Adipose tissue transplantation protects *ob/ob* mice from obesity, normalizes insulin sensitivity and restores fertility

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Abstract

Adipose tissue affects metabolism by secreting various adipokines. Lipodystrophic mice benefit both from leptin replacement therapy and from transplantation of normal fat. Leptin-deficient *Lep^{ob}/Lep^{ob}* (*ob/ob*) mice can also be treated with leptin. Surprisingly, there have been no reports of successful treatment of obese *ob/ob* mice by transplantation of normal white adipose tissue (WAT). If WAT transplantation is ineffective in treating insulin resistance and diabetes in obese individuals, its applicability may be limited in humans as such abnormalities are usually associated with obesity. In the current study, we tested whether WAT transplantation might prevent, and even reverse, abnormalities characteristic of *ob/ob* mice. To assess the preventive potential, 6-week-old *ob/ob* mice were transplanted, subcutaneously, with gonadal fat pads from normal mice. Profound effects on multiple physiological phenotypes were achieved despite leptin levels below 25% of those in control mice. WAT from one donor

reduced body weight gain, and WAT from 4 or 8 donors prevented obesity in *ob/ob* mice. Nonfasting insulin levels and insulin tolerance test were normalized. Corticosterone elevation was also prevented. Finally, WAT from 4 donors restored fertility in *ob/ob* females. The effects of WAT transplantation were long-lasting, with body weight gain suppressed for at least 40 weeks. To assess the therapeutic potential, obese 13-month-old *ob/ob* mice with a long history of leptin deficiency were used. Their body weight decreased by approximately 50% when transplanted with WAT from 8 donors. As in young recipients, transplantation greatly reduced nonfasting insulin, suggesting normalized insulin sensitivity. Thus, WAT transplantation was effective for both prevention and therapy. In the future, WAT transplantation may become a useful alternative to hormone replacement in treating not only lipodystrophy, but also certain types of obesity.

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Introduction

Leptin, a major hormone secreted by white adipose tissue (WAT) (Zhang *et al.* 1994), has been implicated in the regulation of body weight (Baskin *et al.* 2001, Mantzoros 2001), glucose metabolism (Fruhbeck & Salvador 2000, Al-Daghri *et al.* 2002, Ceddia *et al.* 2002) and fertility (Brann *et al.* 2002, Moschos *et al.* 2002). Leptin null, *Lep^{ob}/Lep^{ob}* mice are obese, hyperphagic and insulin resistant (Coleman 1978). Lipodystrophic mice, lacking WAT and therefore having very low leptin levels, are also hyperphagic, insulin resistant and diabetic (Gavrilova *et al.* 2000). Metabolic abnormalities of both *Lep^{ob}/Lep^{ob}* (Pellemounter *et al.* 1995, Harris *et al.* 1998) and lipodystrophic (Colombo *et al.* 2002) mice can be ameliorated by leptin treatment.

Leptin-deficient patients can also be successfully treated with recombinant leptin (Farooqi *et al.* 2002, Oral *et al.*

2002). However, as with insulin injections in diabetes, leptin injections in leptin deficiency are a psychological burden and may result in a suboptimal pattern of the hormone throughout the day. Thus, similarly to replacing insulin via islet transplantation, replacing leptin via WAT transplantation may offer a more physiological pattern of hormone release and obviate the need for a life-long regimen of injections. However, attempts to use WAT transplantation in animal models of leptin deficiency have given mixed results.

Transplantation of normal (Gavrilova *et al.* 2000), but not leptin-deficient (Colombo *et al.* 2002) WAT was successfully used for ameliorating metabolic abnormalities of lipodystrophic mice, indicating that leptin produced by normal WAT grafts is essential for treating lipodystrophic mice. Surprisingly, in contrast to lipodystrophic mice, there have been no reports of successful treatment of *Lep^{ob}/Lep^{ob}* mice by normal WAT transplantation. Early experiments

on WAT transplantation were unable to detect an improvement in metabolic and endocrine characteristics of these mice (Hausberger 1959, Ashwell *et al.* 1977).

