Protein and mRNA Studies of Rat FA1/Pref-1/dlk

GABRIELLA PERSDOTTER HEDLUND
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Abstract

The timing of cell differentiation is important for development and renewal of well functioning organs and tissues. One protein involved in this process is Preadipocyte factor 1 (Pref-1). Most likely, the role of this protein is to maintain cells in an undifferentiated state.

The work presented in this thesis, has employed the rat as an animal model for the studies of Pref-1. Rat models of obesity (Zucker, ZO) and type II diabetes (Goto-Kakizaki, GK) were used to determine metabolic influence on Pref-1 and adipokine mRNA expression in adipose tissues.

The Pref-1 cleavage product was purified from rat amniotic fluid and physicochemically characterised. Concentration of Pref-1 in serum, amniotic fluid and urine was determined by ELISA. Soluble Pref-1 and the compartmentalisation of the protein were highly similar to what had previously been demonstrated in mice and humans.

Immunohistochemistry studies displayed similar staining patterns of Pref-1 in adrenal glands, ovaries and pituitary glands of non-pregnant and pregnant rats. This suggests that pregnancy do not influence the protein expression of Pref-1 in these organs.

In the GK rats, Pref-1 mRNA was altered and a decrease in the visceral compared to subcutaneous adipose depots was demonstrated, in contrast to the ZO rats. Additionally, adiponectin, leptin, IL-6 and TNF-α mRNA levels were altered in the diabetic strain, indicating that this animal model expresses many of the typical features of type II diabetes.

In conclusion, the rat is an appropriate model for studies of FA1/Pref-1/dlk. Pref-1 is highly elevated in fetal and maternal serum during pregnancy. However, the expression of Pref-1 in some endocrine tissues did not alter due to pregnancy. The mRNA expression of Pref-1 was altered between adipose depots and demonstrated to be affected by metabolic disturbances in the animals.

Keywords: Preadipocyte factor 1, delta like protein, Fetal antigen 1, Goto-Kakizaki, Zucker, adipokines, adipose tissue, diabetes, obesity

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Till Stefan, Nils och Moa
List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I  Purification, characterization, and biological compartmentalization of rat fetal antigen 1.  

II  Fetal antigen 1 (FA1) in the adult rat adrenal gland, ovary and pituitary gland.  
In Vivo. 2003 Jan-Feb;17(1):1-4

III  Pref-1 and adipokine expression profiles in adipose tissues of GK and Zucker rats.  
Gabriella Persdotter Hedlund, Jenny Lind and Carina Carlsson  
Manuscript

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Related articles not included in the thesis:


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## Abbreviations

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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AF</td>
<td>Amniotic fluid</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index (bodyweight (kg)/(length (m))²)</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>Dlk</td>
<td>Delta-like protein</td>
</tr>
<tr>
<td>DSL</td>
<td>Delta Serrate LAG2</td>
</tr>
<tr>
<td>ED</td>
<td>Embryonic Day</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FA1</td>
<td>Fetal antigen 1</td>
</tr>
<tr>
<td>FOXA2</td>
<td>fork head transcription factor (hepatocyte nuclear factor 3 beta, HNF-3b)</td>
</tr>
<tr>
<td>GAS1</td>
<td>Growth Arrest Specific protein</td>
</tr>
<tr>
<td>Hes-1</td>
<td>Hairy and enhancer of split 1</td>
</tr>
<tr>
<td>i.m.</td>
<td>Intra-muscular</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intra-peritoneal</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like Growth Factor 1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>MEK/ERK</td>
<td>Mitogen activated protein kinase/Extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>MEN-1</td>
<td>Multiple endocrine neoplasia type 1</td>
</tr>
<tr>
<td>mPref-1/FA1</td>
<td>mouse Preadipocyte factor 1/Fetal Antigen 1</td>
</tr>
<tr>
<td>MS</td>
<td>Maternal Serum</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Pref-1</td>
<td>Preadipocyte factor 1</td>
</tr>
<tr>
<td>RAF</td>
<td>Rat Amniotic fluid</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>rPref-1/FA1</td>
<td>rat Preadipocyte factor 1/Fetal Antigen 1</td>
</tr>
<tr>
<td>SAT</td>
<td>Subcutaneous white adipose tissue</td>
</tr>
<tr>
<td>SCP-1</td>
<td>Stromal cell derived protein</td>
</tr>
<tr>
<td>TACE</td>
<td>TNF-α converting enzyme</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>VAT</td>
<td>Visceral white adipose tissue</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>ZOG</td>
<td>Zona Glomerulosa specific protein</td>
</tr>
</tbody>
</table>
Introduction

During embryonic and fetal development, specialisations of cells are crucial for the development of functional organs and tissues. In adulthood, the renewal of old or dysfunctional cells in the tissue is dependent on a well-controlled cell regeneration to maintain the function of the organ. This differentiation from immature to functional and specialised cells is a stepwise process and dependent of gene regulation and factors expressed by surrounding cells. One group of proteins that is involved in cell differentiation is Epidermal Growth Factor (EGF)-like proteins. EGF-like proteins are proteins containing EGF-like, cysteine-rich repeated motifs (reviewed in Takada et al. 2000). Most of the EGF-like superfamily members are membrane-associated proteins or proteins released extra-cellularly acting through their EGF-like repeats in protein-protein interactions. An example of a well studied EGF-like family member is Notch and its ligands Delta and Serrate of Drosophila which are involved in the decisions of cell fate during development (reviewed in Lewis 1998). Both Delta and Notch are membrane bound proteins and important for cell-cell communication in e.g. neurogenesis. In the late 80’s, a new protein with EGF-like repeats was discovered independently by different research groups in different tissues, namely Preadipocyte factor 1 (Pref-1) or delta like protein (dlk).

Nomenclature

The first discovery of this gene was made in 1987 when Helman and co-workers identified a new gene, named pG2, highly expressed in the human adrenal cortex and medulla (Helman et al. 1987, 1990). In 1988, Fay and colleagues isolated a novel EGF-like protein from second trimester human amniotic fluid, which they assumed to be of fetal origin and thus termed Fetal Antigen 1 (FA1)(Fay et al. 1988). Further independent studies identified a gene by cDNA cloning and gave the gene and corresponding protein a name associated with origin of the cells or tissues studied (Table 1). All these proteins were later identified to be encoded by the same gene, now mainly called dlk1 or Pref-1, and FA1 turned out to be the soluble variant of Pref-1/dlk (Jensen et al. 1994, Lee Y. L. et al. 1995). In this thesis, the full-
length, transmembrane form of the protein will be referred to as Pref-1 and the circulating cleavage-product Pref-1/FA1.

Table 1. Overview of the nomenclature and discovery of Pref-1 in different species and specimen.

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Research group</th>
<th>Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA1</td>
<td>Human</td>
<td>(Fay et al. 1988)</td>
<td>Fetal antigen 1, discovered as a protein purified from human amniotic fluid.</td>
</tr>
<tr>
<td>Dlk</td>
<td>Human</td>
<td>(Laborda et al. 1993)</td>
<td>Delta-like protein, discovered as a gene expressed in a human small cell carcinoma cell line and a mouse fibroblast cell line.</td>
</tr>
<tr>
<td>Pref-1</td>
<td>Mouse</td>
<td>(Smas &amp; Sul 1993)</td>
<td>Preadipocyte factor 1, discovered as a gene in a mouse preadipocyte cell line.</td>
</tr>
<tr>
<td>SCP-1</td>
<td>Mouse</td>
<td>(Maruyama et al. 1993)</td>
<td>Stromal cell derived protein-1, identified as a gene expressed in an adipocytic stromal cell line, encoding a transmembrane protein.</td>
</tr>
<tr>
<td>ZOG</td>
<td>Rat</td>
<td>(Okamoto et al. 1997)</td>
<td>Zona glomerulosa specific factor, discovered as a gene expressed in zona glomerulosa cells in the adrenals of the rat.</td>
</tr>
<tr>
<td>Pref-1</td>
<td>Cattle</td>
<td>(Fahrenkrug et al. 1999)</td>
<td>Identified from a mixed-tissue cDNA library.</td>
</tr>
</tbody>
</table>

The Pref-1 gene is a paternally imprinted gene (Schmidt et al. 2000, Takada et al. 2000) situated on human chromosome 14 (Gubina et al. 1999), mouse chromosome 12 (Gubina et al. 2000), and rat chromosome 6 (Kloting & Kloting 2004). Pref-1 is present in phylogenetically high-ranking species but absent in lower ranking animals. However, the gene product is structurally related to well conserved proteins throughout the animal kingdom (Laborda et al. 1993).
Figure 1. Schematic figure of a full-length transmembrane form of Pref-1, its domains and soluble cleavage products. In mouse preadipocytes, this form of Pref-1 can generate a large cleavage product (50 kDa) known to be biologically active, and a small cleavage product (25 kDa). A possible dimerisation of the protein may be due to interactions between the EGF-like repeats.

Characterization

Human/mouse/rat Pref-1 is a transmembrane, single chained glycoprotein with a molecular weight between 40 and 60 kDa. The approximately 380 amino acids long protein can be divided into an extracellular domain, transmembrane domain and short intracellular part (Figure 1). The extracellular domain consists of six EGF-like repeats, a signal peptide and two cleavage sites (Laborda et al. 1993, Smas & Sul 1993).

