INTRODUCTION & REVIEW OF LITERATURE

CHAPTER 1

1. Introduction and Review of Literature

1.1. Diabetes Mellitus

Ancient Egyptians first recognized the diabetic condition and it was later named as diabetes mellitus (DM) which comes from Latin and Greek as 'to run through (a siphon)' and 'honey', which corresponds to polyuria and glycosuria respectively. DM is a collection of metabolic disorders characterized by hyperglycaemia resulting from defects in insulin secretion, action or both owing to disturbance in carbohydrate, fat and protein metabolism, which in chronic condition results in complication of eyes, kidneys, nerves, blood vessels and heart (Karamanou et al., 2016). Current statistics project that nearly 385 million or 8.3% of adults worldwide have diabetes, which may ascend to 592 million or one in every ten adults by 2035. India covers 86% of the South-East Asia region. Statistically, India is severely afflicted by DM comprising, one fifth of all diabetic adults in the world which is about 65.1 million people in India. This number is estimated to increase to 109 million by 2035 which is 10.1% adult Indian population (Fig 1.1). Apart from this about 50% cases of DM remain undiagnosed which has created an alarming situation for a dynamically developing nation like India (Guariguata et al., 2014).

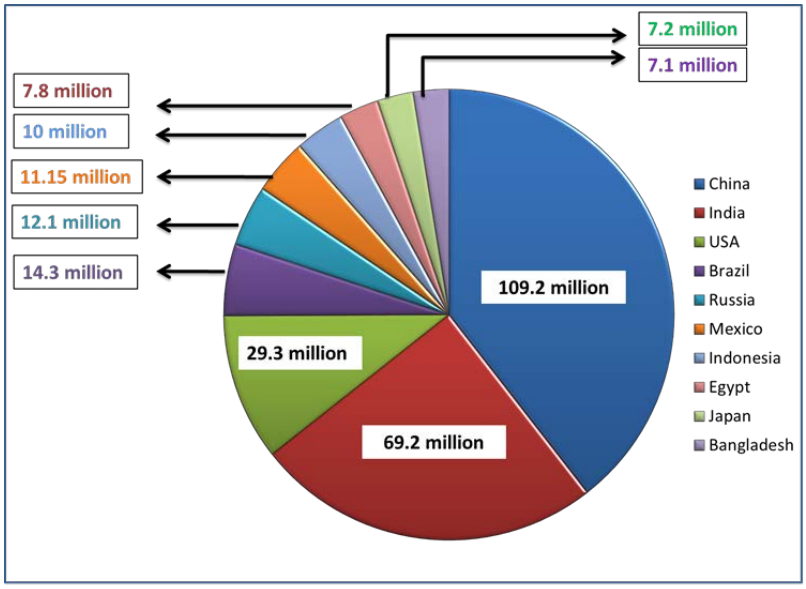


Figure 1: Worldwide epidemiological survey on diabetes as per the IDF -2015.

DM is diagnosed by hyperglycaemia along with thirst, weight loss, polyuria, glycosuria etc., blood glucose levels of 11.1 mmol/l (200 m/dl) at random or 7.0 mmol/l (126 mg/dl) at Fasting confirms DM. Also, an oral glucose tolerance test (OGTT) by drinking 75 g glucose load can diagnose DM

accurately where the 2 h value establishes DM with levels of 11.1 mmol/l (200mg/dl) (Consultation, 1999).

1.2. Types of Diabetes Mellitus

DM is broadly divided into two main types; Type 1 DM and Type 2 DM. Type 1 DM or Insulin dependent DM (IDDM) usually has juvenile onset, characterised by absolute insulin deficiency due to beta cell death. Type 1 DM has the tendency to develop ketosis and ketoacidosis with absolute need to administer insulin. The prime cause for this is autoimmune destruction of beta cells. The onset of this condition is very strongly linked to the genes encoding the human leukocyte antigen (HLA) region within the major histocompatibility complex (MHC) which is now recognized as IDDM1. Mutations in HLA DR haplotypes have been found in for more than 90% patients with Type 1 DM. The insulin gene (*INS*) is the only non-HLA gene has been recognized and associated with type 1 DM which is now designated as IDDM2. Apart from the genetic predisposition the most likely environmental factor that can cause type 1 DM is viral infections. Viruses including mumps, retroviruses, cytomegalovirus, Coxsackie B, Epstein-Barr virus and rubella are associated with type 1 DM. Infection by these can result in expression of class II MHC antigens by the pancreatic beta cells which are recognised by the T-lymphocytes and upon activation, produce cytokines like interleukin (IL)-2, interferon (IFN)-γ and tumour necrotic factor (TNF)-α generating specific T cells specific for the antigens against which the B lymphocytes can produce antibodies are directed against the beta cells resulting in their permanent destruction (Rabinowe and Eisenbarth, 1984; Scobie, 2006).

Type 2 DM or non-insulin-dependent diabetes mellitus (NIDDM) is recognized as a life style disorder and is associated with obesity. Here just like type 1 the main concern is hyperglycaemia but unlike type 1 the manifestation of Type 2 is a result of peripheral insulin resistance and relative insulin deficiency. Incidence of Type 2 diabetes has escalated

worldwide and is associated with increasing urbanisation and economic development resulting in dietary changes and reduced physical work ultimately leading to increase in sedentary lifestyle. India being an emerging economy has observed an alarming increase in the Type 2 DM patients over the last decade. Type 2 DM is characterised by an early incidence of insulin resistance i.e. a defect in insulin action causing hyperglycaemia. Persistent hyperglycaemia can lead to overt diabetes where the beta cells fail to increase their insulin output with respect to the increased blood glucose which then cause beta cell death due to overstrain.

Defect in insulin action can be due to down regulation of insulin receptor and/or problems with binding or post binding of insulin to its receptor which results in decreased glucose transport, its phosphorylation and impaired glycogen synthase activity. Further, insulin release is biphasic; the first phase is the release of insulin already stored in the beta cells in response to the glucose whereas the second phase is the insulin that is synthesized and late released in response to the elevated blood glucose. In Type 2 DM, a diminished first phase and lack of second phase of insulin release is observed. Chronic Type 2 DM patients are at risk of developing serious macro and micro vascular complications (Association, 2003; Scobie, 2006).

Gestational diabetes mellitus is characterised with any level of glucose intolerance during pregnancy. Such individuals are at risk to develop Type 2 DM in future. There are patients that mimic Type 2 diabetic conditions but are rather diagnosed as having a late-onset of immune mediated type 1 diabetes, also known as latent autoimmune diabetes in adults (LADA). Further, there are a collection of genes responsible for beta cell homeostasis, any monogenic defect in such genes may lead to maturity-onset diabetes of the young (MODY). Type 2I diabetes is the characterised with the effect of insulin resistance on brain. There are people with blood glucose levels higher than normal but not as high in diabetes and people

with above normal blood glucose even after a period of fasting. Such people are said to have impaired glucose tolerance (IGT) & impaired fasting glucose (IFG) respectively and are highly susceptible to become diabetic (Borch-Johnsen et al., 1999; Scobie, 2006; Zimmet et al., 1994).

1.3. Prevention of Diabetes Mellitus

Preventive strategies for type 1 DM may include: (i) recognizing and eradicating environmental triggers; (ii) promoting environmental protective factors and (iii) blocking immunological processes causing beta cell death by administering immunosuppressive agents. Preventive strategies for Type 2 DM is broadly related in managing obesity. Any lifestyle modification or pharmaceutical intervention that improves insulin sensitivity and beta cell function can have positive implications in managing Type 2 DM condition. Several studies on people with IGT in China, Finland and United States have provided evidence that lifestyle changes with regular physical activity can reduce the prevalence of Type 2 DM by as much as 58% whereas when they were treated with Metformin, a known insulin sensitizer, it only reduced to 31% (Atkinson and Eisenbarth, 2001; Scobie, 2006; Tuomilehto et al., 2001; Zimmet, 1995).

1.4. Treatment of Diabetes Mellitus

Maintaining a strict diet which is high in complex carbohydrates and proteins with less fats and cholesterols are recommended along with regular exercise becomes effective management of both type 1 and 2 diabetic conditions. An inverse relation of exercise with mortality and morbidity in DM patients along with decrease in insulin doses has been observed. Insulin administration is the only way to manage type 1 diabetes where fast acting and/or slow acting insulin is administered once or twice in a day depending upon the need to manage the hyperglycaemic condition, especially the postprandial high glucose. This along with continuous monitoring of their blood glucose levels using devices like insulin pumps makes it possible for a type 1 diabetic to lead a normal life. However, Type 2 DM is a

therapeutic challenge which requires a combination of drugs, physical activity and diet control for effective management (Association, 2000; Tuomilehto et al., 2001).

Five classes of hypoglycaemic drugs are used to manage the Type 2 DM condition. (i) Sulfonylureas stimulate insulin secretion and make beta cells more sensitive to glucose. There are many drugs in the market e.g. tolbutamide with half-life 3-8 hrs., chlorpropamide with half-life 35 hr. (ii) Metformin is the most popular example of Biguanides that reduces blood glucose by inhibiting hepatic glucose production and elevating peripheral insulin sensitivity. (iii) The absorption of complex carbohydrates from the gastrointestinal (GI) tract are delayed when Glucosidase inhibitors are used and they also help in controlling postprandial high glucose levels. However, metformin and other sulfonylureas are better in lowering blood glucose levels. (iv) Meglitinides produce short insulin bursts in response to glucose which helps restore normal blood glucose in Type 2 DM without causing hypoglycaemia and (v) Thiazolidinediones activate peroxisome proliferator-activated receptor-gamma (PPAR-γ), present in adipose, skeletal muscle and liver tissues. This increases insulin sensitivity by reducing hepatic glucose production and increasing peripheral glucose uptake. They increase uptake of fatty acid into adipocytes thus lowering circulating free fatty acids and triglycerides. They also regulate adipogenesis through PPAR-γ. Thus, improving the glucose metabolism (Gerich, 1989; Marshall, 2003). Apart from these five classes there are two important types which have recently come into the paradigm of diabetes therapeutics. The first of these is glucagon-like peptide-1 (GLP-1) receptor agonist which improve glycaemic control and decrease the risk of hypoglycaemia along with clinically relevant weight loss. Basically GLP-1 increases glucose dependant insulin secretion, decreases glucagon secretion, slows gastric emptying and increase satiety. Further GLP-1 gets degraded by a protease called dipeptidyl peptidase-4 (DPP-4). Hence, DPP-4 resistant mimetics of GLP-1 or DPP-4 inhibitors that increase the activity of endogenous GLP-1 have

been administered for diabetes therapy. Some of the GLP-1 agonists that have been introduced are liraglutide, albiglutide, exenatide, dulaglutide etc. and DPP-4 inhibitors such as sitagliptin, saxagliptin, vildagliptin etc. (Kalra et al., 2016). The second class of drugs are designed to block renal sodium-glucose co-transporter 2 (SGLT-2) that induces glucose excretion and thereby lowering blood glucose levels. The two important SGLT-2 inhibitors that have been approved are canagliflozin and dapagliflozin (Chao, 2014). Finally, insulin is administered in case the condition remains unmanageable even after using the above drugs. This happens when beta cells reserve fall below 15% which occurs in chronic Type 2 condition (Yki-Järvinen, 2001). Another approach in treating diabetes is cell replacement and regeneration based therapies. These have become a hope for every diabetic patient for providing a cure instead of better solutions for managing the diabetic condition.

1.4.1. Whole-pancreas transplantation

Whole-pancreas transplantation was performed worldwide before the advent of islet transplantation. However majority of these were simultaneous pancreas-kidney (SPK) transplantations. The results with SPK patients for survival and functional grafts with complete independence from insulin was 94% and 89% respectively at one year and 81% and 67% respectively at 5 years. Nevertheless pancreas transplantation alone (PTA) remained inferior to SPK. SPK becomes imperative with diabetic patients with advance renal failure. The limitation of this treatment is that the patients remain immunosuppressed throughout their life that makes them susceptible to other pathogens. Also, shortage of donor organs for transplantation has always been a clinical concern but advent and success of islet transplantation has obviated the need for SPK or PTA transplantation thereby improving diabetic therapy (Robertson et al., 2000; Sutherland et al., 2001a; Sutherland et al., 2001b).

1.4.2. Islet transplantation

The first insulin independence achieved after islet transplantation was in 1989 by Dr. Camillio Ricardi at the University of Pittsburgh with insulin independence rate of 50% at one

year but this could not be replicated in type 1 DM patients. It was the Edmonton protocol designed by Dr. James Shapiro that infused life and realistic hope for an insulin free life post islet transplantation for the type 1 DM patients. The protocol was unique as it was designed to transplant 13000 islet equivalents/kg of recipient body weight along with a new immunosuppressive strategy that included a combination of combined sirolimus, tacrolimus and anti-CD25 antibody against rejection and recurrent autoimmunity. The islets were isolated from two or three donors and transplanted into the liver into the main portal vein. With this Shapiro and co-workers in Edmonton achieved insulin independence in seven consecutive patients which increased to more than 85 patients with one year insulin independence rate at more than 80%. This protocol was replicated by many other groups across the world. With the success of Edmonton protocol, the islet transplantation therapy has come a long way but it still has to overcome many hurdles before it can be beneficial for the masses (Robertson, 2004; Shapiro et al., 2000; Shapiro et al., 2017).

1.4.3. Limitations of Islet transplantation

Islets comprise of about 1-2% of the whole pancreatic mass and there are approximately one million islets in each pancreas. The process of isolation of islets from pancreas involved mechanical and chemical damage that inflicts damage to about 50% of the islets. Further it is estimated that as much as 70% transplanted islets undergo cell death due to the host immune response. Normally two batches of half a million islets are transplanted per patient, which means two cadaveric pancreata are required per transplant. This indicates an alarming shortage of transplantable islets and a need for better immunomodulation post transplantation. Hence, alternative sources to generate transplantable islets have become imperative. Various stem cells viz. embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), various mesenchymal stem cells (MSCs) and pancreatic progenitors have been researched for generating new islets as source for islet replacement therapy. Along with stem cell sources

potent extrinsic factors that can effectively stimulate islet neogenesis becomes imperative. In order to better understand islet differentiation it is first important to understand the regulatory machinery and key factors involved (Bruni et al., 2014; Rother and Harlan, 2004; Shapiro et al., 2017).

