Blocking the Wnt pathway, a unifying mechanism for an angiogenic inhibitor in the serine proteinase inhibitor family

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The Wnt pathway regulates multiple biological and pathological processes including angiogenesis and inflammation. Here we identified a unique inhibitor of the Wnt pathway, SERPINA3K, a serine proteinase inhibitor with anti-inflammatory and angiogenic activities. SERPINA3K blocked the Wnt pathway activation induced by a Wnt ligand and by diabetes. Coprecipitation and ligand binding assay showed that SERPINA3K binds to low-density lipoprotein receptor-like protein 6 (LRP6) with a Kd of 10 nM, in the range of its physiological concentration in the retina. Under the same conditions, SERPINA3K did not bind to the frizzled (Fz) receptor or low-density lipoprotein receptor. Further, SERPINA3K bound to LRP6 at the extracellular domain and blocked its dimerization with the Fz receptor induced by a Wnt ligand. The antagonizing activity of SERPINA3K to LRP5/6 was further confirmed by Xenopus axis duplication assay. These results suggest that SERPINA3K is a high-affinity, endogenous antagonist of LRP6. The blockade of Wnt signaling may represent a unifying mechanism for the anti-inflammatory and anti-angiogenic effects of SERPINA3K.

Results

Inhibitory Effects of SERPINA3K on Retinal Inflammation and Wnt Pathway Activation in Diabetic Rats. Two months after the onset of diabetes, streptozotocin (STZ)-induced diabetic rats received an intravitreal injection of purified SERPINA3K. Retinal vascular permeability assay at 48 h following the injection showed that the retinal vascular leakage in the diabetic rats was significantly reduced by SERPINA3K, compared with the contralateral retinas in rats injected with BSA and the control retinas in the diabetic rats without injection (Fig. L4).

STZ-diabetic rats also received an intravitreal injection of adenovirus expressing SERPINA3K (Ad-SA3K, 5 × 1011 pfu/eye). Retinal levels of SERPINA3K were decreased in the injected diabetic rats, compared with that in nondiabetic rats (Fig. 1B and C). The Ad-SA3K injection resulted in overexpression of SERPINA3K in the retinas of the diabetic rats one month after the injection, compared with the eyes injected with control virus (Fig. 1B and C). Retinal levels of VEGF, a major inflammatory and angiogenic factor, as well as a target gene of the Wnt pathway, were significantly increased in the retinas of untreated diabetic rats, compared with nondiabetic rats at the same age (Fig. 1B and C). Cytosolic β-catenin levels in the retina were also significantly decreased, leading to its accumulation (4, 5).

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Different concentrations of SERPINA3K for 8 h (25 mM L-glucose) and high-glucose (HG; 30 mM D-glucose) media with diabetes (33).

Retinal vascular permeability was measured using Evans blue as tracer 48 h after the injection of SERPINA3K, normalized by retinal protein concentrations, and expressed as a percentage of the permeability increase over that of nondiabetic rats (mean \(±\) SD, \(n = 5\)). (B) The diabetic rats received an intravitreal injection of Ad-SA3K, with Ad-LacZ as control. Four weeks after the injection, levels of SERPINA3A, VEGF, and cytosolic β-catenin in the retina were measured by Western blot analysis using 50 μg of retinal proteins from each rat sample. (C) The Western blots shown in B were representative of three independent experiments, and the results were quantified by densitometry, normalized by β-actin levels, and expressed as a percentage of that in nondiabetic control (mean \(±\) SD, \(n = 6\)). (D–G) ARPE19 cells (D and E) and primary HRCEC (F and G) were exposed to low-glucose (LG; 5 mM D-glucose + 25 mM L-glucose) and high-glucose (HG; 30 mM D-glucose) media with different concentrations of SERPINA3K for 8 h (D), 24 h (E and F), or 72 h (G). (D and F) Cytosolic β-catenin levels were measured by Western blot analysis. (E) VEGF secreted into the medium was measured using an ELISA kit (R&D Systems) (mean \(±\) SD, \(n = 3\)). (G) Cell migration of HRECC was measured using Transwell migration assay (mean \(±\) SD, \(n = 10\)). *\(P < 0.05\).

Evoked in the retinas of diabetic rats. The injection of Ad-SA3K mitigated the overexpression of VEGF in the retina and decreased retinal levels of cytosolic β-catenin in diabetic rats, compared with the Ad-LacZ injection control, suggesting an inhibition of the canonical Wnt pathway in diabetes (Fig. 1 B and C).

To evaluate the direct effect of SERPINA3K on the Wnt pathway induced by diabetes, ARPE19 cells were exposed to a medium containing 30 mM D-glucose. Cytosolic β-catenin levels and VEGF secretion were elevated by the high-glucose medium, when compared with the low-glucose control (5 mM D-glucose and 25 mM L-glucose), suggesting that high glucose is responsible for the activation of the Wnt pathway in the diabetic rat retina. SERPINA3K blocked the high-glucose-induced β-catenin accumulation and VEGF secretion in a dose-dependent manner (Fig. 1 D and E).

