Mice Overexpressing Pref-1 are Lean and Diabetic with Developmental Defects: Association with Paternal UPD14

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ABSTRACT

Preadipocyte factor-1 (Pref-1/Dlk1), encoding a transmembrane protein containing EGF-repeats homologous to the Notch/Delta/Serrate family, is a paternally expressed gene localized at homologous imprinted regions of syntenic chromosomes 12, 14, and 18 in mouse, human, and sheep, respectively. Perturbance of imprinted gene expression in human paternal uniparental disomy UPD14 causes growth retardation and bone malformation, whereas maternal UPD14 leads to growth retardation, obesity, scoliosis, and short stature. Here we have generated transgenic mice overexpressing Pref-1 to examine whether Pref-1 is responsible for these symptoms. Pref-1 overexpression results in most of the phenotypes of human pUPD14 and mouse pUPD12, including growth retardation, perinatal lethality, and bone malformation. Moreover, transgenic mice had less fat mass due to decreased adipogenesis. Similar to type II diabetes, these mice exhibited hypertriglyceridemia and impaired insulin homeostasis. These phenotypes establish Pref-1 overexpresssion as the key determinant of human pUPD14 and mouse pUPD12 syndromes. Our studies also suggest pathophysiological implications of Pref-1 in obesity and diabetes.

Introduction

Preadipocyte factor-1 (Pref-1), a transmembrane EGF repeat domain-containing protein, is highly expressed in 3T3-L1 preadipocytes, but its expression is abolished during differentiation into mature fat cells (Smas and Sul, 1993 ; Smas et al., 1997). Although its biological function is not clear, Pref-1 has been used as a preadipocyte marker by various laboratories(Shim et al., 1998 ; Zhou et al., 1997). Recently, it has been well established that PPARγ and C/EBPs play a central role in inducing adipocye gene expression and differentiation(Darlington et al., 1998 ; Gregoire et al., 1998). However, a proper extracellular environment and factors that modulate differentiation are critical but have not been well defined. We previously identified Pref-1 as a key negative regulator of adipogenesis by demonstrating that constitutive expression of Pref-1 inhibits, while downregulation of Pref-1 enhances, 3T3-L1 adipocyte differentiation(Smas and Sul, 1993 ; Smas et al., 1997). Pref-1 is also expressed in various embryonic tissues, and its expression is abolished in most tissues after birth except in preadipocytes, pancreatic beta cells, thymocytes, and cells in the adrenal glands(Carlsson et al., 1997 ; Halder et al., 1998 ; Smas and Sul, 1993). Recently, Pref-1 has been reported to be a paternally-expressed imprinted gene that is reciprocally expressed in relation to the maternally imprinted Gtl2(Schmidt et al, 2000). Paternal monoallelic expression of Pref-1 has also been observed, and the Pref-1 gene is localized at syntenic chromosomes, 12, 18, and 14 in mouse, sheep and human, respectively(Charlier et al., 2001 ; Kobayashi et al., 2000 ; Takada et al., 2000). Studies of imprinted genes often use uniparental disomy (UPD) mouse or human patients, who inherit both alleles of the gene from a single parent. Mouse models of UPD on chromosome 12, and human models of UPD on chromosome 14, suggest that imprinted genes in these regions are important for embryo survival and growth (Fokstuen et al., 1999 ; Georgiades et al., 1998 ;

Sutton et al., 2000 ; Georgiades et al. 2000 ; Jsutton et al., 2000). However, it is not clear whether the developmental defects in these UPD syndromes are due to Pref-1 or other imprinted genes in this region.

In the present report, we generated transgenic mice overexpressing the extracellular domain of Pref-1 as a soluble human immunoglobulin-γ constant region (hFc) fusion protein in adipose tissue. These transgenic mice showed perinatal lethality, growth retardation, bone malformation, and smaller sizes throughout the life span. The overall fat mass and average adipocyte cell size, as well as expression of adipocyte specific genes, was markedly decreased in transgenic mice, indicating that overexpression of Pref-1 causes an impairment of adipogenesis. Furthermore, these mice showed impaired glucose tolerance, hypertriglyceridemia, and reduced insulin sensitivity, a state similar to type II diabetes.

