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“Curcumina en la inhibición de la angiogénesis inducida por epitelio pigmentario de la retina, estudio comparativo con Bevacizumab”

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DEDICATORIA

Dedico este trabajo a quienes dia a dia luchan contra la ceguera.

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Abstract

Choroidal neovascularization is the third leading cause of blindness in the western world. Its etiology remains unknown. However, it has been established that both up-regulation of VEGF and down-regulation of PEDF at the outermost layer of the retina, in the so-called retinal pigment epithelium – choroid complex, is the milestone of this pathological process.

The regulatory mechanisms that ultimately fail to inhibit this pro-angiogenic environment are unknown and current treatments are not completely effective preventing blindness due to this disease. Nevertheless, the transcriptional factor hypoxia induced factor -1 (HIF-1) has been implicated in the pathogenesis of choroidal neovascularization (CNV). The action of HIF-1 upon hypoxia mediated-stabilization is to activate the transcription of specific hypoxia adaptive genes, including VEGF.

Curcumin is a natural diphenol extracted from *Curcuma longa*, and displays a wide range of anti-cancer activities. Among them, inhibition of HIF-1 by yet undefined pathways has been observed in tumor cells. Hence, we aimed to determine the effect of curcumin on hypoxia mediated-choroidal neovascularization.

To this end, we used a bi cellular model, including retinal-pigmented epithelium (RPE) cells and coroidal vascular endothelium (CVE) cells. Firstly, we determined RPE response to hypoxia. Secondly, we explored the response of CVE cells to RPE hypoxia, and the effect that curcumin may elicit in this signaling. Finally, we used an *in vivo* model to further confirm our *in vitro* findings.

As expected, we observed an angiogenic activity in CVE cells in response to RPE hypoxia through soluble factors contained in the conditioned medium. Curcumin was able to inhibit this effect, preventing CVE cell proliferation, migration and hypoxia-induced experimental CNV. The production of VEGF in RPE under hypoxia was also decreased upon curcumin treatment. In an inverse fashion, PEDF expression was upregulated with curcumin treatment. Finally, curcumin was able to inhibit hypoxia RPE-induced angiogenesis *in vivo*.

These data suggest that curcumin could eventually be considered as a possible therapeutic molecule to prevent or treat choroidal neovascular disease and thus, reduce blindness.

Abbreviations and Glossary in alphabetical order

AMD: Age Macular Degeneration. Macular degeneration, often age-related macular degeneration (AMD or ARMD), is a medical condition that usually affects older adults and results in a loss of vision in the center of the visual field (the macula) produced by damage to the retina. It is a major cause of blindness and visual impairment in older adults (>50 years). It occurs in "dry" and "wet" forms. In the wet (exudative and age-related) form, which is more severe, blood vessels grow up from the choroid behind the retina (known as choroidal neovascularization), and the retina can also become detached. The dry form does not produce choroidal neovascularization and rarely leads to blindness.

ARPE-19: ARPE-19 is a spontaneously arising retinal pigment epithelial (RPE) cell line derived in 1986 by Amy Aotaki-Keen from the normal eyes of a 19-year-old male who died from head trauma in a motor vehicle accident. These cells form stable monolayers, which exhibit morphological and functional polarity. ARPE-19 expresses the RPE-specific markers CRALBP and RPE-65.

CNV: Choroidal neovascularization. Consists in the creation of new blood vessels in the choroid layer of the eye. This is a common sign of the wet type of AMD.

CVE: Choroid Vascular Endothelium. Is the endothelium that underlies capillaries of the choroid, which is the vascular layer of the eye. The coroid also contains connective tissue, and lies between the retina and the sclera. The choroid provides oxygen and nourishment to the outer layers of the retina.

DR: Diabetic Retinopathy. Is a disease of the retina caused as complication of Diabetes, as result of microvascular retinal changes and hypoxia. This disease can also establish a pro angiogenic environment in the retina, leading to retinal neovascularization and blindness.

FGF: Fibroblast Growth Factor. A family of small polypeptide growth factors that share several common features, including a strong affinity for HEPARIN, and a central barrel-shaped core region of 140 amino acids that is highly homologous between family members. Although originally studied as proteins that stimulate the growth of fibroblasts, this distinction is no longer a requirement for membership in the fibroblast growth factor family.

HIF-1: Hypoxia-induced Factor 1. A basic helix-loop-helix transcription factor that plays a role in apoptosis. It is composed of two subunits: aryl hydrocarbon receptor nuclear translocator and hypoxia-inducible factor 1, alpha subunit.

HRE: Hypoxia Responsive Element. Binding site for HIF-1α in the promoter region of specific hypoxia-regulated target genes.

PBS: Phosphate Buffer Saline.

RPE: Retinal Pigment Epithelium. Pigmented monolayer of cuboidal cells with phagocytic function, responsible for maintaining the subretinal space and the external hemato-retinal barrier. It also degrades the discs of the outer segments of the photoreceptors.

shRNA: "Short hairpin RNA". Corresponds to small RNA fragments used to attach and degrade specific mRNA via proteasome. This means that a "silenced" gene is achieved, leading to a decrease in the final functional protein.

TER: Trans Epithelial Resistance. The electrical resistance of an epithelium, which means resistance to the passage of an electric current through that epithelium. The inverse quantity is electrical conductance, the ease with which an electric current passes. The unit of electrical resistance is the ohm (Ω).

VEGF-A: Vascular Endothelium Growth Factor-A. The original member of the family of endothelial cell growth factors referred to as VASCULAR ENDOTHELIAL GROWTH FACTORS. Vascular endothelial growth factor-A was originally isolated from tumor cells and referred to as "tumor angiogenesis factor" and "vascular permeability factor". Although expressed at high levels in certain tumor-derived cells, it is produced by a wide variety of cell types, including RPE cells. In addition to stimulating vascular growth and vascular permeability, it may play a role in stimulating vasodilation. Alternative splicing of the mRNA for vascular endothelial growth factor-A originates several isoforms of the protein.

1.1 The imbalance of the growth factors VEGF/PEDF generates choroidal neovascularization in exudative age macular degeneration.

After completion of eye embryonic development, retinal and choroidal vasculature remain quiescent due to the balance of pro and anti-angiogenic factors such as vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF), respectively [1]. However, under pathological conditions, an imbalance of this ratio is produced, favoring angiogenesis. This imbalance is characterized by the production and secretion of VEGF, which induces endothelial cell proliferation in the retina and choroid, and leads to retinal neovascularization and choroidal neovascularization, respectively [1, 2].

One of the most important eye diseases in which this imbalance occurs, is the Age Related Macular Degeneration (AMD) [3]. There are two types of AMD: atrophic and exudative form. The first is characterized by a progressive loss of function of photoreceptors and supporting cells, whereas the second is characterized by the generation of choroidal neovascularization (CNV) [4].

CNV is a pathological condition characterized by abnormal growth of new blood vessels from the vascular layer of the eye, named choroid, from the resident vascular cells. These new vessels grow and extend beyond the choroid through the retinal pigment epithelium (RPE) and increased capillary permeability produces subretinal exudation and hemorrhage. On the other hand, invasion of the RPE occurs through Bruch's membrane, which is the structure responsible for separating the retina from the choroid. Altogether, CNV determines the destruction of the normal architecture and function of the retina. The RPE loses its supporting function of rods and cones, inducing loss of photoreceptors by apoptosis, subretinal fibrosis and, in some cases, irreversible vision loss [3] [5] (see Figure 1).

The mechanism that generates CNV in AMD and other diseases such as multifocal choroiditis is multifactorial and complex, making them difficult to study. Scientific evidence suggests that the RPE is the leading producer of signaling molecules, such as VEGF and PEDF, finally interact with their target cells in the vascular endothelium of the choroid. This homeostatic role of RPE cells is very important, since they are in close contact with the

outer segments of the photoreceptors, participating as macrophages and in the renewal of visual pigments [6]. Additionally, the RPE also senses hypoxia in this microenvironment.

While the etiology of AMD remains unknown, the molecular mechanism that ultimately triggers CNV in AMD has been shown to correspond to an imbalance in the production and secretion of VEGF/PEDF by the RPE. The quiescent state of the choroidal vascular endothelium is maintained by the action of the RPE through these two peptides. These two signals are secreted as soluble forms in the basal domain of the RPE and reach their target cells in the choroidal vascular endothelium [7, 8]. Choroid vascular endothelium, in turn, expresses VEGF receptors, in particular VEGFR2 or KDR receptors that initiate an intracellular signaling cascade that induces proliferation, migration, increased vascular permeability and angiogenesis. Although PEDF receptor has not been well described yet, it is known to be a transmembrane protein of about 85 kD, which has also been found in other vascular cells and in choroid and retinal endothelium [7, 8]. The VEGF/PEDF imbalance produced by the RPE not only occurs in AMD. There is extensive evidence that VEGF levels increase in diabetic retinopathy, retinopathy of prematurity and others [9, 10, 11]. This imbalance stimulates the proliferation, migration and invasion of new vessels from endothelial cells of capillaries into the retina. Molecularly, in RPE cells exposed to hypoxia, the hypoxia inducible transcription factor (HIF-1α) binds the hypoxia response element in the VEGF promoter [12, 13], resulting in upregulation of VEGF. In response to hypoxia, through not well defined mechanisms, PEDF is downregulated.

VEGF, as already mentioned, is the main factor inducing angiogenesis in the retina and choroid [14, 15, 16] .Therefore, anti-VEGF antibodies are currently used for the treatment of CNV. Unfortunately, this treatment is not permanent or fully effective, since it inhibits a proangiogenic signaling pathway, but does not restore the natural retinal antiangiogenic balance generated by the PEDF factor [17, 18, 19].

We have no data on the prevalence of AMD in Chile. However, US data show that the blindness resulting from AMD is increasing due to the gradual aging of the population. In particular, the incidence of AMD increases with age: from less than 1% at ages lower than 60 years to more than 5% for those that are 80 years or older. As population aging increases, the number of AMD cases is also expected to raise [20].

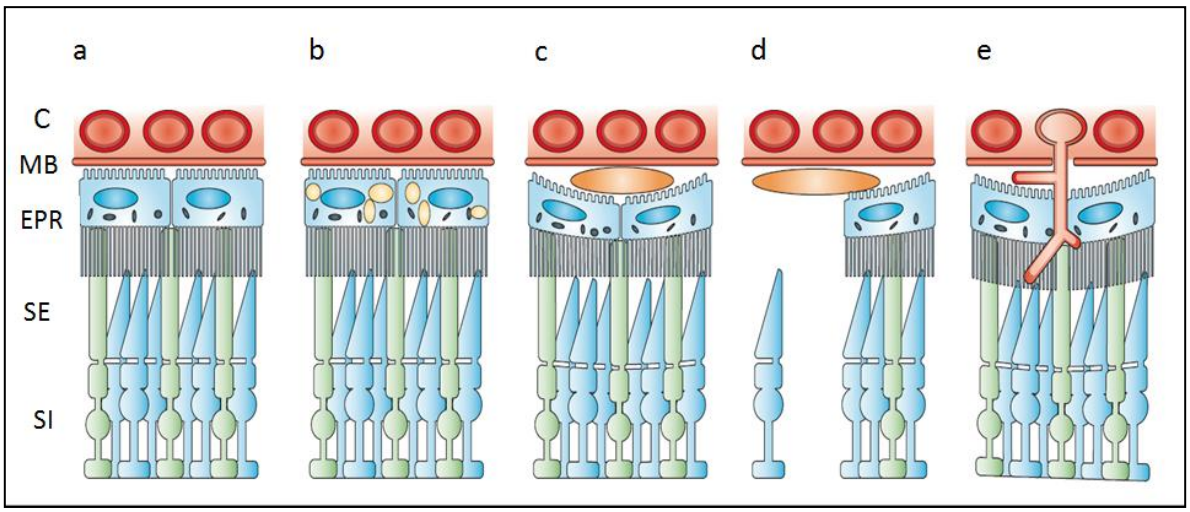

Figure 1. Stages of Age Related Macular Degeneration (AMD). C: choroid, MB: Bruch membrane, RPE: retinal pigment epithelium, SE: outer segments of the photoreceptors, SI: inner segments of photoreceptors. a: normal RPE. b: accumulation of intracytoplasmic granules in early stages of AMD. c: accumulation of amorphous material in the basal extracellular domain, called drusen. d: cell death and loss of SE in the RPE. e: new vessels are formed (choroidal neovascularization). Modified from Nature, 2008 [21].

1.2 Role of VEGF and VEGFR2 receptor in retinal and choroidal neovascularization.

VEGF protein family members include VEGF A, B, C, D, E, and PlGF. Signaling by VEGF-A represents the critical rate-limiting step between normal and pathological angiogenesis in the retina. The VEGF-A gene is located on chromosome 6p21.3 and consists of eight exons and seven introns. This determines the existence of six isoforms of VEGF-A, containing from 121 to 206 amino acids, which are generated by alternative splicing of exons from mRNA. VEGF-165 is the predominant isoform and the primary mediator of neovascularization in the retina and choroid [4, 8, 14, 22]

All VEGF isoforms can bind to two receptors: VEGFR-1 and VEGFR-2. VEGFR-1 has been described to have an inhibitory effect on VEGFR2. VEGFR2 receptor is widely expressed in vascular endothelial cells in the body and is responsible for activating intracellular signaling responses which induce activation and proliferation mediated by VEGF through its tyrosine kinase activity. A third receptor, VEGFR3, is mainly expressed in the lymphatic system [23].

Through interaction with VEGFR2, VEGF triggers signaling pathways that are responsible for retinal and choroidal neovascularization. In this regard, clinical and experimental

evidence has proposed that VEGF is the most important mitogen in the induction of choroidal and retinal vascular endothelial growth [4, 23, 24, 25]. For example, an increase in VEGF expression has been shown to precede proliferative changes in the retina of diabetic rats [26, 27]. Furthermore, it has been established that VEGF is secreted by RPE and is able to stimulate proliferation of vascular endothelial cells through interaction with VEGFR2. In this secretion process, RPE cells are more efficient at the basal pole, i.e. in the vicinity of endothelial cells of the choroid. Consistent with these data, intraocular blockade of VEGF activity with antibodies directed against the protein itself or with inhibitors of tyrosine kinases specific to VEGF receptors, can inhibit CNV in animal models [21, 28]. These results are the biological basis for the development of new anti-VEGF therapies for the treatment of exudative AMD.

In addition to the induction of proliferation, migration and formation of new vessels in vascular cells, VEGF alters the barrier function of the RPE [29, 30]. This was observed methodologically by *in vitro* measurement of the transepithelial resistance (TER) of RPE, mainly determined by the integrity of adherens junctions. This resistance allows the epithelium to function as a barrier to the diffusion of proteins, lipids and cells, constituting the so-called external Blood-Retinal Barrier. In the presence of VEGF, TER decreases, impairing RPE function and facilitating the accumulation of sub-retinal fluid and choroidal endothelial cell migration through the RPE [31, 32].

VEGF has been shown to not only affect the function of the RPE. *In vitro* studies indicate that is a chemotactic factor that attracts endothelial cells from the choroid to the retina [12, 33]. Experimentally, the co-culture of choroidal endothelial cells with RPE increases VEGF production, facilitating the transmigration of cells to the choroid RPE endothelium [30, 34]. In this same system, later reports showed that inhibition of HIF-1α activity in RPE by transfection with a plasmid expressing anti-HIF1-α shRNA inhibits the proliferation, migration and formation of vascular structures of the choroidal microvascular endothelial cells [32]. This biological evidence supports the idea that inhibition of HIF-1α-VEGF signaling pathway also inhibits the formation of new vessels in the retina and choroid.

1.3 HIF-1 is involved in hypoxia adaptation, VEGF expression and angiogenesis

There are multiple data suggesting the involvement of HIF-1 in the pathogenesis of neovascular diseases of the retina. HIF-1 is a transcription factor considered a "master

switch" that mediates cellular adaptive response under hypoxic conditions [35, 36], controling the expression of hypoxia-inducible genes such as VEGF [32, 37, 38]. This is why the role of HIF-1 in the pathological expression of VEGF in retinal neovascular diseases has been so extensively studied [14]. HIF-1 is a basic protein with helix-turn-helix DNA binding domain. It consists of two subunits: HIF-1α (120 kD) and HIF-1β (92 kD). The expression of HIF-1α is finely regulated by cellular oxygen concentration, whereas HIF-1β is constitutively expressed. The retina is the most metabolically active organ of the body, making it highly dependent on oxygen. Variations of retinal oxygen levels are sensed by prolyl hydroxylase enzymes (PHD1-3) that hydroxylate HIF-1α prolines under normoxic conditions sends it to degradation by the proteasome [16]. In the absence of oxygen, prolyl hydroxylase catalytic activity drops, reducing hydroxylation of HIF-1α and promoting stabilization of HIF-1α. Once stabilized, HIF-1α translocates to the nucleus, where it forms a heterodimer with HIF-1β and promotes the expression of their target genes [14, 35]. In this regard, clinical studies have shown that age-related changes in the Bruch membrane leads to atrophy of the choriocapillaris, and a decrease in the oxygen diffusion to the neuroretina, suggesting that retinal hypoxia may be an important factor in the pathogenesis of CNV. In fact, at present, it is thought that outer retinal hypoxia is one of the most important "driving forces" in generating NVC, inducing over-expression of VEGF in RPE cells.

In vitro studies have demonstrated that hypoxia induces upregulation of VEGF in RPE cells by stabilizing HIF-1 [37, 38]. A similar effect was found when retinal cells were exposed to hyperglycemia, suggesting the involvement of HIF-1 in both retinal and choroidal neoangiogenesis. Previous studies using laser Doppler technology to determine choroid blood flow have shown a significantly lower level of macular blood flow in patients with AMD, indirectly demonstrating a possible state of hypoxia in RPE cells [39]. Additionally, we have determined the expression of HIF-1 in choroidal neovascularization associated with AMD and other causes of CNV in *post mortem* studies [12].

In conclusion, retinal hypoxia, through stabilization of HIF-1α, leads to overexpression of VEGF in RPE in various pathological conditions. This growth factor is secreted and reaches vascular endothelial cells in the intra-retinal or choroidal capillaries, where it initiates proliferation, migration, invasion and formation of new vessels in choroidal and retinal endothelial cells [14, 15, 16, 40, 41].

However, the angiogenic balance in the retina is not only established by VEGF. A considerable body of evidence has established that to maintain quiescence in the retina, a balanced expression of pro and antiangiogenic factors is required. The best described antiangiogenic retinal factor is PEDF [19, 42].

1.4 Role of PEDF as antiangiogenic molecule in the retina

PEDF is a 50 kDa glycoprotein, which was initially identified as a neurotrophic factor and has subsequently been shown to be an angiogenesis inhibitor. Structurally, PEDF shares homology sequences with the family of serine proteinase inhibitors (or their acronym, serpin). However, PEDF behaves as a non-inhibitory serpin [17]. It is expressed in multiple tissues and is present in the matrix with a large inter-photoreceptor concentration (250 nM). PEDF is also present in the vitreous and aqueous humor [1]. At the level of the retina, it is mainly expressed by the RPE. In basal conditions, PEDF is secreted and reaches the retinal cells of the choroid and endothelium, interacts with its putative transmembrane receptor (not described yet) and generates signaling cascades to inhibit angiogenesis. This growth factor mainly modulates three signaling pathways: Akt/NFkB, MAP kinases and caspases [17, 43].

It has been recently shown that PEDF displays antiangiogenic activity by specifically inhibiting endothelial cell migration, a key step in angiogenesis. In a mouse model, the expression of PEDF in the retina is regulated positively by hyperoxia [44]. In a retinoblastoma cell line, PEDF is negatively regulated by hypoxia. These findings suggest that PEDF may be a coordinator of vascular retina level functions. Furthermore, it has been determined through immunohistochemistry, that higher levels of PEDF in the retina are located in the photoreceptor layers, corresponding to the avascular layer [45]. In the light of these observations, studies have attempted to upregulate PEDF to inhibit angiogenesis, especially in cancer models. However, the mechanisms regulating expression and secretion of PEDF remain largely unknown. There is an inverse relation between the stabilization of HIF-1 and VEGF expression. In an unspecified way, the activation of the HIF-1-VEGF pathway correlates with a decrease in the synthesis and secretion of PEDF. Interestingly, it has been established in mouse models of retinal neovascularization that there is an unbalanced expression of VEGF and PEDF in the vitreous and retina [42, 44, 46, 47] .

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Angiogenesis is a process that depends on oxygen, and correspondingly, it has been well established that PEDF expression may be regulated by oxygen. Under hypoxic conditions, retinoblastoma tumor cells secrete between 12-5 times less PEDF [19]. This same research group determined, as expected, that the conditioned medium of hypoxic tumor cells (0.5% O_2) is more angiogenic than cells grown in normoxia (21% O_2). Moreover, an angiogenesis promoting effect was obtained when using a blocking antibody for PEDF, changing the medium to the proangiogenic normoxic levels observed in an hypoxic environment [48].

Based on the available data, it has been postulated that PEDF prevents the formation of new vessels in retinal vascular disease models without altering the existing mature vessels. This effect would mainly target cell migration and proliferation, as PEDF inhibits endothelial cell migration *in vitro* in a dose-dependent manner and has a higher inhibitory effect compared to other angiogenesis inhibitors like angiostatin, endostatin, thrombospondin-1 on proliferation and migration of vascular endothelial cells [18, 19, 42]. Correspondingly, in a rat model of ischemia-induced retinal neovascularization, VEGF expression increases in a time course that coincides with a decrease in PEDF levels, therefore leading to an imbalance in the VEGF/ PEDF ratio. In this model, these changes in protein levels are correlated with the development and progression of retinal neovascularization [44, 49].

