A novel model mouse for type 2 diabetes mellitus with early onset and persistent hyperglycemia

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Abstract: Various mouse models of type 2 diabetes have been established, but few of these show early onset and persistent hyperglycemia. We have established a congenic mouse strain (NSY.B6-Tyr+, AY) in which a spontaneous mutation of the agouti yellow (AY) gene, which causes obesity by hyperphagia, was introduced into the NSY strain, which shows increased glucose intolerance with age. This strain has been maintained as a segregating inbred strain by mating obese yellow (AY/a) males with normal black (a/a) females. All yellow males showed marked obesity and hyperglycemia (mean blood glucose level >400 mg/dl) from 10 to 24 weeks of age. The yellow males also showed glucose intolerance and insulin resistance. They provide a potentially valuable model mouse for research into type 2 diabetes, hyperlipidemia, fatty liver, and renal glomerular complications. Yellow female mice also showed marked obesity, but the incidence of diabetes and the severity of various pathological conditions were milder than in yellow males. None of the black mice showed hyperglycemia in either sex. NSY.B6-Tyr+, AY strain has good fertility and does not display inter-male aggression, making them useful as a new model for type 2 diabetes with early onset and persistent hyperglycemia.

Key words: agouti yellow, congenic strain, model mouse, NSY strain, type 2 diabetes mellitus

Introduction

Type 2 diabetes mellitus (T2DM) is a complex metabolic disorder in which disease pathophysiology is determined by the interaction of genetic and environmental factors [1]. T2DM patients show a wide range of pathophysiological features, although peripheral insulin resistance, hyperinsulinemia, and β-cell dysfunction are generally characteristic [1]. Laboratory mice are the most widely used models for human T2DM. Spontaneous T2DM in mice can be either monogenic or polygenic depending on the mouse strain; these different types of T2DM have been exploited to investigate diverse types of human T2DM [2–5]. However, there are few mouse models that show early onset, high incidence, and persistent hyperglycemia; such models would be particu...
larly useful for screening new antidiabetic drugs, for short-term intervention studies, and exploring the mechanisms of diabetic complications.

Two mouse strains, C57BLKS/J-Leprdb (BKS-db) and KK-AY, which show early-onset, high incidence, and persistent hyperglycemia have been extensively investigated [2]. The Leprdb (db) allele is a point mutation of the leptin receptor and has an autosomal recessive mode of inheritance [3]; db/db mice have abnormal splicing of the receptor for the adipocyte-derived hormone leptin [6, 7]. BKS-db/db mice develop hyperglycemia by 2 months of age [8] and are hyperphagic and have reduced energy expenditure, leading to early-onset obesity [9]. Additionally, BKS-db/db mice exhibit fulminant diabetest with persistent hyperglycemia compared with other leptin and leptin receptor deficient mouse strains [2, 4, 8, 10, 11]. For this reason, BKS-db/db mice are of particular value in studies on diabetic nephropathy [12, 13] and diabetic dyslipidaemia, and for screening agents related to insulin sensitizers [5]. However, as homozygous db/db mice of both sexes are infertile, mutant homozygotes have to be obtained by intercrosses of male and female heterozygous (db/+ ) mice; only 25% of the offspring of these crosses are db/db homozygotes.

The agouti yellow (Ay) variant is a long-identified dominant mutant allele; the mutation involves a 150 kb deletion that includes the promoter region of the agouti gene [14]. Agouti yellow mutant mice develop hyperphagia with obesity resulting from antagonism of hypothalamic melanocortin receptor 4 by ectopic expression of the agouti protein [15]. The KK-Ay strain was developed by transferring the Ay allele into the diabetes prone KK strain [2, 3, 13]. This strain displays severe obesity, hyperlipidemia, insulin resistance, and insulin deficiency at about 8 weeks of age, and shows persistent hyperglycemia [11, 16, 17]. It is generally regarded as a suitable model for exploring the mechanisms of obesity-induced T2DM, for studying new antidiabetic drugs [18], and investigating diabetic nephropathy [13, 19]. However, the KK-Ay strain does have some serious disadvantages for experimental use. First, males are very aggressive and show excessive fighting behavior, requiring them to be caged individually. Second, KK-Ay mice have a low reproductive efficiency.

