CHAPTER 3

VEGF Gene Regulation

Marcus Fruttiger*

Abstract

VEGF is best known for its angiogenic properties. Not only does it promote the growth of new blood vessels during embryonic development, it is also important in the adult, where it plays a role in maintaining an adequate supply of oxygen and nutrients to most tissues. *VEGF* gene regulation is controlled by different signalling pathways depending on the context in which it is expressed. Best understood is the induction of VEGF expression by hypoxia in neonates and adults, which represents an adaptive response to metabolic stress. In contrast, the mechanisms that control VEGF expression during embryonic development are currently less clear.

Key Messages

- VEGF is a multifunctional molecule that is regulated by numerous different signalling pathways.
- VEGF expression is induced by hypoxia.
- Hypoxia stimulates *VEGF* transcription by increasing HIF activity.
- Hypoxia stimulates *VEGF* translation by increasing mRNA stability.
- *VEGF* gene expression is also controlled by hypoxia-independent mechanisms, in particular during embryogenesis.

Introduction

More than half a century ago, Michaelson used an ink perfusion technique to visualize the developing retinal vasculature and noticed that capillary growth near veins was much more vigorous than near arteries.[1] He proposed the existence of a vasoformative molecule termed factor X, which is (a) produced by extra-vascular tissue, (b) distributed in a gradient and (c) antagonized by oxygen. As we now know, these criteria are fulfilled by the vascular endothelial growth factor VEGF, also known as VEGFA. A team lead by Eli Keshet was the first to propose that VEGF could be the long elusive factor X that mediates hypoxia-induced vascular growth.[2] This was based on the knowledge that VEGF had already been shown to promote the growth of blood vessels,[3] and on the observation that VEGF mRNA dramatically increases under hypoxic conditions in various cell lines. In addition, it was found that VEGF levels were increased in the hypoxic centre of tumours, suggesting that this factor could mediate the growth of new vessels into tumours. This had important clinical implications, because it was known since the early seventies that tumour growth requires sprouting of new vessels from preexisting vessels, a process known as angiogenesis.[4] The discovery of an angiogenic factor in tumours provided for the first time a molecular target for anti-angiogenesis therapy in the fight against cancer,[5] triggering massive research on VEGF. In 1996 two teams simultaneously reported the

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genetic deletion of *VEGF* in mice.[6,7] Both groups found that the inactivation of just one *VEGF* allele caused early embryonic lethality. This was unexpected and made further experiments technically challenging, as no heterozygous founder mice could be created. It also dramatically illustrated the importance of correct VEGF dosage during embryogenesis. Subsequent research has uncovered a multitude of mechanisms that tightly control VEGF dosage and its biological activity.

VEGF Gene Regulation through the Hypoxia Response Element

The most prominent stimulus for VEGF expression is hypoxia. In hypoxic tissue, VEGF is upregulated, and this stimulates blood vessel growth. Increased blood supply then alleviates the hypoxia, turning VEGF expression off again. This simple negative feedback loop ensures that supply and demand of oxygen in tissue are always adequately matched. But how does this process work at a molecular level? A short sequence in the 5' flanking region of the *VEGF* gene is important for VEGF induction by hypoxia.[8] This sequence element, termed hypoxia response element (HRE), was initially discovered as an enhancer element within the erythropoietin (*EPO*) gene.[9,10] The element is also present in many other hypoxia inducible genes and is a binding site for the transcription factor hypoxia-inducible factor 1 (HIF1). HIF1 is a heterodimer consisting of an alpha and beta subunit, both of which are basic helix-loop-helix PAS domain proteins.[11] The beta subunit is known as the aryl hydrocarbon receptor nuclear translocator (ARNT) and is constitutively expressed, whereas the alpha subunit, HIF1A, is regulated by hypoxia. Although the mRNA encoding HIF1A increases in hypoxic cells, the dominant mechanism of HIF1A regulation is post-translational (Fig. 1). Under normoxic