Current treatment of neovascular AMD and RD includes the use of anti-VEGF antibodies such as bevacizumab. This treatment, however, has no effect on HIF-1α. Therefore, this partial effect on VEGF/PEDF signaling could explain why the effect of this treatment is so short duration and why clinical success has not been achieved in many cases [4, 25].

Currently, there is an intensive search for a strategy that may inhibit angiogenesis at the retinal choroid level, capable of restoring the VEGF/ PEDF balance in order to preserve vision in patients with neovascular diseases of the retina. So far, the regulatory mechanisms that determine the VEGF/ PEDF balance in normal and hypoxia conditions have not been elucidated, which hinders the development of new treatments. While there are established correlations between PEDF, VEGF and HIF-1, it has not been possible to reverse the pathological imbalance. In this sense, a compound which can simultaneously

inhibit the VEGF pathway and potentiate endogenous PEDF levels is required, i.e, a harmless molecule with pleiotropic effects [10, 20, 21, 50].

1.5 Anti-VEGF monoclonal antibodies (Ranibizumab, Bevacizumab) as current treatment for CNV. Need for new molecules

Ranibizumab and Bevacizumab are anti VEGF antibodies used in clinical practice to treat choroidal neovascularization, particularly those affecting the macular area. Bevacizumab is a full 150 kDa antibody having affinity for the VEGF-A isoforms. Ranibizumab corresponds to the *ab fraction (Fab)* of Bevacizumab and has been especially designed for intraocular use. While these antibodies have had much better results than previously available therapies, they do have two major setbacks: only a limited and variable percentage of patients respond favorably to their use and the beneficial effect is transient [10, 20]. This fact is well documented in the SUSTAIN study [51], which evaluated the safety and efficacy of repeated intraocular injections of Ranibizumab for the treatment of neovascular AMD in 249 patients. Only 53% of patients who received multiple intraocular injections of this antibody showed a favorable response by gaining or maintaining their visual acuity. Using statistical modeling, these results predict a large increase in cases of blindness from AMD in the next 40 years [20].

Noteworthy, the possible side effects of the antiangiogenic therapy for CNV and retinal neovascularization, which can lead to vascular or macular atrophy [52]. Systemic side effects may include inhibition of the function of endothelial precursor cells in the retina and choroid. VEGF is a survival factor for all endothelial cells. A decrease in VEGF levels may result in the inability of the resident vasculature to proliferate in response to injury and maintain endothelial fenestration morphology of the choriocapillaris. If the drug enters the systemic circulation from the vitreous, it could block VEGF and thus, interfere with the mobilization of stem cells from the bone marrow. Furthermore, if the CD34+ cells are endothelial precursors derived from bone marrow, they are intended to repair damaged retinal vessels and local inhibition of VEGF can reduce their recruitment to the ischemic region by increasing the area of injury originated by hypoxia [53].

Currently, there are no treatments targeting transcription factor HIF-1α. So far, the failure to inhibit angiogenesis by blocking single molecules has led to the search of drugs having a pleiotropic or polygenic effect. This means that they should be able to restore the

delicate PEDF/VEGF balance through an effect on several target genes and signaling pathways and therefore, permanently inhibit pathologic angiogenesis. To achieve this, it is very important that the compound proves to be harmless to living vascular cells and the rest of the body.

1.6 Curcumin is a natural pleiotropic antiangiogenic molecule.

Curcumin is a natural polyphenol, chemically known as diferuloylmethane ($C_{21}H_{20}O_6$). It is the main active curcuminoid in the plant *Curcuma longa* and has long been used in Chinese and Ayurvedic medicine to treat inflammatory diseases. Other curcuminoids such as bisdemethoxycurcumin and demethoxycurcumin are of the same origin [54]. Curcumin has captured the attention of the scientific community because several studies have demonstrated its antiangiogenic [55] anti inflammatory and antioxidant effects. These effects arise from the ability to inhibit multiple signaling pathways related to protein kinase C, the transcription factor NF-kB, phospholipase A2, arachidonic acid metabolism, Akt, growth factors, HIF-1α and VEGF [54, 56, 57]. Curcumin increases the sensitivity of certain cancers to standard chemotherapies and, additionally, presents low toxicity towards normal cells [58]. There are now at least 45 ongoing clinical trials with curcumin [59], and others have already been completed. These trials have examined the effects of curcumin in different types of carcinomas and conditions associated with inflammation, such as psoriasis and Alzheimer's disease. Most of its effects have been studied in *in vivo* models of cancer but few data exists about curcumin`s effect on non-transformed cells. Moreover, the potential effects on human retinal cells have not been elucidated.

The antiangiogenic effect of curcumin’s has been demonstrated in *in vivo* models of xenografts of several tumor types, including glioblastoma, hepatocellular carcinoma, prostate carcinoma and ovarian cancer. In these transformed cells, curcumin negatively regulates a variety of growth factors, transcription factors and enzymes with proangiogenic activity, such as bFGF, VEGF, angiopoietin-1 and 2, COX-2, matrix metalloproteinase MMP9, NFkB and AP-1 [60]

In transformed cells, curcumin is able to decrease angiogenesis by in vivo induction of fibroblast growth factor (FGF), avoiding corneal vascularization in rabbit [61]. Curcumin also inhibits the migration and differentiation of endothelial cells mediated by FGF, through the inhibition of matrix metalloproteinase gene expression. Additionally, a synthetic

derivative of curcumin, hidrazino curcumin, has proved to be a potent inhibitor of proliferation in bovine aortic endothelial cells, without showing significant toxicity. Curcumin also induces apoptosis *in vitro* in endothelial cells of human umbilical cord (HUVEC), without affecting the viability of fibroblasts, suggesting a mechanism for the selective proliferation vascular cell types. However, the mechanism by which angiogenesis is inhibited has not been fully elucidated and appears to involve the modulation of signaling pathways [62]. For example, treatment of HUVEC cells with curcumin 0.1mM for 8 hours is able to decrease the expression of VEGFR2 mRNA, which is the main mediator of the VEGF angiogenic response in vascular endothelium [63] .

Additionally, curcumin modulates the expression of HIF-1α. In breast cancer (MDA-MB231) and prostate cancer (PC3) cells, curcumin significantly inhibited HIF-1α signaling by decreasing its protein levels and transcriptional activity; an effect that apparently is independent of the proteasome. Furthermore, in the conditioned medium from cells treated with increasing concentrations of curcumin for varying periods of time (0-24h), the production and secretion of VEGF decreases and this effect has been indirectly correlated with an inhibitory effect [64, 65].

To form new vessels, endothelial cells must be able to invade neighboring tissues. Naturally, the RPE has intercellular tight junctions that generate a transepithelial resistance and prevent passage of macromolecules that freely diffuse from fenestrated capillaries of the choriocapillaris to the subretinal space. This barrier also prevents the passage of any vascular endothelial cells and is known as the external blood-retinal barrier. This barrier is penetrated by migrant choroidal neovascular membranes to generate cells in the retina and constitutes a fundamental phenomenon in the pathogenesis of CNV. Under stress conditions, intercellular tight junctions are disrupted in RPE, generating a decrease in trans-epithelial resistance. This facilitates the diffusion of high molecular weight molecules and migration of activated vascular endothelial cells from the retina to the choroid. Curcumin has been shown to have a stabilizing effect on the tight junctions located in corneal epithelium cells, while maintaining intercellular binding proteins such as ZO-1 and preserving normal transepithelial resistance *in vitro*.

These data support the concept that curcumin is innocuous in ocular tissues and cells and is also capable of reversing or inhibiting loss of intercellular junctions. If these results were replicated in the retina, curcumin may hinder the migration of vascular cells through RPE, which is the main pathological process that leads to NVC.

Given the ability of curcumin to decrease the expression of VEGF mediated by HIF- 1α in different types of cancer cells and in models of diabetic retinopathy, we propose that curcumin can downregulate VEGF expression mediated by HIF-1α in a choroidal angiogenesis model *in vitro*. To corroborate our hypothesis, we will study the activity of the HIF-1α-VEGF-PEDF pathway in human RPE cells.

We will use hypoxia to simulate conditions of stress and to induce a pro-angiogenic imbalance, a stimulus which has also been implicated as one of the many drivers of exudative AMD. We will determine the effects of curcumin at the molecular, cellular and *in vivo* levels, and provide a comparison with the effects obtained with a pure competitive blocker of VEGF known as Bevacizumab, which corresponds to the best treatment currently available for these patients. Note the importance of studying this system, because the data we currently have about the effects of curcumin are mainly based on studies with tumor cells, which allows a limited extrapolation to a non-tumor like system such as the retinal and choroidal neovascularization model. Furthermore, the available evidence has not studied a possible relationship between curcumin and the production of endogenous anti-angiogenic PEDF.

From a functional point of view, and due to the lack of established and validated models for studying choroidal neovascularization, we will verify the effects of curcumin in an *in vivo* angiogenesis system, the chicken chorioallantoic membrane. This assay is highly reproducible and has been previously validated. Through this essay, it is possible to directly observe the formation of new blood vessels induced by soluble signals secreted by the RPE under different experimental conditions.

One of the main problems of using curcumin in humans is the low solubility in aqueous phases, poor absorption and tissue bioavailability due to its metabolism in the intestine and liver. To improve the absorption of this polyphenol, the generation of various preparations of oral curcumin have been generated, but are still in the experimental phase [58]. In the case of the eye, this problem does not exist, since we have the possibility to go directly to the target tissue through an intraocular injection.

The results of this thesis are expected to generate new knowledge about the mechanisms that regulate angiogenesis mediated by RPE and provide information about the possible relationship between curcumin and PEDF in this system (see figure 2). Altogether, this evidence would suggest that curcumin, curcuminoids or derivatives are anti-angiogenic

molecules with potential clinical use in retinal diseases such CNV, setting the basis for further preclinical trials in animals or patients at risk of blindness.

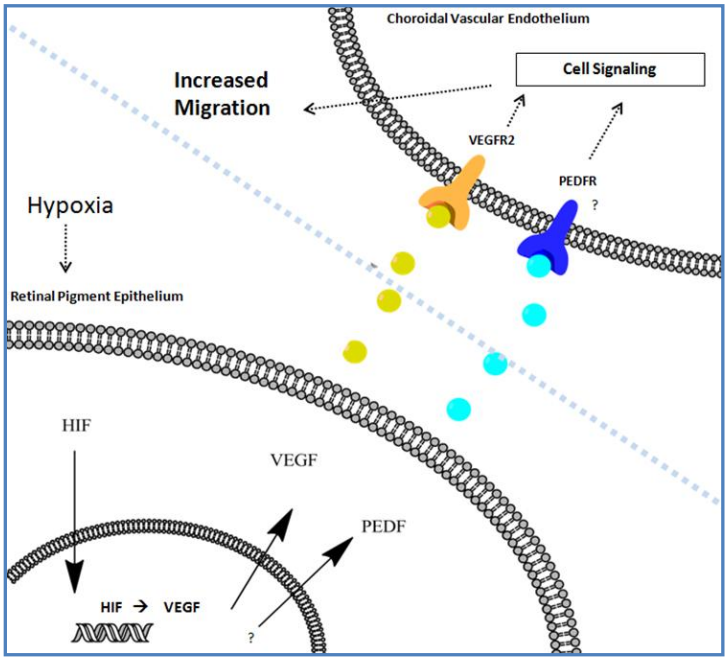


Figure 2. General scheme of the proposed model. Hypoxia stabilizes HIF-1α in the cytoplasm, leading to dimerization of HIF. This transcriptional factor activates expression of target genes, such as the VEGF transcript. VEGF is able to diffuse into the choroid activating signaling pathways in choroidal endothelial cells. This stimulates proliferation, migration and angiogenesis into the retina. PEDF is secreted by RPE cells to inhibit choroidal angiogenesis. An increased VEGF/PEDF ratio is generated leading to choroidal angiogenesis. Curcumin has anti angiogenic effect in other systems, however, the effect on this RPE-CVE cell signaling system remains unknown.

1.7 Summary of the Background

1. Retinal angiogenesis is regulated by the coordinated secretion and production of VEGF and PEDF in RPE cells. These molecules signal to vascular endothelial cells in the choroid and retina.
2. Endogenous PEDF is a secreted glycoprotein that exerts anti-angiogenic effect in the retina.

3. There is an inverse correlation between VEGF and PEDF levels in models of ischemia-induced neovascularization in animals.
4. Curcumin is a natural polyphenol having an anti- angiogenic effect in *in vitro* models of cancer.
5. The effect of curcumin on the activity of HIF-1α and the production-secretion of VEGF and PEDF in RPE cells is unknown.
6. The effect of curcumin on RPE induced- angiogenesis in choroidal endothelial cells is unknown.

Introduction Brief

The quiescent state of the choroidal vascular endothelium is maintained by the action of the retinal pigment epithelium. This action is mediated by the production and secretion of two main proteins: vascular endothelium growth factor (VEGF) and pigment epithelium-derived factor (PEDF). These proteins display pro- and anti-angiogenic functions, respectively. However, conditions that favor a pro-angiogenic state lead to activation of vascular cells, migration and invasion of the retinal pigment epithelium, process known as choroidal neovascularization. The mechanisms underlying this imbalance are multifactorial and complex, but it has been proposed that both choroidal and retinal hypoxia occur. The post-mortem histological samples of choroidal neovascularization further suggest an increased activation of the transcription factor hypoxia-induced factor one (HIF-1) [12, 14]. Interestingly, this protein acts as a sensor of hypoxia and activates VEGF transcription.

Anti-VEGF antibodies are currently being used to treat intraocular cases of choroidal neovascularization. However, its effect is transient and partial, a fact reflected by the need for multiple intraocular injections, together with the complications that this entails. This failure to inhibit angiogenesis in a complete and permanent manner is explained, in part, by the proangiogenic imbalance occurring at the retinal pigment epithelium, which is not reversed by a VEGF neutralizing antibody, such as bevacizumab or ranibizumab. Currently, intensive research is carried out worldwide to develop new anti-angiogenic compounds for the retina [22, 66, 67, 68, 69, 70].

Curcumin is a polyphenol that has anti-angiogenic effects in cancer cells. Curcumin is reportedly harmless for humans and as such, is an interesting candidate for the development of new anti-angiogenic therapies in other diseases. Currently, there are 45 ongoing human studies to determine the effects of curcumin in healthy patients, patients with Alzheimer's disease [60] and in patients with different types of cancer. However, no study has been made in eye diseases.

Curcumin is able to inhibit HIF-1 transcriptional activity and its downstream target genes, such as VEGF. There is also evidence suggesting that curcumin reduces the expression of the VEGF receptor VEGFR2. However, it is unknown what effect it might have on the VEGF/ PEDF balance in the retinal pigment epithelium, or on the induced choroid endothelial cell angiogenesis by RPE secreted factors.

Based on the available evidence, we hypothesized that **Curcumin is an inhibitor of angiogenesis induced by factors derived from the retinal pigment epithelium subjected to hypoxia. This effect would occur by a recovery of the VEGF / PEDF balance**.

To test this hypothesis, we will set an *in vitro* system with both cell types, choroidal vascular endothelial cells (CVE) and retinal pigmented epithelium cells (RPE). To induce a pro-angiogenic state, we will incubate RPE cells of the retina in a hypoxia chamber. In this system, we will study the effect of curcumin on the RPE-CVE signalling at three levels: i) at the molecular level, we will determine HIF, VEGF and PEDF changes at transcriptional and post-transcriptional levels in retinal pigment epithelial cells; ii) at the cellular level we will determine changes in proliferation, migration, invasion and formation of new vessels in choroid endothelial cells in response to signals secreted by retinal pigment epithelium subjected to hypoxia and iii) using an *in vivo* angiogenesis assay (the chicken embryo chorioallantoic membrane assay), we will determine the effects of curcumin on angiogenesis mediated by soluble factors that are secreted by the retinal pigment epithelium under hypoxic conditions. For comparison and to study a possible synergistic effect of curcumin with existing treatments, we will use a known inhibitor of VEGF of common clinical use, Bevacizumab, which is a humanized monoclonal anti-VEGF antibody.

If our hypothesis is correct, the possible implications of our results are two-fold. First, from a mechanistic point of view, our results will provide data regarding the regulation of the VEGF/PEDF balance in pigment epithelial cells of the retina. Second, it may set the foundation for the future development of a formulation of curcumin for the treatment of ocular neovascularization capable of restoring the VEGF/PEDF balance and, therefore, achieve more stable or complete anti-angiogenic effects

Hypothesis

Curcumin inhibits angiogenesis induced by retinal pigment epithelium-secreted factors through the re-establishment of the VEGF/PEDF balance.

General Aim

To study in the retinal pigment epithelium, both *in vitro* and *in vivo*, the anti-angiogenic potential of curcumin on angiogenesis induced by VEGF/PEDF imbalance

Specific Aims

- **Specific Aim 1**. To determine, under hypoxic conditions, the effect of curcumin on the activity of HIF-1α as well as on the expression of VEGF and PEDF in human retinal pigment epithelium cells (RPE)
- **Specific Aim 2**. To evaluate the effect of curcumin in angiogenesis induced by RPE *in vitro*.
- **Specific Aim 3**. To study the effect of curcumin in angiogenesis induced by RPE *in vivo.*

Specific Aim 1

To determine, under hypoxic conditions, the effect of curcumin on the activity of HIF-1α as well as on the expression of VEGF and PEDF in human retinal pigment epithelium cells (RPE).

HIF-1α protein levels and transcriptional activity in ARPE19 cells was determined in normoxia (21% O_2) and hypoxia (1% O_2) by Western blot and reporter assay, respectively. In parallel, the expression of VEGF and PEDF mRNA and protein was evaluated using RT-PCR, qPCR and ELISA, under the same experimental conditions.

To incubate cells in hypoxia, a regulated environment chamber for cell culture that provides 1% O_2 was used. This camera has been used previously to generate hypoxia [54-55]. Since hypoxia is an inducer of HIF-1α and VEGF, and as previously described, there is an inverse relationship between VEGF and PEDF, we expected that cells exposed to hypoxia exhibit greater HIF1-α transcriptional activity, increased VEGF mRNA and protein levels, and decreased PEDF mRNA and protein levels (see Figure 3).

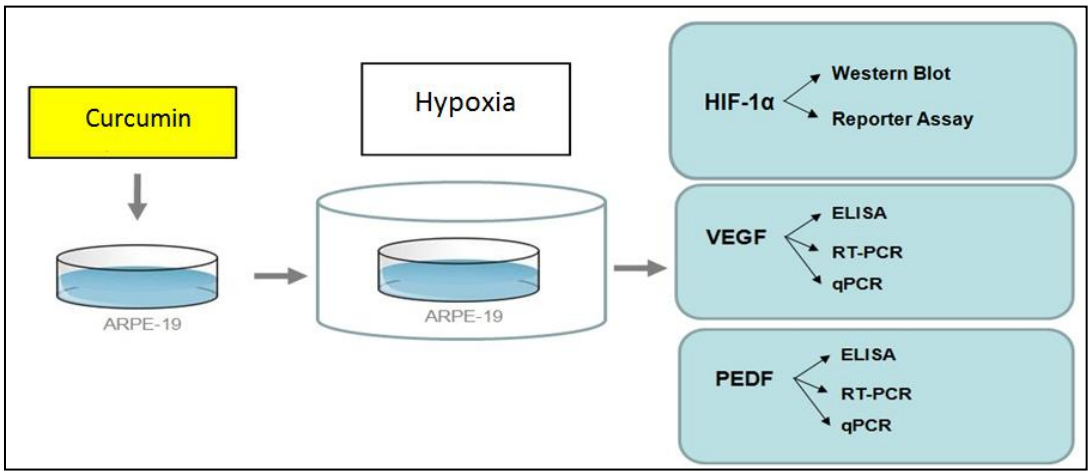


Figure 3. Experimental design for Specific Aim 1. In order to determine the steady state response to hypoxia, ARPE-19 (ARPE) cells were exposed to 1% O_2 and HIF-1 total protein levels were determined by Western Blotting. Additionally, to elucidate changes in HIF-1 transcriptional activity, ARPE cells were transfected with a HRE-luciferase containing plasmid and the reporter gene assay was performed. Transcriptional changes in our two genes of interest, VEGF and PEDF, were studied with RT-PCR. ELISA assays and qPCR were included to quantify changes in mRNA and protein levels.

To determine the effect of curcumin in this system, a curve of time and dose response was conducted. A previous time and dose described was confirmed as optimal, hence ARPE19 cells were incubated for 3 hours with 20 uM curcumin [56]. DMSO was used as vehicle control (curcumin is soluble in organic solvents). We expected that cells treated with curcumin under hypoxia conditions presented less transcriptional activity of HIF-1α, decreased expression of VEGF and greater expression of PEDF than control cells (see Figure 3).

Specific Aim 2

To evaluate the effect of curcumin in angiogenesis induced by RPE *in vitro*.

In order to produce new blood vessels, target cells of the vascular endothelium must proliferate, migrate and invade the Bruch membrane, a basement membrane secreted by RPE. All of these cell functions are necessary for angiogenesis. To determine the effect of curcumin on RPE mediated angiogenesis in a near physiological model, ARPE19 cells were cultured under hypoxic conditions to stimulate VEGF/ PEDF imbalance and angiogenesis was induced using soluble mitogens and chemoattractants.