The drawbacks described above indicate that development of other mouse models is required: in particular, such models should display early-onset, high incidence, and persistent hyperglycemia, should not be aggressive, and should have high reproductive efficiency. We hypothesized that introgression of a spontaneous mutation that causes obesity into a diabetes-prone strain with good breeding and handling characteristics might produce a mouse strain with the required features. We selected the dominant Ay allele and the NSY strain for this purpose. The NSY strain is used as a spontaneous T2DM animal model; it is an inbred strain that was derived from outbred ICR mice by selective breeding for glucose intolerance [20, 21]. The mice are characterized by insulin resistance and impaired insulin secretion without severe obesity and hyperglycemia [21]. Additionally, they breed well and do not have excessive inter-male fighting. Ay heterozygous males are fertile, although heterozygous females are frequently infertile. It is straightforward to obtain similar numbers of yellow obese and non-obese mice from crosses between Ay heterozygous males and wild type females.

In this report, we describe the establishment of a novel T2DM mouse strain that carries the Ay allele on an NSY genetic background. We then investigated the basic pathophysiological features of this novel strain and demonstrate its utility for research into human T2DM.

### Materials and Methods

**Animals**

NSY/Hos (NSY) and C57BL/6JHam-Ay (B6J-Ay) mice were obtained from Japan SLC (Hamamatsu, Japan). The mice were fed a commercial CE-2 diet (CREA Japan, Tokyo, Japan) and had ad libitum access to water. The mice were housed in a pathogen-free facility at the Institute for Laboratory Animal Research, Graduate School of Medicine, Nagoya University, and maintained under a controlled temperature of 23 ± 1°C, humidity of 55 ± 10%, and a light cycle of 12-h light (from 09:00 to 21:00)/12-h dark (from 21:00 to 09:00). Animal care and all experimental procedures were approved by the Animal Experiment Committee, Graduate School of Medicine, Nagoya University, and were conducted according to the Regulations on Animal Experiments of Nagoya University.

**Strategy for congenic strain development**

B6J-Ay mice, which carry the Ay allele on a C57BL/6J (B6J) genetic background, were used. Ay homozygotes die before implantation; heterozygotes are viable and adult mice show a yellow coat color due to the wild type allele (Tyrc+) at the tyrosinase locus. Heterozygous mice show marked obesity, but do not show hyperglycemia when fed a normal diet. B6J-Ay mice have the following genotype at the major coat color loci agouti, tyrosinase-related protein 1 (Tyrc1), and tyrosinase: Ay/a, Tyrc1+/Tyrc1+, Tyrc+/Tyrc+. The genotype of NSY mice at these loci is a/a, Tyrc1+/Tyrc1+, Tyrc+/Tyrc+; the mice show a typical albino coat color. To develop yellow NSY mice,
the $\text{Tyr}^+$ allele and the $\text{A}^+$ allele were introgressed from the B6J-\text{A}^+ into the NSY genetic background.

**Genotyping of Tyr locus**

To detect the albino allele ($\text{Tyr}^-$) derived from the NSY strain, primer sequences for $\text{Tyr}$ were designed using genome assembly data (GRCM39/mm39) as the reference sequence (Supplementary Table 1). Genomic DNA was extracted from mouse pinnas using KAPA Express Extract (Kapa Biosystems, Woburn, MA, USA). PCR amplification was performed using a GoTaq Green Master mix (Promega, Madison, WI, USA). PCR products were purified using the ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA). Purified PCR products were sequenced using the dideoxy chain-termination method with a BigDye$^\text{TM}$ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific), and a 3730xl DNA Analyzer (Thermo Fisher Scientific) by Eurofins DNA sequence service (Eurofins Genomics K.K, Tokyo, Japan).

**Twenty-four-week longitudinal study**

Body weights and blood glucose levels were measured every 2 weeks from 4 to 24 weeks of age under non-fasting conditions. To determine blood glucose levels, a drop of blood (0.6 µl) was taken from the tail tip and assayed with a GluTest analyzer (Sanwakagaku Kenkyusho, Nagoya, Japan). Mice with a blood glucose level higher than 200 mg/dl were considered to be diabetic.

**Oral glucose tolerance test and insulin tolerance test**

Glucose tolerance tests were performed at 12 and 24 weeks of age. Mice were orally administered with a 20% glucose solution (1 g glucose / kg body weight) after 15 h fasting. Blood glucose levels were detected at 0 (fasting), 0.5, 1.0, and 2.0 h after glucose administration using blood collected from the tail. The area under curve (auc) was calculated according to the trapezoid rule, excluding the mice used only for the insulin tolerance test. For the insulin tolerance test, 16 yellow males, 13 black males, 9 yellow females, and 11 black males were used. Data are expressed as means ± SD. Differences in body weights, blood glucose levels, auc, blood insulin levels, blood lipid levels, and liver weights were assessed using t-tests between yellow and black mice in each sex to avoid the clear sex differences for these characteristics. The incidence of diabetes was compared using $\chi^2$-tests. $P$ values <0.05 were regarded as statistically significant.

