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Published Version: doi:10.1073/pnas.0800454105

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Promoter polymorphism of the erythropoietin gene in severe diabetic eye and kidney complications

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Edited by Helen H. Hobbs, University of Texas Southwestern Medical Center, Dallas, TX, and approved March 21, 2008 (received for review January 16, 2008)

Significant morbidity and mortality among patients with diabetes mellitus result largely from a greatly increased incidence of microvascular complications. Proliferative diabetic retinopathy (PDR) and end stage renal disease (ESRD) are two of the most common and severe microvascular complications of diabetes. A high concordance exists in the development of PDR and ESRD in diabetic patients, as well as strong familial aggregation of these complications, suggesting a common underlying genetic mechanism. However, the precise gene(s) and genetic variant(s) involved remain largely unknown. Erythropoietin (EPO) is a potent angiogenic factor observed in the diabetic human and mouse eye. By a combination of case–control association and functional studies, we demonstrate that the T allele of SNP rs1617640 in the promoter of the EPO gene is significantly associated with PDR and ESRD in three European-American cohorts [Utah: \( P = 1.91 \times 10^{-3} \); Genetics of Kidneys in Diabetes (GoKind) Study: \( P = 2.66 \times 10^{-8} \); and Boston: \( P = 2.1 \times 10^{-2} \)]. The EPO concentration in human vitreous body was 7.5-fold higher in normal subjects with the TT risk genotype than in those with the GG genotype. Computational analysis suggests that the risk allele (T) of rs1617640 creates a matrix match with the EVI1/MEL1 or AP1 binding site, accounting for an observed 25-fold enhancement of luciferase reporter expression as compared with the G allele. These results suggest that rs1617640 in the EPO promoter is significantly associated with PDR and ESRD. This study identifies a disease risk-associated gene and potential pathway mediating severe diabetic microvascular complications.

The prevalence of diabetes is steadily increasing, and current U.S. and international figures (18 and 171 million people, respectively) are expected to increase to 30 and 366 million people, respectively, by the year 2030 (1). Despite multiple current therapeutic approaches, diabetic complications remain a major cause of morbidity and mortality. Microvascular complications, including retinopathy and nephropathy, account for the most prevalent and severe morbidity associated with diabetes and may be involved in mediating the increased risk of cardiovascular disease as well. A prominent sight-threatening form of diabetic retinopathy, proliferative diabetic retinopathy (PDR), is the most common and severe cause of new-onset legal blindness in working-aged adults in the United States and accounts for 10% of new-onset blindness overall. Diabetes is also the leading cause of renal insufficiency and end-stage renal disease (ESRD) in the United States and the Western world. Although PDR and ESRD are clearly associated with the degree of hyperglycemia, not all diabetic individuals with poor glycemic control develop advanced retinal or renal complications. Conversely, some patients develop severe complications despite well controlled blood glucose concentrations (2). The underlying etiology of these microvascular complications remains poorly understood.

A well documented high concordance rate (80–90%) between PDR and ESRD underscores a potentially shared pathophysiology (3). PDR, characterized by neovascularization and fibrous proliferation, can ultimately result in tractional retinal detachment and vitreous hemorrhage [supporting information (SI) Fig. S1.4 and B]. The onset of PDR is thought to occur primarily after progressive retinal ischemia produces increased expression of the hypoxia-inducible vascular endothelial growth factor (VEGF), leading to retinal vascular proliferation and permeability (4, 5). Although nearly all patients with diabetes will develop some degree of retinopathy in their lifetimes, PDR occurs only in approximately half (6).

The high degree of familial aggregation and concordance in the development of PDR and ESRD in diabetic patients implies that common genetic factors may be important in susceptibility (or resistance) to these complications (3, 7, 8). However, the causal gene(s) remains largely unknown. A growing number of diabetics develop this complication.


The authors have filed a patent on results described in this paper.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0800454105/DCSupplemental.

\textsuperscript{9}2008 by The National Academy of Sciences of the USA
Genome-wide linkage scans have been performed to detect diabetic nephropathy susceptibility loci. Case–control studies have examined possible roles for various candidate gene single-nucleotide polymorphisms (SNPs), and linkage analysis has suggested a diabetic nephropathy locus on chromosome 3q (9–11). Other loci have also been suggested (reviewed in ref. 11). Recently, several reports implicate a locus at chromosome 7q21 as a modifier of the risk of nephropathy in diabetes (11–14), but a specific gene has not been identified.