The supernatant was collected and used to stimulate the proliferation, migration and invasion of a choroidal vascular endothelial cell line, known as RF/6A (ATCC) in a transwell bicameral system. Proliferation of the RF/6A cells was studied by the MTT assay, following manufacturer's instructions. Tube formation on matrigel poor in growth factors was performed as previously described [71].

To determine the effect of curcumin on these two cellular responses, RPE cells were incubated with 20uM curcumin for 3 hours prior to hypoxia, then supernatant was collected and experiments were conducted in the same fashion (see Figure 4).

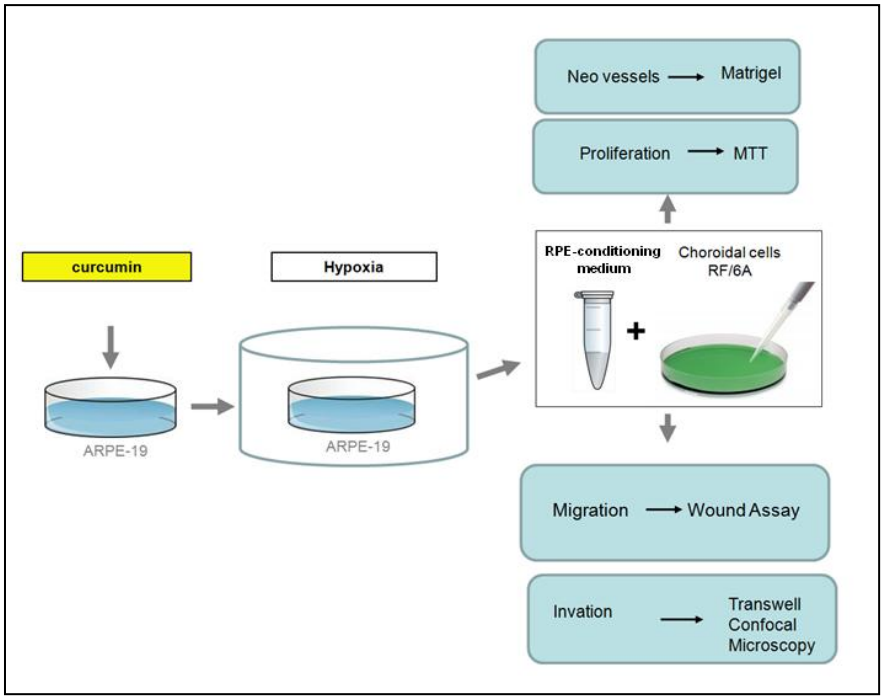


Figure 4. Experimental design for Specific Aim 2. Conditioning medium obtained from ARPE cells was used to stimulate choroidal vascular cells *in vitro* (RF6A cells). Four different methodologies were used to study the response of these vascular cells to soluble factors: i) matrigel, to assess vascular tube formation, ii) MTT, to measure proliferation, iii) the wound-healing assay to visualize migration, and iv) transwell transmigration assays to assess invasion.

The expected results for this specific aim included increased proliferation, migration and angiogenesis in RF/6A cells stimulated with conditioned medium obtained from the supernatant of RPE cells stimulated with hypoxia. After incubation with curcumin, it was expected to have a modulating effect on the VEGF/PEDF balance under hypoxia conditions, reducing or preventing the choroid proangiogenic response. Bevazicumab, an anti-VEGF antibody, was used as a control; it was added to the conditioned medium before the treatment of choroidal endothelial cells. This antibody binds to VEGF and blocks its biological action by preventing the interaction with its receptor.

Specific Aim 3

To study the effect of curcumin in angiogenesis induced by RPE *in vivo.*

To further confirm our *in vitro* findings in a more physiological setting, an *in vivo* neovascular formation assay was used. This assay has been previously reported in the literature as the Chick Chorio Allantoid Membrane (CAM) assay (Figure 5). Using this aproach, a set of experiments were designed in order to study the angiogenic potential of conditioned medium obtained from RPE exposed to hypoxia after curcumin treatment. Briefly, conditioned medium of normoxia and hypoxia treated ARPE19 cells was collected. 300 ul of the supernatant was used to embed a filter paper that was placed under the shell of a fertilized chicken egg. The formation of capillaries stimulated by conditioned medium in the allantoic membrane was quantified directly under light microscopy.

In our *in vivo* model, curcumin was expected to have an inhibitory effect on the formation of capillaries compared to untreated controls.

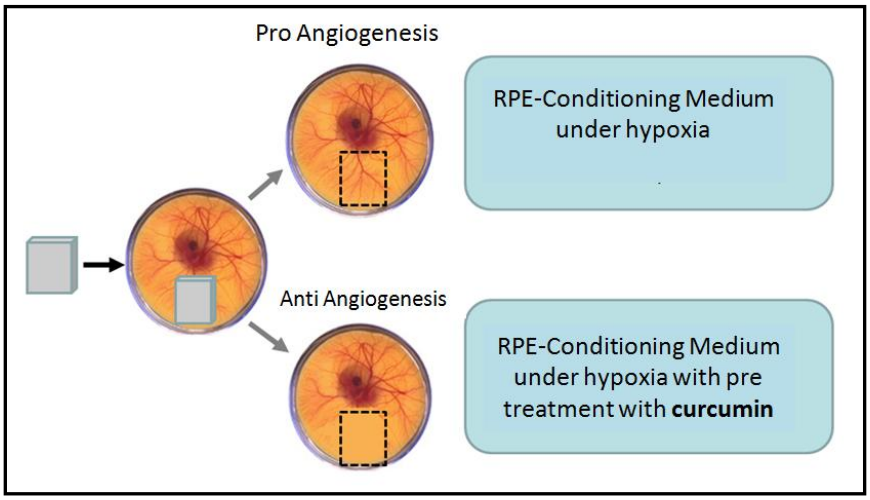


Figure 5. Experimental design for Specific Aim 3 using the chick CAM assay. A nitrocellulose filter of 10 millimeters of diameter was immersed in RPE-conditioned medium obtained from cells exposed to hypoxia. In this case, we expected to observe sprouting of an enhanced vascular network in the area of study. Accordingly, we expected a decrease in blood vessels growth, when conditioned medium was obtained from RPE that was treated with curcumin prior to hypoxia exposure.

BIOLOGICAL INDUSTRIES

Foetal Bovine Serum: catalogue number **04-001-1**

AMERICAN TISSUE AND CELL CULTURE (ATCC)

ATCC ARPE-19: catalogue number CRL-2302. These cells are human retinal pigment epithelium cells. Spontaneously immortalized, they retain most of the phenotypic characteristics of mature RPE cells. These cells are maintained in culture in DMEM-F12 medium and 5% fetal calf serum (Biological Industries®).

ATCC RF/6A cells (CRL-1780™) are choroidal vascular cells. This is a spontaneously immortalized cell line, which has not been genetically modified, obtained from normal rhesus monkey *Macaca mulatta* retinal choroid. This line is grown with fetal bovine serum MEM and 10%.

CORNING

Falcon Culture Dishes: catalogue number 353001. Falcon® 35 mm Cell Culture Dish with Easy-Grip design, TC-treated polystyrene.

BILLUPS ROTHENBERG

Hypoxia Chamber: catalogue number MIC-101 (Modular Incubator Chamber). For physical hypoxia (1% oxygen), a hypoxia chamber (Billups-Rothenberg ®) is used.

R&D SYSTEMS

Human VEGF Quantikine ELISA kit: catalogue number DVE00.

INVITROGEN-SIGMA

Curcumin: obtained from Sigma®, catalogue number C1386. This *Curcuma longa* extract was solubilized in DMSO 10 mg / ml.

Trizol (TRI Reagent): catalogue number T9424.

Deferoxamine: catalogue number D9355.

DMEM: F12 culture media: catalogue number D6421.

Propidium Iodine solution: catalogue number P4868. Prepared 1.0 mg/ml in water. Synonym:3,8-Diamino-5-(3-diethylaminopropyl)-6-diiodide.

CellTracker™ Green CMFDA Dye: catalogue number C7025

CellTracker™ Red CMTPX: catalogue number C34552

Methods

Hypoxia: To achieve hypoxia, a mix of nitrogen 99% and oxygen 1% was perfused into the chamber for 20 minutes, allowing the exchange of air/mix. The Modular Incubator Chamber (MIC-101) utilizes a surface-type seal where all portions of the O-ring are uniformly compressed by a stainless steel ring clamp for a reliable air tight seal. The cylindrical walls and semi-spherical top and bottom provide minimum gas flow resistance.

Cell culture under hypoxia: The cells were kept in DMEM: F12 without serum at all times of hypoxia. The protocol provided by the manufacturer was followed. Briefly, fresh serum free media was incubated overnight in the hypoxia chamber. In parallel, a culture dish of 60 mm with 400.000 RPE cells was prepared for each condition in normoxia. The culture media was removed and cells were washed 3 times with 1 ml of PBS. 3 ml of hypoxic media without serum was placed in each plate exposed to hypoxia. The cells were then placed in the hypoxia chamber, and purged for 20 minutes. After that, the valves of the chamber were closed and the whole unit was placed inside an incubator with 37% for 12 hours. Additionally, two plates with PBS and a mix of antibiotics were placed in the lower level of the chamber to avoid dryness and infection. After 12 hours of incubation, the chamber was opened and cells were immediately processed. Supernatants, if used, were immediately frozen or used directly to stimulate CVE cells.

ELISA: Soluble VEGF expression was determined supernatants of ARPE-19 cells using the Human VEGF Quantikine ELISA (R&D®).

Following the manufacturer's instructions, for cell culture supernatant samples, 50 µL of Assay Diluent RD1W were added to each well. Then, 200 µL of Standard, control, or sample were added per well. The plate was then covered with the adhesive strip provided and incubated for 2 hours at room temperature. Then, the content of each well was aspired and each well was washed with 400 uL Wash Buffer. This process was repeated

three times. The plate was inverted and blotted against clean paper towels and 200 µL of VEGF Conjugate was added to each well. The plate was covered and incubated during 2 hours at room temperature. The washing procedure was repeated and 200 uL of Substrate Solution was added to each well, protecting the plate from light and incubating for 20 minutes at room temperature. Finally, 50 µL of Stop Solution was added to each well. The optical density of each well was determined within 30 minutes, using a microplate reader set at 450 nm. The readings at 540 nm or 570 nm were subtracted from the readings at 450 nm to correct optical imperfections in the plate.

Reporter gene assay for transcriptional activity of HIF-1α: 600.000 ARPE19 cells were transfected with the pGL3-HRE plasmid, which contains three hypoxia responsive elements (HRE) coupled with the gene of luciferase. Hence, HIF-1α binding promotes luciferase transcription. An empy vector was used as control (see Transfections section).

Transfected cells were cultured under hypoxia (1% O_2) normoxia (21% O_2) or in the presence of deferoxamine agent hypoxia simulator. The transcriptional activity of HIF-1α was quantified by measuring the activity of luciferase in luminometer.

Transfections: ARPE19 cells were seeded in culture dishes (10 cms) 24 hours before transfection and grown to 50-70% confluence. They were transfected with FuGENE HD reagent Roche®. The plasmids (2 ug DNA) were dissolved in 150 mL of Optimem medium without serum or antibiotics. The mixture was incubated 5-10 min at room temperature in the presence of FuGENE reagent and 600 uL of complete DMEM:F12 media was added. Cells were incubated overnight under normal culture conditions, and the medium was then replaced with fresh medium. Eight hours after the transfection, the cells were selected with G418 (750 µg/mL) for 15 days, followed by one week of selection with G418 and Higromycin (750 µg/mL). To further verify the expression of luciferase, the transfected cells with the reporter vector pcDNA-HRE-Luc, were seeded in culture dishes in the presence of 1mM IPTG, and treated with the inductor of chemical hypoxia, deferoxamine 200 µM for 18 hours. Finally, lucyferase activity was determined by luminometry.

RNA isolation: Total RNA was isolated from RPE cells using the Trizol® reagent (Invitrogen, Thermo Scientific, USA) at 12 hours post hypoxia, and then reverse transcribed into complementary DNA (cDNA; SuperScript II reverse transcriptase; Invitrogen, Thermo Scientific, USA). All procedures were conducted following the manufacturer's instructions. The quantity and quality of RNA was determined using a

NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The same amount of total RNA was used for reverse transcription using Super-ScripTM II Reverse Transcriptase kit and random primers (Invitrogen). Real-time RT-PCR was performed using SYBR Green Master (Roche Diagnostics GmbH, Mannheim, Germany) in a Light-CyclerH 480 system (Roche Diagnostics GmbH). 18s was used as a housekeeping gene to normalize the mRNA expression levels. The primer sequences were checked using PRIMER BLAST software (from www.ncbi.nlm.nih.gov).

RT-PCR: After RNA isolation described above, The reverse transcription reaction was performed in a final volume of 25 uL, according to the following protocol: 1 ug total RNA; random primers 4 pM; 8 mM dNTPs, buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT) and 8 U/uL of M-MLV reverse transcriptase (Promega, Madison, WI, USA) The mixture of total RNA with random primers was incubated 5 min on ice and then at 70ºC for 15 min. Subsequently, a master mix containing dNTPs, buffer and M-MLV reverse transcriptase was added and incubated for 2 h at 42° C. The PCR reaction was performed using the following final reagent concentrations: cDNA as a template; Primer sense (SN) and antisense (AS) 2 uM; 200 mM dNTPs; 2 mM $MgCl_2$, 0.02 U Taq polymerase/ uL (Promega) and buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl). Electrophoretic migration patterns of the reaction products were analyzed on 2% agarose gels with ethidium bromide in 1X TAE buffer and photographs taken on each gel were exposed to ultraviolet light in a light box. A molecular weight marker of 100 bp DNA was used.

The following primers for VEGF and β-actin, used as a loading control, were used:

Primer Name	Amplicon	Sequence
Aq10029	VEGF	AGGCCAGCACATAGGAGAGA (SN)
Aq 10030	VEGF	ACCGCCTCGGCTTGTCACAT (AS)
Aq05005	β-Actin	CATGAGGTAGTCTGTCAGGTC (SN)
Aq05006	β-Actin	CATGAGGTAGTCTGTCAGGTC (AS)

The thermocycler program used was:

Step 1	94°C	1 min	25 cycles (Step 4)
Step 2	55°C	1 min	
Step 3	72°C	1 min	
Step 5	72°C	5 min	
Step 6	4°C	10 min	
Step 7	25°C	20 hrs	

Real Time PCR: In order to confirm findings using standard semi quantitative PCR techniques, a quantitative approach was carried out using Quantitative PCR (qPCR). The following primers and conditions were used.

Genes	Forward	Reverse	Amplified fragment size (bp)
VEGF-A (total)	ATC CGG GTT TTA TCC CTC TTC	TCT CTC TGG AGC TCT TGC TA	199
VEGF-A $_{189}$	ATA AGT CCT GGA GCG TTC CCT	GCA ACG CGA GTC TGT GTT TT	110
PEDF	CCC AAG CTG AAG CTG AGT TA	CCA TCC TCG TTC CAC TCA AA	160

Table 1. List of primers used for qPCR. Briefly, using the human gene library, three pairs of primers were designed: for VEGF-A (total), VEGF-A (189) and PEDF.

Cell viability: Viability of ARPE-19 cells grown in increasing concentrations and times of curcumin was measured to exclude cytotoxicity. Additionally, viability was measured in the different experimental conditions, to determine the potential anti-proliferative effect of curcumin on these cells, as described previously. Fluorescence-activated cell sorting (FACS) applied in flow cytometry, was used.

FACS analysis: Viable cells do not incorporate propidium iodide. Hence, cell viability is determined counting propidium iodine negative cells by FACS. Briefly, cells are trypsinized studied and centrifuged at 1000 rpm in the presence of complete medium to stop the

enzymatic reaction of trypsin. Cells after two washes with PBS, resuspended in PBS and are permeabilized with propidium iodide (10 ug / ul) and kept on ice until the reading on the cytometer.

Migration and invasion assay in RPE-RF/6A cocultures: The system of co-culturing endothelial RPE cells has been previously described [57]. Migration and invasion assays were made using Costar 8 mm pore Transwell inserts (Costar / Corning, NY), allowing choroidal endothelial cell migration. ARPE-19 cells (50,000) were grown for 6 hours at the lower side of the inserts to form an organized monolayer and 200,000 ARPE-19 cells were cultured at the bottom of the well. Inserts and wells were then exposed to hypoxia and oxidative stress to generate chemo attractants. The RF/6A s cells were labeled with CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) from Molecular Probes (Invitrogen), 1:5000 dilutions, before stocking inside the inserts. This compound is a derivative of fluorescent chloromethyl that diffuses freely through cell membranes. Once inside, this probe reacts with intracellular components and cells are fluorescent and viable for at least 24 hrs after loading. The cell tracker emits a bright green fluorescent signal in the cytoplasm and allows viewing in a fluorescence microscope.

In order to create a monolayer, ARPE cells were seeded on the inferior surface of the transwell, after attachment, the insert was located in its original position and complete cell culture medium was added on top of and under the insert. After 2-4 days of culture, ARPE cells reached confluency, forming a monolayer. Upon maturation of this monolayer, tight juntions must be formed between cell membranes, leading to segregation of different ion and charged molecules from apical to basolateral domains. As a consequence, trans-epithelial electrical resistance (TER) increases, as a hallmark of a mature epithelium and monolayer formation [29]. At this moment, extracelular matrix components are likely to be secreted, producing an *in vitro* Bruch's membrane like structure

Then, RF/6A cells are seeded on the upper surface of the insert. After 6 hours, the RF/ 6A cells were fixed and migration was evaluated under a fluorescence microscope. The test was repeated after pretreatment of ARPE-19 cells with curcumin (see Figure 6).

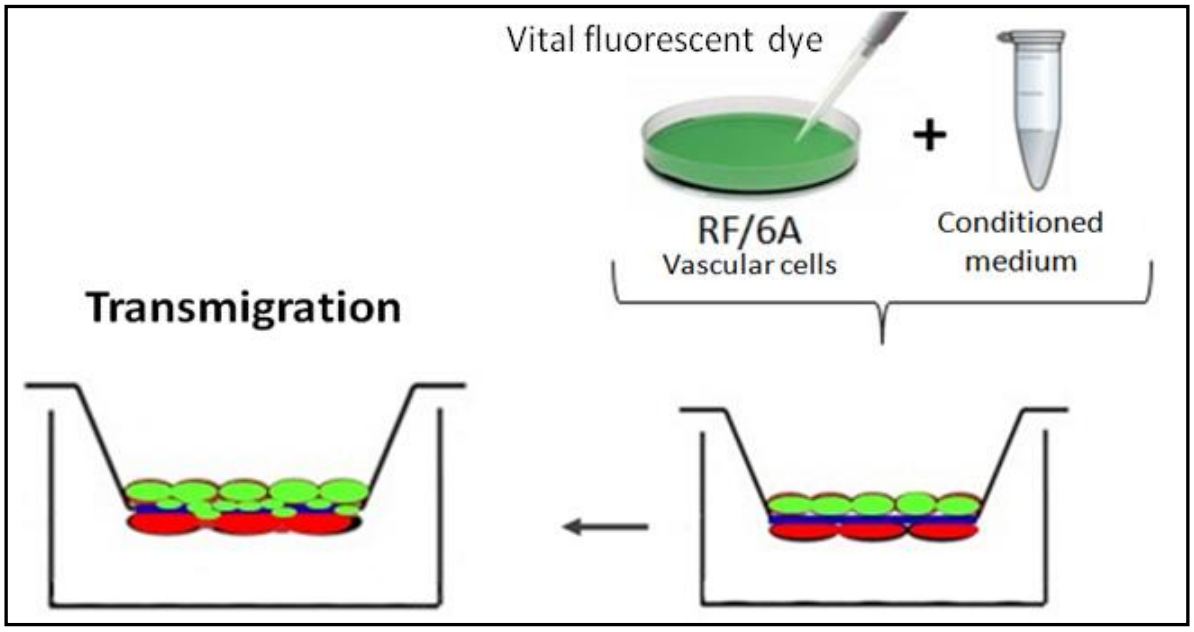


Figure 6. Invasion assay in RPE- RF/6A cocultures. RF/6A cells labeled with Cell Tracker Green® were seeded on top of 8 microns transwell inserts and the migration and invasion of these cells into the underlying RPE cells (red) was observed. To produce chemo-attractants, these RPE cells are previously challenged with hypoxia and oxidative stress. The test is repeated pre-treating the RPE cells with curcumin.

