TARGETING ANGIOGENIC GROWTH FACTORS IN PROLIFERATIVE DIABETIC RETINOPATHY

By

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This document is dedicated to the graduate students of the University of Florida.

ACKNOWLEDGMENTS

Since coming to the United States in August 2001, it has been five years. This was the most challenging five years and there was happy and hard time. To study abroad, especially in the United States, was one of my dreams when I was in high school. Now, with the completed dissertation in hand, I can tell myself: Hao, you made it!

Language has been the biggest obstacle in my study. I was confident about my English, but I came here and found that there is still so much to learn and it still takes time. The study and life for me has been harder than most American students. But I am happily seeing my improvement everyday. I composed my dissertation in English, gave seminars in English and passed the final defense in English; all of these are making me proud.

I thank my mentor, Dr. Maria Grant, for her patient and inspiring guidance in the past four years. Every member in my committee, Dr. Alfred Lewin, Dr. Sean Sullivan and Dr. Stratford May, has given me great suggestions for my dissertation work. I thank everybody in the lab. Dr. Lynn Shaw instructed me in great details during my experiments and dissertation writing. Dr. Aqeela Afzal was also a great help for my bench work. And every other member in the lab has given me great support for my defense.

I thank my parents. They are far away in China but I am sure they are proud and as happy as I am now. They have done everything they could to provide me the best education opportunities and they have always been there encouraging all the way along. I

am the only child in the family and I am thankful that they supported when I decided to study abroad.

I thank Yao for her great help and support. Her love strengthened me during the hardest time. Without her, I could not have overcome all the difficulties and successfully graduated.

There is still a long way ahead, with more challenges and opportunities. I would cherish everything I have had in the University of Florida. The orange and blue will always be a source of courage and confidence. Go Gators!

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TARGETING ANGIOGENIC GROWTH FACTORS IN PROLIFERATIVE DIABETIC RETINOPATHY

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Proliferative diabetic retinopathy is the leading cause of blindness in the working age adults. Pre-retinal angiogenesis is the hallmark of this disease and can lead to vessel leaking, exudate accumulation, hemorrhage, or even retinal detachment. Many growth factors have been identified to promote the vessel growth, physiologically and pathologically. Inhibition of these growth factors can result in less abnormal angiogenesis and potentially prevent the onset of vision impairment. One gene silencing technology, hammerhead ribozyme, was used to inhibit the signaling of these growth factors. Ribozymes are small RNA molecules that can recognize and cleave specific sequence in the target mRNA. Ribozymes against the genes of a number of growth factor receptors, including IGF-1R, insulin receptor, VEGF-R1, VEGF-R2, and multiple integrins, were designed and tested *in vitro* and *in vivo*. All ribozymes were tested by cleavage time courses, kinetic analysis and proved to be capable of cleaving synthetic RNA targets. Then they were transfected in human retinal endothelial cells, and the mRNA levels and

protein levels of the growth factor receptors were reduced. Also the migration, proliferation and tube formation of these cells were inhibited. We used the oxygen-induced retinopathy mouse model to test the ribozymes *in vivo*. The expression of the ribozymes induced significant reductions in the pre-retinal neovascularization levels. To better target the proliferating endothelium *in vivo*, and to minimize the adverse effect of ubiquitous ablation of targeted growth factor receptors, a proliferating endothelial cell-specific promoter was designed. This new promoter was tested with IGF-1R ribozyme and showed specific expression in the proliferating endothelium and significant reduction in the pre-retinal neovascularization levels. Our results suggest that these ribozymes are a useful tool to inhibit the angiogenesis in retinopathy, and the proliferating endothelial cell-specific promoter adds the specificity without losing expression strength.

CHAPTER 1
BACKGROUND

Introduction and Project Aim

Vascular retinopathies, including retinopathy of prematurity, proliferative diabetic retinopathy and age-related macular degeneration, are the leading cause of vision impairment worldwide. Pre-retinal vessel growth is the hallmark for retinopathy of prematurity and proliferative diabetic retinopathy. These new blood vessels are abnormally positioned and are fragile, easy to leak, and can result in hemorrhage and retinal detachment. Currently there is no cure for these diseases. The initiation and maintenance of these pre-retinal blood vessels depend on the involvement of many growth factors. In this project, with the help of a gene silencing technology, hammerhead ribozyme, efforts have been made to target and inhibit the expression of a number of growth factor receptors to reduce the growth factor signaling. Ribozymes are small RNA molecules that can specifically bind to a sequence in the target mRNA and perform cleavage. The genes of IGF-1R, VEGFR-1, VEGFR-2, integrins and insulin receptor have been targeted and the inhibition effects were examined *in vitro* and *in vivo*. To better target the proliferating endothelium *in vivo*, and to minimize the adverse effect of ubiquitous ablation of targeted growth factor receptors, a proliferating endothelial cell-specific promoter was designed and tested. In the basic science point of view, the investigations on the involvement of the growth factors in the pre-retinal angiogenesis can provide useful information about their signaling details; in the clinical application

point of view, this work could also imply new targets and methods for the disease treatment in the future.

The Eye

The Anatomy of the Eye

Optically working like a film camera, the eyes of all the vertebrates are structurally similar. The light enters the eye through the pupil and forms an inverted image on the retina, the light-capturing component that functions like the film in a camera. The cornea and the lens help to focus so that the clearest image is presented on the retina. The white outer surface of the eye ball is termed sclera, which consists of tough but flexible fibrous tissue and provides the mechanical support of the entire eye. The choroid is a layer contained within the sclera, and it is a dense meshwork of blood vessels and other tissues. One of the most important functions of the choroid is to provide nutritional and metabolic support for the retina, which is a neuronal sheet that lies within the choroid. The retina is the most inner surface at the back of the eye. Most of the space in the eye is filled with a gelatinous body, called vitreous. It is surrounded by the lens and the retina and the ciliary body. In the ciliary body, the cells secrete the aqueous fluid into the eye, which contributes to the maintenance of the pressure within the eye.

The Retina

The retina, a layer about 0.4mm in thickness, is primarily composed of neural tissue including five classes of neurons. It spreads out on the interior surface of the back of the eye. The visual pathway is initiated when the light stimulates the photoreceptors that are embedded in the outer retinal layers. The signal is transmitted to bipolar cells and then to ganglion cells. The signal then travels along the axon of the ganglion cells lining

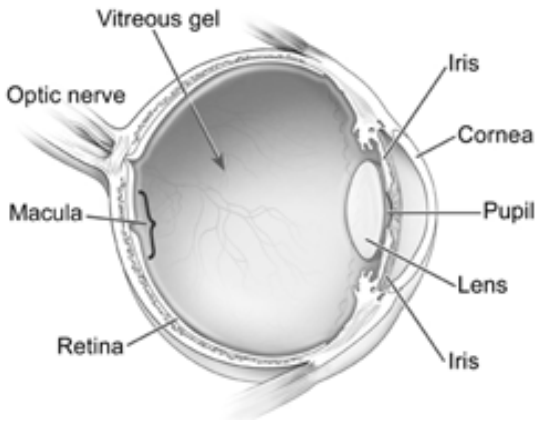

Figure 1.1. Basic structure of human eye (courtesy of National Eye Institute, www.nei.nih.gov).

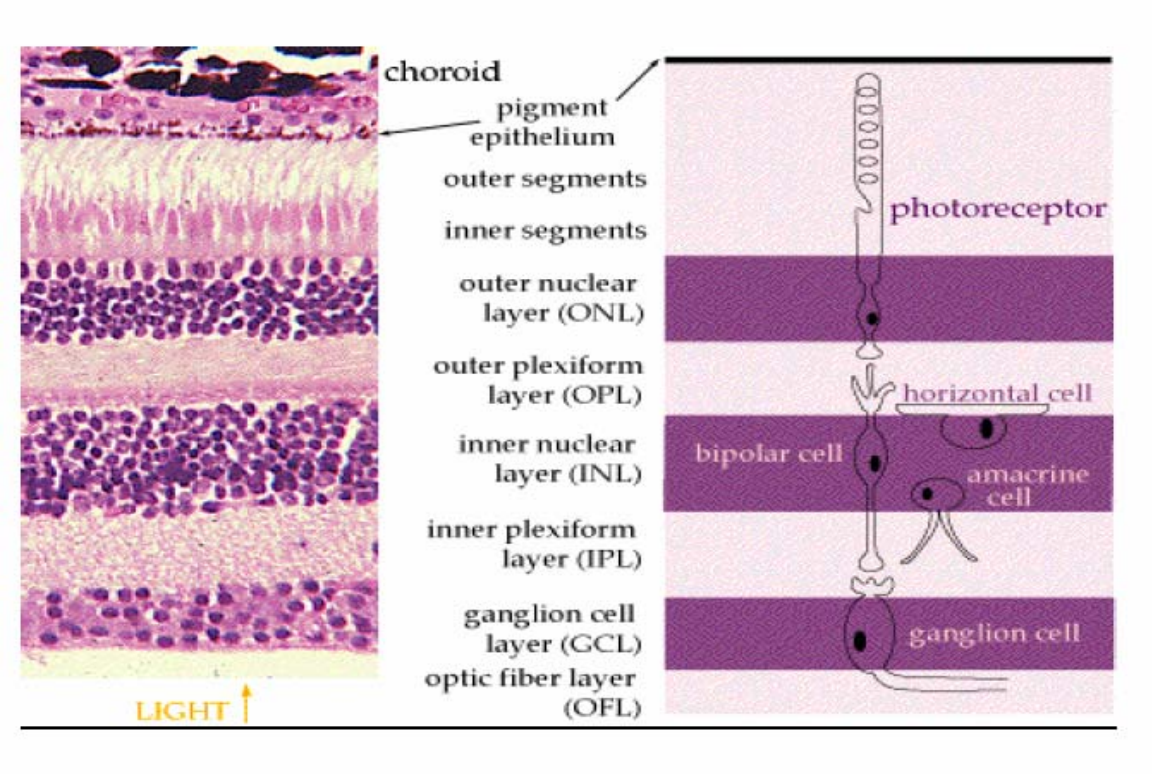

Figure 1.2. Cross section of the retina (http://thalamus.wustl.edu/course/eyeret.html).

the inner surface of the retina to the optic nerve, which penetrates the retina and connects to the brain. There are two more classes of neurons, horizontal cells and amacrine cells. They are both interneuron and assist in signal processing. Horizontal cells primarily contact with photoreceptor axons and bipolar cells in the outer plexiform layer and the

inner nuclear layer, respectively, while amacrine cells contact with bipolar axons primarily in the inner plexiform layer.

Light passes through almost the whole thickness of the retina to be captured by photoreceptors, or the outer segments of the photoreceptor in detail, where the visual pigment molecules for light capturing are located. There are two types of photoreceptors, rods and cones. Rods are specialized to convey variations in light intensity in dim conditions, but they are not able to function in bright light. Cones are specialized for bright light conditions, but they are not as sensitive as rods.

The retina cross section can be divided into multiple layers. The nuclear layers are basically where cell nuclei are located, and the synaptic layers are the place where cells communicate and transmit electric or chemical signals. The retinal pigment epithelium (RPE) functions as the outer blood-retinal barrier (BRB) that shut off the diffusion of large molecules from choroicapillaries. And the retinal vasculature doesn't grow beyond the inner limiting membranes under normal physiological conditions.

The Blood Supply to the Retina

The metabolism in the retina performs in the highest rate in the body. For the same mass of tissue, the metabolic needs of the retina are about seven times that of the brain. In order to meet these high metabolic needs, two separate circulations are involved. They are retinal and choroidal circulations. The larger arteries and veins of the retinal circulation can be seen under an ophthalmoscope, and most of the retinal surface is occupied with a meshwork of retinal capillaries. These capillaries form the inner BRB. The endothelial cells at the capillaries are connected by tight junctions that prevent leakage from the vessels. A lot of proteins or molecules work in the binding of the adjacent cells. Because of the tight junctions, proteins and solutes have to pass through

the apical and the basal membranes of the endothelial cells in order to go into or out of the circulation from or to surrounding tissues. Water, small molecules and dissolved gases can do so, such as glucose, oxygen, carbon dioxide, and so on. But most large molecules, including proteins, cannot pass through freely. The only possible way for them to pass through is through a process of active transport with the help of the proper membrane tunnel proteins. So basically the BRB provides a mechanism of keeping the substance entering the retinal neural tissue in a controlled manner.

The central artery and vein of retinal circulation originate along the optical nerve and extend into the retina from the center of the optical disc. While the choroidal arteries and veins of pass through the sclera at multiple places around the optical nerve, and then they branch into a meshwork of very large capillaries, called choroicapillaries. Large capillaries increase the rate of blood passing through, which keeps the concentration of oxygen high and the concentration of carbon dioxide low, and also quick removes the heat from focused light on the eye bottom. The BRB is not maintained by choroidal circulations, because the cells on the side facing the RPE are fenestrated, and there is no tight junction between these cells. However, the RPE connecting with the choroid have tight-junctions and provide the outer portion of the blood-retinal barrier.

Retinopathies

Retinopathies are diseases that affect the function of retinas. Usually they involve the abnormalities in the vasculatures that nourish the retina. These abnormalities included ectopic angiogenesis, rupture and leakage on the vessels, accumulation of exudates, retina detachment caused by vessel and fibrous tissue contractions, and so on. There are three types of retinopathy clinically identified: age-related macular degeneration (ARMD), which occurs in the elderly people; diabetic retinopathy (DR), which occurs in the

working age people; and retinopathy of prematurity (ROP), which occurs in infants. ARMD more involves the abnormalities in choroicapillaries, while DR and ROP are basically related to the abnormalities of retinal vasculature.

Age-Related Macular Degeneration (ARMD)

ARMD is the leading cause of blindness among those aged over 65 in the western world [1-3]. It affects the outer retina, RPE, Bruch's membrane and the choroids. Thickening of Bruch's membrane is seen in this disease. Our understanding about the pathogenesis has grown in the past decade, but still a lot remains unknown and the current therapy is limited.

Figure 1.3. Normal view vs. ARMD (courtesy of National Eye Institute, www.nei.nih.gov).

The clinical hallmark of ARMD is the appearance of drusen, localized deposits lying between the basement membrane of the RPE and Bruch's membrane. Drusen can be shown as semi-translucent punctuate or yellow-white deposits depending on the stage of the disease. Morphologically drusen are classified as "hard" and "soft". Hard drusen are pinpoint lesions; soft drusen are larger with vague edges and they are easy to become confluent. Drusen can become calcified and they may also regress. Typically clustered drusen are located in the central macula, so they can lead to deficits in macular function

such as color contrast sensitivity, central visual field sensitivity and spatiaotemporal sensitivity [4]. Increased quantity and size of drusen are an independent risk factor for visual loss in ARMD.

Geographic atrophy is also seen in ARMD, which refers to confluent areas of RPE cell death accompanied by overlying photoreceptor atrophy [5]. Geographic atrophy leads to vision impairment, especially the visual function in dark situations [6]. This loss of function is probably because the RPE loss results in reduced nutrients for those photoreceptors that are located in the RPE atrophy areas. Apoptosis in the corresponding area are found [7].

Choroidal (or subretinal) neovascularization (CNV) is a major cause of vision loss in ARMD. As the term itself indicates, CNV refers to the new blood vessel growth from the choroids. It breaks through the Bruch's membrane into the space underneath RPE, or it may further penetrate the RPE layer into the subretinal space. Usually CNV is associated with leakage of fluid and blood. The repeated leakage of blood, serum, and lipid can stimulate fibroglial organization leading to a cicatricial scar [4].

Drusen and CNV can cause irregular elevation of RPE, which can lead to RPE detachment or even RPE tear. RPE detachment can cause visual loss in patients with ARMD [8].

Depending on whether CNV is present, ARMD is classified into the dry form or the wet form. The dry ARMD is nonexudative [4]. This is the early phase of ARMD, and the earliest pathological changes are the appearance of basal laminar deposits between the plasma membrane and basal lamina of the RPE, and the appearance of basal linear deposits located in the inner collagenous zone of Bruch's membrane. The former deposits

are seen in an increase amount in ARMD [9], and the later deposits are only seen in ARMD [10]. Approximately 10 percent of persons with AMD develop the exudative form of the disease, or wet ARMD. Exudative AMD accounts for 80 to 90 percent of cases of severe vision loss related to AMD. CNV occurrence is the hallmark of wet ARMD. CNV is associated with abnormal vessels that leak fluid and blood in the macula, resulting in blurred or distorted central vision. Figure 1.4 is the fundus photograph (A) and fluorescence angiogram (B) of an eye of a patient with exudative ARMD. Note subretinal neovascularisation (A, asterisk) with surrounding hard exudates (arrowheads). On the angiogram (B) the neovascularization is clearly stained by fluorescein (black arrow) [11].

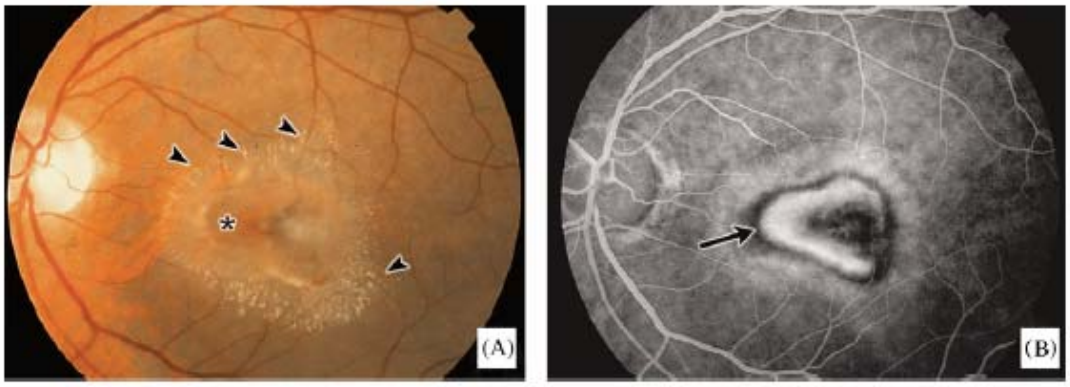

Figure 1.4. Fundus photograph and fluorescence angiogram of ARMD [11].

As for the pathogenesis of ARMD, shortly speaking, Campochiaro and coworkers suggested that the age-related thickening of Bruch's membrane reduces the diffusion of oxygen from the choroid to the RPE and retina [12], and recent evidence suggests that VEGF plays an important role in the development of CNV. VEGF expression was found to be increased in RPE cells of maculae of patients with age-related maculopathy, a condition with a high risk of CNV occurrence [13] and in experimental animal models [14]. VEGF levels in the vitreous of wet ARMD were found to be significantly higher

than healthy controls [15]. Chronic inflammation from drusen may be involved in the development of ARMD [16], but the inflammatory contribution is still controversial.

Retinopathy of Prematurity (ROP)

ROP is an adverse effect of treating those premature neonates in respiratory distress with high oxygen. The high oxygen helps these infants to survive, but it can cause ROP, which will impair their vision. ROP mainly affects premature infants weighing about 1250 grams or less that are born before 31 weeks of gestation. It is one of the most common visual loss diseases in childhood. According to the National Eye Institute, there are about 28,000 infants born weighing 1250 grams or less in the U.S., and among them, 14,000-16,000 of the infants are affected by ROP to some degree. 10% of them need medical treatment and 400-600 infants annually become legally blind of ROP.

The ROP complete progression can be divided into 5 stages. Stage 1 is characterized by a demarcation line between the normal retina (near the optic nerve) and vascularized retina. In stage 2, a ridge of scar tissue rises up from the retina due to growth of abnormal vessels. This ridge forms in place of the demarcation line. In stage 3, the vascular ridge grows due to spread of abnormal vessels and extends into the vitreous. Stages 4 and 5 refer to retinal detachment; stage 4 refers to a partial retinal detachment caused by contraction of the ridge, thus pulling the retina away from the wall of the eye; and stage 5 refers to complete retinal detachment.

ROP is now considered as a two-phase process during the disease development. In the first stage, the high oxygen condition will make the developing retinal blood vessels and especially the developing capillary buds be more “pruned” to drop out. This pathological vessel dropout is an exaggeration of the normal physiologic process, in which there is a constant balance between developing and degenerating capillary buds, as

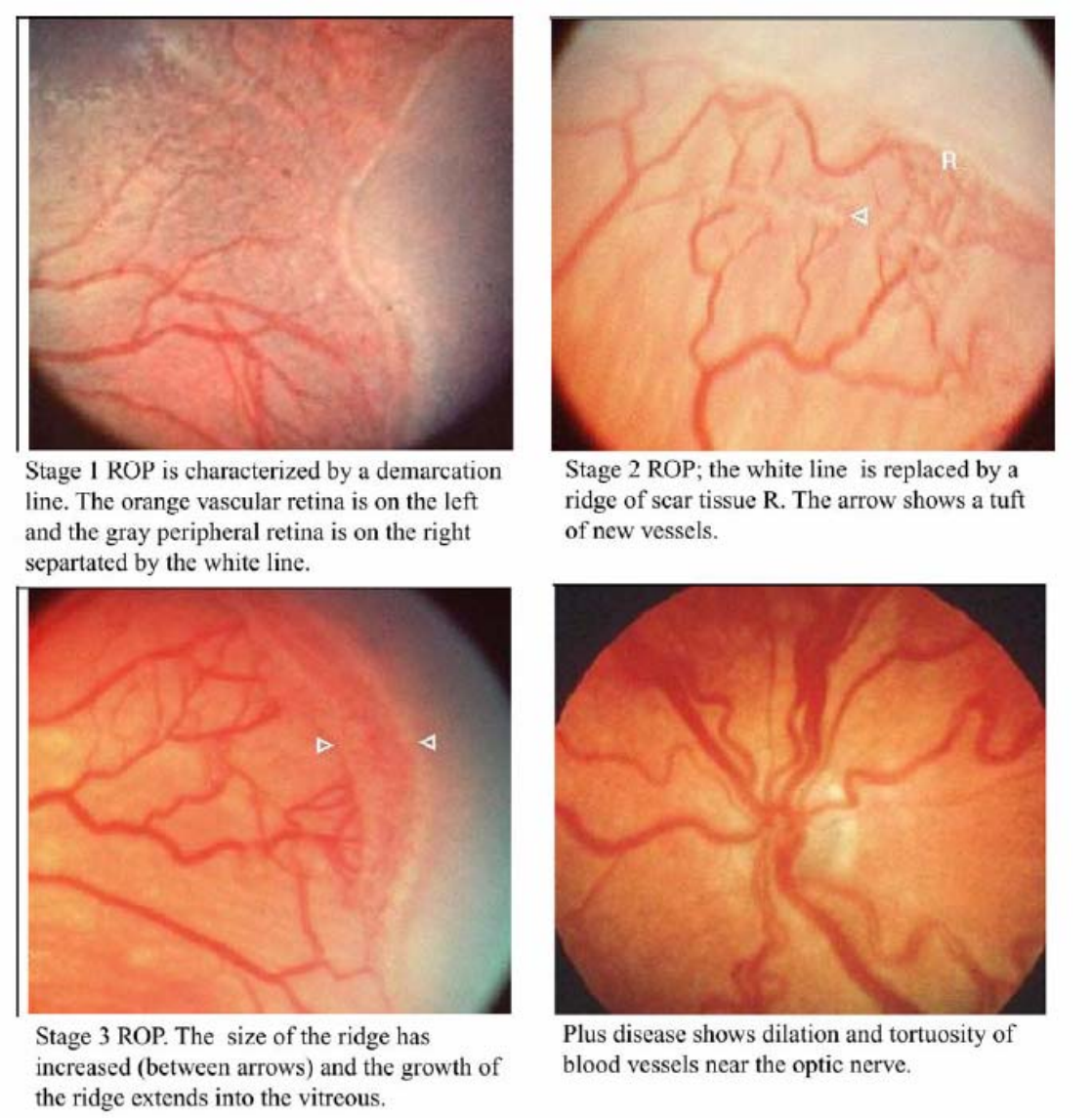

Figure 1.5. Five stages in ROP (courtesy of National Eye Institute, www.nei.nih.gov).

tissue demand changes [17]. In short, the hyperoxic vaso-obliteration occurs in the first stage. When the high oxygen care is complete and the infants survive, they are taken out the high oxygen environment and the second stage occurs. Because of the vessel loss, the tissue becomes hypoxic and the ischemia-induced vaso-proliferation begins. The hypoxia stimulates growth factors increases, especially VEGF. These growth factors play very important roles in the vaso-proliferation. The vaso-proliferation is abnormal in that these new vessels are fragile and leak, scarring the retina and pulling it out of position, which

will lead to retinal folds and retinal detachment. The term babies are less affected by fluctuations in oxygen levels as once the vessels become developed and surrounded by supportive matrix, thus they are no longer susceptible to pruning by hypoxia [18].

Diabetic Retinopathy (DR)

Diabetic retinopathy is one the three major complications of diabetes mellitus (the other two are neuropathy and nephropathy) and occurs in both type I and type II diabetes. DR primarily affects the working age people and is the leading cause of new-onset visual loss in working people in the U.S. and other industrialized countries [19]. DR affects approximately three-fourths of diabetic patients within 15 years after onset of the disease [20]. Retinal neovascularization and macular edema are central features of DR and also the two factors that cause vision loss. The newly-formed vessels are fragile and abnormal and they can leak blood into the center of the eye, blurring vision. Macular edema usually occurs as the disease progresses. The fluid leaks in the center of macular and makes the macula swell, blurring vision. Other characteristics found in DR include basement membrane thickening, pericyte loss, microaneurysms, and so on.

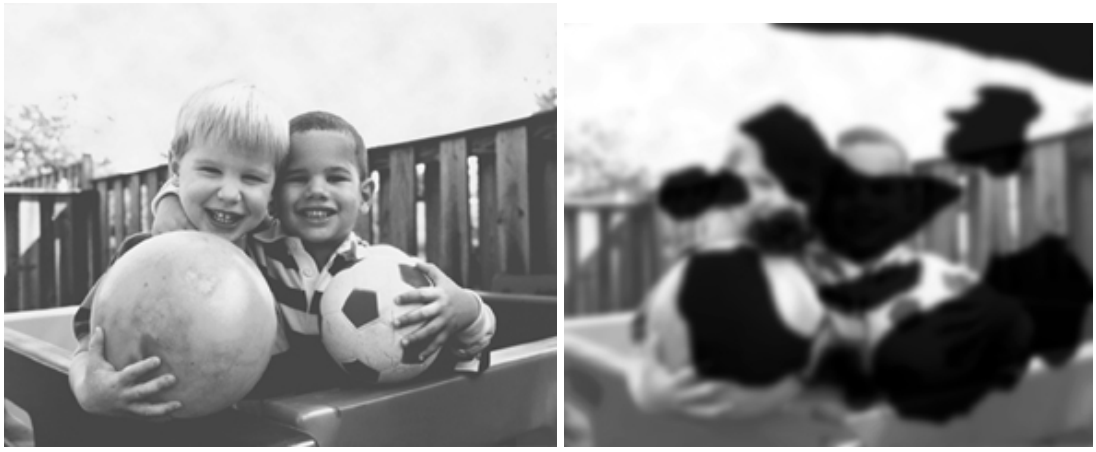

Figure 1.6. Normal view vs. DR (courtesy of National Eye Institute, www.nei.nih.gov).

In the beginning stage of DR, there are no clinically evident symptoms, but the biochemical and cellular alterations are going on in the retinal vasculature. These

alterations include increased adhesion of leukocytes to the vessel wall, alterations in blood flow, basement membrane thickening. These factors are involved in the blockage of the retinal capillaries, which is thought to induce hypoxia and further trigger the overexpression of the angiogenic factors. Other vascular alterations include death of retinal pericytes, subtle increases in vascular permeability, or even the loss of vascular endothelial cells. Following this, the blood and fluid leakage may come. The loss of endothelial cells also leads to acellular capillaries worsening ischemia. With time, more abnormal phenomena occur and they are clinically observable. These abnormalities include microaneurysms, dot/blot hemorrhages, cotton-wool spots, venous beading and vascular loops [20]. The blood and fluid leak out the vessels and accumulate in the retinal tissue, giving rise to exudates. When this occurs in macula, patients will have macular edema and impaired vision. This stage is also called nonproliferative retinopathy. With the progression the disease, next stage is the proliferative retinopathy, featuring the growth of new vessels on the surface of the retina. The new vessels are abnormal, fragile and easy to break. The leaking blood can cloud the vitreous and further impair vision. In more advanced stages, the exaggerated pre-retinal neovascularization can grow from the retinal surface into the vitreous cavity. This can cause retinal detachment can lead to blindness. Proliferative retinopathy typically develops in patients with type I diabetes, whereas nonproliferative retinopathy with macular edema is more common in patients with type II diabetes [20].

Figure 1.7 shows the fundus photograph (A) and fluorescence angiogram (B) of an eye of a patient with non-proliferative diabetic retinopathy. The arrowheads in Panel A

point to intra-retinal hard exudates surrounding areas of leaking microaneurysms (B, white arrows) [11].

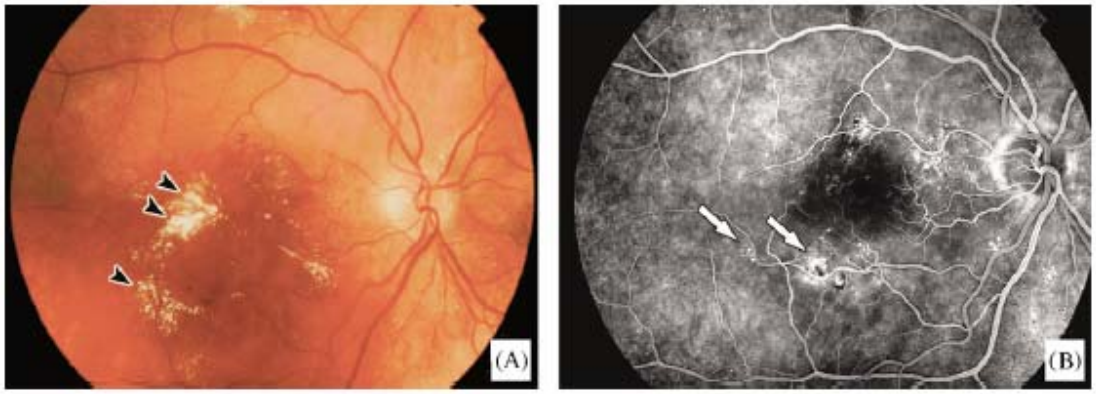

Figure 1.7. Fundus photograph and fluorescence angiogram of non-proliferative DR [11].

Fundus photograph (A) and fluorescence angiogram (B) of an eye of a patient with proliferative diabetic retinopathy is shown in Figure 1.8. Note pre-retinal neovascularization (black arrow) on the optic disc (A), which is extensively leaking fluorescein (B. white arrows) [11].

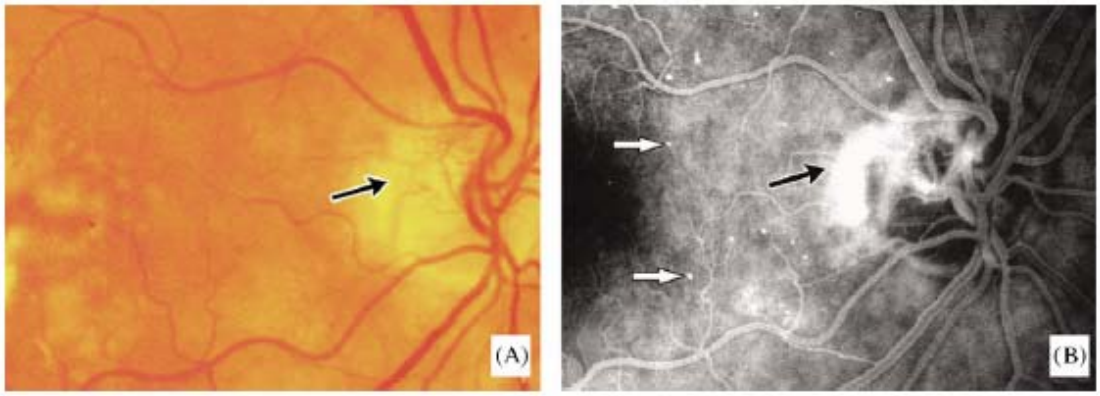

Figure 1.8. Fundus photograph and fluorescence angiogram of proliferative DR [11].

Current Treatments for Retinopathies

Currently the clinical proved treatments for retinopathies are limited, and few drug medications are available. The conventional treatments include laser photocoagulation, cryotherapy, photodynamic therapy, scleral buckle, and vitrectomy. All of them cannot cure the disease, but can only delay the disease progression.

In laser photocoagulation, the doctor places thousands, up to 3,500, small laser burns on the retina. These burns will destroy the normal tissue and decrease the oxygen needs of the retina. The treatment is usually effective, but at the cost of loss of normal tissue, and it reduces peripheral vision, impair night vision and change color perception. The laser photocoagulation is not a cure, as the disease can still progress in spite of treatment. More treatments may be needed to further prevent vision loss. Laser treatment is currently applied in all retinopathies, that is, ROP, DR, and ARMD. Laser is also used to target at the leaking spots, like in severe macular edema, the laser burning is applied in a focal way. When preventing abnormal vessel growth, as in proliferative DR, the laser burning is applied in a scattered way.

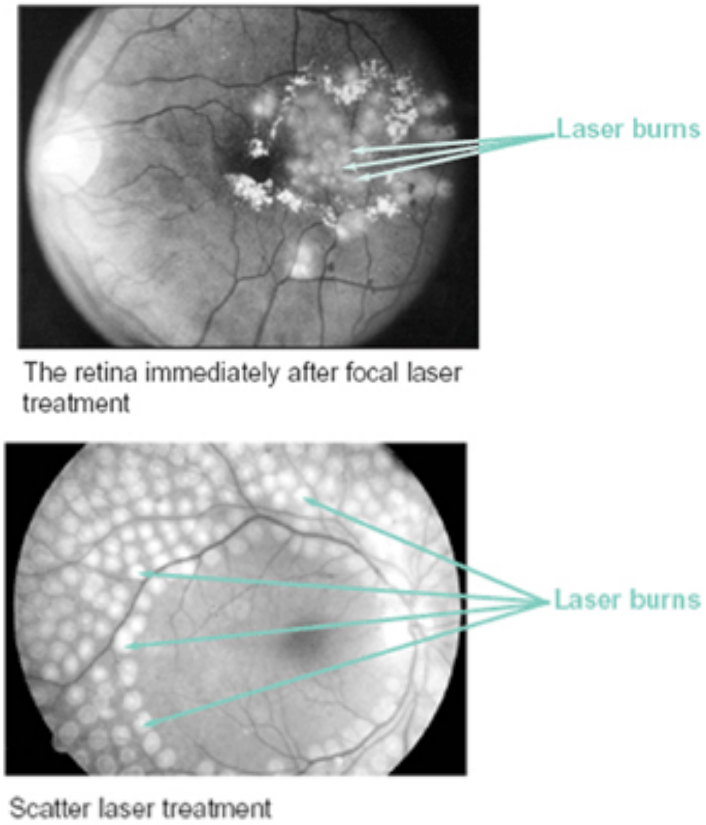

Figure 1.9. Photocoagulation (courtesy of National Eye Institute, www.nei.nih.gov).

Cryotherapy is a procedure in which physicians use an instrument that generates freezing temperature to briefly touch spots on the surface of the eye that overlie the periphery of the retina. It also destroys the tissue and impairs the side vision. Cryotherapy is more used for ROP. In Figure 1.10, cartoon is showing cryotherapy application to the anterior avascular retina. A cold probe is placed on the sclera till an ice ball forms on the retinal surface. Multiple applications are done to cover the entire vascular area. This treatment thins the tissue under the retina and allows easier oxygen diffusion through the retina.

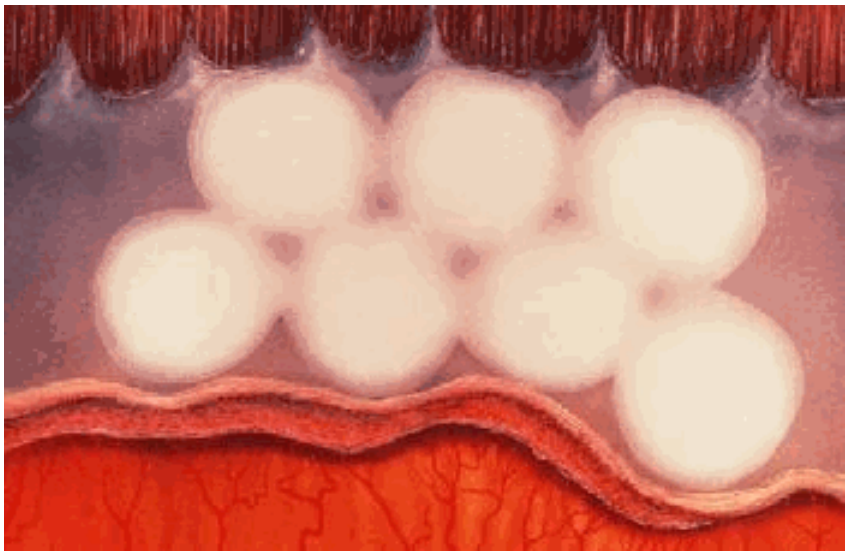

Figure 1.10. Cryotheropy (http://www.checdocs.org/dr_treatment.htm).

In photodynamic therapy, a drug called verteporfin is injected i.v. and perfused to the vasculature in the eye. The drug tends to "stick" to the surface of new blood vessels, and then, a light is shined into the eye for about 90 seconds, and the light activates the drug to destroy the new blood vessels. The advantage of this method is that the drug doesn't destroy the normal tissue surrounding. But the patient needs to avoid bright light for five days because the drug can be activated in their exposed body parts. Photodynamic therapy is more used to treat wet ARMD.

In later stages of ROP, scleral buckle is another treatment option [21]. This involves placing a silicone band around the eye and tightening it. This keeps the vitreous from pulling on the scar tissue and allows the retina to flatten back down onto the wall of the eye. The band will be removed later. In most severe conditions in retinopathies, vitrectomy can be applied, in which the vitreous is removed, scar tissue on the retina peeled back or cut away, and saline solution is replaced for vitreous. The retina reattachment can be seen after this surgical treatment [22].

Pathogenesis of Diabetic Retinopathy

Diabetes mellitus is a serious disease leading to morbidity and mortality as it has long-term complications include macrovascular and microvascular disease. Both type I (characterized by no insulin production) and type II (characterized by insulin resistance) diabetes can have these complications. Retinopathy is one of the microvascular complications. It is believed that the chronic hyperglycemia has a strong relationship with microvascular complications, and clinical research data demonstrates that improved glycemic control contributes to significant microvascular risk reduction [23, 24]. Experiments on animal models also suggest that long-term hyperglycemia is necessary to induce changes in the retinal vasculature [25].

In the retina, GLUT1, which is one of a family of glucose transporters, is responsible for glucose transfer across BRB into the endothelial cell and retinal cells. While in most other cells in the body, insulin assistance is required for internalize glucose; this is not the case with the retina. Excessive transport of glucose through GLUT1 [26], the involvement of GLUT1 in RPE cells [27], and increased density of relocalized GLUT1 in inner BRB [28] have been proposed to be related with intracellular hyperglycemia. Intracellular hyperglycemia in the early stages of diabetes causes

abnormalities in blood flow and increases in vascular permeability. The blood flow changes come from decreased activity of vasodilators, such as nitric oxide, and increased activity of vasoconstrictors such as angiotensin II and endothelin-1 [29]. The increase in vascular permeability comes from VEGF functioning on endothelial cells and changes in extracellular matrix. With time, hyperglycemia can further induce cell loss and progressive capillary occlusion. All these changes will eventually lead to edema, ischemia and hypoxia-induced neovascularization.

To date, there are several hypothesized theories on how hyperglycemia contributes to microvascular damage, or retinopathy. The most common ones are polyol pathway theory, advanced glycation end-products (AGE) theory, oxidative stress theory and PKC activation theory.

Increased Polyol Pathway Flux

As shown in Figure 1.11, glucose is reduced to sorbitol by aldose reductase, and at the same time, nicotinamide-adenine dinucleotide phosphate (NADPH) is oxidized to $NADP^+$. Then sorbitol is oxidized by sorbitol dehydrogenase to fructose, coupled with the reduction of oxidized nicotinamide-adenine dinucleotide (NAD^+) to NADH [29]. So the intracellular high glucose level will result in excess sorbitol, fructose, NADH accumulation and decrease in NADPH. Some damages caused by increase flux through polyol pathway have been proposed to include: activation of protein kinase C [30], contribution of AGE formation [30], decreased activity of Na/K-ATPase [29], and increase in the formation of reactive oxygen species leading to oxidative stress [29].

Production of AGE

AGE are irreversibly cross-linked substances. Intracellular hyperglycemia is possibly the primary initiating event in the formation of intracellular and extracellular

AGE [32, 33]. During formation of AGE, glucose reacts nonenzymatically with the amino group of proteins and other macromolecular to form Schiff bases, which are transformed into Amadori products that eventually lead to AGE formation [34].

When AGE bind their receptors, RAGEs, some abnormal cellular events can occur, including: the stimulation of the production of the vasoconstrictor endothelin-1, VEGF production that is associated with increased permeability, and production of reactive oxygen species. The long-term effects induced by AGE and RAGEs are mostly mediated by transcription factor κB to express cytokines and growth factors [29].

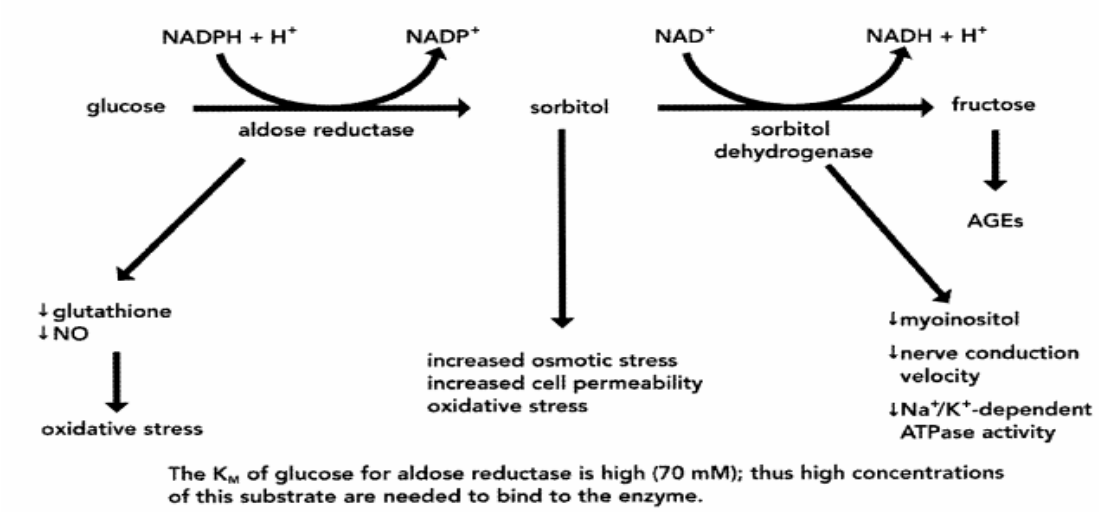

Figure 1.11. Polyol Pathway [31].

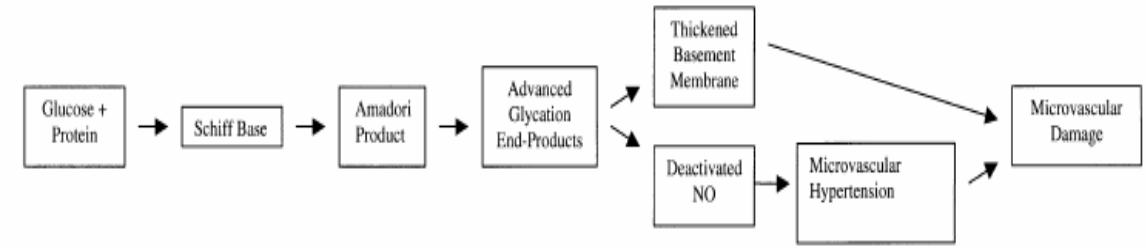

Figure 1.12. AGE formation [31]

There are several adverse alterations in the micro vasculature associated with AGE. AGE formation can contribute to thickening of the basement membrane and to microvascular hypertension by inactivating nitric oxide [31]. The thickening and

hypertension can lead to microvascular leakage and occlusion. AGE can adversely affect vascular permeability, alter the functions of matrix molecules, and alter the functions of vessels, by decreasing the vessel elasticity, increasing fluid filtration across vessels [29], decreasing endothelial cell adhesion [35], and so on.

