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Review

Role of VEGF in organogenesis

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The cardiovascular system, consisting of the heart, blood vessels and hematopoietic cells, is the first organ system to develop in vertebrates and is essential for providing oxygen and nutrients to the embryo and adult organs. Work done predominantly using the mouse and zebrafish as model systems has demonstrated that Vascular Endothelial Growth Factor (VEGF, also known as VEGFA) and its receptors KDR (FLK1/VEGFR2), FLT1 (VEGFR1), NRP1 and NRP2 play essential roles in many different aspects of cardiovascular development, including endothelial cell differentiation, migration and survival as well as heart formation and hematopoiesis. This review will summarize the approaches taken and conclusions reached in dissecting the role of VEGF signalling in vivo during the development of the early cardiovasculature and other organ systems. The VEGF-mediated assembly of a functional vasculature is also a prerequisite for the proper formation of other organs and for tissue homeostasis, because blood vessels deliver oxygen and nutrients and vascular endothelium provides inductive signals to other tissues. Particular emphasis will therefore be placed in this review on the cellular interactions between vascular endothelium and developing organ systems, in addition to a discussion of the role of VEGF in modulating the behavior of nonendothelial cell populations.

Introduction

VEGF is initially expressed at high levels in the yolk sac and in embryonic sites of vessel formation to support the assembly of a cardiovascular system. However, in the embryo, VEGF continues to be expressed after the onset of blood vessel formation, consistent with the idea that it is instructive not only for cardiovascular development, but also for the development of other organ systems. In the adult, VEGF expression becomes restricted to specialized cell types in organs containing fenestrated endothelium, for example the kidney and pituitary.[1] In addition, VEGF is upregulated to mediate physiological angiogenesis during menstruation, ovulation and in wound healing.[2] The expression of VEGF in adults is also induced by environmental stress caused by hypoxia, anemia, myocardial ischemia, and tumor progression to initiate neovascularization.[2]

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Hypoxia upregulates transcription of the gene encoding VEGF (*Vegfa*) by activating the hypoxia-inducible factors HIF1A and HIF2A.[3,4,100] Several major growth factors upregulate VEGF, including epidermal growth factor (EGF), insulin-like growth factor (IGF1), fibroblast growth factor 2 (FGF2; also known as basic FGF), fibroblast growth factor 7 (FGF7; formerly known as keratinocyte growth factor, KGF), platelet-derived growth factor (PDGF) and the tumor growth factors TGFA and TGFB (formerly known as TGF alpha and beta).[5] In addition, inflammatory cytokines and hormones have been shown to induce VEGF expression.[6,7]

The mouse VEGF gene (*Vegfa*) gives rise by alternative mRNA splicing and proteolytic processing to three major isoforms termed VEGF120, VEGF164 and VEGF188.[8,101] The corresponding human VEGF isoforms are one amino acid residue larger and therefore termed VEGF121, VEGF165 and VEGF189. In addition, a number of less abundant human isoforms have been described, including VEGF145 VEGF183, VEGF206. The VEGF isoforms differ not only in their molecular mass, but also in their solubility and receptor binding characteristics. VEGG188/VEGF189 and VEGF206 contain exons 6 and 7, which encode heparin-binding domains and mediate binding to heparan sulphate proteoglycan (HSPG) proteins in the extracellular matrix (ECM), and on the cell surface and ECM. VEGF120/VEGF121 lacks the domains encoded by exons 6 and 7 and is therefore the most diffusible isoform. VEGF164/VEGF165 contains the domain encoded by exon 7, but not exon 6, and accordingly has intermediate properties; 50-70% of this isoform remains associated with the cell surface and ECM. Due to the fact that these isoforms differ in their potential to bind to HSPGs, it is believed that they are differentially distributed in the environment of VEGF-secreting cells. The highest levels of VEGF188 are detected in organs that are vascularized initially by vasculogenesis (e.g., lung, heart and liver), while organs vascularized primarily by angiogenesis, including brain, eye, muscle and kidney have higher levels of VEGF164 and VEGF120.[9] The functional roles of these isoforms in organogenesis are described in more detail below.

Approaches to Study VEGF During Organogenesis

Exon 3 is contained in all known VEGF isoforms. The targeted deletion of a single Vegfa allele by neomycin gene insertion into this exon leads to embryonic lethality due to abnormal blood vessel and heart development between E11 and E12, demonstrating haploinsufficiency.[10,11] Using high G418 selection of the Vegfa+/- neomycin-resistant embryonic stem (ES) cells, Vegfa-/- ES cells could be produced, and these were used to make Vegfa-/- embryos by

tetraploid embryo-ES cell complementation approaches. The resultant Vegfa-/- embryos were even more severely compromised than Vegfa+/- embryos and died between E9.5 and E10 owing to severe defects in vasculogenesis, angiogenesis and heart development, which was accompanied by tissue necrosis/apoptosis.[10]

Given the relatively early mid-gestational lethality associated with targeted VEGF gene inactivation, alternative strategies have been employed to examine the role of VEGF during organogenesis and in the adult: VEGF function has been ablated at the protein level with sequestering antibodies, small molecule inhibitors that interfere with receptor tyrosine kinase signaling, or a soluble truncated chimeric VEGF receptor termed mFLT1(1-3)-IgG, which consists of the FLT1 extracellular domain fused to an IgG-Fc domain and sequesters VEGF protein with high affinity. Ubiquitous inducible ablation of the conditional VEGF allele or administration of mFLT1(1-3)-IgG causes lethality and severe multi-organ abnormalities in neonatal mice.[12] The defects in the lung, heart and kidney compromised the health status of these two types of mice severely, and the specific functions of VEGF in other organs were therefore difficult to deduce.