1.5. Pancreas

Pancreas, a glandular organ is situated in the GI tract. Its function is to secrete both digestive enzymes from the exocrine tissue (95-99%) and hormones from endocrine part (1-2%) of the pancreas (Beger et al., 2009).

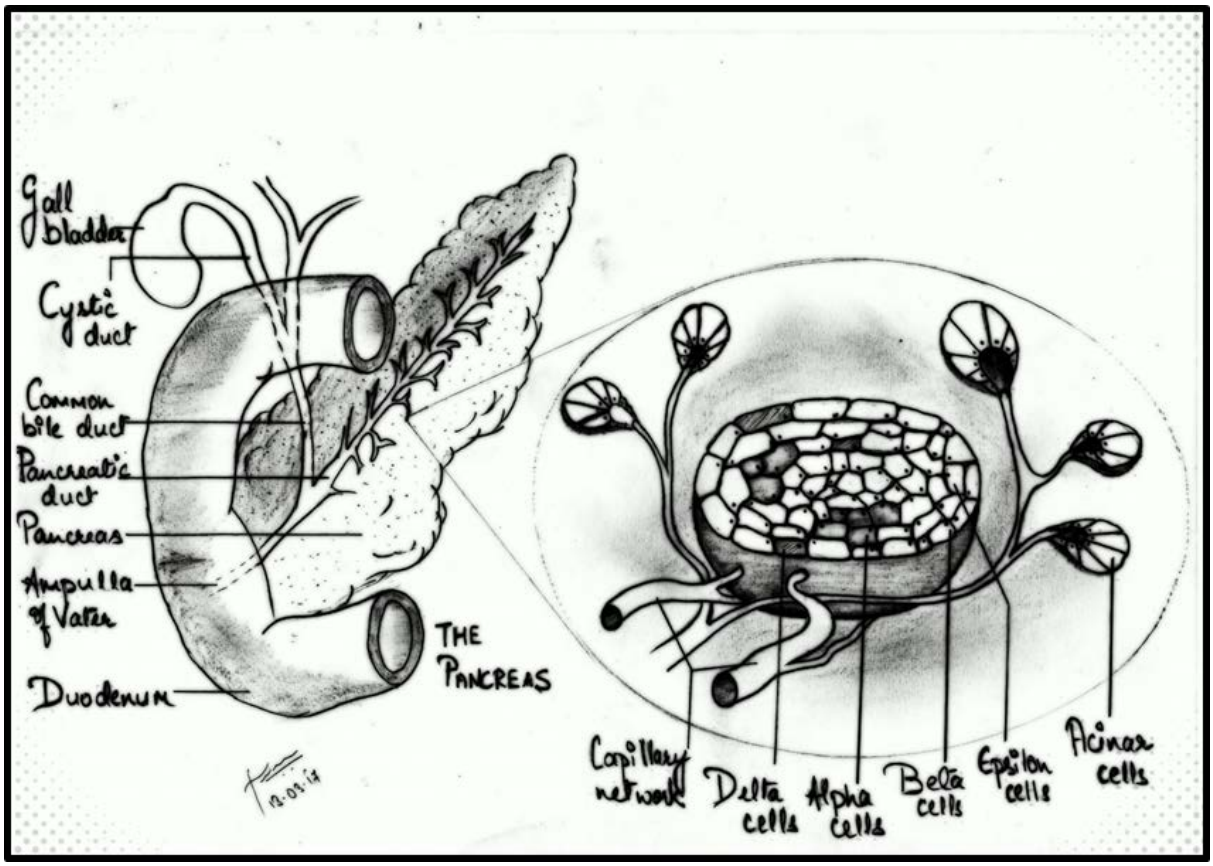


Figure 1.2: Structure of Pancreas and Islet of Langerhans: The figure depicts the gross anatomy and organisation of the pancreas along with architecture of the islet of Langerhans.

It is irregularly shaped and roughly divided into a broad head, body and a narrow tail region. It is located below the stomach, diagonally across the posterior wall of the abdomen, connected to the small intestine at the duodenum (Beger et al., 2009). The endocrine part of

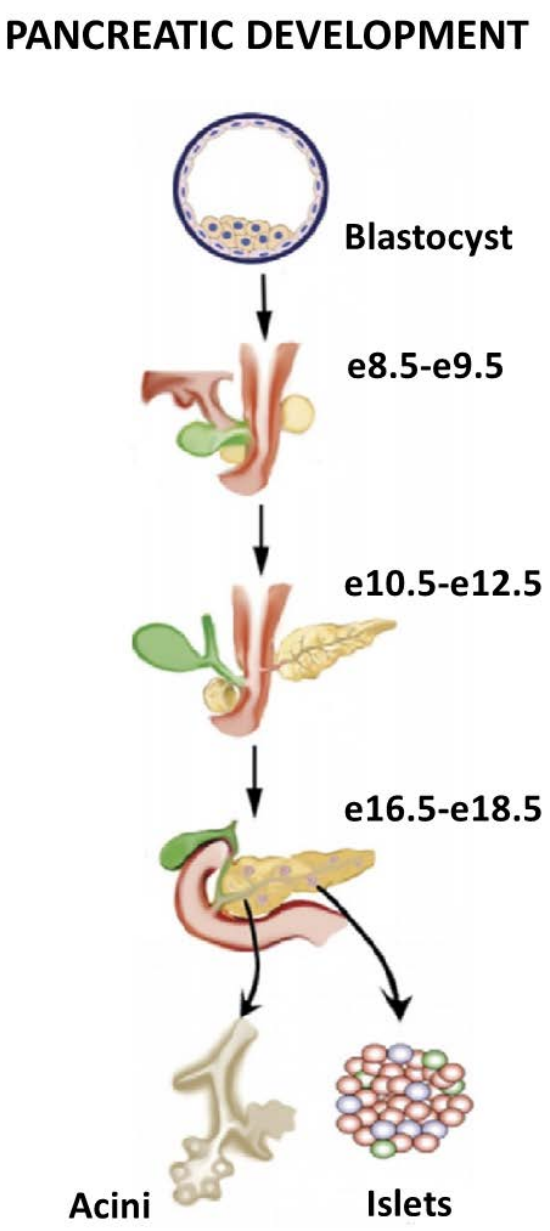
the pancreas comprises of cell clusters termed as Islets of Langerhans coined after the German biologist Paul Langerhans in 1869 (Morrison, 1937). In all the species studied islets comprise of five types of cells namely, first insulin producing beta cells, the most abundant cell type (~70-80%); secondly glucagon producing alpha cells (~20-30%); rest of the three types are scarcely present, they are somatostatin producing delta cells; pancreatic polypeptide producing PP cells and ghrelin producing epsilon cells. Although, the cells present in the islets across species might be the same their cytoarchitecture varies profoundly e.g. in humans & lower primates unlike murine islets where alpha cells are found on the periphery, they are scattered throughout the islets (Fig 1.2) (Joslin and Kahn, 2005).

1.6. Pancreatic development and Islet differentiation

The basic roadmap for pancreatic development consists of four major molecular events which are (i) generation of definitive endoderm/gut epithelium; (ii) pancreatic differentiation; (iii) endocrine specification; and (iv) beta cell differentiation. The most important molecular players are highly conserved between mouse and human. Initiation of pancreatic development, evagination from the primitive foregut along with *Pdx1* expression is observed between e8.5-e9.5 in mouse where as in humans its observed at 3-4 weeks post conception (w.p.c); glucagon expression in the developing pancreas can be observed at e9.5-e10.5 in mice and around 8.5 w.p.c in humans; insulin expression can be observed at e10.5-e11.5 in mice and at 7 w.p.c. in humans. Further, the two pancreatic buds fuse at the base at e12.5 in mice and 8w.p.c. in humans. Formation of acini from ducts and initiation of secondary transition along with somatostatin expression begins at e13.5-e14.5 onwards in mice and 8.5 w.p.c. onwards in humans. Pancreatic polypeptide is expressed at e16.5-e18.5 in mice and 10w.p.c. in humans. Finally, islets formation begins at e18.5 onwards in mice and 12-13 w.p.c. in humans (Fig 1.3). *In vitro* islet differentiation of various stem cells can be carried

out by triggering sequential transcription factors that are responsible in steering the differentiation process as desired (Falin, 1967; Kim and Hebrok, 2001; Slack, 1995).

1.7. Regulators for Pancreatic development and islet differentiation



1.7.1. FOXA2 (HNF3beta)

The winged-helix transcription factor FOXA2 (also known as hepatocyte nuclear factor 3beta [HNF3beta]) is a marker of definitive endoderm. It is an essential marker in islet differentiation from extra pancreatic sources to understand if the differentiation has entered the endoderm lineage (Weinstein et al., 1994). FOXA2 regulate *Pdx1* in beta cells and maintain insulin secretion (Lee et al., 2002). Mice where FOXA2 has been selectively inactivated die from hypoglycaemia as there is significant (>90%) loss of alpha cells but not in their specification (Lee et al., 2005).

Figure 1.3: Developmental pathway for the formation of acini and islets from embryo in mouse (Joglekar et al., 2007).

1.7.2. REG

Research with respect to regeneration of endocrine pancreas and the genes association with it originated the Reg terminology. It is synonymous with Islet Neogenesis associated protein (INGAP). This protein belongs to the Reg family and is also termed as REG3a. REG protein is mitogenic in both islet and ductal cell lines. It is associated with regenerating pancreas. As

the name suggests INGAP triggers islet neogenesis from stem cells (Fleming and Rosenberg, 2007).

1.7.3. PDX1

The pancreatic and duodenal homeobox 1 gene has synonyms such as insulin promoter factor 1 (Ipf1) or islet/duodenum homeobox 1 (IDX1). PDX1 is the pancreatic determination gene which when expressed in the foregut endoderm at e8.5 or 10 somite stage induces the formation of the pancreas and duodenum (Jonsson et al., 2003). PDX1 expression is essential for beta cell differentiation (Ahlgren et al., 1998). It also regulates the expression of insulin gene by binding to its promoter (Ohlsson et al., 1993). Pancreatic agenesis is observed in *Pdx1* knockout mice and point mutations in humans (Johnson et al., 2003). It is known that PDX1 acts in association with other proteins and is essential for beta cell homeostasis (Li et al., 2005b).

1.7.4. NEUROG3

Neurogenin 3 (NEUROG3) is a master key regulator for pancreatic endocrine differentiation as *Neurog3* knockout mice are without islets (Gradwohl et al., 2000). NEUROG3 has been found to be present in pancreatic endocrine progenitors and has been targeted extensively for inducing islet differentiation from various stem cells (Rukstalis and Habener, 2009). Neurog3 is used as a checkpoint marker in all the islet differentiation studies from various stem cells to determine the entry of cells into pancreatic endocrine lineage (Serafimidis et al., 2008).

1.7.5. BETA2/NEUROD

The beta-cell E-box transactivator 2 (BETA2, also known as NEUROD) is a part of binding complex on insulin gene (Naya et al., 1995). *NeuroD* knockout impaired islet morphogenesis after e15.5 suggesting its role at a later stage in development (Naya et al., 1997). NEUROD is needed to direct the endocrine lineage cells towards their respective terminal differentiation (Huang et al., 2000).

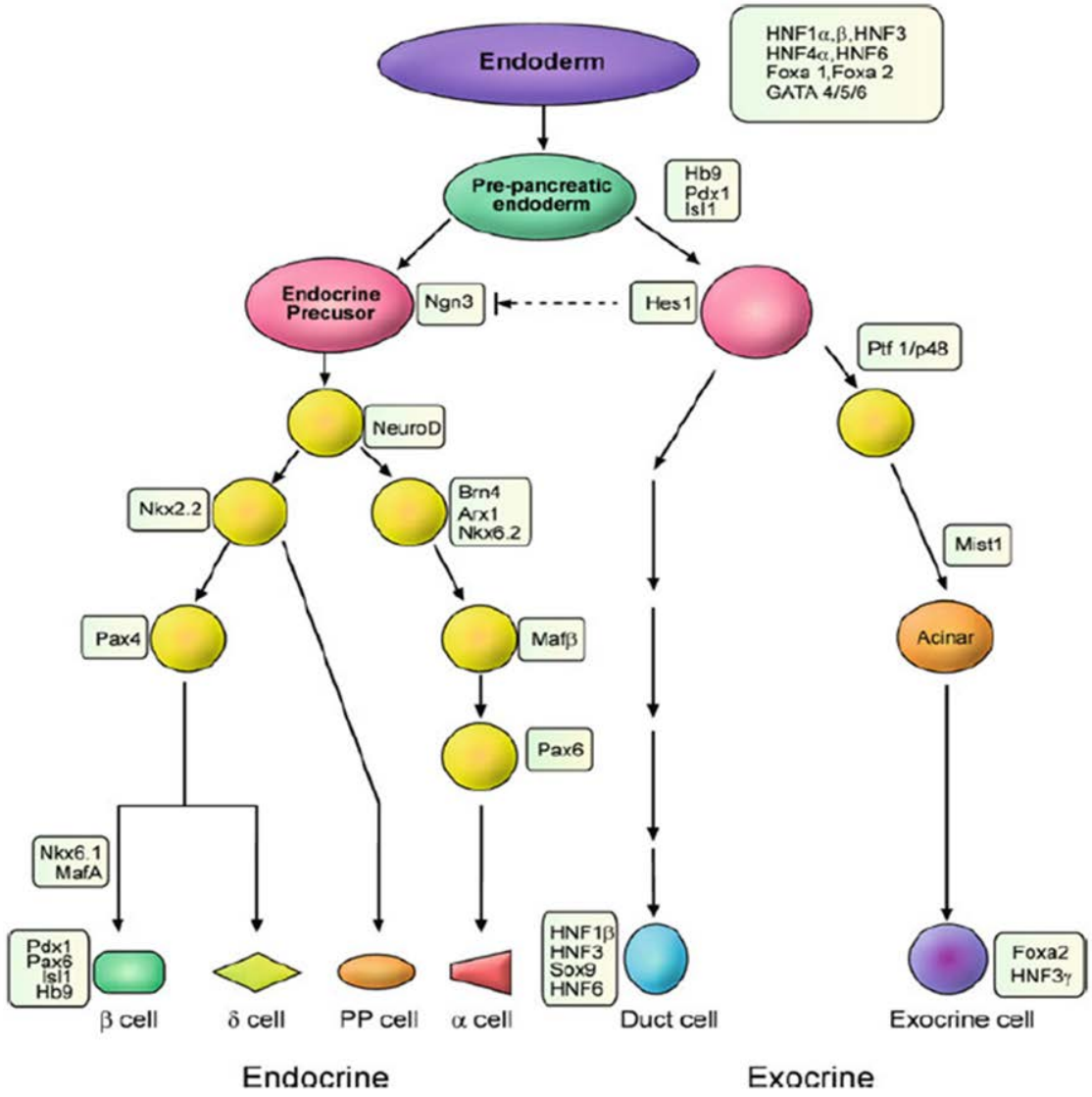


Figure 1.4: Temporal expression of candidate transcription factors involved in the differentiation of pancreatic cells viz. beta cells, delta cells, PP cells, alpha cells, ductal cells and so on from endoderm (Samson and Chan, 2006).

