A Nonautonomous Role for Retinal Frizzled-5 in Regulating Hyaloid Vitreous Vasculature Development

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PURPOSE. Frizzled-5 (Fzd5) is expressed in the developing retina of multiple species and appears to play species-specific roles during eye development. The present study analyzed the effects of tissue-specific deletion of Fzd5 on mammalian eye development.

METHODS. To generate Fzd5 conditional knockout (CKO) mice, Fzd5 +/− mice carrying the Six3-Cre transgene were crossed with Fzd5loxP/loxP mice. To determine which cell lineages in the eye had Cre recombinase activity, Six3-Cre transgenic mice were crossed with ROSA26 reporter mice, and lacZ activity was assayed. Histologic analysis, immunofluorescence, and TUNEL labeling were performed from embryonic day (E)12.5 to postnatal stages to analyze vascularization, cell proliferation, retinal organization, and apoptosis.

RESULTS. On conditional disruption of Fzd5 specifically in the retina, but not in vitreous hyaloid vasculature (VHV), an abnormal accumulation consisting of pericytes and endothelial cells was observed in the vitreous as early as E12.5. The abundant retrolental cells persisted into postnatal stages and appeared as a pigmented intravitreal mass. In Fzd5 CKO mice there was failure of normal apoptosis of the VHV, and cells in the persistent VHV were maintained in the cell cycle up to postnatal day 23. Moreover, morphogenesis of the retina adjacent to the vasculature was disrupted, leading to retinal folds, detachment, and abnormal lamination. This phenotype is similar to that of human eye disease persistent hyperplastic primary vitreous (PHPV).

CONCLUSIONS. Selective loss of Fzd5 in the retina results in PHPV and retinal defects through an apparently cell-nonautonomous effect, revealing a potential requirement for retina-derived signals in regulating the development of the VHV. (Invest Ophthalmol Vis Sci. 2008;49:5561–5567) DOI:10.1167/iovs.08-2226

The Wnt genes encode a large family of secreted glycoproteins that elicit cellular responses by binding with membrane receptors, including the Frizzled (Fzd) and low-density lipoprotein-related receptors 5/6. Ten Fzd family members have been identified in humans and mice. Fzd5 is expressed in the developing retina in zebrafish, Xenopus laevis, chick, and mouse, which suggests a specific role for this receptor in regulating eye development. In zebrafish, Fzd5 mediates non-canonical signaling and promotes eye field formation. 1 In X. laevis, Fzd5 activates the Wnt/β-catenin (canonical) pathway in the retina and controls the neural potential of retinal progenitors by regulating Sox2 expression. 2 In mouse, Fzd5 is highly expressed in developing retina from embryonic day (E)8.5 to postnatal stages 3,4 but is not required for early eye formation or for regulating neural potential of retinal progenitors, 5 suggesting species-specific roles for Fzd5 in eye development. However, disruption of the Fzd5 gene results in embryonic lethality at approximately E10.5 6; thus, the role of Fzd5 at later stages of mammalian eye development is unknown.

During eye development a transient vascular system called the primary vitreous hyaloid vascular system (HVS) is formed. This vascular system provides nutrition to the developing lens and retina before the intraretinal vasculature is formed. Formation of the intraretinal vasculature begins with the entry of the hyaloid artery into the primitive vitreous through the fetal fissure, which then branches in the vitreous to form the vasa hyaloida propria and the tunica vasculosa lentis. 7 In mouse, the formation of HVS starts at E10.5 and is fully formed by E13.5. Because the HVS is a transient capillary network, it undergoes regression through apoptosis at postnatal stages, which ensures the mature vitreous is avascular. Most of the branches of HVS regress by postnatal day (P)10, and the vitreous is completely avascular by P16. 8,9,10 Failure of HVS regression causes the persistence of this fetal vasculature at postnatal stages and the formation of a retrolental mass.

