1. INTRODUCTION & REVIEW LITERATURE

1.1. Pancreas

The mature pancreas is an abdominal gland located behind the stomach connected to the duodenum in humans. The pancreas play a pivotal role for integrating the endocrine system and digestive system in the vertebrates (Roder *et al.* 2016). The pancreas plays an essential role in supporting the homeostasis of the body and proves to be a key player in controlling energy metabolism. Pancreas is functionally and morphologically of dual nature. Both exocrine and endocrine make controlled and regulated pancreatic actions necessary to systematic body function (Thorens 2010).

Human pancreas, a mixed gland, anatomically and functionally comprises of –

Exocrine Pancreas – The exocrine compartment (98% of the total organ mass) secretes enzymes that play a role in digestion and efficient nutrient assimilation, mostly carried by acinar cells surrounding a network system of ducts. Bicarbonate and mucins are actively secreted by mature duct cells. Digestive enzymes are emptied into the duodenum where they facilitate digestion.

Endocrine Pancreas – The endocrine compartment (<2% of the pancreatic tissue) secretes various hormones that play a vital role in regulating the metabolic status of the body, which is carried out by a highly vascularized heterogeneous closely packed innervated cell gatherings termed “The Pancreatic Islets of Langerhans” (Logsdon and Ji 2013).

The pancreatic islets are the endocrine micro-organs comprising the pancreas, which mostly executes hormonal regulation. 3 million islets are distributed in a healthy human which can be circular to oval to irregular shaped. It constitutes about 2 % of the pancreas with about 10-15 % of the vasculature. The closed linked interaction between islets and vascular cells regulates hormone release which establishes a tight control of the glucose metabolism in the body. 5 types of hormone-secreting endocrine cells are found in pancreatic islets. Pancreatic β-cells synthesize and secrete insulin. These cells are most abundantly found compared to other endocrine cells present in the pancreas. β-cells compromise up to 50-70 % percent of cells of the islet in humans whereas α-cells constitute about 20-40 % secreting glucagon. δ-cells and PP cells releases somatostatin and pancreatic polypeptide, respectively and are present less than 10% in humans. The hormone ghrelin is released from epsilon cells which present less than 1

% of all the other endocrine cells of islet of Langerhans. Regulation of storage and metabolism of glucose is maintained by co-ordinated action of hormones insulin and glucagon. A rise in blood glucose level triggers release of insulin which reduced glycemia by directing the glucose load towards storage or metabolism. Glucagon, in contrast, has an opposing function to insulin and promotes glucose release or neogenesis from glycogen during hyperglycaemia (Shahid and Singh 2019).

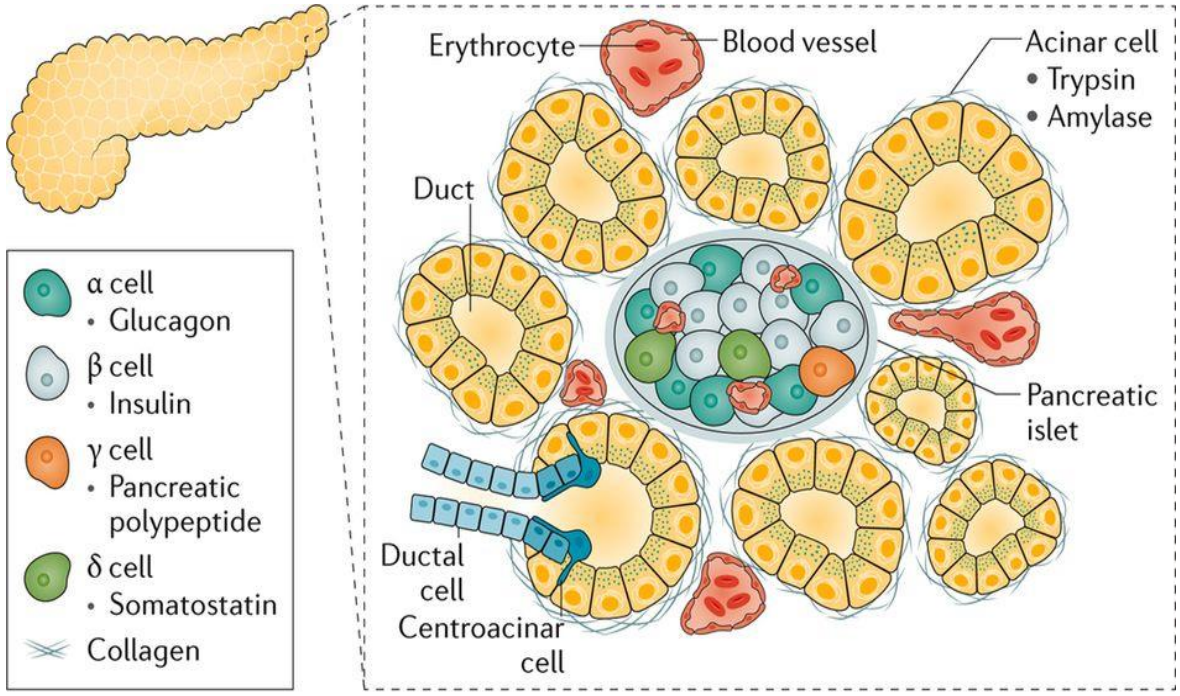

Figure 1. 1: Illustrative anatomy of healthy human pancreas & its cytoarchitecture of exocrine and endocrine cells. The endocrine pancreas is included in four different cell types, containing β-cell (insulin-producing) and α cells (glucagon-producing). The exocrine pancreas is comprised of acinar cells that prepare trypsin and amylase (digestive enzyme). This Figure is adopted from (Ellis *et al.* 2017).

1.2. Various cell types of endocrine nature

The pancreatic islets contain below-mentioned cells types (Figure 1.1):

1.2.1. α-Cells

Alpha cells contribute up to 20% of cells in the pancreatic islets, secreting the peptide hormone called glucagon, which elevates the glucose levels in the blood (Hyperglycaemia). Glucagon executes glycogenolysis by hydrolyzing glycogen to glucose by activating glycogen

phosphorylase in the liver. Additionally,α-cells also release cleavage products such as GLP-1, GLP-2, and glicentin (Roder *et al.* 2016).

1.2.2. β-Cells

Pancreatic β cell is a type of endocrine cell which forms the bulk of the pancreatic endocrine cell mass (50 and 80 %) that synthesize and secrete insulin and amylin. In patients with type I or type II diabetes, β-cell mass and function are diminished, leading to insufficient insulin secretion and hyperglycemia. β-cells secrete insulin which is a 51-aminoacid peptide with strong hypoglycaemic action. The insulin hormone is essential for glucose uptake inside the insulin-dependent cells and thus is redundant for the survival of the organism. Insulin is proteolytically derived from proinsulin. This biologically inactive precursor is cleaved into A and a B chain making the biologically effective insulin molecule, and a C chain (also called "connecting peptide"), which is secreted along with insulin (Fu *et al.* 2013). Pancreatic "Islet Associated Polypeptide" (also termed amylin), a 37-aminoacid peptide associated with calcitonin gene-related peptide (CGRP) is also co secreted by pancreatic β-cell. Insulin is preserved in cytoplasmic secretory vesicles that have a specific morphology with an electron-dense core. Within the granule, insulin is complexed to zinc, forming insulin–zinc hexamers and crystalline granule cores (Wimalawansa 1997).

Elevated local concentrations of zinc are estimated within the islet microvasculature during hyperglycemia. β cell-derived zinc has inhibitory effects on rat pancreatic α-cell glucagon secretion (Zhou *et al.* 2007). Activation of K_{ATP}-channels in the β-cell lines RINm5F and INS-1E by zinc has also been demonstrated. The apparent convergence of the mechanisms behind both insulin and zinc inhibition of glucagon secretion on K_{ATP}-channel suggested that modulation of ion channel activity affects hormone secretion by permitting paracrine signals to have a rapid effect. The presence of zinc benefites in the islet isolation process, where zinc-chelating dyes like dithizone help determine islet yield and purity (Jansson *et al.* 2016).

A pancreatic β-cell is predictable to comprise 9–13.000 secretory granules. With a usual everyday insulin demand of 40 IU and a typical insulin content per granule of 8 femtograms, it can be projected that approx. 1012 secretory granules are secreted from pancreatic β-cells on a daily basis. The mechanism of insulin secretion is majorly glucose-dependent. When the concentration of glucose in the blood increases (>5mM) post meal, there is an uptake of glucose by the pancreatic β-cell, this glucose then gets metabolized to give glucose-6-phosphate (G6P)

and ATP. This ATP then blocks the ATP-sensitive K+ channel, inhibiting the influx of the ions. This inhibition causes depolarization of the cell membrane, which is detected by the voltage-sensitive Ca2+ channels and then Ca2+ influx occurs. These Ca2+ ions cause the exocytosis of the already stored insulin and also causes activation of insulin gene expression via the "Calcium Responsive Element Binding Protein (CREB)" (Goginashvili *et al.* 2015).

Glucose not only stimulates insulin secretion but also increases proinsulin biosynthesis, acting mainly through translational control. Pancreatic β-cells are frequently denoted as "fuel sensors", continuously controlling and reacting to circulating nutrient levels, under the modulatory impact of neurohormonal signals, to release insulin for maintaining glucose homeostasis in the organism. The primary stimulus of insulin secretion is glucose. Increase in blood glucose concentrations resulting in a rise in intracellular glucose levels owing to transport across the β-cell plasma membrane. Glucose-stimulated insulin secretion (GSIS) enhances insulin secretion over the basal release, which is in response to increased extracellular, and eventually intracellular glucose (Komatsu *et al.* 2013). Glucose enters β-cells through specific glucose transporters. It is then phosphorylated by the enzyme glucokinase, which has a high Km for glucose. These primary steps, particularly glucokinase, determine 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 determines metabolic flux through glycolysis(Alejandro *et al.* 2015). Various metabolic coupling factors in the pancreatic β-cell which are engaged in signalling for insulin exocytosis include ATP, NADPH, glutamate, long-chain acyl-CoA, and diacylglycerol. Pulsatile insulin secretion from individual islets appears to follow a dominating pancreatic frequency, where a rhythmic variation in islet secretion is synchronized with oscillations in β-cell cytoplasmic Ca^{2+}. In clusters of β-cells exposed to intermediate stimulatory concentrations of glucose, synchronized oscillations can spread to idle cells as the glucose concentration is increased. This demonstrates β-cell recruitment and intracellular coupling in glucose regulation of insulin release (Rorsman and Ashcroft 2018).

In mature adult islets, insulin secretion occurs very rapidly after glucose administration and is reported to occur with biphasic kinetics. This biphasic pattern of insulin secretion involves the K_{ATP} channel-dependent pathway and another K_{ATP} channel-independent pathway. Both are critically Ca^{2+} influx dependent which affects different pools of insulin-secretory granules. K_{ATP}-dependent pathway executes exocytosis of an "immediately releasable pool" of granules that elicits and represents the first phase response, the K_{ATP} channel-independent pathway,

working in synergy with the K_{ATP}-dependent pathway is responsible for the second phase response (Straub and Sharp 2002). Apart from insulin, c-peptide, zinc, and proteolytic enzymes, the secretory granule includes calcium, adenine nucleotides, biogenic amines, and several supplementary peptide hormones such as chromogranin A and betagranin. (HUTTON *et al.* 1988). Increases and fluctuations in the intracellular Ca^{2+} concentration connected with the mechanism of GSIS can promote the generation of mitochondrial specific ROS (via electron transport chain activity), thought to be critically involved in β-cell dysfunction during the progress of diabetes. Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen, and include superoxide (O2−), hydrogen peroxide (H_2O_2), and hydroxyl free radicals (^{o}OH) which acutely stimulates but chronically induces inhibitory effects on β-cell metabolic pathways and can promote K_{ATP} channel opening resulting in inhibitory effects on insulin secretion. Pancreatic β-cells have inherently low levels of free radical detoxifying and redox-regulating enzymes, like glutathione reductase, glutathione peroxidase, catalase, and thioredoxin, rendering them vulnerable to damage and destruction. Given this, the β-cell while utilizing necessary and positive aspects of ROS production for insulin production and release is susceptible to the damaging effects of unregulated ROS generation and accumulation (Nita and Grzybowski 2016).

1.2.3. Delta-Cells

δ-cells form 5–10% of pancreatic islet volume. The D (or δ) cells secrete somatostatin first isolated from the hypothalamus. This peptide hormone is a potent inhibitor of glucagon and insulin. The hormone occurs in a 28-aminoacid and a 14-aminoacid structure (In't Veld and Marichal 2010).

1.2.4. PP Cells

PP hormone is secreted by the PP cells, which is least studied. In the human pancreas, the relative PP cell mass in the ventral pancreas is significant, establishing equal to 80% of the cells (In't Veld and Marichal 2010).

1.2.5. Epsilon Cells

Epsilon or Ghrelin cell releases hormone ghrelin. Adult pancreatic islets cover < 1% of epsilon cells. The hormone is supposed to be crucial in growth hormone secretion, bio-energetic balance and regulation, however its precise role in pancreatic islet cells has yet to be determined (In't Veld and Marichal 2010).

1.3. Mouse Pancreatic development

The pancreas is an endocrine organ that contains an endocrine and exocrine part that majorly regulates glucose metabolism and enzymatic digestion of food respectively. Perhaps, identifying the molecular signaling mechanism governing pancreas development pathways is an intensive field of research in past few decades. Complex gene regulatory network responsible for pancreas formation in mice. Owing to the limited access to human samples, knowledge of human pancreas development mainly relies on an analysis of embryonic and fetal tissues.

1.3.1. Signaling pathways regulating early pancreas development

In mice, the initial steps of pancreas development take place through sequential morphological changes producing varying cell types that demonstrate the involvement of the foregut endoderm. Pancreas' initial organogenesis can be separated into two phases. In the first phase, multipotent progenitor cells (MPCs) formation and proliferation respond to signals coming from the notochord, mesenchyme, and endothelium, which guides for pancreatic bud formation. These cells then undergo enormous expansion to form a stratified epithelium in which microlumen structures progress (Gittes 2009, Larsen and Grapin-Botton 2017). Meanwhile in the secondary phase, due to the fusion of microlumina generate a central plexus that added rebuilt into a constant ramified epithelial cell organization network, separated into tip and trunk domains areas assigned to bipotent endocrine/ductal progenitor cells fates. Major morphological transformation generates endocrine, exocrine, and ductal cells which develop inter-intra communication with neighboring mesenchyme, endothelium, and neuronal projections (Villasenor *et al.* 2010, Bankaitis *et al.* 2015).

1.3.2. The formation & differentiation of exocrine pancreatic cell type: Acinar

The pancreas contains a huge exocrine compartment, which composes up to more than 95 % of the pancreas which contains acinar and ductal cells. Mesenchyme is important for exocrine differentiation since it secretes factors like follistatin (TGF-β antagonist), which promotes exocrine differentiation but hinders endocrine cell formation. Mature acini contain pyramid-shaped exocrine cells, which express various transcription factors (Figure: 1.2). These specialized cells comprise plenty amount of rough endoplasmic reticulum and secretory granules bearing digestive enzymes, which are imperative for nutrient digestion (Miralles *et al.* 1998).

1.3.3. Generation of ductal epithelium and bipotent cell formation

A rich population of bipotent epithelial cells is found in mouse embryonic pancreas that prominently expresses Hnf1β, Sox9, Pdx-1, and Nkx6.1 and differentiates into either Ngn-3^{+}/Pdx-1^{+} endocrine cells or $Sox9^{+}$/$Hnf1b^{+}$ expressing ductal exocrine cells (Figure: mouse pancreas). Sox9 expression is blocked by Ngn-3, while Hes1 inhibits Ngn-3 expression which stably regulates the determination of cell fate. Notch signaling is a central player in ductal fate and promotes ductal cell formation as well as blocks acinar and endocrine cell differentiation (Seymour 2014).

