Chapter 1 Introduction

1.1. Adiponectin

Adipose tissue is an important location of energy storage and is essential for energy balance. It accumulates triglycerides as a store of energy during nutritional plentitude and provides it as free fatty acids during nutritional deficiency (Kahn, 2000; Spiegelman & Flier, 2001). Basically, adipose tissue as a site of energy storage supports survival in times of malnourishment on the other hands, it is related to metabolic syndromes in the existing nutritionally abundance environment. It is well established that various hormonal signals and nuclear hormone receptors regulate adipose tissue functions (Lowell, 1999; Chawla *et al.*, 2000).

In recent years, adipose tissue is considered as vital endocrine tissue that releases various biologically active substances known as adipocytokines. The major effects of adipocytokines are on food intake, lipids and carbohydrates metabolism and varied other effects in human body (Havel, 2002) (Figure 1.1).

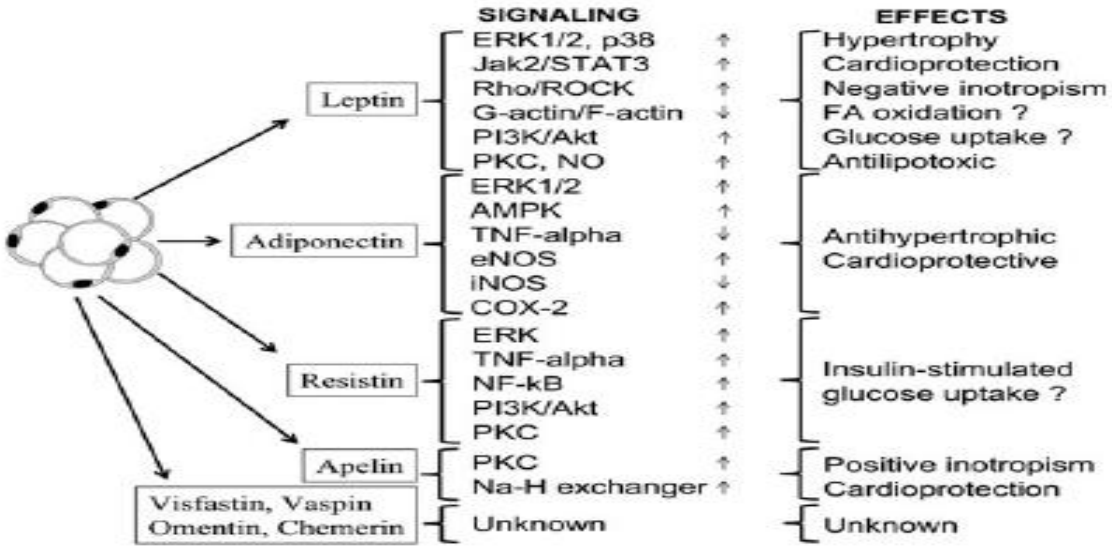

Figure 1.1: Summary of the diversity of cell-signalling responses to adipokines. (Karmazyn *et al.*, 2008)

In mid nineties some studies in adipocytology showed that there is a type of plasma protein that is derived from adipocytes. This was recognized by different research groups separately and later named adiponectin. Since then, adiponectin had also been called other names such as Acrp30 (Scherer *et al.*, 1995) and AdipoQ (Hu E *et al.*, 1996). These two groups of researchers cloned mouse cDNAs for adiponectin by differential display before and after differentiation of mouse 3T3-L1 and 3T3-F442A cells. In another study, human adiponectin cDNA was isolated by large-scale sequencing of a 3'-directed human adipose tissue cDNA library (Maeda *et al.*, 1996). Finally, human adiponectin was purified from plasma as a gelatin binding protein and called GBP28 (Nakano *et al.*, 1996).

Since the discovery of adiponectin, many research reports and reviews have been published regarding its role in the regulation of carbohydrate and lipid metabolism, metabolic syndromes, cardiovascular disorders (Sun *et al*., 2009) (Figure 1. 2).

It has been shown that blood plasma levels of adiponectin decrease in subjects with type II diabetes and obesity especially accumulation of visceral adipose tissue (Hotta *et al.*, 2000; Matsuzawa *et al.*, 2004). Noticeably, blood adiponectin levels were adversely correlated with insulin resistance indexes and serum triglycerides, and episodically with high density lipoprotein (Xita *et al.*, 2005). Furthermore, it has been demonstrated, that patients with reduced adipose tissue mass have obviously higher circulating levels of adiponectin (Delporte *et al.*, 2003; Housova *et al.*, 2005) (Figure 1.3).

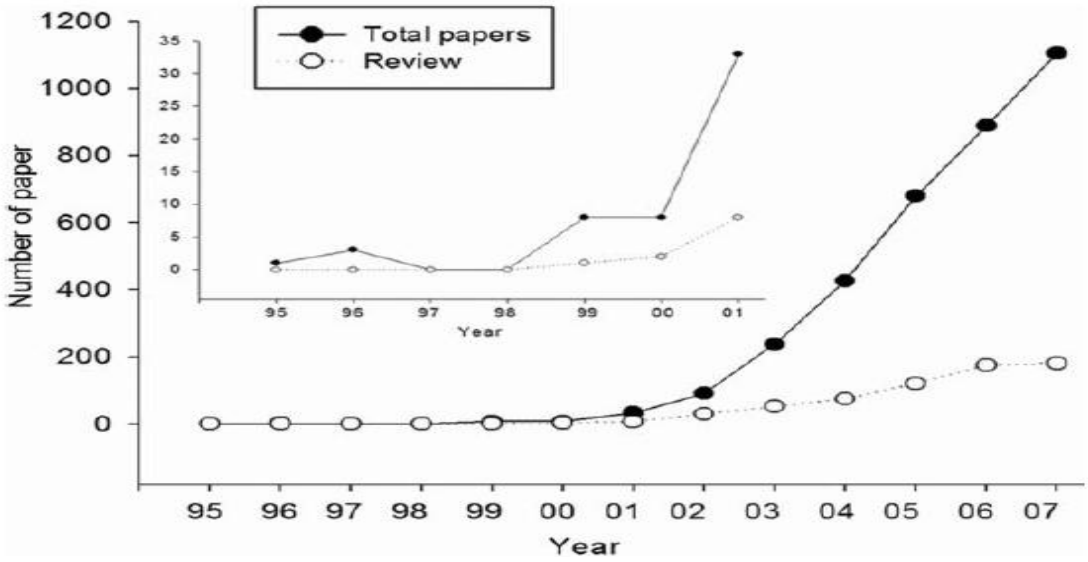

Figure 1.2: Research interest in adiponectin, a protein secreted by adipose tissue. Many research reports and reviews have been published regarding the role of adiponectin in the regulation of metabolism, metabolic syndromes and cardiovascular disorders (The publication was obtained from PubMed by Sun *et al.*, 2009)

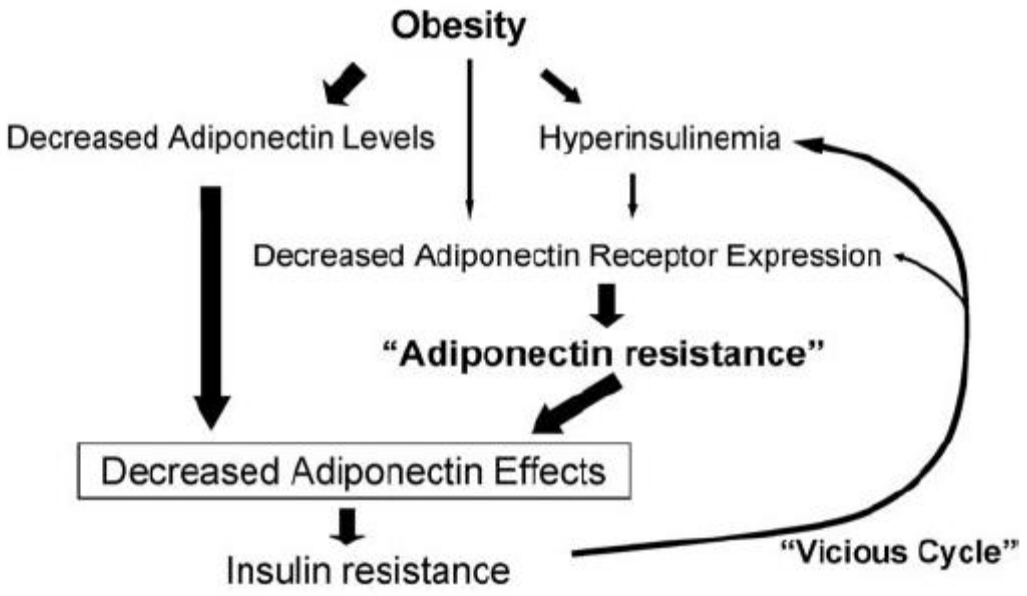

Figure 1.3: The relationships between plasma adiponectin levels, obesity and insulin resistance. It has been shown, that the increase in adipose tissue mass leads to decrease the circulating levels of adiponectin and the expression of its receptors causing insulin resistance (Tsuchida *et al.*, 2004).

