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Wnt Signaling in Vascular Eye Diseases

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Abstract

The Wnt signaling pathway plays a pivotal role in vascular morphogenesis in various organs including the eye. Wnt ligands and receptors are key regulators of ocular angiogenesis both during the eye development and in vascular eye diseases. Wnt signaling participates in regulating multiple vascular beds in the eye including regression of the hyaloid vessels, and development of structured layers of vasculature in the retina. Loss-of-function mutations in Wnt signaling components cause rare genetic eye diseases in humans such as Norrie disease (ND), and familial exudative vitreoretinopathy (FEVR) with defective ocular vasculature. On the other hand, experimental studies in the more prevalent vascular eye diseases, such as wet age-related macular degeneration (AMD), diabetic retinopathy (DR), retinopathy of prematurity (ROP), and corneal neovascularization, suggest that aberrantly increased Wnt signaling is one of the causations for pathological ocular neovascularization, indicating the potential of modulating Wnt signaling to ameliorate pathological angiogenesis in eye diseases. This review recapitulates the key roles of the Wnt signaling pathway during ocular vascular development and in vascular eye diseases, and pharmaceutical approaches targeting the Wnt signaling as potential treatment options.

Keywords

Wnt; development; angiogenesis; Norrie disease; FEVR; AMD; DR; ROP

1 Introduction

The retina is one of the most metabolically active tissues in the body with high-level consumption of nutrients and oxygen. As these high metabolic demands drive the formation of blood vessels in developing eyes, abnormal retinal metabolic changes in eye diseases often precede and trigger vascular pathologies (Joyal et al., 2018; Selvam et al., 2018). The transient fetal vasculature of vitreous develops during early gestation, providing nutrients and oxygen to developing lens and retina until the retinal vasculature forms and gradually replaces the role of the fetal vasculature, which then regresses by apoptosis, usually before birth in humans (Lutty and McLeod, 2018). When fully developed, the adult eyes of most

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mammals are vascularized with two distinct and well-organized vascular networks—the retinal and choroidal vessels (Figure 1). The retinal vessels nourish the inner two-thirds of the retina, whereas the choroidal capillary network adjacent to Bruch’s membrane supplies the outer third of retina, mostly the avascular photoreceptor layer. Vascular formation occurs via either vasculogenesis or angiogenesis. Vasculogenesis begins with clustering and differentiation of the endothelial precursor cells into primitive vessels, defining the patterns of the major vessels and the vessels of endoderm-derived organs (Noden, 1989; Pardanaud et al., 1989). On the other hand, angiogenesis, the formation of new vessels sprouting from existing vessels, plays an important role in the vascularization of ectoderm- and mesoderm-derived organs such as the brain, retina and kidney (Risau, 1997). Since retinal and choroidal neovascularization are major causes of vision loss in eye diseases, the molecular basis for ocular angiogenesis has been intensively studied over the last several decades. These research studies have identified numerous angiogenic factors that regulate ocular angiogenesis both during development and in vascular eye diseases (Chen et al., 2016a; Gariano and Gardner, 2005; Selvam et al., 2018), with hypoxia and vascular endothelial growth factor (VEGF) being the most prominent (Campochiaro, 2015; Das and McGuire, 2003; Penn et al., 2008; Witmer et al., 2003). Yet advances in ocular genetics and experimental studies have also begun to uncover additional signaling pathways other than VEGF, such as Wnt signaling pathway, which may influence ocular vascular development and impact vascular eye diseases.

Since it was first discovered by Nusse and Varmus in the early 1980s (Nusse and Varmus, 1982), the Wnt signaling pathway has been extensively studied in embryogenesis, cancer, bone diseases, neuropsychiatric diseases and cardiovascular diseases (Logan and Nusse, 2004). Genetic studies linked Wnt signaling mutations with rare inherited vascular eye diseases, such as Norrie disease and familial exudative vitreoretinopathy (FEVR), suggesting a pivotal role of Wnt signaling in developmental ocular angiogenesis. Experimental studies in animal models also implicated Wnt signaling in other more prevalent vascular eye diseases, including neovascular age-related macular degeneration (AMD), cornea neovascularization (NV), diabetic retinopathy (DR), and retinopathy of prematurity (ROP). Together these vascular eye diseases may account for more than half of global blindness (Parmalee and Kitajewski, 2008). This review summarizes recent studies on genetics, pathogenesis, and potential experimental therapies related to the Wnt signaling pathway in ocular angiogenesis with a focus on abnormal ocular angiogenesis associated with either deficient or aberrant Wnt signaling.

2 The Wnt Signaling Pathway

The Wnt signaling pathway is fundamentally important in both development and diseases. Wnt proteins are highly conserved in many species, including humans and *Drosophila*, with the name “Wnt” coined as a combination of a segment polarity gene “*Wingless*” in *Drosophila* and pro-oncogene “*Int1*” in humans. Characteristic of secreted glycoproteins, Wnts are cysteine-rich, highly hydrophobic, and contain a cleavable N-terminal signal peptide to guide secretion (Nusse and Clevers, 2017). In mammals, there are 19 different Wnt proteins with varying lengths of approximately 350-400 amino acids. Wnts activate intracellular signaling pathways by binding to one of several frizzled (FZD) family receptors

and, in most cases, a co-receptor, such as lipoprotein receptor-related protein 5 (LRP5) or LRP6 (MacDonald et al., 2009; Nusse and Varmus, 1992).

In general, there are two types of Wnt signaling pathways: the canonical Wnt/ β -catenin signaling pathway, which acts through β -catenin as a transcriptional coactivator, and the non-canonical, β -catenin independent Wnt signaling pathway, which includes the Wnt/ Ca^{2+} pathway and the planar cell polarity (PCP) pathway (MacDonald et al., 2009). Of the two, the canonical Wnt/ β -catenin signaling pathway is the most well-characterized and elucidated, and the mechanisms underlying non-canonical receptor activation are not well understood. Several studies support a role of the non-canonical Wnt/ β -catenin signaling pathway in vascular development through regulation of VEGF availability, and vascular remodeling in response to sheer stress (Franco et al., 2016; Ju et al., 2010; Korn et al., 2014; Stefater et al., 2011). In addition, non-canonical Wnt signaling may also inhibit the canonical Wnt pathway in a receptor- and ligand-dependent manner. For example, the non-canonical ligands Wnt5a and Wnt11 may inhibit the canonical Wnt pathway, in a Ca^{2+} -dependent and -independent manner, respectively (Maye et al., 2004; Mikels and Nusse, 2006). As such, the canonical and non-canonical Wnt pathways are considered to interact as an integrated signaling network rather than acting mutually exclusively (Reis and Liebner, 2013). This review focuses on the most extensively studied canonical Wnt/ β -catenin signaling pathway (referred to here as the Wnt signaling pathway), which plays an indispensable role in developmental and pathological ocular angiogenesis.

2.1 The canonical Wnt signaling and its function

The canonical Wnt signaling pathway acts in a two-state mode: off-state (inactive, without Wnts) and on-state (active, with Wnts). In the absence of Wnt ligands (off-state), β -catenin is recruited to a destruction complex consisting of axin, adenomatous polyposis coli (APC), casein kinase 1 α (CK1 α), and glycogen synthase kinase-3 β (GSK3 β). The sequential phosphorylation of β -catenin by CK1 α and GSK3 β creates a binding site for the ubiquitin E3-ligase β TrCP, targeting β -catenin for ubiquitination and degradation by proteasome in the cytoplasm. In the “on-state”, available Wnt ligands bind to their receptors FZDs and LRP5 (or LRP6), leading to the phosphorylation and activation of dishevelled (DVL) proteins. Active DVL recruits axin to the cell membrane, induces axin degradation, and thereby inhibits GSK3 β activity. Without phosphorylation, β -catenin is stabilized and translocated into the nucleus where it binds to lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factors to activate transcription of Wnt target genes (Figure 2) (Nusse and Clevers, 2017).

The number of recognized Wnt target genes currently amounts to more than 100 (Staal et al., 2004; Willert et al., 2002), including the well-known Yamanaka factors c-Myc (He et al., 1998), Oct4 (Cole et al., 2008), Sox2 (Van Raay et al., 2005), the key cell cycle regulator cyclin D (Tetsu and McCormick, 1999), and angiogenic genes such as vascular endothelial growth factor (VEGF) (Zhang et al., 2001), twist (Howe et al., 2003; Li et al., 2014), and jagged1 (Rodilla et al., 2009). More recently, genome-wide approaches such as RNA-seq and ChIP-seq allow researchers to identify more potential Wnt target genes in a tissue-specific manner (Hatzis et al., 2008; Iyer et al., 2018; Schuijers et al., 2014). As such, the

broad range of Wnt target genes reflects the diverse roles of the Wnt/ β -catenin signaling pathway in the regulation of many biological processes throughout development and disease pathogenesis.

In embryonic developmental stages, the Wnt/ β -catenin pathway plays a crucial role in regulating stem cell fate decision, cell proliferation, differentiation, and migration (Sato et al., 2004; ten Berge et al., 2008; ten Berge et al., 2011). Numerous studies have shown that systemic homozygous knockouts of most key Wnt components in mice are embryonic or perinatal lethal, such as Wnt1 (McMahon and Bradley, 1990), Wnt2 (Monkley et al., 1996), Wnt3 (Liu et al., 1999), APC (Robanus-Maandag et al., 2010), β -catenin (Huelsen et al., 2000), GSK3 β (Hoeflich et al., 2000), Axin1/2 (Zeng et al., 1997), and TCF4 (Korinek et al., 1998). On the other hand, studies using tissue/cell type-specific or inducible knockout model of Wnt signaling components unveiled that, in adults, Wnt pathway also critically regulates tissue homeostasis (Clevers et al., 2014; Kretzschmar and Clevers, 2017), angiogenesis (Dejana, 2010), and inflammation (Ma and Hottiger, 2016). For example, the Wnt/ β -catenin pathway is essential for intestinal homeostasis (Clevers et al., 2014), hair follicle cycling in skin tissue (Lim and Nusse, 2013), and mammary gland plasticity (Alexander et al., 2012).

Since the initial discovery that identified Wnt1 as a proto-oncogene in cancer, a growing number of studies have linked disruption of Wnt/ β -catenin signaling (through either germinal and/or somatic mutations of Wnt pathway components) with many diseases. Loss-of-function mutations in Wnts, LRP6, and FZDs cause developmental disorders including eye and bone defects, whereas upregulated Wnt/ β -catenin signaling often leads to various cancers and is also associated with high bone density, insulin sensitivity and diabetes (Table 1). Table 1 summarizes Wnt-related diseases in organs other than the eyes, whereas later sections of this review will discuss Wnt-related eye diseases specifically in greater details. To name a few, gain-of-function *LRP5* mutations cause high bone density (Boyden et al., 2002; Lara-Castillo and Johnson, 2015; Little et al., 2002; Van Wesenbeeck et al., 2003), and loss-of-function mutations in *LRP5* leads to low bone mass disorder (Ai et al., 2005; Gong et al., 2001b). *WNT1* mutation causes osteogenesis imperfecta (Fahiminiya et al., 2013; Pyott et al., 2013). Other rare disorders associated with loss of Wnt signaling exert additional negative effects on intellectual development such as in Robinow syndrome (Person et al., 2010; White et al., 2018), craniofacial development and odontogenesis in Williams syndrome (Wang et al., 1997), and familial tooth agenesis (Lammi et al., 2004), respectively. Upregulated Wnt signaling resulting from mutations in APC, β -catenin, and axin2 was found in colorectal cancer (Bass et al., 2011; Liu et al., 2000; Morin et al., 1997; Nishisho et al., 1991), hepatocellular carcinoma (de La Coste et al., 1998; Huang et al., 1999; Satoh et al., 2000), lung cancer (Sunaga et al., 2001), and pancreatic cancer (Tanaka et al., 2001). Contrarily, skin cancer shows inactivated Wnt signaling with LEF1 mutation (Takeda et al., 2006). Other diseases are also linked with Wnt signaling abnormality, such as familial adenomatous polyposis (APC mutation, upregulated Wnt signaling) (Kinzler et al., 1991), type II diabetes (TCF4 mutation, down-regulated Wnt signaling) (Florez et al., 2006; Grant et al., 2006), coronary artery disease (LRP6 mutation, down-regulated Wnt signaling) (Mani et al., 2007), and late-onset Alzheimer (LRP6 mutation, down-regulated Wnt signaling) (De Ferrari et al., 2007). Given the strong link between Wnt/ β -catenin signaling and diseases,

many of the Wnt components and regulators are promising pharmaceutical targets by small-molecule inhibitors and activators, particularly for osteoporosis and cancer therapeutics (Anastas and Moon, 2013; He et al., 2017; Huang et al., 2017; McBride et al., 2014).