Angiogenesis Matrigel assay: 0.2 ml of matrigel (BD Biosciences, Bedford, MA) was added to each well of a 24 well plate for cell culture (Falcon, Becton Dickinson, Lincoln Park, NJ) using a cold pipette to prevent bubble formation. The Matrigel was left to polymerize for 1 hour at 37 with 5% CO_2.To measure the angiogenic potential of the conditioned medium in the different experimental conditions, RF/ 6A cells were trypsinized and 40,000 were seeded on the Matrigel in the presence or absence of conditioned medium from ARPE-19 cells cultured under hypoxia, with and without pre-hypoxia treatment. This conditioned medium was collected in a Falcon tube (Falcon, Becton Dickinson, Lincoln Park, NJ) and centrifuged at 3000 X g for 4 minutes to remove cellular debris. This conditioned medium was used to resuspend RF/6A cells, which were seeded on Matrigel poor in growth factors. The cells were periodically observed and photographed using a phase contrast microscope with a 20X objective. Photos were taken using previously published quantitative digital software and 10 representative photos were chosen for the quantification of angiogenesis and angiogenic potential (see Figure 7)

The "Angiogenesis Analyzer" of Image J, allows analysis of cellular networks. Typically, it can detect and analyze the pseudo-vascular organization of endothelial cells cultured in gel medium. Among the tools used to evaluate the anti-angiogenic properties of drugs, the most used is the *in vitro* differentiation of primary endothelial cells cultured in gel (Endothelial Tube Formation Assay (ETFA)). In suitable culture conditions, these cells form structures that can branch and mimic a pseudo-capillary formed *in vitro*. At later stages, this differentiation can lead to a meshed network of different mesh sizes. Although widely used, the interpretation of this assay still remains a problem, especially to obtain a quantitative evaluation of the vessel-like net organization. The Angiogenesis Analyzer is a simple tool to quantify the ETFA experiment images by extracting characteristic information of the network [71].

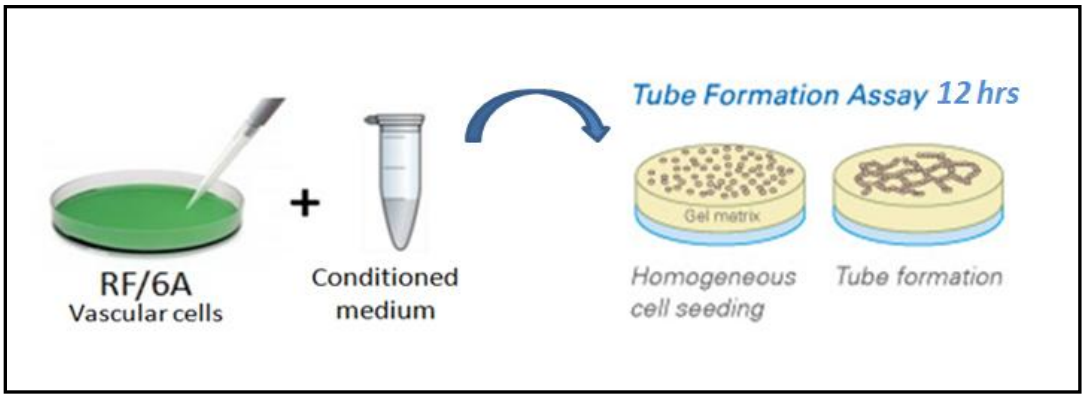


Figure 7. Matrigel Angiogenesis Assay. Choroidal Vascular cells were seeded on matrigel covered culture plates and tube formation was quantified at 12 hours using quantitative digital software.

Chick Embryo Chorioallantoic Membrane (CAM) Assay: The CAM is an extraembryonic membrane which serves as a gas exchange surface and its function is supported by a dense capillary network. Because of its extensive vascularization and easy accessibility, the CAM has been broadly used to study the morphofunctional aspects of the angiogenesis process *in vivo* and to investigate the efficacy and mechanisms of action of proangiogenic and antiangiogenic natural and synthetic molecules. The CAM has long been a favored system for the study of tumor angiogenesis and metastasis, because at this stage the chick immunocompetence system is not fully developed and the conditions for rejection have not been established. The CAM may also be used to verify the ability to inhibit the growth of capillaries by implanting tumors onto the CAM and by comparing tumor growth and vascularization with or without the administration of an antiangiogenic molecule (see Figure 8) [72].

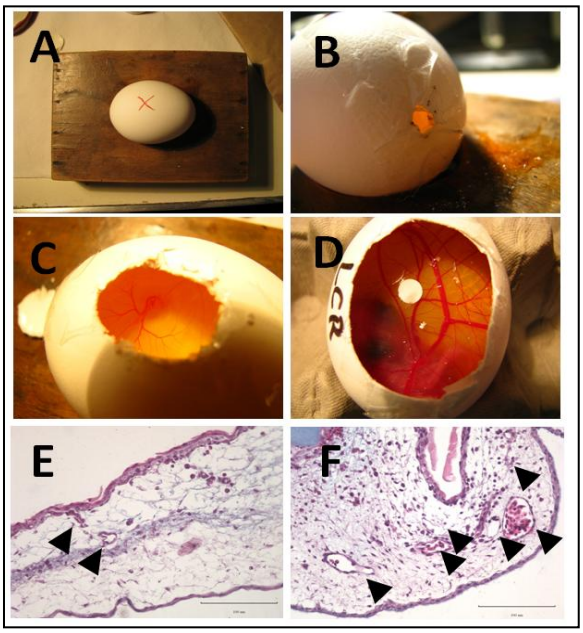


Figure 8. Chick Chorioallantoid Membrane (CAM) assay. In this assay, certified fertilized eggs obtained from our National Health Institute (ISP) are incubated at 37°C. (**A**) In the most upper part of the eggshell a small hole is controllably made to drain albumin. (**B**) At day 7 of incubation, a window is created, leading to clear exposure of the chorio-allantoid membrane. (**C**) A piece of nitrocellulose filter paper previously immersed in the RPE conditioning medium of study is placed on the chorioallantoid membrane (**D**). After 4 days, optical microcopy is used to identify and quantify blood vessel density (**E and F**) in the area where the paper was placed.

Statistical analysis: Statistics were performed using one-way ANOVA and unpaired student t-test using Graph Pad Prism5 software. Significance was accepted at $p < 0.05$.

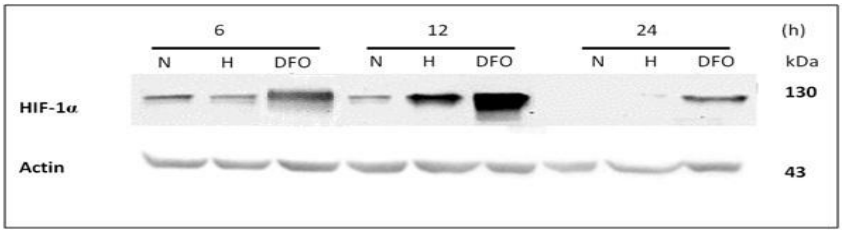
Results

Specific Aim 1 Results

1.1- Hypoxia increases HIF-1 levels in RPE cells

In order to define the time course of HIF-1α, VEGF165 and PEDF induction in RPE cells, kinetics of hypoxia response was performed. Briefly, ARPE cells in culture were exposed to 6, 12 and 24 hrs of hypoxia (1% O_2). Immediately afterwards, total cell extracts were obtained to quantify HIF-1α protein levels by Western Blotting. RNA was also isolated to determine VEGF 165, VEGF 181 and PEDF mRNA levels by semi quantitative RT-PCR. Results indicate that ARPE cells respond to hypoxia by stabilizing HIF-1α protein levels, reaching a peak at 12 hours and obtaining an 8-fold induction, compared to basal levels under normoxia (Figure 9).

A



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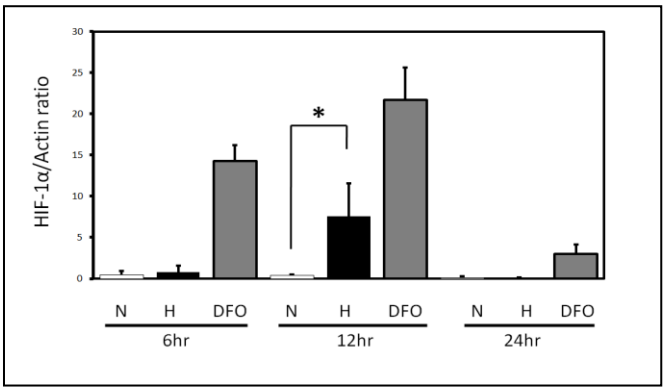


Figure 9. **Time course of hypoxia-induced HIF-1α increase in human retinal pigment epithelium cells.** ARPE-19 cells were plated and left overnight to adhere to cell culture dishes. Cells were then, incubated under normoxia (N) or hypoxia (H) for 6, 12 or 24 hours. A HIF-1α activator, deferoxamine (DFO), was used as positive control. Total cell extracts were obtained, and equal amounts of protein were loaded on a gel. Western blot of nitrocellulose-transferred proteins was performed using antibodies against human HIF-1α. **A**: Representative photograph of Western blot. Actin was used as a loading control. **B**: Values are average ± SEM after quantification performed by densitometric analysis of HIF-1α at different times normalized to actin levels. n=3, (*)p =0.03, ANOVA statistical analysis.

1.2.- Levels of HIF-1α transcriptional activity at 12 hours of hypoxia in ARPE cells.

Given that the most robust induction of VEGF mRNA and HIF-1α stabilization was observed at 12 hours of hypoxia, the transcriptional activity of this protein was tested at this time point. A reporter assay, using a construct with a Hypoxia Responsive Element domain (HRE), which is a specific transcriptional target for HIF-1, coupled with a luciferase gene (reporter gene) was performed. At 12 hours of hypoxia, a 16-fold induction was observed; compared to basal transcriptional activity (Figure 10). Therefore, HIF-1α transcriptional activity is increased at 12 hours of hypoxia in ARPE cells. Pre-treating cells with curcumin 40 µM and 80 µM prevented this hypoxia-induced increase of transcriptional activity. As previously described, chemical induction with DFO induced a saturating effect, which was stronger than the one caused by hypoxia itself.

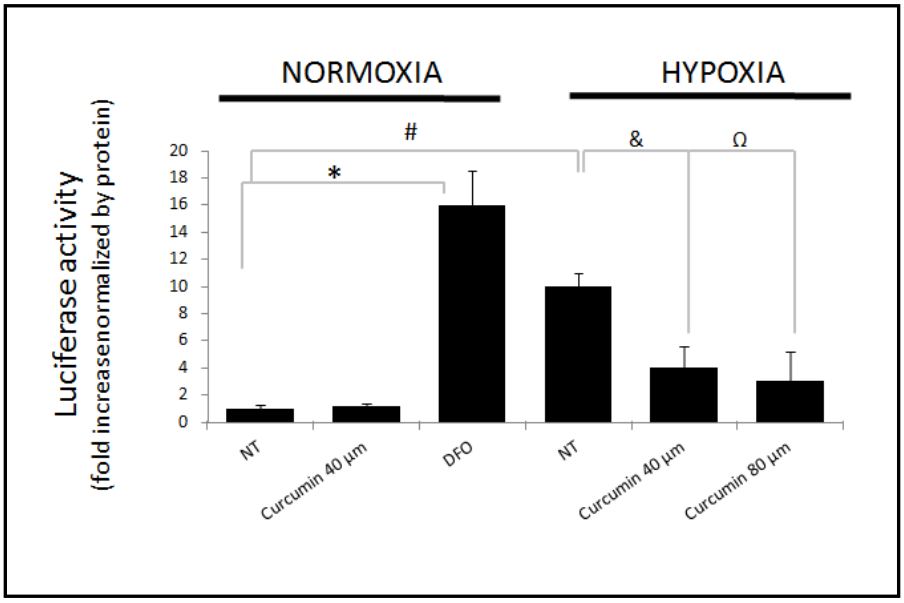


Figure 10. Reporter assay of HIF-1α transcriptional activity at 12 hours of hypoxia in ARPE cells and curcumin effect. ARPE-19 HRE-LUC+ cells were plated and left overnight to adhere to cell culture dishes. Cells were then, incubated under normoxia or hypoxia for 12 hours. A HIF-1α activator deferoxamine (DFO) was used as a positive control. Total luciferase activity was determined with a luminometer. Total values were normalized to total cell lysate protein content. Numbers in the graph are mean values obtained of corrected luciferase activity in each condition (error bars represent SEM). Data presented are from three independent experiments. *p =0.024, #p=0.012, &p=0.01, Ωp=0.02.

1.3 Effect of hypoxia treatment on HIF-1α mRNA levels. Reportedly, HIF-1α levels are regulated post translationally by two specific hydroxylases and further proteosomal degradation [35, 36]. However, only few reports indicate variations in its mRNA levels [73]. Hence, we determined by RT-PCR whether mRNA HIF-1α undergoes variations in our experimental model. Our results indicate that HIF-1α levels remain constant during hypoxia and normoxia in ARPE cells (see Figure 11).

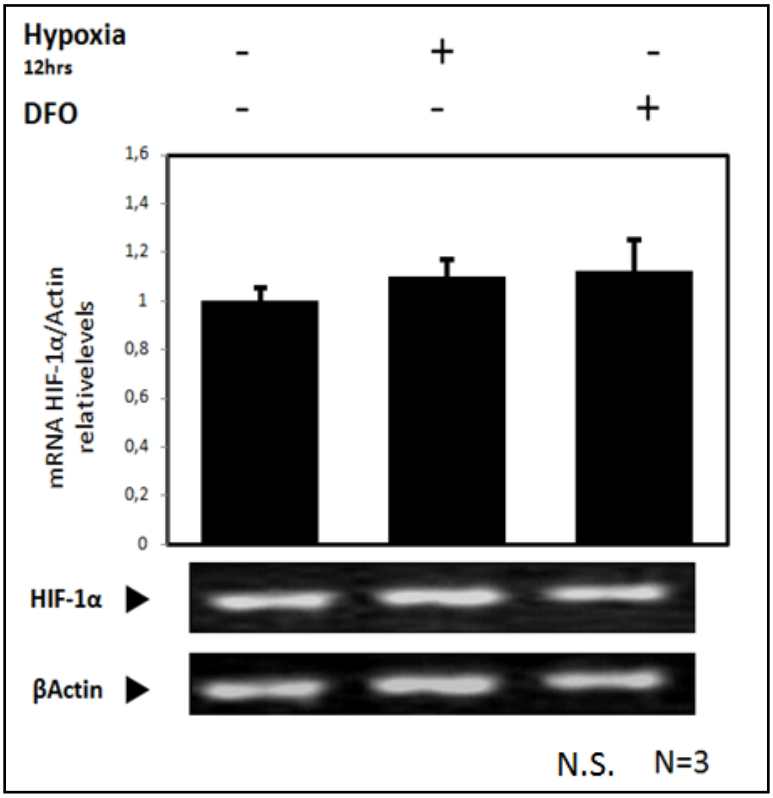
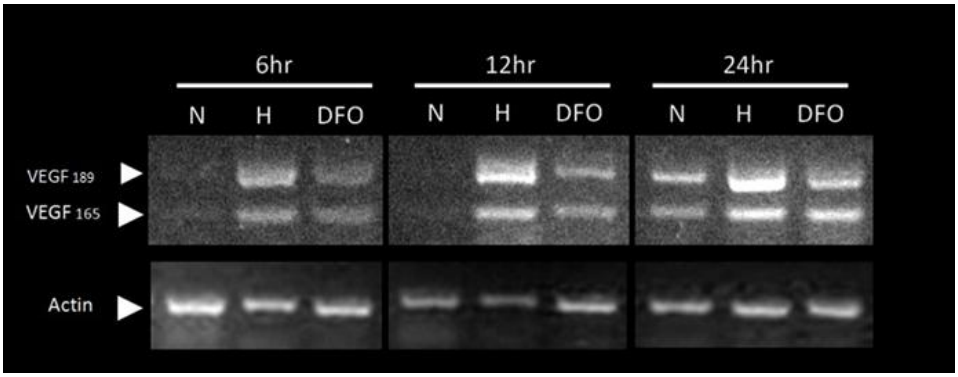


Figure 11. RT-PCR to determine HIF-1α mRNA levels in normoxia and hypoxia. ARPE-19 cells were plated and left overnight to adhere to cell culture dishes. The cells were then incubated under normoxia or hypoxia during 12 hours. A HIF-1a activator deferoxamine (DFO) was used as positive control. Total RNA was isolated using Trizol Reagent as described in methods and RT-PCR was performed. The housekeeping βActin gene was used for normalization. Representative photograph of agarose gel (2%) with PCR products and graph of densitometric analysis of PCR bands are shown. Values in the graph are mean + SEM obtained from three independent experiments.

1.4 Hypoxia-induced VEGF $_{165}$ mRNA increases in human retinal cells. VEGF A, specifically the 189 and 165 isoforms have been implicated in the pathogenesis of choroidal neovascularization. Hence, we evaluated whether hypoxia-induced HIF-1 increase in protein levels and transcriptional activity was enough to increase mRNA levels

of these two VEGF A isoforms. Another hypoxia experiment was done at 6, 12 and 24 hours, and RT-PCR was performed to evaluate VEGF 189 and VEGF 165 mRNA levels. At 12 hours of hypoxia, a significant up regulation of both VEGF A isoforms was observed (see Figure 12)

A



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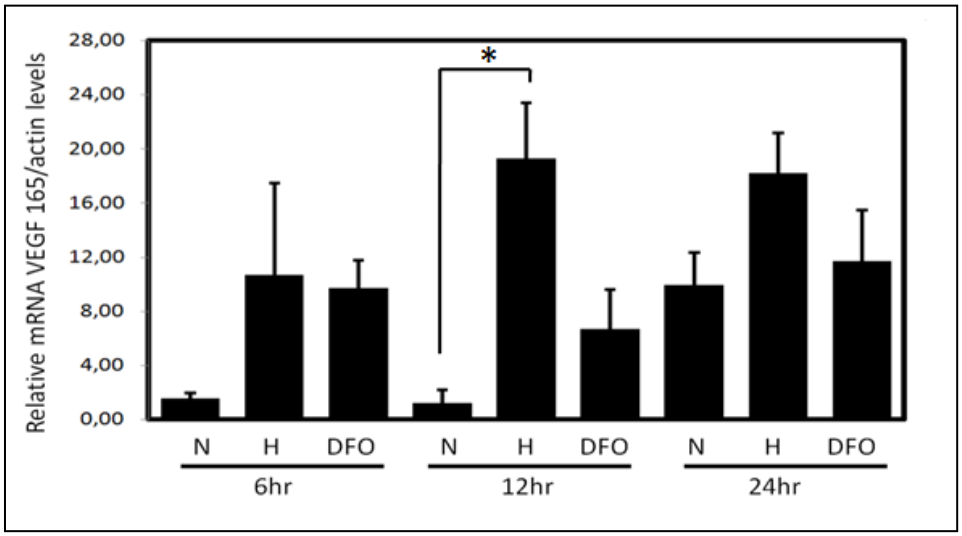


Figure 12. **RT-PCR for 165 and 189 VEGF mRNA in human retinal cells**. ARPE-19 cells were plated and left to adhere overnight on cell culture dishes. After fresh medium replacement, cells were incubated during 6, 12 or 24 hours under normoxia (21% O_2, N), hypoxia (1% O_2, H), or with deferoxamine (DFO) as a positive control. Then, total cell extract was obtained, RNA was isolated and semi quantitative RT-PCR was performed. **A.** Photograph of representative agarose gel (2%). **B.** Graphic of densitometric analysis of RT-PCR agarose gels for VEGF 165 normalized to actin levels at different times. Values in the graph are mean + SEM obtained from three independent experiments. (*)p =0.031, ANOVA statistical analysis.

1.5 Effect of curcumin on VEGF mRNA induced by hypoxia. Curcumin has been shown to display inhibitory properties on HIF-1α in cancer cells [54, 73, 74]. However, no evidence exists in our model. Therefore, another hypoxia experiment was performed and RT-PCR was performed to study variations on hypoxia-induced VEGF mRNA increase. Treatment with curcumin (20 and 50 µM), prior to hypoxia, prevented the hypoxia-induced up regulation of both VEGF A isoforms in ARPE cells, leading to VEGF mRNA levels similar to those observed under basal conditions (see Figure 13).

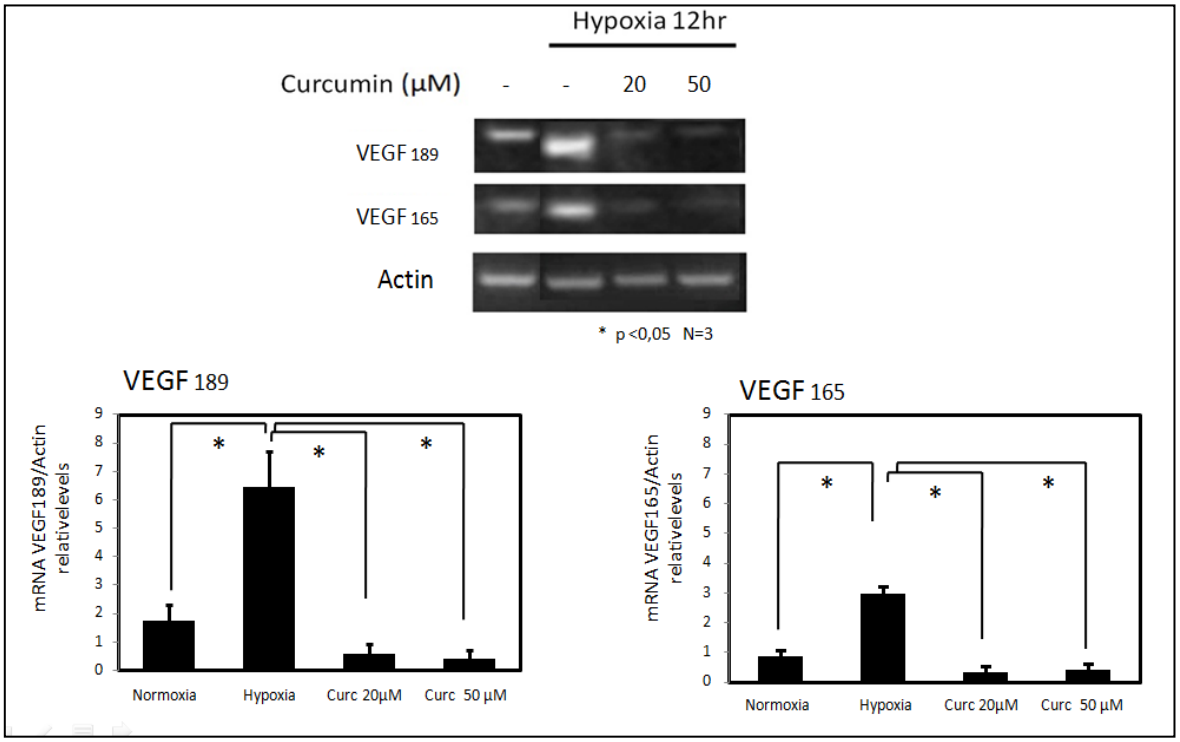


Figure 13. RT-PCR for 165 and 189 VEGF mRNA in human retinal cells treated with curcumin. ARPE-19 cells were plated and left to adhere overnight on standard cell culture dishes. Cells were then incubated with 20 or 50 µM curcumin for 3 hours, and then washed with PBS. Cells not treated with curcumin were incubated in normoxia and hypoxia. Curcumin-treated cells were incubated in hypoxia. All treatments lasted 12 hours. Total cell extract was obtained, RNA was isolated and semi quantitative RT-PCR was performed. Representative photograph of agarose gels. Two graphics of densitometric analysis of RT-PCR agarose gels for 165 and 189 VEGF isoforms are presented. The band density at different times was normalized to actin levels. Values in the graph are mean + SEM obtained from three independent experiments. (*)$p < 0.05$, ANOVA statistical analysis.

