Pathogenic Role of the Wnt Signaling Pathway Activation In Laser-Induced Choroidal Neovascularization

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PURPOSE. Choroidal neovascularization (CNV) is a severe complication of AMD. The Wnt signaling pathway has been shown to mediate angiogenesis. The purpose of this study was to investigate the pathogenic role of the Wnt pathway in CNV and explore the therapeutic potential of a novel Wnt signaling inhibitor in CNV.

METHODS. Adult rats and mice were photocoagulated using diode laser to induce CNV. On the same day, the animals were intravitreally injected with a monoclonal antibody (Mab2F1) blocking LRP6 or nonspecific mouse IgG. The Wnt signaling activation and target gene expression in the eyecup were determined by Western blot analysis. Fundus angiography was used to examine leakage from the laser lesion. CNV areas were measured on choroidal flatmount using FITC-dextran.

RESULTS. Levels of Wnt pathway components and Wnt target gene expression were elevated in both laser-induced CNV rat and mouse eyecups, suggesting activation of the Wnt pathway. Significant suppression of Wnt signaling was observed in the Mab2F1 treatment group. Mab2F1 decreased vascular leakage from CNV lesions and reduced the neovascular area in laser-induced CNV rats. Mab2F1 inhibited the hypoxia-induced activation of Wnt signaling in cultured RPE cells. Mab2F1 also ameliorated retinal inflammation and vascular leakage in the eyecups of very low-density lipoprotein receptor knockout mice, a model of subretinal neovascularization.

CONCLUSIONS. The Wnt pathway is activated in the laser-induced CNV models and plays a pathogenic role in CNV. Blockade of Wnt signaling using an anti-LRP6 antibody has therapeutic potential in CNV. (*Invest Ophthalmol Vis Sci.* 2013;54:141–154) DOI:10.1167/iovs.12-10281

AMD is the leading cause of vision loss in the developed countries.¹ Choroidal neovascularization (CNV) is a severe complication of wet AMD.² While pathogenesis of wet AMD remains elusive, CNV is known as the major cause of sudden and disabling loss of central vision in wet AMD.^{3,4}

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Supported by NIH Grants EY018659, EY012231, EY019309, P20RR024215, and a research award from American Diabetes Association (ADA 7-11-JF-10).

Submitted for publication May 28, 2012; revised September 25 and November 16, 2012; accepted November 28, 2012.

Disclosure: **Y. Hu**, None; **Y. Chen**, None; **M. Lin**, None; **K. Lee**, None; **R.A. Mott**, None; **J. Ma**, None

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The canonical wingless-type MMTV integration site (Wnt) signaling pathway plays a critical role in the regulation of inflammation and angiogenesis.^{5,6} Wnt ligands are secreted, cysteine-rich glycosylated proteins,³ which bind to frizzled (Fz) receptors or to the coreceptor complex of Fz and low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6).^{4–6} Binding of Wnt ligands results in LRP6 phosphorylation and activation,^{7,8} leading to dissociation of the kinase complex containing glycogen synthase kinase-3 β (GSK3 β),⁹ axin and adenomatous polyposis.³ The GSK3 β complex dissociation prevents transcription factor β -catenin from phosphorylation and degradation.¹⁰ Consequently, β -catenin is accumulated in the cytoplasm and translocated into the nucleus, complexes with TCF/LEF family transcription factors,¹¹ regulating expression of Wnt target genes including VEGF, which is the key pathogenic factor in CNV.^{12–15}

Although studies of the pathogenesis and treatment of AMD have been delayed by lacking of ideal animal models,¹⁶ laser-induced CNV rodent models are commonly used to study CNV in wet AMD.^{17–20} Moreover, our previous study has shown that the Wnt signaling pathway is activated in very low-density lipoprotein receptor (VLDLR) knockout (KO) mice, a genetic animal model of subretinal neovascularization (NV).²¹ The present study investigated the role of the Wnt signaling pathway in laser-induced CNV and explored therapeutic potential of a blocker of Wnt signaling in laser-induced CNV and VLDLR KO models.

METHODS

Animals

Care, use, and treatment of experimental animals were in strict agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male C57BL/6J mice and VLDLR KO mice (10 weeks old; The Jackson Laboratory, Bar Harbor, ME) and male Brown Norway rats (8–10 weeks old; Charles River, Wilmington, MA) were used in this study. In all procedures, animals were anesthetized by intramuscular injection of 50 mg/kg ketamine hydrochloride (Vedco, St. Joseph, MO) and 10 mg/kg xylazine (Vedco), and pupils were dilated with topical administration of 1% cyclopentolate (Wilson, Mustang, OK).

