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Insulin Transactivator MafA Regulates Intrathymic Expression of Insulin and Affects Susceptibility to Type 1 Diabetes

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OBJECTIVE—Tissue-specific self-antigens are ectopically expressed within the thymus and play an important role in the induction of central tolerance. Insulin is expressed in both pancreatic islets and the thymus and is considered to be the primary antigen for type 1 diabetes. Here, we report the role of the insulin transactivator MafA in the expression of insulin in the thymus and susceptibility to type 1 diabetes.

RESEARCH DESIGN AND METHODS—The expression profiles of transcriptional factors (*Pdx1*, *NeuroD*, *Mafa*, and *Aire*) in pancreatic islets and the thymus were examined in nonobese diabetic (NOD) and control mice. Thymic *Ins2* expression and serum autoantibodies were examined in *Mafa* knockout mice. Luciferase reporter assay was performed for newly identified polymorphisms of mouse *Mafa* and human *MAFA*. A case-control study was applied for human *MAFA* polymorphisms.

RESULTS—*Mafa*, *Ins2*, and *Aire* expression was detected in the thymus. *Mafa* expression was lower in NOD thymus than in the control and was correlated with *Ins2* expression. Targeted disruption of *MafA* reduced thymic *Ins2* expression and induced autoantibodies against pancreatic islets. Functional polymorphisms of MafA were newly identified in NOD mice and humans, and polymorphisms of human *MAFA* were associated with susceptibility to type 1 diabetes but not to autoimmune thyroid disease.

CONCLUSIONS—These data indicate that functional polymorphisms of MafA are associated with reduced expression of insulin in the thymus and susceptibility to type 1 diabetes in the NOD mouse as well as human type 1 diabetes. ***Diabetes* 59: 2579–2587, 2010**

Type 1 diabetes is caused by autoimmune destruction of insulin-producing β-cells of the pancreas in genetically susceptible individuals (1,2). Susceptibility to type 1 diabetes is under polygenic control, with *IDDM1* in the major histocompatibility complex (MHC) showing the strongest effect (3). In addition to MHC-linked susceptibility, the contribution of several non-MHC genes has been reported (3–6). Most of the non-MHC genes identified to date are immune-regulating genes, which are considered to contribute to type 1 diabetes susceptibility through impaired regulation of autoimmune T-cell activation. Among these are genes encoding cytotoxic T-lymphocyte antigen 4 (*CTLA4*) in humans and mice (7), lymphoid tyrosine phosphatase (*PTPN22*) (8) in humans, and *Cblb* (9) and *Ian4* (10) in rats. Most of these genes are therefore expected to confer susceptibility to autoimmune diseases in general but not to an autoimmune disease in a specific organ, as evidenced by the association of these genes with not only type 1 diabetes but also other autoimmune diseases, such as autoimmune thyroid diseases, rheumatoid arthritis, and/or systemic lupus erythematosus (7,11–13).

In contrast to immune-regulating genes conferring susceptibility to autoimmune diseases through dysregulation of T-cell activation, genes leading to organ specificity are largely unknown, with the only exception being *IDDM2* located in the promoter region of the insulin gene (*INS*). *IDDM2* is most likely to be encoded by a variable-number tandem repeat (VNTR) polymorphism in the *cis*-regulatory region of the insulin gene, which is associated with type 1 diabetes susceptibility through reduced expression of the insulin gene in the thymus (14–17). Accumulating lines of evidence indicate that tissue-specific self-antigens, including insulin, are also expressed within the thymus and play an important role in the induction of central tolerance (18–20). Abnormality in the regulation of intrathymic expression of self-antigen is therefore expected to cause autoimmune disease through impaired negative selection of antigen-specific autoreactive T-cells. Since expression of autoantigens in the thymus is regulated not only by *cis*-regulatory elements in the genes encoding self-antigens, as in the case of *IDDM2*, but also by *trans*-acting factors, tissue-specific transactivators regulating the expression of self-antigens are important candidate genes for organ-specific autoimmune diseases.

MafA has been identified as an islet-enriched transcriptional activator that binds to the RIPE3b1 element in the promoter of the insulin gene and has been postulated to

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regulate insulin transcription in response to serum glucose level in β-cells of the pancreas (21,22). Unlike previously known islet-enriched transcriptional factors, such as Pdx1 and NeuroD/BETA2, which are expressed in non–β-cells as well as in β-cells, the expression of MafA is restricted to only β-cells, suggesting that MafA is responsible for tissue-specific expression of insulin (23). MafA is therefore an important candidate among these transcriptional activators of insulin, leading to organ-specific autoimmunity to pancreatic β-cells by dysregulation of transcription of insulin in the thymus as an organ-specific self-antigen. Here, we report that MafA is expressed in the thymus and colocalizes with and modulates the expression of insulin in the thymus. Functional variants of mouse *Mafa* and human *MAFA* were identified and found to be associated with the expression level of insulin in the thymus and susceptibility to type 1 diabetes.

RESEARCH DESIGN AND METHODS

Female and male nonobese diabetic (NOD)/shi, NOD.nonobese nondiabetic (NON)-Mhc(H2) congenic (NOD.NON-H2), C3H/He, and NSY mice (24) were housed under specific pathogen-free conditions. All experiments were conducted in accordance with the Osaka University Guidelines, which are based on the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. ICR mice with targeted disruption of *Mafa* (Mafa knockout mice) were provided by S.T. (25).

Semiquantitative RT-PCR. Pancreatic islets were isolated from four or five female NOD.NON-H2 or C3H mice by collagenase digestion, as described previously (26). To collect insulitis-free pancreatic islets, NOD.NON-H2 mice, instead of NOD mice, were used for isolation of pancreatic islets for RT-PCR analysis. Total RNA was isolated from mouse pancreatic islets (at 14–16 weeks old) and thymus (at 3–25 days old) using Isogen (Nippon Gene, Toyama, Japan) and treated with 10 units RNase-free DNase I (Takara, Shiga, Japan) to remove genomic DNA. Then, 800 ng total RNA from each sample was subjected to cDNA synthesis using oligo-dT primers (ReverTra Aceα; Toyobo, Tokyo, Japan) (additional supplementary materials and methods for this article can be found in an online appendix, available at http://diabetes.diabetesjournals.org/cgi/content/full/db10-0476/DC1). PCR was performed using Ex *Taq* (Takara) within the log phase of the reaction (24–32 cycles). mRNA levels were measured by nonradioactive RT-PCR and charged-coupled device imaging, as described previously (27).

Immunohistochemical staining. The thymus was embedded in OCT compound (Tissue-TEC; Miles, Elkhart, IN) and frozen by an acetone–dry-ice method. Then, 6-μm-thick frozen sections were cut with a cryostat, placed on slides, and fixed in cold acetone for 10 min. The sections were then rinsed in PBS, incubated for 5 min in 1% Triton X-100, and, after a second rinse, incubated in diluted serum derived from the same animal as the blocking serum. The sections were preincubated with 2.4G2 to block the Fcγ receptor and incubated for 60 min at room temperature with the first antibodies, washed with PBS, and incubated for 60 min on ice with fluorescein-conjugated second antibodies. The first antibodies were guinea pig anti-porcine insulin antibody (Dako Japan, Kyoto, Japan), rabbit anti-mouse MafA antibody (Bethyl Laboratories, Montgomery, TX), and 1:100 diluted sera from Mafa$^{+/+}$ or Mafa$^{-/-}$ mice. Alexa Fluor 488–conjugated anti-rabbit IgG, Alexa Fluor 594–conjugated anti-guinea pig IgG, and Alexa Fluor 488–conjugated anti-mouse IgG were used as second antibodies. The sections were examined using a Provis AX80 (Olympus, Tokyo, Japan).

Genomic sequences. Genomic DNA was amplified by PCR using Takara LA *Taq* (Takara). The refined products were subjected to direct sequencing, utilizing dye termination chemistry with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). To minimize sequencing artifacts, sequencing was performed by direct sequencing of the PCR products, with both strands sequenced at least twice in each strain. The sequences were compared with the sequence of *Mafa* from the BALB/c mouse, which was provided by K.K.

Subjects for molecular scanning and case-control study. A total of 96 unrelated Japanese subjects (16 control subjects, 16 individuals with type 1 diabetes, and 64 individuals with type 2 diabetes) were analyzed for de novo polymorphisms. One hundred and thirty-eight unrelated Japanese individuals (53 male and 85 female subjects) with type 1 diabetes, 347 unrelated Japanese individuals with type 2 diabetes (173 male and 174 female subjects), 190 unrelated Japanese individuals with autoimmune thyroid disease (AITD), and 348 unrelated healthy control subjects were subjected to genotyping. Means ± SD age at onset of type 1 diabetes was 17.4 ± 12.4 years (range 3–59). This study was approved by the appropriate ethical committees, and informed consent was obtained from all participants.

Statistical analysis. Allele frequency was estimated by direct counting. The significance of the difference in distribution of alleles was determined by a Fisher direct probability test. Haplotypes were estimated by the expectation-maximization algorithm (Haploview version 3.32, four gamete rule [28]). Statistical analysis for semiquantitative RT-PCR was performed by unpaired *t* test. Statistical significance was defined as $P < 0.05$.

