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The discovery of the fundamental role of VEGF in the development of the vascular system

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ABSTRACT

In the 1980', vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF) was identified and shown to be a key regulator of endothelial cell proliferation and migration, and vascular permeability. The essential role of VEGF and of VEGF receptors (VEGFRs) for the development of the embryonic vascular system has been established via gene disruption studies. This article summarizes the fundamental contributions that have been made in the early days of angiogenesis research to define the involvement of VEGF/VEGFRs in the developing vasculature.

1. Background

In 1979, Harold D. Dvorak (Fig. 1), working at Harvard Medical School, Boston, testing cell-free supernatants from a variety of human and animal tumor cells in the Miles assay, found that supernatants from nearly all of them generated an intense blue spot due to extravasated Evans blue, whereas those from several normal cells did not (Dvorak et al., 1979). Dvorak called this tumor supernatant permeabilizing activity vascular permeability factor (VPF), with a potency some 50,000 times that of histamine (Dvorak et al., 1992; Senger et al., 1983). However, no amino acid sequence data were obtained, precluding the establishment of the identity of VPF at that time and the affinity of VPF for heparin was substantially lower than that of other typical heparin-binding growth factors, such as basic fibroblast growth factor (bFGF). Senger et al. (Senger et al., 1983) described the partial purification from the conditioned medium of a guinea pig tumor cell line of a protein able to induce vascular leakage in the skin.

Both Napoleone Ferrara (Fig. 2) and Jean Plouet were working as post-doc in Denis Gospodarowicz lab in San Francisco, were the initial discovery of vascular endothelial growth factor (VEGF) was made. Napoleone was working on bovine pituitary folliculo-stellate cells, whereas Jean was working on the AtT-20 bovine cells (Plouet et al., 1989). Napoleone and Jean left Gospodarowicz's lab and continued the work in Genentech Inc., San Francisco, and respectively, in France.

In 1989, Ferrara and William J. Henzel, reported the isolation of a diffusible endothelial cell-specific mitogen from medium conditioned by bovine pituitary follicular cells, which they named VEGF to reflect the restricted target cell specificity of this molecule (Fig. 3). NH_2-terminal amino acid sequencing of purified VEGF proved that this protein was distinct from the known endothelial cell mitogens such as acidic and basic fibroblast growth factors (aFGF and bFGF) and indeed did not match any known protein in available databases (Ferrara and Henzel, 1989). As Ferrara said: “In 1989, we reported the isolation and cloning of a heparin-binding endothelial cell mitogen. This project began while I was a postdoctoral fellow at the University of California, San Francisco in the mid1980s. At that time, I was able to isolate and culture a population of non-hormone secreting cells from bovine pituitary, termed ‘follicular’ or ‘folliculo-stellate’ cells. Earlier investigators noted that they establish intimate contacts with the pituitary perivascular spaces, suggesting a role in the development or maintenance of the pituitary vasculature. In the course of these studies, I discovered that follicular cells release in their culture supernatants an endothelial cell mitogen” (Ferrara, 2016).

In 1989, Connolly and co-workers at Monsanto Company showed that VPF is an endothelial mitogen in vitro and an angiogenic factor in vivo and reported the isolation and sequencing of human VPF from U937 cells (Connolly et al., 1989; Keck et al., 1989); Ferrara reported the isolation of cDNA clones for bovine $VEGF_{164}$ and three human VEGF isoforms: $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ (Leung et al., 1989). These works demonstrated that VEGF and VPF are the same molecule. As Ferrara said: “In 1988, I joined Genentech, where I had the opportunity to pursue the isolation of this mitogen. By early 1989, we were able to determine the amino terminal amino acid sequence of the purified protein. We found that this sequence was unique, since it had no match with known sequences in available databases. Because this molecule appeared to have growth-promoting activity selectively for vascular