CNV Induction

Laser photocoagulation (532 nm, 150–250 mW, 0.01 second, 50 μ m; model diode pumped solid-state; Ellex Medical PTY, Adelaide, Australia) was performed in rat and mouse eyes. Four laser spots were applied in a homodisperse distribution by a standardized manner around the optic disk, using a slit lamp delivery system and a coverslip as a contact lens. The morphologic endpoint of the laser injury was the appearance of a subretinal bubble at the time of laser photocoagulation due to the disruption of Bruch's membrane. Twenty-five Brown Norway rats (Charles River) and 15 C57/BL6

mice were used for laser-induced CNV, with four laser lesions per eye; five rats and five mice were used as untreated controls.

Western Blot Analysis

The eyecups of each mouse/rat were dissected and homogenized. The eyecups of each mouse were combined and homogenized. Protein concentration in the homogenate was measured by the Bradford assay. The equal amount (50 μ g) of total protein from each sample was resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk and separately blotted with primary antibodies. After thorough washes, a peroxidase-conjugated secondary antibody was added, respectively, and incubated with the membrane. The signal was developed with the enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL), and densitometry of the signal bands on digital images was measured by drawing region of the bands of interest and quantified using FluorChem Q software (ProteinSimple, Santa Clara, CA).

Fundus Fluorescein Angiography

Fundus fluorescein angiography was performed using a commercial camera and imaging system (KOWA GENESIS-D system; KOWA Company, Tokyo, Japan) at 1, 2, and 3 weeks after laser photocoagulation. The photographs were captured with a 20-D lens in contact with the fundus camera lens, after an intraperitoneal injection of 0.1 mL of 1% fluorescein sodium (Akorn, Decatur, IL). Lesions were graded on a grade scale based on the spatial and temporal evolution of fluorescein leakage following the documented standard as the following¹⁹: no leakage, faint hyperfluorescence, or speckled fluorescence without leakage; questionable leakage, hyperfluorescent lesion without advancing increase in size or intensity; leaky, hyperfluorescence increasing in intensity but not significantly in size without definite leakage; pathologically significant leakage, hyperfluorescence increasing in intensity and in size with definite leakage.

Fluorescein Angiography and Choroidal Flatmount

Fluorescein angiography was performed 3 weeks after laser photocoagulation. Fluorescein isothiocyanate-conjugated high molecular weight dextran (2%; 10 mL/kg) was intracardially injected into the anesthetized mice/rats. The eyes were dissected and fixed overnight at 4°C with 4% paraformaldehyde in Hanks' balanced saline prepared immediately before use. The RPE/choroid/sclera complexes were isolated and flatmounted. CNV lesions were visualized using a fluorescence microscope (FV 1000; Olympus, Tokyo, Japan). The CNV-related neovascular area was outlined and measured using Java-based image processing software (ImageJ; National Institutes of Health, Bethesda, MD).

Cell Culture

ARPE19, a human RPE cell line, was maintained as described previously.²¹

Quantitative Real-Time Reverse Transcription-PCR

Total RNA was isolated from the eyecup using Trizol according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The total RNA was used for reverse-transcription and amplified by quantitative real-time PCR as described previously.²²

Antibody Preparation

Mab2F1, a monoclonal anti-LRP6 antibody, was raised using a recombinant peptide comprising the E1E2 domain of LRP6. The

antibody was purified from a hybridoma cell line by affinity chromatography protein G (Thermo Scientific, Barrington, IL) and from the ascites fluid of BALB/c mice injected with the 2F1 hybridoma cells. The purified antibody was dialyzed against PBS, sterile-filtered, and administered intraperitoneally. The nonspecific mouse IgG purchased from Vector Laboratories (Burlingame, CA) was subjected to the same procedure as control of Mab2F1.

Intravitreal Injection

Rats were anesthetized with a 10:50 mix of ketamine (100 mg/mL) and xylazine (20 mg/mL). Mice were anesthetized with a 50:50 mix of ketamine (100 mg/mL) and xylazine (20 mg/mL). The pupil was dilated with topical application of phenylephrine (2.5%) and tropicamide (1%). A sclerotomy was created approximately 0.5 mm posterior to the limbus with a blade, and a glass injector (~33 gauge) connected to a syringe filled with 5 μ L (1 μ L for mice) Mab2F1 (3 μ g/ μ L) was introduced through the sclerotomy into the vitreous cavity.

Vascular Permeability Assay

Retinal vascular permeability was quantified by measuring tracer dye leakage from blood vessels into the retina as described previously.²³ Briefly, Evans blue dye (Sigma-Aldrich, St. Louis, MO), 30 mg/mL in PBS, was injected into the femoral vein (30 mg/kg body weight) and allowed to circulate for 120 minutes. Evans blue dye in the circulation was removed by perfusion with PBS. The retinas were carefully dissected and homogenized. Evans blue in the retina was extracted and measured with a spectrophotometer (DU800; Beckman Coulter, Brea, CA). Concentrations of Evans blue in the retina were normalized by total retinal protein concentration.

Statistical Analysis

All of the values in the results were expressed as mean $\pm$ SD. Statistical analyses were performed using Student's *t*-test where appropriate. *P* < 0.05 was considered statistically significant.

