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To the Graduate Council:

I am submitting herewith a thesis written by Rashika Joshi entitled "Agouti Mice Models to Study the Diabetogenicity of FK506 (Tacrolimus)." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

Dr. Roger C. Carroll, Major Professor

We have read this thesis and recommend its acceptance:

Dr. Naima Moustaid Moussa, Dr. John Erby Wilkinson

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Dr. John Erby Wilkinson

Accepted for the Council:

Dr. Anne Mayhew

Vice Provost and
Dean of Graduate Studies

(Original signatures are on file in the Graduate Student Services Office.)

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Agouti Mice Models
to
Study the Diabetogenicity of FK506 (Tacrolimus).

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee Knoxville

Rashika Joshi
August 2001

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Dedication

This thesis is dedicated to my parents Mr. Shiva Prashad Bhattarai and Mrs. Hem Kumari Bhattarai.

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Acknowledgements

I would like to thank my advisor Dr. Roger C. Carroll, who gave me an opportunity to work in his lab under his guidance. Without his support and encouragement this work would not have been possible. I also want to thank my committee members, Dr. Naima Moustaid Moussa and Dr. John Erby Wilkinson for their valuable suggestion and encouragement. Also, thanks to Dr. Michael Zemel for providing the agouti mice and for access to the nutrition department facilities.

I thank my husband Hem Raj Joshi for his support and encouragement during my studies.

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Abstract

Dominant mutations in the promoter region of the mouse agouti gene lead to a syndrome characterized by agouti yellow coat color, obesity, hyperinsulinemia, hyperglycemia, type II diabetes and increased linear growth. The mechanism of agouti modulation of the coat color development as well as obesity involves competitive antagonism of the ? -melanocortin stimulating hormone at its receptor.

Hyperplasia of the pancreatic ? cells in agouti mice precedes the development of obesity. Enhanced insulin secretion is induced in vitro by agouti effects on calcium influx into islet cells. Preliminary testing of insulin producing pancreatic cells has shown that exposure to high doses of an immunosuppressive drug tacrolimus (FK506) altered the secretion of insulin. The hypothesis of the present study is that FK506 treatment of transgenic mice, which either overexpress the agouti gene ubiquitously or in the adipose tissue, may alter expression of agouti mRNA and protein. Agouti mice also have an impairment of the immune system thus, another hypothesis to be tested was if immunosuppression by FK506 would amplify the agouti phenotype.

There was decrease in food intake and glucose levels in the aP2 mice with Tacrolimus (FK506). There was no detection of agouti mRNA or protein in adipose or pancreatic tissue of wild type mice and no significant effect of FK506 on agouti mRNA or protein expression in the aP2 mice. Toxic effects of FK506 at this dose in both mice were not significant.

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I. INTRODUCTION

Background and Characterization of the Agouti Mou se:

The melanocyte is the principal cell type involved in coat color pigmentation. During embryonic development melanocyte precursors (melanoblasts) migrate from the neural tube to the base of developing hair follicles (Billingham and Silvers, 1960; Searle 1968). The melanoblasts differentiate into melanocytes and function as unicellular glands by secreting melanin granules into actively growing hair shafts (Billingham and Silvers, 1960). Eumelanin (brown or black color) and phaeomelanin (yellow or reddish) are two distinct types of melanin produced by melanocytes (Searle1968).

Production of eumelanin and phaeomelanin in the mouse are regulated by the agouti locus on chromosome2. Melanocytes produce eumelanin at first, then switch to phaeomelanin synthesis and return to eumelanin production before the hair growth process is complete which give rise to the true wild -type coat color of mice (agouti pigmentation) (Galbraith, 1964). This pigmentation pattern is called agouti; hair of agouti mice are black or brown at the tip with a subapical band of yellow, and black or brown at the base which give animal an overall grizzled appearance (Searle1968).

Most of the coat colors genes act in a cell-autonomous manner within the melanocyte but agouti usually function s within the microenvironment of the hair follicle. Regulation of the coat color by agouti occurs through direct or indirect intracellular signaling within the follicular environment. There are at least 20 dominant and recessive agouti alleles form a com plex dominance hierarchy. Alleles associated with increased

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phaeomelanin production are generally dominant over alleles associated with agouti pigmentation, which in turn, are dominant over alleles primarily associated with eumelanin production (reviewed in Silver 1979).

The mouse agouti gene was cloned and shown to encode a distinctive 131 amino acid protein with a consensus signal peptide (Michaud et. al., 1993). A human homologue of the mouse agouti gene was cloned that is 85% identical to the mouse agouti gene and 80% identical at the amino acid level (Kwon et. al., 1994; Wilson et. al., 1995). The murine agouti gene is normally expressed only in the neonatal skin, where it regulates melanocyte pigment production. A dominant mutation in the promoter region results in ubiquitous expression of normal agouti protein throughout life in A^y agouti mice. This overexpression leads to a syndrome characterized by yellow coat color as well as obesity, hyperinsulinemia, type II diabetes and an increased incidence of spontaneous and induced tumors (Michaud et. al., 1994).

Mechanism of Agouti Signaling:

The mechanism by which the agouti protein alters function of melanocytes has been well documented while the mechanism by which it causes obesity is not completely understood and there are two different hypothesis for agouti action. The first hypothesis is based on the opposing interactions between agouti and the 13 amino acid peptide ?-melanocyte stimulating hormone (?-MSH). The hormone (?-MSH) causes melanocytes to switch from the synthesis of phaeomelanin to eumelanin (Geschwind et. al., 1966). This effect of ?-MSH is regulated through a Gs-coupled seven transmembrane domain receptor

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(Mountjoy et. al., 1992). ?-MSH is the natural ligand for MC1-R, MC3-R, MC4-R and MC5-R. MC1-R (melanocyte-stimulating hormone receptor) is expressed in melanocytes and has a physiological role in pigmentation (Mountjoy et. al., 1992). Level of cAMP is elevated when ?-melanocyte stimulating hormone binds to its receptor (MC1-R). This results in a switch from eumelanin (black pigmentation) to phaeomelanin (yellow pigmentation) production (see fig 1). This competitive antagonism of melanocortin binding serves as a paradigm for agouti action in obesity (Huszar et. al., 1997, reviewed in Moussa-Moustaid Naima 1999).

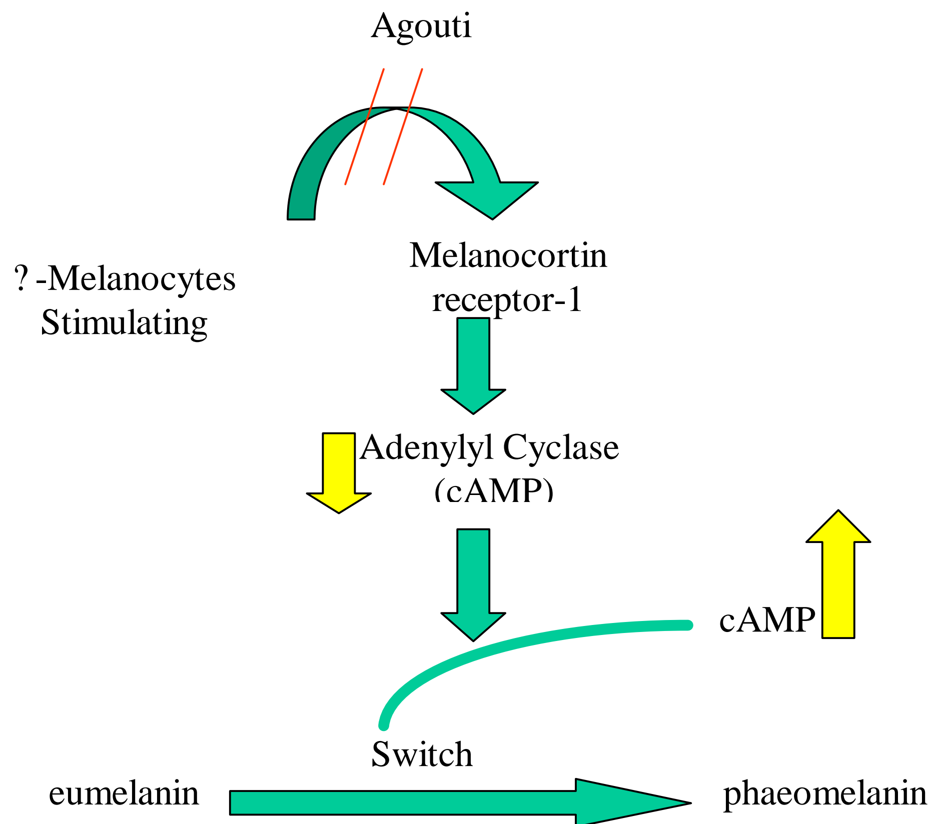

Figure 1: Switch from Eumelanin to Phaeomelanin.

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MC3-R is found in hypothalamic and limbic systems MC4-R is expressed in the brain, and is considered to regulate feeding behavior and metabolism (Dinulescu et. al., 2000). Agouti protein has higher affinity for MC4-R than MC3-R. Antagonism of these melanocortin receptors (MC3-R or MC4-R) could be responsible for the metabolic effects of agouti gene. Deletion of MC4-R gene in mice leads to the development of obesity/hyperinsulinemia (Tota et. al., 1998, Huzar et. al., 1997). In addition, intra-cerebro-ventricular administration of an MC4-R agonist inhibits feeding (Fan et. al., 1997).

The cysteine-rich C-terminal region of agouti protein has the ? -MSH antagonism property (Willard et. al., 1995). Yellow coat color in the dominant agouti mutation is likely to be mediated through antagonism of the ? -MSH receptor, preventing the higher level of intracellular cAMP to switch from phaemelanin to eumelanin pigment production (Lu et. al., 1994).

A second hypothesis is that agouti protein binds to its own receptor and interferes with the action of ? -MSH at an intracellular level (Conklin and Bourne, 1993). The agouti receptor might couple to another G protein to inhibit adenylate cyclase, or affect other intracellular messengers like calcium. This two-receptor hypothesis for agouti gene action has important implications for the multiple roles of overexpressed agouti protein in obesity, hyperinsulinemia and increased tumor susceptibility (Yen et. al., 1994).

Agouti protein and agouti related protein (Agrp) or agouti related transcript (ART) are similar in structure and function, but different in the pattern of distribution (Dinulescu and Cone, 2000). Agrp is usually expressed at the higher levels in the arcuate

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nucleus and acts as the endogenous antagonist of the melanocortin 3 and 4 receptors (MC3-R and MC4-R) (Stanley et. al., 1999). The hypothalamus of the rodent brain expresses agouti related transcript (ART) (Shutter et. al., 1997). The hypothalamus of the obese mice also expresses higher levels of ART and obesity is caused by the over expression of ART in transgenic mice (Ollmann et. al., 1997). Human ART gene (human agouti related protein or hAGRP) can bind and antagonize the hMC3-R and hMC4-R with high affinity compared to MC1-R and MC5-R. It is thought that Agrp functions as a natural antagonist of the neural MC3-R and MC4-R to regulate feeding behavior (Tota et. al., 1998).

Relationship of Agouti to Other Obesity Genes (Leptin):

OB protein which is also known as leptin, is secreted by adipose tissue (Frederich et. al., 1995). Leptin usually circulates in blood and binds to its family of binding proteins and acts on central neural networks that regulate feeding behavior and energy balance. This protein appears to play an important role in control of body fat stores through the regulation of feeding behavior, metabolism, autonomic nervous system and body energy balance (Campfield et. al., 1996). It is secreted and synthesized by adipose tissue in proportion to adipocyte size and number. Like the agouti C terminal region, OB protein also has a C terminal disulfide bond, which appears to play an important role in its activity (Zhang et. al., 1994). OB protein binds to OB-R receptor, which are found to be expressed in the choroid plexus, hypothalamus as well as several peripheral tissues and are localized particularly in adipocytes, macrophages, stomach, placenta and pancreatic islet cell type. There are several forms of OB- receptors, the short form (with a truncated

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intracellular domain) and the long form (with the complete intracellular domain) are the two major forms of the receptor (Campfield et. al., 1996). The long form of the receptor is thought to be the signaling protein that has the biological effects (Tartaglia et. al., 1995).

Agouti regulates adipocyte metabolism including lipogenesis (Jones et. al. 1996) and lipolysis (Xue et. al., 1998). Expression of agouti in adipose tissue increases both adipose tissue and circulating plasma leptin levels (Claycombe et. al., 2000). Leptin synthesis and secretion by agouti was increased due to the increase in leptin mRNA content by agouti and not due to agouti-melanocortin receptor antagonism (Claycombe et. al., 2000). Regulation of an obesity gene product by another obesity gene product and metabolic hormones suggests that these interactions may play an important role in obesity syndromes (Campfield et. al., 1996).

Diabetes, Hyperinsulinemia, and Agouti:

Intracellular calcium ?(Ca^{2+})i? plays an important role in metabolic disorders including obesity and insulin resistance (Draznin et. al., 1987). There is increase in basal ?(Ca^{2+})i? in adipocytes of obese patients (Xue et.al.,1998). Increases in ?(Ca^{2+})i? inhibits insulin stimulated glucose transport. Ca^{2+}-channel antagonism improves cellular insulin sensitivity (Draznin 1993), which may play an important role in insulin resistance by inhibiting the dephosphorylation of insulin sensitive targets. This includes glycogen synthase and the insulin sensitive glucose transporter, which are activated by ?(Ca^{2+})i? -induced phosphorylation and activation of inhibitor 1 through blocking the activation of phosphoserine phosphatase 1 (Begum et. al., 1993). It is possible that ?(Ca^{2+})i? increases

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the activity of protein kinase C that may contribute to insulin resistance via phosphorylation of the insulin receptor ? subunit tyrosine kinase (Bollag et. al., 1987).

