

**TRANSCRIPTIONAL CONTROL OF THE ADIPONECTIN
GENE BY STAT5A**

A Thesis

**Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science**

in

The Department of Biochemistry

**by
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B.S., Louisiana State University, 2005
December, 2008**

ACKNOWLEDGMENTS

The author would like to thank God for all of his blessings. The author would also like to thank his committee members for their time.

Special thanks go to his friends and family for supporting him throughout his time in graduate school. The author also sincerely appreciates the members of his lab for their friendship and support.

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ABSTRACT

Adiponectin is a hormone secreted from adipocytes that plays an important role in insulin sensitivity and fatty acid oxidation. The secretion of adiponectin from adipose tissue has been shown to be attenuated by treatment with prolactin (PRL). PRL is a potent STAT5 activator, and studies have indicated that it can modulate the expression of several genes in adipocytes. In this study, we demonstrate that 3T3-L1 adipocytes treated with PRL exhibit a reduction in adiponectin levels. Furthermore, we identified three putative STAT5 binding sites in the mouse adiponectin promoter and show that only one of these, located at -3809, binds nuclear protein in a PRL dependent manner. Mutation of the STAT5 binding site abrogated PRL dependent protein binding, and supershift analysis revealed that STAT5A and 5B, but not STATs 1 and 3, bind to this site in response to PRL. Promoter/reporter constructs containing the -3809 site were found to be responsive to PRL. Taken together, these data strongly suggest that PRL regulates adiponectin transcription through the binding of STAT5 to the -3809 site.

CHAPTER 1. INTRODUCTION

1.1 Metabolic Syndrome and the Study of Adipocytes

The study of adipocytes is important because of the growing epidemic of obesity in the United States and the world. Obesity is one of a number of interrelated symptoms including, insulin resistance, hypertension, dyslipidemia, and glucose intolerance that are collectively referred to as metabolic syndrome (1). Individuals with metabolic syndrome have an increased risk of developing atherosclerotic cardiovascular disease as well as type II diabetes, and the primary metabolic defects responsible for obesity have been found to exacerbate all aspects of metabolic syndrome (1). Some studies have projected that 50% of the population in the United States will become obese by the year 2025 (2), therefore it is critical to understand the molecular mechanisms that contribute to the pathogenesis of obesity and metabolic syndrome.

Obesity is a condition in which excess adipose tissue accumulates, so it is logical to investigate the primary cell type in adipose tissue, the adipocyte. Mature adipocytes possess three properties that largely distinguish them from other cell types. Firstly, adipocytes are unique in their ability to store large amounts of lipid in the form of triglyceride. Secondly, adipocytes are highly insulin sensitive, which is due largely to their expression of the protein glucose transporter 4 (GLUT4). GLUT4 is an insulin sensitive glucose transporter that is found exclusively in heart, skeletal muscle, and adipocytes. Lastly, studies within the last fifteen years have revealed that adipocytes have important endocrine functions. Indeed, several hormones are made exclusively in adipocytes and referred to as adipokines. Adipokines have been shown to play critical

roles in the metabolic homeostasis of the body, and obese individuals have been reported to have an altered circulating adipokine profile that contributes to the etiology of metabolic syndrome (3). The goal of this thesis is to elucidate whether the transcription of adiponectin, an adipokine hypothesized to have protective effects against metabolic syndrome, is regulated by the latent transcription factor Signal Transducer and Activator of Transcription 5 (STAT5).

1.2 Adipocyte Cell Lines as a Model System

A lot of what is known about adipocyte biology today has been obtained from studies on adipocyte cell lines. Several commonly used adipocyte cell lines were cloned from the mouse embryonic fibroblast cell line 3T3 by Green and colleagues back in the 1970s. Green observed that out of 20 clones taken from the 3T3 stock, 19 of them accumulated lipid to various degrees once confluence was reached (4). Only portions of these confluent cell populations accumulated lipid and subpopulations prone to lipid accumulation were selected and propagated giving rise to the 3T3-L1 and 3T3-F442A cell lines used extensively in studies on adipocyte biology (5). The differentiation process of these cell lines is thought to be similar to native adipocyte formation from preadipocytes, and researchers have found ways to increase the rate of differentiation in these cell lines by treating them with hormone cocktails containing: insulin, isobutylmethylxanthine (a phosphodiesterase inhibitor), dexamethasone (a synthetic glucocorticoid), and fetal bovine serum. Upon differentiation, both cell lines possess the major characteristics of native adipocytes, and experiments have shown that injection of 3T3-F442A cells into athymic mice form fully developed fat pads, which substantiates these cell lines as good model systems (6).

1.3 JAK/STAT Signaling

Adipocytes possess a number of different signaling pathways, including the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway. In this pathway, cytokines bind to a non-enzymatic receptor associated with JAK that dimerizes allowing JAK to transphosphorylate the opposite receptor. STAT proteins then bind to the phosphorylated receptor, and they themselves are then phosphorylated by the receptor associated JAK. Tyrosine phosphorylation of the STAT proteins allows them to dimerize, dissociate, and translocate to the nucleus to modulate gene expression (reviewed in 10).

There are seven STAT proteins, but only five of these, STATs 1, 3, 5A, 5B, and 6 are expressed in mature adipocytes (7). The cytokine prolactin (PRL) strongly activates STAT5 and to a lesser extent STATs 1 and 3 (8). PRL signaling follows the canonical JAK/STAT pathway, but more specifically, when PRL binds the PRL receptor, the receptor recruits JAK2 to phosphorylate the receptor and STAT5. It is largely unknown what proteins are responsible for mediating the translocation of STAT5 through the nuclear pore complex (NPC), but mutational analysis of asparagine 104 on STAT5 has shown that this amino acid is essential for IL-3 dependent nuclear import of STAT5 and suggests a required protein:protein interaction for STAT5 translocation (9).

1.4 STAT 5A and 5B

STAT5 represents two proteins, 5A and 5B, which are encoded for by separate genes on chromosome 11 in mice and are expressed in all tissues at varying levels (10). STAT5A and 5B are capable of forming both homo and heterodimers upon activation, but the significance of this observation remains unclear in the context of gene regulation (11). Both share 96% sequence similarity, and like all STAT proteins, possess a number

of domains including: (in order from N-terminus to C-terminus) an N-terminal domain, coiled-coil domain, DNA binding domain, Src Homology (SH) 3 domain, SH2 domain, tyrosine activation domain, and transcriptional activation domain (TAD). Beginning at the amino terminus, the amino terminal domain forms a ring structure consisting of 5 helices (12). This region has been reported to play an important role in STAT5 dimer and tetramer formation, and mutation of tryptophan 37 within this region has been shown to ablate tetramer formation (13). The coiled-coil domain consists of a flexible region of 4 α helices that protrude from the core structure and serves as a docking site for proteins on STAT proteins including N-Myc interactor (Nmi) on STAT 5B (14). The DNA binding domain has been documented to bind DNA using a β barrel motif similar to those used by the transcription factors p53 and NF- κ B (14). Furthermore, X-ray crystallography studies of STAT5A have revealed that the β barrel motif has a modest number of contact points with bound DNA giving it a dissociation constant in the nanomolar range (15). Next, the SH3 domain is thought to facilitate protein:protein interactions between STAT proteins, although extensive interactions are known to occur among the other domains (16). An SH2 domain lies next to the SH3 domain, and domain swapping studies have suggested that the SH2 domain of each STAT family member is the sole determinant in which STAT proteins are recruited to activated receptors (17). Additionally, the SH2 domain is believed to facilitate the dimerization of STAT proteins by binding to the phosphorylated tyrosine residue on the oppositely bound STAT (18). Furthermore, like other STAT members, STAT5 proteins contain a highly conserved tyrosine residue (Tyr694 on 5A, Tyr699 on 5B) that is required for activation. An additional TAD domain is present at the carboxyl terminus. STAT5A and 5B differ greatly in both the sequence and size of this

domain, and studies have suggested that serine phosphorylation within this region may be required for maximal activation of STAT5 proteins (19).