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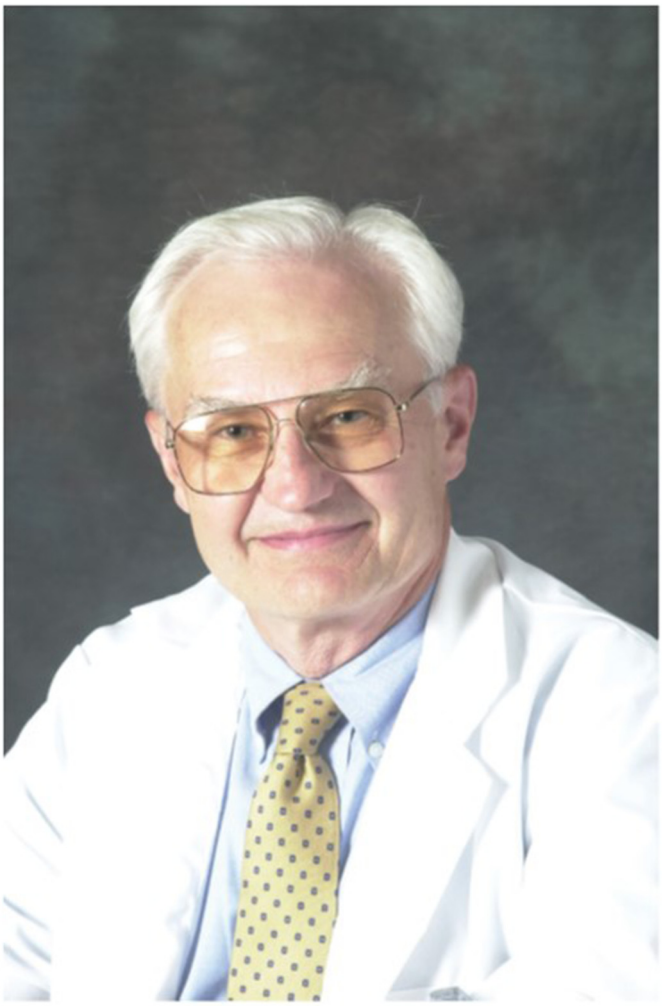

Fig. 1. A port-trait of Harlod F. Dvorak.
(Reproduced from Ribatti, 2007)

Fig. 2. A port-trait of Napoleone Ferrara in his office at the Genentech in San Francisco, California.
(Reproduced from Ribatti, 2011)

endothelial cells, we proposed the name 'vascular endothelial growth factor'. After our paper was accepted for publication, we learned that a group at the Monsanto Company at approximately the same time a

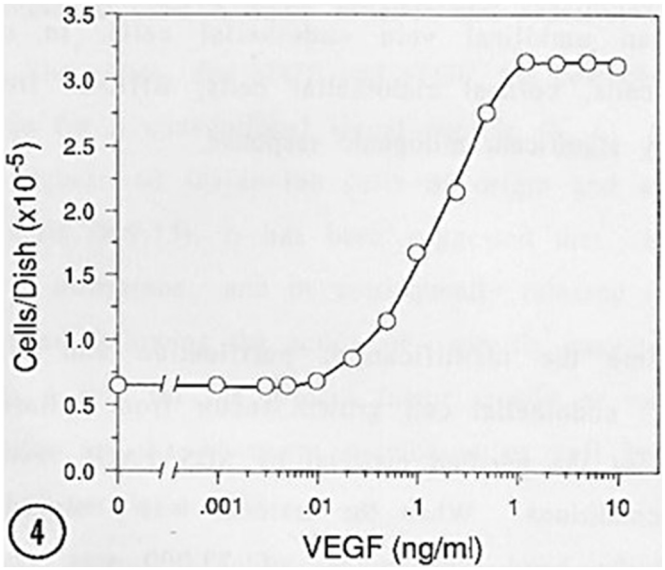

Fig. 3. Dose responsive growth of adrenal-cortex derived capillary endothelial cells in the presence of purified VEGF.
(Reproduced from Ferrara and Henzel, 1989)

manuscript reporting on the cloning of VPF. These investigators described a human clone that encoded a protein identical to VEGF. This group followed up on the earlier work by Senger et al. and was able to isolate and sequence VPF. Therefore, it appeared that the same molecule possesses mitogenic and permeability enhancing activities." (Ferrara, 2016).