The failure to demonstrate WAT graft function in *Lep^{ob}/Lep^{ob}*, in contrast to lipodystrophic recipients, might have resulted from their highly increased fat mass and, hence, a different pattern of signals involved in WAT survival and the regulation of WAT endocrine function. Increased adiposity of *Lep^{ob}/Lep^{ob}* mice might also have affected their leptin sensitivity. If confirmed, such failure of WAT transplantation to treat insulin resistance and diabetes in obese subjects could significantly limit the applicability of WAT transplantation for humans, in whom these metabolic abnormalities are usually associated with obesity (Astrup & Finer 2000). We, however, hypothesized that the early studies were unsuccessful because of technical reasons, including graft rejection (Hausberger 1959) and/or insufficient graft size (Ashwell *et al.* 1977) and that, if modified accordingly, WAT transplantation could be successfully applied for treating metabolic abnormalities in obese *Lep^{ob}/Lep^{ob}* mice.

In the current study, so as to avoid immune rejection, we transplanted congenic WAT, different from the recipient's tissue only by a narrow chromosomal region of the leptin gene, and in order to assure sufficient graft size, we transplanted gonadal fat pads from up to 8 donors into a single recipient. With these modifications, we tested whether leptin-producing WAT could prevent the development of hyperphagia, obesity, insulin resistance and infertility in *Lep^{ob}/Lep^{ob}* mice.

If WAT transplantation is ever to become useful for treating leptin deficiency, two important issues have to be addressed. The first is whether WAT transplants can function long enough to provide a clear alternative to regular injections and/or replaceable pumps. We addressed this issue by following the effects of WAT transplantation for up to 40 weeks. The second issue relevant to the human situation is whether subjects with a long history of leptin deficiency can still be successfully treated by WAT transplantation. We addressed this question by testing the effects of transplantation of WAT into *Lep^{ob}/Lep^{ob}* mice over one year of age.

Materials and Methods

Experimental animals and experimental groups

In the current study, we used female B6.V-*Lep^{ob}*/J mice purchased from The Jackson Laboratory (Bar Harbor, ME, USA). In this strain, the *Lep^{ob}* mutation was backcrossed to the C57BL/6J genetic background for more than 30 generations. Mice were either (1) obese, leptin-deficient *Lep^{ob}/Lep^{ob}*, referred to below as *ob/ob* mice, or (2) lean mice whose genotype was either *Lep^{ob}/+* or *+/+*, referred to in this study as *+/?*.

Most of the experiments in the current study were performed on two sets of mice, introduced into the experiment at 40 days and at 13 months of age. At 40 days of age, *ob/ob* mice just begin to develop obesity, while at 13 months of age, mice have been severely obese for about a year. For the fertility study in mice with advanced obesity, we used 75-day-old instead of 13-month-old *ob/ob* females, because at that age, even normal C57BL/6J females would, for the most part, be infertile. Thus, the young mice were used to test for the preventive effects of the WAT transplantation; the older mice were used to test for the therapeutic effects, i.e. for the ability of the normal WAT transplantation to reverse the *ob/ob* mouse abnormalities.

For each age, we had 6 groups with 4–6 mice in each: a lean *+/?* control group (*+/?*), 4 groups of *ob/ob* mice transplanted with WAT from 1, 2, 4 and 8 lean *+/?* donor mice (FT1, FT2, FT4, FT8), and a group of *ob/ob* mice transplanted with leptin-deficient WAT from *ob/ob* donors (FT0), with a transplant volume equal to the volume of WAT from 3 lean *+/?* donors.

All mice were housed in a specific pathogen-free facility. Animals were fed the NIH 31 diet with 4% fat which was available *ad libitum*, and were maintained on a 14 h light:10 h darkness cycle, with lights on at 0700 h and off at 2100 h. Animal studies followed the guidelines of The Jackson Laboratory and The American Association for Accreditation of Laboratory Animal Care.

WAT transplantation procedure

All donor mice (*+/?* and *ob/ob*) were 2–3 months of age. Their gonadal fat pads were harvested and immediately loaded in a syringe through the plunger end, so that warm ischemia time was less than 10 min. The average volume of WAT from one donor was approximately 0.4 ml. WAT from 1 to 8 donors was transplanted to a single recipient. Recipient mice were anesthetized with isoflurane (Aerrane, Baxter Pharmaceuticals Products Inc., Deerfield, IL, USA), and multiple injections of donors' WAT were made subcutaneously, using 16-gauge needles to minimize adipocyte damage during injections. Injection volume was kept between 100 and 150 µl per injection site. Thus, FT1 animals usually received 3–4 injections, while FT8 mice might have received up to 30 injections. The injections were made in the abdominal region first, and, if needed, additionally into the dorsal region.

