Very Low Density Lipoprotein Receptor, a Negative Regulator of the wnt Signaling Pathway and Choroidal Neovascularization*

Received for publication, December 8, 2006, and in revised form, September 17, 2007 Published, JBC Papers in Press, September 23, 2007, DOI 10.1074/jbc.M611289200

Ying Chen, Yang Hu, Kangmo Lu, John G. Flannery, and Jian-xing Ma

From the Department of Cell Biology, Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104 and Vision Science and the Neuroscience Division, Department of Molecular and Cell Biology, The Helen Wills Neuroscience Institute, University of California, Berkeley, California 94720-3192

Choroidal neovascularization (CNV) in age-related macular degeneration is a leading cause of blindness. Very low density lipoprotein receptor gene knock-out (Vldlr<sup>-/-</sup>) mice have been shown to develop subretinal neovascularization (NV) with an unknown mechanism. The present study showed that in Vldlr<sup>-/-</sup> mice, NV initiated in the choroid and progressed to penetrate the retinal pigment epithelium layer, proliferating in the subretinal space. This phenotype recapitulated what is seen in wet age-related macular degeneration, suggesting that this is a CNV model. The CNV correlated with overexpression of vascular endothelial growth factor in Vldlr<sup>-/-</sup> eyecups and was blocked by a neutralizing antibody against vascular endothelial growth factor receptor-2. The wnt co-receptor LR5/6 expression was significantly up-regulated in Vldlr<sup>-/-</sup> eyecups compared with that in wild-type mice. Significantly, Vldlr<sup>-/-</sup> mice showed impaired phosphorylation of downstream effectors of the wnt signaling pathway, glycogen synthase kinase-3β (GSK-3β), and β-catenin, concomitant with increased levels of free GSK-3β and β-catenin, suggesting an increased activity of the wnt pathway. Down-regulation of VLDLR by small interference RNA resulted in up-regulation of LR5/6 expression and activation of β-catenin in cultured endothelial cells. Furthermore, Dickkopf-1, a specific inhibitor of the wnt pathway, effectively decreased vascular endothelial growth factor and β-catenin levels in the retinal pigment epithelium of Vldlr<sup>-/-</sup> mice and in cells transfected with the VLDLR small interference RNA. These results suggest that VLDLR functions as a negative regulator of CNV, and this function is mediated through the wnt pathway.

Blindness caused by the retinal and choroidal neovascularization (NV)<sup>2</sup> in diabetic retinopathy and in age-related macular degeneration (AMD) remains one of the most urgent medical problems of our times (1, 2). The significance of these blinding disorders is growing as the population ages, and the incidence of diabetes increases. The treatments for AMD are limited by the fact that relatively little is currently understood about the biochemical pathways or pathogenic mechanisms underpinning AMD and consequent choroidal neovascularization (CNV) (3–5).

The retina is supported by two separate vascular networks, the retinal vessels and the choroidal vessels (6, 7). There are two major steps in vascular development, vessel formation and vessel maturation (8, 9). Vessel formation mainly involves endothelial proliferation and migration, forming pericyte-free retinal vasculature (9). Vascular endothelial growth factor (VEGF) is known to play a key growth-promoting role in vessel formation (10, 11).

Wnts are a group of secreted, cysteine-rich glycoproteins that bind to frizzled (Fz) receptors or to the Fz/LDL receptor-related protein 5 or 6 (LR5/6) co-receptors and regulates expression of a number of target genes (12, 13). In the absence of wnts, transcription factor β-catenin is phosphorylated by a protein complex containing glycogen synthase kinase-3β (GSK-3β). The phosphorylated β-catenin is continuously degraded. Upon binding of certain wnts to the Fz-LR5/6 co-receptors, phosphorylation of β-catenin is inhibited, which decreases the degradation of β-catenin and results in its accumulation (14, 15). β-Catenin is then translocated into the nucleus and regulates expression of target genes including VEGF (16–18).

Recent evidence has demonstrated that the wnt signaling pathway plays a role in the regulation of angiogenesis (19, 20). Mutations in the Fz4 or LR5/6 gene in the human have been found to inhibit the normal retinal angiogenesis in the familial exudative vitreoretinopathy patients (21–24), whereas Fz4 knock-out (fz4<sup>-/-</sup>) mice exhibited an incomplete retinal vascularization (25). Moreover, Norrin, a downstream binding ligand of the Fz4 receptor, is associated with the vascular development in the eye, as mutations in or complete knock-out of this gene, as found in Norrie disease, results in congenital absence of intraretinal capillaries and a progressive loss of vessels within the stria vascularis is in the cochlea (25–27). Meanwhile, the key protein in the wnt signaling pathway, β-catenin, has also been implicated in vascular development and remodeling (28). It has...
been shown that the membrane pool of β-catenin is required for mitogenic signaling through the VEGF receptor-2 (VEGFR2)-mediated activation of phosphatidylinositol 3-kinase and Akt (29).

