Proliferative diabetic retinopathy (PDR) is the most severe vision-threatening complication of diabetes. For investigation of genetic association between TCF7L2 and PDR in Caucasian type 2 diabetes mellitus (T2DM) and its functional consequences, 383 T2DM patients with PDR (T2DM-PDR) and 756 T2DM patients without diabetic retinopathy (T2DM-no DR) were genotyped with rs7903146 in TCF7L2. We found that risk allele (T) frequency of rs7903146 was significantly higher in T2DM-PDR patients (allelic \( P = 2.52E-04 \)). In lymphoblastoid cells induced to undergo endoplasmic reticulum (ER) stress by treatment of tunicamycin, higher fold change of TCF7L2 and VEGFA mRNA levels were observed in rs7903146-TT cells than in rs7903146-CC cells (\( P = 0.02 \) for TCF7L2, \( P = 0.004 \) for VEGFA), suggesting that ER stress plays a role in PDR pathogenesis. Silencing TCF7L2 resulted in decreased mRNA levels of both TCF7L2 and VEGFA (\( P < 0.001 \)). Retinas of oxygen-induced retinopathy mice (a model for PDR) had higher TCF7L2 and VEGFA mRNA levels than those of controls (\( P = 2.9E-04 \) for TCF7L2, \( P = 1.9E-07 \) for VEGFA). Together, data from our study show that TCF7L2 is associated with PDR in Caucasian T2DM and suggest that TCF7L2 promotes pathological retinal neovascularization via ER stress–dependent upregulation of VEGFA. *Diabetes* 62:2613–2617, 2013

The prevalence of diabetes mellitus is steadily increasing worldwide. Currently, diabetes mellitus affects 25.8 million people in the U.S. Diabetic retinopathy is the most common cause of diabetes mellitus complications and the leading cause of new cases of blindness among adults aged 20–74 years. Proliferative diabetic retinopathy (PDR) is the most severe vision-threatening complication of diabetes mellitus (1,2). Ischemia-induced angiogenesis and expansion of extracellular matrix in association with neovascularization are the pathological hallmarks in PDR. Neovascularization can ultimately result in vitreous hemorrhage and tractional retinal detachment, producing severe and often irreversible vision loss (3,4). Previous studies demonstrate that severe diabetic retinopathy aggregates in families (5), suggesting that genetic susceptibilities contribute to disease development. However, only a few genes, such as VEGF and EPO, have been identified to be associated with PDR to date (2,6).

Transcription factor 7-like 2 (*TCF7L2* [also known as *TCF4*) is a key component in the Wnt-signaling pathway, which regulates fundamental processes such as vascular development and has been found to mediate pathological neovascularization in proliferative retinopathy (4,7). Common variant rs7903146 in *TCF7L2* has been reported to be strongly associated with type 2 diabetes mellitus (T2DM), and this finding has been replicated in many studies (8,9).

It was reported in some small studies that *TCF7L2* gene is linked with microvascular and macrovascular complications, such as diabetic retinopathy (10), but the mechanism remains unclear. Here, we investigated the role of *TCF7L2* in PDR using a genetic association study and by analyzing the expression of *TCF7L2* and *VEGFA* in human lymphoblastoid cells, ARPE-19 cells, and oxygen-induced retinopathy (OIR) mice.

**RESEARCH DESIGN AND METHODS**

This study was approved by the institutional review boards of University of California, San Diego, and West China Hospital. All subjects gave informed consent prior to participation. A two-stage approach was performed for this study. In stage one, 209 T2DM patients with PDR (T2DM-PDR) and 442 T2DM patients without diabetic retinopathy (T2DM-no DR) were used as a discovery cohort. In stage two, 174 T2DM-PDR and 314 T2DM-no DR patients served as the replication cohort. All patients were of European descent. The control subjects were defined as having no retinopathy and having T2DM for a minimum of 15 years. Characteristics of participants in the study are listed in Supplementary Table 1.

