Chapter 1

Review of Literature
1.1. DIABETES

Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces or it may be culmination of both. Hyperglycaemia, or raised blood sugar, is a common effect of uncontrolled diabetes and over time leads to serious damage to many of the body's systems, especially the nerves and blood vessels. Insulin is a hormone that regulates blood sugar. Diabetes is one of the major causes of premature illness and death worldwide. Mainly, diabetes is of two types Type 1 diabetes and Type 2 diabetes.

Type 1 diabetes mellitus is characterized by loss of the insulin producing β cells of the Islets of Langerhans in the pancreas leading to insulin deficiency. Type 2 diabetes mellitus is characterized by insulin resistance due to reduced responsiveness of the tissue to insulin and is frequently accompanied by subsequent reduced insulin secretion. Type 2 diabetes is a late-onset complex disorder which involves the spectrum of disturbances in metabolic pathways. The risk of developing type 2 diabetes increases with age, obesity and physical inactivity. Type 2 diabetes frequently remains undiagnosed for many years without the classic symptoms. However, if undiagnosed or poorly controlled, type 2 diabetes can lead to the development of chronic complications of micro-vasculature and macro-vasculature. The micro-vascular pathology includes damage in the retina (retinopathy), renal glomerulus (nephropathy) and peripheral nerve (neuropathy). As a consequence of its micro-vascular complications, type 2 diabetes is the leading cause of blindness, end-stage renal disease and neuropathies. Type 2 diabetes is also associated with accelerated macro-vascular disorders, increasing the risk of myocardial infarction and stroke. In addition, type 2 diabetes is most often associated with hypertension that further increases the risk of development of these complications. Type 2 diabetes has become a major health care burden, especially due to these associated vascular complications which has resulted in a large increase in the rates of morbidity and mortality. This accounts for the major fraction of the diabetic population and is most often accompanied with obesity. Obesity is invariably associated with diabetes and a parallel increase in the occurrence of both is evident across all populations (Mokdad et al., 2003 and Zimmet et al., 2001).
1.1.1. Prevalence of Diabetes Epidemic in the World and in India

The prevalence of diabetes has reached epidemic proportions and the world health organization (WHO) has predicted that developing countries will bear the brunt of this epidemic in the 21st century. Currently, more than 70% of people with diabetes live in low- and middle income countries. An estimated 285 million people, corresponding to 6.4% of the world's adult population, has been diagnosed with diabetes in 2010. The number is expected to grow to 438 million by 2030, corresponding to 7.8% of the adult population (Figure 1.1).

While the global prevalence of diabetes is 6.4%, the prevalence varies from 10.2% in the Western Pacific to 3.8% in the African region. With an estimated 50.8 million diabetic population, India leads the world as far as the number of people having diabetes is concerned and the sugar disease is posing an enormous health problem in the country. Calling India the diabetes capital of the world, the International diabetes federation state that there is alarming rise in prevalence of diabetes in India which has gone beyond the epidemic form to a pandemic form. The International Diabetes
Federation estimated that the number of diabetic patients in India has more than doubled from 19 million in 1995 to 40.9 million in 2007. It is projected to increase to 69.9 million by 2025 (International Diabetes Federation. IDF Diabetes Atlas. 4th ed.).

Currently, up to 11 per cent of India’s urban population and 3% of rural population above the ages of 15 have diabetes. The proportion of the urban Indian population will also increase from 33% to 46%. Studies indicate diabetes prevalence in smaller urban centres tends to be about half of that in the larger cities, but it is still twice that of rural areas. In India, a wide range of outcomes for different groups (Mohan et al., 2007) is buried within the average diabetes prevalence of 8%. Prevalence is only 0.7% for non-obese, physically active, rural Indians. It reaches 11% for obese, sedentary, urban Indians; and it peaks at 20% in the Ernakulam district of Kerala, one of India’s most urbanized states. Central obesity which is typified by more upper body adiposity (high waist-to-hip ratio) indicating the deposition of large quantities of abdominal fat, is common in Indians despite low rates of obesity (Ramachandran et al. 2002). Figure 1.2 shows the prevalence of diabetes in different parts of India.

![Figure 1.2: Population based studies showing the prevalence of type 2 diabetes in different parts of India. (Mohan et al., 2007)](image-url)
1.2. THE INSULIN SIGNALING PATHWAY: AN OVERVIEW

The demonstration that insulin acts through the binding to a plasma membrane receptor, dating back from the early '70s, suggested that the altered sensitivity to insulin is observed in obesity, insulin resistance and Type 2 diabetes and could be dependent on a diminished receptor level or weak affinity towards its agonist (Freychet et al., 1971). Since then, the intracellular signaling machinery relaying the metabolic actions of insulin has been elucidated to a good extent, and several of the enzymes taking part in insulin action have been shown to undergo alterations of their physiological function in the insulin resistant state. The discovery of the intracellular steps of insulin signaling has paved the way to intensive investigation aiming at understanding the molecular mechanism responsible for the establishment of the insulin resistant state, both on rodent models and in humans.

The insulin signaling network involves three major pathways, the PI3-kinase, the MAPK, and the Cbl/CAP pathways. These pathways share several signaling elements that intricately affect one another and have some unique characteristics. The MAPK pathway can be considered as a general signaling pathway that is activated by a number of growth factor receptors including insulin, all leading to enhanced cell growth, whereas the PI3-kinase and the Cbl/CAP pathways, triggered by insulin, generate biologic responses that are more unique to insulin action. The action of insulin is initiated by binding to its cognate receptor and activation of the receptor's intrinsic protein tyrosine kinase activity, resulting in the phosphorylation of tyrosine residues located in the cytoplasmic face (Meyts et al., 2002). The activated receptor, in turn, recruits and phosphorylates a panel of substrate molecules. Among these, IRS1 and IRS2 appear to be the adapter molecules playing a major role in the coupling to the PI3K-PKB and MAPK downstream kinases (White, 2002). Tyrosine phosphorylated IRS1/2 recruit the heterodimeric p85/p110 PI3K at the plasma membrane, where it produces the lipid second messenger PIP3, which in turn activates a serine/threonine phosphorylation cascade of PH-domain containing proteins (Alessi and Downes, 1998). PIP3 then phosphorylates PDK1, the serine/threonine protein kinase B (PKB)/Akt, and the atypical protein kinases C ζ and λ isoforms (Vanhaesebroeck and Alessi, 2000, Beeson et al., 2003). Mechanistically, PDK1, PKB and aPKCs, which all contain a PH-domain, are recruited at the plasma membrane by binding to PIP3; thereon, PDK1 phosphorylates PKB and aPKCs on a threonine residue located in the activation loop of
the catalytic domain, causing their activation (Good et al., 1998, Pullen et al., 1998, Stephens et al., 1998). Major targets of activated PKB are GSK-3 (Cross et al., 1995) and AS160 (Kane et al., 2002). Upon PKB-mediated phosphorylation on Serine, GSK-3 is inactivated (Cross et al., 1995). These inactivations, in parallel to protein phosphatase-1 (PP1) activation, relieve the inhibitory phosphorylation of GS, which becomes activated and promotes glycogen synthesis (Brady et al., 1998). PKB also regulates the insulin-stimulated translocation of the glucose transporter GLUT-4 at the plasma membrane, resulting in increased glucose uptake. This pathway involves an inhibitory phosphorylation of the RabGTPase activating protein AS160. Inhibition of AS160 favours the GTP-loaded state of Rab and relieves an inhibitory effect towards GLUT-4 translocation from intracellular compartments to the plasma membrane (Sano et al., 2003). In addition to the role of PKB in controlling GLUT-4 translocation, aPKCs act in parallel or can even be substitutive for PKB (Beeson et al., 2004).

Production and release of glucose by the liver is inhibited by insulin by blocking the pathways of gluconeogenesis and glycogenolysis. It may occur through a direct effect of insulin on the liver (Michael, 2000) as well as by indirect effects of insulin on substrate availability (Bergman and Ander, 2000). Free fatty acid generation from visceral fat can also be influenced by insulin thus affecting glucose metabolism indirectly. This hypothesis is known as “single gateway” (Bergman, 1997). Since visceral fat is less sensitive to insulin than subcutaneous fat, even after a meal there is little suppression of lipolysis by the hormone in this fat depot. This result in direct flux of fatty acids derived from these fat cells through the portal vein to the liver and thus stimulates glucose production and also provides a signal for both insulin action and insulin resistance in the liver. Insulin directly controls the activities of a set of metabolic enzymes by phosphorylation or dephosphorylation and also regulates the expression of genes encoding hepatic enzymes of gluconeogenesis and glycolysis (Pilkis and Granner, 1992). It inhibits the transcription of the gene encoding phosphoenolpyruvate carboxylase, the rate-limiting step in gluconeogenesis (Sutherland et al., 1996). It also decreases transcription of the genes encoding fructose-1, 6-bisphosphatase and glucose-6-phosphatase, and increases transcription of glycolytic enzymes such as glucokinase and pyruvate kinase, and lipogenic enzymes such as fatty acid synthase and acetyl-CoA carboxylase. Although the transcription factors that control the expression of these genes have remained elusive, new data suggest a potential role for the forkhead family
of transcription factors through phosphorylation by Akt-related protein kinases (Nakae et al., 1999), and the PPARg co-activator PGC-1 (Yoon et al., 2001). In normal conditions, the insulin pathway regulates Foxo1 and Foxa2, resulting in regulated glycemia and a balance of hepatic lipid accumulation and oxidation (Wolfrum et al. 2004). Foxa2 also regulates gene expression of rate-limiting enzymes of gluconeogenesis and glycogenolysis in liver and kidney, including phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6PC) and pancreatic-duodenal homeobox factor 1 (PDX1) (Wolfrum et al. 2003). Figure 1.3 gives an overview of insulin regulation of the major metabolic responses.