2. Role of VEGF and VEGFRs in embryonic vasculogenesis and angiogenesis

Formation of the cardiovascular system is one of the earliest events that occurs during embryonic development. In 1996, Ferrara's laboratory (Ferrara et al., 1996) and a collaborative effort between Peter Carmeliet (Fig. 4) in Leuven, Werner Risau (Fig. 5) in Martinsried and then at the "Max Planck Institute" in Bad Nauheim where he was the director, and Andreas Nagy in Toronto (Carmeliet et al., 1996) demonstrated an essential role of VEGF in embryonic vasculogenesis and angiogenesis in the mouse. Inactivation of a single VEGF allele resulted in embryonic lethality between day 11 and 12. The VEGF +/− embryos exhibited a number of developmental anomalies. The forebrain region appeared significantly underdeveloped. In the heart, the outflow region was grossly malformed; the dorsal aortas were rudimentary, and the thickness of the ventricular wall was markedly decreased. The yolk sac

Fig. 4. A port-trait of Peter Carmeliet.

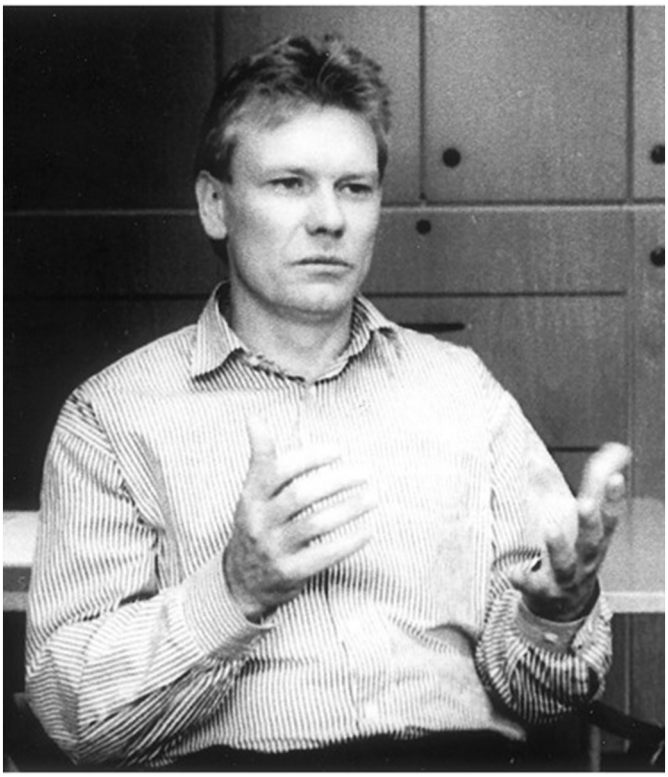

Fig. 5. A port-trait of Verner Risau.
(Reproduced from Hansson et al., 1999)

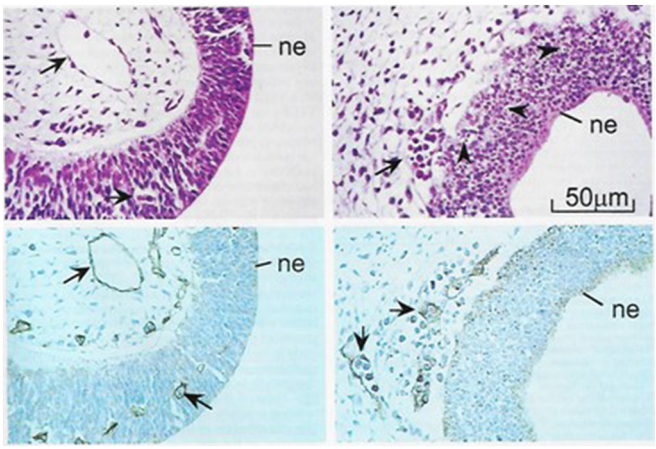

Fig. 6. Hematoxylin and eosin staining (upper panels) and CD34 immunostaining (lower panels) on sections of neuroepithelium (ne) from wild type (left) and VEGF+/− (right) mouse embryos. Arrows indicate blood vessels. Blood vessel lumina can be identified in the mesenchyme adjacent to the ne in both groups. However, they are absent within the ne of the heterozygous embryos. Note also the presence of apoptotic cells in the ne of the heterozygous embryos. This contrasts with the well differentiated and vascularized ne in the wild type.
(Reproduced from Ferrara et al., 1996)