Very low density lipoprotein receptor gene knock-out (Vldlr⁻/⁻) mice were initially created to study the cholesterol pathway. However, Vldlr⁻/⁻ mice have been shown to lack dyslipidemia (30, 31) and as such are of little use in cholesterol research. In a comprehensive ocular phenotype screen, Heckenleivy et al. (32) discovered through fundus examination that Vldlr⁻/⁻ mice develop abnormal subretinal NV. To date, the underlying mechanism by which disruption of the Vldlr gene (knock-out) leads to the subretinal NV has not been elucidated. In the present study we have examined the role of the wnt signaling pathway in the subretinal NV in Vldlr⁻/⁻ mice.

**EXPERIMENTAL PROCEDURES**

**Animals**—Animals were maintained in a 12-h light/12-h dark cycle with an ambient light intensity of 85 ± 18 lux at the cage level. Vldlr⁻/⁻ mice on the C57BL/6 background and wild-type (wt) C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine) were used, treated and cared for in accordance with the statement for the Use of Animals in Ophthalmic and Vision Research set forth by the Association for Research in Vision and Ophthalmology. Vldlr⁻/⁻ mice were genotyped following a PCR protocol recommended by The Jackson Laboratory.

**Cell Culture**—Human umbilical vein endothelial cells (HUVEC) were purchased from the American Type Culture Collection (Manassas, VA). Cell culture reagents, fetal bovine serum, and chemicals were purchased from Invitrogen. ARPE19 cells were maintained in Dulbecco’s modified Eagle’s medium containing 3 mM L-glutamine, 10% fetal bovine serum, and chemicals were purchased from Invitrogen. ARPE19 cells were maintained in Dulbecco’s modified Eagle’s medium containing 3 mM L-glutamine, 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate at 37 °C in an environment containing 95% O₂ and 5% CO₂. HUVEC were cultured in endothelial cell basal medium (EBM-2, Cambrex, NJ) maintained at 37 °C in an environment containing 95% O₂ and 5% CO₂ and supplemented with 5% fetal bovine serum, penicillin/streptomycin, and endothelial cell growth supplement (SingleQuots, Cambrex, NJ). The cells were used in experiments between passage 4 and 6.

**Fluorescein Angiography**—Angiograms were performed using intracardiac injection of 10 mg/ml fluorescein isothiocyanate-conjugated high molecular weight dextran (Sigma, FD-2000S) in deeply anesthetized mice. Eyes were dissected and fixed with 4% paraformaldehyde in Hanks’ balanced saline prepared immediately before use for overnight at 4 °C, and retinas were flat-mounted in Fluoromount-G.

**Immunohistochemistry**—Eyes were enucleated and fixed in 4% paraformaldehyde overnight at 4 °C. Cross-sections (5 μm) were cut and mounted on slides. To reduce autofluorescence background levels, the sections were blocked with 2% mouse serum and 10% normal goat serum in phosphate-buffered saline with 0.3% Triton X-100 for 1 h. Sections were stained with primary antibodies specific for VEGF (Santa Cruz, CA), β-catenin, GSK-3β, phosphorylated GSK-3β, and phosphorylated β-catenin (Cell Signaling, Danvers, MA), LRP5/6 (ABCAM, Cambridge, MA), CD31 (BD Pharmingen), and a
Increased VEGF and VEGFR2 levels in Vldlr−/− eyes. A, the same amount of eyecup proteins from each mouse was separately blotted with antibodies for VEGF and for VEGFR2. The membranes were stripped and rebotted with an anti-β-actin antibody. B, VEGF monomer (23 kDa) and dimer (46 kDa) were semiquantified by densitometry, normalized by β-actin levels, and expressed as the relative ratio of wt to Vldlr−/− (mean ± S.D., n = 3). C, the retina was dissected from the eyecup containing the RPE and choroid. Total RNA was separately isolated from these tissues. Real-time RT-PCR showed elevated VEGF mRNA levels in the Vldlr−/− retinas and choroids (expressed as the wt to Vldlr−/− ratio, mean ± S.D., n = 3). D, VEGFR2 levels were measured by Western blot analysis. E and F, purified neutralizing antibodies (rat IgG) for VEGFR2 and VEGFR3 were separately injected into the subretinal space of Vldlr−/− mice at age of P12. The injected antibodies were detected by staining with a fluorescein isothiocyanate-conjugated goat anti-rat antibody at P21 (green). The retinal vasculature was examined using a monoclonal anti-CD31 antibody (red). Subretinal NV was attenuated by the anti-VEGFR2 antibody but not by the anti-VEGFR3 antibody. Scale bar, 10 μm. ONL, outer nuclear layer; INL, inner nuclear layer.