In the developing eye, Wnt signaling is important for proper formation of both the anterior segment of the eye and neurovascular retina, which has been summarized in several previous review papers. Wnt signaling is tightly regulated in multiple developmental processes including the lens, the retinal pigment epithelium (RPE), the ciliary margin, the dorso-ventral patterning in the optic cup, and the retinal vascular system (Drenser, 2016; Fujimura, 2016). Wnt signaling regulates RPE adhesion, morphogenesis, and pigmentation, and specifically pertains to retinal disease during aging such as age-related macular degeneration (AMD) (Burke, 2008). Crucial effects of Wnt signaling were found in lens development, morphogenesis and fiber differentiation (Bassnett and Sikic, 2017; West-Mays et al., 2010). Wnt signaling pathway in developing retinal vascularization has been linked to ROP and fetal development (Drenser, 2016). Moreover, the emerging role of Wnt signaling in differentiating Muller glial cells to photoreceptors represents an exciting new direction in retinal regeneration (Yao et al., 2016; Yao et al., 2018). This review focuses on the Wnt signaling pathway in ocular vascularization both in developmental context and in genetic and non-genetic vascular eye diseases.

2.2 Norrin: A nonconventional Wnt ligand

The essential role of Wnt signaling in the neuro-retina is evidenced by the broad distribution of conventional Wnt ligands in the retinal cells (de Jongh et al., 2006). Expression of multiple Wnt ligands has been identified in the developing mouse retina (Liu et al., 2003; Yi et al., 2007). Wnt1, -3, -5a, -5b, -7b, and -13 (Wnt2b) are expressed in embryonic and fetal retina, often in a dynamic central-peripheral expression pattern in the neuroblast layer, while Wnt5a, -5b, -10a, and -13 are expressed similarly in the adult retina (Liu et al., 2006; Yi et al., 2007). In the differentiating postnatal (P7) mouse retina, Wnt5a, Wnt5b, and Wnt13 are expressed in the inner nuclear layer in amacrine cells. Wnt13 (Wnt2b) is expressed predominantly in the RPE, and is also detected in the ganglion cells along with the inner retina (Iwai-Takekoshi et al., 2018). Similar expression patterns for Wnt5b and Wnt13 are detected in the adult retina (Liu et al., 2003). However, it was only after the discovery of the unconventional Wnt ligand, norrin, that the role of Wnt signaling in retinal vasculature was finally elucidated.

Although studies in Norrie disease implicated norrin as the protein product of the disease gene in 1990s (Berger et al., 1992), it was not until 2004 that a seminal study characterized norrin as a FZD4 ligand and linked norrin with Wnt signaling and retinal angiogenesis (Xu et al., 2004). Norrin is encoded by the *NDP* (Norrie disease protein) gene, localized at the short arm of the X chromosome (Bleeker-Wagemakers et al., 1985). As a cysteine-rich secreted protein, norrin belongs to the superfamily of growth factors containing a cysteine knot motif (Meitinger et al., 1993). Although norrin has no sequence homology or structural similarity to Wnt proteins, it mimics the receptor recognition characteristic of Wnt proteins (Chang et al., 2015), displays high specificity of binding affinity for FZD4 (but not other FZDs) with nanomolar affinity, and is capable of activating the β -catenin-dependent

canonical Wnt signaling pathway in an LRP5 (but not LRP6)-dependent manner (Xu et al., 2004), to exert a key function in retinal vasculature development (Ye et al., 2010). Norrin is secreted mainly by Müller cells (Seitz et al., 2010; Ye et al., 2011), and partially by endothelial cells in the retina (Lee et al., 2013) and is also found in retinal macrophages (Chen et al., 2011b). A recent study discovered that norrin is a potent trigger of FZD4 ubiquitination and induces internalization of the norrin receptor complex into the endo-lysosomal compartment (Zhang et al., 2017a). Inhibition of ubiquitinated cargo transport strongly impaired norrin/FZD4 signaling and recapitulated central nervous system (CNS) angiogenesis and blood-CNS-barrier defects caused by impaired vascular β -catenin signaling in mice (Zhang et al., 2017a). In addition, norrin/FZD4 signaling also requires another membrane protein, tetraspanin 12 (TSPAN12), which acts as an additional co-receptor to amplify Wnt signaling (Junge et al., 2009; Lai et al., 2017; Luhmann et al., 2005). Together the norrin/FZD4/LRP5/TSPAN12 pathway exhibits unique and indispensable functions in governing retinal angiogenesis (Ohlmann and Tamm, 2012).

2.3 Wnt signaling in vascular endothelial cell function

Angiogenesis requires coordinated regulation of many extracellular and intracellular signals. The Wnt signaling pathway is one of the key regulatory systems in coordinating endothelial cell behavior to govern vascular morphogenesis (Franco et al., 2009; van de Schans et al., 2008; Zerlin et al., 2008). Various Wnt ligands may act as a short-range paracrine signal to mediate many aspects of vascular endothelial cell function and homeostasis. Wnt1 increases proliferation and capillary stability *in vitro* (Cheng et al., 2003; Goodwin et al., 2007; Wright et al., 1999); Wnt2 is important for endothelial cell differentiation and tubular formation (Klein et al., 2008; Wang et al., 2007); Wnt3a promotes proliferation, migration, differentiation, and survival of endothelial cells (de Jesus Perez et al., 2009; Nimmagadda et al., 2007; Wang et al., 2006); and Wnt7b secreted from macrophages mediates endothelial cell apoptosis in hyaloid vessel regression (Lobov et al., 2005).

The importance of Wnt signaling in the development of vasculature is further evidenced by the high expression levels of various frizzled receptors, LRP5/6, and Wnt factors in endothelial cells (Goodwin et al., 2006). Activation of Wnt signaling was discovered during embryonic development in many types of vessels (Corada et al., 2010). Endothelial-specific deletion of β -catenin impairs the development of the embryonic vasculature, leading to defective vascular remodeling and diffuse hemorrhages, thereby resulting in early lethality *in utero* (Cattellino et al., 2003); this suggests that Wnt signaling is an important early step of angiogenesis and that loss-of-function of Wnt signaling impairs angiogenesis. Within the eye, insights into the role of Wnt signaling in ocular angiogenesis were initially revealed mostly by genetic studies in rare inherited eye diseases with ocular vascular abnormalities; thus, in an effort to further understand the role of Wnt signaling in vascular development and vascular eye diseases, it is necessary to introduce the development of ocular vasculature.

3 Development of ocular vasculature

As a specialized sensory organ consisting of multiple tissues, the eye requires blood supply to ensure normal development and visual function. The development of ocular vasculature

depends largely on the ocular tissue demands for oxygen and other nutrients that promote retinal vascular expansion and regression of hyaloid vasculature.

3.1 Development and regression of the hyaloid vasculature

During embryogenesis, the retina is nourished by a transient embryonic vascular bed known as the hyaloid vasculature. This structure develops during embryonic and fetal stages, starting at around 4-6 weeks gestation in humans, to provide blood supply to the growing eye (Lutty and McLeod, 2018). The hyaloid artery (HA) sprouts from the primitive dorsal ophthalmic artery, passing through the fetal fissure into the optic cup (Anand-Apte, 2011). The hyaloid vascular system, a dense intraocular circulatory network comprising the vasa hyaloidea propria (VHP), tunica vasculosa lentis (TVL), and pupillary membrane (PM), transiently nourishes the primary vitreous, immature retina and lens. The VHP branches are derived from the HA, and then anteriorly pass through the vitreous to the lens. The TVL is an arborization of HA that cups the posterior surface of the lens capsule, and the PM is an anastomosis of the TVL and the anterior ciliary arteries that cover the anterior surface of the lens (Anand-Apte, 2011; Hobbs, 2013) (Figure 3A). While lacking veins, the hyaloid vasculature accomplishes its venous drain through the choroidal veins. Along with the lens development, the TVL reaches the anterior surface of the lens and forms a network of vessels in the PM (Fruttiger, 2007; Hartnett, 2013; Saint-Geniez and D'Amore, 2004). In the human embryo, the hyaloid vascular system is complete at approximately the fourth month of gestation and provides nutrients to the developing eye before the complete formation of the retinal vascular system. Afterwards, the regression of the hyaloid vessels starts, coinciding with the development of the retinal vasculature at this stage (Figure 3B) (Anand-Apte, 2011).

During embryonic development, the hyaloid vascular system plays a pivotal role in providing necessary nutrition and oxygen to the retina when the retinal vasculature is not yet fully developed. In humans, the hyaloid vasculature starts to regress when the first retinal vessels appear in mid-gestation. The regression starts with atrophy of the VHP, followed by capillaries of the TVL and PM, and, lastly the HA (Anand-Apte, 2011; Hobbs, 2013). The vessel occlusion by macrophages is an important step for initiating atrophy during the regression process (Anand-Apte, 2011). The complete loss of hyaloid circulation finishes at approximately 35–36 weeks of gestation (Hobbs, 2013). As the hyaloid vasculature regresses, the primary vitreous retracts, and the collagen fibers and other components of the extracellular matrix are produced to form the secondary vitreous. By the sixth month of gestation, the posterior segment is mainly composed of the secondary vitreous, and the primary vitreous is reduced to a small central structure extending from the optic disc to the posterior lens surface, which is referred to as the Cloquet canal (Anand-Apte, 2011; Hobbs, 2013). In mice, where retinal vessel development occurs postnatally, regression of the hyaloid vascular system begins after birth, from about postnatal day 4, and finishes by approximately day 21 (Ito and Yoshioka, 1999) (Figure 3A).

3.2 Retinal vessel development

Beginning at the fourth month of gestation in human embryo, the first retinal vessels appear as solid endothelial cords sprouting from the optic nerve head. By the sixth month of

gestation, the vessels begin lumenization and extend outward radially from the optic disk, migrating peripherally to the ora serrata and the equator by the seventh to eighth month of gestation (Anand-Apte, 2011). Pericytes or mural cells are absent from the developing retinal vessels until two months postnatal. The retinal vascular pattern completely matures by the fifth month after birth (Hildebrand G. D., 2011). The final mature retinal vasculature is composed of three distinct layers of vessels: the superficial capillary in the inner part of the nerve fiber layer, the intermediate layer in the inner plexiform layer, and the deep vascular layers in the outer plexiform layer (Figure 1) (Chen et al., 2016a; Dorrell et al., 2002; Liu et al., 2017)

Unlike in humans, where retinal vessels develop before birth, the retinal vasculature in mice develops postnatally, which provides an ideal time window for experimental studies. Retinal vessels sprout radially from the optic nerve at postnatal day 1 and extend to the periphery in the superficial retina around postnatal day 7-8. From P7 onward, the superficial capillaries start sprouting vertically to form the deep vascular layer and then the intermediate vascular plexus. The deep layer vasculature reaches the retinal periphery at approximately P12, followed by the intermediate plexus between P12 and P15 (Figure 3B) (Chen et al., 2016a; Stahl et al., 2010; Watson et al., 2012). With substantial remodeling and maturation, the retinal vasculature of the mouse eye is completely developed at around 3 weeks postnatal (Chen et al., 2016a; Stahl et al., 2010).

3.3 Neuro-glial-vascular interaction during retinal vessel development

Studies on the retinal vasculature development over the last several decades have enabled the discovery of basic cellular mechanisms underlying vascular development, remodeling, and maturation. While vascular endothelium is a key participant in guiding vascular growth front and the formation of the blood retinal barrier (Bharadwaj et al., 2013; Dorrell and Friedlander, 2006), close interaction of vascular endothelial cells with surrounding neurons, glia, and inflammatory cells is needed to allow coordinated process of vascular growth, remodeling, and repair (Moran et al., 2016; Selvam et al., 2018; Sun and Smith, 2018). During superficial retinal vessel development, as retinal neurons and glial cells differentiate and mature, their metabolic demands increase, creating a radial central-to-peripheral gradient of hypothesized “physiologic hypoxia” that leads to the development of new vessels from the center towards the periphery of the retina (Chan-Ling et al., 1995) (Figure 3C). After new vessels have formed, bringing oxygen and alleviating hypoxia, the vascular growth continues radially towards the peripheral retina (Gariano and Gardner, 2005).