1.6 RT-PCR for HIF-1α mRNA in normoxia and hypoxia after treatment with curcumin. Data from Specific Aim 1 has shown that HIF-1α mRNA levels remain stable during normoxia or hypoxia. In addition, previous reports have shown, in a specific tumor cell line, that curcumin decreases HIF-1α mRNA levels [73]. Currently, no data concerning

curcumin effect in our model is available, thus, different hypoxia experiments were carried out to pretreat cells with 40 and 80 µM curcumin, and RT-PCR for HIF-1α mRNA was performed. As shown in Figure 7, curcumin treatment did not vary HIF-1α mRNA levels (see Figure 14).

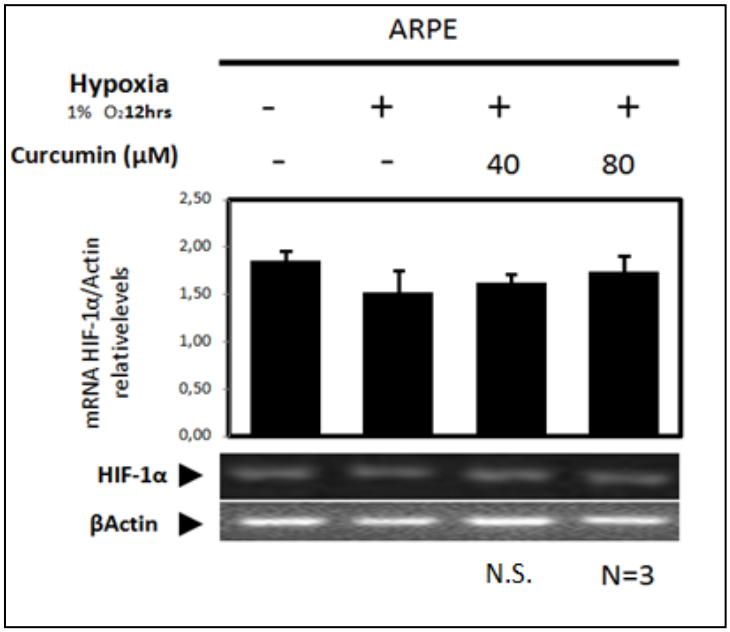


Figure 14. RT-PCR for HIF-1α mRNA in human retinal cells treated with curcumin prior to hypoxia. ARPE-19 cells were plated and left to adhere overnight on standard cell culture dishes. Cells were then incubated with 40 or 80 µM curcumin for 3 hours, and washed with PBS. Cells not treated with curcumin were incubated in normoxia and hypoxia. Curcumin-treated cells were incubated in hypoxia. All treatments lasted 12 hours. Total cell extract was obtained, RNA was isolated and semi quantitative RT-PCR was performed. Representative photographs of 2% agarose gels are shown. Band density was normalized to actin levels at different times. Data shown represent average values from three independent experiments.

1.7.- PEDF levels determined by RT-PCR.

PEDF is a 50 kD glycoprotein that was initially identified as a neurotrophic factor and has subsequently been shown to be an inhibitor of angiogenesis [19]. It is expressed in multiple tissues and is present in the inter-photoreceptor matrix [18]. PEDF is also present in the vitreous and aqueous humor. Recently, PEDF was shown to have anti-angiogenic activity, because it specifically inhibits endothelial cell migration, a key step in angiogenesis [17, 18, 22, 42, 49]. Interestingly, in mouse models of retinal neovascularization it has been established that there is an imbalanced expression of

VEGF and PEDF in vitreous humor and retina [1]. Hence, we determined PEDF mRNA levels in hypoxic conditions and the effect of curcumin on its expression. In presence of 21% O_2, PEDF mRNA levels were detected. Upon hypoxia treatment, PEDF mRNA levels decreased 2-fold. Pretreatment of the cells with 40 µM curcumin inhibited this response, thereby leading to a partial restoration of normoxic PEDF mRNA levels (see Figure 15).

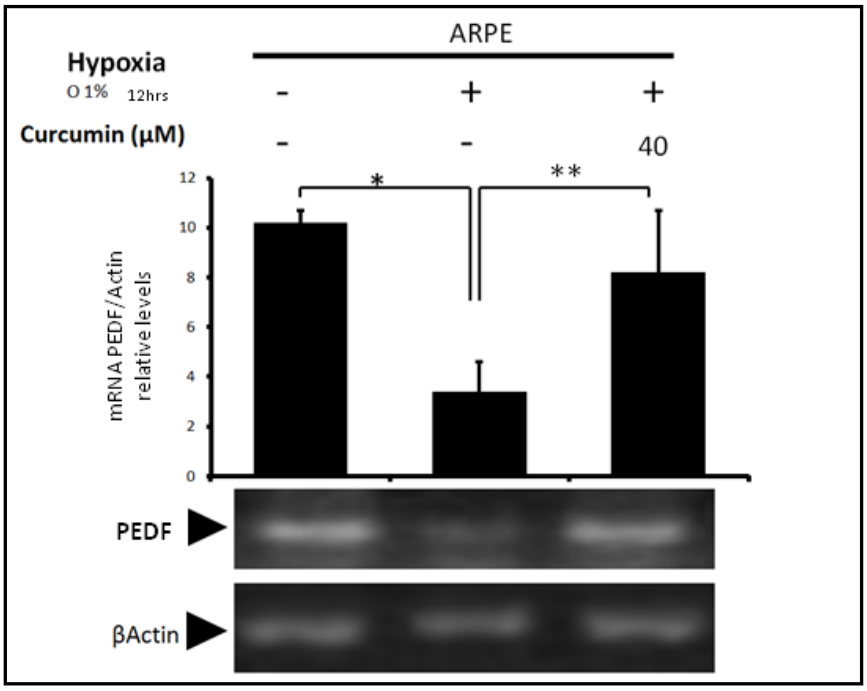


Figure 15. RT-PCR for PEDF mRNA in normoxia, hypoxia and after pretreatment with curcumin. ARPE-19 cells were plated and left overnight to adhere to cell culture dishes . Cells were incubated with 40 uM curcumin for 3 hours, and then cultivated under hypoxia for 12 hours. Total RNA was isolated and qPCR was performed. βActin housekeeping gene was used for normalization. Values in the graph are mean + SEM obtained from three independent experiments. * p = 0.021, **p=0.034 ANOVA statistical analysis.

1.8 qPCR detection of total VEGF-A, VEGF-A_{189} and PEDF

In order to confirm previous findings using standard semi quantitative PCR techniques, a quantitative approach was carried out using Quantitative PCR (qPCR). The primers used in this study are listed in Table 1. Using the same experimental setting, qPCR was used to evaluate total

VEGF-A, VEGF-A_{189} and PEDF. As expected, we observed an increase in VEGF-A and VEGF-A_{189} mRNA levels upon hypoxia, and curcumin was able to prevent this effect. However, the magnitude of the effect was smaller in the case of VEGF-$A_{189,}$ compared to total VEGF-A. Hypoxia during 12 hrs increased nearly 10-fold total VEGF-A mRNA levels, compared to the normoxia condition. This effect was inhibited partially by incubation with 40uM curcumin prior to hypoxia. However, final VEGF-A mRNA levels were significatively higher under hypoxia despite curcumin treatment, compared with normoxia (see Figure 16).

In the same fashion observed for total VEGF-A, hypoxia increased VEGF-A_{189} mRNA levels. However, the magnitude of such increase was lower than that observed for total VEGF-A. This increase was significantly prevented by preincubation with (40uM) curcumin (Figure 17)

Additionally, PEDF mRNA levels showed an opposite change, decreasing upon hypoxia treatment to nearly 50% of the initial normoxic levels. Again, curcumin pretreatment was able to decrease this effect, leading to higher levels or PEDF mRNA, although normoxic levels were not totally recovered (Figure 18).

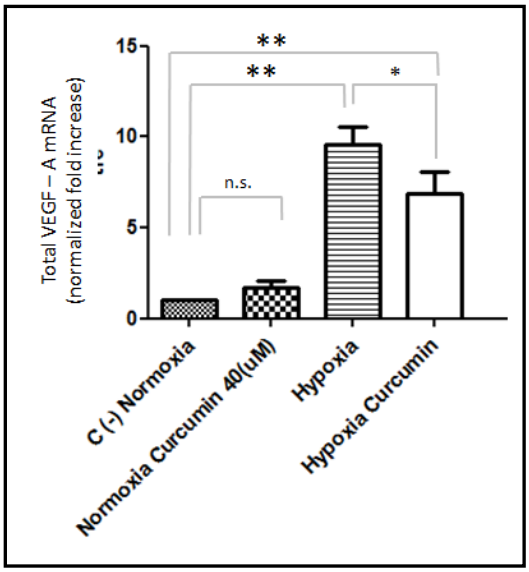


Figure 16. Total VEGF-A mRNA levels by qPCR. RPE cells were incubated with 40µM curcumin for 3 hours, then subjected to hypoxia for 12 hours. Vehicle control [C(-) Normoxia] and positive control (Hypoxia) were not treated with curcumin. Total RNA was isolated and qPCR for total VEGF-A was conducted. *p=0.01, **p=0.001. N=3

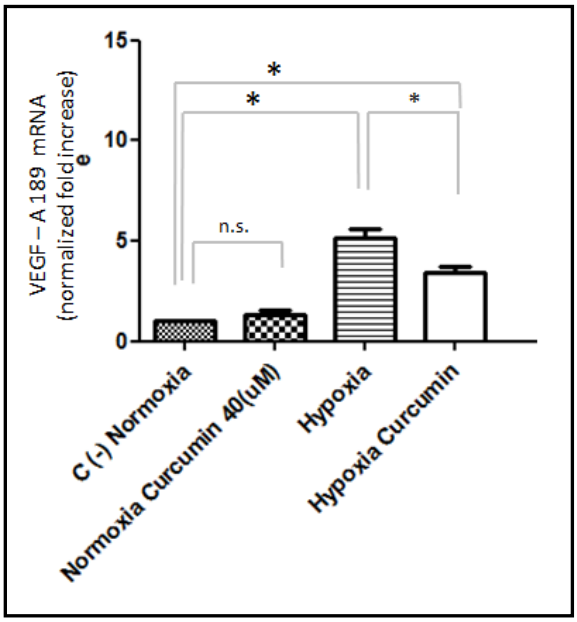


Figure 17. VEGF-A_{189} mRNA levels by qPCR. RPE cells were incubated with 40µM curcumin for 3 hours, then subjected to hypoxia for 12 hours. Vehicle control [C(-) Normoxia] and positive control (Hypoxia) were not treated with curcumin. Total RNA was isolated and qPCR for VEGF-A_{189} was conducted. *p=0.01, **p=0.001. N=3

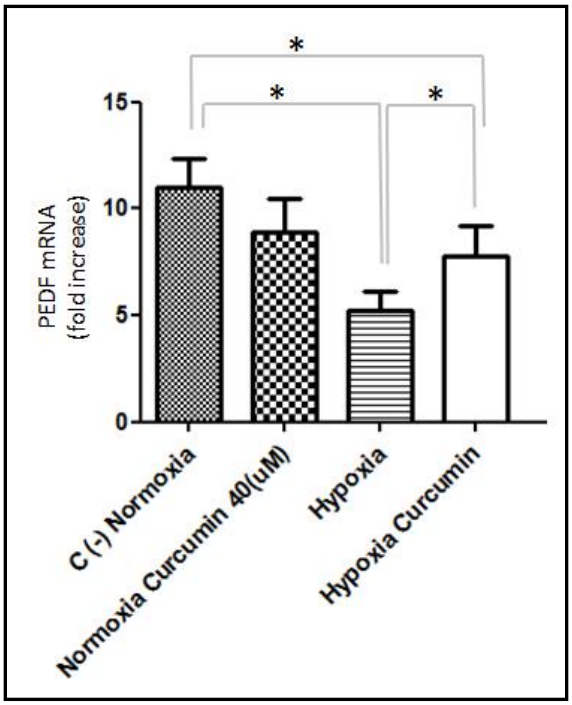


Figure 18 PEDF mRNA levels by qPCR. RPE cells were incubated with 40µM curcumin for 3 hours, then subjected to hypoxia for 12 hours on kept under normoxia. Vehicle control (C(-) Normoxia) and positive control (Hypoxia) were not treated with curcumin. Total RNA was isolated and qPCR for PEDF was conducted. *p=0.01, **p=0.001. N=3

1.9 VEGF-A secretion measurement

To evaluate the effect of pretreatment with curcumin on the secretion of VEGF-A induced by hypoxia in RPE cells, total content of VEGF-A in conditioned medium was measured using a VEGF-ELISA kit that detects all of its isoforms. The range of detection of the ELISA was between 15 pg/ml and 1046 pg/ml. As expected, hypoxia induced a higher release of VEGF-A, reaching nearly 400 pg/ml. The hypoxia-induced secretion of VEGF-A in RPE cells could be partially inhibited by preincubation with 40 µM curcumin. Bevacizumab, an antibody anti VEGF-A, showed a stronger effect, lowering free VEGF-A from the conditioned media, compared to curcumin treatment, and no additive effect was observed when Bevacizumab was added to curcumin pretreated hypoxic conditioned medium (see Figure 19)

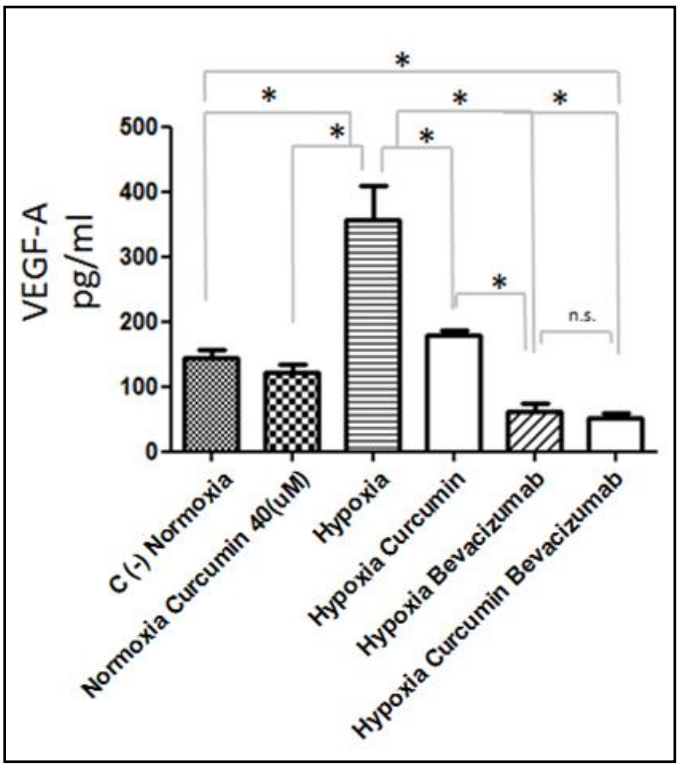


Figure 19. Measurement of VEGF-A secretion with ELISA. The concentration of VEGF-A in the conditioned media derived from treated and untreated RPE cells was measured using an ELISA kit. Briefly, cells were incubated with curcumin or vehicle. After washing the cells, they were cultivated under hypoxia for 12 hours. Then, 1 ml of conditioned medium per plate was collected for measurement in an ELISA plate. *p<0.05. N=3

Summary of Results from Specific Aim 1

1. HIF-1α is stabilized at 12 hours of hypoxia, and HIF-1 transcriptional activity increases 10-fold, compared to basal conditions. 40 or 80 uM curcumin pretreatment is able to partially inhibit this increase induced by hypoxia.
2. Changes in HIF-1α protein levels are not transcriptionally mediated.
3. Total VEGF-A mRNA and VEGF-A_{189} mRNA levels increase upon hypoxia treatment; this can be partially inhibited by curcumin pretreatment.
4. PEDF mRNA decreases upon hypoxia and this can be prevented by curcumin pretreatment, leading to higher levels under hypoxia. However, normoxic level are not reached.
5. VEGF secretion is induced by hypoxia and thus, can be prevented by preincubation with curcumin. Avastin has a stronger effect, decreasing free VEGF-A levels in the conditioned medium.
6. Curcumin prevents hypoxia–induced up-regulation of 165 and 189 VEGF mRNA, both previously known key mediators of choroidal neovascularization.
7. Curcumin does not alter HIF-1α mRNA levels.
8. ARPE cells respond to hypoxia by stabilizing HIF-1α protein levels, reaching a peak at 12 hours and inducing an 8-fold increase with respect to basal levels in normoxia.
9. At 12 hours of hypoxia, a 1.5 fold induction of HIF-1 was observed, compared to basal transcriptional activity.
10. HIF-1α mRNA levels remain constant during hypoxia and normoxia.
11. At 12 hours of hypoxia, a significant up regulation of both VEGF A_{165} and VEGF A_{189} isoforms was observed.
12. Treatment with 20 and 50 µM curcumin prior to hypoxia, prevented the hypoxia-induced up regulation of both VEGF A isoforms in RPE cells, leading to VEGF mRNA levels that are comparable to basal conditions.
13. Curcumin treatment did not vary HIF-1α mRNA levels in RPE cells.

2.1 Viability of ARPE cells in two different culture media during starvation by Trypan Blue method. ARPE cells grow in DMEM:F12 medium and RF6A cells grow in MEM. Since migration was going to be evaluated in RF6A cells, we wondered if MEM medium altered ARPE cell viability under starvation. To answer this question we tested ARPE cell viability in serum-free MEM using Trypan blue. Cell viability was assessed at 12, 24, 48 and 72 hours in the two different culture media. As shown in Figure 9, cell viability showed no changes in any of the culture media studied up to 24 hours. At 48 hours, a notorious trend, yet not significant, was observed. At 72 hours there is a statistically significant difference in cell survival in the different media (see Figure 20).

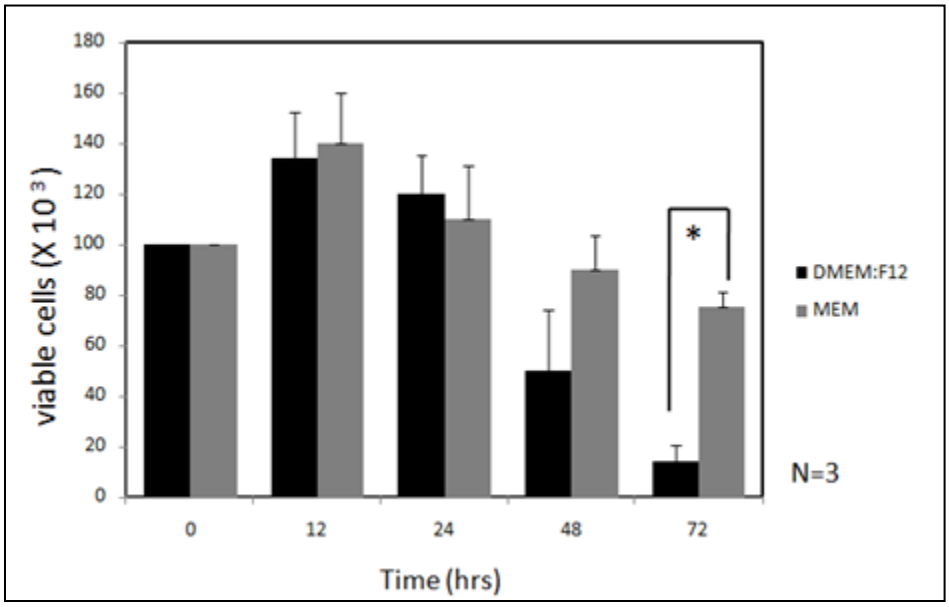


Figure 20. **Viability of ARPE cells in two different culture media during starvation with the Trypan Blue method.** 100.000 ARPE cells were seeded on 6 cm cell culture dishes, and left 4 hours to adhere. After washing with PBS, complete medium was replaced with DMEM:F12 or MEM without serum. Viable cell count was performed at 12, 24, 48 and 72 hours using Trypan blue and cells were quantified in a Neubauer chamber under a light microscope. Graphic shows average values of 3 independent experiments performed in duplicate. Error bars are SEM. (*)p =0.03, ANOVA statistical analysis.

