A Novel Model of Obesity-Related Diabetes: Introgression of the *Lepr^{fa}* Allele of the Zucker Fatty Rat into Nonobese Spontaneously Diabetic Torii (SDT) Rats

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Abstract: An *fa* allele of the leptin receptor gene (*Lepr^{fa}*) of the Zucker fatty rat was introduced into the genome of the Spontaneously Diabetic Torii (SDT) rat, an inbred model of nonobese type 2 diabetes mellitus, through the 'Speed congenic method'. The newly established congenic strain of a SDT rat for *Lepr^{fa}* was maintained by intercrossing between *fa*-heterozygous littermates, and the phenotypes related to obesity and diabetes were investigated till 32 wks of age. SDT *fa/fa* rats of both sexes exhibited obesity, adiposity and insulin resistance associated with hyperphagia from the loss of leptin action. Interestingly, they developed diabetes from 5 wks of age in males and 8 wks in females with the incidences reaching 100% at 16 wks in males and 73% at 32 wks in females. In contrast, heterozygous (+/*fa*) and wild-type (+/+) rats developed spontaneous nonobese diabetes in males from approximately 20 wks, but not in females, as with the original SDT rats. These results indicate that the *fa* gene accelerates the onset of diabetes in SDT rats by making adiposity and/or insulin resistance as potent risk factors for development of their diabetes. The SDT.*Lepr^{fa}* congenic rat strain is expected to be a novel model of obesity-related diabetes and could be a useful tool for studies of the genetic backgrounds of diabetes in response to *fa*-induced obesity.

Key words: congenic strain, diabetes, *fa*, obesity, SDT rat

Introduction

The Spontaneously Diabetic Torii (SDT) rat is known as a useful model of nonobese type 2 diabetes that spontaneously develops hyperglycemia and glucose intolerance in males resulting from decreased insulin

secretion due to β -cell degeneration leading to inadequate β -cell mass in the pancreas [12, 17]. In addition, the diabetes in SDT rats is a polygenetic trait controlled by at least 3 quantitative trait loci (QTLs), *Gisd1*, *Gisd2* and *Gisd3*, localized on chromosomes 1, 2 and X, respectively [11]. Thus, it is clear that the SDT rat

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has a genetic background which produces glucose intolerance associated with decreased insulin secretory capacity.

Type 2 diabetes mellitus is a polygenic disorder that is caused by a metabolic and/or hormonal imbalance between insulin secretion from β -cells and insulin sensitivity in peripheral tissues both of which might be modified by genetic and environmental factors [14, 20]. The decreased sensitivity to insulin leads to an increased requirement for insulin, and is often associated with obesity in which metabolic disturbances are marked in insulin-target organs, such as the liver, muscle and adipose tissues [1, 14]. It is well known that the β -cell response to increased insulin requirements is strongly determined by animals' genetic backgrounds [3, 19, 21].

Based on the above concept, it should be possible to establish a novel model of obesity-related diabetes by introducing the genetic factor of insulin resistance or obesity into the genetic background of the SDT rat. The genetic factor that produces insulin resistance or obesity, an *fa* mutation of the leptin receptor gene (*Lepr^{fa}*) in the Zucker fatty (ZF) rat, is well known [2]. The leptin receptor is mainly expressed in the hypothalamus and is a key molecule that regulates hypothalamic effects on satiety and energy expenditure through binding to leptin, an adipocyte-specific hormone [16, 22]. The fatty phenotype in the ZF (*fa/fa*) rat is characterized by obesity resulting from a defect in appetite control [2] which is caused by an *fa* mutation in the *Lepr* gene, a missense mutation (an A-to-C conversion at nucleotide position 806) in a *MspI* site that results in a single amino acid change from *gln* to *pro* at position 269 [2, 18].

