Vascular Endothelial-cadherin plays a role in regulating the Norrin-FZD4 pathway and can cause Familial Exududative-vitroretinopathy (FEVR).

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VE-cadherin plays a role in regulating Norrin/FZD4 pathway and can cause

Familial Exududative-vitroretinopathy (FEVR)

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Abstract:

FEVR is a genetic disorder that causes abnormal vascularization of the retina, which can lead to retinal detachment and blindness. Mutations in the genes encoding Norrin, LRP5, FZD4 and TSPAN12 can cause FEVR. Interestingly, these genes encode proteins that belong to the Norrin-FZD4 signalling pathway that ultimately activate β-catenin to upregulate gene transcription. Mutations in the pathway are thought to cause FEVR due to the lack of β-catenin translocating to the nucleus, however the mutations described only account for 50% of cases whereas the other half is due to unknown genes. In a cohort of 123 FEVR patients, a proband was found to have a mutation in the vascular endothelial cadherin (VE-cadherin), this mutation is at position 775 where a glycine has been substituted with a serine (G775S).

Our objective is to provide evidence that VE-cadherin (CDH5) plays a role in the Norrin-FZD4 pathway by determining whether wild type or mutant VE-cadherin have an effect on β-catenin binding, determine whether VE-cadhrin (CDH5) interacts with LRP5, and determine whether mutant VE-cadherin has a defect in internalization. In FEVR mouse models, FZD4-/- and TSPAN12-/- mice had increased VE-cadherin protein expression, as well as increased src activation and poly-ubiquination of VE-cadherin.

Immunoprecipitation assays were used to determine whether mutant VE-cadherin had an effect on β-catenin binding. There was no difference in binding between wild-type and mutant VE-cadherin when transfected in HEK293 cells. Cells expressing wild-type VE-cadherin immunoprecipitated with LRP5, whereas G775S did not interact with LRP5. The data suggests that VE-cadherin may play a role in regulating the Norrin-FZD4 pathway and interacting with LRP5.

Introduction:

The retina is a light sensitive nerve tissue found at the back of the eye. It receives images and processes this information to the brain in the form of electrical signals or impulses via the optic nerve (Watanabe T, 1988; Huxlin KR, 1992). Development of a fully functional retina is thus important for maintaining proper vision. Diseases that affect and damage the retina could disrupt its function and ability to send out electrical pulses, which could eventually lead to vision loss or retinal detachment and blindness if not treated properly at its early stages.

Familial exudative vitreo-retinopathy (FEVR) is a genetic disorder that affects the development of the retina and blood vessels which results in impaired delivery of oxygen and nutrients to the retina and the vitreous membrane (Criswick VG,1969; Gow J, 1971). It can affect both eyes but not necessarily with the same intensity.

FEVR is a progressive disease that could get better or worse over time. This condition can be diagnosed from the age of 18 months to the age of 18 years. The severity of the disorder is unpredictable, with some patients suffering from minor defects or problems with vision while in others the condition could lead to retinal detachment and blindness, especially during childhood (Criswick VG,1969; Gow J, 1971, Canny CL1976).

Most cases of FEVR are asymptomatic. When FEVR occurs in its most aggressive form it shows up before the age of 10 years and develops symptoms that causes serious eye problems. Early medical intervention could slow the progression of the disease but because every FEVR case is different medical treatments do not always give the same results in all patients. In some cases, FEVR continues to develop symptoms until the infected individual reaches adolescence, it then stops progressing after the age of 20 years. This rule does not apply to every FEVR case. In patients that suffer from an aggressive form of FEVR, the disorder can start progressing again after an asymptomatic period. FEVR can affect anyone, it does not exclusively affect people of a certain race, ethnicity or age. Almost 1/10,000 people are diagnosed with FEVR every year (Criswick VG,1969; Gow J, 1971, Canny CL1976).

FEVR can induce the formation of a fibrous membrane in the vitreous gel between the lens and the retina. The growth of the fibrous membrane could potentially harm the retina by dragging it and causing it to detach. Eye problems and diseases associated with FEVR include glaucoma, cataracts, and the iris not dilating. (Wolfgang Berger, 2013)

It is possible for a FEVR child to be born with a retinal fold. This results in the abnormal growth of the retina and eventually causes partial blindness. (Wolfgang Berger, 2013)

The main phenotype for FEVR patients is the rapid growth of abnormal blood vessels in the peripheral region of the retina. The bleeding of the abnormal blood vessels results in the appearance of a milky white exudate on the retina. The hemorrhage is responsible for tearing, scarring and eventually detaching the retina. The growth of these blood vessels is associated with abnormal tip cell migration caused by abnormal guidance of filopodial extensions. (Wolfgang Berger, 2013)

Filopodial extensions emerge from specialized endothelial cell positioned at the tips of vascular sprouts and is guided by vascular endothelial growth factor (VEGF) (Holger Gerhardt,2003).

It is known that FEVR occurs in three forms of genetic inheritance: X-linked (Chen ZY,1993) , autosomal dominant and autosomal recessive, where autosomal dominant is the most common form of inheritance (Gow J,1971) .Mutations in the genes low density lipoprotein receptor-related protein 5 (LRP5) (Xia, 2008), frizzled homolog 4 (FZD4) (H Kondo,2003) , NDP (Norrin) (Chen ZY,1993) and the recently discovered tetraspanin 12 (Tspan12) (Harald J. Junge), are known to cause FEVR. The mutations in these genes account for approximately 50% of FEVR cases, however the other 50% of FEVR cases is still unknown.

Mutations in these genes cause FEVR by altering the signaling responsible for normal development and vascularisation of the retina (Figure 1).

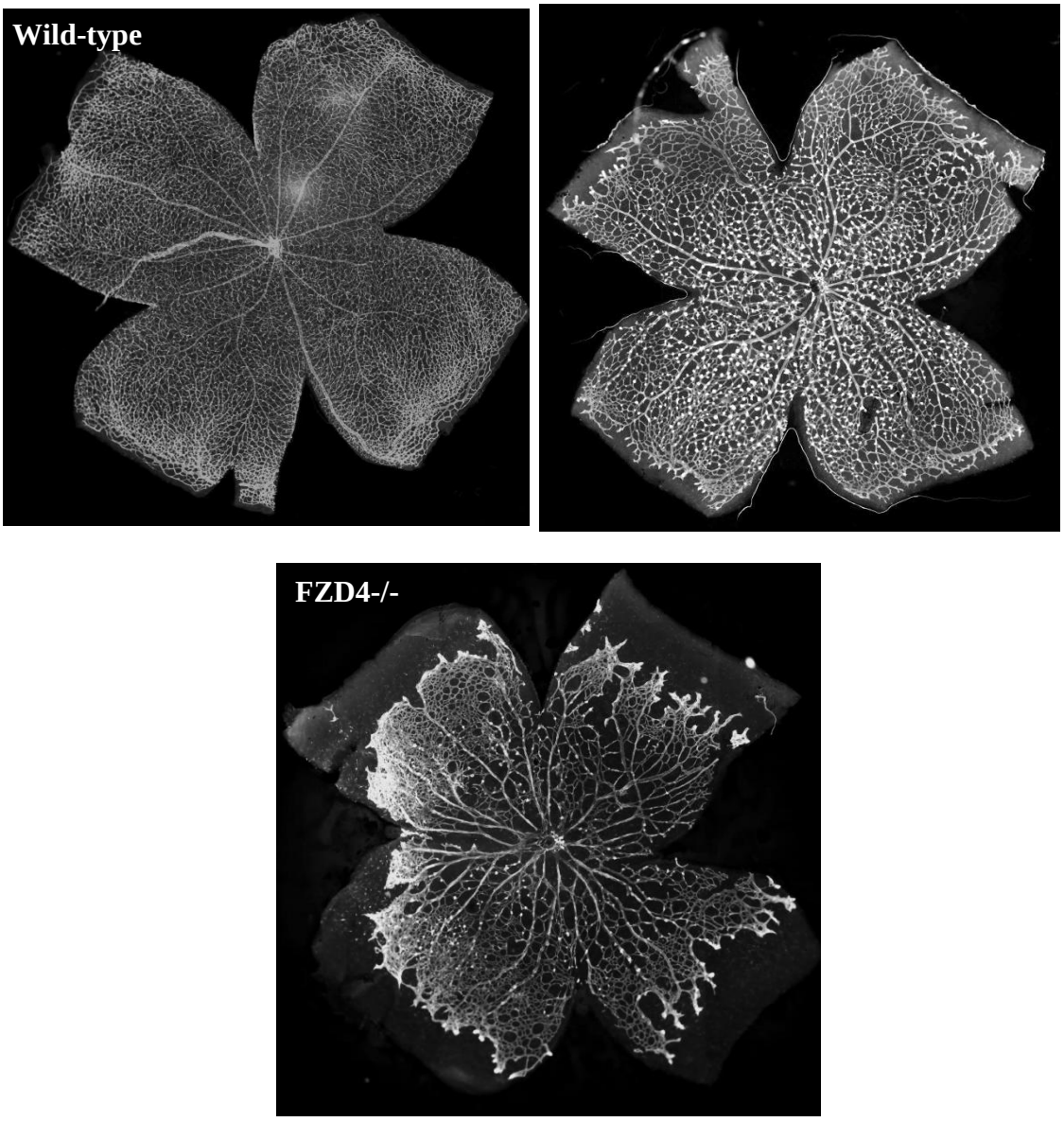


Figure 1. Retina flatmount of wild type and tspan12 null mice at post natal12 (p12). Wild type retina has normal vascularization while tspan12 null has abnormal growth of vessels. Likewise, flatmount retinas for Fzd4 null mice has immediate difference with the vasculature (Chun-hong Xia, 2010).