2.2 Viability of ARPE cells in two different culture media during starvation at 24 hrs by cell cytometry. To further confirm previous findings regarding ARPE cell viability in diferent culture media, a propidium iodine uptake assay was performed. Briefly, viable cells are impermeable to propidium iodine, necrotic cells have a porous membrane, and are highly permeable to propidium iodine and apoptotic cells display an intermediate permeability to propidium iodine. As this compound stains DNA and its signal can be captured and analyzed by cell cytometry, it is a widely used reliable and reproducible method to define viable, apoptotic and necrotic populations within a cell culture,[75, 76, 77]. In agreement with the data obtained with Trypan blue, ARPE cell viability was not affected at 24 hours of starvation in both cell culture media (see Figure 21).

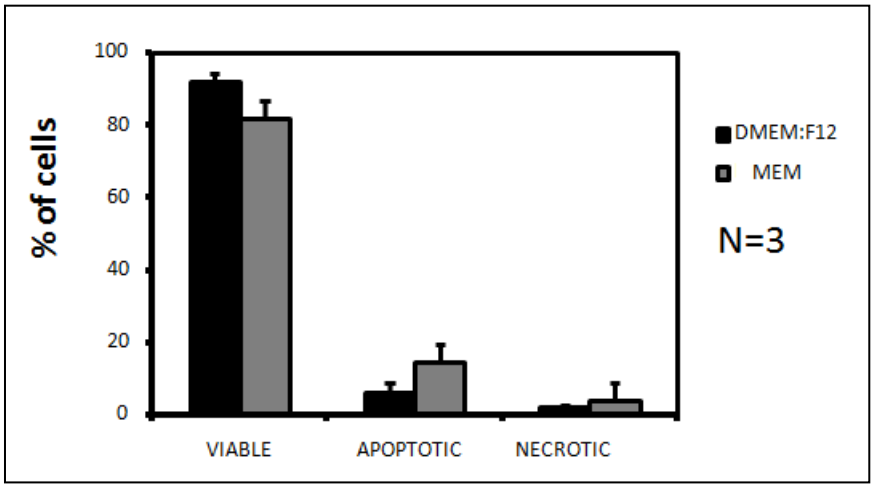


Figure 21. Viability of ARPE cells in two different culture media during starvation at 24 hrs by cell cytometry. 100.000 ARPE cells were seeded on 6 cm cell culture dishes, and left 4 hours to adhere. After washing with PBS, complete medium was replaced with DMEM:F12 or MEM without serum. Viable, necrotic and apoptotic cell count was performed after 24 hours by cell cytometry.

2.3 Wound-healing assay with vascular cells incubated with RPE-conditioning media- treated with curcumin prior to hypoxia. Once methological issues were solved, scratch assays were performed. In these assays, we artificially created a standarized "wound" with a sterile micropipette tip, on a monolayer of choroidal vascular cells. Then, migration of the vascular cells to close the wounded area begins. This process can be monitored in a microscope and registered with a scope camera, allowing measurement of the remaining cell-free area and estimation of the migration rate under diferent stimuli. In this series of experiments, serum-free medium was used as a negative control, in which

less than 20% of wound closure is observed at 24 hours. Complete medium with 10% fetal bovine serum was used as positive control, in which wound closure was 62%. Normoxic ARPE conditioning-medium was used to stimulate vascular cells, which resulted in a wound closure of 42%, suggesting that the conditioning medium contains migration inducer soluble compounds. As expected, hypoxic ARPE conditioning-medium displayed an even higher migration-enhancing effect, leading to wound closure of 60%, comparable to what was observed under stimulation with fetal bovine serum. However, pretreatment of ARPE cells with 20 and 40 µM curcumin for 3 hours, prior to hypoxia, was able to abolish this migration-enhancing effect of hypoxic and normoxic ARPE conditioning medium on choroidal vascular cells (Figures 22 and 23).

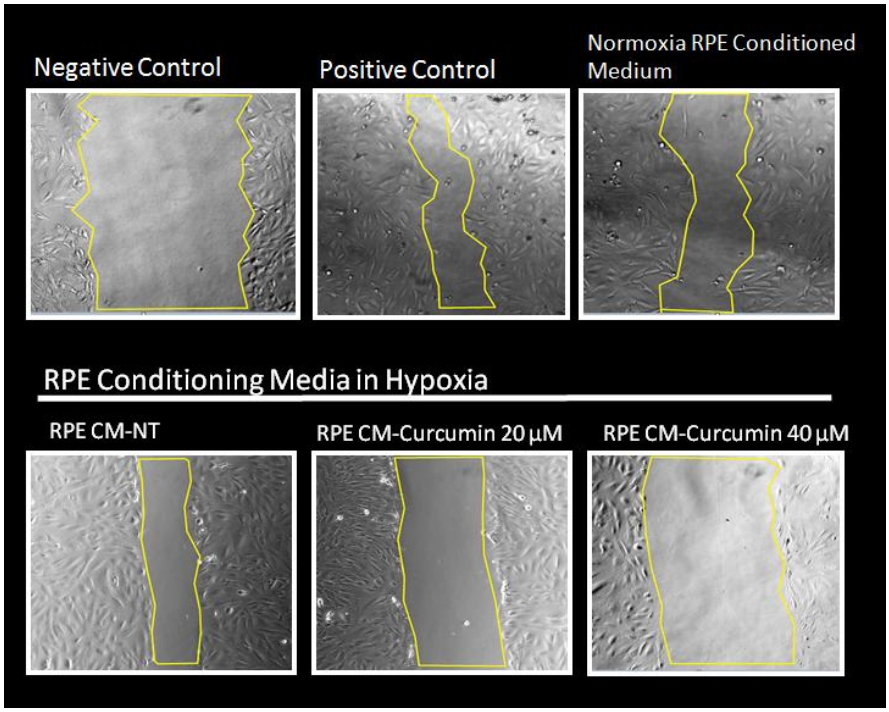


Figure 22. Curcumin inhibits RPE conditioned media-induced migration of vascular cells. 400.000 RF6A cells were seeded on 24-well culture plates and left overnight to adhere and form a monolayer. A scratch was then performed with a sterile micropipette tip. After washing to free all cells in suspension, RPE conditioning media were added and the percentage of wound closure was measured as the cell-free area in 8 micrographs per condition, in triplicate. Photographs show representative fields. Quantification appears in Figure 23.

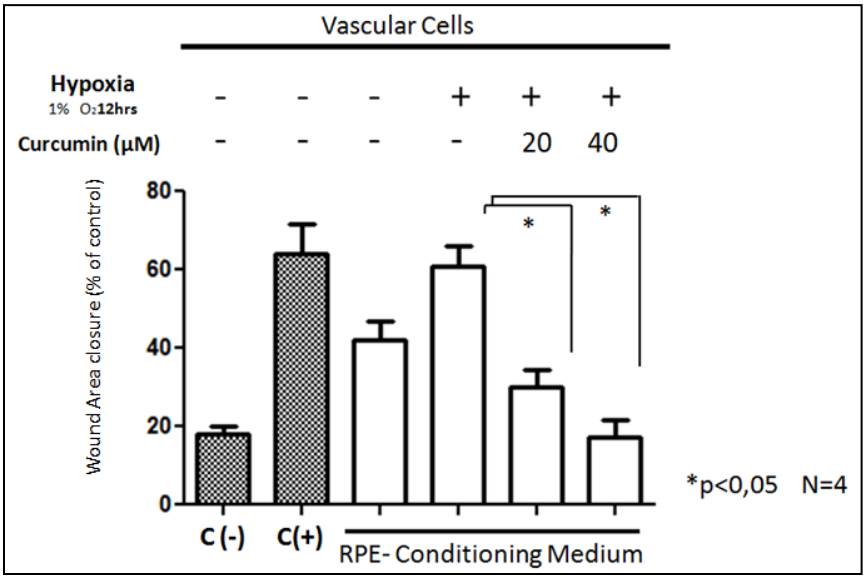


Figure 23. Cell-free area quantified from a sctratch assay of vascular cells incubated with RPE-conditioning media treated with curcumin prior to hypoxia. 400.000 RF6A cells were seeded on 24 well culture plates and left overnight to adhere and form a monolayer. The next day, a scratch shaped wound was performed with a sterile plastic micropipet tip. After washing, RPE conditioning media was added and the percentage of wound closure was measured in 8 micrographs per condition, in triplicate. (*)$p < 0.05$, ANOVA statistical analysis.

2.4 Invasion Assays. Under pathological conditions, vascular cells loose their site-restricted organization, migrating within the choroid and invading the neighbouring Bruch's membrane and retinal pigment epithelium. It is known that overexpression of VEGF leads to a miriad of cell-specific responses in vascular cells, including migration, matrix metalloproteinase secretion and tissue invasion [25, 34, 78]. These events allow vascular cells to cross into the retinal compartment. In order to study this phenomenum in more detail, and to evaluate possible modulation of this process by curcumin, we designed a transmigration experiment of invasion using a transwell co-culture system (see Figure 24)

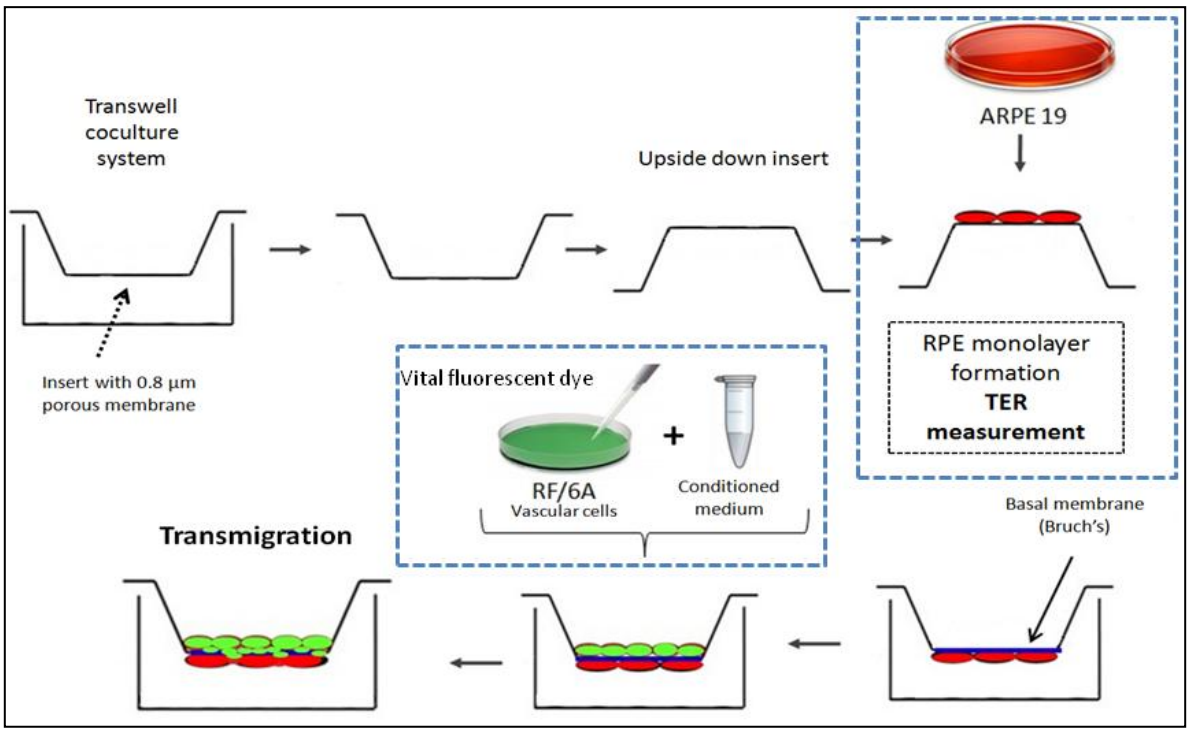


Figure 24. Experimental design of transmigration assay through an ARPE monolayer in transwell chambers. A monolayer or ARPE cells was grown on the inferior surface of the insert of a transwell On top of the artificial RPE monolayer, previously stained vascular cells were seeded and stimulated with RPE-conditioning medium obtained from hypoxia – curcumin experiments. Transmigration of vascular cells was then monitored by confocal microscopy, tracking the fluorescent dye signal from the migrating RF6A cells.

However, in order to perform these experiments, two crucial steps must be given. First, a functional monolayer that offers natural resistance to migrating vascular cells must be achieved. Second, vascular and epithelial cells must be prone to be stained with vital fluorescent dye, in order to distinguish between both cell lineages when transmigration occurs.

2.5 Trans epithelial resistance measurement of ARPE cells grown on the inferior surface of transwell inserts. This is a previously validated method used to study the barrier function of highly differentiated epithelium, such as RPE [29, 30, 79]. In normal conditions, cellular-binding complexes, suchas tight junctions and zonulla ocludens, allow RPE to establish a characteristic high trans-epithelial resistance (TER) due to differential segregation of ions and molecules in highly compartmentalized apical and basolateral membrane domains. In the retinal microenvironment, this physiological TER in the retina is important to inhibit vascular choroidal cell migration through the RPE. Furthermore, it has

been reported that TER breakdown precludes choroidal migration and neovascular formation [30]. Thus, the experimental measurement of TER can indicate the existence of a mature and fully functional epithelium. We therefore aimed to establish the experimental conditions necessary to achieve a high TER that could be used to monitor hypoxia-induced RPE breakdown and choroidal transmigration. Specifically, as we initially designed the experiment, retinal cells were grown in the inferior surface of a transwell insert to achieve the formation of a functional monolayer. After some days of culture, these *in vitro*-formed RPE monolayers were challenged with choroidal cells and supernatants that were seeded on the superior surface of the insert. Transmigration of these cells was stimulated with medium enriched in RPE soluble growth factors, including VEGF 165. Collaboration was established with Dr. Farfan's Laboratory at Luis Calvo Mackenna Children's Hospital, in Santiago, and TER was measured using their facilities and equipment.

Two different cell numbers were assayed, and three consecutive measurements were done at day 0, day 4 and day 8, as shown in Figure 25. According to different references, a surface corrected TER of ±100 Ω, measured with this equipment, may represent a functional monolayer of RPE cells.

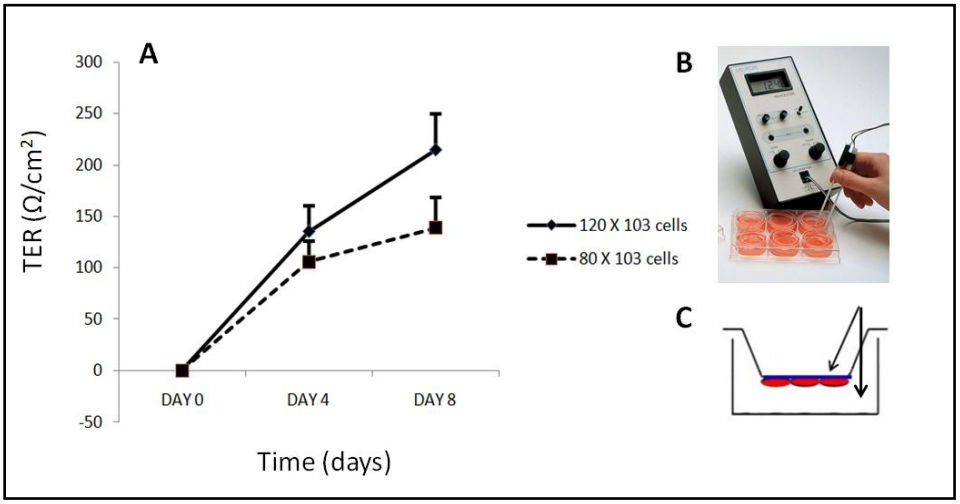
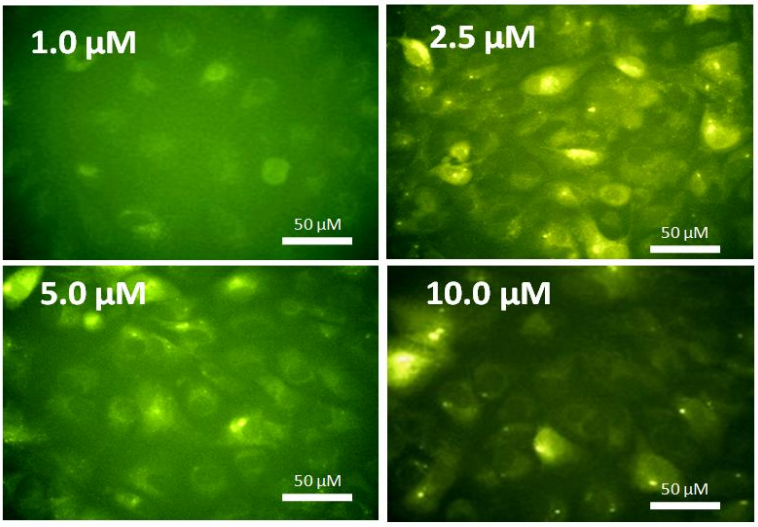


Figure 25. **Transepithelial resistance of a monolayer of ARPE cells.** Two different amounts of cells were plated in the inferior surface of a transwell insert placed upside down. After overnight culture, the inserts were placed in a 24 well plate and grown for 4 and 8 days. **A:** Corrected resistance values. **B:** Photograph of a Millicell ERP measurement system. **C:** Illustration of the position of the two electrodes in the transwell system.

2.6 Vascular and epithelial cell staining using fluorescent dyes. As explained above, cells in a co-culture system must be properly stained in order to follow their migration and invasion behavior. We therefore assessed four different concentrations of fluorescent dye in RF6A and ARPE cells in culture. Both cell lines are stained with low concentrations of the dye, which is adequately detected at least during the first 24 hours after staining without any visible cell toxicity (Figure 26).

A



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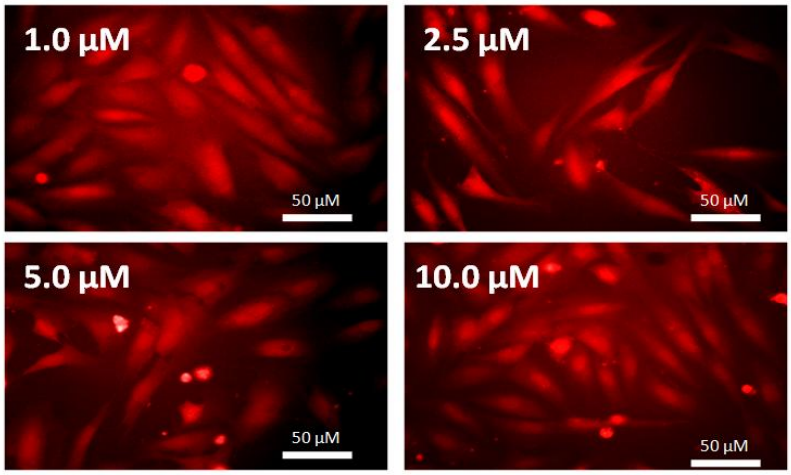

Figure 26. **Vascular and epithelial cell staining using fluorescent dyes.** Cells in culture were incubated with the indicated dye concentrations in serum-free media. A: ARPE-19 cells were stained with green dye and B: RF6A cells were stained with red dye. After careful rinse, cells were kept in the incubator. Samples of each cell type were photographed in a microscope using the

corresponing wave-length filters. Representative photographs are shown. A magnification bar is indicated in each photograph.

2.7 Choroidal vascular cells transmigration through RPE functional epithelium.

In the natural occurring disease of CNV, a characteristic transmigration process occurs. Activated vascular cells migrate through the mature RPE monolayer that anatomically and functionality delimits the choroidal from the subretinal space. We aimed to study the effect of the RPE conditioned medium in an experimental model of trans-RPE migration of vascular cells. Briefly, a monolayer of RPE cells was grown over a semi-porous membrane in a bicameral culture device. Then, vascular cells were seeded and stimulated in the upper chamber, and trans-migration through RPE wass evaluated by counting stained vascular cells in the inferior surface of the membrane with a confocal microscope (see Figure 27). Finally, quantification of stained vascular cells per field were plotted under different experimental conditions (see Figure 28). Incubation with rhVEGF led to an increased transmigration of vascular cells, compared to control. However, the incubation with hypoxic conditioned medium from RPE led to greater transmigration. This could be prevented by preincubating with curcumin, which decreased the angiogenic potential of RPE conditioned medium.