To investigate whether *TCF7L2* is also associated with PDR in type 1 diabetes mellitus (T1DM), 372 T1DM-PDR and 417 T1DM-no DR Caucasian patients were studied. The control subjects were defined as having no retinopathy and having T1DM for a minimum of 15 years.

**Clinical assessment.** Participants underwent detailed eye examinations using the Early Treatment of Diabetic Retinopathy Study (ETDRS) protocol with seven-standard-field stereoscopic fundus photography. Retinopathy status was determined by evaluation of fundus photographs and graded according to clinical ETDRS criteria. Patients with any disk neovascularization, neovascularization elsewhere, vitreous hemorrhage, fibrovascular proliferation, or tractional retinal detachment were considered to have PDR. Retinopathy grading was performed without prior knowledge of genotypes.

**Genotyping.** Genomic DNA was extracted from peripheral blood leukocytes with a Qiagen kit (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. rs7903146 (C/T) in *TCF7L2* was genotyped using single-nucleotide
Recessive 0.081 [1.51 (0.92–2.37)]

**Statistical analysis.** Logistic regression analysis was used to compare the difference between the T2DM-PDR and T2DM–no DR groups. SNP genotyping results were screened for deviation from Hardy-Weinberg equilibrium using χ² tests. The differences in quantitative PCR data were analyzed with independent two-sample t test. Covariable analysis was done by SPSS software. Statistical significance was defined as P < 0.05. Pearson correlation coefficient was used to calculate the correlation between TCF7L2 and VEGFA (16).

**RESULTS**

**Association of TCF7L2-rs7903146 genotypes with PDR.** We performed genetic association study and genotyped a candidate gene TCF7L2 variant rs7903146 in T2DM-PDR case and T2DM–no DR control subjects. Information on demographics of case and control groups is presented in Supplementary Table 1. While there were no significant differences between the T2DM-PDR and T2DM–no DR groups with regard to sex, there was a statistically significant difference in age and BMI between the two groups (P < 0.05). The lower BMI was observed in the T2DM–no DR group.

In the discovery cohort, the T risk allele frequency of rs7903146 in TCF7L2 was significantly higher in patients with PDR (T2DM-PDR, 40.4%) than in patients without diabetic retinopathy (T2DM–no DR, 33.3%) (allelic P = 0.016). We replicated this association in a second T2DM cohort. In this replication cohort, the T-allele frequency of rs7903146 was also significantly higher in T2DM-PDR (42.0%) than in T2DM–no DR (33.4%) (allelic P = 0.008). Together, a combined analysis showed that the risk allele (T) of rs7903146 was strongly associated with T2DM-PDR (allelic P = 2.52E-04, odds ratio [OR] 1.40 [95% CI 1.16–1.68]). After adjustment for BMI, the risk allele (T) of rs7903146 remained significantly associated with T2DM-PDR (adjusted allelic P = 1.06E-03; OR 1.37 [1.14–1.65]). (Table 1).

To test whether this positive association can also be applied to a T1DM population, we genotyped 372 T1DM-PDR patients and 417 T1DM–no DR patients. There is no significant difference for the frequency of rs7903146 T allele between T1DM-PDR (28.0%) and T1DM–no DR (30.3%) (allelic P = 0.300) (Supplementary Table 3).

**ER stress increases VEGFA and TCF7L2 expression.** ER stress, which can be induced with tunicamycin, has been linked to vascular abnormalities in diabetes (17). We therefore tested whether treating human lymphoblastoid...
cells with tunicamycin could alter gene expression profiles of TCF7L2 and VEGFA. After treating human lymphoblastoid cells with tunicamycin, we observed a significantly higher fold change of mRNA levels of TCF7L2 in cells with a TCF7L2 rs7903146-TT risk genotype ($P = 0.02$) (Fig. 1A). Similarly, we observed increased fold change of mRNA level of VEGFA in cells with an rs7903146-TT risk genotype ($P = 0.004$) (Fig. 1B). There was also a positive correlation between the fold change of the expression of TCF7L2 and VEGFA in tunicamycin-treated cells (Pearson $r = 0.8663$) (Fig. 1C).