Endothelial cell migration can be up-regulated by high glucose (32) and Wnt ligand (33). Therefore, we investigated the effect of SERPINA3K on endothelial cell migration using primary human retinal capillary endothelial cells (HRCEC) and a cell line derived from rat endothelial cells, TR-iBRB (34). In both of the cells, the high glucose medium-induced β-catenin accumulation was blocked by SERPINA3K (Fig. 1 F and Fig. S1 B). The Transwell migration of the endothelial cells was up-regulated by high glucose and can be inhibited by SERPINA3K (Fig. 1 G and Fig. S1 N). In TR-iBRB cells, the increase in scratch wound closure induced by high glucose was also attenuated by SERPINA3K (Fig. S1 C–G and M).

It has been reported that phosphorylation of LRP6 is a critical step in the Wnt pathway activation (35, 36). As shown by Western blot analysis using an antibody specific for phosphorylated LRP6 (p-LRP6), phosphorylation of the endogenous LRP6 was induced in the cells exposed to 30 mM glucose for 6 h, compared with that in control cells exposed to low-glucose medium. SERPINA3K at 100 nM decreased the p-LRP6 to a level similar to that in low-glucose control (Fig. 24). In cultured HRCEC, the high-glucose-induced phosphorylation of LRP6 was also reduced by SERPINA3K (Fig. S1 G).

To determine the effect of SERPINA3K on the Wnt pathway activation induced by the Wnt ligand, ARPE19 cells were exposed to a medium containing 50% Wnt3a conditioned medium, with the 50% L medium as control. SERPINA3K blocked the VEGF overexpression induced by Wnt3a (\(P < 0.05\); Fig. 2B). Under the same condition, SERPINA3K also inhibited the Wnt3a-induced endothelial migration in TR-iBRB cells, as shown by Transwell assay and wound-healing assay (Fig. S1 H–N).

To determine if SERPINA3K blocks Wnt signaling activated at the intracellular cascade, the cells were infected with an adenovirus expressing a constitutively active mutant of β-catenin (Ad-S37A), in which the phosphorylation site S37 in β-catenin was mutated. As shown by ELISA, VEGF secretion was induced significantly by Ad-S37A. SERPINA3K at 1,000 nM did not block the VEGF production induced by Ad-S37A (Fig. 2C). To determine whether SERPINA3K regulates the β-catenin degradation rate, LiCl, a GSK-3 inhibitor, was used to activate Wnt signaling at the GSK-3β level. LiCl induced the stabilization and accumulation of β-catenin (Fig. 2D) as well as the β-catenin-dependent reporter gene transcription as shown by the TOPFLASH activity assay, a reporter luciferase assay under the control of a promoter containing TCF binding sites (Fig. 2E). Neither the LiCl-induced β-catenin accumulation nor TOPFLASH activity was inhibited by SERPINA3K (Fig. 2 D and E). These findings suggest that the target of SERPINA3K may be at the Wnt receptor level.

Fig. 1. SERPINA3K blocked Wnt signaling in the retina with diabetic retinopathy and in high-glucose-treated cells. (A) STZ-diabetic rats received an intravitreal injection of 10 μg/eye SERPINA3K and the same amount of BSA into the contralateral eye. Untreated diabetic rats were used as control (DM). Retinal vascular permeability was measured using Evans blue as tracer 48 h after the injection of SERPINA3K, normalized by retinal protein concentrations, and expressed as a percentage of the permeability increase over that of nondiabetic rats (mean \(±\) SD, \(n = 5\)). (B) The diabetic rats received an intravitreal injection of Ad-SA3K, with Ad-LacZ as control. Four weeks after the injection, levels of SERPINA3A, VEGF, and cytosolic β-catenin in the retina were measured by Western blot analysis using 50 μg of retinal proteins from each rat sample. (C) The Western blots shown in B were representative of three independent experiments, and the results were quantified by densitometry, normalized by β-actin levels, and expressed as a percentage of that in nondiabetic control (mean \(±\) SD, \(n = 6\)). (D–G) ARPE19 cells (D and E) and primary HRCEC (F and G) were exposed to low-glucose (LG; 5 mM D-glucose + 25 mM L-glucose) and high-glucose (HG; 30 mM D-glucose) media with different concentrations of SERPINA3K for 8 h (D), 24 h (E and F), or 72 h (G). (D and F) Cytosolic β-catenin levels were measured by Western blot analysis. (E) VEGF secreted into the medium was measured using an ELISA kit (R&D Systems) (mean \(±\) SD, \(n = 3\)). (G) Cell migration of HRECC was measured using Transwell migration assay (mean \(±\) SD, \(n = 10\)). *\(P < 0.05\).