1.1.1. Adiponectin gene characteristics

Adiponectin has been identified as biologically active substance secreted by adipose tissues and organizes the functions of other organs. It has been evident that human adiponectin is encoded by the *ADIPOQ* gene which spans 17 kb on chromosome locus 3q27. *Adiponectin* gene consists of three exons ranging from 18bp to 4277bp in size, with consensus splice sites and two introns of 0.8 and 12kb. The start codon location is in exon 2 whereas the stop codon is located in the exon 3 (Saito *et al.*, 1999; Takahashi *et al.*, 2000).

Interestingly, human chromosome 3q27 has been recognized as the area carrying susceptibility locus for metabolic syndrome and type II diabetes (Kissebah *et al.*, 2000; Vionnet *et al.*, 2000). Adipose tissue has been known as a main source of adiponectin hormone (Scherer *et al.*, 1995). Additionally, adipose tissues produce and release many other biologically active substances that are regarded as adipocytokines (Matsumoto *et al.*, 2002). A study was carried out by Madea *et al.* (1997) to identify the biological characteristic of adipose tissue; they carried out gene expression profile analysis in the abdominal and subcutaneous adipose tissues. Subsequently, they made random complementary DNA (cDNA) sequencing by a 3'-directed cDNA library to determine the active genes. By searching GenBank database, it was found that more than 60% of total genes in 1000 independent clone were recognized as identified genes whereas 40% of total genes were novel and unidentified genes. These results indicated that the adipose tissue contain high percentage of genes encoding secreted proteins. Additionally, the percentage of these genes were higher (30%) in visceral fat tissue than subcutaneous fat tissue (20%) (Funahashi *et al.*, 1999).

1.1.2. Adiponectin protein characteristics

Adiponectin protein consists of three different domains including the variable domain, collagenous domain and globular domain (Shapiro & Scherer, 1998). The amino-terminal domain is variable depending on the species. This is followed by the collagenous domain which includes 22 Gly-X-Y repeats that is similar in sequence to other proteins which called collagen VIII and collagen X. Similarly, the carboxyl–terminal domain of adiponectin is called globular domain which is homologous with other proteins like the complement factor C1q, collagen alpha 1(X), and the brain-specific substance cerebellin what is named globular domain as well (Hu *et al*., 1996) (Figure 1.4).

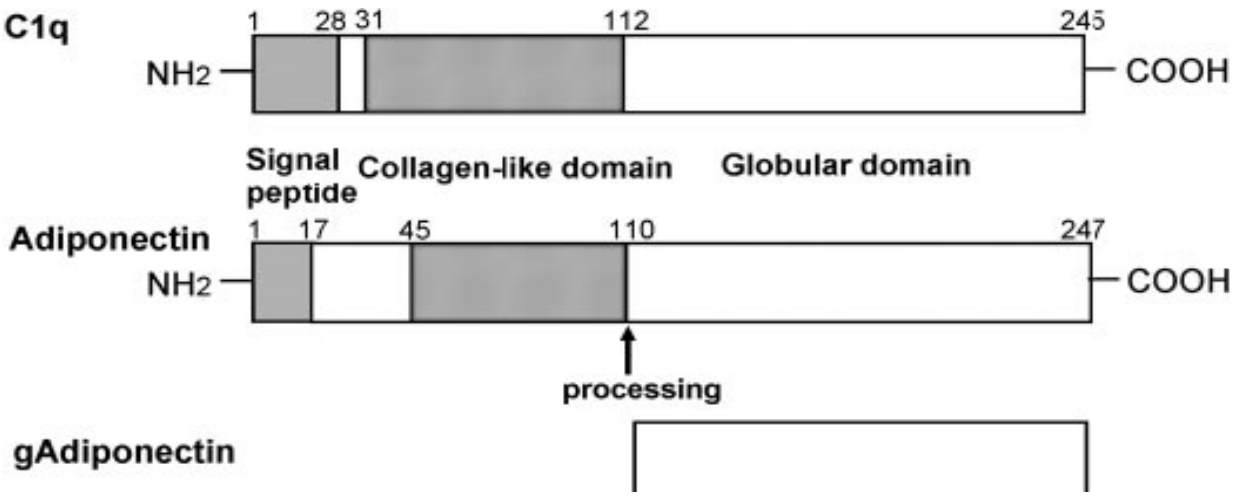

Figure 1.4: Structure and adiponectin domains as described by Kadowaki & Yamauchi (2005). Adiponectin protein consists of variable, collagenous and globular domains staring with adiponecin signal peptide (17 amino acids). The entire molecule of adiponectin can be processed to produce separated globular domain which is normally exist in blood plasma.

Some studies were carried out to characterise the adiponectin protein using different techniques including X-ray and SDS-PAGE analysis. These studies indicated complex structures of adiponectin protein that exist as monomer and multimer. For example, the crystal structure of the globular domain was determined at 2.1 Å resolutions. Interestingly, the structure exposed an unanticipated homology to the necrosis factor (TNF-α) of the family adipocytokines. Despite the differences at the primary level of amino acid sequence, the structural characteristics between TNF-α and globular domain of adiponectin are much conserved. It has been shown that adiponectin globular domain and TNF-α both have ten β strand of folding topology and they are able to form homotrimeric oligomers (Shapiro & Scherer, 1998).

It has been evident by SDS-PAGE analysis that adiponectin exists in blood serum at different molecular weight forming trimer, hexamer and multimer, and a small amount of adiponectin globular domain in trimeric form (Waki *et al.*, 2005). Supposedly, adiponectin globular domain is produced by proteolytic cleavage of the full length adiponectin by activated leukocytes (monocytes and neutrophils) that produce elastase enzyme which is able to catalyze full length adiponectin to produce globular domain in blood serum. However, the physiological significance of *in vivo* adiponectin cleavage by leukocytes enzyme is still to be fully explained (Waki *et al.*, 2005). It has been demonstrated that simple SDS-PAGE under non-reducing and non-heat-denaturing conditions clearly separates multimeric species of adiponectin. Interestingly, specific mutations in adiponectin gene result in an impaired multimerization and secretion of adiponectin from adipocytes (Waki *et al.*, 2003). Additionally, disulfide bond formation by Cys-39 in the variable domain is essential in oligomerization formation of adiponectin. This hypothesis has been proved by expression of mutant adiponectin with a substitution of Cys by Ser at codon 39 which could not form multimers more that trimers (Fruebis *et al.*, 2001; Waki *et al.*, 2003; Onay-Besikci *et al.*, 2004) (Figure 1.5).

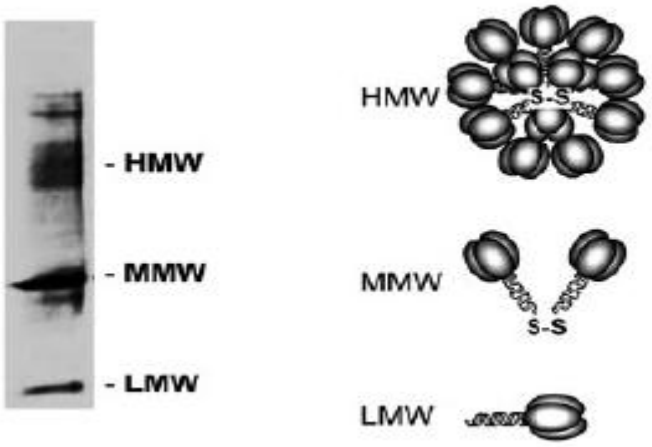

Figure 1.5: Multimer formation of adiponectin. HMW stands for high molecular weight, MMW stands for medium molecular weight, and LMW stands for low molecular weight and S–S stand for disulfide bond (Kadowaki & Yamauchi, 2005).

1.1.3. Glycosylation of adiponectin protein

The post translation modification of adiponectin has been shown to play central role in adiponectin protein stability (Peake *et al.*, 2007). It has been revealed that glucosylgalactosyl residues support the conformation of high molecular weight (HMW) conformation of human plasma adiponectin. Additionally, the HMW isoform includes higher amounts of glucosylgalactosyl residues than the low molecular weight (LMW) isoform, and these saccharides are essential in supporting adiponectin stability *in vivo* (Peake *et al.*, 2007).

It has been documented that hydroxylation and glycosylation of the four lysines in the adiponectin collagenous domain at position 68, 71, 80 and 104 are significant in the biological activity of adiponectin (Wang *et al.*, 2004) (Figure 1.6). Furthermore, post-translation modification on these four lysines has been shown to play an important role in enhancing adiponectin bioactivity especially in inhibiting endogenous glucose production by hepatocytes (Wang *et al.*, 2002). Additionally, adiponectin protein was recognized to be a 2, 8-linked disialic acid-containing protein as well, although the biological role of the disialic acid of adiponectin has not been examined (Sato *et al*., 2001). Mass spectrometry studies have confirmed the existence of post translational modification in human adiponectin and distinguished three additional hydroxylations on Pro71, Pro76 and Pro95 (Richards *et al.*, 2006). The role of the modified lysines in multimer formation has been examined in the secretion of human adiponectin protein produced in mammalian cells. Mutation of modified lysines in the collagenous domain prohibited formation of HMW multimers, while the chemical inhibition of prolyl- and lysyl-hydroxylases prevented formation of hexamers and HMW multimers (Richards *et al.*, 2006).