Disruption of several genes, including Arf, can cause hyperplasia of the HVS even earlier, at embryonic stages, with a subsequent failure of regression. 11 This results in abnormal retrolental tissue that can give rise to cataract, secondary glaucoma, retinal folding, dysplasia, and retinal detachment, 7,9,12 similar to the congenital human eye disease persistent hyperplastic primary vitreous (PHPV). In human patients the mechanisms underlying this disease remain unclear.

To investigate the function of Fzd5 at later stages of mammalian eye development, we conditionally disrupted the Fzd5 gene specifically in the mouse retina. In the absence of retinal Fzd5, we observed hyperplastic hyaloid vitreous vasculature at embryonic stages in the mouse eye. This hyperplastic vitreous vasculature became heavily pigmented and failed to regress at postnatal stages. Retinal abnormalities such as retinal folds, detachment, and disrupted retina lamination occurred in the retina adjacent to the hyperplastic vitreous vasculature. This phenotype is reminiscent of the human eye disease PHPV. Given that Fzd5 was inactivated in the retina but not in the vitreous vasculature tissue, the persistent hyperplastic vitreous vasculature was likely caused by a cell-nonautonomous mechanism. Our data suggest that Fzd5 regulates the expression of retina-derived signals that influence hyaloid vitreous vasculature development in mammals.

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METHODS

Mice and Genotyping

Mice carrying a null allele of the Fzd5 gene (Fzd5<sup>−/−</sup>) were provided by Makoto Taketo.<sup>6</sup> Mice with Boxed Fzd5 alleles (Fzd5<sup>loxP/loxP</sup>) were provided by Hans Clevers.<sup>13</sup> Six3-Cre transgenic mice were provided by Yasuhide Furuta<sup>14</sup> (Cre line 69). Six3-Cre females heterozygous for the Fzd5 null allele (Fzd5<sup>−/−</sup> Six3-Cre) were crossed with Fzd5<sup>loxP/loxP</sup> males to generate conditional Fzd5 mutant embryos (Fzd5<sup>loxP/loxP</sup> Six3-Cre). Littermates carrying a wild-type allele of Fzd5 were used as controls.

PCR was used to identify the genotypes of the embryos and mice, as previously reported.<sup>6,13,14</sup> Primers complementary to neomycin (pn5b, CTA AAG GCC ATG CTC CAG ACT) and to Fzd5 downstream of (Neo, GCG CAT GCT CCA GAC TG) were used to genotype the floxed Fzd5 allele, generating a 700-bp PCR product. The wild-type allele was determined using primers of f5x (AGA GGAGGC TAT ACG TAA CAG GG). The null allele was determined using primers complementary to neomycin (Neo, GCC CAT GCT CCA GAC TG) and to Fzd5 (Fzd5, GAT GAG GAA GAG TGA CAC GA). The Cre gene was identified by using primers Cret159 (TGG ATG CAA CGA GTG ATG AG) and Cre160 (TTC GGC TAT AGC TAA CAG GG).

LacZ Activity

Six3-Cre homozygous transgenic mice were crossed with ROSA-26 reporter mice. The tissue was fixed in 4% paraformaldehyde in PBS for 25 minutes at room temperature, washed in PBS, saturated in 25% sucrose, and embedded in optimum cutting temperature compound (Tissue-Tek; Sakura Finetek, Torrance, CA). LacZ activity was analyzed on cryostat sections (16 µm) at E14.5 and P0 (n = 3 for each age) using X-gal substrate (USB Corporation, Cleveland, OH).

Histologic Analysis

Embryo and eye tissue at ages E12.5, E14.5, E17.5, P0, P8, P10, and P23 were fixed in 4% paraformaldehyde in PBS for 2 hours at room temperature or overnight at 4°C, saturated in 25% sucrose, and embedded as described. Cryostat sections (16 µm) were stained with hematoxylin (Fisher Scientific, Pittsburgh, PA) and eosin (Fisher Scientific). Sections crossing the central area of the eye, where eye and lens sizes were greatest, were used to measure the greatest anteroposterior diameter of the eye and the thickness and diameter of the lens. Thickness of cornea and retina were also measured on these sections. Student’s t-test was used for statistics analysis.