Fig. 2. SERPINA3K blocked Wnt signaling at the cell surface receptor level. (A) ARPE19 cells were exposed to HG or LG medium for 6 h, with 100 nM SERPINA3K or BSA. The same amount of total cellular proteins was blotted with an antibody specific for phosphorylated LRP6 (p-LRP6). (B) The cells were exposed to 50% Wnt3a conditioned medium with 500 nM SERPINA3K or BSA for 24 h. Control cells were treated with the 50% L cell conditioned medium. VEGF secreted into the culture medium was measured by ELISA and expressed as a percentage of control (mean \(±\) SD, \(n = 3\)). (C) The cells were infected with an adenovirus expressing a constitutively active mutant of β-catenin (Ad-S37A) at MOI of 8, with 1,000 nM SERPINA3K or BSA; the same titer of the adenovirus cells were exposed to low-glucose medium. SERPINA3K at 100 nM decreased the p-LRP6 to a level similar to that in low-glucose control (Fig. 2A). In cultured HRCEC, the high-glucose-induced phosphorylation of LRP6 was also reduced by SERPINA3K (Fig. S1 A).

To determine whether SERPINA3K regulates the β-catenin degradation rate, LiCl, a GSK-3 inhibitor, was used to activate Wnt signaling at the GSK-3β level. LiCl induced the stabilization and accumulation of β-catenin (Fig. 2D) as well as the β-catenin-dependent reporter gene transcription as shown by the TOPFLASH activity assay, a reporter luciferase assay under the control of a promoter containing TCF binding sites (Fig. 2E). Neither the LiCl-induced β-catenin accumulation nor TOPFLASH activity was inhibited by SERPINA3K (Fig. 2 D and E). These findings suggest that the target of SERPINA3K may be at the Wnt receptor level.
SERPINA3K Binds to LRPS6 with a High Specificity and Affinity. To reveal the interactions of SERPINA3K with the cell-surface receptors of Wnts, ARPE19 cells were incubated with 100 nM His-tagged SERPINA3K (SA3K-HIS) for 1 h. Following three washes with PBS, the cells were lysed and the plasma membrane solubilized for coprecipitation assay by incubating the cell lysates with Ni resin. After thorough washes, Western blot analysis of the proteins pulled down by the Ni resin showed that LRPS6 was coprecipitated with SERPINA3K-HIS (Fig. 3A). Under the same conditions, Fz4, a Frizzled receptor for Wnt, which is expressed in ARPE19 cells and is known to involve in angiogenesis (12), was not coprecipitated with SERPINA3K (Fig. 3A).

To further confirm the interactions between LRPS6 and SERPINA3K, a His-tagged LRPS6 (LRPS6-HIS) and SERPINA3K (no His tag) were coexpressed in HEK293T cells using plasmid transfection. LRPS6-HIS was precipitated using Ni resin. SERPINA3K was found to coprecipitate with LRPS6-HIS (Fig. 3B). However, in the cells expressing LRPS6 (no His tag) and SERPINA3K-HIS, LRPS6 was also found to coprecipitate with SERPINA3K-HIS, which was pulled down by Ni resin (Fig. 3B). These findings suggested that SERPINA3K specifically binds to LRPS6.

To determine the binding affinity of SERPINA3K to LRPS6, COS cells were transfected with a plasmid expressing LRPS6 and incubated with various concentrations of FITC-conjugated SERPINA3K. The same amount of FITC-SERPINA3K was incubated with the cells transfected with an empty plasmid as background control. The fluorescence intensity in the background control cells was used as nonspecific control and subtracted from the fluorescence in the LRPS6-expressing cells. FITC-SERPINA3K displayed a concentration-dependent and saturable binding onto the cells expressing LRPS6 (Fig. 3C). Scatchard plot analysis revealed a high binding affinity between SERPINA3K and LRPS6 with a dissociate constant ($K_d$) of 10 nM (Fig. 3D). Further, binding of FITC-SERPINA3K onto the LRPS6-transfected cells was competed off by excess amounts of unlabeled SERPINA3K (Fig. 3E), suggesting that the binding is specific.

SERPINA3K Functions as an Antagonist of LRPS6. The LRPS6-transfected HEK293T cells were treated with the 50% Wnt3a conditioned medium in the presence of various concentrations of SERPINA3K for 1 h. Western blot analysis of the cell lysate revealed that Wnt3a induced phosphorylation of LRPS6. SERPINA3K blocked the Wnt3a-induced LRPS6 phosphorylation in a concentration-dependent manner but did not alter total LRPS6 levels (Fig. 4A). Likewise, cytosolic β-catenin levels were elevated by Wnt3a and decreased by SERPINA3K (Fig. 4B).

We also examined the effect of SERPINA3K on the Wnt3a-mediated gene transcription. HEK293T cells were transfected with the TOPFLASH construct. In the transfected HEK293T cells, LRPS6 overexpression induced the TOPFLASH reporter (TCF/LEF) activity, which was further enhanced by the exposure to the Wnt3a conditioned medium (Fig. 4C). The TOPFLASH reporter activities induced by transfection of LRPS6 and Wnt3a were inhibited by SERPINA3K in a concentration-dependent manner (Fig. 4C). These findings indicated that SERPINA3K is an antagonist of LRPS6 and blocks the Wnt ligand-induced Wnt signaling.