Increased VEGF and VEGFR2 levels in Vldlr−/− eyes. A, the same amount of eyecup proteins from each mouse was separately blotted with antibodies for VEGF and for VEGFR2. The membranes were stripped and rebotted with an anti-β-actin antibody. B, VEGF monomer (23 kDa) and dimer (46 kDa) were semiquantified by densitometry, normalized by β-actin levels, and expressed as the relative ratio of wt to Vldlr−/− (mean ± S.D., n = 3). C, the retina was dissected from the eyecup containing the RPE and choroid. Total RNA was separately isolated from these tissues. Real-time RT-PCR showed elevated VEGF mRNA levels in the Vldlr−/− retinas and choroids (expressed as the wt to Vldlr−/− ratio, mean ± S.D., n = 3). D, VEGFR2 levels were measured by Western blot analysis. E and F, purified neutralizing antibodies (rat IgG) for VEGFR2 and VEGFR3 were separately injected into the subretinal space of Vldlr−/− mice at age of P12. The injected antibodies were detected by staining with a fluorescein isothiocyanate-conjugated goat anti-rat antibody at P21 (green). The retinal vasculature was examined using a monoclonal anti-CD31 antibody (red). Subretinal NV was attenuated by the anti-VEGFR2 antibody but not by the anti-VEGFR3 antibody. Scale bar, 10 μm. ONL, outer nuclear layer; INL, inner nuclear layer.

FIGURE 2. Increased VEGF and VEGFR2 levels in Vldlr−/− eyes. A, the same amount of eyecup proteins from each mouse was separately blotted with antibodies for VEGF and for VEGFR2. The membranes were stripped and rebotted with an anti-β-actin antibody. B, VEGF monomer (23 kDa) and dimer (46 kDa) were semiquantified by densitometry, normalized by β-actin levels, and expressed as the relative ratio of wt to Vldlr−/− (mean ± S.D., n = 3). C, the retina was dissected from the eyecup containing the RPE and choroid. Total RNA was separately isolated from these tissues. Real-time RT-PCR showed elevated VEGF mRNA levels in the Vldlr−/− retinas and choroids (expressed as the wt to Vldlr−/− ratio, mean ± S.D., n = 3). D, VEGFR2 levels were measured by Western blot analysis. E and F, purified neutralizing antibodies (rat IgG) for VEGFR2 and VEGFR3 were separately injected into the subretinal space of Vldlr−/− mice at age of P12. The injected antibodies were detected by staining with a fluorescein isothiocyanate-conjugated goat anti-rat antibody at P21 (green). The retinal vasculature was examined using a monoclonal anti-CD31 antibody (red). Subretinal NV was attenuated by the anti-VEGFR2 antibody but not by the anti-VEGFR3 antibody. Scale bar, 10 μm. ONL, outer nuclear layer; INL, inner nuclear layer.

Quantitative Real-time Reverse Transcription (RT)-PCR—Mice eyes were enucleated immediately after death into chilled diethylpyrocarbonate-treated normal saline, and the retinas were dissected. Total RNA was isolated using a commercial kit (Qiagen, Santa Clarita, CA). Primers (VEGF forward and VEGF reverse) 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. Amplicon size and reaction specificity were confirmed by 2.5% agarose gel electrophoresis. All reactions were performed in triplicate. The average Ct (threshold cycle) of fluorescence unit was used to analyze the mRNA levels. The VEGF mRNA levels were normalized by 18 S ribosomal RNA levels. Quantification was calculated as follows: mRNA levels (percent of control) = 2Δ(ΔCt), with
Transfection of Small Interference RNA (siRNA)—The Cy3-labeled siRNA targeting VLDLR was commercially purchased from Ambion (Austin, TX). Transfection was performed using siPORT Amine (Ambion) following the instructions of the manufacturer. Briefly, 5 × 10⁶ HUVEC were incubated with the transfection mixtures containing 100 pmol of the Cy3-labeled siRNA for VLDLR or a Cy3-labeled control siRNA with a scrambled sequence for 24 h at 37°C in 5% CO₂. Twelve hours after the transfection, the cells were washed twice with phosphate-buffered saline to remove transfection mixtures and cultured in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum until they were used.

