Characterization of a novel genetically obese mouse model demonstrating early onset hyperphagia and hyperleptinemia

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OBESITY IS CAUSED BY A DISTURBANCE in energy balance, such as an excessive energy intake or reduced energy expenditure. Many epidemiological studies of obesity have suggested the existence of a link between obesity and symptoms of metabolic syndrome, such as hyperglycemia, hypertension, and hyperlipidemia. These studies have also demonstrated that obesity is a critical risk factor for type 2 diabetes, stroke, and myocardial infarction (2, 6, 17, 28).

Studies examining the mechanisms that contribute to the development of obesity have yielded a large amount of data by drawing comparisons between obese and normal animals. In particular, genetically obese mice produced by gene manipulation or spontaneous mutation have been widely employed as model animals in such studies. To date, agouti yellow, fat (fat/fat), tubby (tub/tub), obese (ob/ob), and diabetic (db/db) mice have been widely studied as monogenic obese mice. The mutation in agouti yellow mice is located in the region of chromosome 2 that encodes the agouti protein. Although homozygous mutation is lethal, heterozygous mutation results in systemic distribution of the agouti protein, which is normally expressed only in skin cells. In obese agouti yellow mice, the agouti protein is expressed ectopically (even in the central nervous system) and antagonizes the anorexigenic effect of α-melanocyte-stimulating hormone (α-MSH) through the melanocortin 4 receptor (MC4R), which results in hyperphagia and obesity (3, 4). The mutation in fat/fat mice is located in the region of chromosome 8 that encodes carboxypeptidase-E (CPE), and this mutation leads to decreased CPE activity and subsequently to elevated levels of the orexigenic peptide melanin. Additionally, because CPE is required for the anorexigenic effect of α-MSH, this effect is decreased in fat/fat mice, and hyperphagia subsequently leads to severe obesity in these mice (4, 10, 39). The mutation in tub/tub mice is located in the region of chromosome 7 that encodes the tubby protein. The tub/tub mouse demonstrates hypophagia despite the existence of obesity, even in heterozygous animals, due to a reduction of spontaneous physical activity and carbohydrate metabolism (4, 10, 13, 25, 51). The mutation in ob/ob mice is located in the region of chromosome 6 that encodes leptin. This mutation induces hyperphagia and severe obesity at an earlier age compared with other obese mouse strains (57). The db/db mouse has a similar phenotype to the ob/ob mouse because the mutation in this mouse affects the leptin receptor gene (5). In addition to these monogenic obese mice, several mouse strains with polygenic obesity, such as the New Zealand obese mouse, have been studied to investigate the mechanisms associated with the development of obesity (23, 44).

Although these genetically obese mice have unique phenotypes, each of these strains (49) as well as mice with diet-induced obesity (22) demonstrate high leptin levels, which indicates the importance of leptin in the manifestation of obesity. Leptin is a 16-kDa adipokine that is secreted mainly by white adipose tissue (WAT) (18). In the central nervous system, leptin activates anorexigenic pro-opiomelanocortin (POMC) neurons and inhibits orexigenic neuropeptide Y (NPY)/agouti-related protein (Agrp) neurons, which results in decreased appetite (46). Additionally, leptin increases energy expenditure by upregulating uncoupling protein 1 (UCP1) expression in brown adipose tissue (BAT) via the activation of the sympathetic nervous system (11). However, despite this antagonistic action of leptin on obesity, leptin is increased in obese individuals. Furthermore, the administration of leptin to obese individuals has no apparent effect, although one very rare form of obesity is induced by the absence of leptin. For these reasons, it is commonly recognized that leptin resistance...
is accompanied by general obesity (18), and many studies have indicated the importance of leptin resistance in the development of obesity. Currently, there are two convincing models of leptin resistance (26). The first model states that leptin resistance is caused by elevated plasma leptin, which induces chronic stimulation of the receptor and the activation of negative feedback pathways to block further leptin signaling. The evidence supporting this model is that leptin can stimulate the expression of suppressor of cytokine signaling-3 (SOCS-3), which directly inhibits leptin signaling, and that the attenuation of SOCS-3 in neurons enhances leptin sensitivity (1, 27). The second model states that dietary fats are responsible for leptin resistance, whereby fats either block leptin signaling directly or activate endoplasmic reticulum stress and inflammation, which can cause impairments in leptin reactivity in neurons (24, 36, 41, 52).

In this study, we screened mice that showed naturally occurring obesity when fed a standard diet from an early stage of life because we thought that another obese mouse model was needed to study the cause of human obesity. We report the development of a novel obese mouse strain with a monogenic mutation and present a detailed characterization of its metabolic and molecular features. We named this mouse Daruma (USA Patent No.: US7,847,147 B2) after the rounded appearance of a well-known traditional Japanese bibelot. The main cause of obesity in this strain is the early onset of leptin resistance. Moreover, our data strongly suggest that the Daruma mouse could be a useful animal model for studying the mechanisms of leptin resistance and the conditions associated with human metabolic syndrome, including type II diabetes, which is similar to the use of other mouse models of monogenic and polygenic obesity.

