International Journal of Obesity (2009) 1–7
 0307-0565/09 $32.00
www.nature.com/ijo

TECHNICAL REPORT

Efficient method for obtaining Lep^{ob}/Lep^{ob}-derived animal models using adipose tissue transplantations

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Background: Leptin-deficient mice (Lep^{ob}/Lep^{ob}, also known as *ob/ob*) are of great importance for studies of obesity, diabetes and other correlated pathologies. Thus, generation of animals carrying the Lep^{ob} gene mutation as well as additional genomic modifications has been essential to associate genes with metabolic diseases. However, the infertility of Lep^{ob}/Lep^{ob} mice impairs this kind of breeding experiment.
Objective: To propose a new method for production of Lep^{ob}/Lep^{ob} animals and Lep^{ob}/Lep^{ob}-derived animal models by restoring the fertility of Lep^{ob}/Lep^{ob} mice in a stable way through white adipose tissue transplantations.
Methods: For this purpose, 1 g of inguinal adipose tissue from lean donors was used in subcutaneous transplantations of Lep^{ob}/Lep^{ob} animals and a crossing strategy was established to generate Lep^{ob}/Lep^{ob}-derived mice.
Results: The presented method reduced by four times the number of animals used to generate double transgenic models (from about 20 to 5 animals per double mutant produced) and minimized the number of genotyping steps (from 3 to 1 genotyping step, reducing the number of *Lep* gene genotyping assays from 83 to 6).
Conclusion: The application of the adipose transplantation technique drastically improves both the production of Lep^{ob}/Lep^{ob} animals and the generation of Lep^{ob}/Lep^{ob}-derived animal models.
International Journal of Obesity (2009) **0**, 000–000. doi:10.1038/ijo.2009.95

Keywords: Lep^{ob}/Lep^{ob}; *ob/ob*; adipose tissue transplantation; breeding; leptin; fertility

Introduction

Obesity is a growing public health problem determined by the interaction of numerous environmental and genetic factors.[1] Several genes and their products have been linked to adiposity.[2] Among these, leptin, the product of the obese gene (*Lep*) has a prominent role.[3] The leptin-deficient mice (Lep^{ob}/Lep^{ob}) exhibit hyperphagia, obesity, hyperglycaemia, insulin resistance and diabetes.[4] Thus, Lep^{ob}/Lep^{ob} animals represent an excellent and very well studied animal model for the study of obesity and obesity-related diseases, such as diabetes, hepatic steatosis and others.[5] In fact, the combination of genetically modified animals with the Lep^{ob}/Lep^{ob} mouse model has proven itself extremely useful for linking other genes with obesity.[6–9]

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Received 2 September 2008; revised 8 February 2009; accepted 17 April 2009

In a recent report, we were able to link kinins to the leptin-mediated control of food intake and energy expenditure by studying the kinin B1 receptor knockout mice on a Lep^{ob}/Lep^{ob} background.[6] Kinins may act through two distinct G-protein-coupled receptors named B1 and B210. Although the kinin B1 receptor is traditionally associated with inflammatory processes and recently to metabolism,[11–13], the kinin B2 receptor displays broader actions in the control of blood pressure and hemostasis.[10,14] In addition, the kinin B2 receptor has been linked to glucose homoeostasis, inducing glucose uptake in rat adipocytes in an insulin-dependent manner.[15] Furthermore, the kinin B2 receptor mRNA is less expressed in the adipose tissue of Lep^{ob}/Lep^{ob} mice.[16] All this motivated us to establish a fast method to obtain double transgenic mice deficient for both leptin and kinin B2 receptor.

Nevertheless, a considerable problem for the establishment of genetically modified strains on Lep^{ob}/Lep^{ob} background is the impaired hypothalamic-pituitary-gonadal axis function in leptin-deficient mice leading to a decrease of reproductive hormones and thus male and female infertility.[17] This is remarkably true in the case of C57BL/6

Journal: IJO ☐ Disk used Despatch Date: 16/5/2009
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mice, the most common genetic background strain used in experiments with the Lep^{ob}/Lep^{ob} mice. The infertility of the Lep^{ob}/Lep^{ob} mice makes breeding experiments to obtain obese mice with a second deficient gene both expensive and time consuming, as Lep^{ob}/Lep^{+} heterozygous animals have to be used for this purpose. Therefore, many crossing steps leading to several genotyping procedures have to be carried out using this traditional method to obtain the desired double homozygous animals.