1.7.6. NKX6.1/NKX2.2

Nkx6.1, a member of NK homeodomain family is present both in the CNS and the pancreas. Nkx6.1 co-localizes with either PDX1 or NEUROG3 in the pancreatic development. Nkx6.1 is a beta cell marker and Nkx6.1 knockout displays significant deficiency of beta cells (Sander et al., 2000). *Nkx2.2* begins to express at e8.5 but later on gets restricted to specific pancreatic cells, which suggests that it might be involved in the terminal differentiation of beta cells (Sussel et al., 1998).

1.7.7. PAX4

Paired box-containing gene 4 (PAX4) has a homeodomain, first observed at e9.5 in the embryonic development. *Pax4* knockout mice lack both beta and delta cells whereas the number of alpha cells compensate for their absence. This suggests that PAX4 is specific and essential to enter the beta cell lineage (Sosa-Pineda, 2004; Sosa-Pineda et al., 1997).

1.7.8. ARX

The aristaless-related homeobox gene (ARX) was first recognized in the mouse CNS and is expressed at e9.5. ARX expression in pancreas is restricted to the endocrine cells and its observed that *Arx* knockout mice lack alpha cells suggesting it's an exclusive terminally differentiated alpha cell marker. ARX promotes alpha cells and oppose beta cells whereas PAX4 promotes beta cells and oppose alpha cells hence their relative levels strict a balance in maintain their populace within the islets (Collombat et al., 2003).

1.7.9. MAFA/MAFB

MAFA is preferentially expressed in beta cells. *MafA* knockout mice develop diabetes. MAFA is a necessary transcription factor for beta cell homeostasis (Nishimura et al., 2006; Zhang et al., 2005a). MAFB is preferentially expressed in alpha cells. Further, the switch from MAFB to MAFA expression is essential for survival and function of beta cells during embryonic development (Nishimura et al., 2006). It has been demonstrated that MAFB is essential for maturation of beta cells by regulating PDX1, NKX6.1 and GLUT2 (Fig 1.4) (Artner et al., 2007).

1.8. Models of Pancreatic Regeneration

1.8.1. Partial Pancreatectomy

Partial Pancreatectomy (40–90%) i.e. surgical removal of a part of the organ remains a classical model for studying pancreatic regeneration. It has been observed that removal of 90% pancreas the mitotic index (MI) of both acinar cell and beta cells increase multiple folds. The MI of acinar cells slow down after a week but the MI of beta cells remains elevated for

up to three weeks suggesting pancreatic regeneration and recovery of its endocrine function. Here both replication of existing beta cells and islet neogenesis from ductal cells were observed (Bonner-Weir et al., 1983; Brockenbrough et al., 1988; Menge et al., 2008).

1.8.2. Duct Ligation

Since the advent of surgical procedures duct ligation is used to study pancreatic obstruction. Duct ligation creates a stressful environment for the pancreas and it is observed that key transcription factors like Pdx1 andNkx2.2 get upregulated during duct ligation facilitating islet neogenesis (Peters et al., 2005; Wang et al., 1995).

1.8.3. Cellophane Wrapping

Cellophane wrapping was developed by Rosenberg and colleagues as a gentler alternative to duct ligation in the early 1980s to study pancreatic duct epithelium hyperplasia associated with pancreatic carcinoma. In this hamster model the head of the pancreas is wrapped with sterile cellophane while avoiding crushing of the tissue or occlusion of the main duct. It was observed that following inflammation new islets had begun to form in 2 weeks with an increase in 2.5 fold islet mass in the pancreas thus reversing diabetic condition in more than 50% of Streptozotocin (STZ) treated animals. Later researchers have used crude preparations of cellophane wrapped pancreas to induce islet neogenesis. Further it was discovered that pancreatic islet neogenesis associated protein was a component of the above preparation and was identical to rat Reg protein, which was later identified in regenerating islets (Rafaeloff et al., 1997; Rosenberg et al., 1983; Rosenberg and Vinik, 1989).

1.8.4. Streptozotocin Treatment

STZ is a toxic glucosamine–nitrosourea compound, which uses GLUT-2 as a transporter to gain entry into the beta cells of the islets of Langerhans. STZ is an alkylating agent that causes DNA damage specifically to the beta cells causing cell death. DNA damage within the beta cells activates Poly (ADP-ribose) (PAR) Polymerase1 (PARP-1) enzyme to use high

energy molecule NAD as its substrate to form PAR chains which then recruit various DNA repair enzymes. If the DNA damage persists, there is more and PARP-1 activation which utilises more and more NAD causing energy depletion within the beta cells which forces the beta cells into apoptosis. STZ can be used in low multiple doses or a single high dose to generate a diabetic animal models (Schnedl et al., 1994). Alloxan is another chemical molecule that operates similarly with different mechanisms. It enters through GLUT2 transporter as its structure is very similar to glucose and it inhibits glucokinase which decreases ATP to ADP ration thereby abating insulin secretion (Lenzen, 2008). These molecules have been popularly used to induce diabetes in experimental animal models. The STZ model reduces beta cell number and causes local inflammation which are both necessary to induce regeneration in pancreas. Many researchers have observed the presence of pancreatic progenitors and islet neogenesis after STZ induction in murine animal models (Pasquali et al., 2006; Pasquali et al., 2008).

1.9. Origin of Islets within the pancreas?

All animals have healing and regenerating capabilities which varies with tissues type 1n different animals. This regenerative capacity within animals could be due to certain potent stem cells or adult tissue specific progenitors that gets triggered and replenish the damaged tissue or it could be due to division of already existing terminally differentiated population (Tanaka and Reddien, 2011). Hence, regardless of the various islet regeneration models the origin of the newly created islets or the pancreatic progenitor source or their exact characteristics has been a highly controversial topic in the field of islet biology.

The mechanisms for islet regeneration include reversible epithelial to mesenchymal transition of existing ductal or acinar cells that may dedifferentiate and redifferentiate into insulin producing beta cells upon insult to pancreas, self-duplication of existing beta cells or awakening of the embryonic developmental program in the existing endocrine pancreatic

cells (Dor et al., 2004; Gershengorn et al., 2004; Xu et al., 2008). Hence, as per the literature pancreatic progenitors can have either acinar, ductal and/or islet source with the pancreatic tissue (Fig 1.5).

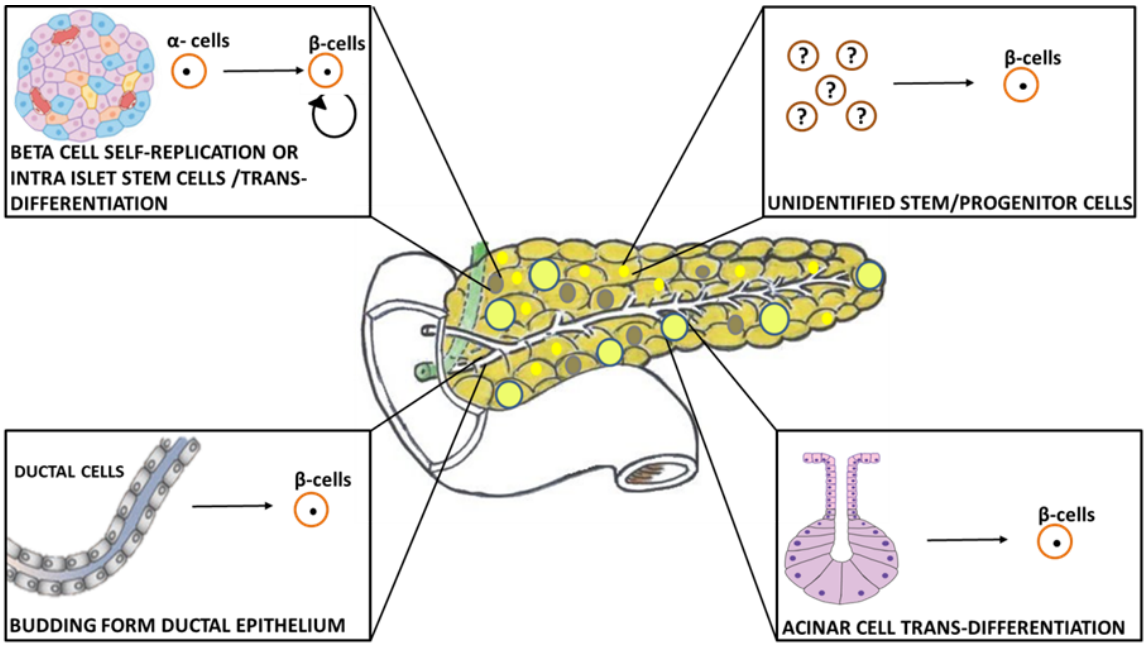


Figure 1.5: Origin of Pancreatic progenitors: Endogenous stem cell sources in different anatomical locations in the pancreas that have the characteristics to replenish beta cells.

1.9.1. Pancreatic progenitors of acinar origin

Genetic lineage-tracing studies have yielded conflicting results with acinar specific promoters. In one such study when a knockin tamoxifen inducible *Ptf1a*$_{\text{CreER}}$ line was used to trace acinar cells in pancreatic duct ligation and duct ligation along with STZ treatment (Pan et al., 2013). It was observed that *Ptf1a*+ positive acinar cells dedifferentiated and gained progenitor characteristics capable to differentiate into both ductal and endocrine mature beta cells at low efficiency. However, in another lineage tracing study of mice models with *Elastase*$_{\text{CreER}}$ in partial pancreatectomy, duct ligation and caerulein-induced pancreatitis demonstrated no acinar to beta cell differentiation (Desai et al., 2007). The current research indicates that the acinar progenitor cells might be conditional with limited intrinsic plasticity. Nevertheless, they can be programmed into assuming endocrine beta cells by introducing key

genes or triggered into differentiating beta cells using growth factors such as Epidermal growth factor (EGF) and Ciliary neurotrophic factor (CNTF) which has been demonstrated to generate insulin producing beta cells in hyperglycaemic diabetic mice by activating Neurog3 through STAT3 signaling cascade (Baeyens et al., 2014). The evidence for acinar progenitor population becomes more robust in vitro where these cells readily acquire progenitor like phenotype differentiating into insulin producing cells upon appropriate induction with growth factors (Baeyens et al., 2005; Minami et al., 2005; Minami and Seino, 2008). However, the acinar progenitor's existence and their role in the pancreatic endocrine regeneration is still a matter of debate.

1.9.2. Pancreatic progenitors of ductal origin

The most acceptable belief in islet biology is that the pancreatic progenitors have a ductal origin. It was Bensley who first reported that small endocrine cell clusters bud from the ducts in the beginning of the 20th century (Bensley and dquo, 1911). Several studies have demonstrated that ductal cells have multipotent progenitor markers and can differentiate into insulin producing islet cell clusters upon stimulation with specific growth factors and culture conditions or after engrafting into kidney capsule (Boj et al., 2015; Huch et al., 2013; Jin et al., 2013; Lee et al., 2013; Rovira et al., 2010; Seaberg et al., 2004). The idea of ductal progenitors become more vivid from the pancreatic development where both endocrine and exocrine cells originate from primitive duct like complexes (Kopinke et al., 2011; Kopinke and Murtaugh, 2010; Kopp et al., 2011b; Solar et al., 2009). It is the *Neurog3* upregulation in the progenitor pool that propels the differentiation towards endocrine lineage while the remaining duct-like complex downregulate *Pdx1* and differentiate into mature ductal tissue (Kopp et al., 2011a; Seymour, 2014). Further, it has been observed that in response to stress and injury pancreatic ductal cells acquire progenitor phenotype (Bonner-Weir et al., 1993; Li et al., 2010; Rovira et al., 2010). Also, recently a lineage tracing study using *Sox9*$_{CreER}$

confirmed that in response to specific culture condition including hyperglycaemia and long term low dose gastrin and EGF can differentiate ductal cells into insulin producing beta cells (Zhang et al., 2016).

1.9.3. Do Islets have their own pool of progenitors/Resident stem cells?

This has been the most controversial topic in the field of islet biology related to progenitors' origin. Till now there have been several reports suggesting existence of a rare intra-islet progenitor population (Seaberg et al., 2004; Smukler et al., 2011; Suzuki et al., 2004). There have been many studies demonstrating production of new beta cells within the islets along with progenitors existence within the damaged islets in the STZ animal model (Fernandes et al., 1997). These progenitors were positive for pdx1 and somatostatin. Similar cells were also observed in nonobese diabetic (NOD) mice model post islet destruction by autoimmune response (Ramiya et al., 2000). Later on Guz et al discovered Glut2+ and Ins+/somatostatin+ dual progenitor population within islets in STZ treated mice (Guz et al., 2001). Further, there is a hypothesis suggesting ductal progenitors migrate within islets to terminally differentiate into the endocrine cells which is the case during pancreatic development. To give support to this hypothesis recently, El-Gohary et al demonstrated that in partial pancreatectomy model intra-islet ductal cells can produce insulin producing beta cells (El-Gohary et al., 2016).

1.9.4. Involvement of other endocrine cell types to β-cell regeneration

The endocrine within the islets of Langerhans are closely associated and have a common developmental lineage which makes them a probable source for new beta cells (Gittes, 2009; Shih et al., 2013). The endocrine cells within the islets are in a very dynamic and flexible state which has been illustrated by many reports over the years demonstrating beta cells self-replication and differentiation of endocrine cells like alpha, delta and epsilon cells into insulin producing beta cells (Al-Hasani et al., 2013; Chera et al., 2014; Collombat and Mansouri, 2009; Collombat et al., 2009; Courtney et al., 2011; Thorel et al., 2010). A Pioneer work

from Herrera's group demonstrated that under the condition of absolute beta cell loss, alpha cells switch fate by dedifferentiating and redifferentiating into beta cells (Thorel et al., 2010).

