

Therapeutic Potential of a Monoclonal Antibody Blocking the Wnt Pathway in Diabetic Retinopathy

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Dysregulation of Wnt/ β -catenin signaling contributes to the development of diabetic retinopathy by inducing retinal inflammation, vascular leakage, and neovascularization. Here, we evaluated the inhibitory effect of a monoclonal antibody (Mab) specific for the E1E2 domain of Wnt coreceptor low-density lipoprotein receptor-related protein 6, Mab2F1, on canonical Wnt signaling and its therapeutic potential for diabetic retinopathy. Mab2F1 displayed robust inhibition on Wnt signaling with a half-maximal inhibitory concentration (IC_{50}) of 20 μ g/mL in retinal pigment epithelial cells. In addition, Mab2F1 also attenuated the accumulation of β -catenin and overexpression of vascular endothelial growth factor, intercellular adhesion molecule-1, and tumor necrosis factor- α induced by high-glucose medium in retinal endothelial cells. In vivo, an intravitreal injection of Mab2F1 significantly reduced retinal vascular leakage and decreased pre-retinal vascular cells in oxygen-induced retinopathy (OIR) rats, demonstrating its inhibitory effects on ischemia-induced retinal neovascularization. Moreover, Mab2F1 blocked the overexpression of the inflammatory/angiogenic factors, attenuated leukostasis, and reduced retinal vascular leakage in both early and late stages of streptozotocin-induced diabetes. In conclusion, Mab2F1 inhibits canonical Wnt signaling, vascular leakage, and inflammation in the retina of diabetic retinopathy models, suggesting its potential to be used as a therapeutic agent in combination with other antiangiogenic compounds. *Diabetes* 61:2948–2957, 2012

Diabetic retinopathy, a leading cause of blindness, is a severe ocular complication of diabetes involving progressive retinal vascular leakage, retinal neovascularization, and retinal detachment in both type 1 and type 2 diabetes (1,2). Multiple factors, such as vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF)- α , connective tissue growth factor, and intercellular adhesion molecule (ICAM)-1, have been shown to play important roles in retinal vascular abnormalities in diabetic retinopathy (1,3,4). In addition, retinal inflammation in a hyperglycemia condition with oxidative stress is believed to play a crucial role in the development of diabetic retinopathy (5–8). Although numerous observations have underscored the association of hyperglycemia with inflammation in diabetic retinopathy, the pathogenic mechanism leading to chronic retinal

inflammation in diabetes is unclear. Recently, the pathogenic role of the canonical Wnt pathway in retinal inflammation in diabetic retinopathy has been established (9).

Wnts, a group of secreted cysteine-rich glycoproteins, bind to a receptor complex consisting of Frizzled receptor and low-density lipoprotein receptor-related protein (LRP)5/6 coreceptor on the cell membrane. Upon Wnt ligand binding, the COOH-terminal-conserved motifs (PPSPXS) of LRP6 are phosphorylated (10,11). The phosphorylation of PPSPXS motifs reduces the cytosolic kinase pool, directly inactivates glycogen synthase kinase (GSK) β , and results in cytosolic β -catenin stabilization and accumulation (12,13). Then, β -catenin is translocated into the nucleus, dimerizes with transcription factors TCF (T-cell factor)/LEF (lymphoid enhancer-binding factor), and activates transcription of target genes including *VEGF*, *ICAM-1*, *TNF- α* , *CTGF*, and *PDGF-BB* (11,14,15). The Wnt coreceptor LRP6 is suggested to play a pivotal role in the Wnt signaling initiation and amplification for the following reasons: 1) binding of Wnt ligand to the LRP6 ectodomain is essential for initiating the Wnt signaling cascades and 2) multiple PPSPXS motifs on the cytoplasm domain of LRP6 serve as signal-amplifying modules (12,16). These facts suggest that LRP6 represents an ideal target for blocking the Wnt pathway.

Our recent studies showed that retinal levels of β -catenin are increased in humans with diabetic retinopathy and in diabetic retinopathy animal models (9). Retinal levels of LRP6 are also increased in the retina of the diabetic retinopathy models. In addition, an intravitreal injection of a Wnt antagonist, Dickkopf (DKK)1, effectively ameliorated diabetic retinopathy in animal models, suggesting a key role of the dysregulation of the Wnt/ β -catenin signaling in the pathogenesis of diabetic retinopathy (9). Furthermore, activation of Wnt signaling in the retina of normal rats by a constitutively active mutant of β -catenin (S37A) was sufficient to induce not only overexpression of angiogenic/inflammatory factors such as VEGF, ICAM-1, TNF- α , and nuclear factor- κ B but also generation of reactive oxygen species (9,17). Together, these previous findings suggest that overactivation of Wnt/ β -catenin signaling plays a pathogenic role in diabetic retinopathy and represents a promising drug target for its treatment.

Based on these previous studies, we hypothesized that blockade of the Wnt signaling provides effective and successful means for drug intervention of diabetic retinopathy. In the current study, we generated a monoclonal antibody (Mab) specific for the first and second propeller domains in the extracellular region of LRP6. We further evaluated its inhibitory effects on the canonical Wnt pathway and its beneficial effects on diabetic retinopathy.

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RESEARCH DESIGN AND METHODS

Luciferase reporter assay. Luciferase reporter assay was performed in human telomerase reverse transcriptase (hTERT)-retinal pigment epithelial (RPE)-1 cells following previously established methods (18,19). hTERT-RPE-1 cells were transfected with 0.25 μ g TOPFLASH (TCF reporter plasmid; *Firefly* luciferase) and 0.05 μ g pRL-TK (pRL reporter plasmid; *Renilla* luciferase) constructs using lipofectamine 2000. To evaluate the inhibitory effect of Mab2F1 on the Wnt1-mediated Wnt-signaling activation, hTERT-RPE cells were transfected with the Wnt1 expression plasmid or an empty vector together with the TOPFLASH reporter plasmid. Four hours posttransfection,

the cells were incubated with fresh media containing Mab2F1 or control IgG. After incubation for 16 h, the cells were lysed and luciferase activity was measured. Luciferase activity was measured using a dual luciferase assay kit (Promega, Madison, WI) following the manufacturer's protocol. *Renilla reniformis* luciferase activity was measured to normalize transfection efficiency. All experiments were performed at least in triplicate.