RESULTS

Activation of the Wnt Signaling Pathway in Rat Eyecups with Laser-Induced CNV

To determine Wnt signaling activation, we measured phosphorylated and total LRP6 levels, and phosphorylated, cytosolic and total β -catenin levels, expression of Wnt target genes including VEGF, c-Myc, Cyclin-D1, ICAM-1, and TNF- α at 7 days postlaser photocoagulation. Phosphorylated LRP6 (p-LRP6) and total LRP6 were significantly increased in rat eyecups with laser-induced CNV, compared with that in the normal control rats (Figs. 1A, 1B). Consistently, phosphorylated β -catenin (p- β -catenin) was significantly decreased, while cytosolic β -catenin (cyto- β -catenin) and total β -catenin were increased in the eyecups with laser-induced CNV (Figs. 1C, 1D), suggesting a decreased β -catenin degradation and subsequent accumulation of β -catenin in the cytoplasm. Another line of evidence supporting the enhanced transcriptional activity of β -catenin was the significantly up-regulated expression of target genes of β -catenin, including VEGF, c-Myc, and Cyclin-D1 in CNV eyecups (Figs. 1E, 1F). In our previous study, inflammatory factors such as ICAM-1 and TNF- α have also been shown to be upregulated by Wnt activation in the retina.²⁴ The present study detected elevated protein levels of ICAM-1 and TNF- α in the CNV rat eyecups, further supporting the activation of the Wnt pathway (Figs. 1G, 1H). Thus, these results suggested that the Wnt signaling pathway is activated in the laser-induced CNV rat model.

FIGURE 1. Activated Wnt signaling in laser-induced CNV rat eyecups. At day 7 postlaser photocoagulation, the same amount (50 µg) of eyecup protein from each rat was used for Western blot analysis. (A) Total LRP6 and p-LRP6. (C) Total β-catenin, p-β-catenin, and cyto-β-catenin. (E) VEGF, c-Myc, and CyclinD1. (G) ICAM-1 and TNF-α. (B, D, F, H) The levels of total LRP6 and p-LRP6 (B); total β-catenin, p-β-catenin, and cyto-β-catenin (D); VEGF, c-Myc, and CyclinD1 (F); ICAM-1 and TNF-α (H) were semiquantified using densitometry and normalized by β-actin levels (mean ± SD, $n = 3$, * $P < 0.05$, ** $P < 0.01$, compared with untreated normal group).

Activation of the Wnt Signaling Pathway in Mouse Eyecups with Laser-Induced CNV

We also investigated the Wnt signaling pathway in laser-induced CNV mouse model. Similar to that in the CNV rats, p-LRP6, and total LRP6 levels were significantly elevated in mouse eyecups with laser-induced CNV (Figs. 2A, 2B). The CNV mice showed decreased p- β -catenin while elevated cyto- β -catenin (Figs. 2C, 2D) levels in the eyecups. Expression of VEGF, c-Myc, Cyclin-D1, ICAM-1, and TNF- α was significantly upregulated in the CNV mice (Figs. 2E-H). These results indicated that the Wnt signaling pathway is activated in the laser-induced CNV mouse model.

An Anti-LRP6 Antibody Inhibits the Activation of Wnt Signaling in Rat Eyecups with Laser-Induced CNV

Mab2F1, a monoclonal anti-LRP6 antibody, was raised using the ligand-binding domain of LRP6. Its specificity and Wnt pathway-blocking activity were confirmed previously.²⁵ To confirm the Wnt signaling pathway activation and evaluate its therapeutic effects, Mab2F1 was intravitreally injected into the rat eyes (15 μ g per eye) with laser-induced CNV at the same day of photocoagulation. Rats in control CNV group were injected with the same amount nonspecific mouse IgG. At 7 days after laser photocoagulation and injection, the Mab2F1-injected group showed lower p-LRP6 and total LRP6 levels than the control CNV rats injected with control IgG (Figs. 3A, 3B). Consistently, Mab2F1 also elevated p- β -catenin and decreased cyto- β -catenin levels in the CNV rat eyecups (Figs. 3C, 3D). Mab2F1 also attenuated overexpression of VEGF, c-Myc, Cyclin-D1, ICAM-1, and TNF- α in the CNV eyecups (Figs. 3E-H), compared with the control CNV group injected with control IgG, suggesting that Wnt signaling plays a key role in the overexpression of these angiogenic and inflammatory factors in this model.

Mab2F1 Inhibits the Activation of Wnt Signaling in Mouse Eyecups with Laser-Induced CNV

Next, we investigated the Mab2F1 effects in laser-induced CNV mice as well. Similar to the observation in rats, the CNV group treated with Mab2F1 showed lower p-LRP6 and total LRP6 levels (Figs. 4A, 4B), elevated p- β -catenin and decreased levels of cyto- β -catenin (Figs. 4C, 4D), and downregulated expression of VEGF, c-Myc, Cyclin-D1, ICAM-1, and TNF- α (Figs. 4E-H), compared with the control CNV group treated with the nonspecific IgG.