1.10. Other Stem cell sources to produce insulin producing cells

Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocysts and are pluripotent in nature (Trounson, 2002). Induced pluripotent stem cells (iPSCs) are generated by inducing expression of Yamanaka factors in somatic cells (Takahashi and Yamanaka, 2006). Both ESCs and iPSCs can differentiate into any of the three germ layers and have been shown to differentiate into insulin producing islet clusters (Trounson, 2002). However, there have been many hurdles with respect to absence of several beta cell markers in the terminally differentiated islet clusters along with below average glucose responsiveness has made the differentiation challenging. Apart from this there has always been an issue of availability, teratoma formation and ethical conundrums related to ESCs (Soria et al., 2001). Their differentiation into beta cells is a four stage process. Stage one is the definitive endoderm formation; stage two is foregut endoderm formation; stage three is the pancreatic determination and the pancreatic progenitor stage and the final stage four is beta cell maturation stage (D'Amour et al., 2006; Yi and Liu, 2013). Further, differentiation of ESCs/iPSCs into functional pancreatic progenitors and their transplantation have provided a potential avenue for beta cell replacement therapy. Adult stem cells of the non-pancreatic origin have been shown to differentiate into insulin producing cells. Mesenchymal stem cells (MSCs) including bone marrow MSCs, umbilical cord blood MSCs, adipose derived MSCs etc. have been widely used to successfully differentiate into islet clusters (Van, 2011). We have summarized various stem cells and there islet differentiation in the table below.

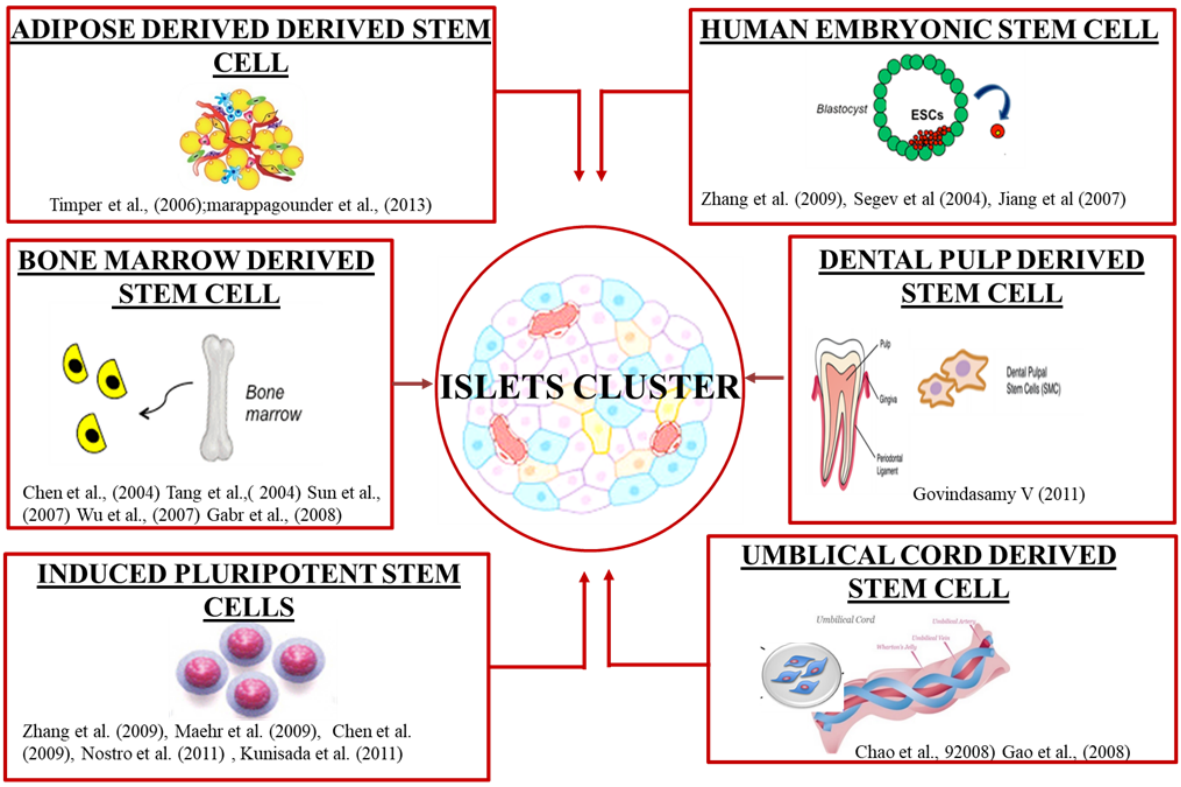


Figure 1.6: Various stem cell sources to generate islet clusters. Embryo, Adipose tissue, Bone marrow, Dental pulp, umbilical cord, adult differentiated cell, and so on had been reported to have the potential to differentiate into islet like cell clusters. Thus, MSCs serve as effective candidate for stem cell therapy for diabetes treatment.

1.11. Extrinsic factors used in generating insulin producing cells

1.11.1. Glucagon-Like Peptide-1:

Glucagon-Like Peptide-1 is an incretin hormone that is processed from proglucagon. Hui et al., in their study in 2001 suggested that GLP-1 is capable of stimulating pancreatic ductal cell lines into insulin producing cells by influencing PDX-1 gene (Hui et al., 2001). This was further augmented by Abraham et al., in their report which disclosed that GLP-1 had insulinotropic effect on the b-cells and it can stimulate b-cell growth and neogenesis from Nestin positive islet-derived progenitor cells by upregulating the expression of PDX-1 gene (Abraham et al., 2002).

1.11.2. Exendin 4:

Exendin-4 is a 39 amino acid protein, which is an agonist of GLP-1 with longer half-life (Eng et al., 1992). Exendin-4 has been used to produce insulin producing cells from various stem

cell sources viz. Wu et al., and Garb et al., demonstrated differentiation of Rat BMSCs into insulin producing cells (Gabr et al., 2008; Wu et al., 2007), Tang et al., used murine BMSCs (Tang et al., 2004b), while Li et al., in 2010 used mouse embryonic stem cells (Oliver et al., 1999). There is another report by Xu et al., which demonstrated pancreatic regeneration by stimulating beta cell proliferation and neogenesis in diabetic rats (Xu et al., 1999).

1.11.3. Fibroblast and Epidermal Growth factors:

Growth factors like Fibroblast growth factor (FGF) family and Epidermal growth factor (EGF) family is very important in cellular growth, differentiation and homeostasis (Herbst, 2004). Their function is mostly concentration dependent i.e. it is required in small concentration for growth of MSCs whereas in higher concentrations they can differentiate stem cells into insulin producing cells Keretinocyte growth factor (KGF), a member of the FGF family is known for pancreatic regeneration and ILC formation (Krakowski et al., 1999) and differentiation of pancreatic duct cells to beta cells by PI3K/AKT pathway (Uzan et al., 2009). In contrast, Dalvi et al, demonstrated that removal of EGF stimulated islet cluster formation (Dalvi et al., 2009). On the other hand, Betacellulin, a 32 kDa glycoprotein is a member of the EGF family (Sasada et al., 1993) and is highly expressed in pancreas and intestine. Several studies suggested the role of Betacellulin in beta cell differentiation (Mashima et al., 1996; Sun et al., 2007c; Zalzman et al., 2005) and β-cell regeneration in 90% pancreaticised rats (Li et al., 2001). There are other reports where Betacellulin has been reported to be often used in combination with Activin A for the production of Insulin producing cells (Mashima et al., 1996; Zalzman et al., 2005).

1.11.4. Activin A:

Activin A is a polypeptide molecule of the Transforming growth factor-β (TGF-β) super family. TGF-β ligands have strongly come into the picture with their role in beta cell homeostasis. Activin A has been extensively used to stimulate stem cells from bone marrow,

liver, pancreas etc. into insulin producing islets through ACT-MAPK-TKK signaling pathway (Brown and Schneyer).

1.11.5. Hepatocyte Growth Factor (HGF):

HGF stimulates cell growth, motility and morphogenesis and is secreted by mesenchymal cells (Zarnegar and Michalopoulos, 1995). HGF has been used as an extrinsic factor to generate IPCs from a variety of cells. It has also been used to differentiate AR42J cell line into IPCs in combination with Activin A (Mashima et al., 1996).

1.11.6. Gastrin:

It is a hormone produced by G cells of the antral part of gastric mucosa and small amounts by the duodenum and the pancreas. In a study, Wang et al, demonstrated that it stimulates islet differentiation of TGF-α induced ductular precursor cells (Wang et al., 1993). Whereas, Tamaki et al., reported that combination treatment of exendin-4 and gastrin preserves beta cell mass by stimulating beta cell growth and differentiation in db/db mice (Tamaki et al.).

1.11.7. Nicotinamide:

Nicotinamide is also called niacinamide or nicotinic acid amide. In 1990s Otonkoski et al. had used nicotinamide as an inducer of endocrine differentiation in cultured human foetal pancreatic cells (Otonkoski et al., 1993). It was shown that treatment of human foetal pancreatic cells with 10 nM nicotinamide resulted in a twofold increase in DNA content and a threefold increase in insulin content associated with development of beta cell outgrowths from undifferentiated epithelial cell clusters. There was also an increase in the expression of the insulin, glucagon, and somatostatin genes (Otonkoski et al., 1993). More recently, in many studies Nicotinamide is used in induction protocols in combination with other stimulants (Chen et al., 2004b; Segev et al., 2004a; Sun et al., 2007c). Nicotinamide is an inhibitor of Poly (ADP-Ribose) Polymerase protein. Okamoto et al., in one of his reports pointed out that on inhibiting PARP activity the protein goes and binds to Reg promoter that initiates islet differentiation (Akiyama et al., 2001). In another such study Ye et al., reported

that Nicotinamide increases *MafA* gene transcription there by promoting differentiation and insulin biogenesis (Ye et al., 2006).

1.12. Essential Signaling pathways during pancreatic development and islet neogenesis include Transforming growth factor (TGF-β) signaling:

The TGF-β family consists of more than 40 different related members and is broadly divided into two subfamilies, The TGF-β-activin and the Bone morphogenetic protein (BMP) family (Brown and Schneyer, 2010). The binding complex on the cell surface consists of two type 1 and two type 2 receptors, the ligand binds to the type 2 receptor and activates the type 1 receptor by phosphorylation which then relays the signal to the intracellular environment via serine-threonine kinase action to a family of Smad , second messenger proteins. These smads that get activated by phosphorylation are also called R Smads; Smad 1,2,3,5 and 8 then forms a complex with Smad 4 which then translocates to the nucleus and regulates gene expressions in association with other transcriptional modifiers. Smads 6 and 7 are inhibitory smads that suppress R Smad's phosphorylation and block the signal through Smad2/3 or Smad 1/5/8 pathways respectively (Fig 1.7) (Pangas and Woodruff, 2000; Shi and Massagué, 2003).

Nodal, a TGF-β family member is essential in patterning of the primitive gut. Nodal downregulation leads to formation of mesoderm. Sonic hedgehog (Shh) mediates between the gut endoderm and the surrounding mesoderm. Downregulation of Shh and activation of Pdx-1 specifies pancreas formation at e8 (Apelqvist et al., 1997; Brown and Schneyer, 2010).

Table1: Summary of Various Stem cells in Islet Differentiation					
Sr. No.	**Sources**	**Agents**	**Time Period (Days)**	**Remarks**	**Authors**
1.	hESCs	β-mercaptoethanol, and bFGF,insulin-transferrin-selenium-fibronectin,N2 & B27supplement, nicotinamide	25	• Enhanced expression of pancreatic genes. • Poly hormonal immature cells with somatostatin and glucagon coexpression.	(Segev et al., 2004b)
2.	hESCs	Insulin-transferrin-selenium-A,monothioglycerol, albumin fraction V, and βmercaptoethanol CDM, activin A retinoic acid	18	• Detection of Sox17 and Brachyury, definitive endoderm markers. • Islets differentiated expressed c-peptide, insulin, glucagon and glut-2 and when transplanted in STZ diabetic mice ameliorated the diabetic condition in 30% mice.	(Jiang et al., 2007)
3.	iPSC from dermal fibroblasts	Activin CHIR CpdC Retinoic Acid SB SB431542 forskolin dexamethasone alk5 inhibitor II NA	25	• 11% INS positive cells but no response to glucose	(Kunisada et al., 2012)
4.	iPSC	Activin wortmannin All-trans Retinoic Acid Fibroblast Growth Factor 7	21	• EGF promoted proliferation of PDX1 positive cells (38%) and some of them expressed FOXA2, SOX9, HNF1b; 25% INS+ cells and glucose responsive	(Zhang et al., 2009)

		Noggin EGF InsulinTransferrinSelenium Exandin4 B Bone Morphogenetic Protein 4 Nicotinamide			
5.	iPSC	Activin Wingless-type, Fibroblast Growth Factor 10, Cyclo All-trans Retinoic Acid , Indolactam V,Exandin4 DAPT (γ-secretase) inhibitor HGF, IGF1	24	• Indolactum V, worked specifically to induce pancreatic progenitors from definitive endoderm; glucose responsive INS positive cells were produced.	(Chen et al., 2009)
6.	iPSC	EB or ML Bone Morphogenetic Protein4, VEGF, Fibroblast Growth Factor, Wingless-type, Activin, Fibroblast Growth Factor 10, Wingless type, Dorsomorphin, Noggin Cyclo All-trans Retinoic Acid; SB, Fibroblast Growth Factor 10 SB431542, Noggin gSIX	20	• 25% c-peptide positive cells, but no glucose response and only 5% c-peptide positive cells derived by using D'amour protocol.	(Nostro et al., 2011)