To achieve a partial loss of VEGF function, different conditional Vegfa alleles containing paired LoxP sites have been created, which can be targeted with CRE recombinase to impair VEGF expression in a spatiotemporally defined manner (Cre/LoxP technology). Two different Cre/LoxP approaches have been used. Firstly, mice in which exon 3 of VEGF is flanked by LoxP sites have been generated.[12] In these mice, CRE-mediated excision deletes exon 3, which is present in all VEGF isoforms, and this results in loss of VEGF production.[10,11] This conditional Vegfa allele can be used to delete VEGF in a cell/tissue specific manner when mated to mouse strains expressing CRE recombinase under an appropriate promoter. An alternative approach was taken to understand the individual roles of the main VEGF isoforms.[101] To create mice expressing only VEGF120, exons 6 and 7 of the Vegfa gene were flanked by LoxP sites and deleted in ES cells by CRE-mediated excision.[13] These targeted ES cells were then used to make mice expressing VEGF120, but not VEGF164 or VEGF188. To create complementary mice expressing VEGF164 or VEGF188 only, a knock-in approach was used: cDNA sequences corresponding to exons 4,5,7,8 (in the case of VEGF164) or 4-8 (in the case of VEGF188) were inserted into the genomic Vegfa locus to remove intervening intron sequences and therefore abolish alternative splicing.[14] Characterization of the phenotypical alterations present in the VEGF isoform-specific mice and the cell/tissue-specific VEGF mutants has contributed greatly to our understanding of VEGF function in organogenesis and in adult life.

Mice that express only the VEGF120 isoform (Vegfa120/120 mice) make normal overall VEGF levels, which permit the formation of sufficient numbers of endothelial cells and therefore vasculogenesis. Accordingly, these mice survive to birth. However, they do show defects in many organ systems, including the heart,[13,15] retina,[14,16,17] bone,[18,19] kidney[20] and lung.[9] Most Vegfa120/120 mice die neonatally due to impaired cardiac function, with only 0.5% of animals living to postnatal day 12.[13] An important consideration in interpreting the defects of Vegfa120/120 mice is that they do not only lack VEGF164 and VEGF188, but also make an increased amount of VEGF120 to compensate for the loss of the other two major splice forms. It has therefore been suggested that the relative balance of the different VEGF isoforms, rather than their absolute levels, is critical for angiogenic vessel patterning.[16] About half of the Vegfa188/188 mice die prenatally due to severe defects in aortic arch remodeling, and the surviving half show defects in artery development in the eye and defects in epiphyseal bone vascularization.[14,15,21] In contrast to Vegfa120/120 and Vegfa188/188 mice, mice that only express VEGF164 (Vegfa164/164) or compound Vegfa120/188 mice develop normally with no discernible phenotypes.[14,16]

The vascular phenotypes of VEGF isoform mutants are likely related to the differential localization of VEGF isoforms in the extracellular space, which provides a control point for regulating vascular branching morphogenesis.[16] Microvessels from mouse embryos expressing only the VEGF120 isoform have an abnormally large diameter and exhibit a decrease in branch formation, which is linked to a delocalization of secreted VEGF protein in the extracellular space. In contrast, mice expressing only the heparin-binding VEGF188 isoform exhibit ectopic vessel branches, which appear long and thin. Intriguingly, the vessel branching and morphogenesis defects of Vegfa120/120 and Vegfa188/188 mice are rescued in compound mutant offspring of these mice that express both the VEGF120 and VEGF188 isoforms, even though they lack VEGF164 (Vegfa120/188 mice). It therefore appears that the growing vasculature migrates along positional guidance cues provided by angiogenic VEGF gradients, which are normally composed of soluble and matrix-binding VEGF isoforms. This VEGF gradient can either be established by the coexpression of VEGF120 and VEGF188 or by VEGF164 alone, as VEGF164 is able to diffuse from the secreting cell, but also binds to and cooperatively signals with extracellular matrix molecules such as heparin sulfate proteoglycans. At the cellular level, VEGF gradients promote the formation of endothelial "tip-cells" at the front of growing vessels, which extend long filopodia presumably to seek out highest VEGF concentrations.[16,17]

Differential VEGF isoform expression does not only control developmental angiogenesis, but is likely to contribute also to physiological and pathological vessel growth in human adults. As an example, the different VEGF isoforms are differentially expressed in humans after submaximal exercise.[22] Moreover, a novel splice variant of VEGF165 termed VEGF165b may provide a natural inhibitor of VEGF165-induced angiogenesis, but is downregulated in renal cell carcinoma, possibly supporting the switch from an anti-angiogenic to a pro-angiogenic phenotype during tumor development.[23] It has recently been demonstrated that VEGF165, but not VEGF121 specifically interacts with β-amyloid plaques in Alzheimer's disease and may prevent β-amyloid- induced formation of neurotoxic reactive oxygen species.[24]

In order to increase our understanding of the individual roles of the three main VEGF isoforms, we have conditionally targeted each of these isoforms to the ubiquitously expressed Rosa26 locus, creating mice in which any one of the three isoforms can be selectively overexpressed in a cell type-specific fashion (Haigh et al., unpublished). Transgene expression is under the transcriptional control of the endogenous Rosa26 promoter, therefore Cre/LoxP mediated excision of the LoxP flanked stop cassette induces expression of each of the VEGF isoforms at the same level, and the activity of the individual isoforms can therefore be directly compared. We are presently dissecting the individual roles of each of these isoforms using several tissue-specific Cre lines to uncover novel roles for the VEGF isoforms in organogenesis and disease.

