Using the Past to Inform the Future: Anti-VEGF Therapy as a Road Map to Develop Novel Therapies for Diabetic Retinopathy

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Therapies targeting vascular endothelial growth factor (VEGF) are revolutionizing the treatment of diabetic retinopathy (DR) and diabetic macular edema (DME). In August 2012, ranibizumab, a monoclonal antibody fragment targeting VEGF designed for ocular use, became the first and only U.S. Food and Drug Administration–approved medical therapy for DME and the first approved treatment in over 25 years. This approval was based on strong preclinical data followed by numerous clinical trials that demonstrate an essential role of VEGF in vascular permeability and angiogenesis in both normal physiology and disease pathology. In this Perspective, we will examine the experimental studies and scientific data that aided in the success of the development of therapies targeting VEGF and consider how these approaches may inform the development of future therapeutics for diabetic eye disease. A multipoint model is proposed, based on well-established drug development principles, with the goal of improving the success of clinical drug development. This model suggests that to provide a validated preclinical target, investigators should demonstrate the following: the role of the target in normal physiology, a causal link to disease pathogenesis, correlation to human disease, and the ability to elicit clinically relevant improvements of disease phenotypes in animal models with multiple, chemically diverse interventions. This model will provide a framework to validate the current preclinical targets and identify novel targets to improve drug development success for DR. Diabetes 62:1808–1815, 2013

For the last 20 years, managing the metabolic deregulation induced by diabetes has been the primary and most effective way to slow the development and progression of microvascular complications including diabetic retinopathy (DR) (1,2). After the appearance of clinically significant vascular lesions and macular edema, laser photoagulation remains an effective approach to slow the loss of visual acuity (3,4). These established approaches have recently been extensively reviewed (rev. in 5). Unfortunately, ~20% of people with type 1 diabetes develop proliferative DR even under intensive metabolic control by exogenous insulin (6), while others have inherent difficulties with maintaining proper euglycemia. Therefore, understanding the causative underlying mechanisms of DR remains of utmost importance in the treatment of this insidious disease. Basic and clinical research into the inflammatory cytokines and proangiogenic signals that drive DR has provided new therapeutic avenues for the treatment of diabetic eye disease. Importantly, anti–vascular endothelial growth factor (VEGF) therapy has revolutionized the treatment of diabetic macular edema (DME). The Diabetic Retinopathy Clinical Research Network suggested that ranibizumab improves visual acuity outcomes in patients with DME. Subsequently, in the RISE clinical trial, 44.8% of patients treated with 0.3 mg ranibizumab for 24 months gained >15 letters improvement in visual acuity vs. 18% of sham-treated patients. In the RIDE study, 45.7% of patients treated with 0.5 mg ranibizumab gained >15 letters vs. 12.3% of sham-treated patients. In addition to increases in visual acuity, improvements were observed in retinal thickness as measured by optical coherence tomography and reduced risk of further vision loss (7). This success has provided much-needed therapeutic options and a blueprint to discover novel treatments for diabetic ocular complications.

VEGF: DUAL ROLE IN PHYSIOLOGY AND PATHOLOGY

Clinical success of anti-VEGF therapy is based on basic scientific research into the mechanisms of angiogenesis, neovascularization, and vascular permeability leading to a broad consensus from the scientific community on the significance and requirement of this growth factor to these defined processes. Exploration in tumor biology led to the hypothesis that diffusible factors provided angiogenic and permeabilizing signals to the tumor vasculature. This led to the seminal hypothesis by Dr. Judah Folkman that inhibition of angiogenesis may be a strategy to halt tumor growth (8). Protein purification and molecular cloning allowed two groups to discover the potent angiogenic and permeabilizing factor: one coining the term vascular permeability factor and the other VEGF (9,10). A review of the biology of VEGF and its receptors on angiogenesis, proliferation, migration, and vascular permeability was performed by Chung and Ferrara (11). Here, we provide a retrospective analysis of the significance of the seminal findings in the development of anti-VEGF therapies and propose a model to apply to the newest set of preclinical targets for DR.