Western blot analysis. Western blot analysis was performed as previously described (19). Mouse anti- β -actin antibody, rabbit anti-TNF- α antibody (Abcam, Cambridge, MA), mouse anti-VEGF antibody, goat anti-ICAM-1 antibody, rabbit anti- β -catenin antibody (Santa Cruz Biotechnologies, Santa

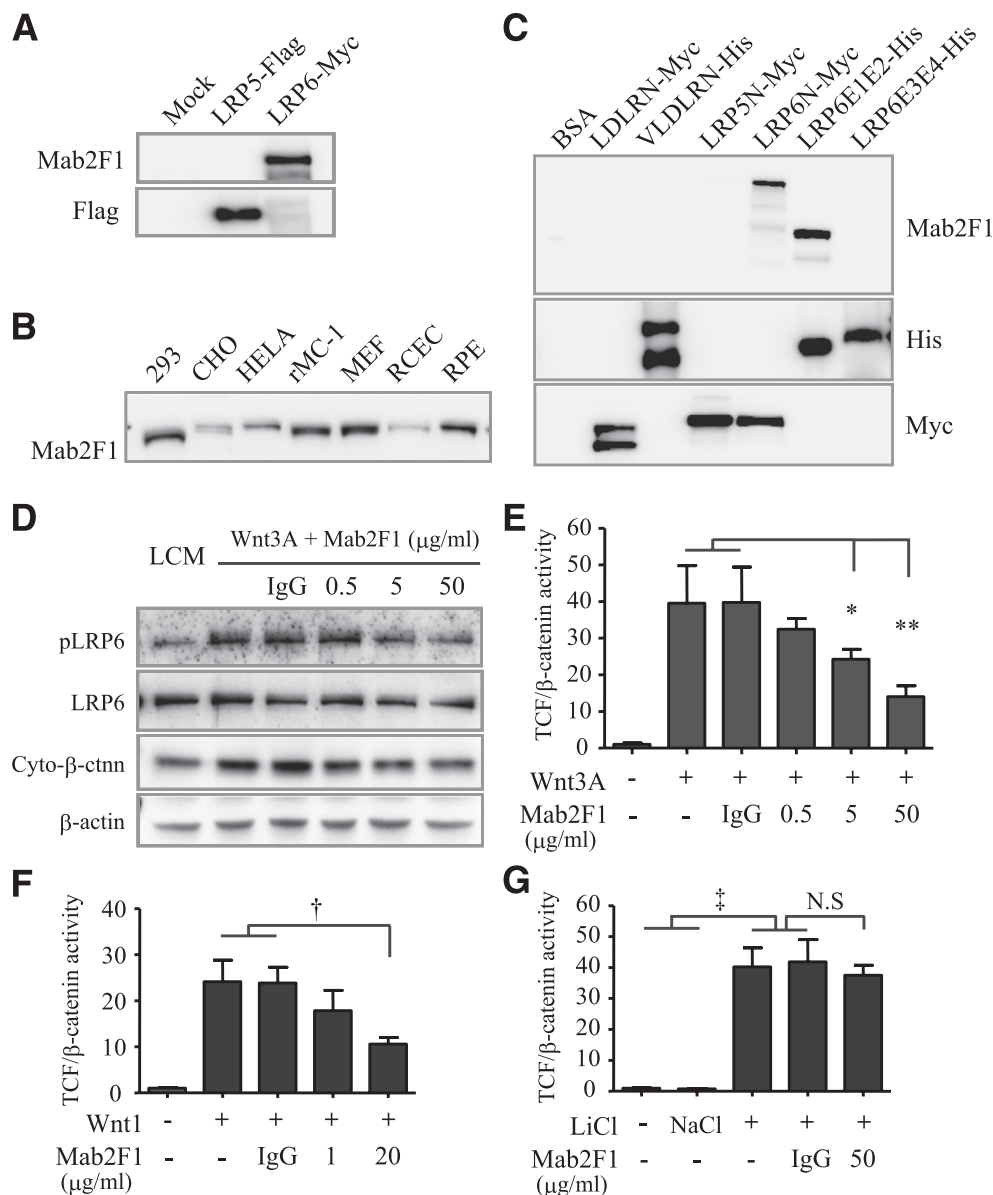


FIG. 1. Inhibitory effect of Mab2F1 on Wnt/ β -catenin signaling at the receptor level. **A:** Total cell lysates (50 μ g) of 293T cells expressing LRP5-Flag and LRP6-Myc were applied for Western blot analysis using Mab2F1 and an anti-Flag antibody (Flag). **B:** Total cell lysates (50 μ g) from each cell line were applied for Western blot analysis using Mab2F1. **C:** Conditioned media containing low-density lipoprotein receptor ectodomain (LDLRN)-Myc, LRP5 ectodomain (LRP5N)-Myc, and LRP6 ectodomain (LRP6N)-Myc and purified recombinant peptides of VLDL receptor ectodomain (VLDLR-N)-His, LRP6E1E2-His, and LRP6E3E4-His were loaded for Western blot analysis using Mab2F1, anti-His, and anti-Myc antibodies. **D:** hTERT-RPE-1 cells were pretreated with Mab2F1 for 30 min and then exposed to 25% Wnt3A-conditioned media (Wnt3A), with L-cell conditioned media (LCM) as a control. After 2 h stimulation, the cell lysates (50 μ g) were subjected to Western blot analysis using antibodies for pLRP6 and for total LRP6. Cytosolic proteins (20 μ g) were isolated and blotted with an antibody for β -catenin (cyto- β -ctnn). **E:** hTERT-RPE-1 cells were transfected with TOPFLASH and pRL-TK plasmids. The cells were then treated with 25% Wnt3A medium and Mab2F1, supplemented with control IgG to reach the same amount of total IgG (50 μ g) in each well for 16 h. **F:** hTERT-RPE-1 cells were transfected with TOPFLASH vectors and a Wnt1-expression plasmid, with an empty vector as control. At 4 h posttransfection, cells were treated with Mab2F1 for 16 h. **G:** hTERT-RPE-1 cells were exposed to 25 mmol/L LiCl to activate Wnt signaling, with NaCl as control. Equal amounts of nonspecific IgG or Mab2F1 (50 μ g/mL) were added to the cells and incubated for 16 h. **E-G:** TCF/ β -catenin activity was then measured using dual-luciferase assay and expressed as relative luciferase units (means \pm SD, $n = 4$; * $P < 0.05$, ** $P < 0.01$, † $P < 0.001$, ‡ $P < 0.0001$). RCEC, retinal capillary endothelial cells. CHO, Chinese hamster ovary. rMC-1, rat Müller cell. MEF, mouse embryonic fibroblast.

Cruz, CA), rabbit anti-phosphorylated LRP6 (pLRP6) (at Ser1490) antibody, and rabbit anti-phosphorylated β -catenin (Ser33/37/Thr41) antibody (Cell Signaling, Danvers, MA) were used for Western blot analysis. Individual protein band was semiquantified by densitometry using the GENETOOLS program (SynGene, Frederick, MD).

Endothelial cell migration assay. Bovine retinal capillary endothelial cells (BRCECs) were cultured in six-well plates to confluency. The BRCEC monolayer was scratched by plastic pipette to generate a zone that is not covered by cells. Then, pictures of no-cell area at the 0-h time point were taken. The scratched cells were treated separately with 20 μ g/mL Mab2F1 and nonspecific control IgG, followed by high-glucose medium. Forty-eight hours postscratch, images of the no-cell zone were taken from at least three non-overlapping areas. Wound closure was quantified by measuring the area of the no-cell zone using the ImageJ (NIH) program.

Tube formation assay. For tube formation assay, Matrigel (BD Bioscience, Bedford, MA) including various growth factors was used. Matrigel was evenly spread into a 24-well plate. Retinal capillary endothelial cells (2.5×10^4 /well) were incubated with control IgG and Mab2F1 at 20 μ g/mL and were then plated onto Matrigel. After 12 h incubation, five individual fields from each well were photographed. Tube formation was quantified by counting branches at points of intercepts.