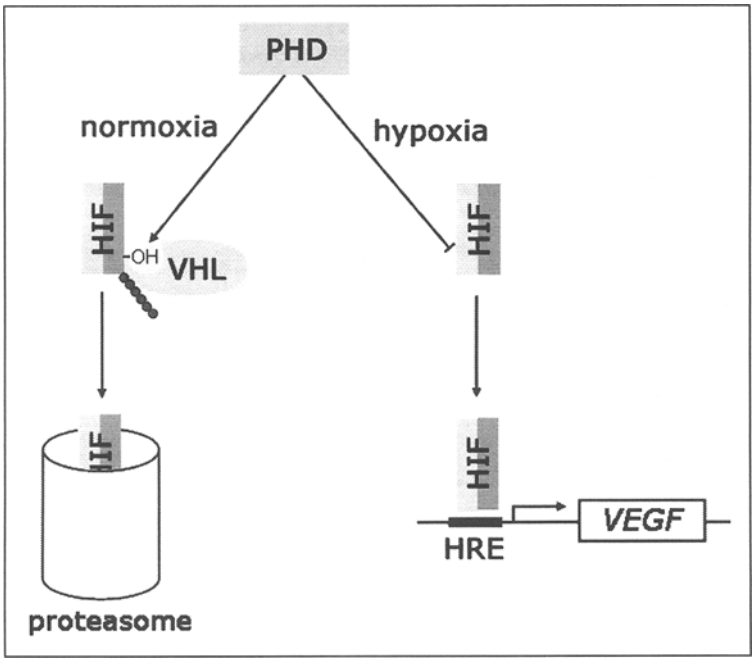

Figure 1. HIF mediated *VEGF* transcription. Under normoxic conditions, hypoxia inducible factor (HIF) is hydroxylated by prolyl hydroxylases (PHDs). This modification facilitates binding of the von Hippel Lindau protein (VHL), resulting in ubiquitination and rapid degradation of HIF. At low oxygen concentration, hydroxylation via PHDs becomes less efficient, resulting in HIF accumulation and HIF binding to a hypoxia response element (HRE) in the *VEGF* promoter.

conditions, HIF1A is swiftly destroyed via proteasomal degradation. However, when oxygen tension falls, this degradation process becomes less efficient and HIF1A protein rapidly accumulates; a sensible strategy, considering that under stressful conditions it might be easier to stop destroying a protein rather than to start producing new protein.

The gene product of the von-Hippel-Lindau (VHL) tumour suppressor gene is critically involved in the degradation of HIF1A. VHL is part of a multi protein complex containing ubiquitin E3 ligase. Binding of VHL to HIF1A results in HIF1A ubiquitination and very rapid proteasomal destruction, making HIF1A one of the most short-lived proteins known. In cells that lack VHL, HIF1A protein levels build up, resulting in increased expression of VEGF and other angiogenic factors. Clinical manifestation of this occurs in von Hippel-Lindau disease, a dominantly inherited familial cancer syndrome predisposing the patient to a variety of malignant and benign tumours. Germline mutations in one allele of VHL do not seem to have any noticeable deleterious effects. However, silencing the second copy of the gene via somatic inactivation or deleterious mutations frequently leads to haemangioblastomas and renal cell carcinomas.

The molecular mechanisms that control binding of VHL to HIF1A are broadly understood and depend on the enzymatic hydroxylation of conserved prolyl residues within the oxygen-dependent degradation domain (ODD) of HIF1A. Deletion of the ODD domain, or mutation of the prolyl residues to alanins, renders HIF resistant to oxygen-induced degradation. The hydroxylation of HIF1A can be carried out by at least three different prolyl hydroxylases, termed PHD1, PHD2 and PHD3 (new names: EGLN1-3). This modification requires the cofactors 2-oxoglutarate, vitamin C, iron and, importantly, molecular oxygen. At low oxygen concentrations, the PHDs become less efficient and HIF1A is no longer hydroxylated, which prevents HIF1A from binding VHL and therefore stops its ubiquitination and degradation; the resulting increased HIF levels ultimately augment VEGF transcription.

