

Diabetic modifier QTLs in F₂ intercrosses carrying homozygous transgene of *TGF-β*

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Abstract When the homozygous active form of porcine *TGF-β1* transgene (*Tgf/Tgf*) (under control of the rat glucagon promoter) is introduced into the nonobese diabetic mouse (NOD) genetic background, the mice develop endocrine and exocrine pancreatic hypoplasia, low serum insulin concentrations, and impaired glucose tolerance. To identify genetic modifiers of the diabetic phenotypes, we crossed hemizygous NOD-*Tgf* with DBA/2J mice (D2) or C3H/HeJ mice (C3H) and used the “transgenic mice” for quantitative trait loci (QTL) analysis. Genome-wide scans of F₂-D *Tgf/Tgf* (D2 × NOD) and F₂-C *Tgf/Tgf* (C3H × NOD), homozygous for the *TGF-β1* transgene, identified six statistically significant modifier QTLs: one QTL (*Tdn1*) in F₂-D *Tgf/Tgf*, and five QTLs (*Tcn1* to *Tcn5*) in F₂-C *Tgf/Tgf*. *Tdn1* (Chr 13, LOD = 4.39), and *Tcn3* (Chr 2, LOD = 4.94) showed linkage to body weight at 8 weeks of age. *Tcn2* (Chr 7, LOD = 4.38) and *Tcn4* (Chr 14, LOD = 3.99 and 3.78) showed linkage to blood glucose (BG) concentrations in *ip*GTT at 30, 0, and 120 min, respectively. *Tcn1* (Chr 1, LOD = 4.41) and *Tcn5* (Chr 18, LOD = 4.99) showed linkage to serum insulin concentrations in *ip*GTT at 30 min. *Tcn2* includes the candidate gene, uncoupling

protein 2 (*Ucp2*), and shows linkage to *Ucp2* mRNA levels in the soleus muscle (LOD = 4.90). Identification of six QTLs for diabetes-related traits in F₂-D *Tgf/Tgf* and F₂-C *Tgf/Tgf* raises the possibility of identifying candidate susceptibility genes and new targets for drug development for human type 2 diabetes.

Abbreviations

| | |
|----------------------------------|-------------------------------------------------------------------------------------------------------------|
| BW | Body weight |
| BG | Blood glucose |
| C3H | C3H/HeJ mice |
| D2 | DBA/2J mice |
| eQTL | Expression quantitative trait loci |
| NOD- <i>Tgf</i> | Hemizygous transgenic NOD mice with <i>TGF-β1</i> |
| F ₁ - <i>Tgf</i> | Hemizygous transgenic F ₁ mice with <i>TGF-β1</i> |
| NOD- <i>Tgf/Tgf</i> | Homozygous transgenic NOD mice with <i>TGF-β1</i> |
| F ₂ -D <i>Tgf/Tgf</i> | Homozygous transgenic F ₂ intercross progeny with <i>TGF-β1</i> between the D2 mice and NOD |
| F ₂ -C <i>Tgf/Tgf</i> | Homozygous transgenic F ₂ intercross progeny with <i>TGF-β1</i> between the C3H/HeJ mice and NOD |
| F ₂ - <i>Tgf/Tgf</i> | Homozygous transgenic F ₂ intercross progeny with <i>TGF-β1</i> |
| Idd | Insulin-dependent diabetes |
| <i>ip</i> GTT | Intraperitoneal glucose tolerance test |
| NOD | Nonobese diabetic mice |
| QTL | Quantitative trait loci |
| T1D | Type 1 diabetes |
| T2D | Type 2 diabetes |

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Introduction

Type 2 diabetes (T2D) is a complex disease in which genetic and environmental factors interact to produce alterations in insulin resistance and insulin secretion, leading to hyperglycemia. To identify the genetic factors that modify diabetic phenotypes, quantitative trait loci (QTL) analyses have been applied to a variety of models of diabetes in different genetic backgrounds (Hirayama et al. 1999; Kido et al. 2000; Komatsu et al. 2002; Leiter et al. 1998; Takeshita et al. 2006; Togawa et al. 2006; Ueda et al. 1999).

We previously generated nonobese diabetic mice (NOD) homozygous for a transgene expressing the active form of porcine *TGF- β 1* (*Tgff/Tgf*) under the control of the rat glucagon promoter (NOD-*Tgff/Tgf*) (Moritani et al. 2005). NOD-*Tgff/Tgf* showed overt diabetes or impaired glucose tolerance and exhibited low insulin concentrations and hypoplasia of endocrine and exocrine pancreatic tissue. In NOD-*Tgff/Tgf*, the active form of *TGF- β 1* is secreted from islet β cells where it suppresses cell cycle progression of endocrine and exocrine pancreatic cells through the induction of *p15^{INK4b}*. It has been reported that T2D patients exhibit reduced islet and β -cell mass (Deng et al. 2004; Sakuraba et al. 2002; Yoon et al. 2003). Genome-wide association studies showed that the locus around the *CDKN2B* gene (encoding *p15^{INK4b}*) was associated with T2D in Caucasians (Scott et al. 2007; Zeggini et al. 2007). NOD-*Tgff/Tgf*, regarded as a transgenic model for diabetes or glucose intolerance due to insulin deficiency, is useful for the identification of modifier genes and improved understanding of the genetic basis of hypoinsulinemic diabetes. We have used F₂ intercross progeny as a tool to unmask QTLs that should contain genetic modifiers of diabetes-related traits.

We performed genome-wide QTL analyses on homozygous transgenic F₂ intercross progeny between DBA/2J mice (D2) and NOD-*Tgff/Tgf* (F₂-D *Tgff/Tgf*), or between C3H/HeJ mice (C3H) and NOD-*Tgff/Tgf* (F₂-C *Tgff/Tgf*). We selected D2 and C3H as strains to cross with NOD for several reasons. First, genomic sequence tag site (STS) markers are well characterized for both strains. Second, each strain has its own genomic diversity. Finally, crossing with different strains should uncover strain-specific QTLs (Roderick and Guidi 1989). We searched for modifier QTLs that changed diabetes-related traits, including body weight (BW), blood glucose (BG) concentrations, and serum insulin concentrations.

Based on genome-wide analyses, we identified six major modifier QTLs, including one QTL in F₂-D *Tgff/Tgf* (Chr 13) and five QTLs in F₂-C *Tgff/Tgf* (Chrs 1, 2, 7, 14, and 18). With respect to the Chr 7 locus showing linkage to BG concentrations in *ipGTT*, data from other replicating studies and the overlap with insulin-dependent diabetes

(*Idd*) 27 prompted us to test for linkage in expression QTLs for *uncoupling protein 2* and 3 (*Ucp2* and *Ucp3*) mRNA levels in soleus muscle.