Generation of Reactive Oxygen Species

The term oxidative stress refers to the imbalance between the production of reactive oxygen species and the normal antioxidant protective mechanisms present to guard tissues from oxidative damage [36]. As discussed above, both polyol pathway and AGE formation can lead to the generation of reactive oxygen species. Glucose also has pro-oxidant properties in the presence of heavy metals and the auto-oxidation of glucose can form free radicals too. These reactive oxygen species can inactive or reduce nitric oxide levels [37].

The reactive oxygen species can result in damaged protein and mitochondrial DNA that have adverse effects on the microvasculature [38], especially leading to increased microvascular permeability [39]. Oxidative stress has been shown to increase intracellular calcium levels, which have been associated with endothelial hyperpermeability of macromolecules [40].

Activation of Diacylglycerol and Protein Kinase C Isoforms

It has been shown that diacylglycerol (DAG) formation can be induced by glucose in cell cultures, animal tissues, and diabetic patients [31]. DAG is very important in the activation of various protein kinase c (PKC) isoforms, with the isoform β being thought to be the most sensitive to changes in DAG levels. PKC-β has been shown to be increased in various vascular tissues following hyperglycemic exposure [41]. PKC-α, PKC-β1 and PKC-β2 are seen to be elevated in the retina during acute and chronic

hyperglycemic states [42]. The consequences induced by PKC activation include increased retinal permeability [43], increased basement matrix protein formation [44], VEGF formation [44], and so on. So PKC may have adverse long-term effects in the vasculature.

Based on the involvement of these pathways, a lot of pathological changes can happen in diabetic retinopathy. Some of the most important changes are covered below. They will lead to edema, ischemia and hypoxia in the retina, which all lead to abnormal neovascularization.

How Does the Change in Retinal Blood Flow Occur?

Hyperglycemia induces changes in retinal blood flow via its effects on vasodilators and vasoconstrictors. Nitric oxide (NO) is one of the most important vasodilators. It is synthesized from L-arginine or L-citrulline in cells via activation of a calcium-dependent nitric oxide synthase (NOS). The NOS isoform produced in endothelium is called eNOS. NO functions by entering smooth muscle cells and activating soluble guanylate cyclase, which will result in increased level of cyclic guanosine 3', 5'-monophosphate (cGMP). cGMP can relax the smooth muscle cells through a decrease in Ca^{2+} and dephosphorylation of myosin light chains [45]. In the hyperglycemic environment, a couple of pathways mentioned above can lead to decreased level of NO. In the polyol pathway as mentioned earlier, sorbitol is produced coupled with the oxidation of NADPH and this reduces NADPH availability, and NADPH is one of the cofactors for NO synthesis. AGE production can lead to subsequent superoxide generation resulting in NO inactivation. PKC activation reduces the capacity of a number of agonists to increase intracellular Ca^{2+} and to stimulate NO production; on the other hand, the superoxide expression may also result from PKC activation.

Endothelin (ET)-1 is a powerful vasoconstrictor. At low concentrations, it induces vasodilation. While at high concentrations, it causes the constrictive response by interacting with its receptors on smooth muscle cells and pericytes in the retinal vasculature. Hyperglycemia-induced PKC activation can enhance ET-1 transcription level [46].

What Causes Retinal Capillary Cell Death?

Pericytes loss and endothelial cells loss are both seen in diabetic retina. The cell death will inevitably lead to microaneurysms and vascular obstruction. Polyol pathway, AGE pathway and oxidative stress are all thought to be associated with cell death. Sorbitol accumulated in polyol pathway may cause hyperosmolality of the cells [47]; accumulated AGE production in the glycation pathway will form cross-links and to generate oxygen-derived free radicals [48]; and the oxidative stress will inactivate NO and cause abnormal chemical changes in DNA structure [49].

What Causes Retinal Ischemia?

Hyperglycemia causes ischemia via several possible mechanisms, including thickened basement membrane, platelet aggregation, leukocyte activation and adherence. Hyperglycemia is sufficient to increase the synthesis of basement membrane components, like fibronectin [50], various types of collagens [51] and vitronectin [52]. Increased number and size of platelet-fibrin thrombi in retinal capillaries have been found in the retina of patients with diabetic retinopathy [53]. Hyperglycemia-induced PKC activation will stimulate platelet-derived factor (PAF) production, which will activate platelets. Activated platelets can produce platelet-derived microparticles, which are involved in the thrombus formation [54]. PAF can also stimulate their receptors on leukocytes rolling on the luminal endothelial membrane and activate them. β2 integrins on activated leukocytes

enable them to adhere tightly to the endothelial cells via binding intercellular adhesion molecule-1 (ICAM-1), while as the same ICAM-1 is also unregulated by PKC activation. And NO downregulation can allow leukocytes to escape from NO control, also leading to leukocyte activation and adherence [54].

Angiogenesis and Growth Factors

Vasculogenesis and Angiogenesis

Small blood vessels consist only of endothelial cells (ECs), whereas larger vessels are surrounded by mural cells (pericytes in medium-sized vessels and smooth muscle cells (SMCs) in large vessels) [55]. Vessels can grow in several ways. Vasculogenesis refers to the formation of blood vessels by endothelial progenitors [55]. It is a process by which the initial vascular tree forms in the yolk sac and aortic arches, and begins immediately following gastrulation when mesodermal cells aggregate into blood islands. Blood islands contain the precursors of hematopoietic and vascular endothelial lineages [56]. Angiogenesis refers to the formation of new vessels formation by sprouting from pre-existing vessels and subsequent stabilization of these sprouts by mural cells. Additional modes of vascular growth include intususception, bridge formation, and vascular splitting, in which invaginations or extensions of the vessel wall form tubes that connect or bifurcate parent vessels [56].

The traditional view is that vessels in the embryo developed from endothelial progenitors, whereas sprouting of vessels in the adult resulted only from division of differentiated ECs. However, recent evidence has shown that endothelial progenitors contribute to vessel growth both in the embryo and in ischemic, malignant or inflamed tissue in the adult. They can even be used therapeutically to stimulate vessel growth in ischemic tissues, a progress called “Therapeutic Vasculogenesis” [57-59]. Although

retinal neovascularization has been thought to be due to proliferation of endothelial cells by angiogenesis, Grant et al. showed that hematopoietic stem cells can enter the circulation and reach the areas of angiogenesis, and clonally differentiate into endothelial cells [60]. In another study, adult Lin(-) hematopoietic stem cells injected intravitreally into neonatal mouse eyes have been shown to interact with retinal astrocytes that serve as a template for retinal angiogenesis [61]. Blood vessels are being modified by endothelial progenitor cells, hematopoietic stem cells or other stem cells, and these cells functionally contribute to physiological and pathological angiogenesis.

Angiogenesis is usually inactivated or kept at low levels in normal tissue of an adult, but may be activated to an excessive state in a number of diseases, such as cancer, psoriasis, arthritis, retinopathy, obesity, asthma, atherosclerosis, and infectious diseases. Cancer is another best known disease that involves pathological angiogenesis that can be potentially targeted for therapy. In 1972 Folkman proposed that solid tumors are dependent on angiogenesis for growth greater than a few millimeters in size, and that increases in tumor diameter require a corresponding increase in vascularization [62]. A critical step during angiogenesis is the local stimulation of endothelial cells by various cytokines and growth factors. Stimulation causes the endothelial cells to lose their contact inhibition, migrate and breach the basement membrane, proliferate, and differentiate to organize into new vessels [63].

Hypoxia-Induced Factor (HIF)

Beyond a size limitation, simple diffusion of oxygen to metabolizing tissues becomes inadequate, and specialized systems of increasing complexity have evolved to meet the demands of oxygen delivery in higher animals [64]. One important role in the systems is angiogenesis, to make new vessels sprouting into the location that blood

delivery is needed. So ischemia or hypoxia is one of the key factors that lead to the initiation of angiogenesis. Exactly how hypoxia induces angiogenesis was however poorly understood. The landmark of hypoxia study in the early 1990s showed that hypoxia could induce expression of platelet-derived growth factor (PDGF) mRNA [65] and vascular endothelial growth factor (VEGF) mRNA in tissue culture [66]. Both PDGF and VEGF are thought to be important growth factors triggering angiogenesis. A large number of genes are involved in different steps in angiogenesis and they are independently responsive to hypoxia in tissue culture. Besides PDGF and VEGF, nitric oxide synthase, fibroblast growth factor, angiopoietins, and matrix metalloproteinases are involved [67-69]. Many of the individual phenotypic processes in angiogenesis such as cell migration or endothelial tube formation can be induced by hypoxia tissue culture [70]. Further study of hypoxia-induced angiogenesis leaded to the discovery of a key transcriptional regulator, hypoxia-inducible factor (HIF)-1 [47, 68, 69, 71].

HIF-1 is a heterodimer DNA-binding factor. HIF-1 consists of an α and β subunits, both of which have a number of isoforms. HIF-1β subunits are constitutive nuclear proteins, while HIF-1α subunits are hypoxia-inducible. There are three isoforms for α subunit. HIF-1α and HIF-2α appear closely related and are both able to interact with hypoxia response elements (HREs) to induce transcriptional activity [72, 73]. In contrast, HIF-3α appears to negatively regulate the response, through an alternatively spliced transcript [74].

The molecular mechanism behind HIF-1 is a pathway that links oxygen availability and the gene expression of various growth factors, especially VEGF. In normoxia and hyperoxia oxygen-dependent prolyl hydroxylases hydroxylate HIF-1 α

proline residues, and this chemical modification leads to a HIF-1 capture by a ubiquitin ligase complex that directs it to the proteasome for destruction. Under hypoxic conditions, HIF-1 α is not hydroxylated, escapes ubiquitination, accumulates and directs pro-angiogenic expression [75].

Vascular Endothelial Growth Factor (VEGF)

VEGF was originally discovered as the vascular permeability factor (VPF) that increased the vascular permeability in the skin [76]. In 1989 Ferrara and Henzel identified a growth factor for endothelial cells from bovine follicular pituitary cells and named it VEGF [77], which was then proved to be identical to VPF [78, 79]. VEGF is the most potent endothelial cell growth factor found to date. In the past two decades, this growth factor has been studied extensively and its key roles in the proliferation, migration, invasion, cell survival, differentiation of endothelial cells and other cell types have been established. It is critical in the normal embryonic development of vasculature and has essential functions in adults during normal physiological events such as would healing, menstrual cycle, even though the mRNA levels of VEGF and its receptors decrease significantly postnatally. Meanwhile, VEGF is also an important factor in numerous pathological situations, many of which involve abnormal angiogenesis, for example, inflammation, retinopathies, psoriasis, and cancer. Targeting VEGF signaling in these diseases has been studied with enthusiasm and a number of novel drugs targeting VEGF are being tested in clinical trials.

VEGF Family and Isoforms

The VEGF gene family consists of multiple variants, including VEGF-A (hereafter referred to as VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor (PlGF-1 and PlGF-2 isoforms). They are secreted glycoproteins

that form homodimers, which belong to a structural superfamily of growth factors, including the platelet derived growth factor (PDGF), characterized by the presence of eight conserved cysteine residues [80, 81]. VEGF-A is believed to be the major stimulator for vascular angiogenesis. VEGF-B is structurally similar to VEGF-A and PlGF is highly abundant in heart, skeletal muscle and pancreas and may regulate endothelial cell functions via a paracrine fashion [82]. VEGF-C and VEGF-D are basically involved in lymphangiogenesis and induce the proliferation and cell survival of lymphatic endothelial cells [83-85]. VEGF-E, encoded by the Orf virus, is structurally similar to VEGF-A, specifically binds to VEGFR-2 and induces angiogenesis [86]. VEGF-F, as a collective name, summarized the variants isolated from snake venoms [87].

The term VEGF refers to a collection of related isoforms expressed from the same gene [88]. The gene encoding VEGF, or VEGF-A, is located on the short arm of chromosome 6 in humans [89] and on chromosome 17 in mice [90]. The *vegf* gene consists of eight exons and seven introns, alternative splicing results in many isoforms. The best studied isoforms in human are VEGF121, VEGF165 and VEGF189. In mice, the homologous counterpart isoforms contain one less amino acid, so mVEGF164 is the corresponding isoform for hVEGF165 [90], for example. In all isoforms, the transcrips of exon 1-5 are all conserved and exon 6 and 7 are where the alternative splicing occurs. Exon 3 and 4 encode the binding domains for VEGFR-1 and VEGFR-2 [91]. Exon 6 and 7 encode two heparin-binding domains, which influence receptor binding and solubility [92]. VEGF189, containing both the exon 6 and 7 transcripts, has high affinity for heparin sulfate and is mostly associated with the cell surface and the extracellular matrix [93]. On the contrary, VEGF165, lacking exon 6, is moderately diffusible; and

VEGF121, lacking both exon 6 and 7, is high diffusible [94]. Recently a new isoform called VEGF165b, a variant of VEGF165, has been identified [95]. The C-terminus of VEGF165b is encoded by exon 9, instead of exon 8 as in VEGF165 and other isoforms [96]. VEGF165b binds to but does not trigger receptor phosphorylation, so it is actually an endogenous inhibitory form of VEGF [96]. This is due to a missing exon 8-encoded C-terminus, which has mitogenic signaling functions. Figure 1.13 [92] shows the alternative splicing among VEGF isoforms.

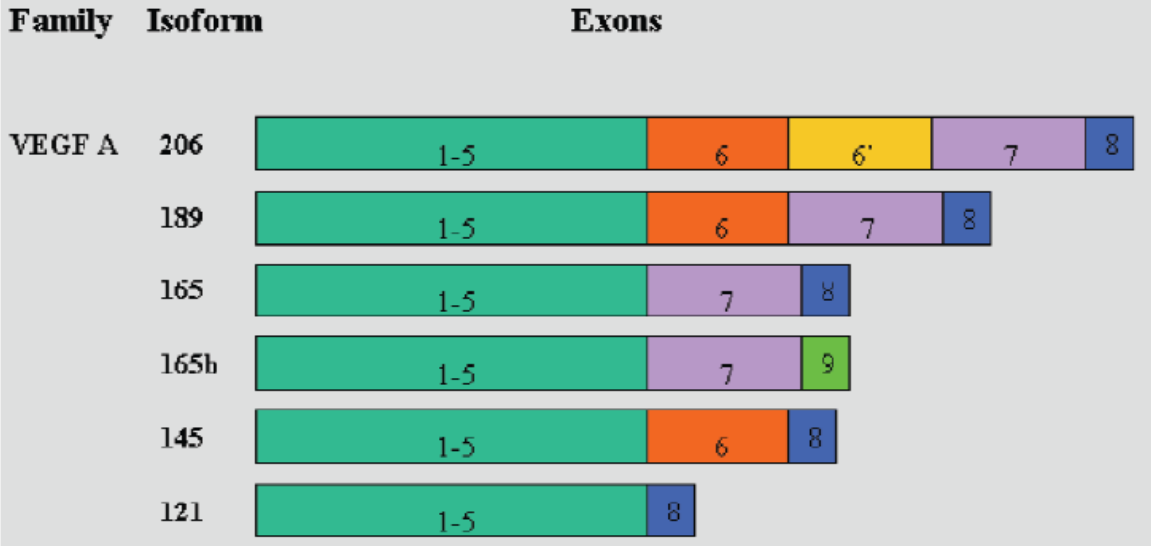

Figure 1.13. VEGF-A isoforms [92].

VEGF Receptors

VEGF binds to three cell surface receptor tyrosine kinases: VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR) and VEGFR-3 (flt-4). VEGFR-1 and VEGFR-2 are primarily located on vascular endothelium while VEGFR-3 is mostly found on lymphatic endothelium. These receptors are structurally similar: all of them contain seven extracellular immnoglobin (Ig)-like domains, a transmembrane domain, a regulatory juxtamembrane domain, and a consensus tyrosine kinase domain interrupted by a kinase-insert domain. The second and third Ig-like domains function as the high-affinity VEGF

binding domain, whereas the first and fourth Ig-like domains regulate ligand binding and facilitate receptor dimerization, respectively [97-99].

VEGFR-1 has a molecular weight of 180 kDa and binds VEGF-A, VEGF-B and PlGF. The affinity of VEGFR-1 for VEGF is ten-fold higher than VEGFR-2 but its tyrosine kinase activity is ten-fold weaker than VEGFR-2 [92]. In the classical views, one of the major functions for VEGFR-1 is to act as a decoy receptor restricting VEGF to bind to VEGFR-2, which is more mitogenic [100]. VEGFR-1 is required for normal blood vessel development during embryogenesis and a VEGFR-1 knock-out is lethal in mice at embryonic day E8.5. The lethality was shown to be associated with an abnormal increase in the number of endothelial progenitors, which is the phenotype as VEGF hyperactivity, indicating a negative regulatory function of VEGFR-1 [101]. Supporting this, a modified form of VEGFR-1 without the tyrosine kinase domain was constructed and found to be compatible with normal vascular development and angiogenesis in transgenic mice [102]. A naturally occurring soluble form of VEGFR-1, called sVEGFR-1 or sFlt-1, is expressed from differential pre-mRNA splicing. sVEGFR-1 has the same ligand affinity as VEGFR-1, but is missing the transmembrane and intracellular domains [103, 104]. It binds to free VEGF and reduces its availability to VEGF receptors, which further suggests its relative, VEGFR-1, as a negative regulator for VEGF signaling. However, VEGFR-1 does mediate VEGF signaling in non-endothelial cells, especially those cells that only express VEGFR-1 as the VEGF receptor, such as monocytes and macrophages [105, 106]. A recent study showed that PlGF signaling mediated by VEGFR-1 in monocytes is associated with the inflammatory reactions [107]. Besides

monocytes, VEGFR-1 signaling is also believed to be important for endothelial progenitors and carcinoma cells.

VEGFR-2, a 230 kDa glycoprotein, is recognized as the primary mediator of VEGF signaling. It regulates endothelial cell proliferation, migration, differentiation, cell survival and vessel permeability and dilation. VEGFR-2 knock-out mice die between E8.5 and E9.5 due to deficiency in blood vessel formation [108], indicating that VEGFR-2 is also crucial for the functions of hematopoietic/endothelial progenitors. VEGFR-3, 170 kDa, binds to VEGF-C and VEGF-D. It is expressed in embryonic endothelial cells but postnatally becomes restricted to the lymphatic endothelium [109].

Apart from these three VEGF receptors, neurophilins (NRPs) can also act as cell surface receptor for VEGF, but in an isoform specific manner. NRP-1, originally identified on neuron cells as a receptor for class 3 semaphorines/collapsins family of neuronal guidance mediators [110], is also expressed on endothelial cells. It lacks the intracellular tyrosine domain and needs to associate VEGFR-1 [111] and/or VEGFR-2 [112] to transduce a signal. It is suggested that NRP-1, as a co-receptor, can form a receptor complex with VEGFR-2 to enhance the binding the signaling of VEGF165 and VEGFR-2 cannot sufficiently transducer the VEGF signaling without NRP-1 [113]. NRP-1 also binds VEGFR-1 forming a ligand-independent complex [111]. NRP-2, lacking an intracellular domain like NRP-1, can also bind to VEGF. It can bind VEGF121, VEGF145 and VEGF165, but NRP-1 cannot bind VEGF145. NRP-2 can also bind to PlGF and can interact with VEGFR-1 [114]. In addition to NRPs, heparin sulfate proteoglycans (HSPGs) can bind to the VEGF isoforms with the heparin binding domains, such as VEGF165 and VEGF189. HSPGs are abundant, highly conserved

components of the cell surface and the extracellular matrix of all cells and have been reported to play a critical role in modulating the differential biological activities of VEGF isoforms [115].

Figure 1.14 [116] demonstrates the binding of VEGF variants to the receptors. In summary, VEGF-A binds to VEGFR-1, VEGFR-2 and the receptor heterodimer; VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3. Notably, PlGF and VEGF-B exclusively bind to VEGFR-1 and VEGF-E exclusively binds to VEGFR-2, which is very useful in receptor specificity studies.

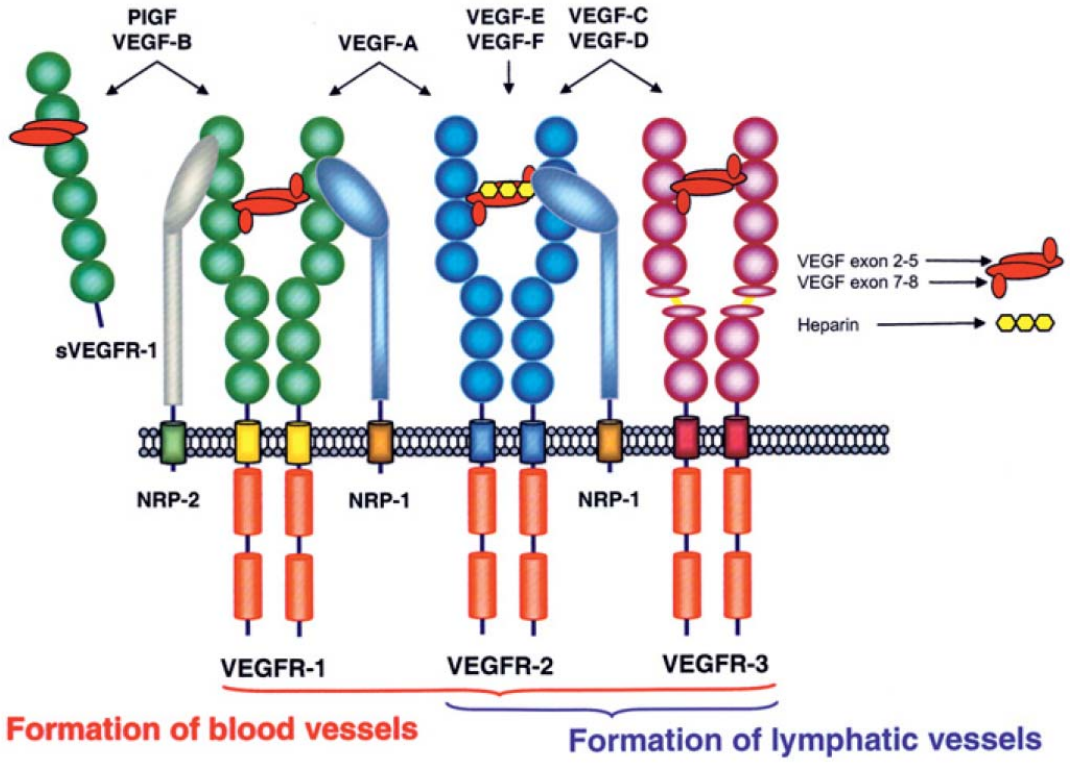

Figure 1.14. VEGF family ligands and their receptors [116].

VEGF Receptor Signaling

As mentioned above, VEGFR-2 is thought to be the major receptor for VEGF signaling in endothelial cells. Upon binding of VEGF, VEGFR-2 is activated by

autophosphorylation, and initiates a number of signaling cascades that induce cell proliferation, migration, survival and/or increase in endothelium permeability.

The cell proliferation induced by VEGFR-2 signaling typically involves MAPK pathways. Activation of VEGFR-2 recruits Grb-2 and activates it, which leads to the activation of Sos, then the activation of Ras, eventually the stimulation of Raf1/MEK/ERK signaling cascade [117]. Activated MAPK pathways will translocate to the nucleus and regulate the gene expression and cell proliferation. VEGFR-2 can also recruit PLCγ-1, and the activation of PLCγ-1 will induce phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis producing 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). The activation of PKC can result from the production of DAG, which further leads to the Ras-independent Raf activation and thus the stimulation of ERK activity [118]. The data demonstrating the requirement of PI3 kinase in the VEGFR-2-induced cell proliferation are conflicting, so the involvement of PI3 kinase is controversial [119, 120]. Cells expressing VEGFR-1 are unable to activate MAPK [121].

VEGF can act as a chemoattractant for endothelial cells so that VEGF signaling is believed to be involved in cell migration. Firstly, the signaling from activated VEGFR-2 can promote focal adhesion kinase (FAK) phosphorylation and recruit it to focal adhesions, together with paxillin and actin-anchoring proteins like talin or vinculin [122, 123]. Therefore the cytoskeleton organization is modified and cell migration is promoted. Secondly, the p38/MAPK pathway can be activated upon VEGF binding to VEGFR-2, and thus may play a role in cell migration and p38 inhibitors can decrease cell migration [124]. Thirdly, the PI3 kinase/Akt pathway can regulate the actin organization and cell migration [125]. Besides VEGFR-2, VEGFR-1 and NRPs have all been implicated in

VEGF-mediated cell migration and invasion [92]. However VEGFR-2 is considered to be the main mediator of cell migration. VEGFR-1 stimulates p38 phosphorylation and has no effect on endothelial cell migration [126].

PI3 kinase/Akt pathway plays an important role in the VEGF-induced cell survival. The phosphorylation of VEGFR-2 can lead to the activation of PI3 kinase and Akt/protein kinase B (PKB). Akt is an anti-apoptotic factor and is sufficient to promote cell survival. It has been reported that the inhibition of PI3 kinase abolished Akt activation and the VEGF-mediated cell survival was also blocked [127]. VE-cadherin and β-catenin can complex with VEGFR-2 and PI3 kinase and form a transient tetramer to promote cell survival [128]. The expression of some anti-apoptotic factors can also be induced by VEGF and contribute to cell survival, for instance, caspase inhibitors Bcl-1 and A1 [129] and IAP (apoptosis inhibitors) family proteins [130]. VEGFR-1 cannot associate with the VE-cadherin complex [128] and does not activate the PI3 kinase/Akt pathway [127], so that it is thought to not be involved in VEGF-induced cell survival.

Originally discovered as a vascular permeability factor, VEGF can also increase the vascular permeability. The administration of VEGF to endothelial cells is shortly followed by the formation of some specialized regions in the cell membrane that are highly permeable to macromolecules [131]. PI3 kinase and p38/MAPK have been suggested to be involved in the increase of membrane permeability [132]. In the established vessels, VEGF also regulates vascular permeability by affecting the components of tight, adherence and gap junctions, such as VE-cadherin, β-catenin and occludin [116]. Another aspect of this interaction is that endothelial NO synthase (eNOS)

can induce the activation of Akt, which further regulates the NO level and leads to vessel dilation and permeabilization [133, 134].

Figure 1.15 [92] summarizes VEGF signaling via VEGFR-2.

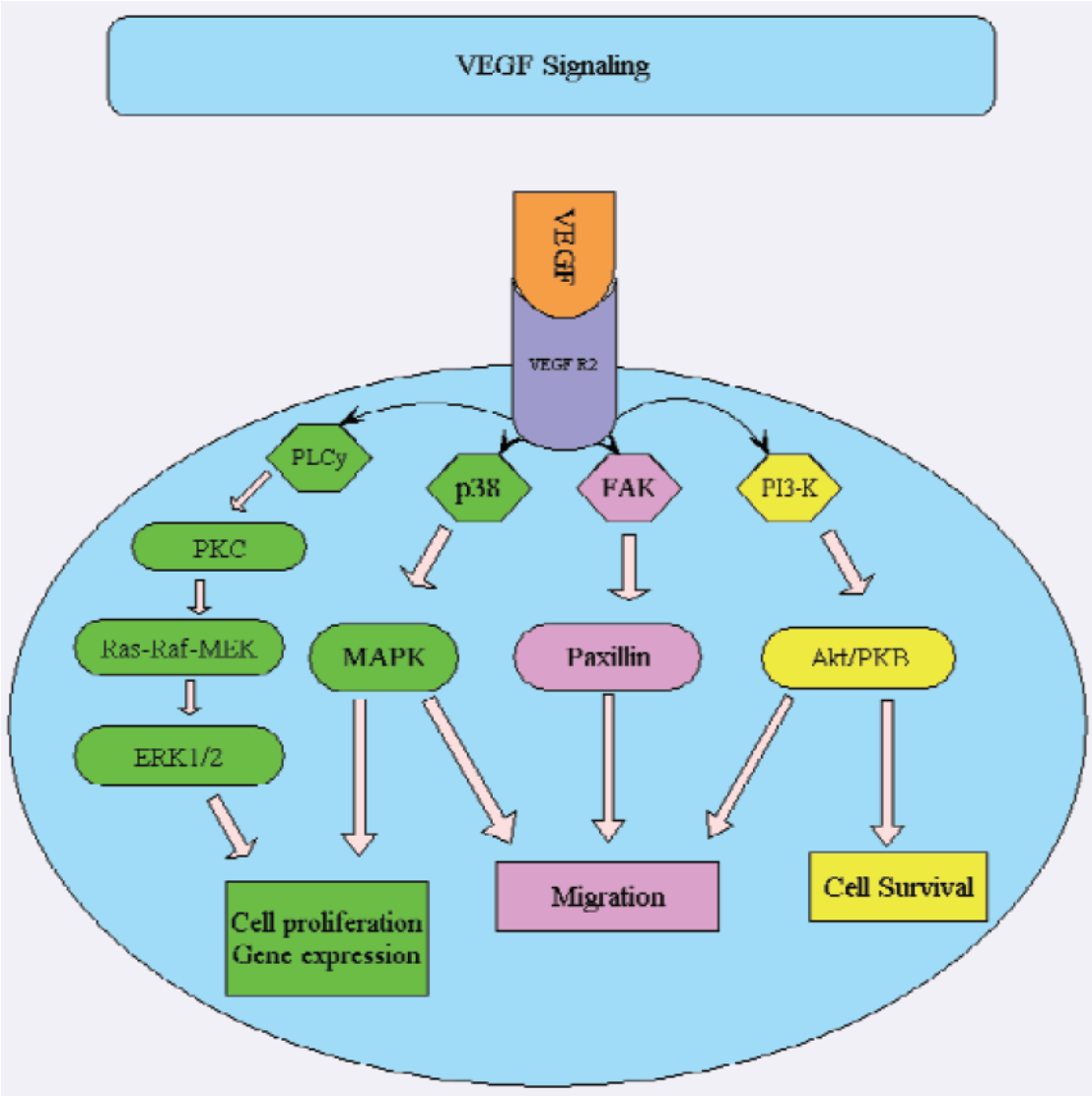

Figure 1.15. VEGF signaling via VEGFR-2 [92].

The Function of VEGF in Ocular Neovascularization

VEGF is thought to play a central role in retinal angiogenesis as supported by data from animal models and clinical investigation. VEGF is upregulated in the retina during neovascularization in animal models with ischemia-induced retinopathy [135-138], and

the VEGF mRNA is increased by three-fold within 12 hours of the onset of relative hypoxia and maintained for many days at higher levels until new vessels start to regress [136]. Patients with active PDR were found to have increased levels of aqueous and vitreous VEGF [139-145]. Higher levels of VEGF expression were also reported in epiretinal neovascular membranes and retinas from PDR patients [146, 147]. However, an interesting finding in the active PDR patients showed that there was a significant decrease in VEGF levels after panretinal laser photocoagulation treatment [140]. Further evidence supporting VEGF's major role in retinal neovascularization comes from VEGF inhibition studies. VEGF receptor chimeric proteins, neutralizing antibodies, and antisense oligonucleotides have successfully showed inhibition effects on neovascularization [148-151].

Based on the evidence, it is widely accepted that VEGF is very important and necessary for retinal neovascularization, but VEGF may not be sufficient for it. Repeated intraocular injections of VEGF or sustained intravitreous release of VEGF in primates results in severe changes to retinal vessels including dilation, leakage, and microaneurysms, but no apparent retinal neovascularization [152, 153]. When VEGF expression is driven by the retinal-specific rhodopsin promoter in the transgenic mice, the development of neovascularization was produced in the deep capillary bed of the retina, and high levels of VEGF expression can further cause retinal traction and detachment [154]. The new vessels grew from the deep capillary bed into the subretinal space. The close proximity of the deep capillary bed to the photoreceptor expressing VEGFs and differential susceptibility of the vascular beds might be an explanation for this vascular growth [155].

The role of VEGF in choroidal neovascularization (CNV) is less clear. Increased VEGF expression was found in fibroblasts and RPE cells of choroidal neovascular membranes surgically removed from patients [146, 156, 157]. And in the animal model of laser-induced CNV, it has been shown that VEGF mRNAs were upregulated in the neovascular lesions [158]. VEGF is thought to be necessary in CNV development because several specific VEGF signaling inhibitors have shown reduced CNV [159-161]. But VEGF is not a sufficient stimulator of CNV because increased expression of VEGF in photoreceptors or RPE cells does not lead to CNV [154, 162].

Basic Fibroblast Growth Factor (bFGF or FGF2)

FGF is a family of heparin-binding growth factors. bFGF has been localized in the adult retina. In the mouse model of ischemia-induced retinopathy, bFGF level is elevated during neovascularization [163]. In the animal model of laser-induced subretinal neovascularization, RPE cells were found to be stained with aFGF and bFGF [164]. In studies on clinical specimen, both elevated and non-significantly-changed levels of bFGF have been reported in the vitreous sample of PDR patients [165, 166], which argues against a major role in retinal neovascularization. Further evidence comes from animal models. In the ischemia-induced retinopathy or laser-induced CNV mouse model, transgenic mice deficient in bFGF developed the same amount of retinal or CNV as the wild-type mice, respectively, indicating bFGF expression may not be necessary in angiogenesis [167, 168]. It has been hypothesized that bFGF will manifest its angiogenic potential when there is cell injury. It is found that bFGF can get access to the extracellular compartment during photoreceptor damage and increased CNV can be stimulated [169].

Angiopoietins

Angiopoietins and their receptors (Tie receptors) are another endothelial-specific system that has been implicated in vascular growth and development. Current understanding about the Tie receptors is that Tie1 signaling is important for vascular integrity and Tie2 signaling is important in remodeling of the developing vessels by maximizing the interactions between endothelial and supporting cells [155]. The ligand for Tie1 has not been identified. Angiopoietin (Ang) 1 and 2 are ligands for Tie2 receptor. Ang1 binds with high affinity and initiates Tie2 phosphorylation and downstream signaling. Ang2 also binds with high affinity, but does not stimulate phosphorylation of Tie2. It looks like Ang2 is a naturally occurring antagonist for Ang1 and Tie2. The interaction of Ang1 and Tie2 is essential for the remodeling function of Tie2 on newly developing vessels. And it has been hypothesized that Ang2 might provide a key destabilizing signal involved in initiating angiogenic remodeling. The Ang2 blockade of Tie2 signaling can disrupt "stabilizing" inputs to ECs, making ECs more responsive to VEGF and thereby stimulating angiogenesis. But when there is no VEGF present, those ECs are prone to apoptosis and the "destabilized" vessels regress [170].

Ang2 mRNA levels have been reported to increase in normal and pathological retinal angiogenesis [171-174]. It has been shown that Ang2 can stimulate a significant upregulation of proteinases in EC [174] that may be important for cell migration during retinal neovascularization.

Platelet-Derived Growth Factor (PDGF)

PDGF, a dimer protein, a potent mitogen and a chemoattractant, has been implicated in angiogenesis. Similar to VEGF, PDGF is another growth factor that is elevated after hypoxia [65]. Recent findings about PDGF include: increased levels of

PDGF-AB was reported in vitreous samples of PDR patients [175]; overexpression of PDGF-B in transgenic mice leads to proliferation of endothelial cells, pericytes and glial cells resulting in traction retinal detachment [176-179]. It has been proposed that PDGF may act in concert with VEGF in ischemic retinopathy [176-178].

Integrins

Integrins are a family of transmembrane proteins that are the major cell surface receptors responsible for the attachment of cells to the extracellular matrix. Structurally, integrins are heterodimeric receptors composed of two subunits, α and β. More than 20 different integrins are formed from the combination of 18 known α subunits and 8 known β subunits. Each integrin binds to its own corresponding extracellular matrix (ECM) and/or cell surface ligand. These include structural ECM proteins, such as collagens, fibronectins, and laminins, as well as provisional ECM proteins that are deposited during tissue remodeling and thrombotic events [180].The first integrin-binding site to be identified was the sequence Arg-Gly-Asp, which is recognized by several integrins. However, other integrins bind to other distinct peptide sequences. While integrins are one of the most essential cell surface components in the body and are present in almost all tissues, no cell expresses all integrins. Indeed the particular integrin types expressed are dependent on the ECM ligands present within the local microenvironment. Even on a given cell type, the specific integrins expressed are also altered to match the concurrent changes within the local ECM. So the expression of integrin is spatially and temporally regulated.

The integrins also function as an anchor for the cytoskeleton. The interaction between the cytoskeleton and the extracellular matrix is responsible for the stability of cell-matrix junctions. There are two categories of cell-matrix junctions: focal adhesion

and hemidesmosome. In focal adhesions the cytoplasmic domains of the β subunits of integrins associate with bundles of actin filaments to anchor the actin cytoskeleton at the cell-matrix junctions. While in hemidesmosome integrins interact with intermediate filaments instead of actin. Hemidesmosome is mostly found in the anchorage of epithelial cells to the basal lamina.

Integrin Signaling

Unlike many cell surface receptors that contain tyrosine kinases, integrins do not contain intrinsic tyrosine kinase activity. Upon ligand binding, the integrins undergo a conformational change into its activation state. The change in activation has been assessed by showing evidence of polymerization, clustering, or the surface exposure of different antibody binding epitopes [181]. Since the cytoplasmic domains of the integrins can bind constitutively to cytoskeletal components such as talin, the conformational change and activation of integrins can result in changes in cytoskeletal protein functions, which will lead to major changes in cell shape and locomotion. On the other hand the activation of integrins can initiate a series of signaling transductions, with the involvement and assembly of a variety of signaling molecules.

A non-receptor protein tyrosine kinase called FAK (focal adhesion kinase) plays a key role in integrin signaling. FAK is localized at the focal adhesion and is rapidly tyrosine auto-phosphorylated following ligand binding by integrins. Besides FAK, members of the Src family or non-receptor protein tyrosine kinases also associate with focal adhesion and are involved in integrin signaling. Src and FAK probably interact with each other, resulting from the binding of the Src SH2 domain to the auto-phosphorylated sites of FAK. Src then phosphorylates additional sites on FAK. In addition to Src, the binding sites for SH2 domain created during FAK phosphorylation are also taken

advantage of by other downstream molecules, for instance, PI-3 kinase and the Grb2-Sos complex. These signaling molecules can form multicomponent signaling complexes that recruit and include small GTPase proteins such as Ras, Rho, Rac. Their involvement and activation will further lead to the activation of a number of signaling cascades. Figure 1.16 [180] demonstrates the integrin signaling via the Akt, ERK and JNK pathways. These signals collaborate to regulate cellular proliferation, migration and survival. And also, many small GTPases like Rho and Rac play critical roles in cytoskeletal remodeling events [180].

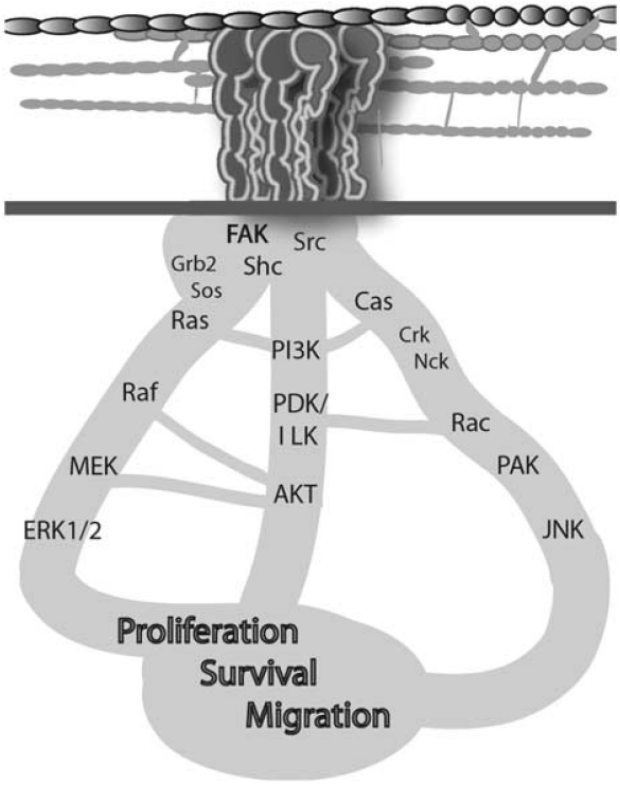

Figure 1.16. The activation of integrins can lead to the signal transduction in a number of pathways. [180].

As mentioned above, integrins need to be activated to serve as a signaling molecule. The activation involves a conformational change that results in an increase in ligand-binding affinity. Proposed in the current model, the inactive form of integrins are

in a folded conformation in which the ligand-binding domain is adjacent to the membrane. When activated, the affinity for the ligand is increased, and ligand occupancy stabilizes the extended conformation of the integrin [182]. Simultaneously, the associated topological change in the transmembrane and cytoplasmic domains makes them separate and bind to intracellular signaling molecules to initiate downstream pathways [182]. According to this model, the conformational change in integrins that induces signaling is the same as the one that is induced by activation. And this activation state can be promoted by both extracellular ligands (so-called "outside-in" signaling) and intracellular signaling molecules ("inside-out" signaling) [182]. The outside-in signaling is usually triggered by ECM ligands and the inside-out signaling molecules are usually the effectors of the activation of growth factor receptors. The ECM (local determiner) and growth factors (systemic and local determiner) can work synergically to enhance the signaling outcome induced by specific integrins in a given cell. Under certain circumstances it is not sufficient to promote cell survival and proliferation until both proper ECM and growth factors are both present.

The activation of integrins, especially those involving the interaction with growth factor receptors, usually occur in lipid-raft microdomains, where cholesterol and glycosphingolipids [183] and intracellular signaling molecules like Src family kinases [184] are relatively concentrated in the cell membrane. These lipid-raft microdomains are distinct from the surrounding membrane in that they restrict the diffusion of the contents. It is suggested that the lipid-raft has other functions [182]. First, they could serve as a physical concentration of pre-assembled molecules for signaling upstream or downstream of the integrin, and the signaling inhibitory molecules could be excluded. Second,

different integrin pools could be separated so that their own distinct function could be better performed. Third, the lipid-raft may also facilitate and/or maintain integrin activation. In addition to help from concentrated pre-assembled molecules, the altered membrane structure, due to the distinct chemical characteristics in the lipid-raft, may favor conformational equilibrium between the inactive and the active forms. It is also proposed that the active integrins might help to generate the lipid-rafts in other models [185].

The integrins can regulate the signaling of growth factor receptors. First the phosphorylation state of the growth factor receptors can be regulated. One example is the interaction between αvβ3 and the epidermal growth factor receptor (EGFR) on human endothelial cells. The adhesion to the ECM mediated by integrin can lead to a low phosphorylated state within the cell, resulting in the phosphorylation of four tyrosine residues but not on the fifth tyrosine which is only phosphorylated by EGF binding. This phosphorylated state is lower than in high concentration of EGF but ECM attachment doesn't occur. This low phosphorylated state is sufficient to induce cell survival but not proliferation. However, if only low concentrations of EGF are present, the ECM attachment can promote the phosphorylation similar to high concentrations of EGF alone [182]. Thus the phosphorylation of EGFR on endothelial cells is not only regulated by ligand binding, but also regulated by integrins. The regulation on growth factor receptors can also occur when integrins interfere with the receptor expression.

