Nanoparticle-Mediated Expression of a Wnt Pathway Inhibitor Ameliorates Ocular Neovascularization

Zhongxiao Wang,* Rui Cheng,* Kyungwon Lee, Puneet Tyagi, Lexi Ding, Uday B. Kompella, Jing Chen, Xun Xu, Jian-xing Ma

Objective—The deficiency of very low-density lipoprotein receptor resulted in Wnt signaling activation and neovascularization in the retina. The present study sought to determine whether the very low-density lipoprotein receptor extracellular domain (VLN) is responsible for the inhibition of Wnt signaling in ocular tissues.

Approach and Results—A plasmid expressing the soluble VLN was encapsulated with poly(lactide-co-glycolide acid) to form VLN nanoparticles (VLN-NP). Nanoparticles containing a plasmid expressing the low-density lipoprotein receptor extracellular domain nanoparticle were used as negative control. MTT, modified Boyden chamber, and Matrigel (TM) assays were used to evaluate the inhibitory effect of VLN-NP on Wnt3a-stimulated endothelial cell proliferation, migration, and tube formation. Vldlr−/− mice, oxygen-induced retinopathy, and alkali burn–induced corneal neovascularization models were used to evaluate the effect of VLN-NP on ocular neovascularization. Wnt reporter mouse (BAT-gal), Western blotting, and luciferase assay were used to evaluate Wnt pathway activity. Our results showed that VLN-NP specifically inhibited Wnt3a-induced endothelial cell proliferation, migration, and tube formation. Intravitreal injection of VLN-NP inhibited abnormal neovascularization in Vldlr−/−, oxygen-induced retinopathy, and alkali burn–induced corneal neovascularization models, compared with low-density lipoprotein receptor extracellular domain nanoparticle. VLN-NP significantly inhibited the phosphorylation of low-density lipoprotein receptor-related protein 6, the accumulation of β-catenin, and the expression of vascular endothelial growth factor in vivo and in vitro.

Conclusions—Taken together, these results suggest that the soluble VLN is a negative regulator of the Wnt pathway and has antiangiogenic activities. Nanoparticle-mediated expression of VLN may thus represent a novel therapeutic approach to treat pathological ocular angiogenesis and potentially other vascular diseases affected by Wnt signaling. (*Arterioscler Thromb Vasc Biol. 2015;35:855-864. DOI: 10.1161/ATVBAHA.114.304627.)

Key Words: eye ■ nanoparticle ■ neovascularization ■ VLDLR ■ Wnt

Ocular neovascularization is a major cause of irreversible visual loss in several eye diseases, such as proliferative diabetic retinopathy, age-related macular degeneration, retinopathy of prematurity, and traumatic corneal injury.1–3 The treatments for these diseases are not satisfactory because of the limited understanding of pathogenesis of ocular neovascularization and the poor drug delivery caused by the ocular barriers.4,5

See cover image

The canonical Wnt signaling pathway regulates a wide array of developmental and physiological processes, including proliferation, migration, differentiation, and apoptosis by activating transcription of multiple target genes.6 Previous studies have reported that the Wnt signaling pathway participates in the regulation of angiogenesis.7 In the canonical Wnt signaling pathway, Wnt ligands bind to a cell surface receptor complex consisting of frizzled receptors and low-density lipoprotein receptor-related protein 5/6 (LRP5/6), leading to stabilization of cytoplasmic β-catenin by attenuating its phosphorylation. Non-phosphorylated β-catenin (n-p-β-catenin) translocates into the nucleus, where it associates with T cell factor (TCF) to activate the transcription of Wnt target genes, including vascular endothelial growth factor (VEGF),8 which is a key causative factor in ocular angiogenesis.9 Although several Wnt pathway inhibitors, such as Dickkopf1, have been identified, the regulation of Wnt signaling is not well understood.

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Several groups have successfully encapsulated naked DNA into biodegradable poly(lactide-co-glycolide acid) nanoparticles for long-term and controlled DNA release.20,21

VLDLR belongs to the low-density lipoprotein receptor (LDLR) gene family and is a highly conserved integral membrane protein consisting of a large ectodomain, a single transmembrane domain, and an intracellular domain.14 The specific functional domain of a large ectodomain, a single transmembrane domain, and an intracellular domain.14 The specific functional domain of VLDLR is known to mediate lipid transport.10 It was reported later that Vldlr−/− mice develop abnormal retinal and subretinal neovascularization.11,12 Our previous study has shown that VLDLR deficiency results in Wnt signaling over-activation in the retina, which is responsible for retinal neovascularization, suggesting an inhibitory effect of VLDLR on Wnt signaling and retinal neovascularization.13 VLDLR belongs to the low-density lipoprotein receptor (LDLR) gene family and is a highly conserved integral membrane protein consisting of a single transmembrane domain, and an intracellular domain.14 The specific functional domain of VLDLR for the interaction with Wnt signaling has not been clearly defined. We hypothesized that the VLDLR N-terminal ectodomain (VLN) is responsible for the inhibitory effect on Wnt signaling, as VLDLR is known to shed VLN into the extracellular space as a soluble peptide.15 Our previous work has shown that VLN is efficient to block Wnt signaling in vitro, whereas the present study generated nanoparticles encapsulated with a plasmid-mediated expression of the soluble VLN with His tag to evaluate the inhibitory effect of VLN on retinal neovascularization and Wnt signaling in vivo. At the same time, nanoparticles expressing the extracellular domain of LDLR (LN) with Myc tag was used as a control because previous studies showed that LDLR knockout does not affect the Wnt signaling pathway or result in neovascular phenotype.17,18