Kim et.al. (1997) conducted a study to determine the effect of purified agouti protein on ?(Ca^{2+})i? in cultured using skeletal myocytes, smooth muscle cells, adipocyte cell lines, as well as in primary human adipocytes, to determine if these effects are mediated through melanocortin receptors. Results suggested that agouti regulation of ?(Ca^{2+})i? in various tissues requires expression of melanocortin receptors.

Studies have shown intracellular Ca^{2+} to play an important role in the metabolic disorder of obesity and insulin resistance (Byyny et. al., 1992, Draznin et. al., 1987). Recombinant agouti protein causes a dose-dependent increase in ?(Ca^{2+})i? in variety of cells including both murine and human adipocytes. Agouti protein stimulates the expression and activity of fatty acid synthase, a key enzyme in deno vo lipogenesis, and increases triglyceride accumulation in 3T3 L1 adipocytes in a Ca^{2+} dependent manner (Jones et. al., 1996).

This result can also be observed by the stimulation of Ca^{2+} influx by KCl and inhibited by a Ca^{2+} channel blocker (Xue et. al., 1999). The relationship between [(Ca^{2+})i] and insulin signal transduction is not well understood. It seems that elevation in [(Ca^{2+})i] may result in insulin resistance by affecting the phosphorylation of glucose transporter type 4 and other insulin-sensitive substrates within the cell (Kim et. al., 1997).

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Agouti and Pancreatic Function :

Obesity increases the risk of diabetes and 80% of type II diabetes are obese, but not all diabetic people are obese and not all obese people are diabetic (Salan 1987). Obesity and diabetes are influenced by independent factors (Barrett-Connor 1989). Obese people have defective glucose tolerance but do not progress to a diabetic state because both insulin resistance and ?-cell defects are required for type II diabetes in mice. Transgenic mice expressing agouti in adipose tissue under the control of adipocyte specific promoter (aP2 promote) become obese if supplemental insulin is concomitantly provided. However supplemental insulin does not produce this effect in their nontransgenic littermates (Mynatt et. al., 1997). Development of obesity in agouti mutant mice may be due to hyperplasia of pancreatic ? cells. Hyperinsulinemia may be a direct effect of agouti action on the pancreas and this hyperinsulinemia, combined with adipocyte agouti expression, may contribute to the obesity syndrome in agouti mutants. This was confirmed in vitro in cultured adipocytes where insulin and agouti exert additive effectson the FAS gene. Insulin resistance is a special feature in type II diabetes and is a characteristic of agouti mutant mice (Xue et. al., 1999).

Agouti mice exhibit some suppression of their immune system. The immunosuppressive drug Tacrolimus (FK506) was used to see if this drug altered the physiologic phenotype of these mice.

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II. IMMUNOSUPPRESSIVE DRUGS

Allogeneic (graft between the animal of same species) transplantation is made possible by the use of immunosuppressive drugs. Three kinds of immunosuppressive drugs are used in clinical transplantation.

1) Corticosteroids: are analogues similar to natural glucocorticoid hormone which is produced by the adrenal cortex and have anti-inflammatory properties.

2) Cytotoxic drugs are commonly used in solid organ transplantation, these drug usually interfer with DNA replication, kill proliferating lymphocytes which are activated by graft alloantigens.

3) The microbial products cyclosporine A, tacrolimus (FK506) and rapamycin derived from different microbes are all potent immunosuppressive agents which act by inhibiting the signaling pathway of T-cell activation.

During 1960s and 1970s, transplant physicians depended on a combination of corticosteroids and cytotoxic drugs to prevent the rejection of transplanted organs. In the early 1970's, introduction of cyclosporine A (CsA) significantly improved the outcome of organ and bone marrow allograft transplantation and advanced therapeutic options in autoimmune diseases (Bierer et. al., 1993). FK506 appears to have a higher therapeutic effect than CsA. FK506 has been used in clinical transplantation trials with encouraging results (Migita et. al., 1999). FK506 and CsA are structurally and chemically unrelated but their mechanism of action are similar. Both FK506 and CsA bind to cytoplasmic peptidyl-prolyl isomerases, which are abundant in all tissue (Bierer et. al., 1993). Cyclosporine binds to cyclophilin and FK506 binds to FK-binding protein (FKBP) (Zenke

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et. al., 1993). Both complexes inhibit the same cytoplasmic serine/threonine phosphatase, calcineurin, which is necessary for the activation, by dephosphorylation, of a T cell-specific transcription factor (Schwaninger et. al., 1993). This transcription factor, NF-AT, is involved in the synthesis of interleukins by activated T-cells. They have a similar effect, inhibiting the action of calcineurin on signal transduction pathways, in other hematopoietic cells (Lyson et. al., 1993). Rapamycin a structural analog of FK506, inhibits cellular function by different molecular mechanisms (Bierer et. al., 1993).

Tacolimus (FK 506):

FK506 (Tacrolimus or Prograf) was discovered in 1984 by scientists at Fujisawa Pharmaceutical Co (Japan) in the fermentation broth of the filamentous bacterium, Streptomyces tsukubaensis. It is a 23-member ring macrolide lactone (Kino et. al., 1987). Chemically it is designated as 5, 6, 8, 11, 12, 13, 14 1,5, 16, 17, 18, 19, 24, 25, 26, 26q-hexadecahydro-5, 19-dihydroxy-3-[2-(4-hydroxy-3-methoxycyclohexyl)1-methylethenyl]-14,16-dimethoxy-4, 10,12,18-tetremethyl-8-(2-propenyl)-15,19-epoxy-3H-pyrido [2,1-c] [1,4] oxaazacyclotricosine-1, 7, 20, 21 (4H, 23H)-tetrone, monohydrate (Walsh 1998). The chemical structure of tacrolimus is shown below fig 2 (Ivery and Weiler 1997).

It has an empirical formula of $C_{44}H_{69}NO_{12}.H_2O$ and a molecular weight of 822.05. Tacrolimus is as white crystal or crystalline powder. It is practically insoluble in water, freely soluble in ethanol, and very soluble in methanol and chloroform (Walsh 1998). Tacrolimus is an important therapeutic agents used in preventing graft rejection during tissue transplantation. It prolongs the survival of the host and transplanted graft in animal transplant models of liver, kidney, heart, bone marrow, small bowels and pancreas,

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lung and trachea, skin, cornea and limb (Siekierka and Signal 1992). The pharmacokinetic parameters of FK506 have been determined following intravenous and oral administration in healthy volunteers, liver transplant, and kidney transplant patients. The whole blood concentration serves as the more appropriate sampling to describe FK506 pharmacokinetics rather than plasma concentration.

Mechanism of Action:

The immunosuppressant FK506 is an extremely potent inhibitor of T-lymphocyte activation (Clipstone and Crabtree 1992). The exact mechanism of action is yet not known (Negulescu et. al., 1994). Studies have shown that the drug becomes active only when bound to specific members of the FK506 binding protein receptors. The FK506- FK506 binding protein receptor complexes interact with a major component of the T-cell antigen receptors signal transduction pathway, the calcium-calmodulin-dependent phosphoprotein

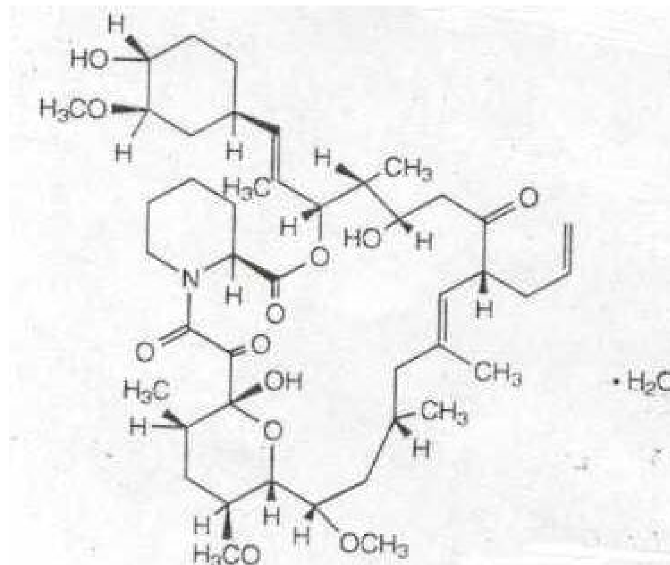

Figure 2: Structure of FK506.

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phosphatase calcineurin (Liu et. al., 1991). FK506, after binding with its binding protein receptors, inhibits the phosphatase activity of calcineurin and prevents transcriptional activation of the interleukin-2 gene (Tocci and Sigal 1992, Luo et. al., 1996). This result in inhibition of the T-lymphocyte activation (i.e. immunosuppression) (Shibasaki et. al., 1996). See figure 3.

Relationship to diabetes:

As a result of calcineurin inhibition, FK506 alters multiple biochemical processes in a variety of cells besides lymphocytes. This may be the cause for its adverse side effects including neurotoxicity, nephrotoxicity, diabetogenicity and gastrointestinal disturbances

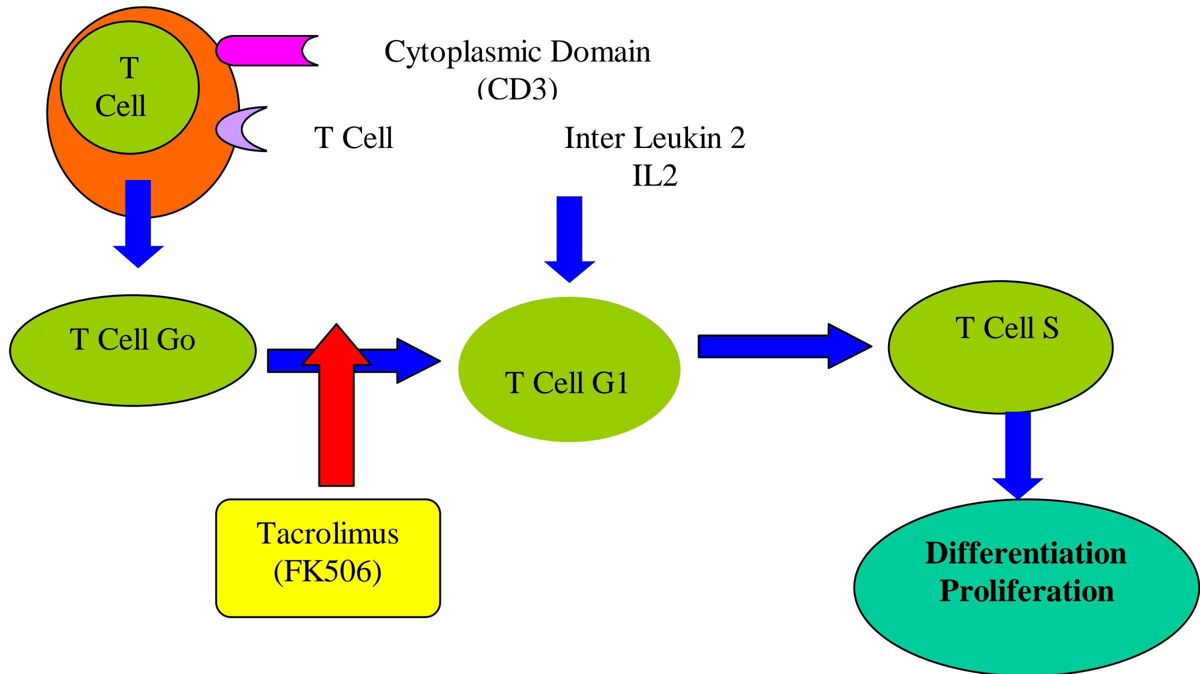

Figure 3: Inhibition of T-cell by FK506

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(Dumont 2000). One of the most common toxicity effects of FK506 is insulin-dependent diabetes mellitus (Jindal et. al., 1997).

Pancreas contains about half a million islets of Langerhans that make the hormones insulin, glucagon, and somatostatin (Thorens and Waeber 1993). Each islet cell functions in such a manner that they make a single hormone: ? - cells make glucagon, ? cells make insulin, and ? cells make somatostatin (Kuby 1994). Insulin is usually secreted from the pancreas in response to the increased blood glucose level arising after a meal (Porte and Pupo 1969). Insulin binds to the surface receptors and stimulates cells to take up glucose and convert it into carbohydrates and fat (Kuby 1994). Destruction of certain insulin-producing cells of pancreas gives rise to insulin dependent diabetes mellitus (IDDM) (Bach 1994).

Individuals with IDDM, have an autoimmune antibody and T-cells respond against insulin, glutamic acid decarboxylase, and other specialized proteins of the pancreatic ? cells. CD8 T cells specific for some ? cells proteins are thought to mediate ?-cell destruction resulting in decreased insulin secreting cells (Robertson 1995). Individual islets become successively infiltrated with lymphocytes, and as ? cells die the structures of the islet degenerates (Porte and Kahn 1989).

FK506 is an immunosuppressive drug, which interrupts calcium-calmodulin-calcineurin signaling pathway in T cells, blocking antigen activation of the T cell early activation genes. It is thought that regulation of insulin gene expression in the ? cell involves a calcium-signaling pathway and clinical use of FK506 has been associated with insulin requiring diabetes mellitus (Lohmann et al., 2000).