RESULTS

Putative transcriptional factors of *Ins2* in thymus. Since insulin, a target gene of MafA, is known to be expressed in both pancreatic islets and the thymus as a primary antigen in type 1 diabetes (29,30), the expression profiles of *Ins1* and *Ins2* in mouse pancreatic islets and neonatal thymus were determined by RT-PCR analysis in control C3H and NOD mice, a model of type 1 diabetes. RNA preparations from neonatal thymus contained the transcript of *Ins2* but not *Ins1*, whereas transcripts of both *Ins1* and *2* were detected in pancreatic islets (Fig. 1*A*), as shown in previous studies (31,32). To search for putative transcriptional regulators of *Ins2* in the thymus, several transcription factors that have been reported to regulate insulin transcription in pancreatic islets and/or the thymus were also examined by RT-PCR analysis. Transcripts of *Mafa* and *Aire*, but not *Pdx1* and *Neurod1*, were detected in the thymus, whereas *Mafa*, *Pdx1*, and *Neurod1*, but not *Aire*, were detected in the pancreatic islets (Fig. 1*A*), indicating a difference in the expression of insulin transactivators between the thymus and pancreatic islets.

Reduced expression of *Mafa* and *Ins2* in NOD thymus. Semiquantitative RT-PCR analyses of *Mafa*, *Ins2*, and *Aire* were performed on thymic cDNA of NOD and control C3H mice aged 3–25 days. We found highest expression of *Mafa* and *Ins2* in the neonatal thymus (3 days of age), and the expression of both transcripts gradually decreased with age (Fig. 1*B* and *C*). In contrast, *Aire* showed stable expression throughout the ages we examined (Fig. 1*D*). In mice of all ages (3–25 days) examined, the expression levels of *Mafa* and *Ins2* were significantly lower in the thymus of NOD mice than in control mice. The largest differences between NOD mice and control mice were observed at 3 days of age, both in *Mafa* (73% reduction, $P < 0.0005$) and *Ins2* (60% reduction, $P < 0.03$) (Fig. 1*B* and *C*). No significant difference was observed between male and female mice (data not shown). In contrast to the marked difference in *Mafa* transcript, *Aire* transcript in NOD mice was comparable with that in control mice, with only a slight difference in 3-day-old neonates (28% reduction, $P < 0.05$) (Fig. 1*D*). The expression level of *Ins2* in the thymus was strongly correlated with that of *Mafa* ($R^2 = 0.809$) (Fig. 1*E*). When the data were stratified by strain, the correlation was stronger in control ($R^2 = 0.863$) than in NOD ($R^2 = 0.393$) mice. No correlation was found between *Ins2* and *Aire* in either control C3H ($R^2 = 0.145$) (Fig. 1*F*) or NOD ($R^2 = 0.135$) mice.

***Mafa* regulates *Ins2* transcription in thymus.** To demonstrate the direct effect of MafA on insulin expression in the thymus, the expression of *Mafa* and *Ins2* in the thymus was studied in *Mafa* knockout mice. *Mafa* transcript was absent in the thymus in homozygous knockout mice (*Mafa*$^{-/-}$) and was significantly decreased in heterozygous knockout mice (*Mafa*$^{+/-}$) compared with that in wild-type littermates (*Mafa*$^{+/+}$) ($P < 0.0001$, ANOVA) (Fig. 1*G*). *Ins2* transcript in the thymus was significantly lower

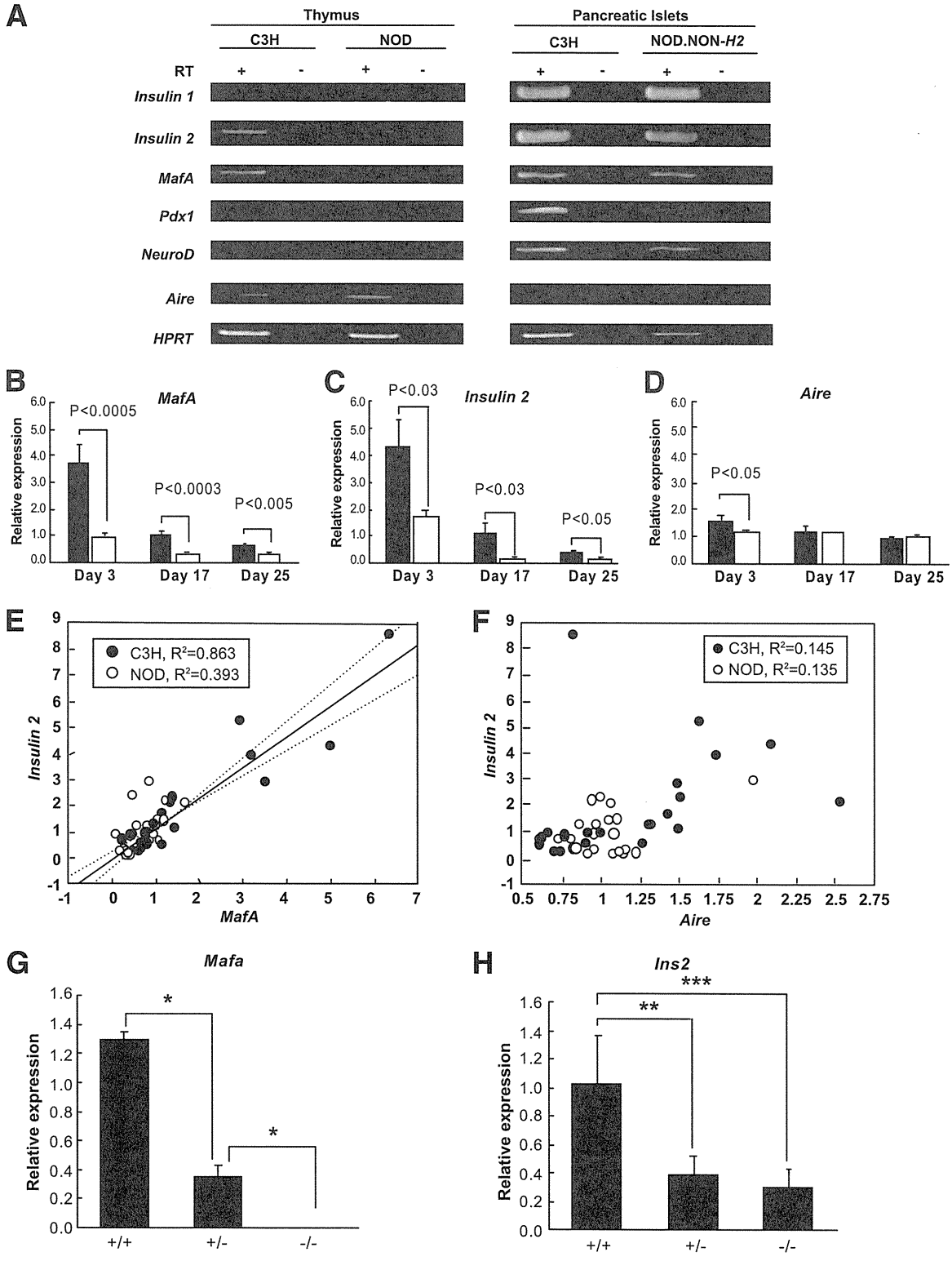

FIG. 1. Regulation of *Ins2* transcription in thymus by *Mafa*. *A*: Gene expression of insulin (*Ins1* and *Ins2*) and putative transcriptional factors of insulin (*Mafa*, *Pdx1*, *Neurod1*, and *Aire*) in thymus and pancreatic islets by RT-PCR. *B–D*: Semiquantitative RT-PCR analysis of expression of *Ins2* (*B*), *Mafa* (*C*), and *Aire* (*D*) in C3H (■) and NOD (□) thymus. Relative mRNA levels normalized to HPRT expression were determined. Data are expressed as means ± SE. *E*: Close correlation of relative expression levels between *Ins2* and *Mafa* expression in thymus. *F*: No correlation between *Ins2* and *Aire* expression in thymus. *G* and *H*: Mafa directly regulates insulin transcription in thymus. Semiquantitative RT-PCR of *Mafa* (*G*) and *Ins2* (*H*) in *Mafa*$^{+/+}$, $^{+/-}$, and $^{-/-}$ mice. Relative mRNA levels normalized to HPRT expression were determined. Data are expressed as means ± SE. **P* = 0.003; ** *P* = 0.001; ****P* < 0.0001, ANOVA.

in homozygous and heterozygous knockout mice than in wild-type littermates ($P = 0.001$, $P = 0.003$, respectively) (Fig. 1*H*). These data clearly indicate a direct link of *Mafa* with *Ins2* transcription in the thymus.