Subretinal Injection of Purified Mouse Dickkopf-1 (DKK1) Protein—Purified DKK1 (R&D Systems, MN) was injected into the subretinal space of the right eye (5 μg/eye), and the same amount of bovine serum albumin (BSA) was injected into the left eye of Vldlr⁻/⁻ mice at age of 4 weeks. Eyeballs were harvested 24 h after the injection, and the eyecups were dissected for analysis.

Statistical Analysis—Data were analyzed by Student’s t tests. Statistical significance was set at p < 0.05.

RESULTS

CNV in Vldlr⁻/⁻ Mice—Vascularization in the retina and choroid in Vldlr⁻/⁻ and wt mice at ages of postnatal day 12 (P12) and 6 weeks were analyzed by both in vivo vessel staining and fluorescein-angiography with fluorescein isothiocyanate-conjugated high molecular weight dextran (25, 34). In the retina of Vldlr⁻/⁻ mice at P12, retinal glial cells were observed to spread into the outer retina, as visualized by GFAP (a Müller cell marker) staining, whereas in wt mice, the GFAP signal was only detected in the inner retina (Fig. 1). Immunostaining of CD31 (an endothelial marker) demonstrated no detectable retinal neovascularization in Vldlr⁻/⁻ mice at age of P12, but GFAP staining clearly demarked retinal neovascularization spread to the outer retina in Vldlr⁻/⁻ mice (Fig. 1, A and B). Concomitantly at this age, choroidal neovascularization can be
The wnt Signal Pathway in CNV

![Image of a graph showing the wnt Signal Pathway in CNV]

At 6 weeks of age, intact RPE layer in wt mice (Fig. 1) demonstrates significantly elevated levels of the VEGF monomer and the co-receptor LRP5/6 in Vldlr-/- mice with that in wt mice (Fig. 4, A and B). In contrast, Fz4 levels were not changed in the eyecups of Vldlr-/- mice (Fig. 4).

At age of P12. Immunostaining of CD31 at the age of 6 weeks demonstrated that an injection of the anti-VEGFR2 antibody abrogated the subretinal NV, whereas the same dose of the anti-VEGFR3 antibody had no effect on subretinal NV, suggesting the role of VEGFR2 in mediating the angiogenic effect of VEGF in this mouse model (Fig. 2, E and F).

Altered VEGF Subcellular Distribution in RPE Cells in Vldlr-/- Mice—Immunohistochemistry detected intense VEGF signal in the retinal regions displaying NV (Fig. 3). High magnification micrographs showed that VEGF signal in the wt RPE was distributed near the surface adjacent to the Bruch's membrane but not near the surface adjacent to photoreceptors, consistent with previous observations of the polarized distribution of VEGF in the RPE (35). In contrast, the VEGF signal in the RPE was detected near the surface adjacent to photoreceptors in some RPE cells in Vldlr-/- mice (Fig. 3).

Vldlr Gene Knock-out Up-regulates LRP5/6 Expression in the Eyecups—Because the wnt signaling pathway is known to play a role in regulation of VEGF expression and in angiogenesis (36–38), we compared the expression levels of the wt receptor Fz4 and the co-receptor LRP5/6 in Vldlr-/- mice with that in wt mice. Western blot and immunohistochemistry analyses showed that LRP5/6 levels were significantly increased in the eyecup of Vldlr-/- mice when compared with the wt eyecup (Fig. 4, A and B). In contrast, Fz4 levels were not changed in the eyecups of Vldlr-/- mice (Fig. 4).

seen to have penetrated through Bruch's membrane and the RPE from the choroid (Fig. 1D), in contrast to the preserved intact RPE layer in wt mice (Fig. 1C), suggesting that the NV is originated from the choroid in this model. At 6 weeks of age, Vldlr-/- mice showed abnormal NV throughout the subretinal space and the photoreceptor layer (Fig. 1, E–H), consistent with the previous observations by Heckenlively et al. (32). By 6 weeks of age, the choroidal vascular network had anastomosed with the retinal vasculature (Fig. 1, F and H). In older Vldlr-/- mice (7 months of age), vasculature accumulated in the subretinal space, similar to the CNV in wet AMD (Fig. 1, I and J).

Overexpression of VEGF and VEGFR2 in Vldlr-/- Eyecups—Because VEGF is widely considered the major angiogenic factor in the retina, we compared VEGF expression in the eyecups of Vldlr-/- mice with those in the age-matched wt mice at both the protein and mRNA levels. Western blot analysis demonstrated significantly elevated levels of the VEGF monomer and dimer in Vldlr-/- eyecups in comparison to the wt eyecups (Fig. 2, A and B). Real-time RT-PCR showed that the increased VEGF expression occurs at the mRNA level in Vldlr-/- mice (Fig. 2C). Moreover, VEGFR2 levels were also elevated in the Vldlr-/- eyecups (Fig. 2D).