The various intermediate phenotypes between the extremes of no nephropathy and diabetic ESRD (Fig. SIC) could affect the results of genome-wide linkage scans. These phenotypes include microalbuminuria without frank proteinuria, proteinuria and/or microalbuminuria with normal renal filtration, frank proteinuria and nephrosis with or without renal insufficiency, and renal failure. Although proteinuria is generally regarded as a key feature of diabetic nephropathy, some large epidemiological studies also report an increase in diabetic non-proteinuric kidney disease. Thus, it is possible that the criteria by which the nonspecific diagnosis “diabetic nephropathy” is defined may have increased phenotypic as well as genetic heterogeneity. Diabetes may harm the eye and kidney through multiple direct and indirect mechanisms. We reasoned that by considering as “cases” only those individuals with the combined severe microvascular complication phenotypes of both ESRD and PDR we would reduce genetic heterogeneity and increase the likelihood of detecting a genetic effect.

To test whether variants in genes involved in angiogenesis increase susceptibility to diabetic microvascular complications, we genotyped 19 SNPs from 11 candidate genes in diabetic patients with or without PDR and ESRD and tested for allelic associations. We also performed functional studies on an EPO promoter, SNP rs1617640, which was associated with diabetic complications in three cohorts.

Results

Genetic Association Study. We genotyped 19 SNPs in 11 genes involved in angiogenesis in 374 patients with both PDR and ESRD and 239 age- and ethnicity-matched diabetic controls from a Utah European-American type 2 diabetes (T2D) cohort (Table S3). No significant association was found (Table S2) except at SNP rs1617640 (Fig. 1A, $P = 1.91 \times 10^{-3}$ for an additive allele-dosage model, $OR_{het} = 1.20 \{0.76, 1.89\}$, $OR_{hom} = 2.01 \{1.23, 3.29\}$, T allele: 63.10% in cases versus 54.18% in controls, Table 1). This association remained significant after Bonferroni correction for multiple comparisons ($P = 0.036$).

To investigate whether rs1617640 was specifically associated with diabetic microvascular complications, rather than complications of T2D per se, we sought to replicate this result in two other independent type 1 diabetes (T1D) cohorts; the GoKinD and Boston cohorts. We chose only European-American individuals in the GoKinD collection and excluded patients or controls with other ethnicities. In total, there were 865 cases in the GoKinD cohort, including 365 T1D patients with both ESRD and PDR, 500 with nephropathy (proteinuria with urine albumin to creatinine ratio $>300$) and retinopathy without progression to PDR and ESRD, and 574 T1D diabetic control patients without retinopathy and nephropathy (16). We observed significant association from patients with PDR and ESRD (Table 1) and only all allele models, $OR_{het} = 1.19 \{0.81, 1.73\}$, $OR_{hom} = 2.38 \{1.60, 3.54\}$, T allele: 63.97% in cases versus 54.18% in controls, Table 1). This association remained significant after Bonferroni correction for multiple comparisons ($P = 0.036$).

Fig. 1. Genetic association. (A) Negative log $P$ values (y axis) from association analyses for 11 SNPs in the chromosome 7q22 region harboring the EPO gene (see also Table S3). The violet squares represent all SNPs around rs1617640 from the Utah T2D cohort. The green square represents rs1617640 from the GoKinD patients who have retinopathy and nephropathy but have not progressed into ESRD. The red square represents rs1617640 from the GoKinD patients who have both PDR and ESRD. The black square represents rs1617640 from the GoKinD cohort. The pink square represents rs1617640 from the Utah T2D cohort. The light blue square represents rs1617640 from the GoKinD patients who have retinopathy and nephropathy but have not progressed into ESRD. The red square represents rs1617640 from the GoKinD patients who have both PDR and ESRD. The black square represents rs1617640 from the GoKinD cohort. (B) Genomic structure and locations of genes between base pairs 99,94 and 100.04 million (National Center for Biotechnology Information build 35). (C) Pairwise $D^2$ and $R^2$ Haploview (38) plots for SNPs in chromosome 7 in the region around the EPO gene when Utah T2D case and control data are used.

another independent Boston European-American T1D cohort. This cohort includes 379 patients who have both PDR and nephropathy and 141 diabetic control patients of European-American ethnicity.