The pathway shown in Figure 1 is probably the best know mechanism of VEGF regulation to date. However, when the HRE in the *VEGF* promoter was deleted in mice, they showed an unexpected phenotype.[12] Consistent with an important role for HIF mediated VEGF expression during development, half of the mutant mice died embryonically or perinatally. The surviving half of mice lived for up to two years, but gained less weight, were infertile and developped adult-onset motor neuron degeneration resembling amyotrophic lateral sclerosis (ALS). Interestingly, these surviving mice show impaired hypoxia-induced VEGF upregulation in the brain and spinal cord, but not in fibroblasts, confirming the previous finding that the response to hypoxia is differentially regulated in an organ- and even cell type-specific manner.[13] However, these in vivo findings contrast a number of in vitro experiments, which had convincingly shown that hypoxic VEGF induction critically depends on HIF and the HRE in many different cell types.[8] It is possible that HIF influences *VEGF* gene expression via elements other than the HRE in the *VEGF* promoter; alternatively, HIF-independent systems may compensate for deficiency in the HRE-mediated hypoxia regulation in some tissues.

Post-Transcriptional Regulation of VEGF

The mRNAs of house keeping genes such as β-globin or GAPDH have half-lives of over 20 hours, whereas the mRNAs of cytokines or transcriptional activators typically are short lived, with half-lives of 10-30 minutes. Stabilization of normally unstable mRNAs in response to stimuli such as hypoxia, growth factors, hormones or second messengers provides a mechanism to regulate protein levels. Accordingly, Levy et al found that hypoxia increases VEGF mRNA half-life by a factor of 3, resulting in 8-30 times higher levels of VEGF mRNA. In comparison, HIF mediated regulation of VEGF transcription increases VEGF mRNA amounts only 2-3 times.[14]

Degradation of mRNA transcripts usually starts with 3'-5' exonucleolytic deadenylation, which removes most or all of the poly(A)-tail. The rest of the mRNA is degraded either by 3'-5' exonucleolytic degradation and/or by removal of the 5' cap, followed by 5'-3' exonucleolytic

degradation.[15] Some mRNAs such as that for insulin-like growth factor are also cleaved by endonucleases, initiating subsequent exonucleolytic degradation.[16] Whilst the proteins that mediate and control this degradation process are only partially understood, a variety of mRNA cis-elements have been identified, which are decisive in determining mRNA stability. In the case of the VEGF mRNA, AU-rich cis-elements (AREs) are important. They are around 50 to 150 nucleotides long with no single conserved consensus motive, but they often contain several copies of the pentamer AUUUA or the nonamer UUAUUUAUU and one or several U-rich stretches. The 3' untranslated region of VEGF mRNA contains a 125bp ARE that is bound by a series of hypoxia-induced proteins,[17] but few have so far been identified. One of the binding partners of the VEGF-ARE is HuR (also known as ELAVL1), a member of the ELAV/Hu family of RNA binding proteins. HuR can stabilize mRNA by displacing or inhibiting factors that cleave or deadenylate ARE-containing transcripts.[18] Interestingly, brain tumours ubiquitously express HuR and display elevated levels of cytokines and angiogenic factors such as VEGF.[19] Poly(A)-binding protein interacting protein 2 (PAIP2) also binds the VEGF-ARE and, via interactions with HuR, stabilizes the VEGF mRNA.[20] A further VEGF-ARE binding protein is zinc finger binding protein 36 (ZFP36L1, also known as TIS11B), but, in contrast to HuR, it has mRNA-destabilizing activity.[21]

