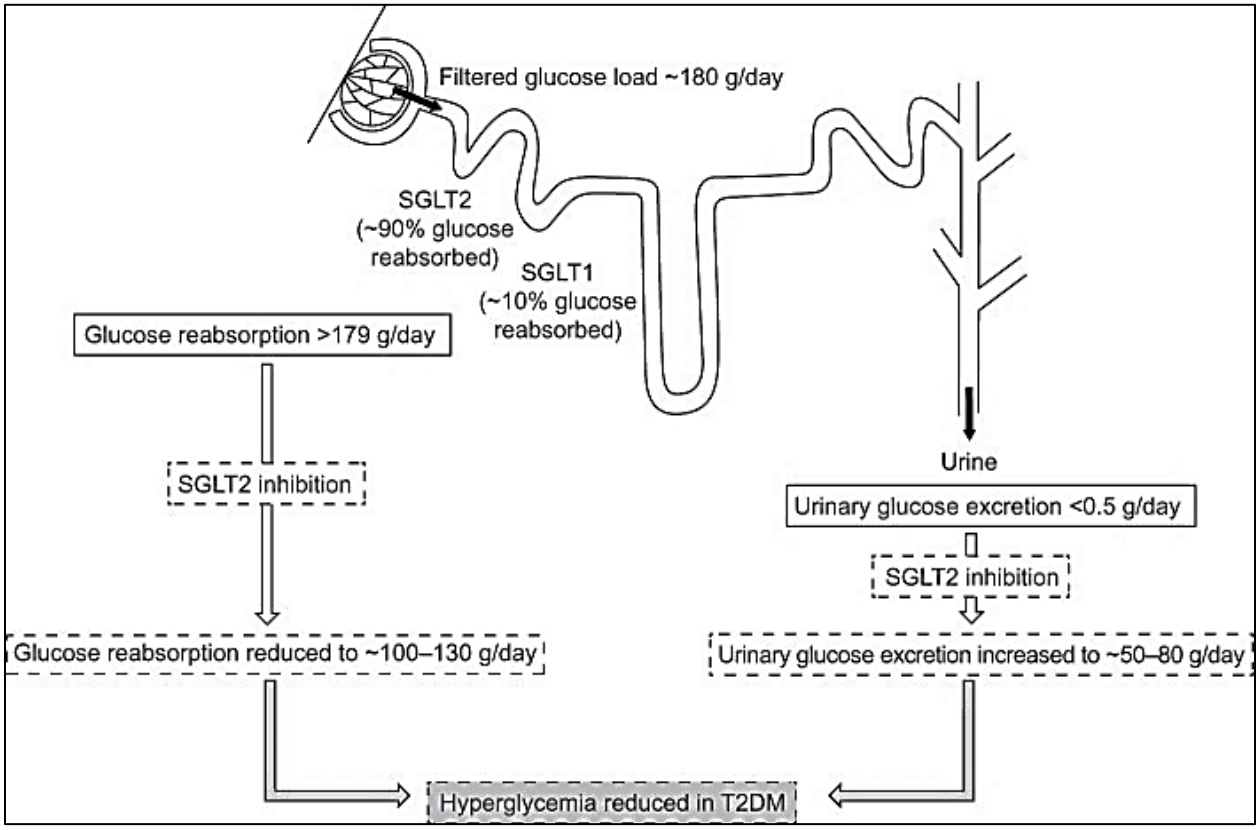

Figure 1.15 Mechanism of action of SGLT2 inhibitors (Nauck 2014)