As for the inside-out signaling, the activation of growth factor receptors is usually the source of signaling. Integrins can be regulated by growth factor receptors in many aspects and cell behavior can be altered. The integrin expression level can be altered, for

instance, the expression level of a number of integrins on endothelial cells are increased by angiogenic growth factors such as FGF-2 [186]. The phosphorylated state of integrins can also be regulated by growth factor receptors. One example is the laminin receptor α6β4, an essential component in the hemidesmosomes, influences epidermal cell attachment to the underlying basal lamina. EGFR can induce the phosphorylation of the cytoplasmic domain of β4 subunit. This results in the cytoplasmic recruitment of Shc, and the activation MAPK and PI3K. More importantly, the change in the phosphorylation state leads to release of the integrin from its ligand, thus the hemidesmosome disassembles, which is a required step for cell proliferation and/or migration [187]. Besides the phosphorylation state, growth factor receptors can also alter the activation state of integrins. For example, it has been shown that VEGF can activate αvβ3 on human umbilical vein endothelial cells, thus the adhesion to ECM is promoted and cell migration follows [188].

Relationships between Integrin and Other Growth Factor Receptors in Angiogenesis

Among the over 20 integrins that have been discovered to date, two of them, αvβ3 and αvβ5, are thought to be especially important for angiogenesis. These integrins are not seen on normal epithelial cells in skin, but are highly expressed on endothelial cells participating in angiogenesis [189]. Only αvβ3 was found in choroidal neovascular membranes from ARMD patients, while both αvβ3 and αvβ5 were found in epiretinal membranes from DR patients [190]. Therefore, retinal and choroidal neovascularization may differ in the integrin requirement. Inhibition studies on integrins further support this. Agents that bind αvβ3 and/or αvβ5 can suppress retinal neovascularization, even though

the effect is modest, but the inhibition of αvβ3 or αvβ5 has no significant effect on choroidal neovascularization [189].

Endothelial cells express at least eight different integrins including αvβ3 and αvβ5 [191], each of them having their own specific ligand. For example, collagen is a ligand for α2β1 while fibrin is a ligand for αvβ3, so that αvβ3 influences adhesion and signaling events of the endothelial cells bound to fibrin [192] but not of those bound to collagen [40, 193]. However, the endothelial cells will eventually become apoptotic when bound with collagen alone via α2β1. The unligated αvβ3 receptors seem to cluster on the cell membrane and colocalize with caspase activity, especially caspase 8 [194]. In addition to αvβ3, many other unligated integrins are likely to induce cell death, this is why integrins could be categorized as dependent receptors under a variety of circumstances.

αvβ3, expressed (although not exclusively) on endothelial cells, has been linked to many angiogenic signaling pathways via the interaction with receptors for a number of growth factors, such as VEGF, EGF, IGF-1, PDGF and insulin. Since VEGF and IGF-1 are the two most important growth factors involved in my dissertation work, I am focusing on the interaction between αvβ3 and VEGFR and IGF-1R.

VEGFR-2 activation by phosphorylation is promoted by αvβ3 [195]. αvβ3 and VEGFR-2 interact and the co-immunoprecipitation of these two receptors has been demonstrated. However VEGFR-2 does not co-immunoprecipitation with the β1 or β5 subunits. VEGFR-2 phosphorylation and mitogenicity are enhanced in cells plated on vitronectin, an αvβ3 ligand, compared with cells plated on fibronectin, an α5β1 ligand, or collagen, an α2β1 ligand; further demonstrating a functional relationship between VEGR-2 and αvβ3. Cell adhesion, migration, soluble ligand binding, and adenovirus gene

transfer mediated by αvβ3 are all enhanced by VEGFR-2 signaling. An anti-β3 integrin antibody reduces VEGFR-2 phosphorylation and PI3 kinase activity suggesting that VEGFR-2 signaling initiated by αvβ3 occurs through the PI3 kinase pathway.

Another molecule, p66 Shc (Src homology 2 domain containing), has been shown to play a key role in the VEGF-αvβ3 interplay during tumor growth and vascularization [196]. The activation state of αvβ3 integrin has a critical function in *in vivo* tumor growth by influencing VEGF expression. By using a non-activable β3, a S752P mutant that cannot cluster, it was found that the stimulation of VEGF expression also depends on αvβ3 clustering. The recruitment of p66 Shc and phosphorylation of β3-associated p66 Shc are enhanced following αvβ3 clustering. The recruitment is not sufficient for αvβ3-mediated effects on VEGF production and tumor vascularization but the phosphorylation is necessary, in that a dominant-negative form of p66 Shc, which is phosphorylation-defective, completely abolished integrin-induced VEGF expression.

IGF-1 is a classic endocrine hormone and systemically synthesized in liver and transported to the peripheral tissues stimulating growth. In addition, IGF-1 is also synthesized locally in peripheral tissue to promote growth in an autocrine/paracrine manner. Similar to VEGF and other growth factors, the extracellular environment contributes to influence the outcome of the hormone signaling. It has been shown that many ECM proteins, such as collagen type I and type IV, fibronectin, thrombospondin, and osteopontin, can modulate the response of various cell types to IGF-1 stimulation via their integrin receptors [181]. The interactions between αvβ3 and IGF-1 on vascular smooth muscle cells (SMC) have been illustrated in great detail and can be used as a good example of how growth factors and integrin signaling influence each other.

When IGF-1 binds to the IGF1-R, IGF1-R will auto-transphosphorylate its two β subunits, and further recruit signaling molecules such as insulin receptor substrate-1 (IRS-1) and Shc, which can transduce the singling into corresponding cascades, such as the PI3K and MAPK pathways. Despite kinases, phosphatases also participate in the signaling modulation. Phosphatases induce dephosphorylation reactions, which can result in either activation or inactivation of signaling molecules. One phosphatase, Src homology 2 containing tyrosine phosphatase (SHP-2), normally transfers to IGF-1R 20 minutes after IGF-1 stimulation, resulting in a decrease in the phosphorylation level of the receptor and subsequent attenuation of MAKP and PI3K activation [181]. However, a premature transfer at 5 minutes and premature attenuation has been found when the ligand occupancy of αvβ3 is blocked [197]. So obviously the properly liganded and activated αvβ3 is a necessary partner in IGF-1R signaling.

Normally when IGF1-R and αvβ3 are activated after ligand binding, SHP-2 will transfer to the phosphorylated β3 subunit first. An adaptor protein, DOK-1, facilitates the transfer. DOK-1 is phosphorylated after IGF-1 stimulation, and the YXXL motifs within its C-terminus domain become capable of binding to SHP-2 via SH-2 domains [198]. Also, DOK-1 contains a phosphotyrosine binding (PTB) domain, which allows it to bind to β3 at a tyrosine that is phosphorylated after αvβ3 activation [199]. Thus DOK-1 mediates SHP-2/β3 association. If the transfer of SHP-2 to β3 is impaired for any reason, SHP-2 will be aberrantly transfer to IGF-1R instead and the premature dephosphorylation of IGF-1R occurs [181].

One SHP substrate, SHPS-1, becomes phosphorylated after IGF-1R activation. It is a single chain transmembrane protein and SHP-2 can bind to it via SH-2 domain. The

transfer of SHP-2 from β3 to phosphorylated SHPS-1 is a necessary step to maintain optimal MAPK and PI3K activation [200]. SHPS-1 also recruits Shc to form a complex that is critical for MAPK and PI3K activation. SHP-2 can activate a Src family kinase via SH-2 domain binding, so that this Src family kinase is recruited to SHPS-1 and phosphorylates Shc in the complex [181]. SHP-2 is further transferred to the appropriate downstream signaling molecules to maintain MAPK and PI3K activation.

αvβ3 has several ECM ligands, such as osteopontin, thrombospondin and vitronectin. For αvβ3 on SMC, the major ECM ligand is vitronectin. The heparin binding domain and RGD (arginine-glycine-asparginine) sequence can both function as the αvβ3 binding site. It is believed that the heparin binding domain is the binding site triggering β3 activation, in that the exposure of cells with the heparin binding domain peptide results in αvβ3 phosphorylation and recruitment of SHP-2 to the plasma membrane [201]. Contrarily, binding of β3 to the RGD sequence has been found to induce the cleavage of β3, thus also the premature recruitment of SHP-2 to IGF-1R and the premature IGF-1R dephosphorylation [202].

Similar to the interaction between integrins and other receptors, it is believed that αvβ3 and IGF1-R signaling occurs within a restricted compartment on the membrane. Integrin-associated protein (IAP) facilitates the formation of this compartment. After IGF-1 exposure, IAP is translocated to the regions where αvβ3 resides [181]. More importantly, IAP can induce an increase in the affinity of αvβ3 for its ligands [203]. The extracellular domain of IAP can associate with SHPS-1 and an antibody disrupting this association prevents IGF-1 stimulation of SHPS-1 phosphorylation and SHP-2 transfer to

SHPS-2 [204]. Therefore, the clustering of αvβ3 and the assembly of a signaling complex involving SHPS-1 may be a crucial in αvβ3 and IGF-1R signaling.

Pigment Epithelium-Derived Factor (PEDF)

The vasculature is normally quiescent under physiological conditions, since there is a balance between the pro-angiogenic and anti-angiogenic factors. Angiogenesis is initiated when there is increase in pro-angiogenic factors and/or decrease in anti-angiogenic factors. PEDF is one of the naturally occurring anti-angiogenic factors.

In the mouse model of retinopathy, it has been shown that hyperoxia results in a decline of VEGF levels with a concomitant expression of PEDF, and the relative hypoxia led to downregulation of PEDF during the angiogenesis process [205]. Systemic or intravitreal administration of PEDF [206, 207] and gene transfer with adenoviral vectors expressing PEDF [176-179] have been reported to decrease the ocular neovascularization levels, In the clinical studies, The vitreous levels of PEDF from PDR patients were found to be lower than normal [208], and the immunochemical staining of PEDF on retinas from PDR patients are much less intense compared with non-PDR [208]. All of these evidence supports that PEDF, an anti-angiogenic factor, may be involved in the suppression of retinopathies.

Insulin-Like Growth Factor (IGF)-1

The discovery of a role of growth hormone (GH)/IGF-1 in DR can be traced back to 1950s. The regression of retinal neovascularization was seen after pituitary infarction [209], and pituitary ablation was even used as a therapeutic method for PDR. More recently, in several studies in patients with PDR, elevated serum and vitreous levels of IGF-1 have been associated with retinal neovascularization [210-212].

In a GH inhibition study, retinal neovascularization was suppressed in transgenic mice expressing a GH antagonist gene and normal mice treated with an inhibitor of GH secretion [213]. This inhibition of neovascularization could be reversed by exogenous administration of IGF-1. IGF-1 also plays a necessary role in normal retinal vascular development. In IGF-1 knockout mice, normal development of the retinal vasculature was arrested despite the presence of VEGF [214]. This also supports the idea that VEGF alone is not sufficient for the development of retinal vessels. Clinically it has been found that the development of ROP in premature infants was strongly associated with a prolonged period of low levels of IGF-1 [214]. This suggests that the critical role IGF-1 plays during normal retina vascular development. Lack of IGF-1 in the early neonatal period leads to the development of avascular retina, and later the proliferative phase of ROP [155]. The function of IGF-1 in CNV is still not clear.

The IGF system includes the IGF-1, IGF-2, the IGF-1 receptor (IGF-1R), and IGF binding proteins (IGFBPs). IGF-1 can be expressed in the liver and utilized systemically as an endocrine, or can be expressed at peripherals and function in autocrine/paracrine mechanisms. The multiple physiologic and pathologic effects of IGF-1 are primarily mediated by IGF-1R, and are also modulated by complex interactions with IGFBPs, which themselves are also modulated at multiple levels.

IGF-1 and IGF-1R

IGFs are synthesized in almost all tissues and have important regulatory function on cell growth, differentiation, and transformation. IGF-1 is the product of the *IGF-1* gene, which has been mapped to chromosome 12 in humans and chromosome 10 in mice [215]. IGF-1 functions in both prenatal and postnatal development and exerts all of its known physiological effects through binding with IGF-1R. Circulating IGF-1 is

generated in the liver under the control of growth hormone [216], and bound with IGFBPs as the endocrine form in the circulation. The IGF-1 produced in other organs and tissues has a lower affinity for IGFBPs, representing autocrine and paracrine forms of IGF-1. The *IGF-1R* gene is located on chromosome 15 in human [215], and IGF-1R is expressed everywhere in the body. The mature receptor is a tetramer consisting of 2 extracellular α-chains and 2 intracellular β-chains with the intracellular tyrosine kinase domain. IGF-1R signaling involves autophosphorylation and subsequent tyrosine phosphorylation of Shc and insulin receptor substrate (IRS) -1, -2, -3, and -4. IRS serves as a docking protein and can activate multiple signaling pathways, including PI3K, Akt, and MAPK. The activation of these signaling pathways will then induces numerous biologic actions of IGF-1 (Figure 1.17 [216]).

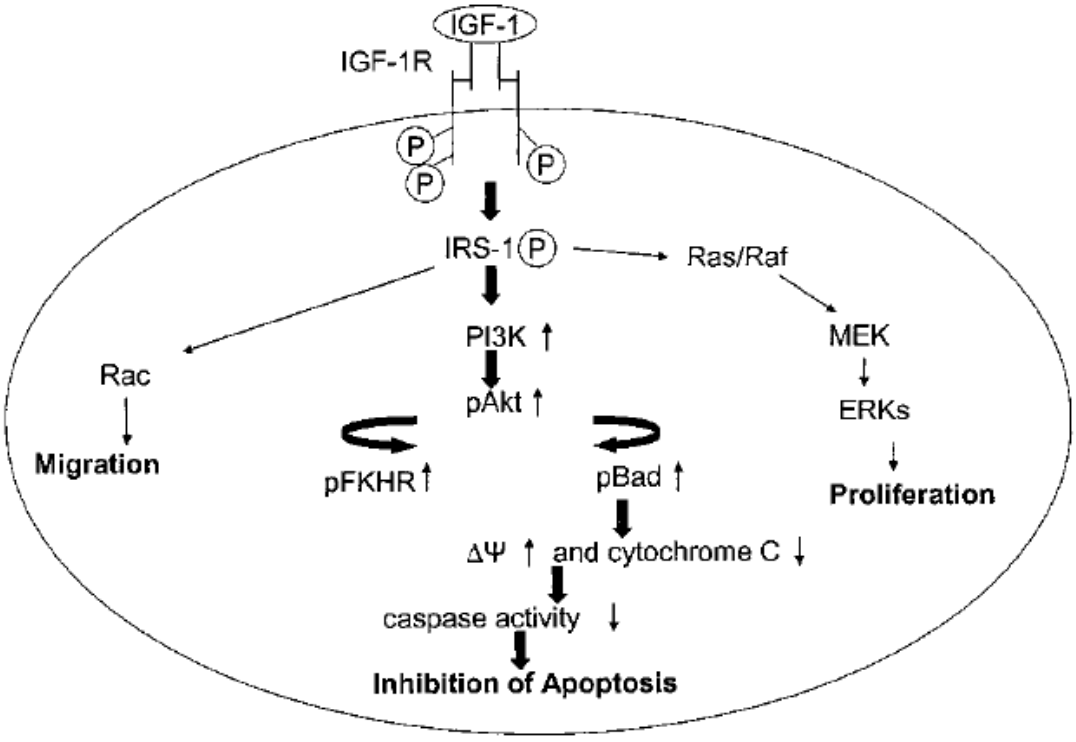

Figure 1.17. IGF-1 signaling transduction [216].

The expression of IGF-1 in ECs is low, but it is expressed both in macrovessel and microvessel ECs. IGF-1 stimulates vascular EC migration and tube formation. IGF-1 is important for promoting retinal angiogenesis, and an IGF-1R antagonist suppresses retinal neovascularization *in vivo* by inhibiting vascular endothelial growth factor (VEGF) signaling [217]. The effect of IGF-1 on ECs is mediated in different signaling pathways. For example, IGF-1-induced nuclear factor-κB (NF-κB) translocation requires both PI3K and extracellular-regulated kinase, while IGF-1-stimulated EC migration requires only PI3K activation [218]. And the IGF-1 effects are also regulated by endothelial nitric oxide synthase (eNOS) expression and VEGF signaling [217].

IGF-1 and IGF-1R are also expressed in vascular smooth muscle cells (VSMCs), and their expressions are regulated by several growth factors in different pathways. Thrombin and serum deprivation, tumor necrosis factor (TNF)-α, and estrogen downregulate IGF-1 mRNA and protein levels; reactive oxygen species (ROS) increases the levels; Ang2 and PDGF have been reported to both increase and decrease the levels. IGF-1 functions as a potent mitogen and antiapoptotic factor and migration stimulator for VSMCs [216]. As for the IGF-1R, its expression can be upregulated by Ang2 via the activation of NF-κB [219]; can be upregulated by fibroblast growth factor (FGF), mediated by the transcriptional factor STAT1, STAT 3 [220]; and the Ras-Raf-MAPK kinase pathway was shown to be required for both of the above growth factor. The cross-talk between IGF-1R and other receptors can also regulate IGF-1 function. For instance, blocking ligand occupancy of αVβ3 integrin receptor results in premature recruitment of SHP-2 to the IGF-1R receptor and reduces IGF-1 signaling [200].

IGFBPs and ALS

At least 6 IGFBPs have been well characterized, and they function as transporter proteins and as storage pools for IGF-1. The expression of IGFBPs is tissue- and developmental stage–specific, and the concentrations of IGFBPs in different body compartments are different. The functions of IGFBPs are regulated in multiple ways, such as phosphorylation, proteolysis, polymerization [221], and cell or matrix association [222] of the IGFBP. All IGFBPs have been shown to inhibit IGF-1 action, but IGFBP-1, -3, and -5 are also shown to stimulate IGF-1 action [223]. Some of IGFBPs' effects might be IGF-1 independent.

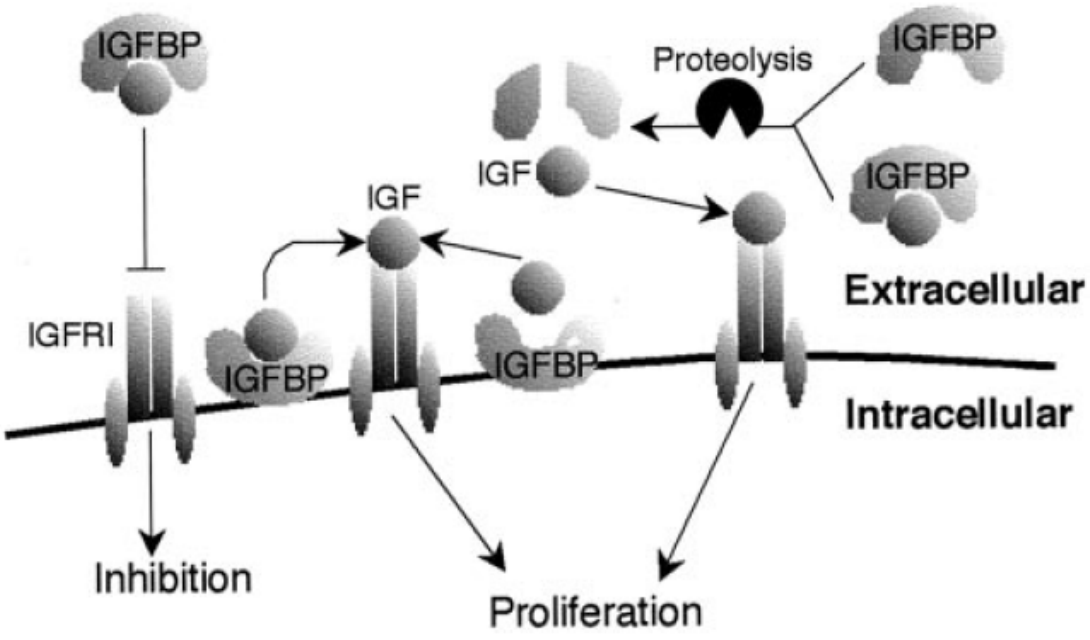

Figure 1.18. Proposed pathway of IGF-dependent IGFBP action [223].

The precursor forms of IGFBPs have secretary signal peptides and mature proteins are all found extracellularly. They all have a conserved amino-terminal domain, a conserved carboxyl-terminal domain and a non-conserved central domain. Both of the amino-terminal and carboxyl-terminal contribute to IGF binding [223], which implies

IGF-binding pocket structure. The major IGF transport function can be attributed to IGFBP-3, the most abundant circulating IGFBP. It carries 75% or more of serum IGF-1 and IGF-2 in heterotrimeric complexes that also contain the acid labile subunit (ALS) [224]. Free or binary-complexes (without ALS) are believed to exit the circulation rapidly, whereas ternary complexes appear to be essentially confined to the vascular compartment. In addition to their effects derived form circulation, IGFBPs also have local actions, both autocrine and paracrine. They have been documented to affect cell mobility and adhesion [225, 226], apoptosis and survival, and cell cycle [227-229]. I will concentrate on IGFBP-3 in this discussion.

IGFBP-3 have both potentiation and inhibition effect on IGF-1 actions. It is thought that IGFBP-3 inhibits IGF-1-mediated effects via its high-affinity sequestration of the IGF-1. But in contrast, preincubation of cells with IGFBP-3 before IGF-1 treatment can lead to the accumulation of cell-bound forms of IGFBP-3 with lowered affinity for IGF [230], which may enhance the presentation of IGF-1 to IGF-1R. But It was also found that cell-bound forms of IGFBP-3 could still attenuate IGF-1-mediated IGF-1R signaling [231]. It has also been reported, based on competitive ligand-binding studies, that IGFBP-3 can interact with IGF-1R, causing inhibition of IGF-1 binding to its receptor [232]. Therefore, the interaction of IGFBP-3 with IGF-1 and IGF-1R signaling system requires further study. Limited digestion from proteases on IGFBP-3 can release IGF-1 from the complex and control the bioavailability of IGF-1. These specific proteases include serine protease, cathepsins, and matrix metalloproteinases [223]. Proteolysis results in IGFBP-3 fragments with decrease affinity for IGF-1, but several studies have shown the inhibition of IGF actions by IGFBP-3 fragments with low affinity

for IGFs [223]. It is not clear whether this inhibition comes form IGF-1 sequestration or from its interaction with IGF-1R. IGFs themselves can also influence the production of IGFBPs and IGFBP-specific proteases, or regulate the activity of these proteases [223]. Figure 1.18 [223] summarizes proposed IGFBP actions that depend on binding of IGFs and modulation of IGF-1R.

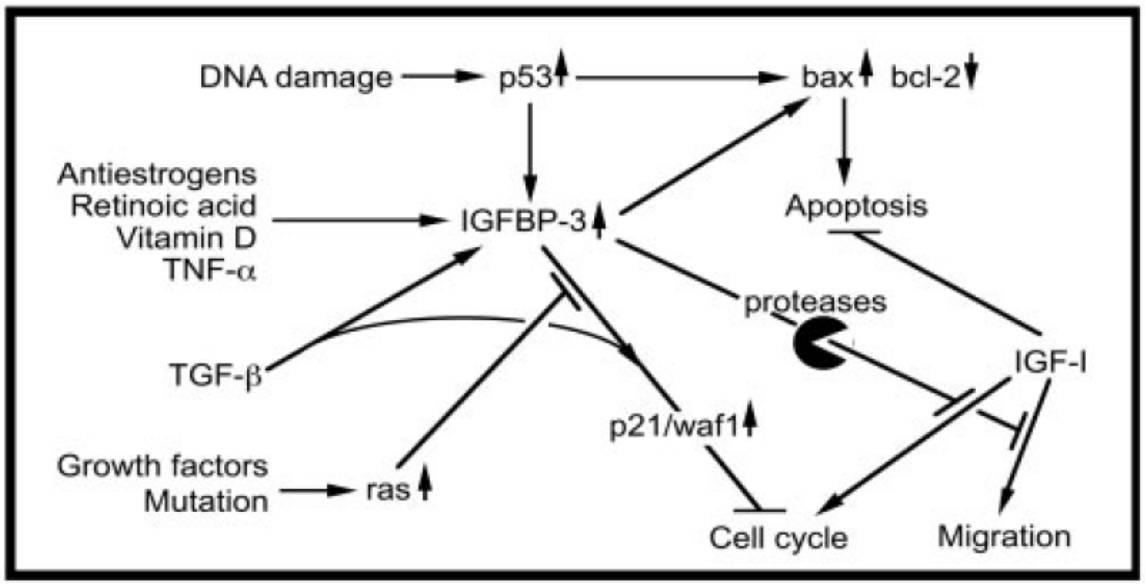

Figure 1.19. Overview of possible IGFBP-3 antiproliferation pathways [223].

IGFBPs also have their own intrinsic bioactivity, without modulating IGF actions, either in the absence of IGFs (IGF-independent effects) or in the presence of IGFs without triggering IGF-1R signaling (IGF-1R-independent effects). Recently there has been particular interest in IGFBP-3's function to induce apoptosis independently of inhibiting the survival functions of IGF-1 [233-236]. Several studies using human breast cancer cells have correlated the induction of IGFBP-3 mRNA and protein expression with growth-inhibitory effects of various antiproliferative agents including TGF-β, retinoic acid [237], antiestrogens [238], vitamin D analogs [239], and TNF-α [240]. IGFBP-3 expression is also upregulated by the transcription factor p53 in colon

carcinoma cells. And in the experiments using antisense IGFBP-3 or specific antibodies to sequester the IGFBP-3, the antiproliferative effects of some of these factors and be partially abrogated [223]. In addition, there is evidence showing that some proteolyzed forms of IGFBP-3 also have IGF-independent effect, especially some IGFBP-3 amino-terminal fragments [223], and they showed little or no affinity for IGFs. This supports the existence of IGF-independent bioactivity. Figure 1.19 [223] summarized some of the proposed pathways of IGFBP-3 independent functions.

IGFBP-3 has IGF-1 independent effects. Interactions of IGFBP-3 with known signaling pathways have been demonstrated. The type V receptor for TGF-β (TβRV) has been shown to be bound with IGFBP-3 relative specifically and may be involved in IGFBP-3 inhibitory signaling [241]. IGFBP-3 has been shown to stimulate the phosphorylation of TβRI of the signaling intermediates Smad2 and Smad3 [242], while TβRV signaling does not involve Smad phosphorylation. All-tans-retinoic acid (RA) is a potent inducer of IGFBP-3 in some cancer cells [223]. The growth-inhibitory effect of RA requires the presence of RA receptor (RAR)-β and can be blocked by retinoid X receptor (RXR)-specific retinoids. IGFBP-3 has been shown to inhibit RA signaling, possibly through enhancing RXR signaling [223]. IGFBP-3 may also interact with PI3-kinase pathway and MAPK pathway. LY294002, an inhibitor of PI3-kinase activity, could block the effects of IGFBP-3 [243]; MAPK/ERK pathway inhibitor, PD98059, can restore the inhibitory effect of IGFBP-3 on DNA synthesis, blocked in cells expressing oncogenic *ras*, in breast epithelial cells [244]. Recently it has been shown that IGFBP-3 strongly up-regulate signal transducer and activator of transcription 1(STAT1) mRNA in the process of chondrocyte differentiation, and phospho-STAT1 protein was shown to

increase and translocate to the nucleus, moreover, the antiproliferative effects of IGFBP-3 in these cells can be ablated in the presence of STAT1 antisense oligonucleotide [245].

The acid-labile subunit (ALS), together with IGFBP-3 and IGF-1, forms the ternary complex as the storage pool in the plasma. ALS is synthesized almost exclusively by the liver, and predominantly stimulated by GH [246]. Presence of ALS after birth is coincident with increased responsiveness to GH resulting from an increase in GH secretion and hepatic GF receptors. After puberty, ALS concentrations basically remain stable throughout adulthood [246]. *ALS* is a single copy gene, containing 2 exons and 1 intron. ALS has no affinity for free IGFs and very low affinity for uncomplexed IGFBP-3, and even its affinity for binary complex (IGF-1 + IGFBP-3) is 300-1000 fold lower that that of IGFBP-3 for IGFs [247]. The ability of ALS to form ternary complex is irreversibly destroyed under acidic conditions. IGFBP-3 and IGFBP5 can both associate with ALS, with the latter being much weaker [246]. The carboxyl-terminal domains of IGFBP-3 and IGFBP5 are important for binding. The association is proposed to happen within the negative-charged sialic acid on the glycan chains of ALS and an 18 amino acid positive-charged domain in IGFBPs [246].

Besides liver, ALS local synthesis may occur in kidney, developing bone, lactating mammary gland, thymus and lung [248, 249]. Their functions are to sequester IGFs into ternary complex. A GH-responsive element of the *ALS* gene transcriptional promoter was identified [250]. This sequence was called ALSGAS1 because of its resemblance with the consensus sequence for γ-interferon activated sequence (GAS). The effects of GH on the *ALS* gene are mediated by the JAK-STAT pathway [251, 252]: the tyrosine kinase JAK2 is recruited to the activated GH receptor complex and

phosphorylates signal transducers and activators of transcription (STAT)-5a and STAT-5b. After dimerization, STAT5 isomers translocate to the nucleus, and activate *ALS* gene transcription by binding to the ALSGAS1 element. The GH signaling pathway leading to increased *ALS* gene transcription is critically dependent on the activation of STAT5 isomers, and is independent of RAS activation.

One the of physiological significances of ALS is to extend the half-lives of IGFs from 10 min when in free form, and 30-90 min when in binary complexes, to more than 12 hours when in ternary complexes [253]. The other important role of ALS is to prevent the non-specific metabolic effects of the IGFs, given that serum IGF concentration is ~1000 fold that of insulin [246]. IGFs in ternary complexes cannot traverse capillary endothelia and activate the insulin receptor, whereas free IGFs and IGFs bound as binary complexes can do so. Incorporation of IGFs into ternary complexes therefore completely restrains the intrinsic insulin-like effects of the IGFs. Null ALS mouse shows significantly reduced circulating IGF-1 and IGFBP-3 concentrations [246], which proves that ALS is absolutely necessary for serum accumulation of both IGF-1 and IGFBP-3.

The Involvement of Insulin Receptor (IR) and IGF-2 in Angiogenesis

IGF-1 primarily binds to IGF-1R, and insulin primarily binds to IR, while IGF-2 can bind to both of the two receptors and its own IGF-2R, as shown in Figure 1.20 [254]. Regarding retinopathy, insulin and IGF-1 have gained more attention. Kondo *et a*t [255], using the Cre-Lox knockout system, found that (1) the retinas of mice develop normally in the absence of endothelial IR or IGF-1R. Presumably, sufficient growth factors (for example, VEGF) are present to facilitate normal development. (2) Under conditions of relative hypoxia and in the presence of endothelial IR/IGF-1R, VEGF, eNOS, and ET-1 are increased, leading to extra-retinal neovascularization. (3) Under conditions of relative

hypoxia and in the absence of endothelial IR or IGF-1R, VEGF, eNOS, and ET-1 are reduced, possibly due to impaired HIF-1 activation or reduced PI3K activity related to IG/IGF-1R [256]. Reduced neovascularization results from less IR/IGF-IR input. And in their experiments, the reduction of VEGF, eNOS, and ET-1 are reduced to a greater extend in IR knockout mouse than IGF-1R knockout mouse, which has brought more emphasis on IR function in the retinopathy, while traditionally IGF-1R is thought to be more important.

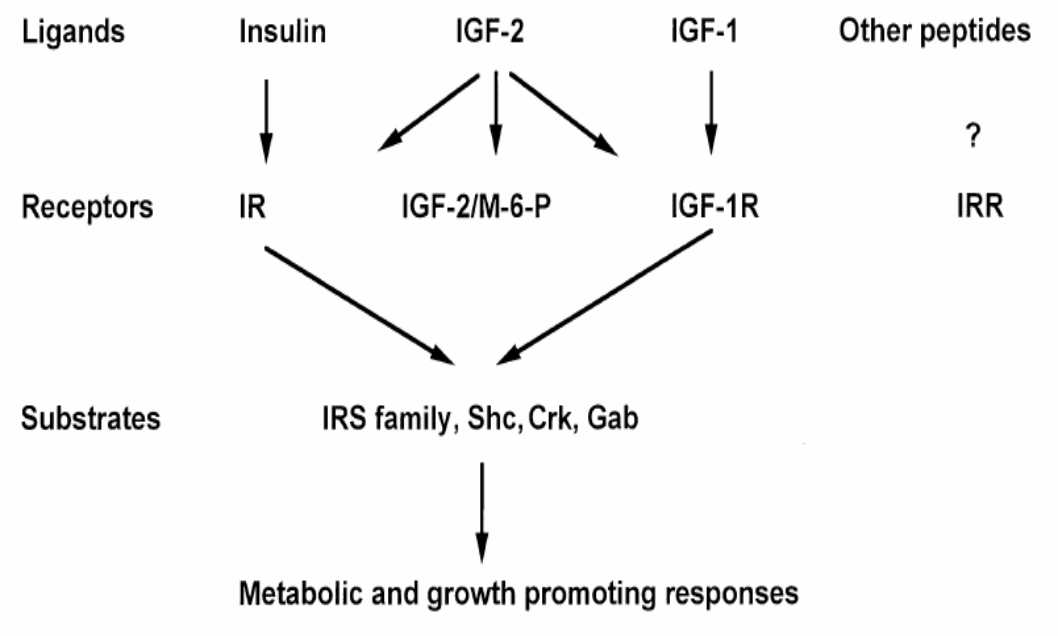

Figure 1.20. The crosstalk between IGF-1, IGF-2 and Insulin signalings [254].

RNA Silencing Technologies

The traditional method to inactive a gene is to create a gene knockout animal model. This process has its advantages, in that it entirely abolishes a gene expression, however, the disadvantages are that it is time consuming, expensive, labor-intensive, and subject to possible failure due to embryonic lethality [257]. RNA silencing technologies, which

inhibit gene expression at the RNA level, are valuable tools to inhibit the

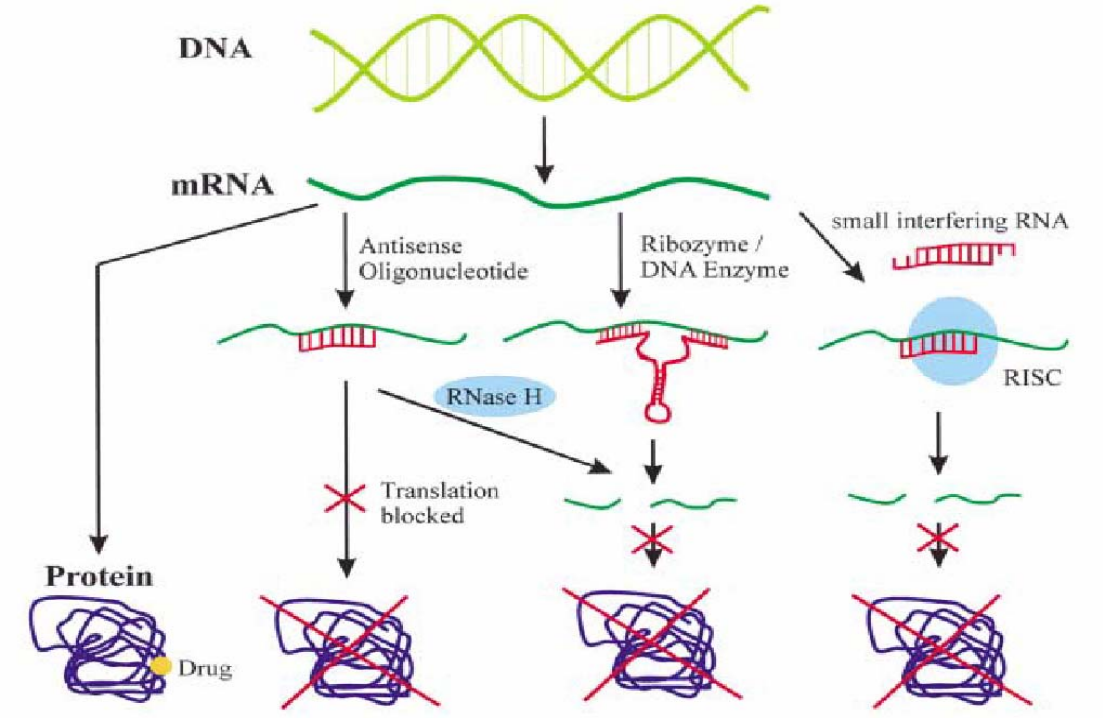

Figure 1.21. Overview of RNA silencing technologies [258].

expression of a target gene in a sequence-specific manner, and may be used for functional genomics, target validation and therapeutic purposes. Theoretically, RNA silencing could be used to cure any disease that is caused by the expression of a deleterious gene [258]. There are three common types of anti-mRNA strategies. Firstly, the use of single stranded antisense oligonucleotides; secondly, the triggering of RNA cleavage through catalytically active oligoribonucleotides referred to as ribozymes; and thirdly, RNA interference induced by small interfering RNA molecules. Figure 1.21 [258] basically summarized the mechanisms of these three kinds of antisense technologies. This scheme also demonstrates the difference between antisense approaches and conventional drugs, most of which bind to proteins and thereby modulate their function. In contrast, RNA silencing agents act at the mRNA level, preventing translation. Antisense-

oligonucleotides pair with their complementary mRNA, whereas ribozymes and DNA enzymes are catalytically active oligonucleotides that not only bind, but can also cleave, their target RNA. RNA interference is a highly efficient method of suppressing gene expression in mammalian cells by the use of 21–23-mer small interfering RNA (siRNA) molecules. These three RNA silencing methods are detailed below.

Antisense Oligonucleotides

The antisense oligonucleotides was first described by Zamecnik and Stephenson who used a 13-mer DNA to inhibit Rous sarcoma virus expression in infected chicken embryonic fibroblasts [259]. The antisense gene silencing naturally occurs in genomic imprinting, in which only one copy of a gene in the mammalian genome is expressed while the other is silenced. It could be the maternally inherited allele or the paternal inherited allele.

Antisense oligonucleotides are complementary to the target mRNA and are usually 15-20 nucleotides in length [258]. There are two major antisense mechanisms that have been proposed [258]. First, RNase H cleaves RNA in the RNA-RNA heteroduplex (or RNA:DNA heteroduplex for antisense DNA oligonucleotides), induced by binding of the antisense oligonucleotides. This results in rapid degradation of the cleaved mRNA products and a reduction in gene expression. Second, translation is arrested by steric blocking the ribosome by the binding of antisense oligonucleotides. When the target sequence is located within the 5’ terminus of a gene, the binding and assembly of the translation machinery can be prevented.

The first step in designing an antisense oligonucleotides is target selection and verification of target site accessibility. Computer programs, like Mfold, perform mRNA

secondary structure analysis. This analysis can generate several mRNA secondary structures centered on our target sequence. If the target is always contained within a stable stem in every structure, this target should be eliminated. In addition to this type of *in silico* analysis of RNA secondary structure, a number of *in vitro* methods have been developed to examine secondary structure in solution. One way is to directly probe the secondary structure of the target RNA with 1-cyclohexyl-(2-morpholinoethylo)cabodiimide metho-*p*-toluene sulfonate (CMCT) [260]. CMCT will mainly modify Us, and to a lesser extent Gs, in single-stranded regions of an RNA molecule. CMCT modification is followed by reverse transcription. Modification of Us and Gs will prevent read-through by reverse transcription, resulting in a pause or stop site at the modified position. When these modification/reverse transcription reaction products are separated on an appropriate electrophoresis gel next to DNA sequencing reactions of the target mRNA region, accessible regions of the target RNA are easily identified. The most sophisticated approach reported so far is to design DNA array to map an RNA for hybridization sites of oligonucleotides [261].

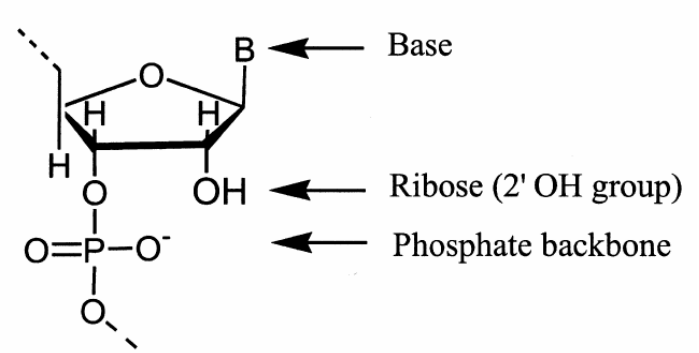

Figure 1.22. Modifications in antisense technology [258].

When designing antisense oligonucleotides, there are some points to consider. Four contiguous guanosine residues should be avoided due to the G-quartets formation

and CpG motifs should be avoided due to potential stimulation of the immune system. In addition, a BLAST search for each oligonucleotide sequence is required to avoid significant homology with other mRNAs that could cause unwanted gene silencing.

Unmodified oligonucleotides are rapidly degraded in biological fluids by nucleases. So one of the major challenges for antisense RNA approaches is the stabilization of RNA oligonucleotides. Chemical modifications of the bases and/or and phospho sugar backbone have been developed to increase resistance against RNase (Figure 1.22 [258]. The major representative of in the first generation modification is the Phosphorothioate (PS) oligonucleotides, in which one of the nonbridge oxygen atoms in the phosphodiester bond is replaced by sulfur [258]. The shortcomings include binding to certain proteins, such as heparin-binding proteins, and their slightly reduced affinity to the complementary RNA sequences [262]. In the second generation, most the emphasis was placed on the 2' hydroxyl group. 2'-O-methyl and 2'-O-methoxyl-ethyl RNA are the most common types of modifications [258]. However, RNase H cleavage can be somewhat reduced or even blocked with these types of modifications, possibly due to the steric blockade. One way to overcome this disadvantage is the gapmer technology [258], in which the 2'-modified nucleotides are placed only at the ends of antisense oligonucleotides. This protects the ends from degradation and a contiguous stretch of at least four or five non-2'-modified residues in the center are sufficient for the activation of RNase H. A variety of modified nucleotides have been developed in the third generation, the antisense oligonucleotides properties such as target affinity, nuclease resistance and pharmacokinetics have been improved [258]. The concept of conformational restriction has been used widely to help enhance binding affinity and biostability.

Ribozymes

Ribozymes, or RNA enzymes, are catalytic molecules that can catalyze the hydrolysis and phosphoryl exchange at the phosphodiester linkages within RNA resulting in cleavage of the RNA strand. There are two types of chemical reactions that are catalyzed during phosphate-group transfer by naturally occurring ribozymes: self-cleaving and self-splicing reactions. The ribozymes that perform self-cleaving reactions include hammerhead, hairpin, hepatitis delta virus (HDV) and *Neurospora* Varkud satellite (VS) ribozymes. They are usually small RNAs of tens of nucleotides in length. The ribozymes that perform self-splicing reactions include self-splicing introns and RNase P. They are much larger in size and usually hundreds of nucleotides in length.

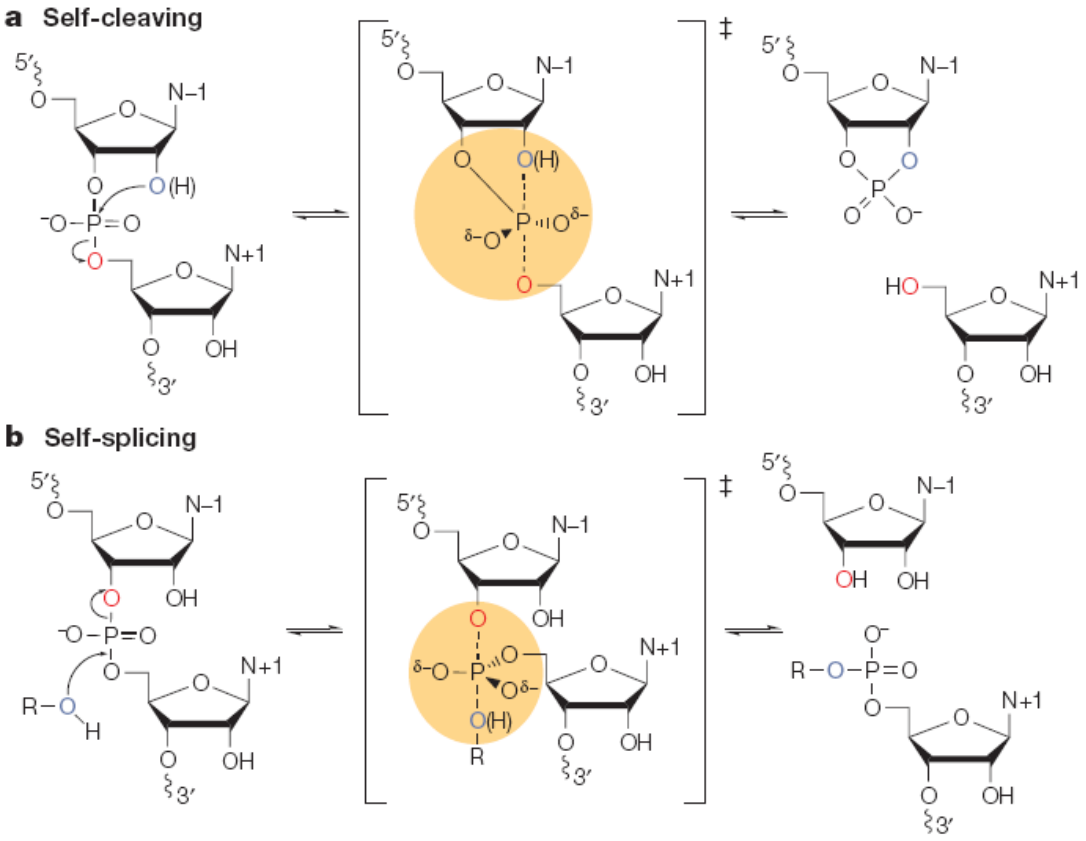

Figure 1.23. Self-cleaving and self-splicing reactions in ribozymes [263].