How hypoxia influences the activity of HuR or other VEGF mRNA-binding proteins remains unclear. An obvious possibility is that the same oxygen sensing machinery that regulates HIF stability feeds into mRNA-degradation mechanisms via interactions with VHL. In fact, there are studies that support such a view. For example, overexpression of VHL in renal carcinoma cells can decrease VEGF mRNA levels via a posttranscriptional mechanism,[22,23] and rapid turnover of mRNA containing AREs depends on ubiquitination and proteasome activity.[24] Furthermore, an association between HuR and VHL was shown in renal cell carcinoma, implicating VHL not only in HIF-mediated VEGF transcription, but also in the cellular signalling events that regulate VEGF mRNA stability.[25] On the other hand, VEGF mRNA can be stabilized by the protein kinase C signalling pathway[26] or the stress signalling pathways via p38MAPK and JNK.[27] Whether these pathways are used to mediate hypoxia-induced VEGF mRNA stabilization is not known.

In addition to mRNA stabilization, the post-transcriptional regulation of VEGF has one more layer of complexity that is based on mRNA translation. Eukaryotic protein synthesis usually depends on binding of the translation initiation complex to the mRNA cap,[28] with ribosomes scanning the 5' untranslated region (UTR) until they encounter the first AUG codon. VEGF possesses an unusually long GC-rich 5' UTR of more than 1000 nucleotides, which can inhibit efficient ribosomal scanning.[29] An alternative, cap-independent mechanism of translation has been identified in picornavirus, which contains long 5' UTRs without caps, but uses elements termed internal ribosomal entry sites (IRES). It is believed that VEGF mRNA with its cumbersome 5' UTR is usually translated efficiently due to the presence of an IRES element in the 5' UTR.[30] The cap-independent translation of VEGF mRNA via the IRES element remains efficient under hypoxic stress, whilst overall cap-dependent protein synthesis is reduced by up to 50%,[30] resulting in a relative increase of VEGF translation.

VEGF Gene Regulation by Transcription Factors

The signalling pathways that regulate cell metabolism and cell growth are intricately linked with the hypoxia-sensing machinery. This is illustrated by the fact that HIF induces angiogenic genes, but also regulates many aspects of anaerobic metabolism, such as the expression of glucose transporter 1 and most of the glycolytic enzymes.[31] VEGF plays a major role in helping to maintain cellular homeostasis in response to hypoxia, acidosis, UV, lack of nutrients and other stresses by stimulating angiogenesis. Accordingly, VEGF expression is not only controlled by hypoxia, but by a complex regulatory network involving many other signalling pathways, including cytokines and growth factors.[32] That the regulation of cell metabolism and the hypoxia-sensing machinery are interconnected is also evidenced in the control of *VEGF* gene

expression: VEGF is rapidly induced upon serum stimulation in normoxic serum-deprived fibroblasts; this response is mediated by the ERK signalling pathway.[33] Activation of this pathway stimulates HIF activity as well,[34] which in turn also induces VEGF transcription.

There are several HIF-independent mechanisms to upregulate VEGF transcription. This is evident from the promoter region of the *VEGF* gene, which contains binding elements for transcription factors such as specific protein 1 (SP1), signal transducer and activator of transcription 3 (STAT3), activator protein 1 (AP1) and many others.[32,35] SP1 is essential for basal transcription of VEGF.[36] Its phosphorylation following ERK activation leads to VEGF mRNA upregulation independent of hypoxia and HIF.[33] It was also shown that SP1 is responsible for the very high levels of constitutively expressed VEGF in many cancer cell lines.[37] Furthermore, tumour suppressors such as p73 or VHL can affect VEGF transcription via SP1.[38,39] In the case of VHL, this is based on direct binding to SP1, which inhibits SP1 activity.[38] Thus, loss of VHL not only induces VEGF via HIF activation (see above), but also via de-repression of SP1. STAT3 is able to directly bind the *VEGF* promoter to upregulate transcription, and a constitutively active mutant form of STAT3 has been found in various types of tumours.[40,41] The presence of four candidate AP1 binding sites in the *VEGF* promoter suggests that AP1 plays a role in VEGF regulation. The AP1 transcription factor is a dimer consisting of basic region-leucine zipper (bZIP) proteins such as Jun and Fos protein family members. It can cooperate with HIF to increase VEGF expression under hypoxic conditions, which involves members of the MAP kinase family.[42] It has also been shown that JUND protects cells from oxidative stress and exerts an anti-angiogenic effect via a HIF-mediated mechanism.[43]