Through studies on an embryonic mouse preadipocyte cell line 3T3-L1, Smas and co-workers discovered multiple alternate splicing forms of Pref-1, resulting in at least four different transmembrane products named
Pref-1A-D, ranging from 50 to 60 kDa. After proteolytic cleavage, these forms can generate several soluble products. However, only the largest form, a 50 kDa product cleaved from Pref-1A and B, seems to inhibit differentiation of preadipocytes in cell-culture (Smas et al. 1994, 1996, 1997, Mei et al. 2002). It has recently been suggested that TNF-α converting enzyme (TACE) is responsible for the proteolytic cleavage of Pref-1 (Wang & Sul 2006). Corresponding as well as additional forms of Pref-1 transcripts and/or proteins have been reported in adult rat and human islets of Langerhans (Carlsson et al. 1997, Friedrichsen et al. 2003) and in human pituitary- and neuroendocrine tumours (Altenberger et al. 2006).

The soluble cleavage products of human/mouse/rat Pref-1, Pref-1/FA1, have molecular weights ranging from 25 to 50 kDa of which 8 % is composed of glycosylation of the protein. The protein has a high content of cysteines, prolines and acidic residues suggesting that Pref-1/FA1 is a compactly folded and hydrophilic protein (Jensen et al. 1993, 1994).

Tissue localisation
During fetal development, Pref-1/FA1 is abundantly expressed by many different cells in the fetus and in cells derived from all three germ layers. In the human fetus, the expression of Pref-1/FA1 is down-regulated in relation to the degree of differentiation and the expression gets restricted to specific predominantly neuroendocrine cells during fetal development (Floridon et al. 2000).

In situ hybridization studies on mouse embryos have revealed expression in pituitary, vertebra (mesenchymal cells), tongue, liver, and lung (Smas & Sul 1993). Immunohistochemistry of human fetal pancreas displayed an increasing number of Pref-1/FA1 positive cells associated with insulin during development (Tornehave et al. 1996). Studies of developing pancreatic anlage from rat embryos have displayed a similar pattern with high Pref-1 mRNA expression and most of the parenchymal cells expressing Pref-1/FA1 at embryonic day (ED) 13 and a restriction to β-cells close to birth (Carlsson et al. 1997).

Other tissues expressing Pref-1/FA1 during development include human respiratory columnar and cuboidal epithelial cells (Floridon et al. 2000). Moreover, cells demonstrated Pref-1/FA1 immunospecific staining in proximal tubules of the kidneys, adrenal cortex, Leydig and Hilus cells of the testis and ovaries, chondroblasts and skeletal muscular myotubes. Neuro- and adenohypophysial cells in early pituitary gland, epidymal epithelium of the plexus coroideus and cells in the floor of 3rd ventricle in diencephalon also stained positive for Pref-1/FA1. In extra embryonic tissues, Pref-1/FA1
has been found in stromal cells around blood islands of the yolk sac and in placental fibroblasts (Floridon et al. 2000).

This expression pattern is even more restricted in adult tissues where Pref-1/FA1 so far has been demonstrated in β-cells of the pancreas (Tornehave et al. 1993 (human), Jensen et al. 1994 (human), Carlsson et al. 1997 (rat)), zona glomerulosa and medulla of human adrenals (Jensen et al. 1994) and in somatotrophic cells in human pituitary (Larsen et al. 1996). In the human reproductive system, Pref-1/FA1 positive staining has been presented in theca interna and Hilus cells in the ovaries and in Leydig cells in the testis (Jensen et al. 1999a). Studies of the central nervous system have displayed Pref-1/FA1-positive neurons in the ventral segmental area, pars compacta of substantia nigra, Edinger-Westphal nuclei and in a few neurons in the reticular part of substantia nigra in mesencephalon. Furthermore, neurons in raphe nuclei and locus coeruleus also demonstrated Pref-1/FA1 positive cells (Jensen et al. 2001).


Compartmentalisation

Prior to the discovery of the different cleavage products of Pref-1 and the data implicating that only the largest soluble form is biologically active, some studies were performed on the distribution of Pref-1/FA1 in different compartments.

Serum from clinically healthy adults contains around 26 ng/ml Pref-1/FA1 with no significant difference between males and females (Jensen et al. 1997). Observations on the quantitative distribution and localisation studies of Pref-1/FA1 in maternal and fetal compartments suggest a fetal origin of the protein (Fay et al. 1988, Tornehave et al. 1989). Studies in mice have demonstrated that during pregnancy, maternal serum levels of Pref-1/FA1 increase dramatically (30 times non-pregnant serum concentrations) during the second half of gestation and reach a peak about two days before partum (Bachmann et al. 1996). Furthermore, the Pref-1 mRNA expression is highly increased at day 14 and at the end of pregnancy in rat islets of Langerhans (Carlsson et al. 1997). Post partum, the Pref-1/FA1 concentration decreases rapidly and after 12 hours the serum concentration is back to non-pregnant levels (Bachmann et al. 1996).

In humans, the renal clearance of Pref-1/FA1 is 11 ml/min (per 100 g body-weight) and T1/2 of circulating Pref-1/FA1 approximately 2.6 h (Jensen et al. 1997). In patients with renal failure, the serum concentration of Pref-1/FA1
is about 10 times higher than in serum from healthy individuals and studies on bilaterally nephrectomized rats indicated five-fold higher serum levels compared to presurgery levels. Jensen and co-workers (1997) concluded that the major extraction of Pref-1/FA1 from the circulation is mediated via renal clearance.

**Biological Function**

Publications on the biological function of Pref-1 have assembled a growing amount of evidence for a regulatory role during cell differentiation, keeping the cells undifferentiated, especially in adipocyte development (Smas & Sul 1993, Smas et al. 1998). One study has, however, suggested that it is the secreted forms of Pref-1/dlk that are down-regulated but the transmembrane forms are in fact up-regulated during the onset of differentiation (Garces et al. 1999).

Furthermore, through studies of haematopoietic cell lines or stromal cell lines with the ability to support haematopoiesis, Pref-1 has been shown to participate in the development of cells from both the myeloid and lymphoid lineage as well as the erythroid lineage (Moore et al. 1997, Bauer et al. 1998, Kaneta et al. 2000, Ohno et al. 2001, Langer et al. 2004, Sakajiri et al. 2005). Moreover, the involvement of Pref-1 has been observed in various course of events such as the development of endocrine pancreas (Tornehave et al. 1993, Carlsson et al. 1997) development/zonation of adrenal cortex (Halder et al. 1998, Raza et al. 1998), wound healing (Samulewicz et al. 2002), myelinisation of nerve cells (Costaglioli et al. 2001), differentiation of skeletal stem cells (Abdallah et al. 2004), activation of satellite cells in skeletal muscles (Crameri et al. 2004), and fetal liver development (Tanimizu et al. 2003) as well as liver regeneration (Tanimizu et al. 2003, Jensen et al. 2004).

Increased expression of Pref-1 has been published in spleen and blood from rats with type 1 diabetes-like features (with respect to a segment containing the Pref-1 gene (Kloting & Kloting 2004)). Moreover, overexpression of Pref-1/FA1 in skeletal muscles was displayed in sheep with muscle hypertrophy (Charlier et al. 2001).

Studies of a plausible tumour suppressor gene MEN1 (Multiple endocrine neoplasia type 1, encoding for the protein Menin), have displayed a decreased Pref-1 mRNA expression when MEN1 was expressed in a transfected endocrine pancreatic cell line. Patients suffering from mutation in the MEN1 gene might develop tumours in several endocrine organs as well as in adipose tissue. Additionally, an increased expression of Pref-1 in tumours with homozygous inactivation of MEN1 was found (Stalberg et al. 2004).
The generation of genetically modified animals is a useful tool for in vivo studies of the function of a protein. However, the body’s extraordinary capacity of compensating for loss or gain of function must be accounted for. A knock-out mouse, lacking the paternal Pref-1, showed malformations resembling the symptoms observed in human maternal uniparental disomy 14 (mUPD14) (Moon et al. 2002). These mice displayed growth retardation, blepharophimosis, skeletal malformation, and obesity. Furthermore, the Pref-1-null animals suffered from enlarged, fatty livers and displayed elevated levels of triglycerides, free fatty acids and cholesterol in serum. Moon and co-workers (2002) suggested that the lack of Pref-1 is responsible for the disturbances in lipid metabolism as well as the other symptoms described above and that Pref-1-null mice may constitute an animal model for studies of human mUPD14.

Lee and co-workers (2003) created a transgenic mouse overexpressing Pref-1/FA1. Animals with overexpression in adipose tissue, displayed reduced fat mass in several depots as well as a decreased mRNA expression of the adipocyte factors adiponectin, leptin and resistin. Furthermore, the mice exhibited glucose intolerance, decreased insulin sensitivity and increased plasma levels of triglycerides. Animals with forced expression of Pref-1/FA1 in the liver also showed a loss in adipose mass, implicating at the endocrine mode of Pref-1/FA1.