Because of the ocular barriers, including the corneal barrier, aqueous barrier, the inner and outer blood–retinal barriers, it is always challenging to deliver large molecules, such as peptides and DNA into the retina. Intravitreal injection is commonly used to deliver genes or proteins into the retina. Because these molecules remain in the eye for only short durations, repetitive injections are needed, which is accompanied by problems, such as cataract, vitreous hemorrhage, and endophthalmitis. Nanoparticles have been applied to improve penetration, controlled and sustained release of drugs, and drug targeting.19 Several groups have successfully encapsulated naked DNA into biodegradable poly(lactide-co-glycolide acid) nanoparticles for long-term and controlled DNA release.20,21

To study the role of VLN in the regulation of Wnt signaling and ocular neovascularization, we encapsulated an expression plasmid of VLN or LN with poly(lactide-co-glycolide acid) polymer to form VLN nanoparticles (VLN-NP)/LN nanoparticles (LN-NP) and evaluated the efficacy of VLN-NP on Wnt3α-induced proliferation, migration, and tube formation of endothelial cells and on ocular neovascularization in ocular neovascularization models, including Vldlr−/− mice, oxygen-induced retinopathy (OIR) model, and alkali burn–induced corneal neovascularization model.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**VLN-NP–Mediated Expression of VLN in Human Retinal Microvascular Endothelial Cells**

VLN-NP and LN-NP had a low polydispersity (≤0.2), indicating a narrow particle size distribution, and were negatively charged. The plasmid loading was 1.1% and 0.9% in VLN-NP and LN-NP, respectively (Table in the online-only Data Supplement).

The conditioned serum-free media (3-fold) from primary human retinal microvascular endothelial cells (HRMEC) were harvested 72 hours after the transfection of VLN-NP and LN-NP and concentrated. As shown by Western blot analysis, both of the anti-His-tag and anti-VLN antibodies detected significant amounts of the VLN peptide with the expected molecular weight (100 kDa) in the conditioned medium from the cells transfected with VLN-NP, but not in that from the cells transfected with LN-NP (Figure 1A). On the contrary, an anti-Myc-tag antibody detected the expression of LN-Myc only from the cells transfected with LN-NP (Figure 1A).

**VLN-NP Inhibits HRMEC Growth Induced by Wnt3a and Small Interfering RNA (siRNA) of VLDLR**

As shown by MTT assay in HRMEC at various time points after transfection, VLN-NP significantly decreased HRMEC viability at 48 and 72 hours post-transfection, compared with the LN-NP–transfected cells (Figure 1B). Furthermore, VLN-NP specifically attenuated Wnt3a-induced HRMEC growth, compared with LN-NP (Figure 1C). In addition, the siRNA knockdown of VLDLR resulted in increased numbers of viable HRMEC, which is consistent with a previous study,22 whereas VLN-NP decreased viable HRMEC, diminishing the effect of the VLDLR siRNA (Figure 1H).

**VLN-NP Suppresses Wnt3a-Stimulated HRMEC Migration and Tube Formation**

HRMEC migration was measured using the transwell chamber assay. As shown in Figure 1D and 1F, Wnt3a-conditioned medium (WCM) stimulated HRMEC migration compared with the L cell–conditioned medium. VLN-NP, but not LN-NP, significantly suppressed the endothelial cell migration induced by WCM.
Tube formation assay demonstrated that WCM induced tubes formation in HRMEC, whereas VLN-NP significantly suppressed WCM-induced endothelial cell tube formation (Figure 1E and 1G).

**VLN-NP Inhibits Wnt3a-Induced Activation of the Wnt Signaling Pathway In Vitro**

To evaluate the modulation of Wnt signaling by VLN in vitro, HRMEC were transfected with VLN-NP or LN-NP. WCM was used to induce Wnt signaling compared with the L cell conditioned medium control. WCM upregulated the levels of phosphorylated LRP6 (p-LRP6), total LRP6 (t-LRP6), n-p-β-catenin, and VEGF, compared with L cell conditioned medium (Figure 2A–2D). VLN-NP dramatically blocked the increases of LRP6, n-p-β-catenin, and VEGF levels induced by WCM (Figure 2A–2D).

Transcriptional activity of TCF/β-catenin was measured using TOPFLASH assay. As shown in Figure 2H, WCM increased luciferase activity by almost 100-fold, whereas VLN-NP reduced the activity by 60%, compared with LN-NP (Figure 2H), suggesting that VLN-NP directly inhibits Wnt signaling induced by Wnt ligand in endothelial cell.

