Functional Analysis of VEGF-B and VEGF-C

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Abbreviations

α-, β-MHC	alpha- and beta-myosin heavy chain
aa	amino acid(s)
AcMNPV	Autographa californica multiply embedded nuclear polyhedrosis virus
aFGF	acidic fibroblast growth factor
AMD	age-related macular degeneration
AMP	adenosine monophosphate
AP-1, -2	activator proteins 1 and 2
βARK	β-adrenergic receptor kinase
BBB	blood-brain barrier
bFGF	basic fibroblast growth factor
BM	basement membrane
bp	base pair(s)
BR3P	Balbiani ring 3 protein
BrdU	Bromodeoxyuridine
BSA	bovine spongiforme
CAM	chorioallantoic membrane
cAMP	cyclic AMP
cDNA	complementary DNA
CIP	calf intestinal phosphatase
CK52	cytokeratin 52 (K14)
CL	corpus luteum
CNP	C-type natriuretic peptide
CSF-1	colony-stimulating factor 1 (M-CSF)
E	embryonic day
EC	endothelial cell
ECM	extracellular matrix
EDRF	endothelium-derived relaxing factor (nitric oxide)
EGF	epidermal growth factor
Epo	erythropoetin
EST	expressed sequence tag
FC	follicular/folliculostellate cells
FCS	fetal calf serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FLK	fetal liver kinase
FLT1	fms-like tyrosine kinase 1 (VEGFR-1)
FLT2, FLT3	fms-like tyrosine kinase 2 and 3
FLT4	fms-like tyrosine kinase 4 (VEGFR-3)
FTU	full transcriptional unit
G-CSF	granulocyte colony-stimulating factor
GAP	GTPase-activating protein
GDP	guanosine-5'-diphosphate
GRB2	growth factor receptor-bound protein 2
GTP	guanosine-5'-triphosphate
H_6	histidine tag
HEL	human erythroleukemia
HEV	high endothelial venule
HF	high five
HGF	hepatocyte growth factor
HIF-1	hypoxia-inducable factor one
HSPG	heparan sulfate proteoglycan
HUVEC	human umbilical vein endothelial cell
IFN_α	interferon alpha
Ig	immunoglobulin
IGF-1	insulin-like growth factor 1
IL	interleukin
IP	immunoprecipitation
IPTG	Isopropyl-β-D-thiogalactopyranosid
K14	keratin 14 (CK52)
kb	Kilobase
KDa	Kilodalton
KDR	kinase insert-domain-containing receptor (VEGFR-2)
KGF	keratiocyte growth factor
LH	luteotrophic hormone
M-CSF	macrophage colony-stimulating factor (CFS-1)
MAP	mitogen-activated protein
NGF	nerve growth factor
o/n	over night
p.c.	post coitum
p.i.	post infection
PA-1	plasminogen activator inhibitor 1
PAE	porcine aortic endothelial
PAF	platelet-activating factor
PAGE	polyacrylamid gelelectrophoresis

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD-ECGF	platelet-derived endothelial cell growth factor
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PECAM-1	platelet-endothelial cell adhesion molecule
PF4	platelet factor 4
PGE	prostaglandin
PH	pleckstrin homology
PI3-K	phosphatidylinositol-3-OH kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC-γ_1	phospholipase C-gamma one
PlGF	placenta growth factor
PMA	phorbol-12-myristate-13-acetate
PTHrP	parathyroid hormone-related protein
Quek	quail endothelial kinase
RIP	rat insulin promoter 1
RT	room temperature
RTK	receptor tyrosine kinase
SDS	sodium dodecylsulphate
Sf	Spodoptera frigiperda
SH2, SH3	Src homology 2 and 3
SHC	Src homology and collagen domain
SOS	Son of Sevenless
SPARC	secreted protein acidic and rich in cytsteine
SV40	simian virus 40
t-PA	tissue-type plasminogen activator
TBS-T	tris-bufferd saline with Tween 20
Tek	tunica interna endothelial cell kinase (Tie-2)
TGF-α, -β	transforming growth factor alpha and beta
Tie	tyrosine kinase with Ig and EGF homology domains
TIMP	tissue inhibitors of metalloproteinases
TNF-α, -β	tumor necrosis factor alpha and beta
TPA	12-0-tetradecanoyl-phorbol-13-acetate
TSH	thyroid-stimulating hormone
TSP-1	thrombospondin 1
u-PA	urokinase plasminogen activator
uPAR	urokinase-type plasminogen activator receptor
UTR	untranslated region
VEGF	vascular endothelial growth factor (VPF)
VPF	vascular permeability factor (VEGF)
VRF	VEGF-related factor (VEGF-B)
VRP	VEGF-related protein (VEGF-C)
X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactoside

1. Summary

Vascular endothelial growth factor (VEGF) is an important regulator of endothelial cell proliferation and migration during embryonic vasculogenesis and angiogenesis as well as in pathological angiogenesis. The recently cloned new factors structurally homologous to VEGF were designated as VEGF-B/VRF and VEGF-C/VRP. The receptor for VEGF-B is unknown. VEGF-C is the ligand for FLT4, a receptor tyrosine kinase whose expression becomes restricted largely to lymphatic endothelia during development and that is related to VEGF receptors FLT1 and KDR.

In this study keratin 14-promoter-directed VEGF-C overexpression in the basal epidermis of transgenic mice was capable of promoting an abundant growth of extensive lymphatic-like vessel structures in the dermis, including large vessel lacunae resembling in their histopathology the human condition known as lymphangioma. Thus, VEGF-C appears to induce selective angiogenesis of the lymphatic vessels *in vivo*. In contrast, preliminary data on mice, which overexpress VEGF-B under the same promoter, does not yet allow us draw any conclusions about its possible biological function.

Recombinant biologically active human VEGF-C was produced using the baculovirus system. Unpurified and purified VEGF-C were used to confirm the interaction of VEGF-C with KDR, a fact recently missed by others. The recombinant protein is going to be used in a large number of future experiments. The production of VEGF-B seems to be intrinsically difficult in non-mammalian cells. Although quantitatively satisfying results could not be obtained yet, the purified growth factor will be used in experiments to identify its receptor.

2. Introduction

In order to react appropriately to environmental changes individual cells in multicellular organisms have to co-ordinate their behaviour. Complex signalling mechanisms have evolved to meet this need. Among other mechanisms the cross-talk between cells is accomplished by signalling molecules.

A simple model of communication between two cells is depicted in Figure 1. A producer cell sends a message by secreting a signalling molecule. When the molecule reaches the target cell, it binds to a receptor and the target cell responds by changing its behaviour.

The ability of a signalling molecule to deliver a particular message to a distinct subpopulation of cells - its specificity - is not only determined by the molecule itself, but also by its receptor and the intracellular signal transduction. The same signalling molecule can convey different messages depending on the receptor type to which it binds on or in the target cell. Further, the same receptor can transduce different messages depending on the intracellular set-up of transduction pathways, which can differ from one cell type to another.

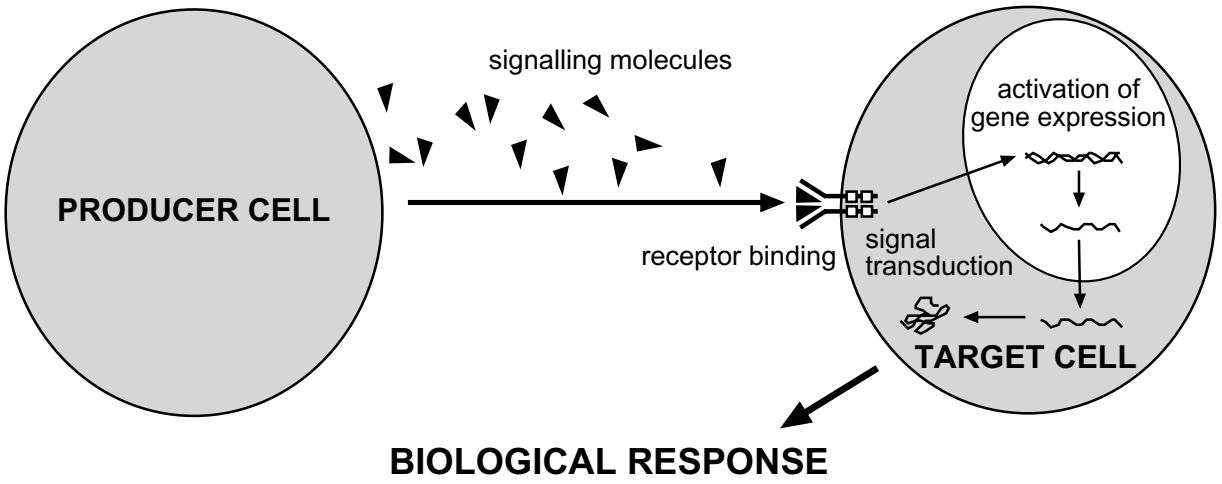

Figure 1. Communication between cells using signalling molecules

2.1. Growth factors and their receptors

Cytokines are secreted proteins with a signalling function. After being secreted, they diffuse in the extracellular matrix and act mainly locally in a paracrine manner, unlike hormones, which are distributed through the circulation. The classification of cytokines into “classical” growth factors, colony-stimulating factors, interleukins, lymphokines, monokines and interferons is rather a left-over from the history of their discovery than a classification according to producer cells and target cells (Nicola, 1994). Many growth factors are rich in extended β-sheets characteristic for group 2 cytokines. Binding of a growth factor to its corresponding cell surface receptor activates complex multistep signal transduction pathways, involving changes in protein phosphorylation, ion fluxes, metabolism, gene expression, protein synthesis and ultimately a biological response (Nicola, 1994). VEGF and its close relatives VEGF-B and VEGF-C form a subfamily within the PDGF family of growth factors (Joukov et al., 1996), which itself belongs to the cystine knot class of cytokines (McDonald et al., 1993).