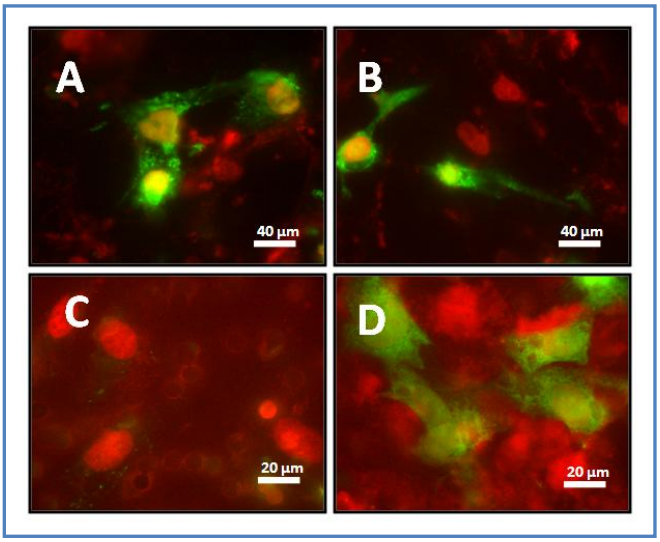

Figure 27. Identification of transmigrating cells in transwell membranes. Choroidal vascular cells, labeled with a green vital fluorescent dye, were seeded in the upper chamber of the transwell device. They were then treated with different conditioned medium. After 12 hours of transmigration, the inferior surface of the membrane was fixed, mounted and photographed with an epifluorescent microcope in order to detect and count the vascular cells that transmigrated through the RPE (red) cells. A: control with normoxic CM, B: Control with rhVEGF, C: Curcumin pretreated hypoxic conditioned media and D: hypoxic conditioned media.

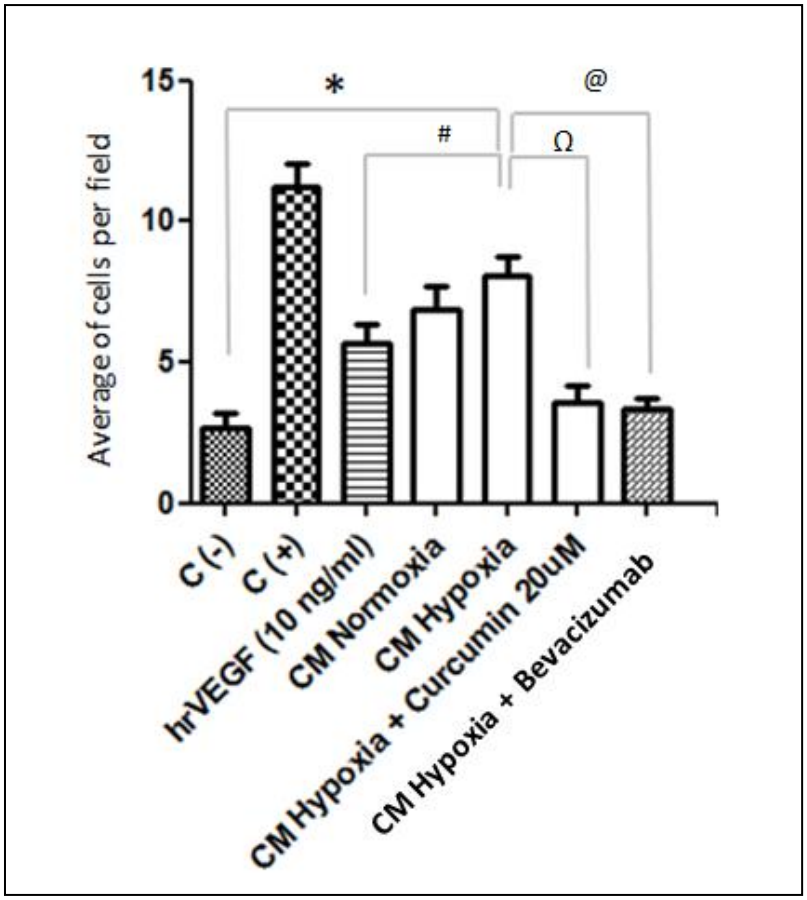

Figure 28. Quantification of transmigrating cells in the inferior surface of the RPE monolayer in a transwell assay. Choroidal vascular cells were stimulated with 5 different conditions, as shown, and seeded on the upper chamber of the insert, where a monolayer of RPE cells was previously grown to achieve confluence, and mature functional properties. Transmigrating cells were counted with epifluorescence microscope and displayed in the graph as average of cells per field. Error bars are shown.p values are: *p=0.21, #p=0.013, Ωp=0.035, @p=0.027

2.8 Digital 3-dimentional evaluation of transmigrating cells

Confocal laser microscopy photographs of the transwell membranes used for transmigration were obtained and then, a 3D reconstruction of the data was performed. As shown in Figure 29, choroidal vascular cells (shown in green) transmigrate through the the membrane, resembling vascular sprouting and neovascular membrane formation that occurs in exudative AMD. This transmigration can be evaluated accurately through

microscopic examination and counting stained cells in the inferior surface, using a standard epifluorescence microscope.

Basically, an increase of transmigration of choroidal cells was observed when were stimulated with conditioned media obtained from RPE cells. After hypoxic exposure, the supernantant displayed higher transmigration of vascular cells.

This induction of an angiogenic response could be partially inhibited when RPE cells were previously incubated with curcumin.

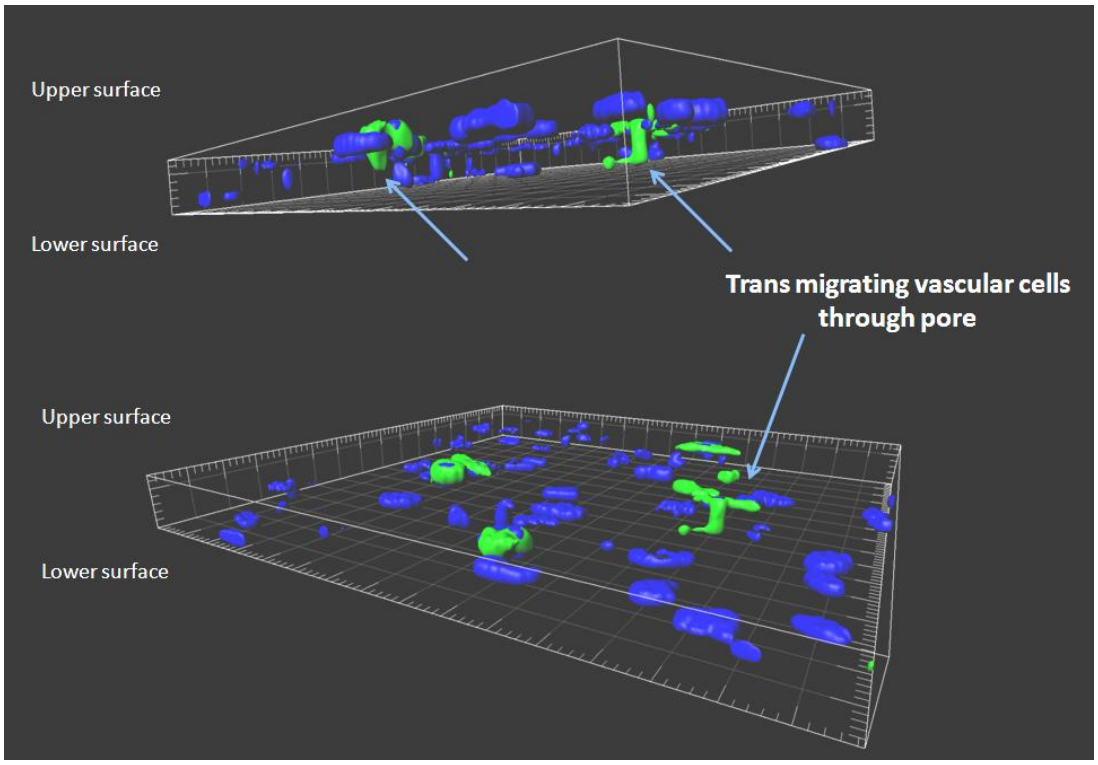

Figure 29. Digital 3D reconstruction of the transmigration assay. **with confocal microscopy scanning images.** Vascular cells are shown in green and RPE cells are shown in blue. In this particular membrane, monolayer is lost due to an artifact of mounting and processing. Hence they are shown as a group of cells on both sides of the membrane. Image processed with Imaris Sofware ®.

2.9 Vascular tube formation assay

Vascular network formation requires interaction and adhesion between vascular cells. The endothelial cells form capillary-like structures in response to angiogenic signals found in

the conditioned media [80]. Tube formation occurs quickly when endothelial cells begin to align themselves and lumen-containing tubules appear. Tubes can be visualized using a phase-contrast inverted microscope. The number of branch sites/nodes, junctions, or the number or length of tubes formed can be easily quantified, and indicate *in vitro* angiogenesis [81].

We used this assay to study the antiangiogenic properties of curcumin. Briefly RF6A were seeded on a culture dish precoated with an extracellular matrix that allows quick adhesion and cell-to-cell contact formation. Hence, cells were seeded with different conditioned media and followed after 12 hours to quantify vascular network formation using a standardized software (see Figure 30) known as Angiogenic Plug In for Image J (NIH, USA) [81] (see Figure 31). This software provides data of a wide array of vascular structures observed in the photographs (see Figure 32). However, only the most important indicators, known as nodes and junctions, were considered for quantification (Figure 33 and Figure 34). Choroid vascular cells treated with human recombinant VEGF (hrVEGF) or hypoxic conditioned medium (vehicle) showed a highly complex vascular network. The angiogenic potential of hypoxic conditioned media of RPE cells was similar and even higher than rhVEGF alone. Preincubation of RPE cells with curcumin led to a lower angiogenic response of the vascular cells treated with its conditioned medium. This effect was very similar to that observed when Bevacizumab (Avastin) was added to the hypoxic conditioned medium. Upon treatment with hypoxic conditioned medium, choroidal vascular cells increase the formation of cell-to-cell junctions (Figure 33) and nodes (Figure 34). This response mimics what can be observed with rhVEGF, but with a higher magnitude. As expected, the conditioned medium of curcumin treated- RPE induced less junction and node formation. This effect parallels Bevacizumab-mediated blockade of VEGF. No additive effect of curcumin and Avastin was observed. This effect is still present in normoxic conditions.

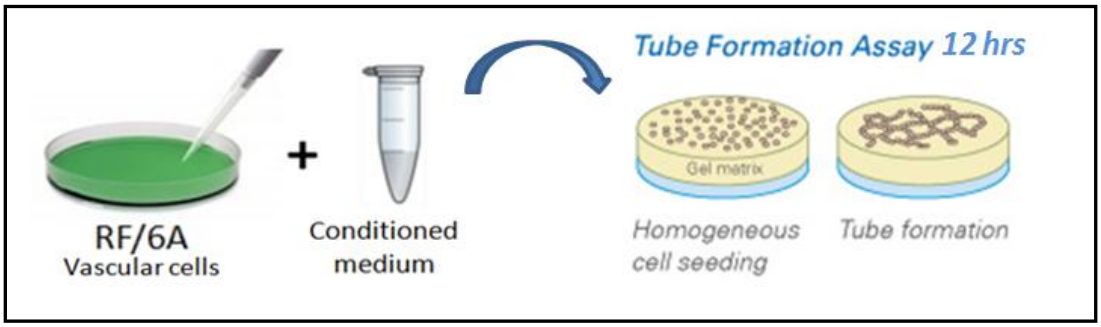

Figure 30. Tube formation assay. RF6A vascular cells were detached, resuspended with each one of the conditioned media under study and seeded on matrigel-covered 24 well plates. After 12 hours of incubation, photographs were taken for digital quantification.

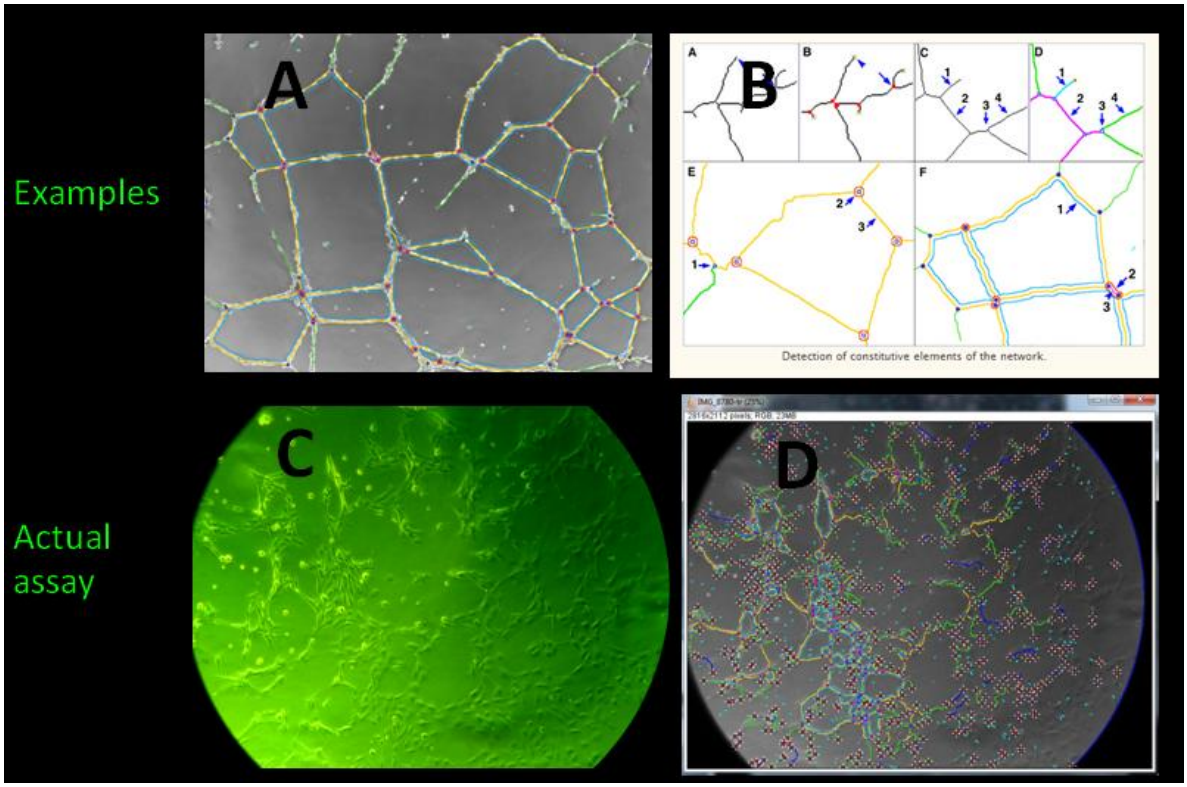

Figure 31. Quantification analysis with Image J. Image J Angiogenesis plug in provides a fast, comprehensive and reliable quantification of vascular networks, as those showed as example in panel A. An algorithm analyzes and simplifies the network in order to identify primary, secondary and more complex structures as those shown in panel B. Our representative photograph (panel C) was comparable to those provided as example, and, as expected, vascular network measurement was feasible, as shown in panel D.

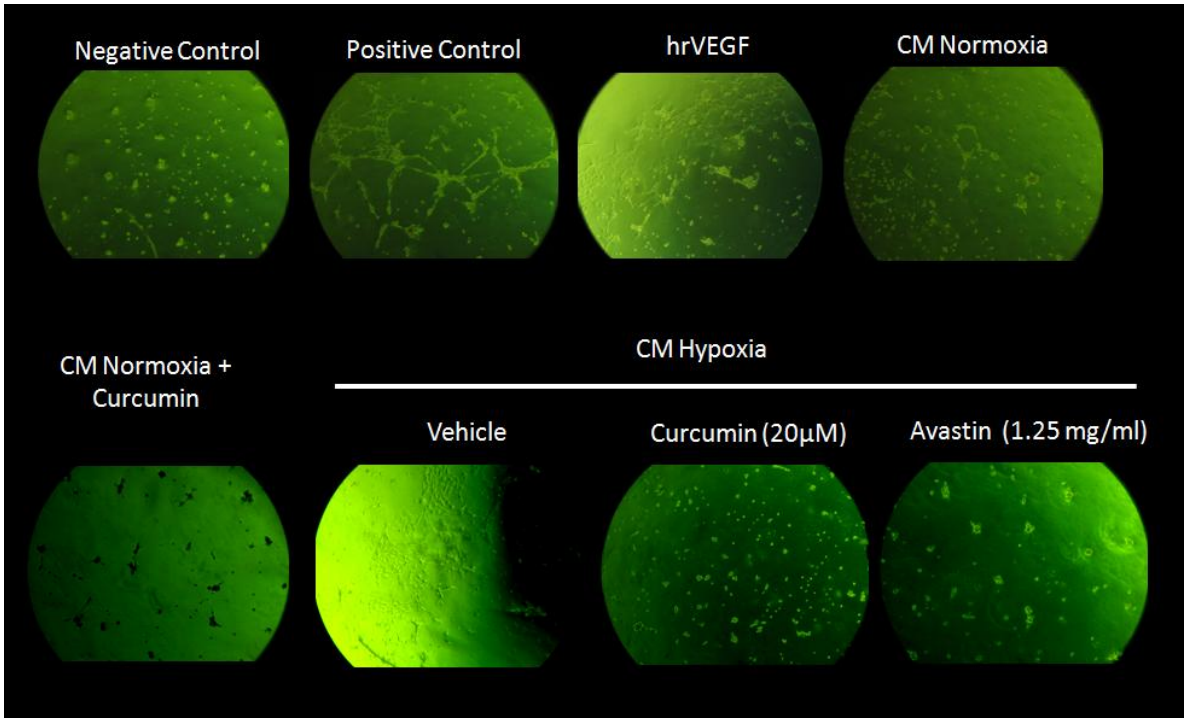

Figure 32. Vascular tube formation assay. RF6A choroidal vascular cells were detached, seeded on matrigel-covered 24 well plates and stimulated with 500 uL of conditioned medium obtained from RPE cells exposed to 12 hrs of hypoxia (CM Hypoxia) that had been previously incubated with 20µM curcumin or vehicle. Avastin was added to Hypoxia CM as a negative control. After 12 hours, 8 photographs per plate were taken to evaluate vascular network formation. Negative Control: DMEM:F12 Medium without serum. Positive Control DMEM:F12 with 10% BCS. hrVEGF: recombinant human VEGF (10 ng/ml). CM Normoxia corresponds to cultures grown with 21 % O_2.

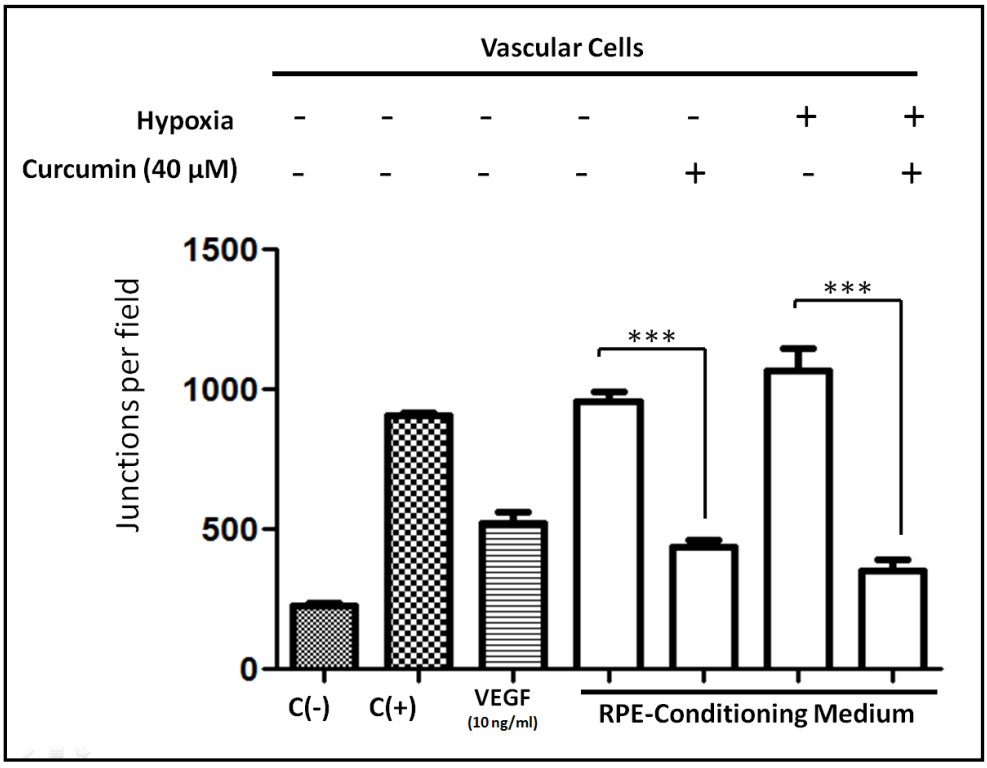

Figure 33. Quantification of tube formation assay: Junctions. Vascular cells were seeded on matrigel- covered 24 well plates and stimulated with the conditioned medium. 8 photographs per condition were taken after 12 hours, digitalized and analyzed with the Image J Software. Average junctions per field are shown. Negative control (C(-)) was fresh culture medium without serum and positive control [C(+)] was culture medium with 5% serum. ***:p =0.001. N=3.