D) Regulation of SGLT2

Modulation of expression and activity of SGLT is regulated by various factors mainly protein kinases. It is observed that high glucose levels decreased the expression and activity of SGLTs and that the SGLT activity was decreased in the brush-border membrane vesicles of diabetic rats (Vallon 2015) (Gallo, Wright et al. 2015). Streptozotocin-induced type 1 diabetes mellitus (T1DM) in mice significantly decreased renal SGLT2 expression (Vallon, Rose et al. 2013). Hyperglycaemia affects glucose reabsorption by SGLT2 and it also affects certain protein kinases that are involved in the regulation of SGLT2. Reports have shown that PKC (Protein kinase C) has been associated with differentially regulating SGLT2 activity across different species. PKC-related diabetic glomerular abnormalities are contributed by PKC-MAPK pathway which also involves ERK, one of the important intracellular signal transduction kinase. SGLT1 contains several consensus sites for regulation by protein kinase A (PKA) and protein kinase C (PKC) (Hirsch, Loo et al. 1996) (Wright, Hirsch et al. 1997). The regulation of SGLT1 by PKC requires the activation of a complex signaling cascade involving p38/mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK)/MAPK, c-Jun N-terminal protein kinase (JNK)/MAPK and phosphoinositide-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) (Pakladok, Hosseinzadeh et al. 2012) (Rexhepaj, Artunc et al. 2007) (Kempe, Siraskar et al. 2010). Diabetes increases the de novo synthesis of diacylglycerol (DAG) and protein kinase C (PKC) activity in the glomeruli from normal or diabetic rats. PKC activates the expression or translocation of SGLTs (Poulsen, Fenton et al. 2015) (Lee, Lee et al. 2007). Three novel phosphorylation sites in rat SGLT2 have also been identified suggesting SGLT2 is also be regulated via phosphorylation. Studies in human embryonic kidney cells expressing human SGLT2 showed that activation of PKA and PKC increased glucose uptake by 225 and 150%, respectively (Ghezzi and Wright 2012).

i. Commercially available SGLT2 inhibitors

SGLT2 inhibitors are commonly referred to as gliflozins and exhibit their effect by altering normal renal physiology by inhibiting action of SGLT2 transporters. It was discovered that C-glucosides bearing a heteroaromatic ring are metabolically more stable SGLT-2 inhibitors than O-glucosides (Nomura, Sakamaki et al. 2010). Phlorizin, a bioactive compound, has been a lead for developing

SGLT inhibitors and also has SGLT1 and SGLT2 inhibitory property (Ehrenkranz, Lewis et al. 2005). O glucoside analogs of phlorizin were developed like T-1095,sergiflozin,remogliflozin but had poor pharmacokinetic stability and insufficient selectivity for SGLT2 (Blaschek 2017). Thus, development of new C-glycosides like dapagliflozin, canagliflozin, empagliflozin with high SGLT2-selectivity, potentially served as analogs of the highly hydrolysis-sensitive O-glycosides (Link and Sorensen 2000) (Meng, Ellsworth et al. 2008).

The first gliflozin named canagliflozin became FDA approved in 2013 which was a novel thiophene derivative of C-glucoside and a highly potent and selective SGLT2 inhibitor. It was developed by Mitsubishi Tanabe Pharma and is marketed under license by Janssen, a division of Johnson & Johnson (FDA.) (Nomura, Sakamaki et al. 2010).

Canagliflozin is an orally active, reversible, and selective inhibitor with 250-fold selectivity toward SGLT2 over sodium-glucose cotransporter 1 (SGLT1) (Nauck 2014). By blocking SGLT2, canagliflozin decreases reabsorption of filtered glucose and reduces the renal threshold for glucose (RTG), thereby elevating the urinary glucose excretion (UGE) and reducing raised plasma glucose (PG) in patients with T2DM (Devineni, Curtin et al. 2013) (Sha, Devineni et al. 2011). It reduces fasting glucose ranging from 0.9 to 2.1mmol/L with daily doses between 50mg and 600mg. Among the available SGLT2 inhibitors, canagliflozin is widely used as doses of 100 and 300 mg once daily Canagliflozin 300 mg has demonstrated improvement of glycemic and nonglycemic parameters including reduction of 0.9% in HbA1c, and is generally well-tolerated in T2DM patients (Rosenstock, Aggarwal et al. 2012). Early weight reduction is seen due to the osmotic diuretic effect, whereas incremental weight loss over subsequent weeks is likely due to caloric loss (Kaushal, Singh et al. 2014). Apart from monotherapy, it has been also used in combination therapy with other antihyperglycemic agents (Rosenstock, Aggarwal et al. 2012). Apart from canagliflozin, there are other gliflozins available Dapagliflozin, Empagliflozin, Ertugliflozin, Ipragliflozin, Luseogliflozin, Sotagliflozin, Tofogliflozin, etc.