**Results**

**Development of NSY.B6-\text{Tyr}^+\text{, A}^+$ strain**

Male B6J-\text{A}+ mice were mated to NSY females to produce F$_1$ mice. Yellow F$_1$ females (\text{A}+/\text{a}, \text{Tygp1}+/\text{Tygp1}+, \text{Tygr}/\text{Tygr}) were backcrossed to NSY males to produce yellow N2 males. Although females heterozygous for \text{A}+ are usually infertile, yellow F$_1$ females were fertile. Therefore, we can replace the Y chromosome of the developing congenic strain from B6J to NSY type. Subsequently, yellow male mice were backcrossed to NSY females to produce the next generation. Finally, brother–sister mating of yellow males and black female mice at the N1 generation yielded homozygosity on an NSY genetic background for the \text{Tyr}+ allele derived from B6J-\text{A}+. We have maintained this new congenic strain (NSY. B6-\text{Tyr}+,\text{A}+) by brother–sister mating of yellow (\text{A}+/\text{a}, \text{Tygp1}+/\text{Tygp1}+, \text{Tygr}/\text{Tygr}) male and black (\text{a}/\text{a}, \text{Tygp1}+/\text{Tygp1}+, \text{Tygr}/\text{Tygr}) female mice (Fig. 1) as a segregating inbred strain because of infertility of yellow females. Individuals of four different phenotypes were used in the
subsequent analysis: yellow (A^{t/a}, Tyr^{+}/Tyr^{+}) males and females; and black (a/a, Tyr^{+}/Tyr^{+}) males and females. The process for establishment of the NSY.B6-Tyr^{+},A^{t} strain is shown in Supplementary Fig. 1. The NSY.B6-Tyr^{+},A^{t} strain showed good reproductive performance with high litter size and early age of first birth (Supplementary Table 2), and there were no problems in maintaining this strain. However, the fertility of yellow males fell after 3 months of age as obesity progressed. Furthermore, the use of groups of males during breeding of the strain did not cause any injuries due to excessive fighting behavior.

Longitudinal study of body weights and blood glucose levels

Body weights of yellow mice were greater than those of black mice from 4 weeks of age to 24 weeks of age in both sexes (Fig. 2). The weight difference between yellow and black mice increased rapidly from 8 to 14 weeks in both sexes. No diabetic mice were identified among black males and females until 24 weeks of age (Fig. 3). Diabetic yellow males were first identified at 6 weeks of age, and all yellow males developed diabetes after 10 weeks of age (Fig. 3) in correspondence with rapid weight gain. After 10 weeks, all yellow males showed persistent hyperglycemia with mean blood glucose levels higher than 400 mg/dl until 24 weeks of age (Fig. 4). Diabetic females were first identified at 12 weeks of age, and the incidence of diabetes gradually increased until 20 weeks of age (Fig. 3). The mean blood glucose levels of yellow females were consistently below

![Fig. 1. Yellow male (left) and black female (right) of the NSY.B6-Tyr^{+},A^{t} strain at 8 weeks of age.](image1)

![Fig. 2. Changes in mean body weights from 4 to 24 weeks of age in NSY.B6-Tyr^{+},A^{t} strain mice. Solid squares, open squares, solid circles, and open circles indicate yellow males (n=19), black males (n=16), yellow females (n=13), and black females (n=11), respectively. Asterisk (*) indicates a significant difference (P<0.05) in a t-test versus black males; hash (#) indicates a significant difference (P<0.05) in a t-test versus black females.](image2)

![Fig. 3. Incidence of diabetes at 4 to 24 weeks of age in NSY.B6-Tyr^{+},A^{t} strain mice. Mice with blood glucose levels higher than 200 mg/dl were considered to be diabetic. Solid squares, open squares, solid circles, and open circles indicate yellow males (n=19), black males (n=16), yellow females (n=13), and black females (n=11), respectively. Asterisk (*) indicates a significant difference (P<0.05) in a \( \chi^2 \)-test versus black males, and hash (#) indicates a significant difference (P<0.05) in a \( \chi^2 \)-test versus black females.](image3)

![Fig. 4. Non-fasting blood glucose levels at 4 to 24 weeks of age in NSY.B6-Tyr^{+},A^{t} strain mice. Solid squares, open squares, solid circles, and open circles indicate yellow males (n=19), black males (n=16), yellow females (n=13), and black females (n=11), respectively. Asterisk (*) indicates a significant difference (P<0.05) in a t-test versus black males, and hash (#) indicates a significant difference (P<0.05) in a t-test versus black females.](image4)
300 mg/dl (Fig. 4), and significantly lower than those in yellow males from 6 to 24 weeks of age, although the test results are not shown in Fig. 4. Two yellow females were normoglycemic until 24 weeks of age despite severe obesity.