Mechanism of Action

As mentioned above, EGF-like proteins act through protein-protein interactions with their EGF-like repeats, either as receptors or as ligands. A cysteine-rich domain called DSL (Delta Serrate LAG2) motif is involved in the receptor-ligand interaction in the Delta-Notch system. A number of attempts have been made to explain how Pref-1 or Pref-1/FA1 is activated and by what receptor (or ligand) it mediates its effects on cell differentiation. Since Pref-1 lacks the DSL motif, it was a surprise that it was demonstrated to bind to Notch (Baladron et al. 2005). The homology between Pref-1/FA1 and ligands of Notch (Delta and Serrate in Drosophila) suggests that Pref-1/FA1 is a ligand. The inhibiting effect on differentiation by the soluble Pref-1/FA1 adds weight to this hypothesis.

FOXA2, a fork head transcription factor, important for the regulation of many genes involved in adipocyte differentiation has been demonstrated to activate the transcription of Pref-1 and thus inhibiting adipocyte differentiation (Wolfrum et al. 2003).

The possibility that Pref-1 acts via the transcription factor Hes-1 (Hairy and enhancer of split 1) expression has been postulated by Kaneta and co-workers (2000) who found an increased cellularity and an increased expres-
sion of Hes-1 in fetal thymus organ cultures treated with a Pref-1 dimer. When Hes-1 deficient thymocytes were treated with dimeric Pref-1, no increase in thymocyte cellularity was displayed. A more recent study has suggested that Pref-1 transcription is down regulated by Hes-1 in the signalling cascade of Notch (Ross et al. 2004). As mentioned above, Baladrón and colleagues (2005) demonstrated an interaction between Notch1 and Pref-1, which can be interpreted as Pref-1 may inhibit Notch1 activation and thereby affect cell differentiation. Previously, this group reported interactions between Pref-1 and Growth arrest specific protein 1 (GAS1) and acrogranin (a precursor of peptides involved in controlling cell proliferation and growth) (Baladron et al. 2002). These results indicate that Pref-1 can also modulate cell growth in addition to cell differentiation.

Moreover, Pref-1 has been reported to suppress the Extracellular-regulated kinase (ERK) signalling cascade by modulating Insulin-like Growth Factor-I receptor activity (Ruiz-Hidalgo et al. 2002, Zhang et al. 2003). However, recently Kim and co-workers published a study where they suggest that the inhibitory action of Pref-1 is via activation of the MEK/ERK pathway without any involvement of IGF-1 (Kim et al. 2007).

The adipose tissue

A function of adipose endocrine tissue is, in addition to energy storage and protection of organs, to participate in the regulation of energy metabolism and homeostasis. Obesity is commonly known as a health hazard, and already more than 250 years ago Carl von Linné is said to have postulated that “man är tyngre när man är ofrisk, än när man är frisk”, meaning you are heavier when you are in poor health, than in good health.

A term for increased risk for developing obesity-related diseases such as type 2-diabetes and cardiovascular diseases is the metabolic syndrome. This syndrome involves three or more of the following features (as defined by National Cholesterol Education Program, USA): obesity, hypertension, glucose intolerance, hypertriglyceridemia, and hyper-cholesterolemia. The adipocyte as well as the whole adipose tissue plays a central role in the development of obesity-related diseases by secretion of proteins, so called adipokines. During the last decade, more than 50 secreted adipokines have been identified. These adipokines are highly heterogeneous in respect to function and affect a broad range of physiological events such as energy balance and lipid metabolism as well as inflammation and angiogenesis (reviewed in Trayhurn 2005).

One of the first identified adipokines, leptin (Farese et al. 1994), has been demonstrated to increase in the circulation with food intake and signals satiety to the centre in the hypothalamus. Leptin is involved not only in the
regulation of energy homeostasis, but also in inflammation and reproduction (reviewed in Otero et al. 2005). The serum level of adiponectin, contrary to most adipokines, decreases with increased adiposity and insulin resistance. The protein seems to prevent insulin resistance and inflammation, and high levels of adiponectin in serum have been connected to a decreased risk for cardiovascular complications (reviewed in Havel 2004, Trujillo & Scherer 2005).

Prior to development of insulin resistance and type 2 diabetes a mild chronic inflammation in adipose tissue has been observed with elevated levels of inflammatory markers in serum (Hotamisligil 1999). For instance the adipokines Interleukin 6 (IL-6) and Tumour Necrosis Factor α (TNF-α) are involved in the inflammatory responses, however, both cytokines have central roles in other events connected to metabolic disorders such as an decreased insulin sensitivity (Hotamisligil 1999, Senn et al. 2002).

Alteration in fat cell mass can be a result of both changes in cell size and cell number. An increase in cell number is acquired by differentiation of preadipocytes to mature adipocytes. An intensive research in the field of adipogenesis has emerged during recent years to identify factors involved in preadipocyte differentiation as well as adipocyte dedifferentiation. Studies of the effect of Pref-1 expression and secretion in preadipocyte differentiation have mainly been performed in cell cultures and to some extent in genetically modified animals (Moon et al. 2002, Lee K. et al. 2003, Bauche et al. 2007).

Laboratory animals

When investigating biological or pathobiological mechanisms, animal models are useful tools for the understanding of complex course of events. The possibility to extrapolate results from an animal model to a target species requires a well-characterised model, both in genotype and phenotype. Nevertheless, potential species differences, when extrapolating results between species, should be kept in mind. The studies in this thesis have used animal models as a tool for investigating biological features of Pref-1 in rats. The results gained in the last study, hopefully can bring additional characteristics to the animal models used in the effort to combat human obesity (Zucker rat) and type 2 diabetes (Goto-Kakizaki rat).

The Goto-Kakizaki rat

The Goto-Kakizaki (GK) rat is an inbred substrain of outbred Wistar stock origin. This strain is widely used as a non-obese model for non-insulin-dependent type 2 diabetes and develops diabetes spontaneously at weaning.
age. The GK rat exhibits mild hyperglycemia, impaired insulin secretion, hepatic and peripheral insulin resistance and a late onset of diabetic complications (Goto et al. 1976).

The Zucker rat
The outbred Zucker rat (Crl:ZUC-Lepr), suffers from a recessive mutation in the fatty gene (fa) encoding for the Leptin receptor. The mutation results in a phenotype with extreme obesity and infertility. The rat is a normoglycemic model with characteristics such as insulin resistance, hyperinsulinemia, hypertriglyceridemia, and hypercholesterolemia (Zucker T.F. & Zucker 1962, Zucker L. M. & Antoniades 1972).
The general aim of the thesis was to characterise protein and mRNA expression of Pref-1/dlk/FA1 in the rat.

The specific aims were:

- To purify and characterise the rat Pref-1/FA1 protein

- To analyse the occurrence of the rat Pref-1/FA1 protein in normal, maternal, and fetal serum, in amniotic fluid and urine and any alterations during pregnancy

- To localise rat Pref-1/FA1 protein in some adult tissues and find possible alterations in Pref-1/FA1 expression in these tissues due to pregnancy or hormone treatment.

- To analyse Pref-1/FA1 in serum and Pref-1 mRNA expression in visceral and subcutaneous fat depots from rats with metabolic disturbances

- To find possible correlations of Pref-1 and adipokines between serum levels and mRNA expression in rats with metabolic disturbances
Materials and Methods

Animals (I-III)

All experimental protocols involving animals were approved by the regional laboratory animal ethics committee in Uppsala, Sweden.

A total of 157 rats were used in study I-III. The different strains and breeds are summarized in table 2.

Table 2. Rats used in study I-III (w=weeks, m=months)

<table>
<thead>
<tr>
<th>Study</th>
<th>Strain</th>
<th>Gender</th>
<th>Age</th>
<th>n</th>
<th>Breeder</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Sprague-Dawley</td>
<td>♀</td>
<td>8 w</td>
<td>86</td>
<td>B&amp;K Universal, Sollentuna, Sweden</td>
</tr>
<tr>
<td>I</td>
<td>Sprague-Dawley</td>
<td>♂</td>
<td>8 w</td>
<td>8</td>
<td>B&amp;K Universal, Sollentuna, Sweden</td>
</tr>
<tr>
<td>II</td>
<td>Sprague-Dawley</td>
<td>♀</td>
<td>10 w</td>
<td>27</td>
<td>B&amp;K Universal, Sollentuna, Sweden</td>
</tr>
<tr>
<td>III</td>
<td>Wistar Furth</td>
<td>♂</td>
<td>3-4 m</td>
<td>10</td>
<td>Scanbur, Sollentuna, Sweden</td>
</tr>
<tr>
<td>III</td>
<td>Goto-Kakizaki</td>
<td>♂</td>
<td>3-4 m</td>
<td>10</td>
<td>Taconic, Ry, Denmark</td>
</tr>
<tr>
<td>III</td>
<td>Zucker obese</td>
<td>♂</td>
<td>3-4 m</td>
<td>8</td>
<td>Charles River Laboratories, Hanover, Germany</td>
</tr>
<tr>
<td></td>
<td>Crl:ZUC-Lepr</td>
<td></td>
<td></td>
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<tr>
<td>III</td>
<td>Zucker lean</td>
<td>♂</td>
<td>3-4 m</td>
<td>8</td>
<td>Charles River Laboratories, Hanover, Germany</td>
</tr>
</tbody>
</table>

The animals were housed in groups of three (II), four (I), or five (III) in Macrolon cages Type IV (59 cm x 38 cm x 20 cm, Scanbur, Køge, Denmark) in the animal facility at the Biomedical Centre, Uppsala University. The rats had free access to tap water and were fed a standard rodent diet (I and II: R36; Lactamin, Stockholm, Sweden; III: R3; B&K Universal, Sollentuna, Sweden) standard food (R36; Lactamin, Stockholm, Sweden). All ani-
mals were kept at 12 hour/12 hour light/dark, room temperature 21(±1)°C and 30-70 % relative humidity.