7.	Adult bone marrow stem cells from the long bones of rats	Nicotinamide, and exendin-4 pancreatic extract, DMSO	17	• Diathiazone (DTZ) positive islet clusters. • Clusters expressed insulin and endocrine specific genes. • Clusters responded to glucose challenge.	(Gabr et al., 2008)
8.	Rat marrow MSCs	β-mercaptoethanol and Nicotinamide	1.4	• Islet-like clusters expressed insulin mRNA and protein. • Clusters responded to glucose challenge and ameliorate hyperglycaemia in STZ diabetic rats.	(Chen et al., 2004a)
9.	Murine bone marrow-derived cells	Nicotinamide and exandin-4	5 to 7	• Differentiated cells expressed pancreatic genes. • Presence of Insulin and c-peptide and insulin release on glucose stimulation was observed. • Transplantation reverted diabetic condition in STZ diabetic mice.	(Tang et al., 2004a)
10.	Human adipose-derived MSCs	Nicotinamide, activin-A, exendin 4,hepatocyte growth factor, pentagastrin, B27 supplement, and N2 supplement	3	• Down-regulation of ABCG-2 and up-regulation of pancreatic developmental transcription factors (Isl-1, Pdx-1 and Ngn3) were observed, together with induction of islet hormones insulin, glucagon, and somatostatin.	(Timper et al., 2006)
11.	Human bone marrow-	B-mercaptoethanol bFGF, EGF, B27betacellulin,	18	• Transdifferentiated cells tested positive for DTZ and insulin. Expressed PDX-1, Neurog3, Pax4, Insulin, and	(Sun et al., 2007b)

	derived MSCs from diabetic patients	activin A, Nicotinamide, B27		Glucagon and responded to glucose stimulation.	
12.	Bone-marrow MSCs from Sprague-Dawly rats	Nicotinamide & Exandin-4	28	• Islets like clusters showed increased cytoplasmic secretory granules in differentiated cells. • Differentiated cells insulin secretion increased by 1.5 fold after glucose challenge in vitro. • After transplantation of islet-like clusters in diabetic rats, islet-like cells expressed islet hormones and lowered glucose levels of diabetic rats during day 6 to day 20	(Wu et al., 2007)
13.	Human umbilical cord blood-derived stem cells	Progesterone, putrescine, laminin, insulin, sodium selenite, nicotinamide, transferring, and fibronectin	7	• Insulin-producing islet-like structures that co-expressed insulin and C-peptide were observed	(Sun et al., 2007a)
14.	Human umbilical cord	Nicotinamide, EGF, Exendin4, Retinoic acid	15	• Islet-like cell clusters appeared 9 days after pancreatic differentiation. 25% cells differentiated and expressed islet-related genes and hormones but were not	(Gao et al., 2008)

	blood-derived MSCs			responsive to glucose challenge.	
15.	MSCs in Wharton's jelly of human umbilical cord	Nicotinamide, and B27, stem cell conditioned medium	28	• Transdifferentiated cells formed islet-like clusters. They expressed Pdx1, Hlxb9, Nkx2.2, Nkx6.1, and Glut-2. Islet-like clusters capable of producing insulin both *in vitro* and *in vivo*.	(Chao et al., 2008)
16.	Dental pulp stromal cells	Insulin-transferrin-selenium (ITS), activin A, sodium butyrate, and 2-mercaptoethanol, BSA, taurine, Glucagon-like peptide (GLP)-1 nicotinamide, and non-essential amino acids (NEAAs)	10	• The identity of clusters was confirmed as islets by DTZ-positive staining, expression of C-peptide, Pdx-1, Pax4, Pax6, Ngn3, and Isl-1. Day 10 clusters released insulin and C-peptide in a glucose-dependent manner.	(Govindasamy et al., 2011)
17.	Ductal Epithelial Cells	ITS supplement BSA, nicotinamide, and keratinocyte growth factor	10	• With 3–4 weeks of culturing. The insulin content per flask increased 10- to 15-fold as the DNA content increased up to 7-fold.	(Bonner-Weir et al., 2000)

				• The cultivated human islet buds were shown by immunofluorescence to consist of cytokeratin 19-positive duct cells and hormone-positive islet cells.	
18.	Intrahepatic biliary epithelial cells (IHBECs)	Insulin–transferrin–sodium selenite nicotinamide ascorbic acid 2-phosphate, dexamethasone EGF, HGF forskolin 3,3,5-triiodo-l-thyromine	26	• About 3% of 4000 cells counted in PDX-1/VP16 transduced cultures stained strongly for C-peptide suggesting that a subpopulation may have the capacity for differentiation. Transduced cells released insulin. IHBECs can be markedly expanded, and then with molecular manipulation a subpopulation of these cells can differentiate towards a b-cell phenotype.	(Nagaya et al., 2009)
19.	Pancreatic Stellate cells (PaSC)	HGF betacellulin nicotinamide exendin-4 ITS exendin-4	12	• Mitoxantrone-resistant cells obtained from lactating rats pancreata have a PaSC phenotype and are able to secrete insulin after cell differentiation	(Mato et al., 2009)
20.	Intra islet precursor cells	Insulin, transferrin, selenium, 2-mercaptoethanol, bovine serum albumin, keratinocyte growth factor (KGF)	4	• A cytokeratin-19 and cytokeratin-7 positive precursor cell population was found scattered throughout the epithelial monolayer. • Upon addition of the KGF, these precursor cells gave rise to islet-like clusters which were confirmed to be islets by marker studies.	(Banerjee and Bhonde, 2003a)

Activin is present in adult murine and human islets along with its receptors and regulatory protein, which indicated that apart from embryonic pancreatic development Activin has an important role to play in adult islets. Activin has been reported to enhance islet function by increasing their glucose responsiveness (Florio et al., 2000; Kim et al., 2000; Totsuka et al., 1988; WADA et al., 1996). Activin has been demonstrated to upregulate Insulin and Glut2 expression along with key transcription factors involved in islet neogenesis and homeostasis i.e. Pdx1, NeuroD, Pax4 and MafA. Hence, it not only aids in beta cells differentiation but also regulates beta cell mass (Brun et al., 2004; Matsuoka et al., 2007; Sosa-Pineda, 2004). Further, it has been observed that Activin A via Smad signaling also downregulates glucagon gene expression in alpha cells in human islets (Mamin and Philippe, 2007; Yasuda et al., 1993). Development of hypoplastic islets and glucose intolerance can be observed in mice with reduced expression of Activin receptors. Similar results are seen when Activin signaling is blocked in transgenic mice (Brown and Schneyer, 2010). Also, blocking Activin signaling *in vitro* during islet differentiation from stem cells completely abolished islet formation (Dadheech et al., 2015). Thus TGF-β signaling is imperative for islet neogenesis, regulating β-cell mass and/or proper insulin release in response to glucose.

Cellular replacement therapies may eventually provide new rays of hopes for a curative treatment for diabetes. The availability of this treatment option is limited due to (1) dearth of cadaveric islet donors (Holland et al., 2009), (2) generation of new islets from stem cell pool could become a limitation as many sources of stem cell cannot yield immature islets and the process takes very long period , and (3) availability of islet neogenic or differentiating agents (Zhang et al., 2005b). However, hurdles of islet transplantation can be significantly improved. (Medical practitioners have started using islet transplantation therapy to treat diabetes in year 2000 (Bretzel et al., 2004).) Stem cell pool is prodigious target that is being exploited to generate islets for clinical intention. Various differentiating agents for this

rationale are now being explored. Biological growth factor like KGF, FGF, GLP-1, and betacellulin (Abraham et al., 2002; Banerjee et al., 2005; Katdare et al., 2004) and chemical agents like Nicotinamide, Activin-A, Exendin-4 (Banerjee and Bhonde, 2003b; Chandra et al., 2009; Xu et al., 1999) are conveniently used by researchers. But the yield of islets after differentiation is not sufficient to overcome the high demand and the high cost of therapy. Hence scientists are now shifting towards the use of dramatic medicinal properties of herbal plants that may possess islet neogenic activity.

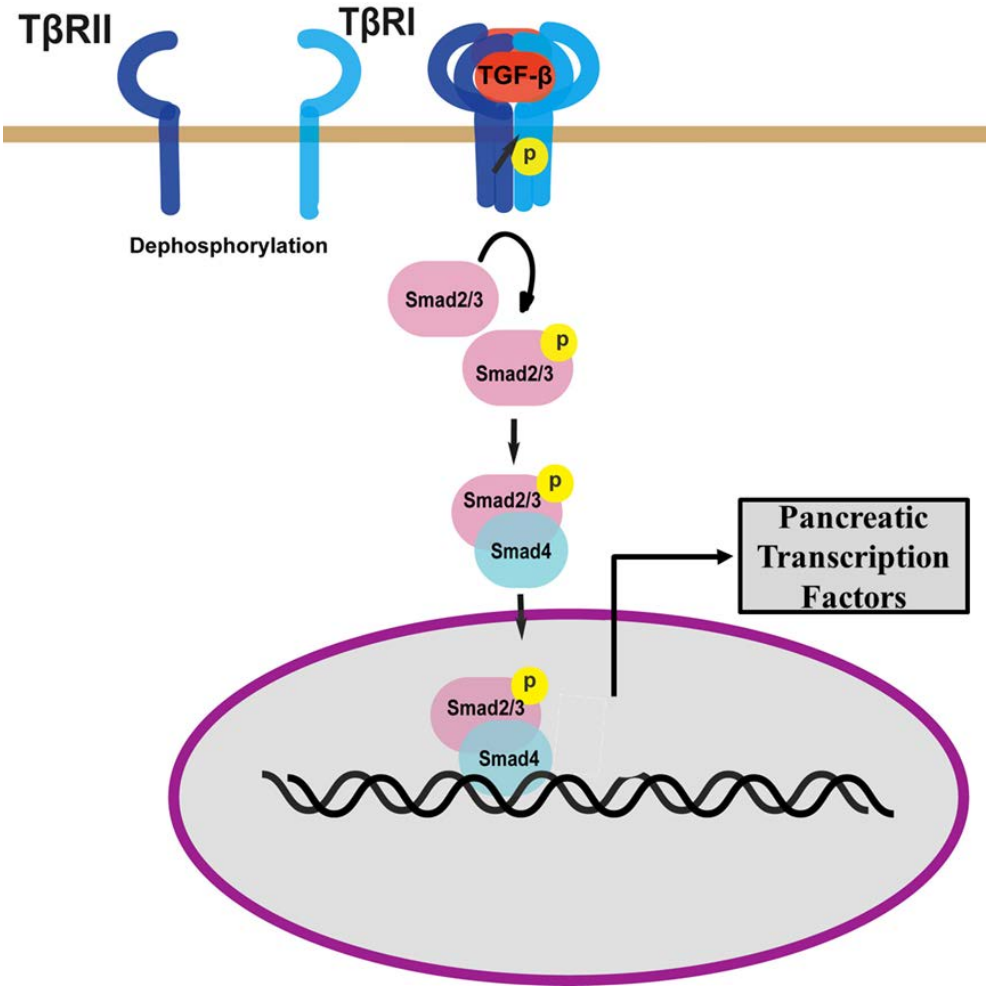


Figure 1.7: TGF-β Smad pathway (Wrighton et al., 2009).

1.13. *Enicostemma littorale* a new target for islet neogenesis

Enicostemma littorale (Gentianaceae) belonging to the family Gentianaceae is a glabrous perennial herb. It grows throughout India up to 1.5 feet height and more frequently near the

sea. It is called as Chota-kirayat or Chota chirayata in Hindi, Mamejavo in Gujarati, Nagajivha in Bengal and Vellarugu or Vallari in Tamil.

Stem/ progenitor differentiation activity of *E. littorale* was demonstrated using human pancreatic carcinoma cell line PANC1 and mouse embryonic fibroblast cell line NIH3T3, which were successfully differentiated into functional insulin producing islet like clusters (Gupta S et al., 2010). Dadheech et al., in 2013 identified the active principal molecule Swertisin, a flavonoid which was responsible for the above islet neogenic property. Swertisin, not only gave 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 normoglyceamic after the transplantation. Further, the molecular mechanism of Swertisin was extensively studied and was found to follow Activin A mediated MEPK-TKK pathway during islet neogenesis. Only few other natural herbal molecules have been reported to have islet neogenic potential, one of them is Conophylline is a vinca alkaloid extracted from the tropical plant *Ervatamia microphylla*. Kojima et al., has demonstrated its role in islet differentiation where the molecule mimics the effect of Activins. Conophylline acting as a ligand for Activin-A was later proved by performing differentiation in presence of Activin-A antagonist. The antagonist blocked islet generation when incubated with Conophylline confirming the mode of differentiation (Kojima and Umezawa, 2006). These are the first few evidences which provided some clue that herbal compounds possess islet neogenic property, thus playing a crucial role in islet differentiation.

1.14. PARP

Poly (ADP-ribose) (PAR) Polymerase 1 (PARP-1), the founding member of the PARP family is the most abundant and ubiquitous nuclear protein present in eukaryotes with the only exception in yeast (Hassa et al., 2006). It is a highly conserved protein with seventeen

members in the PARP family. The classical role of PARP has been characterized in DNA repair and cell death (Krishnakumar and Kraus, 2010b). However, over the decades its role in gene regulation has been well established and further explored.

1.15. PARP-1 structure and enzyme activity

PARP-1is a 116 kDa protein having 1014 amino acids (mice and human) with many independently folded domains (Amé et al., 2004; Schreiber et al., 2006). The various domains from the N terminal to C terminal are as follows: First, the DNA binding domain (DBD) that spans from amino acid 1-372 consists of two zinc finger motifs (FI/Zn1 and FII/Zn2) for binding to DNA, a zinc binding domain (FIII/Zn3) that mediates interdomain contacts for DNA dependent enzyme activation, a nuclear localization signal (NLS) and a caspase-3 cleavage site; second, the auto-modification domain (AMD) from amino acid 372-524, which consists of a BRCA1 C-terminus (BRCT) motif for protein-protein interaction. Third, a WGR motif which has a conserved amino acid sequence of Try, Gly and Arg from amino acid 525-643, may function in nucleic acid binding. Finally, PARP-1 consists of a highly conserved carboxy terminal catalytic domain from amino acid 653-1014. This domain consists of the PARP signature motif which is conserved throughout the PARP family and forms the NAD^+ binding site. It also consists of α-helical PARP regulatory domain (PRD) that interacts with the substrate binding sites and control the PAR chain branching (Krishnakumar and Kraus, 2010b; Luo and Kraus, 2012). The assembly of these domains result into a unique protein that has a variety of functions within the nucleus across genome (Fig 1.8).