Knockout experiments of STAT5A and 5B have revealed both redundant and non-redundant functions for these proteins. STAT5A knockouts exhibited defects in mammary gland development and were infertile (20). The 5A knockout phenotype was strikingly similar to prolactin receptor knockout mice, which suggests that prolactin signals primarily through STAT5A (21). Likewise, male STAT5B knockout mice exhibited a dwarf phenotype that was similar to growth hormone (GH) receptor knockout mice, which suggests that GH signals primarily through STAT5B. STAT5B knockouts were, however, sexually dimorphic because female mice did not exhibit stunted growth (22). STAT5A/5B double knockouts exhibited severe dwarfism in both sexes, were infertile, lacked proper mammary gland development, and had fat pads one-fifth the size of wild type mice. These mice also had immune system defects including a lack of natural killer cells, and numerous T cell and B cell abnormalities (23).

1.5 Prolactin and Growth Hormone

PRL is produced in the lactotroph cells of the anterior pituitary, as well as other locations in the body (24;25). As its name implies, PRL plays a large role in the production of milk in pregnant women, but other roles including modulating lipolysis (26) and adipogenesis (27) have been identified. Interestingly, except in the case of pregnancy, PRL levels in men and women are very similar and it has recently been discovered that PRL is produced by adipose tissue in humans (28). As is the case with other PRL production sites outside of the anterior pituitary, the PRL gene in adipose

tissue is differentially regulated and under the control of a super distal promoter located - 6kb away from the transcription start site (29;30).

GH is another known activator of STAT5, and is produced in the somatotroph cells of the anterior pituitary gland. It is known that GH increases lipolysis and protein synthesis, and decreases lipogenesis and glucose uptake (31). It is also known that chronic exposure to GH results in reduced adipose tissue growth (32). Furthermore, circulating GH levels are reduced during the condition of obesity (33). Overall, there are numerous studies showing that GH is a prominent regulator of adipocyte metabolism (34).

Both GH and PRL activate the phosphatidylinositol (PI) 3 kinase pathway as well as the JAK/STAT pathway. Similarly, insulin utilizes the PI 3 kinase pathway, and studies have shown that the effects of insulin on glucose transport, gluconeogenesis, and lipogenesis are almost completely dependent on the actions of PI 3 kinase (35). Numerous studies have indicated that chronically high levels of GH and PRL can cause insulin resistance (36;37) , and it has been hypothesized that these actions involve the modulation of PI 3 kinase activity. PI 3 kinase is activated via its recruitment to the plasma membrane by Insulin Receptor Substrate (IRS) proteins which, in turn, are activated by the insulin, PRL, and GH receptors, respectively. The structure of PI 3 kinase consists of a heterodimer containing a regulatory p85 subunit and a catalytic p110 subunit, each of which possess multiple isoforms (35). Knockout experiments of the p85 α isoform in mice exhibit a phenotype of increased insulin sensitivity (38). In addition, other experiments have shown that the overexpression of p85 α in cells results in decreased glucose uptake and inhibited PI 3 kinase activity (39). Chronically high levels

of GH have been shown to induce the transcription of the p85 α subunit (40), which may account for the concomitant loss of insulin sensitivity seen with this condition.

1.6 Regulation of the JAK/STAT Pathway

JAK/STAT signaling is regulated by a number of different protein interactions. Dephosphorylation of activated signaling components by phosphatases such as SH2 protein tyrosine phosphatase 1 (SHP1) is one method cells use to regulate the JAK/STAT pathway. SHP1 consists of two tandem SH2 domains as well as a phosphatase domain which has been reported to bind to activated JAK2 (41). Another method of JAK/STAT regulation comes from the activation of Suppressors Of Cytokine Signaling (SOCS) proteins. The SOCS family consists of 8 members, SOCS 1-7 and Cytokine Inducible SH2-containing (CIS) (42). SOCS 1-3 and CIS are activated by PRL (43). Upon activation, SOCS proteins bind to an activated receptor or JAK and inhibit their interaction with downstream signaling components. Additionally, SOCS proteins contain a SOCS box motif that is known to recruit elongin B and C, two proteins involved in the ubiquitination and subsequent proteasomal degradation of bound proteins. Contrary to SOCS proteins, which are primarily activated by cytokines, Protein Inhibitors of Activated STAT (PIAS) proteins are a constitutively expressed class of STAT inhibitors found in a number of cell types. One PIAS protein, PIAS3, has been shown to inhibit STAT5 signaling by binding and interfering with its ability to bind DNA (44). Furthermore, PIAS proteins have been reported to function as Small Ubiquitin like modifier (SUMO) E3 ligases (45). The functional consequence of SUMOylation can vary depending on the targeted protein, but in the case of STATs, it is hypothesized to modulate their activity (46).

1.7 Genes Regulated by STAT5 in Adipocytes

STAT5 has been reported to play a role in the regulation of a number of genes in adipocytes. A previous study has shown that STAT5 plays a role in lipid accumulation in mature adipocytes and showed that STAT5 activation represses the induction of fatty acid synthase (FAS) (47). Furthermore, another study has shown that STAT5 binds to the Acyl CoA oxidase (AOX) promoter and that treatment with STAT5 activators causes an increase in AOX mRNA (48). STAT5 has also been reported to play a role in insulin resistance and can induce the expression of pyruvate dehydrogenase kinase 4 (PDK4) in adipocytes (49). PDK4 is a kinase that is known to phosphorylate and inactivate the pyruvate dehydrogenase complex (PDC), the protein responsible for converting pyruvate into acetyl CoA in mitochondria. Hence, when PDC becomes inactive, glucose levels rise within the cell which leads to hyperglycemia. This observation correlates well with a study that showed that PDK4 activity and expression increases in type II diabetics (50). STAT5 has also been reported to play an important role in the activation of genes encoding milk proteins. In fact, STAT5 was initially named mammary gland factor (MGF), because it was first discovered as an activator of the milk protein β casein (51).

1.8 Protein Interactions with STAT5

A number of studies have shown that proteins that interact with STAT5 can have a modulatory effect on its function. The transcription factor CCAAT Enhancer Binding Protein (C/EBP β) has been found to associate with STAT5 in the presence of the glucocorticoid receptor (GR), which together form a complex that induces the transcription of the β casein gene (52). Additionally, STAT5 has been found to relieve repression of the β casein gene by interacting with Yin-Yang factor 1 (YY1), a protein

that binds next to the STAT5 response element on the β casein promoter and inhibits β casein transcription in the absence of STAT5 (53). Similarly, STAT5 has been reported to form an enhancer complex with an adjacent binding transcription factor, Sp1, in the promoter region of cyclin D2 (54). Moreover, studies have shown that STAT5 interacts with the histone acetylase Creb Binding Protein (CBP)/P300 which serves as a coactivator of the β casein gene (55). This interaction is enhanced by the protein Nmi, which binds not only STAT5 but other members of the STAT family as well (56).

1.9 Adiponectin

Adiponectin is an adipokine that has recently come into the spotlight as a major player in the control of energy homeostasis. The adiponectin gene encodes a protein that circulates in serum as high and low molecular weight oligomers (57). A proteolytic cleavage product known as globular adiponectin has also been reported (58). Low circulating levels of adiponectin have been associated with an increased risk of cardiovascular disease (CVD) (59). Men have a higher incidence of CVD than women (60), and studies have shown that adiponectin levels on average are lower in men than in women (61) which suggests a possible link between low adiponectin levels and the development of CVD. Low adiponectin levels have also been observed in obese patients (62), and mice lacking adiponectin experience severe hepatic insulin resistance (63). Hence, administration of adiponectin to obese animal models with type II diabetes has shown that adiponectin can help alleviate insulin resistance in these models (64). Moreover, a higher ratio of high molecular weight oligomer/total adiponectin levels has been correlated with higher insulin sensitivity, and appears to be more important than total adiponectin levels in regards to insulin sensitivity (65). Adiponectin is also known to

activate AMP activated kinase whose activity involves increasing fatty acid oxidation and increasing glucose transport (66). A recent study also revealed that adiponectin appears to play a role in signaling the allocation of triglyceride to white adipose tissue, away from other storage sites such as the liver and muscle which are considered to be metabolically unhealthy “spill over” sites for fat storage when white adipose tissue becomes unable to store excess triglyceride (67).