Mab2F1 Reduces Leakage from CNV Lesions and Decreases CNV Areas

Fluorescein angiography was commonly used to evaluate the evolution of CNV in AMD patients. Therefore, the present study evaluated CNV by quantifying grades 1 to 4 lesions based on the severity of leakage of fluorescein from the lesions following a documented standard.¹⁹ Fundus fluorescein angiography was performed at 1, 2, and 3 weeks postphotocoagulation (Figs. 5A-D) in rat groups of normal control, untreated CNV, CNV with the control IgG, and CNV with the Mab2F1 treatment. The lesions of different grades were recorded in each group (Fig. 5E). The Mab2F1 treatment significantly reduced the numbers of grade 4 lesions which have pathologically significant leakage from the CNV lesions, at all of the time points analyzed, with an approximate 31% decrease at 3 weeks postinjection, compared with the

untreated CNV group and the CNV group treated with the control IgG (Fig. 5E). Furthermore, we used the choroidal flatmount following fluorescein angiography to evaluate the leakage from the CNV lesions at 3 weeks postphotocoagulation (Figs. 6A-C). The Mab2F1 treatment significantly decreased the neovascular area compared with the untreated CNV and those treated with control IgG (Fig. 6D). Taken together, these findings suggest that Mab2F1 has significant effects on reducing vascular leakage from the CNV lesions and decreasing CNV areas.

Mab2F1 Inhibits the Hypoxia-Induced Activation of Wnt Signaling in ARPE19 Cells

To determine if Mab2F1 has direct inhibition of the Wnt signaling pathway in RPE cells, ARPE19 cells were treated with either nonspecific IgG or Mab2F1 after exposure to CoCl₂, which is used to induce hypoxic conditions in cultured cells.²⁶ Compared with the untreated group, ARPE19 cells treated with CoCl₂ displayed activation of the Wnt signaling pathway as shown by elevated p-LRP6, total LRP6, cyto- β -catenin and total β -catenin, and overexpression of Wnt target genes, including VEGF, c-Myc, Cyclin-D1, and TNF- α (Figs. 7A-F). Compared with nonspecific IgG, Mab2F1 attenuated the hypoxia-induced increases of p-LRP6 and total LRP6 (Figs. 7A, 7B), decrease of p- β -catenin and increase of cyto- β -catenin levels (Figs. 7A-C). Further, Mab2F1 also downregulated expression of VEGF, c-Myc, Cyclin-D1, and TNF- α under hypoxic conditions (Figs. 7D-F). Taken together, these data imply that Mab2F1 directly inhibits activation of the Wnt signaling pathway induced by hypoxia in RPE cells.

Mab2F1 Inhibits the Activation of Wnt Signaling and Reduces Vascular Leakage in VLDLR KO Mouse Eyecups

Our previous study has shown that VLDLR KO mice develop subretinal NV with overactivation of the Wnt signaling pathway in the eyecups, and is considered a model of subretinal NV.²¹ To determine whether Mab2F1 can inhibit the activation of the Wnt signaling pathway in this genetic NV model, Mab2F1 was injected into the vitreous of VLDLR KO mice at 10 weeks old, with the same amount of nonspecific IgG as control. The eyecups were homogenized for Western blotting at 7 days postinjection. The Mab2F1 injection decreased p-LRP6 and total LRP6 (Figs. 8A, 8B) levels, elevated p- β -catenin while decreased cyto- β -catenin levels (Figs. 8C, 8D). Moreover, Mab2F1 also reduced VEGF, c-Myc, Cyclin-D1, and TNF- α levels in the eyecups, compared with the control IgG (Figs. 8E, 8F).

We have also measured the effect of Mab2F1 on retina vascular leakage, as VLDLR KO mice were reported to have increased retinal vascular permeability, compared with age-matched WT mice.²³ Mab2F1 significantly reduced the retinal vascular leakage in VLDLR KO mice, while the control IgG treatment did not result in significant changes in retinal vascular permeability (Fig. 8G). Taken together, these results confirmed that Mab2F1 also inhibited activation of Wnt signaling in the genetic NV model and significantly reduced retina vascular leakage in VLDLR KO mice.

Alternation of Wnt Ligands and Fz Receptor mRNA Levels in Rat Eyecups with Laser-Induced CNV

To determine the mechanism of Wnt signaling activation in laser-induced CNV, we measured mRNA levels of canonical Wnt ligands and Fzs in laser-induced CNV rats and compared

FIGURE 2. Activated Wnt signaling in laser-induced CNV mouse eyecups. At day 7 postlaser photocoagulation, the same amount (50 μ g) of eyecup protein from each mouse was used for Western blot analysis. (A) Total and p-LRP6. (C) Total, p-, and cyto- β -catenin. (E) VEGF, c-Myc, and CyclinD1. (G) ICAM-1 and TNF- α . (B, D, F, H) The results were semiquantified by densitometry and normalized by β -actin levels (mean $\pm$ SD, $n = 3$, $^*P < 0.05$, $^{**}P < 0.01$, compared with untreated normal group).