![Figure 1.3: Overview of the regulation of major metabolic responses in cells by insulin. Binding of insulin to its receptor induces autophosphorylation at a number of tyrosine residues. Certain residues are recognised by the phosphotyrosine-binding (PTB) domain of adaptor proteins termed insulin receptor substrates (IRS), which are recruited to the receptor at the cell membrane. The insulin receptor then phosphorylates IRS molecules at numerous tyrosine residues, some of which are recognised by the Src homology 2 (SH2) domain of the p85 regulatory subunit of a lipid kinase, PI 3-kinase. The catalytic subunit of PI 3-kinase, p110, then phosphorylates PtdIns (4, 5) P2 at the plasma membranes of cells to generate the second messenger PtdIns (3, 4, 5) P3 which stimulates insulin-dependent processes. Binding of insulin to its receptor also causes it to phosphorylate the protein Cbl which is in a complex with the adaptor protein CAP. This result in the Cbl–CAP complex being recruited to a region of the plasma membrane termed a lipid raft. At this location Cbl interacts with the adaptor protein Crk which is constitutively associated with the Rho-family guanine nucleotide exchange factor, C3G. C3G then activates members of the GTP-binding protein family, TC10, which themselves activate unknown effector molecules to promote GLUT4 translocation. Dotted arrows are steps of the pathway that have not yet been fully elucidated. (Lizcano and Alessi et al., 2002)
1.3. LIVER AS A METABOLIC HUB

The liver is one of the insulin target tissue along with the adipose and skeletal tissue. The liver integrates cell-autonomous and cell-nonautonomous mechanisms to control glucose release into the bloodstream. Glucose homeostasis in the body is largely controlled by the liver, and hyperglycemia, as observed in type 2 diabetes, reflects increased hepatic glucose production (Meyer C et al., 1998, Roden M et al., 2001), as well as reduced glucose uptake (Nielsen MF et al., 1998). The role of altered glucagon-to-insulin ratios in hepatic glucose production (HGP) of type 2 diabetics remains unsettled and is of potential therapeutic import. Earlier it was believed that insulin’s ability to reduce hepatic glucose production (HGP) resulted from the direct interaction of the hormone with its receptor in the hepatocyte plasma membrane. This hypothesis was called into question when Prager et al. (Prager et al., 1987) noted that in obese, nondiabetic humans, suppression of glucose production could occur in response to insulin infusion, even when the estimated portal vein insulin concentration did not rise. These results suggested that insulin also reduces hepatic glucose output by indirect mechanisms. Subsequent work by others supported this concept (Sindelar et al., 1996, 1997, Ader and Bergman, 1990), and it is now recognized that insulin can inhibit HGP by both direct and indirect means (Figure 1.4). The indirect actions of insulin on HGP are diverse. Classic mechanisms of indirect control of HGP include release of gluconeogenic precursors from adipose tissue and muscle (FFAs, glycerol, amino acids), adipocytokines (leptin, adiponectin, resistin), neuronal control—possibly mediated through the vagus nerve. In addition, intrahepatic fat plays an important role in promoting HGP, possibly through accumulation of complex phospholipids. Finally, intrahepatic accumulation of resident macrophages has taken center stage in recent years as a potential mechanism of insulin resistance, leading to lipid accumulation and increased HGP. Indeed, the onset of hepatic insulin resistance typically precedes peripheral insulin resistance in humans (Clore JN et al., 1995). The stimulation of gluconeogenesis occurs as a result of increased activity of PEPCK, G 6 Pase and F1, 6 bpase, and the targeted overexpression or knockouts of these enzymes play a major regulatory role in glucose homeostasis (Niswender KD et al., 1997, Sun Y et al., 2002). Glucagon secretion from α cell of the pancreas is diminished by insulin, which in turn causes a reduction in HGP (Ito et al., 1995). Likewise,
nonesterified fatty acid (NEFA) release from the adipocyte is reduced by insulin, and a reduction in the supply of NEFAs to the liver causes an increase in hepatic glycolytic flux, resulting in glucose-6-phosphate exiting the liver after being converted to lactate rather than glucose (Sindelar et al., 1997).

Additionally, the effect of insulin on fat and muscle reduces the supply of gluconeogenic precursors reaching the liver, again reducing HGP (Sindelar et al., 1996, 1998). More recently, insulin’s action in the brain has been postulated to play a role in the regulation of HGP (Obici et al, 2002). It is known that the brain can sense the circulating insulin level (Davis et al., 1995) and that it provides neural input to the liver (Fleur et al., 2000). Further, it has been shown that infusion of insulin into the
third ventricle of rats can reduce glucose production (Obici et al, 2002). Blocking insulin action in the rat hypothalamus impairs the ability of a physiologic rise in circulating insulin to inhibit HGP (Obici et al, 2002). The exploration of insulin’s indirect effects on the liver called into question the physiologic relevance of the hormone’s direct hepatic effect, even though numerous in vitro studies had shown it to exist. Perhaps the best in vivo demonstration of the hormone’s direct effect on the liver comes from studies conducted in the conscious overnight-fasted dog, in which changes in plasma insulin were brought about selectively in the liver using the pancreatic clamp technique. In the absence of any change in arterial plasma glucagon or insulin levels, plasma NEFA level, gluconeogenic precursor load reaching the liver, or insulinization of the brain, hepatic glucose output rose 3-fold when portal vein insulin fell by 75%, and decreased by 50% when portal vein insulin rose by 75% (Sindelar et al., 1998). These data leave no doubt that the liver responds directly, rapidly, and sensitively to the insulin in the plasma perfusing it. Insulin resistance in the liver also leads to accumulation of fats and causes Non alcoholic fatty liver disease (NAFLD), which is identical to that seen in alcoholic fatty liver disease.

The hallmark of the NAFLD is steatosis, histological manifestation of triglycerides within hepatocytes (Figure 1.5). In normal hepatic lipid metabolism, fatty acids are disposed of via either synthesis of TG or oxidation. Fatty acid oxidation in the liver begins with the conversion to acyl-CoA and results in the production of energy (ATP). Several classes of transcription factors, including PPARs and SREBPs, control lipid metabolism in the liver. SREBP-1c is the predominant isoform in the liver, and it regulates other crucial downstream target genes involved in fatty acid and TG synthesis such as fatty acid synthase (FAS), SCD1, and acetylCoA carboxylase (Horton et al., 2002). TNFα is found to be elevated in patients with NASH and increases stepwise from obesity to simple steatosis to NASH (Jarrar et al., 2008). In the liver tissue of NAFLD patients, Crespo et al. (Crespo et al., 2001) identified increased levels of mRNA and a TNFα receptor, p55, and showed that circulating levels correlate with significant fibrosis. In obese (ob/ob) mice fed a high-fat diet, dietary manipulation to favor anti-TNFα pathways and anti-TNFα antibody improved insulin sensitivity, hepatic steatosis, and visible inflammation in the liver (Li et al.,
Pentoxifylline, a phosphodiesterase inhibitor, was shown to prevent production of TNFα, as well as other cytokines, and has had a modest positive effect in small studies of human NAFLD (Harrison, 2006).

Figure 1.5: Current concepts in the pathways of steatosis and steatohepatitis. Many of the current concepts of nonalcoholic fatty liver disease are highlighted. There are three major intersecting components: (1) increased visceral adipose tissue (VAT) and altered systemic and hepatic response to increased insulin (insulin resistance); (2) altered hepatic fatty acid (FA) export, oxidation, and desaturation within the liver; and (3) the initiation and subsequent effects of lipotoxicity. Black arrows highlight the pathways that are known to play significant roles. Gray arrows indicate areas of newer investigation and highlight developing work showing that steatosis and steatohepatitis are not necessarily interdependent processes. Increased caloric intake, in combination with increased saturated fat and fructose consumption, leads to increased VAT; fructose consumption also stimulates de novo lipogenesis (DNL) and does not cause the satiety signaling that occurs with glucose. VAT is a metabolically active tissue that produces numerous proinflammatory cytokines [tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and C-reactive protein (CRP)] and is associated with decreased adiponectin, an adipokine that is anti-inflammatory. Both VAT and hepatic steatosis cause and are caused by hyperinsulinemia and insulin resistance in a feed-forward relationship. VAT also increases delivery of free fatty acids (FFA) to the liver via the portal circulation, resulting in an increased load of FA metabolism within the liver. DNL, increased reesterification of triglycerides, and increased oxidation are all affected. In some patients, compensatory mechanisms to prevent lipotoxicity from the altered FA metabolism fail, and steatohepatitis and fibrosis result. The circulating proinflammatory cytokines from VAT, lack of appropriate desaturation of saturated fatty acids (SFA) to polyunsaturated fatty acids (PUFA), increased reactive oxygen species (ROS) from oxidative stress and endoplasmic reticulum (ER) stress, and increased portal endotoxin have all been speculated to play a role in lipotoxicity. Failure of hepatocyte repair and inflammation perpetuate the process(es) that initiate and promote fibrosis. Abbreviation: VLDL, very low density lipoprotein. (Tiniakos et al., 2010)
1.4. INSULIN RESISTANCE

Insulin resistance is one of the major contributing factors leading to diabetes occurs primarily in the skeletal muscle, adipose tissue and the liver. Numerous investigations over the past decades have revealed a wide array of abnormalities in insulin action in the liver of obese and type 2 diabetic patients, including chronic low grade inflammation, mitochondrial dysfunction, oxidative stress, endoplasmic reticulum stress and defects in various signaling pathways. Insulin resistance has been linked to increased hepatic lipogenesis and steatosis associated with obesity (Leavens and Birnbaum, 2011) (Figure 1.6).