MATERIALS AND METHODS

Animals. All mice were maintained under constant temperature (21–22°C) and 14:10-h light-dark lighting conditions (lights on 0500–1900) with ad libitum access to food and water. In a screen of 13,000 outbred ICR mice, a male mouse that was termed Daruma developed severe obesity from a young age. To breed mice with this trait, the male Daruma mouse was mated with intact females that were maintained in the same colony. Their littersmates were then used for brother-sister or backcross mating to fix the phenotype. Generations four to eight were used for the experiments because the fourth-generation animals exhibited fixed phenotypes at a Mendelian ratio. Mice with body weights exceeding 60 g at 19 wk of age or those with plasma leptin concentrations exceeding 10 ng/ml at 3 wk of age were designated Daruma mice, and the other mice were assigned to the nonobese control group. For the crossbreeding study, obese (ob/ob) mice and diabetic (db/db) mice were obtained from Charles River Laboratories Japan (Yokohama, Japan), and tubby (tub/tub) and fat (fak/fat) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). In all biochemical and molecular biology experiments, we used male mice whenever possible to avoid any influence of sex differences on each parameter. All procedures were performed in accordance with the Japanese Physiological Society’s guidelines for animal care. The authorization number issued by Miyi University Animal Experiment Committee for this research was 2006-051-5.

Growth curves and computed tomography scans for body fat assessment. Body weight was measured weekly from 3 to 23 wk of age to obtain growth curves. X-ray computed tomography (CT) was performed using a LaTheta machine (Aloka System Engineering, Tokyo, Japan) on Daruma and control mice at 15 wk of age following the intraperitoneal injection of pentobarbital sodium (0.75 mg/10 g body wt) for anesthesia.

Histological examination. The histological analyses were performed on Daruma and control mice at 15 wk of age. The liver, pancreas, kidney, WAT, and BAT were removed after decapsulation. These tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 7 days and then transferred to 0.1 M PBS. The tissues were paraffin-embedded, cut into 10-μm thick sections, and stained with hematoxylin and eosin.

Leptin receptor DNA sequence. The leptin receptor sequence was compared between Daruma and wild-type mice using RNA samples from the hypothalamus. Total RNA was extracted from each tissue using an RNasy Micro kit (Qiagen, Tokyo, Japan) and then synthesized into first-strand cDNA using SuperScript III RNase H reverse transcriptase (Invitrogen Japan, Tokyo, Japan). The mouse leptin receptor-specific primers were designed according to the National Center for Biotechnology Information genetic database (accession no. NM_146146). The DNA sequencing reactions were performed using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), and the samples were electrophoresed using an ABI PRISM 3100 Genetic Analyzer (PE Applied Biosystems, Foster City, CA).

Measurement of food intake, plasma triglyceride, total cholesterol, high-density lipoprotein, aminotransferase, alanine aminotransferase, and free fatty acid. Twenty-four-hour food intake was measured in male Daruma and control mice at the ages of 4, 5, 9, and 19 wk. To examine liver function and potential hyperlipidemia, blood samples were collected from Daruma and control mice following decapsulation at the ages of 4, 5, and 9 wk, and these animals had been fasted for 12 h before sampling. Plasma was isolated from the blood samples and stored at −20°C before analysis. From the plasma samples, 10 μl were used to measure the levels of aspartate triglyceride (TG), total cholesterol, alanine aminotransferase (ALT), and aminotransferase (AST). To measure high-density lipoprotein (HDL) cholesterol, a portion of the blood was maintained at 4°C for 20 min. Plasma was obtained from the supernatant and then mixed with fractional liquid for HDL cholesterol, and the samples were then placed at 20°C for 10 min and centrifuged at 3,000 g for 10 min at 20°C. A 10-μl aliquot of the supernatant was used for each sample. All analyses, excluding the free fatty acid (FFA) measurements, were performed using a DRI-CHRM3500V (Fuji Medical Systems, Tokyo, Japan), FFA was measured using an enzyme method and performed by the Mitsubishi Chemical Medience.

Measurement of heart rate, blood pressure, and activity. Heart rate and blood pressure were measured noninvasively using a BP-98A instrument (Softron, Tokyo, Japan). The procedures were performed in conscious mice. Locomotor activity and running activity were measured in individual mice using a passive infrared sensor (Muro-machi, Tokyo, Japan) and a running wheel (Muromach), respectively.

Measurement of plasma glucose, insulin, leptin, and ghrelin. Plasma glucose was measured using a DRI-CHRM3500V instrument (Fuji Medical Systems), and plasma insulin, leptin, and ghrelin were measured using a mouse insulin ELISA kit (Shibayagi, Gunma, Japan), a mouse leptin ELISA kit (Morinaga, Kanagawa, Japan), and an active ghrelin ELISA kit (Mitsubishi Kagaku Iatron, Chiba, Japan), respectively, according to the manufacturers’ protocols. To measure plasma ghrelin, the plasma was immediately obtained after blood collection and mixed with a 1/10 volume of 1 N HCl to prevent decylation. Samples of 10, 5, and 20 μl were used to measure insulin, leptin, and ghrelin, respectively. At the age of 21 wk, each mouse was housed in a metabolic cage, and urine was collected for the glucose measurements. Urinary glucose above 200 mg/dl was considered to indicate the presence of diabetes.

Effect of leptin administration on food intake. Male Daruma and control mice at the ages of 4, 6, and 8 wk were used in the experiment. Leptin at 1 mg/kg body wt was intraperitoneally injected at 1800, and the food intake for 24 h was measured for each individual.