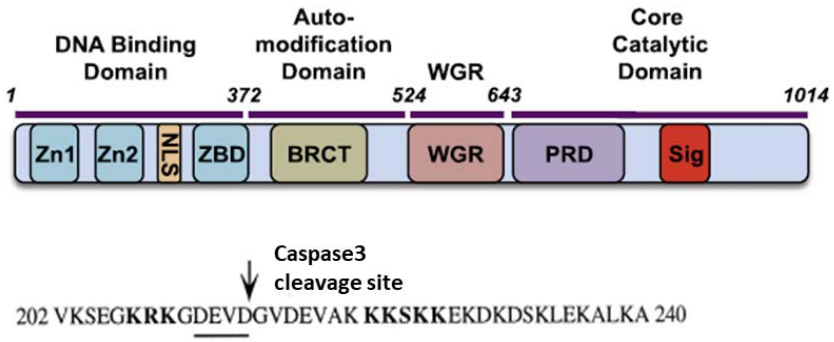


Figure 1.8: Structure of PARP-1: PARP-1 is a nuclear protein of 1014 amino acids having three distinct domains namely DNA binding domain, Auto-modification domain and Core catalytic domain (Kraus and Hottiger, 2013).

1.16. PAR and PARP-1 activation and regulation

PAR, is also identified as the third type of nucleic acid, a long negatively charged polymer that mainly functions in posttranslational modifications, which is produced by catalytic activity of PARP-1 by utilizing NAD^+ molecules as substrate on target proteins (D'amours et al., 1999). The ADP-ribose unites in the PAR polymer (linear or branched) are linked by glycosidic bonds between two ribose units. The PAR polymer can be broken down by poly (ADP-ribose) glycohydrolase (PARG) that has multiple isoforms with both exo and endo (at branching point) activity (Gagné et al., 2006; Gagné et al., 2008; Min and Wang, 2008), ADP-ribose hydrolase3 (ARH3) enzyme having a similar activity as exo PARG (Oka et al., 2006). Also, there are other enzymes of poly and mono (ADP-ribosyl) protein hydrolyase that remove PAR polymers and mono (ADP-ribosyl) protein lyase that cleave the last mono ADP-ribose from the target protein (Hakmé et al., 2008; Hottiger et al., 2010). PARP-1 activation may occur due to any exogenous or endogenous genotoxic stress that could damage DNA and generate

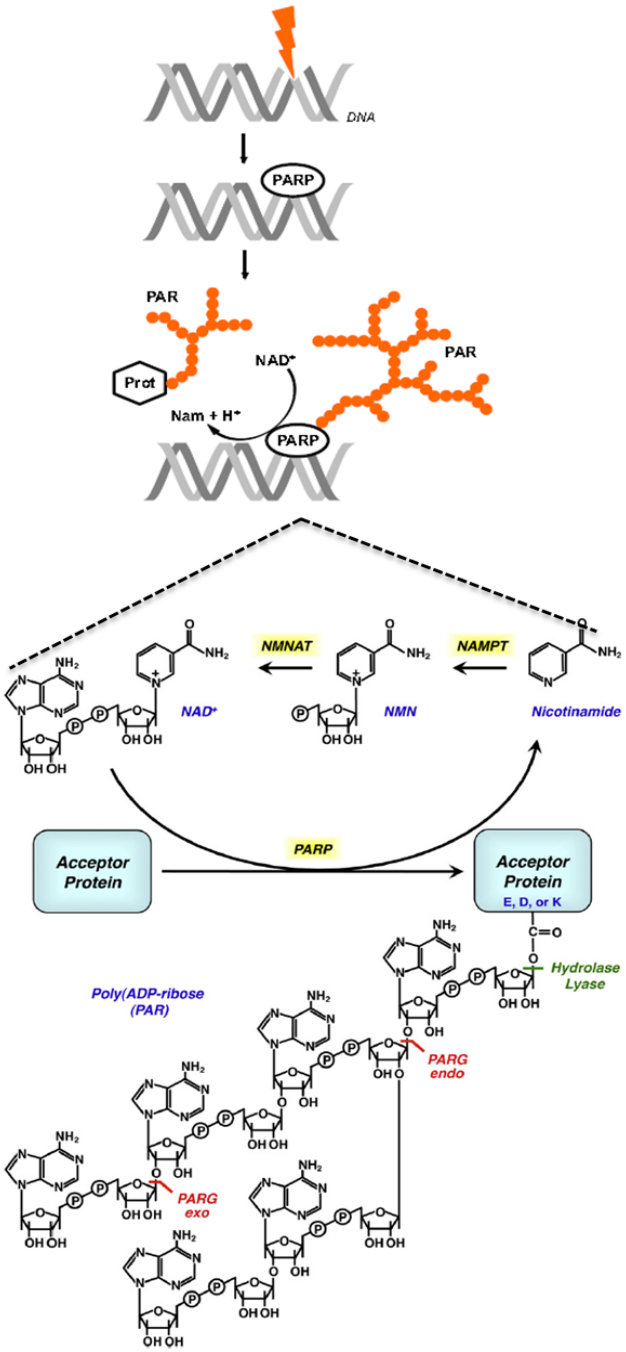


Figure 1.9: Activation of PARP-1 and its regulation: Genotoxic stress activates PARP-1 and Biosynthesis of using NAD+ as substrate. The enzymatic actions of PARP, PARG, (ADP-ribosyl) protein hydrolase, and (ADP-ribosyl) protein lyase are also indicated (Krishnakumar and Kraus, 2010b).

either single or double strand nicks, protein-protein interactions and different forms of DNA structures (Hakmé et al., 2008). PARP-1 can also be activated by nicotinamide mononucleotide adenylyltransferase (NMNAT1) that can locally produce NAD^+ which can be utilized by PARP-1. NMNAt1 is an exclusive nuclear protein that synthesises NAD+ to support nuclear functions of enzymes such as PARP-1 and SIRTs that consume NAD+ as substrate for their functions. PARP-1 recruits NMNAT1 within the nucleus to its function sites where it utilizes the NAD+ produced by NMNAT1 to PARylate its targets (Kim et al., 2004; Kim et al., 2005). Further, post translational modifications can modulate PARP-1 activity. Auto PARylation of PARP-1 inhibits its catalytic activity whereas its phosphorylation by Erk1/2 enhances it (Kauppinen et al., 2006). Also, activation of PARP-1 takes place in response to an inflammatory stimuli induced co-activation of NF-ĸB and p300, where p300 acetylates PARP-1 (Hassa et al., 2003; Hassa et al., 2005). PARP-1 can get SUMOylated at residues Lys 2013 and Lys 486, which get ubiquitylated by the ubiquitin E3lygaseRNF4 resulting in clearance and recycling of PARP-1 from its target promoters (Krishnakumar and Kraus, 2010b; Luo and Kraus, 2012). The N-terminal tail of the H4 can activate PARP-1 to a higher degree than DNA damage. Some of the known targets of PARP-1 catalytic activity includes, histones for example PARylation of H1, H2A and H2B to regulate chromatin structure, various transcription factors, nuclear enzymes and structural proteins (Hakmé et al., 2008; Krishnakumar and Kraus, 2010b). Binding partners that regulate PARP-1's catalytic activity include single and double stranded DNA, histones, nucleosomes and many nuclear proteins including protein complexes that (a) involved in DNA repair e.g. XRCC1; (b) transcriptional regulators; (c) insulators e.g. CTCF and (d) methylate DNA e.g. DNMT1 (Fig 1.9).

1.17. PARP-1 in gene regulation

PARP-1 and the rest of the family have been well explored in the paradigm of DNA repair, cell death and cancer. However, for the last two decades efforts involving elucidation of its role in gene regulation have intensified much more. This area of research has become more exciting as the ubiquitously present nuclear protein PARP-1 have shown functions with respect to chromatin remodelling, regulating various transcription factors and regulating DNA methylation under various conditions of cell proliferation, development and differentiation (Kraus and Lis, 2003; Krishnakumar and Kraus, 2010b). Hence, it has become imperative to explore PARP-1 to understand its impact on various biological processes.

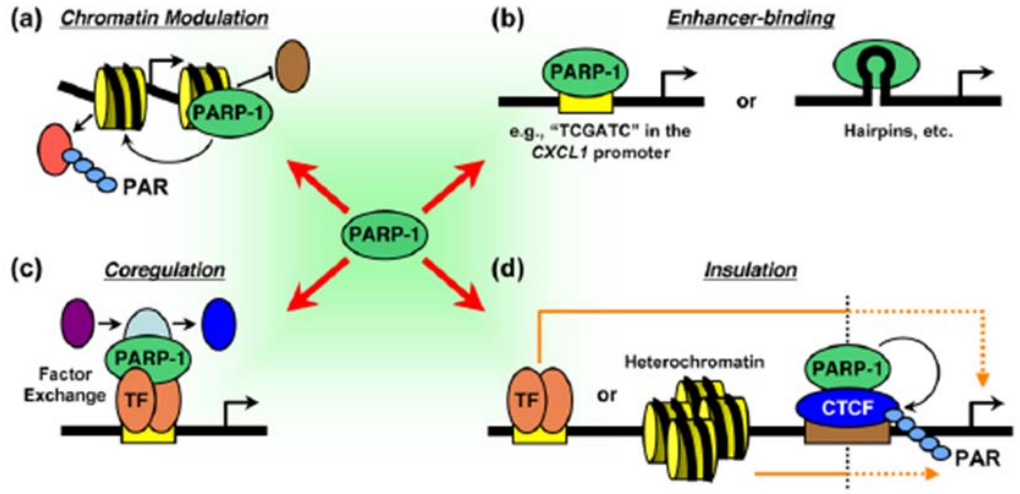


Figure 1.10: Transcriptional regulation by PARP-1: PARP-1 regulates transcription by (a) chromatin modulation; (b) Enhancer binding factor, act as classical DNA binding modulator; (c) act as coactivator or corepressor and function as transcriptional coregulator and (d) can act as insulator (Kraus, 2008).

PARP-1 dependent gene regulation is demonstrated robustly by creating genetic models where the PARP1 gene is knocked out. PARP-1 deficient genetic models have been created for various species viz. mice, fly and worm. It is observed that PARP-1 deletion or depletion in flies (*Drosophila melanogator*) and worms (*Caenorhabditis elegans*) results in gross physiological and developmental phenotypes (St-Laurent et al., 2007; Tulin and Spradling, 2003). Three different PARP-1 knockouts exist for mice targeting different exons. There are no developmental or phenotypic abnormalities in these mice. However, in stress condition

they demonstrate phenotypic changes that point to PARP-1 functions. A gene expression profile from PARP knockout mice from ESCs and liver confirmed that approximately 3.5% of the transcriptome was PARP-1 regulated with almost 70% genes positively modulated by PARP-1 (De Murcia et al., 1997; Shall and de Murcia, 2000). Hence, animal models provide a clear link between PARP-1 gene regulation and physiological functions (Fig 1.10).

1.17.1. PARP-1 in chromatin modulation

PARP-1 has been observed to bind to nucleosome and interact with chromatin domains depending upon the nature of chromatin. Previous studies have demonstrated that both PARP-1 and PAR can modulate chromatin structure e.g. in drosophila the heat shock signaling activates PARP-1 that causes decondensation of chromatin (Petesch and Lis, 2012; Tulin and Spradling, 2003) and alternatively stimulation of macrophages with LPS activates caspase7 which cleaves PARP-1 thereby causing chromatin decondensation (Erener et al., 2012). It was previously suggested that PARP-1 PARylates H1 and thereby causing chromatin decondensation (Poirier et al., 1982). However, recent reports demonstrate that PARP-1 binds the nucleosome where the site overlaps the binding site of the linker H1. Thus, creating a competition between the two for the target gene promoter thereby regulating its expression (Kim et al., 2004). It was earlier reported that PARlylation on core histones occur at glutamate and aspartate residues however recent findings suggests it to be the lysine residues (Altmeyer et al., 2009; Messner et al., 2010). Apart from the core histones PARP-1 has shown to structurally and functionally modulate core histone variants such as H2A variants H2A.Z and macroH2A as part of the nucleosome complex. The interaction between them is associated with inactivation of PARP-1 activity which affects promoters of *Hsp70.1* and *Hsp70.2* (Gibson and Kraus, 2012). Another way of affecting chromatin structure is by modulating the activity and localization of histone and chromatin modifying enzymes. A high correlation between PARP-1 and H3K4me3 i.e. H2 lysine 4 trilemthyl has been observed at

transcriptionally active promoter sites. KDM5B is a histone lysine demethylase enzyme acting on H3K4me3, which is blocked from binding to chromatin and inhibiting its action by PARylation by PARP-1, thus explaining PARP-1's role in regulating the chromatin state at the promoters for both basal and signal driven transcription (Krishnakumar and Kraus, 2010a). Further, interaction between PARP-1 and ATP-dependent nucleosome remodelling enzymes in Drosophila has been reported, where PARylation of ISWI, ATP-dependent nucleosome remodeler inhibits the chromatin binding of ATPase and chromatin condensation activity at the heat shock loci (Sala et al., 2008).

1.17.2. PARP-1 as coregulator of transcription factors

Apart from acting as a chromatin modulator PARP-1 can regulate gene expressions by working along with the RNA Polymerase II transcriptional machinery, other coregulators with specific enzyme activities or promoter specific transcription factors (D'amours et al., 1999; Kraus, 2008; Kraus and Lis, 2003). The effect due to PARP-1's modulation on these activators may either be stimulatory or inhibitory; they may require PARP-1 enzyme activity or require PARP-1 protein to work independent of its enzyme activity and it may vary in a promoter specific manner from cell type to cell type. There is little evidence to suggest PARP-1's regulation on basal transcription machinery and PARlylation of the components of the transcription machinery. However, its known to corregulate RNA polymerase II preinitiation complex prior to binding to transcription factor IID, which demonstrates its DNA damage independent activity (Slattery et al., 1983). PARP-1 is also reported to function independent of its catalytic activity as a scaffold protein to promote and recruit various transcription factors to form a complex at the promoter site for transcription e.g. protein interaction between protein arginine methyltransferase1 (PRMT1) and PARP-1 enhances gene expression in the macrophages (Hassa et al., 2008). Also, as discussed earlier PARP-1 is acetylated by p300 in response to inflammatory response, which promotes functional

cooperation between p300 and p50 subunit of NF-KB (Hassa et al., 2003; Hassa et al., 2005). PARP-1 is also reported to function as an 'exchange factor' that causes the exchange of inhibitory factors to stimulatory factors to the bound transcription factors in a promoter specific manner e.g. during retinoic acid activation PARP-1 felicitates the exchange of Cdk8 positive mediator for Cdk8 negative (Pavri et al., 2005). Hence PARP-1 can coregulate transcription by modulating protein complexes in a promoter specific manner.