Detection of insulin and MafA proteins by immunohistochemical analysis. We next investigated the expression of insulin and MafA protein in the C3H thymus (7 days old) using immunohistochemical analysis. Insulin-positive

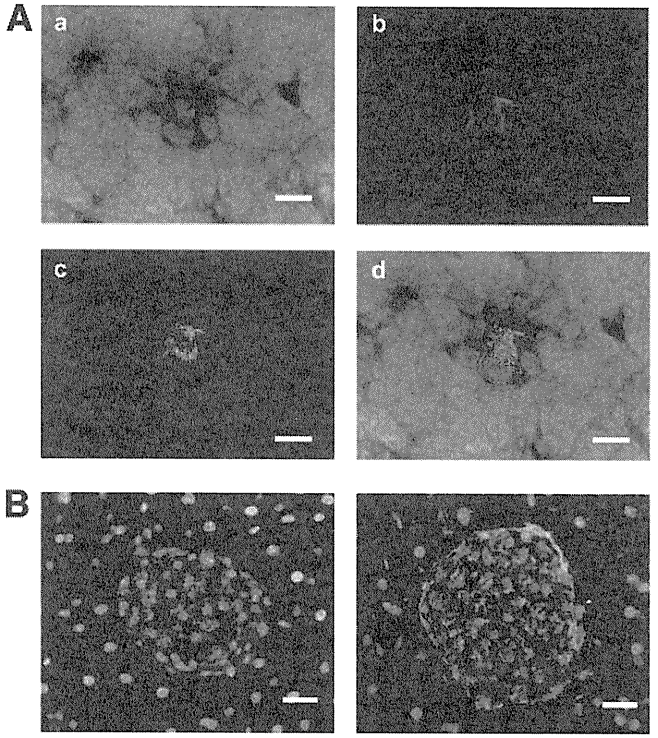

FIG. 2. Immunohistochemical staining of thymic medulla. *A*: Colocalization of insulin and Mafa in thymic medulla (C3H female, 7 days old). Nuclear staining by DAPI (4′,6-diamidino-2-phenylindole) (*a*), anti-insulin antibody (*b*), anti-Mafa antibody (*c*), and merged image (*d*). Bar scale represents 10 μm. *B*: Anti–β-cell antibodies in serum of Mafa$^{-/-}$ mouse. *Mafa*$^{+/+}$ serum (*left panel*) and *Mafa*$^{-/-}$ serum (*right panel*) were used as first antibodies (Alexa Fluor 488, green). Nuclear staining (DAPI, blue). Anti-insulin antibody (Alexa Fluor 594, red). Bar scale represents 100 μm. (A high-quality digital representation of this figure is available in the online issue.)

cells were observed in the thymic medulla and were costained with anti-cytokeratin antibody, suggesting that the site of insulin expression is medullary thymic epithelial cells (supplementary Fig. 1*A*). The thymus was also costained by anti-MafA antibodies and insulin antibodies; MafA staining was observed in the cells positive for insulin, indicating the colocalization of MafA with insulin in the thymus (Fig. 2*A*, supplementary Fig. 1*B*).

Disruption of *Mafa* induces autoantibodies to pancreatic β-cells. To examine the direct link between *Mafa* expression and autoimmunity against the pancreas, mouse pancreas was stained with sera from *Mafa*$^{-/-}$ and *Mafa*$^{+/+}$ mice. The cytoplasm of insulin-positive islet cells was costained with *Mafa*$^{-/-}$ serum but not with *Mafa*$^{+/+}$ serum (Fig. 2*B*), suggesting that *Mafa*$^{-/-}$ serum contains autoantibodies against cytoplasmic proteins of pancreatic β-cells. Mouse IgG-positive cells were also detected in the cells around islets, suggesting that disruption of *Mafa* may induce autoantibodies against not only β-cells but also other cells in the pancreas. Autoantibodies against other endocrine organs, such as thyroid, adrenal, and salivary gland, were not detected in *Mafa*$^{-/-}$ serum (supplementary Fig. 1*C*).

***Mafa* variants were identified in NOD mouse but not other strains.** The entire nucleotide sequence of mouse *Mafa* was identical among the control strains (BALB/c, C3H, and CTS mice) and an animal model of type 2 diabetes (NSY mice) (Fig. 3*A* and *B*). Only NOD mice showed variation in the nucleotide sequence of *Mafa* compared with other strains (BALB/c, C3H, CTS, and NSY mice) (Fig. 3*B*). Ten single nucleotide polymorphisms (SNPs), a 1-bp deletion, and two insertions in the promoter region; one SNP and one 2-bp deletion in the 3′ untranslated region; and one SNP in the 3′ flanking region in the NOD mouse were newly identified (supplementary materials and methods).

Reduced transcriptional activity of Mafa promoter in NOD mouse. NOD *Mafa* promoter activity was significantly lower than that of wild-type mice by 27% ($P < 0.0001$, Student t test) (Fig. 3*C*, *upper panel*, and *D*), indicating that these variants identified in the *Mafa* promoter could contribute to the reduced expression of *Mafa* in NOD mice. When an interval between *Cla*I (*AT/CGAT*, −1,263 bp upstream of the transcription start site) and *Sfi*I (*GGCC*ACTT/G*GGCC*, −399 bp) sites was removed from mMafa-p-luc (mMafa-p-lucΔ*Cla*I-*Sfi*I) (Fig. 3*C*, *lower panel*), the promoter activity appeared to be comparable between NOD and control mice (Fig. 3*E*), indicating the importance of the sequence between the *Cla*I and *Sfi*I sites in the regulation of *Mafa* transcription.

Identification of variants in human MafA gene (*MAFA*). The complete *MAFA* gene in 16 unrelated patients with type 1 diabetes, 16 unrelated control subjects, and 64 unrelated patients with type 2 diabetes was sequenced. In total, 18 SNPs, five insertions/deletions, and VNTRs were newly identified (Genebank accession no. AB205138) (supplementary materials and methods Fig. 4*A*).

The effect of two polymorphisms with a potential functional alteration, an SNP with an amino acid substitution (1377G/T, Gly346Cys), and +69VNTR in the 5′untranslated region (UTR) on the expression and function of MAFA was studied. Luciferase reporter plasmid containing the MAFA response element (MARE) in the promoter region (3 × MARE/RBGP-luc [33]) was transfected into NIH3T3 fibroblast cells (PT67 cells), together with the expression plasmid for hemagglutinin (HA)-tagged MAFA-Gly346 (HA-MAFA-Gly346) or HA-tagged MAFA-Cys346 (HA-MAFA-Cys346). HA-MAFA-Gly346 and HA-MAFA-Cys346 were confirmed to be expressed and phosphorylated at similar levels in the cell lysates by Western blotting, using anti-HA antibody (Fig. 4*B*). HA-MAFA-Gly346 showed a 23% reduction of promoter activity compared with HA-MAFA-Cys346 ($P < 0.003$, Student t test) (Fig. 4*C*), indicating that the 346Cys allele has higher transcriptional activity. The effect of +69VNTR on *MAFA* transcription was also assessed by luciferase assay. Luciferase reporter plasmids containing the cytomegalovirus (CMV)-h*MAFA* promoter (CMV-h*MAFA*-p-luc) (Fig. 4*D*) with each allele of +69VNTR (1R, 2R, or 3R) were transfected into TEC1C6 cells (a thymic medullary epithelial cell line). The allele with one repeat of the VNTR motif (1R) showed significantly higher promoter activity than the allele with two repeats (2R allele) ($P < 0.0001$, ANOVA) (Fig. 4*E*). An allele with three repeats (3R allele) showed significantly lower promoter activity than the 2R allele ($P < 0.0001$). A similar tendency was observed in Min6 cells (a pancreatic β-cell line) with transfection of luciferase reporter plasmids containing h*MAFA*-p-luc (1R vs. 2R: $P < 0.0001$; 2R and 3R: $P < 0.005$) (supplementary Fig. 3).

Gly346Cys was significantly associated with susceptibility to type 1 diabetes, with a significantly lower frequency of the Cys allele in type 1 diabetic than in control subjects (1.8 vs. 5.1%; odds ratio [OR] 0.34 [95% CI 0.13–0.85], $P = 0.0222$) (supplementary Table 1), suggesting that Cys346 of MAFA, which showed higher transcriptional activity than

