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Dual Targeting of Retinal Vasculature in the Mouse Model of Oxygen Induced Retinopathy

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Abstract: Hypoxia-inducible factor-1 (HIF-1) plays crucial roles in retinal neovascularization (NV) by upregulating its target genes, which are involved in anaerobic energy metabolism, angiogenesis, cell survival, cell invasion, and drug resistance. Therefore, it is apparent that the inhibition of HIF-1 activity may be a strategy for treating retinal angiopathies. Many efforts to develop new HIF-1-targeting agents have been made by both academic and pharmaceutical industry laboratories. The future success of these efforts will be a new class of HIF-1-targeting agents, which could be utilized in the treatment of several ocular pathologies. This review focuses on the potential of HIF-1 as a target molecule for the treatment of retinal NV, and on possible strategies to inhibit HIF-1 activity. In addition, we introduce YC-1 as a new anti-HIF-1, anti-neovascular agent in the retinal model. Although YC-1 was originally developed as a potential therapeutic agent for thrombosis and hypertension, recent studies demonstrated that YC-1 suppressed HIF-1 activity and vascular endothelial growth factor expression in retinal microvascular endothelial cells. Moreover, it inhibited retinal NV in the oxygen-induced retinopathy (OIR) mouse model without serious toxicity during the treatment period. Thus, we propose that YC-1 is a good lead compound for the development of new anti-HIF-1, anti-neovascular agents that could be used in the retinal pathologies.

Keywords: Retinal vasculature, angiogenesis, OIR, targeting, HIF-1, YC-1.

INTRODUCTION

Angiogenesis is a complex multistep process of formation of new vessels that is regulated by several growth factors [1] (Fig. **1**). While it is highly instrumental in tissue growth and development in the prenatal period, in the adult organism physiological angiogenesis is less active and is limited to certain organs such as the uterus during the menstrual cycle, pregnancy [2] (Fig. **2A**), or intensely exercising skeletal muscle [3]. Under pathological conditions, however, angiogenesis may become reactivated, for example in tumors [4] and retinopathy [5] and in rheumatoid [6], ischemic heart, and peripheral vascular diseases [7, 8] (Fig. **2A**). Most known angiogenic factors, such as basic fibroblast growth factor (bFGF) [9] and vascular endothelial growth factor (VEGF) [10], are derived from mesenchymal cells that are of either vascular or blood-borne origin. Angiogenesis could be described as either insufficient, such as clinical cases of stroke, coronary heart diseases, and delayed wound healing, or excessive such as pathological cases of retinal NV (Fig. **7B**; right frame) and cancer (Fig. **2B**). Angiogenesis begins with the activation of endothelial cells and proteases (Fig. **3A**), which leads to the degradation of matrix (Fig. **3A**), and subsequent endothelial cell migration (Fig. **3B**), proliferation (Fig. **3C**), and differentiation into capillaries (Fig. **3D-F**) [11]. Formation of arteries, or arteriogenesis, also requires migration and proliferation of vascular smooth muscle cells (VSMCs) [11].

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PHYSIOLOGICAL ANGIOGENESIS

Physiological angiogenesis is a tightly controlled process by a balance in the expression of angiogenic and anti-angiogenic factors (Fig. **1**). A key mechanism underlying physiological angiogenesis is its ability to regenerate the vascular capillary network and to perform vascular remodeling (Fig. **3A-F**). VEGF is unquestionably the Factor X postulated originally by Michaelson as etiological in pathological NV [12]. VEGF is an endothelial cell-specific mitogen and strongly induces both physiological and pathological angiogenesis. Furthermore, VEGF is a hypoxia-inducible gene whose regulation and function have been studied extensively. VEGF plays a key role in physiological angiogenesis, as observed in tissue regeneration, and in pathophysiological angiogenesis, as observed in wound healing, tumor growth, metastasis, psoriasis, and diabetic retinopathy (DR) [13] (Fig. **2A**). VEGF expression is regulated by a variety of stimuli including hypoxia, cobaltous ion, nitric oxide (NO), growth factors, and cytokines [14]. Analysis of the VEGF promoter has uncovered that one HIF-1 binding site (HBS) in its 5'-flanking region functions as a cis-element regulating the hypoxic induction of VEGF (Fig. **4**). Furthermore, VEGF is expressed as several spliced variants, its main human isoforms contain 121 and 165 amino acids. It is now highly accepted that VEGF is the major factor in the genesis of the

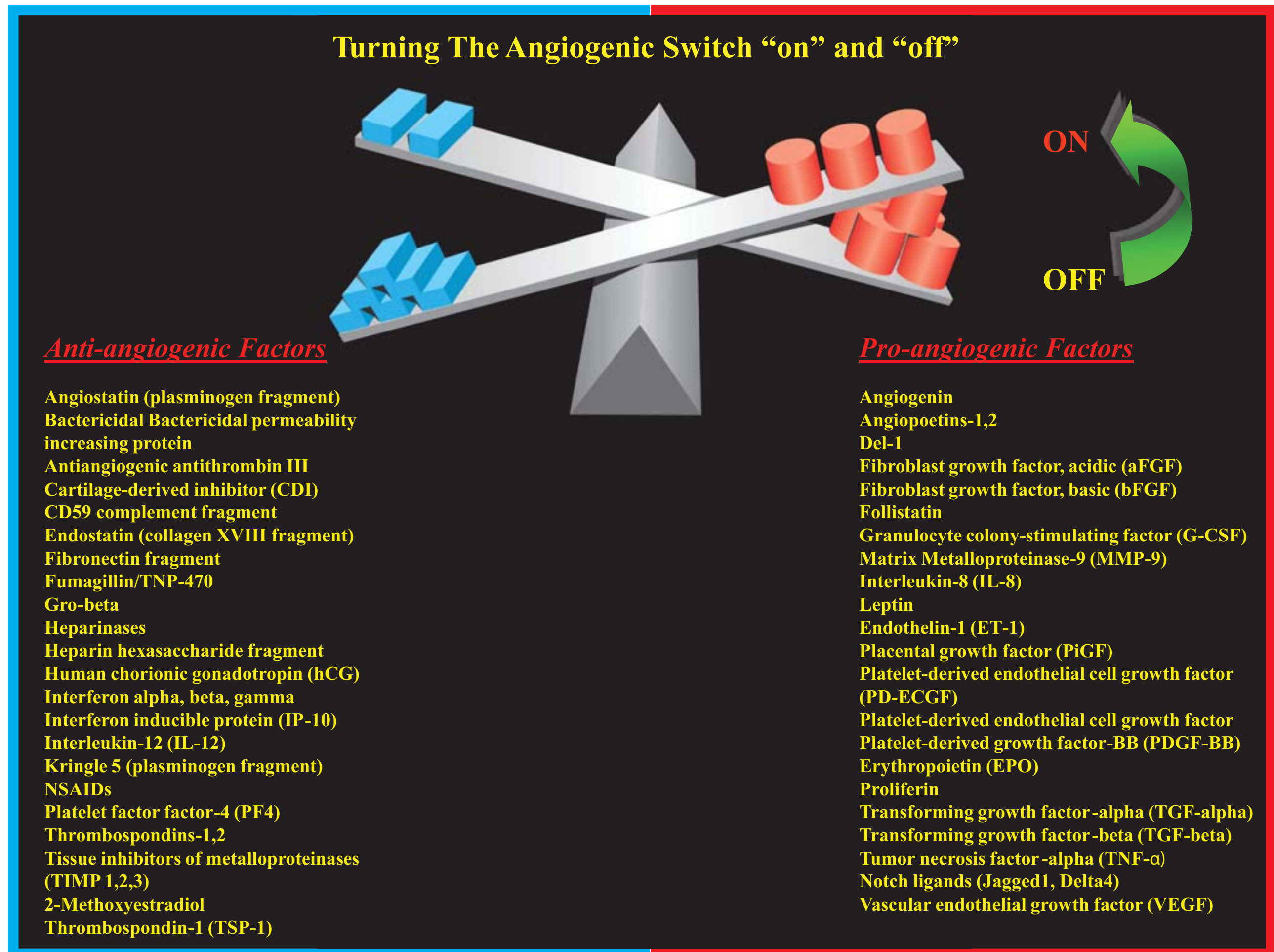
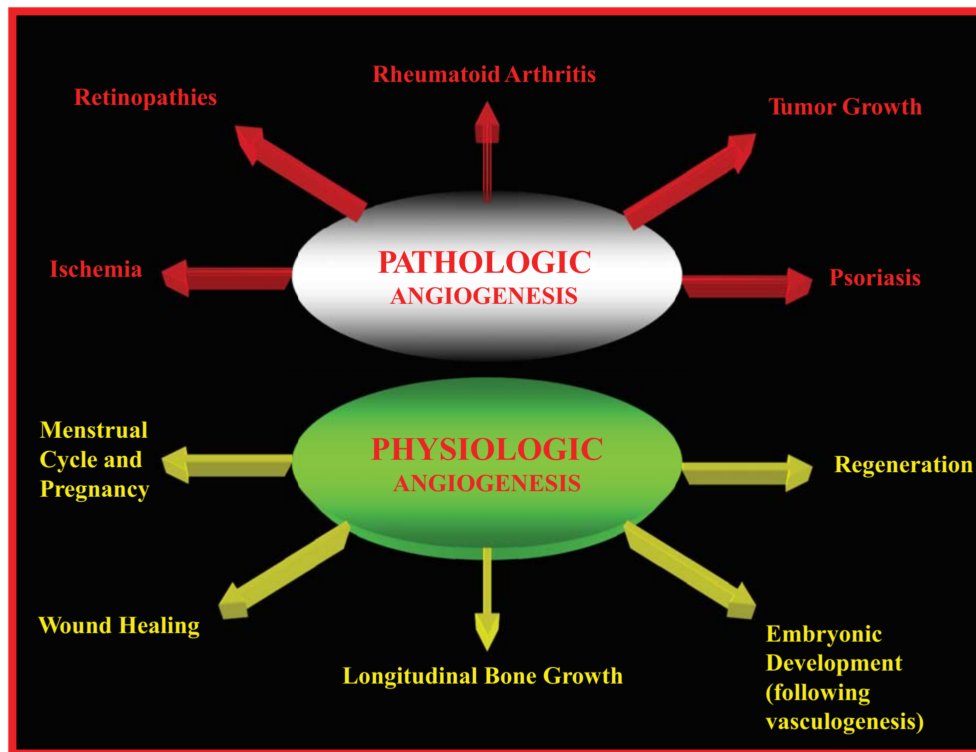