We thus established a congenic line of the SDT rat by introducing the *fa* allele of the ZF rat into the SDT rat genome through the 'Speed Congenic Method' popularized by Markel *et al.* [9] using a PCR technique with DNA markers. In this paper, we describe the process of the establishment of a new congenic strain of the SDT rat and the clinical features related to obesity and diabetes of this congenic strain.

Materials and Methods

Animals and animal handling

SDT rats, the recipient strain, were used from our colony. The ZF rat, the donor of the *fa* allele of the

Lepr gene, was provided by Prof. Komeda of Tokyo Medical University (Tokyo, Japan). Animals were individually housed in a room maintained at $23 \pm 1^\circ\text{C}$ and $55 \pm 10\%$ relative humidity with a 12-h light-dark cycle, and were provided tap water (sterilized by UV light) and food (CRF-1, Charles River Japan Inc., Kanagawa, Japan) *ad libitum*, except when they were fasted for experiments. All the investigations were approved by committees for the humane care and use of animals in our laboratories, in accordance with the Standards Relating to the Care and Management of Experimental Animals (Notification No.6, March 27, 1980 of the Prime Minister's Office in Japan).

Introgression of the fa gene to the SDT rat

Introgression of *fa* gene onto the SDT rat genome was carried out according to a 'Speed Congenic Method' [9], which is a backcrossing of the *fa*-carriers to the recipient SDT rats. Briefly, the *fa*-carriers that were produced by first mating between female SDT rats and one male heterozygous (*+fa*) ZF rat were subsequently backcrossed. The rats for the next generation production were selected from the *fa* allele-carrying progenies with the lowest heterozygous segments based on the genotypes of simple sequence-length polymorphism (SSLP) markers across the entire genome. Congenic rats introgressed with the *fa*-alleles were maintained by intercrossing between heterozygous littermates (*+fa*) to obtain rats having each genotype, *+/+*, *+fa* and *fa/fa*.

Genetic monitoring

SSLP markers were purchased from Research Genetics Inc. (Huntsville, Ala, US) or synthesized based on the sequences published on websites (The Rat Genome Database RatMap, <http://ratmap.gen.gu.se/>), to analyze the polymorphism of the PCR products between the ZF rat and the SDT rat. A total of 226 SSLP markers were selected covering all of the chromosomes, with an average span of 6.2 cM (corresponding to approximately 1,500 cM in total), in order to monitor heterozygosity in the background genome of the backcrossed rats. Genomic DNA extraction, PCR and electrophoresis were performed as described previously [10].

Genotyping of the *fa* locus was performed according to a PCR-restricted fragment length polymorphism (RFLP) method as follows. A primer set to detect the *fa*

allele was designed to cover a 110-bp region containing the *fa* mutation site in the *Lepr* gene with the following sequences: 5'-TATGGAAGTCACAGATGATGG-3' and 5'-CTTACGATTGTAGAATTCTCTAA-3'. This PCR product (110 bp) was digested by a restriction endonuclease *MspI* (Nippon Gene Co., Toyama, Japan) at 37°C for 1 h to cut the *fa* allele, and then electrophoresed on a 4% agarose gel (NuSieve 3:1 agarose, FMC BioProducts Inc., MA, U.S.A.) for genotyping. The *MspI* digestion of the *fa* allele produces two fragments of 77 and 33 bps.