As shown in Figure 1.23 [263], in the self-cleaving reactions, the RNAs catalyze a reversible phosphodiester-cleavage reaction. The nucleophilic attack from the 2'-hydroxyl group results in 5'-hydroxyl and 2'-3'-cyclic phosphate termini. The bridging 5'-oxygen is the leaving group. While in the self-splicing reactions, an exogenous nucleophile attacks on the phosphorus generates a 5'-phosphate and a 3'-hydroxyl termini. The bridging 3'-oxygen is the leaving group. In the first steps of group I intron and group II intron self splicing and the RNase P-mediated cleavage of precursor of tRNAs, the exogenous nucleophiles are, respectively, the 3'-hydroxyl group of exogenous guanosine, the 2'-hydroxyl group of an adenosine in the intron, and the water. They are indicated by the ROH in Figure 1.23 b. The transition states are shown in brackets.

Self Splicing Introns

Self splicing introns can be divided into 2 classes based on the conserved secondary structure and splicing mechanisms: Group I and Group II. Group I is found in a variety of species, including prokaryotes and lower eukaryotes. Except for the *Tetrahymena* large rRNA group I intron, all other known group I introns require a single protein co-factor to provide a scaffold to hold the RNA in the catalytic reaction [264]. Group II introns are found within nuclear pre-mRNA and organelle pre-mRNA [265]. A spliceosome consisting of proteins and small nuclear RNAs (SnRNA) is formed in the catalytic reaction and high concentrations of magnesium and potassium are necessary [265].

The splicing action of both group I and group II introns consists of two similar consecutive transphosphoesterification reactions. In the first step, the 5'-end of the intron is attacked by an exogenous nucleophile, which is the 3'-hydroxyl group of exogenous

guanosine in group I introns, or the 2'-hydroxyl group of an adenosine in group II introns. This results in the cleavage at that site and the addition of the guanosine or adenosine to the 5'-end of the intron. In the second step, the oxygen in the 3'-hydroxyl group of the 3'-end of the up stream exon attacks the 3'-end of the intron. In group I introns, it is a guanosine at the 3'-end of the intron that is attacked. This cleaves the 3'-end of the intron, releasing the intron, and results in ligation of the upstream and downstream exons. Figure 1.24 [263] shows the secondary structure and self splicing steps of group I introns.

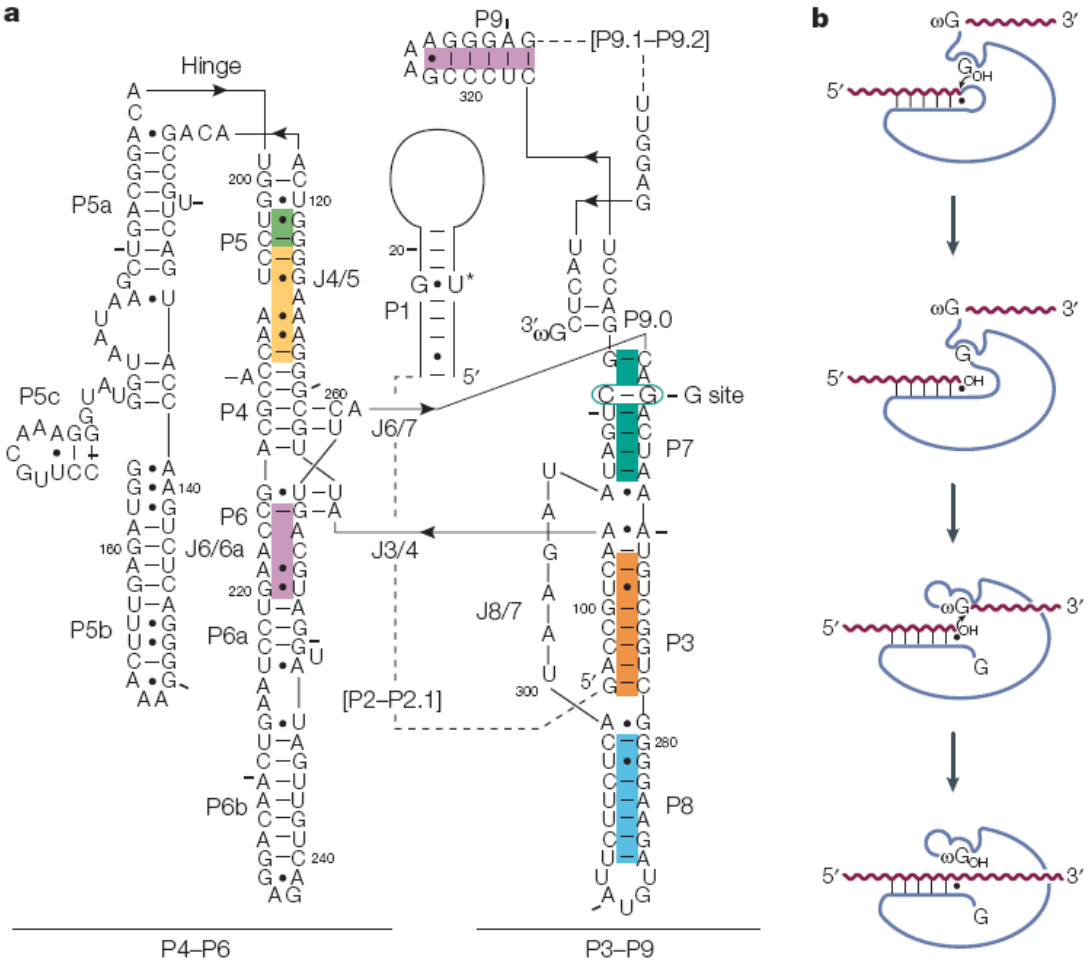

Figure 1.24. Secondary structure and self splicing steps in group I intron [263].

RNase P

RNase P is a ribonucleoprotein complex that removes the 5' leader sequence form precursor tRNAs (ptRNAs) via a hydrolysis reaction. It consists of a catalytic RNA subunit (M1 RNA in *E. coli*) and a protein subunit (C5 protein in *E. coli*) [266, 267]. *In vitro*, M1 RNA can cleave its ptRNA substrate without C5 protein, but the reaction requires high concentrations of Mg^{2+}. However, C5 protein can dramatically increase the rate the cleavage, even at low concentration of Mg^{2+} [268]. *In vivo*, C5 protein is required for RNase P activity and cell viability [266, 267]. Thus both the RNA subunit and the protein subunit are essential for RNAse P function. It has been proposed that C5 protein can facilitate the stabilization of the M1 RNA conformation and also enhance the enzyme and substrate interaction [269, 270].

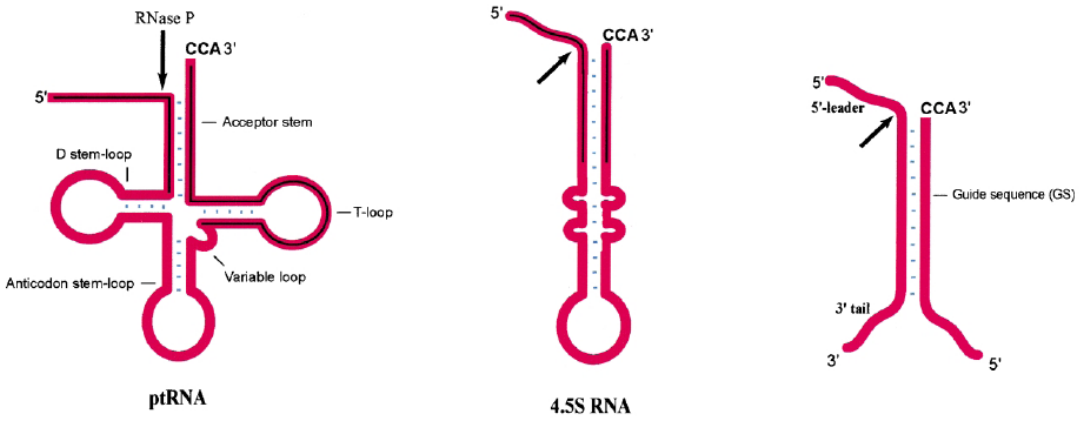

Figure 1.25. Secondary structures of natural and synthetic substrates for RNAse P[275].

All the natural substrates of RNAse P (ptRNAs, precursor of 3.5S RNA and several small RNAs [271-273] in *E. coli*) have a common feature in their secondary structure which includes a 5' leader sequence, and acceptor-stem-like structure and a 3'-CCA sequence. A synthetic external guide sequence (EGS) combined with a CCA sequence has been designed to base pair with a targeted sequence to form a structure very

similar to the natural substrates of RNase P. The M1 RNA from *E. coli* can cleave at this synthetic target site [274]. This EGS-based technology can be used to guide RNAse P to cleave a targeted sequence. Figure 1.25 shows the secondary structures of ptRNA and the 3.8s RNA and the hybridization of the EGS with the targeted sequence [275].

Hammerhead Ribozymes

The hammerhead ribozyme was the first small self-cleaving RNA to be discovered [276, 277], the first ribozyme to be crystallized [278, 279] and the smallest naturally occurring catalytic RNA identified so far. It was found in several plant virus satellite RNAs and is required for the rolling circle mechanism of virus replication [280]. The hammerhead ribozyme cleaves the multimeric transcripts of the circular RNA genome into single genome length strands.

Hammerhead ribozymes are approximately 30-90 bases in length and cleave RNA targets *in trans*. Annealing of the hammerhead ribozyme with the target sequence produces a structure consisting of three stems, a tetra-loop and a conserved catalytic core as shown in the Figure 1.26. Any mutation in the catalytic core will prevent catalytic cleavage. The catalytic core has two functions: it destabilizes the substrate strand by twisting it into a cleavable confirmation, and also binds the metal cofactor (Mg^{2+}) needed for catalysis [278]. The absolute requirement of the target sequence is a NUX cleavage site, where N is any nucleotide and X is any nucleotide except G. The targeting arms of the hammerhead ribozyme bind either side of the U of the NUX site forming stems I and III. GUC has been shown to be the most efficient cleavage site [281], followed by CUC, UUC and AUC. The advantages of hammerhead ribozymes include its small size, easy of cloning and packaging into viral delivery systems, and versatility in target site selection.

In the traditional view, the Mg^{2+} and water are both required in the transesterification reaction. The hydrated magnesium ion can help to provide an environment to facilitate the nucleophilic attack, in which the Mg^{2+} acts as a Lewis acid to coordinate directly with the 2'-hydroxyl and the 5'-leaving oxygen for activation of the nucleophile and for stabilization of the environment. It has been also reported that some monovalent cations (Li^+ and NH_4^+) at higher concentration can substitute for Mg^{2+} [282].

There is another kind of antisense agent called DNA enzyme, which is similar to the hammerhead ribozyme in structure and function but avoids the high susceptibility to nucleases that is common to ribozymes. The best studied DNA enzyme, named "10-23" [283], consists of a catalytic core of 15 nucleotide and two substrate recognition arms. It is highly sequence-specific and can cleave any junction between a purine and a pyrimidine, and its efficiency is similar to hammerhead ribozymes [283].

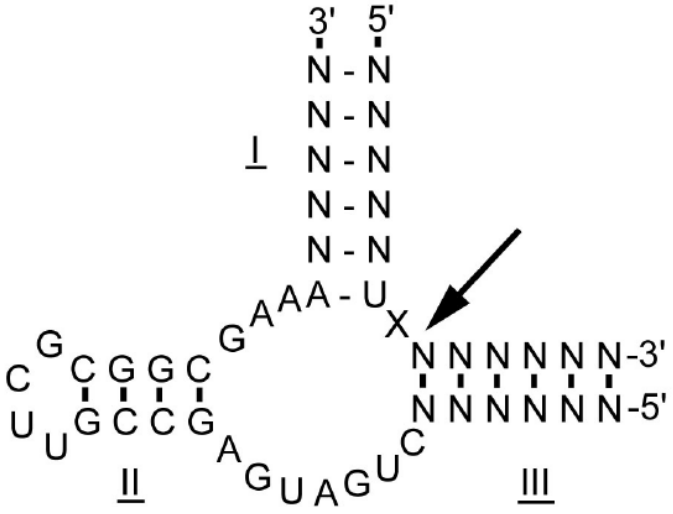

Figure 1.26. Structure of the hammerhead ribozyme.

Hairpin Ribozymes

Similar to the hammerhead ribozyme, the hairpin ribozymes was first derived from tobacco ring spot virus satellite RNA [284].When the hairpin ribozyme binds to the

substrate, a structure with 4 helices and 2 loops is formed. Helix 1 (6 base pairs) and helix 4 (4 base pairs) are where the hairpin hybridizes to the target RNA. In loop A, a BNGUC target sequence is required for cleavage, where B is G, C or U, and N is any nucleotide [285]. Figure 1.27 shows the structure of the binding complex of the hairpin ribozyme and its substrate.

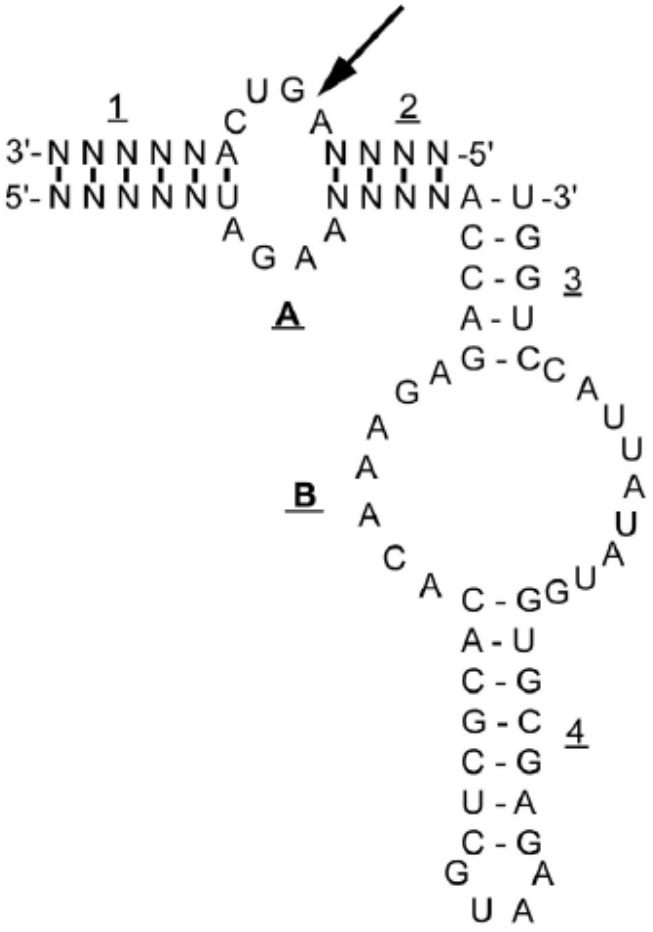

Figure 1.27. Structure of the hairpin ribozyme.

Hairpin and hammerhead ribozymes can also catalyze the ligation of the cleaved products, which is the reverse of the cleavage reaction. The ligation efficiency is much higher for the hairpin than the hammerhead. Another unique feature for the hairpin ribozyme is that it does not require metal ions as cofactors [282, 286].

Hepatitis Delta Virus (HDV) Ribozymes and Neurospora Varkud Satullite (VS) Ribozymes

HDV ribozymes and VS ribozymes also cleave the substrates via self-cleaving reactions. HDV ribozymes are derived from the genomic and the anti-genomic RNAs of HDV [287, 288]. Naturally, the HDV ribozyme cleaves its substrate during the rolling circle replication mechanism of the circular RNA genome, like other self cleaving ribozymes. The VS ribozyme was originated from the mitochondria of certain isolates of *Neurospora* [289]. The self cleaving reactions require a divalent cation but it has also been shown that monovalent cations are be sufficient for the ribozyme to catalyze proficiently [282].

RNA Interference

RNA interference (RNAi) is a naturally occurring process and is a potent sequence-specific mechanism for post-transcriptional gene silencing (PTGS). It was described early in C. elegans [290] and then found to exist throughout nature as an evolutionarily conserved mechanism in eukaryotic cells. RNAi has regulatory roles in gene expression, such as genomic imprinting, translation regulation, alternative splicing, X-chromosome inactivation and RNA editing [291]. In plants and lower organisms RNAi also protects the genome from viruses and insertion of rogue genetic elements, like transposons [292].

Figure 1.28 [293] shows the RNA interference pathways. Long double-stranded (ds)RNA is cleaved by Dicer, an RNase III family member, into short interfering RNAs (siRNAs) in an ATP-dependent reaction. These siRNAs contain an approximately 22-nucleotide (nt) duplexed region and 2-nt unpaired and unphosphorylated 3'-ends. The 5'-end is phosphorylated, which is a crucial requirement for further reactions. In fact, if the

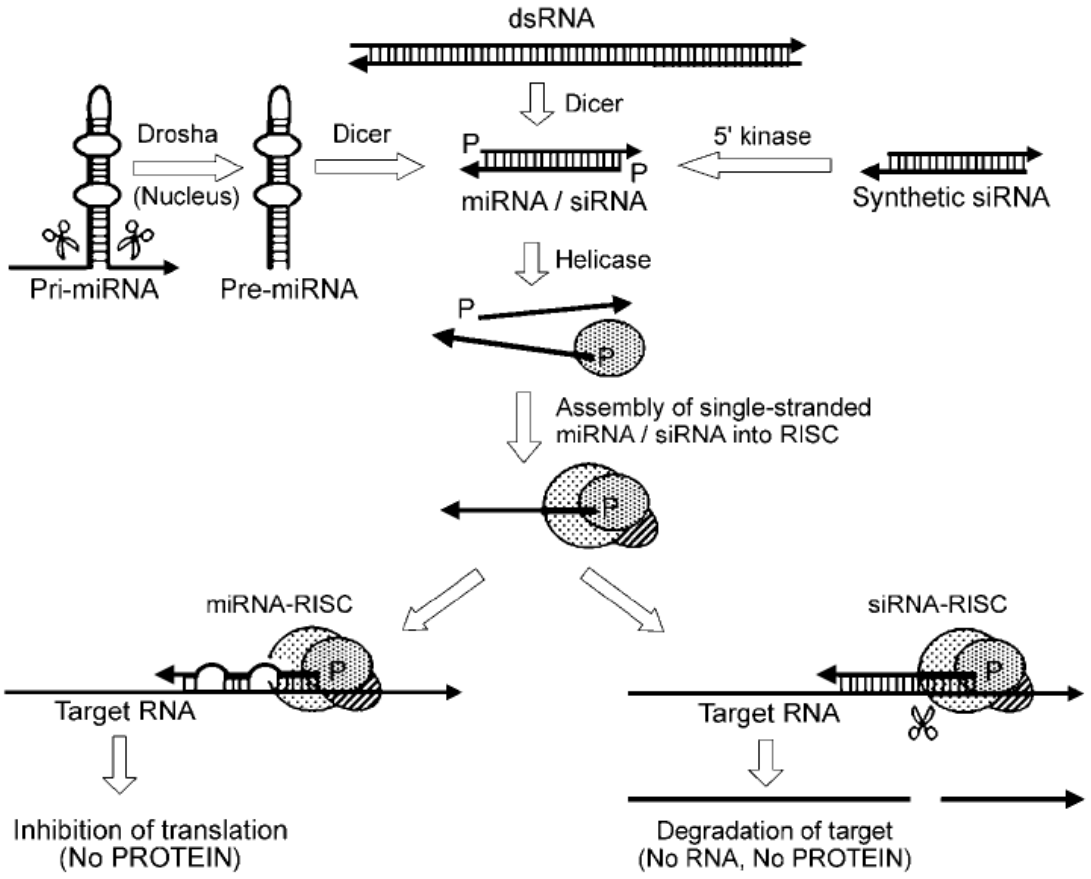

Figure 1.28. RNA interference [293].

siRNA is introduced into human cells as a synthetic molecule, its 5' hydroxyl gets phosphorylated shortly after entry into the cells [294-296]. These siRNAs are then incorporated into the RNA-inducing silencing complex (RISC). Although the uptake of siRNAs by RISC is independent of ATP, the unwinding of the siRNA duplex requires ATP. The unwinding favors the terminus with the lower melting temperature as the start point. Thus the termini containing more A-U base pairs are preferred as the unwinding start point. The strand whose 5'-end is at the start point will be used by RISC as the guide sequence and the other strand is release and degraded. Once unwound, the guide strand positions the RISC/siRNA complex with the mRNA that has a complementary sequence to the siRNA, and the endonucleolytic cleavage of the target mRNA occurs. The target

mRNA is cleaved at the single site in the center of the duplex region between the guide siRNA and the target mRNA. The microRNA (miRNA) pathway is another RNA silencing pathway and is similar to siRNA. miRNA is also approximately 22-nt long, but it is a product of a sequential processing on a single-stranded RNA by two enzymes of the RNaseIII superfamily [297-299]. The long primary transcript (pri-miRNA) is cleaved by a nuclear enzyme, named Drosha in human, into an approximately 70-nt long pre-miRNA. The pri-miRNA is basically a short hairpin RNA (shRNA) and is further processed in the cytoplasm by Dicer to produce the final miRNA. For both siRNAs and miRNAs, the perfect or near-perfect match will lead to the degradation of the target upon association with RICS, and mismatches will repress the translation. It now appears that at least seven continuous complementary base pairs are required for cleavage [300].

Introduction of synthetic siRNAs as a mimic for the Dicer cleavage process triggers the RNAi machinery. In addition, siRNAs or miRNAs produced form shRNA expression cassettes can be cloned into RNA expression vectors to produce the self-complementary hairpin sequences that induce the RNAi pathway. More importantly, the shRNA expressed from a vector could establish long-term silencing of a targeted gene expression. The transcription of shRNA from the vector is usually conducted using an RNA polIII promoter such as the H1 or U6 promoter [301, 302]. U6 promoter strongly favors a G residue at the first position of the transcribed sequence and H1 weakly prefers an A residue [303]. The transcription mediated by polIII promoters terminates after the second or third (less commonly) residue of a “TTTTT” stretch, which results in a 3’-UU tail that forms the 3’-2-nt unpaired overhang end in the hairpin structure after self complementarily annealing of the transcript. Both the preference of first residue and the

3’-2-nt unpaired UU end influence the target site selection. Similar to miRNAs, shRNAs (in the nuclei) are bound by a complex consisting of the nuclear export factor Exportin 5 (Exp5) and the GTP-bound form of the cofactor Ran [304, 305]. For nuclear export, this complex requires an RNA stem of ⩾16 bp, a short 3’-overhang and a terminal loop of ⩾ 6 nt [304, 306]. The efficient cleavage by Dicer requires an RNA of ⩾19 bp and a short 3’-overhang [307]. These prerequisites can be easily met when designing the shRNA expression cassette.

Considering the strand preference of RISC during unwinding, the 3’ end of the guide strand in shRNA is designed tightly base-pair (higher CG contents) and the 5’ end of the guide strand is designed loose base-pair (higher AU contents). As an example shown in Figure 1.29 [303], two GC base pairs at the 3’-end of guide strand (red) are designed. More AU pairs at the 5- end of the guide strand would be appreciated for correct unwinding and even a mismatch can be included. Actually bulges resulting from mismatches are always present in natural pri-miRNAs and they may help to fine-tune the cleavage sites used by Drosha and Dicer and/or may preclude activation of dsRNA-responsive cellular signaling pathway like interferon responses [303]. During the design of the shRNA, it is encouraged to include a bulge close to the 5’ end of the guide strand, which should be done by a introducing a mutation into the to-be-degraded (sense) strand, not into the guide (antisense) strand. It has also been reported that an A residue at position 3 and a U at position 10 of the sense strand can enhance siRNA function significantly. And a G at position 13 of the sense strand may need to be avoided [308]. Figure 1.29 [303] shows the sequence of the designed shRNA, with the reference to

human pre-miR-1 sequence and structure. The blue strand is sense and the red strand is antisense. Arrows mark the Dicer cleavage sites.

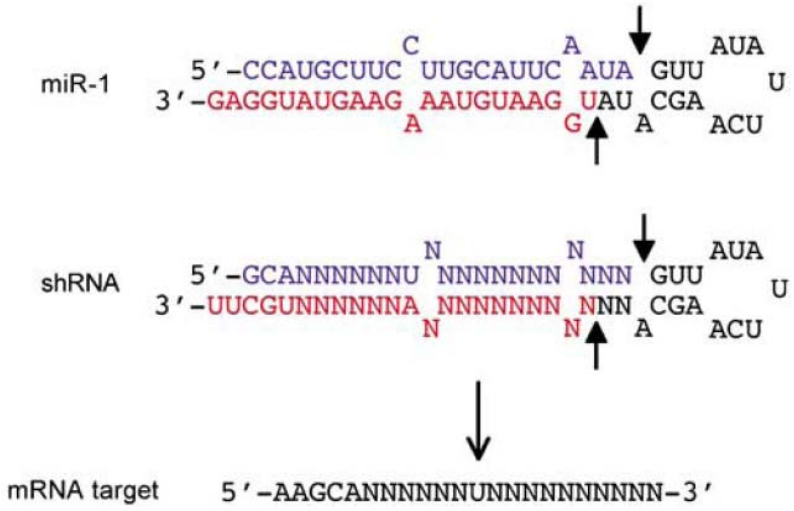

Figure 1.29. Designing artificial shRNA for RNAi [303].

It has also been found that small dsRNA that are 25-30 nt in length requiring RNAi processing appear to be more efficient in inducing RNAi than smaller 22 nt siRNAs [309], which could be due to the fact that Dicer may direct endogenously processed siRNAs and miRNAs to the RISC complex. This gives vector-expressed shRNA an extra advantage over synthetic 22 nt siRNAs. Multiple shRNAs or siRNAs can be introduced into the cell simultaneously, but it is worth keeping in mind that the RNAi machinery can be limiting [310] so that the competence between exogenous shRNAs and endogenous miRNAs, or between exogenous and endogenous siRNAs, for limited amount of Dicer and RISC could occur, which would interfere with the cell's endogenous RNAi pathways. Particularly when the cell is undergoing a cell division, the RNAi machinery could be diluted and adversely affected by inhibiting the gene knock-down mechanism.

RNAi is highly specific to its target and not toxic in almost all situations; however, when designing an siRNA or shRNA, some off-target effects should be considered and avoided. dsRNAs that are 30 nucleotides or longer tend to trigger at least two cellular stress response pathways, both of which will lead to a general and non-specific abrogation of protein synthesis, or even apoptosis [295, 311]. The IFN pathway is usually a mechanism to eliminate virus-infected cells, in which the long dsRNA binds to and activates the dsRNA-activated protein kinase (PKR). PKR can further phosphorylate the translation initiation factor, eIF-2α, and induce global translation inhibition and even apoptosis. In another pathway, dsRNA activates 2'-5' oligoadenylate synthetase. The 2'-5' oligoadenylate will then be formed and bond to and activate RNase I, resulting in non-specific degradation of RNAs. Although siRNA or shRNA, less than 30 nucleotides in length, usually do not activate these stress response pathways. In highly sensitive cell lines and at high concentrations, a subset of interferon genes can be activated [312-314]. In the designing of siRNAs or shRNA, the ones that have significant homology to other irrelevant mRNAs should be avoided. As noted before, a seven consecutive base pairing can be enough to activate the RISC-induced gene silencing. Even the guide strand (antisense) has been designed to introduce RISC to the target site after unwinding, it is still possible that unwinding could initiate from the 5' end of the sense strand and thus sense strand would guide the RISC. The homology of the sense strands should also be checked.

Vector-mediated expression of shRNA can lead to long-term RNAi and the silencing effect has been observed even after two months [302]. The half-life of unmodified siRNAs *in vivo* is only seconds to minutes [315]. The most important reason

for this short half-life is the rapid elimination by kidney filtration due to the small size (~7 kDa). Endogenous serum RNases can degrade the siRNAs limiting the serum half-life to 5-60 minutes. The half-life can be extended in a number of ways, for instance, complexing the siRNAs with other molecules or incorporating them into various types of particles to bypass renal filtration [315-317], chemically modifying the ribose [316, 318-320], or capping the ends of the siRNA [315, 320]. The modification on the ribose usually takes place at the 2'-position; 2'-deoxyribose, 2'-O-metheyl and 2'-fluoro substitutions/modifications have been reported [316, 318-320]. Usually the silencing effects are affected more or less by these modifications but a modified siRNA, with two 2'-O-methyl at the 5' end and four methylated monomers at the 3' end, has been demonstrated to be as active as its unmodified counterpart [321]. Even though siRNAs have the potential to activate interferon pathways, no toxic effects after siRNA application have been observed [258]. There is no strict specific sequence requirement in RNA interference (although there are preferred bases at some positions), and, therefore, the range of target for siRNA is greater that with ribozymes or antisense therapies.

Gene Therapy Overview

With the progress of Human Genome Project, people are reaching a new level of understanding of many biological events, including the etiology of diseases with or without proved treatment. Especially for those diseases currently without treatment, finding the genes that are involved in the initiation and development of the diseases provides new treatment targets.

The most common gene therapy targets are monogenic recessively inherited diseases such as hemophilia [322]. In the treatment of these diseases, gene therapy is designed to introduce a functional gene into a target cell to restore protein production that

is absent or deficient due to the genetic disorder. Conversely in monogenic dominantly inherited diseases like hypercholesteroleamia [323], successful treatment requires the aberrant gene to be silenced, and this is usually done by means of gene-silencing technologies. Cancer, as an acquired genetic disease, is also a good candidate for gene therapy. Apart from expressing functional tumor suppressor genes and silencing activated oncogenes, gene therapy in cancer treatment has also been applied to introduce the expression of immunopotentiation proteins, the expression of a toxic product in transformed cells, and the expression of proteins in healthy cells helping the cell to be resistant to higher doses of chemotherapy [324].

The methods to deliver a gene into cells can be roughly categorized into virus-based system and non-viral system.

Non-Viral Gene Delivery

The gene transfer in non-viral system is in general inefficient and often transient compared with viral vectors, but it has advantages such as low toxicity, simplicity of use and ease of large-scale production. In addition, the transient expression of a therapeutic gene would be desirable in the treatment of certain conditions, such as retinopathy of prematurity. Basically there are three categories of methods for non-viral gene delivery: naked DNA in the form of plasmid, liposomal packaging of the DNA and molecular conjugates.

Naked DNA is the simplest way to delivery a gene. It is not very efficient and can result in prolonged low levels of expression. The simplest way is to inject directly into the tissue of interest or inject systemically from a vessel. The expression level and area are usually limited in a systemic injection due to the rapid degradation by nuclease and clearance by mononuclear phagocyte system. To facilitate the uptake of naked DNA,

several techniques, in addition to simple injection, have been developed. The Gene Gun is a technology to shoot gold particles coated with DNA which allows direct penetration through the cell membrane into the cytoplasm and even the nucleus, bypassing the endosomal compartment [325]. Electroporation, the application of controlled electric fields to facilitate cell permeabilization, is another way to facilitate DNA uptake. Skin and muscle are ideal targets due to the ease of administration. Ultrasound can also increase the permeability of cell membrane to macromolecules like plasmid DNA and has been used to facilitate the gene transfer.

Liposomes are lipid bilayers entrapping a DNA fragment with a fraction of aqueous fluid. It can naturally merge onto cell membrane and initiate the endocytosis process. To improve transfection efficiency, target proteins recognized by cell surface receptors have been included in liposome to facilitate uptake, for example, anti-MHC antibody [326], transferrin [327], and Sendai virus of its F protein [328], which help DNA to escape from endosome into cytoplasm thus to increase DNA transportation to the nucleus. The inclusion of a DNA binding protein on the liposome also enhances transcription by bringing the plasmid DNA into the nucleus [328].

Molecular conjugates are usually a synthetic agent that can bind to DNA and a ligand at the same time [324]. Thus the binding of the ligand to its receptor will initiate the receptor-mediated endocytosis for the complex. This method is more specific for different cell types and receptor types. The synthetic agent needs to be designed accordingly, but this is useful in tissue-specific transfection. The transgene expression in this method tends to be transient and limited by endosomal and lysosomal degeneration.

Viral Gene Delivery

Viral gene delivery systems are based on replicating viruses that can deliver genetic information into the host cell. According to the existence status of the viruses, the virus vectors can be divided into two categories: integrating and non-integrating [329]. Integrating virus include adeno-associated virus, retrovirus, and so on. These viruses can integrate the viral genome into chromosomal DNA so that a life-long expression of transgene could be possibly achieved. Adenovirus and herpes simplex virus fall into the category of non-integrating viruses. They deliver viral genome into the nucleus of targeted cell, however the viral genome remain episomal, so it is possible that the transgene gets diluted during cell divisions.

Generally speaking, genomes of replicating viruses contain coding regions and *cis*-acting regulatory elements. The coding sequences enclose the genetic information of the viral structural and regulatory proteins and are required for propagation, whereas *cis*-acting sequences are essential for packaging of viral genomes and integration into the host cell. To generate a replication-defective viral vector, the coding regions of the virus are replaced by a transgene, leaving the *cis*-acting sequences intact. When a helper plasmid or virus providing the structural viral proteins *in trans* is introduced into the producer cell, production of non-replicating virus particles containing the transgene is established. An ideal viral vector should have these characteristics: 1) The virus genome is relatively simple and easy to manipulate; 2) The viral transduction can yield high vector concentration in the producer cells ($>10^8$ particles /ml); 3) The vector should have no limitation in size capacity; 4) The viral vector can transduce dividing and non-dividing cells; 5) The vector can deliver the transgene as integration in the host cell genome or as segregation being an episome along with cell division so that sustained expression can be

established; 6) The vector has a naïve or modified tissue specificity and the transgene expression can be regulated; 7) The vector produces no or low immune response and allows subsequent re-administration. [330]

The expression specificity can be regulated in many aspects. For tissue specificity, we can pick the virus that has the right tropism specific to some tissue, and in addition tissue-specific promoters can be added to further define the specificity. For spatial specificity, radiation in conjugation with radiation-activated promoter (for example, ergl promoter [331]) would be a good method. Of course the local delivery into the right place is always preferred than systemic administration, if feasible. For temporal specificity, drug-inducible promoters can provide a convenient way to switch the transgene expression on and off. The drug can be used to work on transcription activation or repressor elements to modulate the expression. There are many established drug-regulated gene expression systems, such as rapamycin-regulated gene expression [332] and RU486-regulated gene expression from GAL4 site [333]. And for promoters containing binding site for hormone receptor, heavy metals or cytokines, these specific hormone, heavy metals and cytokines can also be used to induce the expression.

Adeno-Associated Viral (AAV) Vectors

AAV is currently the virus closest to an ideal vector that is under study and application. It belongs to the family of parvovirus; it is non-pathogenic and depends on helper virus (usually adenovirus (Ad) or herpes virus) to proliferate. It is a non-enveloped particle with a size of 20-25 nm and has a vector capacity of 4.7 kb [334]. AAV can infect both dividing and non-dividing cells, with the transduction efficiency best in S-phase of host cell cycle. The viral genome, coded in a single-stranded DNA, has two open-reading frames (ORF). One is *rep*, which is responsible for viral structural proteins,

integration and replication proteins. The other is *cap*, coding for capsid proteins. There are inverted terminal repeats (ITR) at both ends of the genome sized around 150 bp, T-shaped and forming palindromic structure. TR is GC rich and contains a promoter. Due to the integration into the host genome, AAV vector can potentially deliver a long term expression of the transgene. Another advantage of AAV is that it induces overall low immune response. Presence of circulating neutralizing antibodies is in the majority of populations, but they don't prevent re-administration or shut down promoter activity [329]. Small packaging capacity is the number one disadvantage of AAV vectors. Using concatamers, formed by head-to-tail recombination in ITRs, up to 10 kb of transgenes can be packaged for delivery [335], by means of splitting promoter and transgenes sequences over two AAV vectors. But this technology reduces transduction efficiencies.

The infection of a host cell starts when the viral particle binds to its receptor on the cell membrane and initiates the endocytic pathway. The receptor type varies with AAV serotypes. The AAV-2 serotype, the most studied and commonly used serotype, has as its primary receptor heparin sulfate proteoglycans (HSPG) [336]. HSPG is widely expressed in various tissues and this is why AAVs have a wide tropism. There are also co-receptors for AAV-2 to facilitate endocytosis. Fibroblast growth factor receptor-1 (FGFR1), one of the co-receptors, can enhance the virus attachment to the cells [337]. Integrin αvβ3, another co-receptor, can facilitate endocytosis in the clathrin-mediated process, and it may also activate Rac1 and further phosphorylate PIP3 Kinase [338], which leads to microfilaments and microtubes rearrangement to support AAV2 trafficking to the nucleus. After entering the cell, the viral particle is released from the endosome at low pH conditions. Low pH probably induces a conformational change of

viral proteins and thus helps with endosome release and nuclear entry [339]. The viral particle is uncoated in the nucleus, and ssDNA is duplicated into dsDNA by either annealing with a complementary DNA strand from a second AAV or by the host cell machinery. The duplication from ssDNA to dsDNA is the rate-limiting step in AAV transduction. To overcome this, self complementary vector (scAAV) has been designed to expedite this process [340]. With the help of *rep* proteins, the viral genome or the transgene is integrated to a specific site in chromosome 19 via a non-homologous recombination and will be expressed by host cell transcriptional machinery. Some virus may remain episomal and also get expressed. Figure 1.30 [330] summarizes major steps in the AAV internalization and intracellular trafficking.

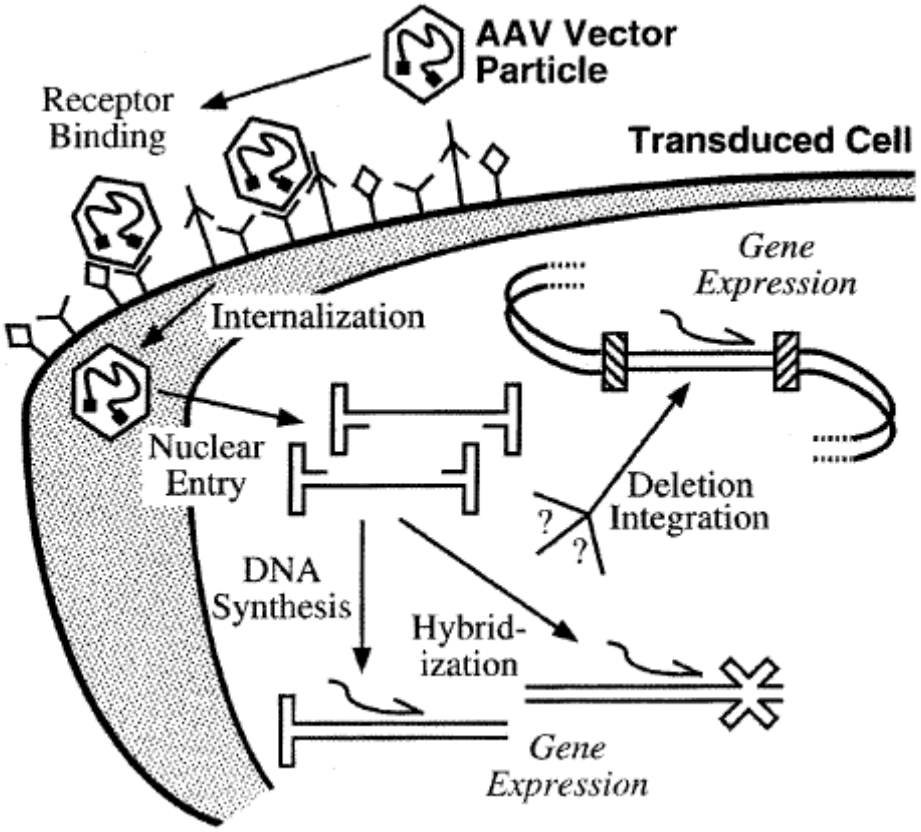

Figure 1.30. AAV internalization and intracellular trafficking [330].

AAV has a number of serotypes. AAV-1 and AAV-4 were isolated from simian sources; AAV-2, -3, -5 were isolated from human clinical specimens; AAV-6 is thought to be the recombination of AAV-1 and AAV-2 (AAV-1's 3' end recombined with AAV-2's 5' end), and AAV-7 and AAV-8 were isolated from rhesus monkey [330]. They have their own tropisms, which are determined by the capsid proteins. For example, AAV-2 is preferred to use for infection of the human eye, spine, while AAV-1 has the highest transduction efficiency in muscle and liver, and AAV-5 has high tropism for retina and is able to transduce airway epithelial cells.

Among all the serotypes, AAV-2 is the most studied and commonly used. As with all the AAV serotypes, the AAV-2 genome has two ORFs, *rep* and *cap*, which span over 90% of the genome. As shown in Figure 1.31 [330] Panel A, in the ORF of *rep*, there are two promoters, p5 and p19, encoding four proteins. Rep 78 and its splicing variant, Rep68 are transcribed from p5. They play important roles in replication, transcriptional control and site-specific integration. Rep52 and its splicing variant, Rep40 are transcribed form p19. They are important for the accumulation of single-stranded genome used for packaging. The other ORF *cap* encodes for VP1, VP2 and VP3 which are transcribed from p40. They are capsid proteins and have pivotal roles in tropism specificity. These three proteins are expressed in the ratio of 1:1:20, making the capsid with icosahedral symmetry. The ITRs at both ends of the viral genome have a couple of functions. The detailed structure and sequence of ITR is shown in Figure 1.31 [330] Panel C. First, the 3' end of the ITR on the 5' end the genome serves as primer in the synthesis of a new DNA strand. Second, ITRs contain Rep binding site (RBS) for Rep78 and Rep68 and

help them work as a helicase and an endonuclease. Third, the terminal resolution site (TRS) is identical to a sequence in chromosome 19, serving as integration sequence [341].