SERPINA3K Inhibits Xenopus Axis Duplication Induced by Wnt Ligand. Wnt/β-catenin signaling induces dorsal axis formation in Xenopus embryos, and the axis duplication assay is a commonly used and reliable method to study the Wnt signaling pathway in vivo (37). Wnt8 and LRPS6ΔN (a constitutively active mutant of LRPS6 without the N-terminal extracellular domain) mRNAs were separately injected into the ventral marginal zone of a four-cell Xenopus embryo. The injections of Wnt8 and LRPS6ΔN resulted in more than 70% embryos with duplicate axis formation (Fig. 4D). The SERPINA3K mRNA injection blocked the Wnt8 mRNA-induced axis duplication by ~50% and did not show any effect on the axis duplication induced by the constitutively active LRPS6ΔN (Fig. 4F). Dickkopf-1 (DKK1), a secreted head inducer and a high-affinity LRPS6 antagonist (1) was used as a positive control, which induced a complete blockade of the Xenopus axis duplication (Fig. 4F).

To further confirm the antagonizing effect of SERPINA3K on Wnt signaling, we examined the XWnt8-mediated induction of Xenopus nodal-related 3 (Xnr3), a directly downstream target of Wnt signaling (37) using RT-PCR. The XWnt8-induced Xnr3 transcription was attenuated by DKK1 and SERPINA3K (Fig. 5B). Taken together, these findings suggest that SERPINA3K inhibits Wnt signaling in the Xenopus embryo model, and the N-terminal extracellular domain of LRPS6 is necessary for the SERPINA3K inhibitory function.

SERPINA3K Binds to the Extracellular Domain of LRPS6. To define the SERPINA3K-binding region in LRPS6, the extracellular portion of SERPINA3K Inhibits Xenopus Axis Duplication Induced by Wnt Ligand. Wnt/β-catenin signaling induces dorsal axis formation in Xenopus embryos, and the axis duplication assay is a commonly used and reliable method to study the Wnt signaling pathway in vivo (37). Wnt8 and LRPS6ΔN (a constitutively active mutant of LRPS6 without the N-terminal extracellular domain) mRNAs were separately injected into the ventral marginal zone of a four-cell Xenopus embryo. The injections of Wnt8 and LRPS6ΔN resulted in more than 70% embryos with duplicate axis formation (Fig. 4D). The SERPINA3K mRNA injection blocked the Wnt8 mRNA-induced axis duplication by ~50% and did not show any effect on the axis duplication induced by the constitutively active LRPS6ΔN (Fig. 4F). Dickkopf-1 (DKK1), a secreted head inducer and a high-affinity LRPS6 antagonist (1) was used as a positive control, which induced a complete blockade of the Xenopus axis duplication (Fig. 4F).

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SERPINA3K Binds to the Extracellular Domain of LRPS6. To define the SERPINA3K-binding region in LRPS6, the extracellular portion of LRPS6 (E1-E4) fused with a Myc-tag (LRPS6N-Myc) was expressed and incubated with the recombinant SERPINA3K-HIS or a control protein, His-tagged cellular retinol-binding protein (CRBP-HIS). After LRPS6N-Myc was immunoprecipitated with the anti-Myc
antibody, SERPINA3K-HIS, but not CRBP-HIS, was coimmunoprecipitated with LRP6N-Myc (Fig. 6A). Furthermore, SERPINA3K-HIS was incubated with LRP6N-Myc or the extracellular region of a control receptor, the N-terminal portion of low-density lipoprotein receptor fused with the Myc-tag (LDLRN-Myc), and pulled down by Ni resin. LRP6N-Myc, but not LDLRN-Myc, was specifically coimmunoprecipitated with SERPINA3K-HIS, as shown by Western blot analysis (Fig. 6B). These observations suggest that SERPINA3K specifically binds to the extracellular domain of LRP6.

SERPINA3K Prevents the LRP6 and Fz Dimerization. Because the dimerization between LRP6 and the Fz receptor is the first step in activation of the Wnt pathway (36), we determined whether SERPINA3K affects the complex formation between the extracellular domains of Fz8 and LRP6 induced by a Wnt ligand. The cysteine-rich domain (CRD) of the Fz8 receptor tagged with an Ig-γ Fc epitope (Fz8CRD-IgG) was expressed in HEK293 cells by transfection of an expression vector, and the conditioned medium was harvested 2 days following the transfection. LRP6N-Myc in the medium was incubated with Fz8CRD-IgG in the presence or absence of purified Wnt1. Fz8CRD-IgG showed only a basal level of dimerization with the LRP6N-Myc in the absence of the added Wnt1 (Fig. 6C). The addition of purified Wnt1 induced a significant dimerization of Fz8 and LRP6, as shown by coprecipitation of Fz8CRD-IgG and LRP6N (Fig. 6C). Inclusion of SERPINA3K in the incubation mixture effectively blocked such dimerization in a concentration-dependent manner (Fig. 6C).