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Table 1. Primer list for TaqMan Gene Expression Assay

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<th>Product</th>
<th>Assay ID</th>
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<tr>
<td>β2-Microglobulin</td>
<td>Mm00437762_ml</td>
</tr>
<tr>
<td>β-ACTIN</td>
<td>Mm00600939_ml</td>
</tr>
<tr>
<td>Carboxypeptidase-E</td>
<td>Mm00516541_ml</td>
</tr>
<tr>
<td>Ornixin/hypocretin</td>
<td>Mm01964030_s1</td>
</tr>
<tr>
<td>NPY</td>
<td>Mm00445771_ml</td>
</tr>
<tr>
<td>Agrp</td>
<td>Mm00475829_g1</td>
</tr>
<tr>
<td>POMC</td>
<td>Mm00435874_ml</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>Mm00436671_ml</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>Mm00456425_ml</td>
</tr>
<tr>
<td>Adrenergic receptor β3</td>
<td>Mm00442669_ml</td>
</tr>
</tbody>
</table>

NPY, neuropeptide Y; Agrp, agouti-related protein; POMC, pro-opiomelanocortin.

Leptin signaling in cultured hypothalamic cells. The hypothalamus was harvested from 4-, 6-, and 8-week-old Daruma and control mice. Dissociation was performed mechanically and enzymatically with papain. Dispersed cells were then suspended in DMEM containing NaHCO₃, penicillin (100 U/ml), and 2% fetal calf serum and seeded onto L-ornithine- and laminin-coated glass cover slips. Two days after culture, all media were changed to new medium containing leptin (10⁻⁷ M) and maintained for 30 min at 37°C. The cells were fixed with 4% paraformaldehyde in 0.1 M PBS for 30 min and then incubated overnight at 4°C with anti-mouse phospho-signal transducer and activator of transcription 3 (stat3) antisum (Cell Signaling Technology) as the primary antibody. The cells were then washed with PBS and incubated with Alexa Fluor 488-conjugated donkey anti-mouse IgG.

Glucose tolerance test and insulin tolerance test. All mice were fasted for 14 h from 1900 to 0900. Glucose (1.5 g·kg⁻¹·mouse⁻¹) was administered at 0900 h intraperitoneally to 8-week-old male and female Daruma and control mice. Blood samples were collected by the tail tip incision method at 0, 30, 60, 120, and 180 min after the glucose injection, and plasma insulin and blood glucose were measured by the method described above.

Insulin tolerance tests were performed in male and female 8-week-old Daruma and control mice. All mice were fasted for 14 h before insulin administration. Insulin (Humulin R; Eli Lilly) was administered intraperitoneally (0.75 mU/g body wt) at 1000. Blood samples were collected in 20-min intervals after the treatment, and blood glucose was measured by the method described above.

Quantitative real-time PCR. Changes in the expression of several genes, which had previously been identified by microarray analysis, in the hypothalamus and BAT of Daruma mice at 3 wk of age relative to control mice were quantified using real-time RT-PCR. Total RNA was extracted from each tissue using an RNeasy Micro kit (Qiagen) and then reverse transcribed to single-stranded cDNA (40–100 ng tissue equivalent) was quantified using a Taqman Gene Expression Master Mix (Applied Biosystems) with the 7300 Real-Time PCR System (Applied Biosystems) and the TaqMan Gene Expression Assay kit (Bio-Rad Laboratories) as well as leptin, according to the method described above.

Pair-feeding experiment. A portion of the 14-day-old pups were killed, and the WAT, BAT, brain, leg muscle, stomach, and liver were removed and weighed. To examine whether hyperphagia inhibition after weaning would prevent obesity in Daruma mice, a pair-feeding experiment was performed. A pair-fed group was allowed restricted dietary access for 3 wk (from 21 to 42 days of age) to ensure that the Daruma mice had the same food intake as the control mice. Subsequently, the animals were fed ad libitum for 14 days. Body weight and food intake were measured weekly and daily, respectively. At the age of 42 days (pair fed for 3 wk), the mice were killed, and their body compositions were assessed. One day before, 3 days after, and 14 days after the return to ad libitum feeding, blood glucose, leptin, and ghrelin were measured according to the methods described above. Wheel-running was performed from 4 to 7 wk of age in a cage that was equipped with a running wheel (20.5 cm diameter).

Phenotype transfer of the ICR-Daruma mice to C57BL/6J background. Female Daruma mice (background: ICR strain) were mated with male wild-type C57BL/6J mice to produce the F₁ generation. The obese mice were selected from crossbreedings between F₁ mice, and these animals were mated with wild-type C57BL/6J mice. Crossbreeding was repeated nine times, which produced Daruma mice on the C57BL/6J background.

Statistical analysis. The data (mean ± SE) were analyzed statistically using ANOVA with Fisher’s post hoc test.

RESULTS

Interbreeding or crossbreeding experiments. First, we developed fourth-generation mice with heavier body weights at the time of weaning; these animals developed obesity at 7 wk of age, and their body weight increased to approximately twofold over that of their littermates. These Daruma mice were mated with female or male wild-type (intact) mice, and, of the 202 mice that were produced, none became obese (0%). Interbreeding these mice produced 660 additional mice, and 159 (24%) of these animals developed obesity. Then, interbreeding these obese mice produced 42 progeny, and all (100%) of these mice developed obesity. Interbreeding the obese mice and their nonobese littermates produced 290 mice, and 139 (48%) of these mice exhibited obesity. The interbreeding results are summarized in Table 2. The proportion of obese progeny approximated a Mendelian ratio, which indicated that the obese phenotype of the Daruma mouse may be attributable to autosomal-recessive inheritance of a single mutated gene.