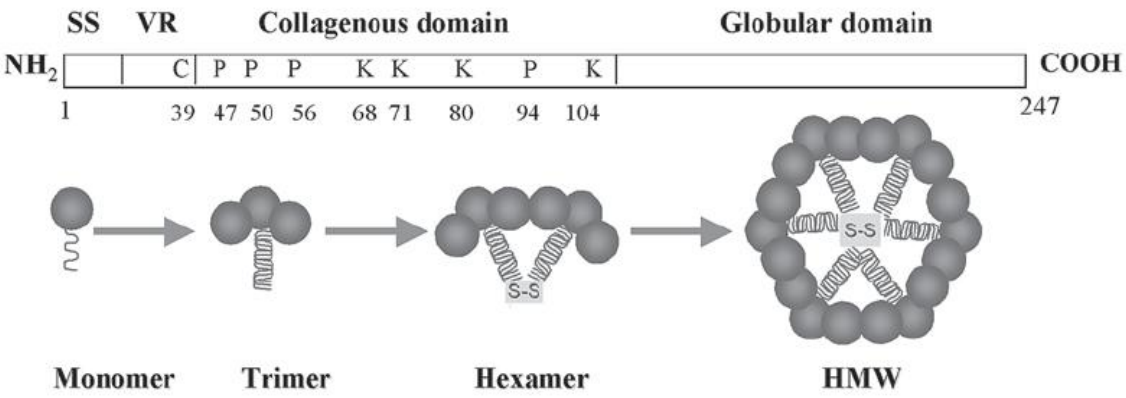

Figure 1.6: Structural characteristics of adiponectin protein. The hydroxylation and glycosylation of the lysines and prolines and disulfide bond formation in the adiponectin collagenous domain are significant in the formation of adiponectin isoforms which is important in its biological activity. The numbers stand for to the sites of hydroxylated prolines or lysines. SS: signal peptide sequence; VR: variable region. "S–S" is disulfide bond mediated by cysteine residue 39 at the variable region. (Xu *et al.*, 2007).

1.1.4. Regulation of *adiponectin* gene expression

Adiponectin levels in blood plasma decrease in humans with abdominal obesity, and correlate negatively with insulin resistance (Arita *et al.*, 1999; Yamamoto *et al.*, 2004). It has been evident that some factors have direct or indirect effects on adiponectin gene expression and plasma adiponectin levels through different stages of adiponectin formation (Figure 1.7). One of these factors is an oxidative stress, which has been suggested to inhibit the expression of *adiponectin* (Furukawa *et al.*, 2004). Furthermore, male humans and rodents have lower plasma adiponectin levels than females, elucidating that sexual hormones control the production of adiponectin. However, the mechanism by which these hormones, such as testosterone and estrogen are affecting on plasma adiponectin level is not well identified (Combs *et al.*, 2003; Xu *et al*., 2005). Other factors may affect directly or indirectly on *adiponectin* gene expression. For example adiponectin expression is up-regulated during the progression of adipocyte differentiation under the effect of insulin (Halleux *et al.*, 2001) and it has been shown that IGF-1 up-regulates adiponectin gene as well, whereas Tumor Necrosis Factor (TNF-α) and glucocorticoids reduce *adiponectin* gene transcription (Halleux *et al*., 2001; Fasshauer *et al*., 2002). This is especially true for Tumor Necrosis Factor (TNF-α) because it is considered as a strong inhibitor of adiponectin promoter activity (Maeda *et al.*, 2001). However, the mechanism of the regulation of *adiponectin* gene expression remains to be studied because the plasma adiponectin level is affected by other factors, including lifestyle, aging and gender.

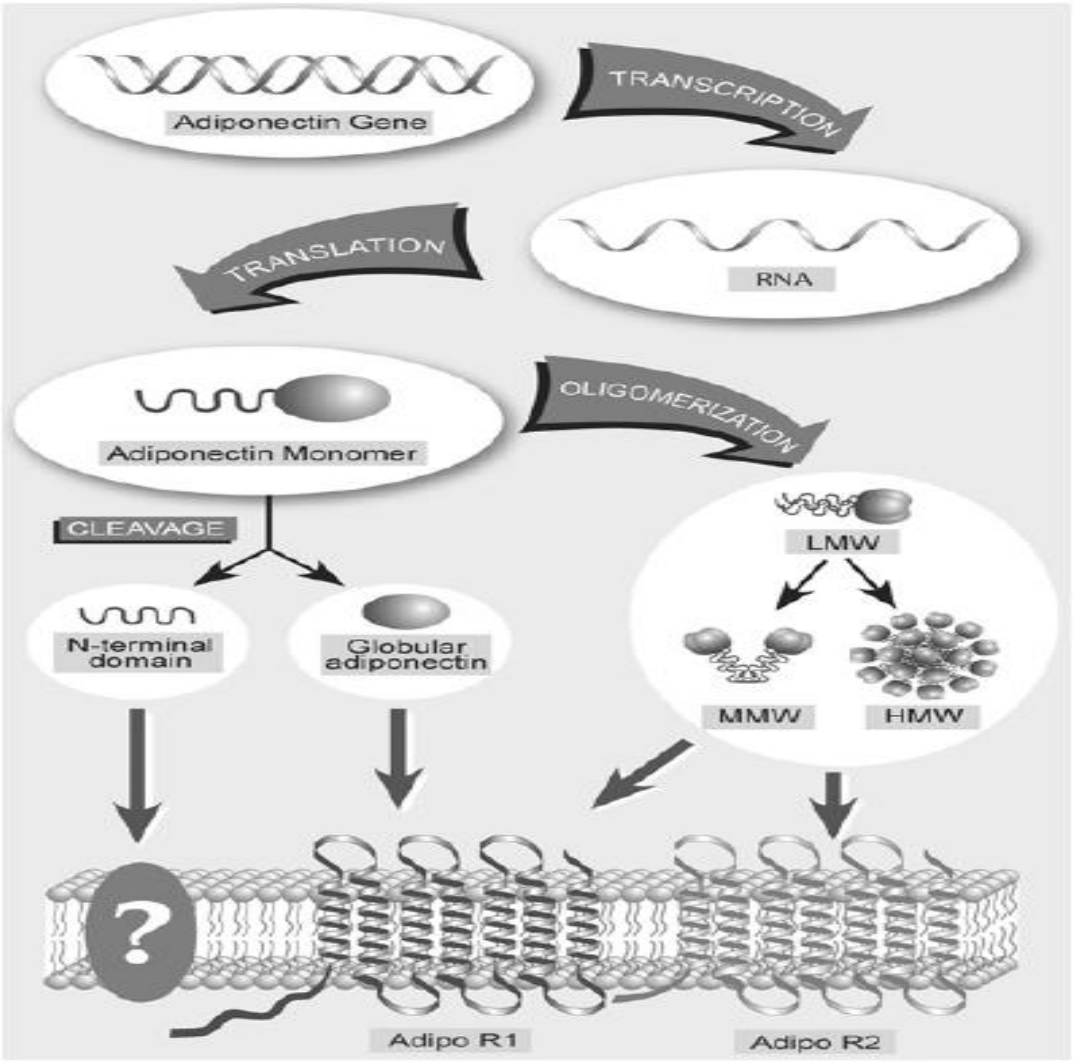

Figure 1.7: Regulation of adiponectin synthesis and function. Adiponectin isoforms influence differently on adiponectin receptors (Adipo R1 and Adipo R2). LMW: Adiponectin trimers, MMW: Adiponectin hexamers, HMW: Adiponectin oligomers, Adipo 1 and Adipo 2: adiponectin receptors (Fang & Sweeney, 2006).

It is also well acknowledged that peroxisome proliferator-activated receptor γ (PPARγ), is identified as a transcriptional activator of various adipocyte-specific genes (Iwaki *et al.*, 2003). Recent studies have shown that adiponectin expression and secretion was improved by the administration of rosiglitazone or pioglitazone which are commonly used to reduce insulin resistance and glucose intolerance in type II diabetes as a PPARγ agonists thiazolidinediones (TZDs) (Yamauchi *et al.*, 2001; Pajvani *et al.*, 2004) (Figure 1.8). These experimental observations suggest that nuclear receptor PPARγ plays an important role in the adiponectin gene expression and secretion from adipocytes especially transcriptional activation by a functional PPARγ receptive element in adiponectin promoter. Study of Wolf (2008) showed that adiponectin binds to thiol protein ERp44 in the lumen of adipocyte endoplasmic reticulum and released by another thiol protein called Ero1-Lalpha. Nuclear receptor PPARγ ligand agonists appear to regulate this retention-release mechanism. The exact mechanism of *adiponectin* gene induction awaits further investigation (Wolf, 2008).