For histological evaluation of transplanted tissue, grafts were removed 3–6 months after transplantation and fixed in 4% formalin in saline; 10-µm paraffin sections were stained with hematoxylin and eosin.

Determination of anti-leptin antibodies in mouse sera

Anti-leptin antibodies were measured by sandwich ELISA. Mice were bled and serum was collected and

diluted 1:1000 in PBS with 1% BSA (pH 7.4). For coating with the capture antibody, a Nunc Immuno Maxisorb plate (Nalge Nunc International, Rochester, NY, USA) was incubated for 2 h at room temperature with goat anti-mouse leptin antibody (AF498, R&D Systems, Minneapolis, MN, USA) at 1 µg/ml in PBS (pH 8.0). The wells of the plate were washed and recombinant mouse leptin (498-OB, R&D Systems), 1 µg/ml in PBS (pH 8.0), was added for 18 h at 4 °C. The plate was washed, blocked by 3% BSA in PBS for 30 min, and the pre-diluted serum samples were added (50 µl per well) for 2 h at room temperature. After washing the plates, 100 µl alkaline phosphatase-conjugated goat anti-mouse Ig (A3562, Sigma, St Louis, MO, USA) diluted 1:5000 in PBS/1% BSA were added for 1 h. Finally, p-nitrophenyl phosphate (Sigma, FAST N2770) was added and the optical density was read at 405 nm.

Body weight and food consumption measurements

Food consumption for each group was measured daily in 2 separate cages, with 2–3 mice in each, by weighing the food remaining in the food hopper.

Plasma hormone and metabolite measurements

To measure plasma leptin levels, we used the Quantikine M, Mouse Leptin ELISA kit (R&D Systems). This kit detected no more than 0.15 ng/ml of a cross-reacting substance in *ob/ob* mice.

Insulin was measured with Sensitive Rat Insulin RIA kit (Linco Research Inc., St Charles, MO, USA). Corticosterone was measured by an ImmuneChem double antibody corticosterone RIA ¹²⁵I kit for rats and mice from ICN Biomedicals, Inc. (Costa Mesa, CA, USA). Plasma glucose was determined with a colorimetric enzymatic kit (Trinder) from Sigma-Aldrich (St Louis, MO, USA).

For the insulin tolerance test, we used 2 U human insulin/kg i.p. (Humalog, Eli Lilly, Indianapolis, IN, USA) and measured blood glucose before, and 15, 30, and 60 min after insulin injection.

Estrous cyclicity measurements

Estrous cycle stages were determined from vaginal smears as described by Nelson and colleagues (1982). Briefly, smears were obtained daily between 1000 and 1300 h. The fire-polished tip of a Pasteur pipette was placed at the vaginal orifice, and a drop of saline was expelled into the vagina, aspirated back, and transferred to a microscope glass. Dry smears were fixed and stained in 2% Giemsa blood stain. Smears were examined microscopically at × 40 magnification and were classified into seven stages: diestrus/proestrus, proestrus, proestrus/estrus, estrus,

Figure 1 Morphology of WAT transplants. (A) Hematoxylin and eosin staining of a representative WAT transplant 3 months after transplantation. The transplant has normal WAT morphology. (B) A higher magnification of the selected region from panel A shows a blood vessel lumen filled with erythrocytes.

metestrus 1, metestrus 2, and diestrus. An estrous cycle was defined as the period between two successive proestrous smears.

Data analysis

Data are expressed as means ± standard error. To assess the effectiveness of WAT transplantation, we used the *t*-test to compare all transplanted groups with the FT0 group. To assess whether the transplantation resulted in a complete reversal of the *ob/ob* phenotype, we used the *t*-test to compare transplanted groups with the lean +/± group.

For body weight, food consumption, and plasma glucose, the analyses were carried out on untransformed data. Because the variance strongly correlated with the mean for insulin and corticosterone data, we applied the *t*-test to log-transformed values for these analyses. Average daily corticosterone levels were calculated by the trapezoid rule (<http://archives.math.utk.edu/visual.calculus/4/approx.1/index.html>) on the log-transformed values. Fertility data were analyzed by the Chi-square test.