To investigate whether the genetic mutation of the Daruma mouse differed from previously identified autosomal recessive

Table 2. Incidence of obesity after mating between obese and nonobese ICR mice

<table>
<thead>
<tr>
<th>Parents</th>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Pups</td>
<td>No. of Obesity</td>
<td>Obesity Incidence, %</td>
</tr>
<tr>
<td>Obesity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (intact)</td>
<td>114</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Obesity</td>
<td>88</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Littermate (nonobesity)</td>
<td>660</td>
<td>159</td>
<td>24</td>
</tr>
<tr>
<td>Obesity</td>
<td>42</td>
<td>42</td>
<td>100</td>
</tr>
<tr>
<td>Obesity</td>
<td>222</td>
<td>106</td>
<td>48</td>
</tr>
<tr>
<td>Littermate (nonobesity)</td>
<td>68</td>
<td>33</td>
<td>49</td>
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</table>
mutations, female heterozygous Daruma mice were crossbred with male heterozygous ob, db, tubby, and fat mice, and 87, 82, 83, and 34 progeny were born, respectively. No obesity was observed in the progeny of the crosses between the Daruma and ob, tubby, or fatty mice, but 11 progeny (13%) from the crossbreed between the Daruma and db mice developed obesity (Table 3).

### Table 3. Interbreeding analysis of mice with obesity

<table>
<thead>
<tr>
<th>Parents</th>
<th>Male</th>
<th>Female</th>
<th>No. of Pups</th>
<th>No. of Obese Pups</th>
<th>Obesity Incidence, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ob</td>
<td>Daruma</td>
<td>87</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Db</td>
<td>Daruma</td>
<td>82</td>
<td>11</td>
<td>13</td>
<td>23.8</td>
</tr>
<tr>
<td>Fat</td>
<td>Daruma</td>
<td>83</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>tubby</td>
<td>Daruma</td>
<td>34</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Appearance and morphological phenotype of the Daruma mouse. The body weight of the Daruma mouse increased significantly after 5 wk of age and reached approximately double the weight of the controls by 19 wk of age (Fig. 1A). There were no significant differences in body weight between nonobese littermates and purchased (wild-type, intact) mice (data not shown). The Daruma mice displayed a clearly obese body form that resembled an oval ball (Fig. 1B). A CT scan of the Daruma mice revealed marked accumulation of adipose tissue surrounding the internal organs (Fig. 1C), and a histological analysis revealed hypertrophic adipocytes in the WAT and lipid droplet accumulation in the liver at 15 wk of age. In contrast, no significant histological differences were evident in the BAT, pancreas, kidney, or muscle (Fig. 1D).

Leptin receptor sequencing in the Daruma mouse. We sequenced the full-length DNA encoding the leptin receptor in the Daruma mouse and in nonobese littermate and wild-type controls. All of the Daruma mice showed mutations at two base positions, in exons 8 and 15, of the leptin receptor gene, which resulted in the substitution of adenine and thymine with guanine and cytosine at positions 1627 and 2810, respectively. Subsequently, these mutations led to the conversion of isoleucine to valine and isoleucine to threonine, respectively, in the protein product (Fig. 2, A and B). Two ICR mice among the ~600 that were examined did not show obesity, although they did carry the mutations within the leptin receptor gene sequence.

Food intake, heart rate, blood pressure, and activity level of the Daruma mice. The daily food intake of the Daruma mouse was significantly increased at 4, 5, 9, and 19 wk of age (Fig. 3A). Additionally, heart rate and blood pressure were significantly higher in Daruma mice than control mice (Fig. 3, B and C), although no significant difference in blood pressure was evident past 8 wk of age (Fig. 3C). The locomotor activity of the Daruma mice was significantly decreased at 13 wk of age (Fig. 3D), and the wheel-running activity of these animals was very low from the weaning period onward (Fig. 3E).

Biochemical analysis of the blood of Daruma mice. Compared with control mice, Daruma mice had higher plasma total cholesterol and HDL cholesterol at 4 wk of age and higher TG at 9 wk of age (Fig. 4, A–C). In addition, plasma ALT in the Daruma mice was significantly increased at 5 and 9 wk of age (Fig. 4D), although no significant changes were observed in plasma AST (Fig. 4E). Plasma FFA were also higher in Daruma mice than controls at 9 and 15 wk of age (Fig. 4F).

Blood glucose, insulin, leptin, and ghrelin levels in Daruma mice. In male Daruma mice, the blood concentrations of both glucose and insulin were significantly higher than those in control mice, and these increases became significant at 3 wk of age and increased further with age (Figs. 5, A and B). In comparison, female Daruma mice exhibited only slight increases in plasma glucose, although high plasma insulin was observed at 7 and 9 wk of age (Fig. 5, A and B). High plasma leptin was observed in both male and female Daruma mice at 3 wk of age, and no significant

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Fig. 1. A: the growth curves of Daruma (●, male n = 15; ▲, female n = 16) and control (○, male n = 15; ▼, female n = 29) mice. Nonobese littermates were used as control mice. *P < 0.01 male and #P < 0.01 female Daruma mice vs. control mice. B: the appearance of the Daruma (left) and control (right) mice at 15 wk of age. C: the computed tomography scan images of male Daruma and control mice at 15 wk of age. Sections of each line are shown below the overview. The white and blue masses indicate bone and adipose tissue, respectively. D: white adipose tissue, liver, brown adipose tissue, pancreas, kidney, and muscle of Daruma and control mice at 15 wk of age. All sections were stained with hematoxylin and eosin.

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Diabetes was present in more than 50% of male Daruma mice (Fig. 5D) at 21 wk of age, although fewer than 10% of female Daruma mice developed diabetes.