Phenotypic characterizations

The animals bred for this study were routinely checked for the appearance of glucosuria using Multi-Sticks (Bayer-Sankyo, Tokyo, Japan) from 4 to 32 weeks of age. The diagnostic criteria for diabetes in this study were the appearance of glucosuria and hyperglycemia (fed blood glucose ≥ 250 mg/dl). At 4, 5, 6, 8, 10, 12, 14, 16, 20, 25 and 32 weeks of age, the animals were weighed, and their blood glucose concentrations were determined under non-fasted conditions using a portable glucose meter (DexterZ, Bayer-Sankyo, Tokyo, Japan) within 1–4 h after the light cycle began. Food consumption was measured at 6 weeks of age. At 14 weeks of age, after overnight fasting, animals were ether-anesthetized, weighed, measured for their body length (from anus to nose), and exsanguinated from the abdominal aorta. Retroperitoneal and interscapular fat pads were removed and weighed. Blood samples collected into heparinized tubes were centrifuged to obtain the supernatant plasma. The plasma glucose concentrations were determined by the glucose oxidase method using an autoanalyzer (HITACHI 7150, HITACHI, Tokyo, Japan) with reagents from Wako Chemical Inc. (Osaka, Japan). The concentrations of plasma insulin and leptin as an immuno-reactant were determined by an enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Morinaga Biochemical Research Laboratory, Yokohama, Japan) for rat insulin and leptin. Body mass index (BMI, g/cm²) was determined by dividing body weight (g) by the square of body length (cm²).

Statistical analysis

The data are shown as means $\pm$ standard deviations (S.D.). The statistical differences between *fa/fa* and *+/+* rats were determined by Student's *t*-test.

Results

Establishment of the congenic strain

First, female SDT rats were crossed with a male heterozygous (*+/fa*) ZF rat to obtain the F1 hybrids carrying the *fa* allele. The heterozygous F1 females (*+/fa*) were backcrossed to male SDT rats, and the N2 generation was obtained. Subsequently, backcross mating was done between the progenies and the recipient SDT rats. In the N5 generation, we obtained 'the best male' in which all of the SSLP markers covering the entire rat genome were genotyped to SDT-homozygous, except for *D5Rat159* flanking *Lepr*. The genetic distance between *D5Rat159* and *Lepr* was 4 cM on the Rat Genome Database RatMap (Table 1). We conducted further backcrosses until the N7 generation. Although 130 male *fa*-carriers were produced among N2–N7 generations, we could not find any with recombination between *D5Rat159* and *Lepr*, suggesting that both loci are closely adjacent at a distance of less than 0.8 cM in this cross. The maximum genetic length of a heterozygous interval was estimated to be 6 cM in 63–69 cM of chromosome 5 (Table 1). The heterozygous interval was considered to be less than approximately 0.3% of the entire rat genome (2,028 cM on the KLAS map (Kyoto Laboratory Animal Science map)) [8]. At present, this congenic strain is maintained by inter-crossing between *fa*-heterozygous littermates.

Obesity-related phenotypes in congenic rats

Gross appearances of the 6-wk-old male SDT *+/+*, *+/fa* and *fa/fa* rats are shown in Fig. 1. The *fa/fa* rats became overtly obese and were distinguishable from wild-type (*+/+*) and heterozygous (*+/fa*) lean rats.

Their growth curves until 32 weeks of age are shown in Figs. 2A and 2B. The body weights of the SDT-*fa/fa* rats of both sexes were higher than those of the wild-type (*+/+*) and heterozygous (*+/fa*) lean rats. The mean peak body weight of the *fa/fa* rats reached the maximum value: 694.9 g for males (at 20 wk) and 708 g for females (at 32 wk in final observation), 1.5-fold and 1.7-fold higher than age-matched wild-type rats, respectively. Wild type (*+/+*) and heterozygous (*+/fa*) lean rats showed similar growth curves to the original SDT rats as reported previously [17]. Also, the BMI as an index of obesity was statistically greater ($P < 0.01$) in *fa/fa* rats of both sexes (0.91 and 0.87 g/cm² in males and