Transplantation of white adipose tissue (WAT) from lean littermates is, however, known to acutely revert the obese phenotype of Lep^{ob}/Lep^{ob} mice.[18,19] Here, we show that WAT transplantations stably restore the fertility of Lep^{ob}/Lep^{ob} mice for at least 1 year. In addition, we propose the use of this simple method to facilitate the generation of Lep^{ob}/Lep^{ob} transgenic mice and the maintenance of Lep^{ob}/Lep^{ob} colonies. This allowed us, in a proof of principle experiment, to generate leptin and kinin B2 receptor double-deficient animals ($Lep^{ob}/Lep^{ob}Bdkrb2^{tm1Jfh}/Bdkrb2^{tm1Jfh}$) with four times less animals and with considerably less genotyping steps when compared with the conventional method.

Methods

Animals

Lep^{ob}/Lep^{ob}, Lep^{ob}/Lep^{+} and $Bdkrb2^{tm1Jfh}/Bdkrb2^{tm1Jfh}$ mice, referred to below as *ob/ob, OB/ob and B2−/−* respectively, all on C57BL/6j genetic background, were obtained from the Centre for the Development of Experimental Animal Models (CEDEME), Federal University of São Paulo (UNIFESP). Mice received commercial rodent food and tap water *ad libitum* (Nuvi Lab, Nuvital Paraná, Brazil) and were maintained under a 12-h light/dark cycle under constant room temperature (22 ± 1°C) and humidity (60 ± 3%) conditions.

Blood Samples

To extract blood sera, the blood of transplanted animals was collected (via venous puncture under anaesthesia with diethyl ether) after 12 h of fasting. Insulin and leptin were measured by commercial kits (Rat/mouse insulin ELISA Kit, Linco Research; Quantikine M, Mouse Leptin, R&D Systems).

Genotyping

Genomic DNA from tail fragments was obtained using the method described by Ren *et al*.[20] Genotyping of the *Lep* gene was performed via restriction fragment length polymorphism using the primer sequences 5′-CCCTGCTCCAGCAG CTGC-3′ and 5′-CATGATTCTTGGGAGCCTGG-3′ and the restriction enzyme *Dde*I. The kinin B2 receptor was genotyped through a tetra-primer PCR with the following oligonucleotides: 5′-TGCCCTCAGCGTGTTCTTCC-3′ (B2 receptor-sense); 5′-GGTCCTGAACACCAACATGG-3′ (B2 receptor anti-sense); 5′-CTTGGGTGGAGAGGCTATTC-3′ (neomycin cassette sense); 5′-AGGTGAGATGACAGGAG ATC-3′ (neomycin cassette anti-sense).

Fertility assay

To analyse the reproductive functionality of males, each male mouse was placed in a box with three females and the frequency of parturition as well as the number of pups of resulting litters was measured by visual inspection. To analyse the fertility of females, each female was placed with one fertile male; pregnancy, parturition and number of pups were verified weekly.

Adipose tissue transplantation

For transplantation, lean littermates (*OB/OB or OB/ob)* were killed and their adipose tissue removed and kept under sterile conditions in PBS. Three months old *ob/ob* receptor animals were anaesthetized with ketamine and xilasine (10 and 150 mg/kg^{-1} body weight, respectively), a dorsal trichotomy was performed, and after antisepsis, four WAT fragments (100–150 mg each) were then introduced subcutaneously through two little skin incisions (0.5 mm) in a total of eight transplantations. The time between the removal and the transplantation of the tissue was not longer than 10 min. The surgical incise was closed and benzylpenicillin and butorphanol were applied subcutaneously (50 000 IU kg^{-1}, and 5 mg kg^{-1}, respectively) following the surgical procedures.