Results

WAT graft histology, survival and immunogenicity

Histological evaluation of adipose tissue grafts 3 months after transplantation reveals that, while some fibroblasts are present, adipocytes are the predominant cell type with characteristic unilocular lipid droplet and eccentric nucleus (Fig. 1A). A significant degree of vascularization is present (Fig. 1B is a higher magnification of the area in the frame on Fig. 1A showing erythrocytes in the lumen of a venule).

Leptin-deficient *ob/ob* mice have never encountered leptin or leptin-derived peptides in their ontogeny. Thus, leptin-reacting clones of B- and T-lymphocytes in these mice might not be depleted during negative selection process in bone marrow and thymus. Therefore, *ob/ob*

Figure 2 Plasma leptin levels in *ob/ob* mice transplanted with WAT. Young (40-day-old) female *ob/ob* mice were transplanted with WAT from 1, 2, 4, or 8 female *+/?* donor mice. One group of *ob/ob* mice was also transplanted with WAT from *ob/ob* donors (FT0). The number of recipients was 5–6 per group. Individual leptin values, as well as means and standard error (S.E.) bars for each group, are shown.

mice might develop a strong immune response to leptin after WAT transplantation. We measured anti-leptin antibodies in sera of WAT recipients 9 months after transplantation. ELISA readings for anti-leptin antibodies in the groups transplanted with normal WAT, FT1 to FT8, were at the background level, which was defined as the levels in the FT0 and *+/?* groups in which no immune response to leptin was expected (data not shown).

WAT transplantation at 40 days of age

We transplanted WAT from 1, 2, 4, or 8 lean *+/?* donors into 40-day-old female *ob/ob* recipients (the FT1, FT2, FT4, and FT8 groups respectively). One group of *ob/ob* recipients was also transplanted with WAT from *ob/ob* mice (FT0 group). Figure 2 shows plasma leptin levels in *ob/ob* recipients 9–14 weeks after transplantation. All groups transplanted with *+/?* WAT had leptin levels significantly higher than the one transplanted with *ob/ob* WAT ($P < 0.001$; *t*-test). Recipients of WAT from 4 *+/?* donors had higher leptin levels than recipients of WAT from 1 donor ($P < 0.001$; *t*-test), and recipients of WAT from 8 donors had higher leptin levels than recipients of WAT from 1 ($P < 0.001$), 2 ($P < 0.001$) and 4 ($P < 0.01$) donors. While plasma leptin levels increased with the amount of transplanted WAT, even mice transplanted with WAT from 8 donors had leptin levels of 1.32 ± 0.04 ng/ml, i.e. only 20% of the 6.72 ± 1.30 ng/ml plasma levels found in lean, age-matched *+/?* control mice ($n = 6$).

Importantly, despite these relatively low leptin levels, the effect of transplantation on body weight was very pronounced and long-lasting. Figure 3A shows FT0 (center) and FT8 (right) mice 9 months after transplantation; both are compared with an age-matched *+/?* mouse (left). Even transplantation of WAT from a single donor

Figure 3 Effect of WAT transplantation on body weight and food consumption. (A) Representative photographs (from left to right) of a normal *+/?* mouse, an FT0 mouse and an FT8 mouse, approximately 9 months after transplantation. (B) Body weights of *ob/ob* mice transplanted with various amounts of normal WAT over the period of 9 months after transplantation. Also shown is body weight of age-matched *+/?* control mice (*+/?*). Data are shown as means $\pm$ S.E., $n = 5$ –6. At all time points, transplanted mice were significantly lighter than the FT0 mice, but heavier than *+/?* controls ($P < 0.05$). (C) Effect of WAT transplantation on food consumption 9–14 weeks after transplantation. Data are shown as means $\pm$ S.E., $n = 2$ (cages per group) * $P < 0.05$, significantly different from FT0; # $P < 0.05$, significantly different from *+/?*.