Transcription factors that directly regulate VEGF transcription, such as HIF, SP1, STAT3 and AP1, are in turn controlled by a complex network of interacting signalling pathways. Apart from the PHD-VHL pathway, the ERK, JNK and p38 MAP kinase pathways can all modulate VEGF expression; moreover, signalling via PI3K, AKT (also known as RAC) and mTOR (also known as FRAP1) plays a role in *VEGF* gene regulation.[32,35] Therefore, the simple concept that hypoxia controls HIF to control VEGF levels had to be broadened to include the idea that VEGF transcription and VEGF translation are being influenced by several other signalling pathways (Fig. 2).

Developmental Control of VEGF Expression

In all animals, small blood vessels and capillaries are organized stochastically, whereas larger vessels form stereotypical vascular trees. This makes sense, as small vessels grow in response to metabolic demand. On the other hand, large vessels look more or less the same in every person, because they were patterned by "hard wired" morphogenetic programs operating during embryonic development. VEGF plays a major role in vessel formation both in physiological and pathological angiogenesis in the adult as well as in the developing embryo. We now know that angiogenesis in the adult is to a large extent hypoxia-driven. But is developmental VEGF expression regulated by hypoxia or hypoxia-independent mechanisms? There is evidence supporting both mechanisms of VEGF-stimulated angiogenesis during development.

The neonatal retina is a particularly illustrative example of a tissue in which hypoxia plays a major role in VEGF regulation and angiogenesis during development. Around birth in mice and before birth in humans, the retinal vasculature emerges from the optic nerve head and covers the inner retinal surface as a centrifugally spreading vascular plexus. This process is tightly controlled by retinal astrocytes, which also emerge from the optic nerve head and migrate ahead of the growing blood vessel (Fig. 3). These specialized glial cells provide a template for the vascular network and strongly express VEGF. However, as soon as they become covered by the oxygen-carrying vessel network, VEGF is downregulated (Fig. 3). This differential expression of VEGF (low in the centre and high in the periphery of the retina) leads to a VEGF gradient across the retina that is important for the correct outgrowth of the retinal vasculature.[44] Labelling with a hypoxia-probe shows that the peripheral, not yet vascularized region of the retina is indeed hypoxic, whereas the vascularized central portion is not (Fig. 3). This

