Expression of VLDLR in the Retina and Evolution of Subretinal Neovascularization in the Knockout Mouse Model’s Retinal Angiomatous Proliferation

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PURPOSE. Very-low-density lipoprotein receptor (VLDLR) in knockout mice (vldlr−/−) has been reported to induce subretinal neovascularization. Therefore, VLDLR expression in the wild-type mouse retina was investigated and the retinal angiogenic process in vldlr−/− mice was characterized.

METHODS. VLDLR expression in the retina and in purified retinal vascular endothelial cells (RECs) and retinal pigment epithelial (RPE) cells was determined by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry. Angiogenic evolution in vldlr−/− mice was examined by fundus fluorescein angiography, histology, double-staining of FITC-dextran perfusion and elastin immunohistochemistry, isoelectin staining, and confocal fluorescence microscopy.

RESULTS. VLDLR mRNA was detected in the wild-type mouse retina and in purified RECs and RPE cells. The VLDLR protein was localized in the RPE layer, vessels in the ganglion cell layer, and around the outer limiting membrane of the retina. The retinal pathogenic process in vldlr−/− mice recapitulates key features of retinal angiomatous proliferation (RAP) in humans, a subtype of neovascular age-related macular degeneration (AMD). These include neovascular growth originating from retinal vessels and progressing to the subretinal space with intraretinal, subretinal, and choroidal angiogenic stages, RPE disruption and Bruch membrane exposure, retinal-choroidal anastomosis, subsequent photoreceptor degeneration, RPE hyperplasia, and subretinal fibrosis at the end stage.

CONCLUSIONS. VLDLR is expressed in the wild-type mouse retina, especially in RECs and RPE cells. The vldlr−/− mouse exhibits histologic and angiographic characteristics of RAP and is a reproducible animal model facilitating studies of the molecular mechanisms of RAP. (Invest Ophthalmol Vis Sci. 2008; 49:407–415) DOI:10.1167/iovs.07-0870

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In neovascular age-related macular degeneration (AMD), choroidal neovascularization (CNV) first proliferates under the Bruch membrane and retinal pigment epithelium and then invades the subretinal space. The leakage of CNV and the subsequent fibrosis eventually destroy the photoreceptor layer. At the end stage, CNV may infiltrate the neurosensory retina and communicate with the retinal circulation, forming what has been referred to as retinal-choroidal anastomosis.4 In recent years, it has been recognized that the reverse occurs in a subset of neovascular AMD, where the angiomatic proliferation originates from retinal vessels and extends posteriorly into the subretinal space. This distinct form of neovascular AMD is termed retinal angiomatous proliferation (RAP),7 and it is present in approximately 12% to 15% of patients with newly diagnosed neovascular AMD.5–6

The neovascular process of RAP has been categorized into three angiogenic stages.2 Stage 1, intraretinal neovascularization (IRN), involves capillary proliferation originating from the deep capillary plexus within the retina. Stage 2, subretinal neovascularization (SRN), is determined by the growth of retinal vessels extending beyond the photoreceptor layer into the subretinal space. Stage 3, CNV, occurs when SRN infiltrates the retinal pigment epithelium, leading to reactive pigment change, formation of retinal-choroidal anastomosis, and subsequent scarring.7 In addition, intraretinal hemorrhage, pigment epithelium detachment, and focal hyperpigmentation are often associated with RAP. Although clinical manifestations and angiogenic sequences of RAP are now better understood, RAP remains difficult to treat and patients have poor prognoses.5,7,8 Different pathologic processes and treatment responses indicate potentially different mechanisms involved in RAP. To date, however, little is known about the etiology and mechanisms of RAP.2,7–9,19 Lack of an appropriate animal model and the poorly understood molecular mechanisms limit the development of a specific treatment strategy to block pathogenic neovascularization.