1.3.4. The formation of pancreatic endocrine progenitor cells

In the secondary transition phase, endocrine multipotent progenitors are obtained from the bipotent trunk epithelium, which transiently expresses Ngn-3 and generates all types of endocrine cells. Extrinsic signals as well as intrinsic gene regulatory complex networks coordinate the generation of mature hormone-producing cells from bipotent trunk epithelium (Gradwohl *et al.* 2000, Gu *et al.* 2002).

Sox9 expressing bipotent progenitors display transiently expression of Ngn-3, a master regulator of the endocrine cell population, which further induces several endocrines -specific transcription factors i.e. NeuroD1, Insm1, Irx1/2, Rfx6, Nkx2.2. A high level of notch signaling promotes Hes1 expression, which is a negative regulator of Ngn-3 expression (Petri *et al.* 2006). Elseways, a minor level of notch signaling directly promote Sox9 expression, which further induced Ngn-3 expression levels and determine endocrine differentiation fate. In bipotent progenitor generate two types of cell differentiation are based on Ngn-3^{High} and Ngn-3^{low} expression. The elevated level of Ngn-3 protein-expressing cells (Ngn-3^{High}) triggers endocrine fate. Thus, they are functionally different from pro-ductal precursors and undergo symmetric cell division to either generate two differentiated endocrine cells and preserve the self-renewing progenitor's population pool (Figure:1.2). Moreover, while the cell in the Ngn-3 high stage is unipotent, it is ambiguous how they prone to generate specialized endocrine subtypes. The timing of Ngn-3 expression might play an important role in determining the cell fate process. In the early stage of mouse pancreas development, NGN-3 induction leads to generating primarily α- cells, however, in a later stage, it supports the formation of delta or β/PP cells. In recent time, several signaling pathways (Wnt/PCP/Notch) has been demonstrated to be involved in regulatory function in endocrine differentiation as well as pancreatic islets cell maturation (Wang *et al.* 2010).

1.3.5. Formation of pancreatic islets of Langerhans

Initiation of the secondary transition phase, differentiate various endocrine cells from the ductal epithelium, migrate into adjacent mesenchyme and coalesce to generate proto-islets (Figure:1.2). The inter and intra connection of the various cells like endothelial, mesenchymal, and neuronal cell encourage to generate functional mature "Islets of Langerhans". This entire process is regulated by continuum activities of various transcription factors and the systemization of the cell dynamics process.

A. Pancreatic endocrine cells delamination

The pancreatic islets formation process requires the delamination of migratory differentiated endocrine cells from trunk epithelium (Figure:1.2). It has been reported that Ngn-3 protein expression in endocrine cells promotes delamination. Additionally, an elevated level of Ngn-3 protein expression converts endocrine precursor into bottle-shaped morphology, proposing that Ngn-3 is crucial in repolarization and triggers epithelial migration into neighboring mesenchyme (Figure:1.2). Cellular dynamics that regulate delamination process, possible mechanisms have been suggested: (1) Epithelial-mesenchymal transition (EMT) (2) asymmetric cell division (Neumüller and Knoblich 2009). Ngn-3 triggers transcription factors of Snail superfamily, (mainly Snail 2 or Slug), which play a very crucial role in EMT, promoting mesenchymal genes and inhibiting an epithelial program, which converts E-cadherin to N-cadherin and expression of the vimentin, which are a key marker of EMT. Signaling molecules such as TGF-β and canonical Wnt which are well studied to regulate EMT in cancer cell metastatic, are also actively involved in developing pancreas (Rukstalis and Habener 2007, Gouzi *et al.* 2011).

B. Pancreatic endocrine cell migration, clustering, and assembly

After the delamination process, endocrine cells travel inside the mesenchyme to generate proto-islets morphology (Figure:1.2). To migrate, endocrine cell external stimulus of small GTPase Rac 1, regulate remodeling of adhesion molecule such as E cadherin-

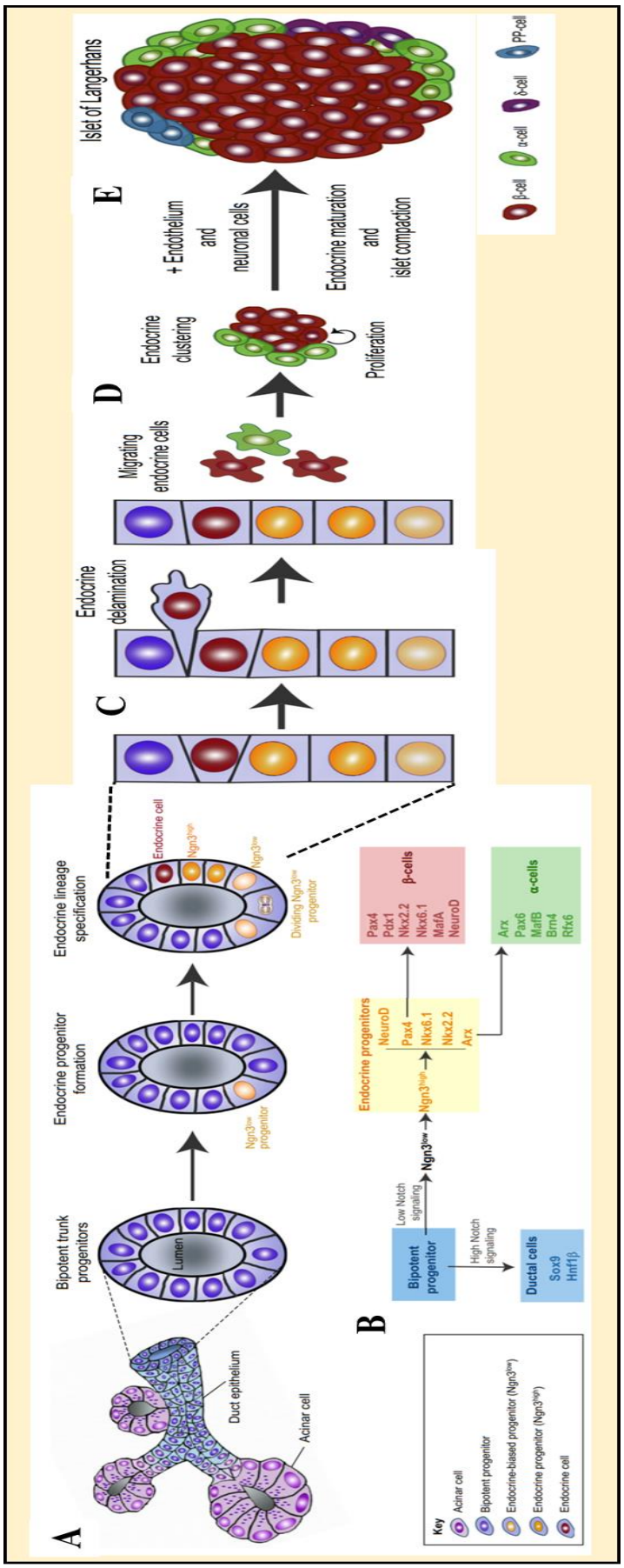

Figure 1. 2: Endocrine lineage establishment & endocrine cells stratification, migration and cluster formation during mouse pancreas development. (A) Endocrine-biased precursor ($Ngn3^{low}$) appear from bipotent lineage inside the trunk epithelium between E12.5 and E16.5. These $Ngn3^{low}$ cells offer ascent to post-mitotic endocrine precursors expressing elevated levels of Ngn3 ($Ngn3^{high}$) that are focused on all endocrine cell types. (B) High or low degrees of Notch signaling encourage the determination of epithelial cells in the direction of bipotent ductal precursor or endocrine precursor, respectively. (C) After differentiation in the epithelium, endocrine cells come out from the epithelial zone by a delamination method and they migrate towards the nearby mesenchyme. (D) Inside the mesenchyme, various endocrine cells aggregate to generate proto-islets. (E) The interlinking of these structures with blood vessels and nerve cells generates the functionally mature pancreatic islets (This figure is adopted and modified from (Bastidas-Ponce *et al.* 2017).

derived cell adhesion (switch from E-cadherin to N-cadherin) and to simultaneously coordinate cell migration. Further, β-cellulin also partially regulates endocrine cell migration (Figure:1.2). The ultimate step of pancreatic islets formation includes an aggregation of endocrine cell populations to generate proto-islets. CAMs and cadherins are two important adhesion molecules in endocrine cell communication and for islet aggregation in pancreatic development. Interestingly, isolated single endocrine cells immediately reaggregate to form pseudo-islets (Dahl *et al.* 1996, Jain and Lammert 2009). Pancreatic islets further adopt a globular shape in which special arrangements for β- cells, which constitute approximately 60-80 % of mature islets, located in central regions while others surrounding cells such as α-and δ-cells, which constitute of remaining part of mature islets. In a nutshell, an inter-intra connection between endocrine and non-endocrine cells within the islet's niches is thought to produce three-dimension morphology that provides a platform for the physiological function of the pancreas. However, islet final 3D architecture and complete maturation status remain unclear (Roscioni *et al.* 2016).

1.4. Human pancreas development

Human embryogenesis covers from egg fertilization to average 8 weeks post-conception (WPC), after that time, the embryo is called a fetus. Human embryonic development is graded into the 23 distinct carnegie stages (CS) (O'rahilly and Müller 2010) (Figure:1.3).

In humans (CS9), the definitive endoderm still tries to communicate with the visceral endoderm of the yolk sac. Moreover, endodermal folding due to SHH (Sonic hedgehog) signaling is first seeming at CS 10 stage. It is also called "Distal endoderm foregut". On the other hand, in human pancreatic development, there has been an absence of "primary transition" (it's also called early pancreatic endocrine differentiation) which is prominent in mouse pancreas development (Villasenor *et al.* 2008). In the human embryo, PDX1 can be detected at CS12 stage. An early appearance of ventral buds and later the dorsal pancreatic bud comes out at CS13 generally 4 weeks of gestation. GATA6 has limited expression in pancreatic progenitor cells which have been defined at a particular time duration, CS16-CS18. GATA4 is the first time expressed in early pancreatic progenitors within the foregut endoderm at period CS12 to CS19 and later CS19 its detection becomes limited to the peripheral "Tip" cells. Moreover, at stage CS13, SOX-9, PDX-1, and GATA4 transcription factors require for ventral and dorsal pancreatic growth (Figure: 1.3). In the human pancreas development process, epithelial branching and polarity have been obscured, evidence proposes that the communication

between controllers of neural migration and element of the extracellular matrix perform a crucial role in pancreatic endoderm cell migration and adhesion (Figure: 1.3). Interestingly, NKX2.2 protein is not detected in the human embryo before endocrine differentiation. While NKX2.2 is predominately present in multipotent progenitors in mice. Further, bifurcation of pancreatic progenitor cell becomes clearly visible by crucial stage CS19 when SOX9+/NKX6.2+/ GATA4- central duct-like structure i.e. "trunks" while the additional surrounding grouped cells i.e. "Tip" cells are SOX9+ /GATA 4 + /NKX6.2+ (Figure:1.3). During human pancreas development, NGN-3 protein expression elevates swiftly after the embryonic stage timed with the generation of fetal pancreatic β-cells, which are the chief islet cell type to display in pancreatic development (Piper *et al.* 2004). Early developmental transcription factor SOX9 is vanished in robust NGN-3 expressing cells and is overlooked in endocrine cells consequently, although it remains in pancreatic ductal cells. Pancreatic islets clusters are finely vascularized by 10 weeks post-conception (WPC), and later at 12-13 weeks post-conception (WPC). Pancreatic islets have resembled that contain beta cells, alpha-cells, delta-cells, and gamma-cells with its unique molecular signature (pancreatic transcription factors) and hormone production. In summary, the researcher does not support an early stage/phase of pancreatic endocrine progenitor differentiation in human pancreatic development analogous to mouse pancreas development & further extensive research is essential to enhance our understanding of human pancreas development (Jennings *et al.* 2013, Jennings *et al.* 2015).

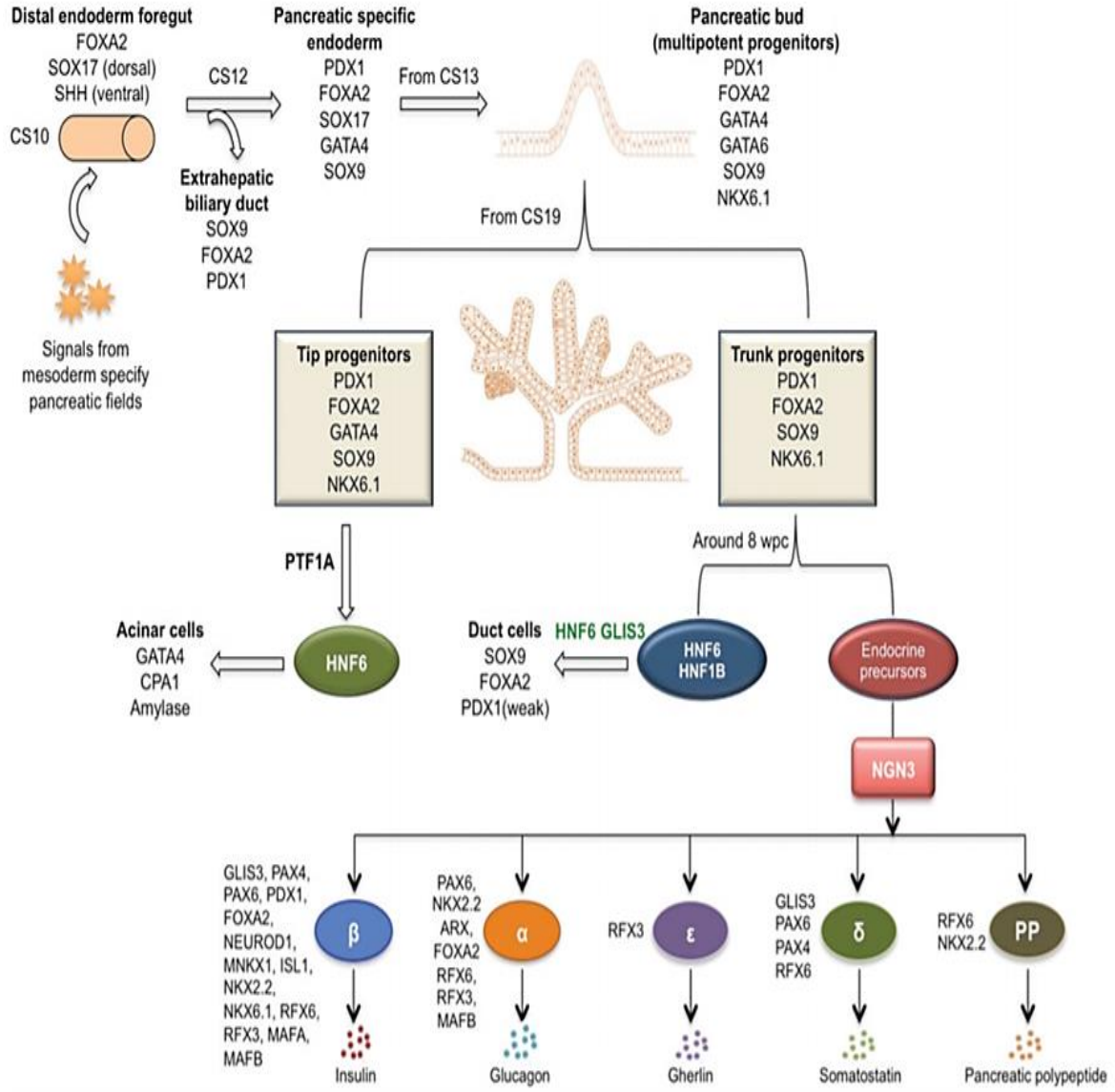

Figure 1. 3: Role of transcription factors in human pancreas development: Transcription factors (TF) regulatory network with a different stage of pancreatic islet development. During early pancreas description and different lineage commitment, precise pancreatic transcription factors along with other vital markers are expressed at respectively pancreatic development stages. CS, carnegie stage; wpc, weeks post conception. This figure is adopted from (Al-Khawaga *et al.* 2018).