To provide additional evidence for the causative role of VEGF overexpression in the CNV in Vldlr-/- mice, we injected neutralizing antibodies (10 μg/eye) specific for VEGFR2 and VEGFR3 separately into the subretinal space of Vldlr-/- mice at age of P12. Immunostaining of CD31 at the age of 6 weeks demonstrated that an injection of the anti-VEGFR2 antibody abrogated the subretinal NV, whereas the same dose of the anti-VEGFR3 antibody had no effect on subretinal NV, suggesting the role of VEGFR2 in mediating the angiogenic effect of VEGF in this mouse model (Fig. 2, E and F).
the RPE cells and HUVEC, with the expected molecular weight as that in HUVEC but not in the macrophages (Fig. 4H).

To reveal if VLDLR regulates VEGF expression at the protein or mRNA level, we have transfected HUVEC with the VLDLR siRNA using an siRNA with scrambled sequence as the negative control. The culture medium was collected and concentrated 48 h after the transfection for VEGF ELISA. VEGF secreted from both the HUVEC transfected with the VLDLR siRNA were significantly higher than in the cells transfected with the control siRNA (Fig. 4I). The VEGF mRNA levels were also significantly elevated by the VLDLR siRNA (Fig. 4J).

**Activation of Downstream Effectors of the wnt Signaling Pathway in Vldlr<sup>−/−</sup> Eyecups**—We determined the activation status of GSK-3β and β-catenin, two downstream effectors of the wnt signaling pathway, by measuring their phosphorylation status in Vldlr<sup>−/−</sup> mouse eyecups. The levels of phosphorylated and free GSK-3β in Vldlr<sup>−/−</sup> mouse eyecups were compared with that in wt by Western blot analysis using specific antibodies. Vldlr<sup>−/−</sup> mouse eyecups showed dramatically decreased levels of phosphorylated GSK-3β but elevated free GSK-3β in the eyecups when compared with that in wt mice (Fig. 5).

Similar to what we observed with GSK-3β, phosphorylation of β-catenin, a downstream target of GSK-3β, was nearly completely abolished in Vldlr<sup>−/−</sup> mouse eyecups as shown by Western blot analysis using an antibody specific for phosphorylated β-catenin (Fig. 6). The free form of β-catenin was increased in Vldlr<sup>−/−</sup> mice when compared with that in wt mice, demonstrating an accumulation of β-catenin in Vldlr<sup>−/−</sup> eyecups (Fig. 6).

**VLDLR Regulates the wnt Signaling in Endothelial Cells**—To further confirm that VLDLR directly regulates the wnt signaling, we first determined if VLDLR and LRP5/6 are located in the same cells. Because VLDLR is well known to express at high levels in endothelial cells (39), we determined the expression of LRP5/6 in HUVEC. Western blot analysis showed that LRP5/6 is expressed at high levels in HUVEC with the same molecular weight as that in the mouse eyecups, but not in rMC-1, a rat Müller cell line (Fig. 7A).

To establish the regulatory function of VLDLR in the activation of the wnt signaling pathway, we used a specific siRNA to

---

**FIGURE 7.** Up-regulation of LRP5/6 expression by the VLDLR siRNA in endothelial cells. A, high levels of LRP5/6 in endothelial cells. Total proteins from wt and Vldlr<sup>−/−</sup> eyecups, cultured HUVEC, and rMC-1 cells were immunoblotted with an antibody specific for LRP5/6. B, down-regulation of VLDLR by a specific siRNA. HUVEC were transfected with the Cy3-labeled VLDLR siRNA or control siRNA. Twenty-four hours after the transfection, 15 µg of total cellular proteins were separately blotted with an anti-VLDLR antibody and anti-LRP5/6 antibody. C, HUVEC were transfected with the Cy3-labeled VLDLR siRNA (C2 and C4) and control siRNA (C1 and C3) to show high transfection efficiency. C1 and C2 are phase contrast images of C3 and C4, respectively. D, the transfected cells were separately stained with the anti-VLDLR antibody (green) (D1 and D2) and with an anti-LRP5/6 antibody (green) (D3 and D4). Red, Cy3 signaling from the siRNAs. Scale bar, 10 µm. Note that LRP5/6 signal was increased by VLDLR siRNA (D4). E, the mRNA levels of LRP5/6 were quantified by real-time RT-PCR and compared using Student’s t-test. Values are the relative LRPE5 and LRPE6 mRNA levels as fold over that in the cells treated with the control siRNA (mean ± S.D., n = 3). *p < 0.05; **p < 0.01.