(FT1), resulting in leptin levels of less than 8% of those in the lean age-matched *+/?* mice, significantly decelerated body weight gain to about halfway between obese and normal (Fig. 3B). Moreover, WAT from 4 and 8 donors, creating plasma leptin levels of only 14% and 20% respectively of the normal, completely prevented body weight gain 16 weeks following transplantation – the period when even lean *+/?* mice, with leptin levels five- to sevenfold higher, were gaining weight. Sixteen weeks after transplantation, the FT4 mice weighed 35.2 ± 1.3 g and FT8 mice weighed 33.0 ± 0.6 g, only 5.5 and 3.3 g respectively more than lean *+/?* mice. At the same time, the differences in body weights between the FT4 and FT8 groups and the FT0 group were more than 30 g. Thirty-nine weeks after the transplantation, body weight of the FT4 group seemed to plateau at 39.8 ± 0.8 g; the FT8 group plateaued at 37.4 ± 0.9 g, i.e. much closer to 29.5 ± 0.6 g – the weight of lean *+/?* mice, than to 85.3 ± 1.5 g – the weight of the FT0 mice (Fig. 3B). Transplantation of *ob/ob* WAT did not have a significant effect on body weight and 8 weeks after transplantation, for example, FT0 and aged-matched intact *ob/ob* mice weighed 58.1 ± 0.7 g and 58.7 ± 1.0 g respectively.

Leptin-deficient *ob/ob* mice are hyperphagic. Nine to fourteen weeks after WAT transplantation, food consumption was substantially reduced (Fig. 3C). All mice, except the FT1 group, had a significantly lower food consumption than the FT0 group (* – significantly different from FT0 ($P < 0.05$; *t*-test)). The effect was so robust that it could be detected with food consumption measured only in 2 cages per group. Food consumption was not, however, fully normalized and the FT2 and the FT8 groups still had a significantly higher food consumption than lean *+/?* mice (# – significantly different from *+/?* ($P < 0.05$; *t*-test)).

The *ob* mutation on the C57BL/6J genetic background is characterized by *hyperinsulinemia* and *normoglycemia* (with only a transient *hyperglycemia* early in life (Coleman & Hummel 1973)). Thus, we tested whether WAT transplantation would prevent the development of hyperinsulinemia. Figure 4A illustrates that nonfasting insulin levels were brought to normal in the FT2, FT4, and FT8 groups by 9–14 weeks after the transplantation. Transplantation of WAT from even a single donor (FT1), resulting in less than 8% of normal plasma leptin, was sufficient to lower insulin levels by approximately fivefold in *ob/ob* mice, indicating that insulin sensitivity was improved. In contrast, insulin levels in *ob/ob* mice transplanted with *ob/ob* WAT were not different from those in age-matched intact *ob/ob* mice, 26.3 ± 8.0 ng/ml and 19.9 ± 2.3 ng/ml respectively, suggesting that the transplantation procedure itself did not have any long-lasting effect on insulin levels.

To confirm that insulin sensitivity was improved in WAT-transplanted mice, we performed insulin tolerance tests, 4 weeks after WAT transplantation, on a separate group of 14-week-old FT2 mice and compared the results with those obtained in age-matched FT0 (*ob/ob* mice

Figure 4 Effect of WAT transplantation on non-fasting plasma insulin levels and insulin sensitivity. (A) Non-fasting insulin levels in WAT-transplanted mice 9–14 weeks after transplantation and in age-matched *+/?* mice. The animals are the same as in Fig. 3B. Data are shown as means $\pm$ S.E., $n = 5$ –6. * $P < 0.05$, significantly different from FT0; # $P < 0.05$, significantly different from *+/?*. (B) An insulin tolerance test was administered 4 weeks after WAT transplantation to separate FT0 and FT2 groups, and to a *+/?* group (3–5 mice per group), when all animals were 14 weeks of age. At all time points after the injection, FT0 mice were significantly more insulin-resistant than both FT2 and *+/?* mice, whereas FT2 mice were not different from control *+/?* mice. Data are shown as means $\pm$ S.E. * $P < 0.05$, significantly different from FT0. Initial blood glucose levels in the FT0 mice, 235 ± 23 mg/dl, were significantly higher than those in FT2 and *+/?* mice, 142 ± 8 and 143 ± 10 mg/dl respectively.

transplanted with *ob/ob* WAT) and lean *+/?* mice. Plasma glucose decreased significantly in both FT2 and *+/?* mice after insulin, and their responses were not different, whereas FT0 mice were insulin resistant (Fig 4B, * – FT0 significantly different from FT2 and *+/?* ($P < 0.05$; *t*-test)).

Hypercorticism is an important feature of the *ob/ob* mouse phenotype (Naeser 1974, Dubuc *et al.* 1975). We measured the levels of the mouse major glucocorticoid, corticosterone, 7–9 months after transplantation. Because of the pronounced diurnal variation, corticosterone measurements were taken around the time of minimum, 0800 h, and around the time of peak, 1800 and 2100 h, concentrations. At all time points, corticosterone levels were significantly higher in the FT0 group than in lean, *+/?* mice and in any WAT-transplanted mice (data not shown). Average diurnal corticosterone levels showed a very similar response to WAT transplantation.