To determine whether the regulation of VLN on VEGF is through the Wnt signaling pathway, we used a monoclonal antibody Mab2F1, which specifically blocks the ligand-binding site of the Wnt coreceptor LRP6.23 The results showed that Mab2F1 successfully blocked the VEGF expression induced by WCM (Figure 2E) and abolished the inhibitory effect of VLN-NP on Wnt3a-induced VEGF upregulation (Figure 2E), suggesting that VLN-NP mediated VEGF suppression acts exclusively through Wnt inhibition. Moreover, an adenovirus expressing a constitutively active mutant of β-catenin (Ad-S37A) was used to induce VEGF overexpression in the absence of Wnt ligand, and the VEGF expression induced by this active mutant of β-catenin was not blocked by VLN-NP as shown at the protein and mRNA levels (Figure 2F and 2G). Together, these results suggest that the suppression of VEGF expression by VLN-NP is through inhibiting the Wnt signaling pathway, which occurs at the LRP6 coreceptor level.

**VLN-NP Mediates Sustained Expression of VLN in the Retina**

To examine the expression and duration of the genes delivered by nanoparticles in the retina, VLN-NP were injected intra-vitreally (50 μg/eye), with LN-NP as control, and then the retinas were dissected at 1, 2, 3, and 4 weeks (3 mice per time point) after the injection. VLN was detected with an expected molecular weight in the retinas injected with VLN-NP at all of the time points analyzed by Western blotting analysis using the anti-His-tag antibody, but not in the retinas injected with LN-NP (Figure 3A).
VLN-NP Has No Detectable Toxicities to the Structure and Function of the Retina

To detect the possible adverse effect of VLN-NP and LN-NP on visual functions, electrophotography recording was used in the treated eyes and eyes 4 weeks after intravitreal injection of VLN-NP and LN-NP. The amplitudes of A- or B- waves of scotopic and photopic electroretinography in the VLN-NP-treated group were not significantly different among the mice injected with VLN-NP, LN-NP, and the control (Figure IC in the online-only Data Supplement).

Possible toxicities of VLN-NP were also evaluated by histological examination. No difference was observed in the number of retinal nuclear layers or thickness of the retina among the mice injected with VLN-NP and LN-NP (Figure IC in the online-only Data Supplement).

VLN-NP Inhibits Retinal Neovascularization in Vldlr−/− Mice

As shown by angiography of the flat-mounted whole retinas, Vldlr−/− mice developed small, irregular in shape (coiled or enlarged) intraretinal neovascular blebs throughout the retina, which is consistent with a previous report24 (Figure 3B). The injection of VLN-NP significantly reduced numbers of intraretinal neovascular blebs in Vldlr−/− eyes compared with those with the LN-NP injection (Figure 3B and 3C).

BS-1 lectin staining of retinal sections showed decreased vascular density and less abnormal vessels in outer nuclear level of the retina in Vldlr−/− retinas after the VLN-NP injection, compared with those in the retina with LN-NP injection (Figure 3D).

VLN-NP Inhibits the Wnt Signaling Pathway in the Retina of Vldlr−/− Mice

Levels of p-LRP6, t-LRP6, n-p-β-catenin and t-β-catenin, and VEGF were significantly elevated in the eyecups of Vldlr−/− mice with LN-NP injection at age of P30, suggesting the activation of Wnt signaling in the Vldlr−/− retinas (Figure 4A–4D). A single injection of VLN-NP into Vldlr−/− vitreous at P10 abolished the upregulation of p-LRP6, t-LRP6, n-p-β-catenin, t-β-catenin, and VEGF levels, suggesting that VLN is the functional domain responsible for the inhibition of the Wnt pathway (Figure 4A–4D).

VLN-NP Alleviates Ischemia-Induced Retinal Neovascularization in OIR Mice

As shown by fluorescein angiography, severe neovascularization and enlarged nonperfusion area were exhibited...
in the flat-mounted retinas in the OIR mice treated with LN-NP, whereas the VLN-NP–treated OIR mice displayed alleviated retinal neovascularization and dramatically smaller nonperfusion areas (Figure 3E and 3F). Moreover, the VLN-NP–treated OIR group developed significantly fewer preretinal vascular cells, in comparison to LN-NP–treated OIR mice (Figure 3G and 3H), supporting an inhibitory effect of VLN-NP on ischemia-induced retinal neovascularization.

VLN-NP Inhibits the Wnt Signaling Pathway in OIR Mice

Our group has previously shown that the Wnt pathway is activated in the retina of OIR model, which contributes to the retinal neovascularization. A single injection VLN-NP attenuated the elevations of p-LRP6, t-LRP6, n-p-β-catenin, t-β-catenin, and VEGF levels in the retina of OIR animals (Figure 4E–4H).

We also evaluated the effect of VLN-NP on Wnt signaling activity using the Wnt reporter mice, BAT-gal transgenic mice that express the β-galactosidase gene under the control of a promoter containing TCF/β-catenin-binding sites. As shown by X-gal staining, the retina with OIR showed more intense blue color compared with that of age-matched normal mice, suggesting Wnt pathway activation induced by OIR. The injection of VLN-NP decreased blue color in the retina of OIR mice compared with LN-NP, as measured by corneal neovascularization clock hour (Figure 5B) and vascularized area (Figure 5C). H&E and CD31 staining of corneal sections showed that the VLN-NP–treated group developed fewer vessels in the cornea with alkali burn compared with untreated and LN-NP–treated groups (Figure 5D).