A study done on Tspan12 showed that the absence of Tspan12 in the retinas of mice moderately slowed down the centrifugal outgrowth vasculature of the nerve fiber layer. It also caused the retina to remain permanently lacking blood vessels

(Harald J.Junge, 2009). These phenotypes resemble the problems that mutated FZD4, LRP5 and Norrin cause in the retina (H Kondo,2003; Chen ZY,1993; Xia, 2008) . Some other phenotypes that have been observed in Norrin-/- mice include the appearance of microaneurysms in the inner nuclear layer sprouting from the nerve fiber layer. The extension of these microaneurysms was also found in Tspan12 -/- mice (Harold J.Junge,2009). The regression of the hyaloid vessels proceed at a slower rate in Tspan12-/- mice as well as in LRP5, FZD4 and Norrin mutant ones.(Harald J.Junge, 2009 ; Xu .Q, 2004; Michael l. Dorrell,2002). Hyaloid vessels are temporary vascular beds that deliver blood to the retina as it develops, any delay in its formation impacts the vascularization of the retina (Magali S.Geiniez, 2004).

A treatment for FEVR has not been developed yet, but certain treatments are constantly used to deal with symptoms of FEVR. One of the treatments is laser photocoagulation, which uses a laser to cauterize ocular blood vessels to attempt to minimize damage. Another method is cryoretinopexy, which uses extreme cold to fix retinal tears. Both methods can relieve the progression of FEVR, with low success rates.

In order to understand how FEVR impacts the retina, it is important to understand how the retina develops. Retinal development starts with the vessels around the optic papilla radiating away from the optic nerve head, then a capillary plexus

assembles between the vessels allowing them to interconnect and form a network (Figure 2) (Michael l.Dorrell, 2002).

In the nerve fiber layer, the newly formed network progressively expands increasing its volume and area. This expansion is usually accompanied with an increase in the complexity of the network. Then the collateral branches start growing from the capillaries located in the superficial plexus and branch out further into the outer retina where they reconnect again laterally.

This reconnection known as anastomose yields a secondary planar vascular plexus, which extends into the inner nuclear layer towards its outer edges. This process is followed by the assembly of the intermediate vascular plexus in between the primary and secondary layers resulting in a three layered architecture (Figure 2)(Michael l.Dorrell,2002; Harald J.Junge,2009; Fruttiger,2007).

In order for normal retinal development and vascularization to take place, canonical Norrin / β -catenin signaling pathway must be activated (Clevers H.2006). β -catenin is a transcriptional co-activator that is responsible for regulating the expression of genes directly linked to development. The ligand Norrin binds to the receptor Frizzled4 and the co-receptor LRP5, Tspan12, an integral membrane protein that is expressed specifically in retinal endothelial cells, stabilizes the receptor complex resulting in a signaling cascade that results in an accumulation of β-catenin translocating from the cytoplasm to the nucleus where it

upregulates transcription of downstream gene targets (Figure 3) (Harald J Junge,2009; Logan CY, 2004).

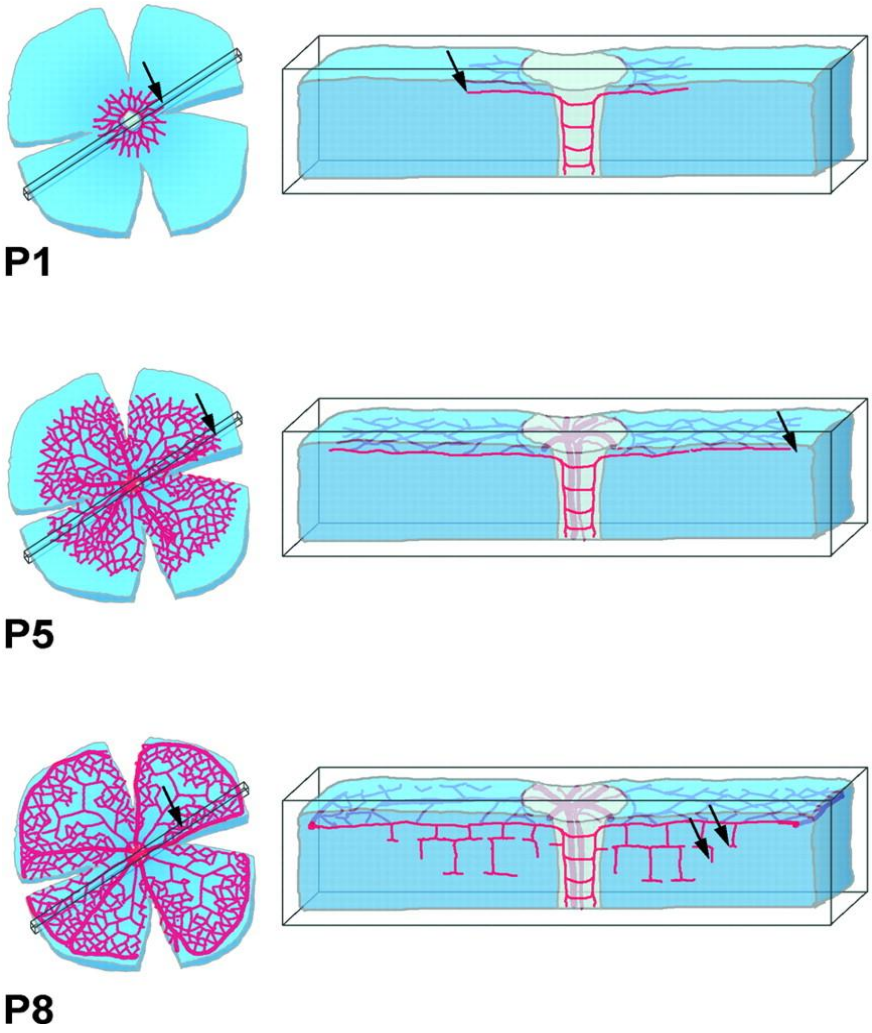


Figure 2. Blood vessels begin sprouting from the optic disc migrating towards the intermediate network and the deep layers. Further branching and interconnection of these vessels forms the plexus. In mice, the retinal vasculature develops during several days postnatal. In humans, development of the retinal vasculature occurs by birth, however is arrested or delayed in developing disorders such as FEVR.(Holger Gerhardt,2003)

LRP5 phosphorylation occurs upon the formation of the Norrin-FZD4-LRP5 complex and is attached to the scaffolding protein Disshevelled (Dvl). The recruitment of the Dvl to the complex also helps Axin bind to the receptors. This process stabilizes β-catenin and allows it to accumulate in the cytoplasm by eliminating the Axin-mediated β-catenin phosphorylation. The dephosphorylated form of β-catenin then translocates into the nucleus where it acts as a transcriptional co-activator and forms complexes with the transcriptional factors TCF/LEF. The attachment of β-catenin to TCF/LEF promotes expression of ligand-activated genes (Figure 3) (He X. ,2004).