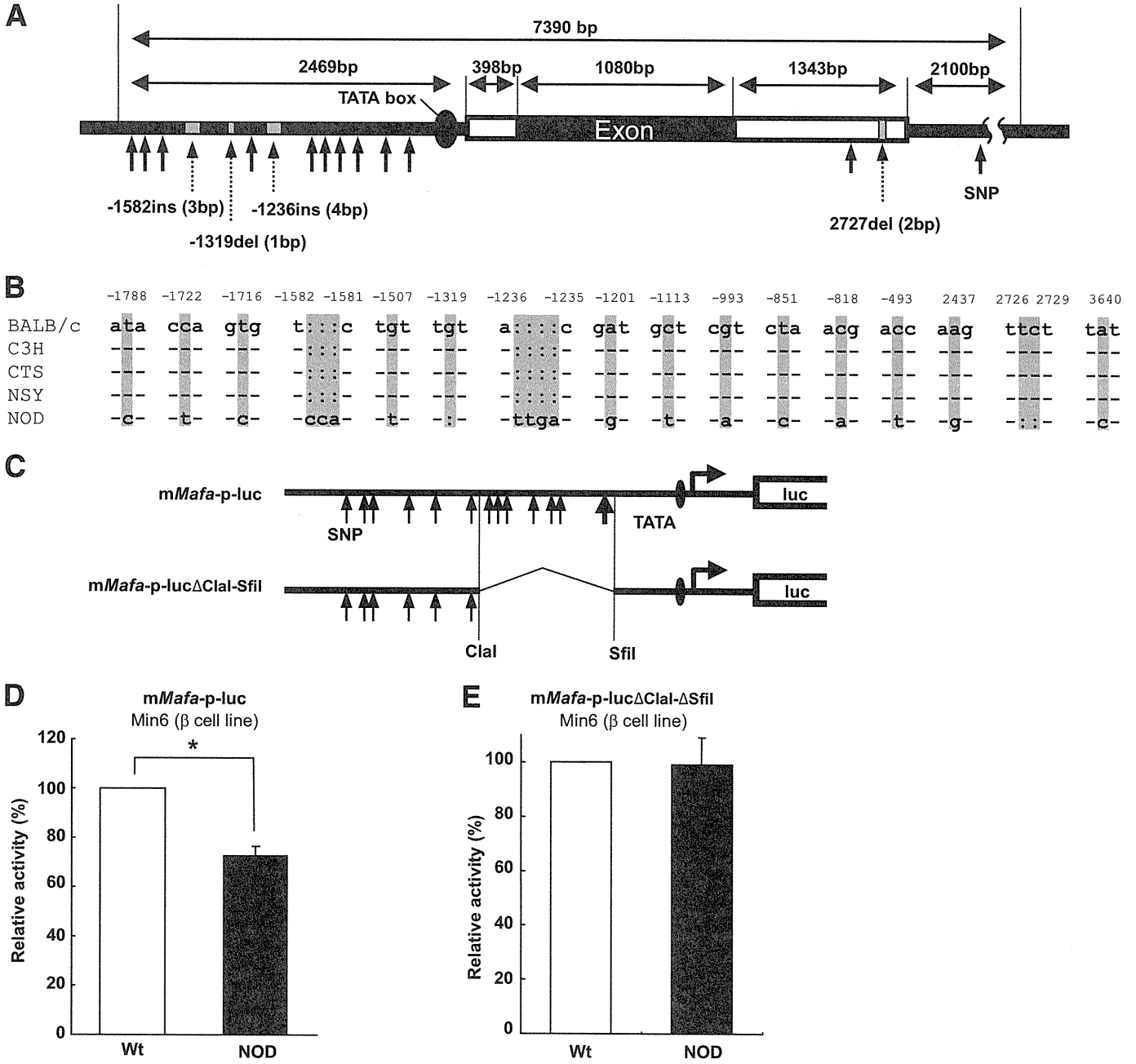

FIG. 3. Reduced promoter activity of *Mafa* in NOD mice. *A*: Localization of sequence variants of *Mafa* in NOD mice. *B*: Comparison of *Mafa* sequences in BALB/c, C3H, CTS, NSY, and NOD mice. Numbers represent position of each variant relative to transcription start site. *C*: Constructs of luciferase reporter plasmids. *D*: Comparison of promoter activity of mMafa-p-luc between BALB/c and NOD mice. Data are expressed as means ± SE. *$P < 0.001$, Student t test. *E*: Comparison of promoter activity of mMafa-p-lucΔ*Cla*I-Δ*Sfi*I between BALB/c and NOD mice. Data are expressed as means ± SE.

Gly346 of MAFA, is protective against type 1 diabetes. Whereas homozygotes with Cys346 were absent from both case and control subjects, heterozygotes with Cys346 were negatively associated with type 1 diabetes (0.33 [0.13–0.83], $P = 0.0196$) (supplementary Table 1), suggesting a dominant mode of protection by Cys346. Given the well-known association of *INS* (*IDDM2*) polymorphisms with type 1 diabetes and insulin expression in the thymus, type 1 diabetic patients were stratified by *INS* polymorphism (patients with the $-23\text{HphI}^{+/+}$ genotype, which is associated with lower transcriptional activity of insulin in the thymus as high-risk subjects and those with $-23\text{HphI}^{+/-}$ as low-risk case subjects). The protective effect of Cys346 was concentrated in patients with high-risk *IDDM2* (high-risk patients versus control subjects, $P = 0.0095$, 0.23 [0.08–0.71]) (supplementary Table 2).

The Gly346Cys polymorphism and +69VNTR were in linkage disequilibrium ($D' = 1.0$), giving rise to three haplotypes (*MAFA-ht1–3*) (Table 1 and supplementary Table 3). A haplotype association study revealed that the frequency of *MAFA-ht3* (1R-Cys) was significantly lower in patients with type 1 diabetes than in control subjects ($P < 0.03$) (supplementary Table 3). When subjects with type 1 diabetes were limited to the high-risk *IDDM2* genotype ($-23\text{HphI}^{+/+}$ as high-risk case subjects), the association was further concentrated (OR 0.23 [95% CI 0.07–0.70], $P =$

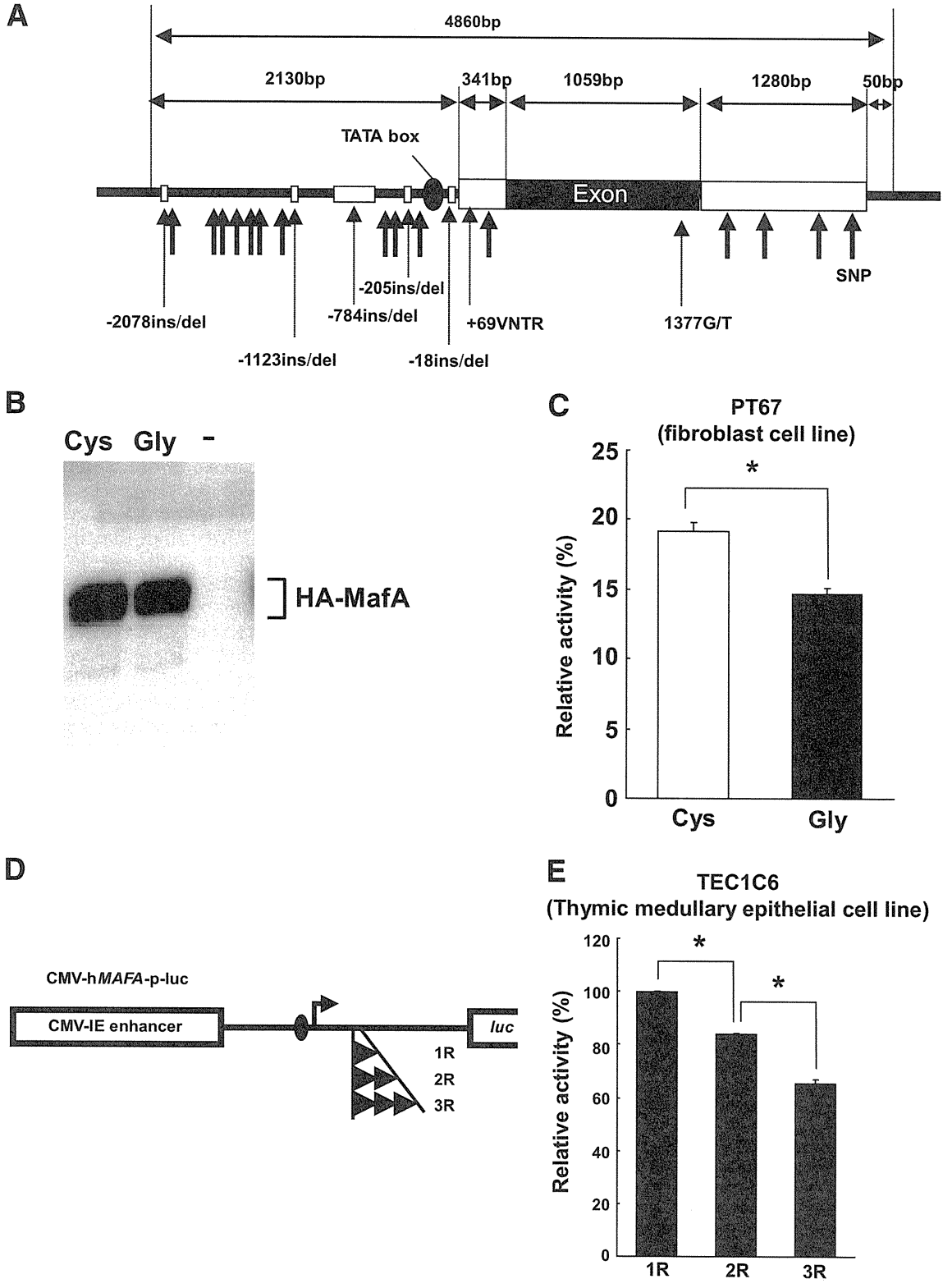

FIG. 4. Gly346Cys and +69VNTR polymorphisms in human *MAFA* are functional. *A*: Localization of sequence variants identified in h*MAFA*. *B*: Western blotting of HA-MafA-Gly346 and HA-MAFA-Cys346 using anti-HA antibody. *C*: MAFA-346Cys showed significantly higher promoter activity than MAFA-346Gly. Data are expressed as means ± SE. **P* < 0.003, Student *t* test. *D*: Constructs of luciferase reporter plasmid containing *MAFA* promoter with each allele of +69VNTR (1R, 2R, and 3R). *E*: 1R allele had significantly higher promoter activity than the 2R or 3R alleles. Data are expressed as means ± SE. **P* < 0.0001, ANOVA.

0.0089) (Table 1). Stratification of cases by HLA haplotype (cases with high-risk *IDDM1* versus control subjects) did not affect the association with type 1 diabetes (data not shown). No association with susceptibility to type 2 diabetes was found for *MAFA* (for Gly346Cys 1.52 [0.85–2.71], *P* = NS) (supplementary Table 4).