**Figure 1.6:** Consequence of insulin resistance in different tissues and its correlation to type 2 diabetes.

Insulin resistance may be either peripheral (which refers to diminished insulin-mediated uptake of glucose by skeletal muscle and depends primarily on the failure of glucose transporter type 4 (GLUT4) expression and translocation to the plasma membrane) or hepatic insulin resistance (which describes impaired suppression of hepatic glucose production, and largely accounts for hyperglycemia and glucose intolerance).
Moreover, intricate signaling crosstalk and lipid/metabolite trafficking between liver and adipose tissues play important roles in controlling metabolic and glucose homeostasis to accommodate organism energy needs.

The targeted disruption of insulin receptor (IR) gene in mice had been shown to develop severe form of diabetes, with massive fatty acid infiltration in the liver (steatosis) and increased production of ketone bodies (ketoacidosis) (Accili et al. 1996; Joshi et al. 1996). Mice lacking insulin receptor substrate 1 (IRS1) only showed mild insulin resistance whereas insulin receptor substrate 2 (IRS2) deficient mice presented marked glucose intolerance and became diabetic (Withers et al. 1998; Kubota et al. 2000, Taniguchi et al. 2005). Moreover, mice with triple heterozygous knockout for IR, IRS1 and IRS2 were found to develop diabetes (Kido et al., 1994). Also, knock in mice expressing a mutant of PDK1 were insulin resistant and hyperglycemic (Bayascas et al. 2008). Obesity-induced insulin resistance is characterized by a loss of insulin sensitivity mediated by factors released from adipocytes, mainly free fatty acids and proteins, termed adipocytokines, which act to control various metabolic functions (Pittas et al., 2004, Hotamisligil et al., 1994, Cheung et al., 1998, Liu et al., 1998) with well-described physiological effects (Boden and Shulman et al., 2002). One such adipocytokine is tumour necrosis factor α (TNFα), which has been identified as a significant contributor to insulin resistance, and its levels have been reported to be increased significantly in obese diabetic individuals and in several animal models of obesity (Katuski et al., 1998, Hotamisligil et al., 1993, Hamann et al., 1995, Hotamisligil et al., 1995, Kern et al., 1995). Other etiological factors have been proposed in the pathogenesis of obesity-induced insulin resistance that includes oxidative stress, mitochondrial dysfunction, intracellular lipid accumulation in skeletal muscle and liver and decreased β-oxidation (Catalan et al. 2009).

1.5. TNFα

TNFα was originally identified as an endogenous factor, induced by inflammation or mitogenic stimulus and was specifically targeted to tumor cells. It is mainly secreted
by macrophages and other cell types and its expression is upregulated in the adipose tissue/skeletal muscle of both obese insulin resistant human subjects and experimental animals. TNFα is a 233 (25.6 kDa) amino acid-long type II transmembrane protein that organizes into stable homotrimerers from which soluble form of TNFα can be released by the metalloprotease TNFα converting enzyme (TACE). Each 17 kDa protomer in the soluble trimer is 157 amino acids long. TNFα has a short (35aa) N-terminal cytoplasmic tail with a phosphorylation site at Ser2. Both the membrane-bound and the soluble forms of TNFα can initiate cell signaling. It induces the activation of predominantly two signaling pathways within the cell – one involving the transcription factor NF-κB (Nuclear Factor-κB) that lies inactive in the cytosol and is activated on TNFα binding to its receptor on the membrane and the other involves the MAPK (Mitogen Activated Protein Kinase) pathway (Sato et al., 2005, Shim et al., 2005, Wang et al., 2001) Both these pathways underlie the basic mechanism(s) of TNFα action in several pathological conditions. The link between IKKβ activity and insulin resistance is less well understood. IKKβ activation leads to NF-κB translocation to the nucleus, resulting in a feed-forward loop that promotes the synthesis of TNFα and other mediators of inflammation that then may cause insulin resistance (Steinberg et al., 2007) (Figure 1.7). Mice heterozygous for IKKβ are protected against the development of insulin resistance in ob/ob mice as well as high-fat diet-associated obesity (Yuan et al., 2001) Selective low-grade activation of the IKKβ/NF-κB pathway in liver cells results in a state of subacute chronic inflammation with increased production of cytokines, such as TNFα and interleukin (IL)-6, and both hepatic and systemic insulin resistance.

Although primarily known to be associated with inflammation, TNFα now occupies a central place in the crosstalk of many pathological states. It plays a major role in muscular abnormalities resulting in muscle wasting (Li and Reid, 2001), idiopathic pulmonary fibrosis (Pantelidis et al., 2001), rheumatoid arthritis (Mulcahy et al., 1996), Crohn’s disease (Akobeng, 2008) and psoriasis (Leonardi et al., 2003). Several other diseases like cerebral malaria (McGuire et al., 1994), asthma (Berry et al., 2007) and cystic fibrosis also have a strong correlation to TNFα levels.
Chronic inflammation is also frequently associated with the metabolic syndrome and TNFα is one of the significant mediators between the two states. Circulatory TNFα levels that are elevated in obese diabetic individuals inhibit several mediators of the insulin signaling cascade and this leads to attenuation of insulin mediated inhibition of hepatic glucose output (Katuski et al., 1998). In fact, whole body infusion with TNFα is associated with significant increase in hepatic glucose output resulting from impaired ability of insulin to suppress hepatic glucose production (Ling et al., 1994, Lang et al., 1992). TNFα is implicated widely in obesity associated insulin resistance impairs the insulin signaling pathway, yet, its role in hepatic gluconeogenesis in the insulin resistant state and the complex underlying mechanisms involved are not well studied (Katuski et al., 1998, Gupta et al., 2007, Rui et al., 2001).

1.5.1. TNFα Induced Inhibition of Insulin Signaling

The molecular mechanism by which TNFα attenuates insulin signaling is not well understood. Presumably, this entails interface between the TNFα and the insulin
signal transduction cascades at an undetermined site. Recent reports have suggested potential mechanisms whereby TNFα impairs insulin signaling. The ability to inhibit insulin signaling by TNFα in in vitro experiments has facilitated the study of the mechanisms involved in this process. Several reports have shown that TNFα interferes with insulin signaling in various cell lines such as hepatocytes, fibroblasts and myeloid cells (Feinstein et al., 1993, Peraldi et al., 1996, Kroder et al., 1996). IRS-1 is one of the direct substrates of the insulin receptor and is necessary for several of the biological function of insulin (White and Kahn, 1994). The tyrosine phosphorylation of IRS-1 induces the binding of several SH2 domain containing proteins. These associations further induce an activation of the protein (for example the PI3 kinase) or modify the compartmentalization of proteins, bringing them close to their substrates (for Grb2-Sos for example). TNFα suppresses the expression of many proteins that are required for insulin-stimulated glucose uptake in adipocytes, such as the insulin receptor (IR), insulin receptor substrate-1 (IRS-1) and GLUT4 (Stephens et al., 1997, Ruan et al, 2002) (Figure 1.8). Although mechanisms such as accelerated proteasomal degradation (e.g. IRS-1) and impaired translation may be important, more is known about how TNFα alters their expression at the transcriptional level. Amongst the first transcription factors shown to be targeted by TNFα signalling in adipocytes was the adipogenic master regulator, peroxisome proliferator-activated receptor gamma (PPARγ) (Miles et al., 1997, Peraldi et al., 1997, Ruan et al., 2003). TNFα can target PPARγ by inhibiting the expression of PPARγ mRNA and also through suppression of its transcriptional activity. The latter can occur by promoting serine phosphorylation of key regulatory residues in the N-terminal domain of PPARγ (e.g. S112 in murine PPARγ 2 or S84 in human PPARγ1) (Hu et al., 1996, Adams et al., 1997). This phosphorylation event can be mediated by JNK and extracellular signal-regulated kinase (ERK1/2) (Hu et al., 1996, Adams et al., 1997) and has been implicated in TNFα induced suppression of PPARγ in hepatic stellate cells (Sung et al., 2004). Furthermore, in vitro over-expression of PPARγ S112A in 3T3-L1 adipocytes prevents the TNFα mediated downregulation of IRS-1 (Iwata et al., 2001), and mice with a homozygous S112A mutation in PPARγ remain insulin-sensitive on a high fat diet (Rangwala et al., 2003).
Hence, TNFα may suppress PPARγ activity in adipocytes by promoting its phosphorylation. TNFα may also inhibit PPARγ activity through activation of the classic TNFα induced transcription factor, NFκB. Specifically, p65/RelA has been suggested to directly bind to PPARγ in complex with its co-activator, PGC1, thereby preventing binding to PPARγ response elements (Suzawa et al., 2003). This is consistent with the observation that p65 can suppresses PPARγ activity in the absence of any NFκB DNA-binding sites (Ruan et al., 2003). Suppression of PPARγ mRNA levels is also observed following treatment with TNFα. How this is achieved remains incompletely understood. One mechanism stems from the suggestion that PPARγ expression is itself up regulated by PPARγ activity. Hence, in TNFα treated adipocytes, downregulation of PPARγ activity is likely to also result in reduced mRNA levels. This would also be true for another adipogenic transcription factor and PPARγ target gene, CCAAT/enhancer binding protein (CEBPα). TNFα also
suppresses CEBPα mRNA expression. Since the GLUT4 promoter contains response elements for, and is regulated by, both PPARγ and CEBPα (Jain et al., 1999), it is likely that TNFα can suppress GLUT4 expression via a PPARγ and CEBPα-dependent mechanism. Whether this is true for all genes that are downregulated in insulin-resistant adipose tissue is unclear.