Rat models of oxygen-induced retinopathy and streptozotocine-induced diabetes. All of the animal experiments were performed in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Brown Norway rats (Charles River Laboratories, Wilmington, MA) were used for the oxygen-induced retinopathy (OIR) model and streptozotocine (STZ)-induced diabetes model following established protocols (9,20).

Retinal vascular permeability assay and leukostasis assay. Retinal vascular permeability was measured using Evans blue–albumin as tracer following an established protocol (21). Leukostasis assay was performed by staining

adherent leukocytes with fluorescein isothiocyanate–conjugated concanavalin-A as previously described (9).

Statistical analysis. The quantitative data were analyzed and compared using Student *t* test or repeated-measures ANOVA. Statistical significance was set at $P < 0.05$.

RESULTS

Mab2F1, an Mab specific for LRP6 E1E2 domains, blocks Wnt/ β -catenin signaling at the Wnt receptor level. A number of clones of Mabs were raised using a recombinant peptide of the E1E2 domains from the human LRP6 extracellular region. From positive-antibody clones, Mab2F1 was selected for further studies based on its specificity and high affinity for LRP6 in various cell lines. Specificity of Mab2F1 for LRP6 was tested by Western blot analysis, which showed that Mab2F1 recognized LRP6 as a single band but not LRP5, the other Wnt coreceptor with 71% amino acid sequence identity to LRP6 in the human. In addition, Mab2F1 recognized endogenous LRP6 in various cell lines derived from different species including human, mouse, rat, and bovine (Fig. 1A and B). As LRP6 belongs to the LDL receptor (LDLR) family, the possible binding of Mab2F1 to other members of the LDLR family such as LDLR and VLDL receptor (VLDLR) was evaluated. Western blot analysis showed that Mab2F1 did not recognize LDLR or VLDLR, suggesting its specificity for LRP6 (Fig. 1C). Furthermore, we confirmed that Mab2F1 recognized the

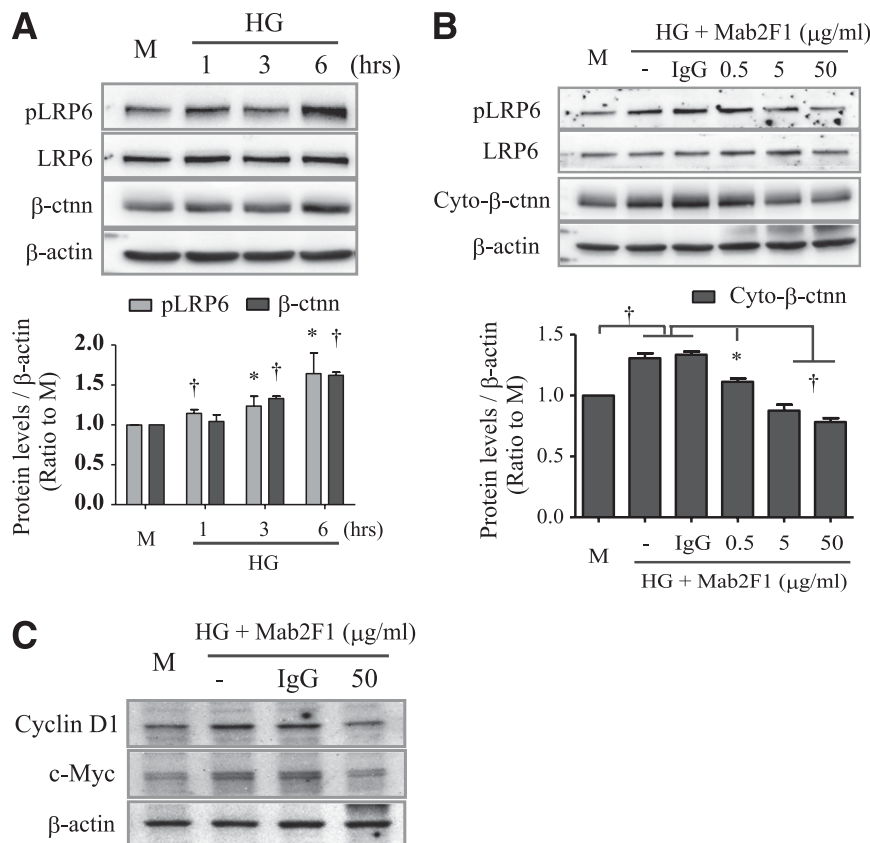


FIG. 2. Inhibitory effect of Mab2F1 on high glucose-activated Wnt/ β -catenin signaling. hTERT-RPE-1 cells were exposed to high-glucose media using 30 mmol/L glucose (HG) with 5 mmol/L glucose and 25 mmol/L mannitol (M) for osmotic control. **A:** The cells were exposed to high glucose for different durations. The levels of pLRP6 and β -catenin were semiquantified by densitometry. **B:** The cells were treated with high-glucose medium with Mab2F1 or control IgG for 6 h. Total cell lysates were used for Western blot analysis using antibodies for pLRP6 and total LRP6. Cytosolic β -catenin (Cyto- β -ctnn) was measured in isolated cytosolic fraction by Western blot analysis, quantified by densitometry, and normalized by β -actin levels (means \pm SD, $n = 4$; * $P < 0.05$, † $P < 0.001$). **C:** The cells were exposed to high glucose for 24 h with 50 μ g/mL Mab2F1 or IgG, followed by Western blot analysis using antibodies specific for cyclin D1 and c-myc.

full-length ectodomain and the E1E2 fragment of LRP6 but not the E3E4 domain, suggesting that the epitope of Mab2F1 is located in the E1E2 domain of LRP6 (Fig. 1C).

We next determined whether Mab2F1 has an inhibitory effect on the canonical Wnt pathway. hTERT-RPE-1, a cell line derived from human RPE cells and expressing endogenous LRP6, was exposed to Wnt3A-conditioned media or was transfected with a plasmid expressing Wnt1 to activate Wnt/ β -catenin signaling. Mab2F1 efficiently inhibited the Wnt3A-induced increase of pLRP6 levels, suggesting inhibited phosphorylation of LRP6 on residue Ser1490, an early step in Wnt pathway activation, while having no effect on total LRP6 levels after a 2-h incubation. Mab2F1 also attenuated the Wnt ligand-induced increase of cytosolic β -catenin levels, suggesting that it attenuated the β -catenin accumulation induced by Wnt ligand. In contrast, nonspecific IgG had no effect on pLRP6 or cytosolic β -catenin levels (Fig. 1D). The effect of Mab2F1 on transcriptional activity of TCF/ β -catenin was evaluated using TOPFLASH activity assay, which measures Luciferase activity driven by a promoter containing TCF/ β -catenin-binding sites. TOPFLASH assay showed that Wnt3A induced Luciferase activity by 40-fold, and the Mab2F1 suppressed the Wnt3A-induced Luciferase activity in a concentration-dependent manner with $IC_{50} \sim 20 \mu\text{g/mL}$ (Fig. 1E). Similarly, Mab2F1 also inhibited the Wnt1-induced TCF/ β -catenin activity (Fig. 1F).