VEGF Signalling in the Developing Cardiovasculature

The phenotypes of the VEGF and VEGF receptor knockouts all demonstrate that VEGF signalling is critically important to promote the differentiation, proliferation, migration and survival of endothelial cells both during development in the adult. In addition, VEGF signalling plays several distinct roles during heart development. In the following paragraphs, we will first describe the cardiovascular phenotypes of the VEGF receptor mutants and then discuss in more detail the role of VEGF signalling in the nonendothelial cell types of the cardiovasculature and the hematopoetic system (Fig. 1).[100,102,103]

Formation of the cardiovasculature. Formation of blood vessels. Migrating mesodermal progenitors that are commonly referred to as hemangioblasts arise soon after gastrulation and the formation of the primitive streak.[25] They establish the blood islands of the extra-embryonic yolk sac, which consist of elongated peripheral endothelial precursor cells (angioblasts) that surround hematopoietic progenitors (hemoblasts). The angioblasts coalesce into primitive vascular channels in a process termed vasculogenesis.[25] Subsequently, the vascular endothelium within these early vessels proliferates and migrates to give rise to new vessel sprouts in a process termed angiogenesis. Vasculogenesis and angiogenesis are repeated in the embryo proper. Nascent vessels subsequently remodel and maturate, which involves the recruitment of pericytes or smooth muscle cells and the development of supporting basement membranes.[26]

Formation of hematopoetic stem cells. In mammals, yolk sac blood islands produce primitive erythrocytes and macrophages as well as progenitors that move on to populate an intra-embryonic region giving rise to definitive hematopoietic stem cells (HSCs); this region is known as the para-aortic splanchopleura (PAS) or aorta-gonads-mesonephros (AGM). The embryonic HSCs populate first the fetal liver and then the bone marrow, which, together with thymus and spleen, produces the hematopoietic cells of the eyrthroid, myeloid and lymphoid cell lineages in the adult.[27]

Formation of the heart. The development of the heart involves a complex series of finely orchestrated cellular and molecular interactions that commences just after gastrulation.[28] Initially, the primitive cardiac mesoderm ingresses along with other mesodermal and endodermal progenitors through the primitive streak and migrates to the anterior-most region of the embryo. Here, the mesodermal progenitors condense between embryonic day (E) 7.0 and 7.5 to form a crescent shaped epithelium often referred to as the cardiac crescent. The endocardium (future endothelium of the heart) invades the myocardium (the future muscle layer of the heart) and the sub-adjacent endoderm. From around E8.0, the bilateral cardiac progenitors coalesce at the ventral midline and fuse to form a linear heart tube consisting of an inner endocardial tube surrounded by a myocardial epithelium. Between E8.0 and E8.5, this linear heart tube begins a series of looping morphogenic events that sets the stage for the formation and positioning of the cardiac chambers that begin functioning to pump blood through the remodeling developing vasculature.

Requirements for VEGF receptors in the formation of a cardiovascular circuit. KDR (FLK1, VEGRF2). The essential role of VEGF signaling in the initial stages of cardiovascular development is underscored by the fact that KDR receptor null embryos, which contain the beta-galactosidase gene in frame with the ATG in exon 1, die at around E8.5, even earlier than *Vegfa-/-* embryos; death is the result of the failure of mesodermal progenitors to differentiate into vascular endothelium, endocardium and hematopoietic cells.[29] To better understand the role of KDR in vasculogenesis and hematopoiesis, the developmental potential of KDR null ES cells was studied in an ES cell-diploid embryo chimera approach.[30] These studies demonstrated that KDR signaling is essential for the migration of hemangioblasts from the primitive streak region of the embryo to the yolk sac, where they form blood islands. In vitro differentiation assays showed that KDR null ES cells can differentiate into hematopoietic and endothelial cells in vitro, although at a reduced frequency,[31] suggesting that the mesodermal progenitors of hematopoietic and endothelial cells do not require KDR signaling for their formation or differentiation, but rather for their migration and expansion.

FLT1 (VEGFR1). The role of FLT1 has been more controversial, as it has been debated whether this VEGF receptor acts as a major transducer of VEGF signals or instead acts as a decoy receptor that modulates the amount of VEGF available to KDR. Recent evidence indicates that the conflicting reports may be due, at least in part, to the fact that the functions and signaling properties of FLT1 differ depending on the developmental stage, cellular context, or alternative splicing. FLT1 can be produced as a full-length receptor with an intracellular tyrosine kinase domain or as a truncated receptor that is either membrane bound via its transmembrane domain or secreted. Mice that are homozygous null for FLT1 die between E8.5 and E9.0 as a result of increased and disorganized blood vessels.[32] This increased and aberrant blood vessel development is caused by an increased commitment of mesodermal progenitors to the endothelial/hematopoetic lineage, which has been postulated to result from excessive KDR signaling in the absence of FLT1.[33] Mice that lack the FLT1 tyrosine kinase domain, but express the extracellular and transmembrane domain of FLT1 develop a normal cardiovasculature, supporting the idea that during development FLT1 signaling is not essential and that the extracellular domain of FLT1 serves to modulate the amount of VEGF that binds to KDR.[34] More recently, mice have been made that lack both the tyrosine kinase domain and transmembrane domain of FLT1 and produce mainly the soluble FLT1 protein.[35] In these mice, VEGF is not recruited efficiently to the plasma membrane and KDR receptor activity is reduced, suggesting that the membrane fixation of FLT1 is essential to recruit VEGF for efficient KDR stimulation. Likely because KDR is necessary for the anterior migration of mesodermal prognitors of the cardiovasculature,[36] these mice suffer from embryonic lethality, particularly in a strain with high endogenous KDR levels.[35] However, a proportion of these mice survive embryonic development, which suggests that the endogenous soluble form of FLT1, termed sFLT1, is able to fulfill some functions encoded by the full-length gene. For example, it has been suggested that sFLT1 is critical to shape VEGF gradients in the environment of sprouting blood vessels.[37] In adults, FLT1 is thought to amplify VEGF-signaling through KDR by cooperative binding of VEGF and the other FLT1-specific ligand, placental growth factor (PGF; also known as PlGF). This signalling pathway has been suggested to operate in bone marrow-derived circulating endothelial progenitors, which play important roles in neoangiogenesis in the adult.[38]