1.10 Adiponectin Receptors

Two receptors for adiponectin have been identified thus far, AdipoR1 and AdipoR2. AdipoR1 is expressed ubiquitously and is most abundant in skeletal muscle, whereas AdipoR2 is primarily expressed in the liver (68). Human and mouse adipoR1 and adipoR2 share 96.8% and 95.2% sequence similarity, respectively (69). The gene for AdipoR1 resides on chromosome 1p36.13-q41 in humans and 1E4 in mice and AdipoR2 is located on chromosome 12p13.31 in humans and 6F1 in mice (70). AdipoR1 and AdipoR2 share 66.7% sequence identity and both are predicted to form 7 transmembrane domain proteins similar to receptors belonging to the G-protein-coupled receptor (GPCR) families. However, GPCR receptors and adiponectin receptors are only distantly related because the N-terminus of the adiponectin receptor resides in the cytosol and the C-terminus resides externally which is opposite to that of typical GPCR receptors. It has also been shown that globular adiponectin binds to AdipoR1 with a higher affinity than AdipoR2 and that full length adiponectin binds to AdipoR2 more tightly than AdipoR1 (71). Human studies have revealed that AdipoR1/R2 mRNA is upregulated after exercise in human skeletal muscle (72). Furthermore, families with a history of type II diabetes have been reported to have lower AdipoR1/R2 expression levels than families without a

history of type II diabetes (73).

1.11 Actions of Adiponectin

A number of studies have shown that adiponectin increases insulin sensitivity and fatty acid oxidation throughout the body. When adiponectin binds to one of its receptors, the protein Adaptor-Protein containing Pleckstrin and phosphotyrosine domains and Leucine zipper motif (APPL1) associates with the receptor and becomes activated (74). Upon activation, APPL1 has been documented to associate with the monomeric GTPase Rab5, which is known to play a major role in endocytosis (75). APPL1 has also been reported to activate the protein kinase LKB1, a known tumor suppressor that lies upstream of AMP kinase (AMPK). AMPK is a heterotrimeric protein that consists of a catalytic α subunit and two regulatory β and γ subunits. AMPK serves as the global energy regulator of the cell by detecting the ratio of 5'-AMP/ATP, where 5'-AMP activates AMPK and 5'-ATP inhibits AMPK. One mechanism in which AMPK increases fatty acid oxidation is by phosphorylating acyl-CoA carboxylase (ACC) (76). Phosphorylated ACC results in a reduction of malonyl-CoA production, an inhibitor of long chain acyl-CoA carnitine palmitoyltransferase 1 (CPT1). Thus, low levels of malonyl-CoA result in increased transport of long chain acyl-CoA into the mitochondrial matrix resulting in increased fatty acid oxidation.

Adiponectin signaling has been reported to activate p38 Mitogen Activated Protein Kinase (MAPK) (77). The MAPK kinases (MAPKK) MEK3 and MEK6 are responsible for activating p38 MAPK, however, it is unknown how MEK3 and MEK6 are activated by adiponectin because they lie downstream from numerous upstream MAPKK kinases (MAPKKK) (78). Activated p38 MAPK phosphorylates and activates

PPAR α , a nuclear receptor that, like PPAR γ , forms an obligate heterodimer with the retinoid X receptor (RXR). PPAR α is responsible for the transcriptional control of numerous enzymes responsible for fatty acid oxidation and is activated not only by p38 MAPK, but ligands such as arachidonic acid derivatives, long chain fatty acids, and fibrates (79). Activated PPAR α upregulates the expression of Fatty Acid Transport Binding Protein (FATP), as well as a host of enzymes involved in β oxidation such as medium chain acyl CoA dehydrogenase (MCAD), acyl CoA Oxidase (ACO), and cytochrome P450 fatty acid ω -hydroxylase (80).

Adiponectin is also responsible for increased insulin sensitivity in numerous cell types. A mechanism for this action has been hypothesized by Wang et al. in C2C12 myotubes that involves prolonging the activation of Insulin Receptor Substrate 1 (IRS-1) in response to insulin by inhibiting S6 kinase, an inhibitor of IRS-1 (81). S6 kinase is activated by the mammalian Target Of Rapamycin (mTOR)/raptor complex, and treatment with rapamycin, an mTOR/raptor inhibitor, decreased the activity of S6 kinase on IRS-1. Interestingly, treatment with adiponectin also inhibited S6 kinase activity on IRS-1 and further experimentation revealed a likely pathway in which AMPK (activated by adiponectin) activates the tuberous sclerosis complex (TSC1/2), which inhibits Ras homology enriched in brain (Rheb), an activator of the mTOR complex. Thus, a possible pathway for the insulin sensitizing effects of adiponectin has been shown in C2C12 myotubes.

1.12 Single Nucleotide Polymorphisms of the Adiponectin Gene

The human adiponectin gene is located on chromosome 3q27, which is a region of DNA in which more than a dozen Single Nucleotide Polymorphisms (SNPs) have been

linked to type II diabetes and metabolic syndrome (82-84). SNPs within the proximal promoter of adiponectin have been identified and it has been observed that individuals with an SNP of G/G at -276 of the transcription start site (the binding site of PPAR γ) have a two fold higher risk of developing type II diabetes when compared to individuals with an SNP of T/T (85). Other more distally located SNPs have been found at -11377 and -11391 and are associated with hypoadiponectinemia and type II diabetes (86). SNPs with metabolic implications within the adiponectin gene itself have also been identified. Individuals with SNP 45 on exon 2 independently or combined with the -276 SNP exhibit an increased risk of obesity and insulin resistance (87). Moreover, individuals with an I164T missense mutation have been observed to have significantly lower levels of adiponectin independent of body mass index (BMI) (88).

1.13 Transcriptional Control of Adiponectin

Numerous studies have examined the transcriptional control of the human adiponectin gene. Deletion analysis of the human adiponectin promoter region has demonstrated that the region from -676 to +41 is sufficient for basal level transcription activity (89). Furthermore, mutation analysis of response elements for Sterol Regulatory Element Binding Protein (SREBP) -423 and CCAAT/enhancer binding protein (C/EBP) at -224 has shown that both sites are necessary for basal level transcription (90). Another study has revealed that there are multiple C/EBP α sites within the first intron of the human adiponectin gene that serve to enhance basal level transcription (91). The adiponectin promoter also contains a Peroxisome Proliferator Activated Receptor γ (PPAR γ) response element (PPRE) at -273 that allows the nuclear receptor PPAR γ to activate the adiponectin gene (92). Activation of the adiponectin gene can also be

achieved through the use of thiazolidinediones, a class of drugs that serve as artificial PPAR γ agonists. Moreover, an orphan nuclear receptor known as Liver Receptor Homolog-1 (LRH-1) has been reported to bind to -229 of the human adiponectin promoter and mutation analysis of this binding site has revealed that it is required for both basal and induced transcription (93).

The promoter region of adiponectin in mice has also been extensively studied. The transcription factor forkhead transcription factor O1 (FOXO1) has been reported to bind the mouse promoter at -670 and -610 and serves to upregulate adiponectin. FOXO1 is a known target of protein kinase B (PKB), a protein kinase activated by insulin and various growth factors (94). Phosphorylation of FOXO1 by PKB results in its exclusion from the nucleus and renders it incapable of inducing the transcription of adiponectin. Moreover, FOXO1 has been found to form a complex with C/EBP α at these sites (95), and this interaction is enhanced by the protein deacetylase Silent Information Regulator 2 Mammalian Ortholog (SIRT1), which deacetylates 3 lysine residues in the forkhead DNA binding domain (96). Furthermore, a study in mice has shown that C/EBP and Nuclear Factor-Y (NF-Y) bind the -73 to -117 region of the adiponectin promoter and that mutation or deletion of this region results in dramatically reduced transcription levels (97).