Materials and methods

Animals

D2 and C3H were purchased from CLEA Japan Inc. (Tokyo, Japan). Hemizygous transgenic NOD mice for *TGF- β 1* (NOD-*Tgf*) were produced as previously described (Moritani et al. 1998). We first created F₁ hybrids by breeding female D2 or C3H with male NOD-*Tgf*. The hemizygous transgenic F₁ mice carrying *TGF- β 1* (F₁-*Tgf*) were then intercrossed to produce F₂ generations. The strategy for creating homozygous transgenic F₂ intercross progeny carrying *TGF- β 1* (F₂-*Tgff/Tgf*) is shown in Fig. 1. In the cross of D2 with NOD-*Tgf*, we selected 241 F₂-*Tgff/Tgf* (F₂-D *Tgff/Tgf*), consisting of 124 males and 117 females, out of a total of 1059 F₂ offspring. In the cross of C3H with NOD-*Tgf*, we selected 198 F₂-*Tgff/Tgf* (F₂-C *Tgff/Tgf*), consisting of 105 males and 93 females, out of a total of 841 F₂ offspring.

All pups were weaned at 4 weeks of age and were kept under pathogen-free conditions. Mice were fed *ad libitum* with standard laboratory chow (MF, Oriental Yeast Corp., Japan) and maintained in a regular light cycle of 12 h/12 h light/dark and the temperature was controlled at 22°C with a relative humidity of 50%. We extracted genomic DNA from tail tips of the F₂ progeny at 4 weeks of age. The genotype of the *TGF- β 1* transgene in F₂-*Tgff/Tgf* was determined by PCR as previously described (Moritani et al. 2005). The experimental protocol for animal use was approved by the Animal Care Committee of the University of Tokushima.

Measurement of phenotypes

We measured seven diabetes-related traits for F₂-*Tgff/Tgf*: nonfasting BW at 8 weeks of age (56 ± 2 days), five BG concentrations in *ipGTT* at 0, 30, 60, 120, and 240 min at 8 weeks of age (56 ± 2 days), and serum insulin concentrations at 30 min after glucose injection when killed at 10 weeks of age (70 ± 2 days). We performed *ipGTT* by injecting glucose (2 mg/g BW) in physiologic saline after fasting for 16 h. The mice were anesthetized with ether when killed and blood samples were collected from the abdominal aorta to determine serum insulin concentrations. The BG concentrations were monitored with a glucose analyzer (Antsence II, Bayer Sankyo Inc., Tokyo, Japan). Serum insulin concentrations were assayed with an ELISA kit using a mouse insulin standard (Morinaga Institute of Biological Science Inc., Kanagawa, Japan).

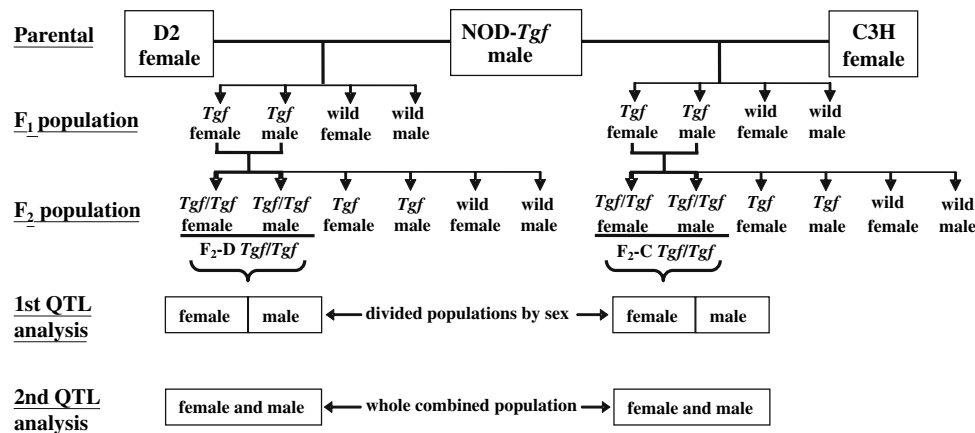


Fig. 1 Methodology for generation of F_2 - $Tgff/Tgff$. We obtained intercross mice by mating female D2 or C3H with male NOD- $Tgff$ to generate F_1 hybrids. The F_1 - $Tgff$ progeny were intercrossed to produce the F_2 generation. Genotyping of the $TGF-\beta 1$ transgene in F_2 - $Tgff/Tgff$ was determined by PCR. We performed genome-wide

QTL scans using R/qtl software. First, F_2 - $Tgff/Tgff$ was divided into male and female subpopulations, and each subpopulation was scanned. Second, male and female F_2 - $Tgff/Tgff$ subpopulations were combined and genome-wide scans were performed

Microsatellite markers

We selected well-validated microsatellite markers differing in size by 2 bp or more with 289 markers between D2 and NOD and 313 markers between C3H and NOD. We obtained the polymorphism information from the mouse genome database at the Mouse Microsatellite Data Base of Japan (<http://www.shigen.nig.ac.jp/mouse/mmdbj/top.jsp>), the Whitehead Institute/MIT Center (<http://www.genome.wi.mit.edu/cgi-bin/mouse/index>), and an online database of the Jackson Laboratory Center (<http://www.informatics.jax.org/>). We purchased microsatellite markers from Applied Biosystems (ABI, Foster City, CA, USA). Primer sequences are available on request.

Genotype analysis

Mice were genotyped for microsatellite markers with a 9700 thermal cycler (ABI) in 10- μ l reaction volumes. Every pair of primers consisted of a fluorescently labeled sense primer, including HEX, FAM, or NED, and a non-labeled antisense primer. PCR conditions were based on the manufacturer's recommendation, and the amplicons were electrophoretically separated with a PRISM 3700 capillary DNA sequencer (ABI) followed by analysis with GeneScan ver.3.0 software (ABI).

Linkage analysis

We performed genome-wide QTL scans using R/qtl software based on the EM algorithm (Broman et al. 2003; Sen and Churchill 2001) as an add-on package for the freely

available statistical language R (Ihaka and Gentleman 1996; <http://www.r-project.org/>). First, F_2 - $Tgff/Tgff$ were divided into male and female subpopulations, and each subpopulation was scanned. Second, all the male and female F_2 - $Tgff/Tgff$ were combined and scanned with sex as a covariate by the covariate-dependent genome scan using reported methods (Ahmadiyeh et al. 2003; Solberg et al. 2004) (Fig. 1). We analyzed the traits using sex as an additive covariate, and an additive and interactive covariate, with R/qtl software. We also performed permutation tests (Churchill and Doerge 1994) with R/qtl software to determine the trait-specific significance thresholds for seven traits. The 99th and 95th percentile points obtained by permutation testing (permutations = 5000) were taken as the significance threshold values.

