

TCF7L2 Variation and Proliferative Diabetic Retinopathy

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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*-rs7903146 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

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primer extension assay (ABI Prism SNaPshot Multiplex kit; Applied Biosystems) on an ABI 3130xl genetic analyzer as previously described (11). The primers used for genotyping are listed in Supplementary Table 2.

Tunicamycin-treated lymphoblastoid cell lines. The human lymphoblastoid cell lines were generated by the method "general protocol for the immortalization of human B-lymphocytes using EBV" (<http://www.unclineberger.org/tissueculture/protocols/b-lymphocytesprotocol>). Nine lymphoblastoid cell lines with the genotypes of *TCF7L2* rs7903146-CC and another nine with the genotypes of *TCF7L2* rs7903146-TT were used. The cells were cultured in RPMI base medium (61870127; Invitrogen) supplemented with 20% FBS and 1×Penicillin-Streptomycin (P0781; Sigma). On the day before treatment, the cells were diluted to $\sim 2 \times 10^6$ /mL. On the day of treatment, tunicamycin (T7765; Sigma) was added to a final concentration of 10 μ g/mL. RNA was extracted at 12 h posttreatment. The total mRNA levels of *TCF7L2* and *VEGFA* were measured by quantitative RT-PCR (qRT-PCR). For monitoring for endoplasmic reticulum (ER) stress in human lymphoblastoid cell lines, RNA expression of XBP1 isoforms was used as a positive marker (12).

Lentivirus-mediated TCF7L2 short hairpin RNA knockdown in ARPE-19 cells. Retinal pigment epithelium ARPE-19 cells were cultured in Dulbecco's modified Eagle's medium/F12 (1:1) base medium (11320; GIBCO) supplemented with 10% FBS and 1×Penicillin-Streptomycin (P0781; Sigma) and 1×MEM NEAA (11140; GIBCO). Short hairpin RNA (shRNA) sets in pLKO.1 clones against *TCF7L2* (NM_030756.1) were purchased from Sigma-Aldrich. ARPE-19 cells were infected with control lentivirus or lentivirus-mediated *TCF7L2* shRNA. RNA was extracted at 48 h posttreatment. The total mRNA levels of *TCF7L2* and *VEGFA* were measured by qRT-PCR. *DNMT1* (DNA methyltransferase 1) gene was used as a control gene to test whether there are off-target effects for the shRNA in our study.

OIR mouse model. Retinal neovascularization was produced in C57BL/6 mice by placing postnatal day 7 (P7) mice and their mothers in an atmosphere of $75 \pm 3\%$ oxygen for 5 days (13). 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 the retinas were removed. Total RNA was isolated from the retinas of OIR mice and littermate controls. *TCF7L2* and *VEGFA* mRNA levels were measured by real-time qRT-PCR.

Real-time quantitative PCR. Total RNA extraction from mouse tissues or human lymphoblastoid cells, cDNA synthesis, and qRT-PCR experiments were performed as previously described (14). Assays were performed in triplicate. Relative mRNA levels were calculated by normalizing results using *GAPDH* and then compared with tissues from wild-type littermates or untreated human lymphoblastoid cells. The primers used for qRT-PCR are listed in Supplementary Table 2.

Western blot analysis. Retinal protein extracts were lysed in radioimmunoprecipitation assay buffer with a mixture of protease inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA). The total protein concentration was quantified by BCA protein assay (15). Twenty micrograms of total protein were used for blotting with anti-phosphorylated (p)-eukaryotic initiation factor (p-eIF2) α (Ser51) (Cell Signaling Technology, Boston, MA). The same membrane was stripped and reblotted with an anti- β -actin antibody (Santa Cruz Biotechnology) as internal control. The intensities of Western blot bands were quantified using the Multi Gauge, version 3.0 software (Fuji Film, Tokyo, Japan).

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 χ^2 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

TABLE 1

Genotype and association results of *TCF7L2*-rs7903146 in patients with T2DM-PDR and T2DM-no DR

	Caucasian (discovery cohort)		Caucasian (replication cohort)		Combined	
	T2DM-PDR	T2DM-no DR	T2DM-PDR	T2DM-no DR	T2DM-PDR	T2DM-no DR
Sample size	209	442	174	314	383	756
CC genotype	75	200	57	144	132	344
CT genotype	99	190	88	130	187	320
TT genotype	35	52	29	40	64	92
Risk allele (T) frequency	0.404	0.333	0.420	0.334	0.411	0.333
HWE <i>P</i> value	0.810	0.505	0.613	0.215	0.871	0.190
Allelic	0.016* [1.36 (1.06–1.74)];		0.008* [1.44 (1.09–1.90)];		2.52E-04* [1.40 (1.16–1.68)];	
	0.018# [1.36 (1.06–1.75)]#		0.023# [1.39 (1.05–1.84)]#		1.06E-03# [1.37 (1.14–1.65)]#	
Dominant	0.024* [1.48 (1.03–2.10)]		0.005* [1.74 (1.16–2.61)]		3.59E-04* [1.59 (1.22–2.07)];	
					1.34E-03# [1.55 (1.19–2.02)]#	
Recessive	0.081 [1.51 (0.92–2.46)]		0.127 [1.37 (0.79–2.37)]		0.035* [1.45 (1.01–2.08)];	
					0.065# [1.41 (0.98–2.02)]#	

Data are *n* or *P* [OR (95% CI)] unless otherwise indicated. *P* calculated from χ^2 test. DR, diabetic retinopathy; HWE, Hardy-Weinberg equilibrium. *Statistically significant. #Adjusted for BMI.