Additionally, to explore whether the loss of VLDLR regulates the expression of LRP5/6 gene expression, we compared LRP5/6 mRNA levels in Vldlr<sup>−/−</sup> eyecups with that in wt mice using quantitative real-time RT-PCR. The mRNA levels of LRP5 and LRP6 in the Vldlr<sup>−/−</sup> eyecups increased by 2-fold over that in wt eyecups, indicating that VLDLR gene knock-out up-regulates LRP5 and LRP6 gene expression (Fig. 4C).

Vldlr Gene Knockdown Up-regulates VEGF Expression in Endothelial Cells and RPE Cells—To identify the cell types in which the regulation of LRP5/6 by VLDLR occurs, we also performed double immunostaining of LRP5/6 and an RPE marker (RDH10) in the Vldlr<sup>−/−</sup> mouse eyecup. The results showed a co-localization of RDH10 and LRP5/6 in the RPE (Fig. 4E, 1–3).

We also examined the expression of LRP5/6 in primary RPE cells from bovine eyes, HUVEC, and in human mono/macrophages. The equal amount of proteins from these lysates was blotted with an anti-LRP5/6 antibody. LRP5/6 was detected in
The wnt Signal Pathway in CNV

knock down the expression of VLDLR in HUVEC and measured the expression levels of LRP5/6 and subcellular localization of β-catenin, which is a downstream effector of the wnt signaling pathway and directly up-regulates VEGF expression in endothelial cells (18, 28). As shown by Western blot analysis and immunocytochemistry, the siRNA specific for VLDLR decreased LRP5/6 levels in HUVEC. In contrast, the siRNA significantly up-regulated LRP5/6 expression in the same cells compared with the cells treated with the same amount of the control siRNA with a scrambled sequence (Fig. 7, B–D). Real-time RT-PCR also showed that the VLDLR siRNA up-regulated LRP5 and LRP6 mRNA levels (Fig. 7E).

Immunocytochemistry using the antibody specific for phospho- rylated β-catenin showed that the treatment of HUVEC with the VLDLR siRNA reduced phosphorylation of β-catenin (Fig. 8, A and B). Immunostaining with an antibody specific for free β-catenin demonstrated increased levels of free β-catenin in cells treated with the VLDLR siRNA compared with that treated with the control siRNA. Moreover, the VLDLR siRNA treatment induced an increased nuclear translocation of β-catenin, a key step in its activation (Fig. 8, C and D). These results indicate that down-regulation of VLDLR alone activates the wnt signaling pathway in retinal endothelial cells.

Inhibition of VEGF Expression by a Specific Inhibitor of the wnt Signaling Pathway in Vldlr−/− Mice—To confirm the association of the wnt pathway activation with VEGF overexpression in Vldlr−/− mice, 5 μg of purified mouse DKK1, a specific inhibitor of the wnt signal pathway by binding with LRP5/6, were injected into the subretinal space of the Vldlr−/− mice into one eye, and the same amount of a control protein (BSA) was injected into the contralateral eye. Twenty-four hours after the injection, β-catenin phosphorylation and VEGF levels in the eyecup were measured by Western blot analysis. The results showed that DKK1 increased phosphorylation of β-catenin, suggesting an inhibited wnt signaling (Fig. 9A). As shown by Western blot analysis and real-time RT-PCR, DKK1 also significantly decreased the VEGF protein and mRNA levels in Vldlr−/− eyecups, correlating with the inhibited wnt signaling (Fig. 9, B and C). Immunohistochemistry using antibodies specific for VEGF and β-catenin showed that a subretinal injection of DKK1 significantly decreased levels of VEGF and β-catenin in the RPE of Vldlr−/− mice compared with the contralateral eyes injected with the same amount of BSA (Fig. 9, D–G).

We have also investigated the regulatory roles of VLDLR and the wnt pathway in VEGF expression in cultured endothelial cells. In HUVEC DKK1 also blocked the VEGF overexpression induced by the VLDLR siRNA (Fig. 9, H and I). Consistent with documented evidence, these new data suggest that the activated wnt signaling pathway is responsible, at least in part, for the VEGF overexpression in Vldlr−/− mice.

