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

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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 × 10−3; Genetics of Kidneys in Diabetes (GoKind) Study: P = 2.66 × 10−8; and Boston: P = 2.1 × 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 EV1/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 currently 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 cardio- and cerebrovascular 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...
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. S1C) 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 nephropathy 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 (15). 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 S1). 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, $\text{OR}_{\text{het}} = 1.20$ [0.76, 1.89], $\text{OR}_{\text{hom}} = 2.01$ [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 ($P = 1.04 \times 10^{-6}$, allelic model, $\text{OR}_{\text{het}} = 1.19$ [0.81, 1.73], $\text{OR}_{\text{hom}} = 2.38$ [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 ($P = 0.033$).

The red squares represent rs1617640 from the GoKinD patients who have both retinopathy and nephropathy but have not progressed into ESRD. The black square represents rs1617640 from the GoKinD patients who have retinopathy and nephropathy but have not progressed into ESRD. 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 yellow squares represent rs1617640 from the Utah T2D cohort. The purple squares represent rs1617640 from another independent Boston European-American T1D cohort.

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 ($D^\prime = 0.89$, 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, $P = 5.00 \times 10^{-4}$, 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, $P = 1.55 \times 10^{-3}$, Table S5). The GTA disease haplotype was also identified in the Boston T1D cohort (38% 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, $P = 1.9 \times 10^{-3}$) 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/MEI1 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/MEI1 or AP1 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.

**Expression of EPO mRNA in Mouse Retina and Kidney.** The db/db mouse model has been extensively used in studying diabetic kidney complications (21). Similarly, the oxygen-induced retinopathy (OIR) mouse model has been used extensively in

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**Table 1. Genotype and association results of rs1617640 from different cohorts**

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Utah T2D, $n = 613$</th>
<th>GoKinD T1D, $n = 1439$</th>
<th>Boston T1D, $n = 520$</th>
<th>All three cohorts, $n = 2,572$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype</td>
<td>Case</td>
<td>Control</td>
<td>Case</td>
<td>Control</td>
</tr>
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<td>Population size, n</td>
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</tr>
<tr>
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<td>335</td>
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<td>54.18</td>
<td>62.95</td>
<td>52.53</td>
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<td>Allelic P values</td>
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<td>2.76E-11</td>
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<td>Recessive P values</td>
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<td>3.27E-5</td>
<td>0.011</td>
<td>1.12E-10</td>
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<tr>
<td>Dominant P values</td>
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<td>6.22E-5</td>
<td>0.276</td>
<td>1.79E-5</td>
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<tr>
<td>ORhet 1.20 [0.758, 1.89]</td>
<td>1.46 [1.09, 1.97]</td>
<td>1.01 [0.639, 1.81]</td>
<td>1.30 [1.04, 1.63]</td>
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</tr>
<tr>
<td>ORhom 2.01 [1.23, 3.29]</td>
<td>2.43 [1.76, 3.35]</td>
<td>1.85 [1.04, 3.32]</td>
<td>2.17 [1.70, 2.76]</td>
<td></td>
</tr>
</tbody>
</table>

GoKinD, Genetics of Kidneys in Diabetes; HWE, Hardy–Weinberg equilibrium; ORhet and ORhom, odds ratio for heterozygous and homozygous states, respectively, with 95% confidence interval in brackets.
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
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/gokinb) 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 Utah 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 tractional 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 and Boston T1D cohorts. To investigate haplotype structure within the EPO region, 16 additional tagging, coding, or potentially functional SNPs in EPO and adjacent genes (10 SNPs in EPO, 3 SNPs in POP7, and 3 SNPs in ZAV) were genotyped in the Utah T2D cohort. A complete list of primers used for each SNP is listed in Table S7.

All SNPs were genotyped by using single-nucleotide primer extension assay (ABI Prism SNPShot Multiplex Kit, Applied Biosystems) on an ABI 3130xl genetic analyzer as previously described (36) and according to manufacturer’s instructions.

Genotyping Error Rate. All SNPs reported in this manuscript had a genotyping success rate > 98% and accuracy > 99% as judged by regenotyping using PCR product sequencing of 20% of samples in all three cohorts.

Statistical Analysis. All SNP genotyping results were screened for deviations from Hardy–Weinberg equilibrium and no SNPs showed significant deviation (P > 0.01). The χ2 test for trend for an additive model or dominant allele model over alleles was performed to assess evidence for association by using PEPI version 4.0.29. Odds ratios and 95% confidence intervals were calculated by using SPSS version 13.0 to estimate disease risk for heterozygotes and homozygotes. Linkage disequilibrium (LD) structure was examined by using Haploview (version 3.29) (37). Default settings were used, creating 95% confidence bounds on D’ and R2 to define pairwise SNPs in strong LD (39). Haploview was also used for allelic association tests.