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. 1A).

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 *XBPI*. 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-eIF2 α , 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.

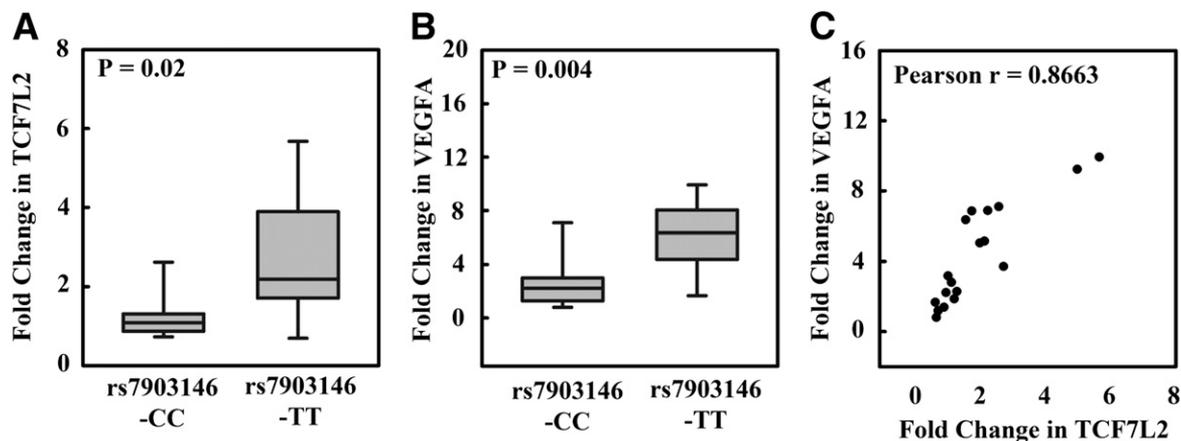


FIG. 1. Quantitative gene expression of *VEGFA* and *TCF7L2* in tunicamycin-treated lymphoblastoid cells. **A:** Compared with that in untreated cells, the *TCF7L2* mRNA level in tunicamycin-treated cells was 1.31 ± 0.18 -fold higher with an rs7903146-CC genotype and 2.68 ± 0.54 -fold higher with an 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 $r = 0.8663$.

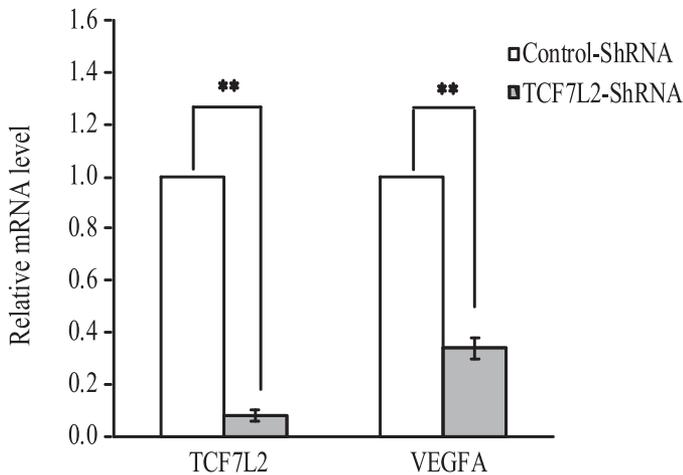


FIG. 2. Quantitative gene expression of *TCF7L2* and *VEGFA* after silencing by *TCF7L2*-shRNA in ARPE-19 cells ($n = 3$). ARPE-19 cells infected with *TCF7L2*-shRNA lentivirus had a 92% decrease in *TCF7L2* gene expression and 66% decrease in *VEGFA* compared with cells infected with control-shRNA lentivirus ($P < 0.001$). ** $P < 0.001$.

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

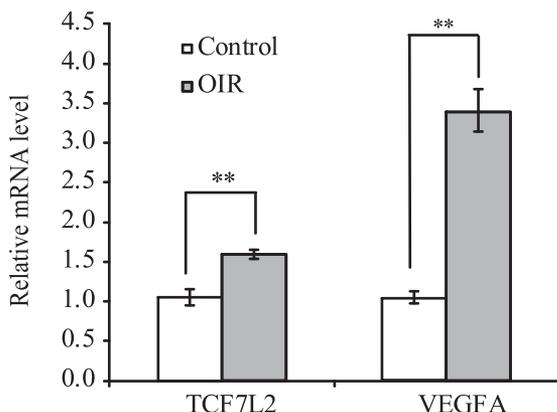


FIG. 3. Quantitative gene expression of *TCF7L2* and *VEGFA* in OIR mouse retinas ($n = 9$). Compared with those in controls, OIR mice retinas had higher *TCF7L2* mRNA expression ($P = 2.90E-04$) and *VEGFA* mRNA expression ($P = 1.90E-07$). ** $P < 0.001$.

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 quantifiably) 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-eIF2 α 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, HbA_{1c} 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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No potential conflicts of interest relevant to this article were reported.

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, designed the study, analyzed and interpreted data, wrote the manuscript, and was the principal investigator. K.Z. is the guarantor of this work and as such had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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