Discussion
A number of serpin members have been identified as endogenous angiogenic inhibitors (20–23). Some of these serpin angiogenic inhibitors have also displayed anti-inflammatory activities (38). However, the receptors or mechanisms responsible for their antiangiogenic and anti-inflammatory activities have not been identified. In the present study, we have identified the first serpin angiogenic inhibitor, SERPINA3K, as an endogenous inhibitor of Wnt signaling and LRP6 antagonist. As Wnt signaling regulates angiogenesis and inflammation processes (11, 12), antagonizing LRP6 may represent a unifying mechanism responsible for the antiangiogenic and anti-inflammatory activities of serpin angiogenic inhibitors.

Wnt signaling is involved in angiogenesis process through regulation of the angiogenic factors such as VEGF (12, 39). Endothelial cell migration is an essential step in angiogenesis (40) and is regulated by Wnt signaling (33). In human retinal vascular diseases, mutations in the Fz4 (Wnt receptor) and LRP5 (Wnt coreceptor) genes have been found to associate with abnormal angiogenesis (12, 41). Our recent study showed that activation of the Wnt pathway also plays a pathogenic role in subretinal neovascularization in an animal model of wet AMD (42). Therefore, Wnts are considered a new class of angiogenic factors (12). Further, we reported that Wnt signaling is activated in the retinas of diabetic animal models and in human donor retinas with diabetic retinopathy. However, blockade of Wnt signaling by DKK1 attenuated retinal inflammation, vascular leakage in diabetic retinopathy models (43). These findings established a pathogenic role of Wnt signaling in diabetic retinopathy. Here, we found that β-catenin is stabilized in both diabetic retinas and in cultured cells exposed to high-glucose medium. Further, phosphorylated LRP6 levels were increased under high-glucose condition. Therefore, these observations further support the role of Wnt signaling in diabetic retinopathy.

There are 19 Wnt ligands, many of which function as agonists of Arrow/LRP5/LRP6 and the Fz receptors, and thus activate Wnt/β-catenin signaling (44). However, some natural inhibitors of the Wnt pathway, such as DKK family members and IGF1BP, have been identified (45–47). Here we showed that SERPINA3K blocks the Wnt pathway activation induced by high glucose and Wnt ligands, but not by LiCl or a constitutively active mutant of β-catenin, suggesting that SERPINA3K inhibits Wnt signaling, possibly at the Wnt receptor level. This assumption was supported by specific binding of SERPINA3K to LRP6. Further, the coimmunoprecipitation assay confirmed the physical association of SERPINA3K with LRP6. Coprecipitation assay of LRP6 and Fz8 showed that binding of SERPINA3K to LRP6 blocks the LRP6-Fz receptor complex formation, suggesting that SERPINA3K functions as an antagonist of LRP6 and an endogenous modulator of the Wnt pathway.

It has been shown that SERPINA3K levels are decreased in the retina, correlating with activation of the Wnt pathway in the retina of a diabetic retinopathy model (28, 43). Further, intravitreal injection of SERPINA3K or injection of adenosine expressing SERPINA3K attenuated the Wnt pathway activation in the retina with diabetic retinopathy. To further prove the hypothesis that the
decreased SERPINA3K levels may contribute to the pathological Wnt pathway activation, we performed siRNA knockdown of SERPINA3K in cultured RPE and endothelial cells. In both of the cell lines, cytosolic β-catenin levels were up-regulated by knockdown of SERPINA3K alone (Fig. S2 A and B). Further, VEGF secretion level in cultured RPE cells and migration of endothelial cells were both increased by knockdown of SERPINA3K (Fig. S2 C and D). Next, we measured transcript levels of several known Wnt target genes, including VEGF, connective tissue growth factor (CTGF), Cyclin D1, and alkaline phosphatase (AP), to reflect the Wnt pathway activation (Fig. S3). Our real-time RT-PCR results showed that knockdown of SERPINA3K alone resulted in up-regulation of mRNA levels of all of these Wnt target genes (Fig. S3). Consistently, the real-time RT-PCR results showed the increased levels of all of these four Wnt target genes in the diabetic rat retinas, providing another support for the Wnt signaling activation in the diabetic retinopathy (Fig. S3). These findings provide further evidence suggesting that decreased SERPINA3K levels contribute, at least in part, to the Wnt pathway activation in diabetic retinopathy.

Xenopus axis duplication is a commonly used model to study Wnt signaling, as the Wnt pathway is known to play a key role in the axis formation (37). In the Xenopus axis duplication assay, SERPINA3K blocked axis duplication induced by Wnt8, which confirmed the inhibitory effect of SERPINA3K on Wnt signaling. This finding suggests that SERPINA3K can antagonize LRP6 in broad cell types. In the Xenopus embryos experiment, SERPINA3K has no effect on Wnt signaling induced by the constitutively active mutant of LRP6 (LRP6ΔN), which is consistent with the observation that SERPINA3K interrupts dimerization between the extracellular domains of LRP6 and the Fz receptor, suggesting that the inhibitory effect of SERPINA3K is dependent on the extracellular domain of LRP6, similar to that of DKK1. SERPINA3K inhibited the functions of Wnt3a (Fig. 4), Wnt8 (Fig. 5), and Wnt1 (Fig. 6), all of which are ligands of LRP6 (1). These findings provide further evidence that LRP6 is the molecular target of SERPINA3K. However, it remains to be investigated which domain in LRP6 the SERPINA3K binds to, and whether SERPINA3K plays a role in embryo development.