LiCl is a known inhibitor of GSK3 β , which constitutively phosphorylates β -catenin and mediates its proteasomal degradation. Thus, Li activates canonical Wnt signaling independent of Wnt ligands, Wnt receptors, and co-receptors. As shown by TOPFLASH assay, 25 mmol/L LiCl markedly induced TCF/ β -catenin activity. Mab2F1 did not inhibit Li-induced TOPFLASH activity, suggesting that the inhibition of Wnt signaling by Mab2F1 occurs at the receptor level (Fig. 1G).

Mab2F1 inhibited high glucose-induced Wnt/ β -catenin signaling. For testing of the inhibitory effect of Mab2F1 on diabetes-induced Wnt/ β -catenin signaling, hTERT-RPE-1 cells were exposed to a high-glucose medium (30 mmol/L), with low-glucose medium (5 mmol/L glucose and 25 mmol/L mannitol) as control. Mab2F1 was added to the medium and incubated with the cells to evaluate its effect on Wnt signaling. Western blot analysis showed that exposure to high glucose for 6 h increased pLRP6 and β -catenin levels (Fig. 2A). Mab2F1 attenuated the high glucose-induced increases of pLRP6 and cytosolic β -catenin levels in a concentration-dependent manner (Fig. 2B). For further confirmation of the activation of Wnt signaling, expression levels of Wnt target genes such as *cyclin D1* and *c-myc* were measured. High-glucose medium upregulated the expression of *cyclin D1* and *c-myc*. Mab2F1 attenuated the high glucose-induced overexpression of *cyclin D1* and *c-myc* (Fig. 2C). These results suggest that Mab2F1 attenuates the high glucose-induced activation of Wnt/ β -catenin signaling and overexpression of its target genes.

Mab2F1 inhibits high glucose-induced overexpression of angiogenic/inflammatory factors in retinal cells. hTERT-RPE-1 cells were exposed to 30 mmol/L glucose to activate Wnt/ β -catenin signaling. We tested whether Mab2F1 inhibits overexpression of angiogenic and inflammatory factors that are regulated by Wnt signaling (17,18,22). In the RPE cells, high-glucose medium induced overexpression of VEGF, ICAM-1, and TNF- α , which are known pathogenic factors in diabetic retinopathy. Mab2F1,

but not nonspecific IgG, suppressed the overexpression of these factors in a concentration-dependent manner (Fig. 3A). At high concentrations, Mab2F1 reduced the levels of VEGF, ICAM-1, and TNF- α to a range of low glucose control. Moreover, compared with low glucose control (5 mmol/L glucose and 25 mmol/L mannitol), 30 mmol/L glucose increased secreted VEGF levels in the culture medium, while Mab2F1 attenuated the increase induced by high glucose. The inhibitory effect of Mab2F1 on the overexpression of these factors in high-glucose media was also confirmed in retinal capillary endothelial cells and Müller cells (Supplementary Fig. 1). These results suggest that Mab2F1 attenuates the high glucose-induced expression of angiogenic and inflammatory factors via inhibition of Wnt/ β -catenin signaling.

Mab2F1 inhibited endothelial cell migration. The effect of Mab2F1 on endothelial cell migration was evaluated by the scratch wound-healing assay and tube formation

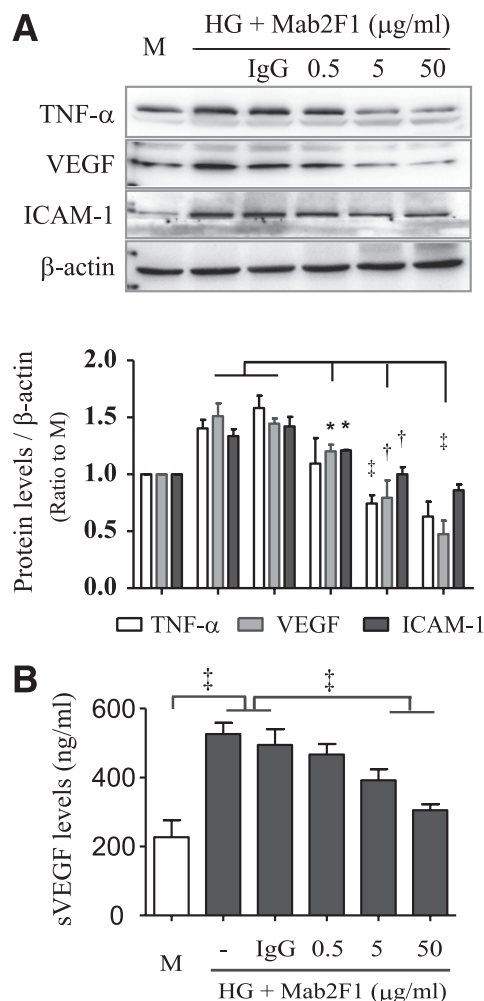


FIG. 3. Inhibitory effect of Mab2F1 on high glucose (HG)-induced overexpression of angiogenic and inflammatory factors. **A:** hTERT-RPE-1 cells were exposed to high glucose for 24 h after 1 h preincubation with Mab2F1. Western blot analysis was performed using antibodies for ICAM-1, TNF- α , and VEGF. Representative blots from at least three independent experiments are shown. Levels were semi-quantified by densitometry and normalized by β -actin levels (means \pm SD, $n = 3$; * $P < 0.05$, † $P < 0.001$, ‡ $P < 0.0001$). **B:** hTERT-RPE-1 cells were exposed to high glucose for 24 h after 1 h preincubation with the Mab2F1. The culture media were collected, and secreted VEGF levels were measured using enzyme-linked immunosorbent assay (means \pm SD, $n = 3$; ‡ $P < 0.0001$). M, mannitol.

assay using primary BRCECs, as endothelial cell migration is an important step in retinal neovascularization. The scratch wound-healing assay showed that high-glucose medium enhanced BRCEC migration, based on the wound area measurement 48 h after the scratch. In the presence of Mab2F1, the high glucose-induced BRCEC wound healing was substantially decreased (Fig. 4A–F). In BRCEC tube formation assay, BRCEC formed a tube-like pattern, after 12 h culture in the Matrigel, which was attenuated by Mab2F1 but not by nonspecific IgG (Fig. 4G–J). Taken together, these results demonstrate that Mab2F1 inhibited endothelial cell migration.

Mab2F1 reduced vascular leakage, inhibited inflammation, and attenuated retinal neovascularization in the retina of the OIR model. One of the hallmarks of diabetic retinopathy is retinal vascular leakage due to breakdown of the blood-retina barrier. OIR rats, a model of ischemia-induced retinopathy, manifest increased retinal vascular permeability due to ischemia-mediated overexpression of proangiogenic factors such as VEGF. For evaluation of inhibitory effect of Mab2F1 on vascular leakage in the OIR model, Mab2F1 was injected intravitreally into the right eye (10 $\mu\text{g}/\text{eye}$) at age of postnatal day (P)12 and the same amount of nonspecific mouse IgG into the contralateral eyes as control. Retinal vascular leakage was measured using the permeability assay at P16, which showed that the eyes injected with Mab2F1 had significantly lower retinal vascular permeability compared with that in eyes injected with control IgG (Fig. 5A). Next, we measured retinal levels of the factors contributing to vascular leakage

and inflammation. Compared with control IgG, Mab2F1 suppressed the expression of ICAM-1, TNF- α , and VEGF in the retina of OIR rats (Fig. 5B and D). Mab2F1 also down-regulated retinal levels of LRP6 and β -catenin, suggesting that Mab2F1 attenuated angiogenic/inflammatory activities in the retina of OIR rats by inhibiting Wnt/ β -catenin-signaling activation (Fig. 5B and C). Moreover, the effect of Mab2F1 on preretinal retinal neovascularization—vascular cells growing into the vitreous cavity, a characteristic of the proliferative diabetic retinopathy—was evaluated. Injection of Mab2F1 at P12 of OIR rats significantly decreased preretinal vascular cells as quantified at P18, suggesting that Mab2F1 suppressed preretinal retinal neovascularization in the OIR rat model (Fig. 5E). Taken together, the results demonstrate that Mab2F1 conferred inhibitory effects on retinal vascular permeability, inflammation, and retinal neovascularization in an ischemia-induced retinopathy model.