1.5.2. TNFα and Lipid Metabolism

The influence of TNFα on lipid metabolism is very complicated and the detailed mechanisms underlying these actions are still not very clear. However, documented data have demonstrated that the mechanisms through which TNFα exerts its effects on lipid metabolism takes place at different levels and different steps, and varied in different cells, tissues, and organs: from increasing free fatty acid (FFA) production to inducing lipolysis, from affecting lipid metabolism-related gene expression to regulating enzymes activity. In addition, TNFα could also affect lipid metabolism by altering the expression and secretion of other adipokines such as leptin, adiponectin, etc.

TNFα increases free fatty acid (FFA) production. FFA is the basic material for neutral triacylglycerols (TAG), the form of lipid droplets in adipocyte synthesis. Generally, there are three FFA sources: (a) from the circulation, (b) from lipolysis of intracellular TAG, or (c) de novo FFA synthesis from glucose (Cawthorn and Sethi et al., 2008). Hence, the availability of relevant substrates and the regulation of several enzymatic pathways might exert on the overall metabolic flux of lipids into TAG. Grunfeld et al. (Grunfeld et al., 1988) showed that bolus intravenous administration of TNFα to normal rats resulted in a rapid stimulation of hepatic FFA de novo synthesis and induced an acute increase in the plasma levels of FFA, which was supposed to be through raising hepatic levels of citrate, an allosteric activator of acetyl-CoA carboxylase. Suppression of liver peroxisomal β-oxidation by inhibiting the activity of peroxisomal fatty acyl-CoA oxidase may also contribute to this process (Beier et al., 1992) Other possible mechanisms include downregulation of the expression of FFA transport protein (FATP), translocase (FAT) in adipose tissue and the FA binding protein (FABP4/aP2), and/or inhibition of the transcript levels and expression of many proteins involved in glyceroneogenesis, de novo FFA synthesis and esterification, which also leads to
impaired triglyceride storage in adipose tissue (Cawthorn and Sethi et al., 2008). However, it is interesting to note that TNFα stimulated plasma FFA production might be diet dependent since TNFα increases plasma FFA in chow-fed rats while in rats fed a high sucrose diet no such phenomenon was observed (Feingold et al., 1990). In liver, TNFα stimulates hepatic fatty acid de novo synthesis through raising hepatic levels of citrate and suppression of liver peroxisomal β-oxidation by inhibiting the activity of peroxisomal fatty acyl-CoA while in adipose tissue; this might be through TG and by regulating FAT and FATP.

1.6. MicroRNAs: ANOTHER REGULATORY DOMAIN FOR DIABETES

While the underlying mechanisms contributing to insulin resistance and metabolic dysregulation remain to be fully elucidated, recent studies have uncovered a key role for small (20–25 nucleotides) noncoding microRNAs (miRNAs) as novel regulators of metabolism (Krützfeldt and Stoffel, 2006). MicroRNAs (miRs or miRNAs) comprise a novel group of small (~22 nucleotide) single stranded non-coding RNAs that are ubiquitously present in plants and animals and act in a sequence specific manner to regulate gene expression at the post transcriptional level by cleavage or translational repression of their target mRNAs. lin-4 and let-7 were the first identified microRNAs (miRNAs) in C. elegans as regulators of the developmental timing in the nematode. Going by numbers, as many as 1212, 679 and 1040 mature microRNAs have been annotated in human, rat and mouse respectively till date and this collectively has been cataloged in the miRNA registry (http://microrna.sanger.ac.uk, V 16.0 September, 2010). With an estimated 3% of human miRNAs targeting hundreds of mRNAs their significance in post-transcriptional gene regulation is unmistakably large (Bartel et al., 2004; Engels and Hutvagner et al., 2006).

1.6.1. MicroRNA Biogenesis and Mechanism of Action

MicroRNA generation is a multi step process, where miRNA genes are first transcribed from genomic loci either as independent units or as part of introns of other protein-coding genes (Du and Zamore, 2005), and like mRNAs, they contain a 5’-cap structure and a poly-A tail (Bracht et al., 2004; Cai et al., 2004). This primary
transcript (pri-miRNA) exists in a hairpin conformation ~200nt long, which is further processed by enzymes downstream. These long RNA precursors (pri-miRNAs) are processed in the nucleus by the RNase, Drosha, which chops off the stem loop to give rise to the subsequent ~70 nucleotide intermediate, the pre-miRNA. Exportin-5 and Ran-GTP export these small duplex pre-miRNA species into the cytoplasm where they are excised by another enzyme, Dicer, to form the functionally active ~22 nucleotide mature miRNA. This duplex miRNA molecule then becomes a part of the RNA induced silencing complex (RISC). In the cytoplasm, one of the strands is eliminated and the other remaining forms the mature miRNA strand.

Figure 1.9: The primary transcripts of miRNAs, called pri-miRNAs, are transcribed as individual miRNA genes, from introns of protein-coding genes, or from polycistronic transcripts. The RNase Drosha further processes the pri-miRNA into 70–100 nucleotide, hairpin-shaped precursors, called pre-miRNA, which are exported from the nucleus by exportin 5. In the cytoplasm, the pre-miRNA is cleaved by Dicer into an miRNA:miRNA* duplex. Assembled into the RISC, the mature miRNA negatively regulates gene expression by either translational repression or mRNA degradation, which is dependent on sequence complementarity between the miRNA and the target mRNA. ORF, open reading frame. (Eva van Rooij, Eric N. Olson, J Clin Invest. 2007)
The first 2-8 bases on the miRNAs form the ‘seed’ sequence that is important deciding factor for the action of specific miRNAs. In plants, miRNAs exhibit a near-perfect match to targets, thereby triggering an RNAi-like mechanism that results in cleavage of target mRNAs, one of the modes of miRNA-mediated mRNA regulation (Jones-Rhoades et al., 2006). In animals, however, miRNAs functions through imperfect base pairing to the 3’ untranslated region (3’UTR) of their target mRNAs and subsequently inhibit translational initiation (Figure 1.9). There are also evidences to show that mRNA destabilization occurs in certain cases (Bagga et al., 2005; Giraldez et al., 2006).

Each miRNA species can inhibit the expression of many genes, while each mRNA can be potentially targeted by several miRNAs. Interestingly, miRNAs display a spatiotemporal pattern of expression and function and therefore are crucial in the regulation of various biological processes in various stages. It is therefore, not surprising that they have been implicated in several diseases like cancer (Hwang and Mendell, 2006, Volina et al., 2006, Gottardo et al, 2007), cardiovascular diseases (Sayed et al., 2007, Thum et al., 2008, Morton et al., 2008), neurological disorders (Saba et al., 2008, Hebert et al., 2008), viral infection (Ghosh et al., 2009, Pfeffer et al., 2004,), asthma (Tan et al., 2007) and diabetes research (Tang et al., 2008, Poy et al., 2004, Lovis et al., 2008).

Unlike in the case of cancer, not much insight has been obtained regarding the contribution of microRNAs in diabetes and the picture that stands as of today is just the beginning towards understanding the complex mechanisms that culminate into the disease phenotype and its complications. Considering the complexity and interplay of several factors that culminate in the manifestation of diabetes, unraveling the contribution of miRNAs in these phenomena will offer significant information in the area of diabetes.