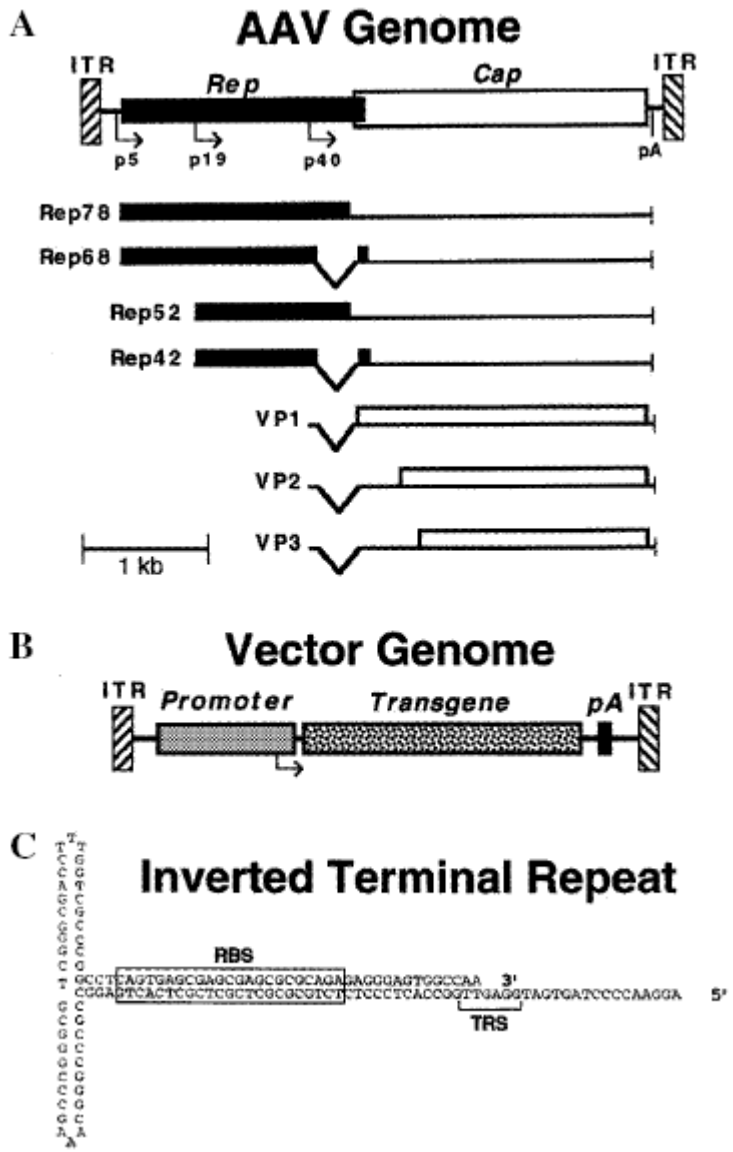

Figure 1.31. AAV2 genome and the vector genome [330].

When making an AAV viral vector, the two ORFs and the viral promoter are all replaced by a transgene and the only *cis* elements needed for AAV integration, packaging and assembly are the ITRs. The vector genome is shown in Figure 1.31 [330] Panel B.

rep and *cap* will be provided *in trans* in another plasmid, and helper virus gene products (E1a, E1b, E2a, E4 and VA RNA from Ad) are also provided *in trans*. Originally the vector production method is to co-transfect the HeLa cells with transgene plasmid, the plasmid providing *rep* and *cap*, and wide type Ad, or to co-transfect human 293 cells with the transgene plasmid, *rep* and *cap* plasmid, and E1-deleted Ad, as the E1gene products can be provided endogenously in 293 cells. Recently helper virus-free system has been designed to minimize the safety issues. See Figure 1.32 [334].

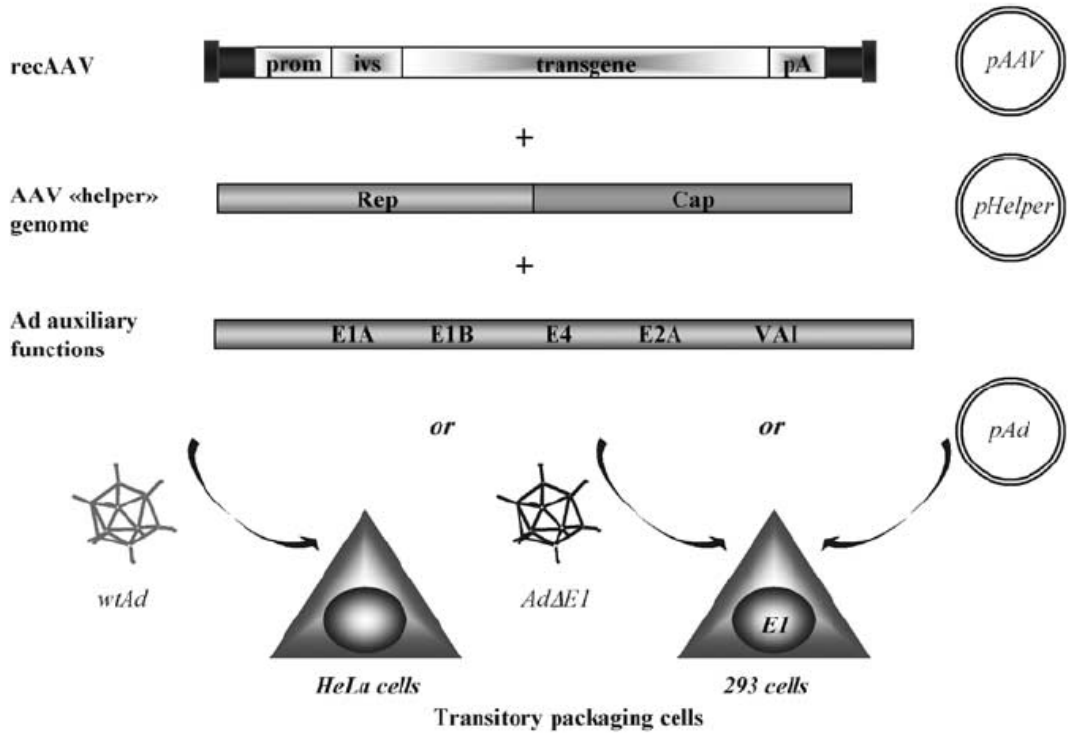

Figure 1.32. Helper virus –free systems in rAAV production [334].

The helper virus-free system has the three-plasmid system and the two-plasmid system [334]. In the three-plasmid system, besides AAV vector plasmid and AAV helper plasmid providing *rep* and *cap* genes, an Ad helper plasmid is introduced to provide the helper virus gene products (E2A, E4 and VA RNA from Ad) and human 293 cells are used as the host cell to provide Ad E1 gene products. The best molar ratio for these three

plasmids is 1:1:1, or 1:1:3 in mass [342]. The two-plasmid system combines the AAV helper plasmid and the Ad helper plasmid into one. The most recent version (called pDF) has 6 different helper plasmids, common in *rep* gene (AAV-2 *rep*) but varying in *cap* genes (AAV-1 to AAV-6 *cap*) [330]. What's more, different fluorescence protein gene is incorporated in the different plasmids. Because there are 5 kinds of most frequently used fluorescence protein genes, the plasmid having AAV-1 *cap* and the plasmid having AAV-6 *cap* use the same fluorescence protein gene, cyan fluorescence protein (CFP). The other four plasmids encode green fluorescence protein (GFP), yellow fluorescence protein (YFP), blue fluorescence protein (BFP), red fluorescence protein (RFP). This two-plasmid system is called Helper virus free, Optically controllable, Two-plasmid-base, or HOT [343]. This system not only minimizes safety issues and simplifies operation, but also adds controllable tropism. Figure 1.33 [330] summarizes the *rep* and *cap* genes, fluorescence and preferred tropism for these 6 pDF helper plasmids.

Helper plasmid	rep *gene*	*AAV1*		*AAV2*		*AAV3*		*AAV4*		*AAV5*		*AAV6*	
		cap	fp	cap	fp	cap	fp	cap	fp	cap	fp	cap	fp
pDP	AAV-2	1	—	2	—	3	—	4	—	5	—	6	—
pDF	AAV-2	1	Cyan	2	Green	3	Yellow	4	Blue	5	Red	6	Cyan
Modified pDF	AAV-4	1	Cyan	2	Green	3	Yellow	4	Blue	5	Red	6	Cyan
Preferred primary or transformed cell tropisms		Human spine, eye, brain, liver, nose, muscle and mouse brain		Human eye, spine, liver, nose, and monkey kidney		Human eye, spine, liver and nose		Human eye, heart, spine and mouse brain		Human heart, nose, muscle and rat liver		Human eye	

Figure 1.33. The 6 pDF helper plasmids in the two-plasmid system [330].

After the production of the AAV vectors, the vector purification can be accomplished in several ways, such as CsCl gradient ultracentrifugation, iodixanol

discontinuous gradient ultracentrifugation, heparin affinity column (for AAV-2), and HPLC [330]. The combined use of heparin affinity column and HPLC for AAV-2 can result in more than 50% recovery and more than 99% purity [344, 345].

Beyond a vector's own tropism, if we want to regulate the transgene expression more specifically or more precisely, some modification on AAV vector can be done. A linker molecule between the virus and the target cell can be incorporated as an indirect modification. The linker molecule can be bispecific antibody or streptarvidin. One example is the antibody F(ab'γ)2 which is used to help AAV-2 capsid to target α2β3 integrin [346]. A direct modification would be the modification on capsid proteins, for instance, the serpin receptor ligand has been incorporated into AAV-2 capsid gene [347]. Besides the modification on external surface of the vectors, tissue specific promoters/enhancers can also be used to regulate the transgene expression is specific tissue.

Adenovirus (Ad) Vectors

Adenoviruses are non-enveloped, dsDNA viruses. Ad vectors can capacitate up to 30 kb of transgene [324], which is larger than AAV vectors. Ad is non-integrating virus and remains as an episomal element in the nucleus. Ad is very efficient at transducing, *in vivo* and *in vitro*, dividing and non-dividing cells and can produce very high titers ($>10^{11}$/ml). Ad vector administration usually induces strong immune response. After intravenous injection, 90% of the vector is degraded in the liver and the remaining viruses have their promoter inactivated [348]. The persisting antibody prevents subsequent administration. Transit immunosuppressive therapies may be needed with Ad vector administration.

The Ad genome has four early transcriptional units (E1, E2, E3, E4), which have regulatory functions, and one late transcript, which encodes the structural proteins. The "gutless" vector contains the inverted terminal repeats (ITR), the packaging sequences around the transgene, and additional "stuffer" DNA to maintain the optimum package size. The necessary viral genes are provided *in trans* by helper virus. Figure 1.34 [324] shows a simple structure of the Ad genome, vector genome and helper virus.

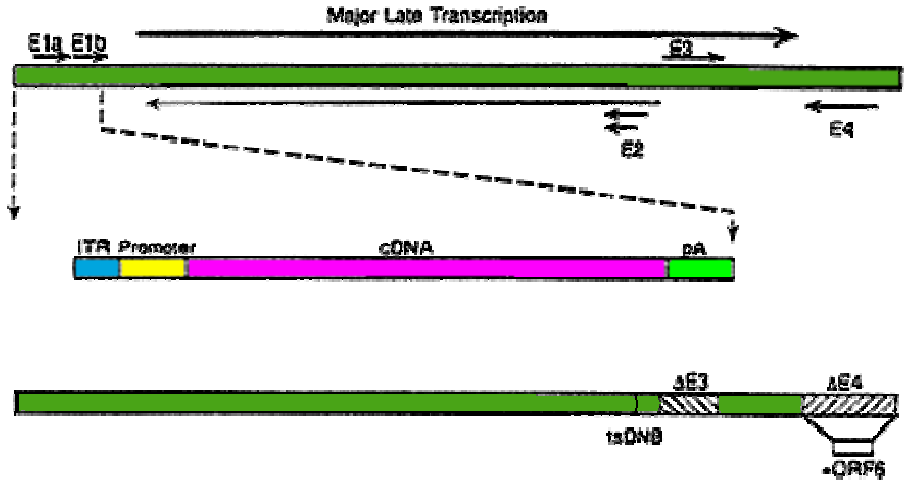

Figure 1.34. Ad genome and the vector genome [324].

During transduction, the fiber protein in Ad binds to cellular receptors, which are usually MHC class I molecules [349] and coxsackievirus-adenovirus receptors (CAR) [350]. Next the penton base protein on Ad binds to the co-receptor, αvβ3 and αvβ5 integrins, and internalize via clathrin-mediated endocytosis. After transport to the nucleus, the transcription of early genes is initiated and interferes with the antiviral defense of host cells. DNA replication is initiated by E2 products. In the late phase, structural proteins are highly expressed and virus assembly starts. Similar to AAV, specific cellular promoters/enhancers are used to direct tissue specific transgene expression.

Retrovirus Vectors

Retroviruses are enveloped, ssRNA viruses. ssRNA needs to be reversely transcribed into dsDNA and then the viral genome can integrate into the host gene but the virus can only target dividing cells. The vector capacity is around 7.5 kb [324]. The vector is easily inactivated by c1 complement protein and anti-α galaetosyl epitope antibody, both of them are present in human sera [351, 352]. The biggest disadvantage for retrovirus vectors is that insertional mutagenesis can possibly occur, because retrovirus (with its own proto-oncogene removed) can transform cells by integrating near a cellular protoncogene and drive inappropriate expression from its 3' long terminal repeats (LTR); or disrupt a tumor suppressor gene.

There are three categories of retrovirus: oncoretrovirus, lentivirus and spumavirus [329]. Oncoretrovirus is the simplest in structure and is the most commonly used. The oncorectrovirus genome contains three genes: *gag* encoding the core proteins, *pol* encoding the reverse transcriptase, *env* encoding the envelope proteins and determining tropism. There are LTR at both ends of the genome. The LTR is comprised of 3 regions, which are U3, R and U5. The LTR is essential for reverse transcription, integration and transcriptional activation as it contains a viral promoter/enhancer. ψ, located between the 5' LTR and the viral genes. This sequence required for packaging. In retrovirus vectors, LTRs and ψ are retained and the viral genome is replaced by a transgene. Transgene expression can be driven by the viral promoter/enhance in the 5' LTR or by other exogenous promoters.

Figure 1.35 [329] shows the genomic structure of MLV DNA, which is the most frequently used vector in oncoretravirus.

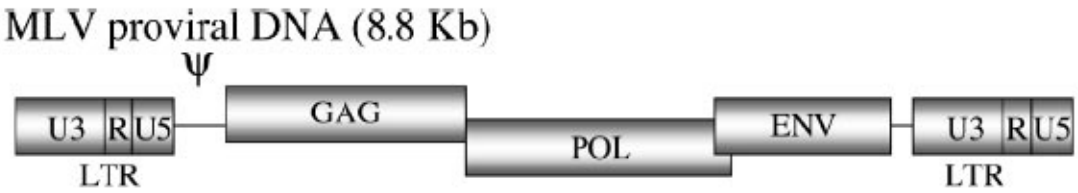

Figure 1.35. MLV genome structure [329].

Herpes Simplex Virus Type 1 (HSV-1) Vectors

HSV-1 are dsDNA, neurotropic viruses, and are good for neural gene transfers. The capacity of HSV-1 is about 40-50 kb [324]. HSV-1 has two life cycles. In the lytic life cycle, the viral genes get expressed shortly after transduction and new viruses are packed and released. In latent life cycle, the virus remains an intranuclear episome and the infected cell functions normally. Structurally, besides envelope, capsid and viral genome, the HSV-1 contains tegument, which is a protein layer between capsid and the envelope. Tegument is essential for viral internalization, resistance from the host cell defense system and transcription activation [329]. The HSV-1 genome has three classes of genes: immediate-early gene, early genes, and late genes.

One kind of plasmid vector derived from HSV-1 is called amplicon. It contains Col E1 ori (an Ecoli origin of replication), Ori S (HSV-1 origin of replication) and HSV-1 packing sequence [324]. The transgene is under the control of an immediate-early promoter. The expression is dependent on helper virus or helper plasmid containing the necessary genes from helper virus. The other kind of HSV-1 vector is the replication-deficient HSV-1, which is made by deletion of one immediate-early gene [324]. The deleted immediate-early gene is provided *in trans*.

CHAPTER 2
METHODS AND MATERIALS

Hammerhead Ribozyme Target Sites

The hammerhead ribozymes designed for this study were all 34 bases in length. They formed three stem structures when bound to target. The targeting arms of the ribozyme bound to either side of the X of the target NUX sequence to form stems I and III. The cleavage target was, therefore, 13 bases in length. Stem II was four base pairs in length and formed a stabilizing tetraloop within the folded ribozyme structure. An internal loop, formed within the ribozyme when it was bound to target, contained the catalytic core of the hammerhead ribozyme.

Choosing a target site in the mRNA sequence was the first step in ribozyme design. Shimayama et al. [281] refined the NUX rule of hammerhead ribozyme target site selection and demonstrated that a GUC site is the most efficient site for cleavage. Then Fritz and colleagues found GUCUU or GUCUA was more efficient cleaved [257]. The initial step in designing a ribozyme wa to search for potential target sites within the mRNA sequence of the target gene. The software package Vector NTi (Invitrogen, Carlsbad, CA) can be used for this purpose. The target mRNA sequence was downloaded into Vector NTi from GeneBank and the sequence was searched for the presence of GUCUU and GUCUA sites. Once potential target sites were identified the next step was to determine target accessibility.

Accessibility of Target Site

All the selected sites were only potential target sites because in the real world the secondary and tertiary structure of mRNA would affect the sequence accessibility significantly. To select the most accessible sites we used Zuker's Mfold program (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/) to examine target accessibility. This program was used to predict the secondary structure of 200 bases of the target mRNA centered on the NUX target sequence. In most cases the program would generate several possible structures. Since the structure of RNA in solution is dynamic, it is possible that the target region would exist in a dynamic equilibrium made up of the structures generated by Mfold and other structures also. For these studies, target sites which were completely or partially accessible within loop structures or at the end of a stem structure were considered accessible.

The accessibility and thermodynamic stability of the ribozyme is also very important. After the most accessible target sites were found by using Mfold, the corresponding ribozyme secondary structure was then examined using the same program. Generally ribozymes fold into one the four types of secondary structures shown in Figure 2.1 [257]. Type A has its two targeting arms completely accessible and forming no internal secondary structure with the rest of the ribozyme. Ribozymes that form only structure A, by Mfold analysis, typically have high catalytic activity. Type B and C structures do have internal secondary structures formed within one or both of the targeting arms but have a higher dG than structure A and, therefore, should have relatively good accessibility to bind to the target and possess relatively high catalytic activity. Ribozymes that form structures like D have lower dG values than structure A

and are more stable. Ribozymes that form structure D may have very low or no catalytic activity.

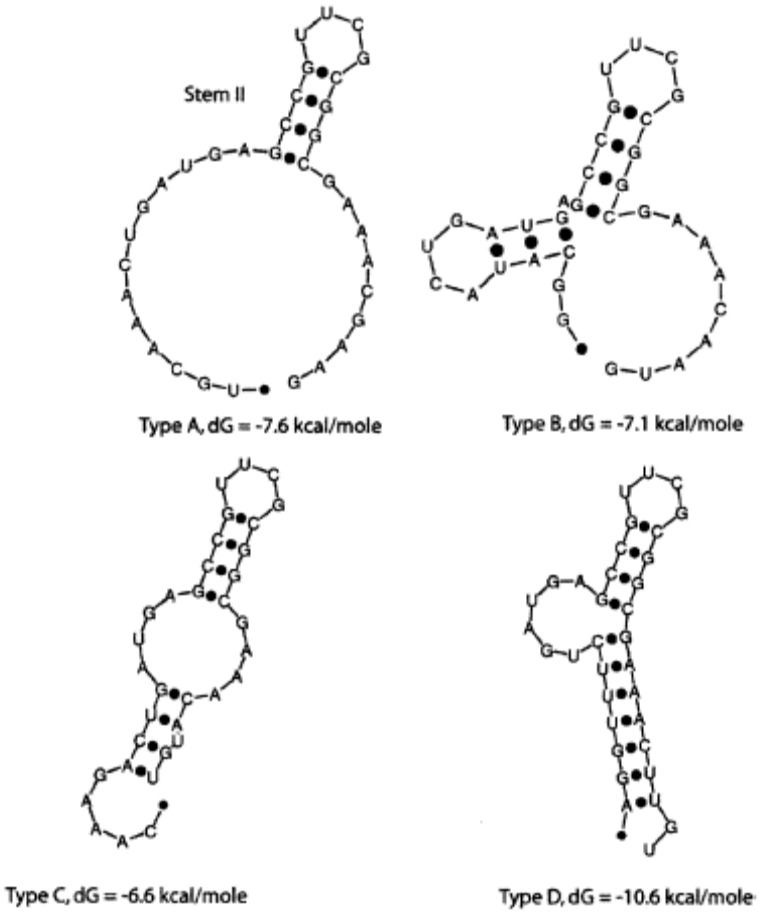

Figure 2.1. Typical structures of hammerhead ribozyme predicted by Mfold [257].

Once an accessible target site and ribozyme were identified the next step was to purchase RNA oligonucleotides corresponding to both the target and the ribozyme in order to perform *in vitro* cleavage analysis to determine the kinetic parameters of the ribozyme.

Kinase of Target Oligonucleotides

For the *in vitro* assays ribozymes and target RNA oligonucleotides were purchased from Dharmacon (Boulder, CO). They were deprotected according to manufacturer's protocols and suspended to a concentration of 300 pmol/µl in TE or water

and stored at -70ºC. The target RNA oligonucleotide was radioactively labeled with ^{32}P at the 5' end. 2 µl of RNA oligo (10 pmol/µl, 20 pmole total) was mixed with 1 µl 10X polynucleotide kinase buffer (Promega, Madison, WI), 1 µl RNasin (Promega, Madison, WI), 1 µl 0.1M DTT (Sigma, St. Louis, MO), 3 µl water, 1 µl [$\gamma^{32}P$] (10µCi) (ICN, Santa Clara, CA) and 1 µl T4 polynucleotide kinase (5 units) (Promega, Madison, WI). Reactions were incubated at 37ºC for 30 minutes, 65 µl of water was added, and the mixture was extracted with 100 µl of phenol/chloroform/isoamyl alcohol solution. The aqueous layer was added to a pre-packed G-50 fine spin column to separate the labeled target oligonucleotide from the unincorporated label. Samples were collected in a 1.5 ml Eppendorf tube (Fisher, Suwanee, GA) and stored at 4ºC. Samples are usable for one week but best when used within 24 hours.

Time Course of Cleavage Reactions for Hammerhead Ribozymes

1 µl of ribozyme (2 pmole total, diluted from 300 pmol/µl stock) was mixed with 13 µl of 400 mM Tris-HCl (Fisher, Suwanee, GA), pH 7.4-7.5, and 88 µl of water. The mixture was incubated at 90ºC for 2 minutes to denature the ribozyme and then held at room temperature for 10 minutes. Next 13 µl of 1:10 RNasin:0.1M DTT and 13 µl of 200 mM $MgCl_2$ (20 mM final) (Sigma, St. Louis, MO) was added and the mixture was incubated at 37ºC for 10 minutes. Cleavage was initiated by addition of 2 µl of target oligonucleotide (1 µl ^{32}P-kinased target plus 1 µl of cold target (20 pmole, 150 nM final)). The reaction was incubated at 37ºC, and time points were taken at 0, 1, 2, 5, 10, 15, 30 and 60 minutes. For each time point 10 µl of the reaction was added to 10 µl of formamide dye mix (90% formamide (Sigma, St. Louis, MO), 50 mM ethylenediamine tetra acetic acid (EDTA) pH 8 (Fisher, Suwanee, GA), 0.05% bromophenol blue (Sigma,

St. Louis, MO) and 0.05% xylene cyanol (Sigma, St. Louis, MO)). Samples were placed on ice then stored at -20ºC. The samples were heat denatured at 90ºC for 5 minutes, cooled on ice, and applied to a 10% PAGE-8 M urea gel (6 µl sample loaded each well) to separate the reaction products. The gel was held at 33 mA until the bromophenol blue moved 60% of the length of the gel. The gel was fixed and dried. The gel was analyzed on a Molecular Dynamics PhosphoImager (Ammersham, Sunnyvale, CA). The time point when 15% of the target was cleaved was determined and used as the endpoint for the multiple turnover kinetic reactions.

Multiple Turnover Kinetics

Reactions were done in a final volume of 20 µl. Ribozyme (0.3 pmol/µl, 15 nM final) in 40 mM Tris-HCl (pH 7.5) was incubated at 65°C for 2 minutes and then at 25°C for 10 minutes. The reactions were supplemented with DTT (20 mM final) and $MgCl_2$ (20 mM final) and 4 units of RNasin, incubated at 37°C for 10 minutes. Adding gradient concentrations of the target oligonucleotide (0-300 pmol/µl; 0-1500 nM final) initiated the cleavage reactions. The reaction tubes were incubated at 37°C for a fixed interval determined in the time course analysis of cleavage. This experiment could also be done with incubation in 1 mM $MgCl_2$ at 25°C. The addition of 20 µl of formamide stopped buffer terminated the reactions. Samples were initially held on ice then stored at -20ºC. Later the samples were heat denatured at 90ºC for 5 minutes, placed on ice and cleavage products were separated on 10% polyacrylamide-8 M urea gels. The gels were analyzed on a Molecular Dynamics PhosphoImager.

Treating ribozymes as classical catalytic enzymes, this experiment was done to determine the kinetic parameters (V_{max}, K_m, k_{cat}). The addition of gradient concentrations of the target oligonucleotide initiated the cleavage reaction and high concentrations of

target saturate the ribozyme's catalytic capability. This fixed interval for reaction incubation was usually how much time it needs to reach the 15% cleavage of the target, at which the cleavage reaction was linear. Thus the average cleavage velocity determined in this interval could be used as the initial reaction velocity. This velocity was determined using the amount of cleavage product divided by the fixed interval of time. The amount of the target cleavage product was determined by autoradiograph. By plotting the cleavage velocity versus the corresponding target concentration, a saturation curve was generated that is a typical Michaelis-Menten equation curve for the enzyme kinetics study. A double-reciprocal plot (also called: Lineweaver-Burke plot) could be further generated to graphically determine the kinetic parameters. This double-reciprocal plot was linear; the slope equaled K_m/V_{max} and the intercept on the Y-axis equaled $1/V_{max}$. Thus V_{max} and K_m could be determined. k_{cat} was the "turnover number" and assigned as the value of V_{max} divided by enzyme total concentration. The enzyme (ribozyme) total concentration was a known value. The two equations are listed below.

$$v_0 = \frac{V_{max} \bullet [S]}{K_m + [S]}$$, Michaelis-Menten Equation;

$$\frac{1}{v_0} = \frac{K_m}{V_{max}} \bullet \frac{1}{[S]} + \frac{1}{V_{max}}$$, Lineweaver-Burke Plot;

where v_0 is the initial reaction velocity, and [S] is the concentration of substrate (oligonucleotide target).

Cloning of the Ribozymes into an rAAV Expression Vector

The rAAV cloning vector was p21NewHp (Figure 2.2) and was described by Shaw and coworkers [353]. This vector was modified from the pTRUF21 plasmid (obtained from the UF Vector Core) by insertion of a self-cleaving hairpin ribozyme

between SpeI and NsiI sites. These sites were immediately downstream of the position where the hammerhead ribozyme is located. The hairpin ribozyme cassette included a cleavage site at its 5'-end that was recognized by the hairpin ribozyme. During transcription, the cytomegalovirus (CMV)/chicken β-actin chimeric enhancer/promoter drived the transcription through the hairpin ribozyme. Self cleavage by the hairpin ribozyme liberated the 3'-end of the hammerhead ribozyme with an additional eight bases at the hammerhead's 3'-end. Cleavage at this position eliminated downstream sequences that could interfere with the hammerhead ribozyme annealing to its target.

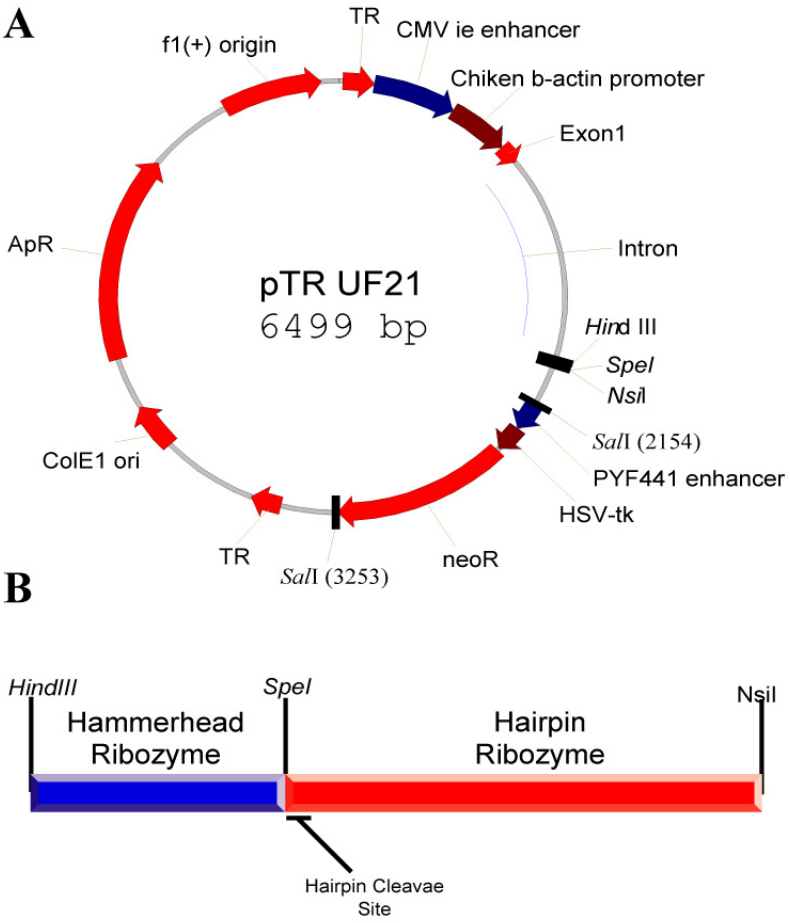

Figure 2.2. The pTRUF21 expression and cloning vector and the orientation and position of the hammerhead and hairpin ribozyme cassette.

For the cloning of one of the hammerhead ribozymes, two synthetic complimentary DNA oligonucleotides with phosphates at the 5'-ends that code for a single hammerhead ribozyme were purchased from Invitrogen (Carlsbad, CA). They were annealed by incubating at 90ºC for 3 minutes then slow cooled to room temperature for about 40 minutes. The annealed product was a double-stranded DNA oligonucleotide with a cut HindIII site at 5' end and a cut SpeI site at 3' end. This double stranded DNA fragment was cloned into the HindIII and SpeI site of p21NewHp.

The plasmid was digested by HindIII and SpeI restriction endonucleases (Promega, Madison, WI) according to manufacturer's protocols. Then the annealed oligonucleotide product was ligated into the HindIII and SpeI sites in the plasmid using DNA T4 Ligase (Promega, Madison, WI) according to manufacturer's protocols. The ligated products were transformed into SURE competent cells (Stratagene, La Jolla, CA) using electroporation. SURE cells were used in order to maintain the integrity of the inverted terminal repeats (TRs).

Screening and Sequencing of the Clones

The ligation mixture-transformed SRUE cells were grown in terrific broth (TB, Sigma, St. Louis, MO) supplemented with ampicillin at 37^{o}C for 16 hours or less. The plasmid DNA was purified using Genelute HP Plasmid Maxiprep Kit (Sigma, St. Louis, MO). The purified plasmids were digested with PstI restriction endonuclease (Promega, Madison, WI) according to manufacturer's protocols to monitor the integrity of TRs. The insertion of the hammerhead ribozyme eliminated a PstI site and loss of this site was used as a diagnostic indicatior of hammerhead insertion into the vector. In addition, we also performed SmaI digests on the plasmids to determine the integrity of the TRs. Plasmids that lacked the PstI site and still retained intact TRs were then sequenced to verify the

presence of the hammerhead ribozyme insert. The plasmids were sequenced using Ladderman Dideoxy Sequencing Kit (TaKaRa Shuzo Co, Japan) according to manufacturer's protocol or sequenced at ICRB Core sequencing facility at UF.

HREC Tissue Culture

Human eyes were obtained from National Disease Research Interchange within 36 hours of death. HRECs were prepared and maintained as previously described [354]. The eyes were placed on a sterile gauze pad (Johnson and Johnson Medical Supplies, Arlington, Texas) in a laminar flow hood and washed with 5 ml of betadine (Fisher, Suwanee, GA), and dissected with sterile scalpels (No. 1, Feather Industries Limited, Japan) and tweezers. Neural retina was isolated from the posterior portion of both eyes clear of RPE layer. The retina was placed on a 53 micron mesh nylon membrane (Tetko Inc, Lab Pack, Kansas City, MO) to separate the endothelial cells. Phosphate buffered saline (PBS) mixed with 2% antibiotic/antimycotic mix (ABAM) (Sigma, St. Louis, MO) was used to wash the retina. While washing, the retina was ground over the nylon membrane by a sterile wooden spatula. Then the remaining retina was transferred to a 20 ml Erlenmeyer flask containing 10 ml of PBS with antibiotics, using a sterile 10 ml pipette. Approximately 1 mg of collagenase (342 units/mg, Worthington Biomedical Corporation, Lakewood, NJ) was added to the flask. The flask was incubated in a 37 °C water bath for 15 minutes, and the contents were stirred every 5 minutes to dissolve the collagenase. Then 20 ml of complete endothelial cell media (250 ml Dulbelco's Modified Eagle Medium (DMEM) low glucose, 250 ml HAM's F12, 10% fetal bovine serum, 15% endothelial cell growth supplement, 15% insulin/transferring/selenium, 2% L-glutamic acid, 2% antibiotic/antimycotic mix) was added to the flask. The cells were washed twice with media and placed into a T25 flask (Fisher, Springfield, NJ) coated with 1% gelatin

(Sigma, St. Louis, MO). For the next 48-72 hours the cells were kept undisturbed so that they could grow and attach to the flask. Then the media was changed and fresh antibiotics were added. The cells were passaged upon reaching confluence. They were washed twice with PBS, then washed with 5 ml of trypsin (Sigma, St. Louis, MO) and then incubated in CO_2 for 45 seconds. The trypsin was neutralized with 2X volume of the complete endothelial cell media. The cells were centrifuged at 1000 rpm in an Eppendorf CT 5810R, resuspended in 6 ml of complete endothelial cell media and transferred to a T75 flask (Fisher, Suwanee, GA). Next 15 ml of complete endothelial cell growth media and fresh antibiotics were added to the T75 flask to culture the cells. Confluent cells used for the studies were those of passages 3-4 and were ascertained positive for acetylated LDL.

Transfection of HRECs with Lipofectamine

HRECs were grown on 150 mm plates to confluence before transfection with the ribozyme expressing plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). For the transfection 728 µl of Opti-MEM I was mixed with 52 µl Lipofectamine 2000 and kept at room temperature for 5 minutes. A second aliquot of 780 µl of Opti-MEM I was mixed with 13 µg of plasmid DNA and held at room temperature for 5 minutes. These two solutions were combined and held at room temperature for 20 minutes. The combined solutions were then added to cell cultures. After 24 hours the media was replaced. Cell cultures were harvest after 72 hours. Transfection efficiency was determined using a rAAV plasmid expressing green fluorescent protein (GFP).

Total RNA Extraction

Trizol LS reagent (Invitrogen, Carlsbad, CA) was used to isolate total RNA from HRECs. Experiments were done following manufacturer's protocol. For the 12-well plate, after media removed, 1.5 ml Trizol was added to each well. The cells were

resuspended and then transferred to 1.5 ml eppendorf tubes and incubated at room temperature for 5 minutes. 80 µl chloroform was added and the mixture was extracted for 30 seconds on a vortex mixer. After incubation at room temperature for 3 minutes, cells were centrifuged at 7400 rpm for 15 minutes. The aqueous layer was then transferred to a fresh tube and 190 µl isopropanol was added. The mixture was incubated for 10 minutes at room temperature, and centrifuged at 7400 rpm for 15 minutes. The supernatant was discarded and the pellet was washed with 380 µl of 75% ethanol. The cells were mixed on a vortex mixer for 15 seconds and centrifuge at 7400 rpm at 15 minutes and the supernatant was discarded. The pellet was then air dried for 10 minutes, dissolved in 25 µl RNase-free water or TE. The product was stored at -70^{o}C, aliquoted at 8 µl in 3 tubes for future use.

Relative Quantitative RT-PCR

Relative quantitative RT-PCR was performed on RNA isolated from HRECs transfected with plasmids expressing ribozymes and the p21NewHp vector expressing no ribozyme. Reverse transcription (RT) reactions were performed using reverse transcriptase (SuperScript from Invitrogen, Carlsbad, CA) and a random hexamer according to manufacturer's protocol. In brief, 8 µl of RNA isolated from transfected HRECs and 2 µl of random hexamer were mixed and incubated at 90^{o}C for 3 minutes and then held on ice for 5 minutes. Then 4 µl 5X RT buffer, 10 mM dNTP and 1 µl RNasin, 0.1 M DTT and the reverse transcriptase were added into the reaction. After a series of incubations at 25^{o}C for 10 minutes, 42^{o}C for 60 minutes and 95^{o}C for 10 minutes, the RT product was complete and stored at -20^{o}C.

For the relative quantitative PCR, the linear range of the amplification of the RT product was determined by using a PCR master mix (1 µl RT product/50 µl, 200 µM

dNTPs, 1 mM $MgCl_2$, 0.4 μM PCR oligonucleotides, 1x *Taq* DNA polymerase buffer (Sigma-Aldrich, St. Louis, MO), 2 U *Taq* DNA polymerase (RED*Taq*; Sigma-Aldrich, St. Louis, MO, 0.5 μCi/50 μl [α^{32}P]-dATP (ICN, Irvine, CA)). 50 μl of the master mix was separated into eight 0.2 ml tubes, and amplification was performed with an annealing temperature of 61°C. Samples were removed at even-numbered cycles starting at cycle 26. For each PCR sample, 5 μl was removed and 2 μl of formamide dye mix was added. The samples were heat denatured at 95°C for 3 minutes, cooled on ice, and applied to a 6% polyacrylamide-8 M urea electrophoresis gel. Dried gels were analyzed on the phosphorescence imager to determine the linear range of amplification. Cycle 34 and cycle 36 was determined to be within the linear range of amplification for IGF-1R and integrin α1 mRNAs, respectively.

In the relative quantitative RT-PCR assays the level of target mRNA was determined within each sample relative to an internal β-actin standard. β-actin mRNA levels were determined with a β-actin primer/competimer oligonucleotide set (QuantumRNA) from Ambion (Austin, TX). The competimer oligonucleotide pair from the β-actin primer set annealed to the same targets as the primer oligonucleotide pair, but they were blocked at their 3' ends to prevent extension. This primer/competimer oligonucleotide set allowed us to determine the ratio of primer to competimer that yields a β-actin PCR fragment that is approximately equimolar to the IGF-1R PCR product. To determine the ratio of the primer/competimer oligonucleotide set necessary to achieve this, PCR reactions were performed as described earlier, and amplification proceeded for 34 cycles (IGF-1R) or 36 cycles (α_1-integrin). The ratio of primer to competimer

oligonucleotide was determined to be 10:1 at a final concentration of 0.4 μM for the combined primer/competimer mixture.

Table 2.1. Sequences of primer pairs and annealing temperatures used in relative quantitative PCR.

mRNA	Primer Pairs		Annealing ºC
IGF-1R	AGGACGGCTACCTTTA CCCGGCACAATTAC	ATCAACAGGACAGC GACGGGCAGAG	61
Integrin α1	GAAAAACTCAATGACT TTCAGCGGC	CCAGTTGTGTAATGC AAATGTCCACA	54
Integrin α3	CGTCGTCTCCGCCTTC AACCTGGAT	GGCCACAGTCACTCC AAGCCACATG	60
Integrin α5	ACCCAGGGTCGGGGG CTTCAACTTA	GCCCCGAACCACTG CAAGGACTTGT	61
Integrin αv	CGCTTCTTCTCTCGGG ACTCCTGCT	CAGATGCTCCAAAC CACTGATGGGA	58

PCR reactions were then performed for IGF-1R, integrin α1 and β-actin simultaneously to determine the relative amount of IGF-1R to β-actin, using the above conditions. Table 2.1 lists the sequences of primer pairs and the annealing temperatures. PCR products were later separated on 6% polyacrylamide-8 M urea gels and analyzed on the phosphorescence imager.

Reverse Transcription–Real Time PCR

For each reverse transcription (RT) reaction, 4 μl of total RNA isolated from HRECs was used with iScript cDNA Synthesis Kit (BioRad, Hercules, CA) following manufacturer's protocol in a 20 μl reaction. 4 μl from the 20μl RT-reaction product was used to perform real-time PCR using iQ SYBR Green Supermix (BioRad, Hercules, CA) according to manufacturer's protocol. Primers from manufacturers were resuspended to 7.5 pmol/μl before addition to the PCR reaction mix. All reactions were performed in duplicate. β-actin PCR primers (Ambion, Austin, TX) were used as the internal normalization control. Real-time PCR was performed on a DNA engine Opticon system

with continuous fluorescence detector (MJ Research, Waltham, MA). Opticon monitor analysis software (MJ Research, Waltham, MA) was used for analysis. For IR, the sequences of primer pairs used were GATGCACCGTCATCAACGGGAGTCTGATC and GGCGCCCCTTGGTTCCTGAAACTTC, and annealing temperature was 58 ºC. For VEGFR-1 and VEGFR-2, the primers were pre-synthesized from manufacturer (R&D, Minneapolis, MN) and the annealing temperatures were both 55 ºC.

Total Protein Extraction

HRECs grown on 150 mm tissue culture plates were washed with PBS and scraped in ice cold phenol-free Hanks balanced salt solution (HBSS) containing 1 mM EDTA. The cells were centrifuged at 1000 rpm for 5 minutes at 4ºC in Eppendorf 5810R centrifuge and 30 μl of lysis buffer (150 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Igepal CA-630 (Sigma St. Louis, MO), 1% protease inhibitor cocktail (Sigma, St. Louis, MO) and 1 mM DTT (Fisher, Suwanee, GA)) was added. The mixture was sonicated for 2 seconds and then centrifuged at 13,200 rpm for 15 minutes at 4ºC. Protein levels in the supernatant were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) according to manufacturer's protocol.

Western Blotting

80 μg of total protein was loaded on a 4%-15% gradient polyacrylamide gel (Criterion; BioRad, Richmond, CA). The gel was electrophoresed at 120 V for 20 minutes to allow for stacking of the samples and then 140 V for 65 minutes to separate proteins. The gel was transferred to a nitrocellulose membrane (Millipore, Bedford, MA) using a blot cell apparatus (BioRad, Richmond, CA) at 80 V for 5 hours on ice in 4°C cold room. The membrane was blocked in TBS containing 0.05% Tween (Sigma St. Louis, MO) and 5% milk for 1 hour at room temperature. Then the membrane was

incubated with primary antibody at 4°C overnight. The membrane was washed again with TBS containing 0.05% Tween and 5% milk for 5 minutes and then incubated with secondary antibody for 1 hour at room temperature. The membrane was washed twice for 5 minutes and twice for 10 minutes with TBS containing 0.05% Tween. Usually the same membrane was also used to detect the internal protein control, β-actin or cofilin. An enhanced chemiluminescence (ECL) Western blot Detection Kit (Amersham Biosciences Ltd., Amersham, UK) was used to visualize the western bands. Standard molecular weight markers (BioRad, Richmond, CA) were loaded on the same gel and used to determine the target protein's molecular weight. The band intensity was analyzed using Scion Image (Scion, Frederick, MD).

Table 2.2 lists the concentration of primary and secondary antibodies and molecular sizes of probed proteins.

Table 2.2. Summary of primary and secondary antibodies used in western blottings.

Protein	Primary Antibody	Secondary Antibody	Molecular Size
IGF-1R	1:2000 rabbit polyclonal anti-human IGF-1R β subunit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA)	1:2000 horseradish peroxidase (HRP)-conjugated mouse anti-rabbit antibody (Santa Cruz)	95.2 kDa
IR	1:100 rabbit polyclonal anti-human IR β subunit IgG antibody (Santa Cruz)	1:1000 mouse anti-rabbit IgG-HRP (Santa Cruz)	95 kDa
β-actin	1:5000 mouse monoclonal anti-β-actin antibody (Sigma)	1:7500 HRP-conjugated rabbit-anti-mouse IgG antibody (Sigma)	42 kDa
Cofilin	1:2000 rabbit anti-cofilin (Cytoskeleton)	1:1000 mouse anti-rabbit IgG-HRP (Santa Cruz)	18 kDa

Flow Cytometry

The protein levels of VEGFR-1 and VEGFR-2 were determined using flow cytometry rather than western blotting. Transfected cells were harvested into single cell

suspensions 48 hours post transfection. After centrifugation at 1500 rpm at 4 ºC for 10 minutes, the cell pellets were suspended in 1 ml of buffer (0.1% BSA in 10 mM NaCl and kept on ice). 10 μg of either VEGFR-1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or VEGFR-2 antibody (Neomarkers, Fremont, CA) was added to the cells. After an incubation on ice for 30 minutes, the cells were washed twice in the buffer and incubated with the secondary antibody for 30 minutes in the dark (22.5 μg of goat anti-rabbit-FITC antibody (Jackson Immuno Research, West Grove, PA) in 1 ml of 0.1% BSA). The cells were washed twice in buffer and 5000 cells were analyzed on a FACScan (BD Biosciences, San Jose, CA).