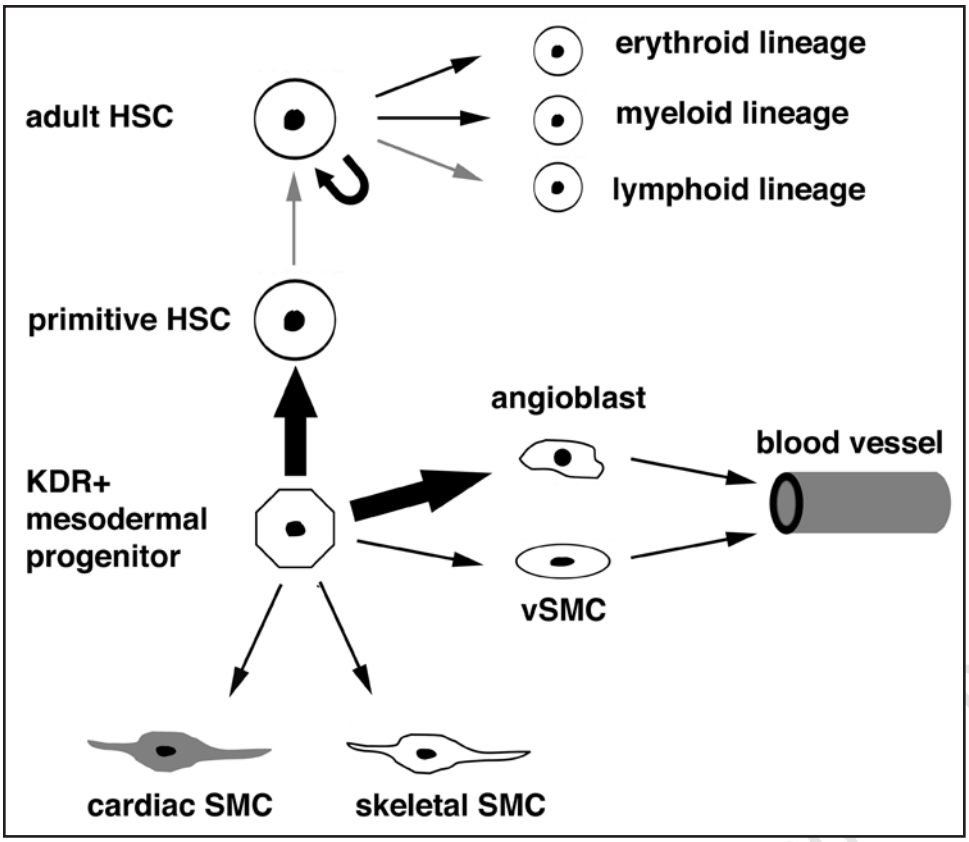

Figure 1. Role of VEGF in endothelial, hematopoetic and muscle differentiation. In the embryo, VEGF signalling promotes differentiation of a KDR+ progenitor cells into the endothelial and hematopoetic lineages. Differentiation decisions skewing progression into these lineages may depend on VEGF signalling acting in concert with transcriptional regulators. Accordingly, VEGF signalling together with TAL1 is thought to promote a hematopoietic/ endothelial cell fate develoment. VEGF plays an autocrine role in adult hematopoetic stem cells, promoting both survival and differentiation. Pathological over expression of VEGF may drive HSC differentiation towards the myeloid or erythroid lineages. VEGF also promotes vessel assembly by stimulating the migration of angioblasts and vascular smooth muscle cells, and by supporting vascular smooth muscle cell recruitment into nascent vessels. VEGF signaling may also promote the formation of two other muscle cell types, cardiac and skeletal muscle; in adults, VEGF supports aspects of muscle cell function, such as protection of muscle cells from ischemia, promoting muscle regeneration and regulating cardiac muscle contractility.

NRP1 and NRP2. The neuropilins NRP1 and NRP2 are transmembrane glycoproteins with adhesive properties that also function as axonal receptors for neuronal guidance cues of the semaphorin family and for specific VEGF isoforms on endothelial and tumor cells; in blood vessels, they are thought to amplify VEGF signaling through KDR.[39,40] Targeted deletion of NRP1 results in embryonic lethality around E12.5 due to defects in aortic arch remodeling, and impaired blood vessel invasion into the neural tube.[41] Compound homozygous null NRP1/NRP2 embryos die between E8.5 and E9.0 due to defective yolk sac vascularization and are intermediate in severity of cardiovascular defects relative to VEGF and KDR homozygous null embryos.[42] Mice that are either compound homozygous for NRP1 deficiency and heterozygous for NRP2 deficiency or vice versa die around E10.5, demonstrating gene dosage sensitivity as previously demonstrated for VEGF.[42]

Role of VEGF signalling in heart development. A small proportion of *Vegfa120/120* mice survive to term, but they then succumb to cardiac defects that resemble ischemic cardiomyopathy as a result of impaired myocardial angiogenesis.[13] The hearts of the *Vegfa120/120* mice are enlarged and show an irregular heartbeat with dysmorphic, weak contractions. To address the paracrine role of the myocardial VEGF source, mice carrying the conditionally targeted *Vegfa* allele[12] were crossed with the *MLC2v-Cre* transgenic line[43] to specifically delete VEGF in the ventricular myocardium.[44] The observed cardiac phenotypes were similar to the ones seen in *Vegfa120/120* mice, implying that the myocardium provides a critical source of VEGF to support the development of the coronary vasculature.