Similar to many other serpins, SERPINA3K has high levels in the circulation (27). Previous studies showed that plasma SERPINA3K levels are approximately 0.4 μg/mL (8 nM) in rats (48). The total SERPINA3K concentration in the serum is close to 10 μg/mL, as shown in our Western blotting result (Fig. S4C). The discrepancy could be due to that SERPINA3K may be bound by a partner in the serum, which blocks the antibody binding site in ELISA. To dissociate SERPINA3K from its binding partner, we pretreated the sample with urea, and, consequently, the ELISA result showed that the serum concentration was 6.9 μg/mL (138 nM), significantly higher than that without urea treatment. However, in the retina and vitreous samples, the effect of urea was not as substantial as in the serum. In the retina, the concentration of SERPINA3K was 565 ng/mg (wet weight; Fig. S4D). In the vitreous, the concentration of SERPINA3K was 94 ng/mg (wet weight; Fig. S4D). These findings showed approximate concentrations of SERPINA3K to be 11 nM in the retina and 2 nM in the vitreous. The retinal concentration is close to the Kd (10 nM) for SERPINA3K to bind with LRP6. In the retina, SERPINA3K is expressed in various cell types, with highest levels in ganglion cells (Fig. S4). Adenovirus injection induced overexpression of SERPINA3K in ganglion cells (Fig. S5). Because SERPINA3K is a secreted protein, overexpressed SERPINA3K can diffuse into other retinal layers to exert its functions.

SERPINA3K is known as a specific inhibitor of tissue kallikrein (27). Our previous studies have suggested that it is also a potent angiogenic inhibitor (25). The present study has established a unique anti-inflammatory activity of SERPINA3K in diabetic retinopathy, as it down-regulated inflammatory cytokines and reduced retinal vascular leakage in diabetic rats. In vitro assays demonstrated the inhibitory effect of SERPINA3K on retinal endothelial cell migration, another evidence for its antiangiogenic effect. However, SERPINA3K did not prevent pericyte loss in the retina of diabetic rats (Fig. S6). These findings, together with previous studies showing the SERPINA3K inhibits retinal neovascularization and ameliorates retinal inflammation (25, 31), suggest that SERPINA3K has therapeutic potential for diabetic retinopathy.

Materials and Methods

Proteins, Plasmids, Adenovirus, Conditioned Medium, Transfection, and Reporter Assay. Expression and purification of SERPINA3K-HIS and CRBP-HIS from E. coli were performed as described previously (49). SERPINA3K-HIS and LRP6-HIS were cloned into the pTriEx1.1 vector (Novagen). SERPINA3K and LRP6 (no His tag) were cloned into the pcDNA3 vector. LRP6-Myc, LDLRN-Myc, and Fz8CRD-IgG were expressed in pCZ20 vectors, and the TOPFLASH vector was constructed as described (50). Fugene 6 (Roche Applied Science) was used for transfection following manufacturer’s protocol.

The recombinant adenovirus was generated using the human adenovirus serotype 5 (Ad5) vectors and the AdEasy XL adenoviral vector system from Stratagene.

The TOPFLASH and renilla luciferase pRL-TK vectors were cotransfected into the cells. TOPFLASH activity was measured using dual luciferase reporter system (Promega) and normalized by renilla luciferase activity.

Experimental Animals. Care, use, and treatment of all of the animals in this study were in strict agreement with the Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental diabetes was induced as described previously (51). The retinal vascular permeability was quantified using the Evans blue-albumin leakage method as described previously (51).
Western Blot Analysis and Immunoprecipitation. Western blot analysis was performed as described previously (31). Antibodies for VEGF, LRPs, and β-catenin were purchased from Santa Cruz Biotechnology and used at 1:1,000, 1:500, and 1:3,000 dilutions, respectively. Antibodies for His-tag (1:1,000) and for β-actin (1:3,000) were purchased from Invitrogen. Antibody for phosphorylated LRPs was purchased from Cell Signaling Technology and used at 1:500 dilution. The monoclonal antibody for SERPINA3K (1:1,000) was generated using the recombinant SERPINA3K through the service with Proteintech Group.

For immunoprecipitation, recombinant proteins, supernatant of cell lysate, and conditioned medium following the manufacturer’s protocol. Brieﬂy, after overnight incubation on a nucrat at 4 °C, the proteins were precipitated and washed six times with washing buffer [PBS for Protein G resin, PBS with 20 mM Tris (pH 8.0), for Ni resin, and TBS-T for the Myc-tag antibody conjugated resin (Pierce)] was added to the cell lysates or conditioned medium following the manufacturer’s protocol. Briefly, after overnight incubation on a nucrat at 4 °C, the proteins were precipitated and washed six times with washing buffer [PBS for Protein G resin, PBS with 20 mM Tris (pH 8.0), for Ni resin, and TBS-T for the Myc-tag antibody conjugated resin (Pierce)] was added to the cell lysates or conditioned medium following the manufacturer’s protocol.