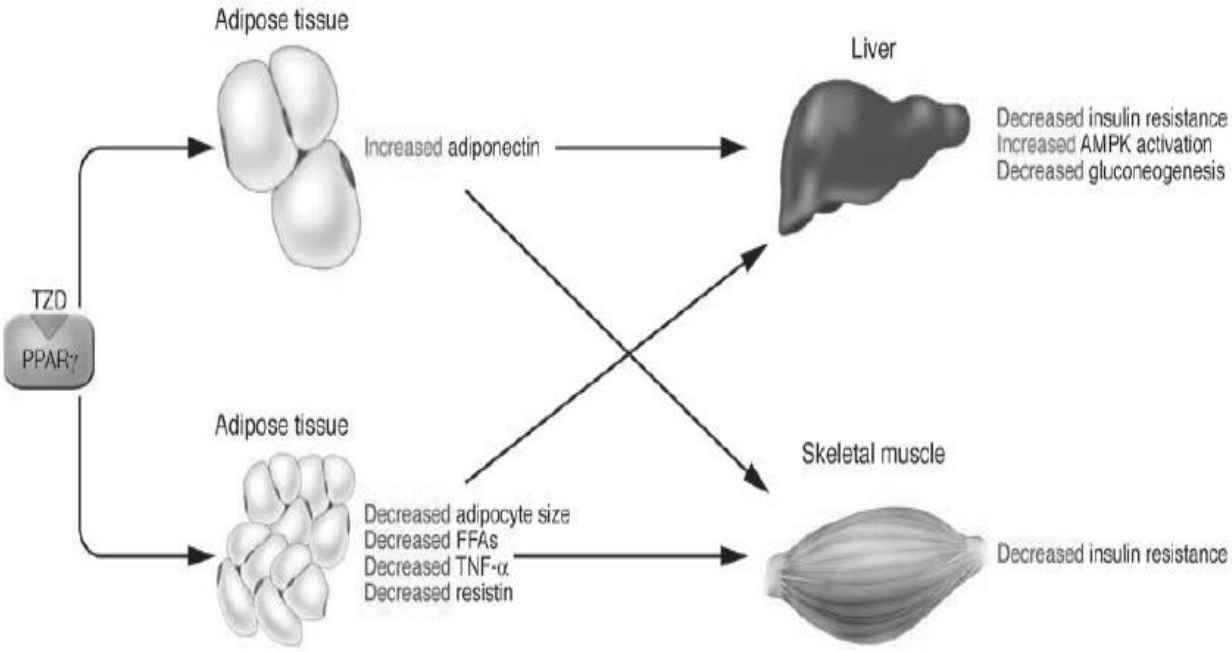

Figure 1.8: PPARγ agonists thiazolidinediones (TZDs) ameliorate insulin resistance and diabetes by both adiponectin-dependent and -independent pathways. (Kadowaki *et al.*, 2006).

1.1.5 *Adiponectin* single nucleotide polymorphisms (SNPs)

Adiponectin levels in blood plasma depend on interactions between genetic factors and environmental factors. For example, SNPs in the *adiponectin* and inactive life style that causes obesity may play a central role in the development of the metabolic syndrome such as insulin resistance and type II diabetes, (Kadowaki & Yamauchi, 2005).

As mentioned, *adiponectin* gene spans 17 kb on chromosome locus 3q27 and human chromosome 3q27 has been identified as a region carrying susceptible locus for metabolic syndrome. Consequently, the *adiponectin* gene is considered a promising susceptibility candidate gene for metabolic syndromes (Kissebah *et al.*, 2000).

Hara *et al.* (2002) explained that one single nucleotide polymorphism (SNP) in the *adiponectin* was associated with reduced plasma adiponectin levels and development of type II diabetes. This SNP is located 276 bp from 5'-terminus of the site of translational start site (Hara *et al.*, 2002) (Figure 1.9). Additionally, the existence of G allele or T allele in locus 276 was associated with lower and higher levels of plasma adiponectin respectively (Hara *et al*., 2002; Fredriksson *et al.*, 2006). Therefore, in the case of SNP 276, the G/G genotype was identified to be linked with increase glucose intolerance (Gonzalez-Sanchez *et al.*, 2005). Additionally, individuals who have G/G genotype have higher risk for type II diabetes compared with those with the T/T genotype. It has been documented that more than 40% of Japanese people have the G/G genotype; this genotype increases vulnerability to type II diabetes due to inherited reduction of plasma adiponectin levels (Hara *et al.*, 2002).

In addition, another SNP was detected, as an existence of G allele in position 45 which has a link with developing glucose intolerance (Filippi *et al.*, 2004). To support

these findings, Menzaghi *et al.*, demonstrated that the presence of genotypes 45G and 276T in *adiponectin* gene are tightly correlated with insulin resistance markers such as waist circumference, body weight and HDL cholesterol (Menzaghi *et al.*, 2002). Another study has been carried out on the French Caucasian population. Same study indicated that there is a clear connection between the presence of SNP 11377 and SNP 1139 in adiponectin promoter and other mutations in exon three with the ability of adipocyte to secrete adiponectin protein. These SNPs may be part of the genetic determination of hypoadiponectinemia that may lead to type II diabetes (Vasseur *et al.*, 2002). In general, these findings provide support the hypothesis that adiponectin may play an essential role in the development of type II diabetes.

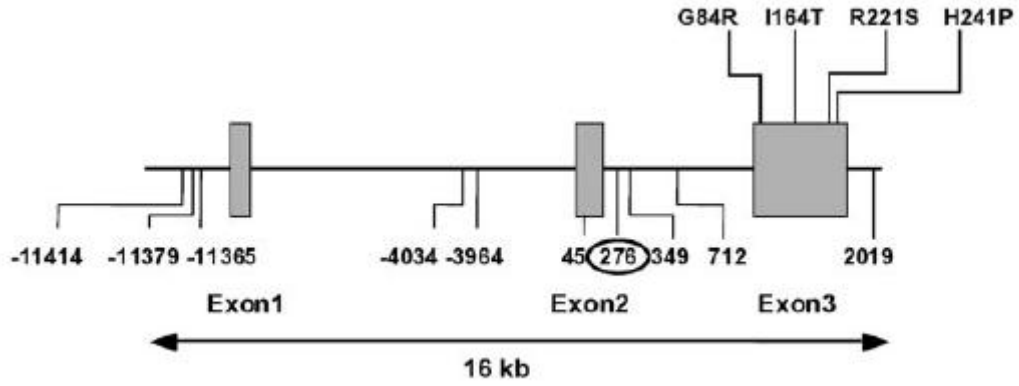

Figure 1.9: Diagram of polymorphic variants of the *adiponectin* gene Single nucleotide polymorphism is located 276 bp from 5'-terminus of the site of translational start site. The existence of G allele or T allele in locus 276 was associated with lower and higher levels of plasma adiponectin respectively. The case of SNP 276, the G/G genotype was identified to be linked with increase glucose intolerance (Kadowaki & Yamauchi, 2005).

1.1.6. Adiponectin biological activity

Adiponectin is secreted from adipocytes into the circulation and has been known as an insulin-sensitizing adipokine with plural functions such as anti-diabetic, anti-atherogenic, cardioprotective properties and anti-inflammatory (Wang *et al.*, 2008). Additionally, it has been found that *adiponectin* gene may be expressed, at a less extent, in other organs due to detection of its expression in hepatocytes (Yoda-Murakami *et al.*, 2001), myotubes (Staiger *et al.*, 2003; Delaigle *et al.*, 2004), bone cells (Berner, 2004) and cardiomyocytes (Pineiro *et al.*, 2005).

Plasma adiponectin levels are relatively higher than other hormone levels (5–30 μg/ml). Under normal physiological conditions, plasma adiponectin presents 0.01% of total plasma proteins within very slight changing (Scherer *et al.*, 1995). Normally, adiponectin is secreted as three oligomeric isoforms, including trimeric, hexameric and the high-molecular weight (HMW) form. The HMW form can consist of at least 18 mers and there are multible biological functions of adiponectin because it has different oligomeric isoforms (Wang *et al.*, 2008) (Figure 1.10).

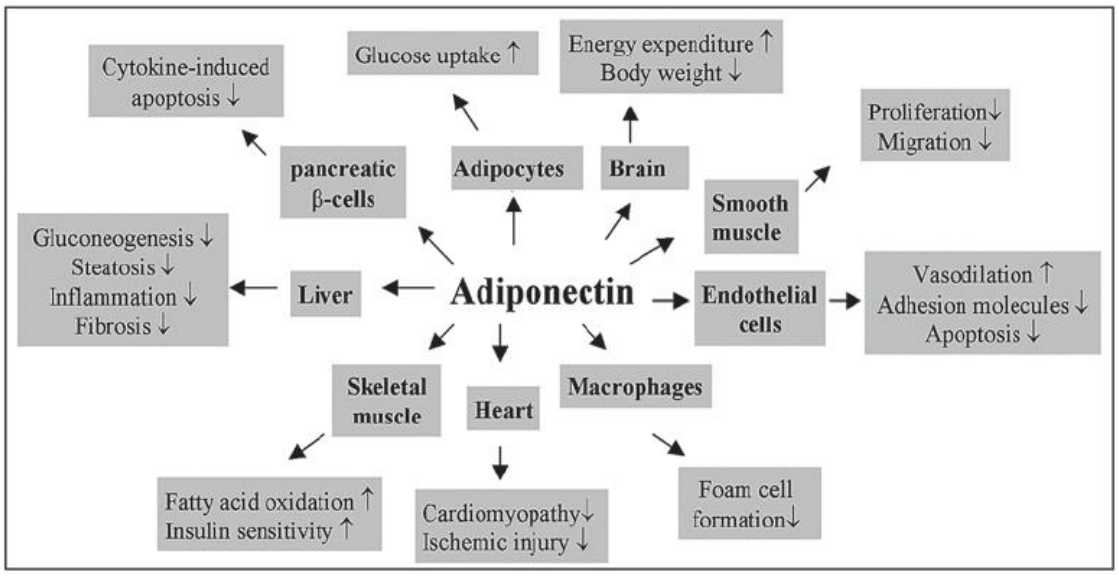

Figure 1.10: Main target organs and bio-activities of adiponectin (Xu *et al.*, 2007).