Materials and Methods

Transgene construct and generation of transgenic mice. The transgene construct was designed for adipose-specific expression of soluble preadipocyte factor -1/hFc fusion protein under the control of the proximal 5.4 kb of the mouse adipocyte lipid-binding protein promoter. To generate the soluble Pref-1/hFc fusion protein, the PCR product encoding the soluble form of Pref-1 (N-terminal 301 amino acids) with the Kozak sequence at the 5′ end and *Eag* I site at the 3′ end was ligated in frame to the RT-PCR product of the human IgGγ-1 heavy chain Fc region sequence encoding C-terminal 235 amino acids. The 7.4 kb transgene fragment was released by digestion with *Hind* III, excised from an agarose gel, and purified with a QIAquick gel extraction kit (Qiagen).

The DNA was microinjected into fertilized eggs of C57BL/6 x FVB mice to produce five founder lines of mice.

Western Blot Analysis. Three μl of serum from 10-week-old wild type and transgenic mice were subjected to electrophoresis through 10% SDS-PAGE and transferred onto nitrocellulose membrane. Pref-1 or Pref-1/hFc fusion protein was probed with a polyclonal rabbit anti-Pref-1 antibody (1:5,000) or anti-human IgG antibody conjugated with peroxidase (1:3,000) and an appropriate secondary antibody before detection using chemiluminescence. Twenty μg of protein isolated from 11-day old whole embryo extract were used for Western blot.

Bone staining. Embryos or mice were inviscerated and fixed in 90% ethanol for at least one week. For the staining of cartilages, samples were stained with 0.01% Alcian blue in 80% ethanol and 20% glacial acetic acid. Then, samples were rehydrated with series of ethanol, 70, 40 and 15% for 2 hours each. For bone staining, samples were incubated with Alizarin red in 1% KOH for 5 days. After several rinsing with 1% KOH, samples were stored in glycerol.

Histology. Renal fat tissues were fixed in Bouin' s fluid and then dehydrated with a series of alcohol washes. Samples were embedded in paraffin and sliced into 8 μm sections. Sections were stained with hematoxylin and eosin. Image capturing and adipocyte volume measurements were performed with NIH image software.

Northern blot analysis and RT-PCR. Total RNA from the tissues was prepared using TriZOL reagent (Invitrogen). Total RNA (15 μg) was subjected to electrophoresis through a 1% formaldehyde-agarose gel in 2.2 M formaldehyde, 20 mM MOPS, 1 mM EDTA, and transferred to nylon membrane (Hybond N, Amersham Pharmacia). After UV cross-linking, the membranes were hybridized with ^{32}P-labeled cDNA probes (aFABP, FAS and SCD-1) in ExpressHyb solution (Clontech). Total RNA from reproductive fat pads from 10 week-old mice was reverse transcribed with SuperScript II (Invitorgen). We amplified endogenous Pref-1 with primers (Forward: 5′-GCCA TCGTCTTTCTCAACAAGTG-3′; Reverse: 5′-GTAA GCATAGGCTTCACTCGATTC-3′) and β-actin with primers (Forward: 5′-TCCTATGTGGGTGACGAGGC-3′;

Reverse:5′-CATGGCTGGGGTGTTGAAGG-3′).

Glucose and insulin tolerance tests. Glucose tolerance tests were performed after an overnight fast. D glucose(2 ㎎/g of body weight) was intraperitoneally injected and glucose levels were monitored before injection and 30, 60, and 120 min after injection using Accu Chek glucometer (Roche). Insulin tolerance tests were performed on 10 12 week old mice following 5 hours fast. Animals were injected intraperitoneally with 0.5 U/kg body weight of insulin (Eli Lilly). Mouse tail blood samples were taken at time 0, 30, 60, 90 and 120 min after injection for measurement of blood glucose levels.

Measurements for Blood Triglycerides and Insulin levels. Serum triglycerides were analyzed by Triglyceride (INT) 10 (Sigma). Insulin levels after overnight fasting were measured by a Linco Rat RIA kit.