In study I, seventy-six female Sprague-Dawley rats were housed with males over night, and mating was confirmed by analysis of vaginal smears the following morning. The day following the overnight mating was designated Day 0 of pregnancy.

Procedures on animals

Sampling of serum, amniotic fluid and urine (I)

Mated rats (n=44) were euthanised with carbon dioxide on selected days of pregnancy. Blood samples were collected in connection with decapitation. Fetuses with placenta and yolk sac were dissected out and rinsed in distilled water. The amniotic membranes were punctured and amniotic fluid emptied in a petri dish and the fluid transferred to plastic tubes. Blood was collected from fetuses by decapitation. The blood samples were kept overnight at 4°C, centrifuged (3000 rpm, 10 min, 4°C) and serum separated (samples were stored at -20°C until analyses were performed). Serum and amniotic fluid samples were pooled separately for each litter except from litters where the amount of amniotic fluid was small (Day 10 to 15 of pregnancy). In these cases the amniotic fluid samples were mixed with fetal serum and treated as amniotic fluid. Serum and amniotic fluid were placed in refrigerator (4°C) over night followed by centrifugation (3000 rpm, 10 min, 4°C) to remove debris and red blood cells. The supernatant was kept at -20°C until used.

For antigen preparation, 16 pregnant Sprague-Dawley rats were euthanised on Days 12-21 of pregnancy with carbon dioxide inhalation followed by decapitation. Amniotic fluids were collected and treated as described above.

Twelve mated and six non-mated female rats were transferred to individual metabolism cages (Scanbur, Køge, Denmark) and kept for 16-20 h for collection of urine on days 0, 6, 13, and 19 of pregnancy. Before the rats were returned to conventional cages, blood samples (50 µl) were obtained from the tip of the tail into heparinized tubes. Serum was collected from blood samples and treated as described above.

Hormone treatment of rats (II)

Eight rats were divided into 4 groups for treatment with hormones via osmotic pumps. The rats were anesthetised using a mixture of Ketamine and
Xylazine (37.5 mg/ml plus 5 mg/ml; 0.2 ml per 100g body weight). Osmotic mini pumps (ALZET 2ML1, Alza Corporation, Palo Alto, CA, USA) were placed subcutaneously close to the dorsal midline. The incision was closed with surgical glue (Histoacryle; Braun, Melsungen, Germany). The pumps were filled with 1.6 IU of human Growth hormone (GH, Genotropin; Pharmacia-Uphjorn, Uppsala, Sweden), or 5 IU ovine Prolactin (PRL; Sigma, St Louis, MO, USA), or 1.6 IU GH + 5 IU PRL, or sterile saline (Apoteket, Uppsala, Sweden) as a vehicle resulting in a total volume of 2 ml. Ten days after implantation, the rats were euthanised by carbon dioxide inhalation followed by cervical dislocation.

Preparation of formalin fixed organs (II)

Nineteen rats, two pregnant (Day 15) and 17 non-pregnant female rats, were euthanised with carbon dioxide inhalation followed by cervical dislocation. The animals were subjected to necropsy and the following endocrine organs were placed in 4% formaldehyde (Merck Darmstadt, Germany): pituitary, pancreas, adrenal glands and ovaries. Bone structures surrounding the pituitaries were decalcified with Parengy’s decalcification solution (Apoteket, Uppsala, Sweden) for two days before embedding. Tissues to be examined were processed through increasing concentrations of alcohol (Solveco, Stockholm, Sweden) to xylene (Prolabo, Fontenay S/Bois, France) and embedded in paraffin wax (Merck, Darmstadt, Germany). The embedded tissues were cut in 4 to 6 µm sections with a microtome (Leitz, Wetslar, Germany) and mounted on slides (Menzel-gläser, Merck, Darmstadt, Germany) pre-treated with 3-aminopropyl-triethoxysilane (Sigma, St Louis, MO, USA).

Adipose tissue isolation and blood sampling (III)

GK, WF, ZO, and ZL rats were anesthetized with Inactin (thiobutabarbitral sodium; 120 mg/kg body weight, i.p.; Research Biochemicals International, Natick, MA, USA). Blood glucose concentrations were measured with test reagent strips (MediSense, Stockholm, Sweden) and serum insulin concentrations with ELISA (Rat Insulin ELISA, Mercodia, Uppsala, Sweden) in venous blood sampled before obtaining the tissue samples. Tissue samples were collected from eight white adipose depots, interscapular brown fat and pancreas (Figure 2). The amount of tissue varied between 15 and 40 mg. All tissue samples were placed in RNAlater stabilizing reagent (Qiagen, Hilden, Germany) in -20°C until RNA isolation. The rats were euthanised by exsanguination during anesthesia.
Purification of Pref-1/FA1 (I)

Rat FA1 was purified from amniotic fluid (AF) obtained at days 12-21 of pregnancy. The antigen was purified by affinity chromatography as described by Bachmann et al (1996) using affinity purified rabbit anti mouse FA1 (mFA1) (kindly donated by Dr. B Teisner, University of Odense, Denmark) coupled to CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden) using the manufacturer’s standard protocol.

Mass Spectrometry (I)

Purified rFA1 was analysed by MALDI mass spectrometry (Kratos III, Kratos, Manchester, UK) according to the manufacturer’s manual.
Amino Acid Analysis (I)

The amino acid composition of rPref-1/FA1 was analyzed with an LKB Alpha-Plus amino acid analyzer (LKB, Stockholm, Sweden) using the standard protein hydrolysate program as described in the manufacturer’s manual. Data collection was done with a Shimadzu CR2-AX integrator (Shimadzu, Kyoto, Japan). The values for threonine, serine, glucosamine and galactosamine were corrected for hydrolytic loss using the standard recovery values 0.96, 0.90, 0.50 and 0.50, respectively. The results were normalized on the basis of the recovery of the internal standard, norleucine. The values for half-cysteine and methionine were only estimates since a performic acid oxidation was not carried out.

Amino acid sequencing of the 28 first N-terminal amino acids of purified rPref-1/FA1 was performed using a protein sequencer (Applied Biosystems 477A Protein Sequencer, Pharmacia, Uppsala, Sweden) as described in the manufacturer’s manual.

Production of antibodies (I and II)

Antisera

Antisera against rat amniotic fluid proteins and antisera against purified rat Pref-1/FA1 (rPref-1/FA1), respectively, were produced in New Zealand White rabbits (weight 2.5 kg, housed at the National Veterinary Institute, Uppsala, Sweden). Pooled rat AF (0.2 ml) or purified rPref/FA1 (app. 25 µg) was mixed with 0.5 ml Freund's Complete Adjuvant (Sigma, St Louis, MO, USA) and administered into rabbits by intra muscular injections. Two weeks later the rabbits were injected i.m. with the respective antigen emulsified with Freund's Incomplete Adjuvant (Sigma, St Louis, Mo, USA). This procedure was repeated twice at two weeks intervals. The rabbits were bled from the marginal ear vein before receiving the booster injections and exsanguinated during surgical anaesthesia ten days after the final injection. The blood samples were kept over night at 4°C, serum was collected and centrifuged (3000 rpm, 10 min, 4°C) to remove debris and red blood cells (samples were stored at -20°C until analyses were performed). The antiserum against rPref/FA1 was absorbed liquid phase, with adult male rat serum and nonadsorbed material from affinity chromatography of amniotic fluid, to monospecificity. The antiserum was found to be monospecific in crossed immuno-electrophoresis using rat AF as antigen.
Biotinylation of antibodies

Biotinylation of antibodies was performed according to manufacturer’s manual by incubating biotin 3-sulfo-N-hydroxysuccinimide ester (Sigma) with affinity-purified rabbit anti-rat FA1 in dimethylsulphoxide (Merck, Darmstadt, Germany). After additional incubation with added NH₄Cl the solution was dialyzed in PBS to eliminate excess of biotin.

Negative controls

Biotinylated rabbit anti-mPref/FA1 was removed from the anti-mPref/FA1 conjugate by affinity chromatography on a rPref/FA1 coupled CNBr-activated Sepharose 4B gel. Protein containing fractions of the non-adsorbed material were analysed in ELISA and used as a negative control in immunohistochemistry.

Positive controls

The biotinylated rabbit anti-mPref/FA1 was eluted from the rPref/FA1 coupled CNBr-activated Sepharose 4B gel and neutralised. The eluted antibodies were tested for activity in ELISA and used as positive controls in immunohistochemistry.