1.1.6.1. Anti-diabetic property of adiponectin

Obesity can be defined as an increase in the total mass of the adipose tissue, and promoting vulnerability to metabolic syndromes such as type II diabetes and cardiovascular diseases. However, the molecular mechanisms for that relationship still need further explanation (Yamauchi *et al.*, 2003). Noticeably, in obese children, the high insulin levels are positively correlated with obesity and glucose intolerance, which may be the main cause of insulin resistance and subsequent development of type II diabetes in adolescents (Young *et al.*, 2000). It has been suggested that reduction in insulin sensitivity may lead to initiate the metabolic syndromes that is also influenced by environmental and genetic factors (Goldstein, 2002) (Figure 1.11).

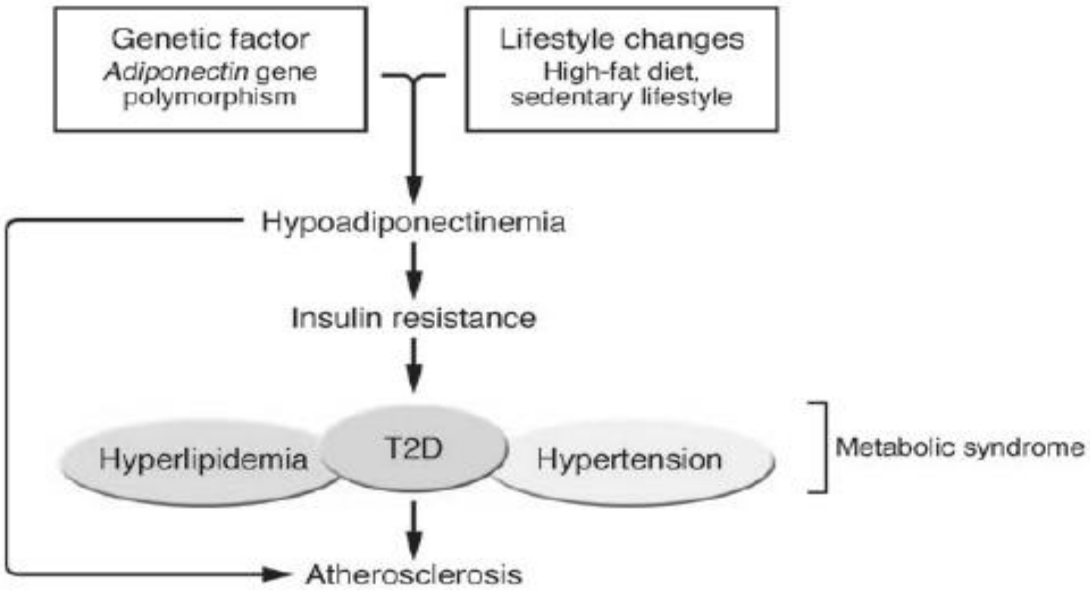

Figure 1.11: The hypothesis of adiponectin effect on metabolic syndromes. The increase in insulin resistance leads to initiate the metabolic syndromes that are influenced by environmental and genetic factors (Kadowaki *et al.*, 2006).

Generally, reduction in insulin sensitivity has been considered to be the key point in individuals with metabolic syndromes including obesity, type II diabetes and cardiovascular disorders (Lindsay *et al*., 2002). It has been shown that the expression of *adiponectin* gene and plasma adiponectin concentrations is negatively correlated with obesity and whole-body insulin resistance (Arita *et al.*, 1999; Hotta *et al*., 2000). Moreover, plasma adiponectin concentrations considerably increase with weight loss (Hotta *et al.*, 2000). However, plasma adiponectin concentrations in case of type II diabetes are lower than in non-diabetic men and women of the same age and body mass index (Hotta *et al*., 2000).

The relationship between adiponectin levels and metabolic syndromes has been explained through the results of thirteen studies with a total of 14,598 participants and 2623 subjects of type II diabetes and confirmed that the elevated adiponectin concentrations are positively correlated with insulin sensitivity (Li *et al.*, 2009). Supporting this finding Lindsay *et al*. (2002) have shown that individuals with elevated adiponectin levels are invulnerable to the risk of type II diabetes compared with individuals with lower levels (Lindsay *et al*., 2002). It has been known that there is an obvious correlation between plasma adiponectin levels and insulin sensitivity. Therefore, insulin resistance can be identified by the reduction in plasma levels of adiponectin before development of obvious diabetes. Moreover, the decline in plasma levels of adiponectin can be observed in severe forms of diabetes and insulin resistance like type II diabetes, gestational diabetes and diabetes associated with lipodystrophy (Lindsay *et al*., 2002; Retnakaran *et al*., 2004; Williams *et al.*, 2004) .

Yamauchi *et al.* (2003) explained that adiponectin has two receptors; AdipoR1 is the most essential form in skeletal muscle whereas AdipoR2 is mediating adiponectin effects in liver. Receptor knockout studies in rodent models indicate an important role of these receptors in mediating the effects of adiponectin. However, these receptors

have different binding affinities of the different adiponectin isoforms (Kadowaki *et al*., 2008) (Figure 1.12). It has been reported that the globular domain of adiponectin is able to induce glucose up take in cell cultures of myocytes and reducing plasma glucose levels in isolated muscles (Maeda *et al.*, 2002; Tomas *et al.*, 2002; Yamauchi *et al.*, 2002). Similar finding has shown that globular domain of adiponectin has an ability to improve translocation of glucose transporter molecules to the cell membrane and subsequently increases glucose uptake in skeletal muscle cells (Ceddia *et al.*, 2005). Additionally, adiponectin has potential effect in lowering blood glucose levels by suppressing endogenous glucose production by the liver through down-regulation of genes expression that code glucose- 6-phosphatase and phosphoenolpyruvate carboxykinase (Berg *et al*., 2001; Combs *et al*., 2001) (Figure 1.13)..

Other studies explained that adiponectin is able to reduce the rate of glycogen synthesis in the skeletal muscle cells. The reduction of glycogen synthesis after adiponectin treatment may be through AMPK activation that can potentially inactivate glycogen synthase (Ceddia *et al*., 2005; Kahn *et al.*, 2005).

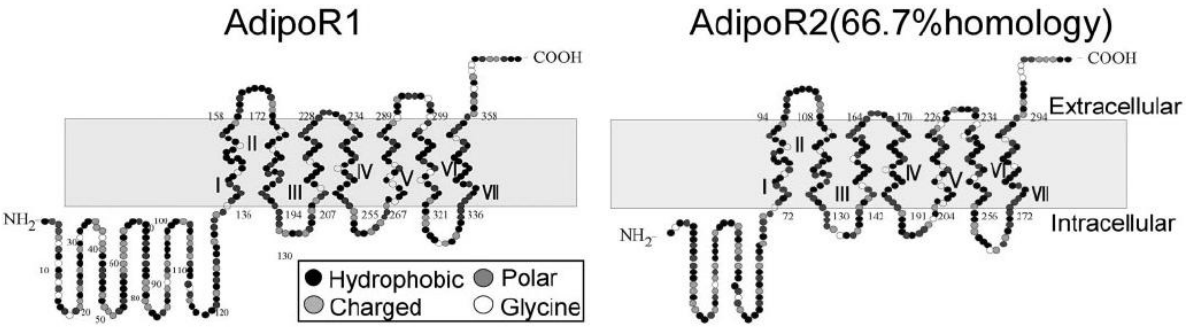

Figure 1.12: Proposed structure of adiponectin receptors as described by Kadowaki and Yamauchi (2005). Adiponectin has two receptors: AdipoR1 is the most essential form in skeletal muscle whereas AdipoR2 is mediating adiponectin effects in liver.