Hidden subdivision (stratification) can induce false-positive associations in case–control studies (17). This effect has been
minimized by our choice of European-American case–control cohorts derived from limited, distinct geographic areas in the United States (GoKinD cohort: Boston, Pittsburgh, and Minnesota; Utah cohort: Salt Lake City; Boston cohort: Boston). In particular, our initial Utah T2D cohort are all Utahns of European descent, known to be genetically homogeneous and very similar genetically to the British and Scandinavian populations from whom they were derived (18, 19). An analysis of subdivision in eight dispersed geographic samples from Utah demonstrated that only 1% of genetic variation can be attributed to subdivision effects (20). This slight degree of subdivision is highly unlikely to cause the strong association observed in the Utah case–control study. Furthermore, it is improbable that the same stratification effect would be seen in the two independent replication cohorts.

**Haplotype Analysis.** rs551238 is in high linkage disequilibrium (LD) with rs1617640 ($D^* = 0.89$, and $R^2 = 0.75$) and also exhibits a significant case–control difference in the Utah cohort ($P = 1.7 \times 10^{-2}$, Table S3). We observed a disease-associated haplotype, TTA, of SNPs rs1617640, rs507392, and rs551238 (59.5% in patients, 49.5% in controls, $P = 5.1 \times 10^{-5}$, Table S5). These SNPs exhibited high LD with each other in both case and control groups (Fig. 1C, which was generated by using Haploview 3.32). Two additional SNPs, rs734908 and rs4729607, are also in high LD with rs1617640, rs507392, and rs551238 in the Utah cohort (Fig. 1), but not in the GoKinD or Boston cohorts, and were therefore excluded from further analysis. We also identified a rare protective haplotype, GTA (2% in cases, 6.8% in controls, $P = 1.55 \times 10^{-5}$, Table S5). The GTA disease haplotype was also identified in the Boston T1D cohort (58% in cases, 48% in controls, $P = 5.9 \times 10^{-3}$), whereas another disease haplotype, TCA, was observed in the GoKinD cohort (20% in cases, 3% in controls, $P = 1.18 \times 10^{-7}$). The GTA protective haplotype was also present in the Boston cohort (2.3% in cases, 6.3% in controls, 1.9 $\times 10^{-3}$ and the GoKinD cohort (1.4% in cases, 5.1% in controls, $P = 0.023$). We identified another protective haplotype (GCC) in the GoKinD cohort (10.1% in cases, 28.2% in controls, $P = 1.17 \times 10^{-6}$). The over-riding difference between disease and protective haplotypes is T versus G at rs1617640. Allele T of rs507392 and allele A of rs551238 were present in both the risk and the protective haplotype, indicating that the T allele in rs1617640 is the main source of association.

**Effects of Different rs1617640 Alleles on EPO Expression.** rs1617640 is located 1,125 bp upstream of the EPO transcription start site. Using MatInspector to scan putative transcription factor binding sites within this region, we found that the risk allele T creates a matrix match with the EVI1/MEL1 or AP1 enhancer binding site (Table S6), suggesting it may confer an increased risk by influencing EPO expression. EPO protein concentrations were 7.5-fold higher in vitreous samples of nondiabetic individuals with the TT genotype compared with those with the GG genotype (Fig. 2, $P = 0.008$). The T allele enhanced luciferase reporter expression by 25-fold compared with that of the G allele (Fig. 3, $P = 4.7 \times 10^{-29}$). This substantial increase in promoter activity is probably specifically due to an EVI1/MEL1 or API binding site created by the T allele, because elimination of this SNP by deletion or substitution of T by other bases (C or A) resulted in markedly decreased reporter expression (Fig. 3). These data suggest that the T allele of rs1617640 plays a significant functional role in EPO expression.
studying retinal neovascularization (22). Therefore, we investigated EPO expression in the db/db and OIR mouse models. Consistent with the hypothesis that increased expression of EPO plays a role in the pathogenesis of diabetic kidney and eye complications, we demonstrated that the expression of EPO was increased ∼3-fold in the kidney of db/db mice (Fig. 4A, \( P = 0.0022 \)) and in the mouse ischemic retina (Fig. 4B, \( P = 1.1 \times 10^{-7} \)).