The remarkable structural alignment among retinal vessels, astrocytes, and neurons suggests their potential contribution as angiogenic guidance mechanisms. Prior to the formation of retinal blood vessels, astrocyte glial cells migrate into the retina from the optic nerve head during embryonic development (Chu et al., 2001). Astrocyte growth in the retina follows the path of radially oriented ganglion cell axons in a platelet-derived growth factor-dependent manner (Dorrell et al., 2002; Gariano et al., 1996) and spatially and temporally overlaps the pattern of vascular growth. It was initially hypothesized that astrocyte-secreted VEGF may guide the filopodia of sprouting endothelial cells (Dorrell et al., 2002; Gariano et al., 1996; Gerhardt et al., 2003; Watanabe and Raff, 1988) (Figure 3C), yet more recent findings found

no developmental vascular disruption after astrocyte-specific deletion of VEGF (Scott et al., 2010; Weidemann et al., 2010). This suggests that astrocytic VEGF is likely more critical for vascular maintenance in pathologic conditions than in development. Other cells surrounding blood vessels, such as retinal neurons and inflammatory cells, may also be capable of governing retinal vascular development. Indeed, neuronal deletion of VEGFR2 in the retina leads to misdirected retinal angiogenesis, suggesting that retinal neurons may titrate VEGF availability to limit angiogenesis (Okabe et al., 2014).

Within the vascular endothelium, the growth and expansion of the superficial vessels depend largely on the interaction between stalk cells, which are mainly responsible for endothelial cell proliferation, and the more motile tip cells, which use filopodia to sense the growth factor gradients to lead cell migration. The NOTCH pathway is crucial in establishing the tip cell phenotype and balancing of the tip/stalk cells in a toggle mechanism tightly integrated with VEGF signaling (Figure 3D) (Benedito et al., 2009; Blanco and Gerhardt, 2013; Hellstrom et al., 2007). Once the superficial vascular layer is fully developed, vertical sprouts start to form and dive towards the outer retina, first to develop the deep vascular layers and then the intermediate vascular layers afterwards (Figure 3B). Development of these deeper vascular layers, however, appears to rely on mechanisms separate from VEGF and NOTCH, with Wnt signaling emerging as one of the most critical pathways based on observations from studies of both human genetics and experimental models.

3.4 Choroidal vessel development

The choroid, a thin and highly vascularized membrane between the retina and the sclera, covers the posterior five-sixths of the human eye. Unlike the retinal vasculature, the choroidal vasculature arises from posterior ciliary arteries, which originate from the ophthalmic artery (Hartnett, 2013). Starting from the fourth week of gestation in humans, the precursors of the choriocapillaris begin to differentiate and form endothelial cells adjacent to the RPE, followed by the formation of a large plexus of primitive vessels and the extension of vascular plexus along the entire exterior surface of the optic cup (Hartnett, 2013; Saint-Geniez and D'Amore, 2004). By the eighth week, the choroidal capillary network is organized through connections to the short posterior ciliary arteries. The choroidal vasculature matures by the fourth month of gestation (Anand-Apte, 2011). As a sinusoidal vascular plexus with highly fenestrated endothelium, the choriocapillaris is the site of the greatest blood flow in the body (Henkind 1979), as it provides 65-85% of the blood volume in the eye. Through diffusion, it nourishes the outer portion of the retina (Bela Anand-Apte, 2011), including photoreceptors and RPE, as well as the fovea, which contains only photoreceptors for high acuity central vision and is devoid of vessels and other retinal neurons.

4 Inherited ocular vascular diseases with Wnt-related mutations

Several inter-related inherited eye diseases, including Norrie disease, familial exudative vitreoretinopathy (FEVR), osteoporosis-pseudoglioma syndrome (OPPG), and Coats' disease, share common pathological features of disturbed ocular angiogenesis. Studies on these diseases identified genetic variations involving different components of the Wnt

signaling pathway, such as the ligand norrin or the receptors FZD4, LRP5, and TSPAN12. These studies substantially enhanced our understanding of the role that Wnt signaling plays in ocular angiogenic processes. A summary of ocular vascular diseases related to genetic deficiency in Wnt signaling is listed in Table 2.

4.1 Norrie disease

Norrie disease is an X-linked recessive disorder, hence affecting primarily male infants, with females diagnosed only in very rare occasions. Norrie disease is a sporadic disease with only about 400 cases reported. First described by a Danish ophthalmologist, Gordon Norrie in 1927 (Andersen and Warburg, 1961), the responsible disease gene (Norrie Disease Protein or *NDP*), which encodes norrin, was not discovered until much later, in the 1990s (Berger et al., 1992). Mutations in *NDP* cause inactivation of the Wnt signaling pathway (Xu et al., 2004). Norrie disease is characterized by the ocular manifestations of incomplete peripheral retinal vasculature, lack of deep retinal vascular layers, and persistent hyaloid vessels (Berger et al., 1992; Drenser et al., 2007). Patients with the disease generally become bilaterally blind with retinal scarring and retraction accompanied by persistent hyaloid vessels. Histopathological abnormalities did not manifest in eyes of an 11-week old fetus with known *NDP* mutations (Parsons et al., 1992); however, retinal detachments could be found in the late third trimester by obstetric ultrasonography (Redmond et al., 1993; Wu et al., 2017), suggesting that the disease process in the eye likely occurs in late gestation. Other than ocular symptoms, about 50% of patients show some degree of progressive mental disorder, often with psychotic features, and approximately one-third of patients develop sensorineural deafness in the second decade of life. In addition, some patients have more complex symptoms, including growth failure and seizures (Berger et al., 1992).

4.2 Familial exudative vitreoretinopathy (FEVR)

FEVR is another rare hereditary eye disorder with progressive vision loss and was first described in 1969 (Criswick and Schepens, 1969). Patients with FEVR show similar but milder and more variable clinical presentations compared with Norrie disease, and the prevalence for FEVR is higher than that in Norrie disease. Additionally, FEVR can often be asymptomatic in patients and may thus go unreported. The symptoms of FEVR can vary widely, including clinically asymptomatic, minimal vascular changes in the peripheral retina, and extensive neovascularization, exudation, and retinal detachments (Figure 4) (Yonekawa et al., 2015). This varying degree of penetrance likely reflects in part the genetically heterogeneous nature of FEVR. The inheritance patterns of FEVR include autosomal dominant, autosomal recessive, and X-linked recessive forms, and they differ depending on in part on the involved genes (de Crecchio et al., 1998; Laqua, 1980; Shastry et al., 1995). The autosomal dominant form is the most common form associated with genetic mutations in *FZD4* (Robitaille et al., 2002), *LRP5* (Toomes et al., 2004), and *TSPAN12* (Poulter et al., 2010). Mutations in autosomal recessive FEVR mostly occur in the region containing the gene for *LRP5* (Jiao et al., 2004), whereas X-linked FEVR is largely due to mutations in the *NDP* gene, similar to Norrie disease (Fullwood et al., 1993; Shastry et al., 1995). Each of these reflects loss-of-function mutations, leading to pathological inactivation of the Wnt signaling pathway, thereby hindering ocular vessel development.

4.3 Osteoporosis—pseudoglioma syndrome (OPPG)

OPPG is a rare autosomal recessive disorder which occurs approximately in one in two million people (Neuhauser et al., 1976), and is sometimes considered a special subtype of FEVR. The disease is characterized by skeletal fragility, which is often recognized during childhood, and by congenital or infancy-onset visual loss due to abnormal blood vessel development in the eye. Affected individuals often have severe bone thinning or osteoporosis, resulting in multiple fractures in bones including vertebrae, and sometimes skull softening. The physical phenotype of OPPG often resembles eye tumors (retinal glioma), hence the name pseudoglioma. Cognitive impairment was also reported in ~25% of patients with OPPG. OPPG is caused by inactivating mutations in *LRP5* (Baron and Kneissel, 2013; Gong et al., 2001b), and hence inactivation of Wnt signaling, which is essential for the development of both bone and retinas.

The *LRP5* gene plays a crucial role in the osteogenesis, with abnormalities linked to both loss- and gain-of-function mutations (Cui et al., 2011; Williams, 2017). Loss-of-function mutations in *LRP5* result in low bone mass disorder in OPPG (Ai et al., 2005; Gong et al., 2001b), while heterozygous missense mutations in *LRP5* have been observed in individuals with dominantly inherited high bone mass, largely due to increased and uninhibited Wnt signaling (Boyden et al., 2002; Lara-Castillo and Johnson, 2015; Little et al., 2002; Van Wesenbeeck et al., 2003). Moreover, studies have found that LRP5 functions not only through the Wnt signaling pathway, but also through other pathways including fatty acid β -oxidation enzyme upregulation (Frey et al., 2015), and Rac1 signaling upregulation to induce activation of mTORC2-AKT signaling in a β -catenin-independent manner in osteoblasts (Esen et al., 2013). Given that Wnt signaling has recently been shown to act in a GSK3 β -dependent, but β -catenin-independent manner to regulate the stability of numerous proteins including Wnt signaling related proteins, metabolic enzymes, signaling molecules and transcription factors (Huang et al., 2015; Koch et al., 2015; Xu et al., 2009), it is possible that LRP5 regulates osteogenesis or potentially even angiogenesis through multiple, cross-linked signaling pathways during development.

4.4 Coats' disease

Another retinal vascular disease genetically linked to Wnt abnormalities is Coats' disease, or exudative retinitis. Children with Coats' disease show progressive development of retinal vascular telangiectasia, microaneurysms, extensive intraretinal and subretinal lipid exudates, and possible exudative retinal detachments. Coats' disease is unilateral in ~90% of the cases, which are mostly isolated, and affects predominantly young males without other systemic symptoms. The genetic causes of the disease are still largely unclear, although Black *et al.* demonstrated a somatic mutation in the *NDP* that was not present within the non-retinal tissue, indicating the possibility of a somatic, locally acquired mutation contributing to this disease process (Black et al., 1999). More recently, Robitaille *et al.* found that loss-of-function mutations of *FZD4* may also contribute to Coats' disease in an autosomal recessive way (Robitaille et al., 2011), and Wu *et al.* discovered haploinsufficiency of *RCBTB1* in both Coats' disease and FEVR (Wu et al., 2016), which is associated with β -catenin stabilization in norrin-Wnt signaling, further indicating the link of Wnt/ β -catenin signaling to vascular abnormalities in Coats' disease and vitreoretinopathies.

5 Norrin/FZD4/LRP5 signaling in ocular vasculature development

5.1 Mouse models of FEVR and Norrie disease

To study the pathogenesis of FEVR and Norrie disease, mice with genetic knockout of diseased genes have been investigated. In both humans and mice, the norrin/FZD4/LRP5 signaling pathway shows direct influence in developing retinal vasculature, as mutations in *NDP* gene cause impaired outgrowth of capillaries from the superficial vascular layer of the retina and lack of intraretinal vessels (Luhmann et al., 2005; Ohlmann et al., 2005; Rehm et al., 2002; Richter et al., 1998). Moreover, the norrin-deficient (*Ndp*^{y/-}) mice modeling Norrie disease reveals persistent hyaloid vessels, which fail to regress postnatally (Rehm et al., 2002; Richter et al., 1998). Similar vascular phenotypes were reported in related *Fzd4*^{-/-} and *Lrp5*^{-/-} mouse models of FEVR (Kato et al., 2002; Xia et al., 2008; Xu et al., 2004), indicating an essential function of norrin-Wnt/ β -catenin signaling in retinal vasculature development.

Together, mice with genetic deficiency in *Lrp5* (*Lrp5*^{-/-}), *Ndp* (*Ndp*^{y/-}), *Fzd4* (*Fzd4*^{-/-}) and *Tspan12* (*Tspan12*^{-/-}) all demonstrate similar ocular vascular abnormalities resembling human phenotypes of FEVR and Norrie disease (Chen et al., 2012; Gal et al., 2014; Hsieh et al., 2005; Kato et al., 2002; Luhmann et al., 2005; Rehm et al., 2002; Robitaille et al., 2002; Toomes et al., 2004; Xia et al., 2008; Xu et al., 2004). Similar to *Lrp5*^{-/-} mice (Figure 5A-D) (Wang et al., 2016), these Wnt-deficient mice all have delayed primary retinal angiogenesis, with more sparse vascular coverage at an early stage of retinal development (postnatal day 7), complete absence of secondary and tertiary vasculature, and persistent hyaloid vessels in adults (Chen et al., 2011b; Chen et al., 2012; Wang et al., 2016; Ye et al., 2009). The absence of inner retinal vessels in *Ndp*^{y/-} and *Lrp5*^{-/-} mice results in consequent retinal hypoxia, which induces increased *Vegfa*, and leads to pathologic glomeruloid vascular structures in the superficial plexus (Luhmann et al., 2005; Rattner et al., 2014; Wang et al., 2016). The abnormal ocular vasculature and the hypoxic microenvironment are associated with malfunction of inner retinal neurons (Schmidt et al., 2008), and hence impaired visual function in *Ndp*^{y/-}, *Fzd4*^{-/-} and *Lrp5*^{-/-} mice, as evidenced by diminished b-wave in electroretinography (Beck et al., 2017; Wang et al., 2016; Xia et al., 2010; Ye et al., 2009). In addition to the defects in retinal vessel growth, *Ndp*^{y/-} mice also have defective vascular patterning in the inner ear, consistent with hearing loss in mice and in humans with Norrie disease (Rehm et al., 2002). *Lrp5*^{-/-} mice also have reduced bone mass, which reflects the abnormalities in osteoblast growth as seen in OPPG patients (Gong et al., 2001a).