Embryo RNA Injection and Animal Explants. Embryo manipulations and RT-PCR were performed as described previously (52). Capped RNAs were synthesized using the mMessage mMachine in vitro transcription kit (Ambion). For secondary axis induction, RNAs were injected into the ventral marginal region of Xenopus embryo at the four-cell stage. The following amounts of RNA were used: 1.6 pg Wnt8c, 1 ng secreted LDL receptor (DLLR-FC), 45 pg DGK7, 80 pg LRPS6, and 1 ng SERPINA3K. The presence of asec.

Statistical Analysis. Student’s t test was used in all statistical analyses, and statistical signiﬁcance was accepted when the P value was less than 0.05.

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Supporting Information

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SI Materials and Methods

Cell Culture. ARPE19, HEK293T, and RGC-5 (a rat retina ganglion cell line) cells were purchased from ATCC and cultured in DMEM containing 10% FBS. Lcells and Lcells stably expressing Wnt3A (L-Wnt3a) were purchased from ATCC and cultured in DMEM containing 10% FBS and 0.4 mg/mL G-418 (Invitrogen). Cells and conditioned medium (1 g/L glucose, 1% FBS) were harvested following the procedure recommended by ATCC. RPE-J cells, a rat RPE cell line, were purchased from ATCC and cultured under ATCC recommended conditions. HRCEC were purchased from Cell Systems Corporation and cultured in the recommended CSC complete medium. rMC-1 cells, a cell line derived from rat retinal Müller cells, were a kind gift from Vijay Sarthy (Northwestern University, Evanston, IL) and cultured in DMEM containing 10% FBS. TR-iBRB cells, a cell line derived from rat retinal capillary endothelial cells, were a kind gift from K. Hosoya and T. Terasaki (Tohoku University, Sendai, Japan) and cultured under recommended conditions (1). The cultured cells were starved in 1 g/L glucose (5 mM) DMEM containing 1% FBS overnight before the addition of proteins or compounds. HRCEC were processed in DMEM/F12 (50:50) with ECGS (Millipore).

Cell Lysate Preparation. For cytosolic β-catenin measurement, cells were lysed by three freeze/thaw cycles followed by centrifugation, and the supernatants were isolated for Western blot analysis. For total cell lysates, harvested cells were sonicated in RIPA (Cell Signaling Technology) buffer containing 1% SDS. For retinal homogenate preparation, the retinas were homogenized in PBS with a protease inhibitor mixture using a soft tissue pestle (Fisher Scientific).

siRNA Transfection. As we described previously (2), the SERPINA3K siRNAs were synthesized by Ambion. The sense sequences (5’ to 3’) for the RNAi were: RRNAi-1, GGCCCAAGAU-AUAAGUGAtt; RNAi-2 CUCUAAAAGUUUACCGCAAtt; control RNAi, GCAUUCAAGGACCCGAGUt. Transfection was performed with the siPORT TM lipid transfection reagent (Ambion) according to the manufacturer’s instruction.

ELISA. VEGF concentrations in culture media were measured using an ELISA kit purchased from R&D Systems according to manufacturer’s protocol. SERPINA3K concentrations were measured using the sandwich ELISA using two monoclonal antibodies for SERPINA3K generated by Proteintech Group.

β-galactosidase Cytochemistry. Cultured cells infected by Ad-LacZ (MOI = 10) were fixed for 5 min at 4°C in PBS containing 2% formaldehyde and 0.2% glutaraldehyde. After the washes with PBS, the cells were incubated in the reaction mixture (1 mg/mL X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgCl2 in PBS) for at least 4 h at 37°C or until the blue cells were visible.

SERPINA3K inhibited Wnt signaling and cell migration in endothelial cells treated with high-glucose media and Wnt ligand. (A) HRCEC were exposed to an HG or LG medium with 100 nM SERPINA3K or BSA for 12 h. The same amount of total cellular proteins was immunoblotted with antibodies specific for phosphorylated LRP6 (p-LRP6) and for total LRP6. (B) TR-iBRB cells, a rat retinal endothelial cell line, were exposed to a HG or LG medium containing 100 nM SERPINA3K or BSA for 24 h. The same amount of cytosolic proteins was immunoblotted with an antibody specific for β-catenin. (C–L) Endothelial cell migration was measured by the scratch wound healing assay. The phase contrast image of the cells (C and H) and the cells with DAPI staining (D and I) showed the original scratch wound. (E–G) The cells were exposed to the high-glucose medium with 100 nM SERPINA3K (G) or BSA (F) for 72 h. Control cells were treated with the L-glucose medium (E). The fluorescence image of DAPI staining (1 μg/mL) was captured. (J–L) The cells were exposed to the Wnt3a conditioned medium with 500 nM SERPINA3K (L) or BSA (K) for 72 h. Control cells were treated with the L-cell conditioned medium (J). The cells migrated into the scratched area were quantified by fluorescent densitometry measuring the DAPI staining signal. (M Left) The quantification result was shown as the relative migration ratio in high-glucose-treated cells (mean ± SD, n = 3). (M Right) The DAPI stained cells in the wound area were shown as the relative migration ratio in the cells treated with Wnt3a (mean ± SD, n = 3). (N) Cell migration rate of TR-iBRB cells was measured using Transwell migration assay (mean ± SD, n = 10). *P < 0.05. (Scale bar: 400 μm.) These findings show that SERPINA3K inhibited Wnt signaling in endothelial cells and blocked endothelial cell migration induced by high glucose and Wnt3a.