Figure 5 Effect of WAT transplantation on plasma corticosterone. Average daily corticosterone levels were calculated by the trapezoid rule. Data are shown as means $\pm$ S.E., $n=5-6$. * $P<0.05$, significantly different from FT0; # $P<0.05$, significantly different from +/?.

All transplanted groups had significantly lower corticosterone levels than the FT0 group, and none of the groups, except the FT2 group, differed in corticosterone levels from the +/?. mice (Fig. 5; * – significantly different from FT0 ($P<0.05$; t -test), # – significantly different from +/?. ($P<0.05$; t -test)).

Female *ob/ob* mice on the C57BL/6J genetic background are infertile (Chehab *et al.* 1996). We tested whether WAT transplantation could restore fertility in these mice. We assessed the presence of estrous cyclicity 7–8 months after transplantation. In the FT0 group, vaginal smears confirmed the lack of estrous cyclicity. In the FT1 group, the small amount of normal WAT was not sufficient to restore estrous cyclicity. In the FT2 group, only 3 out of 6 mice showed some signs of estrous activity, with an average cycle length of 5.9 ± 0.5 days. In the FT4 and FT8 groups, all mice showed estrous cyclicity (4.8 ± 0.3 and 5.5 ± 0.3 days respectively) which was not different from that of +/?. control animals (4.9 ± 0.4 days).

To confirm that estrous cyclicity was indicative of full reproductive competence, we mated a separate group of *ob/ob* mice, 6 weeks after they had been transplanted with WAT from 4 donors. Nine out of twelve transplanted mice became pregnant and produced pups when mated with proven breeder C57BL/6J males – results similar to those in lean +/?. mice where 11 out of 12 mice became pregnant (χ^2 , $P>0.05$). Litter size was slightly lower in FT mice than in +/?. mice, 5.9 ± 0.4 and 7.9 ± 0.7 pups respectively ($P<0.05$). It is noteworthy, however, that while pups from all newly found litters from +/?. dams had visible gastric milk content, pups in a half of the litters (4 out of 9) from the transplanted females had empty stomachs, seemingly due to the females' failure to lactate.

We also tested whether WAT transplantation could restore fertility in *ob/ob* mice that had already developed substantial obesity. For that study, we used 75-day-old female *ob/ob* mice with a body weight of 56.1 ± 0.9 g. The mice were transplanted with WAT from 4 lean +/?.

Figure 6 Plasma leptin levels in *ob/ob* mice transplanted with WAT. Mature (13-month-old) female *ob/ob* mice were transplanted with WAT from 1, 2, 4, or 8 young (2- to 3-month-old) female +/?. donor mice. The number of recipients was 3–4 per group. Individual leptin values, as well as means and S.E. bars for each group, are shown.

donors; 3 months later, when their body weight was 43.4 ± 1.0 g, they were tested for fertility. All 7 transplanted mice became pregnant and continued to produce litters for the next 5–7 months. Average first litter size was 6.6 ± 1.0 pups, not statistically different from 7.2 ± 1.1 pups in the age-matched +/?. mice. In this case, all first litters of transplanted mothers died because, seemingly, females did not lactate. It is noteworthy, however, that some of the consequent pregnancies resulted in a small number of pups (1–2) being weaned.

WAT transplantation at 13 months of age

To test whether late WAT transplantation may reverse the *ob/ob* phenotype, we transplanted WAT from 1, 2, 4, or 8 young lean, +/?. donors into 13-month-old *ob/ob* recipients (FT1, FT2, FT4, and FT8 groups respectively). Figure 6 shows plasma leptin levels in *ob/ob* recipients 10 weeks after transplantation. Groups transplanted with WAT from 2, 4, or 8 donors had leptin levels significantly higher than the group transplanted with WAT from 1 donor ($P<0.05$; t -test). While leptin levels increased with the amount of transplanted WAT (from 2 to 8 donors), the differences among FT2, FT4 and FT 8 groups were not statistically significant ($P>0.05$). Plasma leptin levels were similar to those achieved with the transplantation into young recipients (see Fig. 2). However, even mice transplanted with WAT from 8 donors had leptin levels of 1.32 ± 0.35 ng/ml, i.e. only 15% of 8.67 ± 2.57 ng/ml, the plasma levels found in lean, age-matched +/?. control mice.