Figure 3. Anti-angiogenic activity of very low-density lipoprotein receptor extracellular domain nanoparticle (VLN-NP) in the retina. A, Expression of VLN in the mouse retinas was measured using Western blot analysis at 1, 2, 3, and 4 weeks after an intravitreal injection of VLN-NP. B, Representative retinal angiographs of Vldlr−/− (VKO) mice (upper panels, ×40; lower panels, higher magnification of the boxed areas). VLN-NP was injected into the VKO eyes on P10, with low-density lipoprotein receptor extracellular domain nanoparticle (LN-NP) as control. Retinal angiography was performed on P30. C, Quantification of intraretinal neovascular blebs (IRN blebs) in VKO mice treated with VLN-NP or LN-NP. **P<0.01, n=6. D, Immunostaining of BS-1 lectin (green) in VKO retinal sections. The nuclei were counterstained with DAPI (blue) (×200). Arrows indicate abnormal vessels in the retinas. E–H, VLN-NP or LN-NP was intravitreally injected into oxygen-induced retinopathy (OIR) mice on P12 and retinal neovascularization analyzed at P17. E, Representative retinal angiographs (upper panels, ×40; lower panels, higher magnification of the boxed areas). F, Quantification of nonperfusion area in the retina. **P<0.01, n=6. G, Representative retinal sections with H&E staining. Arrows indicate preretinal vascular cells (×200). H, Quantification of preretinal vascular cells. **P<0.01, n=6. GCL indicates ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; and RPE, retinal pigment epithelium.

VLN-NP Ameliorates Alkali Burn-Induced Corneal Neovascularization

Corneal neovascularization was induced with alkali burn in rats, as shown in corneal images, 7 days after the alkali burn. A single topical treatment of VLN-NP (20 μL, 10 mg/mL) at the procedure day of alkali burn substantially reduced corneal neovascularization induced by alkali burn at day 7 after treatment compared with the same dose of LN-NP (Figure 5A). Quantitatively, VLN-NP significantly reduced neovascularization severity in the alkali burn model compared with LN-NP, as measured by corneal neovascularization clock hour (Figure 5B) and vascularized area (Figure 5C). H&E and CD31 staining of corneal sections showed that the VLN-NP–treated group developed fewer vessels in the cornea with alkali burn compared with untreated and LN-NP–treated groups (Figure 5D).
VLN-NP Suppresses the Wnt Signaling Pathway in the Rat Cornea with Alkali Burn-Induced Neovascularization

As shown in Figure 6, levels of p-LRP6, t-LRP6, n-p-β-cat, t-β-cat, and VEGF in the cornea were significantly elevated in the untreated and LN-NP–treated corneas with alkali burn compared with control without alkali burn. VLN-NP (20 μL, 10 mg/mL) treatment dramatically suppressed the increases of p-LRP6, t-LRP6, n-p-β-cat, t-β-cat, and VEGF (Figure 6A–6D).

The same procedure of alkali burn was performed in BAT-gal transgenic mice, and flat-mounted corneas and corneal sections showed more intense blue color, indicative of active Wnt signaling, after X-gal staining in the alkali burn group, without treatment or with LN treatment compared with no burn group. Moreover, significantly less intense blue color was observed in the VLN-NP–treated corneas with alkali burn (Figure 6E), indicating that VLN-NP inhibited Wnt signaling activity in the cornea with alkali burn–induced neovascularization.

Discussion

Ocular neovascular diseases constitute the most common causes of severe and irreversible vision loss in developed countries. The canonical Wnt signaling pathway plays a vital part in ocular development and diseases, as well as in ischemia-induced retinal neovascularization and laser-induced choroidal neovascularization.

Our group reported that VLDLR deficiency results in overactivation of the Wnt signaling pathway and retinal neovascularization, suggesting a potential role of VLDLR as an endogenous inhibitor of Wnt signaling and ocular neovascularization. Recently, our group revealed that the extracellular domain of VLDLR is essential for its inhibition of ocular neovascularization through down-regulating Wnt signaling.

Additionally, our data also show that nanoparticle-mediated delivery of VLDLR extracellular peptide is an effective approach to treat ocular diseases in mouse models.