Migration Assay

The modified Boyden chamber assay was used to assay the cell ability to migrate to increasing concentrations of growth factors. Trypsin (Trypsin-EDTA solution for endothelial cell culture, Sigma-Aldrich) was used to detach the transfected HRECs into a single cell suspension. After the trypsin was inactivated, cells were washed three times with PBS and suspended in DMEM to a final concentration of 1000 cells/μl. 30,000 cells (30 μl) were added per lower well in the blind-well chemotaxis chamber. A porous polyvinyl- and pyrrolidone-free polycarbonate membrane (12 μm pores) coated with 10% bovine collagen was applied on the wells and the chamber was fully assembled. The chemotaxis chamber was inverted and held in 5% CO_2 and room air at 37°C to allow cell attachment to the membrane. After 4 hours, chambers were then placed upright. 50 μl of a cocktail containing VEGF (25 ng/ml), bFGF (25 ng/ml), and various concentration s (1 ng/ml, 10 ng/ml, or 100 ng/ml) of the specific growth factor required to stimulate migration was added to the upper wells. For example, IGF-1 was used to test the IGF-1R ribozyme, PlGF was used to test the VEGFR-1 ribozymes and VEGF-E was used to test

the VEGFR-2 ribozyme. The chamber was incubated in 5% CO_2 and room air at 37°C overnight. Next, cells on the attachment side (lower wells) were scraped from the membrane, and only those cells that migrated through the pores of the membrane into the upper wells were left. The cells were fixed in methanol and then stained with a modified Wright-Giemsa stain (LeukoStat solution; Fisher Scientific, Springfield, NJ), and finally mounted on glass slides. DMEM served as a negative control of random cell migration, and DMEM with 10% FBS was used as a positive control in each experiment. A minimum of six replicate wells were assayed for each condition. A light microscope was used to count the cells, and the average of the number of cells counted in three separate, high-power (400X) fields was used as a quantitative reflection of the number of migrated cells per well.

Cell Proliferation Assay (BrdU)

The BrdU-incorporation assay was performed following manufacturer's protocol (Roche Applied Science, Indianapolis, IN). For a 96-well plate, transfected cells were added in a final volume of 100 µl/well. The cells were incubated for 48 hours. BrdU was added in the wells to a final concentration of 10 µM, and the cells were incubated for 2 hours. The cell media was removed and 200 µl/well of FixDenat from the kit was added to the cells and the plate was incubated at room temperature for 30 minutes. The FixDenat was removed and 100 µl/well of BrdU antibody-conjugate was added and the plate was incubated for 90 minutes at room temperature. The antibody solution was discarded and the cells were washed with 300 µl/well of washing solution from the kit. The washing solution was removed and 100 µl/well of substrate solution was added. The plate was held at room temperature until color development was sufficient for photometric detection (5-30 minutes).

Tube formation Assay (Matrigel)

Cells were transfected as detailed above. 24 hours after tranfection the cells were trypsinized and 5000 cells were seeded on matrigel (BD Biosciences, San Jose, CA) and incubated at 37^{o}C in 5% CO_2 environment. The cells were photographed every 24 hours.

Proliferating Endothelial-Cell Specific Promoter Constructs

The pLUC1297 and pLUC1298 plasmids were transformed into and isolated from DH5α- E.Coli Bacteria (Qiagen Mega Kit). These plasmids contained the proliferating endothelial-cell specific promoter and a luciferase reporter gene followed by a polyA site. The promoter was composed of a 4X (1297) or 7X (1298) 46-mer of the endothelin enhancer upstream of the human cdc6 promoter. The pLUC1297HHHP and pLUC1298 plasmids contained the IGF-1R hammerhead ribozyme/hairpin ribozyme cassette. A variant of pLUC1298HHHP designated pGE1298HHHP was missing the luciferase reporter gene. The cloning of IGF-1R hammerhead ribozyme with immediate-downstream processing hairpin ribozyme into the vectors was performed as detailed above, but ligation was into the Xba1 and Xho1 sites. The ligation product was transformed in StblII cells and the anti-kanamycin clones were selected. The isolated plasmid DNA (Giga Prep Kit-Qiagen) was sequenced to confirm ribozyme sequences.

Plasmid Formulation for Adult Mouse Eye Gene Transfer

Plasmid DNA was isolated and purified from StblII bacteria (Invitrogen). A cationic lipid (Lipid 89 Genzyme Corporation) in a molar ration of 3:1 lipid:plasmid was used to condense the DNA in 40% ethanol/5% dextrose. A helper lipid mixture composed of lysophospatidylcholine: monoacylglycerol: free fatty acid (1:4:2) (mole/mole/mole) was mixed with the cationic lipid. The lengths of the acyl chain helper lipids were 18:2

and the ratio of cationic lipid to helper lipid mixture was 10:90. Ethanol was removed by dialysis against PBS and the final DNA concentration was 0.5 mg/ml.

Animals

All animals were treated in accordance with the ARVO statement for the use of animals in Ophthalmic and Vision Research and with the Guide for the Care and Use of Laboratory Animals. All protocols were approved by the IACUC of the University of Florida. C57BL6/J timed pregnant mice and adult mice were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were housed in the University of Florida Health Science Center Animal Resources facilities. Ketamine (70 mg/kg body weight) and xylazine (14 mg/kg body weight) mixture was i.p. injected to anesthetize mice before laser treatments or euthanization. Intravitreal injection into 24-hour-old mouse pups was accomplished by placing the pups on a plastic shield on ice in order to anesthetize the pups before injection.

Intravitreal Injection into the Mouse Model of Oxygen-induced Retinopathy (OIR)

Shown in Figure 2.3 is the time course of the mouse model of oxygen-induced retinopathy (OIR). The newborn mouse pups were injected intravitreally with 0.5 μl plasmid (2 mg/ml) in the right eye within 24 hours of birth. Left eyes were used as uninjected controls. Seven days after birth the pups were placed in a chamber that maintained a 75% oxygen environment. After 5 days the 12-day-old pups were returned to normal room air. Return to normal air simulated a hypoxic response that resulted in the onset of retinopathy. This process mimicked human retinopathy of prematurity, and the aberrant neovascularization was very similar to what is seen in diabetic retinopathy patients. Neovascularization was initiated upon return to normal room air on day 12 and peaks on day 17 when the mice were sacrificed and the eyes were removed for further

analysis. Selected animals were perfused with 1.5 ml of 4% paraformaldehyde for immunohistological studies.

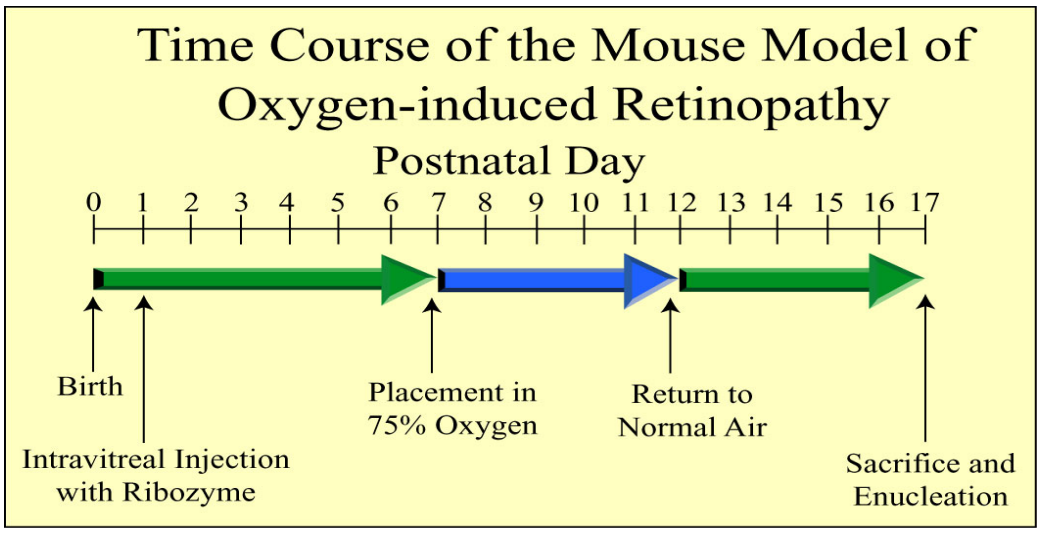

Figure 2.3. Time course of OIR mouse model.

The average number of pre-retinal nuclei per retinal cross section was determined and used as a quantitative measure of the extent of abnormal neovascularization. To prepare the eyes for analysis the eyes were fixed in 4% paraformaldehyde for one hour or in TRUMPS over night, washed in PBS, and embedded in paraffin. Each eye was sliced into 300 serial sections (6 μm thick) sagitally through the cornea parallel to the optic disc. Every thirtieth section was placed on slide and stained with hematoxylin-eosin (H&E). Three blinded individuals counted the number of nuclei in the cross-sections of pre-retinal blood vessels that grew beyond the retinal inner limiting membrane into the vitreous space. The total number of nuclei was used as the indication of neovascularization levels.

Intravitreal Injection into the Adult Mouse Model of Laser-Induced Retinopathy

Figure 2.4 shows the time course of this mouse model. Six to eight week old C57BL/6 mice were intravitreally injected in right eyes with 2 µl of AAV-VEGF, which expressed VEGF and induced neovascularization. Left eyes were uninjected controls.

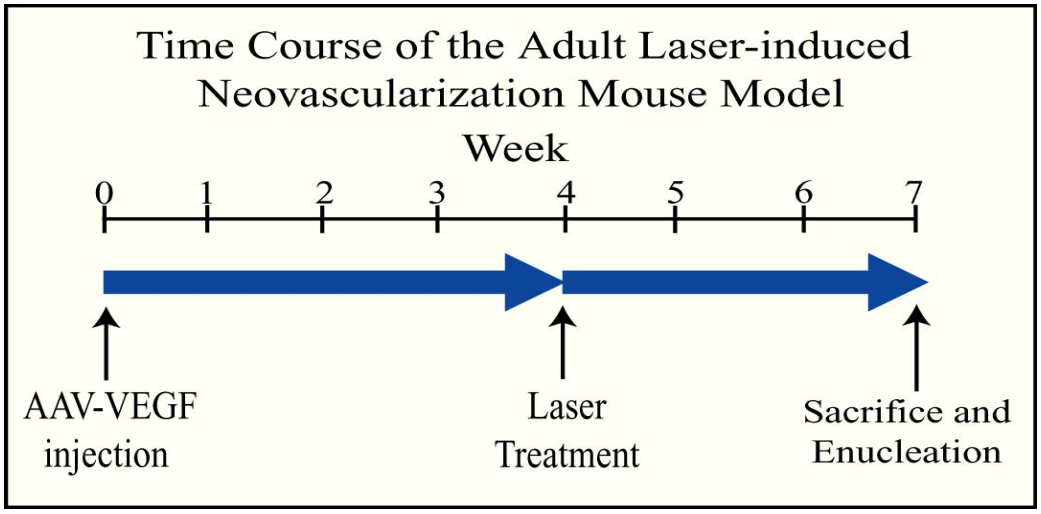

Figure 2.4. Time course of the adult mouse model of laser-induced neovascularization.

Four weeks later the AAV-VEGF injected eyes were subjected to with laser occlusion of the large venous vessels in the retina. An argon green laser system (HGM Corporation, Salt Lake City, UT) with a 78-diopter lens was used for retinal vessel photocoagulation. The blue-green argon laser (wavelength 488-514 nm) was applied to selected venous sites next to the optic nerve. Laser occlusion was performed at the setting of 1 second duration, 50 µm spot size and an average of 600 mW intensity with an average of 44 burns per retina. Immediately afterwards we injected 2 µl of the ribozyme expressing plasmid intravitreally in the same eye that had received laser treatment. Plasmids were formulated in a final concentration of 0.3 mg/ml with a cationic lipid that can facilitate transfection of the adult mouse endothelium. The mice were sacrificed three weeks following laser treatment. 3 ml of 4% paraformaldehyde was perfused in each

mouse. The eyes were enucleated and the retinas removed for immunohistological studies.

Immunohistological Studies

Retinas were placed in 96-well plates and permeabilized with PBS containing 0.2% Triton X-100, 0.1% BSA and 0.1% rabbit serum for 24 hours at 4ºC. Then they were washed in PBS for 24 hours at 4ºC. Anti-luciferase pAb (1:50 dilution of goat polyclonal IgG) (Promega, Madison, WI) was the primary antibody. After a wash in PBS the retina were treated with 0.1% rabbit serum for 24 hours at 4ºC. The secondary antibody was FITC-conjugated rabbit-anti-goat-IgG (green) (1:4000 dilution in PBS) (Sigma, St. Louis, MO). The blood vessels in the flat-mounted retina were labeled with endothelial cell-specific agglutinin conjugated to rhodamine (red) (1:1000 dilution in permeabilization solution, Vector Laboratories, Burlingame, CA). The retinas were imaged using a MRC-1024 Confocal Laser Scanning Microscope at the Optical Microscopy Facility at the University of Florida (Gainesville, FL). ImageJ (ImageJ 1.32j. Wayne Rasband, National Institutes of Health, USA, http://rsb.info.nih.gov/ij/) was used to analyze the images.

Statistical Analysis

Statistical analysis of the data was performed using the Student's t-test (Excel; Microsoft, Redmond, WA). Results were reported in mean ± SE. $P < 0.05$ is deemed significant.

CHAPTER 3
RESULTS

In this project a number of cell surface protein receptors were selected as our ribozyme targets. These ribozymes were tested *in vitro* in HRECs and/or *in vivo* in mouse models of retinal neovascularization. Inactive versions of selected ribozymes were also produced and tested. A proliferating endothelial cell-specific promoter was developed and tested with selected ribozymes.

Ribozyme Design

All hammerhead ribozymes designed in this study were 34 bases in length. They bound to targeted mRNA, formed a three stem structure, and cleaved at the 3' end of the X in the NUX triplet within the target sequence. The target sequences were all 13 bases long with six bases on either side of the X forming ribozyme stems I and III. The design of a ribozyme began with the GeneBank search of the proteins full cDNA sequence and the selection of a 13-base-long target site.

Target Site Selection

Taking the insulin receptor (IR) as an example, the human and mouse IR gene sequences were first retrieved from GeneBank (human accession number NM_000208 and mouse accession number NM_010568). We searched for GUC sites as the candidates of cleavage sites. All these candidate targets were examined *in silico* for accessibility. We eventually chose the 5'-UUACGUCUGAUUC-3' sequence in the human gene and the 5'-GCUUGUCUGAAAU-3'sequence in the mouse gene as cleavage target sites. Figure

3.1 shows the full cDNA sequence of human IR gene and the selected target site is highlighted.

```
ORIGIN
        1 gagaaggacg cgcggccccc agcgctcttg ggggccgcct cggagcatga cccccgcggg
       61 ccagcgccgc gcgcctgatc cgaggagacc ccgcgctccc gcagccatgg gcaccggggg
      121 ccggcggggg gcggcggccg cgccgctgct ggtggcggtg gccgcgctgc tactgggcgc
      181 cgcgggccac ctgtaccccg gagaggtgtg tcccggcatg gatatccgga acaacctcac
      241 taggttgcat gagctggaga attgctctgt catcgaagga cacttgcaga tactcttgat
      301 gttcaaaacg aggcccgaag atttccgaga cctcagtttc cccaaactca tcatgatcac
      361 tgattacttg ctgctcttcc gggtctatgg gctcgagagc ctgaaggacc tgttccccaa
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     1321 actttccttc ttccggaagt tacgtctgat tcgaggagag accttggaaa ttgggaacta
     1381 ctccttctat gccttggaca accagaacct aagggcagctc tgggactgga gcaaacacaa
     1441 cctcaccacc actcagggga aactcttctt ccactataac cccaaactct gcttgtcaga
     1501 aatccacaag atggaagaag tttcaggaac caaggggcgc caggagagaa acgacattgc
     1561 cctgaagacc aatggggaca aggcatcctg tgaaaatgag ttacttaaat tttcttacat
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     2341 aacctcttca ggcactggtg ccgaggaccc taggccatct cggaaacgca ggtcccttgg
     2401 cgatgttggg aatgtgacgg tggccgtgcc cacggtggca gctttcccca acacttcctc
     2461 gaccagcgtg cccacgagtc cggaggagca caggcctttt gagaaggtgg tgaacaagga
     2521 gtcgctggtc atctccggct tgcgacactt cacgggctat cgcatcgagc tgcaggcttg
     2581 caaccaggac acccctgagg aacggtgcag tgtggcagcc tacgtcagtg cgaggaccat
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     2821 cgctctggaa cggggctgca ggctgcgtgg gctgtcaccg gggaactaca gcgtgcgaat
     2881 ccgggccacc tcccttgcgg gcaacggctc ttggacggaa cccacctatt tctacgtgac
     2941 agactattta gacgtcccgt caaatattgc aaaaattatc atcggccccc tcatctttgt
     3001 ctttctcttc agtgttgtga ttggaagtat ttatctattc ctgagaaaga ggcagccaga
     3061 tgggccgctg ggaccgcttt acgcttcttc aaaccctgag tatctcagtg ccagtgatgt
     3121 gtttccatgc tctgtgtacg tgccggacga gtgggaggtg tctcgagaga agatcaccct
     3181 ccttcgagag ctggggcagg gctccttcgg catggtgtat gagggcaatg ccagggacat
     3241 catcaagggt gaggcagaga cccgcgtggc ggtgaagacg gtcaacgagt cagccagtct
     3301 ccgagagcgg attgagttcc tcaatgaggc ctcggtcatg aagggcttca cctgccatca
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     3481 tcctggccgc cctcccccta cccttcaaga gatgattcag atggcggcag agattgctga
     3541 cgggatggcc tacctgaacg ccaagaagtt tgtgcatcgg gacctggcag cgagaaactg
     3601 catggtcgcc catgatttta ctgtcaaaat tggagacttt ggaatgacca gagacatcta
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     3721 ggagtccctg aaggatgggg tcttcaccac ttcttctgac atgtggtcct ttggcgtggt
     3781 cctttgggaa atcaccagct tggcagaaca gccttaccaa ggcctgtcta atgaacaggt
     3841 gttgaaattt gtcatggatg gagggtatct ggatcaaccc gacaactgtc cagagagagt
     3901 cactgacctc atgcgcatgt gctggcaatt caaccccaag atgaggccaa ccttcctgga
     3961 gattgtcaac ctgctcaagg acgacctgca ccccagcttt ccagaggtgt cgttcttcca
     4021 cagcgaggag aacaaggctc ccgagagtga ggagctggag atggagtttg aggacatgga
     4081 gaatgtgccc ctggaccgtt cctcgcactg tcagagggag gaggcggggg gccgggatgg
     4141 agggtcctcg ctgggtttca agcggagcta cgaggaacac atcccttaca cacacatgaa
     4201 cggaggcaag aaaaacgggc ggattctgac cttgcctcgg tccaatcctt cctaacagtg
     4261 cctaccgtgg cgggggcggg caggggttcc cattttcgct ttcctctggt ttgaaagcct
     4321 ctggaaaact caggattctc acgactctac catgtccagt ggagttcaga gatcgttcct
     4381 atacatttct gttcatctta aggtggactc gtttggttac caatttaact agtcctgcag
     4441 aggatttaac tgtgaacctg gagggcaagg ggtttccaca gttgctgctc ctttggggca
     4501 acgacggttt caaaccagga ttttgtgttt tttcgttccc cccacccgcc cccagcagat
     4561 ggaaagaaag cacctgtttt tacaaattct ttttttttct ttttttttct tttttttgct
     4621 ggtgtctgag cttcagtata aaagacaaaa cttcctgttt gtggaacaaa atttcgaaag
     4681 aaaaaaccaa a
```

Figure 3.1. The human IR cDNA sequence with ribozyme target site highlighted.

Accessibility of Target Site

The tertiary structure of the mRNA surrounding the target site will affect the accessibility. The mRNA sequence from 200bp upstream and downstream of the NUX target site was examined with Zuker's Mfold program (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/). Figure 3.2 shows some of the possible secondary structures of the human IR target region predicted by the Mfold program. As shown, the target site (red arrows) is partially within the loops, which indicates, at least, partial accessibility of the site. The ideal situation suggesting complete accessibility of the target would be location of the target completely within loops.

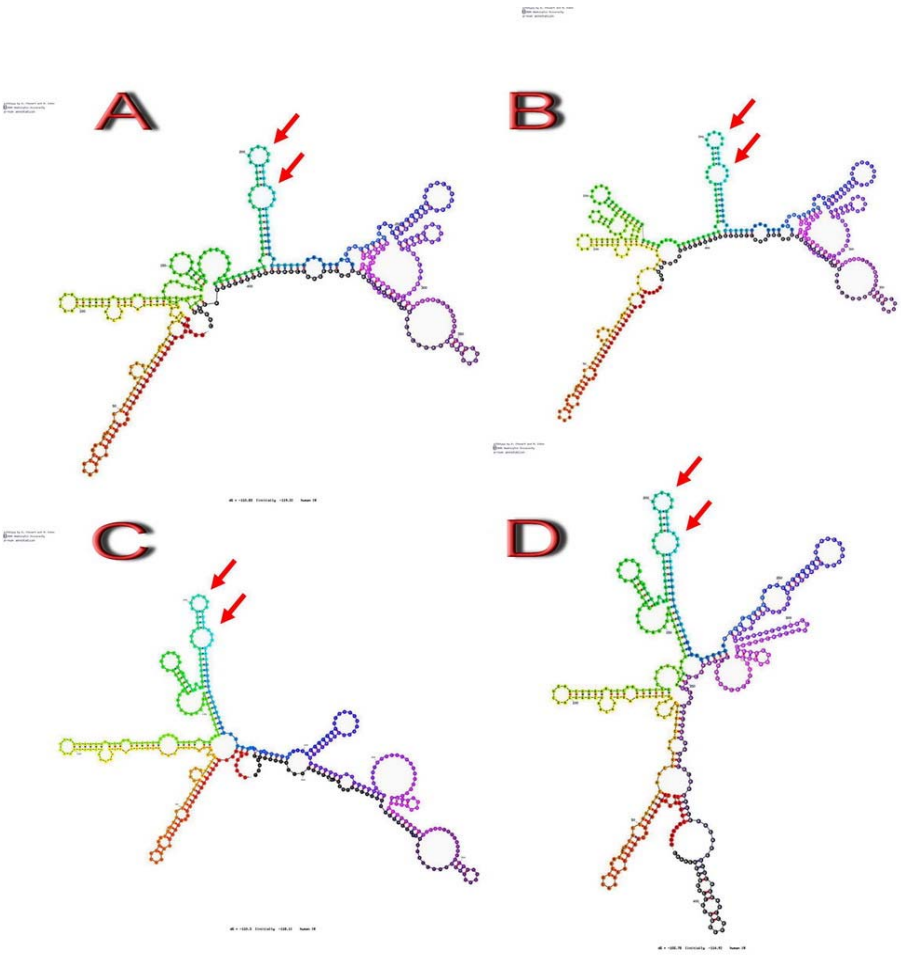

Figure 3.2. Mfold structures predicted for the human IR target region.

After determining if the target site was accessible, it was then necessary to examine the potential folding of the ribozyme by Mfold. The sequence of the ribozyme was generated from the target sequence, which gave the sequence of the 5'- and 3'-six base targeting arms that form stems I and III and the catalytic core and tetraloop sequence that was used in all of the hammerhead ribozymes in this study. Figure 3.3 shows the only Mfold structure predicted for the human IR ribozyme. This structure is a typical type A structure, based on the nomenclature of Fritz, et al. [257]. In this structure both targeting arms are completely accessible and the internal tetraloop has been formed by stem II hybridization. Only this structure being predicted by Mfold indicates that this ribozyme is completely accessible for target arm binding to the mRNA target sequence.

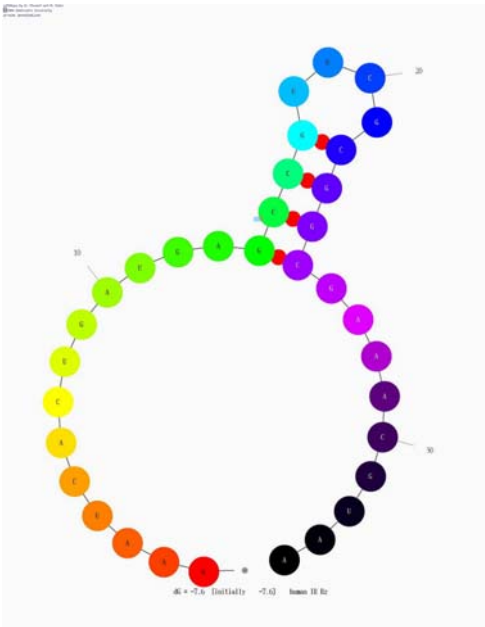

Figure 3.3. Mfold predicted secondary structure of human IR ribozyme.

The mouse target and ribozyme selection and design were performed as above. Figure 3.4 shows the technical structure of the bound complex of the human/mouse insulin receptor ribozymes and their target sequences. The stem I, II, and III in this complex are also marked out.

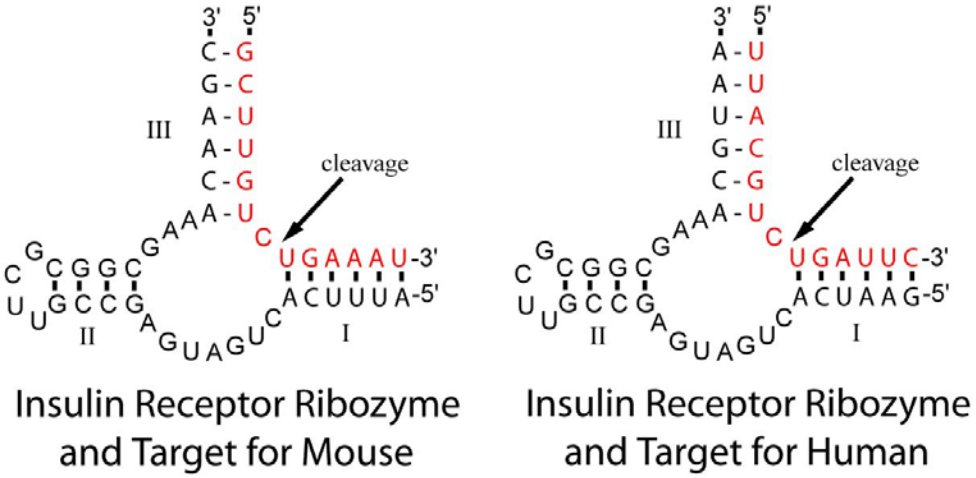

Figure 3.4. The 34-base ribozymes (black) annealed to the 13-base targets (red) for both human and mouse.

Sequences of the Ribozymes and the Targets

Table 3.1 shows all ribozyme sequences and their 13-nucleotide target sequences.

Table 3.1. Summary of ribozyme and target sequences.

Ribozyme	Ribozyme Sequence (5'→3')	Target Sequence (5'→3')
IGF-1R Rz1	CGCAAACUGAUGAGCCG UUCGCGGCGAAACGAAG	Mouse: CUUCGUCUUUGCG
		Rat: CUUUGUCUUUGCA
		Human: CUUCGUCUUUGCA
IGF-1R Rz2	AUGGAACUGAUGAGCCG UUCGCGGCGAAACAUAC	Mouse: GUAUGUCUUCCAU
		Rat: GUAUGUCUUCCAU
		Human: GUAUGUCUUCCAU
IR human	GAAUCACUGAUGAGCCG UUCGCGGCGAAACGUAA	UUACGUCUGAUUC
IR mouse	AUUUCACUGAUGAGCCG UUCGCGGCGAAACAAGC	GCUUGUCUGAAAU
VEGFR-1	CCUAUACUGAUGAGCCG UUCGCGGCGAAACACCC	Mouse: GGGUGUCUAUAGG
		Human: AGGUGUCUAUCAC
VEGFR-2	ACAGAACUGAUGAGCCG UUCGCGGCGAAACCAUG	CAUGGUCUUCUGU
Integrin α1 mouse	CUUAUACUGAUGAGCCG UUCGCGGCGAAACAUCU	AGAUGUCUAUAAG
Integrin α3 mouse	CAUGAACUGAUGAGCCG UUCGCGGCGAAACAUAG	CUAUGUCUUCAUG
Integrin α5 mouse	GUGGCACUGAUGAGCCG UUCGCGGCGAAACAGGA	UCCUGUCUGCCAC
Integrin αv mouse	AACUUGCUGAUGAGCCG UUCGCGGCGAAACCAUU	AAUGGUCCAAGUU

Some mRNA sequences shared significant homology between species so the same ribozyme can target and cleave multiple species. Cleavage occurred on the 3' side of the boxed C and the ribozyme and target complimentary sequences are underlined. The part of the target sequence that is not underlined did not base-pair with the ribozyme. The remainder of the ribozyme sequence formed the catalytic core and stem II of ribozyme.

In Vitro Testing of Ribozymes

The first step in testing a ribozyme was to examine its *in vitro* cleavage activity. 13-base ^{32}P-labeled RNA oligonucleotides were used as the cleavage targets to examine the cleavage activity of the ribozymes.

Time Course of Cleavage

Time course of cleavage analysis gave the first indication of the level of catalytic activity of a ribozyme. To examine a ribozyme's time course of cleavage, synthetic 13-nucleotide-long target RNA oligonucleotides were 5'-end-labeled with ^{32}P and cleaved by ribozyme *in vitro*. The 5' cleavage product was 7 nucleotides in length. Panel A in Figure 3.5 is the autoradiograph of a 10% polyacrylamide-8M urea gel used to separate cleavage products of the human IR ribozyme on the human RNA target oligonucleotide. Panel B is the graphical representation of the data obtained by analysis of the gel in panel A using a PhosphorImage. The IR ribozyme had good catalytic activity since over 80% of the cleavable RNA oligonucleotide was cleaved within 2 minutes. Notice, however, that only approximately 60% of the target RNA was cleaved in this reaction. The remaining target remained uncleaved presumably due to a fraction of the ribozyme was misfolded and therefore inactive. Some of our other ribozymes exhibited more complete cleavage reactions that could reach 90% to 100% cleavage of the target oligonucleotide. The situation for this IR ribozyme was not unusual.

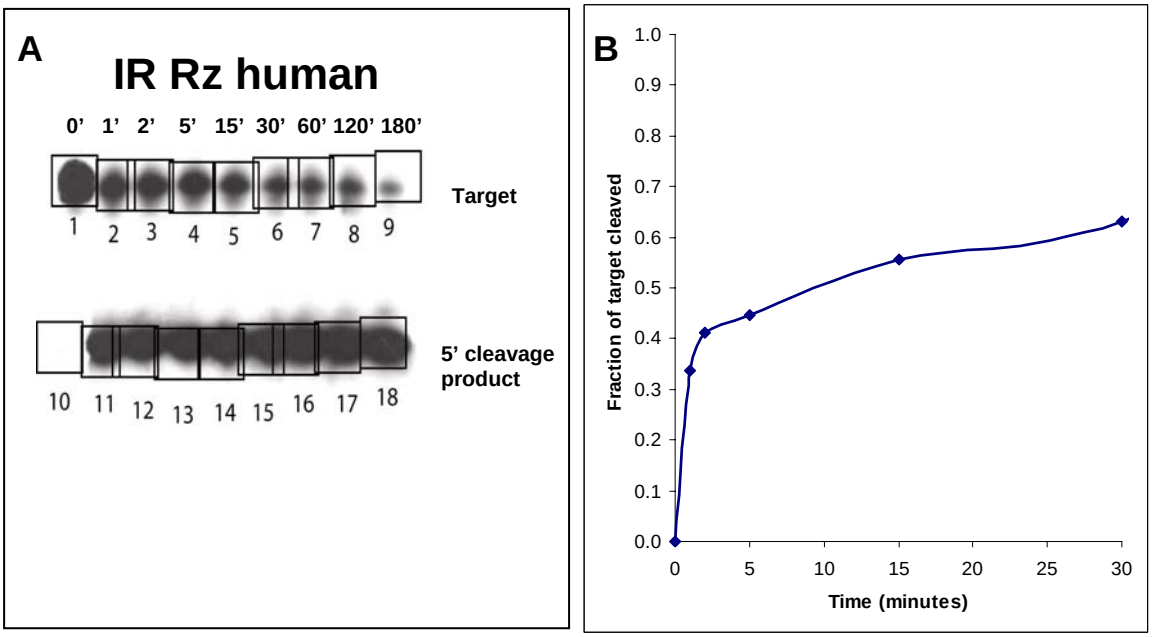

Figure 3.5. Cleave time course of human IR ribozyme.

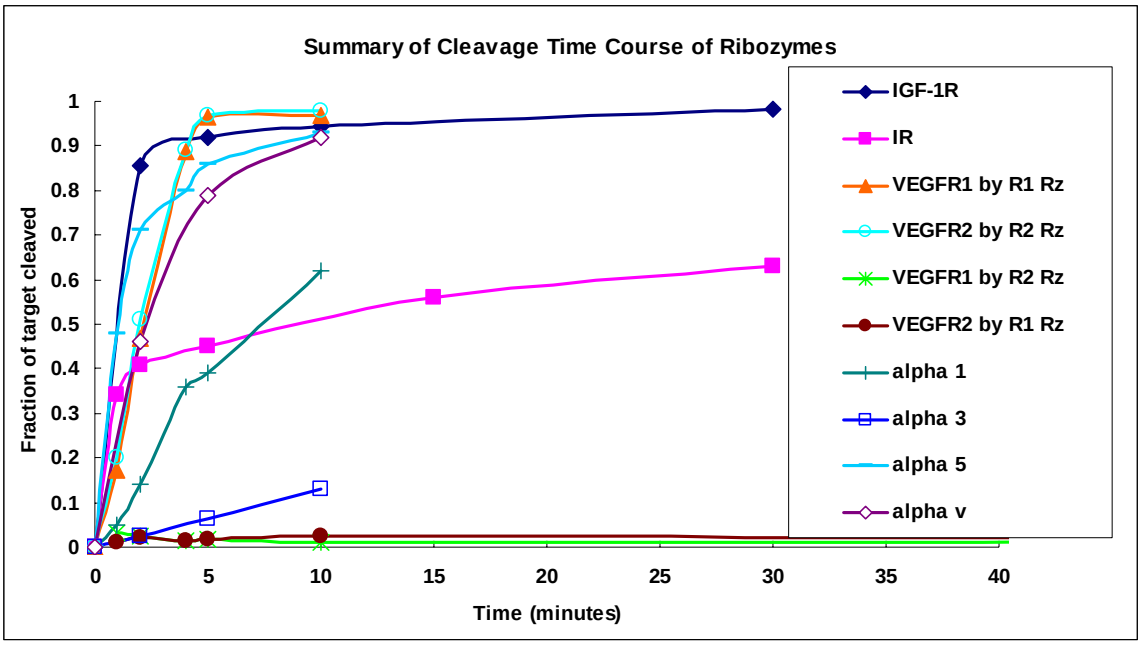

Figure 3.6. Summary of time courses cleavage of the ribozymes generated in this study.

Other ribozymes were tested similarly and their time courses of cleavage are shown in Figure 3.6. For IGF-1R ribozyme, the inactive forms were also tested. As expected, they did not show any cleavage activity (data not shown). We tested VEGFR-1

ribozyme on its own target and also the target of VEGFR-2 ribozyme. Similarly VEGFR-2 ribozyme was also tested with both targets. Both VEGFR-1 and VEGFR-2 ribozymes showed high catalytic activity on their respective target RNA oligonucleotides. Over 90% of the target RNA oligonucleotides were cleaved within 5 minutes. On the other hand, we did not see any cleavage of the VEGFR-1 ribozyme on the VEGFR-2 target or of the VEGFR-2 ribozyme on the VEGFR-1 target. This demonstrated the specificity of these two ribozymes. The cleavage rates of integrin ribozymes varied from lower than 20% cleavage to around 90% cleavage within 10 minutes.

Kinetic Analysis

After the time course cleavage analysis, multi-turnover kinetic analysis was performed to determine the kinetic parameters (V_{max}, K_m, k_{cat}). Figure 3.7 shows the saturation curve and the double-reciprocal plot of the human IR ribozyme kinetic analysis. The slope and interception of the double-reciprocal plot were used to determine the kinetic parameters.

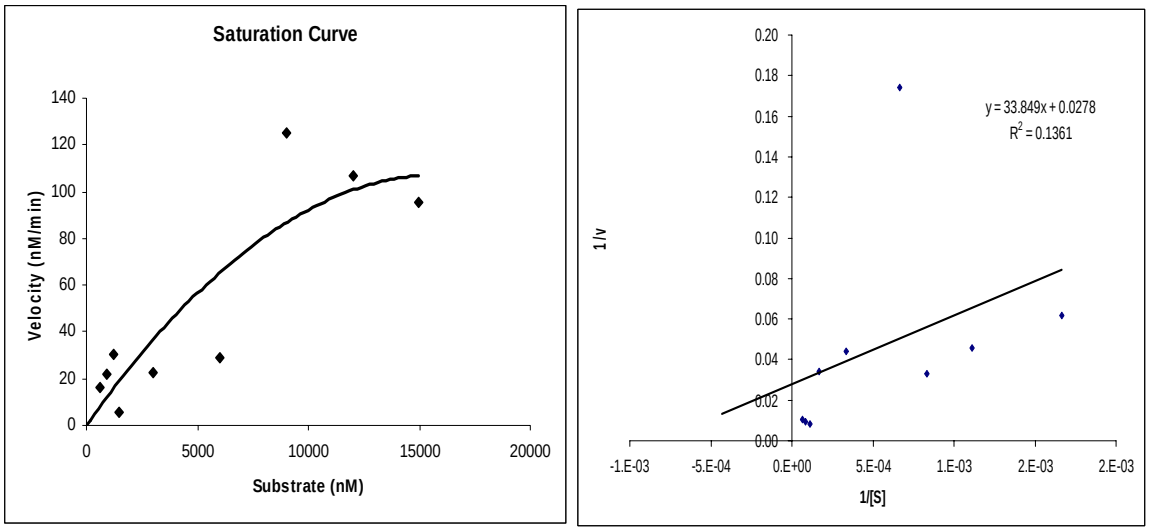

Figure 3.7. Multiple-turnover kinetic analysis of a human IR ribozyme.

Table 3.2 summarizes the kinetic parameters (V_{max}, K_m, k_{cat}) of the ribozymes.

Table 3.2. Summary of ribozyme kinetic data.

Ribozyme	V_{max} (nM/min)	K_m (μM)	k_{cat} (min^{-1})
IGF-1R Rz1	7.0±0.3	47.1±1.7	0.47±0.01
IGF-1R Rz2	2.8±0.60	1.8±0.1	0.2±0.04
IR	35.97	1217.59	2.39808
VEGFR-1	227.3±45.8	5.4±0.58	15.2±3.2
VEGFR-2	356.9±35.2	7.6±0.35	29.6±5.6
Integrin α1	57.05±4.7	25.6±10.0	3.8±0.3
Integrin α3	6.1±1.1	41.6±0.41	57.05±0.07
Integrin α5	322.6±78.6	81.1±18.2	21.5±5.8
Integrin αv	33.4±2.7	5.2±0.2	2.2±0.2

Functional Analysis of Ribozymes in HRECs

Following successful *in vitro* analysis of the ribozymes, we cloned the ribozymes into the p21NewHp plasmid and transfected these plasmids into HRECs. This allowed for the determination of target mRNA levels, target protein expression levels and for the testing of cellular activities related to normal HREC physiological functions such as cell migration, cell proliferation and endothelial tube formation.

Inhibition of mRNA Expression

Messenger RNA was the target of a ribozyme thus mRNA levels were measured after transfection. As an example, IR mRNA levels are shown in Figure 3.8. The mRNA levels were determined using reverse transcription on isolated total cellular mRNA followed by real time PCR on the cDNA products with primer pairs specific for the target mRNAs. The levels of the target were normalized to β-actin mRNA levels. The mRNA levels in mock-transfected cells were set as 100%. Cells expressing the human IR ribozyme showed a signification reduction of 42.4±5.1% in IR mRNA level (P=0.014). The non-transfection or transformation with the p21NewHp vector showed no significant difference in IR mRNA levels compared to the mock-transfected cells (P=0.35 for NT and P=0.16 for vector)

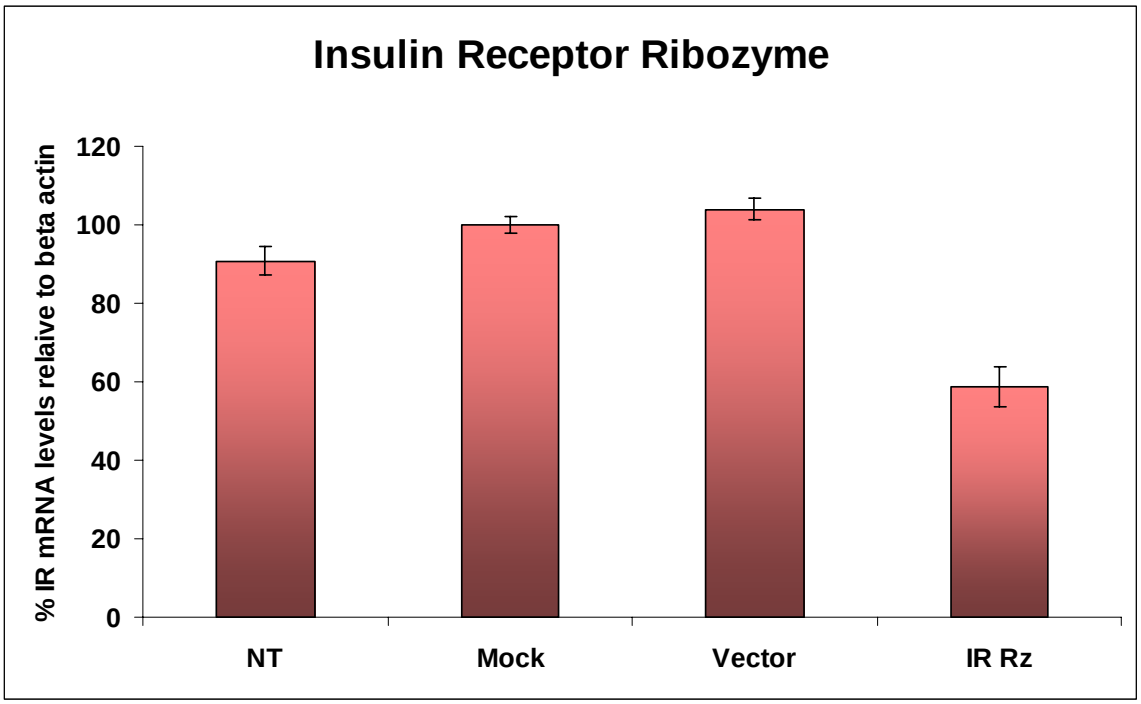

Figure 3.8. Insulin receptor mRNA levels in HRECs.

Table 3.3 summarizes the reduction in mRNA levels in HRECS after transfection with plasmids expressing the indicated ribozymes. Inactive versions of the IGF-1R ribozyme 1 and 2 and the α1 integrin ribozyme were also tested. As expected, expression of these inactive ribozymes resulted in no reduction of target mRNA levels (P>0.1, data not shown).

Table 3.3. Reduction in target mRNA levels in HREC by the ribozymes.