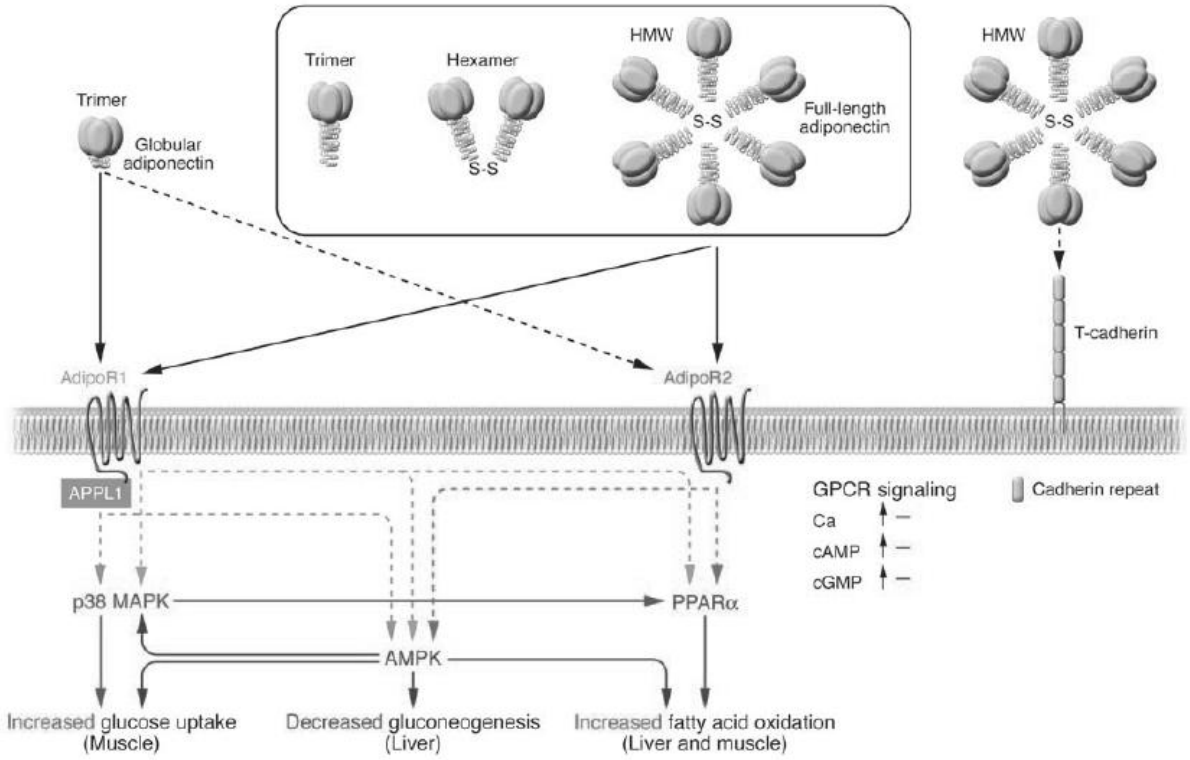

Figure 1.13: Signal transduction by adiponectin receptors. Adiponectin isoforms have different binding affinities with Adipo R1 and Adipo R2 and influence mainly through AMPK pathway (Kadowaki *et al.*, 2006.)

1.1.6.2. Anti-atherogenic, anti-inflammatory and cardioprotective properties of adiponectin

The relationship between plasma adiponectin levels and fatty acids metabolism has been well studied by different groups of researches. It has been shown that adiponectin has an ability to improve glucose consumption in cardiomyocytes cells line, and accelerates fatty acid oxidation in the heart muscle (Pineiro *et al.*, 2005). This finding had been evident by administration with the globular domain of adiponectin which significantly increases fatty acid oxidation in the heart muscle and this effect of adiponectin is proposed to be mediated by AMPK activation (Figure 1.13). Additionally, adiponectin is negatively associated with some of cardiovascular risk factor including heart rate, blood pressure , cholesterol, low-density lipoproteins (LDL) and triglycerides levels and positively associated with high density lipoprotein (HDL) (Onay-Besikci *et al.*, 2004) .

Concomitant with previous findings, adiponectin deficiency is associated with insulin resistance and atherosclerosis development which are both ameliorated by treatment with recombinant adiponectin in the adiponectin knock-out mice (Kubota *et al.*, 2002). Therefore, the local production of adiponectin protein by cardiomyocytes could be contributed in the regulation of cardiac metabolism and function (Pineiro *et al.*, 2005). The beneficial effects of adiponectin on the cardiovascular disorders have confirmed in a number of animal models. Shibata *et al*., (2004) have been reported that *adiponectin* knock-out mice suffered from blood high pressure that lead to increase cardiac hypertrophy and mortality due to decrease myocardium AMPK signaling. Consistent with this finding, a recent study by Liao *et al.* (2005) also showed that adiponectin knock-out mice are more vulnerable to heart failure compared with wild

type mice. Therefore, treatment of adiponectin knock-out mice with supplementation of adiponectin enhanced cardiac hypertrophy.

It is known that adiponectin has anti-inflammatory effect. For example subjects with coronary artery disease have lower plasma levels of adiponectin compared with those who have higher body mass index (BMI) and age (Ouchi *et al.*, 1999; Kumada *et al.*, 2003). Therefore, adiponectin deficiency can be considered as one of the main causes of endothelial dysfunction in any case rather than insulin resistance (Hotta *et al.*, 2001). It has been shown that adiponectin has a central role in the development of vascular diseases due to its roles in suppression of macrophage to produce cell transformation, adhesion of monocyte into endothelium, differentiation of myeloid cells, phagocytosis and production of cytokines by macrophage (Ouchi *et al.*, 1999 & 2001). These findings explain how globular domain of adiponectin can protect against atherosclerosis in a mouse model for atherosclerosis (apolipoprotein E deficient) (Yamauchi *et al.*, 2001) (Figure 1.14).

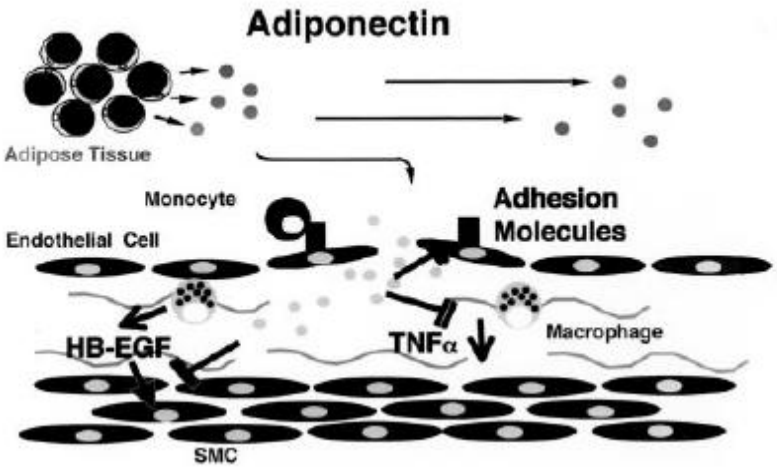

Figure 1.14: Adiponectin functions as anti-atherogenic factor (Matsuzawa *et al.*, 2004).

Adiponectin may play an essential role in the development of cardiovascular diseases that is common in obese individuals. Collectively, circulating adiponectin concentrations are adversely associated with specific factors causing cardiovascular diseases such as overload blood pressure, C-reactive protein concentrations and hyperlipidemia (Iwashima *et al.*, 2004).

1.2. Expression of proteins in prokaryotic and eukaryotic expression systems.

Recombinant proteins can be produced by either prokaryotic or eukaryotic expression systems. *Escherichia coli* have been known as a commonly used prokaryotic expression system, whereas there are many types of eukaryotic expression systems including mammalian cell systems, plant systems, yeasts such as *Saccharomyces cerevisiae* and *Pichia pastoris*, and insect cell systems. Additionally, researchers continue to establish new expression systems such as single-celled slime mold (Arya *et al.*, 2008) and green alga (Heitzer *et al.*, 2007) as eukaryotic system to express many recombinant proteins.

Generally, prokaryotic expression systems such as the *E. coli* were popular due to ease and rapidity of the expression system, with good value of large-scale production of recombinant proteins. On the other hand, the mammalian cells expression system provides proper folding and post-translational modifications of compound proteins. Therefore, mammalian cells still the logical expression system for a lot of eukaryotic proteins that are produced for pharmaceutical purposes, especially, those requiring post-translational modifications. For example, the increase in insulin sensitivity after administration with adiponectin produced by mammalian cells was better than that achieved by adiponectin produced in *E. coli* because mammalian cells have post-translational modifications which enhanced adiponectin bioactivity (Wang *et al.*, 2002).

However, the production of recombinant protein by mammalian cells is usually for small amounts of proteins such as antibodies production.

Basically, the selection of appropriate expression system depends on some of the biological and biochemical characteristics of the protein of interest such as molecular weight, conserved domains as well as the required amount of recombinant protein (Verma *et al*., 1998). Additionally, the advantages and disadvantages of the expression system should be taken in consideration. For example, there are some advantages as well as disadvantages of the yeast as a eukaryotic expression systems compared with *E. coli*. One of the main advantages is that yeast can be grown to very high densities cultures rather than eukaryotic post translation modification. On the other hand, *E. coli* have been used to express eukaryotic genes easy and well understood expression system (Brawner *et al*., 1991; Payne *et al.*, 1990). However, *E. coli* problems were identified as poor glycosylation process and incorrect protein folding, resulting biologically inactive protein rather than insolubility (Binnie *et al*., 1997). Other studies have demonstrated that the use of *Streptomyces spp.* bacteria as host cells to produce eukaryotic proteins was successful in producing medium secreted proteins with correct folding, and the expressed protein was easy to purify by one step process. One of prokaryotic expression systems such as *Streptomyces spp.* emerges as promising alternative to other bacteria such as *Bacillus subtilis* and *E. coli* in term of protein glygosylation (Binnie *et al.*, 1997) However, *Pichia pastoris* still the best in proteins glygosylation process comparing with those produced by *Streptomyces spp.* (Stahl *et al.*, 2003).