Mutations in other Wnt signaling inhibitors, such as *ERG* and *Apcdd1*, may cause vascular phenotypes resembling the Wnt-deficient mice. *ERG* is the ETS (E26 transformation-specific) related gene, which activates the Wnt pathway by promoting β -catenin stability through signals mediated by VE-cadherin and the Wnt receptor FZD4. *ERG*-deficient endothelial cells show decreased Wnt signaling and defective vascular development in the retina, whereas overexpression of *ERG* reduces permeability and increases stability of VEGF-induced blood vessels (Birdsey et al., 2015). Adenomatous polyposis coli downregulated 1 (*APCDD1*), a membrane-bound glycoprotein and a downstream Wnt

target, shows decreased mRNA levels in both *Ndp^{y/-}* and *Lrp5^{-/-}* retinas (Chen et al., 2012). It also functions as an inhibitor of the Wnt signaling pathway by blocking the interaction between Wnt3a and LRP5 (Shimomura et al., 2010; Takahashi et al., 2002). APCDD1-deficient mice exhibit delayed vessel pruning, and APCDD1 mutant endothelial cells precociously form the paracellular component of the barrier, whereas overexpression of APCDD1 in retinal endothelial cells shows reduced vessel density but increased paracellular barrier permeability. Taken together, the Wnt signaling is essential in guiding and maintaining a stable vascular microenvironment. Both loss- of and gain- of function mutations of the Wnt signaling pathway may lead to aberrant endothelial cell activity and abnormal proliferation of vessels.

5.2 Which factors drive Wnt-related retinal vascular defects?

In the retina, both FZD4 and LRP5 are expressed in vascular endothelial cells (Chen et al., 2011b; Ye et al., 2009), suggesting direct Wnt-mediated angiogenic effects on the vessel endothelium (Figure 5E) (Wang et al., 2016). Additional expression of LRP5 was also reported in Muller cells (Xia et al., 2010), where the function of LRP5 is still unclear. While it is generally accepted that the development of superficial layer of retinal vasculature follows a gradient of growth factors such as VEGF, it is unlikely that norrin forms a gradient to guide either superficial or deep vessel formation in the retina, because ectopic expression of norrin driven by a lens promoter completely restored the defective ocular vasculature in Norrie disease mice (Ohlmann et al., 2005). These observations suggest that norrin/FZD4/LRP5 signaling more likely acts as a switch that needs to be turned on to enable angiogenic processes regulated by other factors, particularly in the deep layer network formation, where increased levels of hypoxia-induced VEGF are not sufficient to overcome suppression of deeper vascular network formation in the absence of Wnt signaling (Luhmann et al., 2005; Rattner et al., 2014; Wang et al., 2016).

Substantial efforts have been directed towards elucidating the mechanisms of Wnt-related ocular angiogenic disorders. The absence of norrin/FZD4 pathway results in reduced expression of SoxF family including Sox7, Sox17, and Sox18 in the developing retina (Zhou et al., 2015), with Sox17 suggested to be a genetic controller of the angiogenic program (Ye et al., 2009; Zhang et al., 2017b; Zhou et al., 2015). The single deletion of any of these SoxF members does not affect vascular development in the retina, whereas combined deletion of Sox7, Sox17, and Sox18 leads to a dense capillary plexus with a dramatic loss of radial arteries and veins without reproducing the Wnt-deficient vascular phenotype, and the presence of a single Sox17 allele largely restores arterial identity (Zhou et al., 2015). On the other hand, suppression of claudin-5, a tight-junction protein down-regulated in both *Ndp^{y/-}* and *Lrp5^{-/-}* retinas (Chen et al., 2012; Schafer et al., 2009), partially suppresses developmental retinal angiogenesis (Chen et al., 2011b), however, it is not yet clear whether claudin-5 deficient mice, which show size-dependent loosening of brain vascular integrity (Nitta et al., 2003), may recapitulate the defective ocular vasculature in *Ndp^{y/-}* and *Lrp5^{-/-}* retinas.

Gene expression analysis has identified multiple common down-stream genes dysregulated in both *Ndp^{y/-}* and *Lrp5^{-/-}* retinas (Chen et al., 2012; Schafer et al., 2009), including down

regulation of claudin-5 and upregulation of PLVAP, endothelial markers for vascular leakage, reflecting increased retinal and brain vascular permeability in these mice (Chen et al., 2011b; Wang et al., 2012; Zhang et al., 2017a), which is consistent with the suggested role of Wnt signaling in regulating blood-brain barrier (Daneman et al., 2009; Liebner et al., 2008; Ma et al., 2017; Stenman et al., 2008; Zhang et al., 2017a). It is important to note that Sox7, Sox17, and Sox18 are also crucial in maintaining blood vessel integrity in adulthood, as adult onset vascular endothelial-specific deletion of all three SoxF genes leads to massive edema despite nearly normal vascular architecture (Zhou et al., 2015). Other common genes dysregulated in both *Ndp^{-/-}* and *Lrp5^{-/-}* retinas include an amino acid transporter SLC38A5, apolipoprotein D and aderenomedullin (Chen et al., 2012; Schafer et al., 2009), yet whether these or other proteins mediate Wnt-dependent ocular angiogenesis is not fully clear and awaits further studies.

Other Wnt signaling related pathways, or co-receptors, may also participate in the regulation of retinal angiogenesis. In addition to FZD4, FZD7 is also expressed by endothelial cells and drives retinal angiogenesis via activation of DVL/ β -catenin signaling (Peghaire et al., 2016). FZD7, which controls the crosstalk between Wnt and Notch signaling pathways during postnatal angiogenesis (Peghaire et al., 2016), may function as a coreceptor in concert with FZD4 to interact with secreted Frizzled-related protein-1 (sFRP-1), a proposed Wnt signaling inhibitor, to promote angiogenesis after an ischemic event (Dufourcq et al., 2008). Another potential Wnt-coreceptor is GPR124, an orphan G protein-coupled receptor. GPR124 and RECK, a glycosphosphatidylinositol (GPI)-anchored protein, have been shown to serve as receptors for Wnt7 in collaboration with FZD and LRP co-receptors (Eubelen et al., 2018; Vallon et al., 2018). GPR124/RECK/Wnt7 were found to function in collaboration with norrin/FZD4 signaling to control CNS vascular development and brain-blood barrier regulation (Chang et al., 2017; Cho et al., 2017; Zhou and Nathans, 2014). Considering the close connection and the anatomical similarity between the eye and the brain (London et al., 2013), the GPR124/RECK/Wnt7 signaling may also be relevant in the retinal vascular development. In addition, very low density lipoprotein receptor (VLDLR) was suggested to negatively inhibit the Wnt signaling pathway by targeting LRP6 and thereby suppressing retinal angiogenesis (Chen et al., 2007), yet VLDLR may also function through other Wnt-independent mechanisms, including influencing photoreceptor lipid and glucose energy metabolism (Joyal et al., 2016) and c-Fos induced inflammation (Sun et al., 2017). Moreover, Hippo effectors YAP and TAZ have been shown to interact with DVL to inhibit Wnt/ β -catenin (Varelas et al., 2010), and regulate endothelial cell migration through controlling the Rho family GTPase CDC42 activity (Sakabe et al., 2017).

It is important to note that defects in several other signaling pathways unrelated to Wnt signaling, such as angiopoietin and transforming growth factor- β (TGF- β), lead to similar ocular vascular phenotypes as observed in Wnt-deficient mouse models. Loss of angiopoietin-2 in mice delays development of the superficial plexus and causes complete absence of deeper vascular layers, as well as persistent hyaloid (Hackett et al., 2002), similar to norrin/FZD4/LRP5 deficient mice. Similarly, deletion of TGF- β 1 or vascular endothelium specific knockout of TGF- β receptor causes incomplete formation of superficial retinal vasculature and disruption of deep vascular layers in the retina (Allinson et al., 2012; Arnold et al., 2012). In addition, genetic deletion of girdin, an AKT substrate functioning as an

actin-binding protein, also leads to more sparse superficial vasculature coverage, and incomplete formation of intermediate and deep vascular layers (Ito et al., 2013; Kitamura et al., 2008). Girdin is important in VEGF-dependent endothelial cell migration and tubular formation (Anai et al., 2005; Enomoto et al., 2005; Wang et al., 2017). Given these observations, it is tempting to speculate that these different pathways: norrin/FZD4/LRP5, angiopoietin, TGF β and girdin, may somehow converge to coordinate angiogenic processes. Supporting this notion is the observation that angiopoietin-2 is induced in norrin-treated vascular endothelial cells (Ohlmann et al., 2010). In addition, norrin has weak sequence homology with TGF- β family proteins, and maternal *Xenopus* norrin is suggested as an antagonist of BMP4/TGF- β activities (Xu et al., 2012). Cross-inhibition of norrin and TGF- β was also reported in transgenic mice over-expressing either norrin or TGF- β in the lens (Seitz et al., 2018), suggesting that loss of inhibition of TGF- β may contribute in part to Norrie disease (Xu et al., 2012). So far no genetic mutations in either angiopoietin-2 or TGF- β mutations have been linked with human FEVR. Additional work may shed further light on the cross interaction of these signaling pathways to help uncover the exact molecular mechanisms underlying Wnt signaling that guides the retinal angiogenesis.

5.3 Norrin/Wnt signaling in hyaloid regression

In addition to affecting retinal vasculature, Wnt signaling also influences hyaloid vessel regression. Wnt7b, which activates canonical Wnt signaling, is critical for proper regression of hyaloid vessels (Lobov et al., 2005). In the developing eye, macrophages around hyaloid secrete Wnt7b, which activates Wnt/ β -catenin signaling in adjacent hyaloidal endothelial cells, later stimulating cell cycle entry through FZD4-LRP5, causing programmed cell death, thereby resulting in hyaloid vascular regression. As a result, transgenic mice carrying mutations in *Wnt7b* (*Wnt7b^{dl/dl}*) have persistence of hyaloid vessels (Lobov et al., 2005). Similar to Wnt7b, the binding of norrin to FZD4-LRP5 also plays an important role in the induction of hyaloid vascular regression during development. *Ndp^{y/-}*, *Fzd4^{-/-}* and *Lrp5^{-/-}* mice all show delayed regression of hyaloid vessels supporting this notion (Chen et al., 2012; Kato et al., 2002; Ohlmann et al., 2004; Xia et al., 2008; Xu et al., 2004; Ye et al., 2009). A recent study discovered two genes downstream of Wnt/ β -catenin signaling, *c-Myc* and the cyclin-dependent kinase inhibitor *Cdkn1a*, were required for hyaloid regression through regulation of cell cycle progression and cell death (Nayak et al., 2018). Additionally, the co-receptors LRP5 and LRP6 have overlapping functions on mediating Wnt/ β -catenin signaling in hyaloid vascular endothelial cells (Nayak et al., 2018). Since hyaloid regression and incomplete retinal vessel development often go hand-in-hand, incomplete retinal vascularization in Wnt-deficient retinas can result in retinal tissue hypoxia and elevated VEGF levels (Luhmann et al., 2005; Rattner et al., 2014; Wang et al., 2016), which may contribute to persistence of the hyaloid vasculature. Supporting this VEGF-dependent notion is the observation from mice with retinal neuronal deletion of VEGFR2, which demonstrates massive persistent hyaloid vessels due to excessive VEGF from lack of sequestration by VEGFR2 (Yoshikawa et al., 2016).

6 Aberrant Wnt signaling in other vascular eye diseases

In addition to inherited eye diseases with Wnt-related mutations, aberrantly upregulated Wnt signaling was also suggested as pathogenic in several experimental models of more prevalent eye diseases including retinopathies and age-related macular degeneration, indicating a broad role of Wnt signaling in non-genetic vascular eye diseases.