Very-low-density lipoprotein receptor (VLDLR) is an 86-kDa transmembrane protein initially identified in endothelial cells.20,21 It belongs to the low-density lipoprotein receptor (LDLR) family, which includes LDLR, apolipoprotein E receptor 2 (ApoER2), LDL-related protein (LRP), glycoprotein 330, and LR11.22 The identification of consistent SRN in a germline knockout mouse of the gene encoding VLDLR23,24 has linked VLDLR with retinal neovascularization. The 100% penetration of the retinal phenotype in the VLDLR knockout (vldlr−/−) mouse revealed a strong association between retinal neovascularization and the VLDLR mutation, indicating a prominent inhibitory effect of VLDLR on retinal angiogenesis. This hypothesis is further reinforced by the recent identification of VLDLR as one of the functional candidate genes for a significant association with AMD in humans.25 In addition, microarray screening of hundreds of single nucleotide polymorphisms (SNPs) in 360 patients with AMD and 360 healthy persons replicates a positive association of the VLDLR gene with the mixed phenotype of AMD.26 However, there is no evidence of
the cellular origin of VLDLR in the retina. Therefore, in this study, we first localized the mRNA and protein expression of VLDLR in the wild-type mouse retina. We then further characterized the pathologic angiographic patterns of retinal neovascularization in the vldlr−/− mouse. We have found that spontaneously occurring retinal neovascularization, including IRN, SRN, and CNV, in the vldlr−/− mouse recapitulates the key features of RAP in humans and can serve as a unique animal model of RAP.

**Materials and Methods**

**Animals**

Breeding pairs of mutant mice with targeted deletion of the VLDLR gene (B6; 12957-Vldlrmtm1(Ji); vldlr−/−) were obtained from The Jackson Laboratory (Bar Harbor, ME). The colony was maintained and bred in standardized conditions. Age-matched wild-type (C57BL/6); +/+ mice were used as normal controls. All procedures were performed with strict adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Indiana University Animal Care and Use Committee.

**Isolation of RPE and Retinal Vascular Endothelial Cells**

Mouse retinal pigment epithelial (RPE) cells were isolated using a modified method of Gibbs. Briefly, the eyes harvested from vldlr−/− and wild-type mice (n = 6) at 3 weeks of age and were incubated in high-glucose Dulbecco modified Eagle medium (DMEM) with 2% dispase (Invitrogen, Carlsbad, CA) at 37°C for 45 minutes and then in DMEM containing 10% FBS for 20 minutes at 37°C. The retina was removed, and sheets of RPE were peeled from the choroid. RPE cells were collected by centrifugation at 1500 rpm for 2 minutes.

Mouse retinal vascular endothelial cells (RECs) were isolated by affinity purification using magnetic beads coated with an antibody specific for endothelial cells, anti-platelet/endothelial cell adhesion molecule-1 (anti-PECAM-1) antibody. Briefly, mouse retinas (n = 6) at 4 weeks of age were digested with collagenase type I (1 mg/mL; Worthington, Lakewood, NJ) for 40 minutes at 37°C. Dissociated cells were filtered through a 40-μm nylon membrane (Fisher Scientific, Hanover Park, IL) and were incubated with magnetic beads (Dynal Biotech, Lake Success, NY) precoated with a rat anti–mouse PECAM-1 monoclonal antibody (BD Biosciences, San Jose, CA) for affinity binding. Bead-bound cells were collected in a plate precoated with 2 μg/mL fibronectin (BD Biosciences) for 2 to 3 days in an endothelial cell growth medium containing 20% FBS, 2 mM L-glutamine, 2 mM sodium pyruvate, 20 mM HEPES, 1% nonessential amino acids, 55 U/mL heparin, and 100 μg/mL endothelial growth supplement (Sigma, St. Louis, MO).