Dapagliflozin, another SGLT2 inhibitor was developed with lipophilic ethoxy substituents at the 4-position on the B-ring of phlorizin (Meng, Ellsworth et al. 2008). Dapagliflozin exhibits significantly decreased plasma glucose levels and glycated hemoglobin (HbA1c) and bodyweight reduction (Komoroski, Vachharajani et al. 2009). Dapagliflozin was first approved and marketed

in Europe in 2012, and in 2014 in the USA. Empagliflozin is the third agent in the gliflozin class approved by both the EMA and FDA in 2014, which possesses the highest selectivity for SGLT2 over SGLT1, among the SGLT2 inhibitors on the market (Grempler, Thomas et al. 2012). For dapagliflozin, the inhibition of SGLT2 versus SGLT1 is 1200-fold higher and for canagliflozin a 160-fold. For empagliflozin, a 2700-fold higher inhibitory activity against SGLT2 compared to SGLT1 has been described (Choi 2016)..

SGLT2 inhibitors have proven their potential actions apart from SGLT2 inhibition. Empagliflozin treatment in T2DM patients was found to be related to improved β-cell function (Al Jobori, Daniele et al. 2018). It was demonstrated that empagliflozin may have a favourable effect on preserving β-cell regeneration improving blood glucose homeostasis in type 1 diabetes mellitus (Cheng, Chen et al. 2016). On the other side, canagliflozin also improves β cell function in patients with type 2 diabetes (Polidori, Mari et al. 2014). It was demonstrated that db/db mice treated with canagliflozin significantly preserved β cell mass (Hamamatsu, Fujimoto et al. 2019). So gliflozins have beneficial effects on pancreatic β cells apart from inhibiting SGLT2.

ii. Natural compounds as SGLT2 inhibitors

Natural products have been used for the regulation of normal physiological conditions and the treatment of various metabolic diseases, as a form of nutraceuticals and dietary supplements. Phlorizin, a dihydrochalcone was isolated from the bark of apple trees in 1835. It is the first known natural product with SGLT inhibitory activity. The principal pharmacological action of phlorizin is to produce renal glycosuria and block intestinal glucose absorption through inhibition of the sodium-glucose symporters located in the proximal renal tubule and mucosa of the small intestine. Phlorizin was and still is an important lead compound for the development of SGLT2, SGLT1, and dual SGLT2/SGLT1-inhibitors (Ehrenkranz, Lewis et al. 2005). Phlorizin is found in apple tree leaves and fruits (Gosch, Halbwirth et al. 2010), strawberry fruits (Hilt, Schieber et al. 2003), rose hips (Rosa canina L.) (Hvattum 2002), and in the bark of pear (Pyrus communis L.) (Gosch, Halbwirth et al. 2010). Kurarinone and sophoraflavanone isolated from the roots of Sophora flavescens Ait. (Fabaceae) demonstrated good SGLT-inhibitory activities (Sato, Takeo et al. 2007). Glycosidic flavonoid tiliroside showed inhibition against SGLT1 in the human intestinal

Caco-2 cell line (Goto, Horita et al. 2012). Alkaloid compounds from the leaves of *A. macrophylla* possess SGLT inhibitory potential (Arai, Hirasawa et al. 2010).

The major work of the present thesis focuses on understanding crosstalk between insulin sensitive tissues and pancreatic islets which can add to the knowledge of cell-cell interactions and management of diabetes mellitus which will widen the understanding and add to the knowledge of diabetes therapeutics. Safe and cost-effective herbal and natural repertoire will benefit providing holistic treatment avenues for healthcare management.