Results and Discussion

We generated transgenic mice overexpressing the extracellular domain of Pref-1 as a soluble human Fc fusion protein in adipose tissue under the control of the adipocyte lipid binding protein (aFABP/aP2) promoter. We choose the aFABP promoter because adipose tissue is the largest organ among tissues that express Pref 1 including pancreatic islets, adrenal glands and thymus(Carlsson et al., 1997 ; Kaneta et al., 2000). Multiple forms of transmembrane Pref 1 are generated by alternative splicing and glycosylation (Smas et al., 1994 ; Smas et al., 1997). In addition, proteolytic cleavage of the extracellular domain produces biologically active soluble forms of Pref 1(Smas et al., 1994 ; Smas et al., 1997). Here, we used the large soluble secreted form of Pref 1 fused to human hFc to provide long-range action through the circulation (Fig. 1B). Moreover, Pref 1 has been known to function as a dimer, and the Fc fusion could enhance its dimerization and bioactivity(Kaneta et al., 2000 ; Ohno et al., 2001)

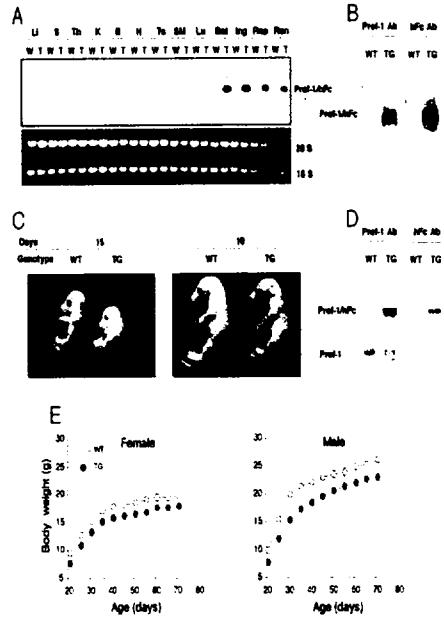

Fig. 1. Mice overexpressing Pref-1 are growth retarded. (A) Northern blot analysis for transgene expression in various tissues. Total mRNA was extracted from the tissues of a 10-week-old transgenic mice (T) and wild type littermates (W) and probed with Pref 1 cDNA. Li, liver; S, spleen; Th, thymus; K, kidney; H, heart; Ts, testis; SM, smooth muscle; Lu, lung; Bat, brown adipose tissue; Ing, inguinal fat pad; Rep, reproductive fat pad; Ren, renal fat pad. (B) Western blot analysis for Pref 1/hFc fusion protein in the serum (3 μl) of transgenic mice. The serum proteins were separated by SDS-PAGE and probed with either Pref-1 or human Fc antibodies. Both antibodies detect the same size (75 kDa) of Pref 1/hFc fusion protein in transgenic mice. The endogenous Pref 1 in serum is not detectable by Western blot. (C) The appearances of wild type and transgenic littermates are compared at E15 and E18, showing the growth retardation of transgenic embryos. (D) Total protein extracts from E11 embryos were subjected to Western blot analysis for Pref-1. Pref 1/hFc fusion protein is detected as a 75 kDa band only in

transgenic mice while multiforms of endogenous Pref-1 are detected at around 50 kDa in both wild type and transgenic mice. (E) Growth curves for male and female wild type (open circle) and transgenic (filled circle) mice fed a chow diet. Body weights of mice measured at five-day intervals are shown; each point represents mean ± standard error of mean from 6-13 mice. Body weights of Pref-1/hFC transgenic mice are significantly lower ($p<0.01$) than those of wild type mice at all ages.

Five transgenic founder lines expressing the transgene at various levels were analyzed. The Pref-1/hFc transgene transcript was exclusively detected in adipose tissues, including inguinal, reproductive, renal, and brown fat (Fig. 1A). We could easily detect the circulating Pref-1/hFc fusion protein in all five transgenic lines, but not in wild type mice, as expected. In this report, studies on the transgenic line with the highest expression level were described. Fewer than one-third of live born mice carried the transgene (32 %, n = 66), and the average litter size was smaller (5.1 ± 0.85, n = 14) compared to wild type (6.8 ± 0.32, n=19, $P < 0.01$), suggesting that Pref 1 overexpression causes perinatal lethality. Examination of E15 and E18 embryos showed that transgenic embryos had growth retardation and short stature (Fig. 1C). Surprisingly, Pref 1/hFc transgene expression was detected at E11, the first time point examined, and further increased during development (Fig. 1D). High Pref 1 transgene expression during early embryogenesis could have caused retardation in pre- and post-natal growth and development. At weaning age of three weeks, transgenic mice had lower body weights than their wild type littermates (Fig. 1E; for males, 7.8 ± 0.41g in transgenic mice, n=8, versus 9.9 ± 0.31g in wild type, n=12, $P < 0.01$; for females, 7.6 ± 0.52g in transgenic mice, n=7, versus 9.4 ± 0.23 g in wild type, n=13, $P < 0.01$). Transgenic mice remained undersized throughout life up to 10 weeks of age ($P < 0.01$). However, transgenic mice had proportionally larger sizes of organs examined including liver, lung, kidney, and thymus (Fig. 2A).