Statistical analysis

Phenotypic data were presented as mean \pm SD or \pm SEM. Statistical analysis was carried out with JMP software ver. 4.05J (SAS Institute Japan Inc., Tokyo, Japan). Phenotypic comparisons in different genotypic groups were performed by the Tukey-Kramer's honestly significant difference test.

Results

BW, BG concentrations, and serum insulin concentrations in parental strains

Table 1 shows the results of comparing BW, BG concentrations in *ip*GTT, and serum insulin concentrations among parental strains of D2, C3H, NOD, NOD- $Tgff/Tgff$, and F_2

Table 1 BW, BG concentrations, and serum insulin concentrations of the parental strains and F₂ progeny

| | Parental | | | | F ₂ population | |
|----------------------------------------|--------------|--------------|---------------|-----------------------------|------------------------------------|------------------------------------|
| | NOD | D2 | C3H | NOD- <i>Tgff/Tgff</i> | F ₂ -D <i>Tgff/Tgff</i> | F ₂ -C <i>Tgff/Tgff</i> |
| Male | | | | | | |
| No. of mice | 5 | 5 | 5 | 5 | 124 | 105 |
| BW (g) at 8 weeks of age | 27.13 ± 1.73 | 24.75 ± 0.35 | 25.63 ± 0.18 | 23.22 ± 2.01* | 27.32 ± 2.51 ^{SS} | 26.53 ± 2.76 ^S |
| BG concentrations in <i>ipGTT</i> (mM) | | | | | | |
| 0 min | 2.3 ± 0.5 | 3.4 ± 1.1 | 4.3 ± 0.6 | 12.6 ± 8.9* | 8.9 ± 5.1 | 7.1 ± 4.0 ^S |
| 30 min | 13.5 ± 5.0 | 15.7 ± 3.4 | 17.1 ± 2.1 | 26.9 ± 9.4* | 23.8 ± 6.4 | 20.2 ± 4.6 ^S |
| 60 min | NT | NT | NT | 30.2 ± 8.1 | 20.7 ± 7.0 ^{SS} | 16.6 ± 5.2 ^{SS} |
| 120 min | NT | NT | NT | 24.6 ± 8.8 | 12.2 ± 6.1 ^{SS} | 9.5 ± 4.8 ^{SS} |
| Serum insulin at 30 min (ng/ml) | 1.17 ± 0.35 | 1.05 ± 0.23 | 1.50 ± 0.93 | 0.39 ± 0.12** | 0.39 ± 0.28 | 0.62 ± 0.42 |
| Female | | | | | | |
| No. of mice | 5 | 5 | 5 | 6 | 117 | 93 |
| BW (g) at 8 weeks of age | 21.61 ± 1.51 | 18.15 ± 0.35 | 17.30 ± 0.61* | 20.26 ± 1.60 | 22.20 ± 1.81 | 21.30 ± 2.20 |
| BG concentrations in <i>ipGTT</i> (mM) | | | | | | |
| 0 min | 2.1 ± 0.4 | 3.0 ± 0.3 | 3.7 ± 0.3 | 3.5 ± 0.9 | 5.4 ± 1.4 ^S | 5.5 ± 2.4 ^S |
| 30 min | 13.6 ± 4.7 | 8.3 ± 1.5 | 8.8 ± 1.8 | 19.2 ± 8.0 ^{##, †} | 18.4 ± 4.7 | 15.7 ± 4.4 |
| 60 min | NT | NT | NT | 14.4 ± 8.4 | 12.3 ± 4.4 | 10.3 ± 4.7 |
| 120 min | NT | NT | NT | 6.8 ± 5.7 | 6.3 ± 2.8 | 6.2 ± 4.1 |
| Serum insulin at 30 min (ng/ml) | 1.10 ± 0.41 | 1.02 ± 0.31 | 0.88 ± 0.31 | 0.16 ± 0.10** | 0.69 ± 0.34 | 0.63 ± 0.30 |

Traits are given as mean ± SD

* Significant difference at $p < 0.05$ vs. NOD in parental strains; ** significant difference at $p < 0.01$, vs. NOD in parental strains

Significant difference at $p < 0.01$ vs. D2 in parental strains

† Significant difference at $p < 0.05$ vs. C3H in parental strains

^S Significant difference at $p < 0.05$ and ^{SS} significant difference at $p < 0.01$, F₂-D *Tgff/Tgff* or F₂-C *Tgff/Tgff* vs. NOD-*Tgff/Tgff*

NT = not tested

progeny of F₂-D *Tgff/Tgff* and F₂-C *Tgff/Tgff*. The male NOD-*Tgff/Tgff* showed decreased BW and increased BG concentrations in *ipGTT* at 0 and 30 min compared to NOD ($p < 0.05$, for both). The female NOD-*Tgff/Tgff* showed increased BG concentrations in *ipGTT* at 30 min compared to D2 and C3H ($p < 0.01$ and < 0.05 , respectively). Female C3H showed decreased BW compared to NOD. Insulin concentrations were not different in any pair of parental strains among NOD, D2, and C3H in either sex. The male and female NOD-*Tgff/Tgff* showed decreased insulin concentrations in *ipGTT* at 30 min compared to NOD ($p < 0.01$, for both).

The male F₂-D *Tgff/Tgff* showed increased BW and decreased BG concentrations in *ipGTT* at 60 and 120 min compared to NOD-*Tgff/Tgff* ($p < 0.01$, for both). The male F₂-C *Tgff/Tgff* showed increased BW ($p < 0.05$) and decreased BG concentrations in *ipGTT* at 0, 30, 60, and 120 min compared to NOD-*Tgff/Tgff* ($p < 0.05$, 0.05, 0.01, and 0.01, respectively). The female F₂-D *Tgff/Tgff* showed increased BG concentrations in *ipGTT* at 0 min compared to NOD-*Tgff/Tgff* ($p < 0.05$). The female F₂-C *Tgff/Tgff*

showed increased BG concentrations in *ipGTT* at 0 min compared to NOD-*Tgff/Tgff* ($p < 0.05$). The male F₂-D *Tgff/Tgff* and the male F₂-C *Tgff/Tgff* did not show significant differences in serum insulin concentrations compared to NOD-*Tgff/Tgff*. BG concentrations in *ipGTT* at 240 min did not show any significant differences (data not shown).