Fig. S2. Effects of SERPINA3K knockdown on the Wnt signaling and downstream events. Cultured RPE-J cells (A and C) and TR-iBRB cells (B and D) were separately transfected with two different RNAi: RNAi-1 and RNAi-2 specific for SERPINA3K and control RNAi for 72 h. (A and B) The same amount of culture media was blotted with the anti-SERPINA3K antibody; the same amount of cytosolic proteins was blotted with an antibody specific for β-catenin. (C) VEGF secreted into the medium was measured using ELISA (mean ± SD, n = 3). (D) Migration of TR-iBRB cells was measured using Transwell migration assay (mean ± SD, n = 10). *P < 0.05.
Fig. S3. Up-regulation of mRNA levels of Wnt target genes in diabetic rat retinas and cultured cells after SERPINA3K knockdown. Transcript levels of four Wnt target genes, including vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF), Cyclin D1, and alkaline phosphatase (AP), were measured by real-time RT-PCR. To normalize the variation of the amount of mRNA in each reaction, 18S rRNA was simultaneously measured in the same sample as an internal control. (A) The forward (F) and reverse (R) primers for each gene were designed spanning at least one exon junction with indicated product size. The quality of PCR products was examined using agarose gel electrophoresis. PCR efficiency for each pair of primers was obtained from the standard curve using serial 1–10 dilutions of the cDNA from reverse transcription and all of the correlation coefficient squared values (R²) were greater than 0.99. (B) In the retinas from rats with STZ-induced diabetes for 3 months, mRNA levels of all of the four Wnt target genes were significantly higher than that in nondiabetic control retinas. (C and D) Cultured TR-iBRB cells (C) and RPE-J cells (D) were separately transfected with RNAi-2 (showing higher knockdown efficiency from Fig. S4) specific for SERPINA3K and control RNAi for 72 h. Compared with the control RNAi, RNAi-2 significantly up-regulated transcription levels of all of four Wnt target genes in both TR-iBRB and RPE-J cells. Values are mean ± SD, n = 3. *P < 0.05, versus the control. These findings suggest that the decrease of SERPINA3K contribute, at least in part, to the Wnt pathway activation in diabetic retinopathy.
Fig. S4. Expression of endogenous SERPINA3K in the retina. (A) Immunofluorescence staining of SERPINA3K (green) in retinal sections showed diffuse SERPINA3K signal in the retina, with intensive staining in the ganglion cell layer. The nuclei were counterstained (red). (Scale bar: 50 μm.) (B) Retinal cells such as RPE-J (RPE cell line), TR-IBRB (retinal endothelial cell line), RGC-5 (ganglion cell line), and rMC-1 (retinal Müller cell line) were seeded in the six-well plate (10⁶ cells/well). For each cell line, 20 μL culture medium was loaded for Western blot analysis using the anti-SERPINA3K antibody. (C) Western blot analysis was performed to estimate the concentration of SERPINA3K in the serum. Different amounts of recombinant SERPINA3K and 1 μL rat serum were loaded and blotted by the anti-SERPINA3K antibody. (D) Concentrations of SERPINA3K in the serum, retina, and vitreous were measured by ELISA (mean ± SD, n = 3). FBS was used as a negative control, and recombinant SERPINA3K was used as a positive control.

Fig. S5. Infection of different retinal cell types by adenovirus. (A and B) Four weeks after the intravitreal injection of Ad-SA3K, the eyeballs were fixed and processed for histology analysis. (A) Immunofluorescence staining with the antibody for SERPINA3K (green) showed overexpression of SERPINA3K in the ganglion layer and also diffused signals in other layers in the Ad-SA3K infected retina, compared with that with the Ad-LacZ (B). (C–F) RGC-5 (C), rMC-1 (D), RPE-J (E), and TR-IBRB (F) were incubated with Ad-LacZ (MOI = 10) for 3 days, and the infected cells showed blue color after X-gal staining, suggesting that adenovirus infects all of the retinal cell lines with different efficiencies. (Scale bar: 100 μm.)
Fig. S6. Lack of effect of SERPINA3K on the diabetes-induced retinal pericyte loss. (A–D) Retinal trypsin digestion assay was performed to count pericytes in the retinas from four experimental groups, including nondiabetic rats (A), rats with STZ-diabetes (DM) for 3 months without injection (B), DM rats injected with Ad-LacZ (C), and DM rats injected with Ad-SA3K (D). The assay has been described in Materials and Methods. (E) Retinal pericytes in each field were averaged and compared (mean ± SD, n = 30). *P < 0.05; NS, P > 0.05. (Scale bar: 50 μm.)