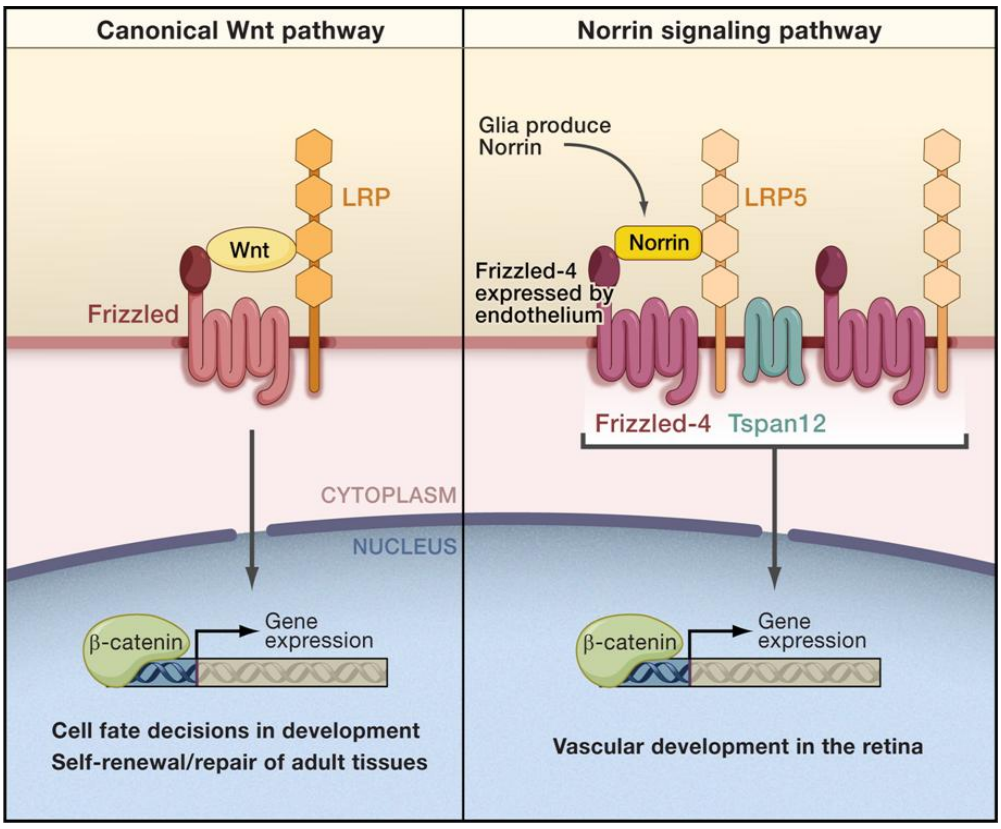


Figure 3. A) A receptor complex forms between FZD4 and the co-receptor LRP5 when the Wnts ligands are available. This process inhibits the Axin-mediated phosphorylation and degradation of β-catenin, giving β-catenin proteins the opportunity to accumulate in the cytoplasm, enter the nucleus and act as a co-activator for TCF which activates Wnt target genes. B) There are many FZD receptors in the plasma membrane, FZD4 is the only receptor that has the ability to bind both Norrins and Wnts even though both are structurally unrelated. The binding of FZD4 to Norrins exclusively happens in the retinal vasculature, where Tspan 12 binds to the FZD4/LRP5 complex to enhance the response to Norrins. The binding of the Norrins allows β-catenin to enter the nucleus and activate target genes responsible for retinal vascularisation. (Junge et al., 2009; Ye et al., 2009)

In the inactivated state, cytoplasmic β-catenin protein is continuously degraded as it is bound to the Axin complex (He X. ,2004; Bryan T, 2009). The Axin complex is composed of several proteins such as glycogen synthase kinase 3 (GSK3), scaffolding protein Axin, ,casein kinase 1 (CK1) and the tumor suppressor *adenomatous polyposis coli* gene product (APC) (He X. ,2004).

The N-terminal region of β-catenin is phosphorylated by CK1 and GSK3 by an E3 ubiquitin ligase subunit (β-Trcp). Phosphorylated β-catenin becomes ubiquitinated by E3 ubiquitin ligase subunit (β-Trcp) and is targeted for destruction by proteosomes. This continuous degradation of β-catenin inhibits its accumulation in the cytoplasm which in turn prevents its translocation into the nucleus where it upregulates Norrin target genes (He X. ,2004; Bryan T, 2009).

The vascular endothelial cadherin (VE-cadherin) originally called cadherin-5 play an important role in retinal development (Suzuki S, 1991). They are responsible for maintaining contact integrity of endothelial cells, and controlling vascular permeability (Breviario F,1995). They are integral membrane proteins located in the plasma membrane within endothelial adherens junctions. Cadherins interact with themselves through their extracellular N-terminal domains as cis-dimers. They are calcium-dependent and regulated through their C-terminal anchorage to β-catenin (Figure 4) (Pertz O, 1999; Boggon TJ,2002; Ahrens T,2002) .

VE-cadherin impacts retinal development through binding to β-catenin/α-catenin complex via the C-terminal end, which allows the association of the cadherin with actin (Wheelock MJ, 2003). In a study that used Chinese hamster ovary cells (CHOk1) to generate mutated forms of VE-cadherins, it was found that VE-cadherins mutated at their tyrosine residues 731 and 658 had their function disrupted (Potter MD, 2005; Lampugnani MG,1997).

There is also, evidence supporting that VE-cadherin restrains endothelial cells proliferation. Researchers found that plating endothelial cells onto substrates that have VE-cadherin extracellular domains inhibits the growth and proliferation of endothelial cells (Caveda L,1996).Various signaling pathways were found to be linked to VE-cadherin, one of those pathways being the β-catenin pathway. β -catenin is known to translocate into the nucleus to activate its target genes. The localization of the β-catenin at the junction may influence its activity by stopping it

from reaching its target genes, which in turn leads to the prevention of gene transcription and cell division (Conacci-Sorrell M, 2002).

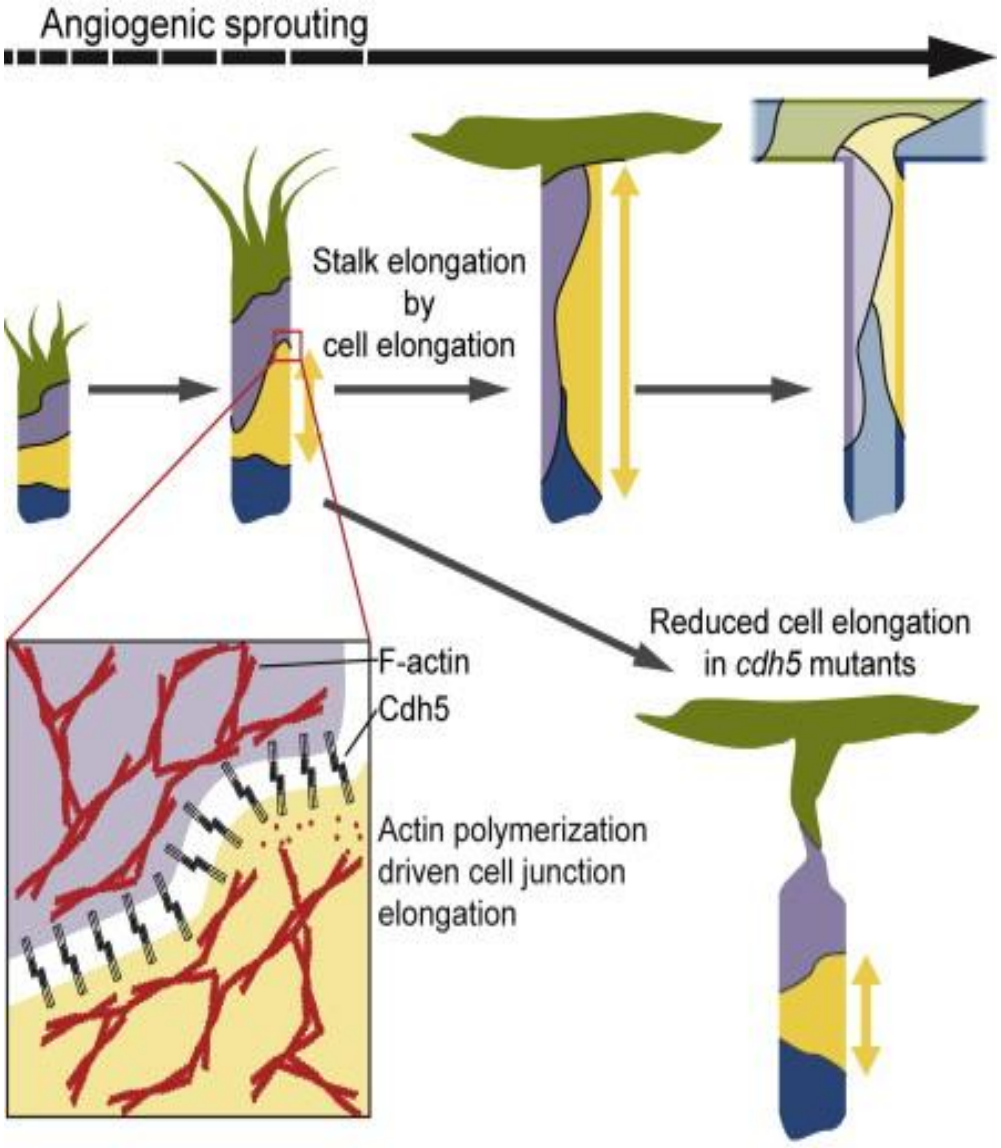


Figure 4. A schematic diagram illustrating the role that VE-Cadherin/CDH5 plays in the progression of angiogenesis and cell migration. (Sauteur et al., 2014, Cell Reports 9, 504–513,2014)

There are studies that support β –catenin's involvement in the Norrin signaling pathway and there is scientific support for the interaction of β -catenin with VE-cadherin, however there is no support connecting β-catenin's role between these two different pathways.