1.6.2. MicroRNAs and Glucose Metabolism

Maintenance of appropriate levels of circulatory glucose levels results from a balance between normal insulin secretion and action. Dysregulation at any step of this fine tuning is responsible for the initiation of Type 1 diabetes and insulin resistance that culminates in Type 2 diabetes. Apart from the various mechanistic regulators of insulin
secretion and action, microRNAs have also emerged as novel regulators of these phenomena and hence are appropriately referred to as “ribo-regulators of glucose homeostasis”. Pancreatic β-cells play a fundamental role in glucose homeostasis, releasing insulin response to glucose levels in the blood. A large number of miRNAs has been implicated in pancreas development including miR-15a/b, miR-16, miR-195, miR-503, miR-541, miR-214, miR-9, miR-124a, miR-7, miR-376 and miR-375 and others. A major player that emerged as a significant mediator of insulin release and thereby of glucose homeostasis is the pancreatic islet specific miRNA, miR-375. It is one of the earliest microRNAs to be identified as possessing a validated functional role in the pancreas where it negatively regulates glucose-stimulated insulin release in a calcium independent manner and its antagonoms (small synthetic chemically engineered oligonucleotide that is used to silence endogenous miRNAs) revert insulin secretion back to the normal (Poy et al., 2004). From a set of its specific predicted targets that included Vti1a (t-SNAREs yeast homologue 1A that is critical in insulin vesicle biogenesis and recycling), Mtpn (myotrophin), MAPK14 (p38 mitogen activated protein kinase), Slc16A2 (monocarboxylic acid transporter member 8) and Mxi1(Max interacting protein 1 with a role in β-cell differentiation), all with a potential role in β-cell function and insulin secretion, the authors found that overexpression of miR-375 led to significant reduced levels of the Mtpn and Vti1a protein; however transfection with 2’-O-Me (2’-O-methyl oligoribonucleotide that inhibits the miRNA) could increase only the levels of Mtpn with no effects on the levels of Vti1a (Poy et al., 2004). The 3’UTR of Mtpn harbors a binding site for miR-375 that when bound inhibits Mtpn expression. This inhibition is not observed when the binding site is mutated to reduce the complimentarity between the microRNA and the Mtpn mRNA. Functionally Mtpn is involved in modulation of the actin network that affects membrane docking and fusion (Tang et al., 2008, Gauthier et al., 2006, Cuellar et al., 2005). This strongly correlates to insulin vesicle exocytosis in the pancreas. Additionally inhibition of Mtpn with siRNA also attenuates insulin release. All these indicate towards a direct sturdy role of miR-375 and its target, myotrophin in insulin release from the pancreas that ultimately determines glucose homeostasis within the body. Quite interestingly in a later study miR-375 was identified as the most abundant intra-islet miRNA (Bravo-Egana et al., 2008).
Recently, miR-375 has also been reported to target PDK1 in the pancreatic islet cell (Ouaamari et al., 2008). Its elevated expression in the pancreatic islets of diabetic Goto-Kakizaki rats indicates towards its role in diabetes. Using INS-1E insulinoma cells and rat primary islets, it was observed that miR-375 directly binds to the 3’UTR of PDK1 and inhibits its protein level. PDK1 in the presence of phosphatidylinositolosites generated by PI3K activates Akt/ PKB. Glucose stimulation of insulin gene expression via PDX-1 also involves the PI3K pathway and a recent experiment in this connection (Ouaamari et al., 2008) unravels a novel angle of regulation of this pathway wherein miR-375 modulates glucose mediated stimulatory effect on insulin gene expression by targeting PDK1. Earlier it was reported that β-cell specific ablation of PDK1 induces diabetes accompanied with a reduction in β-cell mass (Hashimoto et al., 2006). Along with these reports of the roles of miR-375 in pancreatic function, its presence and significance in the pancreas is also substantiated by the fact that its knockdown is accompanied by severe morphological defects in the pancreatic islets in zebrafish (Kloosterman et al., 2007). miR-375, therefore, appears to be the most well studied as far as the regulation of insulin release and glucose homeostasis is concerned. Another microRNA, miR-9 has been reported as a strong candidate and regulator of insulin exocytosis from the pancreas (Plaisance et al., 2006). The pancreatic exocytotic machinery for insulin involves the participation of several proteins that are under direct and/or indirect control of several factors. Elevated levels of miR-9 inversely correlated with glucose stimulated insulin release. This effect of miR-9 on insulin release was preceded by elevated levels of the Rab GTPase effector, Granuphilin/Slp4 (Coppola et al., 2002, Gomi et al., 2005) and this is regulated by the direct miR-9 target, Onecut2 (OC2) that inhibits the expression of Granuphilin/Slp4. While overexpression of OC2 downregulated Granuphilin transcription, it’s silencing by RNAi replicated the effects of miR-9 on granulophilin expression and insulin exocytosis. All these indicate miR-9 to be explicitly involved in insulin exocytosis from the pancreas. Likewise, miR-96 also negatively regulates insulin exocytosis by targeting synaptotagmin like 4(Lovis et al., 2008), and reduces levels of Noc2, which impairs β-cell ability to respond to secretagogues. In a later study, using miRNA microarray, Baroukh et al. (Baroukh et al., 2007) found that miR-124a strongly correlated with mouse pancreatic development suggesting its role in β-cell differentiation. Looking for predicted miR-124a targets,
F Foxa2 emerged and was subsequently validated as the one with an identified role in pancreatic β-cell development. Overexpression of miR-124a inhibited and anti-miR-124a could withdraw this inhibition on Foxa2 and its downstream target, Pdx-1. Under identical conditions, mRNA levels of other significant regulators of pancreatic development and function namely, Kir-6.2 and Sur-1 also depicted identical patterns of expression though this appears not to be a direct effect. It can be said quite conclusively that such miR-124a regulation of Foxa2 and subsequently of Pdx-1, Kir-6.2 and Sur-1 suggests a central role of this miRNA in the fine-tuning of targets that together comprise a major fraction of determiners of global pancreatic development and function. Very recently, Tang et al. employed an identical microarray approach and found 61 glucose regulated miRs from a total of 108 miRs in the mouse insulinoma cell line, MIN6 (Tang et al., 2009). Of these, most of the miRs were upregulated and only few that included miR-296, miR-184 and miR-160 were downregulated. miR-30d that was significantly elevated here was subsequently found to mediate glucose stimulated insulin gene expression while inhibition of this microRNA attenuated this effect. miR-30d overexpression or inhibition by itself, however, did not have any effect on insulin gene transcription. The authors thereby suggest that miR-30d and its targets may be potential regulators of insulin gene expression. Recently Trajkovski et al. (Trajkovski et al., 2011) showed that miR-103 and miR-107 are regulators of insulin sensitivity and they showed increased hepatic expression in rodents and humans with insulin resistance and hepatic steatosis. They showed that miR-103/107 targets Cav1, thereby diminishing the number of insulin receptors in caveolae-enriched plasma membrane microdomains and reducing downstream insulin signaling.

A recent report by Lovis et al. (Lovis et al., 2008) documented the altered miRNA pattern that contributes to free fatty acid (FFA)-induced pancreatic β-cell dysfunction. Microarray analysis revealed several miRNAs to be altered by palmitate in MIN6B1 cells and after appropriate validation and replication studies, only miR-34a and miR-146 were analysed for their further role in the pancreas. Supportively, their levels were also elevated in the islets of diabetic db/db mice that parallels the elevated plasma free fatty acid concentrations. Looking beyond these alterations, it was found that miR-34a is allied to p53 activation that is an inducer of apoptosis in several diseases (Vousden
and Lane, 2007) and also to Bcl2 inhibition (Bommer et al., 2007), especially in the pancreas they are known to be involved in apoptosis of insulinoma cell lines (Wrede et al., 2002, Kim et al., 2005). FFA mediated regulation of p53 via miR-34a, is therefore a novel mode of regulation of pancreatic apoptosis initiated by FFA. Within the islets as well, miR-34a affects hormone secretion by targeting VAMP2, a vesicle protein that is involved in insulin exocytosis. The other miRNA that was identified by Lovis et al. (Lovis et al., 2008) was miR-146 that acts by targeting IRAK1 and TRAF6, both of which are involved in pancreatic β-cell death (Taganov et al., 2006, Dunne et al., 2003, Ortis et al., 2006, Abderrahmani et al., 2007). These miRNAs therefore underlie some of the negative effects of free fatty acids on pancreatic function and survival that mimics the state of obesity associated Type 2 diabetes.

Another miRNA highly elevated in the skeletal muscle of diabetic GK rats is miR-29 that has been implicated in inhibition of insulin action. Adenovirus mediated overexpression of this miRNA in 3T3-L1 adipocytes significantly repressed insulin stimulated glucose uptake that was accompanied by inhibition of insulin stimulated Akt activation. However, the total levels of the Akt protein were unchanged by miR-29 overexpression indicating that Akt is not the direct target gene of miR-29 and the effects of miR-29 on insulin action could involve other mediators (He et al., 2007). Another significant intermediate, Insulin Receptor Substrate-1 (IRS-1) is a major mediator of insulin signaling and its mutation or dysfunction has been associated with diabetes (Baroni et al., 2001, Marini et al., 2003, Zeng et al., 2000). Although in a different context, miR-145 has been recently identified to target and downregulate the IRS-1 (Insulin Receptor Substrate 1) protein in human colon cancer cells (Shi et al., 2007) and this targeting has elaborate effects on the growth and proliferation of these cells. Considering the role that IRS proteins play in insulin signaling and thereby on glucose homeostasis, it may be worthwhile to undertake in depth studies to unravel the role of this miRNA in insulin action, if any.