Traditional method for generation and production of ob/ob*B2−/− mice*

Generation of *ob/ob*B2−/− mice (C57BL/6j Lep^{ob}/Lep^{ob} $Bdkrb2^{tm1Jfh}/Bdkrb2^{tm1Jfh}$) by the traditional method was done by crossing heterozygous *OB/ob* mice with homozygous B2−/− mice (Figure 1a). Selection of heterozygous *OB/ob* parents was done by genotyping. The B2−/− parents were obtained from homozygous breeding pairs. The product (F1) of this cross was genotyped in relation to the *Lep* gene. The double heterozygous animals were then crossed to obtain the F2 generation. According to Mendel's laws, the F2 animals may present nine different genotypes. Three quarters of these animals were lean and had to be genotyped to detect the Lep^{ob} allele and kinin B2 receptor gene alleles. Finally, to obtain the double mutant animal, lean animals heterozygous for the Lep^{ob} allele and homozygous for the kinin B2 receptor knockout (*OB/ob*B2−/−) were selected and mated with each other. The final crossbreeding of this method resulted in 25% obese and 75% lean animals. The lean animals had to be genotyped for the *Lep* gene to replace reproducers for colony maintenance and/or amplification.

Alternative method for ob/ob *mice production*

Restoring fertility by WAT transplantation permits the crossing of *ob/ob* animals to obtain obese animals in F1 in

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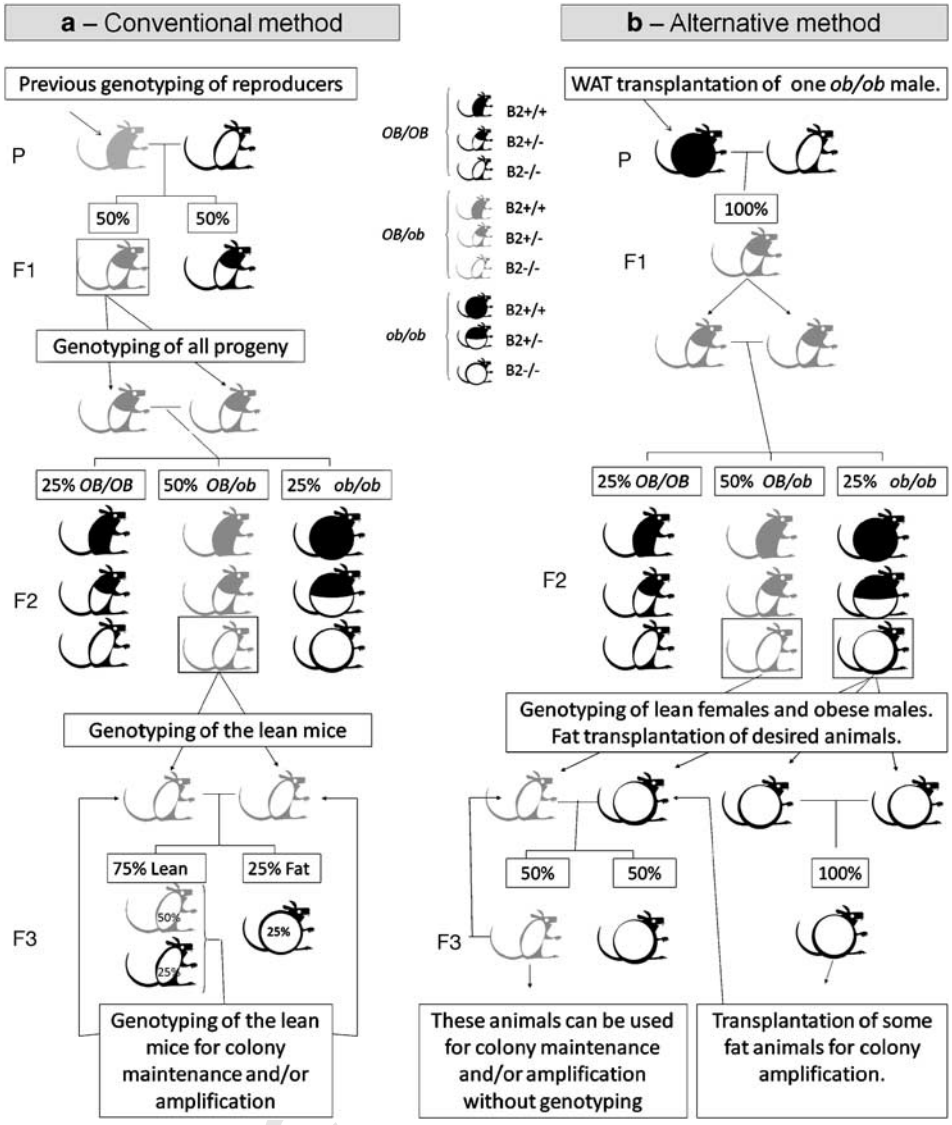