Ocular neovascularization, depending on its location and causation, can have distinct pathogenic mechanisms. However, angiogenic factors, such as VEGF, are commonly involved in almost all types of the ocular neovascularization. Wnt signaling, which is an upstream signaling pathway regulating VEGF, has been shown to play a crucial part in retinal neovascularization formation, and inhibitors of Wnt signaling...
have displayed therapeutic potential for retinal neovascularization diseases. This study demonstrates that Wnt signaling is upregulated in all 3 neovascularization models (Figures 4 and 6), which are consistent with previous studies in Vldlr−/− mice and OIR model. Further, the present study is the first to establish the pathogenic role of Wnt signaling in corneal neovascularization. Although β-catenin was reported to be upregulated in the neovascularized cornea after alkali burn, it was not clear whether the Wnt pathway was activated because β-catenin has multiple functions in addition to participating in Wnt signaling cascade, such as modulating cellular adhesion and cytoskeleton. The present study evaluated the Wnt pathway activity at multiple levels, including phosphorylation of a Wnt coreceptor, LRP6; upregulation of VEGF, a direct target gene of β-catenin; and transcriptional activity of β-catenin in the corneas (Figure 6). All of these assays showed that Wnt signaling was activated in the neovascularized cornea after alkali burn, thus providing further evidence for a key pathogenic role of Wnt signaling in ocular neovascularization. In addition, we also measured the expression of transcription factor TCF in HRMEC. The TCF mRNA was not altered with the treatment of WCM or VLN-NP (Figure III in the online-only Data Supplement). The functional suppression of TCF activity by VLN is supported by our results from TOPFLASH assay (Figure 2H), showing that VLN suppressed Wnt3a-induced TOPFLASH activity, indicative of TCF transcriptional activity. Therefore, the transcriptional function of TCF, rather than its expression level, is relevant to Wnt signaling activity and VLN suppression.

LRP6 and VLDLR both belong to the LDLR family and share significant amino acid sequence identity in their extracellular domains. It has been established that the extracellular domain of LRP6 is the essential binding domain for Wnt ligands and some Wnt signaling inhibitors, such as Dickkopf1, and also required for homodimerization of LRP6 to initialize Wnt signaling. As the extracellular domain of VLDLR can be shed into the extracellular space, we hypothesized that the soluble extracellular domain of VLDLR may function as a soluble cytokine and confer some physiological or pathological functions distinct from its membrane form. Based on the hypothesis, our group dived into the structural basis for the inhibitory effect of VLDLR on Wnt signaling and found that VLN is essential for the inhibition of Wnt signaling. Here we generated a nanoparticle-encapsulated extracellular domain of VLDLR, termed as VLN-NP. Our results show that VLN is sufficient to inhibit Wnt3a-induced HRMEC growth, migration, and tube formation (Figure 1B–1G). Moreover, it was reported that knockdown of VLDLR by siRNA enhanced endothelial cell viability, migration, and tube formation. Our data show that VLN-NP inhibits HRMEC growth induced by...
the VLDLR siRNA (Figure 1H), suggesting that the ectodomain of VLDLR is sufficient to constitute the Wnt-inhibiting function of VLDLR in regulation of Wnt signaling. Three animal models all demonstrated that VLN-NP ameliorate ocular neovascularization and suppress Wnt signaling as shown at the receptor level (LRP6), transcription factor level (β-catenin), and target gene level (VEGF) in vivo. Our data showed that a specific blocker of LRP6 attenuated the VEGF expression induced by WCM, similar to the effect of VLN-NP (Figure 2E). Further, VLN-NP did not decrease VEGF expression induced by a constitutively active mutant of β-catenin (Ad-S37A), which activates Wnt target genes down-stream of LRP6 in HRMEC (Figure 2F and 2G). These results support that the inhibition of VEGF by VLN is achieved through suppressing Wnt signaling at the coreceptor LRP6 level. Our recent publication demonstrated that VLDLR inhibits Wnt signaling through the formation of a heterodimer with LRP6. This heterodimerization blocks the binding of Wnt ligand and the coreceptor LRP6, which is an essential step in the activation of Wnt signaling. The heterodimerization also reduces LRP6 levels on the cell surface by accelerating its internalization and turnover of LRP6, which explains the reduction of total LRP6 in VLN-NP-treated group and nonsignificant changes of the p-LRP6/t-LRP6 ratios in this study. Here, our data showed that the mRNA levels of LRP6 were not changed in vascularized corneas in vivo or with Wnt3a stimulation in vitro, nor by VLN-NP treatment (Figure II in the online-only Data Supplement), indicating that VLN may modulate LRP6 on post-translational level. Taken together, these data indicate that soluble VLN functions as an inhibitor of ocular neovascularization through suppressing Wnt signaling pathway.

The delivery of macromolecules, such as DNA and protein to ocular tissues, especially the posterior segment, is challenging because of the existence of structural barriers. Therefore, the development of nano-sized carriers may represent a promising approach in ocular drug delivery. Although the nanoparticles formulated using poly(lactide-co-glycolide acid) polymers have not been widely used in clinic, they are being extensively used in research because of their sustained release characteristics, biodegradability, biocompatibility, and ability to protect DNA from degradation. Our data showed that VLN-NP mediated substantial and sustained VLN expression in cultured cells and in the retina for >4 weeks after a single injection, confirming effective internalization of the nanoparticles, and that the cargo protein VLN is expressed and released into the extracellular space.

In conclusion, this study suggests that the Wnt signaling pathway plays an important pathogenic role in corneal and retinal neovascularization; nanoparticle-mediated delivery of soluble VLN has therapeutic potential for ocular neovascularization.
diseases and likely through the inhibition of Wnt signaling. However, there are still some unsolved questions that warrant further studies in the future. Previous study showed that Vldlr−/− retinas and downregulation of VLDLR by siRNA resulted in upregulation of LRP6 expression at both the protein and mRNA levels, whereas our data indicate that the Wnt signaling pathway and VLN does not affect LRP6 mRNA level. It is possible that VLDLR regulates LRP6 expression at different levels, depending on cell types, duration of the treatment, and types of the treatments. The present study focuses on the mechanism that VLN regulates LRP6 at the post-translational and types of the treatments. The present study needs to be performed to demonstrate the molecular mechanism by which VLDLR modulates Wnt signaling through downregulating LRP6 at different levels. In addition, although nanoparticle significantly reduces the frequency of injection, there may be still associated risks because of the characteristics of intravitreal injection. Better drug administration remains to be explored. Nevertheless, VLN-NP represents a promising strategy to treat ocular neovascularization, providing an encouraging perspective for clinical usage.