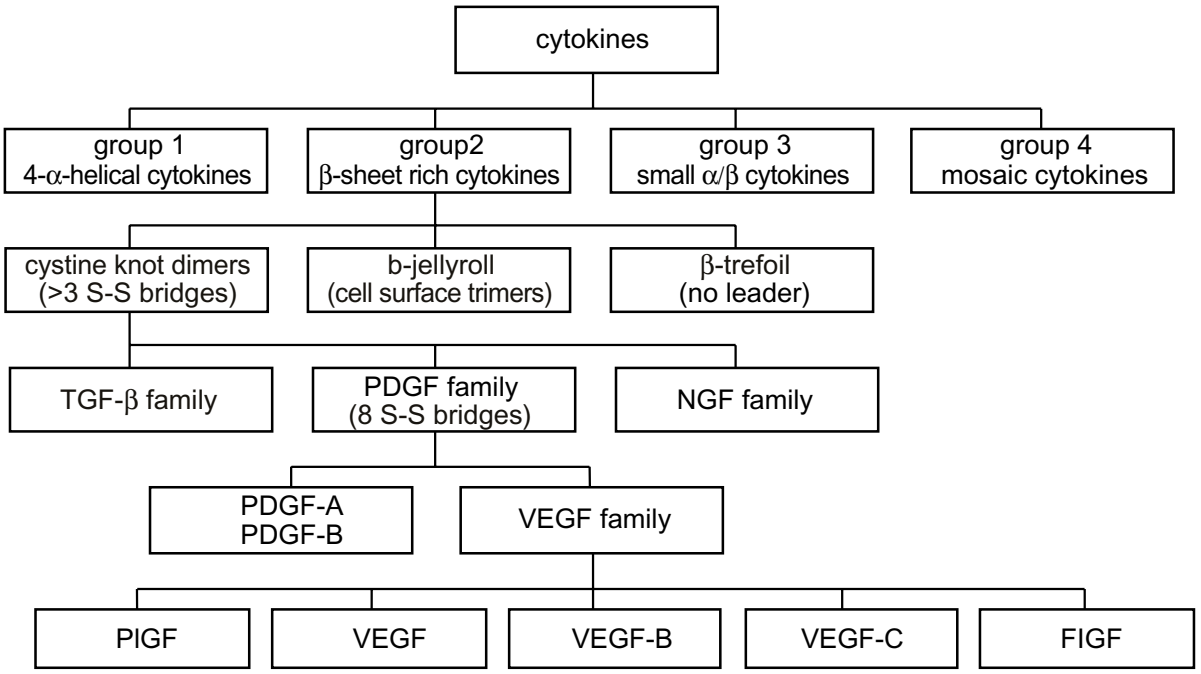

Figure 2. Classification of cytokines

The members of the PDGF family bind to receptors of the class III tyrosine kinase family, that contain multiple immunoglobulin domains in the extracellular region and a split kinase domain in their cytoplasmic part (Ullrich et al., 1990). Receptors of other growth factors include class IV tyrosine kinase receptors (e.g. used by fibroblast growth factor; Basilico et al., 1992) and class V tyrosine kinase receptors (Barbacid, 1993) and serine/threonine kinase receptors (e.g. used by TGF-beta; Wang et al., 1991).

Defects in the signalling pathways of growth factors have been connected to pathophysiological processes that involve uncontrolled cell proliferation, e.g. tumorigenesis (Bishop, 1991). The factor itself can be affected (Cross et al., 1991), its receptor (Yarden et al., 1988) or the intracellular signal transduction pathway (Cantley et al., 1991).

2.2. The interaction between RTKs and their ligands

The receptors for growth factors are transmembrane proteins, whose activation follows the universal mechanism of receptor activation by oligomerisation: Upon binding of the ligand, receptors dimerise or oligomerise inducing a conformational change into their active form, that allows the receptor to transmit the signal through the lipid bilayer of the plasma membrane (Lemmon et al., 1994). Dimerisation of class III tyrosine kinase receptors is thought to result from the dimeric nature of the ligand. So far more then 100 growth factors and 50 RTKs have been cloned and classified (reviewed in Nicola, 1994; van der Geer et al., 1994). A representative overview on RTKs being receptors for growth factors is given in Figure 2 (adapted from Pajusola, 1996). A relationship can be observed between the structure of a growth factor and the type of receptor to which it binds. Nerve growth factors bind to class V tyrosine kinase receptors, members of the PDGF family bind to class III tyrosine kinase receptors, and β-trefoil growth factors like FGFs bind to class IV tyrosine kinase receptors and require heparin-bound FGF for dimerisation and high-affinity binding.

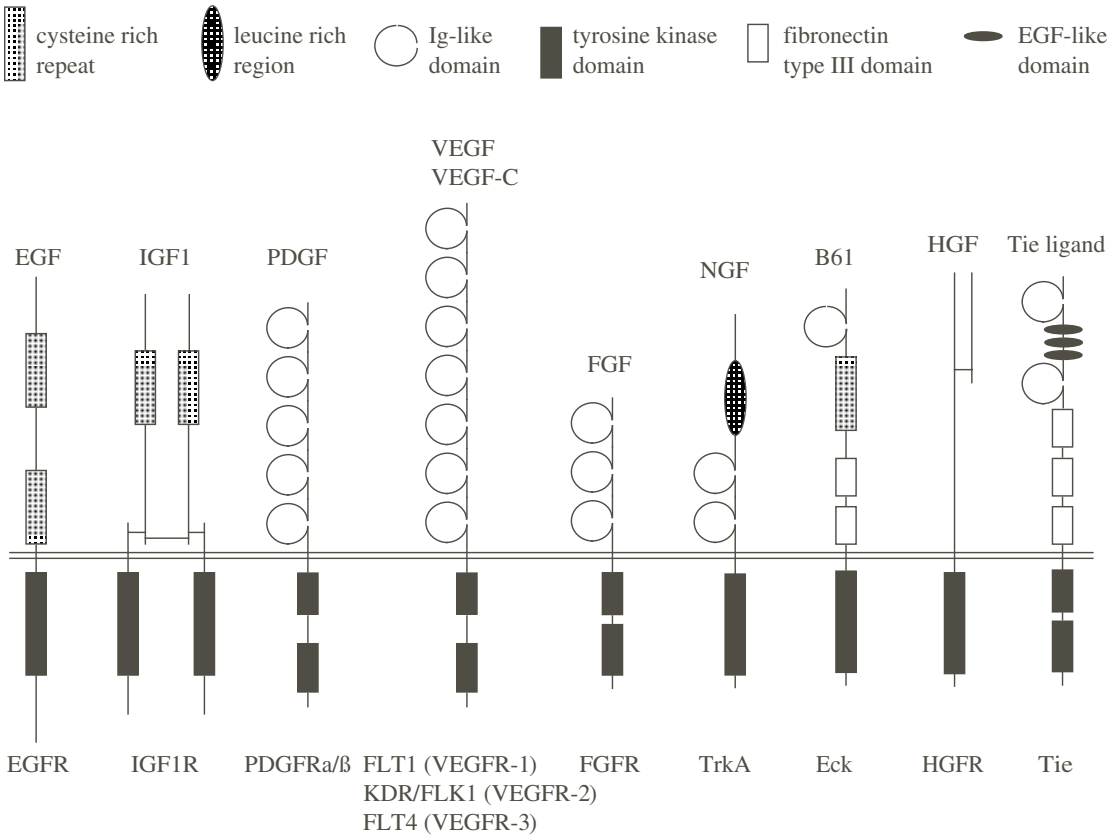

Figure 3. RTKs that are receptors for growth factors

Ligand binding and receptor dimerisation induce the crossphosphorylation of critical tyrosine residues, which are present in the cytoplasmic domain of the RTK (Honegger et al., 1990). Depending on the receptor type, distinct sets of cytoplasmic tyrosine residues are targets for autophosphorylation (reviewed in Carpenter, 1992). These phosphorylation events induce a conformational change within the RTK and create high affinity docking sites for cellular substrates like SH2-domain-containing molecules. SH2-domain-containing molecules bind specifically to certain phosphorylated tyrosines of the RTK recognising three amino acids immediately C-terminal of the tyrosine residue (reviewed in Koch et al., 1991). Additionally, the kinase activity towards cellular substrates is increased upon phosphorylation, e.g. EGF phosphorylates the SH2-domain-containing phospholipase C-γ_1 (PLC-g1; Anderson et al., 1990) and the p85 subunit of phosphatidylinositol-3-kinase (PI3-K; Hu et al., 1992). Not only the activation by phosphorylation increases the activity of PLC-γ_1 and PI3-K, but also the recruitment from the cytoplasm to the plasma membrane, where the substrates of these enzymes are located (Pawson, 1995).

SH3 domain. SH3-domain-containing molecules have not been shown to interact directly with RTKs, but they are involved in most signalling pathways of RTKs as adapter molecules (reviewed in Cohen et al., 1995a). An example is the interaction of the SH3-domain of GRB2 with PXXP-strings of the *ras*-guanine-nucleotide-exchange factor son of sevenless (SOS; Buday et al., 1993). SOS replaces the *ras*-bound GDP by GTP and thus activates the *ras/raf/*MAP kinase pathway.

Pleckstrin homology. Another homology domain involved in membrane localisation and common to signalling molecules is the pleckstrin homology (PH) domain (Haslam et al., 1993), which has been shown to bind to the βγ subunits of heterotrimeric G proteins (Gβγ), and to activate together with PIP_2 the β-adrenergic receptor kinase (bARK; Pitcher et al., 1995).

2.3. The vascular system

2.3.1. The development of the vascular system

2.3.1.1. The cardiovascular system

The development of the cardiovascular system starts soon after gastrulation, and it is the first organ that starts functioning in a developing embryo (Gilbert, 1991). The inner lining of all blood and lymphatic vessels, as well as the heart endocardium, are composed of endothelial cells, which form a single cell layer. The embryonic vascular development can be divided into three phases[1]:

1. Differentiation of certain mesodermal cells into angioblasts
2. Vasculogenesis[2] (differentiation of endothelial cells in situ from angioblasts)
3. Angiogenesis[3] (vascular sprouting from pre-existing vessels)

Differentiation of mesodermal cells into angioblasts. In the splanchnic mesoderm of the embryo, a distinct set of cells differentiates into angioblasts (also called hemangioblasts), which are the precursors for both endothelial cells and blood cells (His, 1900). Evidence has been presented that the mesodermal cells which become angioblasts are determined already before gastrulation, because the blocking of DNA replication and morphogenetic movements during gastrulation did not prevent the emergence of endothelial cells (von Kirschhofer et al., 1994).

Vasculogenesis. The earliest blood vessels of an embryo form by a mechanism called vasculogenesis, which is defined as differentiation of endothelial cells in situ from angioblasts (Risau et al., 1988b). Angioblasts aggregate and form blood islands. Cells at the periphery of the blood island differentiate into endothelial cells; cells in the centre of the blood island differentiate into blood cells.

Heart and major blood vessels. Simultaneously with the development of the blood islands, angioblasts start to differentiate from mesodermal mesenchymal cells forming the early heart rudiment and the major blood vessels, such as the paired dorsal aortae (Pardanaud et al., 1987; Coffin et al., 1988; Coffin et al., 1991).

The vasculature of certain internal organs of endodermal origin (e.g. lung, spleen and pancreas) forms through a similar vasculogenetic mechanism (Auerbach et al., 1985), which was demonstrated e.g. by studies on quail-chicken chimeras (Pardanaud et al., 1989).

Angiogenesis. Angiogenesis, the sprouting of new blood vessels from pre-existent vessels, is responsible for the vascularisation of certain other developing organs, e.g. brain, kidney and limbs (Bär, 1980; Ekblom et al., 1982; Wilson, 1983) and neovascularisation in adults. New capillaries are formed by sprouting from existing capillaries or postcapillary venules, hence involving the proliferation of the existing endothelium in response to an angiogenic stimulus elicited by the tissue to become vascularised. Since sprouting (that is angiogenesis) is the most frequent, but not the only mechanism, by which new capillaries are derived from pre-existing ones, an alternative terminology was proposed by Sherer (Sherer, 1991). Sherer distinguished between angioblastic development (vasculogenesis) and angiotrophic growth, which employs three different mechanisms: sprouting, intussusceptive growth (a large sinusoidal capillary divides into two smaller capillaries[4]) and intercalation (endothelial cells divide within a blood vessel wall to increase its length and diameter; Folkman, 1987).