**Reverse Transcription–Polymerase Chain Reaction**

Total RNA was isolated from RECs, RPE cells, and retinal tissues of animals with 1 mL PBS and then received 1 mL of 50 μg/mL fluorescein-labeled dextran solution (FITC-Dextran, approximately 2 × 10^6 molecular weight; Sigma). The eyes were removed and fixed in 4% PFA at room temperature for 2 hours. Cornea, iris, lens, and neural retina were removed from the eyeball. Six radial cuts were made from the edge of the cornea to the equator; the retinal pigment epithelium-choroid-sclera complex was flat-mounted with the sclera facing down on a glass slide in aqueous coverglass mountant (Aquamount; Andwin Scientific, Addison, IL).

**Immunohistochemistry**

Eyes of vldlr−/− and wild-type mice at ages of 3 and 6 weeks, 10 and 12 months (n = 3 for each group) were embedded in OCT compound (Miles Inc., Elkhart, IN) and immediately frozen at −80°C. Radial sections of 12-μm thickness were cut on a cryostat at −20°C. A goat anti–mouse VLDLR polyclonal antibody (R&D Systems, Minneapolis, MN) and an FITC-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used to visualize VLDLR immunostaining.

Cell nuclei were counterstained with DAPI. A monoclonal antibody against intermediate filament protein (Vimentin) was used as a marker for fibroblasts. A biotinylated rabbit anti–mouse secondary antibody and an avidin-biotin reagent kit (Vectastain Elite ABC; Vector Laboratories) were used with 3,3′ diaminobenzidine (DAB) as a chromogen.

**Fundus Fluorescein Angiography**

Fundus examination and fluorescein angiography (FA) were performed using a fundus camera (FK 30; Carl Zeiss, Oberkochen, Germany) on the vldlr−/− mice at 2, 3, 6, and 10 weeks and at 4, 6, 8, 10, and 12 months of age (n = 3 for each age). The mice were anesthetized (Avertin; 1.25% wt/vol tribromoethanol, 0.8% vol/vol amyl alcohol) 0.02 mL/g body weight. Topical 1% tropicamide and 2.5% phenylephrine were administered for pupillary dilation. For FA evaluation, 25% sodium fluorescein (0.1 mg/kg) was injected intraperitoneally. Fundus was photographed during the late phase of FA.

**Histology**

Eyes were enucleated after the animals were humanely killed and were fixed in 4% paraformaldehyde (PFA) solution overnight at 4°C. After removal of the cornea, iris, and lens, whole eyeballs or posterior eyecups were gradually dehydrated, embedded in paraffin, serially sectioned (6 μm), and stained with hematoxylin and eosin.

**Isolectin Staining and Fluorescence Confocal Microscopy**

A conjugate of isoelectric IB4 from Griffonia simplicifolia (isoelectric IB4 Alexa Fluor 488; Invitrogen) was used in retinal cross-sections and whole flat mounts labeling retinal blood vessels. Frozen retinal sections of two mice from each genotype at 6 weeks of age were fixed in 4% PFA for 30 minutes, blocked with 10% donkey serum, incubated in a 1:150 diluted isoelectric solution containing 1:50 donkey serum overnight at 4°C, and mounted with an antifade DAPI reagent (ProLong Gold; Invitrogen). Retinal sections were visualized under an inverted fluorescence microscope, DM IRB (Leica Microsystems Inc., Bannockburn, IL). For whole retinal flat mounts, eyes of two mice at 16 days, 4 weeks, and 1 year of age were fixed in 1% PFA at 4°C overnight. After blocking, the entire neural retina was incubated in a 1:100 diluted isoelectric solution containing 10% BSA and 10% donkey serum overnight at 4°C, washed with PBS, and mounted with an antifade reagent with DAPI (ProLong Gold; Invitrogen). Retinal flat mounts were then visualized under a fluorescence confocal microscope (LSM-510 Meta confocal microscope system; Carl Zeiss). Stacks of images spanning the entire thickness of the retinal vasculature and three-dimensional images were obtained using image analysis software (LSM Image Browser; Carl Zeiss).