Fig. (1). Angiogenic Stimulators and Angiogenic Inhibitors. In red are the major players. Angiogenesis is regulated by stimulators and inhibitors. In quiescent/normal tissue the angiogenic switch is in balance or off. Angiogenesis can be achieved by increase in stimulators or decrease of inhibitors.

angiogenesis that characterizes the retinal new vessel formation of proliferative DR or ischemic retinal vein occlusion and the destructive NV of the retinopathy of prematurity. When the pivotal role of VEGF in NV became apparent, interest was generated in the use of anti-VEGF therapy for the NV seen in age-related macular degeneration (AMD) and currently this is a major area of clinical and commercial interest. Because VEGF in its various isoforms and other hypoxia induced factors appear also to play an etiological role in macular edema [15-17], the use of anti-VEGF therapy is becoming widely deployed in the treatment of diabetic macular edema (DME) and the macular edema accompanying retinal vein occlusion. VEGF 165 is the isoform most linked to pathological angiogenesis and increased vascular permeability while lower molecular weight isoforms are associated with physiological angiogenesis [18] (Fig. **2A**).

Angiogenesis is a process that takes place at any time and at any site where physiological or pathological tissue mass increases, from embryogenesis to postnatal life [1, 19]. Because of such a flexible nature, angiogenesis has been studied in various experimental settings that can roughly be divided into two large groups: angiogenesis in the embryo and angiogenesis in postnatal life. Embryonic angiogenesis comprises diverse processes, from the generation of cells of vascular components to the formation of higher-order architecture complying with the function and structure of tissues to be vascularized. Angiogenesis in postnatal life provides the advantages of visibility and manipulability of its processes. The most common postnatal angiogenesis is the one induced by tumors, which has indeed contributed to the understanding of molecular mechanisms regulating angiogenesis [20]. The architecture of tumor vasculature is usually far less organized, however, than that attained during embryogenesis, making it unsuitable for studying morphogenetic processes required for constructing an hierarchical vascular architecture. In contrast to these pathological settings, some forms of physiological angiogenesis are initiated in postnatal life. In humans, the posterior part of the eye is nourished by two vascular networks, the choroidal and retinal vascular systems, which nourish the outer and inner layers of the retina, respectively [21-23]. During embryonic development, the inner layer of retinal vasculature is absent and nourishment of the retina is accomplished by choroidal and hyaloid vessels [24]. The hyaloids vasculature is a transient vascular network, which is attached to the lens and undergoes progressive regression as

(**A**)



(**B**)