**Impaired glucose tolerance and insulin resistance**

Blood glucose levels of yellow mice were significantly higher than those of black mice at all the time points before and after glucose challenge in both males and females at 12 weeks of age (Fig. 5A). Blood glucose levels in yellow males after glucose administration were consistently more than 100 mg/dl higher than those of black males. Yellow males exhibited overt impaired glucose tolerance (Fig. 5A). Yellow females also showed glucose intolerance, although the degree of hyperglycemia was less than that of yellow males (Fig. 5A). The AUC of yellow mice at 24 weeks of age was significantly greater than that at 12 weeks in both males and females. In contrast, there were no significant differences in the AUC of black mice of both sexes between 12 weeks and 24 weeks of age (Fig. 6).

**Fig. 5.** Glucose tolerance and insulin sensitivity in NSY.B6-Tyr⁺,Ay strain mice. (A): Changes in blood glucose levels during an oral glucose tolerance test (1 g glucose / kg body weight) at 12 weeks of age. Solid squares, open squares, solid circles, and open circles indicate yellow males (n=19), black males (n=16), yellow females (n=13), and black females (n=11), respectively. (B): Insulin tolerance test (1U insulin / kg body weight) at 18 weeks of age. Solid squares, open squares, solid circles, and open circles indicate yellow males (n=16), black males (n=13), yellow females (n=9), and black females (n=11), respectively. Asterisk (*) indicates a significant difference (P<0.05) in a t-test versus black males, and hash (#) indicates a significant difference (P<0.05) in a t-test versus black females.

**Fig. 6.** AUC during OGTT at 12 and 24 weeks of age in NSY.B6-Tyr⁺,Ay strain mice. Nineteen yellow males, 16 black males, 13 yellow females, and 11 black males were used in this experiment. Asterisk (*) indicates a significant difference (P<0.05) in a t-test versus 12 weeks of age in male mice. Hash (#) indicates a significant difference (P<0.05) in a t-test versus 12 weeks of age in female mice.
Insulin sensitivity as assessed by the insulin tolerance test was impaired in both male and female yellow mice compared with black mice (Fig. 5B). Blood glucose levels at 0.5 h and 1.0 h after insulin injection were significantly lower than those before insulin injection in black males and females. However, blood glucose levels in yellow males and females did not decrease after insulin injection (Fig. 5B). Furthermore, blood insulin levels in both male and female yellow mice were markedly and significantly higher than those of black mice (Table 1). These observations confirm the severe insulin resistance of yellow mice.

**Dyslipidemia**

Blood TG and TC levels of yellow mice were significantly increased compared with those of black mice in both sexes, and this difference was particularly pronounced in the males (Table 1). The livers of all yellow males were clearly enlarged with significantly higher weights than in black males (Table 1). The gross appearance of the livers of yellow males was characterized by a lighter color than in black males (Fig. 7A). Histological examination of the livers of yellow males revealed the presence of large hepatic lipid droplets (Fig. 7B, lower panel); these droplets were not found in the livers of black males (Fig. 7B, upper panel). Blood ALT levels of yellow males were markedly and significantly higher than those of black males (Table 1). Liver weights and blood ALT levels were also significantly elevated in yellow females compared to black females, but to a lesser degree than in yellow males (Table 1). These results indicate that yellow males exhibited hyperlipidemia and fatty liver. By contrast, dyslipidemia was relatively mild in yellow females compared with yellow males.

**Histological analysis of pancreatic islets and kidneys**

There were no serious pathological changes, such as lymphocytic infiltration, in pancreatic islets from the NSY.B6-Tyr*,AY strain (Fig. 8). Islet architecture was...
well preserved at 25 weeks of age (Fig. 8, left panel) except for yellow males; the yellow males had a mixture of normal islets (Fig. 8, middle panel) and islets with unclear borders and invading exocrine tissue (Fig. 8, right panel). Pathological changes were observed in the kidneys of yellow males with expansion and deposition of hyaline-like substances in the glomeruli (Fig. 9, right panel). These changes did not occur in black males (Fig. 9, left panel), suggesting the development of nephropathy in yellow males, but not in black males.