Sequence analysis of the human and murine adiponectin promoters reveal a number of potential transcription factor binding sites that are unpublished. Similar to the human LRH-1 site previously described (74), a potential LRH-1 site is located at -124 of the murine adiponectin promoter that resembles an LRH-1 binding site found at -48 and -55 of the rat Cyp8b1 gene (98) (Table1). Furthermore, the murine adiponectin promoter

contains a potential PPAR γ response element (PPRE) at -554 similar to those found in other genes including the ARE7 site found in the mouse AP2 gene (99) (Table 1). Moreover, a possible NF-Y binding site is present at -380 of the human adiponectin promoter (Table 1), which is similar to the sites already found in the murine adiponectin promoter (78). A comparison of the mouse and human adiponectin promoter regions is presented in figure 1.

Table 1. Comparison of Promoter Response Elements

<u>Gene</u>	<u>LRH-1 Sequence</u>	<u>Position</u>
Human adiponectin	TCAAGGCCT	-229 (ref. 74)
Rat Cyp8b1	GCAAGGTCC	-55 (ref. 79)
Rat Cyp8b1	CCAAGGGCA	-48 (ref. 79)
Mouse adiponectin	GCAAGGGCA	-124 (putative)

<u>Gene</u>	<u>PPRE Sequence</u>	<u>Position</u>
Human adiponectin	GGGCA A AAGTCA	-273 (ref. 74)
Mouse AP2	TGAACT C TGACCC	ARE7 (ref. 80)
Mouse adiponectin	TGAACT C AGGACC	-554 (putative)

<u>Gene</u>	<u>NF-Y Sequence</u>	<u>Position</u>
Mouse Adiponectin	ATTGG	-73 (ref. 78)
Human Adiponectin	ATTGG	-380 (putative)

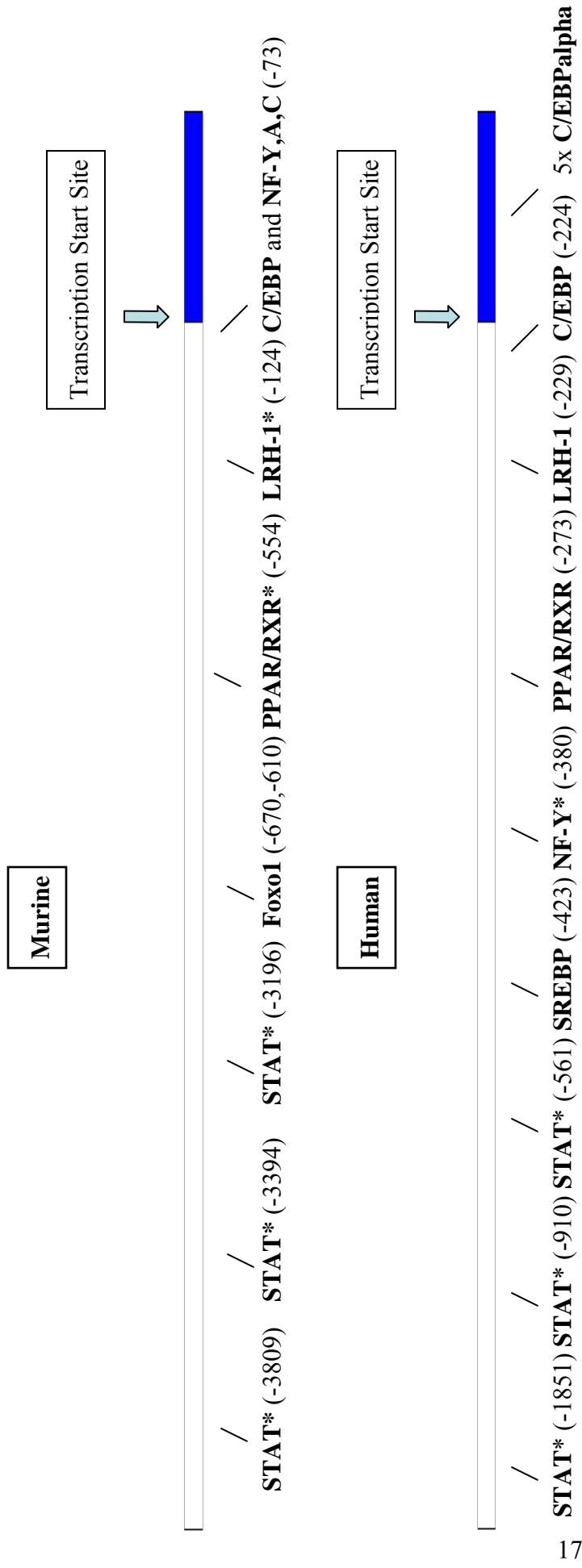


Fig. 1. Comparison of the Murine and Human Adiponectin Promoter Regions. The figure above represents published and potential transcription factor binding sites for the murine and human adiponectin promoter regions. The * symbol denotes unpublished sites found using the www.ensembl.org genome database: murine accession # ENSMUST00000023593, human accession # ENST00000320741.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

STAT5A and adiponectin antibodies were purchased from Santa Cruz Biotechnology. [α - 32 P] dCTP was purchased from Perkin-Elmer and dTTP, dATP, dGTP were purchased from Amersham Biosciences. Klenow and Klenow buffer were purchased from Promega. Fetal bovine serum (FBS) was purchased from Atlanta Biologicals and calf serum was purchased from Biosource. Sheep prolactin was purchased from Sigma. Bicinchoninic Acid (BCA) was purchased from Pierce. Oligonucleotide probes were ordered from Integrated DNA Technologies.

2.2 Cell Culture

Murine 3T3-L1 preadipocytes were plated and grown to 2 days post-confluence in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% bovine serum. Medium was changed every 48 hours. Preadipocytes were differentiated into adipocytes by changing the medium to DMEM containing 10% fetal bovine serum, .5 mM 3-isobutyl-methylxanthine, 1 μ M dexamethasone, and 1.7 μ M insulin (MDI). After 48 hours, medium was replaced with DMEM supplemented with 10% FBS and cells were allowed to differentiate for 5 days. After differentiation, medium was changed every 48 hours and fully differentiated adipocytes were maintained until harvested for experimentation.

2.3 Preparation of Nuclear and Cytosolic Extracts

Cell monolayers were rinsed with PBS buffer and nuclear homogenization buffer (NHB) was added containing 20 mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 1 μ M of

dithiothreitol, 1 uM phenylmethylsulfonyl fluoride, 1 uM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 uM leupeptin, and 2 mM sodium vanadate. Igepal CA-630 (Nonidet P-40) was added to a concentration .15% before each extraction and homogenized 16 times in a Dounce homogenizer. Resulting homogenates were then centrifuged at 1500 rpm for 5 minutes at 4°C. Supernatants were decanted and saved as cytosolic extracts. The nuclear pellets were then resuspended in ½ the volume of NHB used previously and centrifuged as before twice to wash the nuclei. The nuclei pellet was then resuspended in extraction buffer containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5mM MgCl₂, .2mM EDTA, 1 uM dithiothreitol, 1 uM phenylmethylsulfonyl fluoride, 1 uM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 uM leupeptin, 2 mM sodium vanadate, and 25% glycerol. Nuclei were then extracted on ice for 30 minutes to 1 hour with frequent inversion to ensure proper extraction. Afterwards, samples were centrifuged at 10,000 rpm at 4°C for 10 minutes to pelletize cell debris. Supernatants containing the nuclear extracts were then analyzed for protein content using the BCA assay kit, aliquoted, and frozen at -80°C for later use.