1.6.3. MicroRNAs and Lipid Metabolism

It has now been established beyond doubt that alterations in lipid metabolism contribute to insulin resistance and diabetes. Abnormalities of triglyceride storage and
lipolysis in insulin-sensitive tissues are an early manifestation of conditions characterized by insulin resistance. Increased free fatty acid (FFA) flux from adipose tissue to nonadipose tissue, resulting from abnormalities of fat metabolism, participates in and amplifies many of the elementary metabolic derangements that are notable traits of the insulin resistance syndrome and type 2 diabetes (Lewis et al., 2002). The precise biochemical mechanisms whereby fatty acids and cytosolic triglycerides exert their effects resulting in the diabetic phenotype remain poorly understood. With the discovery of microRNAs and emerging evidences of their regulation of lipid metabolism, a new paradigm that was until now not completely unknown is gradually being exposed. Initial studies in this regard began with the identification of miR-14 as a regulator of fat metabolism in Drosophila melanogaster (Xu et al., 2003). miR-14 null animals had increased levels of triglycerides and diacylglycerol that reverted back on increasing the copy numbers of the microRNA. Another microRNA involved in energy homeostasis in Drosophila is miR-278 (Teleman et al., 2006) and miR-278 mutants in spite of having elevated insulin production capacities depict increased circulatory glucose levels indicating a loss of insulin responsiveness. Being prominently expressed in the fat, its involvement in the regulation of fat metabolism is not really unexpected and of the mRNAs that miR-278 targets, expanded was the most noteworthy because of the elevated levels of its mRNA in miR-278 mutants. Expanded loss-of-function mutants cause tissue overgrowth (Blaumueller et al., 2000) in parallel with increased miR-278 expression. miR-278 mutants exhibit a lean phenotype indicating towards its involvement in maintaining a balance between fat accumulation and utilization. Around the same time, Esau et al. (Esau et al., 2006) revealed the role of the liver specific miR-122 as a significant regulator of hepatic lipid metabolism. In normal mice, inhibition of miR-122 with antisense oligonucleotides led to an increase in hepatic fatty acid oxidation accompanied with a decreased rate of fatty acid and cholesterol synthesis in the liver. More importantly the circulatory cholesterol levels were also reduced indicating that miR-122 inhibition may be a significant module for lowering plasma cholesterol levels that is elevated in several metabolic diseases. In an obese mouse model miR-122 inhibition not only lowered plasma cholesterol levels but also significantly improved liver steatosis and the status of several hepatic lipogenic enzymes specifically phosphomevalonate kinase. Such a role of miR-122 in the liver is also
substantiated by an earlier report (Kratzfeldt et al., 2005) wherein the authors have used antagonisirs against miR-122 and concluded that genes of the cholesterol biosynthetic pathway are the most affected by miR-122 and in vivo antagonimsr inhibition of this microRNA significantly reduced circulatory cholesterol levels.

The miRNA paralogs, miR-103 and miR-107 have recently been reviewed (Wilfred et al., 2007) with a prediction of their involvement in metabolism. Of these absolutely conserved vertebrate intronic miRNAs that exist within the pantothenate kinase (PANK) gene, miR-103 genes encode for two mature miRNAs, miR-103-1 and miR-103-2 while the miR-107 gene encodes miR-107 that differs from miR-103 by a single nucleotide. The host gene, PANK catalyses the rate limiting steps of pantothenate phosphorylation during the generation of Coenzyme A (CoA) that is a critical cofactor of several enzymes involved in diverse metabolic pathways. Although there is no experimental report as of now regarding the role of these miRNAs in regulating lipid metabolism; bioinformatics predictions suggest a possible role similar to their host gene, PANK, in regulation of acetyl CoA and lipid metabolism. Such a coordinated and symbiotic function between the host gene and the microRNA is quite expected considering the co-relatable expression patterns between them (Baskerville and Bartel, 2005).

When it comes to lipid metabolism, the adipose tissue emerges as the most critical organ and considering the parallel escalating numbers of obesity and diabetes, this tissue has of late surfaced as a significant mediator of this complex disease. It therefore is even more thrilling to study the regulation of adipogenesis by microRNAs and in an effort in this direction; Esau et al. (Esau et al., 2004) using antisense oligonucleotides studied the role of miRNAs in adipocyte differentiation. Using a human adipocyte model system, the authors profiled the miRNA pattern between preadipocytes and differentiated adipocytes. From the set of differentially regulated miRNAs, miR-143 was singled out particularly since its elevated expression levels paralleled with adipocyte differentiation and inhibition of miR-143 with an antisense oligonucleotide inhibited the same. While hunting around for the targets of this microRNA, the authors reported that ERK5/BMK1 could be one of the possible mediators of the link between miR-143 and adipocyte differentiation and it may be involved in maintaining a balance between proliferation and differentiation of adipocytes (Esau et al., 2004). Although the
authors did not completely dissect out the direct or indirect association between miR-143 and ERK5, they did conclude the possibility of exploiting miR-143 as a potential target for therapeutic intervention for obesity and metabolic diseases. miR-320 increases insulin sensitivity of insulin resistant adipocytes (Ling et al., 2009) and miR-27b impairs human adipocyte differentiation (Karbiener et al., 2009). These miRNAs target conserved core regulatory pathways that are affected both locally and systematically by obesity and by diabetes generally. miR-320 targets p85, which plays a critical role in cell growth by increasing Akt phosphorylation and thus the level of Glut 4 (Ling et al., 2009). Karbiener et al showed that miR-27b targets peroxisome proliferator-activated receptor γ.

1.6.4. MicroRNAs and Diabetic Complications

Almost all forms of diabetes are invariably characterized by end stage specific pathological complications in the renal glomerulus, peripheral nerve and the retina. Another significant long-term diabetic complication is hypertension and heart valve defects which later on manifest as cardiac hypertrophy that is characterized by thickening of the myocardial wall and reduction of the ventricular chambers. Just as microRNAs are critical in the development and progression of diabetes, currently emerging reports also associate altered levels of a range of miRNAs with these diabetic complications. miR-133 is one of the most abundant microRNAs present in the adult cardiac and skeletal muscle in mammals where they are critical in regulating myogenesis. miR-133 was one of the first miRNAs reported to be overexpressed in the hearts of diabetic rabbits and this was accompanied by a parallel increase in the expression of serum response factor (SRF) (Xiao et al., 2007). Serum response factor (SRF) is a member of the MADS (MCM1, AGAMOUS, DEFICIENS, SRF) box family of nuclear transcription factors that interacts as a dimer with a 10 bp AT-rich sequence on the DNA known as the serum response element (SRE) (Miano, 2003, Treisman, 1992). It plays an important role in cardiac development and function and regulates the expression of a wide variety of inducible genes by various stimuli ranging from growth factors to changes in intracellular calcium flux (Johansen et al., 1995). More specifically, SRF has also been implicated in the regulation of genes encoding non-contractile cardiac proteins, including the sarcoplasmic reticulum Ca²⁺ ATPase (SERCA2) and the Na/Ca²⁺ exchanger (NCX1) (Fisher, 1996, Cheng et al., 1999).
Further support for an important role for SRF in cardiac function comes from transgenic experiments in rodent model systems demonstrating that cardiac-specific dysregulation of SRF expression induces cardiac hypertrophy and cardiomyopathies in postnatal animals and this mimics those that are observed during the initial development of congestive heart failure, indicating that SRF is involved in cardiac pathogenesis (Zhang, 2001, 2001). The increase in miR-133 levels in the diabetic heart was also accompanied with a decrease of ERG (ether-a-go-go related gene) and I\textsubscript{kr} (rapid delayed rectifier K\textsuperscript{+} current) protein levels. The increase in the levels of SRF in the diabetic heart is invariably accompanied with a prolonged QT (an indicator of the cardiac electrical activity) syndrome, a potentially dangerous situation that may lead to cardiac arrest (Collins et al., 2006). All these effects could be reversed using miR-133 specific antisense oligonucleotides. Such a decrease in HERG (human ERG) protein levels possibly is responsible for repolarisation, the observed QT prolongation and the associated arrhythmias in diabetic hearts. Apart from these, along with miR-1, miR-133 also determines the pathogenesis of cardiac hypertrophy where both these miRs are significantly downregulated. In a rat model of cardiac hypertrophy, hyperpolarisation activated cyclic nucleotide gated channels encoded by HCN2 and HCN4 were considerably increased accompanied by significant reductions of miR-1/miR-133 levels (Luo et al., 2008). HCN channels generate I\textsubscript{f} (hyperpolarisation activated current) which contributes to the genesis of the cardiac pacemaker activity. Four different HCN genes have been identified (Moosmang et al., 2001). HCN1 is the most rapidly acting channel, HCN4 the slowest with HCN2 and 3 possessing intermediate kinetics (Biel et al., 2002). HCN4 is the most highly expressed in the SA (sino-auricular) node and HCN2 expression is prominent in the atrium, ventricle and SA node. Overexpression of miR-33/miR-1 significantly inhibited the increase in HCN2 and HCN4 levels. Elevated levels of HCN2 and 4 are hallmarks of arrhythmia (Michels et al., 2005, Plotnikov et al., 2008) and subsequently the hyperpolarisation activated current (I\textsubscript{f}) is strikingly increased in animal models of cardiac hypertrophy and heart failure (Stilli et al., 2001, Cerbai et al., 1997, Hoppe et al., 1998). In fact, miR-1 has been shown to be overexpressed in individuals with coronary heart disease (CAD) and its targeted overexpression in normal hearts manifests an identical phenotype associated with arrhythmias (Yang et al., 2007). Mechanistically this microRNA targets KCNJ2 (that
encodes the K+ channel subunit Kir2.1) and GJA1 (encoding connexin 43) that consequently slows down the cardiac conduction resulting in depolarization of the cytoplasmic membrane. All these effects could be attenuated in the presence of an antisense miR-1 inhibitor thereby indicating a role of this microRNA in cardiac physiology. A very recent article on miRNA related myocardial dysfunction as observed in diabetes was reported by Wang et al. (Wang et al., 2009).