The process of sprouting has been divided into five sequential steps (Folkman, 1985) as outlined below:

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1. Sabin divides the vascular development into three phases, according to the mechanism, by which endothelial cells arise. In phase one endothelial cells arise solely by vasculogenesis, in phase two by both vasculogenesis and angiogenesis and in phase three exclusively by angiogenesis (Sabin, 1920).
2. Previously also called primary angiogenesis (Benninghoff et al., 1930).
3. Previously also called secondary angiogenesis (Benninghoff et al., 1930).
4. Also the opposite event - fusion of blood vessels - can be observed and is of major importance in the remodelling of the vascular system during embryogenesis, e.g. the aorta seems to form by fusion of a capillary plexus into a longitudinal tube (Evens, 1909; Wilting et al., 1996b).

1. *Local degradation of the vascular basement membrane.* The importance of proteolytic activity increases with the development of the endothelial basal lamina and is certainly crucial in adult angiogenesis. Capillary endothelial cells are capable of secreting several proteases, which hydrolyse the basal lamina of endothelial cells and certain ECM components (Moscatelli et al., 1988).
2. *Endothelial cells migrate along angiogenic gradients.* The extremely high migratory potential of endothelial cells during development has been shown in quail-chick grafting experiments (Wilms et al., 1991). Gradients of soluble angiogenic factors, ECM-bound angiogenic factors and the specific composition of the ECM have been identified as key factors in the directed migration of both embryonic and adult endothelial cells (reviewed in Wilting et al., 1996b).
3. *Lumen formation.* Two opposite mechanisms for lumen formation have been described, inter- and intracellular lumen formation. Intercellular lumen formation is the extension of the lumen of the pre-existing capillary (Benninghoff et al., 1930). Of minor importance seems to be the intracellular lumen formation as a result of vacuolation (Sabin, 1920). The specific contributions of these two mechanisms to angiogenesis are not settled (Wilting et al., 1996b).
4. *Mitosis of endothelial cells in the midsection of capillary sprouts.* Although vessel formation seems to be possible without mitosis, it was restricted to at least two levels of vascular loops in experiments, that employed endothelial cells, whose capacity to proliferate was destroyed by irradiation/poisoning (Sholley et al., 1984; Koolwijk et al., 1996). Whether under physiological conditions the initial steps of angiogenesis take also place without concurrent mitosis of the involved endothelial cells is still subject of discussion.
5. *Fusion of adjacent sprouts and loop formation.* It is unknown, whether individual capillary sprouts find each other by a directed mechanism or by trial-and-error migration (Folkman, 1985). A directed mechanism would involve concentration gradients and/or adhesion molecules and, indeed, several candidate molecules have been proposed, e.g. Tek/Tie and their ligands (Dumont et al., 1994).

Hyperpermeability. Microvascular hyperpermeability regularly accompanies angiogenesis, and also seems to play a role in its induction. Fenestration and transcytosis are increased in hyperpermeable vessels (Karnovsky et al., 1967; Kohn et al., 1992; Roberts et al., 1995), enabling plasma proteins to leak and to form a new provisional ECM by extravascular clotting, which permits, favours or even induces inward migration of endothelial cells (Dvorak et al., 1995a). The mature ECM, by which the provisional ECM is gradually replaced, subsequently would suppress angiogenesis. Notably several markers of vascular maturation inhibit the proliferation of endothelial cells, e.g. laminin (Kubota et al., 1988; Risau, 1991).

ECM in the development of the vasculature. Migration of the early angioblasts and endothelial cells is essential for the proper development of the vasculature. Several ECM components are endothelial cell-derived, and endothelial cells possess multiple receptors for ECM components, such as integrin receptors for types-I and -IV collagen, laminin, fibronectin[5] and vitronectin (van Mourik et al., 1990). For example, blood islands and early intraembryonic capillaries secrete fibronectin, whose expression is replaced later by laminin (Risau et al., 1988a). Precardiac mesodermal cells, which later differentiate into endocardial endothelial cells, have been shown to migrate along a fibronectin gradient (Linask et al., 1986). Laminin may be involved in the inhibition of proliferation and induction of differentiation of the endothelial cells (Kubota et al., 1988). This illustrates the interdependence between ECM components and the migrational and proliferative behaviour of angioblasts and endothelial cells (Grant et al., 1990).

Many angiogenic factors are able to interact with heparin and heparan sulphates in both free and cell-associated forms, and they seem to play important roles in the regulation of angiogenesis. Certain angiogenic molecules like FGFs require interaction with heparin or heparan sulphate for receptor binding (Yayon et al., 1991). Cell-associated and extracellular-matrix heparan sulphate proteoglycans (ECM-HSPGs) appear to provide sustained release reservoirs for angiogenic molecules having affinity for heparin like VEGFs (Ferrara et al., 1992) and FGFs (Baird et al., 1987), and heparin binding can protect such molecules from degradation. The success of the heparin mimic aluminium sucrose octasulphate (sucralfate) in the treatment of gastric ulcers may be partly based on its ability to protect endogenous bFGF from degradation by gastric acid. The healing of the peptic ulcers seems to depend on the angiogenic activity mediated by the elevated level of endogenous bFGF (Folkman et al., 1991).

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5. Interestingly the two endothelial cell-specific RTKs Tie and Tek have three fibronectin type III domains in their extracellular part (Schnürch et al., 1993).

2.3.1.2. The lymphatic vascular system

The lymphatic vascular system starts to develop shortly after the blood circulation has started, but not much is known about its origin. The theory of a venous origin with a centrifugal spread was first proposed by Sabin (Sabin, 1902, 1909, 1912). According to this theory, lymphatic vessels arise by sprouting from early lymphatic sacs, which themselves develop from large central veins by sprouting at certain locations (around E12 of mouse development). This theory is supported by the expression pattern of FLT4 (Kaipainen et al., 1995). Around E15 lymphatic sprouting from the veins ceases, but extensive growth into the periphery continues. Vessels fuse and remodelling continues before the mature vasculature is formed. This includes the regression of earlier capillaries, possibly by apoptosis (Risau, 1995). Except for the left thoracic duct all links to the venous system are disconnected (Zadvinskis et al., 1992).

Lymphatic capillaries differ from blood capillaries. They are blind-ended, possess generally a larger and more irregular lumen and no continuous basement membrane. Lymphatic endothelial cells show no tight junctions (but instead many patent junctions and “complex adherents”; Schmelz et al., 1994), resulting in a generally higher permeability compared to blood capillaries. Via unique anchoring filaments they are connected to the adjacent connective tissue (Leak, 1970).

Although almost no molecular biological research has been done on lymphangiogenesis, the sequential model for the angiogenesis of blood vessels might probably be adapted for the sprouting of lymphatic vessels as well. However, differences between blood and lymphatic vessels should be accommodated to such model; for example, no basal membrane has to be degraded in lymphangiogenesis, and loop formation is not required.

The present work suggests that lymphangiogenesis may involve at least two distinct signals for the establishment of a lymphatic capillary network: one signal that would stimulate migration/sprouting and another, distinct signal, that would stimulate the proliferation of the involved endothelium 5.1.6..

It has been proposed that also the angiogenesis of blood vessels is dependent on two distinct signals (Folkman, 1984). Anyhow, angiogenesis of blood vessels inevitably seems to involve VEGF, a molecule that carries signals for both migration/sprouting AND proliferation, yet other molecular inducers of angiogenesis might deliver only one signal (migration/sprouting OR proliferation).

2.3.1.3. Diversity of endothelial cells

Endothelial cells are engaged in a variety of functions: endothelial cells in large vessels participate in the control of vascular tone and blood pressure, whereas capillary endothelial cells are mediating the exchange of gas and metabolic products between blood and tissues, as well as the extravasation of immune cells during infection (Geneser, 1986; Stevens et al., 1992).

The capillary endothelial cells therefore show significant heterogeneity. The degree of permeability of different capillaries depends on the type of intercellular junctions, as well as on the cell morphology itself, and determines the molecular weight cut-off in the separation of blood/lymph and tissue. The liver sinusoids contain a discontinuous endothelium with large holes through the cytoplasm, whereas in the brain the capillary endothelial cells are linked to each other by tight junctions and contain only few transport vesicles thus creating the blood-brain barrier (BBB; Geneser, 1986; Stewart et al., 1987; Dejana et al., 1995). Fenestrated capillaries consist of endothelial cells with openings that are closed by a thin diaphragm. These kind of capillaries are found, for example, in the choroid plexus, whose capillaries are responsible for the secretion of the cerebrospinal fluid of the brain ventricles. The high endothelial venules (HEVs) contain endothelial cells with a characteristic morphology. They are found in lymphatic organs and are responsible for the extravasation of mature lymphocytes during their circulation from blood to lymph (Risau, 1995). Leukocytes as well as macrophage extravasation requires various interactions between selectins, integrins and other adhesion molecules, and their respective ligands, expressed on both the leukocytes and the endothelium of the venules and lymphatic capillaries (Tedder et al., 1995). Lymphatic vessels collect and return extra plasma proteins found in the tissue fluid back to the circulation, regulate cell hydration and osmosis. In the immuneresponse, lymphatic vessels provide for macrophages and Langerhans cells an exit pathway from tissues, and later immunoglobulins - produced in the lymph nodes - reach the blood via lymphatic vessels (Geneser, 1986).

2.3.2. Regulation of angiogenesis: Stimulatory and inhibitory factors

Physiological angiogenesis is regulated by a balance involving an interplay of positive and negative angiogenic molecules. Although stimulatory and especially inhibitory angiogenic factors have been almost exclusively discovered in the past decade, their number is already very large. They can be categorised into directly and indirectly acting factors. A factor is classified as indirect if it fails to stimulate endothelial cells *in vitro* despite its angiogenic activity *in vivo*. Additionally, indirect angiogenic factors may not only fail to stimulate endothelial cell proliferation *in vitro*, but actually inhibit it, e.g. TGF-β (Baird et al., 1986; Müller et al., 1987).

This apparent paradox was resolved by demonstrating that TGF-β is a strong chemotattractant and activator for monocytes/macrophages (Wahl et al., 1987), which in turn are able to secrete a variety of inflammatory and angiogenic molecules including VEGF and TNF-α (Leibovich et al., 1987; Berse et al., 1992), and that TGF- induces VEGF and bFGF in fibroblasts and epithelial cells (schematically shown in Figure 4; Pertovaara et al., 1993; Pertovaara et al., 1994).