**FITC-Dextran Perfusion and Elastin Staining**

Anesthetized mice at 3 weeks of age were perfused with 1 mL PBS and then received 1 mL of 50 μg/mL fluorescein-labeled dextran solution (FITC-Dextran, approximately 2 × 10^6 molecular weight; Sigma). The eyes were removed and fixed in 4% PFA at room temperature for 2 hours. Cornea, iris, lens, and neural retina were removed from the eyeball. Six radial cuts were made from the edge of the cornea to the equator; the retinal pigment epithelium-choroid-sclera complex was flat-mounted with the sclera facing down on a glass slide in aqueous coverglass mountant (Aquamount; Andwin Scientific, Addison, IL). After blocking in PBS with 0.5% Triton X-100, 2% BSA, and 10% donkey serum for 30 minutes at room temperature, flat mounts were stained with a polyclonal antibody against mouse elastin (Elastin Products
RESULTS

Expression of VLDLR in RPE and Retinal Endothelial Cells in the Mouse Retina

Although the fact that VLDLR knockout triggers retinal neovascularization clearly indicates its role in retinal angiogenesis, it is unclear whether VLDLR is present, or which cells express VLDLR in the retina. Therefore, our first goal was to determine the expression pattern of the VLDLR in the retina. VLDLR mRNA was detected in neural retinal tissues of adult wild-type mice by RT-PCR (Fig. 1). To verify its cellular origin, RT-PCR was also performed in purified RECs and RPE cells isolated from the wild-type retina. VLDLR mRNA signals were amplified in RECs and RPE cells, confirming cell-specific expression of these cell types (Fig. 1). Corresponding negative amplifications of VLDLR in the retina, RECs, and RPE cells of vldlr−/− mice further validated the specificity of the primer set and confirmed the successful knockout of VLDLR in this mutant (Fig. 1). Simultaneous amplification of GAPDH mRNA signals was included in all reactions as an internal control (Fig. 1).

To examine the cellular distribution of the VLDLR protein in the retina, immunofluorescence localization studies were performed in retinal tissues of three pairs of wild-type and vldlr−/− mice at 2 months of age. Because no report was available about any positive expression of VLDLR in the retina, the antibody’s specificity was first evaluated in the skeletal muscle of wild-type mice given its high levels of immunodetectable VLDLR,

Neovascularization in vldlr−/− Mice

To determine the origin and evolution of retinal neovascularization, retinal histology was examined in serial sections around the onset of the neovascularization at postnatal day (P) 12, P13, P14, P15, and P16, at 3, 6, and 10 weeks, and at 10, 12, and 24 months. Given that Heckenlively et al.23 have characterized the phenotype of subretinal neovascularization well at 3 and 6 weeks and at 3 and 9 months, we focused our attention on the initial and end stages of the neovascularization in vldlr−/− mice. Initial signs of abnormal blood vessel growth were found in the deep capillary bed of OPL, protruding into the avascular zone of the outer nuclear layer (ONL) as early as 14 days of age in vldlr−/− mice (Figs. 3A, 3B). At P15, some of the balloon-shaped new vessel buds with clear lumen, often filled with blood cells, were found to reach the subretinal space, representing the beginning stage of SRN (Figs. 3C, 3D).

Consistent with the previous report,23 classical SRN could be found at 6 weeks of age (data not shown). Typical CNV membranes developed after 6 months of age with tissue destruction in the nearby OPL and ONL (data not shown). The morphology of the CNV at the late stage of 10 months was indistinguishable from the CNV originating from the choroidal vessels (Fig. 3E). Others might have had characteristics of retinal-choroidal anastomosis (Fig. 3F). In addition, the CNV in the 10-month-old vldlr−/− retina was often surrounded by a dense layer of pigmented cells, presumably reactive retinal pigment epithelium (Figs. 3E, 3F). Although the vascular anastomosis was still visible, the neovascular growth was covered by cells and fibrous tissues and became subretinal fibrovascular masses. Interestingly, neovascularization originating from the deep capillary plexus in the OPL of the retinal circulation always grew vertically toward the subretinal space and finally merged with choroidal vessels to form retinal-choroidal anastomoses. Retinal neovascularization in vldlr−/− mice was never found to grow toward the vitreous. This neovascular growth pattern was clearly different from the ischemia-induced retinal neovascularization in diabetes in which retinal vessels grow into the vitreous cavity.