Clear and compelling genetic studies revealed that VEGF contributes a critical and essential role in vascular biology (rev. in 11). Genetic loss-of-function experiments demonstrated that developmental expression of VEGF is required for vasculogenesis and angiogenesis, as single Vegf allele inactivation resulted in embryonic lethality with deficient vascularization of several organs (12,13). Moreover, gene targeting via the Cre-loxP system and administration of a soluble VEGF receptor chimeric protein led to significant increases in mortality and impaired organ development; however, this critical requirement for VEGF...
Generating novel therapies for the indication to treat DR is extremely difficult owing to the long and incremental organ failure associated with diabetes. The first ocular indication for anti-VEGF therapy (FDA approved in 2006) was for neovascular age-related macular degeneration (wet-AMD). The pathogenesis of wet-AMD involves significant choroidal neovascularization and vascular permeability, and strong preclinical data demonstrate that VEGF mediates these effects. The Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular Age-Related Macular Degeneration (MARINA) trial showed that ranibizumab treatment led to a 95% rate of visual stabilization, and nearly 40% of treated patients had a ≥15 letter gain of visual acuity at 12 months. Importantly, these benefits were associated with significant decreases in foveal center point thickness in the ranibizumab-treated patients (26). Additionally, the ANCHOR Study (Anti-VEGF Antibody for the Treatment of Predominantly Classic Choroidal Neovascularization in Age-related Macular Degeneration) demonstrated superior efficacy in treating choroidal neovascularization over photodynamic therapy with verteporfin (27,28). Notably, ocular anti-VEGF therapy was well tolerated with low risk of ocular adverse events and no systemic side effects as seen with intravenous administration for cancer treatment (29). FDA approval in 2010 for macular edema after retinal vein occlusion was granted after the Branch Retinal Vein Occlusion (BRAVO) and Central Retinal Vein Occlusion (CRUISE) phase III trials that demonstrated that 50% of patients gained ≥15 letters in visual acuity after treatment with ranibizumab (30,31).

Initial studies in persons with diabetes began in 2005 with pegaptanib, an aptamer targeting VEGF. This phase II trial demonstrated greater visual acuity improvements after treatment with pegaptanib compared with sham injection patients (32). After this study, numerous clinical trials have demonstrated success of anti-VEGF therapies including ranibizumab, bevacizumab, and aflibercept, which are extensively reviewed elsewhere (33). Subsequent studies examining dosing regimens and dose responses all arrived at the same conclusion; namely, multiple independent pharmacological agents targeting VEGF exhibit robust clinical efficacy. After the RIDE/RISE results, ranibizumab gained approval on 10 August 2012 for DME after evidence of significant visual acuity gains of ≥15 letters for 35–50% of the patients treated with ranibizumab, which correlated with dramatic improvements in retinal edema as measured by optical coherence tomography (7). Although some patients fail to respond to anti-VEGF therapy, this medical treatment has revolutionized ophthalmic care. Clearly, increased vascular permeability and retinal edema contribute to the pathology of DR and loss of visual acuity (34), and various studies targeting VEGF with chemically diverse therapies demonstrate that blocking excess VEGF signaling prevents loss of vision in DR.

While empiricism has gained great advances in therapies with limited mechanistic understanding (e.g., aspirin, penicillin), we propose that the approach used in understanding the role of VEGF in vascular biology provides a learning model for future drug development for DR. An analysis of the development of anti-VEGF therapies may be distilled to a set of principles that may expedite the validation and development of preclinical drug targets for DR prior to large clinical trials in humans. This model has five principles:

1) **Physiology:** knowledge of the target’s role in normal physiology from biochemical and genetic manipulation.
2) Pathology: demonstration of the target’s causal link to pathological features as evident from biochemical and genetic gain-of-function or loss-of-function experiments.
3) Relevance: evidence demonstrating a correlation with disease progression or severity in humans.
4) Reversibility: pharmacological agents targeting the factor must be able to elicit a biologically significant response in vivo in preclinical screens that are clinically meaningful.
5) Repeatability: structurally distinct probes that regulate the target, or another target on the same pathway, demonstrate a similar effectiveness.