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Animal Model (Rats and Mice) Studies Showing Pathophysiological Changes:

Several studies in animal models have shown that the toxicity of FK506 is dosage dependent. Studies in a adult rat model (Ishida et. al., 1997) showed that a high oral dose of FK506 for 13 weeks uniformly induced histopathologic changes in several tissues, including renal and pancreatic injuries. Cataracts were also observed in rats dosed orally with FK506 5mg/kg/day, with an incidence of 25%, where as no cataract formation was observed in the 0.2 or 1mg/kg/day groups. Biochemical parameters, such as sorbitol, Na^{+}/ K^{+}-ATPase, and glutathione in the lens of rat were also evaluated. Five mg/kg/day FK506 led to an increase of sorbitol and a decrease of reduced glutathione but did not affect the Na^{+}/ K^{+}-ATPase activity of the lens. The higher dose of FK506 also gave a very high incidence of neurotoxicity and nephrotoxicity.

FK506 is known to have diabetogenicity mediated through pancreatic injury, which appears as vacuolation of islet cell in rats. High dosages (5mg/kg/day) of FK506 induced an elevation of blood glucose association with glucose intolerance, and decreases of both basal insulin level and insulin content in the pancreas. These changes were parallel with the cataract development in this study. Diabetic parameters did not change in lower dosage groups (Ishida et. al., 1997).

Murase et.al. (1991) studied lower dosage FK506 effects on preventing spontaneous autoimmune diabetes in the BB rat. BB rat is the experimental analogue of human juvenile diabetes mellitus. The diabetes follows specific autoimmune destruction of the ?-cells while leaving ? and ?-cells intact. Intragastric doses of FK506 were given at 1.0 mg/kg/day to 2.0 mg/kg/day in the BB rat treated group. Treatment was started at 30

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days of age and continued for 90 days. Body weight and glycosuria were measured three times a week. Administration of 2.0 mg/kg/day of FK506 completely prevented the development of spontaneous diabetes in BB rat, and greatly reduced the characteristic histopathologic changes in the pancreas. With a lower dose of FK506 (1.0 mg/kg) 15.7% became diabetic and prevention of pancreatic injury was less complete. However, post-therapy relapse rate of diabetes by this dose was low. In the untreated group 75% developed diabetes between the age of 70 and 103 days. This study showed that the higher (2.0 mg/kg/day) dose had beneficial effects, which far outlasted the time of treatment. There was no mortality in this study except for that caused by diabetes, and toxic effects of FK506 at this dose were not noted except for a slight decreased rate of weight gain (Murase et al, 1991).

Toxic and therapeutic effects of FK506 have also been noted in murine studies. Wada et. al., (1991) showed that daily injection of 3.2mg/kg FK506 could protect a BALB/c skin graft in C57BL/6 recipient mice as long as the drug was continued for up to 1 month. With lower dosage of 1mg /kg, some of the animals rejected the skin graft, while higher doses of 5.6 mg/kg, some animals died with an intact skin graft. The cause of death and its relationship to the higher dose of FK506 was not reported.

In contrast a lower dosage or shorter time course of FK506 may be well tolerated in mice and even protect transplanted ?-cells. As a potential protocol for inducing tolerance to islets allografts, Fukuzaki et. al., (1995), showed that induction of tolerance to islet allografts is possible with preoperative donor spleen cell injection and a short course of FK506 treatment. BALB/c and B6 mice were used as a donor and recipients for

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the islet. Islet cells were isolated by collagenase digestion method. These islets were transplanted in the renal subcapsular space of STZ-induced diabetic mice (streptozocin-induced diabetic mice). Hyperglycemia level of >300mg/dl was the criteria indicating that rejection had taken place. Mice were treated for a long and short course of FK506 after transplant. Long course treatment was for 4 weeks after islet transplantation with intramuscular FK506 dosages of 1.0 or 3.2 mg/kg. Short-term injection of FK506 was at a dose of 3.2 or 10 mg/kg intramuscularly for 3 days (days 0 to 2,3 to 5, 5 to 7) after transplant. Significant numbers of recipient mice showed normoglycemia over 90 days following a short course of FK506 treatment. A short course of FK506 treatment after grafting has been found to induce tolerance, avoiding long term use of immunosuppressant. A second experiment was carried out on the timing of the donor spleen cell injection (DSI) and FK506 in order to induce tolerance to islet allografts. There was marked increase in the graft survival after a single injection of FK506 on day 3 and day 5 after DSI. From this experiment it was observed that there is a critical window period for the DSI and FK506 treatment for inducing unresponsiveness. Induction of tolerance is possible before transplantation by administration of donor antigen followed by a short course of FK506 treatment.

Kai et.al. (1993) showed the preventive effect of a low dosage of FK506 on autoimmune insulitis in non-obese diabetic mice. These mice develop diabetes spontaneously as result of an autoimmune response to beta cells in the pancreatic islets. The mice were given FK506 intraperitonelly in a dose of 1.0 mg/kg, every other day, from ages 2 to 12, 2 to 6, and 4 to 12 weeks, the incidence of insulitis and overt diabetes was

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monitored. Effects of FK506 on immune reactions to ? cells were also investigated by using both syngeneic and allogeneic islet transplants. Treatment with FK506 in mice from age 2 weeks completely prevented the onset of overt diabetes, and the incidence of insulitis was reduced to less than 10% at age 30 weeks. However, treatment of mice with FK506 from age 4 weeks was less effective in preventing insulitis and onset of diabetes. In the case of islet transplantation, FK506 treatment of non obese diabetic (NOD) mice from age 2 to 6 weeks prevented autoimmune responses both in syngeneic (tissue that have identical genotype) islets and in allogeneic islets, which share the same H-2 antigen with the nonobese diabetic mouse (Kai et.al. 1993).

Hypothesis:

The hypothesis to be tested in this study is that immunosuppressive calcineurin inhibitors such as FK506, might alter agouti mRNA and/or protein levels in adipose and pancreatic tissues. FK506 could also antagonize signal transduction pathways elicited by agouti protein, which are required for its effects on both tissues. In addition, this study also seeks to determine if the hyper expression of agouti might amplify the pathogenic effects of a nontoxic dosage of FK506.

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III. MATERIALS AND METHOD

Animals:

Agouti mice (aP2) specifically express agouti in adipose tissue under the control of the fatty acid binding promoter. Adipose tissue specific promoter agouti mice (aP2) along with their nontrasgenic littermates were selected for this study. The expression of agouti in aP2 mice is found in skin, white (higher level) and brown adipose tissue, muscle and low levels in the pancreas (Mynatt et. Al., 1997). Male and female mice of four weeks to eight weeks of ages and at least 20 grams of body weight were used in this study. They all were caged in a clean environment with hepta-filtered air to protect the immunosuppressed animals from pathogenic organisms. The mice used in this study were from the breeding colony at the Department of Nutrition, University of Tennessee Knoxville. Each transgenic strain and its normal littermates were divided into two groups housed in cages of 2 each.

Diet/Dosing Tacrolimus:

The mice were allowed free access to the powder diet (Dyets Inc. PA) of 10gm/day/ cage and water. The transgenic and nontransgenic littermates (treated groups) were treated with FK506 oral dosage of up to 0.5-0.7mg/kg/day for 13 weeks by mixing it with powder diet (0.05mg of FK506/10 gm diet). The plasma level of FK506 was assayed by a modified enzyme immunoassay using a mouse monoclonal anti-FK506 antibody (Abbott Diagnostic, Abbott Prk, IL). The calculated average dosage of FK506 of the course of the study was about 0.7mg/kg/day based on the consumption of 3.3gm of food.

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This compares to the human therapeutic dose for immunosuppression of 0.02 mg/kg/day (2 oral tablets/day).

Tissue Sampling:

After 13 weeks of treatment the mice were sacrificed. Immediately after sacrifice fat pads abdominal, perirenal and subscapular fat pads were pooled, pancreas, kidney and the head were collected. Fat and pancreas for the RNA extraction were kept in RNA Later (Ambion, Austin, TX), which acts as a preservative for the RNA. Kidneys, a small portion of the pancreas (50mg), and the head were preserved in Carson Millonig (10 % formalin, sodium phosphate 14.7gm/L, sodium hydroxide 2.15/L) (Biochemical Sciences Inc, Swedesbord, NJ) for the study of pathophysiological changes.

Body weight, Urinanalysis, Blood Glucose/Insulin Levels:

Body weight of the mice was measured at the time of treatment and weekly for 13 weeks. Weekly urinanalysis was done by a semiquantitative analysis strip (chemstrip 2GP Roche Diagnostic Corporation, IN). Glucose detection is based on the enzymatic glucose oxidase/ peroxidase method. A positive reaction is indicated by color change of the strips from yellow to green. The blood glucose level was measured at the time of initial treatment and weekly by Fast Take Glucose Meter Test Strips from Life Scan Johnson and Johnson Company. Blood was obtained by pricking the tail vein (Ishida et. al., 1997). At the time of sacrifice, blood was obtained by cardiac puncture for blood glucose and centrifuged at 1600g for 20 minutes. The plasma was used to determine the circulating

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insulin levels by rat insulin enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI) (Ishida et. al., 1997).

Other Pathophysiologic Changes:

Tissue were routinely fixed in Carson's Millonig (Biochemical Sciences Inc, Swedesbord, NJ), processed, and paraffin-embedded. Histopathological examination of the eye lenses, pancreas and kidney was done to determine any cataract formation, amyloid deposition, or renal changes as evidence of nephrotoxicity in the treated mice as compared to untreated controls.

RNA Preparation and RT-PCR:

Total RNA from adipose tissue and pancreas was prepared using CsCl density centrifugation. Adipose and pancreatic tissues were taken out of the RNA Later solution (Ambion) and weighed. Guanidine Thiocynate with ?-mercaptoethanol were added 10 ml to 1 gram of tissue. The tissues (fat and pancreas) were homogenized using a tissue tearer (Distributed by Brinkmann Co. made in Switzerland by Kinematics Gmbh) for 30 seconds. The homogenized solution was gently layered over the cesium chloride (5ml) in 12ml Sorvall tubes (Sorvall Instrument, Wilmington, DE). The tubes were balanced and sample centrifuged at 40,000 rpm for 18-20 hours at 20°c in a Sorvall Rotor. The supernant was aspirated and the RNA pellet at the bottom of the tube was dissolved in RNase free water. RNA was quantitated by measuring its absorbance at 260/280 nm (UV Visible Recording Spectorphotometer, Shimadza Corporation, Kyoto, Japan), and its quality was assessed after electrophoresis of 3?g of RNA on a 1% agarose gels stained with ethidium bromide.

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Two distinct bands of 28s and 18s consistent with intact RNA were observed in photographs of the gel.

RT-PCR was conducted as described by Kwon et. al., (1994) from the extracted RNA. Construction of cDNA was done using 3?g/5?l of RNA in 50?l reaction. RNA was heated at 65^0C for 5minutes and cooled in ice for 5 minutes. Reverse transcription 10x buffer, dNTP mixture, RNasin ribonuclease inhibitor, random hexamers and AMV reverse transcriptase mix was added at last to the RNA. The cDNA reaction was incubated at room temprature for 10 minute and heated at 42^0C for 60 minutes and at 99^0C for 5 minutes to inactivate AMV and on ice for 5 minutes (Perkin Elmer, Branchburg, NJ). PCR was performed using red Taq polymerase (Sigma, Saint Louis, MO). The conditions for PCR were 1 cycle (94^0C, 5 min), 35 cycles (94^0C, 30 s; 68^0C, 1 min; 72^0C, 1 min) with 0.5 ?M agouti sense (ATGGATGTCACCCGCCTAC) and agouti antisense (CGCAGTTGGGGTTGAGTA) primers (Sigma Genosys, St. Louis, MO). ?-actin primers (Sigma, Genosys, St. Louis, MO) were added to separate PCR reactions as a control for the same cDNA product. The amplified PCR product was then visualized by 1% agarose gel electrophoresis (Kwon et. al., 1994). Autoradiogram results were quantitated by video densitometry on a Lynx 4000 Molecular Biology Workstation (Applied Imaging, Santa Clara, CA, USA). Expression of agouti cDNA was normalized to those of ?-actin and expressed as a ratio of density of agouti bands over density of actin bands in the same sample. ANOVA test was used for statistical analysis of the data.

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Northern Blot Analysis:

Northern blot was performed in the RNase free condition (Autoclave tips and tube, DEPC treated water, and wearing gloves). Total RNA (20?g) from adipose and pancreatic tissues for electrophoreses was prepared by adding 2.5?l sample loading buffer (0.75ml formamide, 0.25ml formaldehyde, 0.15ml 10x MOPS [3-N (Morpholinol) Propane Sulfonic Acid], 0.3 ml glycerol and 0.25% bromophenol blue) per 1?l of RNA sample. Samples were heated at 65°C for 10 minutes then cooled in ice for 5 minutes then electrophoresed through formaldehyde-agarose gels (1 gm of agarose in 74 ml of DEPC treated water, 10 ml of 10x MOPS and 16 ml of 37% formaldehyde). RNA was transferred from gels to the Gene Screen Plus Hybridization Transfer membrane (New life Science, Boston, MA) by capillary transfer method. The membrane was placed in the UV light cross linker chamber and cross-linked for 30 seconds 160mJ/cm^2. The cross-linked membrane was then stained with methylene blue (0.3M Na Acetate Ph 5.2 and 0.03% methylene blue, treated with DEPC). If the 28s and 18s bands were visible, a picture of the membrane was taken. The blot was pre hybridized with ULTRAhyb (Ambion, Austin, TX) and hybridized with radiolabeled agouti probe. The probe was prepared by using Random Primer DNA Labeling System (Gibco BRL life technologies, Gainshburg, MD). After a high (2X SSC, 0.1% SDS) and low stringency (0.1X SSC, 0.1% SDS) wash the blot was exposed to X-ray film for autoradiography. The blot was stripped in a boiling solution of 0.1X SSC and 0.1% SDS for 15 minute with slight agitation. The process of pre hybridization and hybridization was repeated again with a probe for the ?-actin as an internal control to check the quantity and quality of RNA present (Yen et. al., 1994).