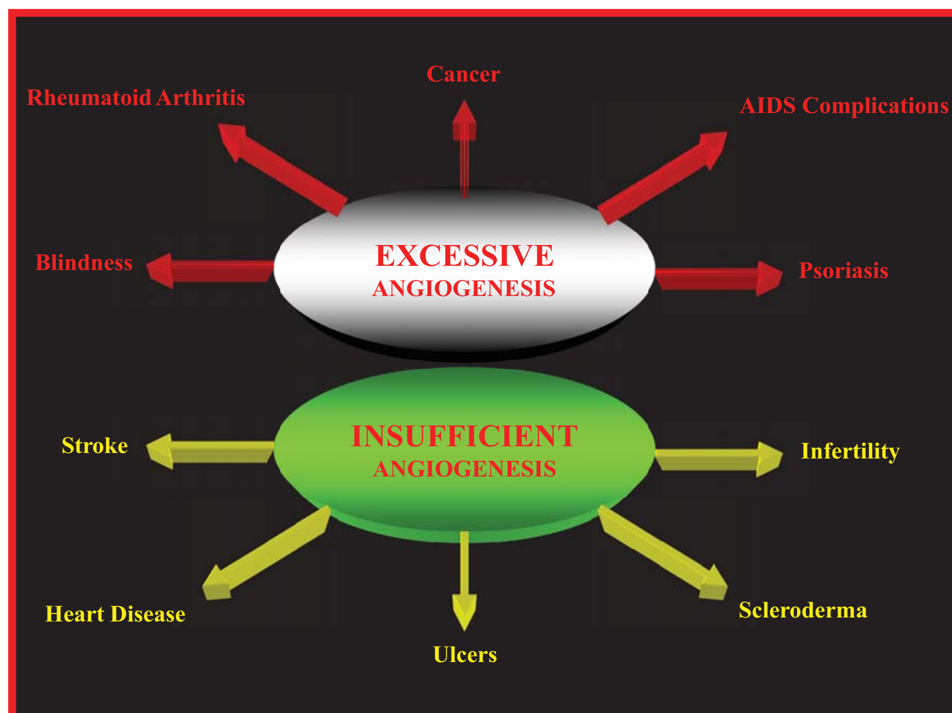
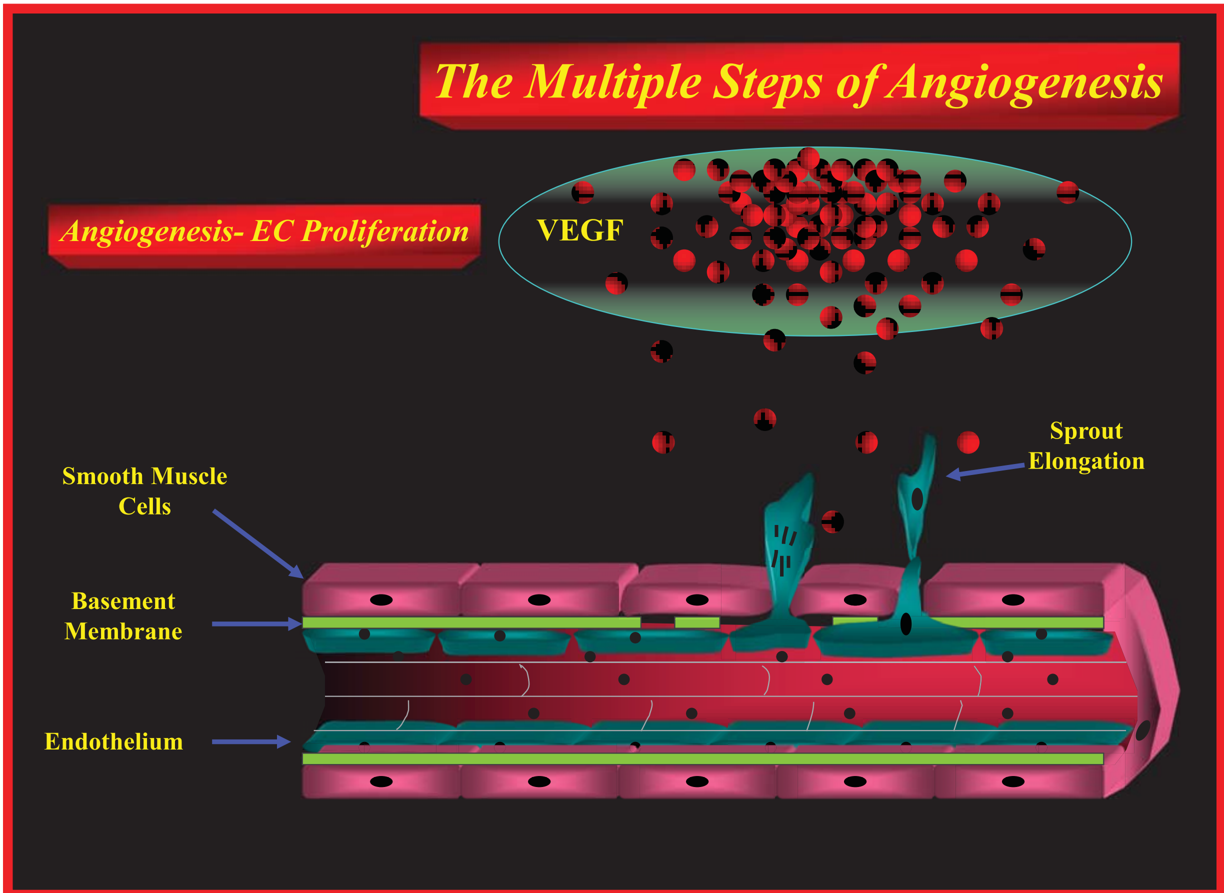
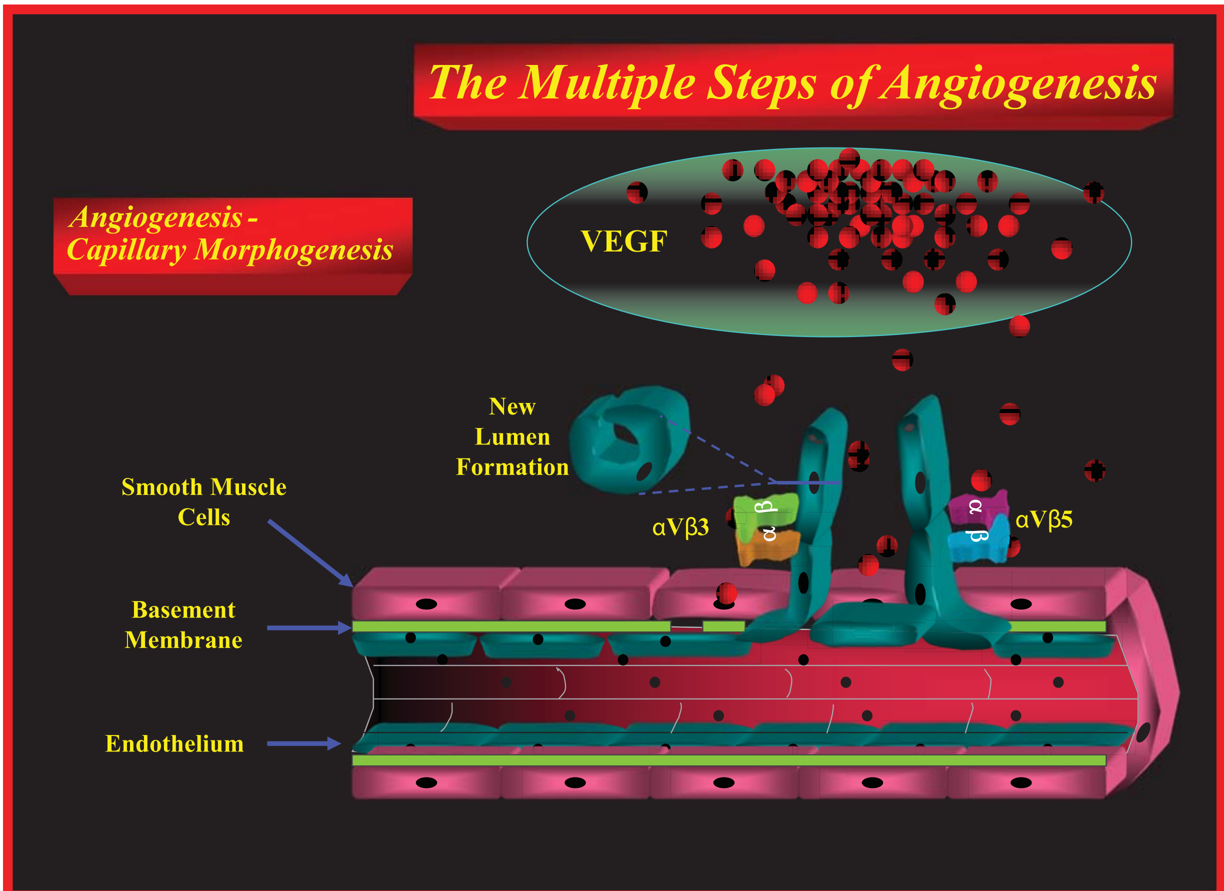


Fig. (2). New blood vessel formation occurs under physiologic and pathologic conditions. This can be characterized as excessive or insufficient. (**A**) Physiologic angiogenesis can be found in reproduction and development as well as in wound healing. Pathologic angiogenesis plays an essential role in tumor growth, in certain chronic inflammatory conditions such as rheumatoid arthritis and in ocular neovascularizing diseases. (**B**) Angiogenesis could be characterized as either excessive (cancer and blindness); or insufficient, as in ischemia and stroke. In the eye, angiogenesis is responsible for common vision-threatening diseases such as PDR, ROP, and secondary glaucoma after branch and central RVO, age-related maculopathy and corneal neovascularization.


The Multiple Steps of Angiogenesis
Angiogenesis- EC Proliferation
VEGF
Sprout
Elongation
Smooth Muscle
Cells
Basement
Membrane
Endothelium


The Multiple Steps of Angiogenesis
Angiogenesis -
Capillary Morphogenesis
VEGF
New
Lumen
Formation
Smooth Muscle
Cells
αVβ3
αVβ5
Basement
Membrane
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(Fig. 3) contd…..