MatInspector Analysis. The possible transcription factor (TF) binding sites were examined in rs1617640 variants by using positional weighting matrices extracted from MatInspector (www.genomatix.de/cgi-bin/matinpector.prom/fimat.fam.pl).

ELISA. Undiluted human vitreous samples were obtained at the time of pars plana vitrectomy surgery for indications of epiretinal membranes, or macular hole from nondiabetic patients. The concentration of EPO in human vitreous body was measured by using a sandwich ELISA. Polyclonal anti-EPO antibody (R&D Systems) was coated on poly(vinyl chloride) microtiter wells in carbonate/bicarbonate buffer (pH 9.6, 0.05 M) at 4°C overnight. After washing, remaining protein-binding sites were blocked with 10% goat serum at room temperature for 2 h. Twenty-five microliters of vitreous sample, or EPO standard sample (Cell Sciences) at different concentrations and 75 µl of 10% goat serum were added to each well and the plate was placed on a horizontal shaker at room temperature for 1 h. After washing, monoclonal anti-EPO antibody (R&D Systems) and horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology) were added. Finally, 3,3’,5,5’-tetramethylbenzidine (TMB) solution was added to develop color. Absorbance was read at 655 nm by a Benchmark Plus microplate reader (Bio-Rad) and the EPO concentration was calculated from a standard curve.

Luciferase Reporter Assays. A DNA fragment containing base pairs −1357 to +180 relative to the EPO transcription site including either the G (G construct) or T allele (T construct) of rs1617640 at −1125 was PCR amplified from genomic DNA of a normal individual by using the following primers: forward, CGGGGTACCAACTCTTGCTGCCTCCAAGGT; reverse, CGCGTGACTGCCCCGGGCTGTCCGGCCTTGTTCCCTTG (Fig. S2). Three other constructs with altered alleles (A, C, or deletion) at rs1617640 were also created. A DNA fragment containing base pairs −963 to +180 relative to the EPO transcription site without rs1617640 [Promoter (short)] was PCR amplified from genomic DNA of the same individual by using the following primers: forward, CCGGGTACCACTCGTGCCTCCAAGGT; reverse, CGCGTGACTGCCCCGGGCTGTCCGGCCTTGTTCCCTTG. All constructs were verified by restriction enzyme digestion and complete bidirectional DNA sequencing. We confirmed that the only difference between the T and G construct was T or G at rs1617640 and the rest of the EPO promoter sequence was identical to that of native human EPO promoter sequence. A positive control plasmid (pGL3-Control Vector) containing a SV40 enhancer and promoter driving luciferase reporter was obtained from Promega.

HEK 293 cells were split into 24-well plates and cotransfected 24 h later with 1 ng 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 done by using the Fugene-6 protocol according to the supplier's specifications (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.

EPO mRNA Level in Oxygen-Induced Retinopathy (OIR) Mouse Model and db/db Mouse Model. Retinal neovascularization was produced in C57BL/6J mice by placing postnatal day 7 (P7) mice and their mothers in an atmosphere of 75 to 3% oxygen for 5 days (40). 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.

db/db mice are among the best characterized and most intensively investigated mouse models for diabetic kidney disease (21). Animals were obtained from The Jackson Laboratory. Two-month-old db/db mice were killed, their kidneys were rapidly removed, and EPO mRNA levels were measured by RT-PCR.

Total RNA was isolated from retinas of the OIR mice and littersmate controls and from the kidney of db/db mice and littersmate controls. The RNA was converted into cDNA (Invitrogen, SuperScript II First-Strand Synthesis System for RT-PCR, catalog no. 18080-051). Fifty nanograms of cDNA was used for real-time PCR (Qiagen, QuantitTect SYBR Green PCR Kit) with the following primers (Mus-EPO-qPCR-L, CCAACCTGCTGTTTTACTAC/Mus-EPO-qPCR-R, CT-CAGCTTTGACACCTTG) for the mouse EPO gene to generate a 166-bp product. GAPDH expression (Mus-GAPDH-qPCR-L2, GTGAAAGTTCGGTGT-GACAGC/Mus-GAPDH-qPCR-R2, GCCGTGAATTGCCCGTGA) was used to normalize EPO expression. The RT-PCR was performed simultaneously for EPO and GAPDH on an ABI 7300 real-time PCR system. RT-PCR conditions were one cycle of 50°C for 2 min, 95°C for 15 min, followed by 35 cycles in which each cycle included 94°C for 15 sec, 58°C for 30 sec, and 72°C for 30 sec.

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