In Situ RNA Hybridization

In situ RNA hybridization was performed as previously described<sup>15</sup> on serial cryostat sections of embryos at E13 through the central region of the eye. Digoxigenin-labeled antisense Fzd5 riboprobe (1 µg/mL) was used for the procedure. To ensure reproducibility, in situ hybridizations were repeated on both eyes from three wild-type embryos.

Immunofluorescence and Apoptosis Analysis

Cryostat sections were processed for immunofluorescence staining using the following primary antibodies: rat anti-PECAM-1 (1:75; BD PharMingen, San Diego, CA), goat anti-Brn3b (1:50; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-proliferating cell nuclear antigen (PCNA: 1:500; DAKO, Carpinteria, CA), rabbit anti–NG2 chondroitin sulfate proteoglycan (1:500; Chemicon, Temecula, CA), mouse anti-Tuj1 (1:1000; Covance, Princeton, NJ), and rabbit anti–Otx2 (1:1500; Chemicon). For PCNA labeling, antigen retrieval with citric acid and sodium citrate buffer (2 mM citric acid, 9 mM sodium citrate) was used. For visualization, sections were incubated with Alexa 568- or Alexa 488–conjugated secondary antibodies (1:1000–1:5000; Molecular Probes, Eugene, OR) and were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Roche, Basel, Switzerland). Cell apoptosis was monitored with a cell detection kit (In Situ Cell Death Detection Kit, Fluorescein; Roche) according to the manufacturer’s instructions. Images were captured using a microscope (E800; Nikon, Tokyo, Japan) equipped with an RT slider digital camera (Diagnostic Instruments, Sterling Heights, MI). Throughout this study mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RESULTS

Abnormal Retrolental Tissue and Retina Dysplasia at Embryonic Stages Caused by Loss of Fzd5

To investigate Fzd5 function during later stages of mouse eye development, Six3-Cre–mediated recombination was used to generate Fzd5 conditional knockout mice. Six3-Cre mice were crossed with ROSA-26 reporter mice to determine which cell lineages exhibit successful Cre-mediated recombination. X-gal staining at E14.5 and P0 (n = 3 for each age) showed that β-galactosidase activity is specifically restricted to the neuroretina and optic nerve (Figs. 1A, 1B, and data not shown) but does not occur in the hyaloid vitreous vasculature tissue and the lens (Fig. 1B). In the original report describing the generation of the Six3-Cre lines, lacZ activity is detected in the vitreous vasculature in a cross with the ROSA-26 reporter.<sup>14</sup> However, this was for the transgenic line Cre-i9 (which has been discontinued), whereas the line used here was Cre-i99, which does not show activity in the vitreous vasculature (Fig. 1B, arrow; Yas Furuta, personal communication, August 7, 2008). Furthermore, in situ hybridization analysis showed that Fzd5 is expressed in the retina and optic stalk (Fig. 1C) but not in cells of the vitreous vasculature (Fig. 1D, arrow).

To investigate the roles of Fzd5 during mouse eye development, the ocular morphology of Fzd5 conditional knockout mice was analyzed at E12.5, E14.5, and E17.5 by hematoxylin and eosin staining. At E12.5 (n = 3) and E14.5 (n = 3), abnormally abundant retrolental mass tissue was observed in the vitreous body of Fzd5<sup>loxP/loxP</sup>;Six3-Cre embryos, possibly representing hyaloid vitreous vasculature tissue (Figs. 2B, 2D, arrowheads). In the Fzd5<sup>loxP/loxP</sup>;Six3-Cre embryos at E14.5, the retina was 51% thinner and the cornea was 120% thicker than in controls, respectively (P < 0.01). The lens diameter was also reduced by 17% in the Fzd5<sup>loxP/loxP</sup>;Six3-Cre embryos (seven eyes analyzed) compared with control (six eyes analyzed), but this was variable and not highly significant (P = 0.037). In addition, the laminar architecture of the neoblasts in the retinal area that was adjacent to the abnormal retrolental mass tissue was disrupted (Fig. 2D, white arrow). This disruption was not observed in the retinal area distant from the retrolental mass tissue. At E17.5 (n = 2), abnormal retrolental mass tissue persisted in the vitreous and the posterior surface of the lens (Fig. 2F, arrowhead), and in some instances this mass tissue extended into the optic nerve. The disorganization of the retina adjacent to the vitreous mass tissue was more obvious at this stage (Fig. 2F, white arrow). Sagittal sections at E14.5 did not reveal defects in optic tissue closure (data not shown). These results suggest that loss of Fzd5 in the retina results in the excessive formation of retrolental mass tissue in the vitreous through a cell-nonautonomous mechanism because neither Fzd5 expression nor Cre recombinase activity was detectable in the vitreous and lens.