FIGURE 3. Mab2F1 inhibited the activation of Wnt signaling and attenuated overexpression of inflammatory factors in laser-induced CNV rat eyecups. Rats were intravitreally injected with 15 μ g Mab2F1 at the same day as the laser photocoagulation, with the same amount nonspecific IgG as control. At day 7 postlaser photocoagulation, the same amount (50 μ g) of eyecup proteins from each rat was immunoblotted with antibodies. (A) Total LRP6 and p-LRP6. (C) Total, p-, and cyto- β -catenin. (E) VEGF, c-Myc, and CyclinD1. (G) ICAM-1 and TNF- α . (B, D, F, H) The results were semiquantified by densitometry and normalized by β -actin levels (mean $\pm$ SD, $n = 3$, * $P < 0.05$, ** $P < 0.01$, compared with IgG-treated control group).

FIGURE 4. Mab2F1 inhibited the activation of Wnt signaling and attenuated overexpression of inflammatory factors in laser-induced CNV mouse eyecups. Mice received an intravitreal injection of 5 μ g Mab2F1 at the same day of laser treatment, with the same amount of nonspecific IgG for control. At day 7 postlaser photocoagulation, the same amount (50 μ g) of eyecup protein from each mouse was immunoblotted with antibodies. (A) Total LRP6 and p-LRP6. (C) Total, p-, and cyto- β -catenin. (E) VEGF, c-Myc, and CyclinD1. (G) ICAM-1 and TNF- α . (B, D, F, H) The individual protein levels were semiquantified with densitometry and normalized by β -actin levels (mean $\pm$ SD, $n = 3$, * $P < 0.05$, ** $P < 0.01$, compared with IgG-treated control group).

FIGURE 5. Mab2F1 decreased numbers of grade 4 lesions in laser-induced CNV rats. Rats were intravitreally injected with 15 μ g Mab2F1 at the same day as the laser treatment, with nonspecific IgG for control. The fluorescein fundus angiograph was examined at day 21 postlaser photocoagulation. (A) Untreated CNV group. (B) CNV rats with nonspecific IgG treatment. (C) CNV rats with Mab2F1 treatment. (D) Numbers of lesions of different grades were recorded at days 7, 14, and 21 postlaser photocoagulation. (E) The grade 4 CNV lesions per rat were examined, quantified, and compared at day 21 (mean $\pm$ SD, $n = 5$, $**P < 0.01$, compared with IgG-treated control group). Red arrows indicate the lesions caused by laser shot.

FIGURE 6. Mab2F1 decreased the neovascular area in laser-induced CNV rats. Rats were intravitreally injected with 15 μ g nonspecific mouse IgG or Mab2F1 at the same day as the laser treatment. The angiographs of CNV lesion in the retina/RPE/choroid wholemount were examined at day 21 postlaser photocoagulation. (A) Untreated control group. (B) Control IgG-treated group. (C) Mab2F1-treated group. (D) The average neovascular areas of CNV lesions were quantified (mean $\pm$ SD, $n = 20$, $^*P < 0.05$, compared with IgG-treated control group) as described in "Methods." Red lines represent the edge of area of CNV lesions.

them with those in control rat eyecups. The results showed that mRNA levels of Wnt3a, Fz1, Fz5, and Fz10 were significantly increased in the eyecups with laser-induced CNV (Figs. 9A, 9B).

DISCUSSION

The results, presented here for the first time, demonstrated that the canonical Wnt signaling pathway is activated in both laser-induced CNV rat and mouse models. The present study also showed that Mab2F1, a novel monoclonal antibody specific for LRP6—a coreceptor in canonical Wnt signaling—alleviated vascular leakage and CNV through inhibition of the Wnt signaling in these CNV models. Furthermore, Mab2F1 also attenuated the activation of Wnt signaling and reduced the retinal vascular leakage in VLDLR KO mice, a genetic model of subretinal NV.²¹ These observations indicate that the activation of the canonical Wnt signaling pathway plays a pathogenic role in CNV and may represent a new therapeutic target for wet AMD.

Laser photocoagulation-induced CNV is considered a wound healing process under ischemia condition,^{27,28} in

which oxidative stress and inflammation play critical roles.^{29–31} dative stress was found to activate the canonical Wnt pathway in the retinas of oxygen-induced retinopathy rats.³² Moreover, inflammation pathways have potent cross-talks with the Wnt signaling pathway,³³ and our previous study showed that inflammation factors such as TNF- α and ICAM-1 are upregulated in the rat retina after activation of the Wnt pathway.²⁴ Our results demonstrate that levels of LRP6, phosphorylated LRP6, and β -catenin are significantly elevated in the eyecups of the laser-induced CNV rodent models, demonstrating activation of Wnt signaling. LRP6 is expressed in multiple cell types such as RPE cells, retina endothelial cells, Müller cells, and photoreceptor cells in the eye (see Supplementary Material and Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10281/-/DCSupplemental>). Thus, the cellular mechanism for Wnt signaling activation in laser-induced CNV may be involved with multiple cell types in the eye. In the present study, we simulated hypoxic condition of laser-induced CNV using CoCl₂ treatment in RPE cells (Fig. 7) and Müller cells (see Supplementary Material and Supplementary Fig. S2, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10281/-/DCSupplemental>). Our results indicated that Wnt signaling is activated by hypoxia in these cells.