2.4 Preparation of Whole-Cell Extracts

Cell monolayers were rinsed with PBS and then harvested in a non-denaturing buffer containing 10mM Tris (pH 7.4), 150 mM NaCl, 1mM EGTA, 1mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, and 1 uM phenylmethylsulfonyl fluoride. A protease inhibitor cocktail was added to this buffer before each experiment consisting of 1 uM phenylmethylsulfonyl fluoride, 1 uM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 uM leupeptin, and 2mM sodium vanadate (a phosphatase inhibitor). Samples were extracted for 30 minutes on ice and centrifuged at 15,000 rpm at 4°C for

15 minutes. Supernatants containing whole cell extracts were analyzed for protein content by bicinchoninic acid (BCA) analysis according to the manufacturer's protocol.

2.5 Western Blotting

Whole cell extracts were run on a 7.5% polyacrylamide gel containing SDS using the Laemmli method (100). Proteins were transferred to nitrocellulose (Bio-Rad) in 25 mM Tris, 192 mM glycine, and 20% methanol under a current of 55 volts for 4 hours at 4°C. After transfer, nitrocellulose membranes were blocked overnight in TBS buffer (.025M Tris, .125M NaCl, .1% Tween 20) containing 4% powdered milk. Nitrocellulose membranes were washed twice for 10 minutes on an orbital shaker and exposed to rabbit derived primary antibodies specific for adiponectin or STAT5A for 1.5 hours. Membranes were then washed for 15 minutes in TBS buffer 3 times and exposed to rabbit specific secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories) for 1.5 hours. Afterwards, membranes were washed for 10 minutes in TBS buffer 3 times and visualized using enhanced chemiluminescence (Pierce) according to the manufacturer's protocol.

2.6 Electrophoretic Mobility Shift Assay (EMSA)

Primary and complementary DNA oligonucleotide strands (Table 2) were combined (10 ug each strand) and heated for 5 minutes in a 95°C water bath. Oligonucleotide strands were allowed to cool and anneal overnight forming double stranded oligonucleotide probes. Oligonucleotide probes were radioactively labeled by combining 4 ug of DNA probe with Klenow, Klenow buffer, 20 uCi α -³²P dCTP, .33 mM dATP, dGTP, dTTP, and incubating the reaction for 15 min. at 30°C. The reaction was stopped by adding 1 ul of .5M EDTA. Probes were purified from the reaction mixture

using micro Bio-Spin 30 chromatography columns (Bio-Rad). Probe purity was measured by running samples on TLC plates (Sigma) in a reservoir of .5 M KH_2PO_4 and exposing the plates to autoradiography film. Specific probe activity was measured by scintillation and typically measured 50,000 cpm/ul. Samples were prepared using the method described by Ritzenthaler (101) and loaded onto a pre-run (200V for 1 hour at 4°C) TBE gel consisting of 6% polyacrylamide/bisacrylamide (40:1 polyacrylamide to bisacrylamide), 90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.0. Cold competition experiments were performed by serial diluting 2 μl of stock unlabeled probe two fold for each successive sample and combining 1 μl of diluted probe with 1 μl radiolabeled probe before incubation with nuclear extracts. Supershift analysis was performed by incubating 1.5 $\mu\text{g}/\mu\text{l}$ of antibody with nuclear extracts for 1 hour at room temperature prior to incubation with the DNA probe.

2.7 Plasmid Construction

The region corresponding to the -3809 site and the 125 bp flanking it were amplified by PCR. PCR primers were designed using the primer 3 program which can be found at the website http://biotools.umassmed.edu/bioapps/primer3_www.cgi. Restriction sites for NheI and HindIII were added to primer ends to facilitate directional cloning (Table 3). PCR reaction conditions consisted of 45 μl of platinum PCR supermix HiFi (Invitrogen), .5 μl of each primer from a 20 picomolar stock, and 100 ng of genomic DNA from 3T3-L1 adipocytes. PCR was performed using an MJ Research PTC-100 machine set to cycle at 1) 94° for 2 minutes 2) 94° for 30 seconds 3) 55° for 30 seconds 4) 68° for four and a half minutes, which was then repeated 35 times beginning with step 2. Amplicons were purified and digested with NheI and HindIII according to the

Table 2. Primary and Complementary DNA strands Used in EMSAs

Site	Primary Strand
-3196	5'-GGAGTGTTCTCAGAAAATGAAAAA-3'
-3394	5'-ACTGGCCTTCGGAGAAAAG-3'
-3809	5'-GAGGCCCTGAATTCTAATTCCCAGAA-3'
β -Casein	5'-GCCATGGTTTTCTTGGAACACTACTAT-3'
Site	Complementary Strand
-3196	5'-TTTTTTCATTTTCTGAGAA-3'
-3394	5'-TGAATTGCTTTTCTCCGAA-3'
-3809	5'-GTGGTTTCTGGGAATTAGAATTCAGG-3'
β -Casein	5'-GAGGCCCTGAATTCTAATTCCCTTAA-3'

manufacturer's instructions (New England Biolabs). The pGL4.27 luciferase vector (Promega) was also digested with NheI and HindIII and ligated to the previously digested amplicons by using T4 DNA ligase according to the manufacturer's instructions (New England Biolabs) giving rise to the PGL4-Adipo construct.

2.8 Transformation and Plasmid Preparation

One Hundred microliters of Dh5 α competent cells (Invitrogen) were diluted five fold in Tris-HCl (pH 7.5) and 1 mM EDTA. One microliter of DNA (containing 1-10ng DNA) was added and cells were incubated on ice for 30 minutes. Cells were then transformed by heat shock for 45 seconds in a 42°C water bath and placed on ice for 2 minutes prior to the addition of .9 ml of room temperature S.O.C. medium. Cells were

then shaken at 225 rpm, 37°C for 1 hour and diluted 1:10 with S.O.C. medium. One hundred microliters from this suspension was then spread on fresh LB plates containing 100 µg/ml of ampicillin before being placed in a 37°C incubator overnight. Colonies from overnight plates were used to inoculate 20 ml of LB broth containing 100 µg/ml of ampicillin and grown for 8 hours. Cells were diluted 1:1000 and grown for 12-16 hours in LB broth containing 100 µg/ml of ampicillin. Afterwards, cells were pelleted by centrifugation at 6,000 x g for 15 minutes at 4°C. Plasmids were isolated from cell pellets using a Qiagen endofree maxi kit according to the manufacturer's instructions.

Table 3. PCR Primers Used in This Study

Name	Sequence	Restriction Site
PGL4-Adipo	5'-GATCGCTAGCATGCAGGGCTCTCTGTTTTG-3'	Nhe1
PGL4-Adipo	5'-GATCAAGCTTGGAGCAACTGGAGCCAACTA-3'	Hind3

2.9 Transfections and Luciferase Assays

3T3-L1 adipocytes were differentiated for 6-8 days. Next, differentiation medium was removed and cells were washed twice with PBS buffer and trypsinized using 5x trypsin (Sigma) for 1 minute. Trypsinization was stopped by re-adding differentiation medium supplemented with 4% glycerol and cells were centrifuged at room temperature for 5 minutes at 90 g. Next, cells were resuspended in 10 ml of differentiation medium supplemented with 4% glycerol and cell density was determined using a hemocytometer. An appropriate number of cells (2×10^6 cells per 100 µl) were then centrifuged at room temperature for 5 minutes at 90 g. Cells were resuspended to a volume containing 2×10^6

cells per 100 μ l in room temperature nucleofector L solution (Amaxa) and 2.5 μ g of PGL4-Adipo was added, along with 250 ng of CMV/renilla vector to control for transfection efficiency. Amaxa cuvettes were loaded with 100 μ l of cell suspension and transfection was performed using program A-033 on an Amaxa Nucleofector II device. After transfection, 500 μ l of pre-warmed differentiation medium was added to the cells in the cuvette and cells were transferred to one well in a 6 well plate containing 2 ml of pre-warmed differentiation medium. Re-plated cells were allowed to grow for 2 hours until medium was changed to DMEM containing 1% calf serum. Upon changing the medium, cells were treated with 450 nM PRL or left untreated and grown for 48 hours. Cells previously treated with PRL were treated again after 24 hours and after 48 hours cells were aspirated and washed twice with PBS buffer before being lysed with 500 μ l of passive lysis buffer (Promega). PGL-4 Adipo activity was quantified by adding 20 μ l of cell lysate to 100 μ l of LARII reagent (Promega) and mixed by pipetting 2-3 times before being placed in a Berthold Detection Systems Sirius luminometer set to automatically read after closing the sample door with a 2 second pre-measurement delay followed by a 10 second measurement interval. Immediately following luciferase detection, co-transfected CMV/renilla vector was measured by adding 100 μ l of Stop and Glo reagent (Promega) and samples were briefly vortexed before being placed back in the luminometer for detection. Relative light units (RLU) were calculated by dividing firefly luciferase activity by renilla luciferase activity.