revealed a substantially reduced number of nucleated red blood cells within the blood islands, indicating that VEGF regulated both vasculogenesis and hematopoiesis. Also, the vitelline veins failed to fuse within the vascular plexus of the yolk sac. Significant defects in the vasculature of other tissues, including placenta and nervous system, was evidenced. In the nervous system of heterozygous embryos at day 10.5, vascular elements could be demonstrated in the mesenchyme, but not in neuroepithelium and the failure of blood vessels ingrowth was accompanied by apoptosis and disorganization of neuroepithelial cells (Fig. 6).

Risau and co-workers found that the spatial and the temporal expression pattern of VEGF mRNA correlated with angiogenesis during embryonic development in the mouse brain (Breier et al., 1992). VEGF mRNA was expressed in the ventricular layer of the developing neuroectoderm, and a concentration gradient of the diffusible VEGF isoforms declining from the ventricular layer towards the perineural vascular plexus causes the ingrowth of capillaries from the vascular plexus towards the angiogenic stimulus provided by VEGF secreting ventricular epithelial cells (Breier et al., 1992).

Flk-1/VEGFR-2 is the first endothelial receptor tyrosine kinase known to be expressed on endothelial cell precursors and plays a central role in regulating embryonic vascular development and tumor angiogenesis. Lineage-tracing experiments demonstrated the presence at the ventral margin of the embryo at 1 h post-gastrulation of a labeled population consisting exclusively of hematopoietic (GATA-1 positive) and endothelial (flk-1/VEGFR-2 positive) lineages (Vogeli et al., 2006). Mice deficient in flk-1/VEGFR-2 died in utero between days 8.5 and 9.5, as a result of an early defect in the development of hematopoietic and endothelial cells and a complete lack of vasculature (Shalaby et al., 1995). These mice fail to develop yolk sac vasculature, indicating that flk-1/VEGFR-2 plays an essential role in survival/growth and differentiation of endothelial cell progenitors.

Risau and co-workers isolated flk-1/VEGFR-2 from proliferating endothelial cells of post-natal mouse brain, when there was maximal endothelial cell proliferation (Millauer, 1993). This VEGF receptor was restricted to capillaries and blood vessels, and when the first vascular sprouts begin to radially invade the neuroectoderm form the perineural plexus, expression of flk-1/VEGFR-2 was high in the perineural vascular plexus and in invading vascular sprouts (Millauer, 1993). In the adult brain, when angiogenesis has ceased, flk-1/VEGFR-2 expression was very low and restricted to the choroid plexus endothelial cells (Kremer et al., 1997). Risau and co-workers (Kabrun et al., 1997) demonstrated that flk-1/VEGFR-2 provides a marker for the earliest detectable hematopoietic precursors. RNA analysis indicated that it was expressed in the yolk sac at day 10–12 of gestation, in the liver throughout fetal life and in embryoid bodies. Moreover, Risau and co-workers (Kappel et al., 2000; Rönicke et al., 1996) performed a functional analysis of the flk-1/VEGFR-2 promoter in vivo demonstrating that flk-1 5′-flanking sequences confer endothelium-specific expression in transfected endothelial cells, and identified by mutational analysis binding sites for SCL/Tal-1, GATA, and Ets transcription factors as critical elements for the endothelium-specific flk-1/VEGFR-2 gene expression in transgenic mice, providing the first evidence that SCL/Tal-1, GATA, and Ets transcription factors act up-stream of flk-1/VEGFR-2 in a combinatorial fashion to determine embryonic blood vessel formation. SCL/Tal-1 activates VEGF expression in the endoderm and mesoderm. In response, angioblasts upregulate the expression of surface flk-1/VEGFR-2 and migrate from the splanchnopleuric mesoderm and differentiate in response to paracrine fibroblast growth factor-2 (FGF-2) and VEGF signaling (Coultas et al., 2005). The loss of both vascular and hematopoietic cells in flk-1/VEGFR-2 mutants, suggests that endothelial and hematopoietic development are linked through a common progenitor, the hemangioblast.