To investigate the availability of glucose in Daruma mice, we performed glucose tolerance tests at the age of 8 wk. Before treatment, there was no significant difference between the plasma glucose levels of fasted Daruma mice and control mice (Fig. 5E). Following glucose administration, blood glucose increased significantly within 30 min and then returned to the pretreatment level after 180 min in both the control mice and female Daruma mice. However, in male Daruma mice, plasma glucose increased within 30 min of glucose administration and stayed at this level for at least 180 min (Fig. 5E). Even when the Daruma mice were fasted for 14 h, plasma insulin before treatment was high in both the male and female mice, and no significant changes were observed following glucose treatment. In control mice, the changes in plasma insulin displayed the same trend as the changes in blood glucose (Fig. 5F). Next, we performed insulin tolerance tests at the age of 12 wk. Administration of insulin significantly reduced blood glucose to ∼30% of that in the control mice regardless of the sex. In Daruma mice, however, blood glucose decreased to ∼60 and 80% of the initial levels in female and male mice, respectively (Fig. 5G).

Administration of leptin resulted in a significant decrease in 24-h food intake in 4-, 6-, and 8-wk-old control mice. In Daruma mice, however, such a significant decrease in food intake was only observed in 4-wk-old mice. There was no significant difference between the saline- and leptin-treated groups in 6- and 8-wk-old Daruma mice (Fig. 5H). In addition, immunohistochemistry revealed that leptin induced phosphorylation of Stat3 in cultured cells from 4- and 6-wk-old control mice; however, in Daruma mice, the phosphorylation of Stat3 was low in 4-wk-old mice, and no phosphorylation was observed in 6-wk-old mice (Fig. 5I).

Obesity-related gene expression at weaning and body weight, plasma, and tissue leptin levels during the preweaning period in Daruma mice. Because the Daruma mice exhibited signs of obesity at the time of weaning, quantitative RT-PCR for mRNAs associated with food regulation and obesity was performed on samples from the hypothalamus and BAT at the time of weaning (3 wk old). In the hypothalamus, NPY, AGRP, and POMC mRNAs were significantly increased in Daruma mice, whereas no differences were observed in the mRNA expression of CPE, hypocretin, or somatostatin (Fig. 6A). Moreover, in BAT, adiponectin and Adrb3 mRNAs were significantly decreased, whereas UCP1 mRNA was increased in Daruma mice compared with controls (Fig. 6B).

Because the Daruma mice had high plasma leptin as well as differences in several mRNAs expressed in the hypothalamus and BAT at the time of weaning, we reexamined in greater detail the changes in body weight observed in the Daruma mice from 12 days of age until weaning, as well as the leptin levels in these mice at 2 wk of age. Although the growth curves for body weight during the nursing period demonstrated no significant differences between Daruma and control mice, a tendency to become overweight was observed in Daruma mice during the preweaning period (Fig. 6C). Plasma leptin was also significantly higher in Daruma mice than in controls as early as 14 days of age (Fig. 6D). Moreover, leptin was significantly higher in the WAT, lungs, and kidneys of Daruma mice compared with controls at 14 days of age (Fig. 6E).
Body composition during development and effect of pair feeding in Daruma mice. When the tissue and body weights of 14-day-old Daruma and control mice were compared, there were no significant differences, except for the BAT weight (Figs. 7, A and B).

The tendency for Daruma mice to become overweight after weaning was completely inhibited by pair feeding. During the pair-feeding period, there was no significant difference in body weight between pair-fed Daruma mice and control mice (Fig. 7C). Following the return to ad libitum feeding, however,
hyperphagia was immediately evident in the Daruma mice (Fig. 7D). Additionally, the body weight of these animals increased rapidly and approached that of the Daruma mice that had been fed ad libitum from the outset within 2 wk (Fig. 7C). Blood glucose in pair-fed Daruma mice remained normal during pair feeding and then increased significantly 2 wk after returning to the ad libitum diet (Fig. 7E). In contrast, plasma leptin in pair-fed Daruma mice was higher than in control mice but lower than in ad libitum-fed Daruma mice. Moreover, 2 wk after returning to ad libitum feeding, plasma leptin reached the level of Daruma mice that had been fed ad libitum from the outset (Fig. 7F). Plasma ghrelin in pair-fed Daruma mice increased significantly compared with wild-type mice and ad libitum-fed Daruma mice during the pair-feeding period (Fig. 7G), whereas plasma ghrelin of ad libitum-fed Daruma mice remained consistently lower than in control mice. After pair feeding, the Daruma mice were returned to an ad libitum diet, and their plasma ghrelin decreased rapidly and reached the level observed in the Daruma mice that had been fed ad libitum from the outset (Fig. 7G). The wheel-running activity levels

Fig. 5. Blood glucose (A), plasma insulin (B), and plasma leptin (C) in the Daruma (♂: male; ▴: female; n = 6) and control (♂: male; ▴: female; n = 5) mice at various ages. The symbol and vertical bar represent the mean ± SE, respectively. *P < 0.01 male and #P < 0.01 female Daruma mice vs. the control mice. D: the incidence of diabetes in Daruma mice. Mice with urinary glucose above 200 mg/dl were considered diabetic. No diabetes was observed in the control mice. Blood glucose (E) and plasma insulin (F) following ip administration of glucose (1.5 g/kg body wt) to male Daruma (♂: male; ▴: female) and control (♂: male; ▴: female) mice. G: blood glucose following ip administration of insulin (0.75 mU/g body wt) to Daruma (♂: male; ▴: female) and control (♂: male; ▴: female) mice. H: food intake for 24 h following ip administration of leptin (filled bars; 1 mg/kg body wt) or saline (open bars). I: phosphorylation of signal transducer and activator of transcription 3 induced by leptin in cultured hypothalamic cells from Daruma and control mice. Scale bar = 10 μm. *P < 0.05 male Daruma vs. male control mice. #P < 0.05 female Daruma vs. female control mice. The symbol and vertical bar represent the mean ± SE, respectively (male: n = 7; female: n = 6).
were identical between the control and pair-fed Daruma mice; however, when ad libitum feeding was reinstated, the Daruma mice demonstrated very low wheel-running activity (Fig. 7H).