Ribozyme	Reduction in mRNA levels	*P* value	RT-PCR method
IGF-1R Rz1	39.5±10.1%	0.003	Relative Quantitative
IGF-1R Rz2	12.7±5.7%	0.003	Relative Quantitative
IR	42.4±6.4%	0.014	Real-time
VEGFR-1	71.1±2.1%	0.0002	Real-time
VEGFR-2	85.1±1.9%	0.0008	Real-time
Integrin α1	32.4±5.0%	<0.01	Relative Quantitative

Protein Levels

IR protein levels were also investigated after transfection of HRECs with the plasmids. The β-subunit of the IR appeared in two bands (around 200 kDa and 90 kDa) as

precursor and mature forms (Figure 3.9). The protein level in non-transfected HRECs was set to 100%. Vector-transfected cells showed no significant reduction. Expression of the human IR ribozyme resulted in a reduction of 20.9±2.1% (P=0.006) in IR protein levels.

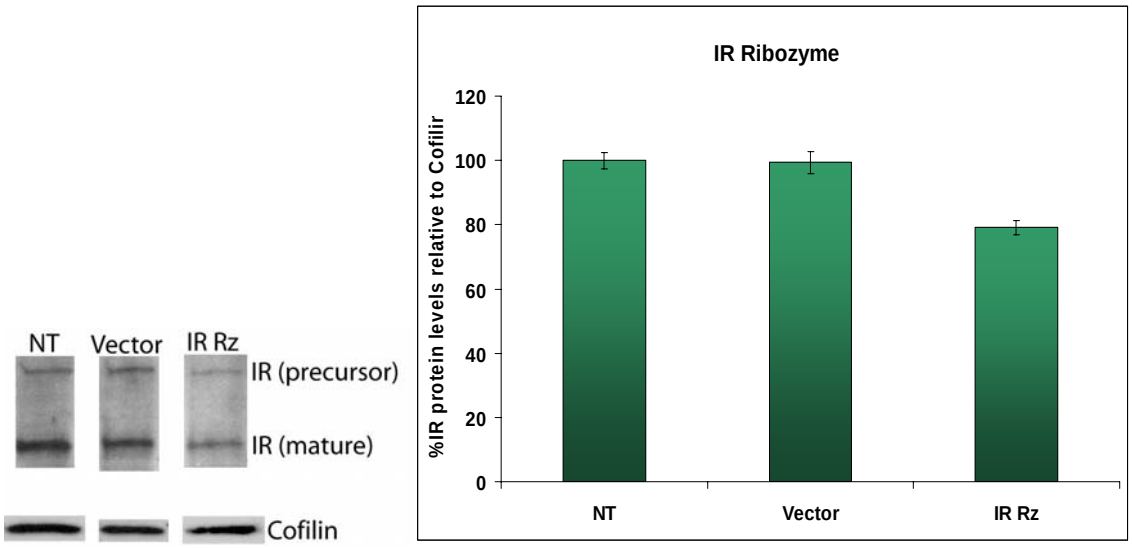

Figure 3.9. Western analysis of IR levels in cells expressing the human IR ribozyme.

While expression of inactive forms of the ribozymes resulted in no reduction in mRNA levels, there was a significant reduction in protein levels (30.8±3.6% for inactive IGF-1R Rz1). This reduction resulted from the antisense binding of the ribozyme to the target mRNA. VEGFR-1 and VEGFR-2 proteins levels were measured by flow cytometry rather than western analysis. As expected, the VEGFR-1 ribozyme reduced both VEGFR-1 mRNA and protein levels and the VEGFR-2 ribozyme reduced both VEGFR-2 mRNA and protein levels. In addition the VEGFR-1 ribozyme reduced the levels of VEGFR-2 mRNA and protein and the VEGFR-2 ribozyme reduced the levels of VEGFR-1 mRNA and protein. These results demonstrated that there is co-regulation between these two receptors.

Table 3.4. Reduction in protein levels by the ribozymes.

Ribozyme	Reduction in protein levels		*P* value
IGF-1R Rz1 active	47.7±10.6%		$5.4x10^{-5}$
IGF-1R Rz1 inactive	30.8±3.6%		$4.6x10^{-5}$
IR	20.9±2.1%		0.006
VEGFR-1	R-1 protein: 66.7%	R-2 protein:34.9%	<0.01
VEGFR-2	R-1 protein: 15.4%	R-2 protein:41.9%	<0.01
VEGFR-1 + VEGFR-2	R-1 protein: 64.1%	R-2 protein:27.9%	<0.01

Migration Assays

Figure 3.10 examines the ability of transfected HRECs to migrate in response to increasing concentrations of IGF-1. The cell migration was examined for HRECs transfected with the vector, or plasmids expressing the IGF-1R ribozyme 1 or ribozyme 2, or the inactive ribozyme 1. Migration assays were performed in a modified Boyden chamber. The active IGF-1R ribozyme 1 and 2 demonstrated a reduction in migration of approximately 91% and 58%, respectively. Inactive ribozyme 1 also showed approximate 51% reduction in cell migrations. This reduction possibly resulted from antisense inhibition of the IGF-1R protein.

The effect of the VEGFR-1 or VEGFR-2 ribozymes on migration was also examined (Figure 3.11). For these assays VEGF-E or placental growth factor (PlGF) was used to stimulate migration of HRECs. VEGF-E specifically binds to VEGFR-2 while PlGF specifically binds to VEGFR-1. The ability of HRECs, transfected with vector DNA, to migrate across a membrane to solutions containing either VEGF-E, PlGF or the heterodimer VEGF-E/PlGF was measured. HRECs expressing the VEGFR-1 ribozyme did not migrate toward PlGF, suggesting that they lacked VEGFR-1. HRECs expressing the VEGFR-2 ribozyme did not migrate toward a VEGF-E suggesting that they lacked VEGFR-2.

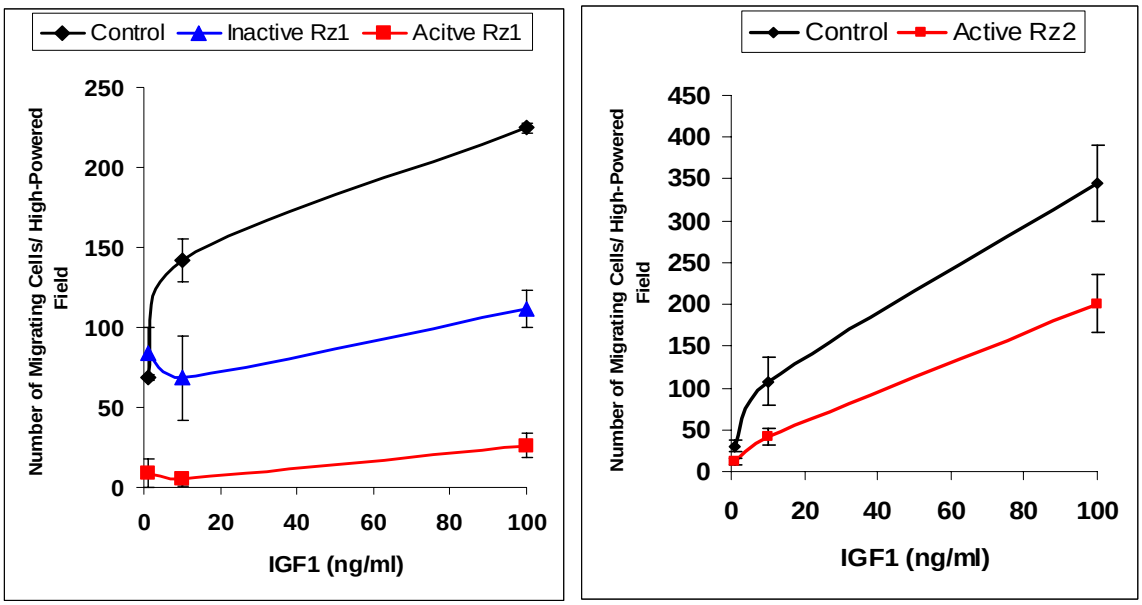

Figure 3.10. HREC migration assays in response to IGF-1.

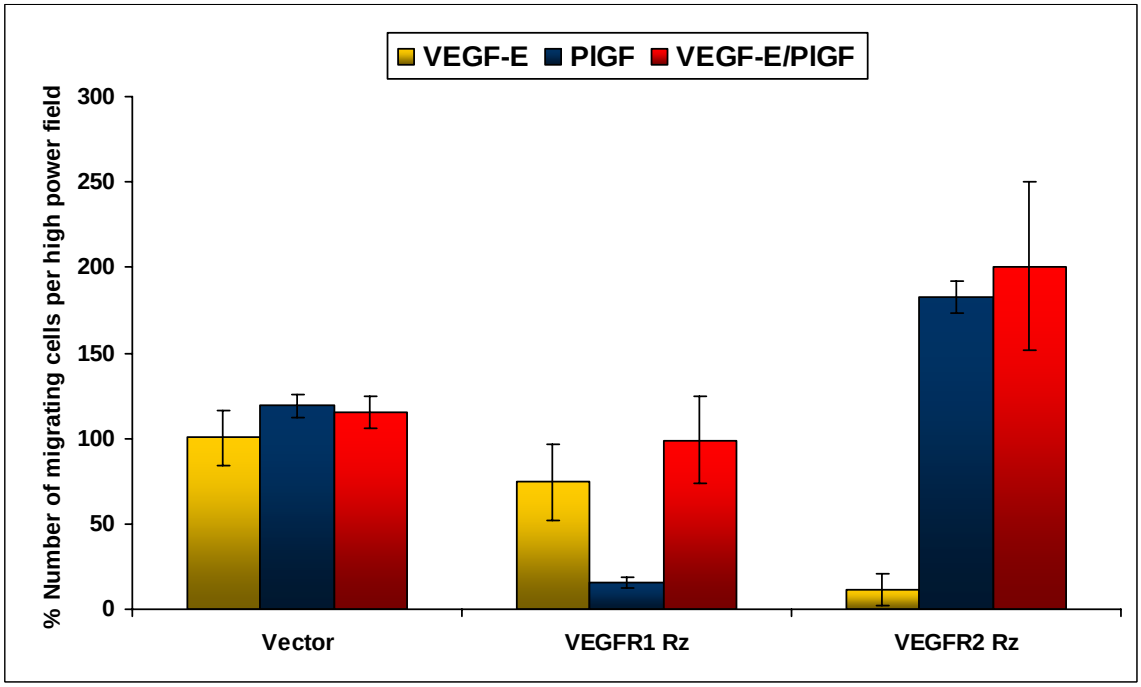

Figure 3.11. Effect of the VEGFR-1 and VEGFR-2 ribozymes on HREC migration.

Cell Proliferation Assays

Cell proliferation was measured by cellular incorporation of bromo-uridine (BrdU). Cells transfected with the vector, or plasmids expressing the VEGFR-1 or

VEGFR-2 ribozymes or the IGF-1R ribozyme were examined. Results are shown in Figure 3.12. The incorporation of BrdU in vector-transfected cells was set to 100%. VEGFR-1 ribozyme expression reduced incorporation by 42.7±12.5% (P=5.1x10^{-4}), VEGFR-2 ribozyme expression reduced incorporation by 50.25±1.9% (P=1.3x10^{-5}), and IGF-1R ribozyme expression reduced incorporation by 83.7±0.7% (P=6.8x10^{-7}).

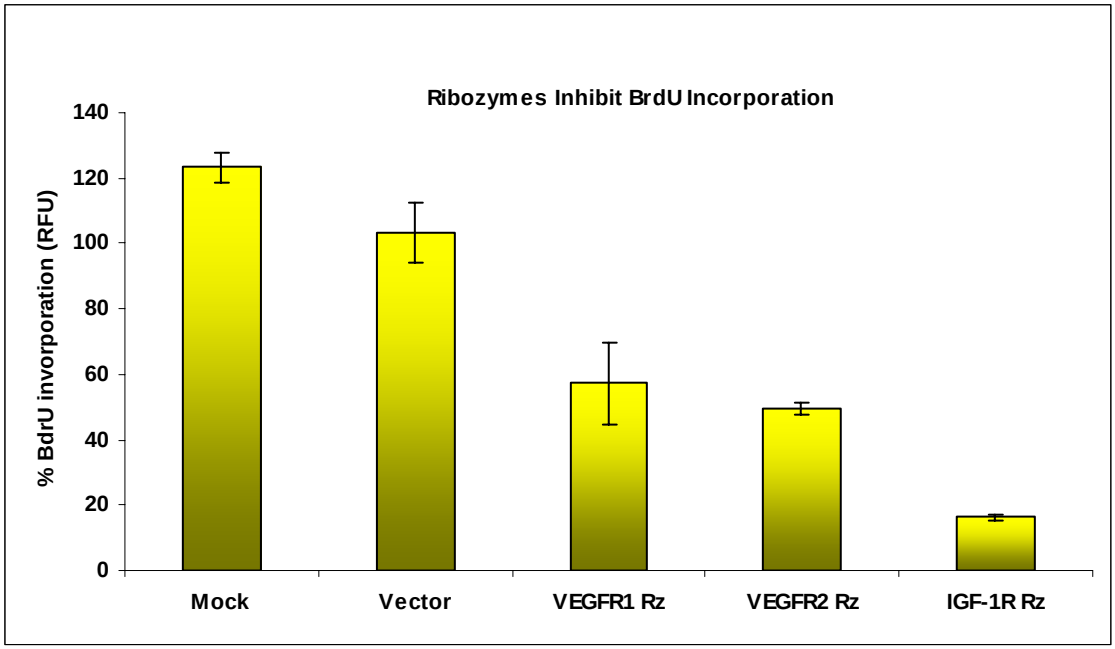

Figure 3.12. Effect of ribozyme expression on cell proliferation.

Tube Formation Assays

The ability of HRECs to form tubes on Matrigel is another basic function of endothelial cells. HRECs would form honeycomb-like structures consisting endothelial tubes naturally when cultured on Matrigel. When cells were transfected with the plasmid expressing the IGF-1R ribozyme, or the VEGFR-1 ribozyme or the VEGFR-2 ribozyme; tube formation was completely inhibited (Figure 3.13). The empty vector transfected cells were used as the control.

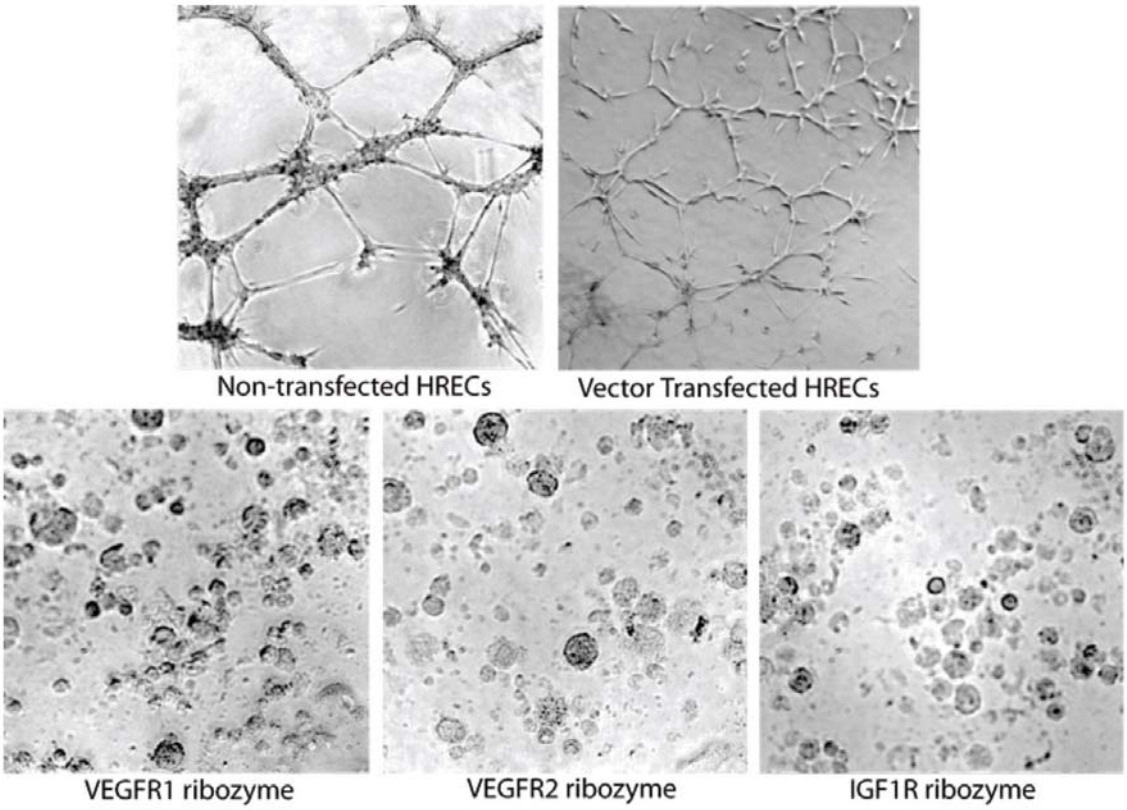

Figure 3.13. Effect of ribozymes on HREC tube formation.

In Vivo Analysis of Ribozymes

The *in vivo* effects of the ribozymes were examined in the mouse model of oxygen-induced retinopathy (OIR). Figure 3.14 is a cross section of mouse pup eye, stained with H&E, from the OIR model. All major anatomical parts of the eye are shown in this figure. Pre-retinal blood vessels (green arrows) grew beyond the retinal inner limiting membrane into the vitreous space. They are the representation of abnormal neovascularization. The measure of aberrant neovascularization was determined by the average number of pre-retinal blood vessel nuclei per section.

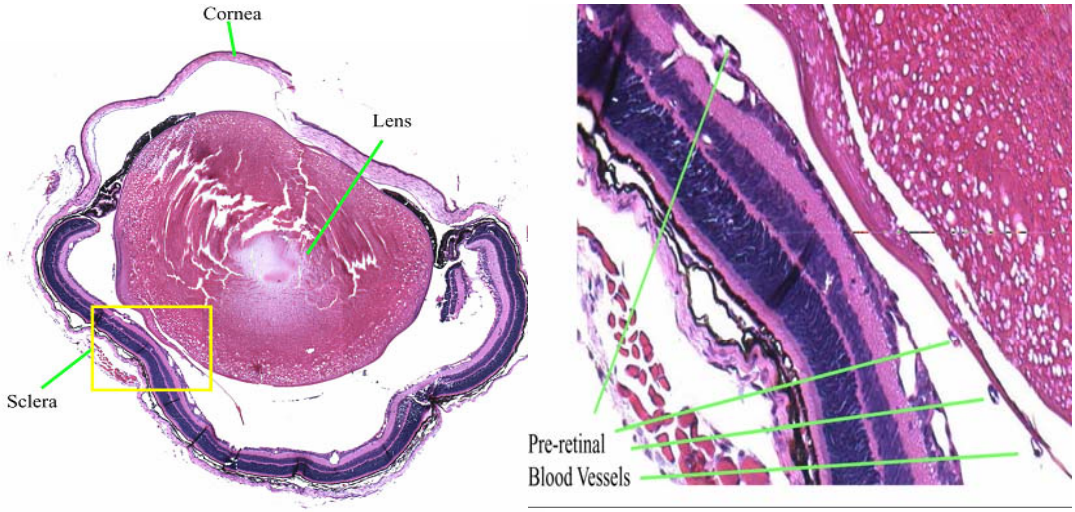

Figure 3.14. Cross section of a mouse eye showing pre-retinal vessels.

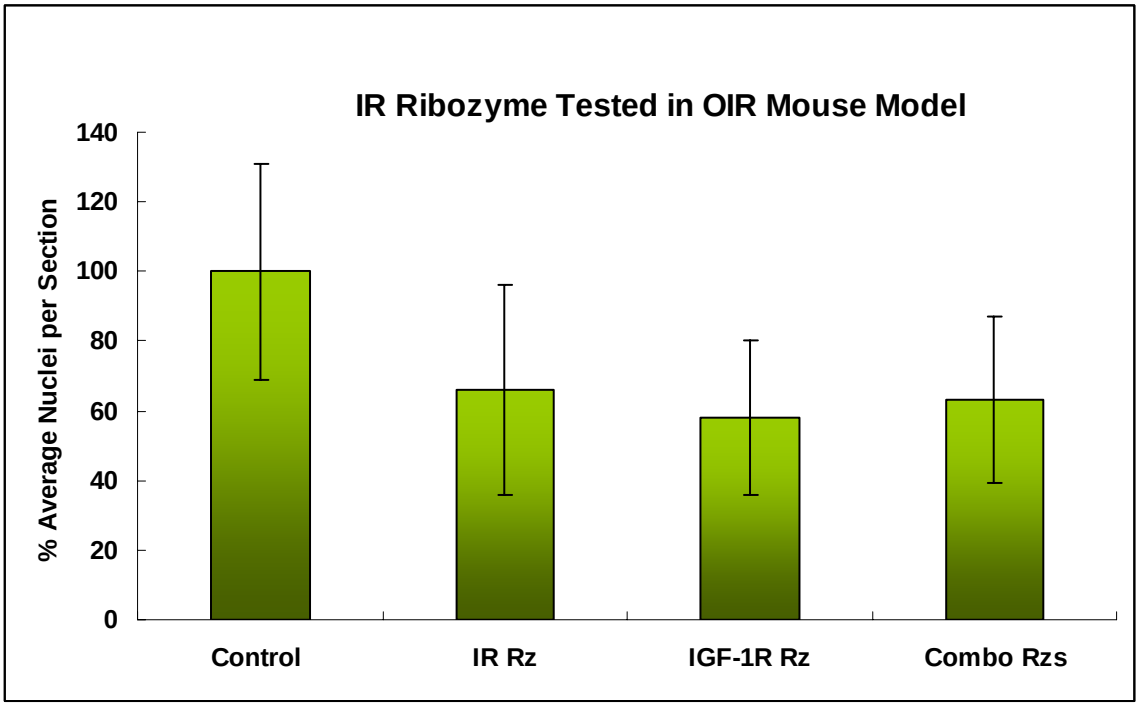

Figure 3.15. Ribozyme reduction of pre-retinal neovascularization in the OIR model.

Table 3.5 summarizes the results of the OIR mouse model assays on all ribozymes tested. Rows separated by solid lines are different groups of mice and dotted lines separate different test litters in the same group. Inactive ribozymes led to reductions in pre-retinal neovascularization to some extent. However, the reduction found with inactive

IGF-1R ribozyme 1 and 2 were minimal or close to significant ($P<0.05$ is considered significant), while the reductions resulted from inactive Integrin ribozymes were significant.

Table 3.5. All ribozymes tested *in vivo*

Ribozyme	Reduction in average nuclei per section	*P* value
IGF-1R Rz1 active	64.7±3.6%	$2.7x10^{-5}$
IGF-1R Rz1 inactive	17.3±5.1%	0.03
IGF-1R Rz2 active	51.7±2.2%	$2.3x10^{-5}$
IGF-1R Rz2 inactive	10.1±13.8%	0.09
VEGFR-1	47.0±5.0%	$5.3x10^{-4}$
VEGFR-2	75.5±5.0%	$7.5x10^{-8}$
Integrin α1 active	88.8±6.4%	$5.44x10^{-7}$
Integrin α1 inactive	46.2±6.3%	$1.7x10^{-3}$
Integrin α3 active	83.5±4.0%	$1.31x10^{-5}$
Integrin α3 inactive	63.7±13.7%	$1.2x10^{-4}$
IR	34.0±30.3%	$8.36x10^{-7}$
IGF-1R	42.0±22.1%	$1.66x10^{-8}$
IR + IGF-1R (Combo)	36.6±24.0%	$1.27x10^{-4}$

Promoter Development

The expression of ribozymes cloned into the p21NewHp vector was driven by the CMV enhancer/chicken β-actin promoter. This promiscuous enhancer/promoter was active in numerous cell lines and under a variety of physiological states. Thus, using this promoter could be a problem since it will result in the expression of the ribozymes in multiple cell types and tissues. The targets of our ribozyme were physiologically required for normal retinal development and function. We only wanted to inhibit the abnormal expression of the target proteins while leaving normal expression alone. But ubiquitous ribozyme expression could lead to the oblation of all normal and abnormal expression and lead to adverse effects. This was observed with the integrin ribozymes.

Integrin Ribozyme Expression *in vivo* with the CMV/β-actin Enhancer Promoter

As detailed in the introduction the various integrin αβ dimers are very important for cell adhesion and migration and they play essential roles in normal eye development. We observed severe structural abnormalities in the eye in the OIR mouse model after injection of plasmids expressing ribozymes to the α1 and the α3 subunits of integrin.

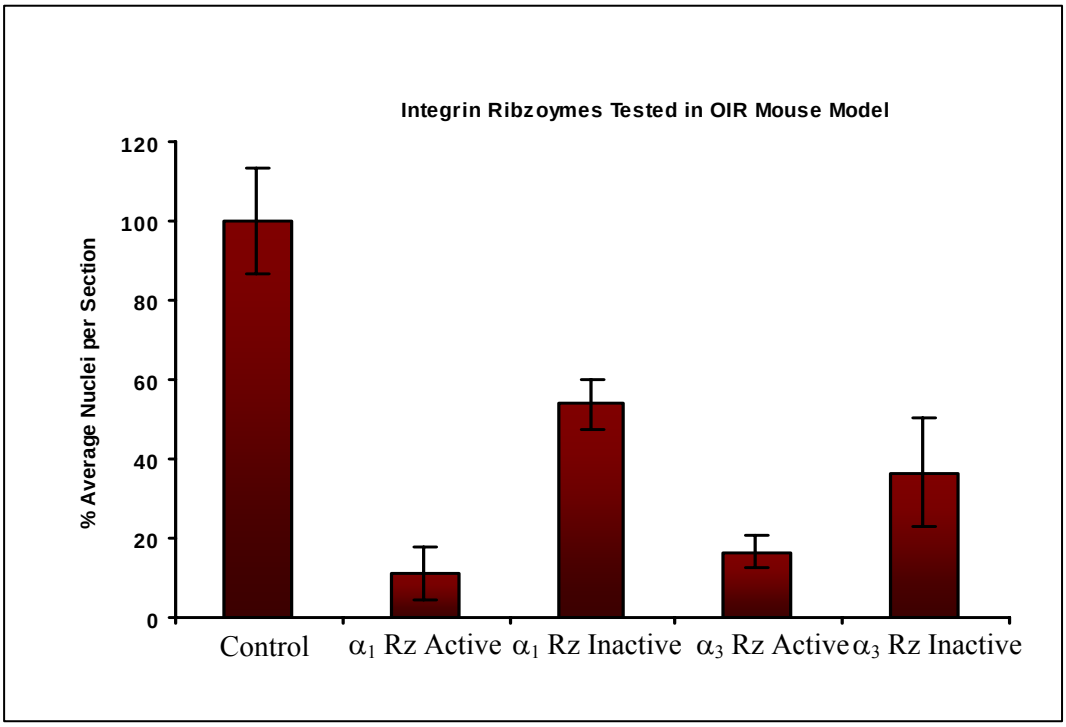

Figure 3.16. Reduction of pre-retinal neovascularization in the OIR mouse model with expression of the α1 or α3 integrin ribozymes.

The α1 and α3 ribozymes significantly reduced the amount of pre-retinal neovascularization in the OIR mouse model (Figure 3.16). Active α1 and α3 ribozymes resulted in 88.8±6.4% (P=5.44x10^{-7}) and 83.5±4.0% (P=1.31x10^{-5}) reduction in pre-retinal neovascularization, respectively. Their inactive forms resulted in a less signification reduction, 46.2±6.3% (P=1.7x10^{-3}) for inactive α1 ribozyme and 63.7±13.7% (P=1.2x10^{-4}) for inactive α3 ribozyme. These reductions with active or inactive ribozymes were much greater than any other ribozymes we tested in the same

mouse model. We also found that even the inactive versions of these two ribozymes could cause structural abnormalities in the developing eye due to antisense inhibition.

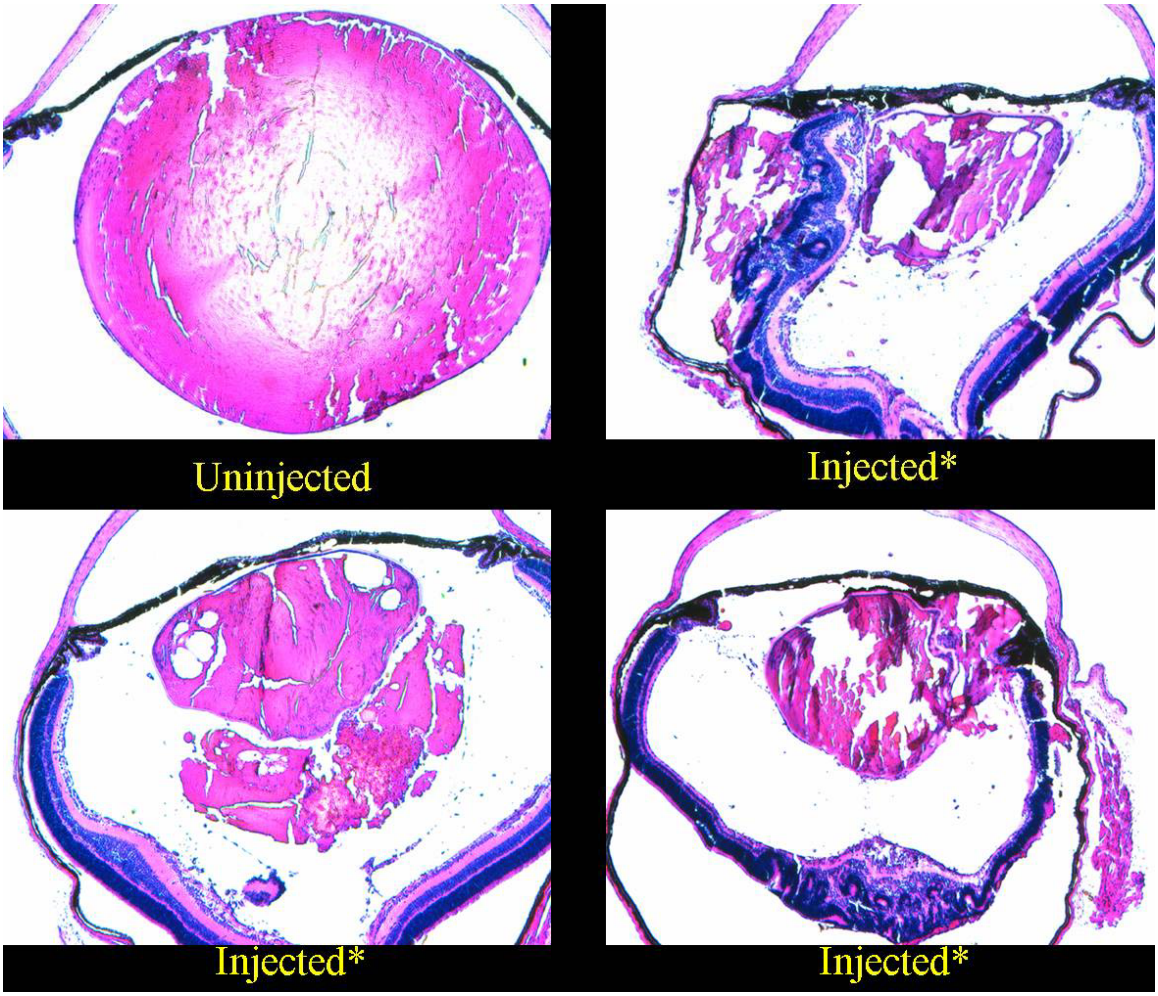

Figure 3.17. Expression of α1 ribozyme in OIR model results in severe deformations of the eye.

Figure 3.17 shows cross sections of a normal uninjected eye and of eyes expressing the integrin ribozymes. Overall the injected eyes showed a number of abnormalities, such as lens separation, retina detachment and closed iris. In addition the injected eyes were smaller than the uninjected eyes (although it is not unusual for intravitreal injection to affect eye size). While integrin ribozyme reduced neovascularization, these integrin ribozymes also significantly inhibited the normal

development of the eye. It is so interesting that the antisense effect of the inactive ribozymes was powerful enough to interfere with the developmental process. Due to the severity of deformation found with the CMV expression of the integrin ribozymes, we decided to use a proliferating endothelial cell specific promoter for integrin ribozyme expression.

The Proliferating Endothelial Cell-Specific Promoter

To overcome potential expression problems with the CMV/β-actin promoter, we cloned and tested our ribozymes in a vector that had a proliferating endothelial cell-specific promoter. Dr. Sullivan designed and constructed this promoter. Using this promoter, we were hoping to only target proliferating endothelial cells while not affecting the quiescent endothelial cells in the developed vasculature and any other cells in the retina.

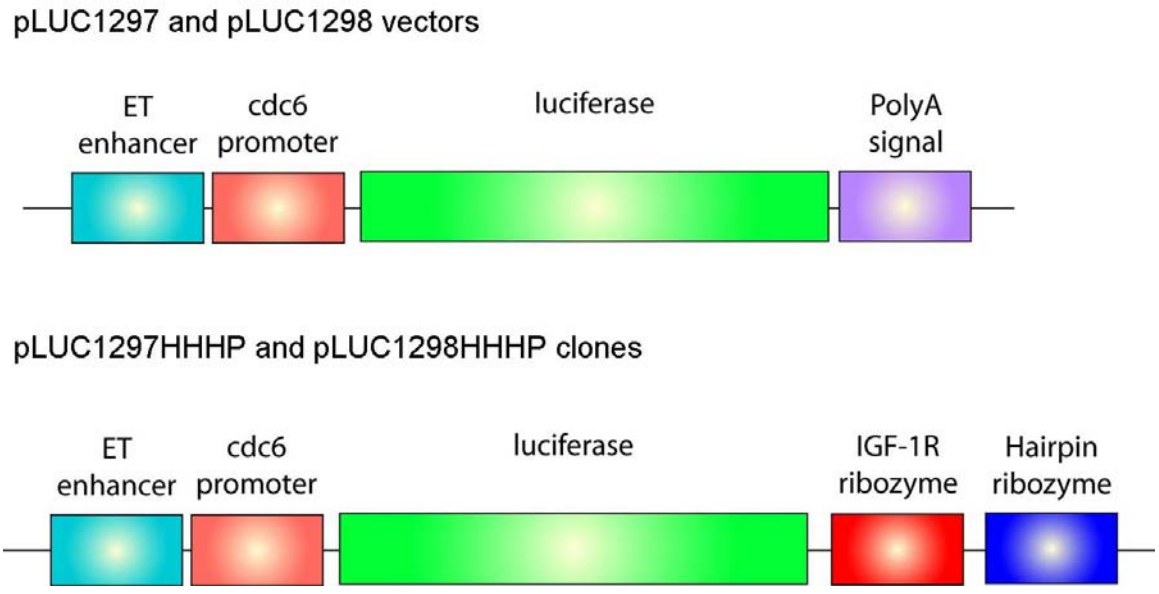

Figure 3.18. pLUC1297/1298 vectors and pLUC1297HHHP/1298HHHP clones

Dr. Sullivan tested a number of enhancers and promoters and eventually found that the combination of endothelin enhancer/cdc6 promoter provided the best specificity

to endothelial cells. Endothelin (ET or ET-1), which is exclusively synthesized by vascular endothelium, is one of the most powerful vasoconstrictors known. Cdc6 is a 30,000-dalton protein essential for the initiation of DNA replication. This protein functions as a regulator at the early steps of DNA replication. It is thought to be involved in the assembly of minichromosome maintenance proteins onto replicating DNA. It localizes in the cell nucleus during cell cycle G_1, but translocates to the cytoplasm at the start of S phase. Quiescent cells in G_0 do not express this protein. Therefore in this specific vector, ET enhancer determined the expression specificity in endothelial cells and cdc6 promoter further narrowed the specificity into proliferating endothelial cells.

Dr. Sullivan produced two specific promoters, both of which had the cdc6 promoter. One vector had a 4X multimer of the ET enhancer, designated pLUC1297, and the other vector had 7X multimer of the ET enhancer, designated pLUC1298. The structure of these promoters is shown in Figure 3.18. Downstream of cdc6 promoter was a luciferase reporter gene followed by PolyA signal. The IGF-1R ribozyme was cloned and these two vectors followed by a self-cleaving hairpin ribozyme that generated a discrete 3'-end to the run-off transcript. (pLUC1297HHHP and pLUC1298HHHP).

The four plasmids, pLUC1297, pLUC1298, pLUC1297HHHP and pLUC1298HHHP, were transfected into HRECs and fibroblast cells to examine the cell-specific expression of luciferase. We tested the expression in two fibroblast cell lines, shown as F1 and F2 in Figure 3.19, and in HRECs from two different donors, shown as HREC 10 (ten-year-old donor) and HREC 14 (fourteen-year-old donor) in Figure 3.19. pLUC1297 and pLUC1298 showed high levels of luciferase expression in HRECs compared with fibroblasts (about 200 times higher in HREC 10). Similarly

pLUC1297HHHP and pLUC1298HHHP also showed higher levels of luciferase in HRECs compared with fibroblasts (about 20 times higher in HREC 10). Luciferase expression was higher in HREC 10 than in HREC 14, which probably resulted from a difference in the donor cells. When comparing pLUC1297HHHP with pLUC1297, or pLUC1298HHHP with pLUC1298, we found that the ribozyme-inserted constructs had much lower luciferase expression level than their parent constructs. This is probably due to the loss of the PolyA signal in the plasmids.

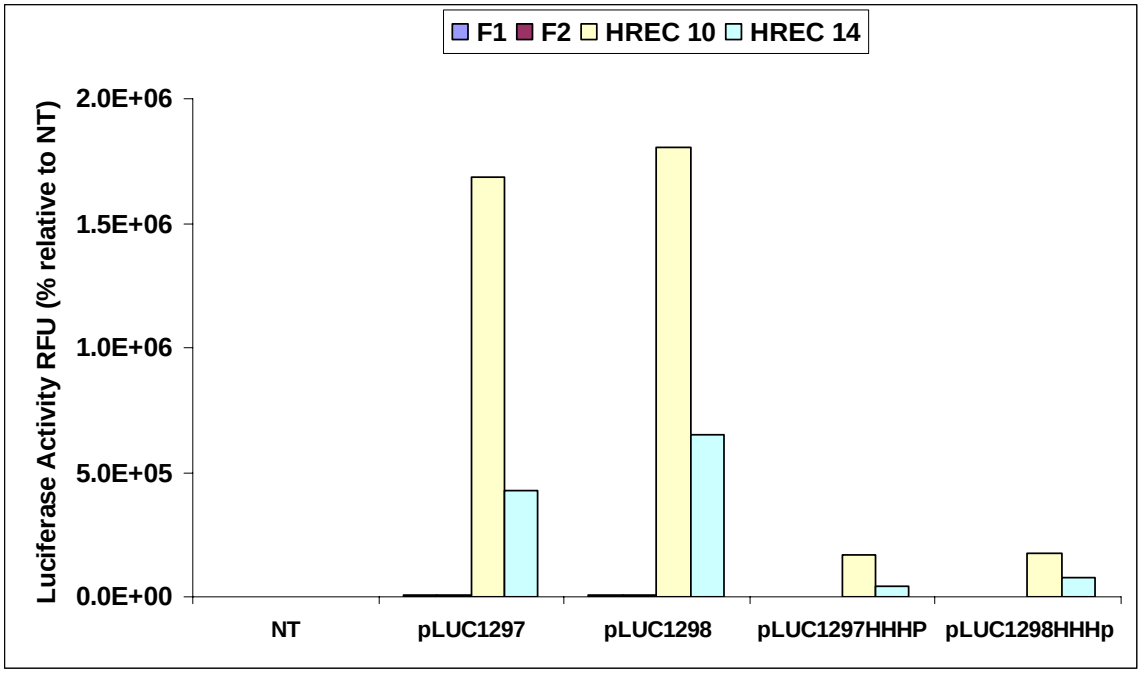

Figure 3.19. Verification of the cell specificity of the proliferating endothelial cell-specific enhancer/promoter.

The New Promoter Tested *in vivo*

We tested the pLUC1298HHHP construct in the OIR mouse model. Figure 3.20 shows confocal images of eyes from these experiments. OS (left eyes) were un-injected eyes and OD (right eyes) were injected eyes. The vessels were labeled with endothelial cell specific agglutinin conjugated with rhodamine, and luciferase expression was

immunofluorescently shown in green by secondary antibody. Uninjected eyes (panels A and C) showed background green fluorescence, while the injected eyes (panels B, D, E and F) showed expression of luciferase only on the vasculature (green and yellow). The magnifications of panel E and F were 400x, and panel F was showing greater detail of the boxed part in panel D. When comparing panel C and D (magnification 200x), there was a greater density of abnormal, small, blood vessels evident in the uninjected eye (panel C) while the injected eye (panel D) showed a lower density of blood vessels on the retina. This suggests that the IGF-1R ribozyme was actively expressed and was reducing pre-retinal neovascularization. This was confirmed by examining H&E stained cross sections that quantitatively showed a reduction in pre-retinal neovascularization in injected eye as detailed below.

Figure 3.21 shows the results of the OIR mouse model injections with the proliferating endothelial cell-specific constructs. Blue bars are injected eyes and brown bars are uninjected eyes. There were five groups of mice, injected with pLUC1297, pLUC1298, pLUC1297HHHP, pLUC1298HHHP, pGE1298HHHP respectively. pGE1298HHHP had the same structure as pLUC1298HHHP except that the luciferase reporter gene was eliminated. Uninjected eyes from all groups were averaged together and the average nuclei number was set as 100%. The eyes injected with pLUC1297 and pLUC1298, compared with uninjected eyes, showed no significant difference as expected (P=0.47, 0.37, respectively). The eyes injected with the ribozyme-expressing constructs showed significant reduction in pre-retinal neovascularization. pLUC1297HHHP showed 48±12% (P=3.58x10^{-7}) reduction; pLC1298HHHP showed 54±11% (P=1.46x10^{-4}) reduction; and pGE1298HHHP showed 59±11% (P=2.89x10^{-10}) reduction.

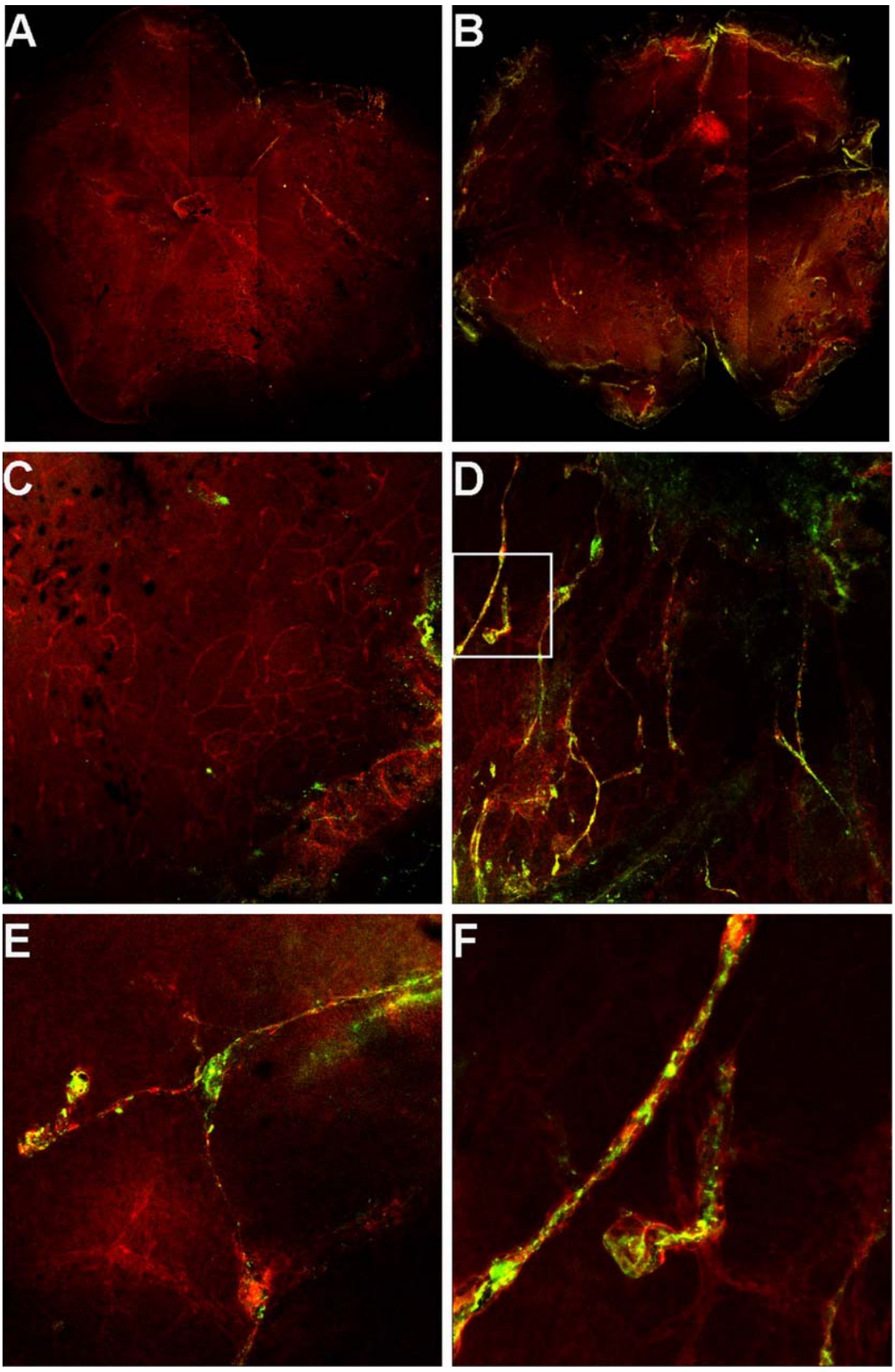

Figure 3.20. The proliferating endothelial cell-specific promoter limits expression of luciferase to the actively proliferating blood vessels in the OIR model.