Our goal is to provide evidence that these two pathways are linked together through β –catenin. We have western blots from Tspan12-/- mice and there was a large abundance of VE-cadherin, suggesting that Tspan12 and VE-cadherin have a direct or indirect role in the development of the retinal vasculature. Another goal is to determine if there is a difference in β-catenin binding between CDH5 and its mutant form G775S, and if the phosphorylation status at tyrosine residues 658 and 731 in both CDH5 and G775 have any impact on β-catenin binding. Lastly, determine the impact of CDH5 on the localization of β-catenin to the nucleus by using immuno-fluorescence.

Materials and methods:

Reagents:

Antibodies for VE-Cadherin, Phospho-VE-Cadherin, Beta-catenin, Active-beta-catenin, actin and DDK-tag antibodies were purchased from Millipore and used according to manufacturer's protocol. Secondary antibodies conjugated with infrared-800 were purchased from Li-Cor. Secondary antibodies conjugated with alexaFluor-488 and alexaFluor-594 were purchased from Molecular Probes. Protein-A Sepharose beads were purchased from Thermo Scientific. Protease Inhibitors with EDTA and Phosphatase Inhibitors were purchased from Roche (Mississauga ,ON). Lipofectamine LTX transfection reagent was purchased from Life Technologies.

Plasmids:

VE-Cadherin and Tspan12 plasmids were purchased from Origene. Genes are in a pCMV6-DDK tag vector. Plasmids expressing FZD4 and LRP5 were a kind gift.

Mouse Lines and Cell Culture:

Mice were housed under Carleton Animal Care Facilities (North Gower, ON) under accordance of live animal ethics (code# 2015-022). Wild-type mice and FEVR mouse models with FZD4 and Tspan12 knockouts were made in C57BL/J backgrounds and obtained from Jackson Laboratories (Farmington, Connecticut). Mouse Brain Endothelial cells (BEND.3), Human Embryonic Kidney 293 cells (HEK293) , Chinese Hamster Ovaries cells k1 (CHOk1) and HeLa cells were

cultured in DMEM with 10% FBS (HEK293) and 20% FBS (BEND3) at 37°C with 5% CO_2. BEND.3 cells were seeded at 25,000 cells per well in a 12-well plate. HEK293 cells were seeded at 100,000 cells per well in a 12-well plate. HeLa cells were seeded at 250,000 cells per well in a 12-well plate. CHOk1 cells were seeded at 250,000 cells per well in a 12-well plate.

Immunofluorescence:

Cells were grown on sterile cover slips in 12 well plates at 37^{o}C overnight and transfected according to protocol for 24h. Transfected cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature and were permeabilized with PBS containing 0.1% (v/v) TX at 4^{o}C for 15 minutes. After washing with 1% BSA/PBS, primary and secondary antibodies were incubated overnight at 4^{o}C. Cover slips were mounted with Mowiol-88 and analyzed on Axiovert 200 microscope at 100X mag.

Flatmounts

Retinas were fixed with 4% PFA and permeabilized with PBS containing 1% (v/v) Triton X-100. Retinas were incubated with lectin conjugated to Alexa-Fluor-594 (endothelial cells) and anti-VE-cadherin and secondary Alexa-Flour-488. Retinas were flatmounted and imaged at 10X using AXIOVERT Z2 microscope containing a digital stage.

Immunoprecipitation:

After removing media and washing cells with ice cold PBS, 0.5 ml of ice-cold 1X cell lysis buffer was added to each well. Plates were left on ice for 5 minutes. Cells were scraped off and transferred into microcentrifuge tubes. Tubes were centrifuged gently (500g/1500 rpm) at 4° for 2 minutes. Pellets were resuspended in 400 µl of lysis buffer. Tubes were placed on ice for 20 minutes then centrifuged for 20 minutes at 14,000 X g, 4^{o}C. Supernatant was transferred into new tubes. 100 µl aliquots were incubated with antibodies at 4^{o}C overnight (100 µl for control, 100 µl with CDH5 antibody, 100 µl with β-catenin antibody). 30µl of 1:1 slurry of Protein A Sepharose were added to each sample and incubated for 1hr at 4°. After incubation, tubes were centrifuged for 30 seconds then the pellet was washed 3 times with 400 µl of 1X cell lysis buffer. Pellets were resuspended in 15µl 2X SDS sample buffer. Samples were heated up to 55^{o}C for 2-5 minutes then centrifuged for 1 minute at 14,000 X g. Samples were processed by SDS-PAGE (10%) and transferred to PVDF membranes. Membranes were blocked and probed with appropriate antibodies at 4°C overnight.

Transfections:

Cells were seeded in 12-well plates. The following day, cells were transfected with 2µg of DNA plasmid for 24hrs according to manufacturer's protocol. Briefly, cells were maintained in DMEM plus FBS in a 37°C incubator with 5% CO_2.

Homogenization:

A volume of 0.1ml homogenization buffer (PBS containing 1% TX-100 (v/v),1X protease inhibitors, 1X phosphatase inhibitors, 2mM EDTA, 1 mM PMSF) to isolated mouse retinas. Tissue was homogenized on ice and sonicated for 10 seconds twice. Samples were incubated on ice for 20 minutes, followed by centrifugation at 14,000 xg for 20 minutes. Protein concentration was determined using BIO-RAD protein assay dye. Equal protein loads were analyzed in wild-type and FEVR mouse models by SDS-PAGE and western blotting.

Results:

FEVR mouse models:
Flatmounts:

To observe the vascularization of retinas and determine whether VE-Cadherin was affected from FEVR mouse models: FZD+/+ FZD4+/- , FZD4-/- , TSPAN12+/+, TSPAN12 +/- and TSPAN12 -/-, C57B/J mice were anesthetized with isoflurane prior to euthanization. Eyes were enucleated, fixed, and permeabilized for immuno-staining. Retinas were incubated with lectin conjugated to Alexa-Fluor-594, which stains for glycoproteins specific to endothelial cells, and anti-VE-cadherin (CDH5) primary and Alexa-Flour-488 secondary (Simon Tual-Chalot ,2013).

Retinas were flatmounted and imaged at 10X using AXIOVERT Z2 microscope containing a digital stage. The stage and program allows you to stitch a mosaic of multiple pictures, allowing you to capture images of the entire tissue rather than individual cells.

Wild-type retinas had normal vascularisation and typical distribution of VE-cadherin in the retina (figure 5a,5b). However, knocking down TSPAN12 caused abnormal vascularization into the intermediate and deep layers of the retinas. Knocking down FZD4 also caused abnormal vascularization into the peripheral region of the retina, however very distinct from TSPAN12-/-. The phenotypes observed in FZD-/- were similar to those reported by Xu et al. (Xu et al.,2004)

The accumulation of VE-cadherin (CDH5) in bundles (figure 5c,5d), was observed in both TSPAN12 null retinas and FZD4 null retinas (figure 5e,5f).