Earlier in development, VEGF is specifically expressed in the atrioventricular (AV) field of the heart tube, soon after the formation of the endocardial cushions that give rise to the heart septa and valves.[45] In support of the idea that VEGF levels are critical for this aspect of heart formation, two to three-fold ubiquitous over expression of VEGF impairs cardiac septation and leads to outflow tract abnormality combined with aberrant trabeculation and myocardial defects.[45] The induction of VEGF in myocardial cells with the tetracycline-inducible system has been used to demonstrate that VEGF can act as a negative regulator of endocardial-to-mesenchymal transformation, which is a critical step in the formation of the endocardial cushions; ischemia-induced VEGF up-regulation has therefore been proposed to play a role in congenital defects of heart septation.[46] *Vegfa120/120* and *Vegfa188/188* mice also display defects in aortic arch remodeling.[15] VEGF signaling through KDR and NRP1 appears to cooperate with plexin D1 and its semaphorin ligand SEMA3C to influence the remodeling of vessels in the developing cardiac outflow tract.[47,48]

Taken together, these studies on the role of VEGF suggest that altering the level of VEGF or the isoform expression profile disrupts vessel formation during heart development at multiple levels. Whilst it was originally thought that KDR is expressed predominantly in the endocardial cells, more recent studies suggest that VEGF/KDR signaling also influences muscle cells at times of cytotoxic stress in the adult, for example when cardiac muscle cells experience hypoxia.[49] Cell fate lineage-marking experiments with the *Rosa26-LacZ* reporter mouse strain and a *Cre* recombinase gene targeted to the *Kdr* locus have demonstrated that KDR+ progenitor cells give rise to cardiac muscle cells and also to skeletal muscle cells (Fig. 1).[50] However, the role of KDR-signaling in modulating the differentiation of KDR+ myogenic progenitor cell populations, in early morphogenic events during heart tube formation and in response to hypoxic stress remains to be genetically determined.

Role of VEGF in vascular smooth muscle cells. In vitro differentiation of embryonic stem (ES) cells has provided a valuable tool to elucidate the molecular mechanisms involved in cell fate differentiation decisions of KDR+ mesodermal progenitor populations.[102] In particular, ES cell differentiation has been used to identify clonal KDR+ hemangioblast cell populations that can give rise to both endothelial and hematopoietic progenitors.[51] More recently, it has been demonstrated that KDR+ cells derived from ES cells can differentiate into both endothelial and smooth muscle actin-positive cells, which assemble into blood vessels when cultured on an appropriate collagen matrix.[52] The addition of VEGF to these ES cell cultures enhanced the formation of endothelial cells, whereas the addition of PDGFBB enhanced the formation of smooth muscle cells. The ES-cell derived KDR+ cells could also give rise to both vascular endothelium and smooth muscle cells in xenograft models.[52] An ES cell-based study has recently looked at the combinatorial effects of KDR and the transcriptional regulator TAL1 during vascular and hematopoietic development. In this study, KDR+ mesodermal progenitor cells and hemangioblast colony forming cells gave rise to smooth muscle cells, and TAL1 inhibited the formation of smooth muscle cells; moreover, expression of TAL1 in a KDR null background rescued both endothelial and hematopoietic differentiation. This work along with other experiments has lead to a model in which KDR marks a mesodermal precursor cell population that forms KDR+ progeny, but can also give rise to non-KDR expressing cell types, such as smooth muscle, cardiac and skeletal muscle cells (Fig. 1).[53] Mesodermal cells that retain KDR expression have a strong potential to develop into vascular endothelial cells, or may adopt hematopoietic activity in the presence of transcriptional regulators such as TAL1 and RUNX1.[53] The presence of KDR on cultured vascular smooth muscle cells was suggested to stimulate their migration in response to VEGF.[54]

VEGF signaling in the hematopoietic system. As described above, VEGF signaling is essential during the initial stages of hematopoietic differentiation in the embryo. To determine the role of VEGF in adult hematopoietic stem cells (HSCs), conditional null VEGF transgenic mice were crossed to mice carrying a *Cre* recombinase transgene under the control of an alpha-interferon inducible promoter (*MX-Cre*).[55] This allowed the selective ablation of VEGF in bone marrow cell isolates and HSCs after administration of α-interferon to the culture medium. In these experiments, VEGF-deficient HSCs and bone marrow mononuclear cells failed to repopulate lethally irradiated hosts, despite the coadministration of a large excess of wild-type supporting cells. These findings demonstrated that a wild-type stromal cell microenvironment is insufficient to rescue engraftment of VEGF-deficient HSC in the bone marrow. VEGF therefore controls HSC survival during hematopoietic repopulation via an internal autocrine-loop mechanism (Fig. 1). VEGF-deficient bone marrow cells also failed to form colonies in vitro, but this defect was rescued by addition of VEGF to the culture medium. A similar rescue effect was observed in vivo, when VEGF-deficient HSCs were transduced with retroviral vectors expressing VEGF, as this restored their ability to repopulate lethally irradiated host mice.

The use of VEGF-selective small molecule receptor antagonists, which can enter the HSC, suggested that both KDR and FLT1 play a role in the VEGF repopulation potential of the HSC.[55] However, in another study it was found that adult mouse hematopoietic stem cells are normally KDR^{-} or KDR^{low}, and that KDR^{+} cells have no long-term reconstitution capacity.[56] These observations may be reconciled in a model in which KDR is essential for the development of HSCs during early embryonic development, but is redundant with FLT1 for HSCs in adult mouse bone marrow. In agreement with this idea, the enforced expression of PIGF in VEGF-deficient bone marrow cells revealed that activation of FLT1 is fully sufficient to rescue HSC survival in vitro and hematopoietic repopulation in vivo.[55] Further support for a model in which FLT1 is required during bone marrow repopulation was provided by experiments using monoclonal antibodies blocking murine FLT1.[57] In order to resolve the controversy surrounding the essential role of the VEGF receptors in HSC function, similar cell specific ablation experiments will need to be performed using conditional null receptor alleles. To determine the requirement for VEGF and its receptors in more mature hematopoietic cell populations, mice carrying conditional VEGF/VEGF-receptor alleles have to be mated to hematopoietic lineage-restricted *Cre* lines.