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Autoradiogram results were quantitated by video densitometry on a Lynx 4000 Molecular Biology Workstation (Applied Imaging, Santa Clara, CA, USA). Expression of agouti mRNA was normalized to those of ?-actin by dividing the density of agouti bands with the density of actin bands in the same sample. The normalized data was compared for significant differences between strains and treatments with using ANOVA statistical analysis.

Western Blot Analysis:

The frozen tissue samples of adipose (100 mg) and pancreas (60mg) were weighed and immediately sonicated in centrifuge tubes for 1 minute using a Vibra Cell Sonicator Bath in 5?l/mg tissue of 1.5 fold concentrated denaturing mix (250 mM Tris HCl pH 6.8, 3% sodium dodecyl sulphate (SDS) 250mM EDTA, 250 mM EGTA, 0.015% bromophenol blue, 15% glycerol) with 3% ?-mercaptoethanol. The samples were then centrifuged for 40 minute at 150,000?g at 200C. Supernanant aliquots of 90?1 were electrophoresed through 6-17% SDS-PAGE gradient gels and transblotted to nitrocellulose membranes according to standard methods. 1% nonfat milk proved superior to 1% bovine serum albumin and was used to block nonspecific binding sites on the nitrocellulose after protein transfer. All washes and incubations with primary and secondary antibodies were per manufacturer's directions (Enhanced Chemiluminescence ECL Kit, purchased from Amersham). ECL detected bound antibodies with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin. The nitrocellulose membranes were treated with rabbit anti mouse agouti (Alpha Diagnostic International, San Antonio,

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TX). The secondary antibody was horseradish peroxidase (HRP) conjugated goat anti rabbit (Jackson Labs, West Groove, PA). Bound antibody was detected by Super Signal West Pico Chemiluminescent Substrate (PIERCE, Rockford, IL). The blot was stripped with stripping buffer (100mM 2-mercaptoethanol, 62.5mM Tris HCl pH 6.0 and 2% SDS) and reprobed with mouse anti glyceraldehyde 3-phosphate dehydrogenase (Sigma, Saint Louis, MO) and visualized with HRP-goat anti mouse IgG as internal control for loading differences.

The band observed by the western blot was confirmed to be agouti by running the same sample through the NuPage Bis-Tris Gels (Novex, San Diego, CA) and blotting it to the PVDF Membrane (Novex, San Diego, CA). The membrane was stained with Coomassie blue stain (25% isopropanol, 10% acetic acid and 0.05% Coomassie blue) and destaining was done with several changes of 10% acetic acid plus 10% methanol solution. The band observed at the level of 14 Kda, after destaining, was cut out and micro-sequenced in 494 Protein Sequence (Applied Biosystem, Foster, CA) with cycles and conditions as recommended by the manufacture.

Data Analysis:

Computerized statistical analysis was performed on data by group comparisons to determine significant differences. The criteria for parametric testing assume samples from normally distributed populations with the same variances. This test is based on the estimates of the population means and standard deviation, the parameters of a normal distribution. Nonparametric tests do not assume samples from a normal population distribution. Ranks Sum Tests automatically rank numeric data, and then compare the

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ranks rather than the original values. The parametric tests used in this study are unpaired t-tests and ANOVA. Nonparametric tests are Mann-Whitney Rank Sum Test and Kruskal-Wallis Analysis of Variance on Ranks. Chi-square or Fisher Exact test was performed to analyze proportions for significant differences. The power or sensitivity, of the test is the probability that the test will detect a difference or effect if there is a differences or effect. Power of 0.80 means that there is an 80% chance of detecting a specified effect with 1-? confidence (i.e,a 95% confidence when ?=0.05). The type of test for each data set is indicated in the figure or table legend or in the text.

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IV. RESULTS

FK506 plasma levels:

In a pilot study after 4 weeks of the diet supplemented with FK506, blood plasma levels of FK506 in the treated (8) mice are 20 times higher than in treated human (transplant patient). This assay was done to make sure that the diet with FK506 achieved a high but nontoxic dosage of FK506. It was confirmed that the mice were taking the food properly and the level of FK506 was high enough. Diet consumed averaged over the treatment course by agouti mice was 3.35 gm and by the nontransgenic littermate is 3.45 gm/day. The average FK506 consumed by agouti treated mice was 0.67mg/kg/day and treated wild type was 0.69mg/kg/day dose for each group

Diet:

Figure A-1 (agouti mice) and Figure A-2 (non transgenic littermates) shows the average food intake per week by the mice throughout the treatment. Initially there was significantly less food intake by both groups of FK506 treated mice, which became insignificant by week 2. This is most likely the result of the mice becoming accustomed to the taste of the FK506. Food uptake remained rather constant although low for the treatment groups throughout the 13 weeks of treatment with no evidence of the expected hyperphagia by the agouti mice. Surprisingly, at the end of the 13 weeks untreated non transgenic littermates averaged less consumption (3gm P=0.001) as compared to the beginning intake. There were also significant differences in the consumption of food in

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treated and untreated agouti mice at the end of the time course (treated ones 2.7gm P= P=0.408 untreated 3.4gm P=0.001). Further studies over a longer time course would be necessary to confirm these effects of FK506 on food intake.

Body Weight:

Figures A-3 (agouti) and A-4 (non transgenic littermates) shows weekly body weight averages for FK506 treated and non-treated groups. Despite the slight reduction in food intake over the course of the experiment, the nontransgenic littermates showed no effect of FK506 treatment on weight gain. At the end of the treatment the agouti mice showed significant differences with respect to FK506 treatment (Figure A-3). The untreated agouti mice gained more weight as compared with the treated agouti (P= 0.020). This may be due to the overall less consumption of the food or an effect of the drug on metabolism or fat deposition.

Blood Glucose Level:

Studies by Mynatt et. al. (1997) had shown that there are no differences in the blood glucose level in the transgenic mice (aP2) 120 ? 7 and 122 ? 11 mg/dl non transgenic littermates respectively. One of the toxic effects of FK506, established in the rat model, is insulin dependent diabetes with attendant elevation of blood glucose due to the injury of the pancreatic cells (Ishida et. al., 1997). Figures A-5 (agouti) and A-6 (non-transgenic littermates) show blood glucose levels (mg/dl), which were low in both the treated and untreated mice at the beginning of the experiment. Which may reflect some stress and acclimatization to a new environment or sampling error. By the second week,

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more normal values were seen for all groups with and without FK506 treatment. At the third week of treatment with FK506, the agouti mice showed a significant and unexplained decrease in the blood glucose level. This returned to normal by the fourth week and remained relatively constant through out the rest of the time course. There is no significant difference at the end of the time course in the blood glucose levels in the treated and untreated groups with this dosage of FK506. The glucose levels were quite variable and reached >200mg/dl in several of the untreated agouti mice. FK506 treated agouti mice had less variable glucose levels and none >200 mg/dl. The high variability resulted in an overall lack of a statistically significant effect of FK506 treatment and no evidence for a diabetic effect.

Plasma Insulin Levels:

Plasma insulin levels were determined using the RIA kit per manufacturer's directions. Data obtained is shown in Table I. Statistical analysis showed that that mean values of the treated mice (agouti mice 0.187 ? 0.0221nmols/ml and wild type 0.220 ? 0.0131nmols/ml, P=0.761) were less than untreated agouti mice 0.200 ? 0.0344nmols/ml or wild type 0.230? 0.069nmols/ml, P=0.122). Plasma insulin level of the agouti and the wild type mice with FK506 are 31?3?U/ml, 36?2?U/ml, respectively. Those without FK506 treatment had insulin levels of: agouti mice 33?5?U/ml and wild type 38? 11?U/ml. Previous studies of plasma insulin level of the aP2 at 25 to 30 weeks were found to be 29?8?U/ml for transgenic mice and 31?8?U/ml for nontransgenic littermates (Mynatt et. al.1997).

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Urinanalysis:

None of the mice showed color change in strips showing that the urine glucose level were in normal range in both treated and untreated agouti mice and their non transgenic littermates.

RT-PCR:

RT-PCR products with agouti primer (Figure A-7) from adipose and pancreatic tissues showed expression of the agouti mRNA in both treated and untreated agouti mice. There was no detectible expression of the agouti in corresponding tissue from non transgenic littermates (Figure A-8) with or without FK506. PCR done with the same cDNA sample with ? actin showed a band for each sample after agarose gel electrophoresis (Figure A-9). Data was analyzed by densitometery for RT-PCR and normalized as mean ? SE of agouti/?-actin densitometry (arbitrary units) for 10 treated versus 10 untreated mice from the aP2 or nontransgenic littermates. The expression of the agouti cDNA in the adipose and pancreatic tissues in the aP2 with and without FK506 was 0.628± 0.168SD (treated) versus 0.587±0.161 (untreated) (P=0.708) and pancreas 1.140± 0.727SD (treated) versus 0.493±0.172 (untreated) (P=0.089). Wild type mice with and without FK506 showed no detectable expression of agouti cDNA in the adipose and the pancreatic tissues.

Northern Blot:

Northern blot analysis of RNA from adipose (Figure A-9) and pancreatic (Figure A-10) tissues of FK506 treated and untreated agouti mice show no difference in the

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expression of the agouti. There was no expression of agouti in the treated wild type mice (Figure A-11, A-12). ?-actin northern analysis of these blots showed similar amounts of RNA within each experiments. Data was analyzed as the normalized mean ? SE of agouti/?-actin densitometry (arbitrary units) for 10 treated versus 10 untreated mice from the aP2 or nontransgenic littermates. The expression of the agouti mRNA in adipose tissue of the aP2 mice with and without FK506 was 1.152? 1.188 SD versus 1.069?0.686 SD (P=0.851) respectively and for pancreas 1.550?1.214 SD versus 3.019?3.092 SD (P=0.179) respectively. Adipose and pancreatic tissues of wild type mice, either with or without FK506, showed no detectable expression of the agouti mRNA and therefore no difference in expression of agouti with FK506 treatment could not be evaluated. We can only conclude that high dosages of FK506 had no effects on the adipose or pancreatic tissue levels of agouti mRNA in the aP2 agouti mice.

Western Blot:

Several attempts at western blotting to analyze agouti protein levels were carried out. SDS solubilized tissue fractions were electrophoresed on SDS PAGE and transblotted by standard protocols as described in Methods. Blocking with bovine serum albumin gave multiple non specific bands which were reduced by using 1% non-fat milk. Expression of the agouti peptide in fat was seen in agouti mice with and without FK506. In view of multiple bands observed, even with 1% non-fat milk, micro-amino acid sequencing the N-terminal sequence 131amino acids of blot bands was used to confirm that the 14.5 kDa band was the agouti peptide and that other higher molecular weights

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band were unrelated. The candidate agouti band was sequenced at least 3 times from the treated and untreated agouti and gave a match to the known sequence for agouti. Despite several attempts at varying extractions and SDS-PAGE transblotting conditions and use of two different antibody preparations for agouti (monoclonal antibody, affinity pure monoclonal antibody from Alpha Diagnostic International, San Antonio, TX) the results were quite variable with problems of nonspecific and variable specific staining making data analysis difficult. Analysis of 3-phospho-glyceraldheyde dehydrogenase (GAPDH) was done to determine equal loading and blotting efficiency. No marked differences were observed in agouti protein levels in FK506 treated mice. As expected, there was no detectable agouti band or amino acid sequence in the 14.5 kDa region SDS-PAGE analysis of wild type mice fat and pancreatic tissue. This is consistent with the RT-PCR and northern blotting results for agouti mRNA. We were also unable to detect agouti peptide in the pancreatic tissue of agouti mice.

Histopathology:

Histopathological study of the pancreatic islet cells was limited. There were four groups of mice; each group had ten mice (treated and untreated) but we did not obtain usable sections on all these mice because of the requirements of tissue for the other studies. Islet cells of six agouti mice with FK506 and four without FK506 were observed. Of 552 total cells observed in agouti mice treated with FK506, 19% were vacuolated. Of the 259 cells observed in agouti without FK506 treatment, significantly fewer 5.9% (Chi-squared test, $p < 0.001$) cells were vacuolated. Islet cells of two wild type mice with and without FK506 (non transgenic littermates) were observed. Of 81 total cells observed in

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wild type mice with FK506, 38% cells were vacuolated and without FK506 of 95 total cells a statistically similar number ($p = 0.949$, Chi-square test) (38%) was observed to be vacuolated. Figure A-14 (A and B) shows typical examples of vacuolation of the islet cells in the treated and non-treated mice. Unexpectedly the agouti mice had significantly less vacuolation than their nontransgenic wild type littermates either with or without treatment ($p < 0.001$). These results suggest some general protective effect of the agouti protein on islet cell.

It had been reported in rats that high 5mg/kg/day dosages of FK506 uniformly caused cataract formation (Ishida et. al., 1997). Studies were done to detect cataract formation after the 0.7mg/kg/day dose FK506 treatment in this study examining 10 agouti mice and 10 nontransgenic littermates. Mild cataracts were observed in two treated agouti mice, Figure A-15 (A). Cataracts were not observed in their littermates, Figure A-15 (B), but this was not statistically significant (Fisher Exact test, $p = 0.474$).

It had also been reported that high doses of FK506 had toxic effects on the kidney with histopathologic changes in glomerulus tissue. In this study, no apparent kidney pathology was observed in either of the FK506 treated groups of mice (data not shown).

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V. DISCUSSION

The purpose of this study was to determine if a high but nontoxic dose of the immunosuppressive drug FK506 alters expression of the agouti gene message and/or protein as well as the agouti phenotype. Conversely, this study sought to determine if overexpression of agouti enhanced or altered the toxic effects of FK506.