Development of Retrolental Mass Tissue in Fzd5<sup>loxP/loxP</sup>;Six3-Cre Mice into Pigmented Hyperplastic Primary Vitreous Vasculature

To determine the identity of the retrolental mass tissue observed in Fzd5<sup>loxP/loxP</sup>;Six3-Cre mice, immunofluorescence stain-
Abundant PECAM-positive cells were also found in the abnormalities, (data not shown), suggesting that the mass tissue in the retina was heavily pigmented by P0 (Fig. 3N, arrowhead); hence, it remains possible that neural crest-derived melanocytes migrate to the vitreous or that cells from the retinal pigment epithelium migrate into the mass at late embryonic stages. Other cell types may be present in the mass at this stage, including cells derived from the retina sclera or optic nerve. We conclude that initially the abnormal retrolental mass tissue in the Fzd5LoxP/+;Six3-Cre eye is hyperplastic primary vitreous vasculature, though additional cell types may ultimately contribute to the phenotype.

Persistence of Retrolental Hyperplastic Vitreous Vasculature at Postnatal Stages

Normally, the primary vitreous vasculature is a transient blood vessel system that regresses through apoptosis at postnatal stages to ensure that the mature vitreous is avascular.\(^9\)\(^{\text{-10}}\) However, the hyperplastic vitreous vasculature in the Fzd5LoxP/+;Six3-Cre eye persisted as a pigmented mass tissue at postnatal stages P0 (n = 7), P10 (n = 4), and even P23 (n = 6); Figs. 4B, 4E, 4H). This hyperplastic vitreous vasculature was occasionally attached to the lens or to the inner surface of the retina (Figs. 4B, 4E). In addition, the eye size in Fzd5LoxP/+;Six3-Cre mice was reduced by 24% (\(P < 0.05\)) compared with control littermates at P0. Morphogenesis of the retina adjacent to the hyperplastic vitreous vasculature was disrupted, leading usually to multiple abnormalities such as partial retinal detachment and occasionally to complete detachment, retinal folds, and abnormal retinal lamination, especially in the inner retinas and rosette structures (data not shown; Figs. 4C, 4E, 4F, 4H, 4I). The retina distant from the hyperplastic vitreous vasculature appeared morphologically normal (Figs. 4E, 4H, and data not shown), suggesting that retinal dysplasia is a secondary

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One possibility was that Fzd5 mutant cells derived from the neural retina or optic nerve migrate to the vitreous to generate the phenotype. However, because endothelial cells and pericytes are derived from the pericocular mesenchyme, do not express Fzd5, and are not present in the retina at E12.5 and E14.5 (Figs. 3B, 3F), these excess endothelial cells and pericytes in the vitreous of Fzd5LoxP/+;Six3-Cre mice were unlikely to have migrated from the retina. In fact, the opposite was observed, with occasional invasion of PECAM-positive endothelial cells and NG2-positive pericytes into Fzd5LoxP/+;Six3-Cre retina (Figs. 3D, 3H, arrows). Furthermore, we confirmed that at the stages between E14.5 and P0, the retrolental cells did not express neural progenitor markers (Pax6, Chx10), markers of retinal neurons (TUJ1, Brn3b), or retinal pigment epithelium (Otx2; see Supplementary Fig. S1, online at http://www.iovs.org/cgi/content/full/49/12/5561/DC1, and data not shown), further suggesting that the excess cells were not derived from the neural retina or retinal pigment epithelium. Bright-field imaging indicated that the retrolental mass tissue was heavily pigmented by P0 (Fig. 3N, arrowhead); hence, it remains possible that neural crest-derived melanocytes migrate to the vitreous or that cells from the retinal pigment epithelium migrate into the mass at late embryonic stages. Other cell types may be present in the mass at this stage, including cells derived from the retina sclera or optic nerve. We conclude that initially the abnormal retrolental mass tissue in the Fzd5LoxP/+;Six3-Cre eye is hyperplastic primary vitreous vasculature, though additional cell types may ultimately contribute to the phenotype.