Comparison of body weight during the preweaning period in male Daruma (●; n = 18) and control (○; n = 18) mice (C) and plasma leptin at 14 and 21 days of age in male Daruma (●; n = 6) and control mice (○; n = 6) (D). The symbol and vertical bar represent the mean ± SE, respectively. E: leptin levels in various tissues of 14-day-old male Daruma (filled bars) and wild-type (open bars) mice. *P < 0.05 Daruma vs. control mice.

Phenotype transfer of the ICR-Daruma mice to C57BL/6J background. The background of the Daruma mice was transferred from ICR to C57BL/6J (Fig. 8). C57BL/6J-Daruma mice showed the same characteristics as ICR-Daruma mice (data not shown).

DISCUSSION

Interbreeding experiments revealed that the incidence of obesity in Daruma mice corresponded to a Mendelian ratio of inheritance (Table 2), which indicated that the obese phenotype was due to autosomal-recessive inheritance of a single-gene mutation. We also succeeded in transferring the phenotype of the ICR-Daruma mice to the C57BL/6J background and produced nine generations of C57BL/6J mice (Fig. 8), which also suggests that the obese phenotype was caused by a single-gene mutation. No obesity was observed in progeny that were produced as a result of interbreeding between nonobese Daruma littermates and heterozygous ob, fat, or tubby mice, which indicates that the gene mutation responsible for obesity in the Daruma mice differs from those previously identified for these mouse strains (Table 3). In contrast, 13% of the pups produced by breeding nonobese Daruma littermate mice to heterozygous db mice developed obesity. If the genetic mutation in the Daruma and db/db mice had been identical, the
Fig. 7. Comparison of body weight (A) and tissue weights (B) between 14-day-old Daruma (filled bars) and control (open bars) mice. The effects of 3 wk pair feeding followed by ad libitum feeding on body weight gain (C) and food intake (D) in Daruma and control mice (○, ◆: ad libitum fed on days 21–56, control mice; ●, ■: ad libitum fed on days 21–56, Daruma mice; △, ▲: pair fed on days 21–42 and then ad libitum fed on days 43–56, Daruma mice). The control mice included both wild-type and heterozygous mice. The pair-feeding periods are represented by horizontal arrow bars. Blood glucose (E), plasma leptin (F), and plasma ghrelin (G) in Daruma mice either fed ad libitum throughout the study (○) or pair fed on days 21–42 and then fed ad libitum (▲) and control mice fed ad libitum throughout the study (○). Mice were killed 1 day before (A), 3 days after (B), or 14 days after returning to (C) ad libitum feeding. Therefore, A, B, and C on the x-axis represent the sampling days shown in C. The relative value for the control is presented. H: wheel-running activity during the pair-feeding and return to ad libitum feeding periods (○: control, ■: Daruma). Comparison of body weight (I) and tissue weights (J) between Daruma (filled bars) and control mice (open bars) mice after 3 wk of pair feeding. The symbol and vertical bar represent the mean ± SE, respectively (n = 8). *P < 0.05 Daruma vs. control mice.
expected rate of obesity would have been -25%, which suggests that possible mechanisms of interaction may exist. All Daruma mice revealed mutations at two base positions, within exons 8 and 15, of the leptin receptor gene (Fig. 2, A and B). These mutations are candidates for the cause of obesity in Daruma mice; however, the whole genome must be sequenced to determine the responsible gene.

The body weight of the Daruma mice increased rapidly after 4 wk of age and reached 60–70 g by 19 wk of age in both sexes (Fig. 1A). Although it may be difficult to compare body weights between the ICR and C57BL/6 mice, early onset obesity can be observed in ob/ob and db/db mice by 5–7 wk of age (19, 31), in contrast to the development of obesity in fat/fat and tub/tub mice at 6–8 and 10 wk of age, respectively (10). Regarding maximum body weight, the Daruma mice were more similar to ob/ob and fat/fat mice, which attain a weight of 60–70 g, than to db/db and tub/tub mice, which attain a final body weight of 50–60 g (10, 38, 40). Therefore, the Daruma mice displayed characteristics of early onset obesity as well as a heavier body weight than other obese mouse models. As in other obese animal models, excess weight gain in the Daruma mice was characterized by visceral fat accumulation throughout the body rather than fat accumulation confined to the axial and inguinal regions (Fig. 1, B and C).