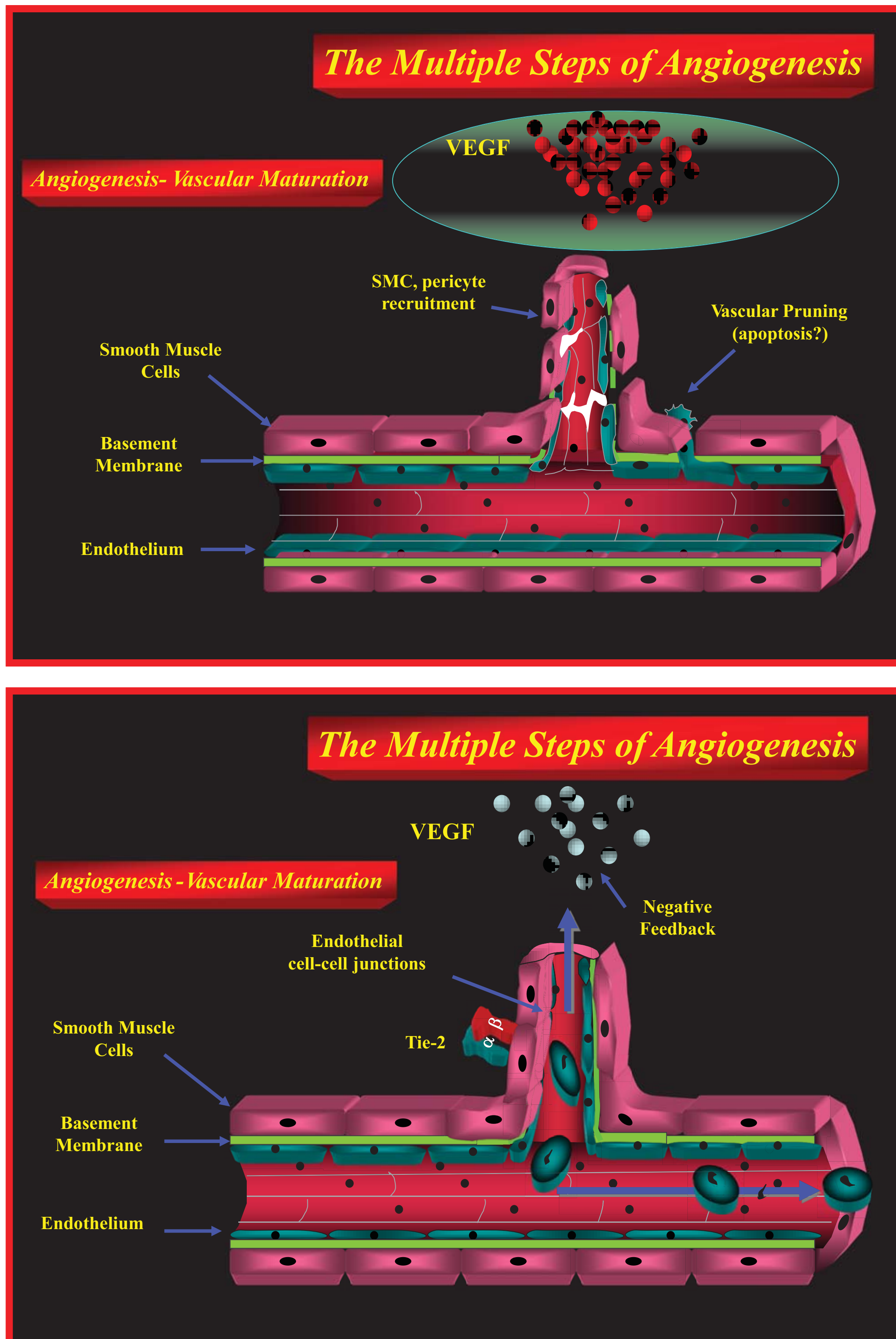

Fig. (3). The Multiple Steps of Angiogenesis. In the adult, new blood vessels arise by sprouting from preexisting vessels. This process can be simplified as follows: I.) Angiogenesis- Basement Membrane (BM) Breakdown: [1] Angiogenic Factors Production; [2] Angiogenic Factors Release; [3] Endothelial Cell Receptors (ECRs) Binding and Intracellular Signaling; [4] Endothelial Cells (ECs) Activation and BM Degradation. II.) Angiogenesis-ECs Migration: [1] ECs Migration; [2] ECs Proliferation. III.) Capillary Morphogenesis: [1] Directional Margination; [2] Extracellular Matrix (ECM) Remodeling. IV.) Vascular Maturation: [1] Tube Formation; [2) Loop Formation; [3] Vascular Maturation; [4] Vascular Stabilization.

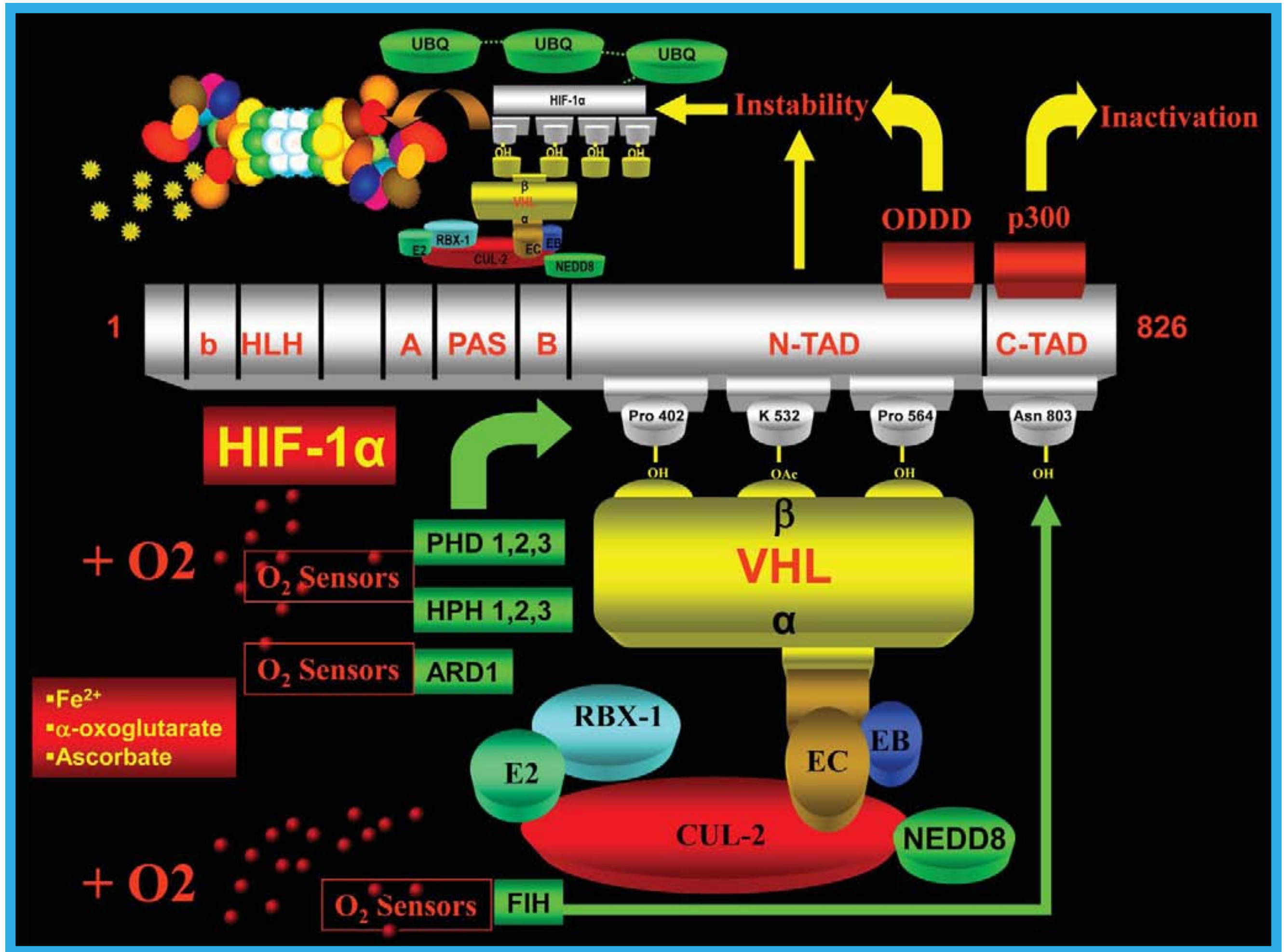

Fig. (4). Hypoxia Inducible Factor-1 (HIF-1) Signaling Pathway and the Regulation of (HIF-1)Protein Stability. In the presence of molecular oxygen (O_2), Prolyl Hydroxylase (PHD) post-translationally modifies hypoxia-inducible transcription factor (HIF)-1α, allowing it to interact with the von Hippel-Lindau (VHL) complex. Prolyl hydroxylase contains an iron moiety, so iron chelation inhibits this activity. VHL is part of a larger complex that includes Elongin-B, Elongin-C, CULLIN 2 (CUL2), RING-Box Protein 1 (RBX1), and Ubiquitin-Conjugating Enzyme (E2). This complex, together with ubiquitin-activating enzyme (E1), mediates the ubiquitylation (Ub) of HIF-1α. The Ub modification targets HIF-1α for degradation. Under hypoxic conditions HIF-1-prolyl hydroxylases are inactive which prevents binding of pVHL. Therefore, HIF-1α escapes ubiquitination and proteasomal degradation, and can be transported to the nucleus where, after dimerization with HIF-1β, it stimulates target genes transcription.

the retinal vasculature develops and matures. Angiogenesis in the mouse retina is a representative example that eventually develops into a highly organized architecture after birth. Because of the feasibility of monitoring and manipulating its entire process, retinal angiogenesis in neonatal mice has provided a valuable model system for investigating various events in angiogenesis, such as endothelial sprouting and morphogenetic organization of vascular networks [25-27].

PATHOLOGICAL ANGIOGENESIS

Ocular pathological angiogenesis may lead to visual impairment and even irreversible blindness in people of all ages worldwide. In addition, pathological angiogenesis in the retina is the leading cause of human blindness resulting from DR, AMD, and retinopathy of prematurity (ROP) [21, 28-30] (Fig. **2A**). These common ocular disorders are characterized by overgrowth of disorganized, leaky and physiologically non-functional retinal vessels, which lead to vision impairment and blindness [21, 28].