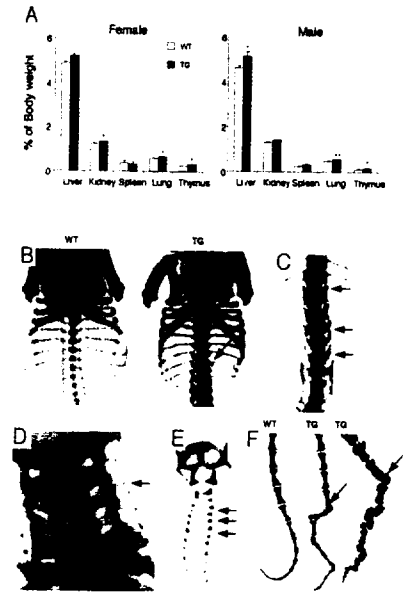

Fig. 2. Organ weights and bone staining. (A) Organ weights of 10 week old mice are presented as percentage of body weights (n = 8-10 per group). Statistically significant differences between the groups are indicated as *, $p < 0.05$ and **, $p < 0.01$. (B) Bone and cartilage staining. Embryos (E17) were stained with Alizarin red for bone and Alcian blue for cartilages. The Pref-1/hFc transgenic embryos had a smaller thoracic cavity with short ribs. Lower thoracic ribs are often fused as indicated by an arrow. (C) Ventral view of lumbar vertebral column. Vertebrae are fused and disorganized resulting in scoliosis. (D) The third cervical vertebra (c3) is branched and the fourth (c4) is a hemivertebra as indicated by arrows. (E) Ventral view of fused tracheal ring cartilages of the transgenic mice. (F) The transgenic mice had kinky tails with disorganized distal vertebrae.

Our transgenic mice also exhibit different skeletal abnormalities. Pref 1/hFc transgenic mice had a smaller thoracic cavity with a shorter circumference of the rib cage which has been reported in human pUPD14 and mouse pUPD12(Cotter et al., 1997 ; Georgiades et al., 2000) (Fig. 2B). The cervical, thoracic and lumbar vertebrae of the Pref 1/hFc transgenic mice were deformed and fused, resulting in scoliosis, and fusion of lower ribs was often observed (Fig. 2B, C, D). The Pref 1/hFc transgenic mice also had fused tracheal rings and developed short, kinky tails with malformation of the distal vertebra column (Fig. 2E, F).

Phenotypes in Pref 1/hFc transgenic mice clearly demonstrate that growth retardation and bone malformation phenotypes of human pUPD14 and mouse pUPD12 syndromes are due to Pref 1 overexpression.

We investigated whether overexpression of Pref 1 inhibits adipose tissue development since human mUPD14 is often associated with obesity(Berends et al., 1999 ; Manzoni et al., 2000) and our previous reports showed that Pref 1 inhibits differentiation of 3T3 L1 preadipocytes into adipocytes in vitro(Smas and Sul, 1993 ; Smas et al., 1994). The transgenic mice had smaller inguinal, renal, and reproductive fat pads at 10 weeks of age (in males, 33, 59 and 27%, respectively; in female 32, 33, and 42%, respectively, $P < 0.01$) (Fig. 3A). Degrees of decrease in fat pad weights were correlated with levels of transgene expression of the founder lines (in midlevel expresser line, 14 and 27 % reduction of inguinal and reproductive fat pad weights).

We further examined fat cell size and number, since decreased fat depot size can result from either hypotrophy or hypoplasia of adipose tissue or a combination of both. The DNA content of adipose tissue reflecting total cell number was not different between wild type and transgenic mice (data not shown). Histological staining of white adipose tissue of 10 week old mice showed that Pref 1/hFc transgenic mice contained a larger number of small cells in adipose tissue than their non transgenic littermates (Fig. 3B).