We did not observe a significant difference in the total expression levels of TCF7L2 in the cell lines with an rs7903146-CC or rs7903146-TT genotype at the baseline or after tunicamycin treatment (at baseline, $P = 0.853$; after tunicamycin treatment, $P = 0.390$) (Supplementary Fig. 1D).

Similarly, there was no significant difference in the total expression level of VEGFA in cell lines with an rs7903146-CC or rs7903146-TT genotype at the baseline ($P = 0.927$). However, there was a significant higher expression of VEGFA in the cell lines with rs7903146-TT than in rs7903146-CC after tunicamycin treatment ($P = 0.004$) (Supplementary Fig. 1B). There was no correlation between total transcripts of TCF7L2 and VEGFA without calculation of fold change (Pearson $r = -0.1$) (Supplementary Fig. 1C).

The changes in gene expression mentioned above were not due to alterations in the present experimental conditions, as the level of a housekeeping gene (GAPDH) remained the same throughout the experiment (Supplementary Fig. 1D) and was used for normalization of TCF7L2 and VEGFA expression in fold change calculations. In the cells treated with tunicamycin, ER stress was induced as shown by alternative splicing of XBP1. Two bands (unspliced and spliced band) were detected for the tunicamycin-treated cells (Supplementary Fig. 2).

Effect of silencing TCF7L2 on VEGFA expression. To validate the role of TCF7L2 in modulation of VEGFA expression, we performed an RNA silencing experiment by knocking down the expression of TCF7L2 in ARPE-19, and we observed concomitant 66% reduction of VEGFA mRNA level ($P < 0.001$) (Fig. 2).

For testing of whether there were off-target effects by shRNA, DNMT1 was used as an endogenous control in our study. The results showed that the expression of DNMT1 gene remained the same before or after shRNA treatment (Supplementary Fig. 3).

**TCF7L2 and VEGFA mRNA levels were elevated in OIR mice.** OIR mice model has been used extensively to study retinal ischemia and PDR. We measured mRNA levels in retinas from the OIR model. TCF7L2 and VEGFA mRNA expression was significantly higher in retinas from OIR mice compared with controls ($P = 2.90E-04$ for TCF7L2; $P = 1.90E-07$ for VEGFA) (Fig. 3). The expression of p-eIF2α, a positive marker for ER stress, was significantly upregulated in OIR mice retinas (1.72 ± 0.22-fold) (Supplementary Fig. 4).

**DISCUSSION**

TCF7L2 is the most significant susceptibility gene for T2DM thus far identified (9,18). However, the association between TCF7L2 and diabetic retinopathy has been conflicting (10,19). The discordant results in these studies may be due to a small sample size or the lack of distinction between the two stages of diabetic retinopathy: non-proliferative diabetic retinopathy (NPDR) and PDR. There are patients with NPDR who never progress to PDR despite poor glycemic control and long-standing diabetes, suggesting that there may be genetic factors predisposing patients to the development of PDR from NPDR. Our study therefore differentiates from other prior studies by taking the most extreme diabetic retinopathy phenotype (most severe phenotype). Here, we found that TCF7L2-rs7903146 is associated not only with T2DM (Supplementary Table 4) but also with PDR in Caucasian patients with T2DM. The allele (T) of rs7903146 is the same risk allele for T2DM-PDR as well as T2DM.

In patients with PDR, the expression of VEGFA is markedly upregulated (20). In our OIR study, the expression of both TCF7L2 and VEGFA was found to be significantly higher compared with controls. These findings provide evidence that TCF7L2, a key component of the Wnt-signaling pathway, plays an important role in pathological neovascularization.