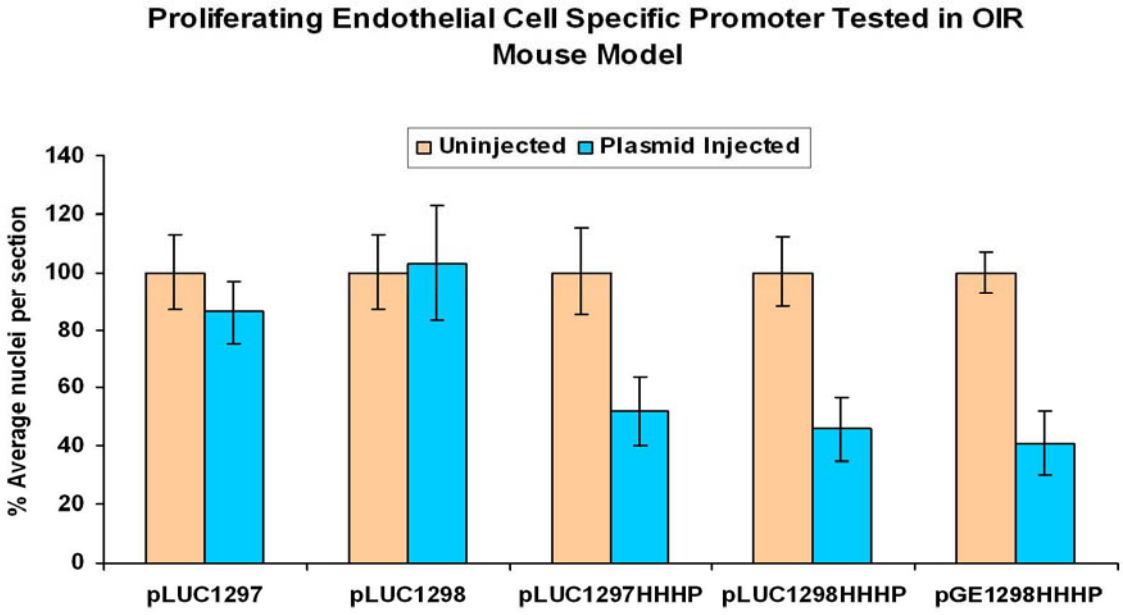

Figure 3.21. Quantitative assessment of the IGF-1R ribozyme's ability to inhibit pre-retinal neovascularization when expressed from the promoter.

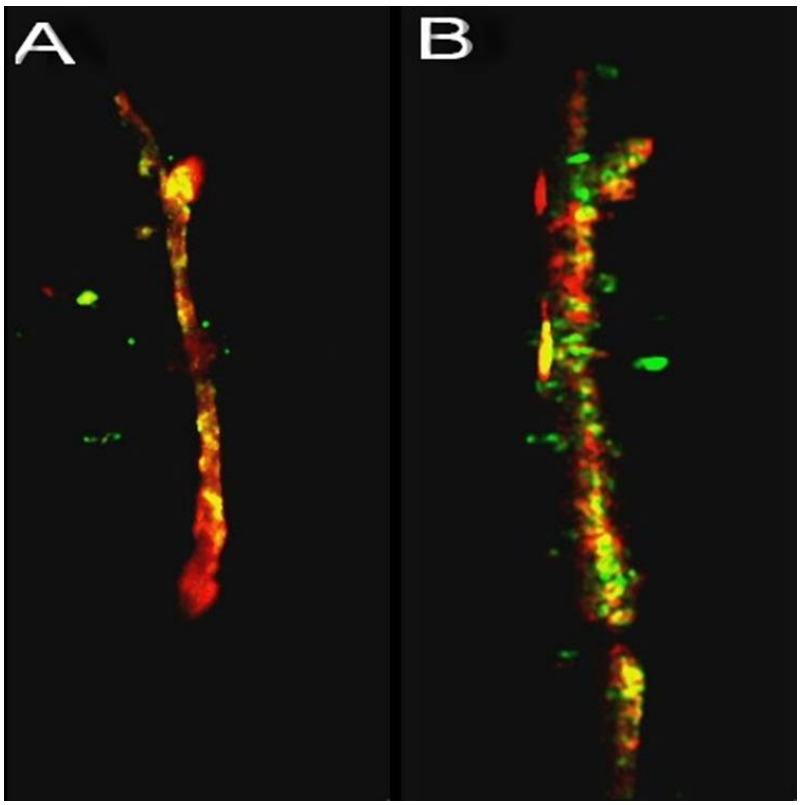

Figure 3.22. New promoter tested in adult mouse model of laser-induced neovascularization.

The proliferating endothelial cell specific constructs were also tested in an adult mouse model of laser-induced neovascularization. The eyes from adult mouse were also stained with endothelial cell specific agglutinin conjugated with rhodamine (red) and a secondary antibody bound to luciferase (green). A video clip was made from the animation of a stack of pictures (400x) focused at same horizontal position but different vertical levels, about 1μm apart between levels, taken using a confocal microscope. Panel A in Figure 3.22 is a snapshot of the clip. A z-projection view of the same vessel was made using imageJ and Panel B is a snapshot of the z-projection view. The green staining was not seen in the interstitial space outside vasculature, but was colocalized with blood vessels (yellow color for colocalization). Also the green staining was in cell-like shape aligned on vessel walls, which indicated the luciferase expression occurred in endothelial cells in these small vessels. For a better view of the colocalizations, please see the supplementary movie clips.

Object 3.1. A blood vessel from the adult mouse model shows the luciferase expression is specific for proliferating endothelial cells.

Object 3.2. The 3-D view of the blood vessel from the adult mouse model.

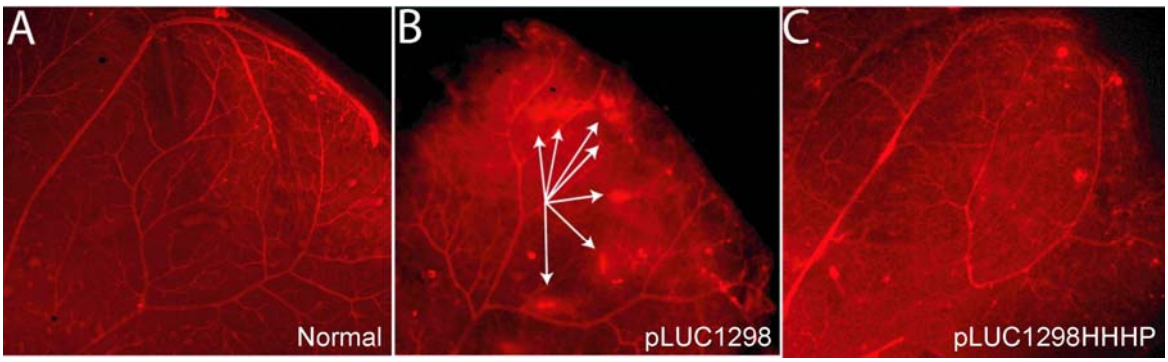

Figure 3.23. The expression of the IGF-1R ribozyme from the new promoter reduced aberrant blood vessel formation in the adult laser model.

We also perfused retina from the adult mouse model with rhodamine-labeled dextran to examine the state of the vasculature on the retina (Figure 3.23). The left panel showed the normal vasculature with no abnormal (leaky) neovascularization. The middle panel is the eye that had neovascularization induced by laser treatment, also injected with empty vector pLUC1298. The hazy areas indicated the leaky small vessel resulting from the abnormal neovascularization. The right panel is the eye treated by laser but injected with pLUC1298HHHP, the IGF-1R ribozyme expressing construct. Compared with normal retina we can still see some hazy areas but there were much less of them in quantity and the size of leaky areas than pLUC1298-injected eye. This indicated the IGF-1R ribozyme inhibited laser-induced neovascularization to some extent.

The New Promoter Tested with Integrin Ribozyme

The proliferating endothelial cell specific promoter was used to express the integrin ribozymes *in vivo*. Five mouse pups were injected with the plasmid in one eye on day 1 of the OIR mouse model as usual. The eye sections are shown in Figure 3.24.

Eye A in Figure 3.24 is the section of an uninjected eye. Eyes B, C, D, E and F are sections from injected eyes. Many abnormalities still existed, such as smaller size in some injected eyes (especially eyes C and D), detached retina from choroid, unusual folding in the retina. Nonetheless, compared with CMV/β-actin-driven expression of the integrin ribozymes, much less eye deformation resulted. The lens in the injected eyes were normal. With the CMV/β-actin promoter no open iris was found on any cross sections. Now, most eyes have an open iris. Eye D also had an open iris but the section shown was too close to the cornea/iris boundary to see the pupil. Eye E looked no difference with a normal eye.

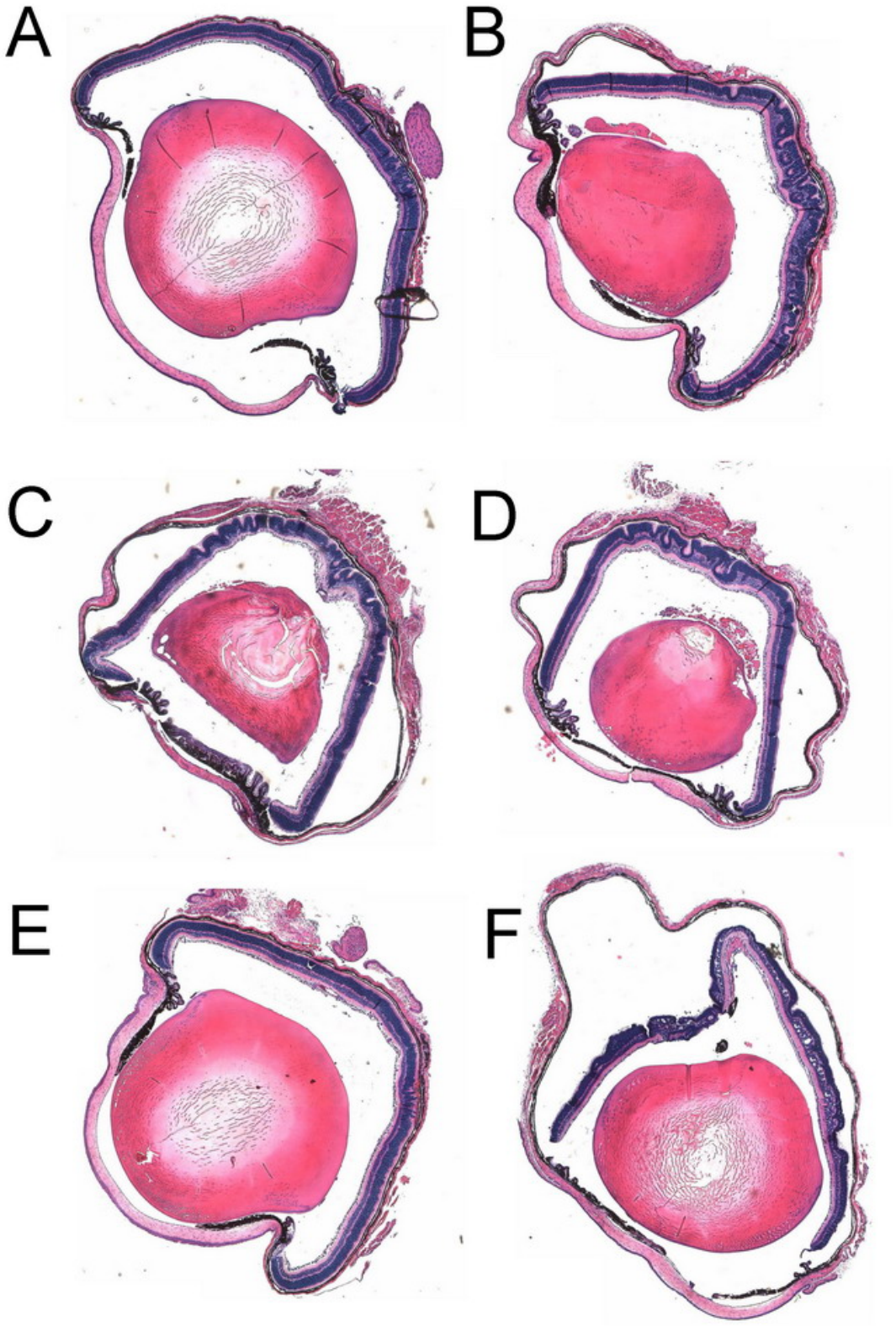

Figure 3.24. Expression of integrin ribozyme driven by proliferating endothelial cell-specific promoter resulted in less eye deformation.

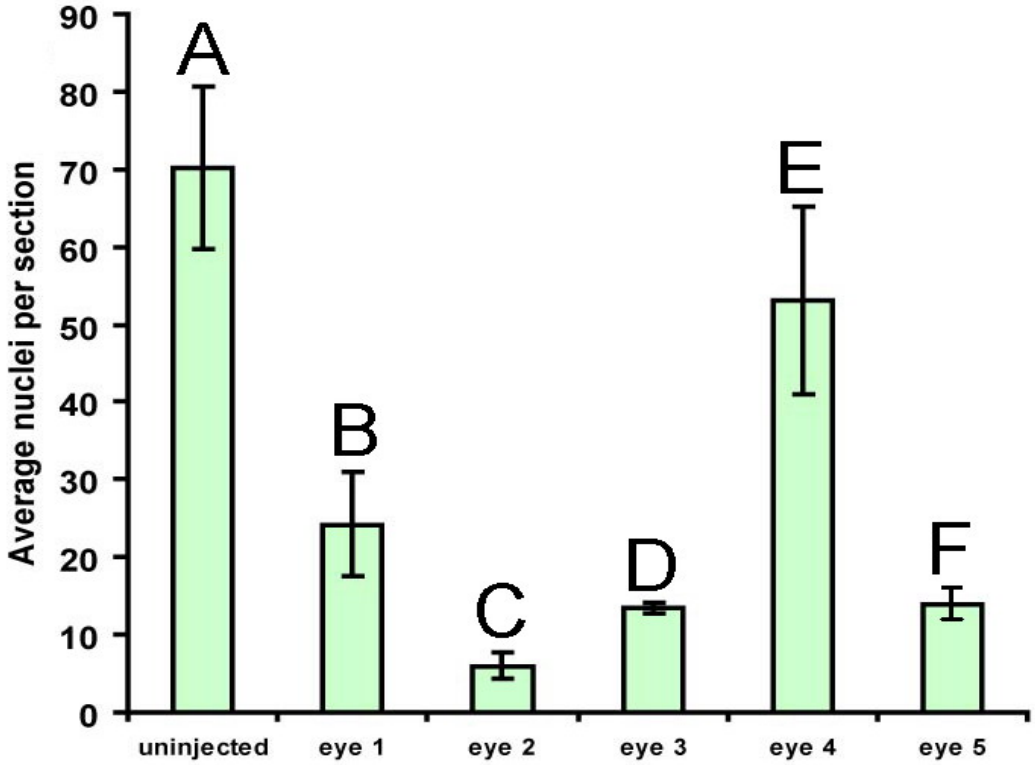

Figure 3.25. Proliferating endothelial cell specific promoter with integrin ribozyme tested in OIR model

The neovascularization quantification results showed different levels of reductions in the injected eyes (24.3±17.1% to 91.4±2.9% as shown in Figure 3.25). Interesting, the "most deformed" eye (eye C) showed greatest reduction, while the "most normal" eye (eye E) showed least reduction.

CHAPTER 4
DISCUSSION

This project involved developing multiple ribozymes and testing them *in vitro* and/or *in vivo*. Our primary focus was to use these ribozymes to inhibit the expression of proteins that play important roles in the abnormal retinal neovascularization. A number of proteins were chosen as our targets in this study, including IGF-1R, IR, VEGFR-1, VEGFR-2, and integrins. They have different functions but are all important for endothelial cell physiology such as proliferation and migration, which are essential in the development of neovascularization.

The developing the testing steps were similar to all these ribozymes. We selected target sites in protein gene sequence, designed the ribozyme accordingly, tested cleavage reactivity *in vitro*, transfected and functionally analyzed ribozymes in HRECs regarding mRNA levels, protein levels, physiological functions of HRECs, and eventually tested in mouse models *in vivo*.

Ribozyme Testing Results and Antisense Effect

All of these ribozymes have been shown to have cleavage reactivity and can reduce the expression of target proteins. All the testing results have been summarized in tables in the previous chapter. Taking IGF-1R ribozyme 1 as an example, more than 90% of the target RNA oligos were cleaved within the first 2 minutes in the cleavage time course study, indicating the ribozyme was highly catalytically active. After HRECs were transfected with the IGF-1R ribozyme, mRNA levels for IGF1-R were reduced by about 40% compared with vector-transfected cells, and the inactive version did not result in any

significant reduction. The IGF-1R ribozyme decreased protein expression by 48% and the inactive version also decreased the protein expression about 31%. This decrease with inactive ribozyme treatment may have been caused by its antisense effect, in which the catalytic-deficient ribozyme cannot cleave the mRNA but still complimentarily binds to target site and physically blocks translation. This effect was not unexpected to exist in studies that involve protein expression and function. For example, in the migration study, we observed 91% reduction in the cell's ability to migrate with active IGF-1R transfection and 58% reduction with inactive IGF-1R transfection, not surprising for the active version.

IGF-1R ribozymes 1 and 2 induced 65% and 52% reductions in the pre-retinal neovascularization levels. Their inactive forms also resulted in reductions; these reductions are minimal or close to significant (P=0.03, P=0.09, respectively), so there was minimal antisense effect in the *in vivo* test. This is not consistent with *in vitro* studies in HRECs but our explanations are: 1) A threshold must be achieved in the reduction of protein expression to see a reduction in functional analysis. The threshold may differ in different species and vary with methods of assay. 2) The number of IGF-1R proteins differs substantially in different situations including but not limited to species, cell phases, study conditions (*in vitro* vs. *in vivo*), and so on. According to Rubini et al. [355], taking mouse fibroblasts as a reference, cells in proliferation have more than 30,000 IGF-1R proteins per cell while quiescent cells only have 15,000 to 20,000 IGF-1R proteins. In our studies, most *in vitro* experiments were done with confluent cultured cells, but *in vivo* experiments were done in developing retinas. So the antisense effect might have been diluted in the mouse model. However the active ribozymes still resulted in

neovascularization reductions in that they can completely cleave and oblate the protein functions versus possibly partial function inhibition with inactive ribozymes blockade. More importantly, the active ribozyme has catalytic ability so it can “process” much more proteins than inactive version to physically block in a 1:1 molar ratio.

VEGFR-1 and VEGFR-2 Interactions

In the traditional view, VEGFR-1 functions as a decoy receptor and negatively regulates VEGFR-2 signaling. This is basically accomplished by VEGFR-1 acting as a sink, binding VEGF ligand, and preventing activation of VEGFR-2. However, some recent data indicate that the kinase activity of VEGFR-1 plays an essential role during pathological angiogenesis and in wound healing, by potentiating VEGFR-2 signaling [100, 356, 357]. It has been accepted that there is cross talk between VEGFR-1 and VEGF-R2 signaling. PI3 kinase [356] and nitric oxide [358]have been proposed to be involved in VEGFR-1 regulation of VEGFR-2. In our studies, we showed that transfection of HRECs with the VEGFR-1 ribozyme down-regulated theVEGFR-2 mRNA. On the other hand, the transfection of HRECs with VEGFR-2 ribozyme also downregulated VEGFR-1 mRNA. This is a further support of intra- and inter-molecular cross talk between the two receptors. In the OIR mouse model, the VEGFR-1 and VEGFR-2 ribozymes both significantly reduced pre-retinal neovascularization, by 47% and 75%, respectively. The inhibition on VEGFR-2 inhibited neovascularization to a greater extent, which is consistent with the major role of VEGFR-2 in promoting endothelial cell proliferation, migration and therefore angiogenesis, even though the interactions between VEGFR-1 and VEGFR-2 exist. Another recognized role for VEGFR-1 kinase activity is its capability of recruiting hematopoietic stem cells from bone marrow precursors [105, 359]. It is possible that the decrease in neovascularization

found from blocking of VEGFR-1 signaling may affect stem cell involvement. In one study, a chimeric protein containing both the VEGF binding domains of VEGFR-1 and VEGFR-2 was constructed and expressed in a murine model of ischemic retinopathy. A single intravitreal injection the chimeric protein resulted in a >90% reduction of retinal neovascularization compared with control eyes [360]. This suggested that a combined targeting of VEGFR-1 and VEGFR-2 may bring about a deeper reduction in retinal neovascularization than either receptor alone. A ribozyme that can target both receptors or an administration of both ribozymes together could be tested.

Besides the interactions inside VEGF system, IGF-1 may also crosstalk with VEGF signaling. We have shown that intravitreal injections of IGF-I result in an acute increase in vascular permeability and vascular engorgement, followed by development of pre-retinal angiogenesis in rabbit eyes [361]. In addition, IGF-1 production by HRECs, in turn, stimulates increased VEGF production [217] and visa versa [362]. It was also reported that elevated IGF-1 levels *in vivo* resulted in an increase in VEGF gene expression. Considering that VEGF and IGF-1 and their receptors can all be expressed by HRECs and both these growth factors have autocrine and paracrine function, it is reasonably to propose that the interaction between these signaling systems do exist and a better picture of their involvement in pre-retinal neovascularization should include their interactions.

Apart from endothelial cells, VEGF and its receptors are expressed in many other cell types, such as inflammatory cells [363]. VEGF may function in an autocrine fashion on these cells. Unlike diabetic retinopathy, the ischemia-induced retinopathy was thought to be inflammatory-free; however, it is now known that inflammatory cells are involved

[364]. These inflammatory cells may participate in the processes of blood-retinal barrier breakdown and neovascularization [115]. The plasmid expressing VEGF receptor ribozymes has a CMV/β-actin promoter and does not exclusively target endothelial cells, so it is possible that the inhibition of VEGF signaling on other cell types also contributed to the overall outcome of neovascularization reduction.

The Proliferating Endothelial Cell Specific Promoters

Expression of the IGF-1 ribozyme by the promiscuous CMV/β-actin promoter did not result in eye deformation like the integrin ribozymes. However, IGF-1 and its receptor still play a major role in vascular development of both mouse and human eyes, thus the indiscriminant loss of IGF-IR could result in altered vascular development and propagate the ischemia observed in ROP infants or OIR mice [365]. Therefore, we also used the cell specific promoter to express the IGF-1R ribozyme *in vitro* and *in vivo*. Our results demonstrated that the cell specific promoter limited expression to HRECs and no expression in fibroblasts. Our *in vivo* results also showed that the new cell specific promoter is active in the rapidly dividing vasculature of the eye.

During construction of the new IGF-1R ribozyme plasmid, it was observed that ligation of the IGF-IR hammerhead ribozyme and the hairpin ribozyme caused the endothelin enhancers to be deleted in several strains of bacteria, DH5, Stable 2s and Top Tens [365]. However, when placed at the 3' end of the luciferase gene, the hammerhead/hairpin ribozyme insertion did not cause the deletion of the endothelin enhancer. Thus the luciferase gene was also playing a role in stabilizing the construct. The insertion of the ribozymes made the vector lose the PolyA tail, which affected the stability of the transcript and reduced the expression levels of luciferase and IGF-1R

ribozyme. This was confirmed *in vitro* in HRECs (Figure 3.17). The luciferase expression from these two plasmids was lower than their parent vectors (pLUC1297, pLUC1298).

The *in vivo* study showed that the luciferase expression from pLUC1298HHHP was exclusive limited to proliferating endothelial cells. The colocalization of luciferase and proliferating endothelial cells was observed both in the OIR mouse model and the adult mouse model of laser-induced neovascularization. However, the reduction of pre-retinal neovascularization from pLUC1298HHHP (54±11%) were comparable with the reduction found with IGF-1R ribozyme driven by CMV/β-actin promoter (65±6%). In addition, expression of the IGF-1R ribozyme from the plasmid pGE1298HHHP, a modified version of pLUC1298HHHP with the deletion of luciferase gene, resulted in 59±11% reduction in pre-retinal neovascularization. Therefore the luciferase gene did not affect the expression of ribozymes.

It is known that systemic administration of plasmid DNA alone by hydrodynamic administration results in initial high levels of expression 24hrs after injection and decreases to 7% of the peak value by day 10 [366, 367]. In our experiments, the luciferase expression and ribozyme activity was observed 17 days after administration in OIR model and 21 days after administration in adult mouse model. It is worth mentioning that the expression of luciferase, and by extension of the IGF-1R ribozyme, from naked plasmid in OIR model or formulated plasmid in the adult mouse model exhibited significant expression through the time courses of the experiments.

The idea of introducing a promoter that is specific for proliferating endothelial cells originated from the integrin ribozyme *in vivo* study, since the ubiquitous knockdown of integrin resulted in severe eye deformation in the OIR mouse model. The expression of

the same integrin ribozyme (against integrin α1 subunit) driven by the specific promoter showed much fewer problems. However, the deformations were still significant. But due to the specificity of the promoter, these deformations must result from affecting endothelial cells at the rapidly proliferating vasculature of the eye. Even though decreases in abnormal neovascularization were found with the cell specific promoter, further refinement of the promoter, if possible, is required when expressing the integrin ribozymes. These problems, found with the integrin ribozymes, result from the roles of the α1 integrin subunit in numerous processes including their direct involvement in angiogenesis. These problems were not found with the IGF-1R ribozyme with either promoter type due to the limited function of this receptor in the developing vasculature of the eye. Therefore, while study of the integrin ribozyme will be useful from functional and developmental points of view, the use of integrins as therapeutic targets probably has limited or little value in the developing eye.

Other Voices on Neovascularization in Diabetic Retinopathy

As summarized earlier in the introduction chapter, there is a tendency to propose that the abnormal neovascularization in diabetic retinopathy is the chronic pathological consequence of hypoxia in the retina. However, oxygen is the not the only nutrient supplied through blood vessels.

Clinically the retinal angiography of diabetic retinopathy patients shows non-perfused capillaries [368], which is indicating that hypoxia taking effect. It has also been reported that hyperoxia improved contrast sensitivity in early diabetic retinopathy [369] and that the supplemental oxygen improved diabetic macular edema [370]. However no studies have directly demonstrated reduction of retinal oxygen levels in humans with diabetes compared with controls [368]. In the animal studies, there was no significant

difference in the pre-retinal oxygenation found between the cats [371] and dogs [372] within 1 year of diabetic onset and the controls. But in the long-term study, one group has reported that the retinal oxygen partial pressure was reduced in cats with 6-8 years of diabetes [373].

Despite the direct evidence in the long-term cat study, most other supporting data of hypoxia are based on the overexpression of growth factors that are regulated by HIF, such as VEGF and PDGF. Even HIF activity was increased in diabetic rats [374], it is not necessary that the increases in growth factor levels are directly linked to HIF. In the diabetic retinas, besides the vascular cells, many other cell types are affected or harmed, including neurons, glial cells and microglial cells [375]. It is possible that the need to maintain neuron-dependent vision motivates angiogenesis to compensate for the nutrient deficiency in the neural retina. In detail, VEGF could increase initially to provide trophic support to neurons through VEGF receptors, but at the cost of increased vascular permeability [368]. The physiological compensation response could convert into a pathological one in a chronic stress situation, and eventually lead to neovascularization and edema due to vascular leakage.

One common problem in hypoxic and nutrition deficient cells is endoplasmic reticulum (ER) stress. ER stress can influence VEGF and PEGF expression levels [376] and thus affect the balance between cell survival and death signals. So it is not unreasonable to hypothesize that ER stress could be a potential target in the treatment of neovascular diabetic retinopathy.

Final Words on RNA Silencing

We used hammerhead ribozymes as a tool to inhibit gene expression. Ribozymes are only one category of RNA silencing technologies. Gene silencing with antisense

oligonucleotides is the earliest discovered and utilized, the easiest to design and has no target sequence requirement other than target accessibility. But, one significant disadvantage is that antisense oligonucleotides function in a 1:1 molar ratio with target mRNAs. This means a significant reduction in translation level may not be achieved without a fairly large amount of antisense oligonucleotides. Ribozymes, however, can catalyze the cleavage of target mRNAs and will be recycled and reused again, thus the dose of the RNA silencing agents can be significantly reduced. However, the sequence requirement for ribozymes is the major obstacle in the development into convenient RNA silencing tools. RNAi, an endogenous and ubiquitous pathway, doesn't have much sequence requirement on targets. RNAi has other advantages such as ease of design, ease of synthesis and high specificity. Silencing with RNAi has been reported to exceed what can be achieved by antisense oligonucleotides or ribozymes [377, 378]. In one head-to-head comparison, it has been shown that siRNAs knocked down gene expression hundreds of time more efficiently than antisense oligonucleotides [379]. RNAi is an attractive alternative as the gene silencing tool in my study.

The antisense oligonucleotides have been studied intensively for the longest time and the first antisense DNA agent is now on the market in the USA and Europe. However its mechanism is still not without controversy and it has been proposed that the therapeutic outcome could be a result of the CpG presence and the consequent immune stimulation in some cell types [380]. Two clinical trials using ribozymes in gene therapy are in progress, in which retroviral vectors, which express ribozymes targeting sequences in human HIV-1 RNA, are transduced in CD4 lymphocytes or CD34 hematopoietic precursors [381, 382]. Just three years after RNAi was shown to work in mammalian

cells, the first Phase I clinical trials using RNAi have started in which RNAi is used to target the VEGF angiogenic pathway in ARMD patients. No evidence for clinical toxicity or disease progression has been shown in these studies conducted by Sirna Therapeutics [292]. These studies indicate that RNA silencing tools, especially ribozymes and RNAi, have a great potential to be used as therapeutic agents.

New ribozyme types have been discovered. One recent report indicated the existence of a metalolite-responsive ribozyme in the mRNA of *glm*S, the *Bacillus subtilis* gene that encodes flucosamine frustose-6-phosphate aminotransferase [383]. The cleavage product is terminated by a cyclic 2'-3' phosphate, very similar to the products of other self cleaving ribozymes, suggesting that the transesterification reaction involves the nucleophilic attack from the 2'-oxygen. In another report, an element in the 3'-flanking region of human β-globin mRNA has been found that self cleaves [384]. The cleavage site is contained within a region that shows some similarity to the hammerhead ribozyme; however the 3'-hydroxyl and 5'-phosphate termini generated in the cleavage reaction imply a different mechanism of cleaving from other self cleaving ribozymes.

As mentioned above, an HIV-directed hammerhead ribozyme has been tested in patients to exploit its ability to inhibit HIV replication [385]. The clinical trial is performed in *ex vivo*, in which the peripheral blood T lymphocytes obtained from the HIV-infected patients are transduced with a retroviral vector coding a hammerhead ribozyme against HIV RNA expression. The transduced lymphocytes are injected back into HIV patients. The results showed that the infusion of gene-altered, activated T-cells is safe, that the transduced cells persist for long intervals and the possible patient long-term survival resulting from the transduced cells [385]. In another study targeted against

hepatitis C virus (HCV) replication, six hammerhead ribozymes were designed that are targeted a conserved region of the plus and minus strands of the HCV genome and were expressed using recombinant adenovirus vectors. Testing in primary hepatocytes obtained from HCV-infected patients showed a beneficial antiviral effect of the ribozymes, and when used together with type 1 interferon, the replication of HCV-polivirus (PV) chimera was inhibited up to 98% [386]. In another study, synthetic and modified hammerhead ribozymes targeting 15 conserved sites at the 5'-untranslated region of HCV RNA were also tested for knock-down efficiency and stability [387], and a significant reduction (40%-90%) in gene expression of a reporter gene following the 5' untranslated region was observed [387]. Ribozymes targeted against hepatitis B virus (HBV) has also been proposed [388]. Modified hepatitis delta virus has been used to target HBV virus through its natural tropism to hepatocytes and the result of transgene delivery was positive [389, 390]. In cancer therapy, fusion proteins have been suggested as a target for ribozymes. The fusion proteins are expressed from chimeric genes resulting from abnormal chromosomal translocations, which shuffle that translocated exons and produce chimeric mRNAs [388]. These are tumor-specific chromosomal abnormalities and only exist in the tumor cells [391], thus they provide a tumor cell-specific target and the normal cells are not targeted. These type of strategies could help to increase the effectiveness of current cancer treatments.

In this study the *in vivo* application of the ribozymes required sufficient expression and stability of the ribozymes to survive the time course of the two animal models. The CMV/β-actin promoter produced qualitative expression of a GFP reporter gene beginning on day P11 of the mouse OIR model and extended beyond day P17 (data

not shown). Thus it is expected that the hammerhead ribozymes are also expressed in a similar manner and can meet the timeframe demand for expression in OIR mouse model. It has been reported that synthetic siRNA, transfected into human cells, show an optimal effect around 24 hours and the RNAi starts to diminish in 4-7 days [392]. So if we use synthetic RNAs (either ribozymes or siRNAs) as the gene silencing tool in the OIR mouse model, the synthetic siRNA will probably not last through the 17-day time course of the experiment. However, a vector expressing shRNA, similar to our ribozyme expression vectors, could be used. In one study, the AAV-cloned shRNA introduced in mouse brain started to silence its target in 4-6 days and the silencing phenotype (Parkinson's disease) reached its peak around two weeks and persisted for nearly two months [393]. Therefore it is probable that vector-expressed shRNAs could be successfully used in the OIR mouse model or even the adult mouse model of laser-induced retinal neovascularization, where the experiment termination is 3-4 weeks after injection.

In our *in vitro* tests of the ribozymes, the ribozyme effects were determined by assaying both mRNA and protein levels using relative quantitative RT-PCR, real-time RT-PCR, western analysis and flow cytometry. We assume the transfection efficiency is consistent throughout all experiments thus did not measure it every time. However, variations may occur. Cell death resulting from transfections could also happen, which may not be the same for the transfection of empty vector, mock transfection, or ribozyme transfection. The normalization with living cell numbers may be useful in a fined measurement of reduced mRNA or proteins levels. In another aspect, the targeted protein could have a relative long half-life thus a modest drop in protein expression might not be

seen in a short-period of time post-transfection. Cullen suggested approach of the introduction of an expression plasmid encoding an epitope-tagged form of the target protein [303]. A western blotting using the antibody against the epitope will be performed to measure the gene silencing effectiveness. In this way, the co-transfection efficiency is technically 100% same for silencing agents and the targets and protein half-life is not an issue any more. The construction of the epitope-tagged form of the targeted protein is laborious and time-consuming; however, this approach could give us an accurate measurement on the effectiveness of the silencing agents. This is especially important in selecting a best-effective silencing agent for therapeutic purpose.

The eye is an ideal target organ for gene therapy, in that it has relatively isolated compartment so that the local delivery of exogenous genes to the eye limits exposure to the rest of the body and reduces the dose. Similar to our injection of ribozymes into the eye, siRNAs have also been injected into the eye intravitreally and were readily diffused throughout the eye and detectable for at least five days [394]. VEGF and its receptors have been attractive targets in RNAi *in vivo* studies in the eye so far. In one study, hVEGF cDNA, expressed by an adenoviral vector, was subretinally injected in both eyes of mice. This was coupled with an siRNA targeting against hVEGF mRNA in one eye, or siRNA targeting against GFP mRNA in the other eye. It was showed that eye injected with hVEGF siRNA had significantly less expression of hVEGF compared with the GFP siRNA control [395]. In the mouse model of CNV induced by laser photocoagulation, the area of CNV at sites of rupture of Bruch's membrane was significantly less in the eyes that were subretinally injected with mVEGF siRNA, compared with GFP siRNA controls [168]. These results directly lead to phase I clinical trials of siRNA against VEGF mRNA

in ARMD patients with subfoveal CNV. In the studies for corneal neovascularization, a systemic administration of siRNAs against VEGF-A, VEGFR-1, or VEGFR-2 using a polymer delivery system was conducted [396]. The polymer was composed of branched polyethylenimine (PEI) as one end, polyethylene glycol (PEG) in the middle and an RGD peptide motif at the other end. This tri-functional polymer can self assemble with negatively charged siRNA into a nanoparticle and RGD peptide will be exposed on the surface. The RGD peptide, a specific ligand for αvβ3 and α5β1 integrins on activated endothelial cells, can introduce the expression of siRNA to the neovasculature. PEF helps to prevent nonspecific binding to other tissues. Thus the siRNA is delivered via ligand-mediated endocytosis. The level of corneal neovascularization was significantly reduced with the administration of VEGF-A, VEGFR-1, or VEGFR-2 siRNAs, and the combination of all the three resulted in further reduction. In another study, an siRNA against VEGFR-1 called Sirna-27, was tested and found to maximally reduce VEGFR-1 levels in cultured endothelial cells compared with other siRNA candidates [394]. This siRNA was further examined in mouse models of retinal and choroidal neovascularization. Sirna-027 significantly reduced VEGFR-1 mRNA levels by 57% or 40% after intravitreal or periocular injection, respectively, as measured by quantitative RT-PCR. In the CNV mouse model, the area of neovascularization was decreased by 45% to 66% after the periocular or intravitreous injection of Sirna-27. And in the ischemic retinopathy mouse model, the intravitreous injection of 1.0 μg of Sirna-027 significantly reduced retinal neovascularization [397]. All these studies used VEGFR-1 siRNAs and demonstrated that VEGFR-1 has an important role in stimulating ocular

neovascularizations, which further argues against the hypothesis that VEGFR-1 is only a decoy receptor that negatively regulates the activity of VEGFR-2.

Apart from eye diseases, siRNAs targeting against the VEGF pathway, or angiogenesis, have been studied in the treatment of cancer, inflammation, and so on [398]. The growth hormones, their receptors, signaling transduction factors, matrix metalloproteases and adhesion molecules have all been used as the RNAi targets. These studies provide strong support that RNAi can be used in novel anti-angiogenesis therapies, from bench to bed.

APPENDIX A
LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
ABAM	Antibiotic/antimycotic mix
Ad	Adenovirus
AGE	Advanced glycation end-products
ALS	Acid labile acid
Ang-1	Angiopoeitin 1
Ang-2	Angiopoeitin 2
ARMD	Age Related Macular Degeneration
ARVO	Association for Research in Vision and Ophthalmology
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BFP	Blue fluorescence protein
BRB	Blood-retinal barrier
BrdU	Bromo-uridine
BSA	Bovine serum albumin
CAR	Coxsackievirus-adenovirus receptor
CFP	Cyan fluorescence protein
cGMP	Cyclic guanosine 3',5'-monophosphate
CHO	Chinese hamster ovary cells
CMCT	1-cyclohexyl-(2-morpholinoethylo)cabodiimide metho-*p*-toluene sulfonate
CMV	Cytomegalovirus
CNV	Choroidal neovascularization
DAG	Diacylglycerol
DMEM	Dubellco's modifeid eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DR	Diabetic retinopahty
DTT	Dithiothreitol
EC	Endothelial cells
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
EGS	External guide sequence
eNOS	Endothelial NO synthase
ER	Endoplasmic reticulum
ET	Endothelin
PAF	Platelet-derived factor

FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GAGs	Glycosaminoglycans
GAS	γ-interferon activated sequence
GC	Guanosine cytosine content
GCL	Ganglion cell layer
GFP	Green fluorescent protein
GH	Growth hormone
GLUT1	Glucose transporter 1
HBSS	Hanks balanced salt solution
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDV	Hepatitis delta virus
H&E	Hematoxylin-eosin
HEK 293	Human embryonic kidney cells
HIF	Hypoxia inducible factor
HIV	Human immunodeficiency virus
HRE	Hypoxia response element
HRECs	Human retinal endothelial cells
HSPGs	Heparin sulfate proteoglycans
HSV-1	Herpes simplex virus type 1
IACUC	Institution Animal Care and Use Committee.
IAP	Integrin-associated protein
ICAM-1	Intercellular adhesion molecule-1
Ig	Immnoglobin
IGF-1	Insulin-like growth factor 1
IGF-1R	Insulin-like growth factor 1 receptor
IGF-2	Insulin-like growth factor 2
IGFBP	Insulin-like growth factor binding protein
ILM	Inner limiting membrane
INL	Inner nuclear layer
IP_3	Inositol 1,4,5-triphosphate
IPL	Inner plexiform layer
IR	Insulin receptor
IRS	Insulin receptor substrate
ITR	Inverted terminal repeats
LTR	Long terminal repeats
miRNA	microRNA
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide-adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB
NFL	Nerve fibre layer
NO	Nitric oxide
NOS	Nitric oxide synthase
NPDR	Non proliferative diabetic retinopathy

NRP	Neurophilins
OIR	Oxygen-induced retinoppathy
OLM	Outer limiting membrane
ONL	Outer nuclear layer
OPL	Outer plexiform layer.
ORF	Open-reading frame
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PDR	Proliferative diabetic retinopathy
PEDF	Pigment epithelium-derived factor
PEG	Polyethylene glycol
PEI	Polyethylenimine
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PlGF	Placental growth factor
PS	Phosphorothioate
PTB	Phosphotyrosine binding
PTGS	Post-transcriptional gene silencing
ptRNA	Precursor tRNA
rAAV	Recombinant adeno associated virus
RA	Retinoic acid
RAGE	Advanced glycation end-products receptor
RAR	Retinoic acid receptor
RFP	Red fluorescence protein
RGD	Arginine-glycine-asparginine
RISC	RNA-inducing silencing complex
ROP	Retinopathy of prematurity
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RNAi	RNA interference
rRNA	Ribosomal RNA
RNasin	Ribonuclease inhibitor
RPE	Retinal pigment epithelium
RT	Reverse transcription
RXR	Retinoid X receptor
Rz	Ribozyme
scAAV	Self complementary AAV
SHP-2	Src homology 2 containing tyrosine phosphatase
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SMCs	Smooth muscle cells
SnRNA	Small nuclear RNA
STAT	Signal transducer and activator of transcription
TBS	Tris buffered saline

TβRV	Type V receptor for transforming growth factor-β
TGF	Transforming growth factor
Tie 1 and 2	Angiopoeitin receptors 1 and 2
TNF	Tumor necrosis factor
tPA	Tissue type plasminogen activator
tRNA	Transfer RNA
TR	Inverted terminal repeats
TRS	Terminal resolution site
uPA	Urokinase type plasminogen inhibitor
VE cadherin	Vascular endothelial cadherins
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor-receptor 1
VEGFR-2	Vascular endothelial growth-receptor 2
VPF	Vascular permeability factor
VS	Varkud satellite
VSMCs	Vascular smooth muscle cells
YFP	Yellow fluorescence protein

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BIOGRAPHICAL SKETCH

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