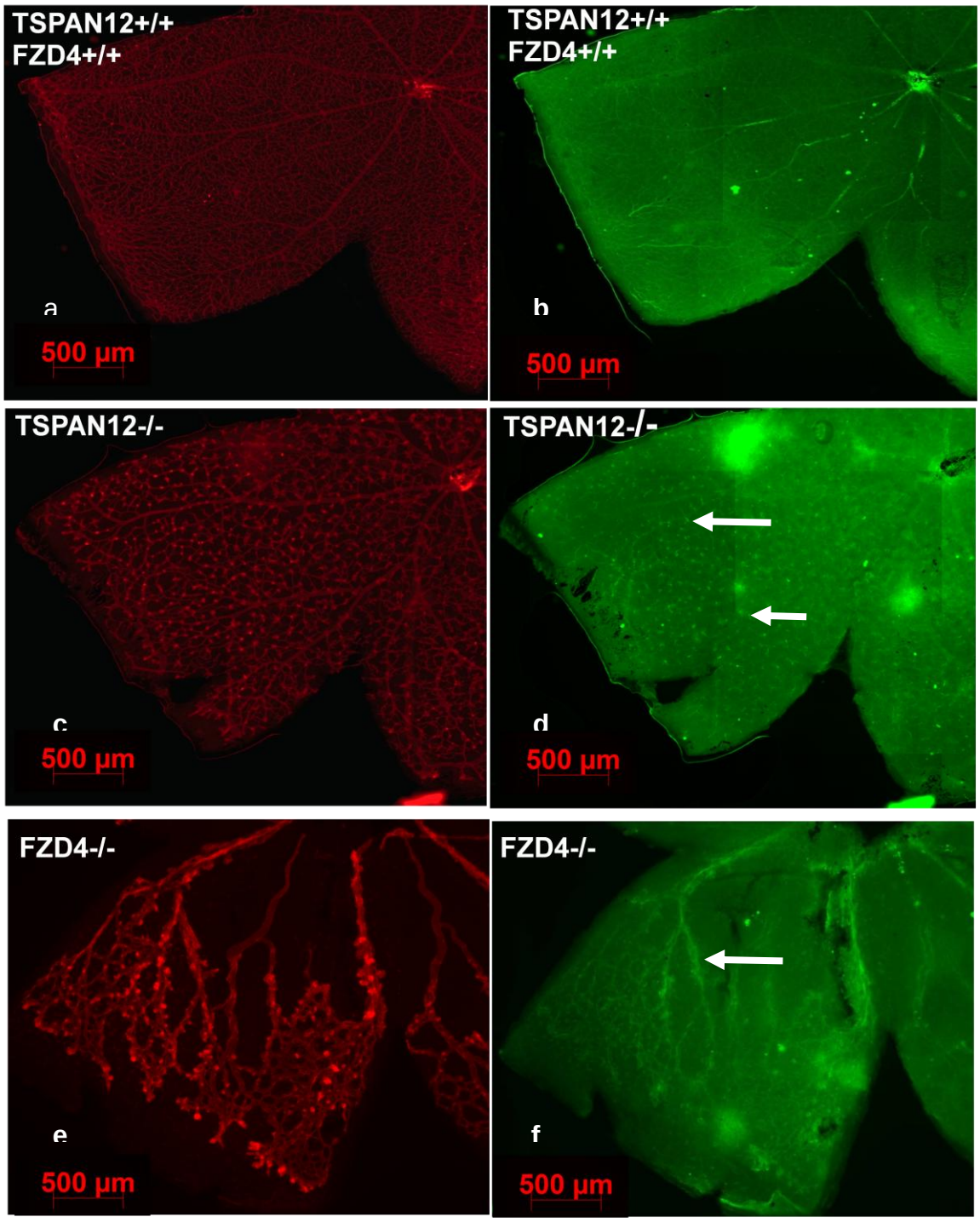


Figure 5. FEVR mouse models have abnormal vasculature and CDH5 co-stains with bundled endothelial cells. TSPAN12 and FZD4 null mice eyes were enucleated at P17

and stained with Lectin-594 and VE-cad (CDH5)-488. White-arrows point to the sites of VE-cadherin (CDH5) colonization in the retina.

Immunoprecipitation and western-blots:

To further elucidate whether VE-cadherin expression is upregulated in C57B/J mice retinas , FEVR mouse models were euthanized at post natal (P17), eyes were enucleated and retinas were homogenized for immunoblotting following immunoprecipitation with the primary anti-body targeted towards the C-terminus of VE-cadherin. Homogenate lysates were loaded with equal amounts of protein. Immunoblots were probed with anti-VE-cadherin. There was a statistically significant increase in the expression of VE-cadherin (CDH5) detected in TSPAN12-/- and FZD4-/- FEVR mouse models, compared to wild-type, while the heterozygous mouse models did not show any significant difference in VE-cadherin (CDH5) expression at P17(figure 6).

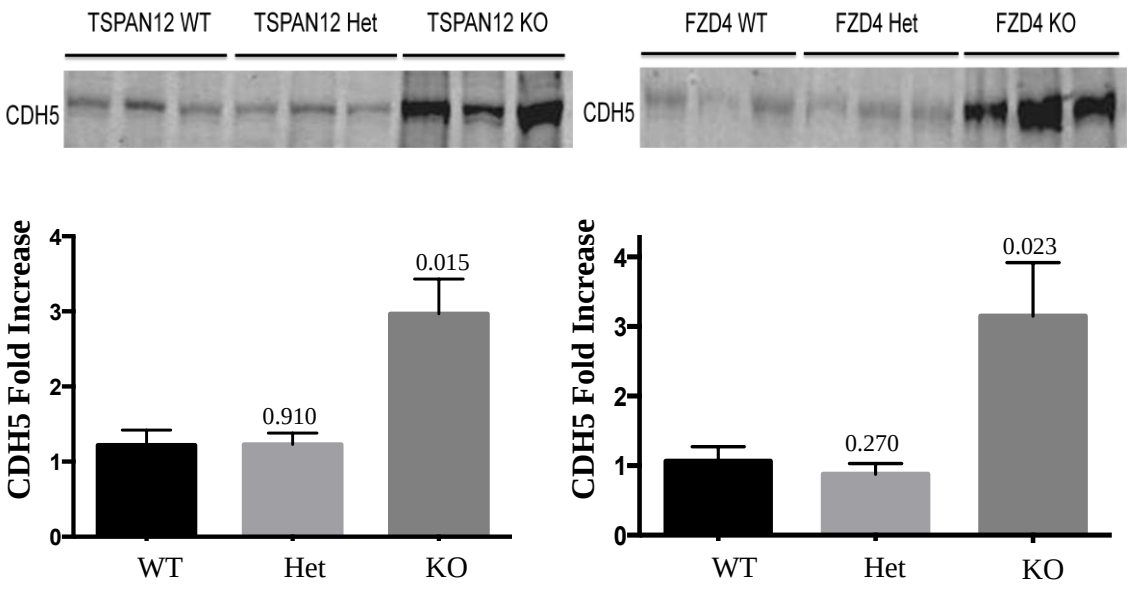


Figure 6. CDH5 is more abundant in FEVR mouse models. TSPAN12 and FZD4 mice were decapitated and eyes were enucleated and homogenized at P17. Equal protein concentrations (50 μg) were separated on a SDS-PAGE. PVDF membranes were blocked and immunblotted for Ve-cadherin (CDH5).

In order to detect ubquitination of VE-cadherin (CDH5), lysates derived from homogenizing retinas at P17 were used for immunoprecipitation with anti-VE-cadherin (CDH5). Immunoprecipitates were analyzed by immunoblotting with anti-ubiquitin. There was no significant difference in ubquitination in FZD4-/- mice, but there was significant increase in ubiquitination in TSPAN12-/- mice (figure7).

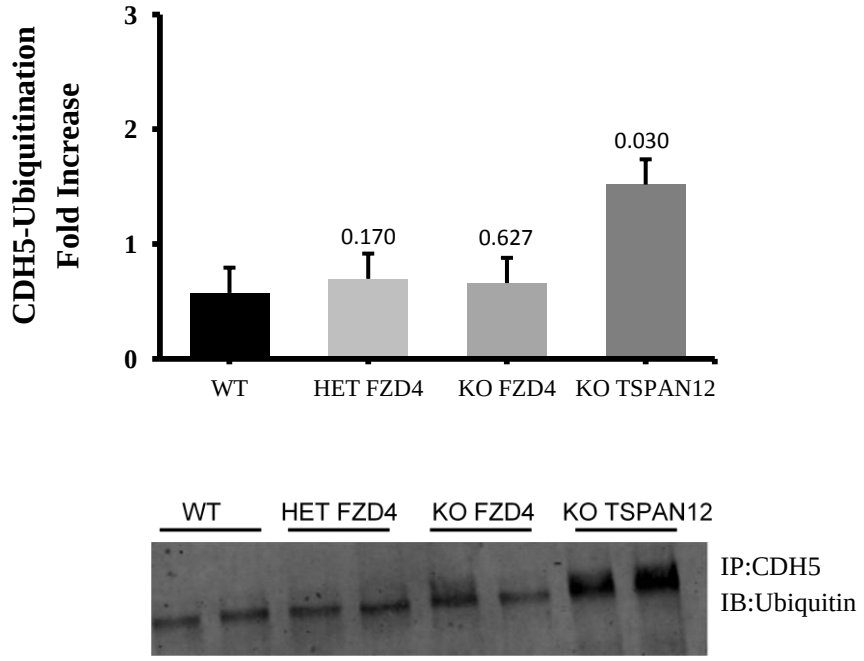
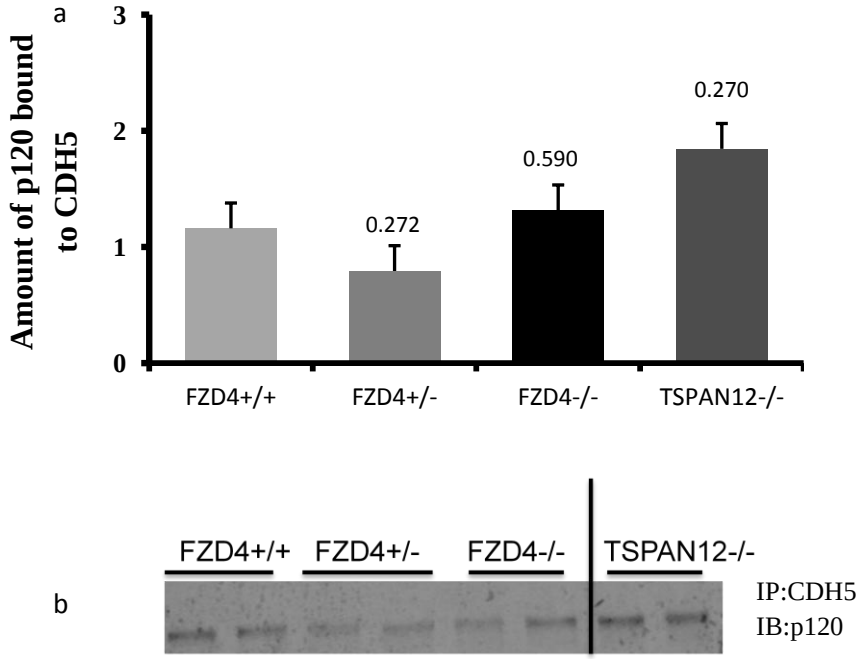