6.1 Wnt signaling in retinopathy of prematurity (ROP)

ROP affects pre-term infants with low birth weight and exposure to high oxygen supplementation. The pathologic progress of ROP starts with arrest of immature retinal development of both vessels and neurons by premature birth (phase I), followed by tissue ischemia, thereby resulting in hypoxia-induced neovascularization (phase II) (Chen and Smith, 2007; Chen et al., 2011a; Hellstrom et al., 2013). In addition to prematurity, hyperoxia (room oxygen level after birth compared with *in utero*, combined with supplemental oxygen) also contributes to the initial delay of vascular growth in both humans and mouse models (Connor et al., 2009; Hartnett and Penn, 2012; Smith et al., 1994), due to suppression of oxygen-regulated angiogenic growth factors, particularly VEGF (Pierce et al., 1995) and erythropoietin (Chen et al., 2008; Chen et al., 2009a). As the retina grows with increased metabolic demands, ischemic retinas become hypoxic and drive increased angiogenic growth factors such as VEGF and erythropoietin, leading to the second proliferative phase (Aiello et al., 1995; Watanabe et al., 2005), which may cause exudates, fibrous scar formation and tractional retinal detachment (Chan-Ling et al., 2018; Fulton et al., 2009; Hansen et al., 2017).

Whereas prematurity itself and oxygen exposure are the major risk factors of ROP, genetic predisposition may also contribute to some cases of ROP. Many signaling pathways were investigated with regard to their roles in the pathogenesis of ROP, most of which relate to hypoxia and inflammation. Among those, a relationship between ROP and the Wnt signaling pathway was discovered through genetic studies. Mutations in *NDP*, *FZD4* and *LRP5* have all been associated with ROP (Dailey et al., 2015; Drenser, 2016; Ells et al., 2010; Hutcheson et al., 2005; Shastri, 2010). Some studies have demonstrated mutations in *NDP* in patients with advanced ROP (Dickinson et al., 2006; Shastri et al., 1997), which may account for 3% of patients with severe ROP (Hiraoka et al., 2001). Yet some limitations may exist for these studies linking Wnt pathway genetic variations with predisposition to ROP. Genetic testing was not always done on all ROP patients, and clinically it is possible to misdiagnose FEVR, or Norrie disease as ROP, due to the similarities in their clinical manifestations (Drenser, 2016; Yonekawa et al., 2017).

In experimental studies of ROP, ectopic over-expression of norrin from either the lens or the RPE promotes physiological vascular regrowth and thereby suppresses pathological vessel growth in a mouse oxygen-induced retinopathy (OIR) model (Ohlmann et al., 2010), a well-established animal model to represent ROP in humans (Figure 6A) (Smith et al., 1994). The effects of norrin in OIR may be exerted by the expression of angiopoietin-2 (*Ang-2*) downstream of norrin, and reflect a norrin-specific regulatory mechanism by *Ang-2*, since Wnt-driven β -catenin signaling does not lead to *Ang-2* up-regulation (Ohlmann et al., 2010; Reis et al., 2012). On the other hand, several studies found that aberrant activation of Wnt

signaling in OIR retinas was a major pathogenic mechanism for retinal neovascularization (Chen et al., 2011b; Chen et al., 2009b; Liu et al., 2013a; Takahashi et al., 2015; Wang et al., 2015; Wang et al., 2014). Our group previously found that up-regulated levels of Wnt ligands, receptors (FZD4 and LRP5) and Wnt/ β -catenin activity during the proliferative stage in the retinas of OIR mice (Figure 6B&C), and that genetic loss of LRP5 or DVL2 significantly suppressed neovascularization in OIR (Chen et al., 2011b). Furthermore, suppression of the aberrantly activated Wnt signaling rescues the phenotypes of the OIR retinas, providing pharmaceutical targets to treat ocular neovascularization diseases (Chen et al., 2011b; Chen et al., 2009b; Takahashi et al., 2015; Wang et al., 2015; Wang et al., 2014). For example, blocking Wnt target protein claudin5 significantly suppresses Wnt pathway-driven endothelial cell sprouting *in vitro* and developmental and pathological vascular growth in OIR *in vivo* (Chen et al., 2011b). Targeting very low-density lipoprotein receptor (VLDLR), an inhibitor of Wnt signaling and retinal neovascularization (Chen et al., 2016b; Chen et al., 2007; Lee et al., 2014), via using the VLDLR N-terminal ectodomain also leads to suppression of OIR vascular response via the inhibition of Wnt signaling (Wang et al., 2015). These studies suggest that Wnt signaling may play dual angiogenic roles in a time-dependent manner during different phases of retinopathy.

6.2 Wnt signaling in age-related macular degeneration (AMD)

AMD is a common cause of irreversible blindness in the elderly (Gehrs et al., 2006), with progressive loss of central fine color vision in the macula (Ardeljan and Chan, 2013). The “dry” or atrophic form features significant RPE degeneration leading to photoreceptor loss. The “wet” or neovascular form of AMD is characterized by choroidal neovascularization (CNV), which invades into the subretinal space and leads to exudation and acute vision loss. Although “wet” AMD only constitutes 10-15% of AMD patients, it accounts for 90% of the AMD-associated vision impairment. The neovascularization in AMD has two etiologic patterns: (1) the most common pattern of CNV refers to the new vessels sprouting from the choroidal vessels, penetrating Bruch’s membrane and growing into the subretinal space (Green, 1999); and (2) abnormal intraretinal vessels derived from the retinal vasculature in a process called retinal angiomatous proliferations (RAP), a special subtype of wet AMD (Brancato et al., 2002). In RAP, new retinal vessels develop from the neurosensory retina and extend outward into the subretinal space, sometimes anastomosing with the choroid-derived vessels (Lafaut et al., 2000). The mechanisms and factors regulating CNV development and formation in “wet” AMD are extensively studied with VEGF identified as a major factor (Grisanti and Tatar, 2008; Krzystolik et al., 2002), and anti-VEGF therapies have become the most common and effective treatment for neovascular AMD (Schmidt-Erfurth and Pruenke, 2007).

Various animal models have been used to study AMD (Grossniklaus et al., 2010), and some studies have suggested that aberrantly activated Wnt signaling may be another pathogenic factor in AMD (Zhou et al., 2010). Activated canonical Wnt signaling pathway was found in both human macular tissues from AMD patients (Tuo et al., 2015), and in a murine laser-induced CNV model (Hu et al., 2013), which is often used to study the angiogenic aspect of wet AMD. Specifically, phosphorylated LRP6 and non-phosphorylated- β -catenin (active form) are up-regulated both in laser-induced CNV model (Hu et al., 2013) and very low

density lipoprotein receptor gene knock-out (*Vldlr*^{-/-}) mice that model RAP, a form of AMD with abnormal intraretinal vessels (Chen et al., 2007). Suppression of Wnt/ β -catenin signaling with either an anti-LRP6 antibody or Wnt inhibitor DKK1 arrests the neovascular lesions in both murine models of CNV and *Vldlr*^{-/-} (Chen et al., 2007; Hu et al., 2013). Wnt7a and Wnt7b also stimulated vascular sprouting from mouse choroidal explants and deletion of both genes decreases the severity of laser-induced CNV (Lin et al., 2018).

More evidence linking Wnt signaling with AMD comes from studies of other Wnt modulators including kallistatin, an inhibitor of the Wnt signaling pathway. Kallistatin is an endogenous anti-angiogenic and anti-inflammatory factor in the serine proteinase inhibitor (SERPIN) family (Zhang et al., 2013), and it inhibits the Wnt signaling pathway by binding to LRP6 (McBride et al., 2014). Circulating levels of kallistatin are down-regulated in the plasma of AMD patients compared to non-AMD subjects (Tuo et al., 2015). On the other hand, some other Wnt signaling modulators, such as WIF-1 and DKK3 exhibit significantly higher levels in the aqueous humor of neovascular AMD patients (Park et al., 2014), whereas DKK1 levels are decreased in the circulation in patients with wet AMD (Qiu et al., 2017a). Cell metabolism is also affected in patients with AMD, as Vallee *et al.* found that up-regulated Wnt signaling was involved in the activation of aerobic glycolysis for production of angiogenic factors in wet AMD patients (Vallee et al., 2017). Wnt signaling is also linked with an RPE epithelial phenotype, especially during aging. The impaired organelle motility with aging may raise low levels of stress which was accompanied with RPE morphology changes, and eventually contributing to aging retinal disease such as AMD (Burke, 2008). Together these findings support a pathogenic role of Wnt signaling in both dry and wet AMD. Suppressing the abnormally activated Wnt signaling pathway in neovascular AMD patients may be a potential way to treat or inhibit the exudative CNV lesions.

6.3 Wnt signaling in diabetic retinopathy (DR)

DR is a common microvascular complication of diabetes, and a leading cause of blindness in the working age population in developed countries. Based on the vascular progression, DR is classified into two stages: non-proliferative DR and proliferative DR (Antonetti et al., 2012; Cheung et al., 2010). Non-proliferative DR is featured with the presence of microaneurysms, hemorrhages, hard exudates, cotton wool spots, venous dilation and beading, while proliferative DR is characterized by neovascularization, pre-retinal hemorrhage, or vitreous hemorrhage (Antonetti et al., 2012). Multiple microvascular pathological changes take place in DR, including leukostasis, pericyte loss, acellular capillaries, progressive vascular occlusions, increased vascular permeability, macular edema, and neovascularization, which ultimately proliferates into the vitreous (Durham and Herman, 2011).

Up-regulated Wnt signaling components have been observed in DR in several studies (Chen and Ma, 2017). Elevated levels of β -catenin in the retinal sections of patients with DR were found compared to non-diabetic controls, indicating an aberrant activation of Wnt signaling in DR (Chen et al., 2009b). Additionally, β -catenin and LRP5/6 levels are up-regulated in two type I diabetic animal models - Akita mice and streptozotocin (STZ)-induced diabetic rats, as well as OIR model which is commonly used to mimic proliferative stage of DR

(Chen et al., 2009b). Elevated LRP6 levels in the vitreous of proliferative DR patients correlate with increased vitreous VEGF levels (Gao et al., 2015; Wirostko et al., 2008). In addition, plasma and vitreous fluid levels of a Wnt inhibitor DKK1 are lower in patients with DR compared with those from non-diabetic patients or diabetic patients without DR (Chen et al., 2009b; Qiu et al., 2014). DKK1 also reduces vascular leakage and neovascularization in OIR model and STZ-induced diabetic models (Chen et al., 2009b). In addition to DKK1, several Wnt signaling inhibitors were evaluated in different animal models of DR to investigate the benefits of blocking the aberrant Wnt signaling. Kallistatin exerts anti-angiogenic and anti-neuroinflammatory effects in Akita mice and OIR mice (Liu et al., 2013b). A monoclonal antibody termed Mab2F1 targeting LRP6 inhibits vascular leakage, and inflammation in three diabetic retinopathy models: OIR model, STZ-induced diabetic model and lens-capsulotomy-induced retinal degeneration in Akita mice (Hombrebueno et al., 2018; Lee et al., 2012). Serine protease inhibitor A3K (SERPINA3K) reduces fibrogenic activity by attenuating the connective tissue growth factor and fibronectin overexpression in retinas of STZ-induced diabetic rats (Zhang et al., 2010b). Finally, interruption of Wnt signaling in Müller cells (conditional β -catenin knockout (KO) in Müller cells) ameliorates ischemia-induced retinal neovascularization in OIR and STZ-induced diabetic model (Zhou et al., 2014a). Together, these Wnt signaling inhibitors improves neovascularization and inflammation in DR, further confirming the causative role of the Wnt signaling in the pathological process of DR.

6.4 Wnt signaling in corneal neovascularization

The avascular nature of the cornea is crucial to maintaining corneal transparency, vision clarity and its angiogenic and lymphangiogenic privilege (Ellenberg et al., 2010). Studies using transgenic mice, which traced fluorescence-labeled angioblasts and blood vessels, show that the cornea is never vascularized during development (Kwiatkowski et al., 2013). Both proangiogenic factors such as VEGF, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and antiangiogenic factors including the semaphorins and netrins, are expressed in the anterior eye (Kwiatkowski et al., 2013). Although the interplay between these factors in the cornea is not completely understood, soluble VEGF receptor-1 (sVEGFR1; also known as sFlt-1), which is highly expressed in the cornea serving as an endogenous VEGF-A trap (Ambati et al., 2006; Kendall and Thomas, 1993), and ectopic expression of VEGFR-3 (Flt-4) by the epithelium (Cursiefen et al., 2006), are both suggested as key modulators inhibiting the invasion of blood vessels into the corneal stroma. In addition, the antiangiogenic factor semaphorin3A also prevents VEGF-induced vascularization in the developing cornea (McKenna et al., 2014). As a key pathway involved in many processes during eye development, the Wnt signaling pathway plays a critical role in epithelial stratification during corneal morphogenesis (Nakatsu et al., 2011; Zhang et al., 2015). Although Wnt signaling is a major regulator of VEGF, its immediate role in the regulation of VEGF-dependent corneal avascularity during normal ocular development remains to be determined. However, several studies have observed the activity of Wnt signaling in corneal neovascularization models (Wang et al., 2015; Zhou et al., 2014b), demonstrating the essential role of the Wnt signaling in maintaining the avascularity of the cornea in disease.