Figure 1 (**a**) Scheme of the conventional method for generation of *ob/ob*B2−/− mice. (**b**) Scheme of the alternative method for generation of *ob/ob*B2−/− mice using adipose tissue transplantations.

100% of cases. In a variant, transplanted homozygous males can be crossed with heterozygous lean females, generating 50% obese animals and 50% of lean heterozygous animals. Identification of the genotype of the progeny by phenotyping avoids any necessity of genotyping to select animals for experiments or colony maintenance and/or amplification.

Alternative method for generation and production of ob/ob*B2−/− mice*

Lep^{ob}/Lep^{ob} (*ob/ob*) male transplanted with WAT were crossed with kinin B2 receptor knockout females (Figure 1b). In the F1, all resulting animals were double heterozygous (*OB/ob*B2+/−) exempting genotyping in the next step. The F2 progeny generated all nine different possible genotypes. For further crosses, we selected the desired animals (*OB/ob*B2−/− females and *ob/ob*B2−/− males and females) genotyping obese animals for the kinin B2 receptor gene and lean females for both genes. Two double mutant males were transplanted with WAT and crossed with some selected females to optimize the crossing scheme. After this step, genotyping was not necessary anymore because all animals in the F3 progeny presented only two genotypes (*OB/ob*B2−/− and *ob/ob*B2−/−) easily distinguishable by their appearance. Some homozygous females were transplanted to improve the production of double mutant mice by crossing two homozygous parents that thus generated offspring with only obese pups.

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Table 1 Results of the traditional ob/obB2−/− crossing scheme (without WAT transplantation)

	P	*F1*	*F2*	*F3*	*Total*
Total animals	6♂ 9	28♂(32) 31♀(32)	44♂(50) 51♀(50)	25♂(25) 23♀(25)	217 animals
No. of selected animals	3♂ *OB/ob*B2+/+(4) 9♀ *OB/OB*B2−/−(9)	7♂ *OB/ob*B2+/− (14) 14♀ *OB/ob*B2+/−(15)	4♂ *OB/ob*B2−/−(6) 7♀ *OB/ob*B2−/−(6)	5♂ *ob/ob*B2−/−(6) 6♀ *ob/ob*B2−/−(6) 4♂ *OB/ob*B2−/−(12) 10♀ *OB/ob*B2−/−(11)	11 obese 5♂+6♀
No. of genotypings	6♂ *Lep* gene	12♂ *Lep* gene 31♀ *Lep* gene	7♂ *Lep* gene 10♀ *Lep* gene 24♂ B2R gene 37♀ B2R gene	10♂ *Lep* gene[a] 17♀ *Lep* gene[a]	93 *Lep* gene 61 B2R gene

[a]Necessary only for maintenance and/or amplification of colony. The number of expected animals is presented in parentheses.

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research. All procedures were approved by the Ethics Committee of the University of Mogi das Cruzes (CEMEA/UMC 02/08).