To clarify whether the association of *MAFA* is limited to autoimmunity against β-cells, an association study with

TABLE 1
Haplotype association of *MAFA* with susceptibility to high-risk type 1 diabetes

Haplotype	Polymorphism		Frequency		OR (95% CI)	*P* value
	+69 VNTR	+1,377 G/T	High-risk case subjects*	Control subjects		
MAFA-ht1	1R	G (Gly)	0.721	0.700	1.11 (0.78–1.53)	NS
MAFA-ht2	Non-1R	G (Gly)	0.266	0.249	1.10 (0.77–1.57)	NS
MAFA-ht3	1R	T (Cys)	0.012	0.051	0.23 (0.07–0.70)	0.0089

*High-risk case subjects: type 1 diabetic patients with the high-risk *IDDM2* allele ($-23Hph^{+/+}$). Fisher direct probability test. NS, not significant.

AITDs (Graves's disease and Hashimoto's thyroiditis) was performed. The Cys allele frequency of *MAFA* Gly346Cys was comparable between subjects with AITDs and control subjects (3.2 vs. 5.1%, P = NS) (Table 2). Among 190 subjects with AITDs, 18 were positive for GAD antibody, indicating autoimmunity against pancreatic β-cells as well as the thyroid gland in these patients. The frequency of the disease-protective Cys allele in AITD patients without anti-islet autoimmunity was similar to that in control subjects (3.5 vs. 5.1%, P = NS), whereas it was similar in AITD patients with anti-islet autoimmunity and type 1 diabetic patients (0.0 vs. 1.8%, P = NS).

DISCUSSION

The organ specificity of autoimmune disease is caused by impaired immunological tolerance to tissue-restricted peripheral antigens. Negative selection of autoreactive T-cells plays an important role in central tolerance to peripheral antigens, which are ectopically expressed in the thymus (34,35). Recent studies strongly suggest that insulin is the primary antigen for type 1 diabetes in humans (36) and the NOD mouse (37) and that the negative selection of insulin-specific T-cells could be impaired in individuals with type 1 diabetes. The expression level of insulin in the human thymus is known to be associated with a regulatory polymorphism in the promoter region of the *INS* gene, *INS-VNTR* (*IDDM2*) (14,15), whereas such variants have not been identified in the NOD mouse. The present study indicates the existence of an alternative mechanism regulating ectopic insulin transcription in the thymus other than *Aire* and *INS-VNTR*. Multiple transcription factors including *Pdx-1*, *Neurod1*, and *Mafa* are reported to be involved in β-cell–specific transcription of insulin (23). Of these transcription factors, only *Mafa* was shown to be expressed in the thymus and to be closely correlated with *Ins2* expression, suggesting that *Mafa* plays a pivotal role in regulating *Ins2* transcription in the thymus. Targeted disruption of *Mafa* resulted in a marked decrease in *Ins2* transcription in the thymus, indicating a direct link of *Mafa* with *Ins2* transcription in the thymus. *MAFA* expression was also confirmed in a thymic cDNA library in humans (supplementary Fig. 4). These results support our hypothesis that MafA is involved in the pathogenesis of type 1 diabetes through the expression of insulin in the thymus and the thymic deletion of insulin-specific autoreactive T-cells.

TABLE 2
Genotype and allele frequencies of *MAFA* +1,377 G/T in AITDs

	Control subjects	AITD subjects	OR (95% CI)	*P* value
n	263	190		
Genotype				
G/G	236 (89.7)	178 (93.7)		
G/T	27 (10.3)	12 (6.3)	0.59 (0.29–1.19)	NS
Allele				
G (Gly)	499 (94.9)	368 (96.8)		
T (Cys)	27 (5.1)	12 (3.2)	0.60 (0.30–1.20)	NS

Data are *n* (%), unless otherwise indicated. Fisher exact probability test. NS, not significant.

MafA has been shown to be a key molecule regulating the tissue-specific expression of insulin in β-cells, but its effect is weak unless it is coexpressed with Pdx1 and NeuroD/BETA2 (38). The expression level of insulin in the thymus is ~100-fold lower than that in pancreatic islets, and it is thought to be sufficient to induce negative selection of autoreactive T-cells in the thymus. In this sense, it is reasonable to speculate that MafA, whose transcriptional activity is modest without coexpression of Pdx1 and NeuroD/Beta2, can control central tolerance to the insulin peptide by regulating insulin transcription in the thymus where Pdx1 and Neurod1 are not expressed. A series of studies showed that newly established medullary thymic epithelial cell (mTEC) clones spontaneously express *Ins2* despite the absence of detectable *Pdx1* (39), and the regulation of *Ins2* transcription in mTEC clones was not coordinated with the expression level of *Aire* (40). Garcia et al. (41) also demonstrated that *Ins2* expression was detected in the thymus of $Pdx1^{-/-}$ mice as well as wild-type controls, further supporting our hypothesis of a *Pdx1*-independent transcriptional mechanism of insulin in the thymus.

Several reports (42–45) have shown that the thymic expression of insulin is *Aire*-dependent, suggesting an essential role of *Aire* in insulin expression in the thymus. Based on the data of our semiquantitative RT-PCR analysis, the expression level of *Aire* was not correlated with that of *Ins2* in either NOD (R^2 = 0.135) or control (R^2 = 0.145) C3H thymus, in contrast to the strong correlation between *Mafa* and *Ins2* (NOD: R^2 = 0.393, C3H: R^2 = 0.863). When the data were restricted to 3-day-old neonatal animals, the expression of *Aire* in the NOD thymus was reduced ($P < 0.05$), and there was a weak correlation between the expression levels of *In2* and *Aire* in the NOD thymus (R^2 = 0.516) but not in the control thymus (R^2 = 0.042, data not shown). The insight derived from these observations is that the expression level of *Ins2* in the control thymus is precisely regulated by *Mafa*, rather than *Aire*, in normal conditions. Taking these results together, we speculate that insulin expression in the thymus is turned on by *Aire* and precisely regulated by *MafA* in normal conditions. In the present study, targeted disruption of *Mafa* led to reduced expression of *Ins2* in the thymus and induced autoantibodies against the pancreas including cytoplasmic protein of pancreatic β-cells, indicating a direct link between *Mafa* and autoimmunity against the pancreas.

Molecular scanning of the human *MAFA* gene revealed several polymorphisms that may affect the function of the gene, including Gly346Cys in the vicinity of the dimerization domain (supplementary Fig. 5) and +69VNTR in the 5′UTR. In vitro studies revealed that the Gly346Cys polymorphism is functional, with the Gly allele showing a 23% reduction in transactivation activity compared with the Cys346 allele. +69VNTR in the 5′UTR was also functional, with the number of repeats of VNTRs being negatively correlated with the promoter activity. Based on a hypothesis-driven association study (46), *MAFA-ht4* containing these two polymorphisms with higher transcriptional activity was significantly associated with protection against type 1 diabetes, and the association was especially concentrated in cases with the high-risk allele ($-23HphI^{+/+}$; *INS-VNTR* class I/class I) at the *IDDM2* locus. These data suggest the involvement of gene-gene interaction of *MAFA* with *INS-VNTR* (*IDDM2*) in type 1 diabetes susceptibility, possibly due to a common underlying mechanism for both genes in the disease pathogenesis of type 1 diabetes. Several non-HLA susceptibility loci for type 1 diabetes have been mapped in recent genome-wide association studies in Caucasian populations (47), but markers located near *MAFA* were not reported to be associated with type 1 diabetes. One possible explanation may be the difference in genetic background between Japanese and Caucasian populations, including *IDDM2*, the susceptibility allele of which shows large ethnic differences in frequency.

In the present study, an association of *MAFA* with type 1 diabetes, but not with AITDs, was observed. Further analysis revealed that the frequency of the disease-protective allele in AITD patients without anti-islet autoimmunity was similar to that in control subjects, whereas the frequency in AITD patients with anti-islet autoimmunity was similar to that in patients with type 1 diabetes, suggesting that *MAFA* is associated with autoimmunity against pancreatic β-cells but not other endocrine organs. These data support our hypothesis that *MAFA* is involved in autoimmunity specific to pancreatic β-cells but not other endocrine organs such as the thyroid gland.

In conclusion, the present study demonstrated that MafA expression in thymic medullary epithelial cells is closely correlated with that of *Ins2*, suggesting that the expression of peripheral-tissue antigens in the thymus is regulated by antigen-specific mechanisms. Reduced expression of *Mafa* and *Ins2* in the NOD thymus could lead insulin-specific T-cells to escape negative selection, resulting in infiltration of insulin-specific T-cells into pancreatic islets and the development of type 1 diabetes. This hypothesis is further supported by the association of regulatory polymorphisms in human *MAFA* with susceptibility to type 1 diabetes. These data suggest a new concept that variation in antigen-specific transcriptional factors plays a critical role in induction of central tolerance to self-antigens, in addition to the more generic role played by Aire.

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No potential conflicts of interest relevant to this article were reported.

S.N. designed this study, researched data, and wrote the manuscript. K.K. designed this study and researched data. Y.K. provided statistical support for human data. N.B. and Y.H. contributed to the animal studies. K.Y. contributed to the animal studies and researched data. T.F. contributed to the animal studies and provided statistical support. S.A. researched data. T.K. and S.T. provided knockout animals and researched data. H.I. reviewed and edited the manuscript and directed the overall project.