Effect of Mab2F1 on retinal vascular leakage and inflammation in early stages of STZ-induced diabetes. To evaluate the beneficial effect of Mab2F1 on diabetic retinopathy, rats with STZ-induced diabetes at 2 weeks after the onset of diabetes received an intravitreal injection of Mab2F1 (20 $\mu\text{g}/\text{eye}$) or the same amount of nonspecific IgG as control. Retinal vascular permeability was measured 1 week after the Mab2F1 injection using the Evans blue-albumin leakage method and compared with the IgG control. The result demonstrated that the eyes injected with Mab2F1 had significantly lower retinal vascular permeability than that in eyes injected with control IgG (Fig. 6A). Retinal

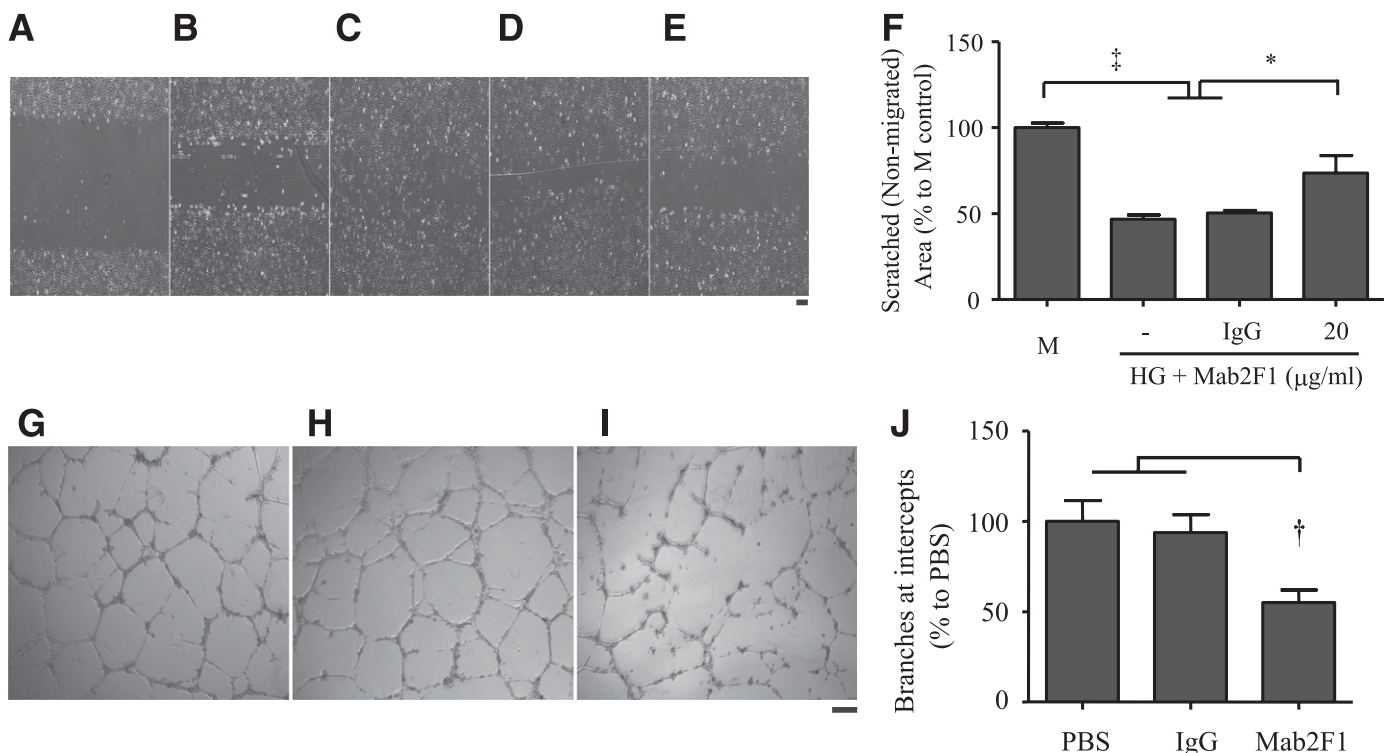


FIG. 4. Inhibitory effect of Mab2F1 on migration and tubular structure formation of endothelial cells. *A–E*: The BRCEC monolayer was scratched and exposed to high glucose (HG) (30 mmol/L) for 48 h with 20 $\mu\text{g}/\text{mL}$ Mab2F1 or nonspecific IgG. Representative images of the scratch area are shown (*A*: 0 h; *B*: mannitol [M]; *C*: high glucose; *D*: high glucose with IgG; *E*: high glucose with Mab2F1). Scale bar = 200 μm . *F*: Scratched areas, which were not covered by migrated cells, were measured using ImageJ (NIH) for the quantification (means \pm SD, $n = 3$; $\ddagger P < 0.001$, $*P < 0.05$). *G–I*: BRCECs were seeded ($2.5 \times 10^4/\text{well}$) onto Matrigel and supplemented with PBS (*G*), 20 $\mu\text{g}/\text{mL}$ nonspecific IgG (*H*), and Mab2F1 (*I*) and grown for 12 h to form a tube-like pattern. Five fields from each well were photographed. Scale bar = 100 μm . *J*: Tube formation was quantified by counting the numbers of branches at points of intercepts (means \pm SD, $n = 3$; $\ddagger P < 0.001$).