VEGF clearly obtained broad scientific consensus as a valid target for vascular angiogenesis and vascular permeability. VEGF was shown to contribute to pathological vascular permeability and retinal edema as well as angiogenesis in preclinical studies, while clinical studies demonstrated a clear correlation with disease. Furthermore, multiple pharmacological agents successfully targeting VEGF showed dramatic improvements in vascular permeability and retinal edema in a range of disease models and clinical studies. Approaches independent of this model have demonstrated success in identifying compounds with great potential for clinical efficacy such as the discovery of the use of fenofibrate for DR (35). Indeed, future studies may provide important mechanistic understanding of the use of these compounds but will not be discussed here. Rather, the model provided by anti-VEGF therapies will be discussed as providing a framework for preclinical research that may improve drug development success and outcomes.

NOVEL TARGETS TO SUPPORT OR REPLACE ANTI-VEGF AGENTS
Although significant success is apparent with anti-VEGF therapy, a number of limitations exist including the need for repeat intraocular injections, the potential for disease rebound upon discontinuing treatment, and the fact that not all patients respond to the treatment, collectively leading to the justification for the investigation of novel therapies for DR. In addition to VEGF, numerous factors have been identified as potential candidates mediating the vascular dysfunction observed in DR. The pathology of the diabetic retina is complex and includes regions lacking vascular perfusion and degeneration as well as regions of vascular growth and responsiveness to VEGF. Figure 1 reveals the cells associated with the retinal capillary and the proposed factors that may contribute to DR. The first category includes targets that are downstream of VEGF signaling. Factors in this category may provide additive benefits to current anti-VEGF strategies through improved pharmacokinetic or drug delivery options such as an orally available alternative to repeat intraocular injections of anti-VEGF mAbs. The second category includes novel proangiogenic factors distinct from VEGF with unique signaling mechanisms that contribute to blood-retinal barrier dysfunction and angiogenesis. Finally, extracellular-inflammatory factors that contribute to vascular permeability and vascular degeneration are discussed. Importantly, drugs for DR may be considered for systemic or local delivery. Local delivery provides a number of potential advantages including the potential for reduced side effects and prolonged drug half-life with the current disadvantage of the need for intraocular injection. The route of drug delivery may have a profound impact on the success of drug development for DR. We apologize in advance to our colleagues with exciting new targets not discussed, as limited examples will be highlighted (Table 1).

**Targets downstream of VEGF signaling.** VEGF requires classical protein kinase C (PKC) and, specifically, PKCβ for signal transduction in vascular endothelium. Both diabetes and VEGF increase retinal PKCβ activity, and an orally active PKCβ-specific inhibitor, ruboxistaurin (RBX), can block VEGF-induced permeability and angiogenesis in rodents (36). PKCβ contributes a critical role in mediating proliferation through retinoblastoma-associated protein phosphorylation (37) and permeability through phosphorylation-induced endocytosis of the tight junction protein occludin (38). Clinical trials to determine the effect of RBX on visual loss in patients with DR demonstrated that the drug was well tolerated and reduced the progression of macular edema, the need for laser, and the risk of sustained moderate visual loss by ~50% (39). However, currently, RBX has not received FDA approval for treatment of DR. PKCβ has been one of the most well characterized DR therapeutic targets since VEGF, and further clinical efficacy studies with appropriately targeted drugs may be warranted.

Recent evidence suggests that atypical PKC (aPKC) isoforms also contribute to VEGF-induced permeability. aPKC contributes to normal developmental physiology by mediating various processes including cellular polarity and barrier establishment (40). Targeting aPKC isoforms both genetically and pharmacologically with siRNA and novel small-molecule inhibitors demonstrates a requirement for aPKC activity in VEGF-induced retinal endothelial permeability. These small molecule inhibitors also prevent VEGF-induced permeability in the rodent retina (41). aPKC may provide an ideal target, as other inflammatory factors such as thrombin, tumor necrosis factor (TNF), and chemokine ligand 2 (CCL2), discussed in more detail below, also signal through aPKC to mediate their effects on endothelial permeability (42–44). Although additional genetic data are needed to validate this target, aPKC represents a common signaling node required for multiple permeabilizing factors and may provide a target that offers improved efficacy over current monotherapies. Signal transduction pathways involved in permeability are summarized in Fig. 2.