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Supplementary Table 1. Genotype and allele frequencies of *MAFA* +1377 G/T (Gly346Cys) in type 1 diabetes (all cases).

	Control n=263 (%)		All cases n=139 (%)		OR [95%CI]	p value
Genotype						
G / G	236	(89.7)	134	(96.4)		
G / T	27	(10.3)	5	(3.6)	0.33 [0.13-0.83]	0.0196
Allele						
G (Gly)	499	(94.9)	273	(98.2)		
T (Cys)	27	(5.1)	5	(1.8)	0.34 [0.13-0.85]	0.0222

Fisher's exact probability test

Supplementary Table 2. Genotype and allele frequencies of *MAFA* +1377 G/T (Gly346Cys) in high-risk type 1 diabetes

	Control n=263 (%)		High-risk cases n=124(%)		OR [95%CI]	p value
Genotype						
G / G	236	(89.7)	121	(98.0)		
G / T	27	(10.3)	3	(2.0)	0.22[0.07-0.69]	0.0083
Allele						
G (Gly)	499	(94.9)	245	(99.0)		
T (Cys)	27	(5.1)	3	(1.0)	0.23 [0.08-0.71]	0.0095

*High-risk cases: type 1 diabetes patients with high-risk *IDDM2* allele (-23Hph+/+)

Fisher's exact probability test

Supplementary Table 3. Haplotype association of *MAFA* with susceptibility to type 1 diabetes (all cases)

	Polymorphism		Frequency			
Haplotype	+69 VNTR	+1377 G/T	All cases	Control	OR [95%CI]	p value
MAFA-ht1	1R	G (Gly)	0.727	0.700	1.12[0.72-1.74]	NS
MAFA-ht2	non 1R	G (Gly)	0.254	0.249	1.02[0.65-1.61]	NS
MAFA-ht3	1R	T (Cys)	0.018	0.051	0.33 [0.13-0.86]	<0.03

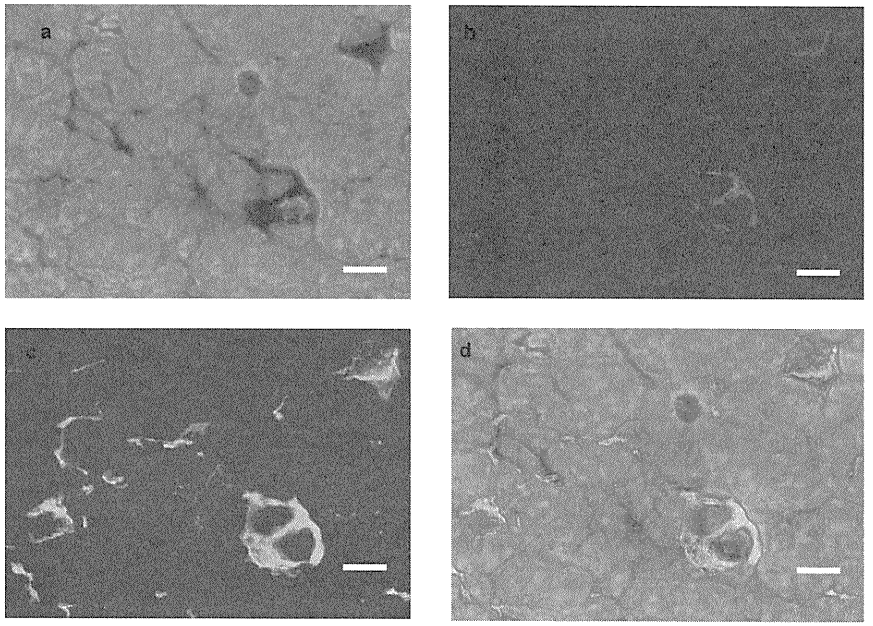
Supplementary Table 4. Genotype and allele frequencies of *MAFA* +1377 G/T (Gly346Cys) in type 2 diabetes

	Control n=425 (%)		Cases n=347 (%)		OR [95%CI]	p value
Genotype						
G / G	392	(92.2)	329	(94.8)		
G / T	33	(7.8)	18	(5.2)	1.54 [0.85-2.77]	NS
Allele						
G (Gly)	817	(96.1)	676	(97.4)		
T (Cys)	33	(3.9)	18	(2.6)	1.52 [0.85-2.71]	NS

Fisher's exact probability test

Supplementary Figure 1.

A

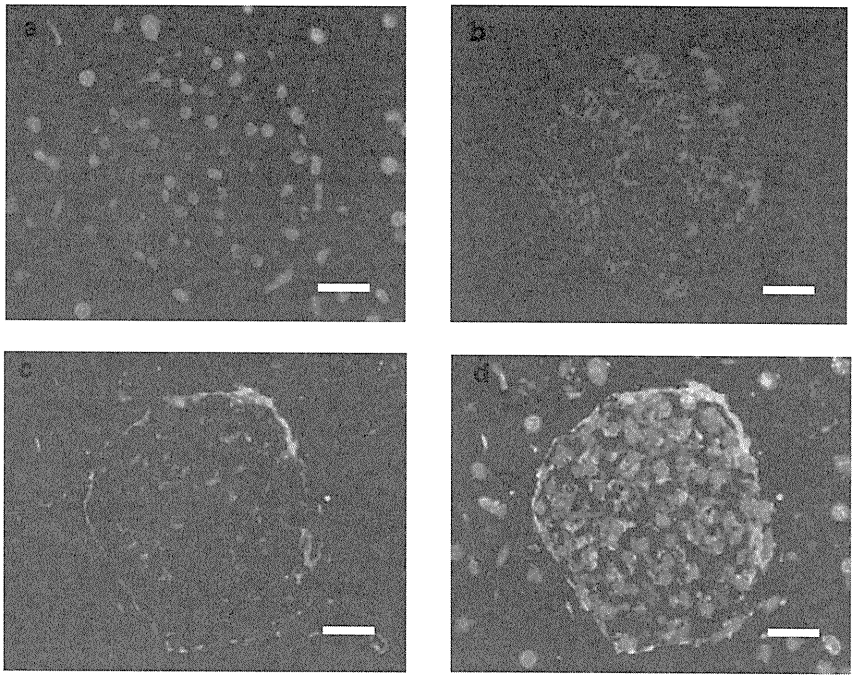


Co-localization of insulin and cytokeratin in thymic medulla (C3H female, 7 days old) (a) Nuclear staining by DAPI, (b) anti-insulin antibody, (c) anti-cytokeratin antibody, and (d) merged image.

Bar scale: 10 μm

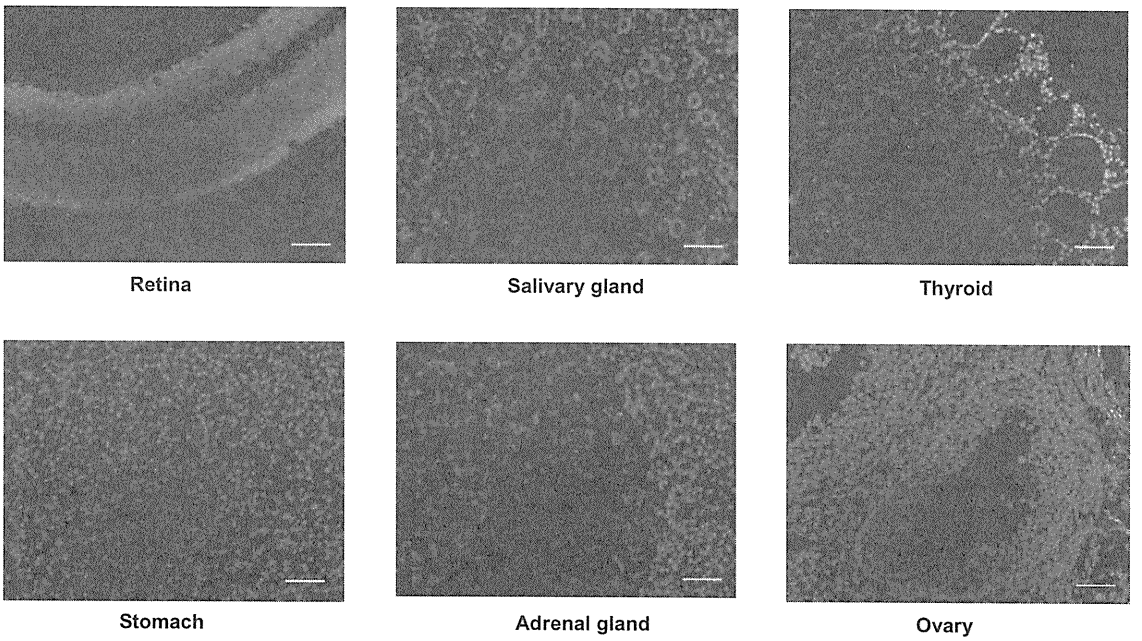
a

B



(a) Nuclear staining by DAPI, (b) anti-insulin antibody, (c) serum from Mafa-/- mice, and (d) merged image.