In addition to the destruction of the ONL, photoreceptor degeneration in vldlr−/− mice was noticeable at 10 months of age, with reduced thickness of the ONL close to the lesion sites compared with the immediate adjacent ONL (Figs. 3E, 3F). Further loss of photoreceptors was evident, with an overall reduction of the ONL thickness at 12 months. The ONL was completely diminished by the age of 24 months in the vldlr−/− retina (data not shown).

Neovascularization in the vldlr−/− Originates from Retinal Vessels

To better visualize the retinal vascular structure, fluorescent dye-conjugated isolectin staining was used to label vessels on retinal sections of 6-week-old wild-type and vldlr−/− mice. Figure 4A shows the normal distribution of retinal vessels in the wild-type retina. Large vessels were found only in the GCL, whereas small capillaries were seen in the inner plexiform layer (IPL), INL, and OPL. No isolectin staining was found in the avascular zone of the ONL or in the inner or outer segments of photoreceptors (Fig. 4A). In contrast, new blood vessels in the vldlr−/− retina clearly invaded the avascular zone and grew.
FIGURE 2. Immunohistochemistry of VLDLR protein expression in the adult wild-type retina. (A, C, E) Green fluorescein signals of VLDLR. (B, D, F) Merged images of nucleus counterstained with DAPI. (A, B) The VLDLR protein was mainly detected in the RPE layer (red arrows) and GCL (white arrows) and around the OLM of the retina (asterisk). (C, D) Magnification (600×) of retinal blood vessels in the GCL, demonstrating the localization of VLDLR immunostaining in endothelial cells. (E, F) The optic nerve also stained positive for VLDLR, with intense signal near the sheath (black arrows). See Supplementary Figure S1, http://www.iovs.org/cgi/content/full/49/1/407/DC1, for nonimmune control and vldlr−/− retina staining.

FIGURE 3. Initial and late stages of retinal neovascularization in vldlr−/− mice. (A, B) New blood vessels originated in the OPL and extended through the ONL as early as P14. (C, D) Balloon-like subretinal neovascularization buds could be seen at P15 with a stem extended from the ONL. The stem might not always have been visible in a cross-section (D, white arrow) but could often be tracked in one of the neighboring serial sections. (E, F) At 10 months, blood vessels could still be seen within the subretinal fibrovascular membranes and OPL. Note the dense pigmentation around the membranes. The ONL above the area of neovascularization was significantly thinner or disappeared completely compared with the ONL away from the lesion site.
into the ONL and subretinal space (Figs. 4B, 4C). New vessel tracks could be traced from the OPL, through the ONL, to the subretinal space and eventually merged with choroidal vessels, forming retinal-retinal anastomoses and retinal-choroidal anastomoses. The density of vascular staining in the GCL, IPL, inner nuclear layer (INL), and OPL appeared comparable between wild-type and vldlr−/− mice (Figs. 4A, 4B).