1.5. Transcriptional control of islet development in the pancreas

Cell differentiation is achieved by the initiation and maintenance of a well-orchestrated gene expression patterns controlled by specific spatial and temporal combinatorial of transcription factors.

1.5.1. Generation of Definitive Endoderm

A. HNFs (Hepatocyte nuclear factors) family of transcription factors

HNFs are important transcription factor networks that control the various gene related to primitive endoderm stage. HNFs stimulates expression of the pancreatic islet transcription factors along with other genes engaged in glucose metabolism and insulin secretion. Transcription factors of HNF families such as HNF1β, HNF-1α, HNF-3β/FOXA2, HNF-6, etc. play an important role in pancreatic islets development and function. Among the most crucial transcription factor is HNF-3β(also known as FOXA2), a marker of definitive endoderm. FOXA2 knockout studies in a mouse model demonstrated hypoglycemia along with hyperinosaemia suggesting that FOXA2 is very crucial for the maintenance of normal glucose homeostasis (Sund *et al.* 2001, Wang *et al.* 2002). The study showed that HNF1β is reported initially in the primitive endoderm and subsequently in the pancreatic epithelium. Its expression eventually becomes restricted to the ductal cells. HNF1α also activates HNF6, indicating a cross-regulatory of these two transcription factors in the early pancreas. Thus, HNF1α and HNF6 may be the precursors for the NGN3+ endocrine progenitors' population (Dassaye *et al.* 2016).

B. SOX9/SOX17

Recent studies show that SOX17 expression is required for definitive endoderm formation and function of the epithelium of the pancreas in mice and humans. SOX17 is a vital transcriptional regulator in the maintenance of progenitor cells in the pancreatic development process by regulating other definitive endoderm factors such as FOXA2 and HNF1β, which are recognized to monitor pancreatic islet function (Kanai-Azuma *et al.* 2002, Jonatan *et al.* 2014). SOX9 play a crucial role in maintaining both multipotent and bipotent pancreatic precursor via signaling pathways such as Notch and FGF. Further, knockout and transgenic mouse model studies demonstrated that SOX9 is required for stimulating pancreatic β-cell differentiation, maintaining pancreatic progenitor cells, and also for identifying pancreatic ductal population (Seymour *et al.* 2007, Seymour 2014).

C. GATA4/GATA6

GATA 4 and GATA6, zinc finger transcription factors, play a crucial role in the determination and differentiation of endoderm as well as mesoderm, including heart, liver, and pancreas. Further, GATA6 and GATA4 are expressed in the multipotent pancreatic progenitors in

embryonic mouse pancreas development. Additionally, studies showed that double GATA6/GATA4 mutant mouse failed to expand epithelium as a result of defects in pancreatic precursor cell (PDX1+) differentiation and proliferation and eventually disrupt pancreas development in embryonic level (embryonic lethal), indicating that GATA4 and GATA6 play important role in controlling pancreas organogenesis by regulating definitive endoderm fates (Decker *et al.* 2006, Minami 2013, Xuan and Sussel 2016).

1.5.2. Formation of Pancreatic Progenitors

D. A. PDX1

Among the earliest transcription factors expressed in the pancreatic progenitors is the pancreatic duodenal homeobox 1(Pdx-1), which is a transcription factor in the Para Hox gene cluster. Loss of PDX1 function results in pancreatic agenesis in humans. The initial pancreatic bud is comprised of Pdx1+ pancreatic precursor cells that co-express HLXB9, HNF6, PTF1A, and NKX6.1, FOXA2, PDX1, and MAFA, indicating that they can be direct regulators of PDX1. HLXB9 and HEX regulate dorsal and ventral pancreatic development respectively. Further, PDX1 is also needed at the mid-gestation stage for the differentiation of pancreatic islet and acinar progenitor cells. In adult stages, PDX1 plays an important role in β-cell function, maintenance, and survival including regulation of insulin expression which results in the silencing of MAFB. PDX1 inhibits the conversion of pancreatic β-cells into α-cells and also appears to be vital in facilitating the effect of insulin on the apoptosis of pancreatic β-cells (Fujimoto and Polonsky 2009).

E. B. PTF1A/P48

Another important transcription factor that is expressed in the pancreatic progenitors is the PTF1A/P48 (pancreas transcription factor 1 complex). PTF1A/P48 is detected throughout the pancreatic epithelium and becomes majorly restricted to an acinar cell. The PTF1A is engaged in the sustenance of exocrine related gene expression such as elastase 1 and amylase. Further, PTF1A/P48 is also expressed in an entirely pancreatic precursor that produces endocrine, exocrine, and ductal progenitor cells. Thus, PTF1A/P48 is important for the specification of early pancreatic progenitors and in the regulation of PDX1 expression (Rodolosse *et al.* 2009).

1.5.3. Establishment of the Endocrine Progenitors

A. NGN3

The most significant of the transcription factors that have been detected as specific for the endocrine part of pancreas development is neurogenin-3 (NGN3). In the adult pancreas, NGN3 expression is almost untraceable. NGN3 positive population function as endocrine progenitor cells and generate all hormone-secreting pancreatic cells. NGN3 is expressed only 2-10% of acinar and duct cells. With respect to upstream network regulating NGN3 expression, binding sites for various transcriptional activators that are widely expressed in the endoderm and pancreatic buds including HNF-1β, HNF-3β, and HNF-6 have been identified in the promoter of NGN3. Several Ngn3 targets have been identified, including paired box gene 4 (PAX4), aristaless-related homeobox (ARX), NEUROD/BETA2, insulinoma-associated antigen 1 (1A-1), NKX2.2, NKX6.1, and NGN3 itself (Villasenor *et al.* 2008).

1.5.4. Endocrine Lineages Specification

A. PAX/ARX Transcription Factors

PAX4 gene is known as the paired box gene 4 encoding protein PAX4. PAX4 is engaged in pancreatic islet development and the differentiation of insulin-producing β-cells. PAX4 is restricted to β- and δ-cells, whereas ARX is expressed in ε- and α-cells. After birth, PAX expression is lacking in pancreatic β-cells and ARX expression remains in adult pancreatic α-cells. Thus in endocrine lineage specification, PAX4 and ARX act downstream NGN3 and repress each other to determine the final proportions of the different endocrine cell types (Murtaugh 2007).

B. NKX Transcription Factors

The two most important members of the Nkx protein family are expressed in the developing pancreas, NKX2.2, and NKX6.1 are participated in pancreatic endocrine lineage differentiation. NKX2.2 is expressed in the pancreatic bud until E13, overlapping with NGN3+ cells. Its expression remains in mature pancreatic α-, β-, and PP-cells. The pattern of NKX6.1 and NKX6.2 expression is similar to that of NKX2.2. It is broadly expressed until E10.5 but is expressed exclusively in β-cells by the end of gestation. NKX2.2 directly activates NKX6.1 transcription (Nelson *et al.* 2007).

1.5.5. Maintenance of pancreatic β-cell Identity

When cells within the islet are differentiated into the hormone-expressing cells, each of these different endocrine cells has to maintain its identity by constantly expressing transcription factors necessary for the maturation and expansion of each cell type.

A general regulator for the expansion and organization of all endocrine cells within the islet is PAX6. PAX6 expression at birth is limited to the pancreatic islet. The inactivation of PAX6 in the mouse embryo reduces the number of endocrine cells, and the few remaining cells appear disorganized and produce low hormone levels. Three transcription factors, NEUROD/BETA2, MAFA, and PDX1, represent specific key players for β-cell maturation and identity. These three factors cooperate to synergistically activate the transcription of the insulin promoter in β-cell (Guney and Gannon 2009).

A. NEUROD/BETA2

NEUROD is expressed in pancreatic β-cells. The bHLH transcription factor BETA2/NEUROD is expressed from E9.5 in scattered pancreatic cells and E14.5 is expressed in NGN3+ cells. NEUROD/BETA2 is a strong inductor of insulin by directly binding to the E-box present in the insulin promoter and regulates its transcription. After birth, its expression becomes restricted to mature β-cells only (Gu *et al.* 2010).

B. MAFA

MAFA gene encodes MAFA protein in humans. The basic leucine zipper MAFA is a β-cell specific transcription factor that binds to the well-characterized insulin promoter. MAFA expression starts at E13.5 in the first insulin-producing cells, and its expression continues in the β-cells to adulthood. MAFA assists in insulin regulation. *MAFA* (gene) has been shown to interact with NEUROD1 and PDX1 to activate the insulin transcription. MAFA works with PDX1 to activate the insulin gene and involved with insulin secretion primarily by maintaining β cell metabolism. The amount of MAFA in the pancreatic β cells is controlled by levels of glucose and oxidative stress. Thus, MAFA is crucial for the maintenance of functional β-cell (Hang and Stein 2011). Dysfunction in pancreatic islet transcription factors might lead to diabetes.

1.6. Diabetes Mellitus

"Diabetes mellitus is a chronic hyperglycemic condition with disturbances of energy metabolism including carbohydrate, fat, and protein metabolism which results in defects in insulin secretion, insulin action, or both."(American Diabetes 2009).It is a complex metabolic disorder considered by a fasting blood glucose level greater than 140mg/dl. Symptoms include polydipsia (increased thirst), polyuria (increased urination), glycosuria (glucose in the urine), polyphagia (increased hunger), and prolonged cases can lead to ketoacidosis, stupor, coma, and death. Diabetes mellitus is caused by several reasons and based on that divided into two types:

Type 1 Diabetes Mellitus- Caused due to loss of β-cell which can be due to idiopathic reasons or autoimmune destruction thus leading to a hypo-insulinemic condition due to the destruction of β-cells (Figure:1.4).

Type 2 Diabetes Mellitus- Caused due to insulin resistance, where insulin is not recognized by its receptors thus inhibiting glucose uptake by tissues. In later stages, type 2 diabetes mellitus get converted to type 1 diabetes where there is insulin deficiency due to β-cell damage (American Diabetes 2009).

1.6.1. Epidemiology

425 million people are suffering from diabetes worldwide will increase to 629 million in 2045. Amongst the 352.1 million people are estimated to have impaired glucose tolerance. In 2045, 9.9% of adults will have diabetes. The vast majority (72.3%) of these people live in low- and middle-income countries. By 2045, several individuals with impaired glucose tolerance are expected to rise to 531.6 million. In 2017, 50.0% (212.4 million) of the people with diabetes in the world were undiagnosed which is predicted to be 51.2% (321.8 million).

In 2017, there were 132,600 newly diagnosed cases of children and adolescents (0-19-year age) with type 1 diabetes. Half (46.6%) of mortality due to diabetes are in people below the age of 60. Almost 4.0 million people (aged group) between 20 and 79 years passed away from diabetes in 2017, corresponding to 1 death every 6 seconds (IDF Diabetes Atlas 2019).

1.6.2. Diagnosis

Diabetes mellitus can be diagnosed by blood glucose measurements. In the presence of characteristic clinical symptoms, diabetes could be diagnosed based on a fasting plasma

glucose (FPG) of ≥126 mg/dl or 2-hour plasma glucose during a glucose tolerance test of ≥200mg/dl. Haemoglobin A1c (HbA1c) is a widely used marker for chronic glycemia that measures the non-enzymatic glycation of hemoglobin and demonstrates average blood glucose levels over a 2-3-month period of time and is highly recommended diagnostic and prognostic marker. The specific characteristic of type 1 diabetes is autoantibody generated towards islet (American Diabetes 2009).

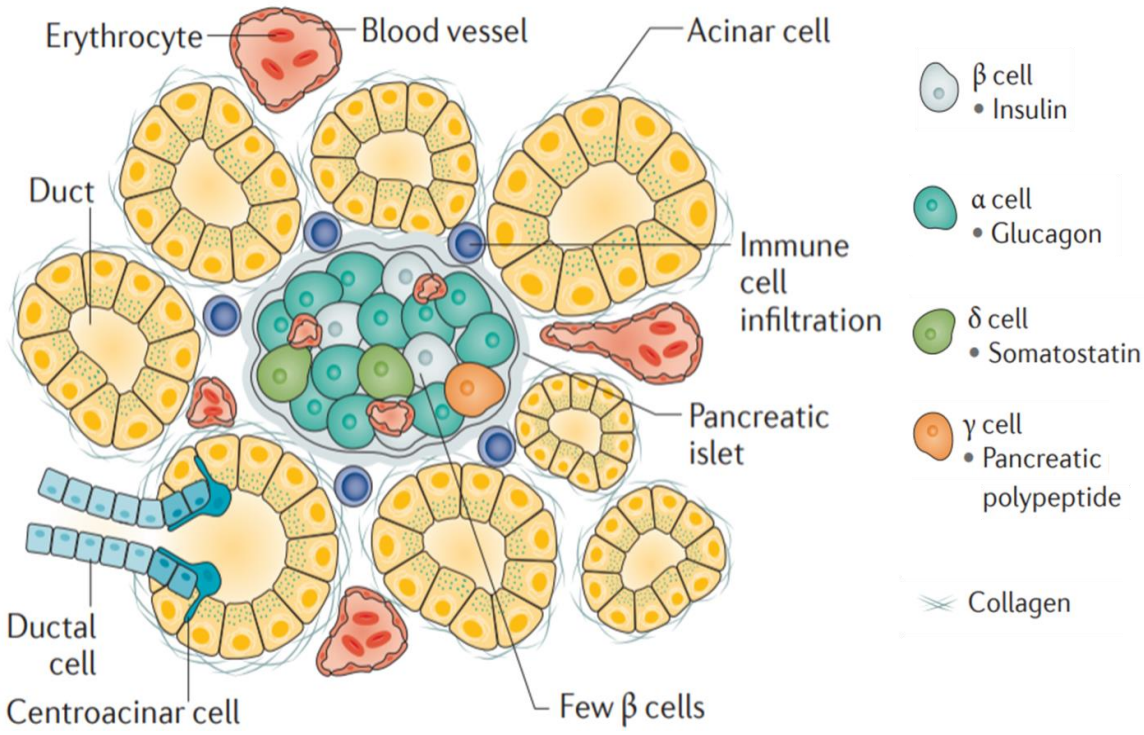

Figure 1. 4: Pancreatic islets in a patient with type-I diabetes mellitus. Very few insulin-producing β-cells are observed in pancreatic islets due to the destruction of β- cell by infiltration immune cells. This figure is adopted from (Ellis *et al.* 2017).

1.7. Type 1 Diabetes

Characterization of type 1 diabetes is a loss of the pancreatic β-cells which leads to insulin deficiency. Destruction of the pancreas is the hallmark of this type of diabetes which constitutes 5%-10% of diabetic subjects (Figure:1.4). The rate of destruction of β-cells is variable with age. More rapid rate progression of β-cell destruction is commonly found in infants and children than adults. A common occurrence of type 1 diabetes accounts for 80%-90% in children and adolescents and hence been termed "juvenile diabetes" because of the recurrent onset in children. Type 1 diabetes can be accompanied by irregular and unpredictable hyperglycaemic levels, with a tendency to ketoacidosis and sometimes with serious

hypoglycaemic levels. Ketoacidosis can be presented as the first symptom commonly in children. In older patients mild fasting hyperglycaemic conditions with lower glucose tolerance which can lead to severe hyperglycemia. During later stages of progressive diabetes very low to no insulin or human c-peptide levels are detected in serum (Klinke 2008). Eisenbarth in 1986, postulated the linear β-cell decline hypothesis (Eisenbarth 1986). However, some reports mention that disease progression in T1D is not a linear process, but rather proceeds at a variable pace in individual patients who conceptualize T1D as a "relapsing-remitting" disease (Von Herrath *et al.* 2007). The effect of complicating factors, such as aging, diet, immune cell metabolism, microbial pathogens, microbiomes, and epigenetic changes, modulate the immune response that destroys β-cells, are still widely researched (American Diabetes 2009).