Mice carrying agouti mutation are expected to exhibit increased body weight compared to their littermate as early as 4 weeks of age (Dicki et. al., 1946, Roberts et. al., 1984). At three months of age, mice overexpressing agouti show approximately twofold increase in body weight compared to control mice (Frigeri et. al., 1988). The obese yellow mice (Avy and ? actin agouti mice) are moderately hyperphagic and eat 10-36% more than the lean littermates (Frigeri et. al., 1988, Yen et. al., 1976). There were no such changes observed in our study with either strain of transgenic and nontransgenic mice with or without FK506 treatment.

At the end of the time course, body weight and blood glucose levels of agouti mice with and without FK506 show significant differences (P= 0.020, P=0.010). Body weight and the blood glucose levels of the untreated agouti mice tended to be higher than the treated ones. A previous study in rats has shown that there is reduction of body weight gain in the 5mg/kg/day FK506 treated animals, which is reversible (Ishida et. al., 1997).

Hyperinsulinemia and hyperplasia of pancreatic ?-cells leads the development of obesity in agouti mutant mice with ubiquitous overexpression of agouti (Warbritton et. al., 1994). Intracellular calcium also plays an important role in the metabolic disorder of obesity and insulin resistance (Byyny et. al., 1992, Draznin et. al., 1988). While

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hyperinsulinemia is detectable at 4 weeks of age in ob/ob mice, Avy/A (BALB/ c ? VY) F1 mice become hyperinsulinemic after 6 weeks of age and exhibit a sexual dimorphism as well as hyperglycemia. By 2 months of age, plasma insulin concentration in yellow Avy/a mice were 4-fold those of controls in females and, 10-fold those of a/a control mice in males (Yen et. al., 1990).

Studies have shown that aP2 mice only become markedly obese with daily injection of insulin. The combination of agouti expression in adipose tissue and insulin treatment induced physiological changes which result in body weight gain similar to other transgenic strains of mice, like BAP20 (?-actin promoter), which ubiquitously express agouti. BAP20 mice are obese and plasma insulin levels are elevated at the age of 12 weeks (Mynatt et. al. 1997).

In this study, plasma insulin level of the agouti mice with FK506 treatment was lower than untreated agouti mice (0.377?0.222, 0.640?0.344) but the observed changes were not significant. Of note, FK506 treatment reduced insulin levels in the aP2 but not wild type mice and the reduced levels were comparable to the untreated or treated wild type mice.

FK506 has been associated with insulin dependent diabetes mellitus during clinical use (Redmon et. al., 1996). FK506 is known to cause diabetogenicity through pancreatic injuries (Ishida et. al., 1997). It has been shown that high dosage (5mg/kg/day) causes the pancreatic injury, which appears as vacuolation of islet cells in rats (Ishida et. al., 1997). In this study, an unexpected difference in vacuolation between the agouti and normal littermates was observed along with a possible protective effect of agouti

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overexpression on preventing FK506 toxicity at this lower dose. However, FK506 toxicity in terms of islet cell vacuolation was not observed in the treated versus untreated wild type mice.

It has been reported (Ishida et. al., 1997) that histopathological examination revealed rupture along with swollen and liquefacient lens fiber in rats treated with high dose of FK506. No pronounced toxic effect of a lower dose of FK506 was observed histopathologically in either aP2 agouti or wild type mice in this study.

The Northern blot and PCR results indicate that there is no effect of FK506 in both strains of mice and limited western blot showed similar result.

The agouti gene is expressed from two different promoters, one active at the midpoint of the hair cycle and other specific for the ventrum, is responsible for generating pigmentation pattern. During the embryonic whisker plate transcript from the hair cycle are detected but before birth it is not detected in other regions of the body, ventral-specific transcripts are detected in the ventral trunk of the embryo and ventral whisker plate. It suggests that agouti gene is regulated by two factors responsible for establishing differences between the dorsal and ventral surfaces of the body during embryogenesis. aP2 mice express very high level of agouti in brown and white adipose tissue. These mice show normal insulin level and do not become obese. This suggests that expression of agouti in adipose tissue is not enough for the mice to be obese (Mynatt et. al., 1997 reviewed Moustaid-Moussa 2000). Daily injection of insulin leads to increases in body weight, so agouti may interact with insulin to induce obesity by upregulating lipogenesis in adipocytes. Signaling pathways that lead to increases in lipogenesis or activation of the

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lipogenic genes by insulin are not known yet. It has been shown that PI 3 kinase signaling pathway, but not the P70 S6 kinase nor the MAP kinase pathway, mediate insulin effect on FAS (Fatty Acid Synthase) (Dong and Hei 1998). None of these studies have shown whether the calcinuerin pathway is also involved in adipose tissue/agouti promoter regulation.

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VI. FUTURE DIRECTIONS

There was decreased food intake and blood glucose level in aP2 mice this may be due to FK506. There was no detection of agouti mRNA or protein in adipose or pancreatic tissue of the wild type mice but agouti was expressed in both tissue of agouti mice. There was no significant effect of FK506 on agouti mRNA or protein expression in the aP2 mice. No significant toxic effect of FK506 at this dosage was observed in both mice.

In future work treatment should be done with other mice strains or rat model for a longer time period. However, there are no rat models overexpressing agouti available. Alternatively, the same model could be tested with different method of treatment (intraperitoneal or intragastric) or higher doses. However, in this study a relatively high dosage of FK506 was used which achieved a 20 fold higher than therapeutic dose as confirmed by analysis of blood levels relative to transplant patients. Another possibility would be to analyze agouti expression in human patient population if suitable tissue sampling and analytical assays could be worked out. Transplant patients using immunosuppressive drug could then be examined for a possible agouti relationship to immunosuppression and human immune cell function. This might expand on the finding of an immune dysfunction in the overexpressing agouti strains.

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APPENDIX

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Appendix A: Figures

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Figure A-1. Powder Diet Consumed by Agouti Mice with and without FK506

10 grams of powder food with and without FK506 was poured in each container daily. Food consumption of mice was measured every day. Weight of the consumed food was obtained by subtracting the weight of the container with remaining food and weight of the empty container. Statistical analysis was performed on the data by ANOVA group comparisons. Lines with triangle represent the agouti mice with FK506 and circles represent without FK506. At the beginning of the treatment there was significant difference (figure A-1) (P=0.008) in consumption of food and the untreated mice consistently consumed more food throughout the time course of the study. The difference was not statistically significant at the end of the treatment time course compared to treated and untreated (P=0.083).

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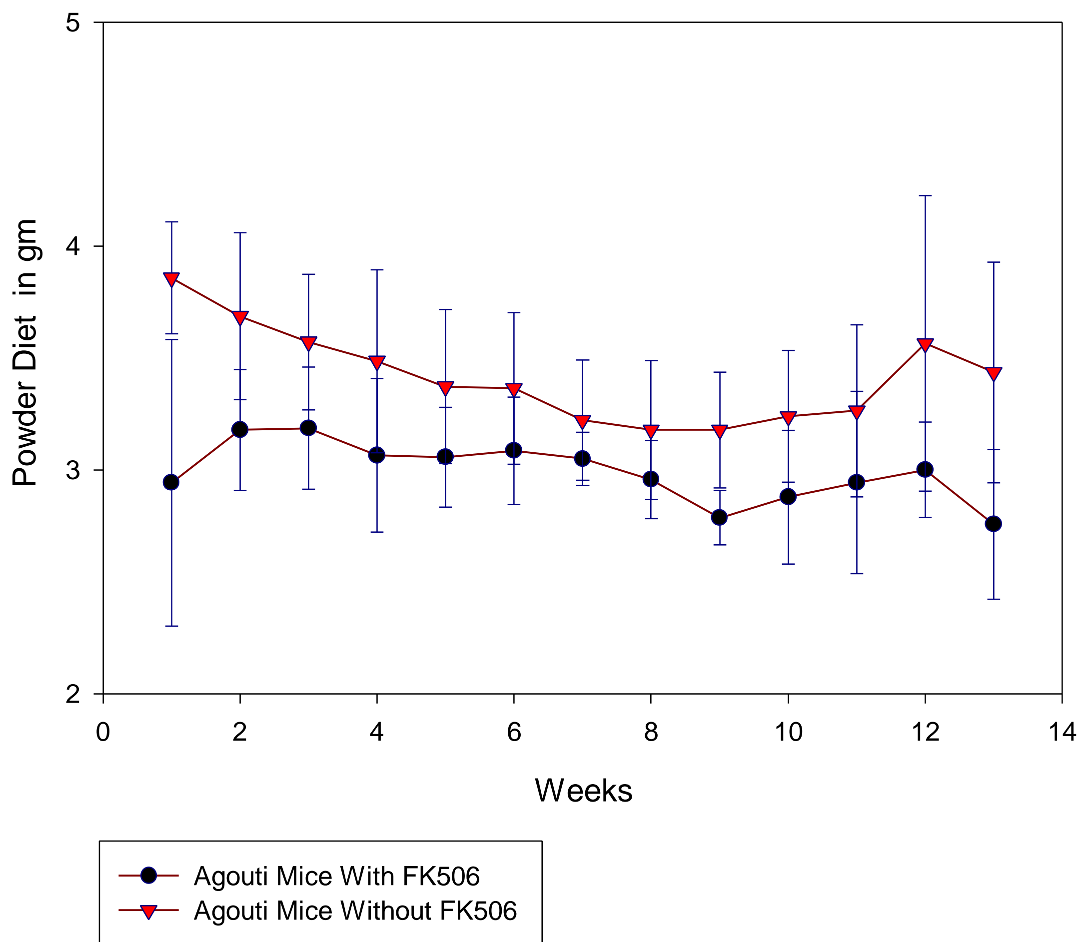

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Figure A-2. Powder Diet Consumed by Wild Type with and without FK506

10 grams of powder food with and without FK506 was poured in each container daily. Food consumption of mice was measured every day. Weight of the consumed food was obtained by subtracting the weight of the container with remaining food and weight of the empty container. Statistical analysis was performed on the data by ANOVA group comparisons. Lines with triangle represent the wild type mice with FK506 and circles represent without FK506. At beginning of the treatment there was significant difference (figure A-2) (P=0.001) in food consumption. However, no differences in food consumption were observed by the end of the treatment compared to treated and untreated (P=0.878).

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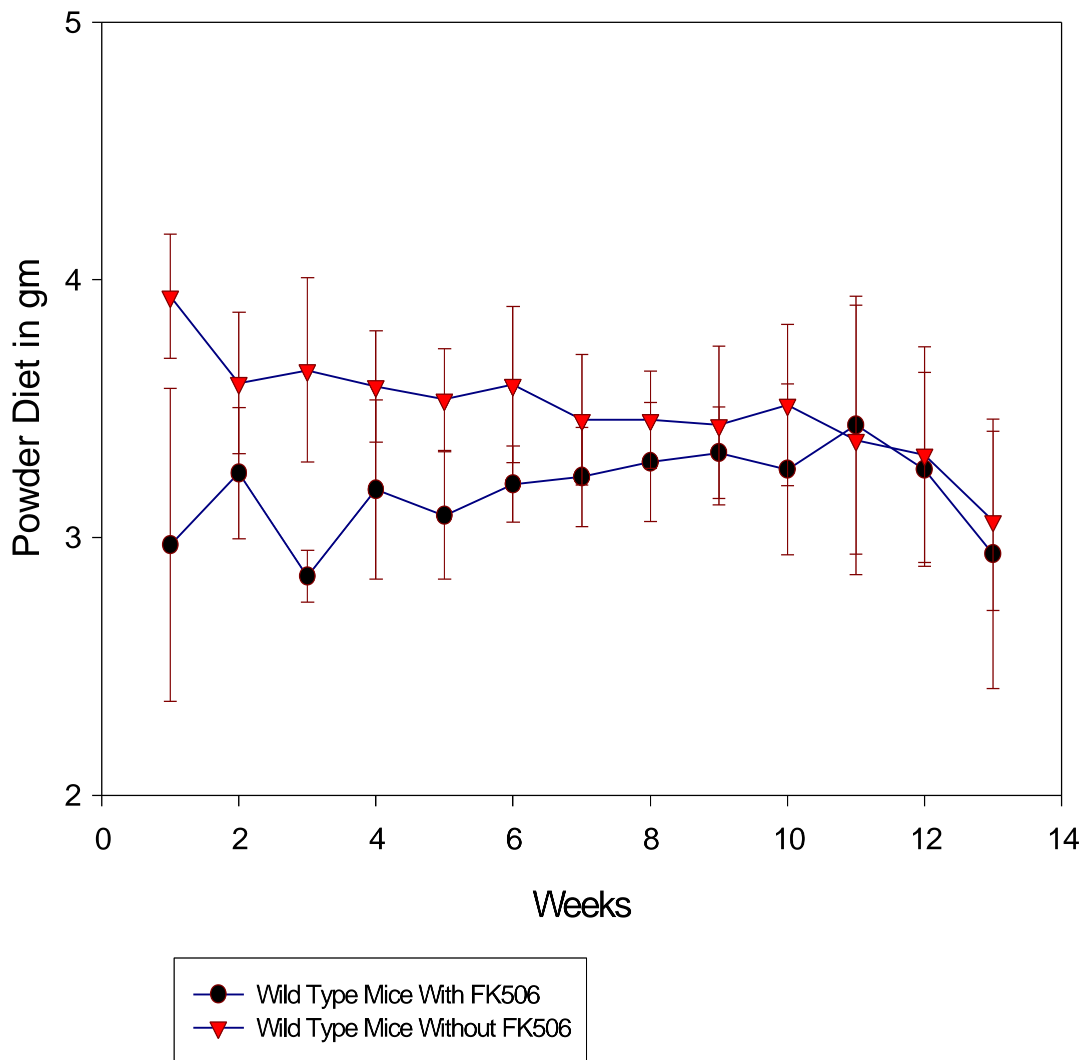

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Figure A-3. Body weight of Agouti Mice with and without FK506

Body weight of the mice was taken weekly. Statistical analysis was performed on the data by group comparisons by ANOVA test. At the beginning both groups of mice started with the same weight ($P=0.553$). By the end of the time course, untreated mice had gained more weight than the FK506 treated mice ($P= 0.020$).