High levels of systemic VEGF can compromise the adult immune system (Fig. 1). For example, long-term continuous-infusion of recombinant VEGF protein or administration of adenoviral VEGF-expressing vectors to adult mice inhibits dendritic cell development, but increases the production of B cells and immature myeloid cells.[58] Moreover, continuous administration of recombinant VEGF or the ubiquitous induction of VEGF in adult mice at pathological levels mimics the profound thymic atrophy observed in tumor-bearing mice and inhibits the production of T-cells.[58,59] These effects may contribute to the ability of tumors to evade normal immune surveillance. The molecular mechanisms behind these adverse effects in the hematopoietic system remains poorly understood, underscoring the need to unravel the effects of VEGF signalling in the hematopoietic system.

VEGF and the Development of Endoderm-Derived Organs

The ability of vascular endothelium and therefore VEGF signaling to modulate the development of two endoderm-derived organs has been well documented: In two recent hallmark publications, the vascular endothelium was demonstrated to play an essential role in the induction of both liver and pancreas.[60,61]

Liver. In a novel liver primordial explant system, it was demonstrated that there is a failure of liver morphogenesis in KDR-deficient tissues, which lack endothelium, and it was concluded that vasculogenic endothelial cells/nascent blood vessels release critical mediators essential for the earliest stages of hepatogenesis.[60] The effects of VEGF on the liver may not be limited to liver endothelial cells, as there have been reports describing VEGF receptors on liver stellate cells,[62] which play a role in liver regeneration in response to injury. High levels of genetically induced or tumor-produced VEGF can change the architecture of the adult liver, resulting in a liver 'peliosis-like' phenotype that is characterized by enlarged hepatic sinusoids, blood pooling, detached sinusoidal endothelial cells and a total disruption of normal liver architecture.[59,63] This phenotype recapitulates the liver pathologies seen in some cancer patients with a high tumor burden. On the other hand, systemically administered VEGF at moderate levels can act on the sinusoidal vasculature (via FLT1) to enhance secretion of hepatocyte growth factor (HGF), which has a protective effect on hepatocytes under times of cytotoxic stress.[64]

Pancreas. In both frog and transgenic mouse models, blood vessel endothelium induces insulin expression in isolated endoderm; moreover, removal of the dorsal aorta prevents insulin expression in frogs, whilst expression of VEGF under the control of the *Pdx1* promoter causes ectopic vascularization in the posterior foregut, which was accompanied by ectopic insulin expression and islet hyperplasia.[61] These studies underscore the fact that blood vessels are not only essential for metabolic sustenance, but also provide inductive signals essential for organogenesis. The nature of these vessel-derived factors remains to be determined. Recently, VEGF has been conditionally deleted in the endocrine pancreas using a *Pdx1 Cre* line and in β-cells using the RIP *Cre* mouse line.[65,66] Neither approach overtly affected pancreatic islet formation, but affected the fine-tuning of blood glucose regulation.[66] However, the β-cell specific ablation of VEGF in the RIP-Tag2 model of tumorigenesis demonstrated the essential role of VEGF in the angiogenic switch necessary for neoplastic progression.[65]

Lung. That VEGF is an important growth factor during lung maturation was demonstrated by several different mouse mutants, including *Vegfa120/120* mice,[9] mice lacking the VEGF transcriptional regulator HIF2A,[67] and mice carrying a mutation in the hypoxia response element within the 5' UTR of the *Vegfa* gene.[68] The upregulation of the VEGF188 isoform is temporally and spatially associated with the maturation of alveolar epithelium in the lung. The phenotype of mice lacking VEGF188 (*Vegfa120/120* mice) suggested that this isoform is produced by the pulmonary epithelium to mediate the assembly and/or stabilization of the highly organized vessel network, which in turn is critical for alveolar development. Specifically, the lungs of *Vegfa120/120* mice are significantly less developed at birth when compared with to their control littermates, as they show a significant reduction in air space and capillaries; moreover, bronchioles and larger vessels are located unusually close to the pleural surface, pulmonary epithelium is less branched and the formation of both primary and secondary septa is reduced. Lungs from *Vegfa120/120* mice at postnatal day 6 showed similar defects, with retarded alveolarization and capillary growth. Administration of VEGF into the amniotic cavity of prematurely born mice can increase surfactant protein production of type 2 pneumocytes, suggesting that VEGF has therapeutic potential to encourage lung maturation in preterm infants.[67] On the other hand, the constitutive expression of VEGF during development increases the growth of pulmonary vessels, disrupts branching morphogenesis in the lung and inhibits of type I pneumocyte differentiation.[69] Inducible lung-specific VEGF expression in the neonate up till six weeks of age using a tetracycline inducible approach caused pulmonary hemorrhage, hemosiderosis, alveolar remodeling changes, and inflammation.[70] Using a similar VEGF inducible system in the lung with slightly later times of VEGF induction demonstrated that VEGF-induced lung inflammation resembles the lung pathology of patients with asthma, raising the possibility that VEGF inhibition in the lung may be of therapeutic benefit in the treatment of asthma or other helper T cell (TH-2) mediated inflammatory disorders elsewhere in the body.[71]