Ob/ob mice exhibit hypotension (33), whereas db/db mice exhibit hypertension (14). Daruma mice demonstrated hypertension before the age of 7 wk (Fig. 3C). Leptin activates the sympathetic nervous system, and the hypotension in ob/ob mice is thought to arise from the loss of this function of leptin (33). In contrast, hypertension in db/db mice arises as a result of autonomic dysfunction characterized by sympathetic hyperactivity and parasympathetic hypoactivity (14), which may occur as a result of the ablative effect of leptin on the development of neuronal circuitry at a young age (12). The observation of hyperleptinemia in the presence of hyperphagia (Fig. 7, D and F) at 3 wk of age and the impairment of leptin signaling in the hypothalamus at 4 wk of age (Fig. 5I) suggest that the cause of hypertension in the Daruma mice is related to sympathetic hyperactivity, which is also the case in db/db mice. This hypothesis is supported by the fact that the Daruma mice displayed a significantly elevated heart rate (Fig. 3B), which is also observed for db/db mice. Moreover, sympathetic hyperactivity results in the downregulation of Adrb3 in BAT (53, 55), and Adrb3 mRNA was downregulated in the BAT of Daruma mice at 3 wk of age (Fig. 6B). A similar decrease in UCP1 mRNA expression in the BAT of db/db mice has also been reported (34). Voluntary running activity is decreased in ob/ob, New Zealand obese (23), db/db (50), and tub/tub mice (13). The Daruma mice demonstrated low locomotor activity and very little running activity (Figs. 3, E and F, and 7F). It is thus likely that these decreased activity levels contribute to the obese phenotype of the Daruma mice.

Hyperlipidemia in the Daruma mice was characterized by elevated total cholesterol, HDL cholesterol, and ALT in young animals (Fig. 4, A, B, and D) and elevated TG and FFA in adults (Fig. 4, C and F). Because elevated plasma FFA can inhibit glucose transporter 4 translocation to the cytomembrane, elevated plasma FFA is strongly associated with insulin resistance and hyperglycemia (7). In contrast, lipoprotein lipase activity in adipose tissue is reduced by hyperinsulinemia (54), which causes decreased fat synthesis and increased blood FFA concentration. In Daruma mice, hyperinsulinemia was observed as early as 3 wk of age (Fig. 5B), when the body weight changes were comparable to those of control mice. At the ages of 9 and 15 wk, the Daruma mice had high FFA (Fig. 4F), which appeared to be related to the impaired action of insulin on adiposity from a young age. ALT in Daruma mice was also significantly increased at 5 and 9 wk of age (Fig. 4D). Elevated FFA and ALT have been observed in many animal strains with genetic obesity, such as ob/ob mice, as well as in animals with diet-induced obesity and in obese humans (15, 22). In contrast, HDL cholesterol in Daruma mice was markedly elevated (Fig. 4B) at a young age, similar to ob/ob and db/db mice (48) but in contrast to other genetic and diet-induced animal models and human obesity (43, 56). On the other hand, no changes were observed in plasma AST of Daruma mice (Fig. 4E), although it is generally increased in animal models of obesity, such as ob/ob and db/db mice (20). Together, these findings imply that differences in dyslipidemia exist among these animal models.

Impaired glucose metabolism at a young age has been reported for db/db and ob/ob mice but not for fat/fat, tub/tub (10), or agouti yellow (37) mice. In ob/ob mice, hyperinsulinemia and hyperglycemia are evident at 17 and 22 days of age, respectively (16), and insulin resistance subsequently occurs at 3–4 wk of age in these mice (21). Db/db mice show hyperinsulinemia and hyperglycemia at 10 days and 4 wk of age, respectively, and insulin resistance is evident at 5 wk of age (9). Because serum insulin was increased in young Daruma mice (Fig. 5B), hyperglycemia and the onset of non-insulin-dependent diabetes mellitus (NIDDM) may have been due to hyperinsulinemia rather than a genetic disorder, similar to db/db and ob/ob mice. The fact that body weight changes and hyperglycemia were not observed in Daruma mice during pair feeding (Fig. 7, C and I) strongly supports this hypothesis, and it is likely that excess weight gain and/or a hyperphagia-induced metabolic disorder was responsible for the development of NIDDM. Unlike ob/ob and db/ob mice, Daruma mice showed no pancreatic histological abnormalities at 9 wk of age (Fig. 1D). In contrast, sexual dimorphism of NIDDM devel-
opment (Fig. 5D) and insulin sensitivity (Fig. 5G) was observed in Daruma mice. In fact, this sexual dimorphism is a general phenomenon in rodents with NIDDM, such as the ob/ob, db/db, and fat/fat mice. In ob/ob and db/db mice, protection from diabetes in female mice can be attributed to low hepatic estrogen sulfotransferase activity, which prevents virilization of liver metabolism (30). Estrogen increases the density of insulin receptor expression on hepatocyte membranes (29), which may offset postreceptor insulin resistance in female rodents. Additionally, such a mechanism could contribute to the mild hyperglycemia and low incidence of diabetes observed in female Daruma mice.

The body composition at the age of 14 days revealed that body weight and tissue weights did not change significantly, except for the BAT weight, in Daruma mice (Fig. 7, A and B). The pair-fed Daruma mice maintained a normal body weight gain compared with control mice that were fed ad libitum beginning at 3 wk of age (Fig. 7, C and D), which indicates that hyperphagia was the primary cause of obesity in the Daruma mice. Plasma ghrelin was elevated during the pair-fed feeding period and decreased to normal following the return to ad libitum feeding (Fig. 7G), which suggests that the Daruma mice may have experienced hunger during the pair-feeding period. Furthermore, body weight rapidly increased following the return to ad libitum feeding in Daruma mice (Fig. 7C). These results also suggest that hyperphagia is a strong preceding factor for obesity in Daruma mice. Although the reason for the development of hyperphagia remains unclear, plasma leptin may be involved in this process. The elevated plasma leptin in the Daruma mice was partially, but not completely, inhibited by pair feeding (Fig. 7F), which demonstrates that plasma leptin increased regardless of whether the Daruma mice consumed a normal amount of food during the pair-feeding period. Pair feeding resulted in significant increases in VAT and BAT; however, other tissue weights and total body weight were not different. Moreover, intraperitoneal injection of leptin did not significantly reduce food intake in Daruma mice (Fig. 5H).