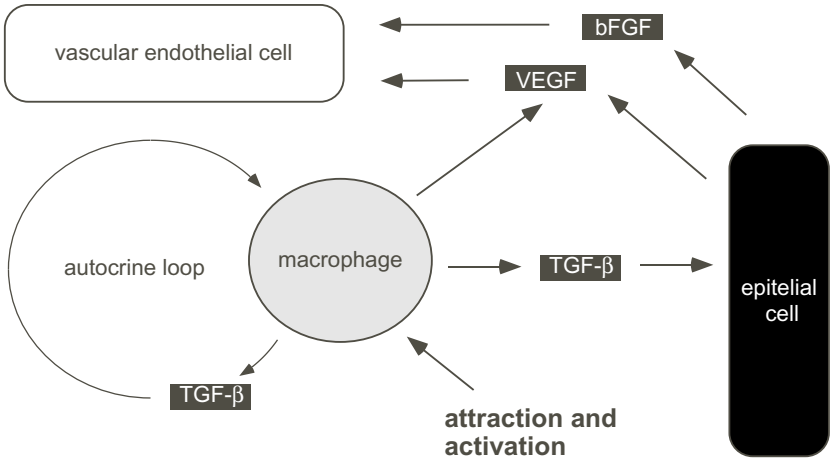

Figure 4. Example of indirect angiogenic activity: TGF-β

Another indirect mechanism is the release of sequestered angiogenic factors by proteolysis. This was shown for cell surface and ECM-bound VEGF (Houck et al., 1992; Park et al., 1993). Inhibitors of angiogenesis often seem to be parts of larger molecules and become activated upon proteolytic cleavage. Interestingly enough, many abundant proteins contain these “cryptic” angiogenic inhibitors. Angiostatin, a potent inhibitor of angiogenesis, is a proteolytic 38-kDa fragment of plasminogen (O'Reilly et al., 1994). Similarly, fibronectin cleavage generates two endothelial cell growth inhibitors (Homandberg et al., 1985). Other recently identified “cryptic” angiogenic inhibitors are collagen-derived and EGF-derived peptides (Tolsma et al., 1993; Nelson et al., 1995), and the moderate anti-angiogenic activity of PF4 can be increased more than 50-fold by proteolytic cleavage (Gupta et al., 1995). The mechanism of rapid generation of large amounts of angiogenic inducers and inhibitors from inactive precursors reflects the requirement for instant upregulation of angiogenesis upon demand as well as for a rigid temporal and spatial limitation, similar to the blood clotting cascade (Furie et al., 1988).

Nevertheless, directly acting angiogenic factors may also act *in vivo* indirectly. It is presently unknown how e.g. FGF elicits angiogenesis *in vivo*. aFGF and bFGF are strong stimulators of vascular endothelial cell growth *in vitro* (Lobb et al., 1985; Shing et al., 1985; Thomas et al., 1985; Montesano et al., 1986), but may have only little direct effects *in vivo*, especially since FGF receptors are not specific to endothelial cells and indirect mechanisms have been described (Pepper et al., 1992; Goto et al., 1993).

The mechanisms underlying the release of angiogenic inducers and inhibitors and of the interactions among them, as well as the integration of their signals inside the endothelial cells, are only beginning to be uncovered. Table 1 gives an overview of well known endogenous angiogenic factors and a selection of less well characterised ones. Table 2 lists several anti-angiogenic substances including some drugs.

Table 1 Angiogenic factors

	references
polypeptides	
acidic fibroblast growth factor (aFGF)	(Lobb et al., 1985; Thomas et al., 1985)
basic fibroblast growth factor (bFGF)	(Shing et al., 1985; Montesano et al., 1986)
angiogenin[a]	(Fett et al., 1985)
vascular endothelial growth factor (VEGF)	(Connolly et al., 1989a; Leung et al., 1989; Plouët et al., 1989)
placental growth factor (PlGF)	(Maglione et al., 1991)
interleukin-8 (IL-8)	(Koch et al., 1992; Strieter et al., 1992)
platelet-activating factor (PAF)	(Montrucchio et al., 1994)
platelet-derived endothelial cell growth factor (PD-ECGF)[b]	(Ishikawa et al., 1989)
granulocyte colony-stimulating factor (G-CSF)	(Bocchietto et al., 1993)
hepatocyte growth factor (HGF)	(Bussolino et al., 1992; Grant et al., 1993a; Rosen et al., 1993)
proliferin	(Jackson et al., 1994)
insulin-like growth factor I (IGF-I)	(Grant et al., 1993c)
epidermal growth factor (EGF)	(Gospodarowicz et al., 1979)
transforming growth factor TGF-α	(Schreiber et al., 1986)
transforming growth factor TGF-β	(Roberts et al., 1986)
platelet-derived growth factor (PDGF)	(Risau et al., 1992)
tumour necrosis factor TNF-α	(Fràter-Schroder et al., 1987; Leibovich et al., 1987)
non-polypeptides	
prostaglandins: PGE_1, PGE_2	(BenEzra, 1978; Form et al., 1983; Graeber et al., 1990)
nicotinamide	(Kull et al., 1987)
adenosine	(Fraser et al., 1979; Dusseau et al., 1986)
1-butyryl glycerol	(Dobson et al., 1990)
degradation products of hyaluronic acid	(West et al., 1985)
(12R)-hydroxyeicosatrienoic acid	(Masferrer et al., 1991)
okadaic acid	(Oikawa et al., 1992)

Cu^{2+} complexed to Gly-His-Lys, ceruloplasmin, heparin or SPARC-derived peptides	(Raju et al., 1984; Lane et al., 1994)
GD3, GM1 (gangliosides)	(Ziche et al., 1992)

a. Angiogenin has ribonucleolytic activity (Shapiro et al., 1986).
b. PD-ECGF has thymidine phosphorylase activity (Moghaddam et al., 1992).

Table 2 Anti-angiogenic factors

	references
polypeptides	
angiostatin (38-kDa fragment of plasminogen)	(O'Reilly et al., 1994)
29-kDa N-terminal and 40-kDa C-terminal fibronectin fragments	(Homandberg et al., 1985)
platelet factor 4 (PF4)/PF4 fragment	(Maione et al., 1990; Gupta et al., 1995)
16-kDa N-terminal prolactin fragment	(Clapp et al., 1993)
protamine	(Taylor et al., 1982)
thrombospondin-1 (TSP-1)	(Rastinejad et al., 1989; Iruela-Arispe et al., 1991)
somatostatin analogue: octreotide	(Grant et al., 1993b)
tissue inhibitors of metalloproteinases: TIMP-1, TIMP-2	(Moses, 1993; Murphy et al., 1993; Johnson et al., 1994)
interferon-α (IFN_α)	(Sidky et al., 1987)
bFGF soluble receptor	(Hanneken et al., 1994)
non-polypeptides	
steroids: medroxyprogesterone, 2-methoxyestradiol, dexamethasone	(Blei et al., 1993; Wolff et al., 1993; Fotsis et al., 1994)
retinoids: Re 80, Am 580, Am 80	(Oikawa et al., 1993)
GPA 1734 (quinolizine derivative, BM synthesis inhibitor)	(Maragoudakis et al., 1988)
cyclic peptide 203 (RGDfV, $\alpha_v\beta_3$ integrin antagonist)	(Brooks et al., 1994)
AGM-1470 (fumagillin derivative)	(Ingber et al., 1990; Oliver et al., 1994)
taxol (microtubule stabiliser)	(Oliver et al., 1994)
pentosan	(Nguyen et al., 1993)
thalidomide	(D'Amato et al., 1994)
placental proliferin-related protein	(Jackson et al., 1994)
GM3 (ganglioside)	(Ziche et al., 1992)

2.3.3. Angiogenesis in the adult organism

Physiological angiogenesis. After the embryonic and postnatal development, the rapid proliferation of the endothelial cells is downregulated almost to quiescence. The turnover time of endothelial cells in the adult is measured in months or years (Denekamp, 1993). However, the capillary endothelial cells maintain their proliferative potential throughout the life of an organism. Almost the only circumstances, under which proliferation is physiologically dramatically upregulated, are the female reproductive cycle and pregnancy (in more detail described under 2.4.1.7.1.).

Pathological angiogenesis. Different pathological conditions involve the neovascularisation of tissues (reviewed in Folkman, 1995a). Neovascularisation can be an accelerator of disease progression (e.g. in many neoplastic diseases) or a mediator of pathogenesis (e.g. in diabetic retinopathy, in more detail described under 2.4.1.7.4.).

2.4. The VEGF subfamily of growth factors

2.4.1. Vascular endothelial growth factor (VEGF)[6]

VEGF is an endothelial cell-specific mitogen that has the ability to promote angiogenesis in several *in vivo* models. By alternative splicing of mRNA, VEGF is made in four different isoforms that differ markedly in their secretion pattern, but have similar biological activities. Expression of VEGF mRNA is temporally and spatially related to the proliferation of blood vessels in a variety of physiological and pathological circumstances, such as embryonic development, formation of the ovarian corpus luteum, wound healing, angiogenesis in solid tumours and in rheumatoid arthritis.

2.4.1.1. Discovery and cloning of VEGF

The two main biological activities of VEGF - mitogenic activity and vascular permeability inducing activity - were described, purified and designated independently as VPF (vascular permeability factor) and VEGF (vascular endothelial growth factor):

Vascular permeability factor. The release of a vascular permeability-increasing agent by guinea pig hepatocarcinoma cells was reported more than 15 years ago (Dvorak et al., 1979). Vascular leakage[7] was subsequently used to monitor purification of VEGF from the supernatant of this (Senger et al., 1983; Senger et al., 1986) and of the human histiocytic lymphoma cell line U-937 (Connolly et al., 1989b). Therefore, the factor was later designated as vascular permeability factor (VPF; Senger et al., 1983) or vasculotropin (Plouët et al., 1989).

Vascular endothelial growth factor. Media conditioned by bovine pituitary follicular/folliculostellate cells (FC) contained an agent that specifically promoted growth of vascular endothelial cells. The mitogenic activity of this agent towards vascular endothelial cells was used to monitor its purification, and on the basis of its target cell selectivity the purified agent was designated VEGF (Ferrara et al., 1989; Gospodarowicz et al., 1989).

Soon after the first descriptions by Ferrara, Gospodarowicz and Conolly, several groups reported the purification of VEGF from different sources: human VEGF from U937 cells (Keck et al., 1989), bovine VEGF from pituitary folliculostellate cells (Leung et al., 1989) and AtT-20 pituitary tumour cells (Plouët et al., 1989).

The first human VEGF cDNAs were cloned from a phorbol ester-activated HL60 promyelocytic leukaemia cell library (Leung et al., 1989) and a histiocytic lymphoma cell line U937 library (Connolly et al., 1989b); both cDNAs were screened with oligonucleotides designed on the basis of the amino acid sequence of the previously purified protein.

VEGF-like sequences have also been found in two parapoxvirus genomes. It is thought that they were acquired from a mammalian host and mediate now capillary proliferation in infected goats and sheep (Lyttle et al., 1994).

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6. Reviews on VEGF: Ferrara et al., 1992; Klagsbrun and D'Amore, in press; Thomas, 1996.
7. As assay the extravasation of an intradermally applied protein-bound dye in guinea pig skin was used (Connolly et al., 1989b).