Table 1. Genetic monitoring of rat chromosome 5 in SDT.*Lepr^{fa}* congenic rats

cM ^{a)}	Locus			
0	D5Rat116			
7	D5Mgh2			
12	D5Rat123			
18	D5Mgh1			
19	D5Rat126			
41	D5Rat8	D5Mit1		
44	D5Rat12	D5Mit2		
45	D5Rat104	D5Mgh6		
51	D5Rat85	D5Rat20	D5Mit11	
52	D5Rat19			
54	D5Rat150	D5Rat149		
56	D5Wox3	D5Rat98	D5Rat92	D5Rat88
58	D5Rat24			
62	D5Rat154			
63	D5Rat71	D5Rat156	D5Rat155	
65	<i>Lepr</i> *			
69	D5Rat159*	D5Rat27		
71	D5Rat95	D5Rat65		
72	D5Rat165	D5Rat108		
78	D5Rat34	D5Rat33	D5Rat203	
80	D5Rat36			
86	D5Rat89	D5Rat43	D5Rat37	
88	D5Rat39	D5Mgh8	D5Mgh14	
92	D5Rat93	D5Rat69		
93	D5Rat41			
97	D5Rat56			
103	D5Rat47			
105	D5Mit15			
106	D5Rat48			
109	D5Mgh16	D5Mgh15		

a) Genetic distance of each locus was based on the integrated linkage map of the Rat Genome Database RatMap. Asterisks indicate loci heterozygous in the N7 backcrossed generation. Maximum genetic length of a heterozygous interval was estimated to be 6 cM in the 63–69 cM of chromosome 5.

females, respectively) than in lean rats (0.75 and 0.58 g/cm² in males and females, respectively) at 14 weeks of age (Figs. 2C and 2D). Retroperitoneal and interscapular fat pads, as indexes of adiposity, were markedly heavier in *fa/fa* rats of both sexes (26.2 and 10.1 g, respectively, in males and 29.1 and 16.6 g, respectively, in females) than in wild-type (+/+) rats (11.0 and 1.9 g, respectively, in males and 8.2 and 1.5 g, respectively, in females) (Figs. 2E and 2F). Food consumption in *fa/fa* rats of both sexes was significantly increased ($P < 0.01$) to approximately double at 8 weeks compared with that in lean rats (Figs. 2G and 2H).

Fig. 1. The dorsal appearance of male SDT.*Lepr^{fa}* congenic rats at 6 weeks of age. Littermates of +/+ (left), +/fa (middle) and *fa/fa* (right) are shown.

Male and female heterozygous (+/fa) and wild-type (+/+) rats showed no differences in the above phenotypes during their life span.

Diabetes-related phenotypes in congenic rats

The cumulative incidence of the onset of diabetes (glucosuria and hyperglycemia) is shown in Figs. 3A and 3B. Male *fa/fa* rats developed diabetes from 5 weeks of age, with the cumulative incidence of diabetes reaching up to 82% at 8 weeks of age and 100% by 16 weeks of age (Fig. 3A). Female *fa/fa* rats developed diabetes with a lower incidence (73% at 32 weeks of age) than the male *fa/fa* rats (Fig. 3B). In contrast to *fa/fa* rats, +/+ and +/fa rats of both sexes showed similar phenotypes to the original SDT rats as reported previously [11, 12, 17]. Male +/+ and +/fa rats exhibited delayed onset of diabetes (18 weeks) and the incidence reached 100% at 29 weeks of age. Female +/+ and +/fa rats did not develop diabetes until 32 weeks of age (Figs. 3A and 3B).

Changes in blood glucose levels under non-fasting conditions are shown in Figs. 3C and 4D. The male *fa/fa* rats showed higher blood glucose levels than the male lean (+/+, +/fa) rats after 6 weeks of age, and became more hyperglycemic by 8 weeks of age (Fig. 3C). The lean male rats were hyperglycemic after 20 weeks of age (Fig. 3C), as with the original SDT rats [17]. In female *fa/fa* rats, the average blood glucose levels were slightly but significantly elevated after 6

Fig. 2. Obesity-related phenotypes in SDT.*Lep^{fa}* congenic rats. Growth curve until 32 weeks of age in males (A) and females (B); black boxes, *fa/fa* homozygous rats; white triangles with dotted lines, *+/-* heterozygous rats; white circles, *+/+* wild type rats. Data are expressed as the mean $\pm$ S.D. of 6–9 rats for each point. Statistical differences between *fa/fa* and *+/+* rats were observed after 5 weeks of age in both sexes. Body mass indexes in males (C) and females (D) and fat pad weights in males (E) and females (F) at 14 weeks of age. Food consumption in males (G) and females (H) at 6 weeks of age. Data are expressed as the mean $\pm$ S.D. of 4–8 rats for each genotype. Asterisks indicate statistical differences ($P < 0.01$) to the wild-type.

weeks of age compared with those in the female lean rats (Fig. 3D).