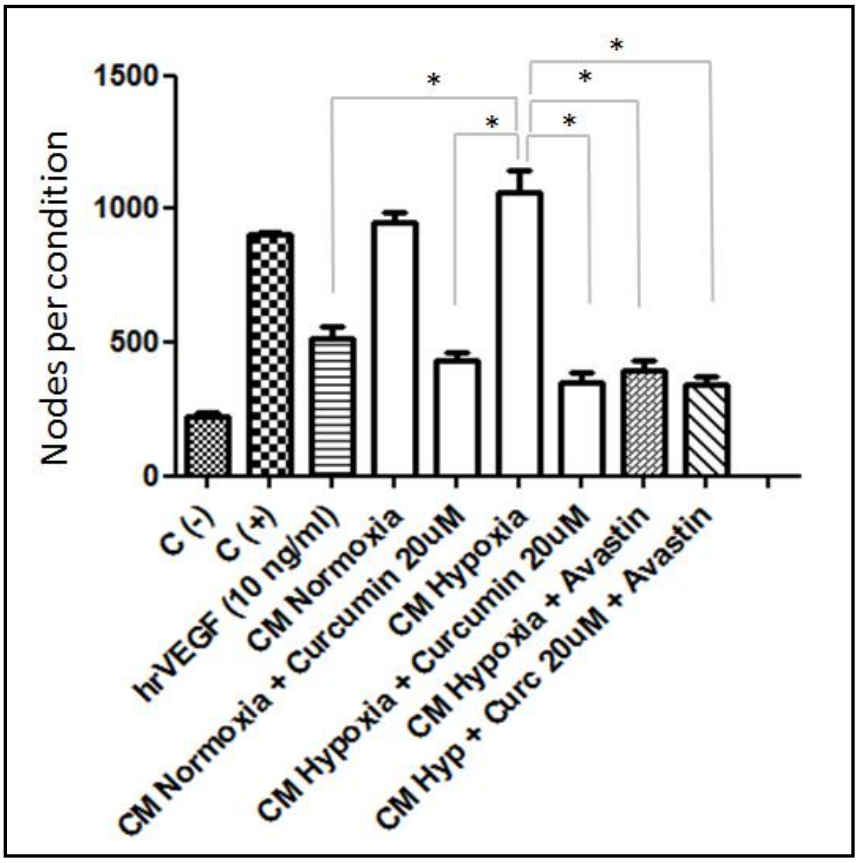

Figure 34. Quantification of tube formation assay: Nodes. Vascular cells were seeded on matrigel-covered 24 well plates and stimulated with different conditioned media 8 photographs per condition were taken after 12 hours, digitalized and analyzed with the Image J Software. Average nodes per field are shown. Negative control [C(-)] was fresh culture medium without serum and, positive control [C(+)] was culture medium with 5% serum. *: $p < 0.05$. N=4.

2.10 Curcumin inhibits hypoxia induced- proliferation of vascular cells.

In the hypoxic retina, RPE signals through soluble factors, such as VEGF. Vascular cells are a specific target for this molecule, leading to an angiogenic response which comprises several functions, including proliferation. We therefore evaluated the effect of soluble factors secreted by RPE cells exposed to hypoxia on the mitotic potential of vascular cells, using Tryphan blue. As expected, hypoxic conditioned medium induces proliferation of vascular cells. This effect can be significantly inhibited by preincubating the RPE cells with 40µM curcumin, prior to hypoxia (Figure 35).

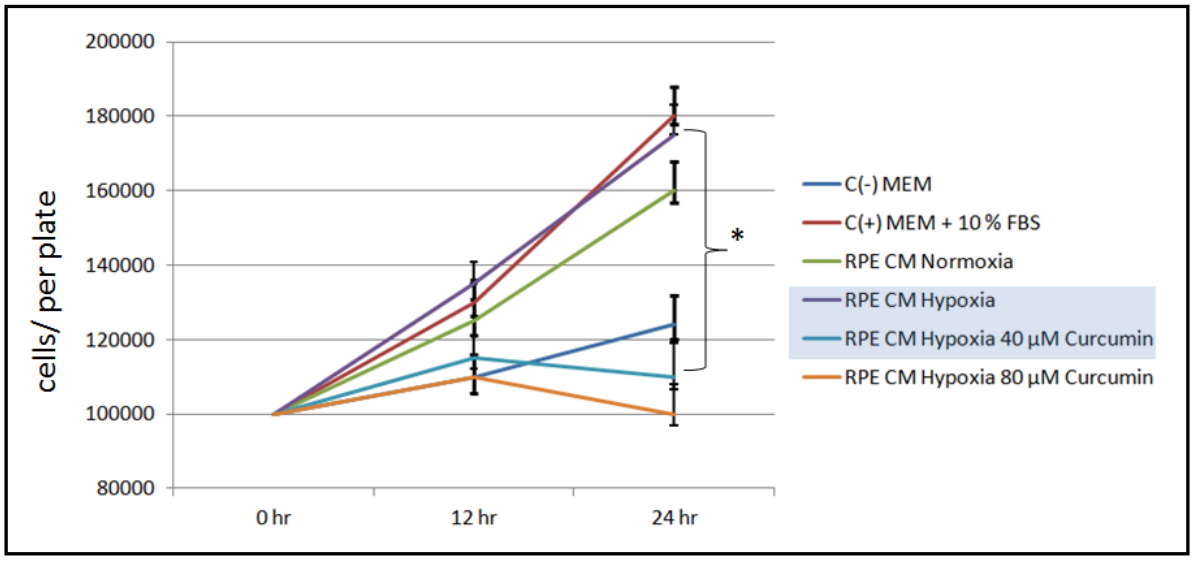

Figure 35. Quantification of viable cells treated with different types of conditioned medium. 100.000 vascular cells were seeded on 6 cm culture dishes and stimulated with experimental conditioned medium (CM), including CM obtained after 12 hours of hypoxia, and CM obtained after hypoxia, but pretreated with 40 µM and 80 µM curcumin. At 12 and 24 hours, cells were detached with trypsin- EDTA, and Trypan blue staining was performed to count viable cells under light microscopy. *p=0.013. ANOVA statistical analysis.

Summary of Results of Specific Aim 2

1. In normoxia and hypoxia, RPE cells **induce migration** of retinal vascular cells through soluble mediators.
2. Preincubation of RPE cells with **curcumin is able to inhibit the migration** induced by RPE-conditioning medium.
3. Curcumin inhibits RPE-induced **transmigration** of choroidal vascular cells through a functional monolayer of RPE.
4. Curcumin inhibits hypoxic RPE-induced **angiogenesis** and **proliferation** in choroidal cells.

5. Transmigration assays are already standarized to test the invasion properties of the vascular cells in the presence of conditioning medium of hypoxia-treated RPE cells.

Specific Aim 3 Results

In vivo angiogenic potential of RPE conditioned medium and the effect of curcumin

In order to study *in vivo* effect of curcumin on angiogenesis, a Chick CAM assay was performed to evaluate the angiogenic response of chorio-allantoid membrane to conditioned medium obtained from RPE cells under the described experimental conditions (Methods). The results demonstrate a basal angiogenic potential of normoxic RPE conditioned medium, which accordingly to ELISA and migration assays, demonstrate the basal secretion of proangiogenic factors in our culture system. As expected, hypoxia induced an increase in this angiogenic potential, leading to an increase of blood vessel density. This hypoxia-induced release of proangiognic factors could be prevented by preincubating the RPE cells with 40 µM curcumin, prior to hypoxia (see Figure 36).

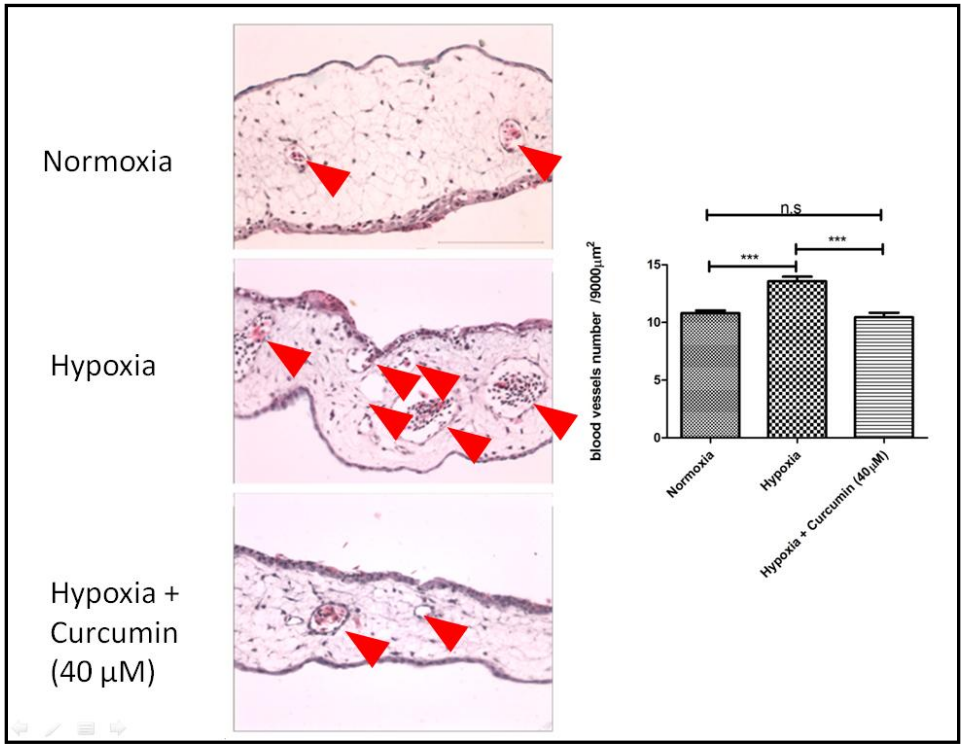

Figure 36. *In vivo* angiogenic potential of RPE conditioned medium and the effect of curcumin. After the chick CAM assay was performed blood vessels per field were counted and averaged. Twelve fields per condition were counted and values were average from 3 independent experiments. *** p=0.001.

Discussion

In the present study, we evaluated the effect of curcumin as a potential anti angiogenic molecule in the pathological process of choroidal neovascularization (CNV) driven by secreted VEGF produced in the retinal pigment epithelium (RPE) of the retina. Our results demonstrated a strong anti-angiogenic effect of curcumin in RPE cells and choroidal vascular endothelial cells (CVE). Indeed, curcumin treated- RPE cells show a decrease in HIF-1α transcriptional activity and downregulation of VEGF mRNA and secretion. In parallel, curcumin treatment inhibited hypoxia-dependent downregulation of PEDF mRNA. Moreover, the anti-angiogenic effect of curcumin was corroborated with a lower angiogenic activity of CVE when exposed to RPE-derived conditioned medium.

CNV is characterized by abnormal growth of new blood vessels from the vascular layer of the eye in the context of exudative age macular degeneration (AMD). These new vessels grow and extend beyond the choroid through the retinal pigment epithelium leading to distortion of the normal architecture and function of the retina. Although the primary molecular mechanism that triggers this pathology remains unknown, many pathways have been described to convey into one same clinical manifestation. One of the components associated with CNV genesis is hypoxia. HIF 1-α, the main regulator of hypoxia, can activate the transcription of various angiogenic genes, including VEGF and ICAM-1, and plays a pivotal role in angiogenesis. HIF-1α expression was detected in surgically excised human CNV membranes [33] and its level is elevated in the eyes of laser-induced CNV animal models [82], while in pharmacologically or genetically HIF-1α-depleted mice, CNV is significantly suppressed, with a reduction of intraocular VEGF. Here, our results demonstrated that HIF-1α activation by hypoxia in RPE leads to higher VEGF secretion to the extracellular matrix, inducing angiogenesis in CVE. This effect was significantly suppressed by curcumin, which is compatible with previous results showing that curcumin or its derivates, had the ability to down-regulate HIF-1α and VEGF expression in vascular endothelial cells and blocked angiogenesis *in vitro* [83]. However in our research, we proposed a different approach to study CNV disease. Instead of targeting the effector cell, like CVE, we aimed at inhibiting the CNV process before it occurs; that is, by inhibiting VEGF over-expression in the RPE . To determine if curcumin downregulation of RPE-secreted angiogenic molecules correlated with a significant inhibiton of CVE, we used a bi-cellular model in which RPE cells were treated with curcumin and challenged with hypoxia

to induce a pro-angiogenic state. We then used the conditioned medium of these cells to stimulate angiogenesis of CVE cells *in vitro* and chorioallantoid vascular cells *in vivo*.

We were able to describe a strong anti-angiogenic effect of curcumin-treated RPE conditioned medium, which correlated with less vascular tube formation structures, lower migration, lower proliferation and significantly lower invasion potential of CVE cells.

Curcumin is a naturally occurring compound present in turmeric, and has been known to possess anti-inflammatory, antioxidant, and anti-cancer activities. Therefore, it has been used for the treatment of inflammatory diseases, diabetes and depression, among others [54]. Accumulating experimental evidence suggests that curcumin interferes with a variety of molecules which are involved in cancer development and progression, including HIF-1α and VEGF. One of the most important setbacks in the development of a curcumin-based therapy has been its low solubility in aqueous phase and poor bioavailability. In this study, we used DMSO as a vehicle and performed toxicity curves of the vehicle and curcumin. According to previous reports [84, 85], retinal pigment cells displayed a different sensitivity range than that reported for cancer cells. Indeed, we observed toxic effects in RPE cells with concentrations above 80 µM. The HIF-1α and VEGF inhibition was observed with concentrations between 20µM and 40 µM. This narrow therapeutic range has set a challenge for future research regarding curcumin as a potential therapy. Therefore, a controlled drug delivery system is required in order to preserve beneficial concentrations of this drug or its analogs, in the vitreous.

Current treatment of AMD consists basically in the use of repetitive intra-vitreal injections of VEGF-blocking antibodies, since VEGF has been the most important molecule identified as a precursor of this disease. Indeed, VEGF is a potent angiogenic stimulator that promotes proliferation and migration of vascular endothelial cells and enhances vascular permeability [8]. However in this study, a different strategy is proposed, focusing on the mechanisms that upregulates pathologically the production and secretion of VEGF. VEGF is produced as a result of HIF-1α transcriptional activity, and oxygen-deprived RPE has been implicated in the pathogenesis of AMD by increasing the secretion of proangiogenic molecules [16]. In the same direction as previously reported results, we provide evidence that in retinal pigment cells, HIF-1 is activated after exposure to short periods of hypoxia. As expected, increase in HIF-1 levels led to an increase in its transcriptional activity. Increase of HIF-1 protein levels was accompanied by a significant increase of VEGF mRNA, effect that could be prevented by preincubating RPE cells with curcumin. Notably,

curcumin inhibited $VEGF_{165}$ and $VEGF_{189}$ mRNA induced by hypoxia, leading to lower VEGF levels in the conditioned medium and also prevented hypoxia-induced release of angiogenic factors. Hence, vascular cell activation and enhanced migration is also inhibited as a result of stimulation with RPE conditioned medium induced by hypoxia. This could probably,be due to the secretion of endogenous antiangiogenic molecules, like PEDF, that are naturally downregulated in hypoxic conditions and by curcumin.

In order to induce VEGF overexpression, which has been described as the main molecular mediator of AMD, we activated HIF-1 transcriptional activity by exposing RPE cells to hypoxia. As expected, increase of HIF-1 levels and its transcriptional activity is not precluded by changes in HIF-1α mRNA, which corroborates what has been previously described in cancer cells regarding its post-translational regulation through the inactivation by hydroxylases and proteasomal degradation [35]. Furthermore, increase in HIF-1 protein levels was accompanied by a significant increase in VEGF mRNA levels. Notably, the retinal cell response is associated with vascular cell activation and enhanced migration. These observations suggest a cross-talk between epithelial and vascular cells via soluble factors secreted into the extracellular media, and that are present in the conditioning medium. Remarkably, curcumin significantly inhibited both VEGF mRNA increase and the enhanced migration of vascular cells. Although further confirmation with measurement of VEGF and PEDF factors in conditioning media is required, the observation of a negative effect on cellular migration suggests an inhibitory effect of curcumin on the HIF-VEGF activation pathway (see model in Figure 37).

CNV is a process that mimics tumoral outgrowth and angiogenic response. In this aspect, our findings are consistent with other recent reports. Specifically, Tan et al (2014) investigated the anti-metastatic effect of curcumin on K1 papillary thyroid cancer cells, as well as its potential mechanisms. The results of this study showed that curcumin effectively inhibits the upregulation of hypoxia-induced reactive oxygen species (ROS) and significantly decreases HIF-1α mRNA and protein expression levels. Although our results did not show variation in HIF-1α mRNA levels, Tan an co-workers demonstrated that curcumin also decreases the DNA binding ability of HIF-1α to the hypoxia response element (HRE), confirming our findings. They concluded that curcumin possesses a potent anti-metastatic effect and might be an effective tumoristatic agent for the treatment of aggressive papillary thyroid cancers [65]. In addition, studies performed with glioblastoma cells treated with curcumin reported inhibition of HIF-1α mRNA (13). We were not able to

detect variations in HIF-1α mRNA upon curcumin treatment; thus, further research is needed to elucidate the mechanisms of action of curcumin on different cell types.

In order to corroborate our *in vitro* findings about the anti-angiogenic activity of curcumin, we used and *in vivo* model of angiogenesis, known as Chick CAM assay. Here, we observed that the conditioned medium obtained from curcumin-treated RPE cells had a significant anti-angiogenic effect compared to non-treated controls. This effect correlated well with our previous i*n vitro* assays..

Interestingly, another paper has recently confirmed the anti-angiogenic potential of curcumin, but in a different system. Nagaraju et al (2014), hypothesized that curcumin and its synthetic analogues EF31 and UBS109, disrupted angiogenesis in pancreatic cancer cells through modulation of HIF-1α and NF-kappaB. They used conditioned medium from pancreatic cancer cells exposed to curcumin and its analogues. Using *in vitro* assays, they observed significantly impaired angiogenesis in HUVEC tube assembly assay, compared to untreated cell medium. *In vivo*, EF31 and UBS109 blocked the vascularization of subcutaneous matrigel plugs. This study also described significant inhibition of VEGF, angiopoietin 1, angiopoietin 2, platelet derived growth factor, COX-2, and TGFbeta secretion under curcumin treatment [64]. Another related paper by Duan et al. (2014) aimed at determining whether curcumin exerted effects on the hypoxia-induced malignant biological behavior of hepatocarcinoma cells under hypoxic conditions. They used $CoCl_2$ to establish *in vitro,* a chemical hypoxia model in HepG2 cells. In this setting, curcumin significantly decreased HIF-1α protein levels leading to lower cell proliferation, migration and invasiveness, in a very similar fashion as that observed in our transmigration assays [56]. To our knowledge, this is the first study that has used an *in vitro* and *in vivo* approach to compare curcumin and a monoclonal antibody in the inhibition of choroidal neovascularization. In our model, curcumin has shown to have a protective effect on RPE cells, preventing the damage induced by hypoxia. But curcumin may elicit protection to another type of damage, such as oxidative stress. Using patient-derived RPEs with the AMD-associated background (AMD-RPEs cells), Chang and coworkers (2014) showed that curcumin caused a significant reduction of ROS. Pretreatment with curcumin protected AMD-RPEs cells from H_2O_2-induced cell death and also increased the cyto-protective effect against oxidative stress generated by H_2O_2 through the reduction of ROS levels. They also demonstrated a downregulation of VEGF, among other molecules under curcumin treatment. Considering that AMD-RPE cells have a decreased antioxidant

defense, they concluded that curcumin may be a suitable drug that can effectively protect from oxidative stress, representing an effective option for macular degeneration therapy and an agent against aging-associated oxidative stress.

In summary, we propose two new concepts in the current state of research for permanent therapy for AMD. On the one hand, blockade of VEGF secretion at a pre transcriptional level is possible. This can be obtained targeting HIF-1 which is the main switch of hypoxic adaptive response. This strategy can probably lead to stronger or longer lasting inhibition of VEGF, slowing or preventing exudative AMD. On the other hand, curcumin, an ancient dyphenol molecule that has shown an interesting potential as a co-adjuvant therapy for some types of cancer,, displays new and strong effects in retinal cells, preventing HIF-1 activation and VEGF production. Further studies must determine the usefulness of this molecule in the therapy of exudative AMD.

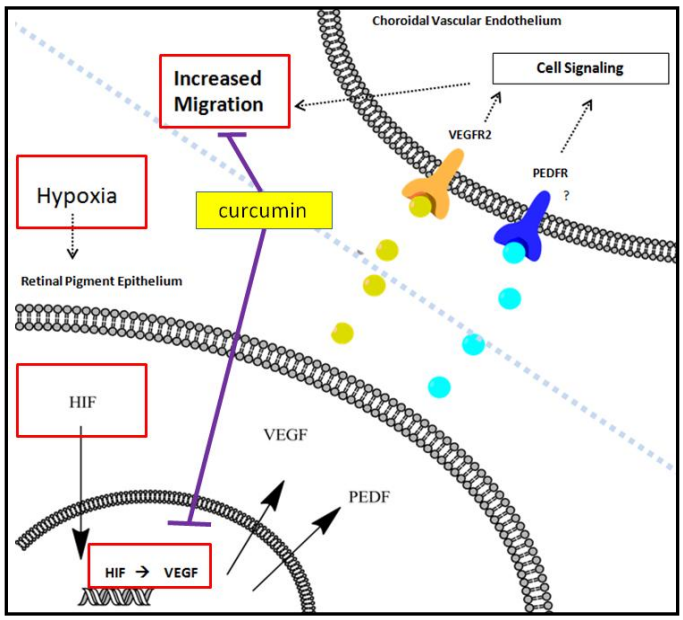

Figure 37.**Model of anti-angiogenic effect of curcumin in RPE and choroid vascular cells.** Hypoxia in the retinal pigment epithelium induces HIF stabilization and transcriptional activity. As a result, VEGF is produced and secreted, targeting vascular cells and initiating multiple cell signaling cascades. Therefore, the angiogenic cell response increases, leading to migration, proliferation, transmigration and vascular tube formation. This signaling events can be blocked by curcumin, which decreases HIF-1α transcriptional activity and inhibits VEGF production.

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