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Disclosures

None.

References


**Significance**

The present study is the first to establish the causative role of activation of Wnt signaling in corneal neovascularization, as well as in retinal neovascularization in Vdldr−/− and oxygen-induced retinopathy models. Extracellular domain of very low-density lipoprotein receptor is essential to inhibit pathological Wnt signaling and suppress aberrant ocular neovascularization, providing a new endogenous inhibitor of Wnt signaling and a potential drug target. In addition, this study also shows that nanoparticle is an efficient delivery way for intraocular administration, which may offer a promising approach in ocular drug delivery.
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Materials and Methods

Construction of expression vectors. A cDNA sequence encoding the soluble extracellular domain of very low-density lipoprotein receptor (VLN) was cloned into pcDNA6 and fused with a sequence encoding a secretion signal peptide at the amino terminus and with a Histidine hexamer tag at the carboxyl terminus. An expression plasmid of the soluble extracellular domain of low-density lipoprotein receptor (LN) with a Myc tag at the carboxyl terminus was a generous gift from Dr. Xi He at Harvard University.

Nanoparticle preparation and characterization. The VLN or LN expression plasmid solution (3 mg/ml) was added to the Poly (lactic-co-glycolic acid) polymer (Resomer 502H, Evonik Industries, Germany) solution. After a few centrifugation and wash steps, the final pellet was redispersed in 10 ml of double distilled water and frozen at -80°C. The frozen nanoparticles were lyophilized for ~18 hr (Labconco Lyophilizer, MO). The physical properties of the nanoparticles were measured, such as the particle size and zeta potential, polydispersity index and plasmid loading. Lyophilized nanoparticles (1 mg) were dispersed in 1 ml of water, and the particle size and zeta potential were measured using the Malvern Nanosizer (Malvern Inc., PA). Plasmid loading was estimated by UV spectrophotometry. One mg of nanoparticles was dissolved in 1 ml of dichloromethane by vortexing for 2 hr. The dichloromethane was evaporated in a nitrogen evaporator (Multivap, Organomotion, MA). Following evaporation of dichloromethane, 1 ml of double distilled water was added to the tube and vortexed further for 2 hr. The aqueous dispersion was then centrifuged at 15,000 rpm for 15 min and absorbance of the supernatant aqueous layer measured at 260 nm.

Electroretinogram recording. Mice were dark-adapted for at least 12 hr. The mice were anesthetized and pupils dilated with topical application of 2.5% phenylephrine and 1% tropicamide. Electroretinogram (ERG) responses were recorded with a platinum needle electrode placed on the surface of the cornea. A reference electrode was positioned in the mouth and a ground electrode to the tail. The duration of light stimulation was 10 ms. The band pass was set at 0.3–500 Hz. Fourteen responses were recorded and averaged with flash intervals of 20 s. For quantitative analysis, the B-wave amplitude was measured between A- and B-wave peaks. The ERG waveforms of both eyes in the same animal were simultaneously recorded and compared using the right-to-left eye ratio of B-wave amplitude.

Cell culture, and the preparation of conditioned medium. Human retinal microvascular endothelial cells (HRMEC) were purchased from Cell Systems (Kirkland, WA) and cultured in Endothelial Cell Growth Medium MV containing Endothelial Cell Growth Supplement (PromoCell, Heidelberg, Germany). Human telomerase reverse transcriptase (hTERT)–retinal pigment epithelial (RPE)-1 cells (hTERT-RPE-1), mouse L cells and L cells stably expressing Wnt3a (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum. Wnt3a conditional medium (WCM) was obtained from a L
cell line stably expressing human Wnt3a, and control L cell conditional medium (LCM) was collected from L cells (ATCC, Manassas, VA).

**Transfection, infection and Mab2F1 treatment in HRMEC.** Transfection of nanoparticles was performed as followed: HRMEC were starved with serum-free medium overnight, and then incubated with the nanoparticles (10 µg/well in 6-well plate) for 8 hr in the basal medium without serum and antibiotics. The transfected cells were incubated in endothelial cell medium at 37°C for 72 hr following the transfection. Transfection of siRNA was performed using an siRNA targeting VLDLR (Ambion, TX) and an siRNA with a scrambled sequence (Ambion, TX). HRMEC were incubated with transfection mixtures containing 100 pmol of the VLDLR or control siRNA for 24 hr. Then the cells were washed with phosphate-buffered saline (PBS) to remove the transfection mixture and cultured in endothelial cell medium at 37°C for further experiment.