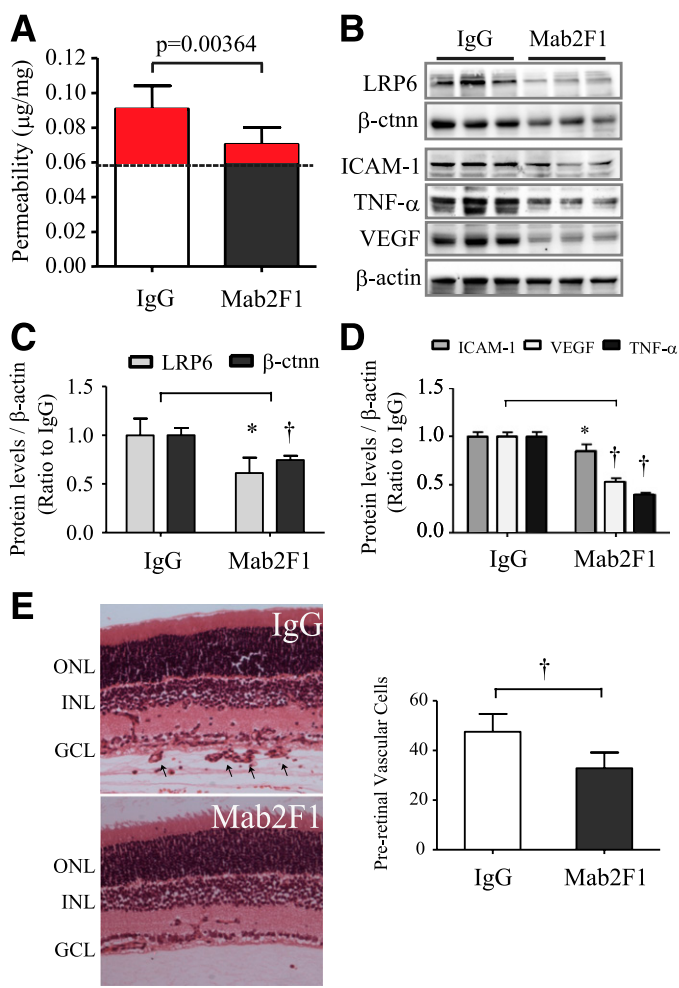


FIG. 5. Inhibitory effects of Mab2F1 on retinal vascular leakage, inflammation, and retinal neovascularization in the OIR model. **A:** OIR rats received an intravitreal injection of 10 µg Mab2F1 per eye and the same amount of nonspecific IgG to the contralateral eyes at the age of P12. Retinal vascular permeability was measured at P16 using Evans blue as a tracer, normalized by total retinal protein concentrations, and expressed as micrograms of Evans blue per milligram of retinal proteins (means \pm SD, $n = 8$; $P < 0.001$). The dotted line indicates the basal level of vascular permeability in age-matched normal animals. **B–D:** The retinas were harvested at P16, and the same amount of retinal proteins (50 µg) was loaded for Western blot analysis using antibodies specific for LRP6, β -catenin (β -ctnn), ICAM-1, VEGF, and TNF- α and normalized by β -actin levels (means \pm SD, $n = 3$; $*P < 0.05$, $\dagger P < 0.001$). **E:** Pre-retinal vascular cells, indicated by arrows, were counted in eight noncontinuous sections per eye and averaged as previously described (39). The average numbers of preretinal vascular cells in the eyes injected with Mab2F1 were compared with those injected with control IgG using paired Student t test (means \pm SD, $n = 6$; $\dagger P < 0.001$). Scale bar = 50 µm. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. (A high-quality digital representation of this figure is available in the online issue.)

inflammation such as leukostasis is another hallmark of diabetic retinopathy. To determine the effect of Mab2F1 on retinal inflammation, we performed leukostasis assay 2 weeks after the Mab2F1 injection in the retinas of rats with STZ-induced diabetes. Compared with nondiabetic control, untreated STZ and IgG-treated STZ rats showed significantly increased numbers of adherent leukocytes in retinal vasculature (Fig. 6B). The number of adherent leukocytes was significantly decreased in the diabetic group injected with Mab2F1, suggesting that Mab2F1 has an inhibitory effect on retinal inflammation (Fig. 6B). As ICAM-1 and TNF- α play

important roles in retinal inflammation in diabetes, we further measured retinal levels of ICAM-1 and TNF- α using Western blot analysis. The STZ rats showed overexpression of ICAM-1 and TNF- α in the retina compared with that in nondiabetic rats. Mab2F1, but not the nonspecific IgG, suppressed the overexpression of ICAM-1 and TNF- α in the diabetic retina (Fig. 6C). Mab2F1 also attenuated the overexpression of platelet-derived growth factor (PDGF)-BB in the retina of diabetic rats (Supplementary Fig. 2). In addition, retinal levels of total LRP6 were significantly elevated in the retina of rats with STZ-induced diabetes compared with those in nondiabetic rats. LRP6 levels in the retina were decreased in the diabetic rats injected with Mab2F1 but not in those injected with nonspecific IgG. These results suggest that activation of the Wnt pathway in the diabetic retina may be in part due to overexpression of LRP6 and that Mab2F1 suppresses overexpression of ICAM-1 and TNF- α through downregulation of LRP6. Taken together, the results demonstrate that Mab2F1 has beneficial effects on diabetic retinopathy, as it attenuates vascular leakage and inflammation in the diabetic retina.

Effect of Mab2F1 on retinal vascular leakage, leukostasis, and glial stress in late stages of STZ-induced diabetes. Retinal pathologies in the retina and effects of Mab2F1 were evaluated in rats with STZ-induced diabetes at 3 months post-onset of diabetes. Rats with diabetes for 3 months received an intravitreal injection of Mab2F1 (50 µg/eye) or the same amount of nonspecific IgG as control. Physiological parameters of diabetic rats including blood glucose (milligrams per deciliter) and body weight (grams) were measured at 3 months after STZ administration (Supplementary Table 1). Retinal vascular permeability was measured at 2.5 weeks after the Mab2F1 injection in the rats with diabetes for 3 months. The result demonstrated that the eyes injected with Mab2F1 had significantly lower vascular permeability than those injected with control IgG (Fig. 7A). Next, we performed leukostasis assay at 4 weeks after the Mab2F1 injection in the rats with diabetes for 3 months. The number of adherent leukocytes was significantly decreased in the diabetic group injected with Mab2F1 compared with that in rats injected with control IgG, suggesting that Mab2F1 has an inhibitory effect on retinal inflammation in late stages of diabetes (Fig. 7B). Retinal Müller glial dysfunction in diabetic retina usually alters expression of glial fibrillary acidic protein (GFAP), indicative of an increase in metabolic stress, vascular permeability, and cell survival. In accordance with the inhibitory effect of Mab2F1 on expression of angiogenic/inflammatory factors in cultured Müller cells (Supplementary Fig. 2), Mab2F1 decreased GFAP labeling in the retinal Müller glia cells 4 weeks after the Mab2F1 injection compared with control IgG (Fig. 7C). Taken together, the results demonstrate that Mab2F1 has beneficial effects on late stages of diabetic retinopathy.