FIGURE 7. Mab2F1 inhibited the activation of Wnt signaling induced by hypoxia in ARPE19 cells. ARPE19 cells were exposed to 200 μ M CoCl₂ for 2 hours, followed by incubation with 100 μ g/mL Mab2F1 or nonspecific mouse IgG for 24 hours. (A) Total LRP6, p-LRP6, and total, p- β -catenin and cyto- β -catenin. (D) VEGF, c-Myc, CyclinD-1, ICAM-1, and TNF- α were measured by Western blot analysis using specific antibodies. (B, C, E, F) These protein levels were semi-quantified with densitometry and normalized by β -actin levels (mean $\pm$ SD, $n = 3$, * $P < 0.05$, ** $P < 0.01$, compared with CoCl₂-treated ARPE19 cells incubated with IgG group).

FIGURE 8. Mab2F1 inhibited the activation of Wnt signaling and reduced retinal vascular leakage in VLDLR KO mice. VLDLR^{-/-} mice were intravitreally injected with 5 μ g/eye control IgG or Mab2F1. At day 7 postinjection, the same amount (50 μ g) of eyecup proteins from each mouse was blotted with antibodies. (A) Total LRP6 and p-LRP6, (C) Non-p- and cyto- β -catenin, (E) VEGF, c-Myc, CyclinD-1, and TNF- α . (B, D, F) These proteins were semiquantified with densitometry and normalized by β -actin levels (mean $\pm$ SD, $n = 3$, * $P < 0.05$, ** $P < 0.01$). (G) Retinal vascular permeability was measured using the Evans blue-albumin leakage method at day 7 postinjection in age-matched wt C57BL/6 group, untreated VLDLR^{-/-} group, control IgG-treated VLDLR^{-/-} group, and Mab2F1-treated VLDLR^{-/-} group. The vascular permeability was normalized by total protein concentrations in the retina (mean $\pm$ SD, $n = 3$, * $P < 0.05$, compared with IgG-treated control group).

FIGURE 9. mRNA levels of canonical Wnt ligands and Fz receptors in the eyecups of laser-induced CNV rats. Total RNA was isolated from the eyecups of CNV rats at day 7 postlaser photocoagulation and age-matched normal rats. Real-time RT-PCR was performed to measure mRNA levels. (A) Canonical Wnt ligands. (B) Fz receptors as indicated (mean $\pm$ SD, $n = 4$, * $P < 0.05$, ** $P < 0.01$, compared with un-treated normal control group).

Another important finding in this study is that Mab2F1 significantly inhibits the activation of Wnt signaling in laser-induced CNV models, a genetic subretinal NV animal model and cultured RPE cells, resulting in the downregulation of inflammatory factors and reduced vascular leakage. The canonical Wnt pathway can be blocked at different levels (i.e., secreted Wnt ligands, Fzs, and LRP5/6 receptors in the cell membrane; Axin, GSK3 β , and β -catenin in the cytoplasm; and β -catenin and TCF/LEF in the nucleus). Since there are 19 Wnts and 10 Fzs but only 2 coreceptors (LRP5/6),³⁴ LRP5/6 are considered ideal and specific targets for blocking the canonical Wnt pathway.³⁵ Modulation of LRP5/6 is more feasible than regulation of the intracellular components of the canonical Wnt pathway. Similar to Dickkopf-related protein 1 (DKK1)—a well-known inhibitor of Wnt signaling,^{36–38}—Mab2F1 has specific interactions with the extracellular domain of LRP6,

and thus blocks the activation of LRP6 and consequently, inhibiting the Wnt signaling pathway. This result is consistent with previous studies using anti-LRP6 antibodies in cancer cells,^{35,39} suggesting that using anti-LRP6 antibodies represent an effective strategy to block the Wnt pathway. Moreover, the present results showed that Mab2F1 suppressed total LRP6 levels both in vivo and in vitro after activation of Wnt signaling. In our previous study, we made a similar observation that Mab2F1 reduces total LRP6 levels in hTERT-RPE-1 cells.⁴⁰ Similar phenomena have been reported in other antibodies. For example, an anti-EGFR antibody was shown to induce internalization of EGFR.⁴¹ However, the mechanism of the total LRP6 level decrease induced by Mab2F1 binding demands further study, which may be caused by the antibody binding-induced conformational change, accelerating endocytosis and degradation of LRP6. Although VEGF and other inflammatory

factors are regulated by multiple signaling pathways in laser-induced CNV model, the inhibitory effects of Mab2F1 indicated that Wnt signaling pathway is at least one of the major pathways contributing to the overexpression of these angiogenic and inflammatory factors in this model. Taken together, our results indicate that Mab2F1 is a novel, specific inhibitor of the canonical Wnt pathway and has therapeutic potential for wet AMD via blockade of multiple inflammatory and angiogenic factors including TNF- α , ICAM-1, and VEGF.