Even these relatively low leptin levels had a profound effect on body weight (Fig. 7). Eighteen weeks after transplantation, the FT4 and FT8 mice lost 40% and 50% respectively of their initial body weight. In contrast to young animals, however, WAT from 1 donor (the FT1 group) was not sufficient to affect the body weight of middle-aged recipients.

Figure 7 Effect of WAT transplantation on body weight of middle-aged *ob/ob* mice. Body weights of WAT-transplanted mice are shown during the 18 weeks after transplantation. Data are shown as means $\pm$ S.E., $n=3-4$. Starting from 4 weeks after transplantation, the FT2, FT4, and FT8 groups weighed significantly less than the FT0 group ($P<0.05$).

WAT transplantation into mature obese mice completely restored normal insulin levels in the FT4 and FT8 groups 8 to 12 weeks after transplantation (Fig. 8, * – significantly different from FT0 ($P<0.05$; t -test), # – significantly different from +/? ($P<0.05$; t -test)). As in young animals, the transplantation procedure had little effect on insulin levels, which were 17.9 ± 8.0 ng/ml and 23.9 ± 5.9 ng/ml in FT0 and in intact *ob/ob* mice respectively.

Discussion

WAT transplantation may prove to be an effective way of providing leptin for leptin-deficient individuals. However, an apparent lack of positive effects of WAT transplantation on *Lep^{ob}/Lep^{ob}* (Hausberger 1959, Ashwell *et al.* 1977), in contrast to lipodystrophic (Gavrilova *et al.* 2000, Colombo *et al.* 2002) mice, raised the possibility that the

Figure 8 Effect of WAT transplantation on non-fasting plasma insulin. Non-fasting insulin levels in WAT-transplanted mice 8 to 12 weeks after transplantation and in age-matched +/? mice. The animals are the same as in Fig. 7 plus an age-matched +/? control group. Data are shown as means $\pm$ S.E., $n=3-4$. * $P<0.05$, significantly different from FT0; # $P<0.05$, significantly different from +/?.

procedure might not be effective for treating obesity, insulin resistance and diabetes in obese leptin-deficient mice. If WAT transplantation were indeed ineffective in treating metabolic abnormalities in obese subjects, it could significantly limit the usefulness of the procedure in humans.

The current study is the first to show that in *Lep^{ob}/Lep^{ob}* mice, WAT transplantation is effective as both a preventive and a curative measure. In young, 40-day-old animals, WAT transplantation prevented further development of obesity and mostly normalized food consumption. It also normalized insulin and corticosterone levels. Finally, in both 40-day-old mice with mild obesity and 75-day-old mice with substantial obesity, WAT transplantation led to fertility.

In 13-month-old mice weighing over 80 g, transplantation of as little as 2–3 ml normal WAT led to an up to two-fold reduction in body weight, and to the normalization of insulin levels.

Thus, WAT transplantation markedly improved, or even completely reversed, various aspects of the *Lep^{ob}/Lep^{ob}* phenotype. The ameliorating effect of WAT transplantation persisted for an extended period of at least 20 to 40 weeks. The procedure was effective in both young mice and mice with a long history of leptin deficiency. Finally, WAT transplantation had a pronounced effect on the *Lep^{ob}/Lep^{ob}* phenotype, despite relatively low leptin levels of only 10% to 20% of those in the +/? mice.

The molecular nature of the *Lep^{ob}/Lep^{ob}* phenotype remained unknown until the leptin gene was cloned (Zhang *et al.* 1994). The parabiosis study, in which a *Lep^{ob}/Lep^{ob}* partner of a lean animal lost weight, suggested that a missing circulating anorectic factor was the primary defect in the *Lep^{ob}/Lep^{ob}* mouse (Coleman 1973). However, the lack of the effect of grafted WAT on the *Lep^{ob}/Lep^{ob}* phenotype (Hausberger 1959) and a WAT cross-transplantation study (Ashwell *et al.* 1977), seemingly excluded WAT as a site of the primary defect. Most likely, previous attempts to detect the effect of WAT transplantation on the *Lep^{ob}/Lep^{ob}* recipient failed due either to immune rejection, when transplantation was attempted in genetically heterogeneous animals (Hausberger 1959), or to the small size of the graft (Ashwell *et al.* 1977).