VEGF is essential in the development of retinal NV as one of the most potent angiogenic stimulators and vascular permeability factors. Inappropriate VEGF expression levels are associated with many diseases involving NV (Fig. **7B**; right frame). In the early stage of the disease, retinal vascular permeability can increase even before the appearance of clinical retinopathy [31, 32]. Retinal vascular leakage and thickening of the retina lead to DMO. In the late stage of DR, abnormal increases in vascular permeability result from retinal ischemia due to nonperfusion of the retina or a decrease in oxygen tension [33, 34] (Fig. **7A**; left column, and Fig. **7B**; right frame). During this stage, over-proliferation of capillary endothelial cells results in retinal NV, abnormal formation of new vessels in the retina and in

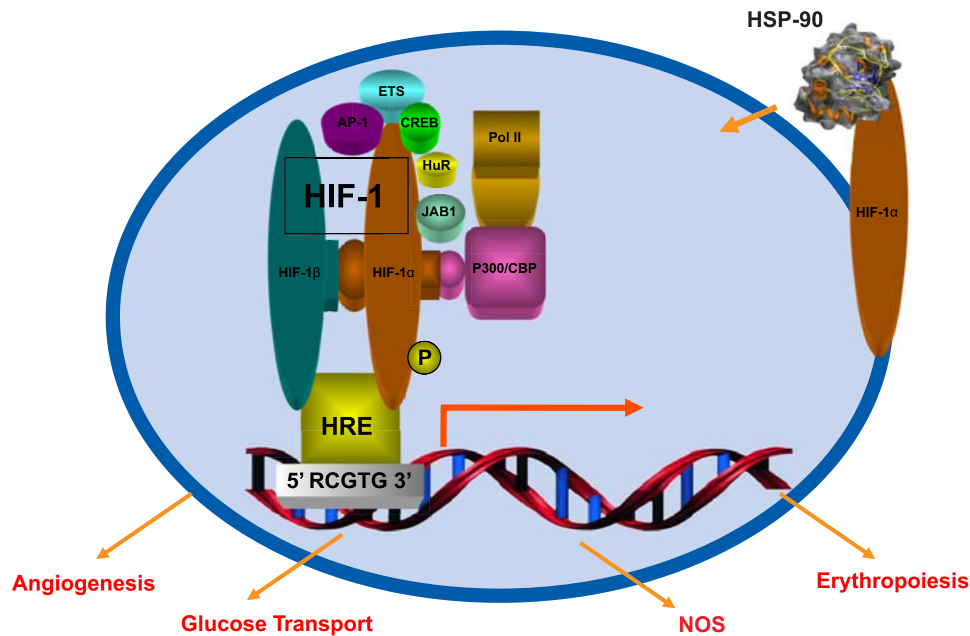

Fig. (5). The Stabilization and the Nuclear Translocation of HIF-1α. Under hypoxia, prolyl hydroxylase enzymes cannot be activated. Therefore, HIF-1α protein escapes degradation, therefore, it is, accumulated, stabilized, initiates a multistep pathway of activation that includes nuclear translocation *via* the assistance of the HSP90 chaperone, phosphorylation heterodimerization with its partner HIF-1β (formation of the stable heterodimer), transcriptional activation by the recruitment and the interaction of transcriptional coactivators CBP/p300, binding to DNA polymerase II, forming the HIF-1 nuclear complex and subsequent binding to cognate HREs of target genes. HREs have the minimal core sequence 5'-RCGTG-3' and are adjacent to auxiliary motifs specifying the responsive genes.

the vitreous, leading to proliferative diabetic retinopathy (PDR) [35, 36]. Additionally, during the late stages of DR, the ischemia-induced pathological angiogenesis ultimately causes severe vitreous cavity bleeding and/or retinal detachment, resulting in severe vision loss. Angiogenic factors, such as VEGF, play a prominent role in promoting retinal NV. Retinal ischemia is the major driving force behind the induction of VEGF, which plays a crucial role in ocular pathogenesis [12, 37, 38]. VEGF has a profound impact on multiple functions in endothelial cells, such as proliferation, migration, survival, tube formation, and vascular permeability [39, 40] (Fig. **3A-E**). Previous studies have indicated that VEGF is an important mediator of NV induced by hypoxic retinopathies [41]. VEGF is implicated strongly in the development of retinal and iris NV in PDR. Eyes with nonproliferative and PDR showed an upregulated expression of VEGF and its mRNA by the retina [42, 43]. It has been reported that there is increased VEGF production in both vitreous [44], and ocular fluids [45] of patients with DR. Suppression of VEGF receptor interaction, VEGF expression and VEGF-induced signaling has been shown to inhibit NV in animal models of retinal ischemia. In ROP, high O_2 perfusion leads to termination of hyaloid vessel regression and the formation of premature retinal vasculature [21, 46]. A rodent model has been developed to imitate human ROP in which newborn mice are exposed to hyperoxia [47]. Upon return to normoxia, the infant retina is relatively hypoxic leading to pathological angiogenesis. Many lines of evidence support the idea that alteration of O_2 levels is the key driving force of initiating pathological NV. These include: [1] Pathological retinal angiogenesis occurs in association with retinal ischemia in several diseases [2, 48, 49]. Development of retinal vasculature in embryos is determined by O_2 gradients [3, 21, 50]. Hypoxia-inducible VEGF expression levels are spatiotemporally coupled to retinal NV [4, 21, 46, 50, 51]. Anti-VEGF agents show remarkable therapeutic efficacy in both animal and human ophthalmologic disorders characterized by retinal NV [21, 46, 50-52]. These findings identify hypoxia-induced VEGF as the key angiogenic molecule responsible for retinal NV. Indeed, the newly formed retinal vessels in AMD, DR and ROP share unique features with VEGF-induced vascular networks. For example, pathological retinal vessels are premature, highly disorganized, and leaky. In fact, AMD, DR and ROP all exhibit the clinical manifestations of severe retinal edema, leading to impairment of vision [23]. High VEGF levels in ischemic retinas might reflect a compensatory mechanism by which VEGF intends to improve O_2 delivery during retinal hypoxia. However, the VEGF-induced vasculature consists of disorganized and leaky primitive vascular networks leading to retinal edema and impairment of vision [53]. Thus, VEGF has become an attractive key target for development of new drugs against ocular diseases. Not surprisingly, both animal studies and clinical experiences demonstrate that blockage of VEGF signaling is a valid approach for the treatment of AMD although the therapeutic efficacy for DR and ROP needs further validation in appropriate animal models. VEGF binds to both Vascular Endothelial Growth Factor Receptor-1 (VEGFR-1) and Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2) mainly distributed in endothelial cells and it is VEGFR-2 that mediates active angiogenic and vascular permeability functions [54]. Thus, VEGFR-2 has become an attractive therapeutic target for treatments of several common human diseases including cancer and AMD.

Fig. (6). Hypoxia-Inducible Factor-1-Transcriptionally-Activated Genes and Their Critical Roles in Diverse Cellular Functions. Hypoxia Inducible Factor-1 (HIF-1); 6-Phosphofructo-2-kinase/Fructose-2,6-Biphosphatase-3 (PFKBF3); Adenylate Kinase 3 (AK3); Adrenomedullin (ADM); Aldolase A (ALDA); Aldolase C (ALDC); Artrial Natriuretic Peptide/Factor (ANP, ANF); Cathepsin D (CATHD); CITED2 (p35srj); Connective Tissue Growth Factor (CTGF); DEC1 (Stra13; Sharp2); Ecto-5'-Nucleotidase (5'NT, CD73); Endocrine Gland-Derived VEGF (EG-VEGF); Endoglin (ENG) (CD105); Endothelin-1 (ET-1); Enolase 1 (ENO1); Erythropoietin (EPO); Ferrochelatase (FECH); Fibronectin 1 (FN1); GADD153 (CHOP); Glucose Transporter 1 (GLUT1); Glucose Transporter 3 (GLUT3); Glucose-6-phosphate Isomerase (GPI; AMF; Neuroleukin); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Hepatic Growth Factor (HGF); Hexokinase 1 (HK1); Hexokinase 2 (HK2); HIF-1 Prolyl Hydroxylase 1 (PHD1); Inducible Nitric Oxide Synthase (iNOS; NOS II); Inhibitor of Differentiation/DNA Binding 2 (ID2); Integrin β2 Subunit (CD18); Intestinal Trefoil Factor (ITF); Lactate Dehydrogenase A (LDHA); Late Envelope Protein 1 (LEP1); LDL-Receptor-Related Protein 1 (LRP1); Leptin (LEP); Matrix Metalloproteinase-2 (MMP-2); MIC2 (CD99); N-myc Downstream Regulated Gene 1 (Cap43; NDRG1); Nucleophosmin (NPM1); Nuclear orphan steroid receptor (NUR77); Cyclin-dependent kinase inhibitor 1 (p21; WAF-1/CIP1); Cyclin-dependent kinase inhibitor 1B (p27); Tumor protein p53 (Li-Fraumeni syndrome) (p53); PKR protein kinase (p58; IPK); Pentraxin 3 (PTX3); P-glycoprotein (MDR1); Phosphofructokinase C (PFK-C); Phosphofructokinase L (PFKL); Phosphoglycerate Kinase 1 (PGK1); Plasminogen-activator Inhibitor-1 (PAI-1); Peroxisome proliferator activated receptor γ (PPARγ2); Presenilin-1 (Psen1); Presenilin-2 (Psen2); Prolyl-4-Hydroxylase α-1 (4-PHα-1); Pyruvate Kinase M (PKM); DNA damage response 2 (REDD2); HIF-1 responsive (RTP801; REDD1); Stromal Cell-Derived Factor-1 (SDF-1; CXCL12); Transforming Growth Factor-α (TGF-α); Transforming Growth Factor- β1 (TGF-β1); Transforming Growth Factor- β2 (TGF-β2); Transforming Growth Factor- β3 (TGF-β3); Transferrin (CD142; Thromboplastin); Transferrin Receptor (TfR); Transglutaminase 2 (TGase2); Triose Phosphate Isomerase (TPI); Urokinase Plasminogen Activator Receptor (UPAR); Vascular Endothelial Growth Factor (VEGF); VEGFR-1 (Flt-1); Vimentin (VIM); Viral-like 30 Element (VL30); X-box binding protein 1 (XBP1); α1β-Adrenergic Receptor (α1β-AR).