2.4.1.2. Genomic structure and splice variants of VEGF

Promoter, transcriptional control elements and transcriptional start site. The hVEGF gene is located in chromosome 6p12 (Vincenti et al., 1996; Wei et al., 1996). It has a single major transcriptional start site 1038 bp upstream from the ATG initiation codon. The 5'-UTR and 2.4 kb of the TATA-less promoter have been sequenced, revealing three SP1 binding sites immediately in front of the transcriptional start site and four AP-1 and two AP-2 binding sites located around the transcriptional start site (Tischer et al., 1989). Recently regulatory elements, located within the 5'-UTR, have been described (Cohen et al., 1996).

Alternative splicing and resulting isoforms/alternative polyadenylation of mRNA. The VEGF gene is organised in eight exons (see Figure 16.), and the its coding region spans approximately 14 kb (Tischer et al., 1989). Four different isoforms are the result of alternative splicing of the mRNA. The shortest isoform, $VEGF_{121}$, is encoded by exons 1-5 and 8, $VEGF_{165}$ includes additionally exon 7. $VEGF_{189}$ and $VEGF_{206}$ mRNAs contain all 8 exons, and the usage of a variable 5'-splice donor site within exon 6 creates the difference between the $VEGF_{189}$ and $VEGF_{206}$ mRNA. The $VEGF_{206}$ mRNA is an extremely rare species as it was isolated only once from a human fetal liver cDNA library (Houck et al., 1991) and the corresponding form is not present in mouse (Shima et al., 1996). All four mRNA isoforms contain the sequence encoding the hydrophobic signal peptide of 26 amino acids for secretion (Tischer et al., 1989; Houck et al., 1991). Every intron-exon boundary possesses classical consensus splicing signals. The majority of transcripts for $VEGF_{165}$ contain 1.9 kb of 3'-UTR (2.2 kb for murine mRNA) resulting in mRNA transcripts around 3.9 kb (Conn et al., 1990), although four potential polyadenylation sites are present, and differential termination of transcription has been reported (Levy et al., 1995).

Initially, $VEGF_{165}$ was thought to be the predominant isoform in all tissues except placenta, where $VEGF_{l2l}$ is the most abundant species (Houck et al., 1991). Although $VEGF_{189}$ has been shown to predominate in rat heart and lung (Bacic et al., 1995), and $VEGF_{121}$ seems to be expressed by several tissues and cell lines in similar amounts as $VEGF_{165}$ (Minchenko et al., 1994; Bacic et al., 1995), in this work the term "main form" is used for $VEGF_{165}$. The longest isoform ($VEGF_{206}$) was expressed in cell culture (Houck et al., 1991), but evidence of its *in vivo*-existence is still lacking.

Mouse VEGF gene structure. The mVEGF gene structure is very similar to the human VEGF gene structure (Shima et al., 1996); compare Figure 16. with Figure 17.. Three isoforms ($VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$) have been shown to be generated by alternative splicing. The existence of a fourth splice variant coding for a putative 145 amino acid peptide (corresponding to the longest human splice variant) is unlikely, a premature stop-codon would be created by the frame shift resulting from the use of the alternative 5'-splice donor site (Shima et al., 1996).

2.4.1.3. Molecular characterisation

VEGFs are dimeric proteins. The main form has a molecular weight of approximately 45 kDa and it is composed of the 165 amino acid isoform (Ferrara et al., 1989). The minor human VEGF isoforms contain 121 and 189 amino acids following signal sequence cleavage. Compared with $VEGF_{165}$, $VEGF_{121}$ lacks 44 amino acids; $VEGF_{189}$ has an insertion of 24 largely cationic amino acids and $VEGF_{206}$ has an additional insertion of 17 amino acids resulting in different affinities for endogenous polyanions such as cell surface heparan sulphates (Figure 15; Park et al., 1993). Rodent and bovine VEGF monomers are shorter by one amino acid. The VEGF monomers have a single glycosylation site at Asp 75 of the mature protein, but glycosylation is not necessary for biological activity (Yeo et al., 1991; Claffey et al., 1995), although it is important for the efficient secretion of VEGF.

Heterodimers have been described in the mouse, which are composed of $VEGF_{121}$ and $VEGF_{164}$ (Breier et al., 1992), and the rat glioma cell line GS-9L secretes heterodimers between $VEGF_{164}$ and PlGF, which were shown to be mitogenically only 3 times less active towards human umbilical vein endothelial cells (HUVEC) than $VEGF_{164}$ homodimers from the same source (DiSalvo et al., 1995).

The amino-acid sequence of VEGF has a weak homology (~15-20%) to PDGF-A and B and their viral counterpart v-Sis (Leung et al., 1989; Conn et al., 1990), a higher homology to VEGF-C/VRP (~30%; Joukov et al., 1996; Lee et al., 1996) and VEGF-B/VRF (43%; Grimmond et al., 1996; Olofsson et al., 1996a) and the highest homology to $PlGF_{131}$ (53%; Maglione et al., 1991). In all these molecules the 8 cysteine residues characteristic for members of the PDGF family are conserved. These cysteines are located in the receptor binding domain and are involved in the formation both intra- and interchain disulphide bridges. Thus the folding pattern of these proteins is likely to be similar, resulting in a typical compact cysteine-bonded knot and physical properties like relative stability against heat, acids and mild proteolysis (Thomas, 1996). The crystal structure and mutational analysis of PDGF-BB have shown that the 2nd and the 4th cysteine residue are involved in the dimer formation in an antiparallel fashion (Andersson et al., 1992; Oefner et al., 1992). The same cysteine residues (aa 25 and 67) have been shown to be involved in the interchain disulphide bridge formation in VEGF (Pötgens et al., 1994). Non-covalently linked PDGF-BB, in which the interchain-forming cysteines were mutated, retained biological activity (Kenney et al., 1994), whereas the biological activity of VEGF is abolished upon disruption of its interchain disulphide bonds (Pötgens et al., 1994).

By mutational analysis the acidic amino acids Asp63, Glu64 and Glu67 have been identified as FLT1 receptor binding determinants, whereas the basic residues Arg82, Lys84 and His86 were critical for FLK1/KDR binding (Keyt et al., 1996b).

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PDGF-A    ........................MRTLACLLLLGCGYLAHVLAEEAEIPREVIERLARSQIHSIRDLQRLLEIDSVGSEDSLD.TSL.
PDGF-B    ........................MNRCWA.LFLSLCCYLRLVSAEGDPIPEELYEMLSDHSIRSFDDLQRLLHGDP.GEEDGAELDL..
PlGF131   ..................................................................MPVMRLFPC..FLQLLAGLAL...P
VEGF165   ..................................................................MNFLLS..WVHWSLALLLYLHH
VEGF-B167 ..................................................................MSPLLR..RLLLAALLQLAPAQ
VEGF-C    MHLLGFFSVACSLLAAALLPGPR.EAPAAAAAFESGLDLSDAEPDAGEATAYASKDLEEQLRSVSSVDELMTVLYPEYWKMYKCQLRKGGWQ

PDGF-A    ..AHGVHATKHVPEKRPL..PIRRKRSI......EEAVPAVCKTRTVIYEIPRSQVDPTSANFLIWPPCVEVKRCTGCCNTSSVKCQPSRVH
PDGF-B    ..MTRSHSGGELES......LARGRRSLGSLTIAEPAMIAECKTRTEVFEISRRLIDRTNANFLVWPPCVEVQRCSGCCNNRNVQCRPTQVQ
PlGF131   AVPPQQW.........ALSAGNGSSEVEVVPFQE.VWGRSYCRALERLVDVVSEYPS..EVEHMFSPSCVSLLRCTGCCGDENLHCVPVETA
VEGF165   AKWSQAA.........PMAEGGGQNHHEVVKFMD.VYQRSYCHPIETLVDIFQEYPD..EIEYIFKPSCVPLMRCGGCCNDEGLECVPTEES
VEGF-B167 APVSQP..............DAPGHQRKVVSWID.VYTRATCQPREVVVPLTVELMG..TVAKQLVPSCVTVQRCGGCCPDDGLECVPTGQH
VEGF-C    HNREQANLNSRTEE..TIKFAAAHYNTEILKSIDNEWRKTQCMPREVCIDVGKEFGV..ATNTFFKPPCVSVYRCGGCCNSEGLQCMNTSTS