DISCUSSION

Our previous studies showed that the Wnt/ β -catenin pathway is overactivated in the retina of diabetic patients and diabetic animal models (9). Furthermore, injection of DKK1, a specific inhibitor of Wnt signaling, can ameliorate retinal inflammation and vascular leakage in diabetic retinopathy models (9). These findings suggest that Wnt signaling plays a pathogenic role in diabetic retinopathy and represents a potential therapeutic target (9,17,23). Although Frizzled 4 is known to regulate retinal

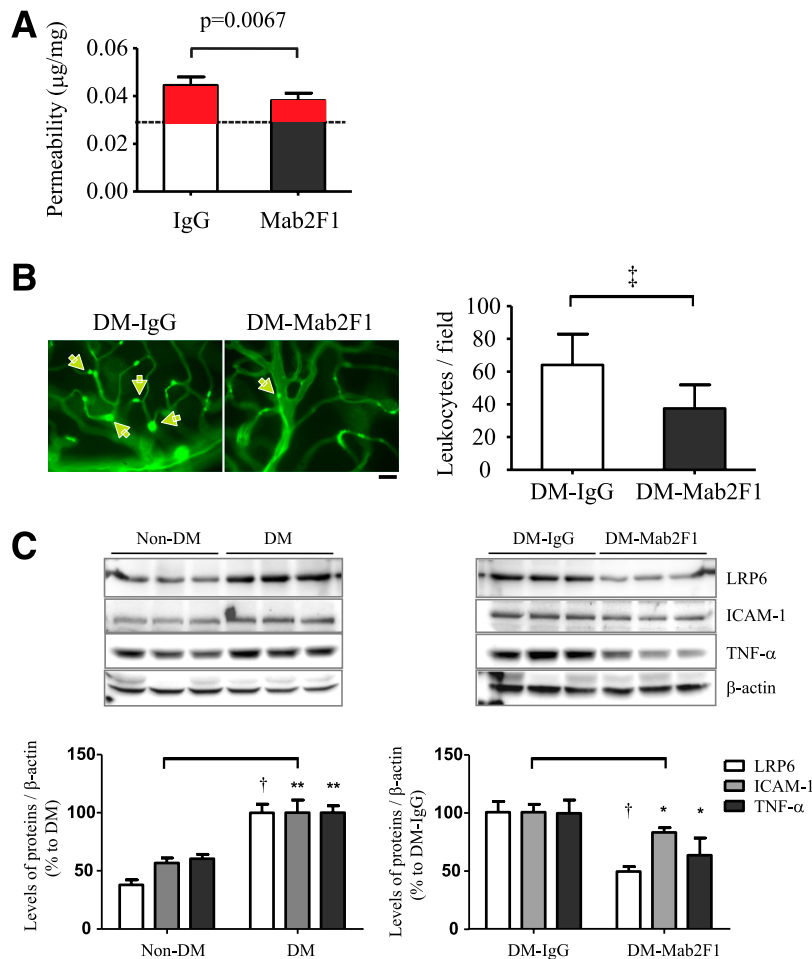


FIG. 6. Inhibitory effect of Mab2F1 on retinal vascular leakage and inflammation in the early stage of STZ-induced diabetes (DM). Rats with STZ-induced diabetes at 2 weeks post-onset of diabetes received an intravitreal injection of Mab2F1 (20 µg/eye) or the same amount of nonspecific IgG as control. **A:** Retinal vascular permeability was measured at 1 week post-injection of the antibody (means ± SD, $n = 6$; $P = 0.00667$). The dotted line indicates the basal level of vascular permeability in age-matched normal animals. **B:** Leukostasis assay was performed in diabetic rats treated with control IgG (DM-IgG) or with Mab2F1 (DM-Mab2F1) at 2 weeks after the injection of Mab2F1. Adherent leukocytes were stained with fluorescein isothiocyanate-concanavalin-A and visualized under fluorescence microscope. Adherent leukocytes were counted in four random fields of each retina for quantification (means ± SD, $n = 5$; $‡P < 0.0001$). Scale bar = 20 µm. **C:** Two weeks after the intravitreal injection, the retinas were dissected from the indicated groups. The same amount of retinal proteins was loaded for Western blot analysis to measure expression levels of LRP6, ICAM-1, and TNF-α, quantified by densitometry, and normalized by β-actin levels (means ± SD, $n = 3$; $*P < 0.05$, $**P < 0.01$, $‡P < 0.001$). (A high-quality digital representation of this figure is available in the online issue.)

vasculature formation, its expression level is not changed in diabetic retina (9,24,25). Furthermore, high-glucose medium increased expression of Wnt ligands, including Wnt1, Wnt4, and Wnt5b and LRP6, but did not change Frizzled receptor expression in endothelial cells (9,26). These studies suggest that LRP6 is a suitable target for blocking Wnt signaling. Despite the well-studied molecular cascade of Wnt signaling, an effective strategy to block the Wnt pathway has not been established for the therapeutic application in diabetic retinopathy. Although natural inhibitors of the Wnt pathway, such as DKK family members, serine proteinase inhibitor (SERPIN)A3K, and insulin-like growth factor 1 binding protein (IGF1BP) have been identified, there are limitations in their clinical applications, including low stability and high costs of production (15,27,28). The current study reports for the first time that an anti-LRP6 Mab attenuates retinal vascular leakage and retinal inflammation in diabetic retinopathy models. In addition, our results provide further support for a causative role of Wnt/β-catenin signaling overactivation in the development of diabetic retinopathy and the

indispensability of LRP6 in this context. These observations establish that blocking LRP6 is a promising new strategy for the treatment of diseases caused by Wnt pathway activation.

Here, we demonstrate that Mab2F1, specific for the first and second propeller domains of LRP6 (E1E2), inhibits Wnt signaling as well as expression of angiogenic and inflammatory factors in various retinal cells. Based on its inhibitory effects on Wnt signaling and expression of inflammatory factors, we further evaluated the beneficial effects of Mab2F1 on diabetic retinopathy in animal models. First, in both OIR and diabetic rats, Mab2F1 significantly reduced retinal vascular leakage, which is the primary cause of diabetic macular edema, the number one cause of vision loss in diabetic patients (29). Second, Mab2F1 suppressed retinal leukostasis, a key inflammatory change that can lead to impaired endothelium, vascular leakage, and closure of capillaries, which subsequently results in local ischemia. Toward the mechanism for its effects on retinal vascular leakage and leukostasis, our in vitro and in vivo results showed that Mab2F1 downregulates expression of VEGF, ICAM-1, and TNF-α by