Plasma biochemistry in fasted congenic rats at 14 weeks of age

Plasma biochemistry data in fasted congenic rats at 14 weeks of age are shown in Fig. 4. The *fa/fa* rats of both sexes exhibited hyperinsulinemia (Figs. 4C and 4D) and hyperleptinemia (Figs. 4G and 4H). The insulin/glucose ratio was markedly increased in *fa/fa* rats of both sexes compared with the wild-type (*+/+*) rats, indicating marked insulin resistance in the *fa/fa* rats (Figs. 4E and 4F).

Fig. 3. Diabetes-related phenotypes in SDT.*Lep^{fa}* congenic rats. Cumulative incidence of diabetes development in males (A) and females (B) until 32 weeks of age; black lines, *fa/fa* homozygous rats (males=11, females=11); dotted lines, *+/-* heterozygous rats (males=20, females=4); solid lines, *+/+* wild-type rats (males=11, females=17). The cumulative incidence in female *+/+* and *+/-* rats was 0% till 32 weeks of age, as hidden by X-axis indicating age in weeks. Changes in blood glucose levels until 32 weeks of age in males (C) and females (D); black boxes, *fa/fa* homozygous rats; white triangles with dotted lines, *+/-* heterozygous rats; white circles, *+/+* wild-type rats. Data are expressed as the mean $\pm$ S.D. for each point. Statistical differences ($P < 0.01$) between *fa/fa* and *+/+* rats were observed from 6 to 20 weeks of age in males and after 6 weeks of age in females.

Discussion

In this study, we established a new congenic rat strain produced by introgression of the *fa* allele of a ZF rat onto the SDT rat genome. This congenic rat spontaneously develops diabetes associated with obesity in *fa*-homozygous rats.

The *fa* mutation in the rat leptin receptor accounts for the fatty phenotype in ZF *fa/fa* rats resulting from impaired appetite control [2]. In this study, SDT *fa/fa* rats of both sexes exhibited obesity with adiposity. Hyperphagia and hyperleptinemia were also observed in the *fa/fa* rats of both sexes as compared with the wild-type and heterozygous lean rats, suggesting that impaired appetite control by leptin resistance in the hypothalamus exists in these rats. This indicates that the *fa* gene evoked a similar phenotype in the SDT background as observed in ZF rats.

Fig. 4. Plasma biochemistry of fasted SDT.*Lepr^ffa* congenic rats at 14 weeks of age. Glucose (A, B), insulin (C, D) and leptin (G, H) concentrations in plasma and insulin/glucose ratio (ng/mg) in 1 ml plasma (E, F) of males (A, C, E and G) and females (B, D, F and H) are shown. Data are expressed as the mean $\pm$ S.D. of 4–6 rats for each genotype. Asterisks indicate statistical differences ($P < 0.01$) to the wild-type.

In contrast, ZF *fa/fa* rats of both sexes do not develop diabetes during their lifespan when fed a normal chow *ad libitum* in similar environmental conditions to those used in our investigations [3, 15, 23], although they show an impaired glucose tolerance and other signs of insulin resistance associated with obesity. We found that SDT *fa/fa* rats spontaneously developed hyperglycemia after 5 weeks of age in males and after 8 weeks of age in females, the onset of which is markedly earlier than in wild-type and heterozygous rats. The cumulative incidence reached 100% in males by 16 weeks of age and 73% in females at 32 weeks of age. These results indicate that the *fa* gene accelerates the onset of diabetes in SDT rats. SDT *fa/fa* rats had higher fasting plasma insulin concentrations with an increased

insulin/glucose ratio than did lean rats, suggesting that they had increased insulin resistance. Insulin resistance is a major risk factor and diabetes develops only when β -cells fail to compensate for increased insulin demand due to hormone resistance [21]. Inadequate β -cell mass is found in SDT rats [12], which appears to be determined by the genetic background of these congenic rats. Thus, the obesity and/or insulin resistance induced by the *fa* gene are regarded as potent risk factors for accelerating diabetes development in SDT rats.