Results

To produce *ob/ob* mice using the conventional method, the use of heterozygous animals is mandatory. To obtain these mice, however, the Lep^{ob} mutation has to be detected by genotyping. If a double mutant animal is desired, the additional mutant allele has to be identified using additional genotyping procedures. Employing this traditional method, we generated *ob/ob*B2−/− knockout mice (Figure 1a and Table 1). For this purpose, three male *OB/ob*B2+/+ and nine female *OB/OB*B2−/− were selected as progenitors (P) after genotyping. Resulting double heterozygous animals (*OB/ob*B2+/−) animals in F1 were then inbred after genotyping. In F2, all lean animals were genotyped for the kinin B2 receptor gene and the selected knockout animals for the kinin B2 receptor gene were additionally genotyped for the *Lep* gene. Thus, four male and seven female *OB/ob*B2−/− were selected for a new crossing step. In total, 217 animals were used to obtain 11 *ob/ob*B2−/− mutant animals. Furthermore, 83 genotyping reactions for the 1*Lep* gene and 61 genotyping reactions for the kinin B2 receptor gene were employed to obtain the desired animals (Table 1).

To accelerate both production of *ob/ob* mice and the crossing of mice to obtain double homozygous *ob/ob*B2−/− animals, we proposed an alternative method by restoring the fertility of *ob/ob* animals by transplanting 1 g of inguinal WAT from lean littermates. This operation is very simple and rapid, and consists in introducing under sterile conditions, small fragments of WAT subcutaneously into the receptor mice through two small incisions in their skin.

After surgery, leptin started to be detected in the serum (Figure 2a) and all animals quickly lost weight in comparison to sham controls (Figure 2b). In addition, blood insulin levels were normalized after 60 days (Figure 2c). All 10 *ob/ob* males transplanted with WAT had their fertility completely restored, being able to fertilize different lean females (Figure 2d). All fertilized females had normal parturitions almost every month, and the resulting litters and pups had normal sizes (Figure 2e). Most transplanted males maintained their fertility for at least 1 year after surgery (Figure 2f).

To restore fertility in females, we transplanted eight female *ob/ob* mice with 1 g of WAT each. Most females transplanted had normal reproductive life with monthly parturitions, with an average of eight pups per litter. However, a few females exhibited cannibalistic behaviour mostly during their first litter. Furthermore, one mouse had four litters and then stopped reproducing, and another displayed tissue rejection of the grafted WAT and was unfertile.

In a model experiment, restored fertility of *ob/ob* mice was used to accelerate *ob/ob* animal production, as well as the production of a double transgenic animal, *ob/ob*B2−/−. The general scheme for this experiment is shown in Figure 1b. Three weeks after surgery, a transplanted *ob/ob*B2+/+ male was crossed with three *OB/OB*B2−/− females. The resulting heterozygous *OB/ob*B2+/− mice from F1 were inbred without genotyping to produce 74 animals with diverse genotypes. All 36 females from this generation were genotyped for the B2 receptor gene, revealing two double mutant (*ob/ob*B2−/−) and six lean B2−/−. All six lean females were genotyped for the *Lep* gene, resulting in four heterozygous *OBob*B2−/− females. Nine obese males were genotyped for the B2 receptor gene, showing two double mutant males (*ob/ob*B2−/−).

A combined strategy was then used to maximize the number of obese animals in F3. Both double mutant females and males were transplanted with WAT from lean siblings of the same generation and each transplanted male was paired with one transplanted female and two of the *OB/ob*B2−/− females. In the progeny, 14 males and 13 females were double mutants. Furthermore, five male and six female animals were *OB/ob*B2−/− in this generation.

In this experiment, a total of 136 animals were used to obtain 27 *ob/ob*B2−/− double mutant animals. Only 48