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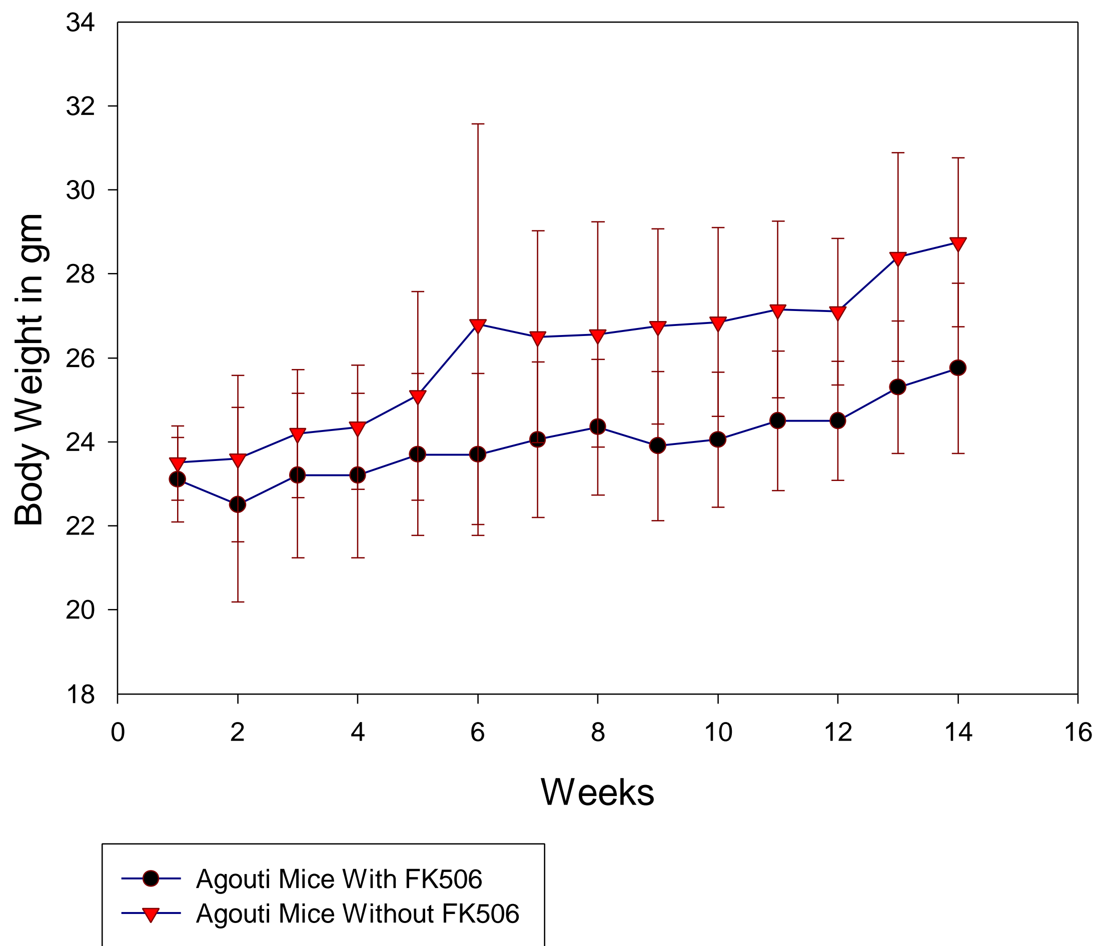

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Figure A-4. Body weight of Wild Type with and without FK506

Body weight of the mice was taken weekly. Statistical analysis was performed on the data by group comparisons by ANOVA test. At the beginning both mice groups started with no statistically significant difference in weights ($P=0.194$). At the end of the treatment time course there were no significant differences observed in the weights of the treated vs untreated mice ($P= 0.419$)

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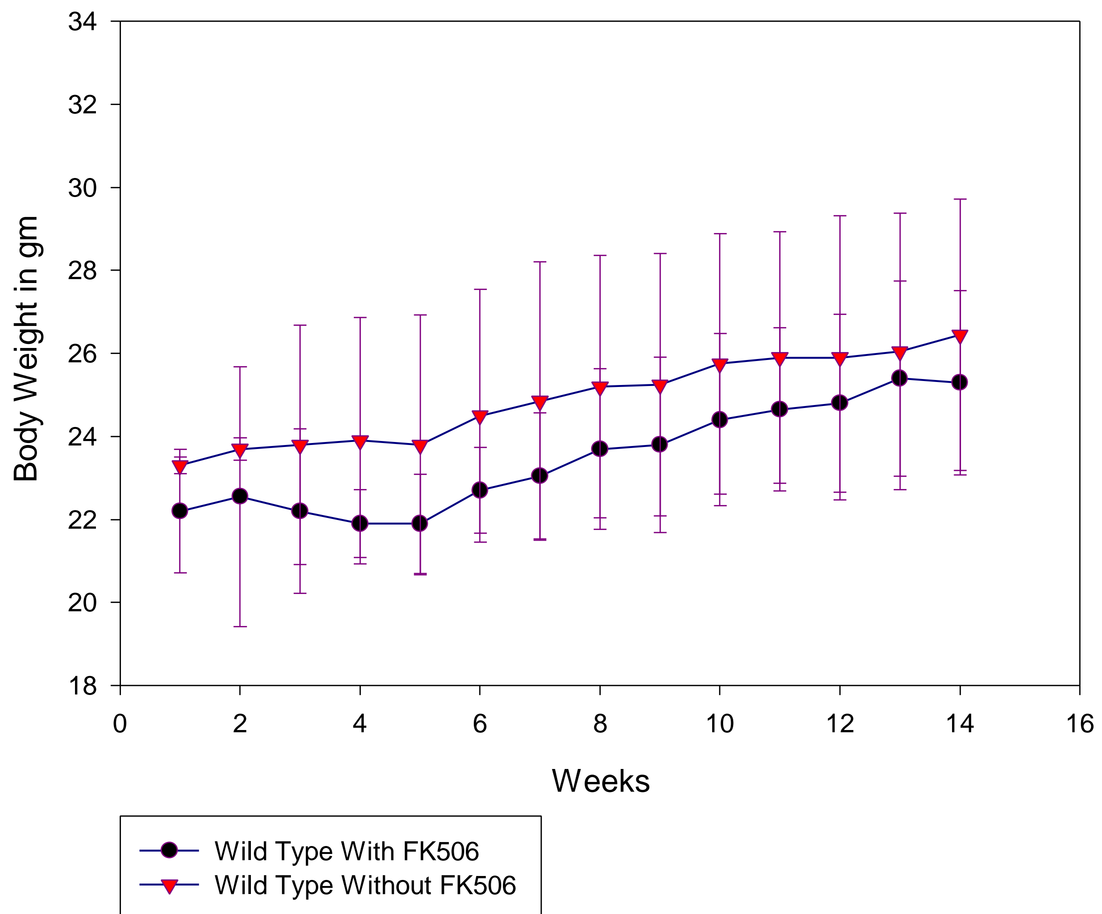

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Figure A-5. Blood Glucose Level of the Agouti Mice with and without FK506

The blood glucose level was checked weekly in fed mice by using glucometric strips with blood obtained by pricking the tail vein. Data is plotted as average blood glucose +/- SD. Average blood glucose levels were higher and more variable for the untreated mice throughout the time course of treatment. Statistical analysis of the data done at the beginning of the treatment P=0.214 and at the end of treatment P=0.134 showed no significant effect of the FK506 treatment.

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Blood Glucose Level (mg/dl) Of Agouti Mice With and Without FK506

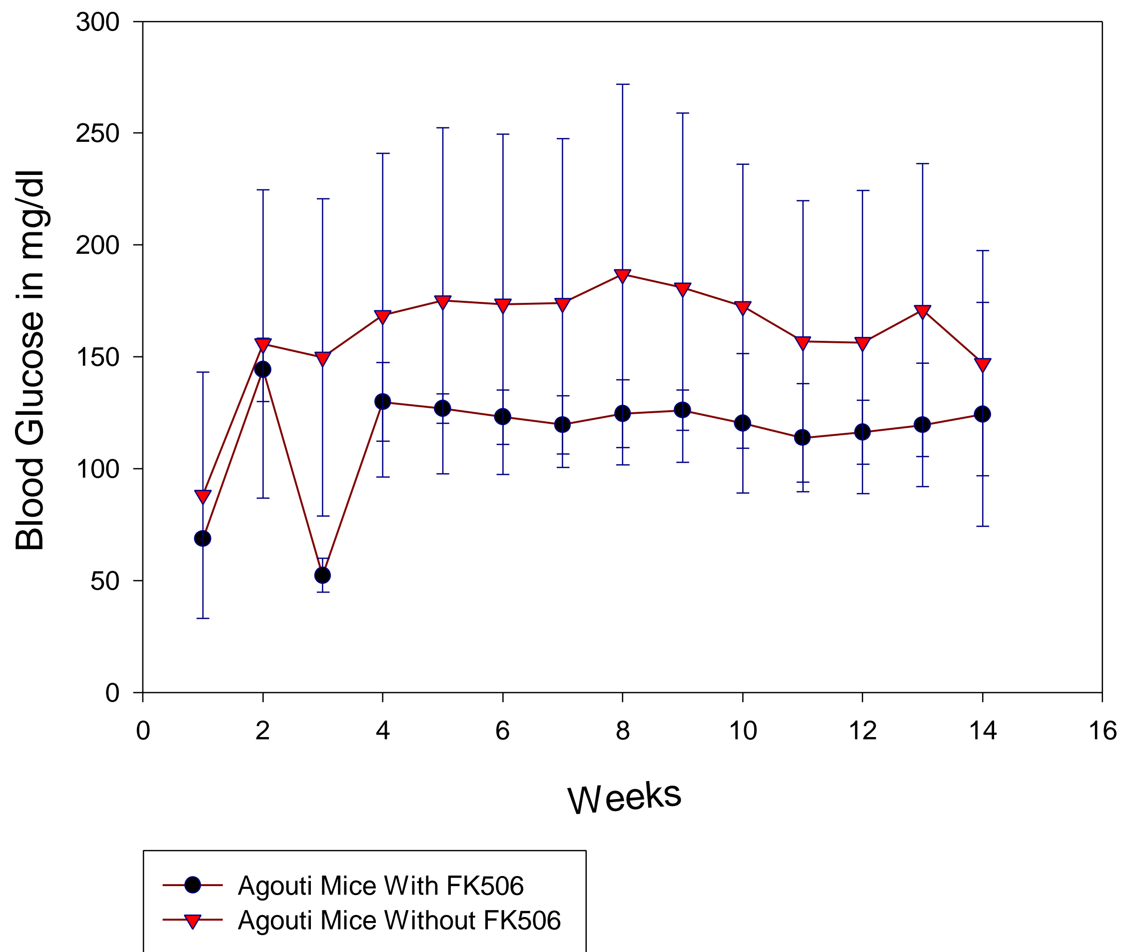

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Figure A-6. Blood Glucose Level of the Wild Type with and without FK506

The blood glucose level was checked weekly in fed mice using glucometric strips with blood obtained by pricking the tail vein. Data is plotted as average blood glucose +/- SD. The average blood glucose levels in the treatment group were lower throughout the time course.

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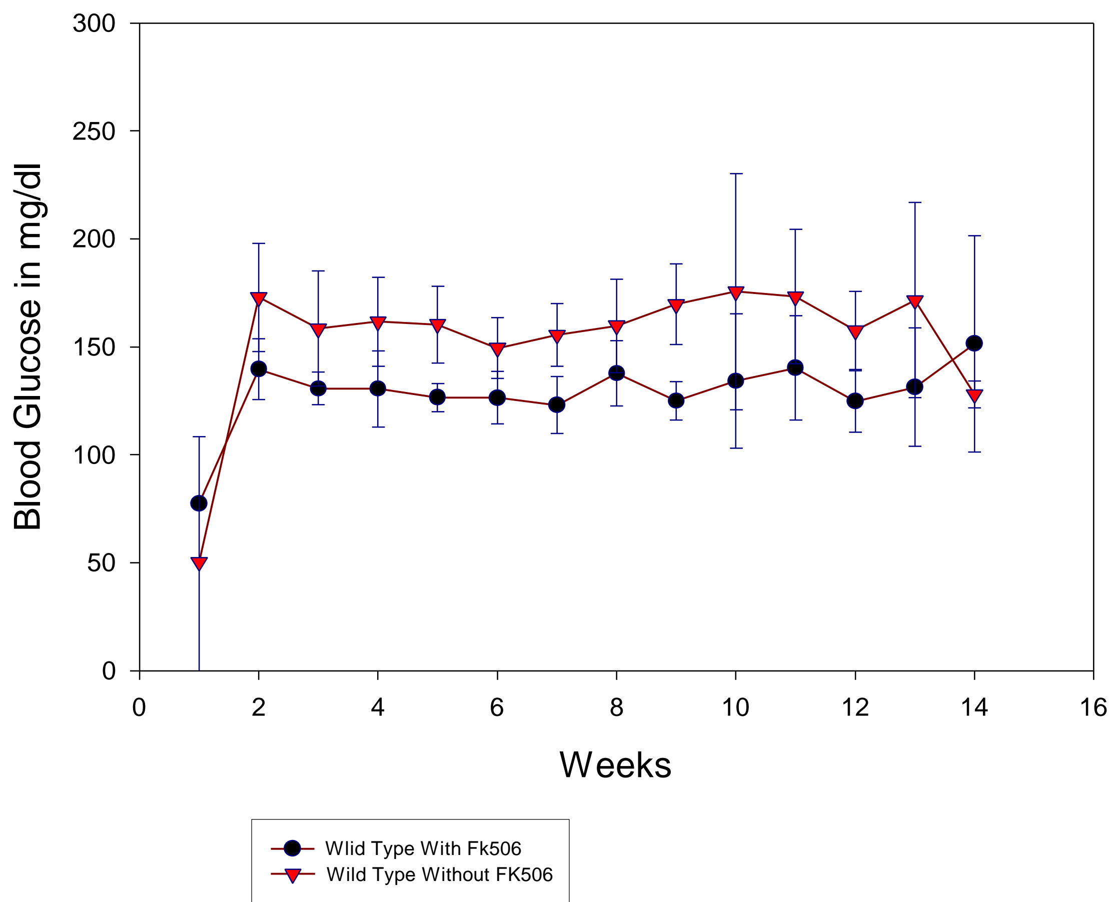

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Figure A-7. RT-PCR of cDNA from the Fat and Pancreatic Tissue of the Agouti Mice and their Littermates.