C

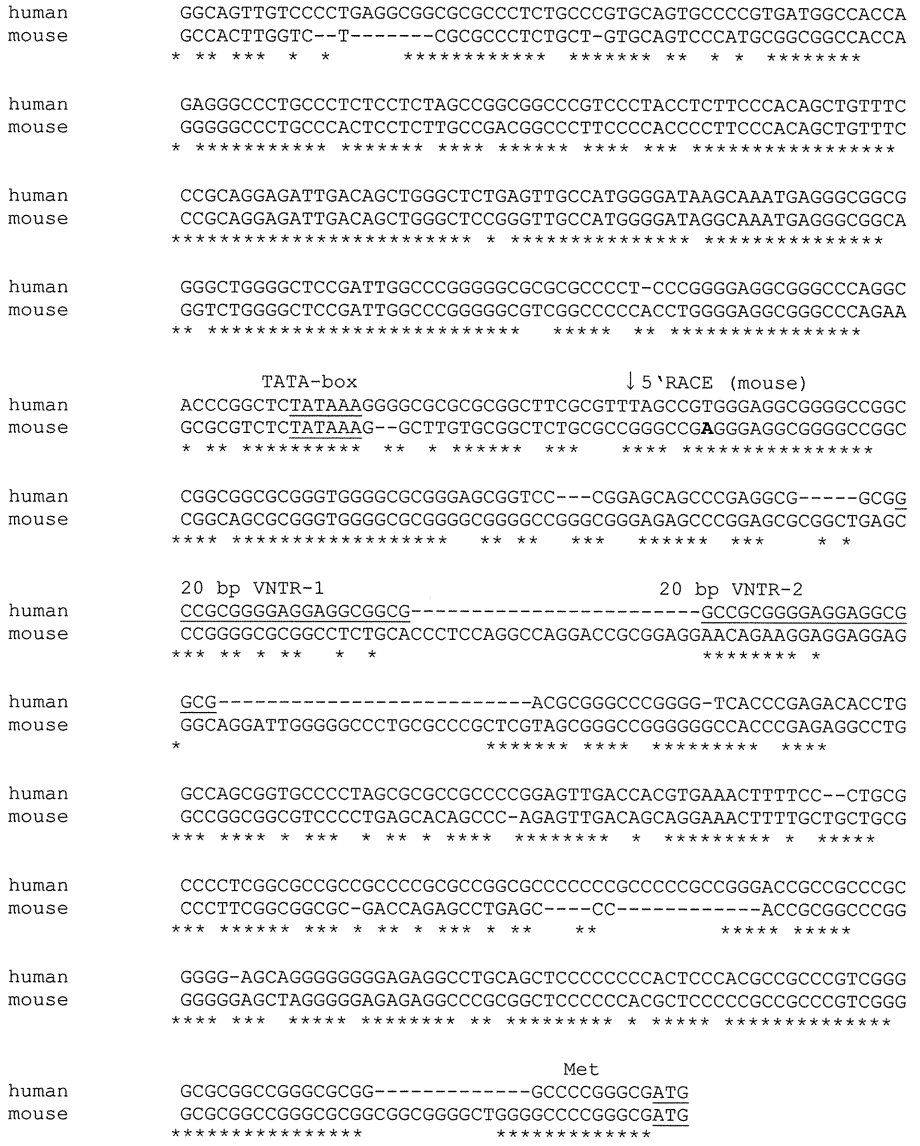


No autoantibody was detected in retina, salivary glands, thyroid, stomach, adrenal gland, and ovary by staining with Mafa -/- serum.

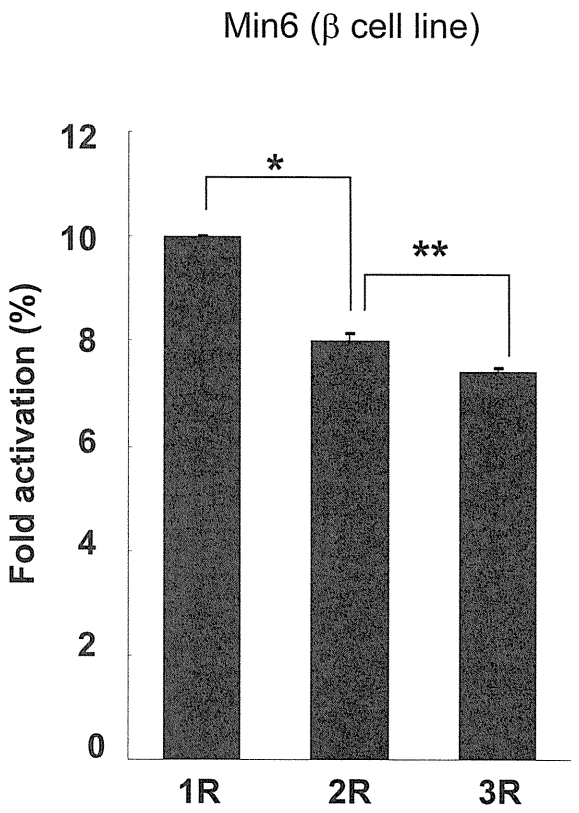
Blue: DAPI for nuclear staining
Green: mouse IgG for autoantibody

Bar scale: 50 μm

Supplementary Figure 2.

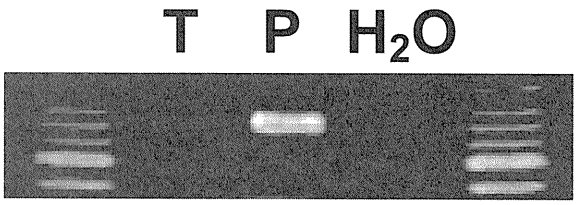


Supplementary Figure 3.



Mean ±SEM, * p<0.0001,**p<0.005 ANOVA

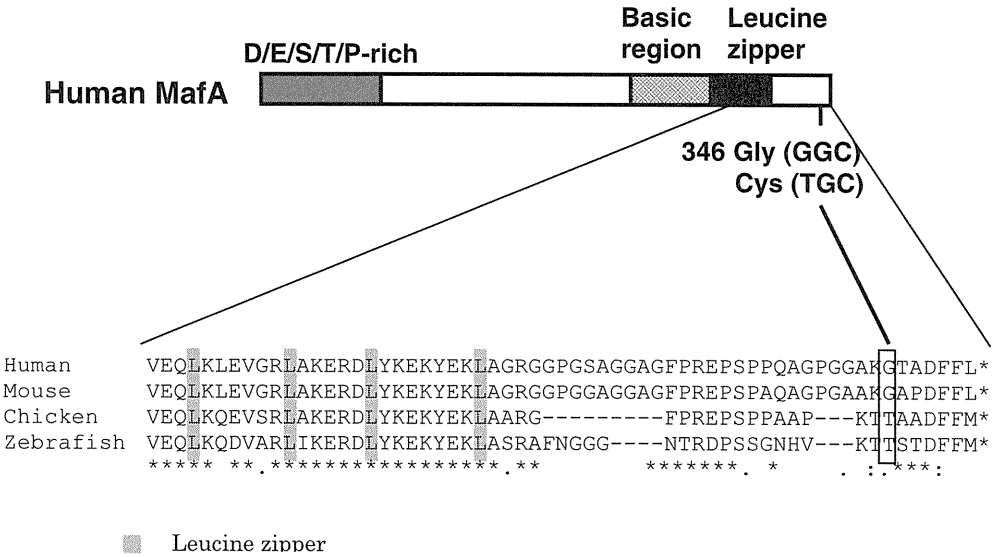
Supplementary Figure 4.



T: human thymic cDNA library

P: human pancreatic cDNA library

Supplementary Figure 5.



Supplementary materials and methods

cDNA was amplified by PCR with the following primers:

Aire	(Forward)	5'-GCGATGCAGTGTGTGTGGCGATGG-3'
	(Reverse)	5'-TCTGGATGGCCCACTGCAGGATGC-3'
Hprt	(Forward)	5'-CTCGAAGTGTTGGATACAGC-3'
	(Reverse)	5'-TGGCCTATAGGCTCATAGTG-3'
Pdx1	(Forward)	5'-CTCGCTGGGATCACTGGAGCA-3'
	(Reverse)	5'-GCTTTGGTGGATTTCATCCACGG-3'
Neurod1	(Forward)	5'-CTTGGCCAAAAACTACATTTGG-3'
	(Reverse)	5'-GGAGTAGGGGTGTACCGGGAA-3'
Ins1	(Forward)	5'-CCATCAGCAAGCAGGTCATTGTTT-3'
	(Reverse)	5'-GCGGGACTTGGGTGTGTAGAAGAA-3'
Ins2	(Forward)	5'-CCATCAGCAAGCAGGTTATTGTTTC-3'
	(Reverse)	5'-CAGCTCCAG TTGTGCCACTTGTG-3'

Immunohistochemical staining

Universal negative control for rabbit primary antigen (DAKO Japan Co., Kyoto, Japan) was used for insulin staining as recommended by DAKO, and rabbit polyclonal IgG (Abcam Co., Tokyo, Japan) was used for MafA staining as isotype control.