Figure7. ubquitination of VE-cadherin (CDH5) is more abundant in FEVR mouse models. TSPAN12 and FZD4 mice were decapitated and eyes were enucleated and homogenized at P17. Equal protein concentrations (50 μg) were separated on a SDS-PAGE. PVDF membranes were blocked and immunblotted with anti-ubiquitin.

VE-cadherin binds p120 , a protein that belongs to the catenin family, that regulates the stability of VE-cadherin (CDH5) at the plasma membrane by inhibiting internalization ,ubiquitination inevitably its degradation. (Anastasiadis, 2007)

Equal amounts of protein from P17 FEVR mouse models were immunoblotted for p120, to test interaction with VE-cadherin (CDH5) (figure 8a, 8b). Briefly, homogenates were immunoprecipitated with anti-VE-cadherin and eluates were immunoblotted with p120 antibodies. VE-cadherin (CDH5) was found to bind p120 normally in equal amounts in TSPAN12-/- retinas and FZD4-/- retinas. Additionally, difference in active β-catenin and total β-catenin amounts was investigated. Figure 8c showed that there was no difference in β-catenin activity in FZD4 (not shown) and TSPAN12 mouse models.



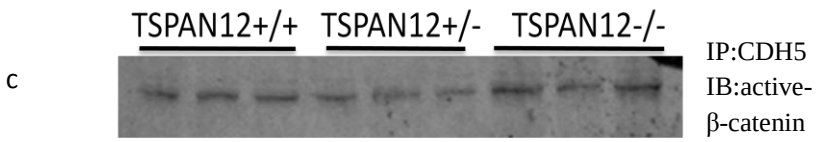


Figure8. FZD4 and TSPAN12 FEVR mouse models have no difference in active β-catenin and p120 interaction with VE-cadherin (CDH5) . TSPAN12 and FZD4 mice were decapitated and eyes were enucleated and homogenized at P17. Equal protein concentrations were separated on a SDS-PAGE. PVDF membranes were blocked and immunblotted with (a, b) anti-p120 (c) anti-active β-catenin.

TSPAN12-/- (figure9a,9c) and FZD4-/- (figure9b,9d) mice had increased phospho-SRC but not total SRC levels (figure 9). To detect changes in phospho-src levels in TSPAN12 and FZD4 mouse models, lysates derived from homogenized retinas were immunoblotted for phospho-src and total src.

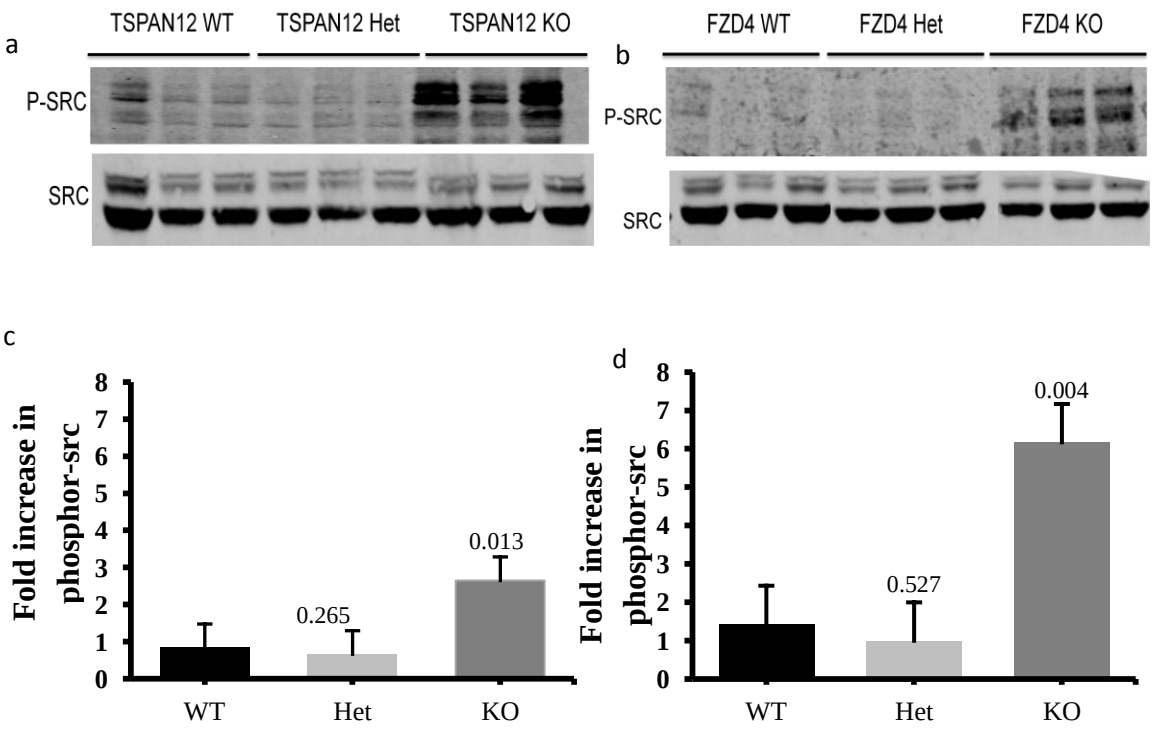


Figure 9. Phospho-SRC is more abundant in FEVR mouse models. TSPAN12 and FZD4 mice were decapitated and eyes were enucleated and homogenized at P17. Equal protein concentrations were separated on a SDS-PAGE. Membranes were blocked and immunblotted for phospho-SRC and Total SRC.

HEK293 Cells Transfections:

G775S does not interact with LRP5 and has no effect on β-Catenin binding:

To investigate whether there is a difference in β-catenin binding between wild-type VE-cadherin (CDH5) and G775S VE-cadherin, HEK293 cells were transfected with plasmids for 24 hours and immunoprecipitated with anti-VE-cadherin. There was no difference in the binding of β-catenin for both wild-type VE-cadherin (CDH5) and the mutant G775S VE-cadherin (figure10e).

FZD4	-	✓	✓	✓	✓	✓
LRP5	-	✓	✓	✓	✓	✓
CDH5	-	-	✓	✓	-	-
G775S	-	-	-	-	✓	✓
GSK-X	-	-	-	✓	-	✓

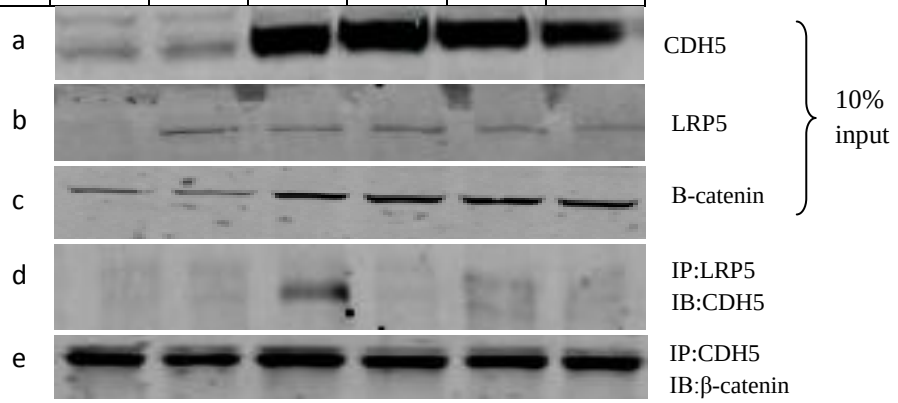


Figure10. CDH5 interacts with LRP5 and G775S does not. HEK293 cells were transfected with plasmids for 24h. GSK-X (10μM) was added 10min prior to harvest. Cell lysates were incubated with primary antibodies for 4 hr. IPs were washed and separated on a 10% SDS-PAGE and immunoblotted with antibodies shown above.