The recombinant Ad-S37A virus was generated to express a constitutively active mutant of β-catenin, which activates Wnt target gene independent of Wnt ligands. HRMEC were infected with Ad-S37A or Ad-GFP (control virus) for 24 hr and then the medium was replaced with fresh culture medium and the cells were cultured for additional 48 hr.

Mab2F1 was raised using a recombinant peptide of the E1E2 domain from the human LRP6 extracellular region. Mab2F1 was added into the medium 4 hr before WCM with a final concentration of 50 µg/ml.

**TOPFLASH assay.** Luciferase reporter assay was performed in hTERT-RPE-1 cells following previously established methods. hTERT–RPE-1 cells were transfected with 0.25 µg TOPFLASH (TCF reporter plasmid; Firefly luciferase) and 0.05 µg pRL-TK (pRL reporter plasmid; Renilla luciferase) constructs using lipopectamine 2000. To evaluate the inhibitory effect of VLN-NP on the Wnt3a-mediated Wnt-signaling activation, hTERT-RPE cells were transfected with VLN-NP/LN-NP together with the TOPFLASH reporter plasmid. After incubation for 48 hr, 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.

**EC viability, migration and tube formation assays.** HRMEC transfected with different nanoparticles were treated with WCM or LCM, and the cell viability was determined using MTT assay (Roche, South San Francisco, CA) according to the manufacturer's instruction.

The cell migration assay was performed using a modified Boyden chamber apparatus (Corning, NY). Briefly, HRMEC (1 × 10⁵/well) were transfected with VLN-NP or LN-NP for 72 hr and then seeded in the upper chamber and incubated for 12 hr. Serum-free medium with WCM or LCM was added into the lower chamber. Cells that had migrated through the membrane were stained and quantified.
Tube formation assay was performed using Matrigel assay (BD Bioscience, San Jose, CA). HRMEC (5×10^4/well) were transfected with VLN-NP or LN-NP for 72 hr and subsequently seeded on Matrigel-coated plates in a serum-free medium containing WCM or LCM. The cells were incubated at 37°C for 6 hr. The degree of tube formation was viewed under a microscope and quantified by counting the branching points per field as described.

**Animals.** C57BL/6J mice, Vldlr^−/− mice and BAT-gal transgenic mice were purchased from Jackson Laboratories (Bar Harbor, ME). Sprague Dawley (SD) rats weighing between 180 and 200 g were purchased from Charles River (San Diego, CA). All animals were maintained in a 12-hr light/dark cycle. All processes including care, use and treatment of the animals were in strict agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and Guidelines for Animal Care and Use dictated by the University of Oklahoma Health Sciences Center.

**Vldlr^−/− mice, OIR model and intravitreal administration of nanoparticles.** VLN-NP and LN-NP were dissolved in saline and injected intravitreally (50 µg/eye) to Vldlr^−/− mice at postnatal day (P) 10. The mice were euthanized at P30, and the eyecups were dissected for analysis. The OIR model was induced in C57BL/6J mice and in BAT-gal Wnt reporter mice, which express the LacZ reporter gene under the control of a promoter containing TCF/β-catenin-binding sites, as described previously. Briefly, mice were exposed to 75% oxygen from P7 to P12 and then returned to room air. VLN-NP or LN-NP was injected intravitreally (50 µg/eye) at P12. OIR mice and normoxia control mice were sacrificed at P17 for analysis.

**Retinal angiography and quantification of pre-retinal vascular cells.** Mice were anesthetized and perfused with 50 mg/mL high-molecular weight FITC-dextran (2×10^6, Sigma-Aldrich, St. Louis, MO) as described by Smith et al. The retinas were dissected and flat-mounted, and the vasculature was then examined under a fluorescence microscope (CKX41 Olympus, Center Valley, PA) by an operator masked to treatment allocation. Paraffin-embedded eyes were stained with hematoxylin and eosin (H&E) to quantify the pre-retinal vascular cells following a documented protocol.

**Alkali burn-induced corneal NV.** Alkali burn-induced corneal NV was generated following a documented protocol. Briefly, a round filter paper (2 mm in diameter) was soaked in 1 N sodium hydroxide, and then placed on the center of the cornea of the right eye of anesthetized rats for 30 seconds followed by a rinse with 10 ml PBS. A single topical treatment of VLN-NP or LN-NP (20 µl/eye, 10 mg/ml) was performed 1 hr after alkali burn. Corneal NV was examined and imaged at day 7 after the treatment. The quantification of corneal NV was performed by NV clock hours and NV area. The cornea was divided into 12 portions, similar to the hours on a clock, and the NV clock hour was measured by corresponding the range of corneal NV to the number of hours designated. The NV area (A) was calculated using: A=C/12 × 3.1416 [r^2 - (r - L)^2]. (C is the clock hours of NV; r is the radius of rat cornea. r=3.5 mm, taken from the measurements of 20 rat corneas; L is the average length of corneal NV). The same
procedure of alkali burn was performed in Bat-gal mice, and Wnt signaling activity in the cornea was detected using X-gal staining at day 7.

**X-gal staining.** BAT-gal transgenic mice were euthanized, and the eyecups were dissected and fixed. Frozen tissue sections (30 µm) were cut and incubated in a β-galactosidase solution [5 mM K$_3$Fe(CN)$_6$, 5 mM K$_4$Fe(CN)$_6$ 3H$_2$O, 2 mM MgCl$_2$, 0.02% NP-40, 0.1% sodium deoxycholate, 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside] for 24 hr in a humidified chamber and subsequently mounted on slides.