DISCUSSION

Angiogenesis in ocular tissues is a delicately coordinated process (6, 9). Abnormal angiogenesis (neovascularization) in the retina or subretinal space is a common cause of vision loss in a number of ocular disorders, but its pathogenesis is not fully understood. The present study demonstrates that Vldlr knock-out results in up-regulation of LRP5/6 expression in the retina and RPE and abnormal activation of the wnt signaling pathway, which mediates the overexpression of VEGF and CNV in Vldlr−/− mice. Furthermore, down-regulation of VLDLR alone by siRNA results in activation of the wnt signaling and VEGF overexpression, which can be blocked by a specific inhibitor of the wnt pathway. These observations for the first time established the function of VLDLR as a negative regulator of the wnt signaling pathway and revealed a role of VLDLR in the regulation of angiogenesis.

It was reported previously that Vldlr−/− mice develop subretinal NV (32). The mechanism for the subretinal NV has not been investigated. The present study has demonstrated that the subretinal vasculature becomes a connected vascular network fusing the inner retina and choroidal vessels. As early as postnatal day 12, choroidal vessels begin to penetrate Bruch’s membrane and the RPE layer before retinal NV, suggesting that NV originates from the choroid in this mouse model. Moreover, NV progresses continuously in the subretinal space. These observations suggest that the Vldlr−/− mouse is a CNV model.

VEGF is known as a major angiogenic factor, promoting normal and abnormal angiogenesis (11). RPE cells are known to express and secrete high levels of VEGF (40, 41). Moreover, it is known that secretion of VEGF from the RPE cells is polarized, i.e. RPE cells secrete VEGF toward the choroid, which is proposed to be essential for maintaining the fenestration status of choroidal vessels (35, 42, 43). In the Vldlr−/− RPE, the polarized secretion of VEGF is also disturbed as immunohistochemistry showed that some of VEGF signals were distributed near the surface adjacent to photoreceptors. The possible secretion of VEGF toward the photoreceptor layer in the Vldlr−/− mice may contribute to subretinal NV. This is supported by the
accumulation of β-catenin. Our results showed that silencing of VLDLR expression by siRNA results in increased LRP5/6 levels and β-catenin phosphorylation in cultured cells. These findings indicate that VLDLR gene knock-out is a causative factor of the wnt signaling activation.

VLDLR is a member of the LDL receptor gene family (44, 45). Unlike the LDL receptor, however, VLDLR has a widespread expression in many tissues (46). VLDLR is well known for its role in lipoprotein uptake and metabolism (44, 47). The present study is the first to reveal its potential role in angiogenesis regulation. The results that knockdown of VLDLR by siRNA resulted in VEGF overexpression provides direct evidence supporting the causative role of VLDLR deficiency in CNV.

To determine whether the activation of the wnt signaling pathway mediates the CNV in Vldlr−/− mice, we have used a DKK1, a wnt antagonist acting through specifically binding to the Frizzled co-receptors LRP5/6 (48, 49). The results indicated that DKK1 effectively blocked VEGF overexpression in the Vldlr−/− eyecups and attenuated VEGF overexpression induced by the VLDLR siRNA in cultured cells. These experiments indicate that VEGF overexpression induced by VLDLR deficiency is mediated through the wnt pathway.

Both of VLDLR and LRP5/6 belong to the LDL receptor gene family (50, 51), but the interactions between VLDLR and LRP5/6 have not been reported previously. Several ligands for VLDLR have been identified previously, including plasminogen inhibitor type 1, thrombospondin-1, and tissue factor pathway inhibitor (52). Furthermore, VLDLR has been shown to mediate LDL-induced plasminogen inhibitor type-1 gene transcription via regulating a transcription factor, namely LDL-inducible factor-1 (53). Our results that LRP5 and LRP6 mRNA levels are increased in Vldlr−/− eyecups suggest that VLDLR regulates LRP5/6 gene expression, possibly at the transcriptional level. These findings suggest that VLDLR may be also coupled with some intracellular signaling pathways, through which VLDLR reg-

\[\text{FIGURE 9. Inhibition of the wnt signaling and VEGF expression by DKK1.} \]

results showing that blocking VEGFR2 by subretinal injection of the neutralizing antibody inhibits subretinal NV.

Recent studies have established the role of the wnt signal in the regulation of angiogenesis (16,18 –20,28). However, the role of the wnt signaling in CNV has not been investigated. Our results showed that Vldlr−/− mice have increased levels of LRP5/6, a co-receptor of wnts, and abolished phosphorylation of GSK-3β. Furthermore, phosphorylation of β-catenin, a downstream effector of the wnt signaling pathway, is also abolished in Vldlr−/− eyecups, which resulted in stabilization and accumulation of β-catenin. Our results showed that silencing of VLDLR expression by siRNA results in increased LRP5/6 levels and β-catenin phosphorylation in cultured cells. These findings indicate that VLDLR gene knock-out is a causative factor of the wnt signaling activation.