Corneal neovascularization, a sight-threatening condition, refers to the formation of new vessels from the pericorneal plexus into the normally avascular corneal tissue as a result of oxygen deprivation (Abdelfattah et al., 2015). Maintaining avascularity of the corneal stroma is an important aspect of corneal pathophysiology for corneal transparency and optimal vision. Diseases associated with corneal neovascularization include inflammatory disorders, corneal graft rejection, infectious keratitis, contact lens-related hypoxia, alkali burns, stromal ulceration, aniridia, and limbal stem cell deficiency (Beebe, 2008; Ma et al., 2006). Several animal models have been established to study corneal neovascularization, including the chemical (alkali) burn model, suture injury model, and some genetic spontaneous corneal neovascularization models (Liu et al., 2017). In these models, phosphorylation of LRP6 and active β -catenin is increased in the corneas of mice and rats after chemical burns (Wang et al., 2015). Additionally, the FZD4 receptor is upregulated in the corneas of rats after corneal suture surgery (Zong et al., 2016). Whereas overexpression of PAX6 produces an abnormal cornea with altered epithelial cell morphology and neovascularization in mice (Davis and Piatigorsky, 2011), Wnt7a controls the corneal epithelium differentiation and avascular feature of cornea through PAX6 (Ouyang et al., 2014). Further, PAX6 is also an important regulator of the Wnt signaling through its interaction with β -catenin and DKK3 (Forsdahl et al., 2014; Fujimura et al., 2015). Therefore, Wnt signaling appears to be closely intertwined with PAX6 to mediate the pathological process of corneal neovascularization.

Inhibiting the aberrant Wnt signaling pathway in the vascularized cornea may provide a feasible approach to reduce neovessel growth. A soluble VLDLR N-terminal ectodomain reduces pathological corneal neovascularization in alkali burn model by blocking the phosphorylation of the receptor LRP6 (Wang et al., 2015). Serine proteinase inhibitor SERPINA3K (Zhou et al., 2014b; Zhu et al., 2014) and curcumin (Kim et al., 2010; Zhang et al., 2017c) also suppress corneal neovascularization via the inhibition of Wnt Signaling and VEGF in corneal suture models. In addition, SERPINA3K ameliorates the corneal oxidative injury induced by 4-hydroxynonenal through the inhibition of the ROS system and Wnt signaling pathway (Zhang et al., 2010a; Zheng et al., 2017). The monoclonal antibody Mab2F1 and its humanized version H1L1 targeting LRP6 reduce the area of corneal neovascularization and opacity (Qiu et al., 2018). Taken together, activated Wnt signaling plays a pivotal role in the formation of corneal neovascularization, providing a potential molecular target for prevention.

7 Wnt signaling as a therapeutic target for ocular angiogenesis diseases

7.1 Pro- vs. anti-Wnt signaling: The need for fine-tuning

Due to the involvement of Wnt signaling in cancer, bone diseases and other diseases, development of therapeutics targeting Wnt signaling has received great interests (Humphries and Mlodzik, 2017; Katoh, 2017; Krishnamurthy and Kurzrock, 2018). Wnt signaling is abnormally active in most cancer, therefore anti-Wnt therapies are potential candidates for development of treatment in oncology. Some of these include targeting Wnt ligands, receptors, tankyrase (ADP-ribosyl transferase that promotes axin degradation), or β -catenin (Gurney et al., 2012; Katoh, 2017). On the other hand, since Wnt signaling is deficient in osteoporosis, pro-Wnt signaling is thereby needed for osteoporotic patients by targeting Wnt

antagonists such as DKK1, SOST (sclerostin), or GSK3 β , with current clinical trials focusing mostly on SOST-targeting therapies (McClung et al., 2014). In the eyes, as deficiency of norrin/Wnt signaling leads to FEVR and Norrie disease, and aberrant upregulation of Wnt signaling detrimentally affects other eye diseases including proliferative retinopathies and AMD, fine tuning of the Wnt signaling cascade in a disease- and context-dependent manner will be necessary for future development of potential treatment approaches.

7.2 Pro-Wnt signaling approaches to promote ocular vascular development

Eye diseases with deficient Wnt signaling such as Norrie disease and FEVR are characterized with severe defects in vessel morphology and visual impairment. Currently, there are no effective therapies available to prevent or treat the defective intra-retina vasculature in these diseases. Because the disease mutations occur on the ligand and receptor levels, one potential therapeutic approach is to target downstream Wnt signaling factors, thereby bypassing the genetic defects. In our previous study, a well-known Wnt signaling downstream activator, lithium, which functions as a GSK3 β inhibitor, was used to bypass the genetic defects of *Lrp5*^{-/-} mice, and successfully reverses the vascular and visual dysfunction in *Lrp5*^{-/-} eyes partially (Wang et al., 2016). Intraperitoneal injection of lithium restores Wnt signaling back to normal levels in the *Lrp5*^{-/-} retina, which accelerates the development of retinal vasculature, restores intra-retinal vessel network, and promotes hyaloid vessel regression (Figure 7A-C). Lithium also partially rescues visual function in the *Lrp5*^{-/-} retina with increased *b*-wave sensitivity in electroretinogram (Figure 7D). The successful normalization of ocular vessels in *Lrp5*^{-/-} mice suggests the possibility of a pro-Wnt signaling approach in treating Wnt-deficient vascular eye diseases by adapting existing Wnt activators such as lithium, which is FDA-approved for treating mental disorders.

Similarly, exogenous norrin has shown great value in promoting retinal vascular growth. Genetic overexpression of norrin driven by lens promoter restores the vascular defects in the retinas of mice with Norrie disease (Ohlmann et al., 2005), and also promotes vascular regrowth and inhibits pathological neovascularization in OIR mice (Ohlmann et al., 2010). Recombinant norrin treatment also reduces avascular area and neovascularization in OIR mice (Ohlmann et al., 2010; Tokunaga et al., 2013; Zeilbeck et al., 2016).

The protective effects of Wnt signaling extend beyond retinal vascular repair to neuroprotection in the retina as well (Hackam, 2005; Ohlmann and Tamm, 2012; Yi et al., 2007). Wnt3a protects retinal ganglion cells (RGC) from elevated eye pressure (Fragoso et al., 2011), and protects photoreceptors in a mouse model of inherited retinal degeneration (Patel et al., 2015). Wnt14 inhibits death of retinal precursor cells (Mizukami et al., 2009). Norrin promotes RGC survival by increasing the number of surviving RGC axons in the optic nerve and decreasing apoptotic death of retinal neurons in staurosporine and NMDA (N-methyl-D-aspartate)-induced RGC damage models (Dailey et al., 2017a; Lin et al., 2009; Seitz et al., 2010). Early phase recombinant norrin treatment also improves RGC survival in OIR model (Dailey et al., 2017a, b). Together Wnt signaling may serve as promising targets to develop strategies that induce intraretinal vessel formation in ischemic retinopathies, or

promote RGC survival in retinal neuronal diseases such as glaucoma (Hackam, 2005; Ohlmann and Tamm, 2012; Yi et al., 2007).

7.3 Anti-Wnt signaling approaches to suppress pathological neovascularization

For retinopathies with pathologically over-activated Wnt signaling, inhibitors of Wnt signaling may be beneficial in treating vascular abnormalities in OIR, AMD, DR or corneal neovascularization (Zhang and Ma, 2010). These inhibitors include endogenous proteins, such as DKK1 (Chen et al., 2007; Chen et al., 2009b), kallistatin (Liu et al., 2013b; McBride et al., 2017), VLDLR (Chen et al., 2007; Wang et al., 2015), pigment epithelium-derived factor (PEDF) (Park et al., 2011), peroxisome proliferator-activated receptor- α (PPAR α) (Cheng et al., 2016), antibodies targeting Wnt signaling receptors such as Mab2F1 (anti-LRP6) (Lee et al., 2012), 1.99.25 (anti-FZD4) (Paes et al., 2011), and Ab 2D10 (anti-TSPAN12) (Bucher et al., 2018; Bucher et al., 2017), and microRNAs such as miRNA-150 (Liu et al., 2015) and miRNA-184 (Takahashi et al., 2015). Endogenous angiogenic inhibitors constitute a major category in ocular neovascularization therapy since the imbalance between angiogenic stimulators and inhibitors is a major contributor to the pathological ocular angiogenesis induced by various stressors, such as hypoxia or ischemia, inflammation and tumor (Zhang and Ma, 2007). Monoclonal antibodies targeting Wnt components may bind to their targets with high specificity. This class of therapeutics possesses many clinical benefits for treating angiogenic and inflammatory ocular diseases (Rodrigues et al., 2009). MicroRNAs (miRNAs) also play a critical role in the pathogenesis of angiogenesis (Mastropasqua et al., 2014), and those microRNAs targeting Wnt signaling components are potential candidates to develop as therapeutics for vascular eye diseases.

In Table 3, we summarized and categorized different Wnt signaling regulators into 3 groups, by the signaling components they target and intercept as follows: extracellular modulators targeting ligand/receptor complex, intracellular modulators targeting GSK3 β /axin/APC complex, and inner nuclear modulators targeting transcription factor TCF/LEF-1. Extracellular inhibitors usually block the first step of Wnt signaling activation, by interacting with either Wnt ligands or receptors. Wnt inhibitory factor 1/2 (WIF-1/2), SFRPs and cerberus (CER1) all bind Wnt ligands to prevent their binding to receptors FZD and LRP5/6 (Hsieh et al., 1999; Kawano and Kypta, 2003; Piccolo et al., 1999), whereas sclerostin binds the receptor LRP5/6 to compete with the Wnt ligands binding (Li et al., 2005; Semenov and He, 2006). However, these inhibitors are not evaluated in ocular vascular diseases and their efficacy remains to be determined.

The DKK family also interacts with LRP6 and another transmembrane protein Kremen1/2 (Krm1/2) to form a complex, which facilitates the internalization and degradation of LRP6, blocking its binding to Wnt ligands (Bafico et al., 2001; Mao et al., 2002; Niehrs, 2006). DKK1 was evaluated in OIR, STZ-induced diabetes model and *Vldlr*^{-/-} mice. DKK1 inhibits increased Wnt signaling and reduces pathological neovascularization and inflammation in those animal models (Chen et al., 2007; Chen et al., 2009b; Tokunaga et al., 2013). Interestingly, DKK2 plays an opposite role by promoting angiogenesis via a signaling cascade involving LRP6-mediated APC/Asef2/Cdc42 activation (Min et al., 2011). In addition, a human serine proteinase inhibitor, kallistatin, is recognized as a novel inhibitor

for LRP6, and significantly suppresses retinal neovascularization and inflammation in OIR mice (Liu et al., 2013b; Zhang et al., 2013). Kallistatin also inhibits retinal neuroinflammation and vascular leakage in Akita mice, a type I diabetes model (Liu et al., 2013b). SERPINA3K, another novel inhibitor targeting LRP6, ameliorates inflammation and retinal neovascularization in OIR model (Zhang et al., 2009; Zhang et al., 2010b; Zhou et al., 2014b). PEDF, an anti-angiogenic factor (Longeras et al., 2012), is a novel canonical Wnt inhibitor via binding to LRP6 (Park et al., 2011). Additionally, a PEDF-derived peptide reduces retinal neovascularization through blocking the mobilization of bone marrow-derived endothelial progenitor cells (Barnstable and Tombran-Tink, 2004). PPAR α , another LRP6 inhibitor, reduces ocular neovascularization, neuronal death and pericyte loss in rodent models of OIR, STZ-induced diabetic retinopathy, and laser-induced CNV (Chen et al., 2013; Cheng et al., 2016; Deng et al., 2017; Ding et al., 2014; Moran et al., 2014; Moran and Ma, 2015; Qiu et al., 2017b; Wang et al., 2014). Curcumin reduces corneal neovascularization in a suture model via the inhibition of LRP6 activity (Kim et al., 2010; Zhang et al., 2017c). The key effects of VLDLR in retinal vascular development are well-established by the pathologic subretinal vessel growth in *Vldlr*^{-/-} mice. Upregulation of Wnt signaling in *Vldlr*^{-/-} retinas was suggested to be partially responsible for its abnormal neovascularization (Chen et al., 2007). VLDLR and its N-terminal extracellular domain function as inhibitors for LRP6, and inhibit abnormal vessel formation in models of OIR and alkali-burned corneal neovascularization (Chen et al., 2007; Wang et al., 2015).