ROLE OF HIF-1 IN ADAPTATION TO HYPOXIA

Oxygen functions as a scavenger to remove harmful electron and hydrogen ions, both of which are waste by-products that remain after mitochondrial oxidative phosphorylation. Hence, when oxygen delivery is disrupted in hematological diseases, pulmonary, and cardiovascular, this energy metabolism is seriously impaired. Therefore, eukaryotes have developed several adaptive mechanisms to

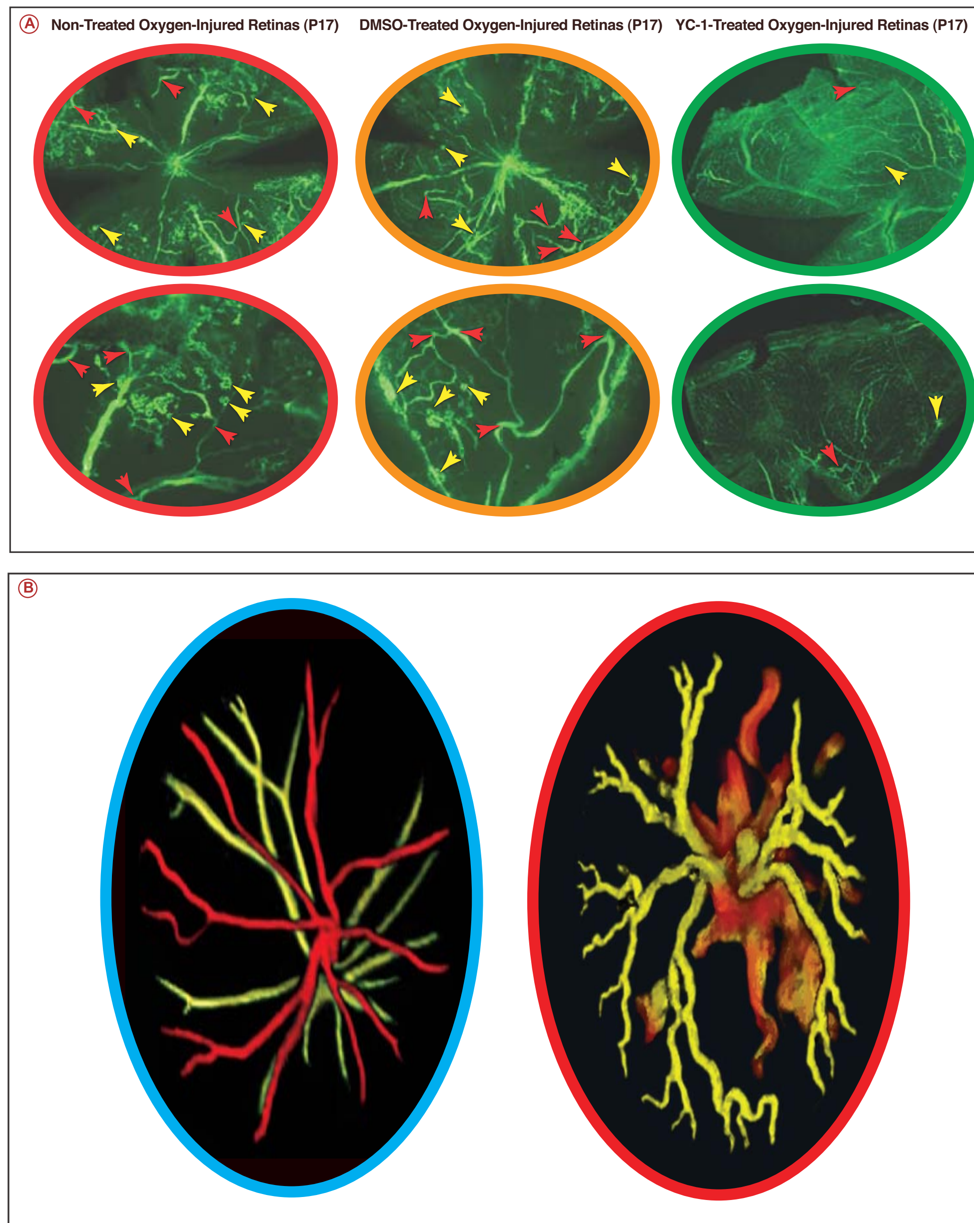

Fig. (7). (**A**) **Quantitative Assessment of NV in P17 Mice Exposed to a Cycle of Hyperoxia and Room Air.** Representative images of whole-mount retina preparations on P17 of different mice groups. Column 1: Retina from P17 non-treated oxygen-injured group (n=8 retinas) shows an engorged tortuous appearance of the blood vessels, large central avascular area (yellow arrows), and high presence of blood vessel tufts (red arrows). Column 2: Retina of oxygen-exposed mouse that was injected with the DMSO, immediately after removal from 5-day treatment of 75% oxygen, on P12 and P15 and sacrificed on P17 (n=11). The retinas show profound vascular engorgement with apparent vaso-obliteration and neovascular tuft formation. The avascularized area has the same size after injection of the vehicle. The size of the central avascularized areas remained almost unaffected by DMSO. Column 3: Retina from P17 mice that have undergone the oxygen-induced model of retinopathy, subsequently given double injections of YC-1 on P12 and P15, and later sacrificed on P17 (n=11). The retinas display a normal vascular pattern similar to (**A**). When compared to non-treated ischemic retinas, the structure of the retinal vasculature was significantly better preserved; the avascularized areas and the growth of new vascular tufts were significantly decreased after a double intravitreal injection-regimen of YC-1. Note that there is a significant decrease in the degree of retinal NV in YC-1-treated retina compared with non-treated oxygen-injured and DMSO-treated mice (***$P < 0.001$). Scale bars: A1, B1, and C1 = 100 µm; A2, B2, and C2 = 200 µm. (**B**) **Retinal neovascularization as presented in the artist's rendition to provide a three-dimensional view to assist in interpretation of histological section of retinal neovascularization.** Left column (blue frame) represents the color-coded image of the ocular vasculature in a P17 retina. Certain regions were color coded during image processing according to their depth. Yellow = hyaloidal vessels, red = retinal vasculature. Right column (red frame) exhibits the ocular pathologies in a mouse model of OIR at P17. Evidence for pre-retinal neovascularization is indicated by the enlarged regions of retinal vessels, in addition to the hyaloidal and retinal vascular tortuosity that persists. The retinal vasculature of the optic disc area is often affected in the OIR model, which was substantiated by the evidence of abnormal vascularization in this area.

enable cells to survive in oxygen-depleted conditions. Hypoxic adaptation includes reflex hyperventilation, the increased production of red blood cells, and new vessel formation, which in combination lead to increased oxygen delivery from the atmosphere to tissues [55]. At the cellular level, adaptation involves a switch of energy metabolism from oxidative phosphorylation to anaerobic glycolysis, which increases glucose uptake, and expressions of stress proteins related to cell survival or death [56].