**Western blot analysis and immunohistochemistry.** Western blot analysis was performed as described previously$^{11}$. Antibodies specific for p-LRP6 (Ser1490) and non-p-β-catenin (Ser33/37/Thr41) were purchased from Cell Signaling (Danvers, MA). Antibodies for LRP6 (C-10), VEGF (A-20) and Myc-tag (9E10) were purchased from Santa Cruz (Santa Cruz, CA). Anti-β-actin and anti-His tag antibodies were purchased from Abcam (Cambridge, MA). Monoclonal antibodies specific for VLN (anti-VLN) were generated via a contracted service by Proteintech Group (Chicago, IL) using the purified recombinant VLN peptide. Western blotting images were captured by a Chemi Genius Image Station (SynGene, Frederick, MD). Individual protein band was semi-quantified by densitometry using the Image J program.

Frozen retinal and corneal sections (8 µm) were immunostained with indicated antibodies, and the nuclei were counterstained with DAPI (Vector Laboratories; Burlingame, CA). Anti-CD31 antibody (R&D System, Minneapolis, MN) was used as the primary antibodies. Alexa Fluor® 488-AffiniPure Donkey Anti-Goat IgG (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA) was used as the secondary antibody. Fluorescein labeled Griffonia Simplicifolia Lectin I (Vector, Burlingame, CA) was used to detect BS-1 lectin.

**Quantitative Real-time Reverse Transcription (RT)-PCR.** Rat corneas were dissected and lysed in TRizol® RNA Isolation Reagents (Life Technologies, NY). Total RNA was isolated using a commercial kit (Qiagen, Santa Clarita, CA). Primers were designed from the cDNA sequences spanning > 1-kb introns using the Primer3 software. Total RNA (1.0 µg) was used for RT reactions, and 1 µl of the RT product and 3 pmol of primers were used for real-time PCR with a SYBR Green PCR Master Mix (Applied Biosystems). Fluorescence changes were monitored after each cycle. All reactions were performed in triplicate. The average CT (threshold cycle) of fluorescence unit was used to analyze the mRNA levels. The mRNA levels were normalized by 18S ribosomal RNA levels. Specific primers used were as follows: human VEGF: Forward: 5’-CTGTCTAATGCCCCTGGACC-3’; Reverse: 5’-ACGCGAGTCTGTGTTTTTGC-3’; rat VEGF: Forward: 5’-CAATGATGAAGCTGGAGT-3’; Reverse: 5’-AATGCTTTCTCCGCTCTGAA-3’; human LRP6: Forward: 5’-GAGTTGGATCAACCCAGACGG-3’; Reverse: 5’-CGACTTTGAACCATCCATTCC-3’; rat LRP6: Forward: 5’-CAGCATTGAACGTGCAACA-3’; Reverse: 5’-GGGCATCCACACACAAAACC-3’; human TCF3: Forward: 5’-GAGCCACACCAACCTGACA-3’; Reverse: 5’-GTGACACGGTGCTGAGATT
Statistical analysis. Experiments were performed at least three times, and representative data were shown. All of values were expressed as mean ± SD. Differences among three or more groups were analyzed for statistical significance using one-way ANOVA, and Tukey’s multiple comparisons test was used to analyze the differences between two groups. Statistical significance was set at $P < 0.05$.

References

Suppl. Table I. Characterization of blank nanoparticles and nanoparticles loaded with plasmids.

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>Particle Size (nm)</th>
<th>Polydispersity index</th>
<th>Zeta Potential (mV)</th>
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<tbody>
<tr>
<td>Blank nanoparticles</td>
<td>278.2</td>
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<td>-18.2</td>
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Suppl. Fig. I. ERG response and retinal histology in the eyes injected with VLN-NP and LN-NP. Adult mice received an intravitreal injection of VLN-NP or LN-NP. A-B: ERG was recorded from six mice per group at 4 weeks after the injection of VLN-NP and LN-NP. Amplitudes of A- and B-waves from scotopic (A) and photopic (B) ERG were averaged and compared (mean±SD, n=6). C: Retinal sections were stained with H&E 4 weeks after the injection of VLN-NP or LN-NP (×400). GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer; RPE: retinal pigment epithelium.
Suppl. Fig. II. LRP6 mRNA expression under experimental conditions. A: HRMEC were transfected with VLN-NP or LN-NP for 48 hr and treated with LCM or WCM for 4 hr. LRP6 mRNA levels were measured by q-PCR and normalized by 18S. NS: not significant, n=3. B: Rat corneas were lysed in TRIzol and LRP6 mRNA levels in different indicated groups were measured by q-PCR and normalized by 18S. NS: not significant, n=6.
Suppl. Fig. III. TCF expression in HRMEC under experimental conditions. HRMEC were transfected with VLN-NP or LN-NP for 48 hr and treated with LCM or WCM for 24 hr. TCF3 mRNA levels were measured by q-PCR and normalized by 18S. NS: not significant, n=3.