In addition to Wnt inhibitors, several neutralizing antibodies against Wnt receptors were developed to inhibit Wnt signaling activity in certain eye vascular diseases. Some examples include Mab2F1 targeting LRP6 (Lee et al., 2012), 1.99.25 targeting FZD4 (Paes et al., 2011), and Ab 2D10 targeting TSPAN12 (Bucher et al., 2018; Bucher et al., 2017). These antibodies were evaluated in mice in separate studies using OIR model, STZ-induced diabetes model, lens-capsulotomy-induced retinal degeneration in Akita mice, and *Vldlr*^{-/-} mice models, and have significant vaso-suppressive effects in both OIR and *Vldlr*^{-/-} mice (Bucher et al., 2017; Hombrebueno et al., 2018; Paes et al., 2011), inhibition of vascular leakage and leukostasis in diabetic retinopathy model (Lee et al., 2012), and suppression of inflammation and protection against neuronal degeneration in Akita mice with lens capsulotomy (Hombrebueno et al., 2018). In addition to antibodies, miRNAs have been widely investigated and hold potential as ocular therapeutics (Mastropasqua et al., 2014). MiRNAs are single-stranded, small (19~24 nucleotides) non-coding RNA molecules (Landgraf et al., 2007) and serve as negative regulators of gene expression at the post-transcriptional level by partially binding complementary to the 3' untranslated region (3'UTR) of target mRNAs (Xu, 2009). Several miRNAs have been shown to negatively modulate Wnt receptors in experimental models. MiR-150 and miR-184 inhibit Wnt receptors FZD4 and FZD7 expression, respectively, and both miRNAs suppress pathologic neovascularization in animal models (Liu et al., 2015; Takahashi et al., 2015). Because miRNAs may target multiple target mRNAs, it is worth noting that the effects of these miRNAs may also act through additional targets other than FZD receptors.

The intracellular Wnt antagonists act mainly on factors downstream of the Wnt signaling cascade in the cytoplasm or nucleus. GSK3 β , axin and APC form the secondary Wnt signaling complex which leads to β -catenin phosphorylation and degradation, thus

preventing its translocation into the nucleus (Kimelman and Xu, 2006). This secondary complex also provides an optimal pharmaceutical target for potential Wnt modulators, such as endostatin. For eye diseases related with over-activated Wnt signaling such as AMD and DR, endostatin directly inhibits β -catenin to reduce VEGF-dependent retinal vascular permeability, neovascularization and retinal detachment (Dixelius et al., 2003; Takahashi et al., 2003). Lastly, other factors such as Chibby in the nucleus may act on the interaction between β -catenin and the transcription factor TCF/LEF-1 to inhibit the target gene expression (Takemaru et al., 2003). Whether such inhibitors have therapeutic effects on ocular vascular diseases awaits further studies.

Together, a large number of studies provide compelling evidence supporting both pro-Wnt and anti-Wnt approaches as potential therapeutics for vascular eye diseases in a disease- and disease phase-dependent context. The Wnt signaling pathway is indispensable for normal development of ocular vasculature, and mutations in Wnt components are genetically linked with multiple pediatric vascular eye diseases. As such pro-Wnt signaling will be beneficial in these circumstances and also in the early vaso-obliterative phase of ischemic retinopathy. On the other hand, aberrant activation of Wnt signaling is associated with pathological neovascularization in proliferative retinopathies and AMD, and thus anti-Wnt signaling may offer a new therapeutic strategy for treating these diseases.

8 Conclusions and Future Directions

The development and homeostasis of ocular vasculature rely on multiple growth factors controlled by their respective signaling pathways, including VEGF, angiopoietin, TGF β , NOTCH and Wnt. This review focuses on the Wnt signaling pathway, an indispensable pathway regulating physiological and pathological vessels in the eye. Loss-of-function in Wnt signaling causes impaired intra-retinal vasculature, delayed vessel pruning, secondary pathologic vascular proliferation, and persistent hyaloid vessels in congenital diseases such as FEVR and Norrie disease. Whereas the development of superficial retinal vascular plexus is relatively well understood, it is still a challenge to fully elucidate the molecular mechanisms driving the formation of deeper vascular networks in the retina, where Wnt signaling plays a critical role. Which factors are downstream of Wnt signaling and essential for the deeper retinal vessel formation? Does Wnt signaling converge or intersect with other signaling pathways to coordinate this process? These questions await further studies. Additional work in both experimental models and potential discovery of new disease genes linked to FEVR may provide new insight. On the translational aspect, although our studies showed that pro-Wnt signaling lithium treatment partially rescues the FEVR phenotype in the mouse model, potential translation of this work into humans may face considerable challenges. Whether effective treatments in mouse models will be effective in humans is a major question. Another obstacle is the proper time window of pro-Wnt treatment, because the development of retinal vessels in humans occurs *in utero*, unlike in mice where it happens after birth. Nevertheless, evaluating similar concepts of pro-Wnt treatment in the Norrie disease model may offer additional benefits in restoring chronic hearing loss to improve patient's quality of life.

In addition to impaired retinal angiogenesis, deficient Wnt signaling also leads to breakdown of blood-retinal barrier, hence increased vascular leakage and hemorrhage. This also occurs in the central nervous system where the integrity of blood-brain barrier is regulated in part by Wnt signaling. Further investigation of Wnt signaling may help provide clues to the molecular basis controlling blood-retinal barrier, which is highly relevant for retinal edema. As a protective barrier to exclude potential pathogen and toxins, the existence of blood-retinal and blood-brain barrier also represents a major obstacle for drug delivery to reach the retina or the brain to treat diseases affecting these regions. Future work on potential ways to modulate Wnt pathway and temporarily opening up blood-retinal or blood-brain barrier may help facilitate drug delivery to the retina and brain.

In ischemic proliferative retinopathies and AMD, on the other hand, pathological activation of the Wnt signaling often results in excessive pathological neovascularization and ocular inflammation. Inhibition of Wnt signaling in these conditions has been largely effective in experimental models. These studies necessitate precise targeting of Wnt signaling to fine tune its proper levels in order to treat different eye diseases or different phases of diseases. For example, a pro-Wnt signaling approach is needed when Wnt signaling is deficient during incomplete developmental angiogenesis and during vaso-obliteration phase in retinopathy; however, an anti-Wnt signaling approach is necessary for direct inhibition of pathological neovascularization. With many Wnt modulators developed and currently in clinical trials for cancer and osteoporosis application, it is tempting to adapt some of these molecules to assess their efficacy in the ocular setting, in addition to those being developed specifically for vascular eye diseases. Among those, antibodies against Wnt receptors seem to hold great promise as they are well tolerated and longer lasting. Future advances in ocular drug delivery including potential cell-penetrating topical drug delivery formulation for small molecule modulators, and slow-release system or longer-lasting formulas may offer additional advantage that will allow us to better treat and manage the vascular eye diseases impacting the vision of both children and adults.

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Figure 1. A schematic illustration of the ocular vasculature

Middle: A schematic cross-section through an eye showing the retinal vasculature lining the inner surface of the retina and the choroid vessels between the retina and the sclera. Right: An enlarged cross sectional illustration of the eye showing detailed structure of the retinal and choroidal vasculature. Three layers of retinal vessels are embedded among retinal neurons: the superficial retinal vasculature lies in the NFL; the intermediate and deep retinal vascular networks align along each sides of the INL. The choroidal vessels between RPE and sclera serve to supply blood to the outer portion of the retina. GCL: ganglion cell layer; INL: inner nuclear layer; NFL: nerve fiber layer; ONL: outer nuclear layer; RPE: retinal pigment epithelium. Enlarged images on the left depict retinal and choroidal vascular cast from mouse eyes. Scale bar: 100 μ m. Figure was adapted with permission from (Chen et al., 2016a).

Figure 2. Overview of Wnt signaling pathway.

(A) In the absence of Wnt or when Wnt is prevented to bind to FZD and LRP5/6 (off state), cytoplasmic β -catenin is targeted by the APC/Axin/GSK3 β complex and CK1, which together coordinate phosphorylation of β -catenin, leading to its ubiquitination and proteosomal degradation. In this off state, the Wnt responsive genes (target genes) are repressed by TCF-TEL/Groucho and HDAC. (B) In the on-state with presence of Wnt ligands or Norrin, LRP5/6 is brought into the receptor complex with Wnt-bound FZD, which recruits DVL, leading to sequential phosphorylation of LRP5/6 by GSK3 and CK1, and recruitment of Axin. The destruction of APC/Axin/GSK3 β complex releases β -catenin and prevents its phosphorylation, allowing β -catenin to stabilize and accumulate in the cytoplasm in a non-phosphorylated form. Stabilized β -catenin subsequently translocates into the nucleus where it promotes transcription of Wnt target genes upon binding to TCF. APC, adenomatous polyposis coli; CK1, casein kinase 1; DVL, Dishevelled; FZD, Frizzled; GSK3, glycogen synthase kinase 3; HDAC, histone deacetylases; LRP, low-density lipoprotein receptor-related protein; TCF, T cell factor; TLE, transducing like enhancer of split 1.

Figure 3. Schematic of retinal vascular development.

(A) Cross-sectional diagram showing the hyaloid vascular system in the developing eye. In human, the hyaloid vessels regress before birth; whereas in mouse eye the process of hyaloid vessel regression occurs postnatally. (B) Cross-sectional diagrams of the developing eye showing the temporal development of the primary (superficial layer), secondary (deep layer), and tertiary (intermediate layer) retinal vessels. Solid red lines depict retinal blood vessels; broken red lines represent the regressing hyaloid vasculature. Deep, deep layer of retinal vessels; HA, hyaloid artery; Int, intermediate layer of retinal vessels; PM, pupillary membrane; Sup, superficial layer of retinal vessels; TVL, tunica vasculosa lentis; VHP, vasa hyaloidea propria. (C) A scheme of retinal vascular growth in mice that originates from the optic nerve head after birth and grows radially towards the peripheral of the retina, and reaches the edge of the retina around postnatal day (P) 8. The growth of the superficial vascular plexus of retinal vessels (red) follows a hypothesized central-to-peripheral physiologic hypoxia wave and an astrocytic template (green). Purple area indicates vascularized retina, and grey shaded area indicates hypoxic retina. (D) Left: A cartoon of retinal blood vessels with nearby interacting astrocytes and retinal ganglion cells (RGC). Right: RGCs secrete platelet-derived growth factor alpha (PDGFA) guiding the growth of astrocytes, which express PDGF alpha-receptor (PDGFR- α). Astrocytes in turn act as a template and secrete VEGF to guide the growing vascular front. The growth of endothelial tip

cells and stalk cells is balanced by DLL/NOTCH pathway. Panel C was adapted with permission from (Chen et al., 2016a).

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Figure 4. Fundus images and fundus fluorescein angiography (FFA) images from a FEVR patient.

(A) Peripheral fundus images of a 40-year-old FEVR patient. The black arrow in the right eye (OD) shows lesion areas, and the black arrow head in the left eye (OS) indicates a retinal hole and regional detachment. (B) FFA mages of the FEVR patient. The white arrow in the right eye (OD) shows areas of lesion and incomplete retinal vessels, and the white arrow head in the left eye (OS) indicates a retinal hole and regional detachment. Image courtesy of Dr. Lexi Ding at Xiangya Hospital, Central South University, China.

Figure 5. Defective ocular vasculature in *Lrp5*^{-/-} mouse model of FEVR.

(A) Representative images of the whole flat-mounted retinas at P7 from WT and *Lrp5*^{-/-} mice with vasculature stained with isolectin (IB₄, red), showing delayed retinal vascular development in *Lrp5*^{-/-} mice. (B) Whole flat-mounted retinas at P17 from WT and *Lrp5*^{-/-} mice stained with IB₄, showing pathologic glomeruloid vascular structures in the superficial plexus of *Lrp5*^{-/-} retina. (C) 3D re-construction of retinal vascular network demonstrating normal three layers in WT, and complete lack of intermediate and deep layers in *Lrp5*^{-/-} retina with thickened vascular sprouts. S: superficial layer; I: intermediate layer; D: deep

layer. **(D)** Isolated hyaloid vessels stained with DAPI (blue) shows persistent hyaloid in *Lrp5*^{-/-} eyes. **(E)** A model of Norrin/FZD4/LRP5 interaction. Norrin is secreted by Müller glia cells in the retina, and binds Wnt receptors FZD4/LRP5 expressed by vascular endothelial cells to regulate angiogenesis. Scale bar: 1000 μm (**A, B, & D**); 50 μm (**C**). Panels A-D were adapted with permission from (Wang et al., 2016).

Figure 6. Aberrantly upregulated Wnt ligands and receptor activity in oxygen-induced retinopathy.