The Wnt signaling pathway has been reported to mediate multiple biological and pathological processes including inflammation, angiogenesis and fibrosis.⁵ Although the laser-induced CNV is an inflammatory and angiogenic process, the implication of the Wnt pathway in CNV has not been established. The present study not only demonstrated the activation of the Wnt pathway at multiple levels including Wnt ligands, LRP6, and β -catenin, but also showed that blockade of Wnt signaling can attenuate the inflammation and CNV. These observations support the pathogenic role of Wnt signaling activation in CNV. Previously, our studies showed that the Wnt pathway plays a pathogenic role in diabetic retinopathy.³² Therefore, identification of activation of the Wnt signaling pathway in laser-induced CNV in this study may provide important insights into the molecular mechanisms for ocular inflammation and neovascular diseases such as AMD. These results also suggest that blockade of Wnt signaling can simultaneously downregulate multiple inflammatory and angiogenic factors, which should be more efficient in amelioration of inflammation and NV. The Wnt pathway may become a promising target for the drug treatment of diabetic retinopathy and wet AMD.

References

- Munoz B, Klein R, Rodriguez J, Snyder R, West SK. Prevalence of age-related macular degeneration in a population-based sample of Hispanic people in Arizona: Proyecto VER. *Arch Ophthalmol*. 2005;123:1575-1580.
- Edwards AO, Ritter R III, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. *Science*. 2005;308:421-424.
- Orsulic S, Peifer M. Cell-cell signalling: wingless lands at last. *Curr Biol*. 1996;6:1363-1367.
- Tamai K, Zeng X, Liu C, et al. A mechanism for Wnt coreceptor activation. *Mol Cell*. 2004;13:149-156.
- Miller JR. The Wnts. *Genome Biol*. 2002;3:REVIEWS3001.
- Dale TC. Signal transduction by the Wnt family of ligands. *Biochem J*. 1998;329(part 2):209-223.
- Bilic J, Huang YL, Davidson G, et al. Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. *Science*. 2007;316:1619-1622.
- Liu G, Bafico A, Harris VK, Aaronson SA. A novel mechanism for Wnt activation of canonical signaling through the LRP6 receptor. *Mol Cell Biol*. 2003;23:5825-5835.
- Piao S, Lee SH, Kim H, et al. Direct inhibition of GSK3 β by the phosphorylated cytoplasmic domain of LRP6 in Wnt/ β -catenin signaling. *PLoS One*. 2008;3:e4046.
- Wu G, Huang H, Garcia Abreu J, He X. Inhibition of GSK3 phosphorylation of β -catenin via phosphorylated PPPSPXS motifs of Wnt coreceptor LRP6. *PLoS One*. 2009;4:e4926.
- Beagle B, Johnson GV. Differential modulation of TCF/LEF-1 activity by the soluble LRP6-ICD. *PLoS One*. 2010;5:e11821.
- Ojesina AI. The role of β -catenin in regulating angiogenesis in Wilms tumor. *J Pediatr Surg*. 2004;39:1446-1447; author reply 1447.
- Tachikawa K, Schroder O, Frey G, Briggs SP, Sera T. Regulation of the endogenous VEGF-A gene by exogenous designed regulatory proteins. *Proc Natl Acad Sci U S A*. 2004;101:15225-15230.
- Easwaran V, Lee SH, Inge L, et al. β -Catenin regulates vascular endothelial growth factor expression in colon cancer. *Cancer Res*. 2003;63:3145-3153.
- Lichtlen PD, Lam T, Nork M, Streit T, Urech DM. Relative contribution of VEGF and TNF- α in the cynomolgus laser-induced CNV model: comparing efficacy of bevacizumab, adalimumab and ESBA105. *Invest Ophthalmol Vis Sci*. 2010;51:4738-4745.
- Lu M, Adamis AP. Molecular biology of choroidal neovascularization. *Ophthalmol Clin North Am*. 2006;19:323-334.
- Miller H, Miller B, Ishibashi T, Ryan SJ. Pathogenesis of laser-induced choroidal subretinal neovascularization. *Invest Ophthalmol Vis Sci*. 1990;31:899-908.
- Toma HS, Barnett JM, Penn JS, Kim SJ. Improved assessment of laser-induced choroidal neovascularization. *Microvasc Res*. 2010;80:295-302.
- Sheets KG, Zhou Y, Ertel MK, et al. Neuroprotectin D1 attenuates laser-induced choroidal neovascularization in mouse. *Mol Vis*. 2010;16:320-329.
- Takehana Y, Kurokawa T, Kitamura T, et al. Suppression of laser-induced choroidal neovascularization by oral tranilast in the rat. *Invest Ophthalmol Vis Sci*. 1999;40:459-466.
- Chen Y, Hu Y, Lu K, Flannery JG, Ma JX. Very low density lipoprotein receptor, a negative regulator of the wnt signaling pathway and choroidal neovascularization. *J Biol Chem*. 2007;282:34420-34428.
- Zhou T, He X, Cheng R, et al. Implication of dysregulation of the canonical wingless-type MMTV integration site (WNT) pathway in diabetic nephropathy. *Diabetologia*. 2012;55:255-266.
- Chen Y, Hu Y, Moiseyev G, Zhou KK, Chen D, Ma JX. Photoreceptor degeneration and retinal inflammation induced by very low-density lipoprotein receptor deficiency. *Microvasc Res*. 2009;78:119-127.
- Zhou T, Hu Y, Chen Y, et al. The pathogenic role of the canonical Wnt pathway in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2010;51:4371-4379.
- Zhou T, Zhou KK, Lee K, et al. The role of lipid peroxidation products and oxidative stress in activation of the canonical wingless-type MMTV integration site (WNT) pathway in a rat model of diabetic retinopathy. *Diabetologia*. 2011;54:459-468.
- Zhang XZ, Jiang JB, Luo XQ, et al. Effect of vascular endothelial growth factor small interfering RNA (siRNA) on retinal microvascular endothelial cells under hypoxia condition in vitro [in Chinese]. *Zhonghua Er Ke Za Zhi*. 2009;47:457-461.
- Falkenstein IA, Cheng L, Wong-Staal F, et al. Toxicity and intraocular properties of a novel long-acting anti-proliferative and anti-angiogenic compound IMS2186. *Curr Eye Res*. 2008;33:599-609.
- Muther PS, Semkova I, Schmidt K, et al. Conditions of retinal glial and inflammatory cell activation after irradiation in a GFP-chimeric mouse model. *Invest Ophthalmol Vis Sci*. 2010;51:4831-4839.
- Tsubota K. Oxidative stress and inflammation: hypothesis for the mechanism of aging [in Japanese]. *Nihon Ganka Gakkai Zasshi*. 2007;111:193-205; discussion 206.
- Zhuang P, Shen Y, Lin BQ, Zhang WY, Chiou GC. Effect of quercetin on formation of choroidal neovascularization (CNV) in age-related macular degeneration (AMD). *Yan Ke Xue Bao*. 2011;26:23-29.
- Shi X, Semkova I, Muther PS, Dell S, Kociok N, Joussen AM. Inhibition of TNF- α reduces laser-induced choroidal neovascularization. *Exp Eye Res*. 2006;83:1325-1334.