RT-PCR products were size-fractionated in agarose gels and stained with ethidium bromide. First lane indicates the size in bp of the 100bp ladder molecular weight marker. Remaining lane is the 0.4Kb fragment is amplified using oligonucleotide primer specific for agouti. Second lane is the positive control, from three to seven lanes is PCR product of the fat tissue with FK506 and remaining lane eight to twelve is without FK506. The 0.8Kb fragment, amplified using oligonucleotide primers specific for the actin mRNA, serves as a positive control. Panel A RT-PCR of RNA from adipose tissue: Agouti mice and their non transgenic littermates with and without FK506. Panel B Shows RT-PCR of RNA from pancreatic tissue: Agouti mice and their non transgenic littermates with and without FK506. Oligonucleatide primer specific for agouti. Oligonucleatide primer specific to actin. On the left hand side of the Panel A and B band with an arrow is 0.5Kb and those with the line is 0.4Kb and 0.8Kb.

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Panel A

Adipose Tissue

M C +FK506 -FK506

1 2 3 4 5 6 7 8 9 10 11 12

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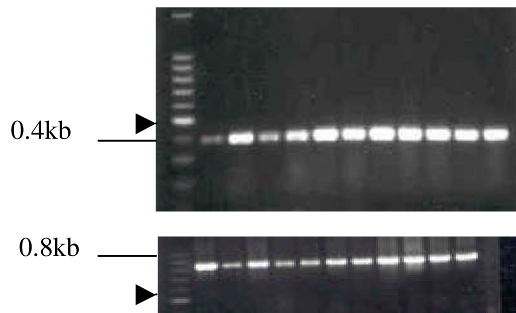

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Panel B

Pancreatic Tissue

M C +FK506 -FK506

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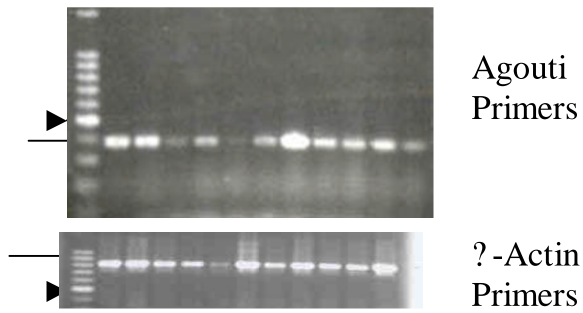

Agouti Primers

?-Actin Primers

M C +FK506 -FK506

1 2 3 4 5 6 7 8 9 10 11 12

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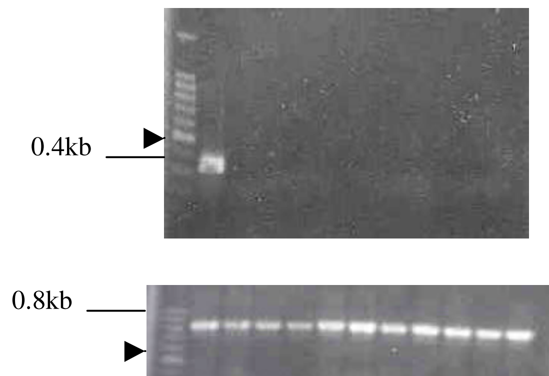

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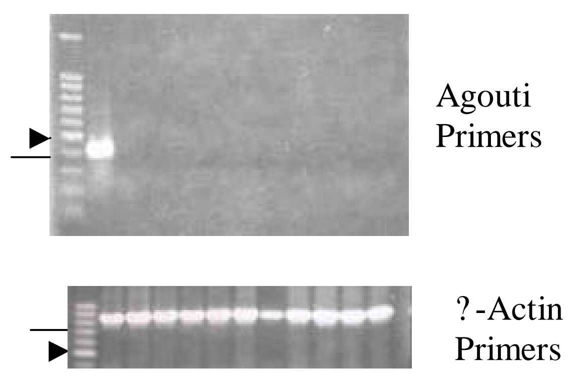
M C +FK506 -FK506

1 2 3 4 5 6 7 8 9 10 11 12



Agouti Primers

?-Actin Primers

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Figure A-8. Northern Blot Analysis for Fat Tissue of Agouti Mice with and without FK506

The agouti cDNA was ^{32}P-labeled and hybridized to poly $(A)^{+}$RNA (20?g per lane) from the fat tissue with and without FK506. Upper panel five treated and untreated and lower panels five treated and untreated mice. The first lane from the left is the positive control of agouti; lane two to six is the RNA from the fat tissue with FK506. Remaining lanes (seven to eleven) are the ones without FK506. The nylon membrane was again hybridized with actin to see the quality and quantity of the extracted RNA. Data are presented as the mean ? SE of Agouti/?-actin for ten treated and untreated mice. There is no difference in the expression of the Agouti in adipose in treated and untreated (P= 0.851).

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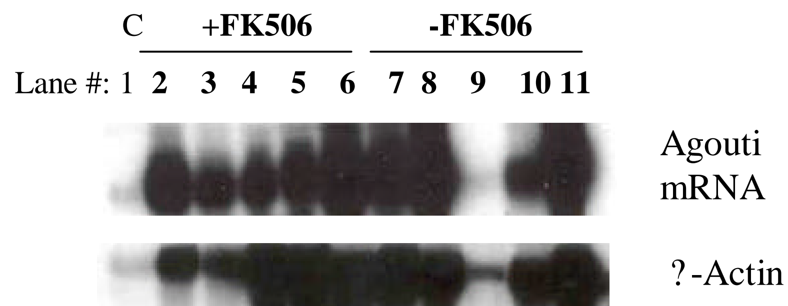
Adipose Tissue



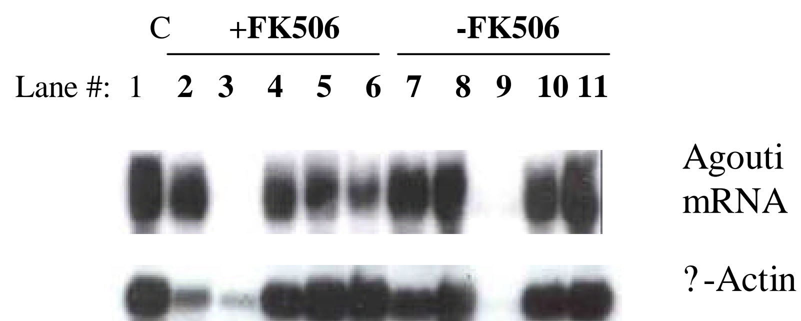


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Figure A-9. Northern Blot Analysis of Pancreatic Tissue of Agouti Mice with and without FK506

The agouti cDNA was ^{32}P-labeled and hybridized to poly $(A)^{+}$RNA (20? g per lane) from the pancreatic tissue with and without FK506. Upper panel five treated and untreated and lower panels five treated and untreated mice. The first lane from the left is the positive control, two to six lanes is the RNA from the fat tissue with FK506. Remaining lanes (seven to eleven) are the ones without FK506. The nylon membrane was again hybridized with actin to see the quality and quantity of the extracted RNA. Data are presented as the mean? SE of Agouti/? -actin for ten treated and untreated mice. There is no difference in the expression of the agouti in pancreas from the treated and untreated mice (P = 0.179).

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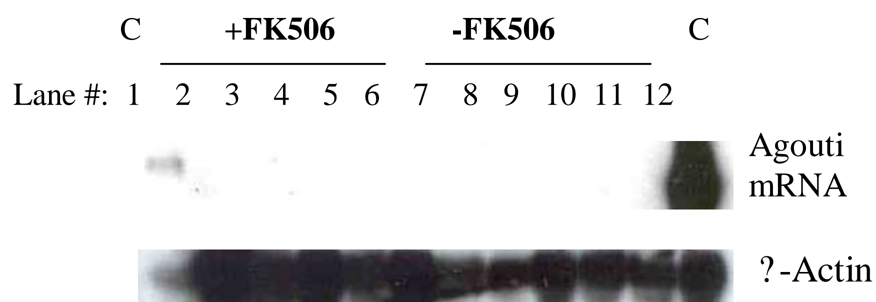
Adipose Tissue



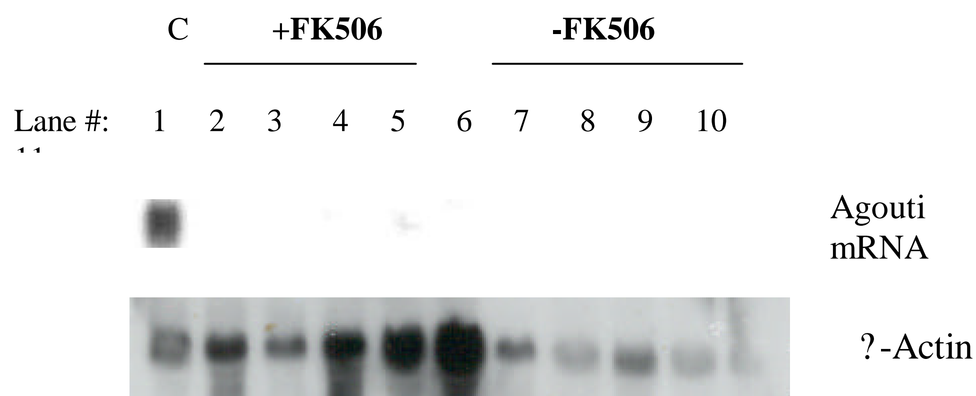


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Figure A-10. Northern Blot Analysis of Fat Tissue of Wild Type with and withou t FK506

The agouti cDNA was ^{32}P-labeled and hybridized to poly $(A)^{+}$RNA (20? g per lane) from the fat tissue with and without FK506. Upper panel five treated and untreated and lower panels five treated and untreated mice. The first and last lane for upper panel is the positive control for agouti two to six lane is the RNA from the fat tissue with FK506, seven to eleven lane is RNA from fat without FK506. First lane of lower panel is positive control two to six lane is the RNA from fat tissue with FK506 and the remaining lane is without FK506. The nylon membrane was again hybridized with actin to observe the quality and quantity of the extracted RNA. Data are presented as the mean? SE of Agouti/? -actin for ten treated and ten untreated mice. Statistical analysis (P=0.149).

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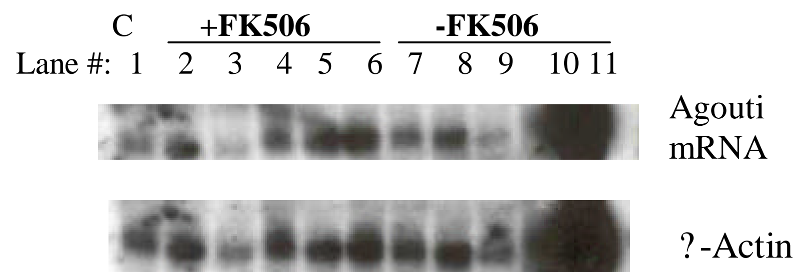
Pancreatic Tissue



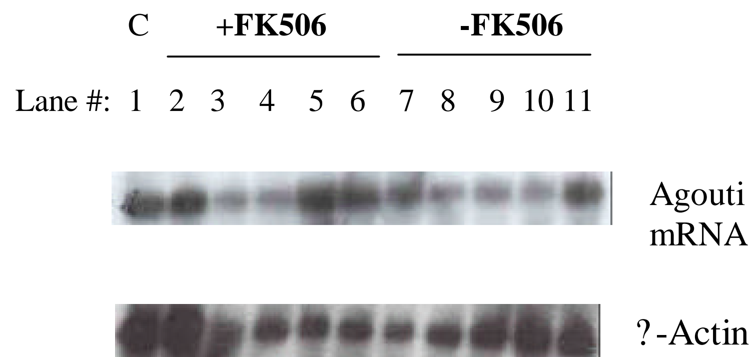


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Figure A-11. Northern Blot Analysis of Pancreatic Tissue of Wild Type with and without FK506

The agouti cDNA was ^{32}P-labeled and hybridized to poly $(A)^{+}$RNA (20? g per lane) from pancreatic tissue with and without FK506. Upper panel five treated and untreated and lower panels five treated and untreated mice. The first lane is the positive control, two to six lanes is the RNA from the pancreatic tissue with FK506, and seven to eleven lane is without FK506. There is no expression of the agouti in treated and untreated ones. The nylon membrane was again hybridized with actin to see the quality and quantity of the extracted RNA. Data are presented as the mean? SE of Agouti/? -actin for ten treated and ten untreated mice. Statistical analysis (P=0.450).