**Discussion**

We have developed a novel congenic strain (NSY. B6-Tyr$^+$,Ay) in which the Ay and Tyr$^+$ alleles from B6J-Ay have been introgressed into the NSY genetic background. All yellow NSY males exhibited persistent hyperglycemia with severe obesity from 10 to 24 weeks of age (Figs. 2–4); they also exhibited impaired glucose tolerance (Fig. 5A), insulin resistance (Fig. 5B), fasting hyperinsulinemia (Table 1), and an absence of serious damage in pancreatic islets (Fig. 8). These characteristics indicate that the yellow NSY males have a high incidence of early-onset diabetes with persistent hyperglycemia associated with obesity as originally intended by introgression of the Ay allele (Supplementary Table 3). We anticipate that these mice will prove to be a useful model for screening of antidiabetic drugs. Mean blood glucose levels remained above 400 mg/dl from 10 to 24 weeks of age. Further analysis of blood glucose levels and diabetes-related phenotypes after 24 weeks of age is needed to clarify the longitudinal outcome of diabetes in this model. Dietary factors are regarded as an important environmental factor in T2DM in both humans and model mice [3, 4, 22]. NSY mice fed a high sucrose diet showed increased body weight, liver steatosis, and

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**Fig. 8.** Comparative histology of HE-stained pancreas of black and yellow male NSY.B6-Tyr$^+$,Ay strain mice at 25 weeks of age.

**Fig. 9.** Comparative histology of PAS-stained kidney of black male and yellow male NSY.B6-Tyr$^+$,Ay strain mice at 25 weeks of age.
higher glucose intolerance compared to mice fed a high-fat diet [23, 24]. The effect of environmental factors, such as high-sucrose diets, on the severity and outcome of diabetes in yellow NSY mice will provide useful information on gene-environment interactions in diabetes.

Leptin prevents insulin resistance via activation of AMP kinase that reduces lipid deposition in insulin-sensitive tissues. However, the absence of leptin signaling is very rare in human T2DM [25]. Therefore, the results obtained from leptin-signaling-deficient rodent models might not fully reflect the pathogenesis of human T2DM [25]. By contrast, yellow NSY males exhibit severe hyperlipidemia and fatty livers (Table 1, Fig. 7) and have intact leptin signaling similar to that of Kk- mouse [26]. Thus, these mice are a useful model for diabetic dyslipidemia. The histological changes observed in kidneys of yellow NSY males include the expansion and deposition of hyaline-like substances in the glomeruli (Fig. 9), suggesting that these mice may be a suitable model for diabetic nephropathy. In addition, we identified age-dependent worsening in glucose AUC (Fig. 6), presumably due to a persistent hyperglycemia and glucose toxicity. B6- mouse are hypertensive compared to C57BL/6J- strain (B6- mice) that show similarly pronounced obesity [27, 28]. Further comprehensive analyses of pathophysiology of pancreas and kidney, including complications caused by persistent hyperglycemia, are needed in NSY.B6-Tyr+, mouse.

Sexual dimorphism of diabetic phenotypes has been previously identified [29]. Yellow females showed a consistently lower incidence of diabetes and lower blood glucose levels compared with yellow males in the NSY. B6-Tyr+, mouse (Figs. 3 and 4), like many other T2DM model mice [29]. Estrogen has been shown to increase insulin content and glucose-stimulated insulin secretion in isolated mouse pancreatic islets, suggesting an active role for this hormone in glucose homeostasis [30]. Several studies have highlighted the beneficial role of estrogen in glucose homeostasis in rodents [30–32]. Sex hormones such as estrogen may have some role in the observed sexual dimorphism for diabetic phenotypes in the NSY.B6-Tyr+, mouse, although this needs to be confirmed in further studies.

B6J is the most widely used inbred mouse strain, particularly as the genetic background for congenic strains carrying spontaneous mutations or artificially targeted mutations. Therefore, we can combine multiple mutations on the B6J background for use in a variety of research areas including obesity and cardiovascular studies. Although several strains with obesity, such as B6J-ob, B6J-db, and B6J- mouse, show mild and transient hyperglycemia [8], few mouse strains exhibit persistent hyperglycemia on this genetic background. Development of new gene editing technologies, for instance the CRISPR/Cas9 system in mice [33, 34], enable the rapid and effective introduction of mutations into selected strains. It should be feasible to generate modified NSY.B6-Tyr+, mouse strains with persistent hyperglycemia in which other targeted mutations have been introduced. The NSY. B6-Tyr+, mouse has good reproductive efficiency, oocyte collection and in vitro fertilization can be carried out by conventional methods. In addition, there are no particular difficulties in artificial manipulation of fertilized eggs of NSY.B6-Tyr+, mouse. NSY.B6-Tyr+, mouse strains with introduced targeted mutations using the CRISPR/Cas9 system will provide a powerful tool for exploring the molecular mechanisms of antidiabetic drugs and dissecting genetic factors relevant to diabetic complications.

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