VEGF in Mesoderm-Derived Organs

Kidney. The main functional unit of the kidney is the glomerular filtration barrier consisting of visceral epithelial cells termed podocytes, fenestrated endothelial cells and an intervening glomerular basement membrane. This filtration barrier is responsible for the purification of the blood; it allows water and small solutes to pass freely into the urinary space, but ensures retention of critical blood proteins such as albumin and blood clotting factors. In the developing glomerulus, all three main VEGF isoforms are highly expressed in presumptive and mature podocytes.[72] Gene targeting in the mouse has demonstrated that VEGF is required for the development and maintenance of the glomerular filtration barrier. Podocyte-specific deletion of one conditional *Vegfa* allele develop symptoms that resemble glomerular endotheliosis, with excessive protein content in the urine.[73] Podocyte-specific deletion of both conditional *Vegfa* alleles leads to congenital nephropathy and perinatal lethality.[73] *Vegfa120/120* mice display smaller glomeruli and have fewer capillary loops.[20] Conversely, podocyte-specific over-expression of VEGF164 causes a phenotype that resembles HIV-associated collapsing glomerulopathy.[73] For a more detailed review of VEGF and its receptors in glomerular development and pathologies see the review article by Eremina and Quaggin.[74]

Skeletal Muscle. As mentioned above, cell fate lineage-marking experiments have demonstrated that KDR+ progenitor cells can give rise to skeletal muscle cells during development (Fig. 1).[50] In the adult, VEGF expression is markedly enhanced in regenerating muscle fibres,[75] and it is thought that VEGF may directly act on skeletal muscle cells.[76] KDR and FLT1 are both expressed by a myoblast cell line termed C2C12. Treatment of C2C12 cells with VEGF results in a significant induction of myoglobin mRNA, and VEGF-induced myoglobin mRNA expression is completely abolished when VEGF treatment was combined with administration of a VEGF-receptor tyrosine kinase inhibitor. In addition, ischemic muscles expressing exogenously administered VEGF vectors become deeply red in color, due predominately to upregulation of myoglobin expression in the skeletal muscle rather than increased vascularization or vascular leakage.[75] Given these intriguing findings, VEGF signaling may have a broader role in muscle cell biology than was originally assumed (Fig. 1). Given the fact that VEGF therapy is already being used in several clinical trials for treating hindlimb and cardiac ischemia with mixed degrees of success, more work needs to be done using in vivo models to elucidate the biological role of VEGF in muscle cell development and in pathological conditions.

Bone. During endochondral bone formation, the cartilage anlagen develop as an avascular tissue until around E14.5 in the mouse, when proliferating chondrocytes begin to hypertrophy and secrete pro-angiogenic factors such as VEGF.[77,78,104] The upregulation of VEGF correlates with the time of angiogenic invasion into the cartilage anlagen and sets in motion a complex developmental process, in which the cartilage is remodeled into trabecular bone. Inhibition of VEGF by administration of soluble chimeric VEGF receptor protein to 24-day-old mice was found to inhibit blood vessel invasion into the hypertrophic zone of long bone growth plates, impaired trabecular bone formation and caused an expansion of the hypertrophic zone.[77] Initial attempts to conditionally delete VEGF in the developing cartilage using a collagen-2 promoter-driven *Cre* gene resulted in similar phenotypes in heterozygous null mice; however the ectopic expression of CRE in the myocardium in this particular transgenic line compromised VEGF expression during heart development

and therefore caused embryonic lethality in homozygously targeted mice[78] (see above). More recently, a different *Cre* line, also driven by the collagen 2 promoter, was used to ablate the conditional VEGF allele specifically and selectively in cartilage; the resultant VEGF mutants lacked angiogenic invasion into the developing cartilage, and, in addition, showed extensive chondrocyte death.[79] This observation raised the possibility that VEGF promotes the survival and differentiation of chondrocytes firstly by attracting a blood supply, and secondly in a cell autonomous mechanism that operates independently of blood vessels. Analysis of *Vegfa120/120* mice indicated an essential role of the VEGF164 and VEGF188 isoforms in the initial invasion and remodeling of growth plate during bone formation.[18,19] *Vegfa188/188* mice, on the other hand, display knee joint dysplasia and dwarfism due to disrupted development of the growth plates and secondary ossification centers. At the cellular level, this phenotype is in part caused by impaired vascularization of the epiphysis, which increases hypoxia and therefore causes massive chondrocyte death in the interior of the epiphyseal cartilage. The VEGF188 isoform alone is not only inefficient in promoting epiphyseal vascularization, but is also insufficient to regulate chondrocyte proliferation and survival responses to hypoxia.[21] Taken together, these studies suggest that VEGF regulates the differentiation of hypertrophic chondrocytes, osteoblasts and osteoclasts. However, the VEGF receptors that regulate bone cell responses to VEGF remain to be identified.

VEGF in Ectoderm-Derived Organs (Skin and Nervous System)

Skin. The main source of VEGF production in the skin is the epidermis, consisting of keratinocytes.[80] In order to determine the role of keratinocyte-derived VEGF in skin development as well as in adult skin function and pathologies, the conditional *Vegfa* allele has been inactivated in epidermal keratinocytes with the *K5 Cre* mouse line, in which CRE recombinase expression is driven by the keratin 5 promoter.[12,81] These skin-specific VEGF mutant mice formed a normal skin capillary system and displayed normal skin and hair development, demonstrating that keratinocyte-derived VEGF is not essential for skin development. However, healing of full-thickness skin wounds in adult animals lacking epidermal VEGF expression was delayed, and these animals showed a marked resistance to skin carcinoma formation induced by chemical or genetic means.[81] Vice versa, overexpression of VEGF in epidermal keratinocytes under the control of the keratin 5 regulatory sequences increased susceptibility to chemically induced carcinogenesis.[82] Other model systems relying on the transgenic delivery of VEGF to the skin have resulted in an inflammatory skin condition with many of the cellular and molecular features of psoriasis.[83] These studies have underscored the contribution of VEGF signalling to several skin pathologies.