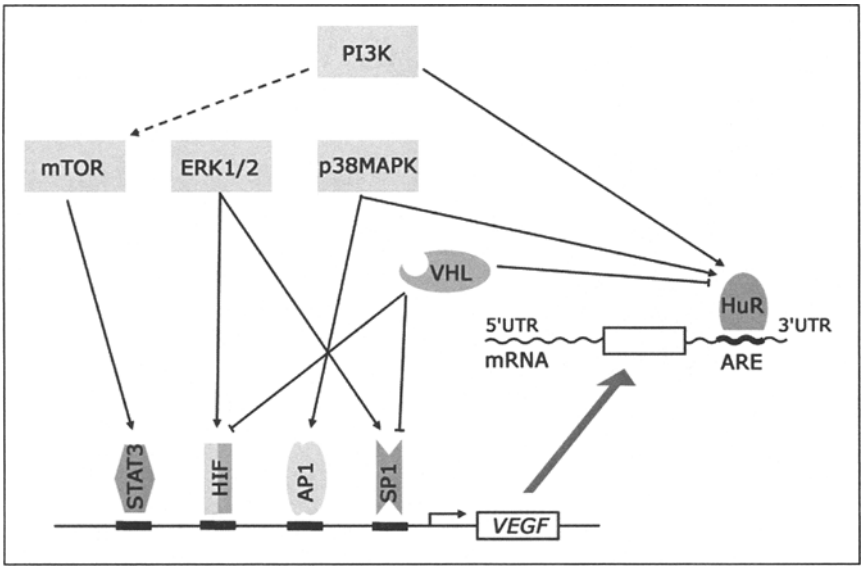

Figure 2. VEGF expression is regulated at the level of mRNA transcription and mRNA stability. VEGF transcription is induced by binding of diverse transcription factors to their recognition elements in the *VEGF* promoter. *VEGF* mRNA stability is increased by mRNA stabilizing proteins such as HuR (ELAVL1). A complex network of signalling pathways including different kinase pathways and VHL can influence both mRNA transcription and mRNA stability of *VEGF*.

difference in tissue oxygenation explains the high levels of VEGF mRNA in the periphery and the low levels in the central vascularized portion of the retina.[45]

It seems that the hypoxia-driven *VEGF* gene expression of peripheral retinal astrocytes does not depend on the HRE in the *VEGF* promoter, because the retinal vasculature develops normally in mice that lack this sequence in their genome.[46] It is therefore likely that the high levels of VEGF mRNA in peripheral retinal astrocytes are due to increased mRNA stability. Furthermore, retinal astrocytes express much higher levels of VEGF than the underlying neurons, even though both cell populations are likely to experience more or less the same oxygen tension. Retinal astrocytes may therefore be more sensitive to hypoxia than neurons; one could envisage a mechanism involving increased basal activity of the *VEGF* promoter, caused, for example, by increased SP1 levels in retinal astrocytes, but this has not yet been investigated.

It is conceivable that in the embryo, similar to the neonatal mouse retina, rapidly expanding tissue outgrows its vascular support and becomes hypoxic, inducing VEGF expression. Consistent with this idea, the use of hypoxia probes has revealed that the brain is hypoxic during embryogenesis.[47] In some brain regions, hypoxia-probed labelled areas correspond to areas prominent in HIF1A—and VEGF-immunoreactivity.[48] However, it is unlikely that a hypoxia-controlled mechanism is sufficient to pattern the entire embryonic vasculature. For example, VEGF plays a crucial role very early during development to promote the propagation of the endothelial precursor lineage and in the formation of the first major vessels, the dorsal aorta and the cardinal veins. Yet, these processes occur at a stage when the embryo is still small enough for oxygenation to occur via diffusion from the vascularised yolk sac. In these situations, VEGF is expressed in stereotypical and discrete areas to stimulate reproducible vascular patterning events. These expression patterns are likely "hard-wired", i.e., controlled by hypoxia-independent pathways. Consistent with this notion, sonic hedgehog is secreted from the notochord in zebrafish to induce VEGF expression in adjacent somites,