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effect caused by the interaction between the hyperplastic vitreous vasculature and the adjacent retina and is not directly caused by the loss of Fzd5 in the retina.

**Disorganization of Ganglion Cell Layer Adjacent to the Hyperplastic Vitreous Vasculature Showing an Increase in Cell Death**

Given that morphogenesis of the retina adjacent to the hyperplastic vitreous vasculature was disrupted and the lamination of the ganglion cell layer (GCL) appeared to be most severely affected (Figs. 4C, 4E, 4F, 4H), we further analyzed the morphology of the GCL by performing immunostaining for the ganglion cell marker Brn-3b. The GCL was formed properly in control Fzd5LoxP/H11001;Six3-Cre mice (n/H11005 2) (Supplementary Fig. S1E). However, in Fzd5LoxP/H11002;Six3-Cre mice (n/H11005 4), the lamination of the GCL was severely disrupted and mixed with the neuroblast layer (Supplementary Fig. S1F).

The dysplasia of the retina adjacent to the hyperplastic vitreous vasculature suggested that cell survival of the dysplastic retina could be affected; therefore, TUNEL staining was performed at P0 to monitor cell apoptosis. In the retinas of Fzd5LoxP+/Six3-Cre mice (n = 3), only a few TUNEL-positive cells were observed in the GCL (Figs. 5A–C). However, many TUNEL-positive cells were observed in the disorganized area of the GCL in Fzd5LoxP+/Six3-Cre mice (n = 4) (Figs. 5D–F), which suggested that cell death was increased in disorganized areas of the retina.

**Abnormal Apoptosis and Proliferation of the Hyperplastic Vitreous Vasculature Associated with Failure of Vascular Regression**

The primary vitreous vasculature normally regresses through apoptosis beginning at P4 and peaking at P7 to P8, with all the vasculature regressed by P16.8–10 Inhibition of apoptosis can result in persistence of the vitreous vasculature.16–18 In Fzd5LoxP+/Six3-Cre mice, the vitreous vasculature was persistent even at P23 (Figs. 4H, 4I). We therefore assessed apoptosis in the vitreous vasculature to determine whether it might be reduced. In control Fzd5LoxP+/Six3-Cre mice, a few remaining vasculature cells were observed on the posterior surface of the lens and in the vitreous at P10 (Figs. 6A, 6B), and these were TUNEL positive (Figs. 6C, 6D), consistent with previously re-
ported results. However, no TUNEL labeling was detectable in the hyperplastic vitreous vasculature cells of Fzd5LoxP/;Six3-Cre mice at P10 (n = 4) and P23 (n = 3; Figs. 6G–I and data not shown). The hyperplastic vitreous vasculature in the Fzd5LoxP/;Six3-Cre eye failed to regress; we tested the cell proliferation status of these cells by immunostaining for PCNA, which is expressed in all phases of the cell cycle. In control mice at P8 and P10, PCNA-positive cells were observed in the inner nuclear layer of the retina but not in the vitreous (Figs. 7A–C and data not shown). In contrast, in Fzd5LoxP/;Six3-Cre eyes, the hyperplastic vitreous vasculature cells were PCNA positive (P8, n = 2; P10, n = 3; Figs. 7D–F and data not shown). Thus, persistence of the vitreous vasculature in Fzd5LoxP/;Six3-Cre mice may be attributed to the failure of normal apoptosis and to ongoing proliferation.