Immunoelectrophoresis (I)

Crossed immunoelectrophoresis and rocket immunoelectrophoresis were performed essentially as described by Jensen et al. (1993) and Fay et al. (1988). The antibodies (rabbit anti mFA1 antiserum (Bachmann et al. 1996), rabbit anti rFA1 or rabbit anti rat amniotic fluid (rAF)) were used in the gels at concentrations of 1:25 antibody:gel (v:v); 1:50 (v:v); or 1:125 (v:v), respectively. Mouse FA1 as well as rAF or purified rFA1 were used as antigens. Rocket immunoelectrophoresis (Laurell 1972) was used to monitor rFA1 during affinity chromatography.

Crossed tandem immunoelectrophoresis (Kröll 1973) was performed as described by Krogh and Hau (1992) using rabbit anti rFA1 or rabbit anti mFA1 (Bachmann et al. 1996) at a concentration of 1:25 antibody:gel (v:v) in the second dimension gels. Undiluted amniotic fluids from pregnant mice and rats (10µl) were used as the antigen preparations.
Immunohistochemistry (II)

The sections were deparaffinised and rehydrated in xylene and decreasing concentrations of ethanol. To quench endogenous peroxidase activity, slides were incubated in 3% hydrogen peroxide followed by washing in PBS (pH 7.4). Sections were incubated with primary antibody (rabbit anti-rFA1) for 45 min at 37°C. After washing, the sections were incubated for 30 min in 37°C with the second antibody horseradish peroxidase-conjugated goat anti-rabbit IgG (whole molecule, Sigma, Steinheim, Germany) and thereafter washed again. 3,3’-diaminobenzidine (DAB) (Sigma, Steinheim, Germany) was used as the chromatogenic substrate and the reaction was stopped by washing in distilled water.

Negative controls were incubated with PBS and bovine serum albumin instead of the primary antibody. The sections were counterstained with haematoxyline and eosin (Papanicolau’s solution, Harris hematoxyline solution, and Certistain, Eosine Y, Merck, Darmstadt). The specimens were then dehydrated in ethanol (Solveco, Stockholm, Sweden) and xylene (Prolabo, Fontenay S/Bois, France). The sections were mounted with cover glasses (Menzel-gläser, Merck, Darmstadt, Germany) with a mountant (Floramount mountant, BDH, Poole, England).

Enzyme-Linked Immunosorbent Assay (ELISA)

Pref-1/FA1 ELISA (I and III)

The ELISA developed was a capture assay based on Krogh et al (1996). Different concentrations of antibody-solutions and chromatogenic solutions were tested to obtain the optimal concentrations as well as incubation times. In brief, microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with the capture antibody rabbit anti-mouseFA1 (the antibodies were kindly donated by Dr. B. Teisner, University of Odense, Odense, Denmark) in study I. In study III, the antibodies used (rabbit anti-ratPref-1/FA1) were produced in study II. The plates were incubated over night in moist chamber (4°C) followed by washing steps. Test samples and standard series (rat amniotic fluid in two fold dilution series in concentrations from 0.186 to 2.73 ng/ml were added to the wells. After incubation over night (4°C, in moist chamber) and additional washing steps, the plates were incubated with biotinylated affinity purified rabbit-anti mPref/FA1 (kindly donated by Dr. B. Teisner, University of Odense, Odense, Denmark). To develop the reaction, peroxidase-conjugated streptavidin (Sigma, St Louis, MO, USA) and H2O2 together with O-phenyldiamine (Sigma, St. Louis, MO, USA) were used.
The optical density was measured in a Labsystems Multiskan RC (Thermo, Electron Corporation, Vantaa, Finland) at 492 nm.

**Leptin, TNF-α and IL-6 ELISA (III)**

A sandwich Rat Leptin ELISA kit (Linco Research, St Charles, MO, USA) was used according to the manufacturer’s manual. In brief, serum samples were added to precoated plates. After washing, the samples were incubated with biotinylated antibody, horseradish peroxidase and, finally, tetramethylbenzidine. The absorbance was measured at 450 nm. Leptin standard samples and quality controls were supplied by the manufacturer.

**Radioimmunoassay (RIA)**

**Adiponectin RIA (III)**

A Mouse Adiponectin RIA kit (Linco Research, St Charles, MO, USA) was used to measure serum concentrations of adiponectin. The RIA was performed according to the manufacturer’s manual. Briefly, samples were diluted in assay buffer and incubated with ¹²⁵I adiponectin and adiponectin antibody in tubes. After 24 hours, normal rabbit serum (30%) and Precipitating Reagent were added and the mixtures were incubated and centrifuged. The supernatants were decanted and the remaining pellets were counted in a gamma counter. Supplied murine adiponectin standards and quality controls were used for calculations and assay control.

**Real time PCR (III)**

**RNA isolation and cDNA synthesis**

RNA was isolated using RNeasy Lipid Tissue Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s manual. In brief, tissue samples were homogenized in QIAzol Lysis Reagent and chloroform was added. After incubation and centrifugation, the upper RNA containing phase was mixed with ethanol (70%), transferred to RNeasy Mini Spin column and centrifuged. After washing, treatment with DNase (Qiagen) and additional washing steps, the RNA was eluted with RNase-free water and stored at -70 °C until use. The purity and concentration of the samples were determined by spectrophotometry. cDNA was synthesized using Reverse Transcription System (Promega, Madison, WI, USA).

**Real time Polymerase Chain Reaction (PCR)**
Real-time PCR was performed using a LightCycler Instrument (Roche, Basel, Switzerland) and SYBR Green JumpStart Taq ReadyMix (Sigma, St Louis, MO, USA). Specific primers for the target genes were purchased from TIB Molbiol Syntheselabor (Berlin, Germany) and were designed to span an exon to exon to avoid amplification of genomic DNA (Table 3). Amplifications were performed with 0.5 or 1 µl cDNA sample, 0.5 µM of each primer, 5 µl SYBR Green and water added to achieve a total volume of 10 µl. Primers for glucose-6-phosphate dehydrogenase (G6PDH) were used as internal standard after evaluation of different housekeeping genes, including tata binding protein (TBP) and porphobilinogen deaminase haem biosynthetic enzyme (PBG).

Table 3. Target genes used in PCR

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequence</th>
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<tr>
<td>G6PDH</td>
<td>ATT GAC CAC TAC CTG GGC AA (forward)</td>
</tr>
<tr>
<td></td>
<td>GAG ATA CAC TTC AAC ACT TTG ACC T (reverse)</td>
</tr>
<tr>
<td>Pref-1</td>
<td>CAA TGG TTC TCC CTG CCA G (forward)</td>
</tr>
<tr>
<td></td>
<td>TGT TGG TCA CGA TCT CAC AGA A (reverse)</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>GAG AAG GGA GAC GCA GGT GT (forward)</td>
</tr>
<tr>
<td></td>
<td>GCT GAA TGC TGA GTG ATA CAT GTA AG (reverse)</td>
</tr>
<tr>
<td>Leptin</td>
<td>GAC ATT TCA CAC AGG CAG TCG (forward)</td>
</tr>
<tr>
<td></td>
<td>GCA AGC TGG TGA GGA TCT GT (reverse)</td>
</tr>
<tr>
<td>TNF-α</td>
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</tr>
<tr>
<td></td>
<td>AGA CCC TCA CAC TCA GAT CAT CTT (reverse)</td>
</tr>
<tr>
<td>IL-6</td>
<td>CAG AAT TGC CAT TGC ACA ACT C (forward)</td>
</tr>
<tr>
<td></td>
<td>CTG ACA GTG CAT CAT CGC TGT T (reverse)</td>
</tr>
</tbody>
</table>

Statistical analysis

For statistical analyses in study I, analyses of variance were conducted in Excel (Microsoft Corporation, Redmond, WA, USA) and \( P \) values <0.05 were considered significant.
In study III statistical calculations were performed in SPSS for Windows version 12.0.1 (SPSS, Chicago, IL, USA). One-way ANOVA and Bonferroni post-hoc test were used for statistical analysis of body weight, blood glucose and serum insulin. For mRNA expression and protein serum concentrations, a two-way ANOVA were used to detect differences between strains. For calculation of significant differences between strains for a certain depot, one-way ANOVA or Student’s unpaired t-test were used.

To calculate mean mRNA expression in visceral or subcutaneous fat depots (named VAT and SAT, respectively), the values from measured visceral (psoas, renal and pancreatic fat) or subcutaneous (abdominal, throat and neck fat) depots were used to calculate mean values.

Some serum samples did not contain a detectable leptin concentration and a few were above the maximal measurable concentration. These samples were assigned the minimum or maximum detectable values for the assay, respectively, for further statistical processing.
Results

Study I

Imunochemical analysis of rat Pref-1/FA1

Concentrated rat Pref-1/FA1 (10 µl, ~200 µg/ml) purified by immunospecific affinity chromatography revealed four precipitates (one strong and three weak) when tested against unabsorbed rabbit anti rPref-1/FA1 antiserum or rabbit anti rAF in crossed immunoelectrophoresis. However, when the antiserum preparations were diluted five times or more, only one precipitate remained visible. When tested against anti rPref-1/FA1 absorbed with a mixture of normal rat serum and amniotic fluid depleted of Pref-1/FA1 by immunoadsorption, or against monospecific anti mPref-1/FA1, only one precipitate was seen with undiluted rPref-1/FA1 as the antigen. The tandem crossed immunoelectrophoresis analysis of mPref-1/FA1 and rPref-1/FA1, using absorbed anti rPref-1/FA1 antibodies in the second dimensional gel, demonstrated complete fusion of precipitates without spur formation indicating immunochemical identity between mouse and rat Pref-1/FA1 (see Figure 1, paper I).