CHAPTER 3. RESULTS

3.1 Prolactin Treatment Results in a Time Dependent Decrease in Adiponectin Expression in 3T3-L1 Adipocytes

Previous studies have shown that PRL can cause a decrease in adiponectin expression in human adipose tissue explants as well as mice (102). PRL is a potent STAT5 activator, therefore we hypothesized that STAT5 may play a role in the hormonal induced down regulation of adiponectin expression. To determine if fully differentiated 3T3-L1 adipocytes were chronically treated every 24 hours with 1 nM PRL for 96 hours. Cells were harvested at various time points and western blot analysis was performed on whole cell extracts (Fig. 2). These studies revealed that adiponectin levels were

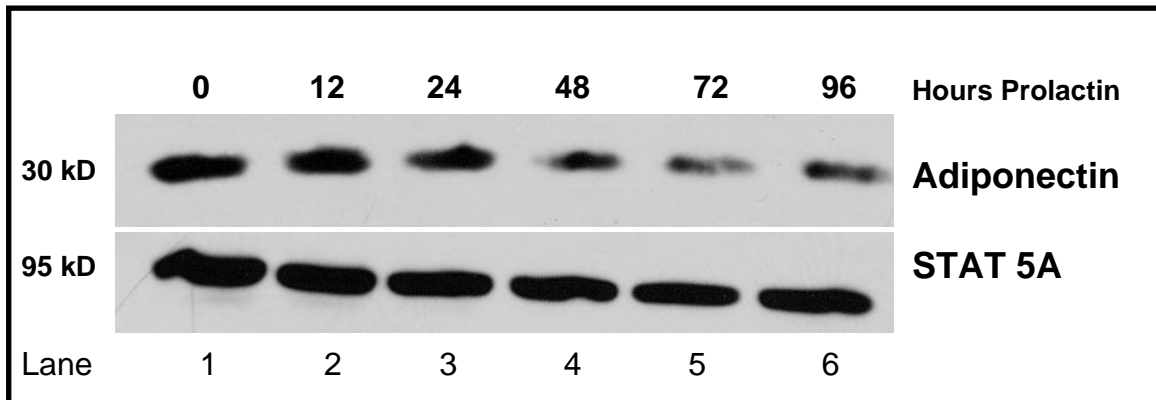


Fig. 2. PRL treatment decreases the expression of adiponectin in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were maintained in DMEM containing 10% FBS and treated with 1 nM PRL every 24 hours until harvested at the times indicated. Forty micrograms of whole cell extract were subjected to SDS-PAGE and transferred to nitrocellulose for western blot analysis. STAT5A levels are shown as a loading control. This is a representative experiment independently performed 2 times.

decreased within 48 hours and remain lowered for the duration of PRL treatment.

STAT5A levels are shown to indicate even loading of protein samples.

3.2 Prolactin Treatment Induces STAT5 Binding of the Murine Adiponectin Promoter in vitro

To determine if the down regulation of adiponectin was due to transcriptional repression by PRL activated STAT5, we analyzed the first 4,000 bp of the adiponectin promoter for consensus STAT5 binding sites (TTC NNN GAA). Three consensus STAT5 binding sites were identified at -3196, -3394, and -3809 bp from the transcription start site (Table 4). To evaluate if these sites bound protein in response to PRL, EMSA analysis was performed using nuclear extracts obtained from serum deprived cells treated with 20 nM PRL for 15 minutes. As seen in figure 3, the site at -3809 binds to PRL induced nuclear protein complexes, where as the sites at -3196 and -3394 sites do not bind protein in response to PRL. The well characterized prolactin responsive STAT5

Table 4. PRL Responsive Sequences

<u>Position</u>	<u>Sequences</u>	<u>PRL-Responsive</u>
-3196	TTC TCA GAA	No
-3394	TTC GGA GAA	No
-3809	TTC TGG GAA	Yes
-3809 Mutant	TTA AGG GAA	No
β -Casein	TTC TAG GAA	Yes

binding site at -119 of the β casein promoter was analyzed as a positive control (Fig. 3, lane 8). Nuclear extracts obtained from untreated cells were used as a negative control and did not result in protein binding to the -3809 site. To show binding specificity for the

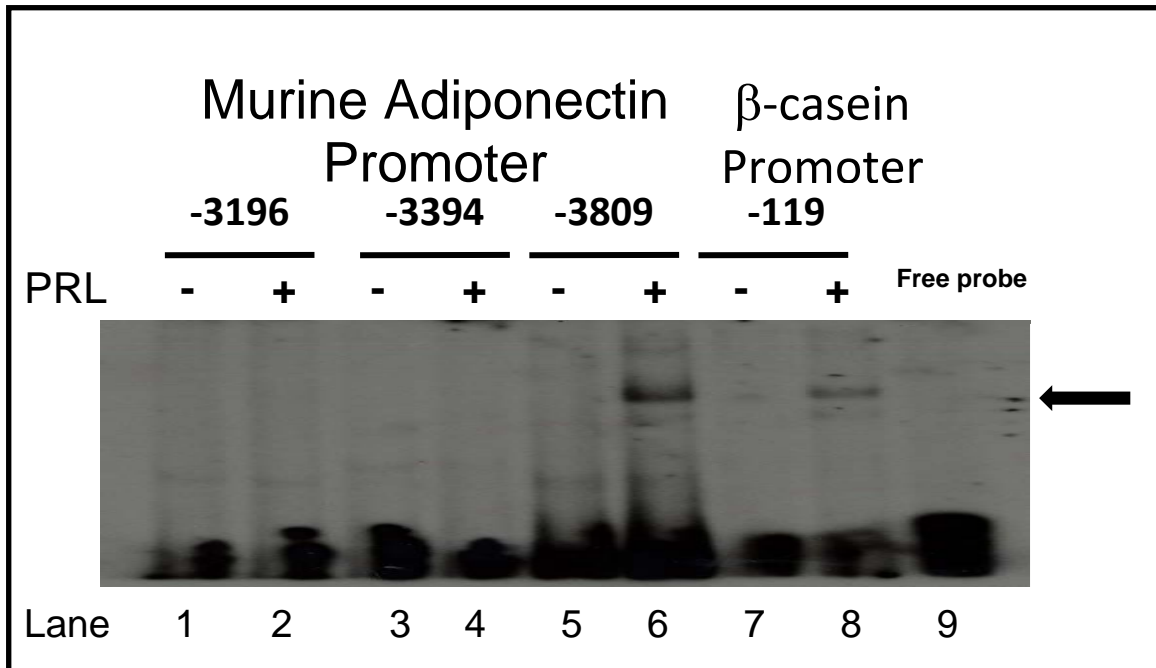


Fig. 3. PRL stimulates protein binding to the -3809 site in the mouse adiponectin promoter. Nuclear extracts were prepared from serum deprived 3T3-L1 adipocytes untreated or treated with 20 nM PRL for 15 minutes. Radiolabeled probes corresponding to potential STAT5 binding sites with an activity of 50,000 cpm/ml were incubated with 10 μg nuclear extracts and subjected to EMSA analysis to analyze for binding activity. A probe corresponding to a known STAT5 binding site in β casein promoter is included as a positive control. This is a representative experiment independently performed two times.