However, following the return to ad libitum feeding, plasma leptin further increased, which suggests that complete leptin resistance was not acquired during pair feeding, although hyperphagia may have advanced the onset of leptin resistance after the return to ad libitum feeding. This phenomenon may be analogous to diet-induced obesity in experimental animals.

The analysis of hypothalamic gene expression by real-time PCR revealed that NPY and Agpr were upregulated at the time of weaning in Daruma mice compared with control mice (Fig. 6A). Therefore, hyperphagia may have begun during the preweaning period and could have been induced by appetite-stimulating peptides in the hypothalamus of the Daruma mice. However, the expression of POMC mRNA, which is a precursor of the anorexigenic peptide α-MSH, also increased at weaning in Daruma mice. α-MSH is an endogenous agonist for MC4R, and Agpr is its antagonist. In Daruma mice, Agpr would block the effect of α-MSH, resulting in overeating. NPY/Agpr and POMC neurons in the arcuate nucleus express the long-form leptin receptor (ObR-b), and peripheral leptin decreases appetite by stimulating POMC neurons and inhibiting NPY/Agpr neurons (46). The reduction of Stat3 phosphorylation in hypothalamic cells (Fig. 5I) suggests that leptin signaling was impaired in Daruma mice, resulting in leptin resistance from an early stage. These results may contribute that the food intake of Daruma mice significantly increased after weaning.

Leptin also regulates energy expenditure and physical activity. Leptin activates the sympathetic nerves innervating BAT through Adrb3 to produce heat by increasing UCP1 expression, whereas UCP1 mRNA expression is decreased in ob/ob and db/db mice (32). Leptin also affects the cardiovascular system by increasing blood pressure and heart rate (42). Additionally, leptin increases locomotor activity (8), whereas spontaneous physical activity is decreased in ob/ob and db/db mice (45, 47). Furthermore, voluntary running activity is decreased in ob/ob mice (23), which is likely a consequence of leptin antagonist overexpression in the hypothalamus (35). These observations suggest that the lack of leptin activity results in severe obesity and the development of metabolic syndrome in both humans and animals with an appropriate genetic mutation. The physiological and metabolic features observed in the Daruma mice, such as hyperleptinemia (Fig. 7F), hyperphagia (Fig. 1A), hypertension (Fig. 3C), and hyperactivity (Fig. 7H) at a young age, could theoretically be explained by a lack of leptin activity. The attenuation of leptin’s effects on food intake and the phosphorylation of Stat3 in Daruma mice (Fig. 5, H and I) are consistent with this hypothesis.

Based on these data, we formulated two hypotheses regarding leptin resistance to account for the obesity observed in the Daruma mice. The first hypothesis is that obesity in these animals is fundamentally caused by leptin overproduction in WAT. In this case, although the responsiveness to leptin is normal, WAT leptin production may be elevated through an increase in positive factors and a decrease in negative factors that regulate leptin production. Moreover, the consequent hyperleptinemia also likely downregulates the expression of the leptin receptor. Therefore, leptin resistance develops by 3 wk of age in these animals and may result in the observed severe hyperphagia and obesity. The second hypothesis is that the fundamental cause of obesity in Daruma mice is related to congenital leptin resistance. Under this hypothesis, reduced hyperleptinemia also likely downregulates the expression of the leptin receptor. Therefore, leptin resistance develops by 3 wk of age in these animals and may result in the observed severe hyperphagia and obesity. These observations suggest that the lack of leptin activity results in severe obesity and the development of metabolic syndrome in both humans and animals with an appropriate genetic mutation. The physiological and metabolic features observed in the Daruma mice, such as hyperleptinemia (Fig. 7F), hyperphagia (Fig. 1A), hypertension (Fig. 3C), and hyperactivity (Fig. 7H) at a young age, could theoretically be explained by a lack of leptin activity. The attenuation of leptin’s effects on food intake and the phosphorylation of Stat3 in Daruma mice (Fig. 5, H and I) are consistent with this hypothesis.

In summary, we have reported a novel monogenic mouse model of obesity that exhibits autosomal-recessive inheritance, which we have termed Daruma. Daruma mice develop hyperleptinemia during the preweaning period and partial leptin resistance by 3 wk of age. The development of leptin resistance subsequently results in severe hyperphagia and obesity. Then, as the development of obesity progresses, the Daruma mice exhibit hyperlipidemia, hypertension, hyperglycemia, hypoactivity, and NIDDM related to glucose intolerance and impaired...
insulin secretion. This new mouse model recapitulates many of the metabolic abnormalities observed in human NIDDM and should prove to be a valuable tool for investigating the fundamental causes of leptin resistance and identifying the molecular defects that underlie obesity.

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**DISCLOSURES**

Makoto Bannai is an employee of Ajinomoto Co., Inc.

**AUTHOR CONTRIBUTIONS**

Author contributions: K.N., K.M., Y.S., and R.O. performed experiments; K.N. and M.B. analyzed data; K.N., M.B., K.M., Y.S., and N.M. interpreted results of experiments; K.N. and M.B. prepared figures; K.N., M.B., and N.M. edited and revised manuscript; M.B. and N.M. drafted manuscript; M.B., K.N., and M.B. analyzed data; K.N., M.B., K.M., Y.S., and N.M. interpreted the metabolic abnormalities observed in human NIDDM and insulin secretion. This new mouse model recapitulates many of the genetic defects that underlie obesity and identifies the molecular defects that underlie obesity.

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