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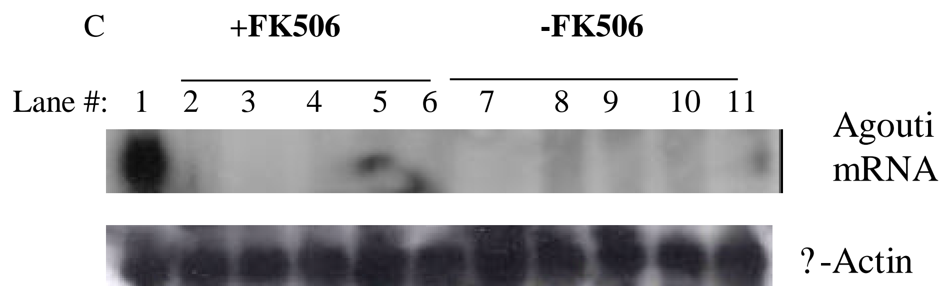
Pancreatic Tissue



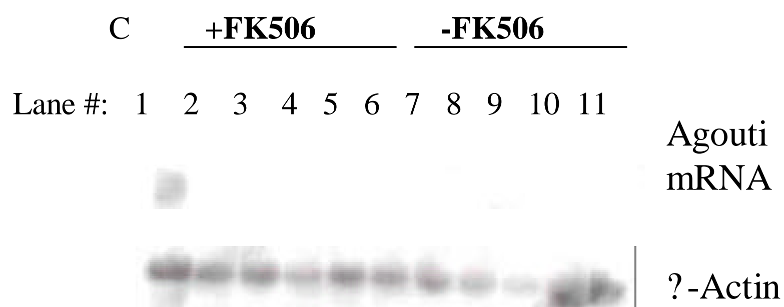


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Figure A-12: Western Blot Analysis of the Fat Tissue of Agouti Mice with and without FK506

The frozen fat tissue was treated with 1.5 fold concentrated denaturing buffer and the sample ultracentrifuged to remove insoluble. The extract is loaded on the gradient gel and a western blot with rabbit anti mouse agouti was performed. The first lane is the low molecular weight marker; second lane is the positive control. From lane three to seven is with FK506 and lane nine to twelve is without FK506, which shows no differences in the expression of the peptides.

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Agouti Adipose Tissue

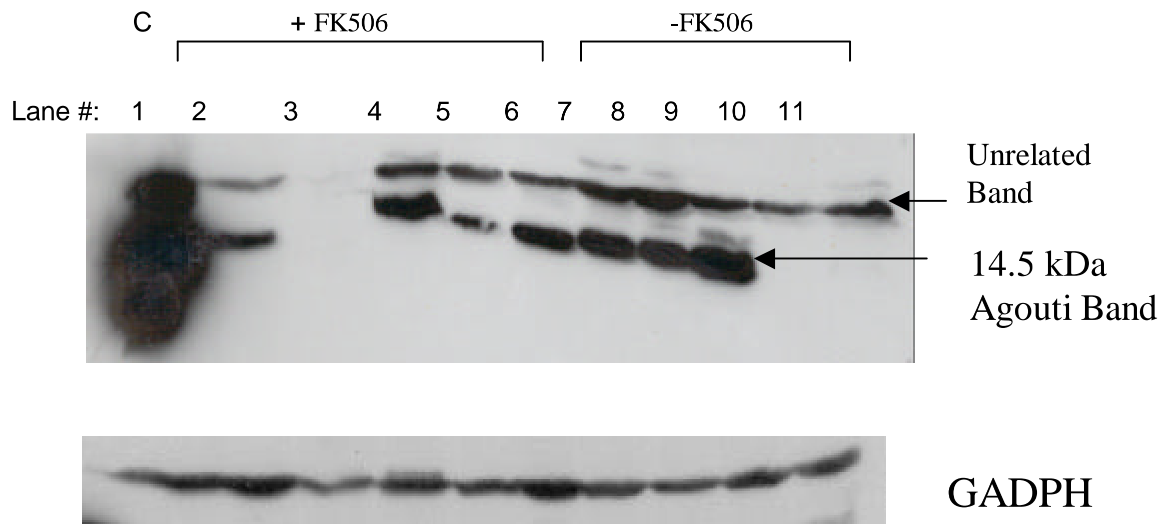

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Figure A-13: Western Blot Analysis of the Fat Tissue of Wild Type Mice with and without FK506

The frozen fat tissue was treated with 1.5% denaturing buffer, the sample was ultracentrifuged to remove the insoluble. The extract is loaded in the gradient gel and a western blot with rabbit anti mouse agouti was performed. The first lane is the low weight molecular marker; lane second is the positive control. From lanes three to seven lane is with FK506 and lane nine to twelve lane is without FK506, which shows no differences in the expression of the peptides.

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Wild Type

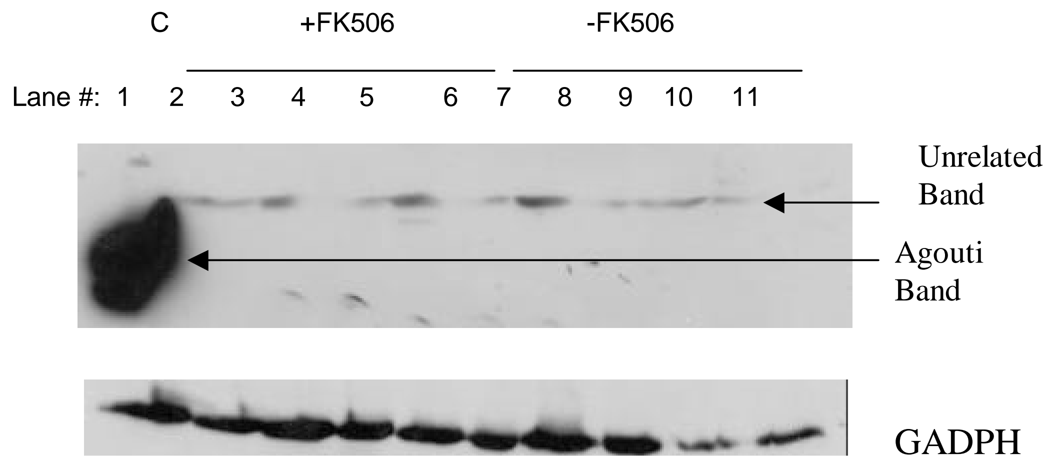

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Figure A-14: A Cut Section of the islet cells of the agouti and their nontrasngenic littermates with and without FK506.

Vacuolation of the islet cells are present in both treated and untreated subjects.

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Cut Section of the Pancreatic Islet Cells

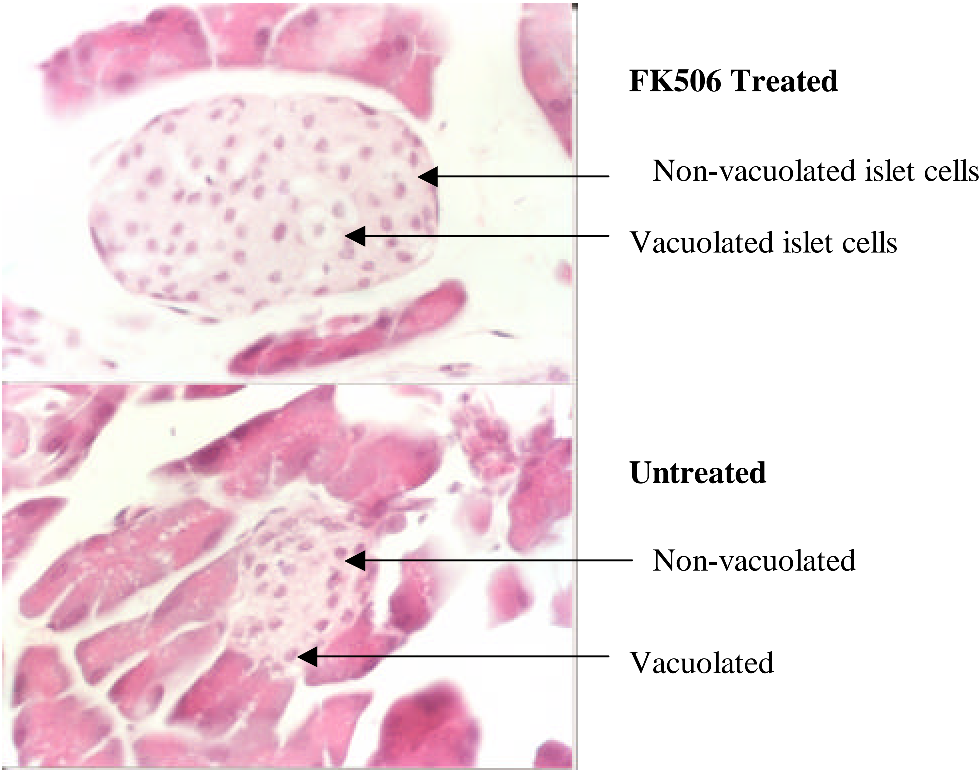

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Figure A-15: A Cut section of the eye (lens).

A. shows an irregular pattern with swollen lens fiber cells large vacuoles in the subcapsular cortex. B. normal eye lens.

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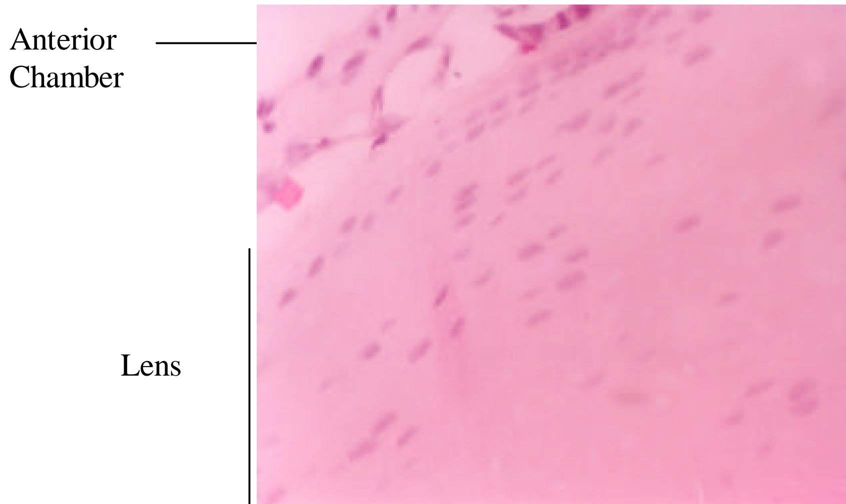
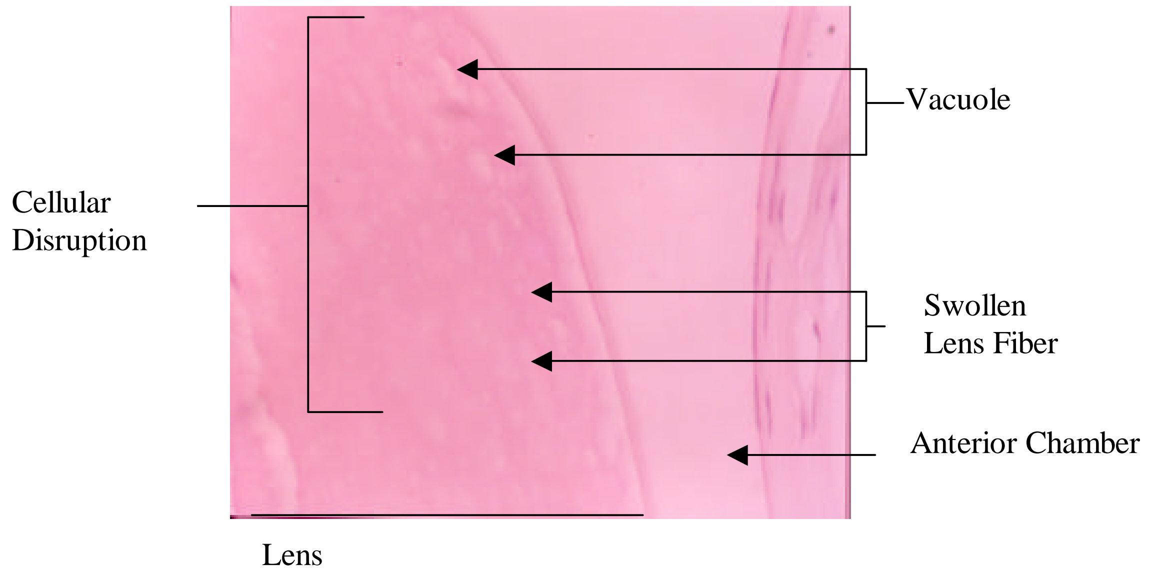
Cut Section of Normal Eye Lens



Cut Section of Eye Showing Cataract



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Appendix B: Table

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Table 1. Plasma Glucose Level of the Agouti Expressed Mice and their Littermates with and without FK506

Blood was collected at the time of sacrifice by heart puncture; plasma was collected by centrifugation. Plasma glucose level was measured using EIA kit. Statistical analysis shows the (P=0.761 of the treated mice and untreated P=0.122) mean plasma glucose value of the treated mice are significantly lower than those of the untreated ones.

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Mice	Mean	Std Dev	SEM
Ag With FK506	0.187 nmol/ml	0.221	0.0698
Ag Without FK506	0.220 nmol/ml	0.0131	0.109
Wild Type with FK506	0.200 nmol/ml	0.0344	0.01414
Wild Type without FK506	0.230 nmol/ml	0.069	0.0532

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VITA

Rashika Joshi was born and raised in Nepal. She received Medical degree from Beijing Medical University, China in 1993. She entered the University of Tennessee Graduate School of Comparative and Experimental Medicine in August of 1998.

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