Nervous system. There are several intriguing similarities between the nervous system and the vasculature. For example, both organ systems form dense networks throughout the entire body and, in both cases, initially primitive networks undergo remodeling and maturation processes to refine their hierarchical organization and integration with other tissues; moreover, both organ systems use signalling ligands and receptors from shared protein families.[84,85] Of particular interest is the presence of NRP1 on both neurons and endothelial cells, as it plays a pivotal role in neuronal guidance by acting as a semaphorin receptor and also acts as a cofactor for VEGF164-mediated KDR signaling. More recently, the role of semaphorins in the vasculature and of VEGF in the nervous system has become the focus of much research activity. For example, the administration of VEGF to the developing CNS improved intraneural angiogenesis, which in turn promoted neurogenesis, and the application of VEGF to experimental models of nerve injury enhanced nerve regeneration after axotomy.[86,87] Several in vitro experiments demonstrated more direct effects of VEGF in several aspects of neuronal biology, consistent with a model of a direct autocrine effect of VEGF on neurons and glial cells. For example, VEGF was shown to rescue neuronal cultures from ischemic and glutamate induced neurotoxicity and to act like a neurotrophic factor in stimulating axonal outgrowth and glial growth.[88,89]

VEGF/KDR signalling has been targeted in neural progenitor cells and their progeny with a nestin promoter-driven *Cre* recombinase transgene to inactivate a conditional null *Vegfa* allele[12] in combination with a hypomorphic *Vegfa* allele,[90] as well as inactivating a conditional null *Kdr* allele.[91] Mice with intermediate levels of VEGF activity showed decreased blood vessel branching and a reduced vascular density in the cortex and retina, and this impaired the structural organization of the cortex and retinal thickening.[91] Severe reductions in VEGF levels decreased vascularity severely and resulted in hypoxia, which led to the degeneration of the cerebral cortex and caused neonatal lethality.[91,92] In contrast, the deletion of KDR in neurons did not obviously impair neuronal development, suggesting that the major developmental role of VEGF in the nervous system is that of providing a paracrine factor for the vasculature. However, VEGF164 does control specific aspects of neuronal patterning, as it is essential for the migration of facial branchiomotor neuron cell bodies.[93] Interestingly, initial experiments suggest that NRP1, rather than a NRP1/KDR complex, controls the VEGF response in this neuronal migration pathway.[105]

Despite the lack of an obvious neuronal phenotype of mice specifically lacking KDR-expression in the nervous system, we have recently isolated definitive neuronal stem cells (dNSCs) from embryonic and adult brains of these mice and cultured these cells in vitro to produce neurospheres. Interestingly, dNSCs isolated from the KDR null mutant brains showed a 50% reduction in the capacity to form neurospheres and a dramatic increase in the number of dNSCs undergoing apoptosis.[94] These results imply that in an environment where growth factors are limited, KDR plays a cell autonomous role in NSC survival that is normally overcome in vivo, i.e. in the brains of mice lacking KDR.[91] Further support for an important role of environmental issues in VEGF signaling and neuronal function has come from studies that have demonstrated that hippocampal sources of VEGF-A may link neurogenesis with enhanced learning and memory potential.[95] In this study, enriched external environments result in increased hippocampal VEGF expression, increased neurogenesis and improved cognitive performance. Exogenously administered retroviral sources of VEGF alone caused similar affects on learning and memory. This is mediated in part by direct signaling through KDR present on rat hippocampal neurons.

From a clinical perspective, altered VEGF levels and vascular development have been associated with neurodegenerative disorders, ischemic cerebral and spinal cord injury, and diabetic and ischemic neuropathy.[96,97] VEGF has also recently been implicated in playing

a causal role in the pathogenesis of amyotrophic lateral sclerosis (ALS).[68] In a mouse model of ALS, injections of lentiviral vectors expressing VEGF into various muscles delayed disease onset and disease progression and extended the survival time by 30% in ALS prone mice.[98] VEGF is thought to modulate neuronal development and function indirectly through its paracrine effects on the vasculature, which provides essential neurotrophic support during nervous system development and homeostasis. More recently, it has been demonstrated that transgenic expression of KDR on motor neurons prolonged the survival of mice that are genetically prone to develop ALS.[99] For a more extensive review on the role of VEGF signaling in the nervous system.[105]

Future Perspectives

The phenotypical consequences of altering the overall levels of VEGF and/or its isoforms have made it very clear that this growth factor plays many diverse and essential roles in normal organogenesis beyond its initial role in the establishment of the cardiovasculature. It is therefore not surprising that aberrant VEGF signaling has been implicated in various human pathological conditions, including the growth and metastasis of tumors, deregulated immune cell surveillance, intraocular neovascular syndromes, enhanced inflammatory responses in arthritis and brain edema, as well as neurological disorders such as cerebral and spinal trauma, ischemic and diabetic neuropathy and amyotrophic lateral sclerosis. A better understanding of the physiological role of VEGF signaling during normal organogenesis will undoubtedly lead to novel insights into the pathenogenesis of these diseases, and it may even lead to novel therapeutic approaches to combat these life threatening and debilitating diseases. The multitude of phenotypes that have been documented as a result of altering VEGF levels in the mouse have already provided a great deal of insight into several human pathologies ranging from cancer progression to amyotrophic lateral sclerosis. Some of the immediate challenges for the future include: (1) to obtain a better understanding of the molecular mechanism of VEGF signaling plays in vascular endothelium and hematopoietic cells (i.e. identification of direct signaling targets, transcriptional modulators and target genes); and (2) to understand the role of VEGF in modulating nonendothelial/nonhematopoietic cell types (i.e., through the analysis of cell/tissue specific knock-outs of VEGF receptors). More long-term challenges include the molecular characterization of signaling pathways that can modulate the activity of VEGF and the development of effective therapeutic approaches to modulate VEGF signaling in human diseases.

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