Mass spectrometry

Analysis of the intact rPref-1/FA1 glycoprotein showed a population of molecules with molecular masses ranging from 28 to 36 kDa with a majority of the material (95%) appearing around 33 kDa.

Amino acid analysis of rat Pref-1/FA1

The sequence of the 28 first N-terminal amino acids of the purified rPref-1/FA1 showed 100 % identity to the corresponding section of rat Pref-1 (aa 24–60, GI:16758572; Figure 2, Paper I), 86 % identity to mouse dlk (GI:83816918) and 82 % identity to human dlk (GI:74136023). The identity
between the first 28 amino acids of mouse and human dlk was 71%. The amino acid composition of the purified rPref-1/FA1 showed 90-100% identity to the corresponding part of rat Pref-1 and ZOG (aa 24–304; GI: 3097285).

Rat Pref-1/FA1 ELISA
The sensitivity of the ELISA, set to the most diluted standard concentration, was 0.186 ng/ml (the background plus 2SD was below this concentration). The coefficient of variation (CV) was 5.1% between plates. The mean CV within plates was 2.1%.

Levels of rat Pref-1/FA1 in different compartments

Pref-1/FA1 in serum and amniotic fluid
The concentration of rPref-1/FA1 in serum of non-pregnant adult rats was approximately 0.01 µg/ml. During the second half of gestation, the serum levels of Pref-1/FA1 in pregnant rats increased dramatically and reached a peak around day 18. The concentration of Pref-1/FA1 in fetal serum and amniotic fluid was about two orders of magnitude higher (approximately 10 µg/ml and 18 µg/ml, respectively). The level of Pref-1/FA1 peaked one day earlier in fetal serum than in maternal serum and amniotic fluid. Compared to normal serum, the levels in fetal serum and amniotic fluid were approximately 1800 and 1000 times higher, respectively (Figure 3).

The relationship between maternal serum concentration of rPref-1/FA1 and the number of fetuses (corrected for day of pregnancy) demonstrated that the concentration of Pref-1/FA1 in maternal rat serum displayed a weak positive correlation to the number of fetuses (n=41; r²= 0.19, p< 0.002).

After delivery, the concentration of Pref-1/FA1 in maternal serum rapidly decreased but in the neonates the serum concentration remained high for 7 days (unpublished observations).

Pref-1/FA1 in urine compared with corresponding serum samples
Rat Pref-1/FA1 could be quantified in all collected samples by the ELISA. There was no significant difference between serum and urine levels in non-pregnant female and pregnant rats until late pregnancy (Table 2 in Paper I). However, on day 19 of pregnancy, significantly increased urine and serum levels were recorded and the concentration of rPref-1/FA1 was significantly higher in urine than in serum (p<0.01). The average concentration of rPref-1/FA1 in urine was approximately twice that in serum and in individual animals it was consistently higher in urine than in serum.
Figure 3. Diagram illustrating the differences in concentration of Pref-1/FA1 between non-pregnant (●), pregnant (■), and fetal serum (●) and amniotic fluid (□). The dots correspond to mean values of samples collected day 17–19 of pregnancy and from non-pregnant female rats.

Study II

Immunohistochemistry

An indirect immunoperoxidase staining technique was used to demonstrate any presence of Pref-1/FA1 in the below mentioned endocrine tissues. The localisation results obtained from the hormone-treated rats as well as pregnant rats did not differ quantitatively from non-pregnant rats.

Pref-1/FA1 was found in cortex as well as medulla of the adrenal glands. In the cortex, the staining was most pronounced in the zona glomerulosa and less intense in the zona fasciculata and zona reticularis. The colour intensity decreased gradually towards the medulla where most cells demonstrated the presence of Pref-1/FA1 in a distinct area close to the cell nucleus (see Figure 1, paper II).

In the pituitary gland, immunospecific staining was located to chromatophilic cells in the pars distalis of the adenohypophysis and to some cells in the pars intermedia (see Figure 3, paper II)
In the ovaries the staining pattern varied between animals. In some animals no staining was observed but in other animals staining of varying intensity could be found in some granulosa lutein cells associated with degenerating follicles (see Figure 2, paper II).

No staining was detected in any of the rat pancreatic glands.

Study III

Evaluation of internal standard for PCR

The mean CV was 1.6 % between runs. The mean CV between duplicates was 0.5 %.

The Goto-Kakizaki rat

Body weight, blood glucose and serum insulin concentrations

GK rats had increased blood glucose concentrations compared to the other strains (Table 2).

mRNA expression of adipokines

Pref-1

In the GK rats, the mean expression of all subcutaneous tissues (SAT) was increased compared to the mean expression of all visceral adipose tissues (VAT) in contrast to ZO and control rats (WF and ZL; Figure 1A, paper III). When all depots were analyzed, a significant difference between strains was present (Figure 1A, paper III). The highest expression of Pref-1 was demonstrated in sternum fat depots from both strains which displayed up to 140 times elevated expression compared to the other depots (Figure 1B, paper III). No significant difference was detected in serum concentrations of Pref-1/FA1 when the different strains were compared (Figure 6A, paper III).

Adiponectin

Compared to control rats, the GK rats had decreased expression of adiponectin mRNA in both VAT and SAT (Figure 2A, paper III). This was also the case in general for all the specific depots and reached statistical significance in epididymal, renal and abdominal fat pads (Figure 2B, paper III). The epididymal fat pad had an increased expression compared to the pancreatic fat pad of the control rats, whereas no differences were displayed within the GK rats. In contrast serum concentrations of adiponectin were increased in the GK rat compared to control (Figure 6B, paper III).
Leptin

The mRNA expression of leptin was altered between the adipose tissues of the GK rats and the control (Figure 3A, paper III). In contrast to the control rat, which demonstrated increased leptin mRNA in SAT compared to VAT no difference was demonstrated in the GK rat (Figure 3B, paper III). The serum leptin concentration was decreased in GK rats compared to the control strain (Figure 6C, paper III).

IL-6

There was a dramatic increase of IL-6 mRNA in adipose tissues of the GK rats in both VAT and SAT compared to controls (Figure 4A, paper III). A general increase was demonstrated in all depots of the GK rat which attained statistical significance in the psoas and the neck depots (Figure 4B, paper III). The GK rat had increased levels of IL-6 mRNA also compared to the two other strains in the study (ZO, ZL). The concentration of IL-6 in serum was only detectable in a few samples, data not shown.

Figure 4 Correlation between TNF-α and IL-6 mRNA expression in the GK rats. ♦ represents one depot sample with a value for relative TNF-α and IL-6 mRNA expression.

TNF-α

Except for sternum, a general increase was demonstrated in specific depots, which only attained statistical significance in the renal depot (Figure 5B, paper III). No detectable serum concentrations of TNF-α was demonstrated, data not shown.
The Obese Zucker Rat

Body weight, blood glucose and serum insulin concentrations

The ZO rats had increased body weight as well as serum insulin concentrations compared to their lean counterparts, ZL (Table 2, paper III).

mRNA expression of adipokines

Pref-1

In contrast to the GK rats, SAT from the obese Zucker rats was decreased compared to VAT (Figure 1C, paper III). Compared to lean controls and the GK decreased levels were demonstrated in general in subcutaneous depots reaching statistical significance only in throat depots. However, within the ZO rat, the pancreatic depot demonstrated the highest levels of Pref-1 mRNA, statistically different from other visceral depots and compared to the ZL pancreatic depot (Figure 1D, paper III). Within the ZL rat, the throat depot had statistically significant increased Pref-1 mRNA levels compared to all other subcutaneous depots.

Adiponectin

The ZO rat had decreased expression of adiponectin mRNA in both VAT and SAT compared to controls (Figure 2C, paper III). A general decreased expression was demonstrated in all adipose depots and attained statistical significance in all depots except the pancreas and abdominal depots (p<0.08, Figure 2D, paper III). Within the ZL rat increased adiponectin mRNA was present in the epididymal depot compared to the renal depot. No statistical significant difference in serum concentrations were demonstrated in the Zucker strains compared to GK and WF (Figure 6B, paper III).

Leptin

Leptin mRNA expression was increased in both VAT and SAT in the ZO compared to control (Figure 3C, paper III). All adipose depots demonstrated increased levels, except for the sternal and epididymal depots, and reached statistical significance in the renal, neck, and throat depots (Figure 3D, paper III). As expected, serum leptin concentration of the ZO strain were increased compared to ZL rats and the other two strains of the study (Figure 6C, paper III).

IL-6

In the ZO rat, the mRNA expression of IL-6 mRNA was in general increased compared to lean controls (Figure 4C, paper III). All specific adipose depots
had increased levels which only attained statistical significance in the renal, sternum and BAT depots (Figure 4D, paper III). The concentration of IL-6 in serum of the ZO was only detectable in a few samples, data not shown.

**TNF-α**

The TNF-α mRNA expression was different in general and between the SAT depots between ZO and ZL rats (Figure 5C, paper III). In specific depots a general decrease was demonstrated in the SAT tissues, but only reached statistical significance in the neck depot (Figure 4D, paper III). Within the ZL strain, a decrease was demonstrated between the epididymal depots and the other visceral depots (Figure 5D, paper III). No detectable serum concentrations of TNF-α were demonstrated, data not shown.