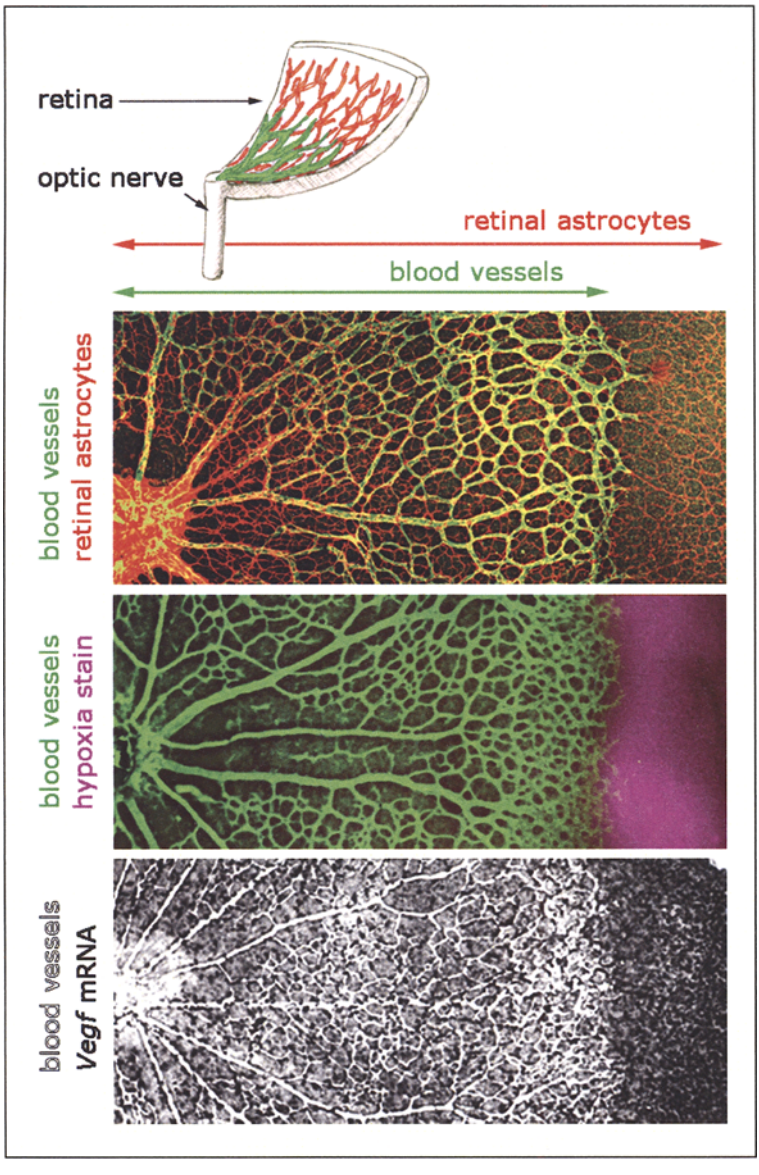

Figure 3. *VEGF* expression during retinal vascularisation. Astrocytes and blood vessels invade the newborn retina from the optic nerve (A). Retinal astrocytes serve as a template and VEGF source for the growing retinal vasculature (B). Astrocytes in the peripheral, not yet vascularized part of the retina experience hypoxia (C) and express *VEGF* at higher levels than astrocytes in the vascularized portion of the retina (D). The resulting VEGF gradient stimulates vessel growth towards the periphery.

which in turn leads to the formation of the dorsal aorta.[49] Moreover, during brainstem development, VEGF is expressed in stereotypical longitudinal stripes reminiscent of neurogenesis pattern well after the brain has assembled a vascular network to counteract the emergence of

hypoxia; these expression patterns likely reflect a role in the development of neurons (and glia), rather than a role in blood vessel growth.[50] The signalling pathways that control VEGF expression during vasculogenesis are only beginning to be understood (see Chapter by L.C5. Goldie, M.K. Nix and K.K. Hirschi), and the pathways controlling VEGF expression during neuronal development are not known at all.

Conclusions

VEGF is a versatile molecule with different functions in different settings. It acts as a survival factor, mitogen and guidance molecule for endothelial cells. It regulates angiogenesis in response to metabolic needs in the adult, but also participates in the formation of the vascular tree during embryogenesis. In addition, VEGF can regulate vessel permeability and plays a role in neuronal development and neuroprotection.[50-52] With such a varied array of biological functions it is not surprising that the regulation of *VEGF* gene expression is diverse. During development, hypoxia-independent pathways likely control VEGF expression to induce the formation of the stereotypically patterned elements of the vascular tree, whereas hypoxia-induced VEGF expression can fine tune the shape and density of capillary networks. Importantly, *VEGF* gene expression is controlled by a complex network of intersecting signalling pathways containing several dozen growth factors and cytokines, many of which induce VEGF expression in cancer cells.[53] Future anti-angiogenic cancer therapies attempting to manipulate VEGF expression will have to take this complexity into account, as cancer cells may achieve VEGF upregulation both through HIF-related mechanisms as well as hypoxia-independent pathways, which are normally used to control VEGF expression during development. The hardwired mechanisms of VEGF regulation are poorly understood and pose a major challenge for future research.

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