-3809 site, EMSA analysis was performed using probes with a mutated STAT5 binding site (Table 4). Mutation of the consensus STAT5 binding site resulted in the ablation of PRL responsive protein binding to the -3809 site (Fig. 4). Additionally, cold competition experiments were performed and showed that increasing concentrations of unlabeled -3809 probe were able to compete for PRL dependent protein binding (Fig. 5), and that unlabeled β casein probe was also able to compete for protein binding (Fig. 5, lane 9).

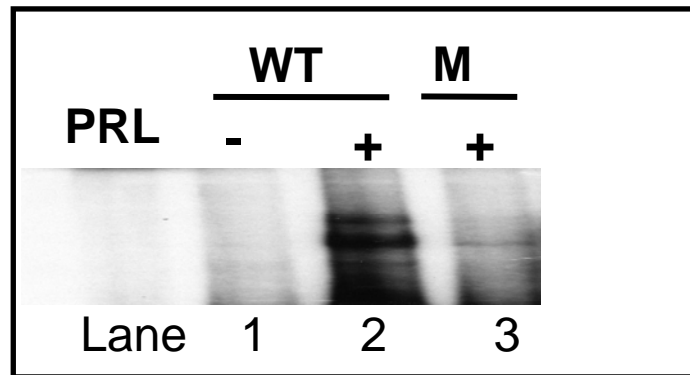


Fig. 4. Mutation of the consensus STAT5 binding sequence ablates PRL induced protein binding to the -3809 site. Wildtype and mutant probes (Table 3) were incubated with 10 μ g PRL treated nuclear extracts and subjected to EMSA analysis. This is a representative experiment individually performed two times.

To determine if PRL activated STAT proteins bound to the -3809 site, we performed supershift analysis using antibodies specific for STAT proteins known to be activated by PRL. As shown in figure 6, an antibody for STAT5A partially shifted the PRL induced complex more than the antibody for STAT5B. This may be due to PRL preferentially activating STAT5A more than STAT5B (11). Nonetheless, these shifts indicate that both STAT5A and STAT5B bind the -3809 site in response to PRL in vitro. Furthermore, antibodies specific for STATs 1 and 3 were unable to shift the PRL activated complex, which indicates that these proteins do not bind to the -3809 site in response to PRL treatment.

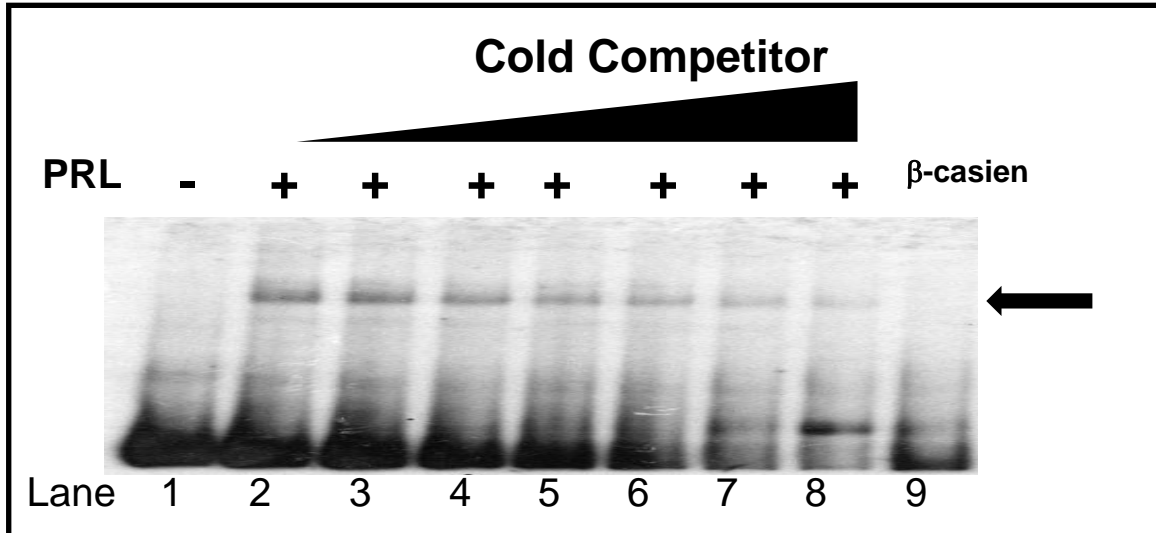


Fig. 5. Cold competition analysis reveals that unlabeled probe can compete for PRL activated protein-DNA complexes. ³²P radiolabeled probe with an activity of 50,000 cpm/ml was serial diluted with 50 nM to 5 μ M of unlabeled probe and combined with 10 μ g of PRL treated nuclear extracts. Five micromoles of unlabeled β casein probe was used in lane 9. Complexes were resolved by EMSA. This is a representative experiment independently performed two times.

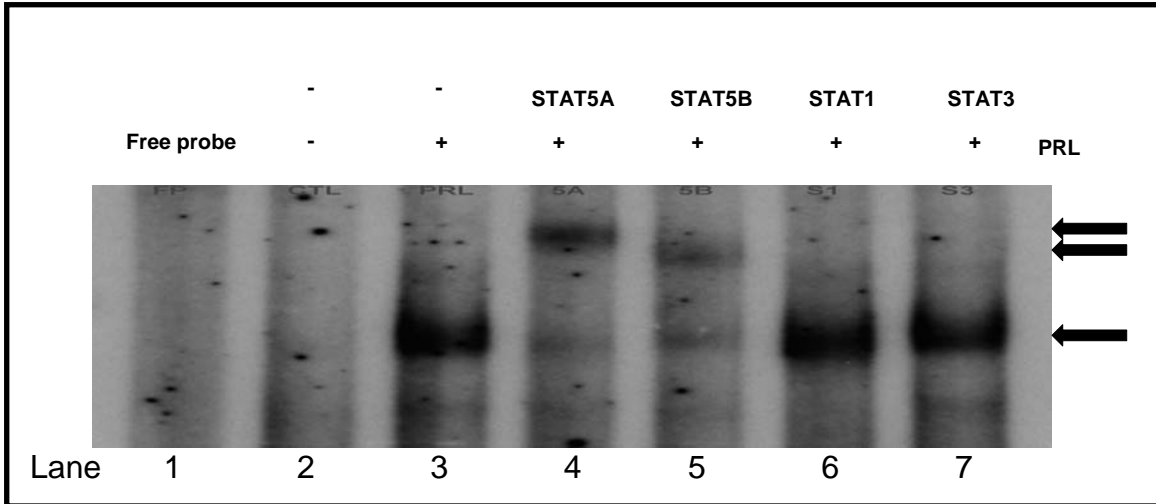


Fig. 6. PRL induces STAT5A and STAT5B binding to the -3809 site in the mouse adiponectin promoter. Ten micrograms of PRL treated nuclear extract was incubated with 1.5 ug antibody specific for indicated STAT proteins for 1 hour at room temperature prior to incubation with 50,000 cpm/ml of radiolabeled -3809 probe. Complexes were resolved by EMSA. This is a representative experiment independently performed three times.

3.3 Luciferase Assay

In order to determine if the adiponectin promoter was responsive to PRL treatment in living cells, fully differentiated 3T3-L1 adipocytes were transiently transfected with the PGL4-Adipo vector containing the -3809 site and the 125 bp surrounding it. Cells were left untreated or treated with 450 nM of PRL for 48 hours and luciferase activity was measured using a Berthold Detection Systems Sirius luminometer. PRL treated cells exhibited an increase in luciferase activity compared to cells left untreated (Fig 7). Thus, stimulation with 450 nM of PRL appears to cause an increase in PGL4-Adipo activity.