Abnormal expression and signaling of many angiogenic factors are some of the many impaired parameters of diabetes and several cardiovascular diseases correlate to insufficient myocardial angiogenesis that is mediated by these abnormal angiogenic factors. By employing a miRNA microarray, these authors report that of all the miRs altered in diabetic myocardial microvascular endothelial cells (MMVECs) as compared to normal MMVECs, miR-320 emerged as a potential mediator with a predicted target list that includes several angiogenic factors and their receptors namely VEGF, IGF-1, IGF-1R and FGF (Wang et al., 2009) that are significant mediators of diabetic cardiomyopathy (Chen et al., 2005, Shan et al., 2003). This revelation by Wang et al. (Wang et al., 2009) of elevated levels of miR-320 in diabetic MMVECs was also accompanied by decreased proliferation and migration rates that amazingly reverted back in the presence of the miR-320 inhibitor. Such a correlation between elevated levels of miR-320 and decreased IGF-1 and IGF-1R levels possibly underlies impaired angiogenesis in diabetes. All these indicate that although the current literature regarding these aspects is at a very nascent stage, miRs are critical in the proper functioning of the heart and thereby implicated in cardiac pathophysiology. A very significant diabetic complication is that of the kidney where the membrane of the glomerulus shows extreme thickening and gets hypertrophied (Ziyadeh, 1993) possibly due to accumulation of extracellular matrix (ECM) proteins namely collagen 1α1 and 2. The ECM proteins are an integral part of the capillary basement membrane and mesangial matrix and they majorly include various types of collagens, laminin, fibronectin, and proteoglycans (Price and Hudson, 1987). Zampetaki et al showed that there is a strong correlation between miR-126 levels and the onset of diabetic vascular complications (Zampetaki et al., 2010). Interestingly this miRNA is significantly increased in patients suffering of coronary artery disease
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and delivery of this miRNA by apoptotic bodies protects against diet-induced atherosclerosis (Fichtlscherer et al., 2010). This indicates that miR-126 can be used as both a biomarker for early detection of vascular complications of diabetes and as a possible RNA-based therapeutic for diabetes induced atherosclerosis.

A very strong underlying factor behind the accumulation of these ECM proteins as is observed in a diabetic kidney is the transforming growth factor β1 (TGF-β). The diversely bared and still being discovered regulatory roles of microRNAs in the pathogenesis of various diseases and the almost exclusive presence of at least five microRNAs in the kidney (Yin et al., 2004) undoubtedly indicate towards their involvement in kidney function and disease. A recent article has depicted the role of miR-192 in the kidney and in the pathogenesis of diabetic nephropathy (Kato et al., 2007). Using microarray analysis, it was found that collagen 1α1 mRNA is increased by TGF β in mouse mesangial cells with a concomitant decrease in the mRNA levels of E-box repressors, δEF-1 and Smad-interacting protein 1 (SIP1). While looking for the possible roles of miRs in these phenomena, the authors found that miR-192 levels were elevated by TGF β in these cells and interestingly, SIP1 is a validated target of miR-192 (Kato et al., 2007). Both SIP1 and δEF-1 are repressors of Col1α2 expression and this repression is withdrawn under diabetic conditions initiated by TGF β. Since TGF β elevates the levels of miR-192 and downregulates SIP1, a target of miR-192, the authors concluded that the observed increases in collagen synthesis and accumulation observed in a diabetic kidney was due to the elimination of the SIP1 repression of collagen expression due to increased miR-192 levels. Such correlations were also observed in a streptozotocin induced diabetic mouse model as well as transgenic db/db diabetic mice. Kidney glomeruli in both these induced and transgenic diabetic species depicted elevated levels of TGF-β1, collagen 1 and 2 and miR-192. All these observations suggest that small non-coding miRs, in this case miR-192 and their inhibitors, could possibly be targets of diabetic nephropathy and other associated diabetic complications. Another matrix protein that is excessively accumulated in the diabetic kidney is fibronectin. Fibronectin, a large glycoprotein consisting of two similar polypeptide chains, is a key component of the mesangial matrix (Romberger, 1997). It may exist in a soluble dimeric form or as oligomers of fibronectin or a highly
insoluble fibrillar form in the extracellular matrix. The latter form has been shown to
modulate various biological processes such as cell adhesion, migration, and
differentiation (Schwarzbauer et al., 1999). In a recent article, Wang et al., (Wang et al.,
2008) reported that in cultured human and mouse mesangial cells exposed to high
glucose and transforming growth factor β as well as in a mouse diabetic nephropathic
model, miR-377 was consistently upregulated. In a computational study, fibronectin did
not emerge as a direct predicted target of miR-377 but two proteins namely p21-
activated kinase and superoxide dismutase, which enhanced fibronectin production
surfaced as miR-377 targets. Experimentally too, an increase of miR-377 led to reduced
levels of these two proteins. So, although indirectly, elevated levels of miR-377 in turn
increases fibronectin levels that accumulate in the kidney matrix and this emerges as
phenotype of diabetic nephropathy.

Basically, four main hypotheses are associated with hyperglycemia induced diabetic
complications, namely increased polyol pathway flux, increased advanced glycation end
product (AGE) formation, activation of protein kinase C (PKC), and increased
hexosamine pathway flux (Brownlee, 2001). Advanced glycation end products (AGEs)
act via their receptors (RAGEs) and interact and modify several intracellular proteins
and other extracellular matrix components that then depict altered functions. Another
well studied ligand that interacts with RAGE is the proinflammatory peptide, S100b that
belongs to the S100/ calgranul in family (Schmidt et al., 1994, Hofmann et al., 1999) and
these interactions are critical in inflammation and diabetic atherosclerosis (Hofmann et
al., 1999, Bucciarelli et al., 2002). Mechanistically along these lines, binding of S100b to
its receptor significantly inhibited the expression of miR-16 in human THP-1 monocytic
cells that consequently altered the mRNA stability of the inflammatory gene,
cyclooxygenase-2 (COX-2) by binding to its 3’UTR (Shanmugam et al., 2008). An
interesting protein that modulates this interaction is the heterogeneous nuclear
ribonuclear protein K (hnRNPK) that binds to the COX-2 promoter in the nucleus.
Exposure to S100b mimics the diabetic milieu and this displaces the nuclear hnRNPK
that translocates to the cytoplasm and interacts with the 3’UTR and prevents the binding
of miR-16. Such an intracellular crosstalk between microRNAs and RNA binding
proteins may underlie the acute regulation of diverse genes particularly those related to
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inflammation under diabetic conditions. This attains significance considering the fact that studies over the couple of years has revealed a strong association of diabetes and inflammation. Considering that each miR can target several genes and each gene can also be regulated by several miRs (Miranda et al., 2006), the regulatory story of miRs becomes even more complicated. The pathogenesis of a disease as complex as diabetes adds on to the complexity of these studies. Yet emerging evidences suggest that miRs play significant roles in insulin production, action and secretion and also in diverse aspects of glucose and lipid metabolism (Table 1.1). Most importantly, microarray studies have highlighted an altered prolife of microRNA expression in insulin target tissues in *invitro* and *in vivo* diabetic models. All these indicate that microRNAs are critical in the pathogenesis and progression of diabetes and its complications and appropriate therapeutic intervention targeted towards their altered levels may offer novel valuable tools for the treatment of a metabolic disease as complicated as diabetes.