1.2.1. Expression in *Escherichia coli*

The production of recombinant protein is necessary for pharmaceutical application like drug and vaccine discovery and for industrial purposes like some

enzymes. The expression system should be able to produce recombinant protein dependably with simple purification. Additionally, the recombinant protein should show the same folding characteristics and possess bioactivity as well as its native state. It has been known that *E. coli* is the prokaryotic expression system that usually used to produce high quantities of heterologous proteins (Hanning & Makrides, 1998; Braun *et al.*, 2002) and has previously been used effectively in high- quantity protein expression and purification researches (Bussow *et al.*, 2005). Additionally, Goulding and Perry (2003) reported that *E. coli* is the common choice for studies that interested in high recombinant protein quantities, rapid expression, and easy purification of proteins.

E. coli strain is highly important in producing recombinant protein considering its ability to keep up the recombinant plasmid stable will contribute in producing good quantity of the recombinant protein (Blattner *et al.*, 1997). The complete genome sequence of *E. coli* K-12 was published by Blattner *et al.* (1997). The total size of *E. coli* K-12 genome sequence was 4,639,221-base pair including 4288 protein-coding genes and 38 % of these genes have no recognized functions (Blattner *et al.*, 1997). Another study has shown that important biological information can be obtained when comparative studies were published for operons distribution in *E. coli* K12 genome rather than genes orientations and genes arrangement across and within the clusters (Yang & Sze, 2009). Additionally, some strains of *E. coli* BL21 contain positive mutations that enhance formation of cytoplasmic disulfide bond developing protein expression level and reduction inclusion bodies formation (Chart *et al.*, 2000).

1.2.1.1. The features of *E. coli* expression system

Basically, several genetic factors need to be present in expression systems. These factors include transcriptional promoters, translation initiation regions, origin of

replication, transcriptional and translational terminators and the specific antibiotic resistance gene (Jonasson *et al*., 2002; Sorensen *et al.*, 2002).

It has been evidenced that the transcriptional promoter in the expression system is essential for controlling high production of recombinant protein. For example, promoter induction can be used for high expression rate by the widely used inducer IPTG (isopropyl-beta-dthiogalactopyranoside) in addition to the chemical or thermal inducers (Hannig & Makrides, 1998) (Figure 1.15). On the other hand the case of lowering transcriptional rate is important when the expression gene initiate a cellular stress condition this can be achieved by the absence of the specific inducer. Therefore, the transcription rate will be in the basal level and it can be minimized in the presence of the specific repressor (Baneyx, 1999; Jonasson *et al*., 2002).

The mainly available antibiotic resistance markers present resistance to ampicillin, chloramphenicol, tetracycline and kanamycin in the expression system. For example, the *bla* gene encodes *β*-lactamase that provides the resistance to ampicillin. Then, the *β*-lactamase catalyses the *β*-lactam rings when it is secreted to the periplasm. Collectively, there are two possibilities for Ampicillin degradation in the culture medium, one of them by secreted *β*-lactamase and another by acidic environment in high-density cultures (Connell *et al.*, 2003).

In the mRNA transcript, there are two sequences that are important for ribosomal binding site called start codon and Shine–Dalgarno sequence. The ribosomal binding is essential to initiate translation process (Sorensen *et al.*, 2002). Additionally, the start codon is located 7±2 nucleotides downstream from the sequence which is AUG in the expression systems (Ringquist *et al.*, 1992). It has been shown that the best translation start is obtained from mRNA transcripts with the sequence of Shine–Dalgarno UAAGGAGG. The high content of adenine and thymine is important in improving the secondary structure of ribosomal binding site which is essential in

translation initiation (Laursen *et al*., 2002). Interestingly, few codons downstream the start codon has a particular effect on the efficiency of translation initiation and highly expressed genes were characterized by the abundant of adenine (Stenstrom *et al.*, 2001).

1.2.1.2. Formation of inclusion bodies and protein folding

It has been defined that inclusion bodies are incorrect folded protein aggregates produced at high expression rate, heat shock, and unbalance between protein solubilization and aggregation (Singh & Panda 2005). Inclusion bodies are the main problems in protein expression, reducing the capacity to obtain proteins by recombinant DNA technology. Principally, protein folding in native three dimensional structures represents one of the essential factors that determine the biological activity of proteins. However, inclusion bodies are commonly formed in prokaryotic expression systems reducing the chance to get soluble and biologically active proteins.

Several factors control the formation of inclusion bodies in *E. coli* expression system that can be optimized to get high yield of recombinant protein with low cost. These factors can be optimized all together or individually such as expression rate, temperature, solubility of tag-technology, host metabolism and co-expression of plasmid-encoded chaperones (Jonasson *et al.*, 2002).

The mechanism of inclusion bodies formation and its precise structure is not well understood. Previous studies explained that the aggregates of inclusion bodies have been examined using simple microscopy and electron microscopy as dense particles missing distinct structure (Carrio *et al.*, 2000). However, the prevention of *in vivo* protein folding will lead to the accumulation of folding intermediates into protein aggregates as inclusion bodies. The aggregates of inclusion bodies are not inactive compounds but work as *in vivo* transitory pool for intermediates folded proteins. This

process is almost happened in case of stress conditions like heat shock (Villaverde & Carrio, 2003).

Further studies showed that both in prokaryotic and eukaryotic cells, some chaperone systems help the correct folding of proteins during translation process in the cytoplasm (Sorensen & Mortensen, 2005). For example, the developing polypeptide chain can be protected against unwanted interactions by the connection between cellular chaperone tools and the ribosomes. Additionally, the early folding during translation process is important for the native folding of proteins that have different domains (Frydman, 2001). Therefore, the co-expression of chaperones-encoding genes has been considered as a possible approach to enhance protein folding, since the bacterial chaperones have been shown to play an important role in preventing aggregation of host proteins (Schwarz *et al.*, 1996). This approach seems to be fascinating but the possibility for the improvement of protein solubility by chaperones co-expressing is still not guaranteed (Ehrnsperger *et al*., 1997) because some of *E. coli* chaperones help in correct folding whereas, the others chaperones prevent protein aggregation (Veinger *et al.*, 1998).

In vitro, the recombinant protein can be recovered from purified aggregates using chemical compounds such as urea and guadinium hydrochloride. The refolding of recombinant protein can be achieved by dilution in the presence of urea or guadinium hydrochloride and the dialysis (Middelberg, 2002) or using on-column refolding methods (Sorensen *et al.*, 2003). Despite most published studies suggesting that the dialysis methods is the best to eliminate denaturing chemicals but there is a critical step that should be considered. A dialysis process exposes the protein solution to a gradient reduction in denaturing agents leading to exposure of the refolded protein for long time to intermediate concentration of denaturing agent which is about 2 to 4M of guanidine

hydrochloride or urea. At these concentrations of denaturing agents the incomplete folded protein will tend to aggregate again (Kelly & Price, 1991).

There are several strategies for expression of correctly folded eukaryotic proteins in *E. coli* expression system such as lowering translation rates to give the nascent polypeptide more time of folding in correct form. This strategy can be achieved by reducing the temperature after induction or reduce the concentration of IPTG (Braun *et al.*, 2002; Hammarstrom *et al.*, 2002; Shih *et al.*, 2002). Other alternate strategy which is successful and commonly used to enhance the expression of soluble proteins is the fusion with solubility enhancing proteins such as glutathione- S-transferase, thioredoxin and maltose binding protein (Dyson *et al.*, 2004).

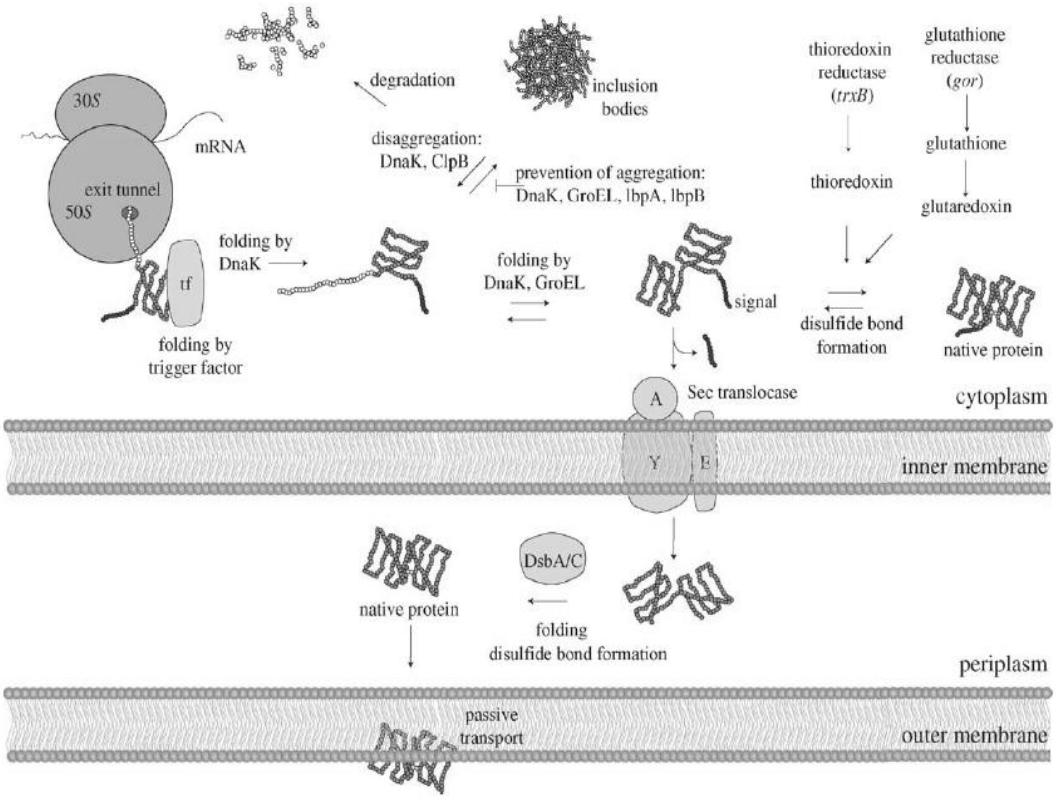

Figure 1.15: The pathway of protein folding and secretion in *E. coli* as described by Sorensen & Mortensen (2005). Protein production as inclusion bodies accumulates in *E.coli* cytoplasm whilst protein production as soluble protein secrets to periplasm. The native proteins of *E.coli* such as glutathione- S-transferase, thioredoxin and maltose binding protein enhance recombinant protein solubility and disulfide bond formation.