32. Chen Y, Hu Y, Zhou T, et al. Activation of the Wnt pathway plays a pathogenic role in diabetic retinopathy in humans and animal models. *Am J Pathol*. 2009;175:2676–2685.
33. Jasielska M, Semkova I, Shi X, et al. Differential role of tumor necrosis factor (TNF)- α receptors in the development of choroidal neovascularization. *Invest Ophthalmol Vis Sci*. 2010;51:3874–3883.
34. Mi K, Johnson GV. Role of the intracellular domains of LRP5 and LRP6 in activating the Wnt canonical pathway. *J Cell Biochem*. 2005;95:328–338.
35. Gong Y, Bourhis E, Chiu C, et al. Wnt isoform-specific interactions with coreceptor specify inhibition or potentiation of signaling by LRP6 antibodies. *PLoS One*. 2010;5:e12682.
36. Glinka A, Wu W, Delius H, Monaghan AP, Blumenstock C, Niehrs C. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature*. 1998;391:357–362.
37. Sakane H, Yamamoto H, Kikuchi A. LRP6 is internalized by Dkk1 to suppress its phosphorylation in the lipid raft and is recycled for reuse. *J Cell Sci*. 2010;123:360–368.
38. Bafico A, Liu G, Yaniv A, Gazit A, Aaronson SA. Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. *Nat Cell Biol*. 2001;3:683–686.
39. Ettenberg SA, Charlat O, Daley MP, et al. Inhibition of tumorigenesis driven by different Wnt proteins requires blockade of distinct ligand-binding regions by LRP6 antibodies. *Proc Natl Acad Sci U S A*. 2010;107:15473–15478.
40. Lee K, Hu Y, Ding L, et al. Therapeutic potential of a monoclonal antibody blocking the wnt pathway in diabetic retinopathy. *Diabetes*. 2012;61:2948–2957.
41. Berger C, Madhus IH, Stang E. Cetuximab in combination with anti-human IgG antibodies efficiently down-regulates the EGF receptor by macropinocytosis. *Exp Cell Res*. 2012;318:2578–2591.