1.7.1. Idiopathic type 1 diabetes

Idiopathic is a rare form of type 1 diabetes of unknown origin, less severe than autoimmune type 1 diabetes, and is not due to autoimmunity has been reported. Several patients with this category are of African or Asian descent and suffer from fluctuating marks of insulin deficiency and episodic ketoacidosis (Piñero-Piloña and Raskin 2001).

1.7.2. Fulminant type 1 diabetes

This is a different type 1 diabetes, initially defined in the year 2000, and has a number of general characteristics with idiopathic type 1 diabetes being non-immune formed. It is characterized by ketoacidosis soon after the onset of hyperglycemia, high glucose levels (≥ 288 mg/dL) with undetectable levels of serum c-peptide, an indicator of endogenous insulin secretion. It has been described mainly in East Asian countries and accounted for approximately 20% of acute-onset type 1 diabetes patients in Japan (5000-7000 cases) with an extremely rapid and almost complete β-cell destruction resulting in nearly no residual insulin secretion (Kharroubi and Darwish 2015). Both genetic and environmental factors, especially viral infections, have been implicated in the disease. An anti-viral immune response may trigger the destruction of pancreatic β-cells through the accelerated immune reaction with no detectable autoantibodies against pancreatic β-cells. Association of fulminant type 1 diabetes with pregnancy has also been reported.

1.7.3. Causes of T1D

A. Genetic

Autoimmune type 1 diabetes has strong HLA associations, with linkage to DR and DQ genes and development of the first islet auto-antibody. Children homozygous for HLA-DR3-DQ2 had glutamic acid decarboxylase autoantibodies (GADA) as the first autoantibody and children with the HLA-DR4-DQ8 haplotype tended to have insulin autoantibodies as the first autoantibody. Other HLA class II molecules also contribute to the initial trigger of β-cell auto-immunities like HLA-DRB3, HLA-DRB4, and HLA-DRB5 associated with β-cell autoantibodies and increased risk of type 1 diabetes. Frequencies of the INS rs689 risk genotype A/A and the A allele were increased in children with insulin autoantibodies with HLA-DR4-DQ8 haplotype thus confirming the contribution of polymorphism of INS gene towards etiology of T1D. Insulin autoantibodies-only are associated with HLA-DR4-DQ8, HLA-DR4, or HLA-DQ8 and PTPN22, SH2B3, ERBB3, RGS1, and INS, and HLA-DR3-DQ2 or HLA-DR4-DQ8 with PTPN22, ERBB3, RGS1, and SIRPG; whereas GADA-only antibodies in individuals with HLA-DR3-DQ2/ HLA-DR3-DQ2 were associated with CCR7, TNFAIP3, SH2B3, and CD226 (Ilonen *et al.* 2019).

HLA-DR/DQ alleles can be either predisposing or protective. When type 1diabetes 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. The major occurrence of type 1 diabetes is mainly due to the autoimmune destruction of the pancreatic β-cells. Immune responses are mediated through both T-cell mediated inflammatory response as well as a humoral (B cell) response. The hallmark of type 1 diabetes is the presence of autoantibodies against the pancreatic islet cells which contain islet cell autoantibodies, and autoantibodies to insulin (IAA), protein tyrosine phosphatase (IA2 and IA2β), glutamic acid decarboxylase (GAD, GAD65) along with zinc transporter protein (ZnT8A). These pancreatic autoantibodies could be detected in the serum of these patients quite a few times before the onset of the disease thus making them a hallmark of this type (Jerram and Leslie 2017).

There are more than 50 susceptibility loci that contribute to the likelihood of developing type 1diabetes. Data from a genome-wide association study, meta-analysis, and SNP profiling association than 100 SNPs. Other causal genes may include IL27 which encodes the cytokine interleukin 27 which modulates the differentiation and activity of various T-cell subsets and

has shown involvement in human type 1 diabetes. Another gene is BAD which is a pro-apoptotic (BH3-only) crucial regulator of the mitochondrial apoptotic pathway. The pro-apoptotic effects of BAD induce β-cell dysfunction. When BAD is phosphorylated in the β cell, it has a role in glucose-stimulated insulin secretion and nutrient-induced β-cell expansion. CD69 is suggested to have proinflammatory pathogenic effects in several autoimmune diseases like rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, and autoimmune thyroid disease also play a role in type 1 diabetes. PRCKQ encodes the protein kinase c theta, which is involved in the interaction between T cells and antigen-presenting cells and affects cytokine secretion from cytotoxic T cells. Apart from psoriasis, it plays a role in T1D.CLEC16A, ERBB3, and CTSH are expressed in immune and β cells, which also play a crucial role in T1D pathogenesis (Pociot *et al.* 2010).

B. Environmental

Though genetic predisposition is of prime importance in type 1 diabetes, several environmental factors have been implicated in the etiology of the disease. Viral factors comprise rubella, viral infection with a herpes virus, enterovirus, rotavirus, cytomegalovirus, a retrovirus. One of the most important viruses implicated is enteroviruses from studies in animal models and human beings. Higher expression of enteroviral VP1 protein immunoreactivity is found in the β-cells of children with type 1 diabetes. It is suggested that enteroviral infections during pregnancy might result in persistent infection and islet autoimmunity in the mother and offspring. Enteroviruses act as triggers of islet autoimmunity promoting the progression of type 1 diabetes. Apart from this, bacterial infections may also cause pancreatic lesions and thus forms a risk factor for T1D autoimmune triggers (Filippi and von Herrath 2008, Kharroubi and Darwish 2015). The number of environmental factors for type 1 diabetes (such as early juvenile diet, and use certain antibiotics) are interconnected with the human microbiome. Gut microbes not only influence lipid and glucose metabolism but also influence immunity and inflammation outside of the intestine on a systemic level. Lower microbial diversity in children with islet autoimmunity before progression to diabetes, compared with healthy controls is reported (Rewers and Ludvigsson 2016). The popular concept depicts “the hygiene hypothesis” that the incidence of autoimmune diseases might be rising because of a decreasing frequency of childhood infections due to improved hygiene. Higher birth weight and rapid weight gain during the age of 12–18 months have also been linked to type 1 diabetes (Rook 2007).

C. Chemicals and Drugs

Toxins in foods or water might activate autoimmune mechanisms in genetically susceptible individuals and exposure to toxins might result in pancreatic islet cell death. Some chemicals and drugs selectively destroy pancreatic cells. Pyrinuron, a rodenticide, and streptozotocin, an antineoplastic agent selectively destroys pancreatic β-cells. Monoclonal antibodies like nivolumab and pembrolizumab used for the treatment of cancer have been reported to occasionally induce autoimmune diabetes. Reports have suggested a link between type 1diabetes and exposure to water containing nitrates, nitrites, or nitrosamines (Burrack *et al.* 2017).

1.7.4. Symptoms

The onset of type 1 diabetes produces unexpectedly and can develop symptoms like polydipsia, polyuria, lethargy, extreme tiredness, polyphagia, unexplained weight loss, delay- wound healing, frequent fungal and bacterial infections and blurred vision with severe dehydration and diabetic ketoacidosis in children and adolescents (Kharroubi and Darwish 2015).

1.7.5. Type 1 Diabetes Management

Diabetes management aims at restoring carbohydrate metabolism with effective control of blood glucose, blood pressure, and lipid profile to avoid long term diabetic complications. With various lifestyle management and proper medication type 1 diabetes is manageable.

A. Diabetic diet and lifestyle modification

People with type 1 diabetes are advised to follow an individualized eating plan; a diet that produces more controllable glycaemic variability rather than a pre-decided one. It is critical to maintaining normal blood sugars because poorly controlled diabetes also leads to high blood pressure and other long-term complications. Lower carbohydrate intake and lower dietary carbohydrate-to-fat ratio are associated with several beneficial metabolic observations, such as lower variability in the blood glucose concentrations, lower blood pressure, and higher HDL-cholesterol concentration (Subramanian *et al.* 2000).

B. Insulin

The primary aim of the treatment of type 1 diabetes mellitus is an external replacement of the functions of β-cells to achieve near normoglycemia as close to the normal range as possible. Thus, exogenous insulin supply which can mimic physiological insulin-action can serve the

therapeutic purpose which can prevent long term macro and micro complications. Insulin is a protein hormone that is given as a treatment for high blood sugar levels. It is also given with glucose to treat high potassium levels. Typically administered as an injection under the skin but some forms can also be given intravenously or intramuscularly. Some who cannot attain satisfactory glucose regulation by traditional insulin injection can do so with a suitable pump (McCall and Farhy 2013). Since the discovery of insulin, insulin preparations have come a long way from purified animal insulins to human insulins produced by genetically modified organisms to insulin analogs that enable improved insulin-action profiles and variable glucose levels. The human insulins come in different types: rapid-acting (regular) insulin, slow-acting neutral protamine hagedorn (NPH) insulin, or zinc-based insulin (Hermansen *et al.* 2004).

(I) Regular insulin

Structurally regular insulin is the same as insulin produced by β cells which consist of six monomers of insulin, each of which consists of an A chain and a B chain linked by two disulfide bridges positioned around a zinc ion and form a hexamer. Glucose lowering effect of the dissociated monomers from hexamer is immediate when these hexamers reach the bloodstream. But the delayed onset of hexamer dissociation causes a miss-match in the regular profile action of insulin and thus a varying glycemic index (Weiss *et al.* 2000).

(II) Zinc and NPH insulins

Zinc and NPH (Neutral Protamine Hagedorn) insulins are formed by the addition of zinc or protamine, respectively, to regular insulin which causes their action profile to be prolonged. NPH insulins are suspensions that require shaking for reconstitution before injection which causes a major drawback of action variability. Basal insulin analogs were subsequently developed and introduced into clinical practice to achieve more improved and predictable glucose absorption profiles and efficacy (Owens 2011).

Various modifications in the basal insulin have been used to create two types of insulin analogs: those that are more readily absorbed from the injection site and therefore act faster than natural insulin injected (prandial insulin); and those that are released slowly over between 8 and 24 hours, intended to supply the basal level of insulin during the day and particularly at nighttime (basal insulin). The first rapid-acting insulin analogs were designed creating less-stable insulin hexamers that would more readily become monomeric, thus moving into the bloodstream more rapidly after subcutaneous injection than human regular insulin. Namely three rapid-acting

insulin analogs are commercially available: insulin lispro, insulin aspart, and insulin glulisine. Insulin glargine and insulin detemir were the first long-acting insulin analogs that were designed to provide more stable basal insulin-action profiles and longer, as well as better, 24 hours coverage of the insulin needs of patients compared with human rapid-acting insulins. Degludec insulin is an ultra-long-acting insulin analog (Sanlioglu *et al.* 2013).

Achieving normoglycemia without hypoglycemia and excessive weight gain in patients with type1 diabetes remains an elusive goal, but the advent of insulin analogs has a large effect, enabling intensive insulin therapy without being too disruptive to daily life. Currently, the vast majority of patients suffering from type 1 diabetes are treated by exogenous insulin injection but are still associated with the slow progression of micro- and macro-angiopathic secondary complications of the disease with a risk of hypoglycemic episodes which can prove fatal. Two different procedures whole pancreas transplantation or isolated islet transplantation have embarked on the clinical reality for the restoration of euglycemia without the administration of exogenous insulin in patients with type 1 diabetes (Brown *et al.* 2011).

1.8. Pancreatic transplantation

Pancreas transplants may be beneficial in people with extremely labile type 1 diabetes (Lerner 2008). Type 1 diabetic patient who needs a regular supply of insulin shows a remarkable improvement in the glycemic index when transplanted with a healthy pancreas. This treatment follows vascularized pancreas transplantation that can establish normal glucose as well as glycosylated hemoglobin levels in type 1 diabetic patients. William Kelly and Richard Lillehei, in 1966 performed the first vascularized pancreas transplant (Kelly *et al.* 1967). However, surgery and accompanying immunosuppression have fatal consequences than continued insulin replacement therapy. At times, 10-20% pancreatic transplantation requires laparotomy. Hence, limiting the scope of pancreas transplantation and only used with or sometimes after a kidney transplant. The reports suggest that pancreatic graft survival usually lasts a year with a success rate of about 83%. Also, better survival has been observed in the simultaneous pancreas-kidney transplant group of patients. Immunological loss in the first year after transplant for simultaneous pancreas-kidney, pancreas after a kidney, and pancreas alone are 1.8%, 3.7%, and 6% respectively. Apart from surgical complications, the bright side is that pancreatic transplantation increases the survival of uremic diabetic patients as compared to uremic diabetic patients on dialysis or with kidney transplantation alone (Júnior *et al.* 2015).

1.8.1. Artificial pancreas

Continuous glucose sensors and insulin pumps have allowed steady progress for the development of artificial pancreas which is also referred to as a closed-loop system. The artificial pancreas is a broad term for different bio-engineering strategies in development to achieve various glycemic requirements. The bio-engineering approaches leading to the attainment of the artificial pancreas are (1) medical equipment approach with real-time monitoring and suppling of insulin via insulin pump (2) the physiological approach with engineered stem cells to be integrated into the body to achieve normalized blood glucose (Lal *et al.* 2019).

1.8.2. Islet transplantation

The first report of islet transplantation in animals dated back in 1972 while that of successful human islet transplantation is in 1989. However, it has taken around 2 decades to clinically convert islet transplantation as a viable treatment for type 1 diabetes. Since Shapiro's landmark report in 2000, islet transplantation procedures and execution has improved tremendously (Shapiro *et al.* 2000). The cases of islet transplantation peaked in 2002 with around 100 islet transplants per year with a fall till 2008. However, technical improvements in collagenase products add the support of Clinical Islet Transplantation (CIT) consortium trials again led to an increase in this activity (Piemonti and Pileggi 2013). Once the islets are transplanted usually into a person's liver, it begins to produce insulin which actively regulates the glycemic index in the blood. If the cells are from a genetically unidentical donor the person's immune system will generate an immune reaction common to any transplant rejection. To prevent these immunosuppressant drugs are used. A combination of immunosuppressive drugs is applied in the Edmonton protocol (Shapiro *et al.* 2006). There are tremendous advances made in this field over the past decade which have progressively improved insulin-independent achievements. Nonetheless, the transition from the pre-clinical stage to clinical routine practice has faced several issues like alloimmune rejection, autoimmune recurrence, the toxicity of immunosuppressive medications, to the in hospitability of the liver itself as a site of implantation, relative inefficiency of the islet isolation process, the progressive loss of islet function over time and the need for multiple donors. Some of these issues can be overcome by various approaches like islets encapsulation to prevent graft rejection or intake of immunosuppressant or islets differentiation from stem cells (Bottino *et al.* 2018).

1.8.3. Islet encapsulation

Cell encapsulation technology is an emerging technique where islets for transplant are physically protected from the patient's immune response, thereby sustaining islet survival without the need for chronic immune suppression but also avoiding stem cell dissemination (Pathak *et al.* 2019) [More details are described in chapter number:6 (introduction section)].

1.9. Regenerative medicine

Pancreas transplantation caused morbidity, acute rejection in receiving patients. Even allographic pancreatectomy along with chronic immunosuppression was used but it caused infection and risk to haematological cancers (Gruessner and Gruessner 2013). Hence, to counter these drawbacks, regenerative medicine and tissue/organ engineering showed great potentials for managing diabetes. Regenerative medicine targets to gain the normal function of the damaged or diseased tissue. The tissue restoration is achieved by the administration of living tissue or cells directly or with specialized supporting material (Langer and Vacanti 1993). Regenerative medicine began with islet transplantation from cadavers almost five decades ago (Sutherland *et al.* 1977). Cadaveric islet transplantation primarily improved with the Edmonton protocol by accomplishing normoglycemia in 88% and 71% after the 1st and 2nd year respectively (Shapiro *et al.* 2000, Hering *et al.* 2016). Later, Ahearn et al used the trans-hepatic portal infusion method for islet transplantation. However, portal vein thrombosis and bleeding were the side effects in this method (Ahearn *et al.* 2015). Hence, the development of novel methods and a large number of biological resources are being considered to overcome insufficiency in islet/pancreas tissue, immune-rejections during transplantation strategies.