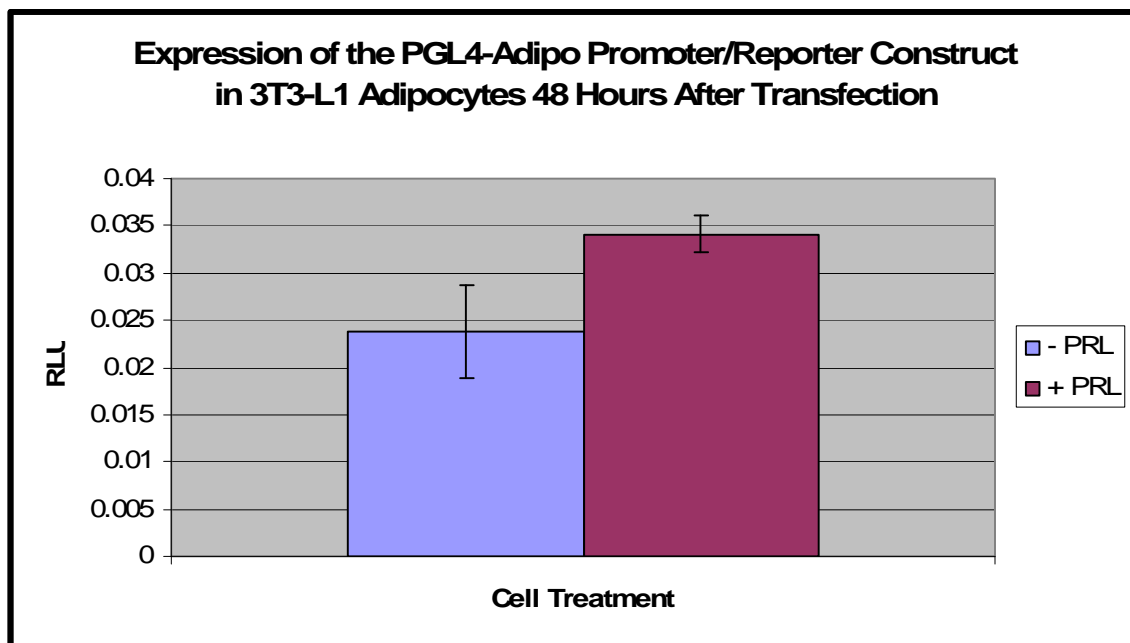


Fig. 7. Treatment with PRL modulates adiponectin promoter/reporter construct activity in 3T3-L1 adipocytes. Fully differentiated adipocytes were transiently transfected with the PGL-4 Adipo vector containing the -3809 site and the 125 bp flanking it. PRL treated cells were stimulated with 450 nM of PRL for 48 hours and a CMV/renilla vector was co-transfected to account for transfection efficiency. Relative light units (RLU) were calculated by dividing firefly luciferase activity by renilla luciferase activity. Results were done in triplicate. This is a representative experiment independently performed 3 times.

CHAPTER 4. DISCUSSION

4.1 Summary

Adiponectin levels in 3T3-L1 adipocyte whole cell extracts were shown to decrease over time in response to chronic PRL treatment. PRL is a potent STAT5 activator, therefore the mouse adiponectin promoter was scanned for potential STAT5 binding sites. After scanning, three classical STAT5 binding sites were found and EMSA analysis revealed that one site, at -3809, bound protein complexes in response to PRL. Four independent pieces of data showed that STAT5 specifically bound to the -3809 site. First, cold competition experiments showed that the prolactin induced protein complex could be specifically competed for by unlabeled oligonucleotide probe corresponding to the -3809 site. Secondly, unlabeled oligonucleotide probe corresponding to the well characterized STAT5 binding site in the β casein promoter was also able to compete for PRL induced complex binding. Moreover, EMSA supershift analysis showed that both STATs 5A and 5B specifically bound to this site, where as STATs 1 and 3 did not. Lastly, mutation of the STAT5 binding site resulted in the ablation of PRL induced protein complex binding which suggests that a classical STAT5 binding site is necessary for protein complex formation.

Luciferase experiments indicated an elevation in PGL4-Adipo activity upon stimulation with PRL. These results may be an artifact of the PGL4-Adipo vector, (which uses the -3809 site and the 125 bp surrounding it to drive the expression of the luciferase gene) because the full length adiponectin promoter may contain response elements that work in cooperation with the STAT5 binding site to silence adiponectin gene transcription. Furthermore, dose dependency experiments (data not shown) demonstrated

that 450 nM of PRL caused the most potent activation of STAT5, however, lower doses of PRL could potentially have a different effect on PGL4-Adipo activity.

4.2 Possible Mechanisms of Adiponectin Regulation

The reduction in adiponectin protein levels is likely due to STAT5 binding to the adiponectin promoter, which eventually causes the down regulation of the adiponectin gene. Yet, adiponectin protein levels do not decrease until roughly 48 hours after PRL treatment. The cause of this delay may be due to the stability of adiponectin mRNA, however, since western blot analysis was done using cells maintained in DMEM containing 10% FBS, the upregulation of the adiponectin gene by transcription factors activated by growth factors in the media cannot be ruled out. Therefore, luciferase assays were performed using 1% calf to help minimize the effects that other growth factors may have on adiponectin gene expression.

Similar studies have been performed on a STAT5 binding site approximately 4000 bp away from the transcription start site of the Interleukin 2 Receptor α (IL2R α) (103). This site not only contains a consensus STAT5 binding site, but a partial STAT5 binding site 11 bp away which has been shown to facilitate the tetramerization of STAT5 proteins (104). In addition, mutation of N-terminal STAT5 residues ablated tetramer formation and resulted in the loss of STAT5 activity on the IL2R α gene. Interestingly, we have observed that the site found at -3809 also contains a partial STAT5 binding site located 6 bp away (Table 5). Extensive studies have been made showing enhanced STAT5 binding activity to tandem STAT5 binding sites (105), and it has been suggested that oligomerization of STAT proteins may contribute to DNA binding specificity (106), therefore binding to the -3809 site may be due to the presence of a partial STAT5 binding

site. Other studies have shown that activation of STAT5 alone cannot modulate the expression of the IL2R α gene (107), and that cooperation with other transcription factors, such as Elf-1, are necessary for full activity. Thus, the activity of STAT5 on the adiponectin gene likely involves the recruitment of a number of proteins that work in cooperation to turn off adiponectin gene transcription.

Table 5. Human and Murine Adiponectin Promoter STAT5 Binding Sites with Surrounding Bases

Sequence	Location from Transcription Start Site
5'-ggaactccccacaccccag ttctcagaat tcacgtgcaaggtcttcc-3'	- 561 (Human)
5'-tgccactcaagcctaact ttctaggaa cacctaagtggtggcagct-3'	- 910 (Human)
5'-cctactttgtgccaggttctat ttctaggaa ctaagggatacaactttgaa-3'	- 1851 (Human)
5'-cagtggaagtcagctcaggag ttctcagaa aatgaaaaatagctgggca-3'	- 3196 (Murine)
5'-caaatgacttatctgatacactggcct tcggagaaa aagcaagtcacacctcatt-3'	- 3394 (Murine)
5'-gttgtagccatcatgtggt ttctgggaa attagaat ttcagggc cctctgc-3'	- 3809 (Murine)

4.3 Potential Impact of STAT5 Regulation of Adiponectin

Prolactin release from the pituitary is pulsatile and occurs approximately every 95 minutes in men (108). Continuous PRL release is inhibited by dopamine, which signals through the D2 receptor (D2R) on the surface of lactotrophs. Studies have shown that D2R expression in obese animal models is lowered, and that subsequent activation of the D2R in these models results in reduced body mass (109). Interestingly, it has been documented that pulsatile prolactin release in obese women is elevated in proportion to the amount of visceral fat excess (110), which may be the result of lowered dopaminergic

inhibition of PRL release. Furthermore, plasma PRL concentrations are highest during sleep (111), and studies have indicated that individuals with increased abdominal circumference (an indicator of visceral fat content), experience enhanced insulin resistance during sleep (112). Therefore, taking into account the data presented in this thesis, it can be hypothesized that elevated PRL levels during the condition of obesity causes a decrease in the amount of adiponectin released by adipocytes. As previously mentioned (56), lowered levels of adiponectin over time may result in the unnatural storage of triglyceride in muscle and liver which is a condition heavily correlated with the development of metabolic syndrome. Thus, lowered levels of adiponectin due to the actions of STAT5 present a possible mechanism for the development of metabolic syndrome.

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