**Angiogenesis targets distinct from VEGF signaling.** From the success of anti-VEGF therapy, other factors that regulate angiogenesis and permeability have been examined as potential therapeutic targets to treat DR. Angiopoietins (Ang) have a clearly defined role in vascular development and physiology. The angiopoietins consists of several ligands and receptors, but the focus has been primarily on Ang1 and Ang2 and the receptor Tie2. Importantly, Ang1 and Ang2 have antagonistic roles upon receptor binding, as Ang1 is an agonist for Tie2, while Ang2 acts as a competitive antagonist of the Tie2 receptor (45). The signaling axis of Ang-Tie2 is essential for angiogenesis as Ang2 promotes vessel destabilization and initiates angiogenesis. Alternatively, Ang1 stabilizes vessels following angiogenesis, and has been implicated in pathological angiogenesis (45). Importantly, double blockade of VEGF and Ang2 with a chimeric decoy receptor has added benefit over VEGF and Ang2 blockade alone in inhibition of tumor angiogenesis and vascular leakage (46). Clinically, Ang2 concentrations are elevated in the vitreous of patients with PDR (47), while neutralizing antibody that
blocks Ang2 prevents ocular angiogenesis in animal models of oxygen-induced retinopathy (48). In addition, Ang1 is a retinal vasoprotectant and exhibits anti-inflammatory activity in animal models of diabetes (49). Strong preclinical genetic and biochemical data supporting a role for Ang-Tie2 axis in physiological and pathological angiogenesis and permeability support targeting this pathway to promote vascular stabilization and reduce vascular leak. Evidence for changes in this pathway in human disease progression would support further drug development. The human protein tyrosine phosphatase β (HPTPβ) has emerged as a regulator of Tie2 signaling and provides a potential point of therapeutic intervention (50).

Platelet-derived growth factor (PDGF) has been suggested as a potent proangiogenic molecule, which is also important for the establishment of the blood-retinal barrier. The literature regarding PDGF’s role in retinal angiogenesis and DR pathogenesis is complex and even contradictory. Whole-body genetic loss-of-function studies demonstrate that PDGF and PDGFRβ are required for vascular development (51). Importantly, for blood-retinal barrier development, PDGF signaling in pericytes is imperative for pericyte survival, and hyperglycemia-induced defects in PDGFRβ signaling in pericytes lead to blood-retinal barrier dysfunction. BKR, bradykinin receptor.

FIG. 1. Extracellular signaling implicated in the pathogenesis of DR. This cartoon illustration of the neurovascular retina provides a summary of the factors implicated in DR and highlighted in this Perspective. Receptors for permeabilizing factors, such as VEGF, are expressed on the endothelial cells. The factors may be secreted in a paracrine fashion by the surrounding glial cells or from microglia or inflammatory cells (not shown). Proangiogenic factors such as Ang2 contribute to pathological angiogenesis and vascular permeability along with VEGF. Inflammatory factors, such as TNF and CCL2, represent another class of factors that may contribute to the increased retinal vascular endothelial permeability. Wnt signaling during endothelial maturation promotes differentiation, while aberrant Wnt signaling increases vascular permeability. In proliferative DR, vascular hemorrhage and erythrocyte lysis increase extracellular carbonic anhydrase in the blood vessel lumen and activate the kinin-kallikrein system promoting vascular permeability. In addition, PDGF signaling in pericytes is imperative for pericyte survival, and hyperglycemia-induced defects in PDGFRβ signaling in pericytes lead to blood-retinal barrier dysfunction. BKR, bradykinin receptor.

observation was mechanistically verified in a diabetic mouse model in which hyperglycemia induces loss of PDGF signaling in pericytes through the activation of PKCδ/Src homology phosphatase-1, leading to pericyte apoptosis and vascular dysfunction (53). These data suggest that PDGF supplementation may be protective in DR. Conversely, studies have demonstrated elevated vitreous concentration of PDGFAB in diabetic patients (54). Indeed, in mouse retina, excessive PDGFA from neuronal cells leads to proliferative disease (55). PDGF-specific antibody can enhance anti-VEGF therapy in models of ocular neovascularization and cause vessel regression, as some new vessels become refractory to sustained VEGF deprivation (56). Together, these studies suggest that PDGF is a well-validated preclinical target according to our model; however, additional studies are needed to determine how to effectively modulate the PDGF axis to prevent the pathogenesis of DR, as both supplementation and inhibition of PDGF may improve the DR phenotype depending on the specific vascular microenvironment.