1.9.1. Reprogramming of pancreatic β-cells and islet neogenesis

The endocrine pancreas is a slow turnover tissue with a relatively long lifespan (rodents 2–3 months) (Finegood *et al.* 1995). During early adulthood in mice, the pancreatectomy of over half of the pancreas showed significant regeneration with the formation of new lobes and increased existing β-cell and acinar cell proliferation (Bonner-Weir *et al.* 1993, Li *et al.* 2010). But with age, the regenerative plasticity within ducts has been lost leading to less neogenesis (Tellez *et al.* 2016). Similar properties have also been seen in human pancreatic β cells after the neonatal period (Gregg *et al.* 2012). Hence, strategies to induce pancreatic β cell replication and several cell cycles modifying molecules were used in mice and humans. But, apart from molecules like Harmine, Aminopyrazine, and SerpineB1, most of the molecules

failed to work in human system and required extensive research (Aguayo-Mazzucato and Bonner-Weir 2018). An alternative and complementary strategy of trans-differentiation is used for the generation of β-cell mass by induction of its differentiation. On the other hand, trans-differentiation has been defined as the direct conversion of a terminally differentiated cell type into another cell type (pancreatic-β cells) (Bonner-Weir *et al.* 2004). The concept of trans-differentiation relies on the induction of the expression of master regulatory transcription factors able to control the transition from one cell type to pancreatic β cell (Lysy *et al.* 2013, Cito *et al.* 2018). Specifically, trans-differentiation has been applied to the following cell types to redirect it to insulin-producing cells (Peloso *et al.* 2018):

- **Pancreatic endocrine α-cells:** Common endodermic origin of α-cells and β-cells. Ectopic overexpression of the Pax4 or the loss of Aristaless-Related Homeobox (Arx) in α-cells during the pancreas development causes trans-differentiation of α-cells to β-cells (Courtney *et al.* 2013).
- **Pancreatic exocrine acinar cells:** The overexpression of certain transcription factors like PDX1, NGN3, and MAFA represented acinar to pancreatic β cell differentiation and restored normoglycemia in STZ-induced diabetic mice model (Courtney *et al.* 2013).
- **Hepatic cells:** Hepatocytes and β-cells have analogous glucose-sensing systems that have been targeted for trans-differentiation into pancreatic β-cells by inducing the expression of endogenous PDX-1 and the expression of other β-cell markers in liver cells to show substantial insulin production (both hepatic and plasma immunoreactive insulin) (Ferber *et al.* 2000).
- **Small intestinal cells:** In 2002, Yoshida et al showed PDX1 overexpression leads to the differentiation of intestinal epithelial cells into insulin-producing cells (Yoshida *et al.* 2002). Moreover, Transient expression of PDX1, MAFA, and NGN3 in intestinal crypt cells promoted rapid differentiation into insulin-secreting endocrine cells (Pancreatic islets) below the crypt base (Chen *et al.* 2014).

1.9.2. Stem Cell-Based Therapies

Neogenesis is usually considered as newly formed islets by differentiation from stem/progenitor cells; these progenitors may have arisen from dedifferentiated duct cells (Bonner-Weir *et al.* 2004). Due to the multi-potent property of stem cells, investigators are immensely exploring their potentials in T1D using both adult stem cells (mesenchymal stem

cells) and pluripotent stem cells (Skyler 2018). Soria et al initiated the generation of functional β-cell clusters from embryonic stem cells as replacement therapy (Soria *et al.* 2000). Generation of insulin-producing cells from human pluripotent stem cells *in vitro* depicted diabetes reversal in mice upon transplantation and such evidence is growing in recent years that directs the potentials of stem cells in regenerative medicine (Rezania *et al.* 2014).

The reason why the focus has shifted to using islets derived from self-stem cells as a treatment for diabetes mellitus is that such pancreatic islets will act as natural glucose sensors thus maintaining the blood glucose level at its optimum by appropriate secretion of insulin and glucagon. This transplant will be autogenic and thus will not be rejected by the patient's immune system. Previous studies have shown promising results concerning different stem cells being able to differentiate into functional islets *in vitro* upon being induced by appropriate factors (e.g. activin A, Exendine-4). Several research groups showed great potentials of differentiation of hBMSCs into insulin-producing islet-like cell clusters under appropriate conditions. (Sun *et al.* 2007, Xie *et al.* 2009, Milanesi *et al.* 2011, Wong 2011, Zanini *et al.* 2011, Milanesi *et al.* 2012, Gabr *et al.* 2013, Marappagounder *et al.* 2013, Pokrywczynska *et al.* 2015). Accordingly, various stem cells as described below can provide as a source of differentiated islets [more details are described in section 1.14 (Table)].

A. Human embryonic stem cells

Human embryonic stem cells (hESCs) are pluripotent cells giving rise to all somatic cells in a developing embryo. Therefore, hESCs can be a potential source that can be used to generate new β-cells for transplantation into T1D patients. Various molecular cues that mimic stages of β cell development have been identified and thus hESCs can be differentiated into pancreatic progenitor, endocrine progenitor, and insulin-producing β-cell by expressing various pancreatic transcription factors like PDX, MAFA, NEUROD1, NEUROG3, and PAX4. This approach has been utilized in many studies (Agulnick *et al.* 2015, Kim *et al.* 2016, Jacobson and Tzanakakis 2017) (Figure:1.5).

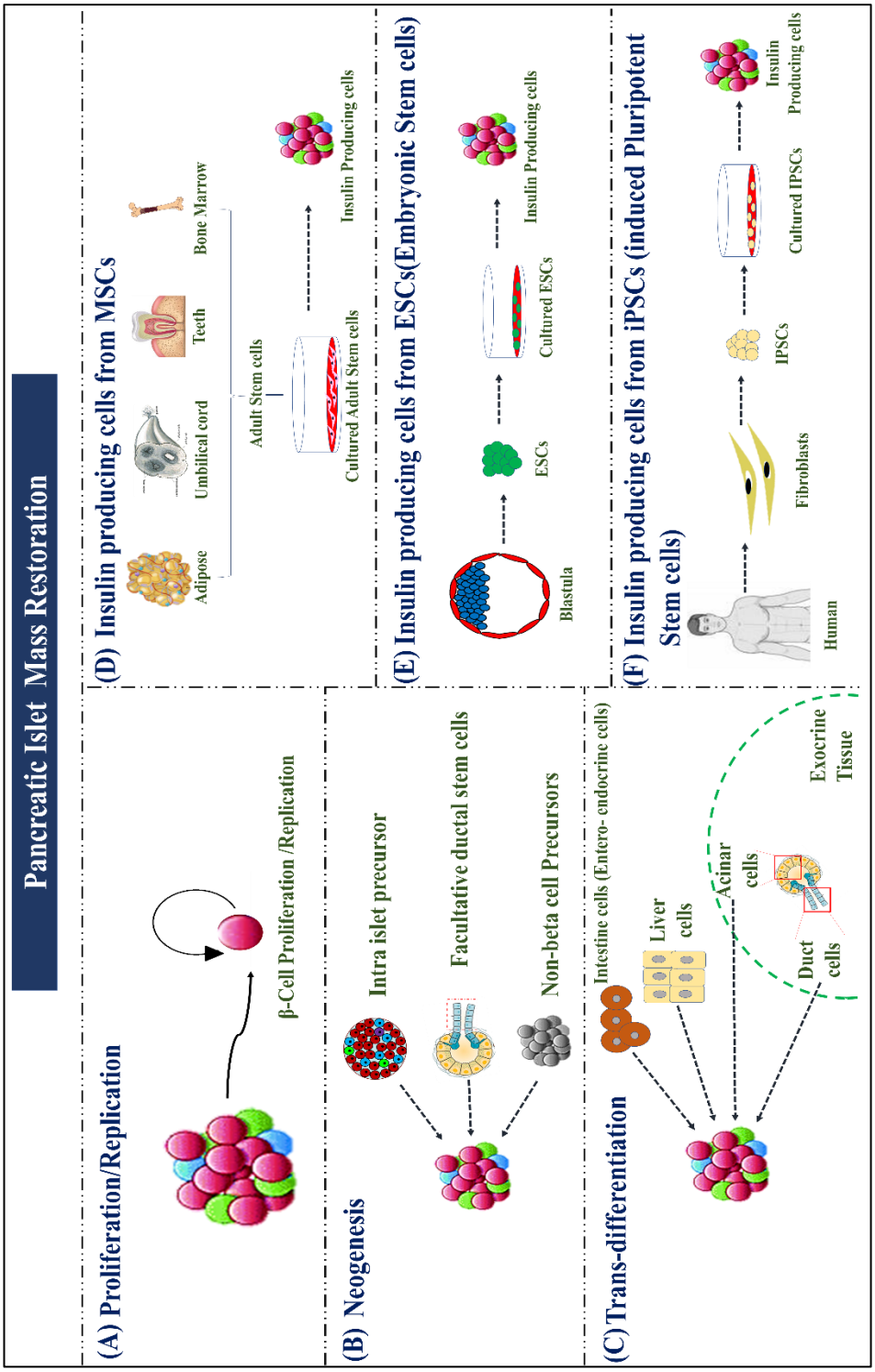

Figure 1. 5: Different strategies for pancreas islet (β-cell) mass regeneration / replacement (A) pancreatic β-cell proliferation/replication; (B) Islet neogenesis from various progenitors (C) Trans-differentiation, (D) pancreatic β cell from different mesenchymal stem cell sources (E) Insulin- producing cells from embryonic stem cells and iPSCs (F). This figure is adopted and modified from (Márquez-Aguirre *et al.* 2015).

B. Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) are specialized pluripotent stem cells generated from somatic cells which can be differentiated into functional β cells. T1D-specific iPSCs have been generated by using the yamanaka transcription factors OCT3/4, SOX2, C-MYC, and KLF4.

Additionally, iPSC-derived cells express pancreatic islet-specific markers such as PDX1, NGN-3, NKX6.1, MAFA, and GLUT2, and reversed diabetes in rodent animals' model (Millman *et al.* 2016, Southard *et al.* 2018, Yabe *et al.* 2019) (Figure:1.5).

Several adult stem cells like human bone marrow-derived stem cells, human adipose-derived stem cell, human dental pulp derived mesenchymal stem cells, etc., which are promising candidates of islet neogenesis [more details are described in section 1.14](Figure:1.5).

1.10. human Bone Marrow Mesenchymal Stem Cells (hBMSCs)

Mesenchymal stem cells have been initially described as clonal, plastic adherent cells from bone marrow with the ability to differentiate into adipocytes, chondrocytes, and osteoblasts, a process also known as trilineage differentiation. These Stem Cells have been identified in various other tissues including adipose tissue. In addition to their ability to differentiate into adipocytes, osteoblast, and chondrocytes, these stem cells were also found to adopt a neural and hepatic phenotype *in vitro* and *in vivo.* Bone marrow mesenchymal stem cells are large mononuclear cells they differ from hematopoietic stem cells concerning differentiation potential, cluster of differentiation markers (CD markers). For *in vitro* isolation and culture of hBMSCs following three criteria have to be fulfilled (Akiyama *et al.* 2012, Kobolak *et al.* 2016):

1. Cells should show self-renewal.
2. Cells should express CD markers that belong to the lineage of mesenchymal stem cells concomitantly they should also be negative for expression of CD markers other than those of mesenchymal origin. CD markers for the identification of hBMSCs (Table 1.1).
3. Cells should differentiate *in vitro* into adipocytes, osteocytes, and chondrocytes.

CD markers for characterization of hBMSCs:

CD 105: Transmembrane type III receptor belonging to TGF-β superfamily. Plays an important role in differentiation.

CD44: Also, known as the hermes antigen, it's a receptor for hyaluronic acid, osteopontin, etc.

CD markers for characterization of hematopoietic stem cells:

CD31: Platelet endothelial cell adhesion molecule (PECAM-1) expressed in hematopoietic stem cells.

CD34: Sialomucin expressed in the early stages of haematopoiesis in hematopoietic stem cells. (Kobolak *et al.* 2016)

Positive CD markers	Negative CD markers
CD 105	CD 31
CD 90	CD 34, CD45
CD 44	CD14, CD19, CD11b
CD 73	HLA-DR

Table 1. 1: Positive and negative CD surface markers for identification of hBMSCs (Akiyama *et al.* 2012, Kobolak *et al.* 2016).

Advantages of using hBMSCs include:

- Reduced ethical issues as compared to embryonic stem cells
- Ease of availability (Vija *et al.* 2009, Pokrywczynska *et al.* 2015)
- Low immunogenicity (Vija *et al.* 2009)
- Cells maintain stemness in diabetic patients too (Sun *et al.* 2007)

Several cell-intrinsic molecular profiles such as epigenetic modification, chromatin remodeling, transcription factors regulators during islet differentiation can also be tweaked apart from using external factors such as encapsulation or transplantation with mesenchymal stem cells to increase pancreatic yield and transplanted islet longevity. This can be achieved by introducing better differentiating agents or by inhibiting the barriers such as micro RNAs that come across efficient islet differentiation.

1.11. MicroRNAs (microRNAs)

MicroRNAs are a family of tiny, non-coding 21–26 nucleotide special RNAs that generally negative regulator of gene expression in a sequence-specific manner. They are transcribed by RNA polymerase II and the genes for microRNAs are present either in intronic sequences or they are present in the untranslated regions of a transcribed gene (He and Hannon 2004, Chen and Rajewsky 2007).

1.11.1. Biogenesis of microRNAs

Biogenesis of micro RNA involves two processing events in animals as shown in the figure:1.6. In the first, the nascent microRNA transcripts (pri-microRNA) are processed into ~70-nucleotide precursors (pre-microRNA) within the nucleus by an endoribonuclease III called drosha along with other accessory proteins like pasha. (Lee *et al.* 2002, Lee *et al.* 2003). In the

second event that follows, this precursor is transferred to the cytoplasm via exportin 5 (in a GTP-dependent mechanism) here it is cleaved to generate ~21–25-nucleotide mature microRNAs by another endoribonuclease III called DICER (Lee *et al.* 2002).