PDGF-A    HRSVKVAKVEYVRKKPKLKEVQVRLEEHLECACATTSLNPDYREEDTDVR
PDGF-B    LRPVQVRKIEIVRKKPIFKKATVTLEDHLACKCETVAAARPVTRSPGGSQEQRAKTPQTRVTIRTVRVRRPPKGKHRKFKHTHDKTALKE…
PlGF131   NVTMQLLKIRSG..DRP.SYVELTFSQHVRCECRPLREKMKPERCGDAVPRR
VEGF165   NITMQIMRIKPH..QGQ.HIGEMSFLQHNKCECRPKKDR...........ARQENPCGPCSERRK...HLFVQDPQTCKCSCKNT
          ..................................................DSRCKARQL...EL...NERTCRCDKPRR
VEGF-B167 QVRMQILMIRYPSSQ....LGEMSLEEHSQCECRPKKKD......SAVKPDSPRPLCPRCTQHHQ...RP...DPRTCRCRCRRR
          ..............................................SFLRCQGRGL...EL...NPDTCRCRKLRR
VEGF-C    YLSKTLFEITVPLSQGP.KPVTISFANHTSCRCMSKLDV.YRQVHSIIRRS.LPATLPQCQAANKTCPTNYMWNNHICRCLAQE
          ..................................LAQEDFMFSSDAGDDSTDGFHDICG.PNK...EL...DEETCQCVCRAG
          ..........................................................LRPASCG.PHK...EL...DRNSCQCVCKNK
          ..........................................................LFPSQCG.ANR...EF...DENTCQCVCKRT
          ....................................................CP.RNQ...PL...NPGKCACECTES
          ..................................................PQKCLLKGK...KF...HHQTCSCYRR
          ..........................................................PCTNRQKACEPGFSYSEEVCRCVPSYWKRPQMS
```

Figure 5. Amino acid sequence alignment: hVEGF family members to hPDGF-A and B

Heparin binding. $VEGF_{121}$ is a weakly acidic polypeptide that does not bind to heparin due to the lack of the heparin-binding domain encoded by exons 6 and 7. In contrast, $VEGF_{165}$ is basic and binds to heparin. $VEGF_{189}$ and $VEGF_{206}$ are even more basic and bind to heparin with greater affinity (Houck et al., 1992). The differences in affinity for heparin affect the fate of the VEGF isoforms (Houck et al., 1992; Park et al., 1993). $VEGF_{121}$ is secreted and is freely diffusible in the medium of transfected cells. $VEGF_{165}$ is also secreted but a significant fraction remains bound to heparin-containing proteoglycans. The longer forms are almost completely bound to the cell surface and extracellular matrix (ECM). They interact probably not only with heparin-like molecules, but also with cell surface proteins distinct from HSPGs via cell surface retention sequences encoded by exon 6 (Boensch et al., 1995). A release of the $VEGF_{165}$ and $VEGF_{189}$ isoforms can be achieved by heparin or heparinases. Notably $VEGF_{189}$ as such seems not to have the same angiogenic activity as the shorter VEGF isoforms (Wilting et al., 1996b), although this might be solely due to its complete sequestration. It is thought to be a latent form of VEGF, capable of being activated by proteolytic mechanisms. Plasmin is able to cleave its C-terminus after Arg 110, releasing a diffusible proteolytic fragment of 34 kDa with 100 fold reduced activity compared to VEGF (Houck et al., 1992; Park et al., 1993). Although the carboxy-terminal domain (aa 111-165) of

VEGF was reported to be critical for mitogenic activity (Keyt et al., 1996a), recombinant $VEGF_{121}$ was fully mitogenic in other studies (Kondo et al., 1995); but comparisons might be difficult due to different experimental settings.

2.4.1.4. VEGF receptors

Two tyrosine kinases, FLT1 and KDR, have been identified as VEGF receptors (de Vries et al., 1992; Terman et al., 1992a; Millauer et al., 1993; Quinn et al., 1993); Figure 6.. FLT1 (fms-like-tyrosine kinase 1) was recently renamed as VEGFR-1 (vascular endothelial growth factor receptor 1; Shibuya et al., 1990) and KDR (kinase insert domain containing receptor) as VEGFR-2 (vascular endothelial growth factor receptor 2; Terman et al., 1991). Non-tyrosine kinase receptors distinct from FLT1 and KDR have been reported recently (Soker et al., 1996).

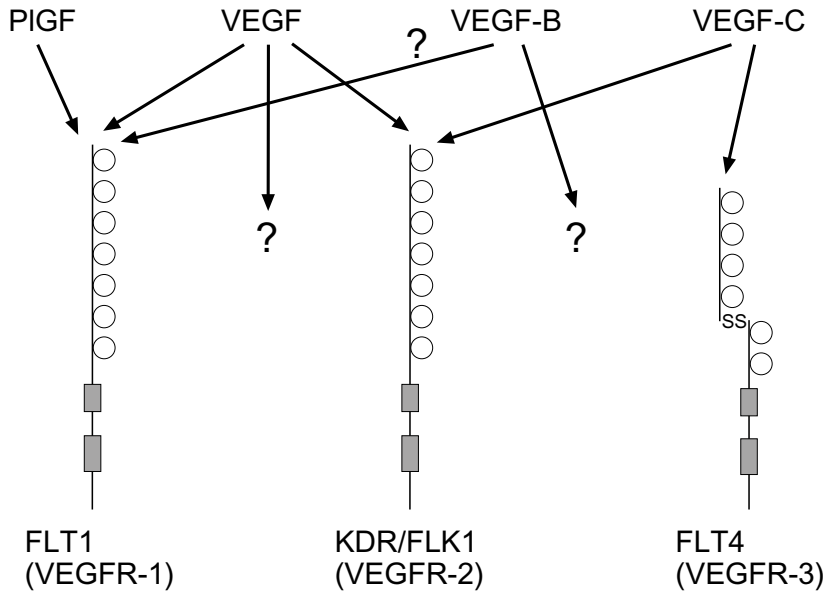

Figure 6. VEGF family members and their receptors

2.4.1.4.1. Cloning and chromosomal localisation of FLT1 and KDR

FLT1. FLT1 was originally cloned from a human genomic library using the v-ros tyrosine kinase as a hybridization probe under low stringency conditions, and the full length clone was subsequently isolated from a placental cDNA library (Shibuya et al., 1990). The mouse and rat homologues of FLT1 have also been cloned (Finnerty et al., 1993; Choi et al., 1994; Yamane et al., 1994). The FLT1 gene localises to chromosome 13q12 in humans near to the FLT3 gene (Shibuya et al., 1990). A similar linkage is seen on the mouse chromosome 5 (Rosnet et al., 1993).

KDR/FLK1. KDR was cloned from a human endothelial cell cDNA library (Terman et al., 1991), its murine homologue (named FLK1, fetal liver kinase 1) from primitive hematopoietic (Matthews et al., 1991) and embryonic neuroepithelial cells (Oelrichs et al., 1993). The rat homologue (named Tkr-III) was obtained from an aortic cDNA library (Sarzani et al., 1992). The FLK1 gene lies on mouse chromosome 5 in a cluster of class III RTKs closely linked to the genes for c-Kit and PDGF receptor A (Matthews et al., 1991). This linkage is not conserved in humans, as the genes for c-kit and PDGF receptor A map to q11-4q13, whereas KDR maps to chromosome 4q31.2-4q324 in a region devoid of any known tyrosine kinase growth factor receptor (Terman et al., 1992b).

2.4.1.4.2. Molecular characteristics of FLT1 and KDR

The FLT1 and KDR proteins belong to the PDGF class of receptor tyrosine kinases and form together with FLT4 (which does not bind VEGF) the FLT-subfamily of endothelial cell receptors (see Mustonen et al., 1995). The FLT-subfamily differs from other members of the PDGF family having 7 instead of 5 extracellular immunoglobulin-like loops in the extracellular domain. During evolution, the FLT family may have emerged via a duplication of the two immunoglobulin domains adjacent to the membrane, a mechanism already proposed for the emergence of the PDGFβ-receptor (Claesson-Welsh et al., 1988; Shibuya et al., 1990).

In spite of its name, FLT3 (also called FLK2) does not belong to the FLT-family. FLT3 is mainly expressed in hematopoietic cells and contains 5 extracellular Ig-like loops, thus being more related to c-Fms (the receptor for M-CSF/CSF-1) and the c-Kit/stem cell factor receptors of the PDGFR-family (Rosnet et al., 1991a; Rosnet et al., 1991b).

The overall amino acid sequence identity between FLT1 and KDR is 44% leading to very similar physical characteristics. The homology between the human and murine forms of both receptors is approximately 85% (Matthews et al., 1991). Both FLT1 and KDR have a hydrophobic leader peptide, seven immunoglobulin-like domains in the extracellular domain, a single transmembrane domain, and a consensus tyrosine kinase sequence interrupted by a kinase-insert domain. FLT1 and KDR share about 30% sequence identity with c-Fms (Shibuya et al., 1990; Matthews et al., 1991; Terman et al., 1991; de Vries et al., 1992; Terman et al., 1992a; Millauer et al., 1993; Quinn et al., 1993).

FLT1. FLT1 is produced as a 1338 amino acid residue precursor with a predicted 22 amino acid signal peptide. The extracellular domain of FLT1 is composed of 736 amino acids; its transmembrane spanning domain is 22 amino acids and its cytoplasmic domain is 558 amino acids long. FLT1 contains 13 potential N-glycosylation sites, several of which appear to be utilised since the observed molecular weight of 190-200 kDa in SDS-PAGE is about 25% higher than the predicted 150 kDa (Shibuya et al., 1990; de Vries et al., 1992). Recently, a cDNA encoding a truncated form of FLT1 has been cloned from HUVEC cells, which misses the seventh Ig-like domain, the cytoplasmic domain and the transmembrane sequence (Kendall et al., 1993). This soluble FLT1 protein competes with FLT1 for VEGF and might be a physiological downregulator of VEGF action.

KDR. KDR is expressed as a 1356 amino acid precursor with a predicted 19 amino acid signal peptide. Its extracellular domain is 745 amino acids long after signal peptide cleavage; its transmembrane domain is 25 amino acids and its cytoplasmic domain 567 amino acids long. KDR contains 18 potential N-linked glycosylation sites. Its observed molecular weight is 190 -200 kDa on SDS-PAGE, whereas its predicted molecular weight is only 150 kDa suggesting that, similar to FLT1, some of the glycosylation sites in KDR are actually utilised (Matthews et al., 1991; Terman et al., 1991; Terman et al., 1992a). Four tyrosine autophosphorylation sites were identified in the cytoplasmic domain of KDR, two of which are located in the kinase insert domain (Dougher-Vermazen et al., 1994). KDR does not contain classical consensus sequences for SH2 domain binding. On the other hand, the protein sequence around Y1054 and Y1059 is conserved between FLT1 and FLT4, indicating the existence of an extended recognition motif.

Binding characteristics. Both FLT1 and KDR have been shown to bind VEGF with high affinity[8], although KDR has a somewhat lower affinity for VEGF than FLT1 does. FLT4, a close homologue to FLT1 and KDR, was shown not to bind VEGF, but instead VEGF-C (Joukov et al., 1996; Lee et al., 1996). Cultured endothelial cells express 3000-60000 KDR receptors/cell and 500-3000 FLT1 receptors/cell (Bikfalvi et al., 1991; Myoken et al., 1991; Olander et al., 1991). Recently, the ligand-recognition of FLT1 was localised to the second Ig-like domain in "domain-swapping" experiments (Davis-Smyth et al., in press).

Modulation of VEGF-receptor interaction by heparan sulphate proteoglycans (HSPGs). HSPGs and other heparin-like molecules are integral components of both cell surfaces and ECM. They have been shown to mediate and influence receptor binding of several growth factors. Also VEGF receptor binding is affected by cell-surface heparan sulphates in a complex fashion, depending on the heparin binding properties of the particular VEGF isoform, on the specific VEGF receptor type involved, and on the amount and composition of heparin-like molecules that are present on the cell surface and the ECM (Gitay-Goren et al., 1992; Tessler et al., 1994). Consequently, the effect of heparin on receptor binding of VEGF *in vitro* shows a concentration optimum. E.g. FLT1 binding of VEGF was claimed to be strongly reduced already at low heparin concentrations, whereas KDR binds VEGF more strongly at high heparin concentrations (Terman et al., 1994). Notably, not only the interaction of the longer VEGF isoforms with their receptors is modulated by heparin, but also $VEGF_{121}$ binding (Cohen et al., 1995b).

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8. K_D(FLT1) = 1-20 pM (de Vries et al., 1992), K_D(KDR) = 75 pM (Plouët et al., 1990; Vaisman et al., 1990; Terman et al., 1992a; Waltenberger et al., 1994), K_D(FLK1) for hVEGF = 500-600 pM (indicating that binding is perhaps partially species-specific; Millauer et al., 1993).

Novel receptors. Binding sites for VEGF of somewhat lower affinity have been described on human endothelial and tumour cells (K_D ~200 pM), but nothing is known about the structure and function of these receptors. They interact with $VEGF_{165}$, but not with $VEGF_{121}$, and apparently exon 7 encoded sequences are important for this interaction (Soker et al., 1996; Omura et al., in preparation). In the light of both old and recent results (Clauss et al., 1990; Barleon et al., 1996) the single class of binding sites identified on mononuclear phagocytes (Shen et al., 1993) represents FLT1, although its affinity was almost two magnitudes lower than expected (K_D ~300-500 pM).

2.4.1.5. VEGF expression