VE-cadherin(CDH5) immunprecipitated with LRP5 in the absence of the drug GSK-X, but no interaction between LRP5 and VE-cadherin was detected when the drug GSK-X was added. Mutant G775S did not interact at all with LRP5 under all conditions (figure

10d). Figure 10e shows that VE-cadherin and G775S bound β-catenin in a similar manner .

Discussion:

Familial excudative vitreoretinopathy (FEVR) is a genetic disorder that disrupts normal vascularization of the retina. About 50% of FEVR cases are caused by mutations in TSPAN12, FZD4, LRP5 and NDP which encodes the ligand Norrin (Harald J. Junge,2009) . These genes encode the Norrin/FZD4 signaling pathway that regulates β-catenin activation and its translocalization into the nucleus where it acts as a transcription factor.

Gene mutations in FEVR are thought to be due to a defective β-catenin signalling pathway and translocating into the nucleus. In an attempt to identify other causable genes of FEVR from a cohort of 123 FEVR patients, a proband was detected to have a point mutation in CDH5 (VE-cadherin) on chromosome 16 in humans. This is quite plausible since VE-cadherin plays a role in endothelial cell migration, proliferation, vaculogenesis, angiogenesis and it also interacts with β-catenin. This study was to determine whether VE-cadherin could cause FEVR by playing a role in regulating the Norrin/FZD4 pathway.

Abnormal vascularization of retinas in mice carrying a loss of function mutation (TSPAN12-/- or FZD-/-) has been shown in previous studies (Xin Ye,2010), and flatmount images support their findings. FEVR mouse models show dilated primary arteries and veins, and clusters of endothelial cells that have reached

intermediate layers of the retina from the vitreal surface and stopped migrating into deeper layers. The lack of vascularization into the peripheral region of the retina, as seen from the FZD4-/- (figure 5e), and the lack of vascularization into the intermediate and deep layers, as seen from TSPAN12-/- (figure 5c), suggests that there is abnormal migration and proliferation of endothelial cells.

To determine whether VE-Cadherin was affected in mouse models, we probed flatmounts with conjugated lectin to Alexafluor-594, to visualize the vasculature, and anti-VE-caderin(CDH5) followed by GAM-488 (Figure 5b, 5d, 5f).

Interestingly, in FEVR mouse models, TSPAN12 and FZD4 null flatmounts have different abnormal structure, suggesting that FZD4 and TSPAN12 have other functions along with activating β-catenin. This is most likely by activating independent signalling pathways. VE-cadherin appeared to be more abundant and co-localized to the abnormal vessels in the FEVR mouse models (figure 5d, 5f).

This suggests that the Norrin/FZD4 pathway plays a role in dictating whether VE-cadherin functions to maintain endothelial integrity, as seen in the wild-type, or whether endothelial cells are active in cellular migration and vessel formation.

To further elucidate whether VE-cadherin expression is upregulated, FEVR mouse models were euthanized at P17, eyes were enucleated and retinas were homogenized for immunoblotting (Figure 6). The fact that VE-cadherin expression increased in FZD4 and TSPAN12 null mice at P17, compared to wild-

type and heterozygotes suggests that VE-cadherin is involved in retinal vascular development, but is downstream from the Norrin/FZD4 pathway.

Endothelial cells appear to be stuck in one phase of their development and unable to move on to the next stage of their process. This however does not clarify whether VE-cadherin is directly or indirectly involved with Norrin/FZD4 pathway, nor does it determine whether there is a difference in function between wild-type and mutant VE-cadherin.

VE-cadherin (CDH5) is localized to the plasma membrane in an inactive form where it interacts with itself in homophillic dimers to maintain endothelial integrity (Elisabetta Dejana,2008).

Upon cellular stimulus to promote cell migration, proliferation, angiogenesis or vasculogenesis; VE-cadherin disassembles and becomes internalized where it is either degraded or recycled back to the plasma membrane. The factors affecting internalization of VE-cadherin include its interaction with p120, its phosphorylation status through SRC signalling, and turnover, via ubiquitination and subsequent targeting to the proteasome.

To determine whether there were any functional differences between wild-type or mutant VE-cadherin, we investigated whether there was an effect on localization of VE-cadherin, if there were any differences in phosphorylation status and whether it was internalized for degradation through the proteasome.

To determine cellular localization of wild-type and mutant VE-cadherin, CHO-K1, HeLA and HEK293 cell lines were transfected with wild-type or mutant VE-cadherin and fixed for visualization. These results were inconclusive as these cell lines are not endothelial cells and transfections either lead to toxicity or rapid turnover of transient expressing cells.

Due to tissue size of the retina, visualization of individual cells was difficult. We are attempting to trypsinize FEVR mouse model retinas in effort to culture individual primary endothelial cells. This is a protocol that requires optimization and mouse retinas.

The amount of ubiquitination of VE-cadherin in FEVR mouse models was analyzed next (Figure 7). Briefly, FEVR mouse model retinas were homogenized at P17 and immunoprecipitated with anti-VE-cadherin. Immunoprecipitates were analyzed by immunoblotting with anti-ubiquitin. There was a significant increase in ubiquitination only in TSPAN12-/- mice.

The increased ubiquitination of VE-cadherin in TSPAN12-/- mice suggests that VE-cadherin is targeted for the proteasome, however it is not degraded at a proportional rate or the lack of TSPAN12 causes unregulated transcriptional expression of VE-cadherin.

Again, this confirms that VE-cadherin regulation is downstream of the Norrin/FZD4 pathway, and that defective turnover/recycling of VE-cadherin could

cause FEVR. The difference in ubiquitination levels between TSPAN12 and FZD4 null mice, support that these two proteins may have independent functions other than the Norrin/FZD4 pathway.

One study supports a theory that TSPAN12 forms domains at the plasma membrane where it stabilizes interactions. Another study showed that TSPAN12 regulates retinal vasculature through the induction of Norrin/FZD4 pathway by increasing the cells response to the ligand Norrin (Harald J,2009), this could possibly be due to the formation of TSPAN12 domains. This ultimately results in increased levels of active β-catenin, and could possibly add to the 'pool' of β-catenin that VE-cadherin can bind to. It is also possible that TSPAN12 plays a role in stabilizing VE-cadherin at the plasma membrane and preventing its degradation, possibly through these TSPAN12 domains.

In addition to binding β-catenin, the cytoplasmic tail of VE-cadherin (CDH5) binds other catenins that belong to the armadillo family of proteins which help establish strong cell-cell adhesion and association of VE-cadherin with the actin cytoskeleton. One of these catenins is p120 (Pokutta and Weis, 2007 ; Hartsock and Nelson, 2008).

We wanted to see if the increased in ubquitination is caused by deregulation of p120 binding to VE-cadherin(CDH5), since several studies showed how the catenin p120 regulates VE-cadherin(CDH5) expression by determining cadherin availability at cell junctions (Davis *et al.*, 2003 ; 2007 ; Chiasson and Kowalczyk,

2008) and the loss of its function would lead to rapid degradation of VE-cadherin via endosomal-lysosomal pathways (Anastasiadis, 2007), so in a way p120 inhibits VE-cadherin (CDH5) internalization and degradation, which is the reason why p120 binding to VE-cadherin was tested in our study.

VE-cadherin was found to bind p120 normally in equal amounts in TSPAN12-/- retinas and FZD4-/- retinas. This reflects that p120 binding to VE-cadherin is not influenced by the Norrin/FZD4 pathway and that p120 is not contributing to the increased ubquitination of VE-cadherin(CDH5).

Vascular permeability is stimulated by endogenous pathways that influence the distribution and function of VE-cadherin, one of these endogenous pathways is the vascular endothelial growth factor (VEGF) pathway, which regulates VE-cadherin internalization and increases vascular permeability through inducing src-phosphorylation of Y658/Y731 leading to clathrin-dependent endocytosis of VE-cadherin (CDH5). This internalization process requires the recruitment of arrestinβ-2 to VE-cadherin (Gavard J,2006; Elisabetta Dejana, 2008).