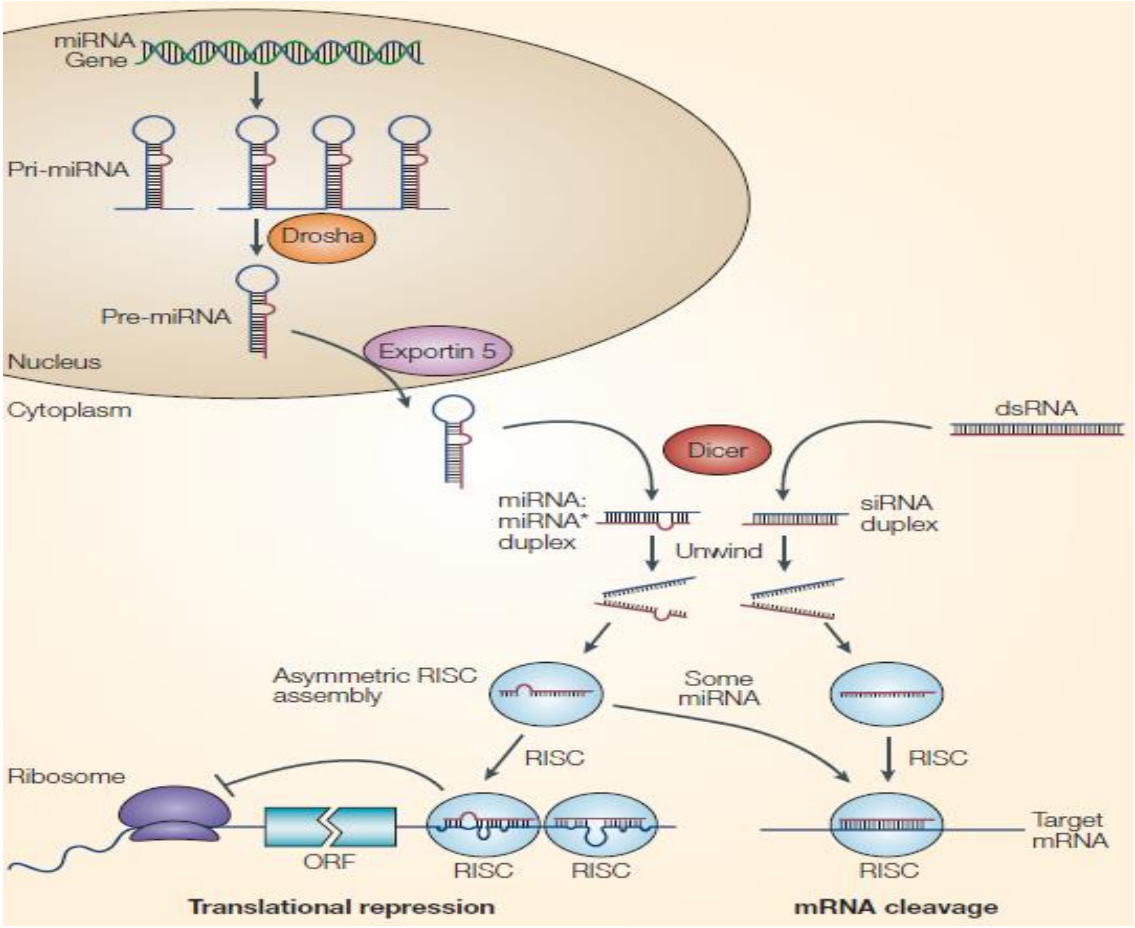

Figure 1. 6: MicroRNA biosynthesis and mode of action. This figure adapted from (He and Hannon 2004).

Only one strand of the microRNA: microRNA* duplex is bringing together into the RNA-induced silencing complex (RISC) for translational inhibition or mRNA cleavage, relying, on the degree of complementarity among the tiny microRNA and its target mRNA (Khvorova *et al.* 2003) (Figure:1.6). Method of translation repression depends on the level of complementarity shared between the target sequence and the microRNA sequence, in case of high complementarity the mRNA degradation is initiated while in case of low complementarity the translational machinery is halted (He and Hannon 2004). microRNA mediated gene expression control is more predominant in plants, where the mRNA is degraded after

microRNA binds to its target site thus decreasing the expression of the gene (Chen and Rajewsky 2007).

1.11.2. Role of microRNA in the pancreas

Studies have shown that microRNAs play a vital role in directly or indirectly regulating the development, regeneration, and functions of pancreatic islets (Poy *et al.* 2004, Rabe *et al.* 2013), β-cell differentiation (Baroukh *et al.* 2007)(Figure:1.7).

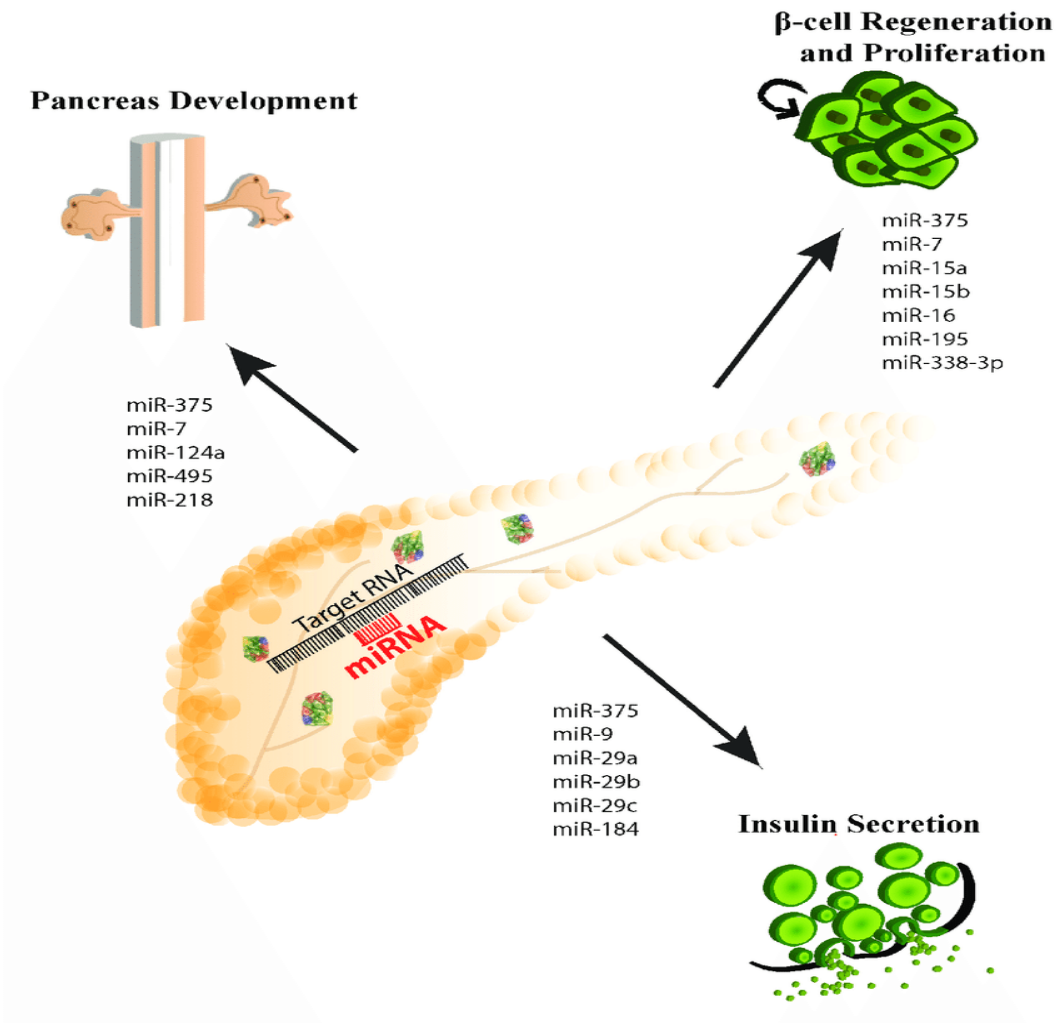

Figure 1. 7: microRNA involved in pancreas development, β-cell regeneration/ proliferation, and insulin secretion. This figure is adopted from (Rabe *et al.* 2013).

MicroRNA profiling of the human fetal pancreas revealed numerous microRNAs which have been implicated to play a role in pancreatic development. The potential targets and functions of these microRNAs have also been proposed, these microRNAs have been enlisted in table 1.2. Similar studies have profiled the dynamic microRNAs expression and subsequent

expression of lineage-specific markers during the *in vitro* hESCs into ILCCs (Figure:1.8) the protocol followed was designed to recapitulate the *in vivo* pancreatic organogenesis pathway, giving us an indication that microRNAs are very likely to play a crucial role in mediating the commitment of stem cells into islet-like lineage (Chen *et al.* 2011, Wei *et al.* 2013).

microRNAs	Function related to pancreas
miR-7	Expressed in pancreatic endocrine cells. (foetal and adult)
miR-375	Targets the protein myotrophin, which is involved in glucose-induced insulin secretion. Also, it targets the transcription of insulin in β-cell.
miR-9	Expressed during pancreatic islet development, targets transcription factor onecut-2, impairs glucose-induced insulin secretion.
miR-195, miR-16, miR-15a, miR-15b	Role in pancreatic regeneration & target NGN-3
miR-124a	Regulation of insulin secretion machinery and targets FOXA2.
miR-146a	The expression is high in db/db obese mice, contributes to fatty acid-induced β-cell dysfunction.
miR-503	Expressed in a pattern similar to that of miR-375.
miR-376a	Highly expressed during islet's development.
miR-21, miR-34a	Expression induced by proinflammatory cytokines in human islets. Contribute to fatty acid-induced β-cell dysfunction.
miR-96	Increased expression of granulophilin, which is a negative regulator of insulin secretion.

Table 1. 2: MicroRNAs involved in the development of the human pancreas (Rosero *et al.* 2010, Chen *et al.* 2011).

Overexpression of microRNAs, as well as specific microRNA knockdown *in vitro*, resulted in alteration in the expression of key transcriptions factors leading to a discernible change in differentiation pattern as well as maturation, this further strengthens the possibility of microRNAs being key regulators of pancreatic islet differentiation and maturation. Review of literature revealed that the involvement of microRNA mediated translational regulation has been discovered in hBMSC fate determination into osteoblasts or adipocytes *in vitro* (Baglìo *et al.* 2013, Sun *et al.* 2014, Huang *et al.* 2016), No studies to our knowledge have shown the involvement of microRNA in the differentiation of hBMSCs into ILCCs. MicroRNAs like

miR-375 has been proven to directly affect insulin secretion and synthesis from β-cells. They have been found to indirectly control energy metabolism also, secondary complications associated with diabetes has been attributed to specific microRNAs (Poy *et al.* 2004, Xiao *et al.* 2007, Li 2014).

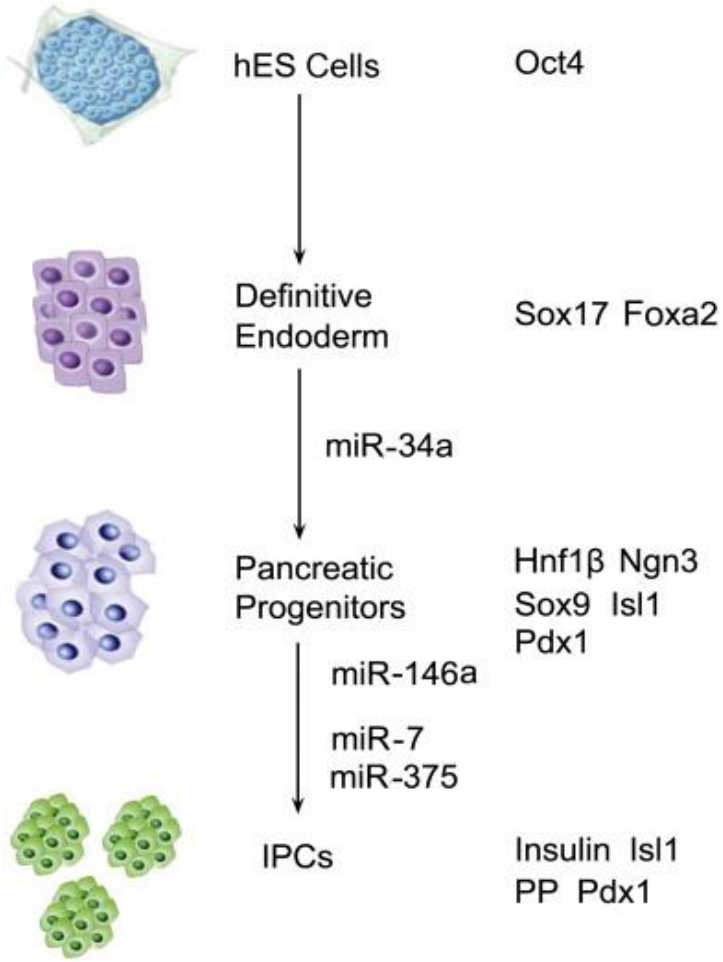

Figure 1. 8: microRNAs expressed during *in vitro* differentiation of human embryonic stem cells into insulin producing cell clusters. This figure is adopted from (Wei *et al.* 2013).

1.11.3. MicroRNA manipulation strategy:

MicroRNAs provide several benefits such as small size, fast synthesis, resistance to nuclease activity, and long half-life/bioactivity. Thus making it an ideal substitute for growth factors and inhibitors to guide differentiation towards any particular cell type (Greve *et al.* 2013).

Two main approaches to manipulating microRNAs (1) microRNA overexpression (Gain of function) (2) microRNA knockdown (Loss of function).

A. micro RNA overexpression:

Several technologies are available for the gain of function approach by overexpressing microRNA such as (a) microRNA mimic (b) microRNA precursors (c) microRNA biogenesis enhancement. Amongst all technologies, microRNA mimics can be easily synthesized and delivered, making them convenient to use for both *in vitro* and *in vivo* study. Oligonucleotide-based strategies involve in miRNA mimics synthesis, which has the same nucleotide sequences as endogenous miRNAs. The major drawback of microRNA mimics is low transfection efficiency, especially in 3D cell clusters and the transfection is generally transient (Zhang *et al.* 2013).

B. microRNA knockdown:

Various techniques applicable for the loss of function approach by inhibiting microRNA for example (a) antisense miRNA oligonucleotides (AMOs), including 2′-O-methyl group modified AMO, (b) Antagomir, (c) locked nucleic acid (LNA), (d) phosphorodiamidate morpholino oligonucleotide (PMO),(e) peptide nucleic acid (PNA), (f) miRNA sponge (g) microRNA protector (h) small molecule inhibitors by complementary binding to the mature miRNA (Zhang *et al.* 2013). Anti-miRNA oligonucleotides are one of the most common strategies in miRNA based therapy; in this strategy, anti-miRNA oligonucleotides specifically bind to miRNA molecules and subsequently prevent the binding of miRNAs to their target genes. An excellent miRNA inhibitor demands to have several functions, including high affinity to the target microRNA, high specificity, low toxicity (for both *in vitro* and *in vivo* condition), resistance to exonucleases, easy delivery method and low cost for synthesis. LNA (Lock Nucleic Acid) is modified oligonucleotides mediated highly specific microRNA inhibitor with several advantages over other existing microRNA inhibitors (Ørom *et al.* 2006)[more details about LNA are described in chapter number:6].

1.12. chemical agents promoting pancreatic islet differentiation

Bioactive molecules have provided a dynamic chemical tool to control cellular proliferation, differentiation by modulation of gene regulation, signal transduction pathways, and even metabolism. Several small bioactive molecules, growth, and differentiating factors trigger pancreatic β-cell differentiation from embryonic and mesenchymal stem cell sources. These small molecules (e.g. retinoic acid, wortmannin, cyclopamine, and sodium butyrate) and large molecules (e.g. betacellulin, activin A, bone morphogenic protein (BMP4), FGF, EGF, IGF, KGF, HGF, noggin, TGF-β, WNT3A) are assumed to promote the initial phase of definitive

endoderm generation and ultimately to the maturation of functional pancreatic endocrine cells. A clear understanding of diverse small and large bioactive molecules and their function benefit us to establish an efficient protocol for pancreatic islet differentiation (Kumar *et al.* 2014). At this point, we describe important small and large molecules promoting the differentiation of stem cells into pancreatic islets.

1.12.1. Activin A and Betacellulin

Activin A is a cytokine associated with the TGF-β family (Woodruff and Mather 1995, Risbridger *et al.* 2001) and regulates cell proliferation, apoptosis, and differentiation (Sulyok *et al.* 2004). Activin A plays a unique role in several biological systems including differentiation into pancreatic islet (Totsuka *et al.* 1988), neural cells (Hashimot *et al.* 1990), mesoderm cells (Albano *et al.* 1990) pituitary cells (BILEZIKJIAN *et al.* 1990) and erythroid (Hemopoiesis) (Eto *et al.* 1987). Betacellulin is a glycoprotein and a member of EGF family protein isolated from mouse pancreatic insulinoma cell line (Sasada *et al.* 1993). It is widely expressed in adult and fetal pancreas (Silver *et al.* 2005) and intestine (Yamamoto *et al.* 2000). Numerous researchers demonstrated that pancreatic islet differentiation using activin A, along with other growth and differentiating factors (β-cellulin), to the culture medium of hBMSCs promoted the formation of pancreatic progenitor's stage during the pancreatic islet differentiation (Yu *et al.* 2007, Gabr *et al.* 2013, Gabr *et al.* 2014).