(A) Schematic illustration of oxygen-induced retinopathy modeling retinopathy of prematurity and proliferative aspect of diabetic retinopathy. Neonatal mice with nursing mothers are exposed to oxygen from postnatal day (P) 7 to P12. Retinas are usually isolated at P17 to visualize vessels. (B) Localization of Wnt receptor Frizzled4 and Wnt activity in the OIR retina. Retinopathy is induced in wild type mice or TOP-Gal Wnt reporter mice. P17 wild type retinas with OIR are stained with Frizzled4 (magenta), Isolectin B₄ (red,

vessel) and CSF1-R (green, microglia). P17 retinas of TOP-Gal Wnt reporter mice with OIR are stained with β -Galactosidase (magenta, lacZ expression for Wnt activity) and Isolectin B₄ (red, vessel). Both Frizzled4 and Wnt activity (lacZ) show co-localization with pathologic neovessels (red). (C) Expression of Wnt ligands *Wnt3a*, *Wnt7a*, *Wnt10a* and *Norrin* in P17 wild type retinas exposed to OIR compared with age matched mice raised in room air. Figure was adapted with permission from (Chen et al., 2011b).

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Figure 7. Pro-Wnt treatment of lithium in FEVR model of *Lrp5*^{-/-} mice.

(A) Representative isolectin (IB₄, red) stained images of the whole flat-mounted retinas at P7 from *Lrp5*^{-/-} mice treated with daily lithium chloride (LiCl, 10 mg/kg body weight) or control (vehicle) from birth, showing promotion of vessel development by lithium. White dashed lines indicate retina edge. Yellow lines indicate the vascularized area edge. (B) 3D re-construction of retinal vascular network from P17 retinas of *Lrp5*^{-/-} mice treated with control (vehicle) or LiCl stained with IB₄, showing partial restoration of deep layer vasculature by lithium treatment. (C) Isolated hyaloid vessels stained with DAPI (blue) from

P8 retinas of *Lrp5*^{-/-} mice treated with control (vehicle) or LiCl, showing improved hyaloid regression by lithium. **(D)** Electroretinography (ERG) shows the partially rescued visual function of *Lrp5*^{-/-} mice treated with LiCl compared with control (vehicle), with quantification of *b*-wave amplitude, normalized as $\log$ of WT controls. Scale bar: 1000 μm (**A, C**); 50 μm (**B**). Figure was adapted with permission from (Wang et al., 2016).

Table 1.

Wnt-related human diseases in organs other than the eye.

Disease category	Disease/Syndrome	Mutated Wnt components (Protein/ Gene)	Effects on Wnt Signaling Activity	Reference
Developmental disorders	High Bone density	<i>LRP5/LRP5</i>	Downregulated	(Boyden et al., 2002; Lara-Castillo and Johnson, 2015; Little et al., 2002; Van Wesenbeeck et al., 2003)
	Osteogenesis imperfecta	Wnt-1/ <i>WNT1</i>	Downregulated	(Fahiminiya et al., 2013; Pyott et al., 2013)
	Osteoporosis-pseudoglioma syndrome	<i>LRP5/LRP5</i>	Downregulated	(Ai et al., 2005; Gong et al., 2001b)
	Robinow syndrome	Frizzled-2/ <i>FZD2</i>	Downregulated	(White et al., 2018)
		Wnt-5a/ <i>WNT5A</i>	Downregulated	(Person et al., 2010)
		<i>DVL-1/DVL1</i>	Downregulated	(White et al., 2015)
		<i>DVL-3/DVL3</i>	Downregulated	(White et al., 2016)
	Williams syndrome	Frizzled-9/ <i>FZD9</i>	Downregulated	(Wang et al., 1997)
	Autosomal-dominant oligodontia	<i>LRP6/LRP6</i>	Downregulated	(Massink et al., 2015)
	Familial tooth agenesis	<i>Axin-2/AXIN2</i>	Downregulated	(Lammi et al., 2004)
	Tetra-Amelia	Wnt-3/ <i>WNT3</i>	Downregulated	(Niemann et al., 2004)
Cancer	Colorectal cancer	<i>APC/APC</i>	Upregulated	(Nishisho et al., 1991)
		β -catenin/ <i>CTNNB1</i>	Upregulated	(Morin et al., 1997)
		<i>Axin-2/AXIN2</i>	Upregulated	(Liu et al., 2000)
		<i>TCF7L2(TCF4)/TCF7L2</i>	Upregulated	(Bass et al., 2011)
	Hepatocellular carcinoma	β -catenin/ <i>CTNNB1</i>	Upregulated	(de La Coste et al., 1998; Huang et al., 1999)
		<i>Axin-1/AXIN1</i>	Upregulated	(Satoh et al., 2000)
	Lung cancer	β -catenin/ <i>CTNNB1</i>	Upregulated	(Sunaga et al., 2001)
	Medulloblastoma	β -catenin/ <i>CTNNB1</i>	Upregulated	(Zurawel et al., 1998)
	Ovarian cancer	β -catenin/ <i>CTNNB1</i>	Upregulated	(Palacios and Gamallo, 1998)
	Pancreatic Cancer	β -catenin/ <i>CTNNB1</i>	Upregulated	(Tanaka et al., 2001)
	Skin tumor	<i>LEF1/LEF1</i>	Downregulated	(Takeda et al., 2006)
Others	Familial adenomatous polyposis	<i>APC/APC</i>	Upregulated	(Kinzler et al., 1991)
	Type II diabetes	<i>TCF7L2(TCF4)/TCF7L2</i>	Downregulated	(Florez et al., 2006; Grant et al., 2006)
	Coronary artery disease	<i>LRP6/LRP6</i>	Downregulated	(Mani et al., 2007)
	Late-onset Alzheimer	<i>LRP6/LRP6</i>	Downregulated	(De Ferrari et al., 2007)

Table 2:

Inherited vascular eye diseases associated with mutations in Wnt signaling components

Diseases	Mutant Genes	Ocular Pathologies	Inheritance Pattern	Citations
Norrie disease	<i>NDP</i>	Fibrovascular masses (pseudoglioma), cataract and the retrolental tissue. Anterior and posterior synechiae, corneal opacification, iris atrophy and vitreous hemorrhage, retinal detachment (frequently)	XR	(Berger et al., 1992; Chen et al., 1992)
FEVR	<i>NDP</i>	Peripheral temporal retinal avascular zone ± congenital retinal folds, macular ectopia, fibrous tissue band at ora serrate	XR	(Chen et al., 1993)
	<i>LRP5</i>		AD, AR	(Qin et al., 2005; Toomes et al., 2004)
	<i>FZD4</i>		AD, AR	(Qin et al., 2005; Robitaille et al., 2002)
	<i>TSPAN12</i>		AD, AR	(Nikopoulos et al., 2010; Poulter et al., 2010)
OPPG	<i>LRP5</i>	Retrolental masses, Hyperplasias of the vitreous, corneal opacities, and secondary glaucoma	AR	(Gong et al., 2001b)
PHPV	<i>NDP</i>	Fibrotic white stalk with hyaloid vessels extending from optic disk to posterior lens capsule	XR	(Shastri, 2009)
	<i>FZD4</i>		AD, AR	
Coats' disease	<i>NDP</i>	Unilateral retinal telangiectasia, exudative fibrosis	unclear	(Black et al., 1999)
	<i>FZD4</i>		AD	(Robitaille et al., 2011)
<i>NDP</i> -related ROP	<i>NDP</i>	Retinal neovascularization (NV), fibrous proliferation, end-stage retrolental fibroplasia	XR	(Hutcheson et al., 2005; Shastri et al., 1997)

FEVR: familial exudative vitreoretinopathy; OPPG: Osteoporosis-pseudoglioma syndrome; PHPV: persistent hyperplastic primary vitreous; XR: X-linked recessive; AD: autosomal dominant; AR: autosomal recessive.

Table 3.
Pharmacological modulators of Wnt signaling evaluated in rodent models of vascular eye diseases

Name	Related Diseases	Target	Effects on signaling	Animal Models Evaluated	Effects on phenotype	Citations
Lithium	FEVR	GSK3 β	Activation	<i>Lrp5</i> ^{-/-}	Improves intra-retinal vasculature, hyaloid vessel persistent, visual function	(Wang et al., 2016)
Norrin	Norrie disease	FZD4	Activation	<i>Ndp</i> ^{-/-}	Induces growth of ocular capillaries and restores normal retinal angiogenesis	(Dailey et al., 2017a; Ohlmann et al., 2005; Ohlmann et al., 2010; Tokunaga et al., 2013; Zeilbeck et al., 2016)
				OIR	Promotes vascular regrowth after oxygen-induced retinal vessel loss and suppresses retinopathy	
DKK1	ROP, DR, wet-AMD, RAP	LRP6	Inhibition	OIR	Reduces retinal avascular area and NV	(Chen et al., 2007; Chen et al., 2009b; Tokunaga et al., 2013)
				STZ-induced diabetes	Ameliorates retinal inflammation, vascular leakage, and NV	
				<i>Vldlr</i> ^{-/-} mice	Inhibits VEGF expression and pathologic NV	
Kallistatin	ROP, DR	LRP6	Inhibition	OIR	Inhibits retinal NV and inflammatory cytokines	(Liu et al., 2013b)
				Akita cross kallistatin-transgenic mice	Inhibits retinal neuroinflammation and vascular leakage	
Mab2F1, HIL1 (anti-LRP6 antibody)	ROP, DR, corneal NV	LRP6	Inhibition	OIR	Reduces vascular leakage, inflammation, and retinal NV	(Lee et al., 2012; Qiu et al., 2018)
				STZ-induced diabetes corneal NV	Inhibits retinal vascular leakage, inflammation, leukostasis and glial stress Reduces corneal NV and opacity	
SERPINA 3K	ROP, DR, corneal NV	LRP6	Inhibition	OIR	Inhibits inflammation and retinal NV, protects tight junction	(Zhang et al., 2010a; Zhang et al., 2009; Zhang et al., 2010b; Zhou et al., 2014b)
				STZ-induced diabetes	Antifibrogenic activity	
				corneal NV suture model	Inhibits corneal NV and inflammation	

Name	Related Diseases	Target	Effects on signaling	Animal Models Evaluated	Effects on phenotype	Citations
Curcumin	Corneal NV	LRP6	Inhibition	corneal NV suture model	Inhibits corneal NV	(Kim et al., 2010; Zhang et al., 2017c)
PEDF	ROP	LRP6	Inhibition	OIR	Inhibits retinal NV	(Gao et al., 2017; Longeras et al., 2012; Park et al., 2011)
PPAR α	ROP, DR, wet-AMD	LRP6	Inhibition	OIR	Inhibits retinal NV, reduces neuronal death and microvascular impairment	(Chen et al., 2013; Cheng et al., 2016; Deng et al., 2017; Ding et al., 2014; Moran et al., 2014; Moran and Ma, 2015; Qiu et al., 2017b; Wang et al., 2014)
				STZ-induced diabetes	Inhibits capillary pericytes loss, Ameliorates leukostasis and vascular leakage	
				Laser-induced CNV	Reduces CNV volume	
VLDLR	ROP, corneal NV, wet-AMD, RAP	LRP6	Inhibition	OIR	Inhibits retinal NV	(Chen et al., 2007; Wang et al., 2015)
				Alkali burn-induced corneal NV	Suppresses corneal NV	
1.99.25 (anti-FZD-4 antibody)	ROP, DR, RAP	FZD4	Inhibition	<i>Vldlr</i> ^{-/-}	Inhibits pathologic NV	(Paes et al., 2011)
				OIR	Inhibits physiological (vascular recovery), but not pathologic, angiogenesis	
miR-150	ROP, DR, wet-AMD	FZD4	Inhibition	OIR	Suppresses pathologic retinal NV	(Liu et al., 2015)
				Laser-induced CNV	Suppresses pathologic choroidal NV	
miR-184	ROP, DR	FZD7	Inhibition	OIR	Suppresses pathologic retinal NV	(Takahashi et al., 2015)
Ab 2D10 (anti-TSPAN12 antibody)	ROP, DR,	TSPAN12	Inhibition	OIR	Inhibits retinal avascular area and NV	(Bucher et al., 2018; Bucher et al., 2017)
				<i>Vldlr</i> ^{-/-}	Reduces subretinal NV	
Endostatin	ROP, DR	β -Catenin	Inhibition	Transgenic mice with inducible expression of VEGF in the retina	Reduces VEGF-induced vascular permeability, retinal NV, and retinal detachment	(Takahashi et al., 2003)
				OIR	Inhibits retinal NV	

FEVR: familial exudative vitreoretinopathy; ROP: retinopathy of prematurity; DR: diabetic retinopathy; AMD: age-related macular degeneration; OIR: oxygen-induced retinopathy; NV: neovascularization.