**Pref-1 mRNA expression in BAT**

The Leptin mRNA expression of the BAT depot was statistically significantly decreased compared to all other WAT depots within the strain (Figure 3B and D, paper III).

In the obese Zucker rats, both the expressions of Pref-1 and adiponectin mRNA were decreased in BAT compared to BAT from the lean control (Figure 1D and 2D, paper III), and the expressions of Leptin and IL-6 mRNA increased in comparison to lean controls (Figure 3D and 4D, paper III).
Physicochemically characterisation of Pref-1/FA1

Pref-1/FA1 was isolated from rat amniotic fluid using monospecific rabbit anti-mPref-1/FA1. In crossed tandem immunoelectrophoresis using rabbit anti-mPref-1/FA1 and rabbit anti-rPref-1/FA1 antibodies, rat Pref-1/FA1 showed immunochemical identity with mouse Pref-1/FA1.

Analyses by mass spectrometry of the intact rPref-1/FA1 glycoprotein displayed a heterogeneous protein with respect to size. A majority of the molecules demonstrated molecular masses near 33 kDa but the span of all molecules ranged from 28 to 36 kDa. If the molecular mass is calculated from the amino acid composition the result is 34 kDa. Results from SDS-PAGE and size chromatography (data not shown) support the conclusion that Pref-1/FA1 varies in glycosylation and chain length. These findings are in agreement with previous reports on human Pref-1/FA1 (Jensen et al. 1994) and mouse Pref-1/FA1 (Krogh et al. 1997).

The amino acid composition of rPref-1/FA1 was found to be identical with the corresponding first 28 amino acid sequence of the N-terminus of Pref-1 in rat (Carlsson et al. 1997) when unidentified amino acids are assumed to be cystein or tryptophan. Rat Pref-1/FA1 showed 86 % identity with mouse Pref-1/FA1 and 82 % identity to human Pref-1/FA1 where the identity between mouse and human Pref-1/FA1 is 71 %. However, because the present analysis was restricted to 28 of a total of approximately 260 amino acids, the results may be different from analyses of the entire molecules.

Concentrations of Pref-1/FA1 in biological compartments

The concentrations of Pref-1/FA1 in different compartments were measured with ELISA. In serum from non-pregnant adult rats, the average concentration was 0.01±0.003 µg/ml. In pregnant rats, the serum concentration of Pref-1/FA1 increased dramatically during the second half of gestation, with a maximum (20 times higher concentration than normal serum) around pregnancy day 18 and was positively correlated to the number of fetuses. The concentration in fetal serum peaked one day earlier (day 17) and the concentration was about 1800 times higher compared to non-pregnant serum. After
delivery, the concentration in maternal serum decreased rapidly during the first day but the concentration remained high in the neonates (unpublished data).

The Pref-1/FA1 concentration in urine of pregnant rats (day 0, 6, 13 and 19) followed approximately the same pattern as in the corresponding serum until day 13 of pregnancy (p=0.058). The differences between concentrations in urine and serum were not significant until day 19 (p=0.008). The ratio of Pref-1/FA1 in urine versus maternal serum at day 19 was approximately 2:1, which can be interpreted as Pref-1/FA1 being concentrated in the kidneys.

These results are consistent with previous results, which suggest that Pref-1/FA1 from fetal urine is the major source of Pref-1/FA1 in amniotic fluid. The fact that serum levels of Pref-1/FA1 in pregnant mice is positively correlated with the number of fetuses adds weight to the theory that most of the Pref-1/FA1 in maternal circulation is produced in the fetus and passes from the fetal circulation or AF to the maternal circulation (Bachmann et al. 1996). However, studies in islets of Langerhans in pregnant rats demonstrated an dramatic increase in Pref-1 mRNA expression day 14 and prior to birth, indicating an altered release due to pregnancy per se in this tissue. The organs from pregnant rats studied in study II (ovary, adrenals, pancreas and pituitary) did not alter in protein expression compared to non-pregnant controls. Nevertheless, it is conceivable that these or other organs increase, or decrease, their secretion of Pref-1/FA1 due to pregnancy, an intriguing question for further investigations.

The circulating Pref-1/FA1 in non-pregnant rats probably originates from the cells in endocrine tissues where Pref-1/FA1 has been localized, e.g. adrenopituitary gland, adrenal gland, spleen, β-cells from islets of Langerhans in the pancreas and adipose tissue (see Figure 1) (Jensen et al. 1993, Smas & Sul 1993, Larsen et al. 1996, Tornehave et al. 1996, 1999a). The presence of Pref-1 mRNA in blood (Kloting & Kloting 2004), and assumed but low protein expression, do not contradict this theory by the reason of discovered alterations in serum levels that correlates to pregnancy (study I and (Fay et al. 1988, Tornehave et al. 1989, Bachmann et al. 1996)) and Pref-1 expressing tumours in patients with small cell lung cancer (Harken Jensen et al. 1999). In study III, no significant differences in Pref-1/FA1 serum concentrations were detected between the GK and WF nor ZO and ZL. This suggests that there is no correlation between the circulating levels of Pref-1/FA1 and the metabolic state of these animals, maybe rather a difference between strains.

The possible endocrine effect of circulating Pref-1/FA1 is poorly investigated. However, the reduction in adipose tissue mass seen in transgenic mice overexpressing Pref-1/FA1 in the liver can be interpreted as an endocrine action on adipocyte differentiation by the liver originated Pref-1/FA1. How the highly elevated levels seen in maternal circulation might affect the maternal tissues remains to be elucidated. The high levels might be a result of
tissue spill-over and/or spill-over from the fetal circulation. However, a much more exiting possibility is an effect on distant tissues and a possible control by Pref-1/FA1 on various cell differentiation events.

Localisation of Pref-1/FA1

The localisation studies using the indirect immunoperoxidase staining technique displayed staining in the adrenals, pituitary gland and ovaries. Since the present work was done with anti Pref-1/FA1 antibodies there ought to be agreement with localisation results obtained using antibodies against the transmembrane Pref-1. By contrast, results obtained using antibodies against only the intracellular part of Pref-1 may not necessarily be in agreement with results obtained using Pref-1/FA1-antibodies.

Localisation studies on ZOG in adult and fetal rat adrenals have been published (Okamoto et al. 1997, Halder et al. 1998, 1998). Pref-1-positive cells were detected in the adrenogonadal primordium at the early embryonic stage and at embryonic day 14.5, Pref-1-positive cells gradually formed the adrenal primordium. The distribution of Pref-1-positive cells in adult rat adrenals showed pronounced staining in the glomerulosa zone and the medulla. These studies suggest that Pref-1 is involved in the differentiation of the adrenocortical tissue and that the Pref-1 down-regulation is essential to trigger adrenocortical cell proliferation. In study II, the most pronounced stainings in the adrenals were seen in the zona glomerulosa and in the medulla. In the medulla, the staining was concentrated to dot-like patterns inside the cell, near the nucleus. This site corresponds to the Golgi zone, which may indicate that these cells synthesise Pref-1/FA1. These results are at variance with results published on human Pref-1/FA1 localisation by Jensen and co-workers (1993), where staining could be found in the cortex but not in the medulla.

Pref-1/FA1 was further demonstrated in the chromophilic cells of the adenopituitary which agrees with findings in the human (Larsen et al. 1996) and in the mouse (Bachmann et al. 1996). Immunohistochemical analyses show co-localisation with growth hormone in the pituitary (Larsen et al. 1996). Pref-1/FA1 was also detected in ganglia around the pituitary (results not shown), which seems to agree well with a study on rat and human central nervous systems (Jensen et al. 2001) where Pref-1/FA1 has been localised to neurons in Edinger-Westphal’s nucleus, substantia nigra, ventral tegmental area, locus coeruleus and raphe nuclei in both species.

In the ovaries, granular staining was clearly identified in some of the rats. However, the intensity of the staining was weaker than in the pituitary and the adrenals and had varying intensity. The staining was restricted to certain granulosa lutein cells associated with degenerating follicles and probably dependent on the age of the corpus luteum. In the healthy human ovary, Pref-1/FA1 has been localised in theca interna, hilus cells and in luteinized
cells in corpus luteum. Follicular fluid samples from women in *in vitro* fertilisation programs demonstrated variations in Pref-1/FA1 concentration between follicles but no significant correlation to the corresponding serum concentration. These results suggest that Pref-1/FA1 in follicular fluid is secreted locally from adjacent cells (Jensen *et al.* 1999a). No variation in serum levels during the menstrual cycle has been detected (Jensen *et al.* 1997). Pref-1/FA1 has been demonstrated in seminal plasma as well (Jensen *et al.* 1999a). Other so called pregnancy-associated proteins show relatively high concentrations in follicular fluid and seminal fluid (Chard 1985). The biological role of Pref-1/FA1 in the reproductive system has not been investigated. However, the inhibiting role of Pref-1/FA1 on cell differentiation in other tissues suggests that the presence of Pref-1/FA1 in the microenvironment of the ovum controls or inhibits inappropriate cell differentiation.