HIF-1 is a transcription factor that controls the hypoxic response and oxygen homeostasis in mammalian cells [57]. HIF-1 plays a critical role by regulating genes, which are involved in angiogenesis [58] erythropoiesis [59], energy metabolism, glycolysis, and apoptotic and proliferative responses to ischemia and hypoxia [57] (Fig. **6**). HIF-1 is a member of the Basic Helix-Loop-Helix/PER-ARNT-SIM (bHLH/PAS) superfamily of transcription factors [60] (Fig. **4**). HIF-1 is a heterodimer composed of two bHLH proteins of the PAS family, HIF-1 and ARNT (HIF-1β) (Fig. **5**). The HIF-1ß subunit is constitutively expressed and unregulated by oxygen tension [61]. HIF-1α is composed of 826 amino acids [59]. Its N terminal contains the bHLH and the PAS domains, which are essential for dimerization and DNA binding [62], and its C-terminal contains two transactivation domains and a nuclear localization signal [63]. The central portion of HIF-1α (amino acid. 401-603) contains an oxygen-dependent degradation domain (ODDD), which determines the stability of HIF-1α protein [64] (Fig. **4**). The regulation of HIF-1 occurs at the transcriptional, translational, post-translational, protein-protein interaction and degradation. The mechanism by which HIF-1α protein is upregulated by hypoxia has been well characterized [65]. In aerobic conditions, HIF-1-prolyl hydroxylases modify two proline residues in the amino acid motif LXXLAP located at either end of ODDD [66, 67]. Under normoxic conditions, HIF-1α subunits are very unstable and are rapidly targeted for degradation in the 26S proteasome [68] (Fig. **4**). Under normoxia, proline hydroxylation in the ODDD of HIF-1α mediates HIF-1α binding to the pVHL, a part of the E3 ubiquitin ligase protein complex. In turn, von Hippel Lindau (pVHL) assembles a complex with E3 ubiquitin ligase that targets HIF for polyubiquitination and subsequent proteosomal degradation [68] (Fig. **4**). Since the enzymatic reaction of prolyl hydroxylation requires oxygen as a substrate, hypoxia limits this hydroxylation, thereby precluding the binding of pVHL and thus leading to the stabilization of HIF-1α [69]. Therefore, active HIF-1 is primarily expressed during hypoxia. Hence, exposure to hypoxia results in a rapid increase of HIF-1α protein in most cells [70] *in vivo* and *in vitro* [65]. In addition to prolyl hydroxylation, the acetylation of a lysine residue (Lys532) within the ODDD also mechanistically regulates HIF-1α stability. Lysine acetylation also enhances the interaction between HIF-1α and pVHL, since the expression of arrest-defective protein 1 (ARD1), an HIF-1α acetyltransferase, decreases under hypoxic conditions, HIF-1α escapes from acetylation and becomes stable [71]. Taken together, when intracellular oxygen reaches a critically low threshold, HIF-1α subunits are rapidly protected from proteosomal degradation, allowing HIF-1α and HIF-1ß subunits to associate and form the active HIF-1 transcription factor [70]. HIF-1 transcription factor binds to the hypoxia response element (HRE) in hypoxia-responsive target genes, and triggers a global role in the transcriptional regulation and the expression of a variety of hypoxia-responsive target genes, and triggers the transcription of many genes such as VEGF [72], inducible nitric oxide synthase (iNOS) [73], erythropoietin (EPO) [59], and endothelin-1 (ET-1) [74] (Figs. **5**, **6**). It has been established that there is a temporal and spatial correlations between the expression of HIF-1 and VEGF in an animal model of retinal NV [75]. Homology searches in the gene bank and cloning experiments found other members of this family, such as HIF-2α (endothelial PAS protein-1 or MOP2) [76] and HIF-3α [77]. HIF-2α is highly similar to HIF-1α in protein structure, but exhibits restricted tissue-specific expression. HIF-2α is also tightly regulated by oxygen tension and its complex with HIF-1β appears to be directly involved in hypoxic gene regulation, as is HIF-1α [78] (Fig. **4**). However, although HIF-3α is homologous to HIF-1α, the physiological role of HIF-3α is uncertain. It could function as a negative regulator of hypoxia-inducible gene expression [79].

PHARMACOLOGICAL APPROACHES FOR AN HIF-1-TARGETING ANTI-ANGIOGENIC, ANTI-NEOVASCULAR THERAPY

Several approaches have been examined experimentally to chemically target cellular processes that regulate HIF-1α expression. For example, heat shock protein-90 (HSP90) is a molecular chaperone involved in the folding of HIF-1α protein [80, 81]. A previous study of the HSP90 inhibitor geldanamycin showed that HIF-1α can be destabilized when HSP90 binding is inhibited [82]. Furthermore, previous data have indicated that RNAi targeting HIF-1α could inhibit the retinal NV by approximately 65% through down-regulating the expression of HIF-1α and VEGF in the murine retinas, which may provide a powerful and novel therapeutic tool for ischemic-induced retinal diseases [101]. These data indicate that the application of HIF-1α Small interfering RNA (siRNA) and VEGF siRNA technology holds great potential as a novel therapeutic for retinal NV. In addition, the oncogenes rat sarcoma viral oncogene homolog (Ras) [83] and Src [63] are also responsible for stabilizing HIF-1α. Pharmacological agents that inhibit the activity of the Ras oncogene, such as the farnesyl transferase inhibitors, could potentially exert anti-neovascular properties by inhibiting HIF-1; moreover, the inhibition of Src kinase could be targeted for HIF-1 inhibitor development. On the other hand, tumor suppressor genes, such as phosphatase and tensin homolog (PTEN), have been reported to inhibit HIF-1α expression. PTEN protein also inhibits the cellular process of HIF-1α stabilization by antagonizing the phosphatidyl-3-inositol kinase (PI3K) pathway. Therefore, it is possible that pharmacologic approaches taken to activate these tumor suppressor genes may also be applicable to the inhibition of HIF-1. Furthermore, a growing body of evidence indicates that the PI3K pathway modulates both the protein expression and the transcriptional activity of HIF-1α [84]. PI3 kinase inhibition is considered as an attractive pathway to modulate the development of angiogenesis in the eye. Several studies have indicated that LY294002 exerts its anti-angiogenic effect in a dose-dependent manner, without perturbing existing vessels. Moreover, growth factors reported to show this effect, include epidermal growth factor (EGF) [85],

insulin-like growth factor (IGF-1) [86], and insulin-I [87]. These growth factors are known to bind to their receptors and to activate receptor tyrosine kinases, which in turn activate the phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway.

ANTI-HIF-1, ANTI-ANGIOGENIC, AND ANTI-NEOVASCULAR EFFECTS OF YC-1: THERAPEUTIC CONSEQUENCES AND PERSPECTIVES

YC-1; (3-(5'-hydroxymethyl-2'furyl)-1-benzyl indazole) is a small molecule HIF-1 inhibitor, which potentiates soluble guanylyl cyclase (sGC) stimulation. YC-1 was first described by Ko *et al*. 1994 [88]. In isolated platelets, YC-1 inhibits platelet aggregation, adenosine triphosphate (ATP) release, phosphoinositide breakdown, and the elevation of intracellular free calcium. These pharmacological actions of YC-1 are derived from the activation of platelet sGC and the elevation of cyclic guanosine monophosphate (cGMP). In mice, YC-1 was found to prevent intravascular thrombus formation by inhibiting platelet aggregation [88]. In platelets and vascular smooth muscle, the administration of YC-1 is accompanied by an increase in the intracellular cGMP concentration, which occurs *via* the stimulation of sGC [89]. Similarly, YC-1 was found to activate sGC in vascular and corpus cavernosal smooth muscle cells, promoting vasodilation and penile erection [90]. Biochemical studies on expressed sGC showed that the stimulatory effect of YC-1 on sGC is enhanced by the physiologic sGC activators nitric oxide (NO) and carbon monoxide (CO) [91, 92]. Indeed, combined treatment with YC-1 and natural sGC activators was found to synergistically inhibit platelet aggregation and to relax arterial smooth muscle. Therefore, YC-1 is viewed as a research tool for investigating sGC- and cGMP-mediated cellular processes, and as a potential drug for treating platelet-rich thrombosis, vasospasm, or male erectile dysfunction. Previous data have indicated that YC-1 has novel effects on HIF-1α expression and tumor promotion. Initially, YC-1 was used as a NO mimic to examine the effects of NO signaling on hypoxic response in Hep3B hepatoma cells. YC-1 was found to block the induction of EPO and VEGF mRNAs, and to suppress the DNA-binding activity of HIF-1 [93]. Furthermore, it was also found to inhibit non-hypoxic HIF-1α induction by cobalt or desferrioxamine. However, sGC inhibitors failed to block these effects of YC-1 on HIF-1α, and further treatment with 8-bromo-cGMP also failed to inhibit the hypoxic induction of HIF-1α. These results indicate that the HIF-1-inhibitory effect of YC-1 is unlikely to be mediated by sGC/cGMP signal transduction, rather that the YC-1 effect is probably achieved by a novel cellular process linked with the oxygen-sensing pathway [93]. Since HIF-1 plays a crucial role in angiogenesis, YC-1, as a novel HIF-1 inhibitor, could be further developed as a novel anti-angiogenic and anti-neovascular agent targeting HIF-1 and retinal NV.