To obtain an overview of the retinal vascular system, isoclin staining was also performed on retinal whole mounts with the photoreceptor layer facing up. Because the choroidal vessels and retinal pigment epithelium were removed in the preparation, all labeling vasculatures were within the neural retina. Figure 5 depicts low-magnification images of the whole retina from 4-week-old wild-type (G) and vldlr−/− (H) mice. In contrast to the age-matched wild-type retina, numerous brightly stained angiomatous growths (89–97 per retina) were densely distributed throughout the retina of the vldlr−/− mutant (Fig. 5H). At a higher magnification of merged Z-stack images under a confocal fluorescence microscope acquired by continuous scanning of the whole depth of the retina, many neovascular tufts were found connecting with retinal vessels (Fig. 5I). Neovascular buds were also observed originating from the superficial retinal vessels near the ganglion cell layer (Fig. 5J), indicating that neovascularization arose from small and large blood vessels at different depths of the retinal vasculature. In addition, clumps of RPE cells were found adhering to the neovascular tufts in the subretinal space (Fig. 5K). The subretinal neovascular tufts merged with each other and became larger vascular tangles covered with more RPE cells at 1 year of age (Fig. 5L). The number of stained angiomatous growths was actually decreased in the whole mount because of the merging (data not shown).

To unequivocally determine the evolution of the neovascularization at an early stage, we examined the initial neovascular growth on retinal whole mounts from P16 vldlr−/− mice. Confocal images of two subretinal neovascular buls at various depths are shown in Figures 5A through 5K. Two balloon-like vascular bulbs grew far away from the retinal capillary plexus (Fig. 5A) and toward the avascular zone of the photoreceptor layer. A thin stem at the base of both bulbs could be traced down, connecting to the deep layer of retinal vascular network (Figs. 5B, 5C). Additional small vascular sprouts representing early neovascular growth already protruding into the avascular zone, but not as deep as the two bulbs, were also noticeable (Figs. 5B, 5C). Figure 5F depicts a merged Z-stack image of multiple scanning of the two neovascular bulbs through the whole depth of the retina. The connection and sprout of neovascular growths could be traced to retinal vessels and better visualized in three-dimensional images (data not shown).

These results confirm that subretinal neovascularization in vldlr−/− mice originates from retinal vessels. The size of the neovascular bulbs increased significantly with age. The morphology of the neovascular growths transitioned from initial vascular buds to balloon-shaped bulbs and then to mature angiomatous-like vascular tangles. Although some neovascular bulbs reached the subretinal space, new vascular sprouts continued to emerge not only from the deep layer of the capillary network but also from large retinal vessels in the superficial vascular layer. Therefore, there was significant overlap of the intraretinal and subretinal neovascularization processes.

**Figure 4.** Isoclin staining of blood vessels in retinal cross-sections. (A) Normal retinal vasculature was distributed in the GCL, INL, and OPL in the wild-type retina at 6 weeks of age. (B, C) New blood vessels were stained in lesion sites (yellow arrows) of the vldlr−/− retina at 6 weeks of age. The new vessels extended from the OPL (subsite arrow), through the ONL, to the subretinal lesion sites, and merged with the choroidal vasculature. (C) A merged image of phase contrast and (B).
months. Fluorescein leakage was still significant at 6 months of age. These results of early and peak stages of retinal neovascularization are consistent with the previous findings by Heckenlively et al. In addition, we found a significant reduction of leaking spots after 8 months, though large rigid vascular tangles were still observable (Fig. 7C). The fluorescein leakage was hardly noticeable at 12 months (Fig. 7D), and the fundus was filled with pink spots and patches because of the loss of normal pigmentation. These findings are consistent with the data of isolectin staining.

To verify whether the reduction of fluorescein leakage in the late stage may represent vascular closure and fibrotic scar formation, immunohistochemistry was performed on retinal sections to detect the presence of vimentin as a marker for fibroblasts. Significant vimentin-positive staining was localized at the retinal lesion sites of the vldlr mice at 12 months (Fig. 8B). No positive staining was found in retinal tissues from age-matched wild-type mice (Fig. 8A). The presence of fibroblasts in the lesion sites at this stage and the absence of fluorescein leakage indicated fibrosis formation in the vldlr retina. Positive fibroblast staining was also noted at 3 months, but it was less extensive (data not shown). The findings of reduced to absent fluorescein leakage (Figs. 7C, 7D), vimentin-positive staining (Fig. 8B), and concealed neovascularization (Figs. 1E, 1F) at the late stage suggested that the neovascular structure became more mature, less leaky, was confined in scars, or all of these.