Pref-1/FA1 has been described in β-cells of the islets of Langerhans in the human pancreas (Tornehave *et al.* 1993, Jensen *et al.* 1994) in the mouse and rat pancreas (Bachmann *et al.* 1996, Carlsson *et al.* 1997). Treatment *in vitro* with GH and PRL has been found to stimulate the expression of Pref-1 mRNA in isolated rat neonatal islets of Langerhans (Carlsson *et al.* 1997). It was thus unexpected that we failed to demonstrate the presence of Pref-1/FA1 in rat pancreas and that there was no stimulatory effect on Pref-1/FA1 in the GH and PRL treated rats neither in localisation studies or serum analyses (data not shown). Studies on human circulating Pref-1/FA1 levels have shown no differences between healthy adults and patients suffering from overproduction of GH or GH deficiency (Andersen *et al.* 2001). However, when patients with abnormal production of GH were treated for overexpression/deficiency during weeks or month, the levels of Pref-1/FA1 demonstrated a corresponding decrease/increase to GH levels. An explanation for absence of increased concentrations of Pref-1/FA1 in the hormone treated rats in the present study may be that the duration of the treatment was too short or that small changes in Pref-1/FA1 serum levels were cleared from the blood by the kidneys due to an increased glomerular filtration caused by GH and IGF-I. Another explanation is that the expected up-regulation of Pref-1 does not necessarily give an increase of Pref-1/FA1 analogous to what has been seen in adipocyte maturation where the transmembrane form increased when the cleavage product decreased initially (Garces *et al.* 1999). In line with this as reported by Friedrichsen and co-workers, (2003) *in vitro* stimulation with GH and Prolactin, increases Pref-1 mRNA in neonatal islets of Langerhans, but do not affect the release of Pref-1/FA1 from these cells.

**Pref-1 expression in WAT**

White adipose tissue is a heterogeneous tissue. The different depots in the body exhibit differences in expression of adipokines, blood flow, and metabolic activity. This indicates a varying physiological impact on metabolic
features in the body depending on depot. Comparing the four rat strains used in study III, the diabetic GK rat is clearly different from the obese Zucker as well as the control strains WF and ZL. The GK rat displayed altered Pref-1, decreased levels of adiponectin mRNA and increased expression of IL-6.

An altered expression of Pref-1 mRNA in adipose tissue can, hypothetically, be due to changes in preadipocyte number and thus an indication of a change in preadipocyte/adipocyte composition. However, it cannot be excluded that other cells present in adipose tissue (cells associated with stromal, vascular, and nerve tissues as well as macrophages) express Pref-1 mRNA. Moreover, a well vascularised depot, could in theory display an increased mRNA expression due to Pref-1 expressing cells in blood (Kloting & Kloting 2004). Still, the possible increase of Pref-1 expression due to more blood cells in the tissue is likely negligible.

Increased adipose tissue mass is due to an increased cell size (hypertrophy) and/or an increased cell number (hyperplasia) of adipocytes. In ZO rats, the enlargement is probably due to hypertrophy of adipocytes (Johnson et al. 1971, Johnson & Hirsch 1972) and in a recently published study, larger adipocytes in psoas, pancreatic, and abdominal fat depots were found in ZO rats than in the lean controls (Kampf et al. 2005). The decreased expressions in subcutaneous depots of Pref-1 mRNA in the ZO rats demonstrated in this study do not coincide with the increased size of adipocytes. However, an increased turnover of preadipocytes compared to lean controls cannot be excluded.

The decreased expression of Pref-1 mRNA in VAT compared to SAT in the GK rats might reflect differences in adipocyte/preadipocyte composition between the two types of depots. If this is the case, low levels of Pref-1 could be expected in the visceral depots due to lack of preadipocytes because of the elevated adiposity of the depot. This hypothesis is supported by the obese Pref-1-null mice which displays hypertrophy of adipocytes compared to the wild-type (Moon et al. 2002). Moreover, another interesting finding was the high expression of Pref-1 mRNA in the sternal fat compared to all other depots in the GK and WF rats. Further research is needed to clarify if this is due to high contents of preadipocytes or other Pref-1 expressing cells.

Adiponectin seems to possess qualities with beneficial effects on metabolic disturbances such as insulin sensitivity and lipid metabolism. In study III, the adiponectin mRNA expression was affected by the diabetic state in the GK rat and by the increased adiposity in the obese Zucker rats compared to the respective controls. In a study on mice with a genetically modified expressions of adiponectin, Pref-1 mRNA expression was increased in transgenic mice and adiponectin knock-outs exhibited a decreased Pref-1 expression in adipose tissue (Bauche et al. 2007).

Taken together, a clear correlation between Pref-1 expression and the adipokines analysed in the present study can not be done without further studies. To be able to speculate about a relationship between Pref-1 and adi-
pokine expression in this study, a number of assumptions have to be made. First, a “normal” adipose tissue has a certain preadipocyte/adipocyte composition and abnormal alterations in cell number or cell size occur due to metabolic disturbances. Second, this composition is reflected by a “healthy” expression profile of adipokines. The third assumption is that Pref-1 expression is decreased in adipose tissue when the tissue is enlarged due to hypertrophy (and that hypertrophy per se involves differentiation to mature fat cells resulting in less number of preadipocytes. Maybe, an increase in adipocyte number can result in an increased Pref-1 expression as a result of increased recruitment of preadipocytes).

If this would indeed be the case, Pref-1 expression is altered when the adipose tissue becomes “unhealthy”, with increasing adiposity and altered levels of adipokine expression resulting in a changed adipokine expression profile such as decreased levels of adiponectin and increased TNF-α and IL-6 mRNAs.

The heterogeneity of WAT
More or less frequently, a generalisation is made in the literature by assuming that analysis of an individual visceral or subcutaneous depot is representative of these depots in general. In study III, significant differences in mRNA expression of adipokines in the same strain were demonstrated between depots originating from visceral as well as subcutaneous types. This emphasises the inappropriateness of making general conclusions applied on visceral fat as such, on the basis of studies of only one depot. Moreover, high divergences and lack of significant differences between VAT and SAT stress the heterogeneity within strains. This is further stressed by the lack of consistence in depot expression patterns of IL-6 and TNF-α between individual animals within the GK and ZO strain. This reflection highlights the need for further studies on adipose tissues, and their physio-pathological effects in the body.

Pref-1 expression in BAT
Brown adipose tissue is an organ involved in production of heat. In recent years, an involvement in total-body energy balance has come to focus. Animal studies have demonstrated that loss of BAT function results in obesity and vice versa (reviewed in Cannon & Nedergaard 2004). In rodents and other small mammals, the BAT depots persist throughout life. A possible regulator of Pref-1 expression in brown adipocytes is necdin, a growth suppressor regulated by insulin receptor substrates. Studies have demonstrated a decreased expression of Pref-1 due to suppressed expression of necdin, resulting in adipocyte differentiation (Tseng et al. 2005). An interpretation of the decreased expression of Pref-1 in the obese Zucker rat could be, as
above, smaller numbers of preadipocytes due to the increased differentiation of adipocytes, as a result of the known atrophy observed in the obese Zucker strain (Johnson et al. 1971, Johnson & Hirsch 1972, Seydoux et al. 1990). The colour of the brown adipose depots in the obese rats was less brown than in lean controls (unpublished observation). A hypothetic theory is that increased fat deposition in the brown adipocytes contributes to the more light impression.
Conclusions

The studies presented in this thesis deal with the rat as an animal model for the studies of Pref-1/dlk/FA1. New and interesting information regarding some Pref-1 mRNA expression and protein distribution are summarised below:

- The physicochemical characteristics as well as the compartmentalisation of soluble Pref-1 are highly similar to what has been found previously in the mouse and in the human. This strengthen that the rat is an appropriate animal model for studies of Pref-1 and soluble Pref-1.

- During late rat pregnancy, the high levels of Pref-1 in maternal serum can partly be due to a crossover from fetal serum and/or amniotic fluid with extremely elevated levels of soluble Pref-1. However, a possible increase of Pref-1 secretion in several maternal tissues due to pregnancy per se must be accounted for.

- Non-pregnant, pregnant and hormone treated (Growth hormone and/or prolactin) rats display similar immunohistochemical staining patterns in adrenal glands, ovaries and pituitary glands suggesting that pregnancy hormones, and GH and/or prolactin do not influence the expression of soluble Pref-1 in these organs.

- The levels of Pref-1 in serum of Goto-Kakizaki and Zucker rats is not likely affected by the metabolic disturbances these animal suffers from (type II diabetes and obesity respectively).

- The expression of Pref-1 mRNA in adipose tissues and that of several adipokines is markedly changed of the diabetic GK rat. Furthermore, the concentration of adiponectin and leptin in serum is altered indicating that this model expresses many of the typical features of type 2 diabetes seen in man.
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References


Mei B, Zhao L, Chen L & Sul HS 2002 Only the large soluble form of preadipocyte factor-1 (Pref-1), but not the small soluble and membrane forms, inhibits adipocyte differentiation: role of alternative splicing. Biochem J 364 137-144.


Schmidt JV, Matteson PG, Jones BK, Guan XJ & Tilghman SM 2000 The Dlk1 and Gt1 genes are linked and reciprocally imprinted. Genes Dev 14 1997-2002.


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