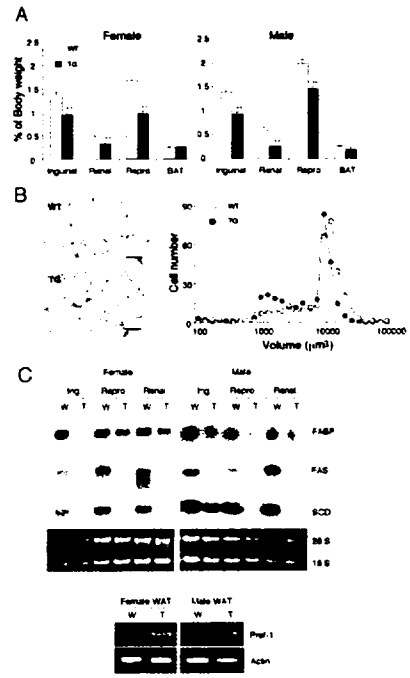

Fig. 3. Inhibition of adipogenesis by Pref 1 overexpression. (A) Fat depots from 10 week old mice are presented as percentage of body weights (n = 6 per group). Inguinal, inguinal fat pad; renal, renal fat pad; repro, epididymal or parametrial fat pad from male and female mice, respectively; BAT, brown adipose tissue. (B) Paraffin embedded sections of white adipose tissue (from renal fat pads) from 10-week old male mice were stained with hematoxylin and eosin. Scale bar, 50 μm. The volume of at least 300 cells per sample (mean of 4 mice per group) was determined with the NIH image software. The distribution of the volume is shown in the graph. (C) Northern blot analysis for adipocyte marker expression in adipose tissue. Total RNA from three different fat pads were probed with adipocyte marker cDNAs. Endogenous Pref 1 expression analyzed by RT-PCR. A primer set as shown in Methods was designed to amplify only the endogenous Pref-1 message. β actin was used as an internal control.

Moreover, northern blot analysis showed that Pref 1/hFc transgenic mice had decreased expression of the adipocyte markers, aFABP, FAS (fatty acid synthase), and SCD (stearoyl-CoA desaturase1) in the three different fat pads examined (Fig. 3C). In contrast, expression of endogenous Pref-1, a preadipocyte marker(Shimomura et al., 1998 ; Zhou et al., 1999), was increased in Pref 1/hFc transgenic mice as detected by RT-PCR (Fig. 3C). Our data clearly indicate that adipocyte differentiation in all regions of adipose tissue was impaired by Pref-1/hFc.

A muscle hypertrophy and lean phenotype of callipyge sheep are caused by overexpression of a number of paternally-imprinted genes including Pref 1 at the region homologous to mouse UPD12 and human UPD14(Charlier et al., 2001 ; Freking et al., 1998 ; Takada et al., 2000). Our transgenic mice overexpressing Pref 1 are lean, as in callipyge sheep, demonstrating the contribution of Pref-1 to callipyge phenotype. On the other hand, we did not observe muscle hypertrophy in Pref-1 transgenic mice as judged by carcass weight (data not shown). It is possible that another paternally imprinted gene in this region, *PEG11*, may be responsible for muscle hypertrophy observed in callipyge. This may be due to the fact that, unlike in rodents and humans, only a non-cleavable transmembrane form of Pref 1, Pref 1C, was reported to be present in a muscle specific manner in sheep(Charlier et al., 2001).

To determine if overexpression of Pref-1 and decreased fat mass in transgenic mice affect insulin and glucose homeostasis, we carried out insulin and glucose tolerance tests. Wild type and transgenic mice had similar basal levels of glucose after a 20-hour fast. Transgenic mice expressing the Pref 1/hFc showed higher levels of blood glucose when glucose tolerance tests were performed (Fig. 4A). Insulin tolerance tests showed that the transgenic mice had overt insulin insensitivity (Fig. 4B). Levels of insulin after 24 hours fasting were higher (0.22 ± 0.04 ng/ml in wild type versus 0.36 ± 0.02 ng/ml in transgenic