**DISCUSSION**

In this study, we localized the mRNA and protein expression of VLDLR in the wild-type mouse retina. Further characterization of the pathologic angiogenic process in the vldlr mouse revealed that the neovascularization originated from retinal microvasculature. The progression of the neovascular growth
Retinal Neovascularization in \textit{vldlr}^{−/−} Model’s RAP

VLDLR in Retinal Angiogenesis

Although the VLDLR gene was cloned more than a decade ago,\textsuperscript{20} most research concerning it has been devoted to the biological significance in lipoprotein metabolism and thrombosis. The discovery that VLDLR knockout triggers subretinal neovascularization in mice clearly indicated its potential role in retinal angiogenesis. However, no information was available regarding what cells in the retina express VLDLR. Therefore, we sought first to determine the presence and cellular origins of VLDLR in the retina. With the use of RT-PCR and immunocytochemistry, we have demonstrated the presence of VLDLR in the mouse retina. We have also identified the origins of VLDLR from two main cell types, RECs and RPE cells. Although the cellular origin of the intense VLDLR immunostaining around OLM is unclear, one possibility is Müller cells. The staining pattern matches well with adherence junctions among Müller cell outer processes. In addition, Müller cell expression of VLDLR has been reported in rats (Loewen N, et al. IOVS 2006;47:ARVO Abstract 1432). It has been reported that VLDLR mRNA is highly abundant in the heart, skeletal muscle, adipose tissue, brain, and macrophages.\textsuperscript{29-30} VLDLR protein is detected in the endothelial cells of capillaries and small arterioles\textsuperscript{21} and in vascular smooth muscle cells.\textsuperscript{31} Our results of positive VLDLR expression in RECs and vessels in the GCL are consistent with these findings. In addition, the identification of RPE cells expressing VLDLR revealed a potential role of VLDLR in RPE cells because retinal pigment epithelium is a critical component in the homeostasis of proangiogenesis and antiangiogenesis.

In addition to serving as a receptor for VLDL, VLDLR binds with several ligands, including IDL and chylomicrons, lipoprotein lipase, receptor-associated protein, thrombospordin-1, urokinase plasminogen activator, plasminogen activator inhibitor-1 complex, and other proteinase-serpin complexes.\textsuperscript{22-32} Although the primary role of the VLDLR is related to lipid metabolism, this receptor has functions other than that in lipid metabolism.\textsuperscript{22} As coreceptors, VLDLR and ApoE receptor are essential components of the Reelin signaling pathway regulating neuronal migration and synaptic plasticity.\textsuperscript{33-35} It has also been shown that the tissue factor pathway inhibitor can associate with VLDLR, inhibiting endothelial cell proliferation in vitro.\textsuperscript{36-38} However, because of the complexity and high homology of the LDL receptor family and the lack of specific antagonists, it has been challenging to dissect the exact function of VLDL, especially with different ligands. Creation of a genetic VLDLR knockout mouse provides a valuable tool for studying the function of VLDLR in vivo. Full penetration of the retinal phenotype with no other detectable abnormality in \textit{vldlr}^{−/−} mice offers a reproducible animal model for subretinal neovascularization research. Mapping of VLDLR expression in the retina, especially in the cells other than RECs, is significant and indicates additional cellular targets of neovascularization in \textit{vldlr}^{−/−} mice. These findings provide fundamental knowledge for studying the biological functions of VLDLR in the retina.