Six major QTLs in F₂-D *Tgff/Tgff* and F₂-C *Tgff/Tgff*

We performed genome scans in male and female subpopulations to map the QTLs responsible for the conditional effects. Based on the threshold values assessed by the permutation test (Supplementary Table 1), we identified one significant QTL in the male F₂-D *Tgff/Tgff* on Chr 13 and five significant QTLs in the male or female F₂-C *Tgff/Tgff* on Chrs 1 and 7 (female subpopulation) and on Chr 2, 14, and 18 (male subpopulation) (Table 2). In the male F₂-D *Tgff/Tgff* subpopulation, only one QTL on Chr 13 at *D13Mit54*, which was designated as *Tdn1* (homozygous *Tgff-β1* transgenic F₂ intercross progeny between D2 and

Table 2 QTLs by genome-wide scans and LOD scores in covariate analysis with sex as a covariate in six QTLs

| Locus | | | | | LOD score of divided F ₂ population by sex | | | | | Combined population with sex | | |
|-----------------------------------------------|-----|------------------|------|--------|-------------------------------------------------------|----------------------------|--------|--------|---------|------------------------------|-------------------------------|---------------------------|
| Designated name | Chr | Closest marker | cM | Sex | BW at 8 weeks of age | BG concentrations in ipGTT | | | | Serum insulin at 30 min | LOD no covariate ^a | LOD with sex ^b |
| | | | | | | 0 min | 30 min | 60 min | 120 min | | | |
| F ₂ -D <i>Tgff/Tgf</i> <i>Tdn1</i> | 13 | <i>D13Mit54</i> | 27.5 | male | 4.39* | 0.27 | 0.17 | 0.58 | 0.15 | 0.27 | 2.20 | 5.77 |
| | | | | female | 0.27 | 0.46 | 0.69 | 0.46 | 0.32 | 0.49 | | |
| <i>Tcn1</i> | 1 | <i>D1Mit18</i> | 22.3 | male | 3.66 | 0.04 | 0.01 | 0.15 | 0.15 | 1.06 | 3.73 | 4.18 |
| | | | | female | 2.10 | 0.38 | 0.39 | 0.03 | 0.21 | 4.41* | | |
| F ₂ -C <i>Tgff/Tgf</i> <i>Tcn2</i> | 7 | <i>D7Mit321</i> | 54.0 | male | 0.45 | 0.22 | 0.71 | 0.86 | 0.61 | 1.24 | 4.17 | 4.75 |
| | | | | female | 0.70 | 0.74 | 4.38* | 2.40 | 1.32 | 1.22 | | |
| <i>Tcn3</i> | 2 | <i>D2Mit287</i> | 75.6 | male | 4.94** | 0.64 | 0.35 | 0.22 | 0.11 | 1.64 | 6.46 | 7.01 |
| | | | | female | 1.50 | 0.49 | 1.63 | 0.70 | 0.32 | 0.28 | | |
| <i>Tcn4</i> | 14 | <i>D14Mit5</i> | 33.8 | male | 0.62 | 3.25 | 2.65 | 3.15 | 3.78* | 0.11 | 5.47 | 5.77 |
| | | | | female | 0.20 | 0.79 | 0.09 | 0.18 | 1.77 | 0.83 | | |
| <i>Tcn5</i> | 18 | <i>D14Mit123</i> | 38.3 | male | 0.12 | 3.99* | 1.83 | 2.10 | 2.93 | 0.01 | 4.40 | 5.91 |
| | | | | female | 0.29 | 0.66 | 0.07 | 0.38 | 1.86 | 1.08 | | |
| <i>Tcn5</i> | 18 | <i>D18Mit7</i> | 43.6 | male | 0.01 | 0.49 | 0.98 | 0.55 | 0.44 | 4.99** | 2.28 | 6.42 |
| | | | | female | 0.56 | 0.46 | 1.15 | 0.23 | 0.04 | 0.22 | | |

* Level of significance at $p < 0.05$; ** level of significance at $p < 0.01$

^a Result of LOD score with sex as additive covariate (no interactive covariates)

^b Result of LOD score with sex as additive and interactive covariates

NOD 1), showed linkage to BW at 8 weeks of age (LOD = 4.39) (Fig. 2A).

In the female F₂-C *Tgff/Tgf* subpopulation, two significant QTLs on Chr 1 and 7 were identified. One QTL on Chr 1 at *D1Mit18*, designated as *Tcn1* (homozygous *Tgff-β1* transgenic F₂ intercross progeny between C3H and NOD 1), showed linkage to serum insulin at 30 min (LOD = 4.41) (Fig. 2B). The other QTL on Chr 7 at *D7Mit321*, which was designated as *Tcn2*, showed linkage to BG concentrations in ipGTT at 30 min (LOD = 4.38) (Fig. 2C). In the male F₂-C *Tgff/Tgf* subpopulation, three significant QTLs on Chr 2, 14, and 18 were identified. One QTL on Chr 2 at *D2Mit287*, which was designated as *Tcn3*, showed linkage to BW at 8 weeks of age (LOD = 4.94) (Fig. 2D). On Chr 14, two interdependent QTLs at *D14Mit123* and *D14Mit5*, which were designated as *Tcn4*, showed linkage to BG concentrations in ipGTT at 0 and 120 min (LOD = 3.99, 3.78, respectively) (Fig. 2E). The two peaks were separated by only 4.5 cM. One QTL on Chr 18 at *D18Mit7* (designated as *Tcn5*) showed linkage to serum insulin at 30 min (LOD = 4.99) (Fig. 2F). *Tcn3*, which showed linkage to BW at 8 weeks of age, and *Tcn5*, which showed linkage to serum insulin at 30 min, showed significance levels at $p < 0.01$, and others (*Tdn1*, *Tcn1*, 2, and 4) satisfied significance threshold levels at $p < 0.05$.