VEGF expression is temporally and spatially coupled to the proliferation of blood vessels in the developing embryo (Breier et al., 1992). It starts at the same time as FLK1/KDR expression (E 7.0 in the mouse development) in the juxtaposed endoderm (Dumont et al., 1995). At E7.5 VEGF expression can be found in the entire endoderm. During further development VEGF mRNA can be found in various epithelial tissues adjacent to FLK1/KDR- and FLT1-expressing endothelium, e.g. primary vasculature, premordial heart, kidney and brain (Breier et al., 1992; Jakeman et al., 1993; Yamaguchi et al., 1993; Dumont et al., 1995). After the embryonic and postnatal development, the endothelium becomes quiescent and, correspondingly, the expression of VEGF declines to a very low level. Despite of no angiogenic activity, VEGF expression persists in adults in distinct tissues, where a specialised endothelium with an increased vessel permeability is required. It has been proposed that VEGF signalling is needed for the differentiation and maintenance of this specialised, fenestrated endothelium and perhaps generally for the “baseline” permeability of endothelial cell layers (Berse et al., 1992; Breier et al., 1992; Brown et al., 1992a; Millauer et al., 1993; Risau, 1995).

Kidney. VEGF is expressed at the time of embryonic kidney angiogenesis, but persists in the adult only in the kidney glomerular epithelium, which is adjacent to the fenestrated endothelium expressing both FLT1 and FLK1/KDR (Breier et al., 1992; Simon et al., 1995). These structures, of course, are specialised to ultrafiltration.

Brain. VEGF is transiently highly expressed in the ventricular neuroepithelium and the choroid plexus epithelium of the embryonic brain, and a VEGF concentration gradient probably induces brain vascularisation by sprouting of endothelial cells from the perineural plexus that covers the neural tube (Risau et al., 1988a; Breier et al., 1992; Millauer et al., 1993). In the adult brain the choroid plexus epithelium continues to express VEGF at modest levels, whereas elsewhere the mRNA is reduced to very low levels (Breier et al., 1992; Hatva et al., 1995). The VEGF expressing endothelium is - like in the kidney - in immediate vicinity to FLK1/KDR expressing epithelial cells, arguing for a specific role of FLK1/KDR signalling through VEGF in the maintenance of this specialised endothelium.

Placenta. VEGF expression has been observed in the developing ovarian follicles, in the capillaries of the forming corpus luteum, in the endometrium undergoing regeneration and the labyrinth region of the placenta during the implantation of the early embryo. These tissues acquire new capillary networks and VEGF seems to provide the necessary angiogenic stimulus (Phillips et al., 1990; Jakeman et al., 1992; Park et al., 1993; Shweiki et al., 1993). Strong VEGF expression by multinucleated trophoblast cells (which separate fetal and maternal blood vessels in the labyrinth region of the placenta) has been - like in the kidney - connected to the frequent fenestrations of the adjacent endothelial cells (Shweiki et al., 1993).

Liver, lung and heart. VEGF is highly expressed by epithelial cells of the adult lung and by cardiac myocytes (Berse et al., 1992). In these tissues an elevated level of the 189 amino acid isoform of VEGF can be found, suggesting, that it might have a specific function in these organs (Breier et al., 1992).

What are the producer cells? Although a great variety of normal cell types have been shown to express the VEGF mRNAs, including macrophages, neurons, myocytes and many epithelial cells (Berse et al., 1992; Ferrara et al., 1992; Peters et al., 1993), there are no reports of vascular endothelial cells expressing VEGF *in vivo* (Shifren et al., 1994). This strongly argues that VEGF is mainly a paracrine mediator of angiogenesis.

2.4.1.6. VEGF receptor expression

Whereas VEGF can be expressed by a variety of cell types, VEGF receptors are almost exclusively found on endothelial cells, both quiescent and proliferating. The expression of both VEGF receptors is regulated by cell differentiation, as well as by external stimuli. In the case of FLT1, a 1 kb fragment of the promoter has been shown to direct endothelial cell-specific gene expression (Morishita et al., 1995). A putative regulation of the VEGF receptor genes by hypoxia has been studied, but the results are not nearly as coherent as for VEGF. *In vivo*, KDR and FLT1 were upregulated upon hypoxia (Thieme et al., 1995), whereas, at least *in vitro*, KDR was downregulated (Takagi et al., in press-a). The KDR gene is unlikely to be directly responsive to hypoxia. *In vitro,* KDR upregulation was induced by medium that was conditioned by myoblasts under hypoxic conditions, whereas direct hypoxia showed no effect (Brogi et al., 1996).

2.4.1.6.1. FLT1

FLT1 expression is largely restricted to endothelial cells and their progenitors (Jakeman et al., 1992; Jakeman et al., 1993; Peters et al., 1993). It starts at E8.0 during mouse development in the extraembryonic mesoderm, probably in the cells that develop later into the spongiotrophoblast cells of the placenta, which show a very high expression of FLT1 (Dumont et al., 1995). In the embryo proper, FLT1 mRNA can be found first in the embryonic mesoderm, which gives rise to the heart and the major embryonic vessels (Yamaguchi et al., 1993). Later FLT1 is seen in the endothelial cells of the blood vessels and capillaries of the developing organs, a pattern very similar to FLK1 expression (Breier et al., 1992; Simon et al., 1995). However, in the developing placenta FLT1 and FLK1/KDR are not colocalised, since FLT1 is present in the spongiotrophoblast layer, whereas FLK1/KDR is found in the labyrinthine layer nearby the VEGF expressing epithelium. The fact that FLT1 is not always located in the immediate vicinity of VEGF expressing cells was taken to support the idea, that the short VEGF isoform might primarily interact with FLT1 in order to elicit a chemotactic response, whereas FLK1/KDR might be activated mainly by the long, cell-associated VEGF isoforms, thereby stimulating proliferation (Breier et al., 1995; Dumont et al., 1995).

In adult tissues the same local correlation is seen between VEGF and FLT1 expression. In adult rats FLT1 is most highly expressed in the lung.

2.4.1.6.2. Responses to FLT1 stimulation.

Our knowledge of the signal transduction by VEGF receptors is limited. VEGF receptors are endogenously expressed on endothelial cells and the set-up of the signal transduction machinery in endothelial cells is probably very different from other frequently used cell types like fibroblasts. Endothelial cells show among themselves a great diversity, which additionally can account for inconsistencies among the published data. Several endothelial cells express more than one VEGF receptor, which makes it difficult to ascribe a certain response to one of them (Yamane et al., 1994; Guo et al., 1995).

At present, little is know about the intracellular signalling pathways that follow binding of VEGF to FLT1 and the resulting cellular responses. FLT1 shows an exceptional behaviour: it binds VEGF with very high affinity, but many attempts failed to show its autophosphorylation upon ligand binding (de Vries et al., 1992; Waltenberger et al., 1994; Yamane et al., 1994). The only report so far of tyrosine phosphorylation comes from FLT1-overexpressing mouse fibroblast (NIH3T3) cells (Seetharam et al., 1995), but similar to FLT1-overexpressing porcine aortic endothelial (PAE) cells, no mitogenic response was seen upon phosphorylation. The failure to elicit many biological responses via FLT1 comparable to KDR/FLK1 has been independently reported in several publications and is yet unexplained (Waltenberger et al., 1994). PlGF, which also binds FLT1, fails to provoke clearcut cellular responses (Park et al., 1994). In spite of this, several intracellular transduction pathways are activated by FLT1 stimulation, including Ca^{2+} signalling (Plouët et al., 1990; Myoken et al., 1991; de Vries et al., 1992), apparently through PLCγ phosphorylation (Seetharam et al., 1995) and phosphorylation of the GAP complex. MAP kinase could not be activated by VEGF in FLT1-overexpressing NIH3T3 cells, whereas it was activated in sinusoidal liver endothelial cells (Seetharam et al., 1995), where the response might have been mediated by FLK1/KDR, which is also expressed by these cells.

The role of FLT1 signalling for vascular endothelial cells remains mysterious and to be determined. Recently, several reports showed the importance of heterodimerisation for ligands (Pötgens et al., 1994) as well as for receptors (Wrana et al., 1994; Karunagaran et al., 1996). Indeed, FLT1 homodimers might have - similar to e.g. ErbB3 - no direct ligand, and an active role of FLT1 might be confined to combinatorial interactions with other receptors.

Already some time ago it has been proposed that FLT1 might play a role in the physiological establishment and further maintenance of an organised vascular system (Shibuya et al., 1990), a function that might remain unnoticed *in vitro*, but which is supported by the FLT1 knock-out phenotype (Fong et al., 1995).

It has been also proposed that FLT1 mediates the chemotactic and FLK1/KDR the proliferative response to VEGF. Monocytes express VEGF receptors (Clauss et al., 1990) and their activation and migration seems to be mediated by VEGF signalling via FLT1[9] (Barleon et al., 1996). It is unknown whether the migrational stimulus for endothelial cells is also transduced via FLT1. Some *in vivo*-data is compatible with this hypothesis, for example the differences between the expression patterns of FLT1 and FLK1/KDR in the developing placenta (Dumont et al., 1995) and the strong overexpression of FLT1 by endothelial cells invading a healing wound (Peters et al., 1993). On the other hand, FLT1-mediated migration could never be demonstrated in response to VEGF *in vitro*. Recently, receptor-specific mutants of VEGF have been generated (Keyt et al., 1996b), which could be helpful clarifying the role of FLT1.

2.4.1.6.3. FLK1/KDR

The expression of FLK1/KDR starts very early during development (mouse E7.0) in both embryonic and extraembryonic tissues. It occurs in the embryonic mesoderm, that later gives rise to the heart and in the future yolk sac mesoderm, i.e. in regions from which the vasculature emerges. FLK1/KDR may therefore be a marker for the hemangioblast, a hypothetical precursor cell for endothelial and hematopoietic cells. VEGF on the other hand might be an early inducer of these cells, since its expression starts simultaneously with FLK1/KDR in tissues surrounding first the emerging and later the expanding vasculature.

FLK1/KDR becomes restricted to the endothelial lineage during the blood island formation (Yamaguchi et al., 1993; Dumont et al., 1995; Flamme et al., 1995a). Expression of FLK1/KDR continues in differentiated arteries, veins and lymphatic vessels, whereas it decreases in differentiated capillary endothelial cells, which maintain the option of upregulating FLK1/KDR upon stimulation by high VEGF levels (Wilting et al., 1996b).

The interplay between KDR and its ligand VEGF in the vascularisation of developing organs by angiogenic sprouting has been demonstrated for the brain and the kidney. During brain development vascular sprouts of the perineural plexus invade the neuroectoderm by an angiogenic process. During this phase VEGF is expressed in the choroid plexus and in the ventricular layer, whereas FLK1 is expressed in the angiogenic sprouts (Breier et al., 1992; Millauer et al., 1993). A similar distribution of VEGF and its receptors is seen in the developing kidney (Breier et al., 1992; Simon et al., 1995) and in animals that locally overexpress VEGF from a retroviral vector with the result of a (most probably KDR-mediated) regional hypervascularisation (Flamme et al., 1995b).

The fact that FLK1/KDR expression persists in certain differentiated vessels has lead to the concept of a dual role for VEGF, being on the one hand an angiogenic factor for certain endothelial cells, and on the other hand a survival- or maintenance factor for others (Dumont et al., 1995).

2.4.1.6.4. Responses to FLK1/KDR stimulation