In this study, for transplantation we used WAT from congenic mice, differing genetically from the *ob/ob* mouse only in having at least one normal copy of the leptin gene. Since transplantation of syngeneic, leptin-deficient WAT into *ob/ob* mice (the FT0 group) did not affect body weight, insulin levels and fertility as the transplantation of a similar volume of normal WAT did, the effects of transplantation should be attributed to leptin produced by normal adipocytes.

As leptin is a major adipostatic hormone, we expected the normal transplants to be stimulated through feedback

mechanisms eventually to support normal leptin levels. This, however, was not the case as even *ob/ob* mice transplanted with WAT from 8 lean +/- donors failed to achieve normal leptin levels. Several factors might have contributed to this apparent discrepancy. First, we might not have used an optimal WAT for transplantation. Even though plasma leptin levels were reported not to be different in +/- and *Lep^{ob}/+* mice (Chung *et al.* 1998), under the conditions of high leptin demand per unit of tissue, *Lep^{ob}/+* WAT might possibly not perform as well as +/- WAT. Also, despite seemingly normal morphology, transplant viability and transplantation site might not be optimal. Another contributing factor might be the transplants' limited capacity for growth. Finally, leptin may not be the only adipostatic hormone, and endogenous WAT, although leptin deficient, might have overproduced some other adipostatic hormones. To distinguish between these possibilities, different transplantation modalities should be attempted, including transplantation into leptin-deficient lipodystrophic mice.

Interestingly, the *Lep^{ob}/Lep^{ob}* phenotype was significantly ameliorated, or even reversed, at leptin levels several-fold lower than those found in lean +/- mice. These results may indicate either that even low leptin levels are sufficient for normal physiology or that mice never exposed to leptin have a very high sensitivity to the hormone. If the latter explanation were correct, elevated sensitivity to leptin would be expected to dissipate after an extended period of exposure to leptin. The effect of transplants on body weight, however, remained stable for as long as 9 months (Fig. 3B), thus arguing against this explanation. Low levels of transgenically expressed human leptin (supposedly from birth) have also been shown to normalize physiology in mice having no endogenous mouse leptin. Such mice were somewhat heavier than nontransgenic lean controls, but they were fertile and had normal insulin and corticosterone levels (Ioffe *et al.* 1998). While the above-mentioned observations support the notion that even low leptin levels are practically sufficient for normal physiology, we still cannot exclude a possibility that some developmental imprinting makes *Lep^{ob}/Lep^{ob}* mice permanently hypersensitive to leptin (Bouret *et al.* 2004).

The majority of abnormalities characteristic of *ob/ob* mice, such as extreme obesity and insulin resistance, were readily preventable in 40-day-old and reversible in 13-month-old mice. Similar, very low leptin levels (see Figs 2 and 6) were effective in restoring insulin sensitivity in young and in old *ob/ob* mice. This observation suggests that the lack of life-long exposure to leptin prevented the development of leptin resistance with age in *ob/ob* mice in contrast to some other rodents (Ma *et al.* 2002). The lack of leptin at early ages, up to 40 days, may, however, result in some permanent developmental defects, including compromised lactation.

A WAT transplantation technique similar to the one described in this study may be used for assessing the

relative contribution of adipocyte-derived endocrine factors to metabolic regulation. For example, it has been shown that in lipodystrophic mice, insulin resistance and hyperglycemia may be substantially alleviated by transplanting normal, but not leptin-deficient WAT (Gavrilova *et al.* 2000, Colombo *et al.* 2002). The technique may also be used for detecting novel adipocyte-derived endocrine factors, as has recently been reported for acyl CoA:diacylglycerol acyltransferase 1 knock-out mice (Chen *et al.* 2003).

Our results extend the applicability of WAT transplantation from treating lipodystrophy to preventing and treating the *Lep^{ob}/Lep^{ob}* syndrome. Thus, the excessive adiposity *per se* does not seem to have an adverse effect on the effectiveness of WAT transplantation to cure adipose-related endocrine abnormalities. WAT transplantation may, in the future, become a better option than a hormone replacement therapy because it provides the advantage of continuous hormone release without the disadvantages of daily injections and the repeated monitoring and adjusting of an exogenous hormone.

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