Covariate-dependent genome scans with sex as a covariate in the six QTLs

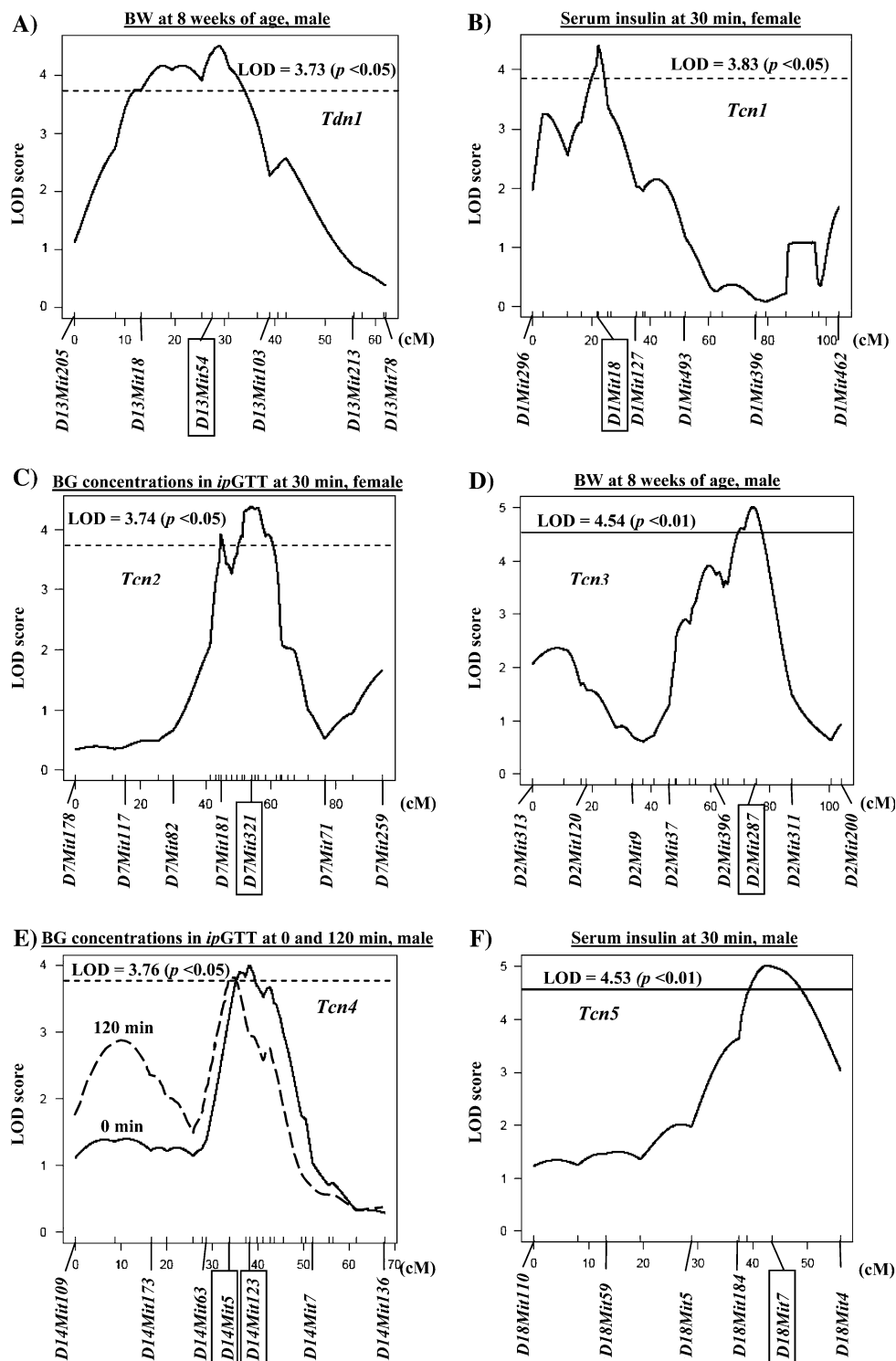
The far right column in Table 2 shows the results of the covariate-dependent scans for the combined population of male and female F₂-*Tgff/Tgf* with sex as a covariate. All loci showed higher LOD scores with sex as an additive and interactive covariate than those without sex as an interactive covariate, suggesting a sex-dependent effect. *Tdn1* at *D13Mit54* and *Tcn5* at *D18Mit7* showed clear differences in LOD scores with sex as an additive and interactive covariate.

Effect of the genotype on traits in the significant QTLs

To evaluate genotypic effects of the six significant QTLs, mice were divided according to genotypes of the marker of the QTLs for *D2/D2* (D2 homozygote), *D2/NOD* (heterozygote), and *NOD/NOD* (NOD homozygote) in F₂-D *Tgff/Tgf* and *C3H/C3H* (C3H homozygote), *C3H/NOD* (heterozygote), and *NOD/NOD* in F₂-C *Tgff/Tgf* (Table 3).

For BW, at *Tdn1* in F₂-D *Tgff/Tgf*, the *NOD* allele at this locus increased BW in a dominant manner (Fig. 3A). At *Tcn3* in F₂-C *Tgff/Tgf*, the *C3H* allele at this locus increased BW in a dominant manner (Fig. 3B). For

Fig. 2 Identification of six significant QTLs on Chr 13 in F_2 -D $Tgfl/Tgf$ and on Chrs 1, 2, 7, 14, and 18 in F_2 -C $Tgfl/Tgf$. The boxed markers denote the maximum LOD scores for markers for each locus. Solid lines and dotted lines denote the significance levels of LOD scores at $p < 0.01$ and $p < 0.05$, respectively. **A** Interval mapping on Chr 13 for BW at 8 weeks of age. Fifteen microsatellite markers were assayed. **B** Interval mapping on Chr 1 for serum insulin concentrations in ip GTT at 30 min. Twenty-two microsatellite markers were assayed. **C** Interval mapping on Chr 7 for BG concentrations in ip GTT at 30 min. Thirty-one microsatellite markers were assayed. **D** Interval mapping on Chr 2 for BW at 8 weeks. Twenty-three microsatellite markers were assayed. **E** Interval mapping on Chr 14 for BG concentrations in ip GTT at 0 and 120 min. Twenty-eight microsatellite markers were assayed. **F** Interval mapping on Chr 18 for BG concentrations in ip GTT at 120 min. Nine microsatellite markers were assayed



serum insulin at 30 min, at *Tcn1* in F_2 -C $Tgfl/Tgf$, the C3H allele at this locus increased serum insulin concentrations in an additive manner (Fig. 3C). At *Tcn5* in F_2 -C $Tgfl/Tgf$, the C3H allele at this locus increased serum insulin at 30 min in a recessive manner (Fig. 3D).

For BG concentrations in ip GTT, at *Tcn2* in F_2 -C $Tgfl/Tgf$, the C3H allele at this locus increased BG concentrations in a dominant manner (Fig. 4A). At *Tcn4*, the NOD allele at this locus increased BG concentrations in a recessive manner (Fig. 4B, C).

Table 3 BW, BG concentrations, and serum insulin concentrations in six QTLs in *F₂-Tg/Tgf* progeny among three genotypes between D2 and NOD or C3H and NOD