1.2.2. *Pichia pastoris* expression system

Yeasts are eukaryotic microorganisms belong to the kingdom Fungi that includes about 1,500 species. Reproduction in yeasts is different depending on the species. For example, *Kluyveromyces lactis* and *Saccharomyces cerevisiae* reproduce asexually by budding. However several yeasts reproduce by binary fission such as *Pichia pastoris* and *Hansenula polymorpha* (Doelle, 1994). Yeast cellular size varied depending on the species as well. Normally, yeast size measures about 3-4µm in diameter, although some yeast size can reach over 40 µm. Some budding yeasts appear to be as multi-cellular organism through forming filament of joined budding cells. However, most yeasts are unicellular micro-organisms (Doelle, 1994). *Saccharomyces cerevisiae* was one of the commonly used species of yeast in baking and fermenting beverages. It is also used in cell biology research as model eukaryotic microorganisms. Last two decades, the interest in yeast genetic engineering has increased to produce high yield and good quality recombinant proteins by certain yeast strains as eukaryotic expression system (Faber *et al.*, 1995). There are some of methylotrophic yeast that has been used as expression system such as *Pichia pastoris*, *Candida boldmu* and *Hansenula polymorpham* and *Kluyveromyces lactis* (Bergkamp *et al.*, 1992; Gellissen & Melber, 1996).

Pichia pastoris had been used for industrial production of single cell protein from methanol. For this purpose, *P. pastoris* had been grown at continuous high density cultures on a media that was designed by Phillips Petroleum Company but the production of single cell protein was invaluable because of high cost of methanol (Cregg *et al.*, 1993). Subsequently, Phillips Petroleum Company with the Salk Institute Biotechnology/Industrial Associates used *P. pastoris* as an expression system for recombinant protein production. Since then, *P. pastoris* was considered as an important

organism in biotechnology processes because methylotrophic *P. pastoris* successfully produced heterologous proteins and become an option for industrial purposes and academic studies (Lin-Cereghino & Cregg 1999; Cereghino & Cregg, 2000). Methanol utilizing yeast have been used as an important expression system because it has many advantages over other expression systems moreover, it has an ability to use methanol as a source of energy and carbon.

1.2.2.1. Advantages of using *P. pastoris* as an expression system

It has been known that *Escherichia coli* are frequently used as appropriate expression system, due to its easy manipulation and growth. However, the growing requires of new recombinant biomedical proteins need to develop the existing production systems and establish new systems. For several thousands of years, yeast has been used for fermentations in large scales comparing with relatively recent usage of *E. coli* in large scale fermentations. It has been known that yeast can normally be grown in cultures of high cell density than bacteria and are highly adaptable to processes of continuous fermentation (Cregg & Higgins, 1995).

The production of recombinant protein in yeast as eukaryotic systems like *P. pastoris*, provides several advantages over other expression systems. For example, in batch fermentation rather that continuous fermentation, *P. pastoris* is well-known for its high productivity of heterologous recombinant proteins (Faber *et al.*, 1995). Interestingly, *P. pastoris* is able to grow at high culture density on minimal medium and secrete the heterologous protein that is simple to purify. For example, Boze and coworkers obtained significantly higher levels of expression in a bioreactor, compared with the shake flask culture. In this study, the increase in recombinant protein concentration was thirty fold in bioreactor culture as compared with shake flask culture and the pH and pO2 were controlled at high cell density continuous fed-batch process

(Boze *et al.*, 2001). In addition, *P. pastoris* can grow in optimal level either in aerobic and anaerobic conditions. For example, in the presence of high glucose concentrations in the media, yeasts produce alcohol aerobically rather than producing biomass via the citric acid cycle. On the other hand, increasing concentrations of glucose accelerates the process of glycolysis which results in the production of considerable amounts of ATP. This process reduces the need of oxidative phosphorylation done by the citric acid cycle through electron transport chain and then decreases the demand of oxygen (Thomson *et al.*, 2005).

It has been shown that *P. pastoris* performs many of the higher eukaryotic post-translational modifications such as protein folding, proteolytic processing, disulfide bond formation and glycosylation with little risk of contamination with viral DNAs, oncogenic factors and endotoxins (Romanos *et al.*, 1992). However, probably the most important characteristic of *P. pastoris* as host micro-organism is the existence of a strong and tightly regulated promoter from the alcohol oxidase 1 gene, *PAOX1* (Cregg *et al.*, 2000).

P. pastoris has been used to successfully produce high yield of recombinant proteins such as vaccines and therapeutic proteins (Asami *et al.*, 2000). The selection of *P. pastoris* as expression system depends on its advantages over other yeasts like the budding yeast, *Saccharomyces cerevisiae*. One of these advantages is *P. pastoris* does not ferment sugars to ethanol that can accumulate in the media and suppress protein production. Another advantage is the methylotrophic yeast used alcohol oxidase (AOX) in the first step of methanol utilization and *AOX* gene sequences has been developed as a highly expressing promoter for heterologous protein expression (Bruin *et al.*, 2005). However, unwanted glycosylation can occur in *P. pastoris*, but it is reported to be less challenging than it is with the *S. cerevisiae* (Byrne *et al.*, 2005).

1.2.2.2. *Pichia pastoris* promoters

The oxidation of methanol to formaldehyde is catalyzed by alcohol oxidase which is the first step in the pathway of methanol utilization in methylotrophic yeasts (Harder & Veenhuis, 1989). There are two alcohol oxidase genes (*AOX1* and *AOX2)* in the *P. pastoris* genome that encode similar protein containing 663 amino acids. The similarity between these two genes in the level of amino acids sequence is 97% homologous and in the level of nucleotide sequence for protein coding region is 92% (Koutz *et al.*, 1989). Despite these homologies the expression levels of the recombinant protein using the *AOX1* as promoter is higher than the expression level when *AOX2* is used as promoter (Mochizuki *et al.*, 2001). Supporting this finding, in the yeast cells, *AOX1* gene is responsible for more than 90% of the enzyme activity compared with *AOX2,* which is responsible for less than 10% of enzyme activity (Harder & Veenhuis, 1989). The variation in the AOX1 and AOX2 activities may be due to the variation was observed in the protein-coding regions (Koutz *et al.*, 1989).

Recently, some attempts have been made to develop new alternative promoters to *PAOX1.* The sequence of glyceraldehide 3-phosphate dehydrogenase was used as promoter (*PGAP*) in successful expression of some heterologous proteins but not in the case when the expressed heterologus protein is toxic to the cells (Waterham *et al.*, 1997). Another choice for promoters is the sequence of the formaldehyde dehydrogenase gene (*FLD1).* The enzyme encoded by *FLD1* gene is play a central role in the methanol utilization as carbon source and the methylated amines metabolism as nitrogen source. Subsequently, *FLD1* promoter can be induced by either methylamine as nitrogen source or by methanol as carbon source (Shen *et al.*, 1998). It has been shown that the expression level of *AOX1* promoter when methanol was used as a source of carbon was similar with the expression level of *FLD1* promoter when methylamine and methanol were used as a source of nitrogen and carbon (Resina *et al.*, 2004).

Methanol concentration is one of the most important key parameters in *P. pastoris* expression system. Adjusting this parameter is important to avoid methanol toxicity when it is used at high levels and the low expression levels when it used in low levels. Maintenance of a regular methanol concentration during the induction phase has positive effects on the level of protein expression (Cereghino & Cregg, 2000; Zhang *et al.*, 2000; Hellwig *et al.*, 2001).

1.3. Objectives of study:

The ability of adiponectin to increase insulin sensitivity in conjunction with its anti-inflammatory and anti-atherogenic properties has made it a promising therapeutic tool for some of metabolic syndromes. The production of the recombinant human adiponectin protein with comparative biological activity would provide useful information in term of protein structure, function and bioactivity in future studies. The current project is carried out with the following objectives:

1- To construct and express human *adiponectin* gene in two different expression systems: *P. pastoris* as eukaryotic expression system and *E. coli* as a prokaryotic expression system.

2- To analyze recombinant human adiponectin bioactivity and compare the effect of the different recombinant protein on some of blood parameters using mice as model system.

3- To optimize the expression of the recombinant human adiponection by *P. pastoris*.

4- To study the effect of recombinant human adiponectin on gene expression of related genes to explore the possible mechanism of action.