In a study demonstrated by demeterco et al., 2000 to examine the effect of activin A and betacellulin on pancreatic islet differentiation, it was observed that betacellulin promoted and increased participation of stem cells in islet differentiation, while activin A was responsible for increased insulin content in islet differentiation. Further, several reports suggested enhanced insulin production employing activin A as a consequence of stem cell differentiation. Wang *et al.*, Johannesson *et al.*, and D'Amour *et al.* supplemented activin A, in combination with WNT3A, to the induction media of hESCs and established the improved formation of the definitive endoderm stage during the pancreatic islet differentiation (D'Amour *et al.* 2006, Johannesson *et al.* 2009, Wang *et al.* 2011). Additionally, Cai *et al.*, 2010 showed that activin A induction of human ES cells, in combination with the retinoic acid, triggered the expression of the all-important pancreatic endocrine specific genes in over 70 % of cells in culture (Cai *et al.* 2010). Hence, activin A and β-cellulin have developed outstanding importance in the development of islet differentiation protocols. The researcher has demonstrated that activin A combination with sodium butyrate, promotes differentiation of human ES to pancreatic islets

during the early stages of endoderm development (Jiang *et al.* 2007). Although, the precise mechanism by which activin A treatment in combination with sodium butyrate induces differentiation during definitive endoderm formation is obscure.

In current years, the focus of islet biology research has largely shifted to the development of a highly efficient and novel step-wise protocol to direct pancreatic differentiation from various stem cell sources using a combination of activin A, β-cellulin, and wortmannin to induce definitive endoderm formation (Zhang *et al.* 2009). However, the researcher also observed that sustained exposure to high levels of activin A induces definitive endoderm formation and pancreatic endoderm formation from stem cell sources (D'Amour *et al.* 2005, Tada *et al.* 2005, Yasunaga *et al.* 2005, Wang *et al.* 2011). Thus, it can be recorded that activin A plays a central role in pancreatic endoderm formation in stem cell differentiation.

1.12.2. Exendin-4

Exendin-4 is an approximately 4.2 kDa long-acting glucagon-like peptide1 (GLP-1) bio-similar, which was firstly isolated from salivary venom of Gila monster lizard (Eng *et al.* 1992). Various reports have been demonstrated to elucidate the impact of exendin-4 on improving glucose tolerance in pre-clinical research because of its possibility to stimulate pancreatic β-cell proliferation and enhance insulin release (Tourrel *et al.* 2001, Yang *et al.* 2006). Xu *et al.*,1999 confirmed that exendin-4 triggered pancreatic β cell proliferation and islet neogenesis in a type-II diabetic rat model (Xu *et al.* 1999). Fusco et al., 2017 demonstrated that exendin-4 stimulated pancreatic β cell proliferation by epidermal growth factor receptor (EGFR) signaling (Fusco *et al.* 2017).

However, the precise molecular mechanism of exendin-4 in pancreatic β-cell replication is not fully understood. Further, Defronzo et al., 2005 reveled that exendin-4 improved glycemic index in type-2 diabetes patients (DeFronzo *et al.* 2005). It is notable here that there are various islet differentiation protocols utilized exendin-4 along and in combination with other growth factors for generating ILCCs from a different source of stem cells. It has been reported that exendin-4 remarkably enhances the islet differentiation from Wharton's jelly mesenchymal stem cells via the induction of several pancreatic islet transcription factors (Kassem *et al.* 2016). The addition of exendin-4 in the last phase of islet differentiation from hBMSCs stimulated the generation of functional 3D- ILCCs (Xin *et al.* 2016). [more information related to exendin-4 function in islet differentiation, see the section number:1.14 (Table)]

1.12.3. FGF, EGF, KGF, and HFG

FGF, EGF, and HFG play a crucial role in cellular differentiation, proliferation, migration, and regeneration. FGF, EGF, and HGF act by binding FGF receptor, EGF receptor, and HGF receptor respectively on the outer surface of a cell which initiates various intrinsic receptor tyrosine kinase activity by carrying out signal transduction cascade and eventually leading to numerous morphological, physiological and biochemical alteration in cells (Zalesna *et al.* 2017). In mammals, approximately 22 FGF family members have been recognized, all are cell signaling multifunctional proteins interacting with heparin and heparin sulfate for stimulating mitogen, endocrine, and regulatory effects in cells (Ornitz and Itoh 2001). FGF has a close connection link among pancreas and different organs, for example, liver, lung, and thyroid. Previously many researchers proved that high BMP concentration in stem cell differentiation media is essential for generating hepatic lineage population, while minute BMP concentration along with FGF is required for the pancreatic islet differentiation from embryonic stem cells (Deutsch *et al.* 2001, Zaret and Grompe 2008). The absence of FGFs in islet differentiation medium negatively affects pancreatic β cell differentiation from hESCs (Bhushan *et al.* 2001, Hart *et al.* 2003). FGF and EGF regulate pancreatic islet differentiation from stem cells in a concentration and time-dependent manner. Interestingly, very low concentration of FGF and EGF are utilized in stem cell maintenance media for stem cell proliferation and promotes liver formation while at high concentration stimulates pancreatic islet formation via increased pancreatic transcription factors expression such as PDX-1 and NKX6.1 (Chiang and Melton 2003, Ameri *et al.* 2010, Zhang *et al.* 2013). Moreover, the intermediate concentration of FGF boosts the 3D cell clustering formation in ILCCs due to its chemo-attractant properties (Hardikar *et al.* 2003), however, the absence of EGF in culture media allowed the generation of 3D cell clusters (Dalvi *et al.* 2009). Further, johnnesson et al., demonstrated that only FGF is capable to trigger low insulin transcript without supplement of retinoic acid, indicating retinoic acid plays an important role in pancreatic islet differentiation (Johannesson *et al.* 2009).

KGF, one of the important members of the FGF family is identified for pancreatic islet differentiation and function by PI3K/AKT signaling pathway from stem cells and pancreatic ductal cells (Krakowski *et al.* 1999, Uzan *et al.* 2009). Kroon *et al.*, 2008 demonstrated that hESCs is differentiated into definitive endoderm formation and eventually insulin expressing

cell clusters using KGF along with activin A as key islet differentiating molecules (Kroon *et al.* 2008).

HGF is also known as scatter factors and secreted by mesenchymal cells in order to control cell proliferation, cell differentiation, and cell motility (Johnson *et al.* 1993). HGF has been utilized extensively for differentiation, maturation, and maintaining the function of pancreatic β-cells from various stem cells (Otonkoski *et al.* 1994, Hayek *et al.* 1995, Otonkoski *et al.* 1996). In a study performed by mashima *et al.*,1996 it was shown that trans-differentiation pancreatic acinar cells into insulin-producing cells using HGF along with activin A (Mashima *et al.* 1996). Further, wang *et. al.,* 2004 demonstrated that HGF differentiated pancreatic islets derived epithelial cells into insulin hormone expressing cells without the formation of islet-like cell clusters, suggesting that only the use of HGF is inadequate in ameliorating pancreatic islet function (Wang *et al.* 2004).

Thus, attentive use of FGF and EGF along with other differentiation factors like activin A, retinoic acid may be crucial in successful pancreatic islet differentiation from various stem cell sources. Additional examples regarding FGF and EGF in pancreatic β cell differentiation are given in section number:1.14

1.12.4. Retinoic acid (RA)

Retinoic acid is a metabolite of vitamin A and plays a key role in the growth and embryonic development of all higher animals (Duester 2008). Several reports demonstrated that all-trans-retinoic acid, a majorly occurring retinoic acid is needed for an early stage of islet differentiation and for expressing important pancreatic transcription factors like *PDX-1, NGN-3* during pancreatic β cell differentiation process from embryonic stem cells (D'Amour *et al.* 2006, Vallier *et al.* 2009, Cai *et al.* 2010, Mfopou *et al.* 2010). Further, many research groups proved that only basal level of PDX-1 transcript was observed when only retinoic acid is utilized, while, the combination of retinoic acid with other differentiating molecules such as activin A, nicotinamide remarkably increase *PDX-1, INS, GCG, PPY* transcript level in pancreatic islet differentiation (Shi *et al.* 2005, Zhang *et al.* 2009, Massumi *et al.* 2016). However, D'Amour *et. al.,*2006 observed that lack of retinoic acid in differentiation culture media fail to express important pancreatic transcription factors like *NGN3, INS, GCG* transcript (D'Amour *et al.* 2006).

1.12.5. Sodium Butyrate

Sodium butyrate, a short-chain fatty acid, acts as a chromatin remodeling agent that promotes cell differentiation and inhibits cell proliferation and dedifferentiation by induction and repression of various gene expression (Kruh 1981, Haumaitre *et al.* 2008). Several reports demonstrated that integration of sodium butyrate and activin A triggers the expression of definitive endoderm specific markers such as HNF4 α and FOXA2 during islet differentiation of ILCCs using embryonic stem cells and murine adipose tissue-derived mesenchymal stem cells (Jiang *et al.* 2007, Chandra *et al.* 2009). The addition of sodium butyrate in the early phase of islet differentiation medium enhances the secretion of insulin and glucagon while removal of it from the differentiation medium drastically decreases the *PDX-1* transcript level (Powers *et al.* 1988, Goicoa *et al.* 2006).

1.12.6. Nicotinamide

Nicotinamide is an active form of vitaminB3 and inhibitor of Poly (ADP-Ribose) Polymerase protein (PARP). As noted previously, a researcher has utilized 10 nM nicotinamide as a stimulator of pancreatic endocrine progenitor differentiation into mature and function pancreatic β-cells by increasing expression of *INS, GCG,* and *SST* transcript level from epithelium cell clusters in cultured human fetal pancreatic cells system (Otonkoski *et al.* 1993). Cho Y M *et.al.,* 2008 proved that ideal combination of nicotinamide and betacellulin efficiently induced and maintained PDX-1 expressing cells during pancreatic islet differentiation from hESCs (Cho *et al.* 2008). The study performed by Ye *et.al. 2006,* demonstrated nicotinamide induced islet differentiation and insulin biogenesis by increasing MAFA transcript levels (Diana *et al.* 2006). The combination of nicotinamide, along with EGF effectively triggers the formation of NKX6.1 positive population from pluripotent stem cells (Nostro *et al.* 2015).

1.13. Antidiabetic activity of isolated compounds from *Enicostemma littorale*:

Many compounds have been isolated and reported from *Enicostemma littorale (EL)*. Five alkaloids, two sterols, and volatile oil have been reported by Natarajan and Prasad (Natarajan PN 1972). Seven flavonoids including swertisin have been reported by Ghoshal *et.al.*,1980 (Ghosal 1980) and swertiamarin a gentiopicroside was isolated from ethyl acetate extract (Desai *et al.* 1966). Further swertisin and swertiamarin were successfully isolated from EL and proved potent insulin sensitizer and adipogenic inhibitor property of swertiamarin in NIDDM rats and hepatic steatosis models (Fatty liver condition)(Patel *et al.* 2013, Patel *et al.* 2016). Swertisin has been proved as a potent inducer of islet differentiation from stem/progenitors.

Antidiabetic activity of *Enicostemma littorale* (EL) has been well reported by our lab (Vijayvargia *et al.* 2000, Maroo *et al.* 2002, Maroo *et al.* 2003, Maroo *et al.* 2003, Vasu *et al.* 2003, Vasu *et al.* 2005, Bhatt *et al.* 2009, Bhatt *et al.* 2011, Bhatt *et al.* 2012, Srivastava *et al.* 2016).

1.13.1. Swertisin as pancreatic islet neogenic agent*:*

Swertisin, one of the bioactive components of EL has been meticulously investigated in our lab for its effective pancreatic islet neogenic and anti-hyperglycemic properties (Gupta S *et al.* 2010, Dadheech *et al.* 2013, Dadheech *et al.* 2015, Srivastava *et al.* 2018). Stem cells/ progenitor differentiation activity of *Enicostemma littorale* has also been reported where human pancreatic carcinoma cells PANC-1 and mouse embryonic fibroblast cells NIH3T3 were efficiently differentiated into functional insulin-producing islet clusters (Gupta S *et al.* 2010), which when transplanted into diabetic mice model demonstrated restoration of glucose homeostasis (Dadheech *et al.* 2013). Dadheech *et al*., in 2015 identified the active principle molecule swertisin, a flavonoid that was responsible for the above islet neogenic property. Swertisin, not only gave a better yield of islets but it was also superior in terms of the amount of insulin released after a glucose challenge. Further, the islets generated using swertisin were transplanted into streptozotocin treated diabetic BALB/c mice, which became normoglycemic after the transplantation. Further, the molecular mechanism of swertisin in islet neogenesis was found to follow the activin A mediated MEPK-TKK pathway. Swertisin raised the level of insulin transcripts with persistent down-regulation of progenitor markers within three days post (PPx) partial pancreatectomized mice model (Dadheech *et al.* 2015). Swertisin has also demonstrated its potent islet differentiation property in mouse bone marrow-derived mesenchymal stem cells (*in vitro*), which after transplantation ameliorates diabetic condition in streptozotocin (STZ) mice *in vivo*. Further, swertisin exhibited islet neogenic potential from pancreatic resident endocrine progenitors (PREPs) stem cell source (Srivastava *et al.* 2019). Moreover, when swertisin administered in STZ diabetic mice (*in vivo*), it triggered the resident pancreatic progenitors to replenish and recover the endocrine function by increasing islet formation (Srivastava *et al.* 2018). All these properties of swertisin make it an ideal candidate for a novel therapeutic intervention in treating diabetes mellitus. Hence, presently islet differentiating activity of swertisin has been explored with human mesenchymal stem cells and further our efforts are in the direction of designing potent islet therapy using plant-derived compounds for effective diabetes treatment.

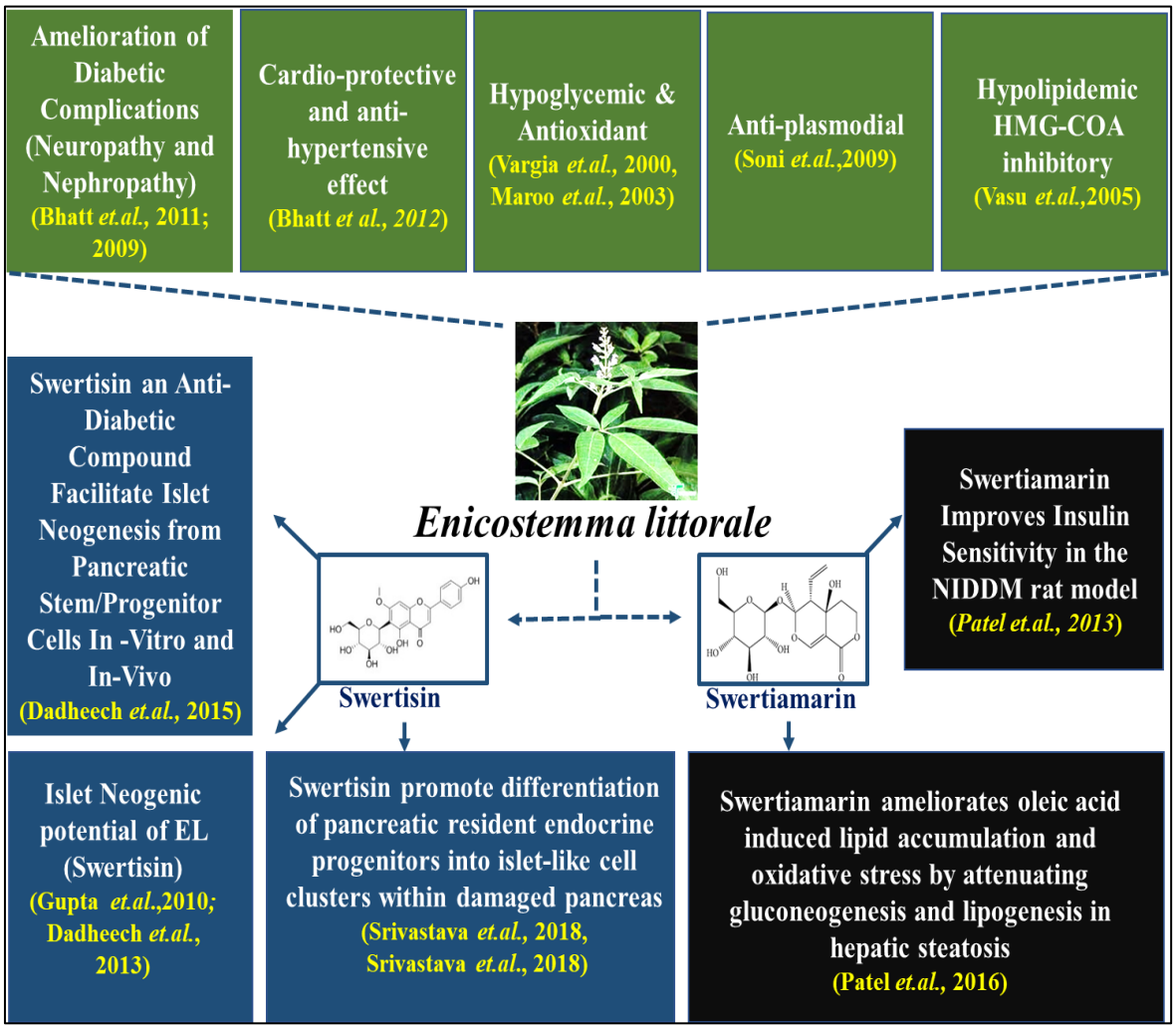

Figure 1. 9: Role of swertisin,swertiamarin, and *Enicostemma littorale* (EL) in the treatment of type-I and type-II Diabetes (Our lab published reports).