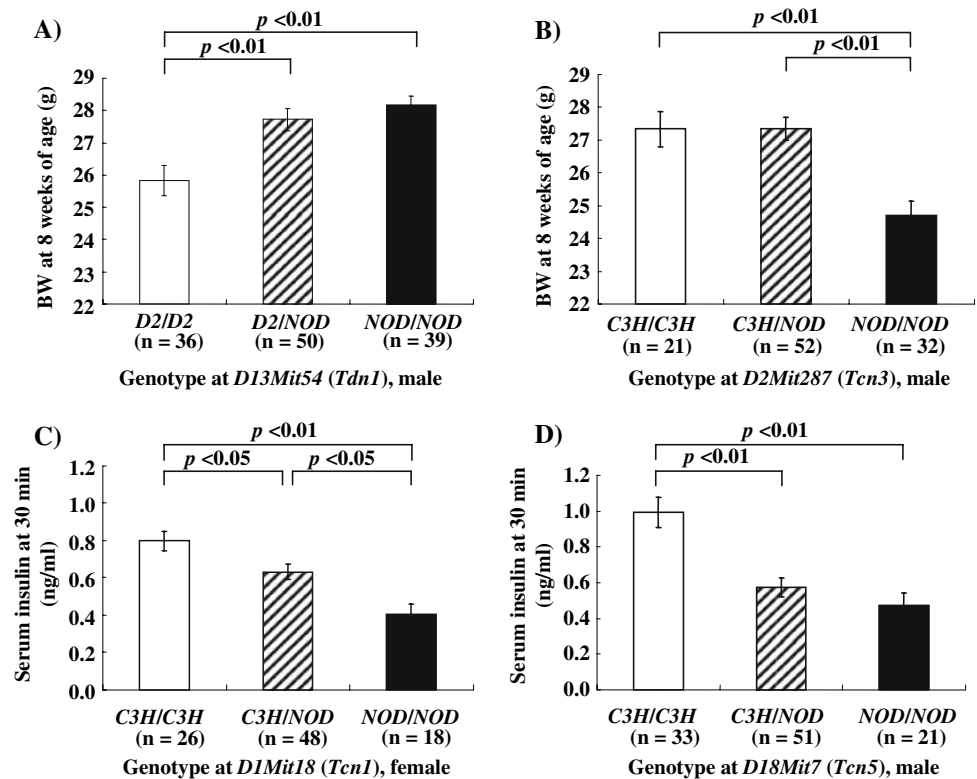
| Cross | Designated name | Sex | Chr | Closest marker | Phenotype | Maximum LOD score | BW at 8 weeks of age (g) | | | <i>p</i> value |
|-------------------------------|-----------------|--------|-----|------------------|-----------------------------------------------|-------------------|---------------------------------|---------------------------------|---------------------------------|--------------------------------------------------------|
| | | | | | | | D2/D2 | D2/NOD | NOD/NOD | |
| <i>F₂-D Tg/Tgf</i> | <i>Tdn1</i> | male | 13 | <i>D13Mit54</i> | BW at 8 weeks of age | 4.39 | 25.8 ± 0.5 (<i>n</i> = 36) | 27.7 ± 0.3 (<i>n</i> = 50) | 28.2 ± 0.4 (<i>n</i> = 39) | D2/D2 vs. NOD/NOD <0.01 D2/D2 vs. NOD/NOD <0.01 |
| <i>F₂-C Tg/Tgf</i> | <i>Tcn3</i> | male | 2 | <i>D2Mit287</i> | BW at 8 weeks of age | 4.94 | 27.3 ± 0.5 (<i>n</i> = 21) | 27.3 ± 0.3 (<i>n</i> = 45) | 24.7 ± 0.4 (<i>n</i> = 27) | C3H/C3H vs. NOD/NOD <0.01 C3H/C3H vs. NOD/NOD <0.01 |
| <i>F₂-C Tg/Tgf</i> | <i>Tcn2</i> | female | 7 | <i>D7Mit321</i> | BG concentrations in <i>ip</i> GTT at 30 min | 4.38 | 16.5 ± 0.7 (<i>n</i> = 21) | 17.2 ± 0.7 (<i>n</i> = 45) | 12.7 ± 0.7 (<i>n</i> = 27) | C3H/C3H vs. NOD/NOD <0.01 C3H/C3H vs. NOD/NOD <0.01 |
| <i>F₂-C Tg/Tgf</i> | <i>Tcn4</i> | male | 14 | <i>D14Mit123</i> | BG concentrations in <i>ip</i> GTT at 0 min | 3.99 | 6.2 ± 0.2 (<i>n</i> = 28) | 6.2 ± 0.2 (<i>n</i> = 50) | 9.8 ± 1.3 (<i>n</i> = 27) | C3H/C3H vs. NOD/NOD <0.01 C3H/C3H vs. NOD/NOD <0.01 |
| <i>F₂-C Tg/Tgf</i> | <i>Tcn5</i> | male | 14 | <i>D14Mit5</i> | BG concentrations in <i>ip</i> GTT at 120 min | 3.78 | 8.1 ± 0.8 (<i>n</i> = 30) | 8.8 ± 0.6 (<i>n</i> = 47) | 12.6 ± 0.8 (<i>n</i> = 28) | C3H/C3H vs. NOD/NOD <0.01 C3H/C3H vs. NOD/NOD <0.01 |
| <i>F₂-C Tg/Tgf</i> | <i>Tcn1</i> | female | 1 | <i>D1Mit18</i> | Serum insulin at 30 min | 4.41 | 0.80 ± 0.05 (<i>n</i> = 26) | 0.63 ± 0.04 (<i>n</i> = 48) | 0.41 ± 0.05 (<i>n</i> = 18) | C3H/C3H vs. NOD/NOD <0.01 C3H/C3H vs. NOD/NOD <0.05 |
| <i>F₂-C Tg/Tgf</i> | <i>Tcn5</i> | male | 18 | <i>D18Mit7</i> | Serum insulin at 30 min | 4.99 | 0.99 ± 0.08 (<i>n</i> = 20) | 0.57 ± 0.05 (<i>n</i> = 51) | 0.47 ± 0.07 (<i>n</i> = 32) | C3H/C3H vs. NOD/NOD <0.01 C3H/C3H vs. NOD/NOD <0.01 |

Mice were grouped according to the marker that showed linkage with the significant LOD score

Traits are given as mean ± SEM

p values denote the significance level in the Tukey-Kramer honestly significant difference test

Fig. 3 Comparison of BW and serum insulin concentrations according to the genotype of the marker for each QTL. White columns denote mice homozygous for the D2 allele (*D2/D2*) or C3H allele (*C3H/C3H*). Striped columns denote mice heterozygous for the D2 and NOD alleles (*D2/NOD*) or C3H and NOD alleles (*C3H/NOD*). Black columns denote mice homozygous for the NOD allele (*NOD/NOD*). Error bars indicate SEM. **A** BW at 8 weeks of age in male *F₂-D Tgff/Tgff* according to the genotype at *D13Mit54*. **B** BW at 8 weeks of age in male *F₂-C Tgff/Tgff* according to the genotype at *D2Mit287*. **C** Serum insulin concentrations at 30 min in female *F₂-C Tgff/Tgff* according to the genotype at *D1Mit18*. **D** Serum insulin concentrations at 30 min in male *F₂-C Tgff/Tgff* is shown according to the genotype at *D18Mit7*



Discussion

Rodent models of human disease have been widely used to dissect the determinants of polygenic pathologies such as T2D. In this study, we took advantage of genetically determined diabetes and glucose intolerance due to insulin deficiency using the transgenic model of *NOD-Tgff/Tgff* to dissect genetic modifier QTLs. *NOD* have been studied as a model of human type 1 diabetes (T1D) and are under the control of multiple *Idd* loci (*Idd1-Idd27*) (Chen et al. 2005; Deruytter et al. 2004; Hall et al. 2003; Mathews et al. 2003; Reifsnyder et al. 2005; Rogner et al. 2001; Serreze and Leiter 2001). It was recently reported that *NOD* express mRNA profiles common to both T1D and T2D, suggesting they share a common molecular etiology (Chaparro et al. 2006). We analyzed *F₂-Tgff/Tgff* to identify the gene(s) that modify the severity of diabetes, impaired glucose tolerance, and/or insulin deficiency.