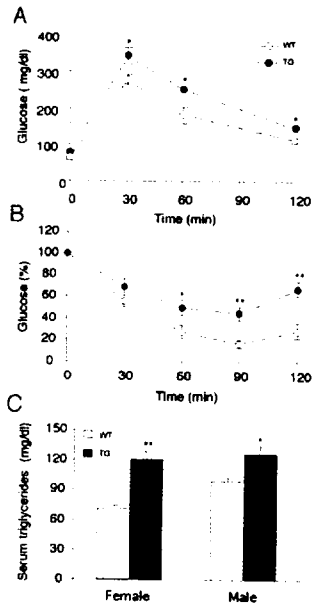

Fig. 4. Insulin resistance and hypertriglycemia in mice overexpressing Pref-1. (A) Glucose tolerance test. Overnight-fasted mice were given an intraperitoneal injection of glucose (2 mg/g body weight). Blood samples were collected from the tail at indicated time points and analyzed for glucose concentration. Results are means ± S.E.M. from six animals in each group. (B) Insulin tolerance test. Insulin (0.5 U/kg body weight) was intraperitoneally injected after fasting for five hours. Data are presented as mean percent of 0 min glucose value ± S.E.M. from six mice in each group. (C) Serum triglyceride levels in *ad libitum* fed 10 week old mice (n = 5 per group). Statistically significant differences between the groups are indicated as *, $p < 0.05$ and **, $p < 0.01$.

mice, n= 4 in each group, $P < 0.05$) in 8 to 10 week-old transgenic mice. We also measured serum triglyceride levels since insulin resistance, hyperglycemia, and hyperinsulinemia have all been associated with dyslipidemia(Bettenidge et al., 2000 ; Cullen et al., 1999). Plasma triglyceride levels (Fig. 4C) were higher in transgenic mice than in wild type littermates (for

males, 125.9 ± 8.06 mg/dl in transgenic mice, n=5, versus 98.3 ± 3.22 mg/dl in wild type, n=5, $P < 0.05$; for females, 120.1 ± 8.20 mg/dl in transgenic mice, n=5, versus 70.9 ± 3.95 mg/dl in wild type, n=5, $P < 0.01$). These data suggest that overexpression of Pref-1 promotes development of a state similar to type II diabetes. Recent studies showed that Pref-1 is expressed in islets of Langerhans of fetal, neonatal, and pregnant rats(Carlsson et al., 1997). Furthermore, growth hormone or prolactin-induced β-cell growth was correlated with increased Pref-1 expression, suggesting Pref-1 as a downstream mediator of prolactin on β-cell proliferation in gestational diabetes (Carlsson et al., 1997). In this context, it is possible that circulating Pref-1 in Pref-1/hFc transgenic mice could directly increase β-cell proliferation and insulin production. On the other hand, severe loss of fat mass in the transgenic mice has been reported to accompany hypertriglyceridemia and type II diabetes in severe lipoatrophic mouse models(Shimomara et al., 1998 ; Zhou et al., 1999). Insulin insensitivity in Pref-1/hFc transgenic mice, therefore, may be an indirect effect of decreased fat mass. It is interesting to examine whether paternal UPD14 patients would have diabetes as shown in Pref-1/hFc transgenic mice.

Our Pref-1 transgenic mice show the most of symptoms of biallelic expression of imprinted genes in the homologous regions in mouse pUPD12 and human pUPD14, establishing Pref-1 overexpresssion as a key determinant of mouse pUPD12 and human pUPD14 syndromes. The decreased fat mass and adipogenesis as well as type II diabetes observed in Pref-1 transgenic mice suggest pathophysiological implications of Pref-1 in obesity and diabetes. Because functional nullisomy in human mUPD14 is often associated with growth retardation, scoliosis and obesity, it would be interesting to find whether Pref-1 knockout mice would show similar phenotypes observed in mouse mUPD12 and human mUPD14.

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Abbreviations footnote : Pref-1(Preadipocyte factor-1), UPD (uniparental disomy), EGF (epidermal growth factor), PPAR (peroxisome proliferater activated receptor), C/EBP (CCAAT/enhancer binding protein), Gtl2 (Gene trap locus 2), aFABP (adipocyte fatty acid binding protein), FAS (fatty acid synthase), and SCD-1 (stearoyl-CoA desaturase-1)