1.14. Islet differentiation from various sources of adult mesenchymal stem cells

Sr. No.	Stem cells source	Differentiation/Growth Factors (Culture medium)	Duration (in Days)	Important Remarks	References
1	Human Bone-Marrow derived Mesenchymal Stem Cells (hBMSCs)	1% BSA, ITS, activin-A, taurine, sodium butyrate, hepatocyte growth factor (HGF), exendin-4, activin A, non-essential amino acids (NEAA), nicotinamide 2 % B27 and N2, nicotinamide	21	• The combination of activin A with exendin-4 enhanced important transcription factors such as *PDX1, NGN3, NKX2.2*, along with pancreatic islet hormone specific gene transcription *(INS,GCG*). • hBMSCs derived ILCCs exhibited insulin and c-peptide release in glucose concentration-dependent manner *in vitro*.	(Jafarian *et al.* 2014)
2	Human Bone-Marrow derived Mesenchymal Stem Cells (BM-MSCs) and Human islet-mesenchymal stem cells (HI-MSC)	β-cellulin, activin-A, nicotinamide, Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), glutamine 2, activin-A, glucagon-like peptide-1, retinoic acid, platelet Lysate (PL)	21	• A similar kind of pattern was observed during islet-like cell cluster formation in both types of MSCs. However, HI-MSCs derived ILCCs secreted more amount of insulin as compared to BM-MSCs derived ILCCs. • HI-MSCs derived ILCCs showed expression of PDX1, GLUT-2, insulin, and c-peptide while BM-MSCs derived ILCCs demonstrated expression of only GLUT-2 and insulin.	(Zanini *et al.* 2011)

3	Human Bone-Marrow derived Mesenchymal Stem Cells (hBMSCs)	β-mercaptoethanol, Basic Fibroblast Growth Factor (bFGF), Epidermal Growth Factor (EGF), 1% nonessential amino acids, B27 supplement, β-cellulin, activin-A, nicotinamide	18	• An increase in the concentration of activin A / β-cellulin up to 30ng/ml remarkably enhanced differentiated islet functionality. It was observed that an increase in the concentration of differentiation factors beyond certain limits did not change the efficacy of pancreatic islet differentiated cells. • Islet like cell clusters displayed human c peptide-protein expression by flow cytometry.	(Czubak *et al.* 2014)
4	Human Bone-Marrow derived Mesenchymal Stem Cells (hBMSCs)	β-mercaptoethanol, Basic Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), B27 supplement, β-cellulin, activin-A, nicotinamide, and L-glutamine	18	• Diabetic patients derived hBMSCs were used for islet differentiation and differentiated ILCCs tested positive for DTZ staining. • ILCCs demonstrated gene expresses of important transcription factors such as *PDX-1*, *NGN-3*, and *PAX-4*. • hBMSCs-derived ILCCs showed insulin and glucagon gene transcription and *in vitro* insulin released in response to different concentrations of glucose.	(Yu *et al.* 2007)
5	Human Bone-Marrow derived Mesenchymal Stem Cells (hBMSCs)	Basic Fibroblast Growth Factor (bFGF), Epidermal Growth Factor (EGF), 1% nonessential amino acids, B27 Supplement, β-cellulin, activin-A,	16-20 days	• Islet like cell aggregates exhibited positive immunostaining for insulin and glucagon. • They developed an islet encapsulation system with a refillable polymer construct using 3D printing and nano-gland fabrication for better islet functionality and longevity.	(Sabek *et al.* 2016)

		nicotinamide, L-glutamine			
6	Human Bone Marrow Mesenchymal stem cells (hBMSCs)	β-mercaptoethanol, Epidermal Growth Factor (EGF), Basic Fibroblast Growth Factor (FGF), β-cellulin, activin-A, B27 supplement, 1% nonessential amino acids, and nicotinamide	18	• β-mercaptoethanol was supplemented for stimulating PDX-1 gene transcription and EGF along with FGF were utilized for promoting proinsulin biosynthesis. • hBMSCs derived ILCCs showed gene expression of pancreatic endocrine specific transcription factors and further release of c-peptide in response to a glucose challenge.	(Gabr *et al.* 2014)
7	Human Bone Marrow Mesenchymal Stem Cells (hBMSCs)	β-mercaptoethanol, Epidermal Growth Factor (EGF), Basic Fibroblast Growth Factor (FGF) β-cellulin, activin-A, B27 supplement, nicotinamide, 1% nonessential amino acids, L-glutamine	18	• Microscopic examination revealed the presence of only 5-10 % insulin-positive cells in differentiated ILCCs. • Differentiated ILCCs showed c-peptide secretion in response to rising glucose concentrations • When differentiated ILCCs transplanted into a diabetic mouse model, it showed normoglycemia up to 3 months.	(Gabr *et al.* 2013)
8	Human Bone Marrow Mesenchymal Stem Cells (hBMSCs)	1% DMSO, high glucose-DMEM with 10% FBS	17	• Differentiated ILCCs showed pancreatic islet-specific genes like *NGN-3*, *PDX-1*, *NEUROD*, and *PAX4* and 3-fold rises of human c-peptide release in response to a high glucose challenge.	(Tsai *et al.* 2014)

				• The transplantation of hBMSCs derived ILCCs into the STZ diabetic rat model demonstrated remarkably reduced hyperglycemia, however, it failed to achieve complete normoglycemia.	
9	Human Bone-Marrow derived Mesenchymal Stem Cells (hBMSCs)	Fibroblast Growth Factor (bFGF), 1% DMSO, Epidermal Growth Factor (EGF), exendin-4, activin-A, HGF, nicotinamide	15	• ILCCs exhibited co-expression of human insulin and c-peptide by an immunostaining method. Further, it expressed various genes associated with pancreatic development like *PDX-1, NGN-3, NKX6.1*.etc. • hBMSCs derived ILCCs restored normoglycemia *in vivo* when transplanted in STZ-induced diabetic nude mice.	(Xie *et al.* 2009)
10	Human Bone-Marrow derived Mesenchymal Stem Cells (hBMSCs)	1% DMSO, HG/LG DMEM medium supplemented with 10% FBS	20	• Trans-differentiated cells displayed an expression of insulin, c-peptide, somatostatin, and pancreatic polypeptide. • Insulin-producing clusters transplanted into the renal subcapsular space of diabetic mice and maintained normoglycemia for up to 90 days.	(Oh *et al.* 2004)
11	Human Bone-Marrow Mesenchymal Stem Cells (hBMSCs)	5% FBS, nicotinamide, exendin-4	29	• ~15% of insulin-expressing cells and ~6% of c-peptide-expressing cells were observed in differentiated ILCCs. • *In-vitro* differentiated ILCCs transplanted in diabetic mice ameliorated the hyperglycemia condition.	(Xin *et al.* 2016)

12	Bone Marrow Mesenchymal Stem Cells of Human First-Trimester (hBMSCs)	β-mercaptoethanol, Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF), Hepatocyte Growth Factors (HGF), B27 supplement, nicotinamide, BSA, β-cellulin, zinc acetate, exendin-4	16	• Islet like cell clusters displayed genes expression profile of all-important pancreatic transcription factors. They also secreted a high level of human c-peptide (2.200±0.468 pmol) and human insulin (2.245 ±0.222 pmol) with high glucose challenge. • Xenograft (*in vivo*) study revealed that differentiated ILCCs normalized the FBS level for at least 70 days in the diabetic mice model.	(Zhang *et al.* 2010)
13	hBMSCs of human first-trimester abortus foetus (hfBMSCs)	β-mercaptoethanol, Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), B27 supplement, nicotinamide, 0.5% BSA, β-cellulin, exendin-4, activin A	16	• ILCCs obtained from non-adherence and adherence induction groups showed positive dithizone staining and gene expression related to pancreatic development like *PDX-1, NGN-3, NEUROD1, NKX6.1/2.2*, Insulin, Glucagon, etc. However, significant-high differentiated ILCCs yield was observed in the non-adherence induction group as compared to the adherence induction group. • Differentiated ILCCs also demonstrated *in vivo* functionality.	(Zhang and Dou 2014)

14	Human Bone Marrow derived Mesenchymal Stem cell (hBMSCs) and Human Adipose tissue derived Mesenchymal stem cells(hADSCs)	Trichostatin-A, glucagon-like peptide-1 (GLP-1), 10 % FBS, DMEM F12	10	• In this islet differentiation protocol "Trichostatin-A" was used as a key chromatin remodelling factor. • ILCCs obtained from both sources MSCs, confirmed the expression of pancreatic endocrine-related gene expressions such as *PDX-1*, *RFX-6*, *NEUROD1*, insulin, and c-peptide. Additionally, they secreted insulin and c-peptide both *in vitro* and *in vivo*. However, no difference between hBMSCs and hADSCs was observed in the context of islet differentiation potential.	(Gabr *et al.* 2017)
15	Human Bone Marrow-derived Mesenchymal Stem Cell (hBMSCs) and Human Adipose tissue derived Mesenchymal Stem Cells (hADSCs)	Retinoic acid, DMEM-LG, N2 & B27 supplements, EGF, activin A, exendin-4, nicotinamide	12	• ILCCs obtained from hBMSCs demonstrated high expression of genes like *PDX-1*, *NGN-3*, and Insulin as compared to ILCCs derived from hADSCs. • Moreover, ILCCs derived from both hBMSCs and ADSCs exhibited insulin secretion *in vitro*. However, significantly high insulin secretion was observed in hBMSCs-derived ILLCs as compare to hADSCs-derived ILCCs, suggested that hBMSCs have a superior stem cell source for pancreatic islet differentiation than hADSCs.	(Marappagounder *et al.* 2013)

16	Human foetal pancreatic progenitor cells	Nicotinamide, all-trans retinoic acid, activin-A and glucagon-like peptide-1 (GLP)	28	• ILCCs generated using suspension culture showed higher insulin expression and secretion as compare to ILCCs produced by non-suspension culture. • The differentiated ILCCs expressed almost all vital genes related to pancreatic islets such as insulin, glucagon, glucose transporters 1 & 2 along with voltage-dependent calcium channel (VDCC). Further, ILCCs maintained euglycemia in diabetic nude mice after transplantation.	(Zhang *et al.* 2013)
17	Human Adipose-derived Mesenchymal Stem Cells (hADSCs)	1% BSA, ITS, activin A, Sodium butyrate, β-mercaptoethanol, Fibroblast Growth Factors (FGF), taurine, glucagon-like peptide 1, nicotinamide, 1% non-essential amino acid.	14	• hADSCs-derived ILLCs showed gene expression of definitive endoderm, pancreatic endocrine markers such as S*OX 17, HNF3β* and *PDX-1, NGN-3, NEUROD1* respectively by qPCR. • Differentiated ILCCs demonstrated *in vitro* and *in vivo* functionality (using macro-capsule).	(Chandra *et al.* 2011)
18	Human Adipose-derived Mesenchymal Stem Cells (hADSCs)	Nicotinamide, activin-A, exendin-4, Hepatocyte Growth Factor (HGF), Pentagastrin, N2 & B27 Supplement	3	• hADSCs were triggered to differentiate into a pancreatic endocrine linage with a short period (3 days) by specialized culture medium. • hADSC derived ILCCs showed gene expression of NGN-3, and islet hormones including insulin, glucagon.	(Timper *et al.* 2006)

19	Human Umbilical Cord Blood-derived Mesenchymal Stem Cells	Nicotinamide, Epidermal Growth Factor (EGF), exendin-4, retinoic acid	15	• Islet-like cell clusters expressed pancreatic islet marker *PDX-1*, *NGN-3*, Insulin, glucagon, *GLUT-2* within 9 days of islet differentiation. • Approximately 25% of cells from ILCCs showed insulin expression. However, differentiated ILCCs didn't release insulin in response to various glucose challenges.	(Gao *et al.* 2008)
20	Human Umbilical Cord Blood-derived Stem Cells	Nicotinamide, ITS, progesterone, putrescine, laminin, fibronectin, N2 and B27 supplements	7	• ILCCs co-expressed insulin and c-peptide after 7 days of islet differentiation • Western blot results demonstrated that high protein expression of c-peptide and low protein expression of OCT4 in differentiated ILCCs.	(Sun *et al.* 2007)
21	Human Amino-derived Mesenchymal Stem Cells (hAMSCs)	ITS, taurine, nicotinamide, GLP-1	10	• Differentiated ILCCs derived from hAMSCs exhibited genes and protein expression of NGN-3, insulin, glucagon, somatostatin by qPCR, immunocytochemistry respectively. • The encapsulation of the ILCCs in macrocapsules (PU-PVP) and further its transplantation in the diabetic mice model lead to the restoration of normoglycemia.	(Kadam *et al.* 2010)
22	Human Dental pulp stromal Cells	ITS, activin-A, sodium butyrate, β-mercaptoethanol, BSA, taurine, Glucagon-like peptide-1(GLP-1), nicotinamide, non-	10	• Day 10^{th} differentiated ILCCS were proved as pancreatic islets by gene expression of *PDX-1*, *NGN-3*, *PAX-6/4*, c-peptide along with DTZ positive staining.	(Govindasamy *et al.* 2011)

		essential amino-acids (NEAAs), DMEM-KO		• Moreover, *in vitro* functionality was confirmed by insulin and C-peptide release assay in day 10 differentiated ILCCs.	
23	Human Postnatal Dental Pulp Stem Cells (DPSCs) & Stem Cells from Human Exfoliated Deciduous teeth (SHED)	ITS, activin-A, sodium butyrate, glucagon-like peptide-1 (GLP-1), nicotinamide, taurine, BSA, DMEM-KO	10	• SHED generated more ILCCs as compared with the DPSCs source. • STZ diabetic mice transplanted with encapsulation devices including SHEM-derived ILCCs were restored to normoglycemia more than 60 days without the use of immunosuppression drugs.	(Kanafi *et al.* 2013)
24	Mesenchymal Stem Cells (MSC) in Wharton's Jelly of Human Umbilical Cord	Nicotinamide, B27 Supplement, 2% FBS, Stem Cell Conditioned Medium (SCM)	28	• Transdifferentiated cells formed 3D islet-like cell clusters (aggregate) at the end-stage of islet differentiation. Further, they remarkably expressed endocrine pancreatic genes such as *PDX-1, NKX2.2, NKX6.1, GLUT2*, and insulin. • HUMSCs derived ILCCs produced and secreted insulin *in vitro* and *in vivo*.	(Chao *et al.* 2008)