We observed differences in BW at 8 weeks of age and in BG concentrations in *ipGTT* in the *F₂-D Tgff/Tgff* or *F₂-C Tgff/Tgff* compared with *NOD-Tgff/Tgff*, respectively. Both *F₂-D Tgff/Tgff* and *F₂-C Tgff/Tgff* showed the higher BW ($p < 0.01$, 0.05 , respectively) than *NOD-Tgff/Tgff* in the male subpopulation. The measured traits were distributed more diversely in *F₂-D Tgff/Tgff* and *F₂-C Tgff/Tgff* than in those parental strains. These results suggest that the difference of diabetes-related phenotypes is strongly

dependent on the genetic backgrounds of C3H and D2. The genetic polymorphisms between D2 and *NOD* or between C3H and *NOD* would account for the differences in BW, BG concentrations, or insulin concentrations.

We examined subpopulations in the *F₂* population divided by sex. These subpopulations should have excluded false QTLs by standardization of the structure of the *F₂* populations. We detected six QTLs, including one QTL on Chr 13 (*Tdn1*) in the male *F₂-D Tgff/Tgff* subpopulation and five QTLs in *F₂-C Tgff/Tgff*, including QTLs on Chr 1 (*Tcn1*) and 7 (*Tcn2*) in the female *F₂-C Tgff/Tgff* subpopulation as well as those on Chr 2 (*Tcn3*), 14 (*Tcn4*), and 18 (*Tcn5*) in the male *F₂-C Tgff/Tgff* subpopulation. We detected subpopulation-dependent significant QTLs in one phenotype except *Tcn4* that modify BW, BG concentrations, or serum insulin concentrations. No common QTL was observed in two *F₂* populations, suggesting that most QTLs were strain- and sex-specific QTLs, especially in *F₂-C Tgff/Tgff*.

QTL studies in a variety of diabetic models have reported many QTLs (Hirayama et al. 1999; Kido et al. 2000; Komatsu et al. 2002; Leiter et al. 1998; Moritani et al. 2005; Takeshita et al. 2006; Ueda et al. 1999). Three (*Tcn1*, *Tcn2*, and *Tcn4*) of six QTLs identified in this study overlapped QTLs in previous reports. The QTL on Chr 1 at *D1Mit18* (*Tcn1*) that affected serum insulin concentrations in the female *F₂-C Tgff/Tgff* apparently coincided with the

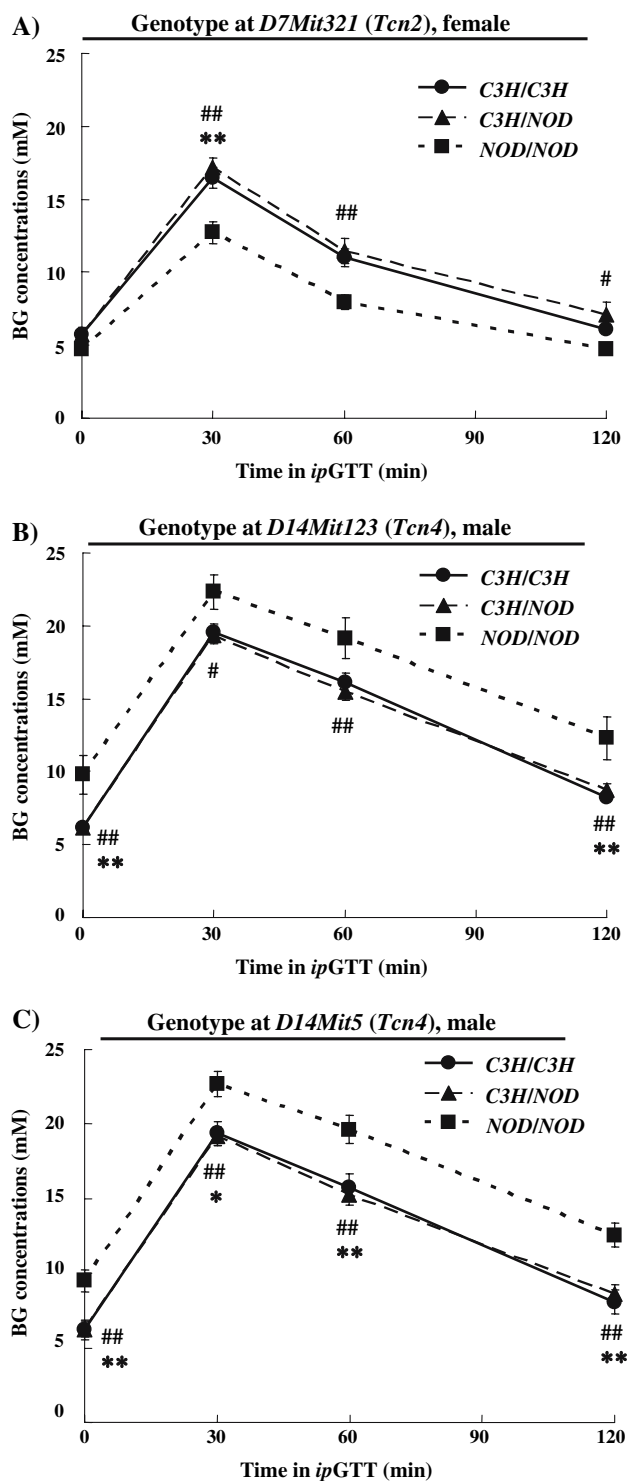


Fig. 4 Comparison of BG concentrations in ipGTT after 16 h of fasting, according to the genotypes at *Tcn2* and *Tcn4*. Closed circles denote mice homozygous for the C3H allele (*C3H/C3H*). Closed triangles denote mice heterozygous for the C3H and NOD alleles (*C3H/NOD*). Closed squares denote mice homozygous for the NOD allele (*NOD/NOD*). **A** BG concentrations in ipGTT at *D7Mit321* (*Tcn2*). **B** BG concentrations in ipGTT at *D14Mit123* (*Tcn4*). **C** BG concentrations in ipGTT at *D14Mit5* (*Tcn4*). Error bars indicate SEM. *C3H/C3H* vs. *NOD/NOD*: * $p < 0.01$, ** $p < 0.01$. *C3H/NOD* vs. *NOD/NOD*: # $p < 0.05$, ## $p < 0.01$

QTL reported in F_2 ($B6^{IR} \times 129^{IR}$) mice with a heterozygous insulin receptor mutation showing linkage to insulin resistance (Kido et al. 2000). Although the QTLs identified in both studies are located in apparent proximity on Chr 1 at *D1Mit19* (36.9 cM, data from Mouse Genome Informatics) in F_2 ($B6^{IR} \times 129^{IR}$) and at *D1Mit18* (29.7 cM, data from Mouse Genome Informatics) in F_2 -C *Tgfl/Tgf*, they are separated from each other by 7.2 cM, and the former linkage peak is very broad. F_2 intercross progeny of different pairs of strains are supposed to be associated with different sets of polymorphisms. *Insulin receptor substrate 1* (*Irs1*: 57.0 cM, 82 Mb) and *Inositol polyphosphate-5-phosphatase D* (*Inpp5d*: 57.0 cM, 89 Mb), which can modify insulin signal transduction, are separated from *D1Mit18* by 27.3 cM and not regarded as the genes responsible for *Tcn1* locus. All of these suggest that different causal genes are responsible for two different phenotypes of insulin resistance and secretion.