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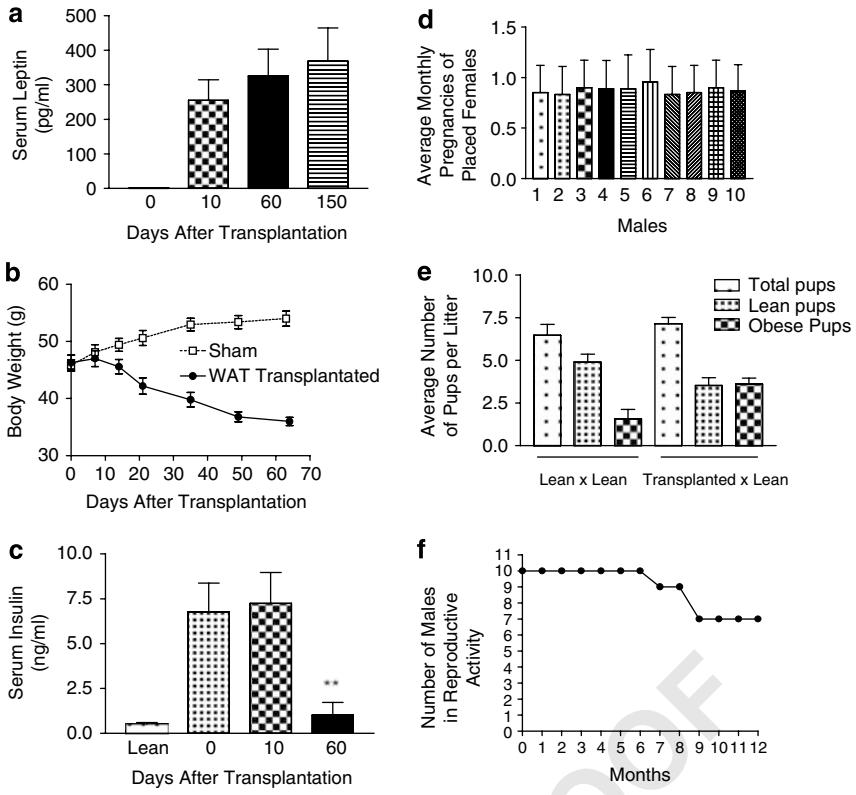

Figure 2 Phenotype alterations after WAT transplantation of male *ob/ob* mice. (**a**) Serum leptin normalization of WAT-transplanted animals. (**b**) Body weight loss. (**c**) Serum insulin normalization. (**d**) Number of average monthly pregnancies per female over a 5- to 6-month period after placing WAT-transplanted males (animals 1 to 10) with three females each. (**e**) Comparison of litter sizes between transplanted and lean animals. (**f**) Stability of restored male fertility. Number of males in reproductive activity (in a group of 10 transplanted animals) was measured during 12 months.

Table 2 Results of alternative *ob/ob*B2−/− crossing scheme with WAT transplantation

	P	*F1*	*F2*	*F3*	*Total*
Total animals	1♂ 3♀	9♂(10) 11♀(10)	38♂(39) 36♀(39)	19♂(21) 19♀(21)	136 animals
No. of selected animals	1♂ *ob/ob*B2+/+ 3♀ *OB/OB*B2−/−	9♂ *OB/ob*B2+/−(10) 11♀ *OB/ob*B2+/−(10)	2♂ *ob/ob*B2−/−(2) 2♀ *ob/ob*B2−/−(2) 4♀ *OB/ob*B2−/−(5)	14♂ *ob/ob*B2−/−(14) 13♀ *ob/ob*B2−/−(14) 5♂ *OB/ob*B2−/−(7) 6♀ *OB/ob*B2−/−(7)	27 obese 14♂+13♀
No. of genotypings	0	0	9♂B2R gene 36♀B2R gene 6♀ *Lep* gene	0	6 *Lep* gene 45 B2R gene
No. of transplantations	1♂	0	2♂ 2♀	7♂[a] 6♀[a]	10♂ 8♀

[a]Necessary only for maintenance and/or amplification of colony. The number of expected animals is presented in parentheses.

genotyping reactions for the B2 kinin receptor gene and six for the *Lep*(Table 2) procedures were performed.

Discussion

In this study, we propose an alternative method for the production of *ob/ob* animals and the generation of experimental animal models to study obesity by crossing *ob/ob* mice with other genetically modified mice.

To compare the conventional method to the method reported here, *ob/ob*B2−/− double mutant mice were generated. According to our results, the WAT transplantation protocol turned to be easier, cheaper and more efficient than the traditional procedure. Several aspects presented here support this conclusion:

(1) The initial genotyping of the P-generation to detect *OB/ob* lean parents is not needed, as obese homozygous animals were used for crossing.