1.17.3. DNA methylation and insulator function of PARP-1

With respect to DNA methylation PARP-1 regulates the expression and activity of DNA methytransferase Dmnt1. PARP-1 PARylates Dmnt1 which inactivates it (Caiafa et al., 2009; Caiafa and Zlatanova, 2009; Reale et al., 2005). Further, PARP-1 has been demonstrated to play important role in interacting with methylcytosine deoxygenase Tet2 that catalyses the conversion of methylcytosine (5mc) to 5-hydroxymethylcytosine (5hmc) during the generation of iPSCs from somatic cells. 5hmc triggers an epigenetic program that induce transcription in the pluripotency loci (Doege et al., 2012). DNA methylation has also been implicated in insulator function as it can regulate the binding of CTCF to insulators. DNA elements that organize the genome into discrete regulatory units either by preventing heterochromatization or by limiting the effect of enhancers on promoters are known as Insulators (Kraus, 2008). PARP-1 has been reported to PARylate CTCF, a DNA binding protein that functions at insulators and CTCF may promote automodification of PARP-1 (Guastafierro et al., 2008; Zampieri et al., 2012).

1.18. PARP-1 in Stem cells and Differentiation

PARP-1's role in regulating stem cell function could be confirmed form the PARP-1 knockout mice models where it was observed that 10% of the genes related to metabolism, signal transduction and cell cycle in the ESCs were altered due to the absence of PARP-1; also there were more genes that were downregulated which elucidate the importance of PARP-1 in ESCs (Ogino et al., 2007). In another study, PARP-1 has demonstrated to regulate

the binding of Sox2 to the promoter of FGF4 which influences differentiation in stem cells. PARP-1 directly PARylates Sox2, a pluripotency marker for the ESCs at the FGF4 enhancer site thus dissociating and leading to its degradation resulting in enhanced expression of FGF4 to initiate a specific cell fate (Gao et al., 2009). Another example of PARP-1 action in regulating stem cells has been discussed above with respect to DNA methylation where it can regulate methylation of methylcytosine that triggers an epigenetic cascade of events that leads to the generation of iPSCs from somatic cells (Doege et al., 2012). Further, maintaining telomere length becomes essential during proliferation for stem cells. PARP-1 regulates its length through PARylation and depletion of PARP-1 induces rapid shortening of telomere length (Tong et al., 2001). Further, PARP-1 coregulates retinoic acid (RA) and influences RA dependent growth and development. In another study it was demonstrated that PARP-1 binds to PPAR-γ on the promoter of its target genes and acts as an exchange factor by swapping NCoR1 co-repressor with p300 co-activator leading to PPAR-γ related gene expressions during adipogenesis (Lehmann et al., 2015). It was earlier reported that PARP2 also plays a role of co-activator for PPAR-γ during adipogenesis and lipid metabolism (Bai et al., 2007). Similar function of PARP-1 is observed in activation of neuronal differentiation with respect to HES1 gene where PARP-1 exchanges the co-repressor complex and allows HES1 to be phosphorylated by CAMKIIδ (Ju et al., 2004). PARP-1 has been reported to induce neurogenesis from hESCs by regulating FGF-ERK1/2 (Yoo et al., 2011).

As discussed previously the TGF-β signalling is pivotal during islet differentiation and neogenesis from stem cells (Brown and Schneyer, 2010; Kim and Hebrok, 2001). There have been many reports that PARP-1 plays a crucial role in regulating this pathway (Takasawa and Okamoto, 2002). Classically, TGF-β is known to play important role in proliferation, differentiation and migration in embryonic development and adult tissue homeostasis (Watabe and Miyazono, 2009). In the Smad mediated TGF-β pathway, activation

(phosphorylation) of Smad2 and 3 forms a complex with Smad4 which gets translocated in to the nucleus and regulates specific gene expression (Brown and Schneyer, 2010). The first study of PARP-1 and TGF-β signaling was in oogenesis and folliculogenesis that demonstrated that poly(ADP-ribosyl)ation is essential and leads to enhanced expression of genes (TGF-β super family) in mouse ovaries (Qian et al., 2010). However, the first study of PARP-1-smad interaction reported that PARP-1 PARylates Smad3 and Smad4 which dissociates the complex from DNA and abates the Smad-specific gene responses and TGF-β induced epithelial to mesenchymal transition (Lönn et al., 2010). Whereas, in another completely opposite report it was reported that PARP-1 PARylates Smad3 and activates it to form Smad complex which increases the Smad3 specific gene expression which was abolished by either using PARP-1 inhibitors or depleting PARP-1 transcripts by using specific siRNA (Huang et al., 2011). PARP-1's role in regulating the TGF-β receptors were also reported where it was demonstrated that PARP-1 negatively regulated the expression of TGF-β receptors in human CD4+ T cells thereby regulating the TGF-β signal transduction (Zhang et al., 2013). In a more recent study, PARP2 accompanied PARP-1 to PARylate Smad3 and negatively regulate its function whereas PARG positively regulated Smad3 function by de-PARylating it during TGF-β signaling (Dahl et al., 2014). These reports demonstrate PARP-1's role in regulating TGF-β signaling in both positive and negative manner.

PARP-1 have also been associated with HIF (Hypoxia inducible factors) that regulate genes from various cellular processes viz. cell growth, metabolism, death etc. (Gonzalez-Flores et al., 2014). In lower organisms like *Drosophila melanogaster* (Hanai et al., 1998) and *Dictyostelium discoideum* (Jubin et al., 2017; Rajawat et al., 2011) PARP-1 has been reported to play an important role in the regulation of their developmental program.

1.19. PARP-1 in Diabetes

PARP-1 has been reported to regulate transcription factors and modulate their gene expressions within the pancreas which has highlighted PARP-1's role in influencing several important pancreatic functions. Also, the fact that *PARP-1* gene expression in the pancreas is the least when compared to every other tissue in the human body, gives us a profound insight into its importance in maintaining pancreatic homeostasis (Fig 1.11) (Fagerberg et al., 2014).

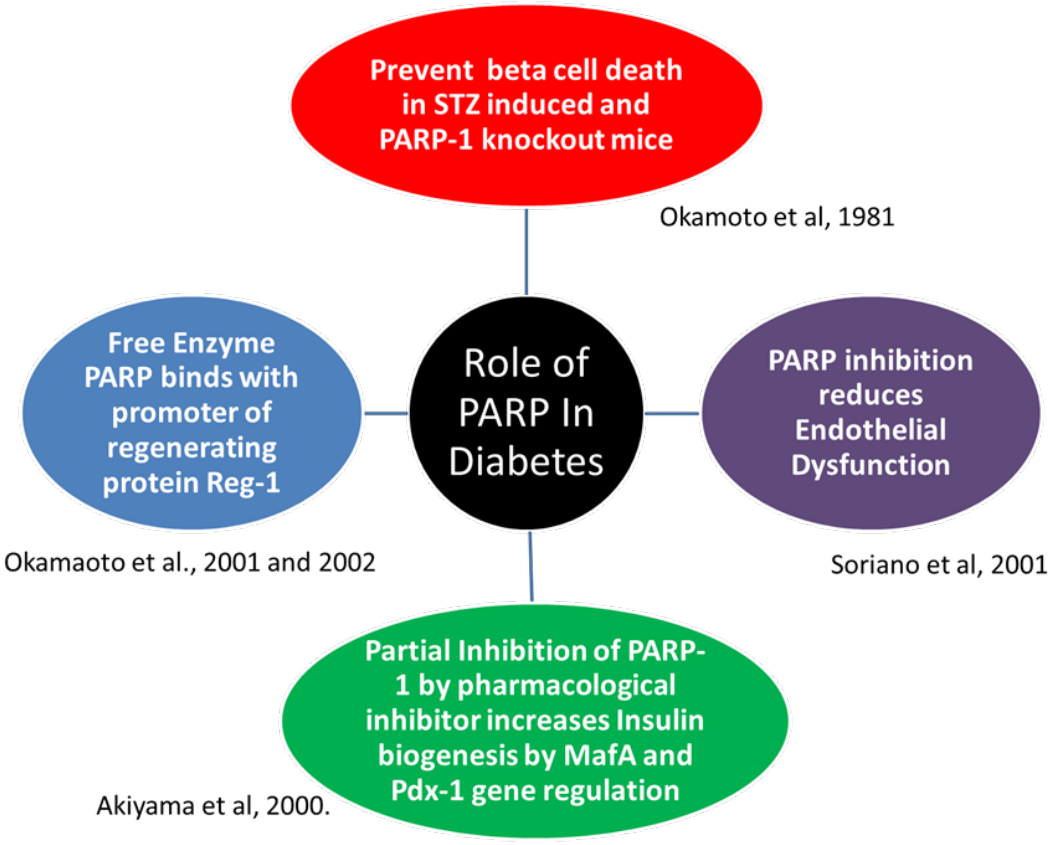


Figure 1.11: Role of PARP in Diabetes: Reports suggest role of PARP in pancreatic regeneration, regulating insulin biogenesis, resistance against chemical insults for beta cell death and reduction in diabetic complications.

1.19.1. Learning from PARP-1 knockout mice model with respect to Diabetes

Three different PARP-1 knockout mice have been generated by deleting exon1, exon 2 and exon4 respectively (Shall and de Murcia, 2000). It was observed and confirmed throughout the three variations of the PARP-1 knockout mice models that after the exposure to STZ the animals remain protected against the development of insulin dependent diabetes mellitus with

defective NF-KB induction (Oliver et al., 1999). STZ induces genotoxic stress in the beta cells in a cell specific manner. Excessive DNA damage overactivates PARP-1, which consumes cellular NAD+ thus depleting energy pool leading to cell death but in absence of PARP-1 cell survives with accumulation of DNA breaks (Burkart et al., 1999; Masutani et al., 1999; Pieper et al., 1999). This was further confirmed in an *in vitro* study where islets from PARP-1 null mice were isolated and exposed to DNA damaging compounds such as nitric oxide (NO) or reactive oxygen intermediates (ROI) to provide a direct relation between PARP activation, NAD+ depletion and cell death. It was observed that islets from PARP-1 null mice did not show NAD+ depletion when exposed to DNA damaging radicals and are more resistant to toxicity of NO or ROI (Heller et al., 1995). Another important discovery in this respect was the use of PARP inhibitors. It was demonstrated that in the STZ model NAD+ depletion can be prevented by administering nicotinamide or 3-aminobenzamide (Schein et al., 1967; Uchigata et al., 1982; Yamamoto and Okamoto, 1980).

The mechanism of protection from developing diabetes is the same as in the PARP-1 null mice model. However, the animals that were treated with PARP inhibitors developed very incidence of pancreatic islet beta cell tumours (Rakieten et al., 1971; Yamagami et al., 1985). In PARP-1 knockout mice model it was also observed that all the functions related to inflammatory responses with respect to macrophages were maintained. While PARP-1 deficiency protects beta cells against cytokine induced cell death, it does not inhibit cytokine mediated DNA damage or reduction in insulin secretion (Andreone et al., 2012). These results were highly interesting as it lead the use of PARP inhibitors in studies with respect to islet neogenesis.

1.19.2. The Okamoto model of pancreatic islet regeneration, insulin signaling and beta cell death:

1.19.2.1. PARP-1 in islet regeneration and Reg gene:

Towards the end of the 19th century Mering and Minkowski reported that dogs became diabetic followed by pancreatectomy, which lead the direction in diabetic research to explore active pancreatic principle for treating diabetes (Karamanou et al., 2016). Okamoto in 1984 performed 90% pancreatectomy in Wistar rats and administered them with PARP inhibitors niconiamide or 3-aminobenzamide intraperitoneally. PARP inhibitors ameliorated diabetic condition and the islets of Langerhans became significantly large that stained excessively with insulin (Yonemura et al., 1984). Further, cDNA library from the regenerating islets were prepared and a novel gene later named as *REG* gene for the regenerating pancreas was identified. When REG protein (1mg/kg/day) was injected intraperitoneally in 90% pancreatectomised rats, it was observed that after one month there was significant amelioration in fasting plasma blood glucose levels. Also, it was observed that the islets in the control animals with 90% pancreatectomy were destroyed after 2 months whereas Reg protein treated islets were enlarged and densely stained for insulin (Watanabe et al., 1994). This study confirmed that Reg protein induced regeneration and/or replication in rat islets. In another study it was demonstrated that overexpressing Reg protein receptor increased BrdU uptake by RINm5F cells in presence of Reg protein (0.3-100nM). However, mRNA expression was found to be unchanged during islet regeneration suggesting that Reg gene regulated regeneration and not its receptor (Kobayashi et al., 2000). It was further reported that Reg gene was activated by PARP inhibitors (Akiyama et al., 2001). This was the first study that directly implicated PARP-1 in regulation of Reg gene and demonstrated its gene regulating activity. Here, in presence of NAD+ Reg gene expression was downregulated whereas PARP inhibitor nicotinamide or 3-aminobenzamide quenched the effect of NAD+ and increased Reg gene expression. The study confirms PARP-1 to be a part of the

transcriptional complex which it self-regulates by auto-PARylation. To explain the above findings in simpler terms, PARP-1 interacts with the nuclear proteins forming the transcription complex on Reg gene. It negatively regulates Reg's expression by auto-PARylating itself which is observed when NAD+ is provided that aids in PARylation. However, incorporating PARP inhibitors prevents auto-PARylation thus enhancing Reg gene expression (Akiyama et al., 2001). In times of genotoxic stress PARP-1 gets activated in response to DNA damage for its repair. Here, PARP-1 auto-PARylates itself inhibiting the formation transcriptional complex and abating Reg expression but upon administration of PARP inhibitors PARP is not auto-PARylate which stabilizes the transcriptional complex for *Reg* expression. This also explains the above observation of islet regeneration in 90% pancreatectomy in presence of PARP inhibitors. This interaction of PARP-1 with Reg gene is the Okamoto model for beta cell regeneration (Fig 1.12) (Takasawa and Okamoto, 2002).