Genomic sequence of mouse Mafa

The transcription start site of mouse *Mafa* was determined by rapid amplification of cDNA ends (5'RACE) analysis of polyA RNA isolated from Min6 cells (mouse insulinoma cell line). Several clones, starting from 398bp upstream of the first ATG and 27bp downstream of the TATA box, were obtained. The 3' untranslated region (UTR, 1343bp) of mouse *Mafa* was estimated by searching the expressed sequence tag (EST) containing both the 3' flanking sequence of the *Mafa* open reading frame (ORF) and the poly A site from a database. Genomic DNA was extracted from liver tissue by a standard phenol-chloroform method. A total of 17 pairs of primers were designed using Primer3 Input (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi), so that the promoter region (approximately 2400bp upstream from the transcription initiation site), an exon, and the 3′-flanking region of the *Mafa* gene were covered by 17 segments.

Newly identified polymorphisms of Mafa in NOD mouse

Ten SNPs (-1788T/C, -1722C/T, -1716T/C, -1507G/T, -1201A/G, -1113C/T, -993G/A, -851T/C, -818C/A, and -493C/T relative to the transcription start site), a 1bp deletion (-1319del) and two insertions (-1582insCCA and -1236insTTGA) in the promoter region, one SNP (2437A/G) and one 2bp deletion (2727del) in the 3' untranslated region, and one SNP (3640A/C) in the 3' flanking region were newly identified in the NOD mouse.

Subjects for case-control studies

The diagnosis of type 1 diabetes was defined based on both clinical features and laboratory data. All the patients were ketosis-prone and lacked endogenous insulin secretion as judged by a C-peptide level of <3.3 nmol/day, and needed four or more insulin injections per day. Individuals with type 2 diabetes were diagnosed according to World Health Organization criteria. Control subjects had normal glucose tolerance and no family history of diabetes or other autoimmune diseases. All individuals with type 1 diabetes or type 2 diabetes and control subjects were of Japanese origin and resided in the Osaka area (western Japan). Glutamic acid decarboxylase antibody (GAD Ab) was measured by a commercially available RIA kit using ^{125}I-labeled recombinant human GAD65 as a tracer reagent (Cosmic, Tokyo, Japan). Samples were defined as GAD Ab positive above a threshold of 10 units/ml as suggested in previous reports (Maruyama et al. *J Clin Endocrinol Metab* 93:2115-21, 2008).

Screening for human MAFA polymorphisms

Genomic DNA was extracted from peripheral blood leukocytes using proteinase K as

described previously, and polymorphisms in the complete *MAFA* gene were detected by sequencing. The transcription start site of *MAFA* was estimated by alignment of sequences between human and mouse (341bp upstream to first ATG and 29bp downstream to TATA box, Supplementary Fig. 2). The 3' UTR (1280bp) of human *MAFA* was estimated by searching EST containing both the 3' flanking sequence of *MAFA* ORF and the poly A site from a database. Primers purchased from Vector Asian Pacific Corporation were designed to determine the sequence of the complete *MAFA* gene. Polymerase chain reaction (PCR) amplification was performed in a total reaction volume of 20 μ l containing the following reagents: 4 ng genomic DNA, 5 pmol of each primer, 0.5 unit LA Taq polymerase (Takara, Shiga, Japan), 2 x PCR buffer containing 5mM $MgCl_2$, and 8mM dNTP mixture. Amplification was carried out in a Takara thermal cycler. PCR products were visualized by 2% agarose gel electrophoresis with ethidium bromide staining, and purified using a gel extraction kit (QIAGEN) for mice and PCR96 Cleanup plates (Millipore) for humans, before the sequence reaction. Sequencing was performed using an ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Newly identified polymorphisms were located at the following positions (numbers are relative to transcription start site, Fig. 4A); -2078ins/del (2bp), -2065T/C, -1764C/A, -1761T/C, -1719C/G, -1670G/A, -1486T/C, -1123ins/del (1bp), -955C/T, -784ins/del (103bp), -282G/T, -263G/A, -205ins/del (1bp), -109C/G, -18ins/del (2bp), +41A/G, +69VNTR (GCCGCGGGGAGGAGGCGGCG, 1 repeat (1R) to 3 repeats (3R)), +132A/G, +153T/A, +1377G/T(Gly346Cys), +1430G/T, +1630T/A, +1716C/T, and +2279T/A.

Genotyping of human MAFA polymorphisms

Gene Scan analysis was performed for genotyping of +69VNTR. The forward primer was labeled with FAM for the polymerase chain reaction, and analyzed by an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Restriction fragment length polymorphism analysis using an ApaLI (New England Biolab, MA, USA) was performed for genotyping the Gly346Cys polymorphism, and the result was confirmed by using a Taqman System (Applied Biosystems) in some of the subjects. All genotyping data were double-scored to minimize error. The overall genotype call rate was 98.1% (+69VNTR) and 96.9% (Gly346Cys). The genotype distribution of each polymorphism was compatible with Hardy-Weinberg equilibrium ($p>0.05$, chi-squared test).

Luciferase assay

To construct the reporter plasmid, m-Mafa-p-luc, the promoter region of mouse Mafa (-2500~first ATG) was amplified from Balb/c or NOD genomic DNA by PCR. The fragments were inserted into KpnI-HindII sites of the pGL2-basic plasmid (Promega). Min6 cells were grown in a 24-well plate and transfected with a total of 3.2 μg of plasmids (2.8 μg luciferase plasmid, 0.4 μg pER-Rluc), using 8 μl Lipofectamine2000. The construction of 3xMARE/RBGP-luc is described in a previous paper {Kataoka, 1996 #84}. The expression plasmid for human MAFA with both alleles of the Gly346Cys variant was constructed by inserting a *NotI* fragment excised from pGEM-T-easy/h-*MAFA* into the pHygEF2 mammalian expression vector. To construct hemagglutinin (HA)-tagged human *MAFA*, a double-stranded oligonucleotide encoding the HA epitope tag sequence was inserted into the 5'-*EcoR*I site of pGEM-T-easy/h-*MAFA*. The resultant HA-h-*MAFA* fragment (XbaI-SpeI) was inserted into pHygEF2 to obtain pHygEF2/HA-h-*MAFA*. PT67 cells (derived from NIH3T3 cells) grown in a 24-well plate were transfected with a total of 1.5 μg plasmids (0.2 μg luciferase plasmid, 0.4 μg pHygEF2 vector, 0.8 μg expression plasmids and 0.1 μg pEF-*R*luc) using 7.5 μl Superfect (Qiagen). Cells were harvested 24 h after transfection. To construct the reporter plasmid, CMV-h-MAFA-p-luc, the human MAFA promoter region containing the 1R, 2R or 3R allele (-130~+321) was amplified from human genomic DNA by PCR, and the CMV immediate early enhancer was conjugated with upstream of the fragments. These fragments were inserted into the pGL2-basic plasmid (Promega). TEC1C6 (thymic medullary epithelial cell line) cells grown in a 24-well plate were transfected with a total of 1.6 μg plasmids (0.4 μg luciferase plasmid, 0.8 μg expression plasmid, and 0.4 μg pEF-Rluc) using 1.6 μl Lipofectamine2000 (Invitrogen). Twenty four hours after transfection in each experiment, firefly and Renilla luciferase activities were measured using a Dual Luciferase Assay System (Promega).

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ARTICLE

Direct evidence for susceptibility genes for type 2 diabetes on mouse chromosomes 11 and 14

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Abstract
Aims/hypothesis Diabetogenic loci for type 2 diabetes have been mapped to mouse chromosome (Chr) 11 and 14 in the Nagoya–Shibata–Yasuda (NSY) mouse, an animal model of type 2 diabetes. We aimed to obtain direct evidence of these genes on each chromosome and to clarify their function and interaction in conferring susceptibility to type 2 diabetes.
Methods We established three consomic strains homozygous for diabetogenic NSY-Chr11, NSY-Chr14 or both on the control C3H background (C3H-11^{NSY}, C3H-14^{NSY} and C3H-$11^{NSY}14^{NSY}$, respectively), and monitored diabetes-related phenotypes longitudinally. The glucokinase gene was sequenced as a positional candidate gene on Chr11.
Results C3H-11^{NSY} mice showed hyperglycaemia associated with impaired insulin secretion and age-dependent insulin resistance without obesity. C3H-14^{NSY} mice exhibited hyperglycaemia mainly due to insulin resistance, with a slight increase in percentage body fat. C3H-$11^{NSY}14^{NSY}$ double consomic mice showed marked hyperglycaemia and obesity, which was not observed in single consomic strains. Sequences of the glucokinase gene were allelically variant between NSY and C3H mice.
Conclusions/interpretation These data provide direct evidence that Chr11 and Chr14 harbour major susceptibility genes for type 2 diabetes. These two chromosomes interact to cause more severe hyperglycaemia and obesity, which was not observed with the presence of either single chromosome, indicating different modes of gene–gene interaction depending on the phenotype. Marked changes in the phenotypes retained in the consomic strains will facilitate fine mapping and the identification of the responsible genes and their interaction with each other, other genes and environmental factors.

Keywords Complex trait · Interaction · Mouse chromosome 11 · Mouse chromosome 14 · *Nidd1n* · *Nidd2n* · *Nidd4n* · NSY mouse

Abbreviations

BAT	Brown adipose tissue
Chr	Chromosome
gAUC	Area under the glucose concentration curve
ipGTT	Intraperitoneal glucose tolerance test
ITT	Insulin tolerance test
NSY	Nagoya–Shibata–Yasuda
QTL	Quantitative trait locus
SNP	Single nucleotide polymorphism

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