(2) WAT transplantation of *ob/ob* animals leads to 100% heterozygous (*OB/ob*B2+/−) offspring in the F1 generation,

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eliminating again the necessity of genotyping animals. In the traditional method only half of the animals of F1 are heterozygous. We were thus able to obtain more double heterozygous animals for a second crossing, obviously increasing the frequency of double mutants in F2.

(3) Breeding of double heterozygous animals leads to all possible phenotypes in F2. However, using our method, only the lean females were genotyped for the *Lep* gene for subsequent breeding. The *ob/ob* males were phenotypically selected and transplanted. In fact, genotyping the F2 generation for the *Lep* gene is not necessary. Two methods can be used to generate double-deficient mice without *Lep* gene genotyping. In one variant, both male and female obese mice in F2 are selected and transplanted (leading to 100% double transgenic mice in F3). In another variant, homozygous transplanted male mice are crossed with lean females of F2 and the phenotype of their pups is used to elucidate the genotype of the mother.

(4) Use of transplanted obese males leads to 50% obese mice in the F3 generation, instead of 25% as in the traditional method. In addition, all lean mice in this generation are homozygous for the B2 kinin receptor knockout allele and heterozygous for the Lep^{ob} gene mutation. These animals are not only the preferential lean controls for experimental purposes, but can also be used for the reposition of female reproducers for colony maintenance and/or amplification without any genotyping steps.

(5) The total number of animals used in our method is by far smaller than in the traditional method. Using two transplanted obese females and four heterozygous lean females in F2, a total of 136 animals were used to obtain 27 double-mutant animals in F3 (five animals per double mutant). Compared with that, using the traditional method, we needed 217 animals to obtain 11 double-mutant obese animals (about 20 animals per double mutant).

The method suggested here is especially efficient when isogenic animals are used to generate *ob/ob* mice. However, our method brings a unique advantage in comparison to the traditional method when the animals to be crossed with the *ob/ob* mice are on a different background. In this case, when the use of littermates as experimental controls is mandatory, the conventional method yields not more than 6.25% double mutant mice and their respective controls in the F2 generation. Our method allows the transplantation and breeding of obese heterozygous, which result in up to 25% double-mutant mice in the F3 generation, in addition to all the necessary littermate controls.

When compared with other alternative methods of restoring fertility of *ob/ob* mice, like backcrossing to the BALB/cj background or injections of leptin,[21–23] the stability of restored male fertility and the simplicity/efficiency of our method considerably facilitates this task.

One of our concerns during the design of the protocol proposed here was immune rejection. For example, a graft from males into females could not be accepted because female B and T cells are capable of recognizing proteins derived from the mouse male H-Y epitope (minor histocompatibility antigene).[24] It is also important to note that leptin mutation did not arise in C57BL/6 but rather was backcrossed in this strain, thus elevating the risk of rejection of a graft from *OB/ob* heterozygote mice into *ob/ob* mice. We therefore used congenic animals from the same gender and injected a sufficient volume of WAT to avoid immune rejection and its effects.[18,19] However, some animals gradually became infertile (Figure 2f), which could be associated to increased graft rejection. Even though, our data showed that infertility occurred in a small percentage of the transplanted animals and thus does not compromise the advantages observed in the method.

It must be also pointed out that the chromosomal position of the target mutations to be combined with the Lep^{ob} allele is an issue to be observed, when applying this method. If the second gene mutation is on the same chromosome of the *Lep* gene (chromosome 6 in the mouse), obtaining double mutants by crossings becomes extremely difficult as they may segregate together. In this situation, the desired animals result only from cromosomal crossing over, and thus the probability of success of the method is directly correlated to the distance between the two genes on the same chromosome.

In conclusion, we were able to show that WAT transplantation stably rescues the infertility of *ob/ob* mice for a period of at least 1 year. This could be used to facilitate the generation of *ob/ob* mice with an additional mutation and thus reduce costs, labour and the number of killed animals used in *ob/ob* colony generation and maintenance.

Acknowledgements

We thank MM Oliveira (Universidade de Mogi das Cruzes) and JB Pesquero (UNIFESP, Brazil) for continuing support. This study was supported by grants from FAPESP (06/59081-6), CNPq and CAPES (PROCAD-0216/05-4).

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