VLDLR is a member of the LDL receptor gene family (44, 45). Unlike the LDL receptor, however, VLDLR has a widespread expression in many tissues (46). VLDLR is well known for its role in lipoprotein uptake and metabolism (44, 47). The present study is the first to reveal its potential role in angiogenesis regulation. The results that knockdown of VLDLR by siRNA resulted in VEGF overexpression provides direct evidence supporting the causative role of VLDLR deficiency in CNV.

To determine whether the activation of the wnt signaling pathway mediates the CNV in Vldlr−/− mice, we have used a DKK1, a wnt antagonist acting through specifically binding to the Frizzled co-receptors LRP5/6 (48, 49). The results indicated that DKK1 effectively blocked VEGF overexpression in the Vldlr−/− eyecups and attenuated VEGF overexpression induced by the VLDLR siRNA in cultured cells. These experiments indicate that VEGF overexpression induced by VLDLR deficiency is mediated through the wnt pathway.

Both of VLDLR and LRP5/6 belong to the LDL receptor gene family (50, 51), but the interactions between VLDLR and LRP5/6 have not been reported previously. Several ligands for VLDLR have been identified previously, including plasminogen inhibitor type 1, thrombospondin-1, and tissue factor pathway inhibitor (52). Furthermore, VLDLR has been shown to mediate LDL-induced plasminogen inhibitor type-1 gene transcription via regulating a transcription factor, namely LDL-inducible factor-1 (53). Our results that LRP5 and LRP6 mRNA levels are increased in Vldlr−/− eyecups suggest that VLDLR regulates LRP5/6 gene expression, possibly at the transcriptional level. These findings suggest that VLDLR may be also coupled with some intracellular signaling pathways, through which VLDLR reg-
ulates expression of target genes such as LRP5/6 in addition to its function in lipoprotein metabolism.

A recent genetic study reported a possible association of variations in the VLDLR, LRP6, and VEGF genes with AMD in human patients (54), suggesting a possible functional linkage between VLDLR and LRP5/6 in the retina and RPE. Furthermore, the pattern of CNV in the Vldlr−/− mouse recapitulates what is seen in wet AMD. Based on this notion, the Vldlr−/− mouse may be considered a model of CNV associated with AMD.

Acknowledgment—We thank Dr. Zengping Zhu at ImClone System, Inc. for kindly providing the neutralizing antibodies for VEGFR2 and VEGFR3.

REFERENCES

1. Anonyms (2004) *Morb. Mortal Wkly. Rep.* 53, 1069–1071
2. Congdon, N., O'Colmain, B., Klaver, C. C., Klein, R., Munoz, B., Friedman, D. S., Kempen, J., Taylor, H. R., and Mitchell, P. (2004) *Arch. Ophthalmol.* 122, 477–485
3. Edwards, A. O., Ritter, R., III, Abel, K. J., Manning, A., Panhuysen, C., and Channing, C. S., Kim, H., Lee, M. M., Park, Y. B., and Kim, H. S. (2006) *Arterioscler. Thromb. Vasc. Biol.* 26, 91–98
4. Carmeliet, P., Vultaggio, F., Moons, L., Lasserre, B., Lampugnani, M. G., Moens, L., Sera, T. (2004) *Arterioscler. Thromb. Vasc. Biol.* 24, 2328–2333
5. Kondo, H., Hayashi, H., Oshima, K., Tahira, T., and Hayashi, K. (2003) *Investig. Ophthalmol. Vis. Sci.* 31, 2450–2457
6. Even, J. L., Zhang, D. S., Brown, M. C., Burgess, B., Halpin, C., Berger, W., Morton, C. C., Core, D. P., and Chen, Z. Y. (2002) *J. Neurosci.* 22, 4286–4292
7. Easwaran, V., Lee, S. H., Inge, L., Guo, L., Goldbeck, C., Garrett, E., Wiesbeck, G., Aoki, K., Robinson, K., Sullivan, M., Martinez, J., Nakamuta, M., Ishimura-Oka, K., and Chan, L. (1999) *Arterioscler. Thromb.* 19, 5163–5172
8. Heckenlively, J. R., Hawes, N. L., Friedlander, M., Lusinowicz-S, H., Rurd, R., Davison, M., and Chang, B. (2003) *Retina* 23, 518–522
9. He, X., Yang, Y., Zhong, X., Yang, Y., and He, X. (2002) *Cell* 113, 149–156
10. Hotta, K., Miyazaki, Y., Kato, T., and Nakamura, M. (2004) *Genomics* 29, 329–335