![FIG. 1. Quantitative gene expression of VEGFA and TCF7L2 in tunicamycin-treated lymphoblastoid cells. A: Compared with that in untreated cells, the TCF7L2 mRNA level in tunicamycin-treated cells was 1.31 ± 0.18-fold higher with an rs7903146-CC genotype and 2.68 ± 0.54-fold higher with an rs7903146-TT ($P = 0.02$). B: The VEGFA mRNA level in tunicamycin-treated cells was 2.66 ± 0.60-fold higher in rs7903146-CC cells than that in untreated cells and 6.09 ± 0.86-fold higher in rs7903146-TT cells than that in untreated cells ($P = 0.004$). C: The fold change of VEGFA mRNA level correlated well with that of TCF7L2 mRNA level, Pearson $r = 0.8663$.](diabetes.diabetesjournals.org)
ER stress pathway has been linked to vascular abnormalities in diabetes and diabetic retinopathy (17,21). VEGFA is one of the target genes of TCF7L2 (22). To study whether ER stress played a role in TCF7L2 and VEGFA expression, we tested the expression of TCF7L2 and VEGFA in the tunicamycin-treated lymphoblastoid cell lines stratified with genotypes of rs7903146. We showed that after treatment of human lymphoblastoid cells with tunicamycin, a more significant increase in TCF7L2 and VEGFA expression was observed in cells with an rs7903146 TT risk allele than in those with a nonrisk CC allele (P = 0.02 and P = 0.004, respectively). There are two TCF7L2-binding sites in the VEGFA promoter region, which may play a role in upregulating VEGFA transcription through TCF7L2 binding (23). Our results are correlated with those of a previous study that showed that the expression of VEGFA in human lymphoblastoid cell lines is induced after treatment with an ER stressor (12). ER stress is also an important feature of chronic metabolic disease that is linked to both metabolic and immune regulation (24). It has been reported that lymphoblastoid cells from T2DM subjects with the genotypes of TT/TC have a higher expression of TCF7L2 in basal condition (25).

One of the plausible explanations is that the lymphoblastoid cells from T2DM subjects may already suffer from ER stress and that the T risk allele confers additional susceptibility to ER stress. The results indicate that ER stress may play an important role in triggering the increase of the expression of TCF7L2 and VEGFA.

The OIR mouse model has been widely used in studies related to PDR (13). In this model, neovascularization and proliferative retinopathy develop reliably (and quantitatively) over 17 days (26). Activation of ER stress and increased VEGFA can be observed in OIR mice models (15). We found that the ER stress–positive marker p-eIF2α increased in OIR mice retinas. OIR mice retinas had higher TCF7L2 and VEGFA mRNA levels than those of controls. These findings suggested that TCF7L2 may be a potential mediator of retinal neovascularization activated by ER stress during retinal neovascularization.

Some evidence indicates that other risk factors, such as BMI, are implicated in the pathogenesis of diabetic retinopathy (27,28). Our study demonstrated a relationship between higher BMI and an increased risk of T2DM-PDR.

There are some limitations in our study: 1) human lymphoblastoid cell lines are a reasonable choice of cells to study the effect of genotype, but they will only be a surrogate model for cells in retina; 2) tunicamycin is a strong chemical ER stressor and not entirely relevant in vivo; and 3) it will be valuable to analyze more covariables like T2DM onset, Hba1c levels (uncontrolled glycemia), fasting glucose, fatty acid, lipid profile, or blood pressure.

In summary, our study shows that TCF7L2 is associated with PDR in Caucasian T2DM subjects. Our results demonstrate that TCF7L2 expression is altered under ER stress and promote pathological retinal neovascularization via upregulation of VEGFA. Our findings also provide genetic evidence further implicating the Wnt/β-catenin–signaling pathway in the pathogenesis of PDR and suggest that TCF7L2 may serve as a potential therapeutic target for the treatment of PDR.

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J.Lu., L.Z., and A.Y.C. designed the study, performed the study, and analyzed and interpreted data. L.Z., A.Y.C., and J.Le. wrote the manuscript. X.Z., J.Zhu, J.Zha., H.O., H.L., Y.S., J.Le., S.P., P.X.S., and S.S. performed the study. Y.Z. designed the study and analyzed and interpreted data. M.G.R. supervised the overall project and designed the study. K.Z. supervised the overall project and designed the study, and analyzed and interpreted data. L.Z., A.Y.C. designed the study, performed the study, and analyzed and interpreted data. L.Z., A.Y.C. designed the study, performed the study, and analyzed and interpreted data.
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