Recently, we have demonstrated that YC-1 suppressed vascular endothelial cell proliferation, migration and tube formation, while it significantly increased the proteasome activity [94, 95]. These data revealed that YC-1 possesses pleiotropic effects, which target several anti-angiogenic mechanisms in the retinal cell model and the retinal explant culture setting. These effects included; [1] inhibition of the angiogenic phenotype, by inhibiting proliferation, migration, tubule and new vessel formation; [2] suppression of HIF-1α protein levels, accumulation, and stability; [3] blocking HIF-1α nuclear shuttling mechanism; [4] promoting HIF-1α-proteasomal degradation; [5] inhibit mRNA and protein levels of VEGF, EPO, ET-1; [6] may directly or indirectly suppress the transcriptional and the translational levels of HIF-2α and matrix metalloproteinase-9 (MMP-9); and [7] induce a G0/G1 cell-cycle arrest. Furthermore, in a 3D collagen matrix model using mouse retinal explants cultured under normoxic and hypoxic conditions, YC-1; [1] inhibited outgrowth of new vessel sprouts; [2] reduced VEGF expression; [3] dramatically decreased the vessels immunoreactivities for CD31 and von Willebrand factor (vWF); and [4] was highly effective in reducing the vascular density within the retina, compared to controls. Throughout our most recent investigation [96]; we have demonstrated that YC-1 exhibited pleiotropic effects, which influenced both mechanisms; angiogenesis and vascular repair, *via* the inhibition of HIF-1α. YC-1 inhibited pathological retinal NV (pathological angiogenesis) by exhibiting anti-angiogenic activities, which impaired ischemia-induced expression of HIF-1 and its further downstream angiogenic molecules, such as VEGF, EPO, ET-1, leading to the inhibition of NV in the retina (Fig. **7A**). Concomitantly, YC-1 promoted physiological revascularization (RV) (physiological angiogenesis) and exhibited vascular repair properties in the avascular retina by impairing ischemia-induced expression of HIF-1 and its downstream anti-angiogenic molecules in the avascular retinas, such as iNOS (Fig. **7A**).

Our findings indicate that YC-1 has conducted a "rescue operation" by executing the following assignments; [1] prevention and/or inhibition of pathological retinal NV; *via* the inhibition of HIF-1 and its downstream pro-angiogenic molecules; [2] concomitant promotion of retinal physiological RV; *via* the inhibition of iNOS. Therefore, the retinal vasculature of the YC-1-treated retinas has returned to its homeostatic level, which resembled the appearance of the retinal plexus during normal development (Fig. **7B**; left frame). This therapeutic modality represents a "double-whammy" effect for YC-1 on retinal NV. Therefore, this modality represents the use of intravitreous YC-1 in the management of retinovascular diseases, particularly those with ongoing intraretinal vascular development, such as ROP. There may be a more favorable therapeutic index for YC-1 in treating retinopathy than for an anti-angiogenic agent that suppresses all vessel growth. Our own studies have demonstrated that YC-1 exerted notable *in vivo* anti-angiogenic effects, which could be exploited as valuable therapeutic potentials to inhibit retinal NV in the ischemic retina. Moreover, we have demonstrated that YC-1 selectively inhibits pathological NV and statistically reduced the retinopathy score when compared to the DMSO-treated retinas (Fig. **8**); while concomitantly promotes physiological RV in a mouse model of OIR (Fig. **7A**) [96]. Furthermore, our studies have investigated the efficacy of YC-1 in modulating iNOS expression as a therapeutic modality to target retinal NV *in vivo,* and examined its therapeutic potentials as a HIF-1 and an iNOS inhibitor. Moreover, YC-1 treatment did not cause any toxicity or impair innate immunity during its treatment period. Thus, we believe that YC-1 should be regarded as a good lead compound for the

development of novel anti-angiogenic, anti-neovascular agent for the treatment of retinal NV. Such pleiotropic effects of YC-1, which are noticeably different from the relatively single effects of other angiogenesis inhibitors, can be reasonably inferred to allow better *in vivo* and even better clinical outcome as a novel anti-angiogenic agent.

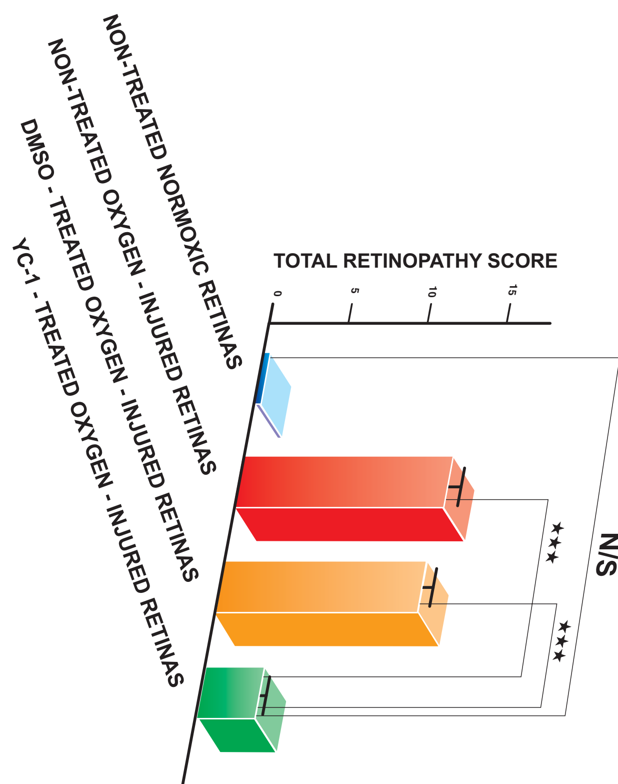

Fig. (8). Total Retinopathy Scores in the Normal and the Experimental OIR Groups Animals (P17) treated with YC-1 after hyperoxia had significantly less total retinopathy scores than oxygen-injured retinas. Average retinopathy score of room air-reared mice (blue bar graph). Average retinopathy score of mice exposed to 75% oxygen (red bar graph). Average retinopathy score of mice exposed to 75% oxygen that had double-injections of DMSO (orange bar graph). Average retinopathy score of mice exposed to 75% oxygen and had a double-injection regimen of YC-1 (100 μM) on P12 and P15 (green bar graph). (***P<0.001) and (**P < 0.01), as compared to DMSO-treated ischemic control.

DOES YC-1 PROMOTE DEVELOPMENTAL GENE EXPRESSION?

In this review, we are proposing for the first time that YC-1 may have promoted the expression of specific developmental genes that are associated with physiological angiogenesis; hence it enhanced the development and growth of blood vessels. This is a highly plausible concept, since the most recent data have indicated that YC-1 significantly enhances NK cell populations differentiated from human umbilical cord blood hematopoietic stem cells (HSCs) [97]. NK cells increased by YC-1 display both phenotypic and functional features of fully mature NK (mNK) cells. Data have indicated that YC-1 enhanced NK cell differentiation through the activation of p38 mitogen-activated protein kinase (MAPK), which is involved in NK cell differentiation. Furthermore, previous data have indicated that YC-1 has an influence on the differentiation of stem cells into myocardial cells and the differentiation of mouse bone marrow-derived mesenchymal stem cells (BMSCs) into osteoblast through cyclic guanosine monophosphate (cGMP)-dependent mechanisms [98, 99], and affects the maturation of monocyte-derived dendritic cells in a cGMP-independent manner [100]. Therefore, we could hypothesize that YC-1 might affect the differentiation of the other precursor cells that are involved in physiological RV of the retina and the development, growth and the formation of healthy blood vessels. We propose that YC-1 regulates specific developmental programs in the window following initial blood vessel development and formation commitment and before cell-type differentiation, with YC-1 serving initially as a key component of opposing co-activator and co-repressor complexes that are recruited in a gene-specific fashion that is required for dictating specific programs of gene expression during mammalian angiogenesis. We consider it likely that YC-1 activates a cohort of developmental gene targets, and/or specific substrates that are likely to be biologically important. A second, intriguing aspect of regulation of developmental gene expression; is whether such regulation is temporally regulated and cell-type-specific.

CONCLUSION

It is clear that the modulation of HIF-1 activity can be an excellent strategy for the treatment of ischemia-related retinal pathologies. Moreover, HIF-1 activity upregulation may promote cell survival during hypoxia or ischemia, and increase angiogenesis at oxygen-deficient tissues. The inhibition of HIF-1 activity could prevent angiogenic activity and the survival of the pathological retinal tissue with ischemic regions. Thus, HIF-1 inhibitors may be widely useful as therapeutic agents for the treatment of various diseases associated the over-activation of HIF-1, such as retinal NV and other ocular angiogenesis-related diseases. Much effort is being expended to develop new HIF-1-targeting agents by academia and the pharmaceutical industry. The success of these efforts will result in a new drug class, namely, HIF-1-targeting anti-angiogenic and anti-neovascular agents, which hopefully will improve the prognoses of many diabetic patients.

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ABBREVIATIONS

NV	=	Neovascularization
RV	=	Revascularization
HIF-1	=	Hypoxia Inducible Factor-1
VEGF	=	Vascular Endothelial Growth Factor

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