**Discussion**

The present study indicates an association between a functional EPO promoter polymorphism (rs1617640) and diabetic microvascular complications of the eye and kidney. The T risk allele is the common allele with comparable allele frequency among European, Asian, and African populations, indicating the risk allele may be an ancestral allele (23). Our study design is confined to a single ethnic group (European-American); therefore, the conclusion may not be generalizable to other ethnic groups and heterogeneity might be encountered when combining T1D and T2D. Further replication studies are needed.

EPO is located on 7q21, and our results are consistent with previous reports that chromosome 7q21 harbors a locus for increased susceptibility to diabetic nephropathy (11–14). The risk allele (T) is associated with elevated EPO in the vitreous body of the human eye and mouse models of diabetic eye and kidney complications.

EPO encodes a potent angiogenic factor expressed in the retina and kidney, and high EPO concentrations in human vitreous body are strongly associated with PDR (22). In addition, the EPO mRNA concentration is increased in the mouse model of ischemia-induced retinal neovascularization (Fig. 4B), and this neovascularization is suppressed by inhibition of EPO (22). EPO receptor immunoreactivity was strongly detected in neovascular tissues of PDR eyes (24). These findings combined with our case–control association study and EPO expression profiles in human vitreous body and luciferase promoter assays suggest that the TT variant in diabetic patients may convey increased EPO expression, resulting in an increased risk for the development of diabetic complications in the retina and kidney. Conversely, our results imply that localized EPO inhibition within the eye might have therapeutic potential for treatment or prevention of PDR.

Previous studies have suggested that variation of gene expression between alleles is common, and this variation is heritable and may contribute to human variability, such as disease susceptibility and drug resistance (for detail see review 25) (26–29). Almost one-third of promoter variants may alter gene expression and result in phenotypic variation (30). The effects of different alleles (T versus G) in the SNP rs1617640 of the EPO promoter on its expression are consistent with this notion.

EPO also maintains a functional role in erythropoiesis and is widely used to treat anemia resulting from renal failure or chemotherapy. In the United States, erythropoietin represents the largest single drug expense for the Center for Medicare and Medicaid Services, with spending at approximately one billion dollars per year (31). Patients with anemia attributable to chronic renal disease (many of whom have diabetes) and receive frequent dosing of EPO to maintain higher hemoglobin levels (13.5 g/dl) have a higher rate of cardiovascular complications than patients who maintain a lower hemoglobin level (11.3 g/dl) (32). A similar effect of EPO on accelerating the decline of kidney function had been suggested by earlier studies (33). Furthermore, prevalence and severity of PDR have worsened with increased EPO dosing (34). Recent work indicates that administration of EPO early in retinal vascular development protects retinal neuron and vasculature, whereas administration of EPO during retinal capillary nonperfusion associated with hypoxia markedly worsens retinal neovascularization in OIR mice (35). Retinal capillary nonperfusion associated with hypoxia is a hallmark of PDR in humans. Our genotype results suggest that caution may be warranted when maintaining higher hemoglobin concentrations by using exogenous EPO treatment in diabetic patients, because it might accelerate progression to PDR or ESRD.

Severe diabetic microvascular complications, including both PDR and ESRD, are also strong predictors of cardiovascular disease and mortality and are associated with a 10-year survival rate of <10% (32). Thus, identification of patients with strong genetic risk and implementation of preventive measures are of considerable importance. Our findings support a key role for EPO in genetic susceptibility to PDR and ESRD and identify a
potentially pathogenetic mechanism for advanced diabetic eye and kidney complications.