1.19.2.2. Insulin promoter activity, MAFA expression and PARP inhibition

Earlier studies of PARP inhibitors (Nicotinamide and 3-aminobenzamide) in regeneration of pancreatic tissue have lead their use in islet differentiation from stem/progenitors to increase beta cell mass and enhanced insulin biosynthesis. It was reported that exposure of high glucose concentration to INS-1 beta cells reduced insulin gene transcription. Administration of low potency PARP inhibitors ameliorated the INS-1 gene transcription by restoring the insulin promoter activity. Further, along with increase in the gene and protein expression of MafA, site directed mutagenesis confirmed the PARP inhibitor response to the A2/C1 promoter binding element that serves as a MafA binding site for the *Ins1* gene. However, no effect on PDX1 protein levels or binding activity was observed. Thus confirming their action of increasing insulin biosynthesis by regulation MafA gene transcription (Ye et al., 2006).

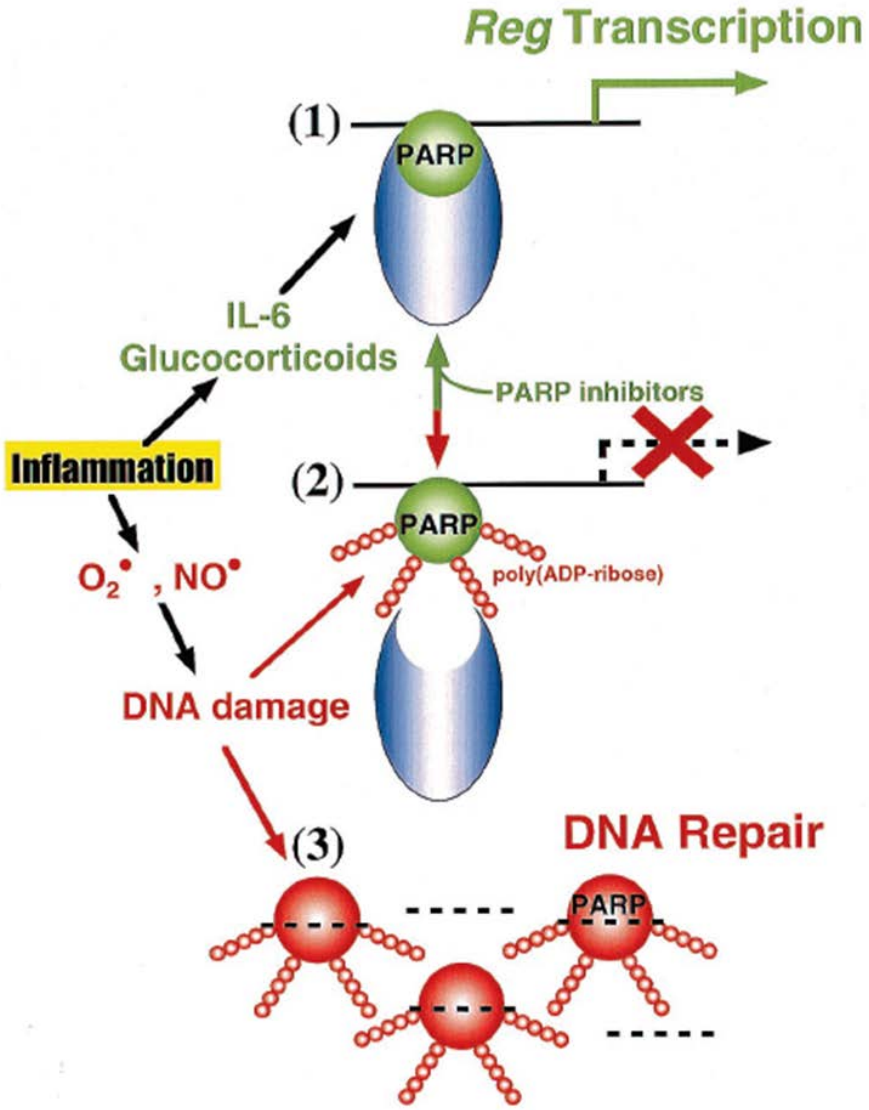


Figure 1.12: Okamoto's model for the role of PARP in Reg gene transcriptional control: Inflammation causes formation of Reg gene transcription complex which gets stabilize in presence of PARP inhibitors and there is transcription of Reg gene. However, on DNA damage PARylation of PARP causes inhibition of formation of transcription complex and if there is massive DNA damage as PARP is rapidly activated for DNA repair there is no transcription complex formation at all (Akiyama et al., 2001).

1.19.2.3. cADPR and CD38 signaling in insulin release

Cyclic ADP-ribose (cADPR) is generated from NAD+ and in pancreatic beta cells by ADP-ribosyl cyclase, it acts as a secondary messenger for intracellular Ca^{2+} mobilization for insulin secretion. Hence, DNA damage induced PARP-1 activation drains NAD+ levels which decreases cADPR synthesis resulting in abatement of insulin secretion from beta cells.

The classical insulin secretion model states that upon glucose uptake by cells, ATP is produced via Glycolysis and Krebs cycle which inhibits potassium channels within the beta cells that causes accumulation of K^+ that depolarises the membrane resulting in opening of Ca^{2+} channels leading to intake of Ca^{2+} ions within the beta cells which stimulates the release of insulin (Fu et al., 2013). Okamoto et al., in 1993 proposed another model for glucose induced insulin secretion by cADPR release of intracellular Ca^{2+} from endoplasmic reticulum (ER). In this study, to provide evidence for glucose stimulated cADPR accumulation in beta cells wistar rats and C57BL/6J mouse islets were treated with 2.8 mM and 20 mM glucose respectively. It was observed that islets incubated with higher glucose concentration accumulated higher cADPR. Further, it was observed that in response to cADPR the pancreatic islet microsomes released Ca2+ in a cell free system. However, Inositol1,4,5-triphosphate (IP3) did not cause Ca2+ release from the same suggesting islet microsomes respond to cADPR and not IP3. Direct effect of cADPR on insulin secretion was observed on digitonin-permeabilized pancreatic islets which were exposed to cADPR and Ca2+ which individually induced insulin secretion but IP3 did not. Also, the combined addition of cADPR and Ca^{2+} did not improve insulin secretion against their individual treatment. cADPR-induced insulin secretion was inhibited by EGTA which suggests its role via intracellular Ca^{2+} signaling. Hence, these studies provide enough evidence that insulin signaling in beta cells is mediated via cADPR mediated Ca^{2+} mobilization from islet microsomes (Takasawa et al., 1998; Takasawa et al., 1993).

1.19.2.4. PARP-1 in beta cell death

The underlying model for beta cell death has been streamlined into a single cascade of events; genotoxic stress due to effect of viruses or inflammation leading immunological responses, toxic free radicals or exogenous chemicals activate PARP-1 which depletes NAD+ pool that may lead to beta cell death. If there is no overactivation of PARP then it is cleaved

by caspases and inactivated which allows cells to undergo apoptosis otherwise it follows the necrotic pathway. Therefore, PARP inhibitors are effective in preventing necrosis but not apoptosis. The choice between the cell death pathway depends on the extent of cell damage, differences in death signals and species of cells (Takasawa and Okamoto, 2002).

1.20. PARP-1 in Diabetic Complications

The main cause of diabetic complications is persistent hyperglycaemia that leads to glucotoxicity. Hyperglycaemia causes increased superoxide production from the mitochondria and inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity. As GAPDH activity goes down there is an accumulation of glycolysis intermediates that are fed into four pathways of hyperglycaemic damage namely the polyol, hexosamine, Protein Kinase C and advanced glycation end products pathways. This causes increased flux of dihydroxyacetone phosphate (DHAP) to diacylglycerol (DAG), PKC activator and of triose phosphate to methylglyoxal, important advances glycated end products (AGE) precursor. Also increased modification of proteins by O-linked N-acetylglucosamine (GlucNAc) upon conversion of fructose-6-phosphate to UDP-N-acetylglucosamine and consumpsion of NADPH and depletion of GSH due to increased glucose flux by polyol pathway (Brownlee, 2001).

1.20.1. PARP-1 in diabetic endothelial dysfunction

The above pathways describes formation of oxygen and nitrogen derived free radicals such as superoxide anion and peroxynitrite which cause DNA damage induced activation of PARP (Garcia Soriano et al., 2001). Intracellular PARP activation in endothelial cells and vascular smooth muscle cells is observed within two weeks of diabetes and onset of endothelial dysfunction is seen with 4^{th} week. It was further observed that administration of PARP inhibitors restored normal vascular function and restored the established diabetic endothelial dysfunction. This was also observed in the in vitro diabetic blood vessels which increased their endothelium dependent responsiveness to relaxant (Garcia Soriano et al., 2001; Soriano

et al., 2001). Similar results were observed with autoimmune model for diabetes, which was associated with loss of NAD+ and NADPH in the vascular tissue that is reversed by PARP inhibitors (Pacher et al., 2002). Protective effect of PARP inhibition on vascular endothelial seems to be having the following mechanisms; conservation of NAD+, the cellular energy pool, prevention or inhibition of pro-inflammatory pathways which are suppressed by PARP inhibition which causes suppression of NF-KB activation. Another important mechanism of PARP inhibition is related to major pathways of hyperglycaemic damage trigger by inhibition of GAPDH by PARylation. Hence, PARP inhibition restores GAPDH activity, thereby suppressing glucotoxicity (Du et al., 2003; Kiss and Szabo, 2005; Mabley and Soriano, 2005; Soriano et al., 2001).

1.20.2. PARP-1 in diabetic retinopathy

PARP activation in diabetic retinal microvessels suggest direct link of PARP to diabetic retinopathy (Obrosova et al., 2004b). In an early study, bovine retinal endothelial cells (BRECs) and pericytes were incubated with 5 mM (normal) and 25 mM (high) glucose for five days with and without PJ34, a potent PARP inhibitor. PJ34 rescued retinal capillary endothelial cells from cell death. It was observed that PARP in BRECs acted as a coactivator of NF-KB and interacted with its subunits p50 and p65. Further, it was observed that PJ34 blocked interaction of PARP to p50 subunit of NF-KB which causes its inactivation, inhibited intracellular activation of adhesion molecule-1 and subsequent leukostasis (Zheng et al., 2004). In another long term study, diabetes was induced by STZ in Lewis rats and treated with PARP inhibitor PJ34 for 9 months. PARP activity was visibly increased after 2 months in the whole retina, retinal capillary endothelial cells and pericytes. At 9 months capillaries had significant DNA damage and were Tunel positive along with presence of acellular capillaries in STZ animals, whereas PJ34 treated rats significantly inhibited the progression of diabetic retinopathy by preventing death of retinal microvascular cells including acellular

capillaries and pericyte ghosts (Zheng et al., 2004). In a similar study, in C57/BL6 mice single dose STZ injection was administered to induce diabetes. PJ34 was given for 6 months. Diabetic mice showed loss of pericytes and acellular capillaries whereas PJ34 protected and inhibited the formation of retinopathy (Kiss and Szabo, 2005). These studies demonstrate that PARP activation is key factor in diabetic retinopathy which can be effectively reversed by administering PAPR inhibitors.

1.20.3. PARP in diabetic nephropathy

It was observed that 6 months treatment with nicotinamide, a PARP inhibitor reduced the deposition of IgG in the glomerulus of STZ treated diabetic rat model (Wahlberg et al., 1985). Similarly it has also been demonstrated that PARP activation observed in the kidney tubules in the STZ diabetic rats can be abated by administering two structurally different PARP inhibitors 3-aminobenzamide and 1,5-isoquinolinediol which also downregulate the Endothelin-1 and ET receptors in the renal cortex, which was upregulated by PARP in diabetic condition (Minchenko et al., 2003). In another study, a comparison between PARP-1 knockout and STZ treated diabetic mice was performed to analyse the extent of pathogenesis of diabetic nephropathy with respect to presence of PARP-1. It was observed that PARP-1 deficiency reduced urinary albumin, which reduced kidney hypertrophy, decreased mesangial expansion and collagen deposition. Although these results were non-significant they definitely indicate role of PARP, where improvement in diabetic nephropathy with PARP-1 deficiency was observed (Shevalye et al., 2010).

1.20.4. PARP in diabetic Neuropathy

Obrosova et al., in 2004 first suggested PARP-1 as a therapeutic target for treating diabetic neuropathy. It is observed that PARP-1 knockout mice are protected against sensory nerve conduction slowing and nerve energy failure that manifest in PARP-1 wild type and galactose fed mice. It was observed that PARP inhibitors could ameliorate blood flow, conductance and energy deficit in the nerves (Li et al., 2004; Obrosova et al., 2004a). The same group further

demonstrated that endothelial and schwaan cells show early accumulation of PAR in response to high glucose suggesting involvement of PARP in peripheral diabetic neuropathy (PDN). They further suggested low dose PARP inhibitor (as long term exposure to PARP inhibitors can cause cellular aging and genome instability) in combination with vasodilators as a combined therapeutic approach for treating PDN (Li et al., 2005a; Li et al., 2004). In recent times there have been many studies with different PARP inhibitors (Actovegin®, Nicotinamide, 4-amino 1,8 naphthalimide etc.) for treating PDN in combination with certain antioxidants like melatonin and resveratrol (Dieckmann et al., 2012; Negi et al., 2010a; Negi et al., 2010b; Sharma et al., 2009; Sharma et al., 2008).

1.20.5. PARP in diabetic Cardiomyopathy

In various models of myocardial-ischemia-reperfusion injuries oxidative stress induced PARP pathway was observed to play a key role (Szabo et al., 2002; Zingarelli et al., 1998). In the diabetic rat model PARP activation controls the degree of myocardial infarction and myocardial contractile failure (Xiao et al., 2004). It is observed that in both STZ induced diabetic model and nonobese diabetic rat and mice models PARP activation leads to cardiac dysfunction. However, treatment with PARP inhibitors restored vascular responsiveness and cardiac function significantly. It was observed that the protective effect of PARP inhibitors persisted for several weeks beyond discontinuation of treatment which could have implications in designing of therapy with PARP inhibitors (Pacher et al., 2002). Thus, PARP inhibitors have cardio protective attribute as they can conserve myocardial energy pool and prevent upregulation of proinflammtory pathways viz. cytokines, adhesion molecules, mononuclear cell infiltration etc. (Szabo et al., 2002; Zingarelli et al., 1998).