Table 1.1: List of miRNAs in Diabetes (Selene et al., 2011, Pandey et al., 2009)

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Tissue</th>
<th>Relevance to diabetes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-375</td>
<td>Pancreas</td>
<td>Expressed in pancreatic development. Regulates insulin secretion in β-cells and increases their death by lipoapoptosis, as it regulates this cell viability and proliferation. Upregulated in β-cells of T2D patients. Its deletion causes severe insulin-deficient diabetes in <em>ob</em>/<em>ob</em> mice.</td>
<td>Poy et al., 2004, Lynn et al., 2007, Jogelkar et al., 2009, Kloosterman et al., 2007, Li et al., 2010, Ouaamari et al., 2008, Xia et al., 2010, Poy et al., 2009, Zhao et al., 2010, Avnit-Sagi et al., 2009</td>
</tr>
<tr>
<td>miR-29</td>
<td>Adipose, Liver</td>
<td>Induced by high glucose and high insulin. Overexpression leads to insulin resistance and Gluconeogenesis</td>
<td>Herrera et al., 2009, Herrera et al., 2010, He et al., 2007, Pandey et al., 2011</td>
</tr>
<tr>
<td>miR-143</td>
<td>Adipose</td>
<td>Participates in adipocyte differentiation and is induced in adipogenesis and downregulated in obesity.</td>
<td>Xi et al., 2009, Esau et al., 2004</td>
</tr>
<tr>
<td>miR-9</td>
<td>Pancreas, Cardiac muscle</td>
<td>Expressed in pancreatic development. Impairs insulin secretion in β-cells and is upregulated in cardiomyocytes of STZ-induced diabetic mice.</td>
<td>Jogelkar et al., 2009, Plaisance et al., 2006</td>
</tr>
<tr>
<td>miR-124a</td>
<td>Pancreas</td>
<td>Uregulated by glucose. Regulates the insulin exocytosis pathway, causing exaggerated insulin release when no glucose is available but reduced glucose-induced insulin secretion.</td>
<td>Baroukh et al., 2007, Lovis et al., 2008, Tang et al., 2009</td>
</tr>
<tr>
<td>miRNA</td>
<td>Tissue</td>
<td>Relevance to diabetes</td>
<td>References</td>
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<tr>
<td>miR-195</td>
<td>Pancreas, Liver</td>
<td>Expressed in pancreatic development and upregulated in liver of diabetic rats.</td>
<td>Joglekar et al., 2007, Herrera et al., 2010</td>
</tr>
<tr>
<td>miR-192</td>
<td>Kidney</td>
<td>Induced by transforming growth factor-β and highly expressed in glomeruli of STZ-induced diabetic mice. Targets SIP1.</td>
<td>Kato et al., 2007</td>
</tr>
<tr>
<td>miR-222</td>
<td>Adipose</td>
<td>Upregulated in response to high glucose in adipose tissue of diabetic rats.</td>
<td>Herrera et al., 2010</td>
</tr>
<tr>
<td>miR-126</td>
<td>Pancreas, Skeletal muscle</td>
<td>Expressed in pancreatic development. Upregulated in skeletal muscle of GK rats and in livers of ob/ob mice compared with STZ mice.</td>
<td>Lynn et al., 2007, Li et al., 2009, Huang et al., 2009</td>
</tr>
<tr>
<td>miR-133a</td>
<td>Cardiac, Skeletal muscle</td>
<td>Overexpressed in rabbit diabetic heart, where it induces prolongation of QT interval. Downregulated in cardiac hypertrophy in mouse and human hearts and in hearts of STZ-induced diabetic mice. Also reduced in human skeletal muscle in T2D. High fasting glucose associates with lower expression of this miRNA.</td>
<td>Granjon et al., 2009, Liu et al., 2007, Care et al., 2007, Xiao et al., 2007, Fred et al., 2010</td>
</tr>
<tr>
<td>miR-296</td>
<td>Pancreas</td>
<td>Expressed in β-cell islets and upregulated by glucose.</td>
<td>Ouaamari et al., 2008</td>
</tr>
<tr>
<td>miR-96</td>
<td>Pancreas</td>
<td>Negatively regulates insulin exocytosis through upregulation of granuphilin.</td>
<td>Lovis et al., 2008</td>
</tr>
<tr>
<td>miR-34a</td>
<td>Pancreas, Liver</td>
<td>Increases in β-cells in response to palmitate, making them more susceptible to death by apoptosis and inhibiting nutrient-induced insulin secretion. Upregulated in the livers of STZ-induced diabetic mice. Found in the bloodstream and can differentiate between nondiabetic and early T2D patients.</td>
<td>Lovis et al., 2008, Li et al., 2009, Kong et al., 2011, Roggli et al., 2010</td>
</tr>
<tr>
<td>miR-146b</td>
<td>Pancreas</td>
<td>Contributes to increased apoptosis of β-cells. Expression induced by cytokines and sodium palmitate.</td>
<td>Fred et al., 2010, Roggli et al., 2010</td>
</tr>
<tr>
<td>miR-30d</td>
<td>Adipose</td>
<td>Upregulated in presence of high glucose, upregulates insulin gene transcription.</td>
<td>Tang et al., 2009</td>
</tr>
<tr>
<td>miR-320</td>
<td>Cardiac vascular endothelium</td>
<td>Upregulated in GK rats with impaired angiogenesis.</td>
<td>Wang et al., 2009</td>
</tr>
<tr>
<td>miR-103</td>
<td>Pancreas, Liver</td>
<td>Overexpression accelerates adipogenesis. Reduced in response to TNF-α. Downregulated in obesity. Upregulated in liver of ob/ob mice (vs. STZ-induced diabetic mice) and diabetic rats.</td>
<td>Herrera et al., 2010, Xie et al., 2009, Li et al., 2009</td>
</tr>
<tr>
<td>miR-107</td>
<td>Pancreas, Adipose</td>
<td>Overexpression accelerates adipogenesis. Reduced in response to TNF-α. Upregulated in β-cells in presence of high glucose.</td>
<td>Tang et al., 2009, Xie et al., 2009</td>
</tr>
<tr>
<td>miRNA</td>
<td>Tissue</td>
<td>Relevance to diabetes</td>
<td>References</td>
</tr>
<tr>
<td>---------</td>
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<tr>
<td>miR-1</td>
<td>Cardiac, Skeletal muscle</td>
<td>Regulates cardiac arrhythmogenic potential. Upregulated by high glucose in cardiomyocytes, where it accelerates apoptosis. High levels found in ventricle of diabetic patients. Impaired insulin response in skeletal muscle of T2D patients. Significantly downregulated in the heart of STZ-induced diabetic mice.</td>
<td>Granjon et al., 2009, Feng et al., 2010, Xiao et al., 2007, Shan et al., 2010, Yang et al., 2007</td>
</tr>
<tr>
<td>miR-223</td>
<td>Heart</td>
<td>Upregulated in the insulin-resistant heart, where it increases glucose uptake through increase of Glut4.</td>
<td>Lu et al., 2010</td>
</tr>
<tr>
<td>miR-125</td>
<td>Liver, Vascular tissue</td>
<td>Upregulated in liver of hyperglycemic rats. Increase of this miRNA results in a proinflammatory diabetic phenotype in vascular smooth muscle cells.</td>
<td>Villeneuve et al., 2010, Herrera et al., 2009</td>
</tr>
<tr>
<td>miR-27</td>
<td>Adipose</td>
<td>Impairs human adipocyte differentiation and targets peroxisome proliferator–activated receptor γ. Upregulated in adipose tissue of diabetic rats and by glucose in 3T3 adipocytes.</td>
<td>Herrera et al., 2010, Karbiener et al., 2009</td>
</tr>
<tr>
<td>miR-216a, miR-217</td>
<td>Kidney</td>
<td>Highly expressed in kidney and upregulated by transforming growth factor-β. Activates Akt signaling through targeting of PTEN.</td>
<td>Kato et al., 2009, 2010</td>
</tr>
<tr>
<td>miR-122</td>
<td>Liver</td>
<td>Suppression in liver results in reduced fatty acid accumulation. Downregulated in liver of STZ-induced diabetic mice.</td>
<td>Li et al., 2009, Esau et al., 2006</td>
</tr>
<tr>
<td>miR-320</td>
<td>Adipose, Vascular endothelium</td>
<td>Highly upregulated in insulin-resistant adipocytes. Targets p85, leading to increased insulin resistance in adipocytes. Upregulated in myocardial microvascular cells in GK rats, where it impairs angiogenesis.</td>
<td>Ling et al., 2009, Wang et al., 2009</td>
</tr>
<tr>
<td>miR-21</td>
<td>Pancreas, Liver</td>
<td>Upregulated by nuclear factor-κB and fatty acids in liver, leading to downregulation of its target PTEN. Induced by interleukin-1β and TNF-α in pancreatic islets. Expression increased in rats on high-fat diet and in liver of T2D patients. Overexpression reduces maximal glucose-induced insulin release in β-cells.</td>
<td>Vinciguerra et al., 2009, Roggli et al., 2010</td>
</tr>
<tr>
<td>miR-206</td>
<td>Cardiac, Skeletal muscle</td>
<td>Upregulated in skeletal muscle of diabetic and prediabetic patients. Upregulated by high glucose in cardiomyocytes. Accelerates cardiomyocyte apoptosis.</td>
<td>Gallagher et al., 2010, Shan et al., 2010</td>
</tr>
<tr>
<td>miR-93</td>
<td>Vascular endothelium</td>
<td>Downregulated by high glucose through downregulation of its host gene MCM7.</td>
<td>Long et al., 2010</td>
</tr>
<tr>
<td>miR-181d</td>
<td>Liver</td>
<td>Most effective miRNA at reducing intracellular lipid content of hepatocytes.</td>
<td>Whittaker et al., 2010</td>
</tr>
</tbody>
</table>
Aims and Objectives
AIMS AND OBJECTIVES

The study proposes to attain the following specific objectives:

1. To analyse the gene expression profiling in HepG2 cells by TNFα.
2. Exploring the molecular mechanism by which TNFα induces insulin resistance in liver.
3. To study the differential spectrum of microRNAs in control and diabetic mice liver.
4. To functionally evaluate the role of miRNA-target interaction as the potential targets for therapeutic intervention in diabetic conditions, using in vitro models.