Materials and Methods

Cases and Controls. This study was approved by the GoKinD Access Committee, University of Utah, and Joslin Diabetic Center Institutional Review Boards. All subjects provided informed consent before participation. The GoKinD study is a collection of North American probands with T1D, with and without diabetic nephropathy. Presence or absence of retinopathy has been documented in most participating individuals. Panretinal laser treatment is used as an indication of PDR. Details of the GoKinD study are available online (www.jdrf.org/go kidn) and are published elsewhere (16). Boston patients were diagnosed and enrolled at the Joslin Diabetic Center, and Utah patients were diagnosed and enrolled at the Moran Eye Center and renal dialysis facilities of the University of Utah. The Boston and GoKinD cohorts were collected independently. All patients are Americans of European descent. Patients underwent detailed eye examinations using the Early Treatment of Diabetic Retinopathy Study (ETDRS) protocol of seven-standard-field stereoscopic fundus photography. Retinopathy status was determined by evaluation of fundus photographs and graded according to clinical ETDRS criteria [no retinopathy, mild nonproliferative diabetic retinopathy (NPDR), moderate NPDR, severe NPDR, very severe NPDR, PDR less than high risk, and PDR with high risk characteristics]. Patients with any disk neovascularization, neovascularization elsewhere, vitreous hemorrhage, fibrovascular proliferation, or tracial retinal detachment were considered to have PDR. Retinopathy grade was assigned without prior knowledge of genotypes. The Boston and Utah diabetic controls enrolled individuals with T1D or T2D for a minimum of 15 years without any nephropathy or retinopathy. A subject was considered to have definite diabetic nephropathy if he/she had proteinuria with a urine albumin to creatinine ratio > 300 (normal range < 30) or ESRD, defined as Stage V chronic kidney disease on dialysis or renal transplantation. The case subjects had both ESRD and a history of PDR unless indicated otherwise. Patient characteristics of the cohorts are listed in Table S1.

Genotyping. Genomic DNA was extracted from peripheral blood leukocytes. We selected 19 SNPs from 11 candidate genes of angiogenesis based on International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort. SNP rs1617640, which showed association based on initial analysis, was genotyped in the GoKinD International HapMap Center d’Etude du Polymorphisme Humain data. Initial genotyping was carried out in the Utah T2D cohort.

EPO mRNA Level in Oxygen-Induced Retinopathy (OIR) Mouse Model and db/db Mouse Model. Retinal neovascularization was produced in C57BL/6 mice by placing postnatal day 7 (P7) mice and their mothers in an atmosphere of 75% O2 and 25% N2 for 5 days. Oxygen concentration was automatically monitored and controlled by an oxygen controller (BioSpherix). At P12, the mice were returned to room air for 5 days. At P17 the mice were killed and their eyes were rapidly removed and dissected. The retina was removed and EPO mRNA levels were measured by RT-PCR.

HEK 293 cells were split into 24-well plates and cotransfected 24 hours later with 1 μg of the transfection control Renilla luciferase plasmid pTK-RL (Promega) and 200 ng of one of the following plasmids: pGL3-control plasmid, promoter (short) plasmid, promoter (G) construct, promoter (T) construct, promoter (A), promoter (C) and rs1617640 deletion (DEL) constructs. Transfections (n = 12) were performed using the FuGENE 6 protocol according to the manufacturer’s instructions (Roche Applied Science). Forty-eight hours after transfection, cells were washed with PBS twice and luciferase activities were measured with the Dual-Luciferase Assay Kit (Promega). Fold induction was derived relative to normalized reporter activity.
obtaining eye donor tissues. We thank Diana Lim for editorial assistance. The following investigators are members of the Genetics of Diabetes and Diabetic Complication Study Group: Xiang Ma, D. Joshua Cameron, Melvin Rabena, Alex Goldfarb-Rumyantzev, Nick Banerjee, Hilary Keenan, Nick Mamalis, Charles Perez, and Bradley J. Katz. We acknowledge the following grant support: National Institutes of Health Grants R01EY14428, R01EY14448, P01EY014800, and GCRC M01 RR00064, Foundation Fighting Blindness, the Macular Vision Research Foundation, Veterans Affairs Merit Award, and Research to Prevent Blindness (to K.Z.); Burroughs Wellcome Fund Clinical Science Award in Translational Research and a Lew R. Wasserman Merit Award (to K.Z.); National Institutes of Health Grant DK54931 and the Juvenile Diabetes Research Foundation (to M.R.P.); Children’s Health Research Center (S.P.); M.D./Ph.D. Program, University of Utah (R.C. and C.R.); National Institutes of Health Grants GM59290 and HL070048 (to L.B.J.); Juvenile Diabetes Research Foundation (S.T.); and National Heart, Lung, and Blood Institute, H.A. and Edna Benning Foundation, Juvenile Diabetes Research Foundation, American Heart Association, Burroughs Wellcome Fund Clinical Science Award in Translational Research, and Flight Attendant Medical Research Institute (D.Y.L.).