Genetic loss of flt-1/VEGFR-1 leads to vessel dys-morphogenesis and overgrowth (Fong et al., 1995; Kearney, 2002). Fong et al. (Fong et al., 1995) showed that Flt-1/VEGFR-1 null mutant mice die in utero due to an overgrowth of endothelial cells and disorganization of blood vessels, suggesting that Flt-1/VEGFR-1 has a negative regulatory role in vascular development. However, mice expressing the VEGFR-1 extracellular and transmembrane domains, but lacking the tyrosine kinase domain develop a normal vasculature (Hiratsuka et al., 1998).

Chappell et al. (Chappell et al., 2009) demonstrated that analysis of developing vessel with perturbed flt-1/VEGFR-1 function revealed misguided emerging sprouts and transgenic soluble flt-1 (sFlt-1)

Fig. 7. VEGF/VEGFRs in the development of the vascular system: milestones.

rescued sprout guidance parameters. Studies using receptor-selective VEGF mutants demonstrated that flt/VEGFR-1 cooperates with flk-1/VEGFR-2 in inducing gene expression in endothelial cells in vitro, although no unique expression pattern was observed in response to flt/VEGFR-1 alone (Yang et al., 2002).

More recent advances have introduced the concept of functional specialization of endothelial cells during the sprouting process in the so-called endothelial tip cells distinct from the endothelial stalk cells, and have highlighted important roles for components of the Notch and Wnt signaling in the regulation of sprouting angiogenesis. VEGF promotes endothelial motility, filopodia extension and proliferation, and under the control of Notch signaling, whether endothelial cells become tip cells or stalk cells. Tip cell migration depends on a gradient of VEGF, whereas stalk cell proliferation is regulated by VEGF concentration (Gerhardt et al., 2003). The leading tip cell responds to a VEGF gradient by migrating outward from the parent vessel up the gradient (Ruhrberg, 2002). VEGF induces the formation and extension of filopodia as well as the expression of Dll4 protein in the tip cells, and filopodia engage with those of a nearby tip cell to form a “bridge” and the formation of a new vessel (Bentley et al., 2009). As the vessel elongates, the stalk cell proliferate in response to VEGF, creates a lumen, synthesizes a basement membrane and associates with pericytes, and increases the mass and surface of the growing vessel. VEGF stimulates tip cell induction and filopodia formation via flk-1/VEGFR-2 which is abundant on filopodia, whereas flk-1/VEGFR-2 blockade is associated with sprouting defects (Phng and Gerhardt, 2009). Moreover, activation of Cdc 42 by VEGF triggers filopodia formation (De Smet et al., 2009).

flt/VEGFR-1 expression is induced by Notch signaling to reduce VEGF ligand availability, preventing tip cell outward migration. flt/VEGFR-1 is predominantly expressed in stalk cells, is involved in guidance and limiting tip cell formation, and loss of flt/VEGFR-1 increases sprouting and vascularization (Chappell and Bautch, 2010; Chappell et al., 2009). During both mouse and zebrafish angiogenesis, VEGFR-3 is most strongly expressed in the leading tip cell and is down-regulated by Notch signaling in the stalk cell (Shawber et al., 2007; Siekmann and Lawson, 2007).

The work from Kari Alitalo (Fig. 7) lab in Helsinki, was central in the characterization of VEGF-C/VEGFR-3 restricted largely to the lymphatic endothelium (Kukk et al., 1996; Ribatti, 2018). VEGFR-3 may play a role in disorders involving the lymphatic system and angiogenesis and may be of potential use in drug targeting, in vivo imaging of the lymphatic vessels and in therapeutic lymphangiogenesis. VEGF-C binds to VEGFR-3, expressed on lymphatic endothelium, and has been implicated in lymphangiogenesis. Overexpression of VEGF-C and VEGF-D in transgenic mice induces the formation of hyperplastic lymphatic vessels. Conversely, VEGFR-3 gene inactivation results in embryonic death due to abnormal remodeling of the primary vascular plexus (Dumont, 1998) and inhibition of VEGF-C and/or VEGF-D by overexpression of a soluble form of VEGFR-3 in the skin of transgenic mice leads to inhibition of lymphatic vessel growth (Jussila and Alitalo, 2002). Transgenic inactivation of both VEGF-C alleles results in prenatal death: endothelial cells commit to the lymphatic lineage, but do not sprout from veins (Karkkainen et al., 2004).