FEVR mouse model P17 homogenates were probed for phospho-Y658, however the antibody was poor and produced inconclusive results. We then reprobed immunoblots with anti-active SRC (phospho-SRC) and anti-SRC (total SRC). FEVR mouse models have increased levels of phospho-SRC suggesting that FZD4 and TSPAN12 help attenuate the SRC signalling pathway, as well as maintaining the Norrin/FZD4 pathway.

The regulation of the SRC pathway through the VEGF receptor and how it regulates VE-cadherin is still poorly understood, however our data suggest that there is cross talk between the Norrin/FZD4 and the VEGF/SRC signalling pathway, possibly through VE-cadherin.

Our initial hypothesis suggested that VE-cadherin contributes to the active pool of β-catenin produced from the FZD4 pathway (figure 10e). However this hypothesis was rejected when HEK293 cells were transfected and immuoprecipitated with anti VE-cadherin or anti- G775S and probed for β-catenin. We saw no difference in β-catenin binding between wild-type versus mutant form of VE-cadherin, nor was there difference when using GSK-Inhibitor X in producing active β-catenin. GSK-inhibitor X is a drug that inhibits GSKβ-3, and mimics activation of the Norrin/FZD4 pathway.

Interestinlgy, LRP5 immunoprecipitated with wild-type VE-cadherin, where there was little to no interaction with G775S mutant VE-cadherin. There was a loss of interaction with wild-type VE-cadherin and LRP5 when GSKβ-3 was inhibited, suggesting that VE-cadherin could possibly function to regulate LRP5 at the plasma membrane and prepare LRP5 for Norrin activation and interaction with FZD4. This result also suggests that mutant G775S could possibly lead to FEVR by the loss of LRP5 interaction, possibly by diminishing the activity level in the presence of Norrin.

The interaction between VE-cadherin (CDH5) and LRP5 changes our initial model (figure 11). Further work could involve testing to see if there was an interaction between VE-cadherin and other elements of the Norrin/FZD4 pathway such asTSPAN12 or FZD4.

In conclusion this study provided evidence that VE-cadherin plays an effective role in regulating Norrin/FZD4 pathway by interacting with LRP5, and not a independent signalling event, as previously reported and that it is a potential new gene that could cause FEVR. We demonstrate that VE-cadherin expression levels could be a potential quick marker to identify new FEVR patients.

Future studies can be directed towards investigating VE-cadherin interaction with other components of the Norrin/FZD4 pathway, and investigating whether wild-type versus mutant influence actin remodelling. This experiment would require transfecting a retinal endothelial cell culture line and determine the localization of the VE-cadherin and actin via immune-fluorescence.

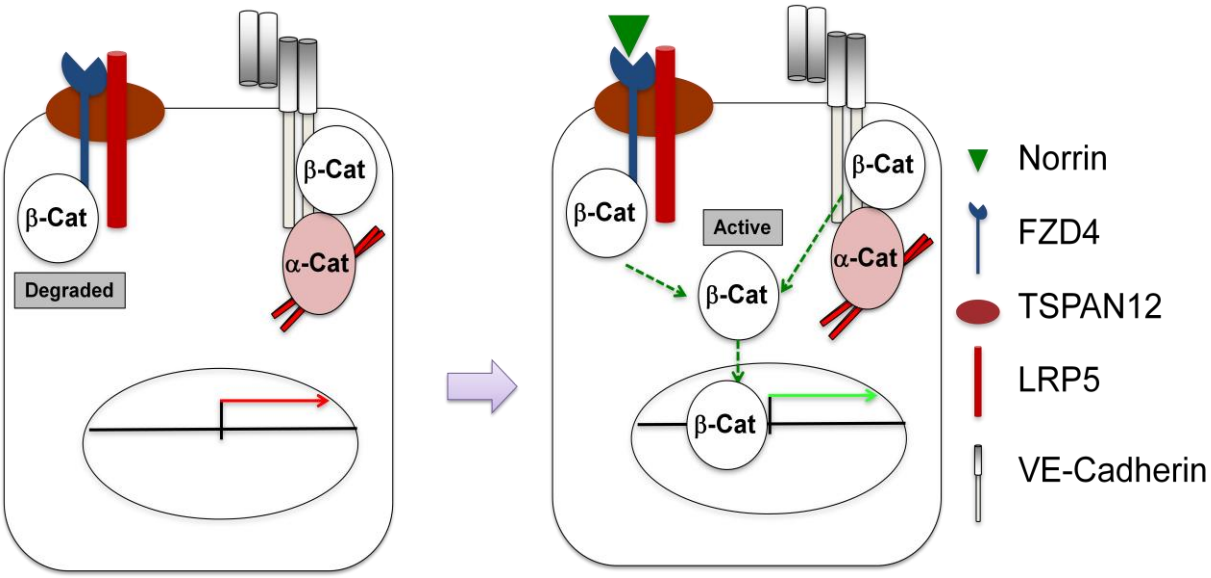


Figure 11. Original hypothesis of β -catenin inactivation leading to FEVR. In the absence of Norrin, β-catenin is sequestered in a signalsome complex which leads to β-catenin degradation. CDH5/VE-Cadherin binds β-catenin in a quiescent state and maintains endothelial barrier integrity. In the presence of Norrin, β-catenin is activated in a stable form where it translocates to the nucleus to regulate gene transcription. We hypothesized that CDH5 contributes to the active pool of β-catenin.

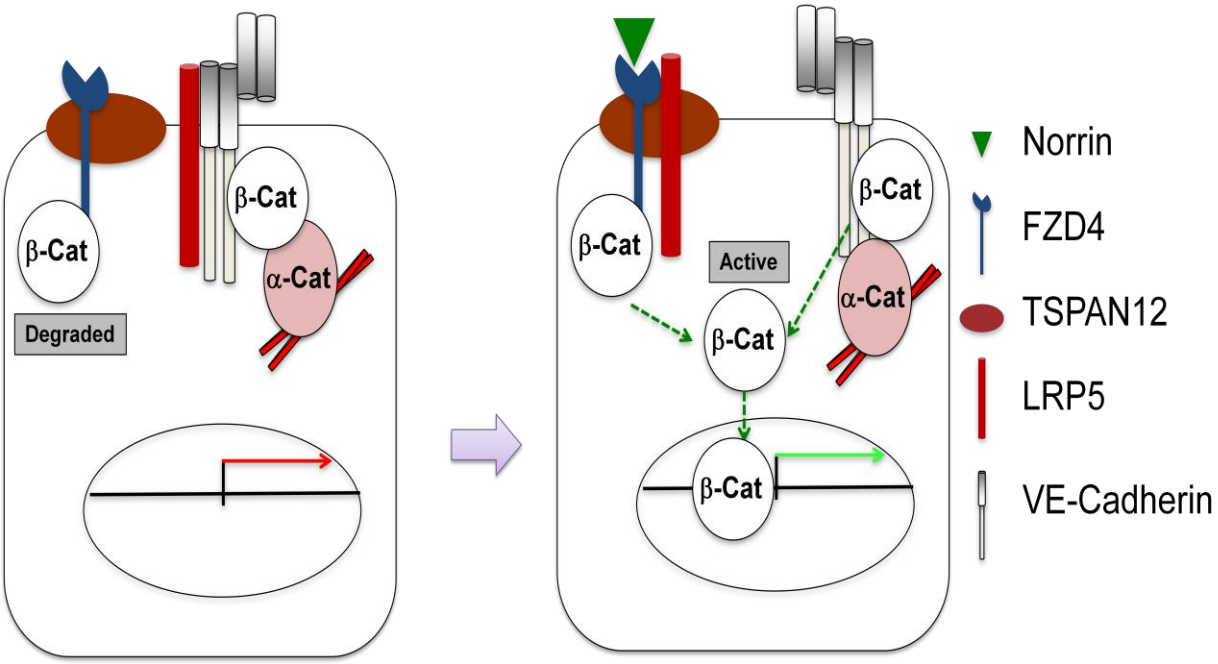


Figure 12. New schematic hypothesis of β -catenin inactivation leading to FEVR. In the absence of Norrin, β-catenin is sequestered in a signalsome complex, which leads to β-catenin degradation. CDH5/VE-Cadherin binds β-catenin in a quiescent state and maintains endothelial barrier integrity where it also interacts with LRP5. In the presence of Norrin, VE-cadherin and LRP5 dissociate where LRP5 acts as a co-receptor for Norrin and leads to β-catenin activation. Active to β-catenin translocates to the nucleus to regulate gene transcription. VE-cadherin interaction with LRP5 may be important to recruit LRP5 to FZD4 forming co-receptors and activating β-catenin.

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