QTL on Chr 7 at *D7Mit321* (*Tcn2*) that affect BG concentrations in ipGTT at 30 min in the female F_2 -C *Tgfl/Tgf* coincided with the QTL reported in F_2 ($KK-A^y \times PWK$) mice showing linkage to BG concentrations (Komatsu et al. 2002). The linkage peak (*D7Mit130*) reported by Komatsu et al. is separated by about 2.6 cM from our peak of *D7Mit321*. The QTL on Chr 14 at *D14Mit123* to *D14Mit5* (*Tcn4*), affecting BG concentrations in ipGTT at 0 and 120 min in the male F_2 -C *Tgfl/Tgf*, coincided with the QTL reported in F_2 ($NSY \times C3H$) mice showing linkage to BG concentrations and serum insulin concentrations (Ueda et al. 1999). This F_2 ($NSY \times C3H$) mouse locus (*D14Mit5*) coincided with our LOD score peak at *D14Mit5*, which suggests the presence of a modifier QTL around *Tcn4*.

Tcn1, *Tcn2*, and *Tcn4* contain several putative candidate susceptibility genes for diabetes. These candidates include the following: growth differentiation factor 8 (*Mstn*; myostatin), heat shock protein 1 (*Hspe1*; chaperonin) on Chr 1 (*Tcn1*), *UCP2* and 3, calcitonin/calcitonin-related polypeptide α (*Calca*) on Chr 7 (*Tcn2*), phosphoenolpyruvate carboxykinase 2 (*Pck2*; known to be involved in gluconeogenesis), and gonadotropin releasing hormone 1 (*Gnrh-1*) on Chr 14 (*Tcn4*). The remaining three QTLs (*Tdn1*, *Tcn3*, and *Tcn5*) in this study did not overlap any other previously reported QTLs and were specific to *NOD-Tgfl/Tgf*. Interestingly, two of these new QTLs (*Tcn3* and *Tcn5*) show higher levels of significance ($p < 0.01$). *Tdn1*, *Tcn3*, and *Tcn5* contain several putative candidate susceptibility genes, including prolactin (*Prl*), docking protein 3 (*Dok3*) on Chr 13 (*Tdn1*), growth hormone releasing hormone (*Ghrh*), nonagouti (a regulator of melanogenesis), hepatic nuclear factor 4 alpha (*Hnf4a*) on Chr 2 (*Tcn3*), and myelin basic protein (*Mbp*), melanocortin 4 receptor (*Mc4r*) on Chr 18 (*Tcn5*). Their possible roles in T2D

remain largely unknown, and further study, including production of congenic, transgenic, or knockout mice, or candidate gene expression analysis, will be necessary to confirm their roles as susceptibility gene(s) for T2D.

Out of *Idd1-Idd27*, *Idd13* (Chr 2), *Idd14* (Chr 13), *Idd26* (Chr 1), and *Idd27* (Chr 7) were the same loci as *Tcn3*, *Tdn1*, *Tcn1*, and *Tcn2*, respectively, in our study. Other QTLs (*Tcn4* and *Tcn5*) were not included in the *Idd* loci. Among six identified QTLs, we regard *Tcn2* as a particularly important QTL for the following reasons: First, this locus shows replicated linkage evidence in previous reports. Second, it overlaps locus *Idd27*. Finally, it contains putative candidate susceptibility genes for T2D, i.e., *Ucp2* and *Ucp3*, known to be important for energy dissipation as heat (Dalgaard and Pedersen 2001).

Taking the *Ucp2* and *Ucp3* genes as putative candidates, we used an expression quantitative trait loci (eQTL) analysis by regarding their mRNA levels in soleus muscle as quantitative traits (see supplementary materials). We found that *Ucp2* mRNA level was linked to the *Tcn2* locus (LOD = 4.90) (Supplementary Table 2 and Fig. 1), while *Ucp3* mRNA level was not linked. Covariate analysis suggested the possibility that *Ucp2* mRNA is a putative susceptibility gene in the *Tcn2* locus. The peak of linkage curve of *Ucp2* mRNA was, however, shifted by about 7.8 cM toward the telomeric side of Chr 7 from the peak of *Tcn2*. In addition, *Tcn2* showed a recessive mode of inheritance for BG concentrations, but *Ucp2* expression showed a dominant mode of inheritance for NOD allele. This shift might be due to the limited number of female F_2 -C *Tgf/Tgf*. The different modes of inheritance might be due to the epistatic effects of other genes in the F_2 genetic background. The candidate genetic polymorphisms in *Tcn2* conferring disease susceptibility to T2D include *cis*-regulation of *Ucp2* mRNA levels in soleus muscle. The possibility that the *Ucp2* gene is one of the candidate genes in *Tcn2* has to be confirmed by congenic mice. The six QTLs, including *Tcn2*, have the potential to provide targets to control diabetes-related traits, including BW, BG concentrations, and serum insulin concentrations. However, it is not clear whether these QTLs are specifically influenced by *TGF- β 1* transgene. To clarify whether the identified QTLs modify the diabetic phenotypes specifically induced by the *TGF- β 1* transgene, it is necessary to perform genome-wide QTL analysis using F_2 intercross progeny without the *TGF- β 1* transgene.

We conclude that genome-wide scans of the F_2 population divided by sex identified six significant modifier QTLs, *Tdn1* (Chr 13) in the male F_2 -D *Tgf/Tgf* subpopulation, *Tcn1* (Chr 1), *Tcn2* (Chr 7) in the female F_2 -C *Tgf/Tgf* subpopulation, and *Tcn3* (Chr 2), *Tcn4* (Chr 14), and *Tcn5* (Chr 18) in the male F_2 -C *Tgf/Tgf* subpopulation for BW, BG concentrations, and serum insulin concentrations. *Tcn2* showed linkage to BG concentrations and *Ucp2*

mRNA levels, and covariate analysis showed that *Tcn2* depends on the *Ucp2* mRNA levels. Therefore, the *Ucp2* gene and other candidate genes in the six QTLs remain to be examined for the presence of functional SNPs that could modify diabetes-related traits.

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