Several laboratories have suggested that the Wnt signaling axis contributes a fundamental role in both normal and disease physiology and therefore represents an exciting pharmacological target for diabetic eye disease. Wnts are a large family of secreted proteins that have a wide range of functions including the regulation of
endothelial gene expression and angiogenesis. Upon Wnt ligand binding to the Frizzled receptor and recruitment of LDL receptor–related peptide (Lrp)5/6, β-catenin is stabilized and trans-locates to the nucleus, where it binds TCF/Lef transcription factors and induces specific gene transcription. Gain- and loss-of-function experiments clearly demonstrate that normal vascular development requires Wnt signaling, specifically in the retina, where several targeted mutations of this signaling axis have drastic retinal vascular defects (57). Wnt also contributes to the pathophysiology of ischemic retinopathies. Deletion of Lrp5 and downstream signaling molecule dishevelled2 decreases the formation of pathological neovascularization in the oxygen-induced retinopathy model (58). Several endogenous antiangiogenic inhibitors of Wnt signaling have become potential therapies to inhibit aberrant Wnt signaling. SERPINA3K binds to Lrp6 and antagonizes Wnt signaling preventing the vascular permeability and inflammation seen in diabetic rats (59). Inhibition with an mAb to Lrp6 also prevents vascular leakage and inflammation of the retina of diabetic animals (60). Evidence suggests that pigment epithelium–derived factor (PEDF) acts as an antiangiogenic factor in the retina of rodents (61). The effect of PEDF may be attributed to antagonism of VEGF signaling preventing the vascular permeability and inflammation seen in diabetic rats (62). Inhibition with an mAb to Lrp6 also prevents vascular leakage and inflammation of the retina of diabetic animals (63). Inhibition with an mAb to Lrp6 also prevents vascular leakage and inflammation of the retina of diabetic animals (64). Additional studies are needed to identify the pathophysiological role of IGF-1/GH signaling and the relationship to disease progression. In addition to GH/IGF-1, erythropoietin (Epo) is elevated in the vitreous of patients with proliferative DR (47). Epo enhances pathological retinal angiogenesis in mice in the oxygen-induced retinopathy model (65), while small interfering RNA–mediated knockdown of Epo suppresses retinal neovascularization (66), suggesting a pathogenic nature for Epo. Conversely, intravitreal injection of Epo inhibits retinal barrier breakdown in diabetic animals (67). Clearly, there is a lack of consensus on the pathogenicity of Epo, and further preclinical studies are needed to validate these targets. Blood-retinal barrier dysfunction induced by inflammatory cytokines and proteases. In addition to proangiogenic signals, inflammatory cytokines represent novel targets for DR. Experimental evidence indicates that cytokines, in addition to VEGF, also contribute to vascular permeability in DR. A series of elegant experiments demonstrated that leukostasis occurs during streptozotocin-induced diabetes in mouse and rat models and that deletion of the gene for the adhesion protein ICAM or its leukocyte-binding partner CD18 ameliorated leukostasis (68). Furthermore, in an acute (1 week) model of diabetes, vascular permeability, leukostasis, CD18, and ICAM expression, as well as nuclear factor-κB activation, were all normalized by high-dose aspirin or the cyclooxygenase-2 inhibitor meloxicam and by a soluble TNF receptor/Fc hybrid (entanercept), suggesting that TNF and cyclooxygenase-2 contribute to DR (69). A recent report provides...
insight into how VEGF and TNF may both contribute to different phases of vascular dysfunction in DR. Using mice with TNF gene deletion, the investigators demonstrate that diabetes-induced permeability was unaffected at 1 month; at 3 months, changes in permeability are partially reduced, while at 6 months the permeability defect is ameliorated (70). Furthermore, in humans elevated interleukin (IL)-1β and TNF levels have been associated with proliferative DR (71,72) and TNF with nonproliferative DR (73). In addition, anti-TNF therapies led to significant improvement in visual acuity compared with placebo-treated eyes in a controlled trial involving patients refractory to other treatments (74). ILs and other inflammatory factors may also contribute to DR; monocyte chemotactic protein-1 (MCP)-1 (or CCL2) along with IL-6 and IL-8 were found elevated in patients with retinopathies associated with inflammation after retinal detachment (75), and MCP-1 contributes important roles in leukocyte infiltration and vascular inflammation after retinal detachment (76). The contribution of inflammatory cytokines and chemokines to DR is complex and early in the discovery phase. What factors are relevant to human DR and at what phase of the disease process remain to be elucidated, but targeting inflammatory cytokines and chemokines offers an inviting option to control DR. However, the effectiveness of monotherapies targeting specific inflammatory cytokines in the face of the wide array of factors changed in DR remains to be determined.