**DISCUSSION**

Fzd5 is highly expressed in the developing mouse retina from E8.5 to postnatal stages. We show that selective loss of Fzd5 in the retina affects the normal development of the hyaloid vitreous vasculature and suggest that this occurs through a cell-nonautonomous mechanism. Loss of Fzd5 in the retina causes abnormal accumulation of the hyaloid vitreous vasculature at embryonic stages and persistence of a retrolental mass after birth. Notably, these ocular defects closely resemble those observed in human PHPV.

PHPV is a congenital human eye disease that occurs in infancy and childhood. It is characterized by the presence of pigmented retrolental mass tissue caused by persistent hyperplastic primary vitreous vasculature in the vitreous body. This...
retrolental mass tissue attaches to the inner neuroretina or the posterior surface of the lens, or both, resulting in various ocular abnormalities, such as retinal folds, retinal detachment, abnormal retinal lamination, and lens destruction. These ocular abnormalities lead to vision loss and even blindness in humans, but the pathogenesis of the disease is unclear.

Several mouse models have shown early hyperplasia and persistence of the HVS. For example, the Arf tumor-suppressor gene is normally expressed in perivascular cells of the HVS from E12.5 through P5 and constrains their proliferation by blocking Pdgfrb-dependent signals. Arf mutant mice show abnormal early proliferation, with the appearance of a retrolental mass as early as E14.5 that persists through postnatal stages. TGF-β knockout mice also exhibit overgrowth of the vitreous vasculature at embryonic stages because of the loss of lens-derived TGF-β, which normally inhibits the proliferation of vasculature cells. In addition, alterations in retinoic acid signaling can affect the migration of pericellular mesenchyme cells that contribute to the vitreous vasculature, causing an accumulation of cells in the vitreous. In each of these cases affecting cell proliferation or cell migration, HVS hyperplasia occurs at embryonic stages, as in the PHPV phenotype caused by conditional disruption of Fzd5 in the retina.

Subsequently, the HVS normally regresses during the early postnatal period through apoptosis. Disruption of cell death regulators (Bax, Bak) or genes normally required for macrophages to trigger apoptosis of vascular endothelial cells of the HVS prevents vascular regression. In some cases, defects in the formation of the retinal vasculature can lead to abnormal persistence of the HVS, presumably to accommodate hypoxic demand from the developing retina. This is observed with a loss of function of Frizzled-4, norrin, VEGF isoform, and collagen XVIII. Although failure of regression was observed in the conditional Fzd5 knockout, this was likely secondary to the early hyperplasia and loss of normal organization of the HVS.

In our mouse model, Fzd5 was specifically removed from the retina but not from the lens or vitreous vascular tissue.
Therefore, the PHPV phenotype observed in Fzd5 conditional knockout mice is likely caused by altered signals from the retina in the absence of Fzd5. Although retinal dysplasia was observed in the retina adjacent to the hyperplastic vitreous vasculature, regions of the retina that were distant from the hyperplastic vitreous vasculature appeared to be morphologically normal. Therefore, the retinal dysplasia phenotype seems to be a secondary defect caused by the hyperplastic vitreous vasculature, which is a common feature of PHPV.

We found that retinal Fzd5 expression is required for preventing abnormal accumulation of the vitreous vasculature, but the mechanism underlying this apparently nonautonomous effect remains unclear. One possibility is that retinal Fzd5 may be involved in regulating the expression of secreted proangiogenesis factors such as VEGF or angiogenesis factors such as placental growth factor, which participate in governing the formation, regression, or both of the vitreous vasculature.27-29 A second plausible mechanism is that Fzd5 signaling in the retina may affect the periocular mesenchyme, which is initially formed by cells originating from neural crest and mesoderm30-32 and provides multiple cell lineages that contribute to the vitreous vasculature.32,33 Further studies will shed light on the exact cellular and molecular mechanisms governing vitreous vasculature formation and regression and on how this is altered in the absence of retinal Fzd5.

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