Age-Related Subretinal Neovascularization in \textit{vldlr}^{−/−} Mice Models RAP AMD in Humans

Although numerous animal models across multiple species have been developed to study the pathogenesis of AMD, most
models of CNV originate from the choroidal vascular system. Another set of neovascularization models is pathogenic growth of retinal vessels into vitreous, which mimics the neovascular process in diabetic retinopathy. In some transgenic mice expressing VEGF or PDGF-B in the photoreceptors, neovascularization could be induced in the retina.\(^\text{38,39}\) It has been shown that the neovascularization in VEGF transgenic mice originated from the deep vascular bed and grew beneath the photoreceptors into the subretinal space.\(^\text{38}\) This model showed that VEGF overexpression in the photoreceptors was sufficient to produce IRN and SRN. However, unlike RAP, no retinal-choroidal anastomosis or involvement of CNV in the late stage has been reported. PDGF-B overexpression induced the formation of a highly vascularized cell mass containing endothelial cells, pericytes, and glia in the superficial retina.\(^\text{39}\) However, the phenotype was different from that in RAP. Studies from these experimental models suggest that additional factor(s) may be necessary for the growth and penetration of vascular cells into the deep layer of the retina. Lack of appropriate animal models that recapitulate the pathogenic process in RAP AMD limits the study of RAP.

In the present study, using different approaches, we carefully examined the pathogenic progression of the neovascularization in the \(vldlr^-/-\) mouse retina. Our results revealed that the retinal phenotype in \(vldlr^-/-\) mice recapitulated many key features of RAP AMD in humans. First, as presented in Figures 3, 4, and 5, we have unequivocally demonstrated that the angiogenesis in the \(vldlr^-/-\) mouse retina originates from the OPL of the retinal vessels, not from choroidal vessels. Second, retinal vessels grow toward the subretinal space with clear IRN at approximately P14 to P30, equivalent to stage 1 IRN in RAP AMD, and then subretinal neovascularization between P16 to P180 (6 months) corresponding to stage 2 SRN in RAP AMD (Figs. 3, 5). Third, the angiomatic morphology of neovascular growth in the retina and the subretinal space, shown in retinal whole mount isoelectin staining (Fig. 4), is similar to that in RAP AMD. Fourth, neovascular growth eventually disrupts retinal pigment epithelium (Figs. 3, 6), similar to pigment detachment seen in RAP AMD. Fifth, at approximately 10 months, two vessel systems eventually merge and form retinal-choroidal anastomoses with the typical morphology of CNV (Fig. 3), comparable to stage 3 CNV in RAP AMD. As in RAP, though end-stage CNV is indistinguishable from the CNV originating from choroidal vessels, the late onset and the angiogenic process in the \(vldlr^-/-\) mouse retina are clearly different from those of other CNV models. Sixth, the neovascular growth is hyperpermeable (Fig. 7). Seventh, intraretinal hemorrhage is also found in the \(vldlr^-/-\) mouse retina in the initial report by Heckenlively et al.\(^\text{33}\) Eighth, subsequent photoreceptor degeneration occurs at a late stage in the \(vldlr^-/-\) retina. Ninth, RPE cells surround the CNV (Figs. 3E, 3F), similar to focal hyperpigmentation in RAP. Tenth, significant fibrosis formation takes place at the end stage of the neovascularization (Fig. 8). These data demonstrate that the retinal neovascularization in the \(vldlr^-/-\) mutant imitates the entire angiogenic process of the retinal phenotype in RAP and can serve as a reliable and reproducible animal model for RAP AMD. This notion was also supported by a recent publication studying biochemical alterations in the retinas of \(vldlr^-/-\) mice.\(^\text{40}\) As with other animal models, the \(vldlr^-/-\) mutant has its own limitations and may not reflect every aspect seen in human RAP AMD. However, the significant resemblance of the origin and evolution of subretinal neovascularization in \(vldlr^-/-\) mice provides a valuable animal model facilitating studies of the molecular mechanisms of retinal angiogenesis and of the evolution of novel antiangiogenic therapy originating from retinal vessels. It can also serve as a model to screen for candidate drugs to treat RAP AMD.

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References

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