Both the wild-type (+/+) and heterozygous (+/*fa*) rats showed no significant differences in the characteristics of obesity- and diabetes-related phenotypes, and exhibited spontaneous diabetes without obesity after 18 weeks of age in males, while the females were nondiabetic at least until 32 weeks of age (the end of the observation period). Their age-related phenotypes were similar to those in the recipient SDT rats. This is the biological evidence that the genetic background of this congenic strain was sufficiently replaced by the SDT rat genome. In addition, no effect of the heterozygosity for the *fa* allele is expected to be in the SDT rat genome as well as in the Zucker background.

Although a large number of rat strains having the *Lepr* gene mutation have been reported [4–6, 15], the SDT-*fa* congenic strain we established is important in terms of having the genetic background of the nonobese type 2 diabetes of the SDT rat. Diabetic phenotypes of SDT *fa/fa* rats described in this study may partially resemble those of Zucker Diabetic Fatty (*fa/fa*) rats exhibiting a severe form of obese diabetes [15]. For example, both *fa/fa* males of SDT and ZDF rats display similar magnitudes of glycemia and insulinemia; blood glucose levels and plasma insulin concentrations were 474.8 ± 140.4 mg/dl and 8.79 ± 3.22 ng/ml, respectively, in the SDT *fa/fa* rats at 12–14 weeks of age (Figs. 3C and 4C) and 504 ± 84 mg/dl and 8.8 ± 3.0 ng/ml in ZDF *fa/fa* rats at 10–13 weeks of age as reported by Peterson *et al.* [15]. However, the SDT background contains genetic defects which impair insulin secretory capacity to glucose stimuli rather than insulin resistance [11]. Further characterization of this congenic rat might be required not only to define a phenotypic difference among the several obese diabetes models but also to understand the heterogeneous pathology of obesity-related diabetes involved with genetic combinations of the SDT-background-derived

defects and the *Lepr* gene mutation.

It is well known that genetic background strongly influences susceptibility to diabetes [3, 13, 19]. Chung *et al.* studied two rat models of diabetic and non-diabetic strains having the *Lepr^{fa}* mutation [3] and determined three QTLs for the variability in the susceptibility to obese diabetes [3]. Of these QTLs, interestingly, the locus regulating adiposity and the number of pancreatic islets was mapped on chromosome 1, positionally overlapping a highly significant QTL, *Gisdt1*, for glucose intolerance in the SDT rat [11]. The SDT-*fa* congenic rat might contribute to the identification of the susceptibility genes to diabetes with or without obesity.

In summary, we established a novel congenic rat strain of the SDT rat by introgression of *Lepr^{fa}* into the SDT rat genome resulting in obesity due to polyphagia. This congenic rat strain might be a useful tool for studying the underlying mechanisms of diabetes development with or without obesity. At present, this congenic strain is maintained as a segregating inbred strain for *Lepr^{fa}* by intercrossing (sister-brother mating). This congenic strain has been designated 'SDT.Cg-*Lepr^{fa}*' according to the rules for the Nomenclature of Mouse and Rat Strains, because the ZF rat used as the donor for *Lepr^{fa}* was an 'outbred' strain. In practice we call it the 'SDT fatty rat', in the same manner as other fatty rats having the *Lepr^{fa}* mutation such as the Zucker fatty, Zucker diabetic fatty (ZDF) and Wistar fatty.

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