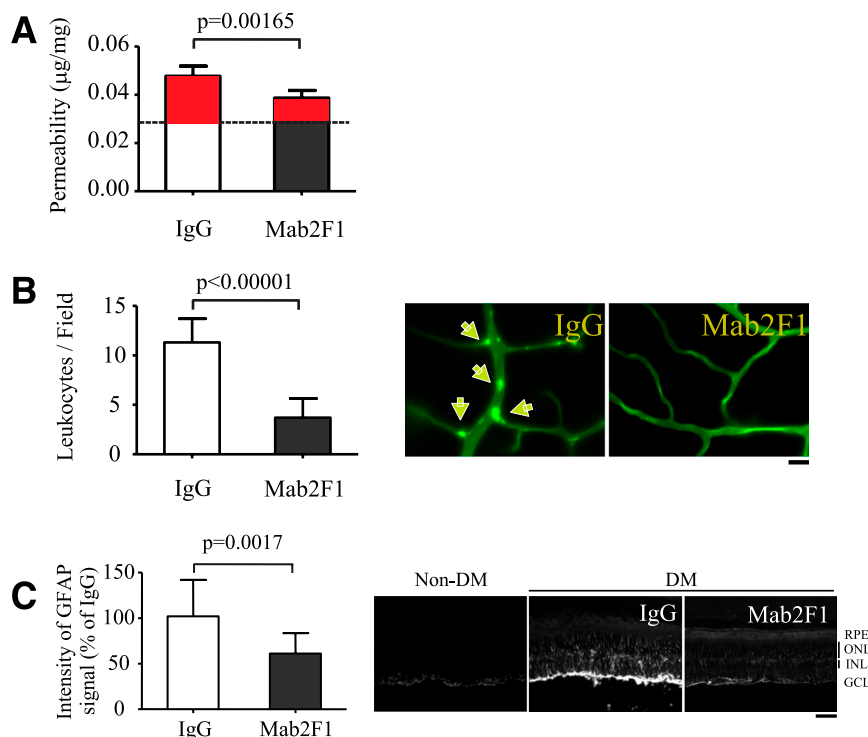


FIG. 7. Inhibitory effect of Mab2F1 on retinal vascular leakage, inflammation, and glial stress in the late stage of STZ-induced diabetes. For studies of extensive retinal pathologies and long-term effects of Mab2F1, rats with STZ-induced diabetes at 3 months post-onset of diabetes received an intravitreal injection of Mab2F1 (50 µg/eye) or the same amount of nonspecific IgG as control. **A:** Retinal vascular permeability was measured at 2.5 weeks postinjection of the antibody (means \pm SD, $n = 6$; $P = 0.00165$). The dotted line indicates the basal level of vascular permeability in age-matched normal animals. **B:** Leukostasis assay was performed in the indicated groups: diabetic rats treated with IgG or Mab2F1 at 4 weeks after the injection of Mab2F1. Adherent leukocytes were stained with fluorescein isothiocyanate-concanavalin-A and visualized under fluorescence microscope at $\times 40$ magnification. Quantification of the adherent leukocytes was performed from $\times 40$ magnification images. Adherent leukocytes were counted in four random fields of each retina for quantification (means \pm SD, $n = 5$; $P < 0.00001$). Scale bar = 20 µm. **C:** Frozen retinal sections from eyes injected with control IgG and Mab2F1 were immunostained using an anti-GFAP antibody. GFAP labeling in diabetes-induced retinal Müller glia was quantified after subtraction of that of basal level signal in nondiabetic retina. GFAP signal intensities were quantified and averaged in fifteen random retinal sections from each rat using ImageJ (means \pm SD, $n = 3$; $P = 0.0017$). Scale bar = 60 µm. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. (A high-quality digital representation of this figure is available in the online issue.)

inhibiting the Wnt pathway, which has been shown to play important roles in retinal inflammation in diabetes (5,30). Moreover, retinal trypsin digestion assay showed that Mab2F1 ameliorated formation of acellular capillaries in diabetic retina, supporting the concept that Mab2F1 suppresses expression of angiogenic/inflammatory factors and alleviates injury of retinal capillaries in diabetes (Supplementary Fig. 6).

The canonical Wnt pathway is a conserved signaling pathway that uses a single effector, multifunctional transcription activator β -catenin, to regulate expression of a number of target genes (10). Toward the upstream cascade of Wnt signaling, however, diverse spatiotemporal activations of the Wnt pathway arise from multiple combinations among 19 Wnt ligands, 10 Frizzled receptors, and 2 coreceptors, providing numerous diversities (31). These diversities dampen the therapeutic approaches to inhibit the Wnt pathway via blockage of Wnt ligands or Frizzled receptors. Based on the following facts, however, we hypothesized that LRP6 is an ideal target for blocking Wnt signaling. 1) The canonical Wnt pathway requires one of the two coreceptors LRP5 and LRP6 (12,13,32). 2) Knockout of LRP6 generates more severe phenotypes than knockout of LRP5, suggesting that LRP6 plays a more important role than LRP5 in Wnt signaling. 3) LRP6 has a large extracellular domain, which is accessible extracellularly by antibodies or inhibitors. 4) Our

previous studies clearly demonstrated that blocking LRP6 by DKK1 or by SERPINA3 K is sufficient to attenuate Wnt signaling induced by diabetes and to ameliorate diabetic retinopathy in animal models (9,15). Although the exact molecular target in Wnt signaling regulated by diabetes is uncertain, the present and previous studies suggest that phosphorylation of LRP6 induced by lipid oxidation product (4-hydroxynonenal) and by high-glucose medium is sufficient to induce Wnt-signaling activation (18,23). These findings strongly suggest that blocking LRP6 provides effective means to inhibit the canonical Wnt pathway.

LRP6 is a type 1 single transmembrane receptor with a large ectodomain composed of four similar epidermal growth factor-like repeats (E1–E4) with Tyr-Trp-Thr-Asp (YWTD) propeller domains. Wnt1 preferentially binds to E1 domain, and E3 is sufficient for Wnt3A binding (33). Despite their different binding sites on LRP6, both the Wnt1- and Wnt3A-induced TOPFLASH activations can be inhibited by Mab2F1. Our *in vivo* studies using OIR and STZ rat models showed that Mab2F1 not only inhibits the activation of the canonical Wnt pathway but also down-regulates total LRP6 levels (1 week after the injection). However, our cell culture results showed that Mab2F1 blocks the phosphorylation of LRP6 but does not decrease total LRP6 levels after 6 h treatment. Total LRP6 levels in hTERT-RPE-1 cells after exposure to Mab2F1 for different

durations showed that with short incubation durations (4, 6, 12, and 24 h), Mab2F1 elevated total LRP6 levels, while it decreased LRP6 levels at 36- and 48-h incubation durations (Supplementary Fig. 3). These results suggest that a long-time treatment with Mab2F1 not only inhibits the activation of LRP6 but also decreases its total levels.

Recent clinical studies showed that anti-VEGF compounds have promising therapeutic effects on age-related macular degeneration (34). In diabetic retinopathy, however, the anti-VEGF compounds are not as effective as in age-related macular degeneration (35). A possible reason is that diabetic retinopathy is a complex and multifactorial disorder. Multiple growth factors, in addition to VEGF, are known to play roles in diabetic retinopathy. Therefore, blocking VEGF alone may not be sufficient for ameliorating diabetic retinopathy. This assumption is supported by the observation that inhibition of platelet-derived growth factor (PDGF)-B and PDGF receptor (PDGFR) β improved antiangiogenic effect of the anti-VEGF antibody, suggesting that simultaneous inhibition of multiple angiogenic and inflammatory factors may improve efficacy (5,36). Since the Wnt pathway regulates multiple inflammatory and angiogenic factors, such as ICAM-1, PDGF, VEGF, TNF- α , matrix metalloproteinase-2, and cyclooxygenase 2, which are implicated in diabetic retinopathy, the anti-LRP6 antibody may be a promising treatment for diabetic retinopathy (37,38). The Supplementary Data indicate that intravitreal injection of Mab2F1 did not alter retinal structure or electroretinogram responses 6 weeks after the injection, suggesting that the antibody itself or blocking LRP6 does not interfere with retinal function or cell survival (Supplementary Fig. 4).

In conclusion, our findings demonstrate that an anti-LRP6 Mab has beneficial effects in diabetic retinopathy. Furthermore, this study provides a strong rationale for investigating antibody-based, LRP6-targeted therapies in diseases associated with Wnt-signaling activation and/or overexpression of LRP6.

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K.L. researched data and wrote the manuscript. Y.H., L.D., and Y.C. researched data. Y.T. researched data and contributed to discussion. R.M. researched data. J.-x.M. researched data and wrote the manuscript. J.-x.M. 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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