Aberrant protease signaling is a relatively new area of investigation for DR. Some of the factors recently identified are actually well-known molecules in human physiology. One promising target is the kinin-kallikrein system, which contributes an essential role in inflammation, blood pressure control, and pain through bradykinin activation. Importantly, complement component C1-inhibitor is an important physiological inhibitor of plasma kallikrein. Deficiency of C1-inhibitor leads to plasma kallikrein and bradykinin activation and robust vascular permeability responses (77). Gao et al. (78) detected carbonic anhydrase (CA-1) and multiple components of the complement and kinin-kallikrein system in the vitreous of patients with DR. Importantly, carbonic anhydrase leads to alkalization of the vitreous initiating bradykinin-kallikrein-system and retinal permeability that may be prevented by a systemically administered PK inhibitor (79). The bradykinin-kallikrein system fits our model of a well-validated target linked to human disease, as prior literature has implicated this system in both normal and disease physiology, and multiple pharmacological agents effectively reverse aspects of DR in relevant animal models. Current clinical trials are underway to determine efficacy of kallikrein inhibition in humans to treat DME and vascular hemorrhage.

TABLE 1
List of preclinical targets and their current state of development from ClinicalTrials.gov

| Target | Types of pharmacological agents | Clinical name (Trade name) | Eye indication |
|--------|---------------------------------|-----------------------------|---------------|
| VEGF   | mAb fragment                    | Ranibizumab (Lucentis)      | Approved wet-AMD, BRVO, CRVO, DME |
| VEGF   | mAb                             | Bevacizumab (Avastin)       | No approval; off-label use common |
| VEGF   | Fusion protein                  | Aflibercept (Elyea)         | Approved wet-AMD, CRVO, phase 3 DME, phase 3 BRVO |
| VEGF   | Aptamer                         | Pegaptanib (Macugen)        | Approved wet-AMD, phase 4 DME, phase 4 BRVO, phase 2 CRVO |
| Classical PKC isoforms | Small molecule | Ruboxistaurin | Halted |
| GH/IGF-1 | Peptide                     | Octreotide | Phase 3 NPDR and PDR |
| TNF    | mAb                             | Infliximab                  | Phase 3 DME, phase 2 AMD |
| PDGF   | Aptamer                         | E10030                      | Phase 2 wet-AMD |
| PEDF   | Adenoviral vector               | AdGVPEDF.11D                | Phase 1 wet-AMD |
| Endostatin and angioatin | Lentiviral vector | RetinoStat | Phase 1 AMD |
| Erythropoietin | Recombinant protein | EPO | Preclinical |
| Angiopoietin and Tie-2 | Small molecule, mAb, peptide, fusion protein | n/a | Preclinical |
| Atypical PKC isoforms | Small molecule | n/a | Preclinical |
| Wnt/Lrp | mAb Lrp6                       | n/a                         | Preclinical |
| Plasma kallikrein | Small molecule | n/a | Preclinical |
| Aldose reductase | Small molecule | Tolrestat | Failed due to toxicity |

Please visit ClinicalTrials.gov for detailed synopses associated with these select molecules and their current trials.

CONCLUSIONS
In this Perspective, a model for preclinical target identification and drug development is proposed to validate clinical trials based on the discovery and success of anti-VEGF therapy for diabetic eye disease. This model encompasses five principles, amassed by extensive preclinical research and supported by numerous independent laboratories that collectively provide compelling evidence for a drug target in human disease. This model aims to help guide preclinical experiments for potential drug targets and reduce the failure rate of agents entering clinical trials. Knowingly, many of the principles discussed in this Perspective have been integral to drug development for some time, but reviewing how these approaches have aided in the success of the development of the anti-VEGF therapies may provide a blueprint for the development of novel approaches to treat DR. Indeed, there are different approaches that may lead to validation of a preclinical target; however, in our perspective, the elucidation of physiologically relevant factors and defining their contribution to the pathogenesis of DR are clearly worth the efforts.
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