3. Concluding remarks

VEGF was identified and isolated as an endothelial-specific mitogen that has the capacity to induce physiological and pathological angiogenesis . Owing to the absolute requirement for VEGF during embryogenesis, several studies have focused on elucidating the physiological requirement for VEGF signaling pathways in early vascular development (Fig. 8). Moreover, research on developmental VEGF signaling

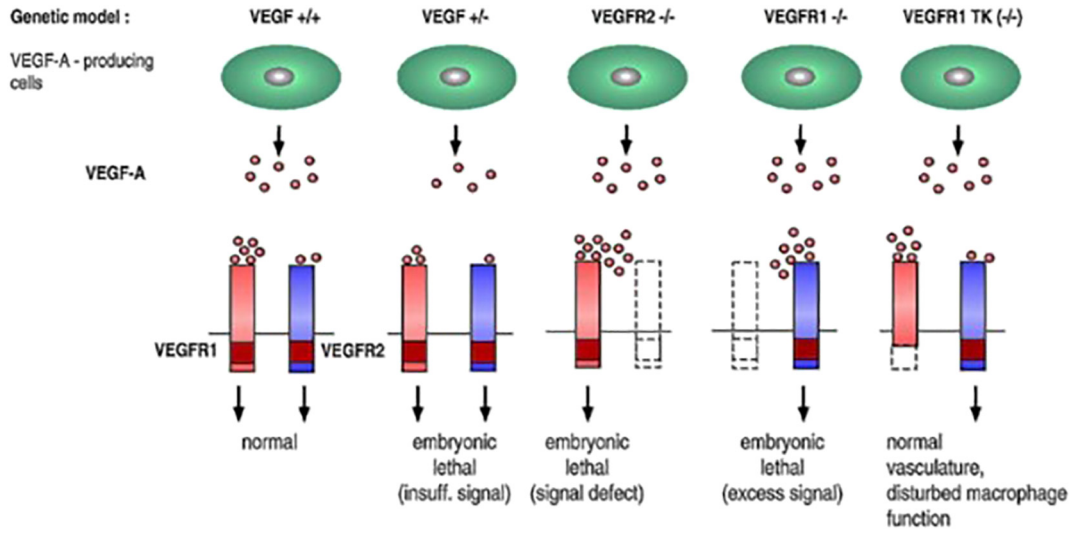

Fig. 8. Genetic models indicate negative regulatory role of VEGFR-1 during embryogenesis. The different genetic models (VEGF + / +, VEGF + /etc. are indicated at the top) and their effects on VEGF-A production (indicated by red circles) or VEGFR expression, are outlined. VEGFR-1 is indicated in red andVEGFR-2 in blue. Unfilled symbols with broken lines indicate elimination by gene targeting. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
(Reproduced from Shibuya and Claessonwelsh, 2006)

pathways has improved our knowledge of neoangiogenesis in the adult in both physiological and pathological conditions. Ferrara reported that inhibition of VEGF-induced angiogenesis by specific monoclonal antibodies resulted in a dramatic suppression of the growth of a variety of tumors *in vivo*. These findings provided the first direct evidence that inhibition of angiogenesis may suppress tumor growth and blocking VEGF action could have therapeutic value for a variety of malignancies (Presta et al., 1997). Due to its central role in tumor angiogenesis, the VEGF/VEGFRs pathway has been a major focus of basic research and drug development in the field of oncology. The first antiangiogenic agent approved by the FDA was bevacizumab (Avastin, Genentech Inc.), a humanized version of an anti-VEGF monoclonal antibody used in early proof of concept studies (Kim et al., 1993; Presta et al., 1997).

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