VEGF binding stimulates rapid and strong tyrosine phosphorylation of FLK1/KDR (Myoken et al., 1991; Millauer et al., 1993; Quinn et al., 1993) and induces cytosolic Ca^{2+} fluxes (Bikfalvi et al., 1991; Brock et al., 1991; Quinn et al., 1993). FLK1/KDR has different signal transduction properties from FLT1, in that its activation leads to a strong biological response, that is seen in both transfected cells and in cells endogenously expressing the receptor (Millauer et al., 1993; Waltenberger et al., 1994; D'Angelo et al., 1995). KDR-transfected PAE cells and a clone of Balb/c3T3 A31 cells (a fibroblast cell line of endothelial cell morphology and behaviour) undergo edge ruffling and other changes of cell

9. Probably also $PlGF_{152}$ uses this receptor to induce monocyte migration (Barleon et al., 1996).

morphology after stimulation with VEGF, in addition to mitotic induction and chemotaxis (Enomoto et al., 1994; Waltenberger et al., 1994), whereas FLT1-transfected PAE cells do not exhibit any of these responses. The exclusive role of FLK1/KDR as the responsible receptor for mitogenesis and proliferation is underlined by the fact that mutated VEGF, which binds FLK1/KDR, but not FLT1, retains its full mitogenic activity (Keyt et al., 1996b).

It seems to be dependent on the cellular background, which signalling pathways are initiated by KDR stimulation. Tyrosine phosphorylation of PLCγ, Ras-GAP and PI3-K can be observed upon VEGF stimulation of bovine aortic endothelial cells, but not upon VEGF stimulation of KDR-transfected PAE cells. This failure might be either due to the different cellular background or, alternatively, the phosphorylation events in bovine aortic cells might have been mediated by FLT1 (Guo et al., 1995). In brain capillary endothelial cells and hepatic sinusoidal endothelial cells, KDR-stimulation by VEGF activates the MAP kinase and elicits a strong proliferative response (Yamane et al., 1994; D'Angelo et al., 1995). Although FLT1 is expressed by the same cells, at least in hepatic sinusoidal endothelial cells MAP kinase activation did not involve phosphorylation of FLT1 (Yamane et al., 1994).

2.4.1.7. VEGF and VEGF receptor expression in neovascular processes in the adult

2.4.1.7.1. Female reproductive cycle and pregnancy

Angiogenesis occurs in a hormonally regulated manner during the female reproductive cycle and pregnancy in the ovary and the uterus (reviewed in Reynolds et al., 1992). In the first third of the ovarian cycle the growing corpus luteum (CL) becomes vascularised by invading blood vessels. During luteolysis and thereafter the newly formed vessels regress. The endothelial cells change the expression pattern of adhesion molecules and detach from the basement membrane, thus leaving areas devoid of the covering cell monolayer. A second mechanism of blood vessel regression is the contraction and occlusion of arterioles and small arteries upon proliferation of smooth muscle cells (Modlich et al., 1996). Hormonally regulated angiogenesis occurs also in the proliferating and regressing endometrium and especially prominently upon implantation of the embryo (Cullinan-Bove et al., 1993).

2.4.1.7.2. Exercise induced angiogenesis

Angiogenesis in the heart as a response to physical exercise and its benefical effects have been noticed long time ago (Tomanek, 1970; Przyklenk et al., 1985). Still under discussion is the question, how the neovascular response is elicited and whether exercise-induced neovascularisation compensates completely for the increased metabolic demands of myocyte hyperplasia in the heart muscle (Mall et al., 1990; Tomanek et al., 1994).

2.4.1.7.3. Wound healing

In wound healing angiogenesis is most prominent during the first seven days after wounding, and this correlates temporarily and spatially with the expression of angiogenic factors. VEGF and its receptor FLT1 are highly overexpressed during wound healing: initially VEGF is expressed by the keratinocytes adjacent to the wound edges and later by invading keratinocytes that migrate to cover the wound surface, and also by macrophages, probably induced by hypoxia (Brown et al., 1992b; Shweiki et al., 1992; Peters et al., 1993). FLT1, on the other hand, is expressed by the endothelial cells of the invading blood vessels (Peters et al., 1993). In a diabetic mouse model delayed wound healing could be correlated with insufficient VEGF expression (Frank et al., 1995).

2.4.1.7.4. Ocular neovascularisation

Ocular neovascularisation is the most common cause of blindness and dominates many eye diseases, among others age-related macular degeneration (AMD; Bressler et al., 1994) and diabetic retinopathy (Olk et al., 1993). Stimulated by VEGF new capillaries in the retina invade the vitreous, bleed, and cause blindness (Adamis et al., 1994; Aiello et al., 1994; Malecaze et al., 1994). In a mouse model for proliferative retinopathy, hypoxia-induced VEGF expression was followed by neovascularisation (Pierce et al., 1995). Anti-angiogenic therapy (using either neutralising antibodies against VEGF or a soluble form of the FLT1 receptor) successfully inhibited ocular neovascularisation in a mouse and a monkey model (Aiello et al., 1995; Adamis et al., 1996). It is interesting that already in 1948 a theory was proposed,

which assigned a putative diffusible factor, secreted by the retina, as the cause for retinal and iris neovascularisation (Michaelson, 1948).

2.4.1.7.5. Ischemic heart and limbs

Collateral vessel formation occurs as a response of the body to arterial occlusions, e.g. after myocardial infarction in coronary heart disease (Shammas et al., 1993). The angiogenic stimulus may be partially due to the oxygen deficiency created by the clogged artery in the affected tissue. In animal models therapeutic benefits could be achieved by supporting the perfusion and neovascular response in ischemic heart or limb through intramuscular or intraarterial VEGF application (Banai et al., 1994; Takeshita et al., 1994). Recently, somatic gene therapy with a VEGF expression vector apparently resulted in angiogenesis in a human patient (Isner et al., 1996). Another potential application of VEGF gene therapy would be the limitation of restenosis after coronary angioplasty by the acceleration of healing of the endothelium with the consequent inhibition of smooth muscle cell proliferation (Finkel et al., 1995).

2.4.1.7.6. Rheumatoid arthritis

Angiogenesis associated with the proliferation of inflammatory synovial tissue is most likely VEGF-mediated. Synovial tissue and macrophages could be identified as sources of the elevated VEGF levels (Koch et al., 1994). Inhibition of angiogenesis was suggested as a possible treatment of the disease, based on an animal model (Oliver et al., 1994).

2.4.1.7.7. Skin diseases

Recent studies suggest that VEGF is a main mediator of angiogenesis in several skin disorders (Detmar et al., 1994; Brown et al., 1995b; Brown et al., 1995c). Microvascular hyperpermeability and angioproliferation - both characterising psoriatic epidermis - seem to be caused by an autocrine mechanism, in which keratinocytes produce TGF-α, which in turn induces their own VEGF expression (Detmar et al., 1994). It will be seen, whether the results on transgenic mice overexpressing VEGF in the epidermis support the concept of the role of VEGF in psoriasis (personal communication by Michael Detmar). Other pathological conditions, in which VEGF upregulation was seen in the skin include bullous pemphigoid, dermatitis herpetiformis, erythema multiforme (Brown et al., 1995b) and the delayed hypersensitivity skin reactions (Brown et al., 1995c).

2.4.1.7.8. Graves' syndrome

Recently Sato added Graves' syndrome of exophthalmic goiter to the increasingly long list of disorders, which are thought to be connected with VEGF overexpression (Sato et al., 1995a).

2.4.1.7.9. Tumour angiogenesis

Diffusion of oxygen sets an upper limit for the size of solid tumours unless the tumour meets its need of oxygen by stimulating nearby capillary endothelial cells to proliferate and to migrate towards the tumour, resulting in its vascularisation. Before switching to this angiogenic phenotype, a growing tumour reaches its size limit of several mm^3, when net proliferation and necrosis/apoptosis are balanced (Folkman, 1971; Gimbrone et al., 1972). This usually happens before diagnosis, hence such tumours/metastases are called dormant (Holmgren et al., 1995). Many molecules are involved in tumour angiogenesis (Auerbach et al., 1984; Blood et al., 1990; Klagsbrun et al., 1991; Liotta et al., 1991; Folkman et al., 1992), and the common clinical patterns in cancer metastasis have been associated with different expression patterns of angiogenic stimulators and inhibitors. Surprisingly, the release of angiostatin, a potent inhibitor of angiogenesis, is triggered by certain tumours. It has a much longer half life in the circulation than most angiogenic stimulators which might be secreted by the tumour, and consequently, vascularisation and growth of distant metastases are suppressed as long as the primary tumour is not removed (O'Reilly et al., 1994; Folkman, 1995a).

The "angiogenic switch". Both upregulation of positive angiogenic signals and downregulation of negative angiogenic signals is required for the "angiogenic switch" (Rastinejad et al., 1989). According to this balance hypothesis a quiescent endothelium is constantly and actively restrained from proliferating. Some tissues express constitutively angiogenic inducers in the absence of any new blood vessel growth, arguing for the existence of general suppressory mechanisms.

Indeed, evidence for angiogenesis suppressor genes have been presented (Parangi et al., 1995). Rather than being predetermined, the angiogenic phenotype is a discrete characteristic (similar to the loss of contact inhibition or immortalisation), for which individual cells are selected in the microevolutionary process connected with the establishment of the fully malignant phenotype (Hanahan et al., 1996). In transgenic mouse models the angiogenic switch was identified in a discrete stage of tumour progression, occurring after the development of carcinoma-in-situ cells, but prior to the appearance of solid tumours (Folkman et al., 1989; Kandel et al., 1991). Biopsies of human breast and cervical cancers of different malignancy grades revealed a remarkable similarity to the mouse models, in that an angiogenic switch was already apparent in the mid- to late dysplastic stage (Guidi et al., 1995). Although the angiogenic switch is one of the indispensable genetic lesions required for rapid tumour growth, it is not the only requirement (Graeber et al., 1996; Kinzler et al., 1996).

Proteases. Many proteases are involved in tumour angiogenesis and metastasis, since degradation of the basement membrane underlying the endothelium and the ECM are important steps in cell migration. The plasminogen system includes urokinase plasminogen activator (u-PA) and tissue type plasminogen activator (t-PA) which can cleave plasminogen to release active plasmin. Plasmin has a variety of proteolytic activities including degradation of many ECM and basement membrane components, as well as activation of certain growth factors and inhibitors by proteolytic cleavage (reviewed in Plow et al., 1995). Endothelial cells of both blood and lymphatic vessels are induced to secrete components of the plasminogen system in response to angiogenic factors like VEGF and TNF-α (Pepper et al., 1994; Mandriota et al., 1995; Koolwijk et al., 1996).

Surprisingly, in knock-out mice for u-PA, t-PA or plasminogen activator inhibitor the development of the vasculature was not affected (Carmeliet et al., 1994). On the other hand, application of urokinase receptor antagonists slowed down tumour progression in mice (Min et al., 1996).

The p53 connection. Even vascularised tumours eventually reach an equilibrium with net cell birth equalling net cell death (both necrosis and apoptosis) in the regions farthest away from the tumour vasculature (Kinzler et al., 1996). Interestingly, the hypoxia-induced apoptosis/growth arrest is p53-dependent, although the underlying mechanism is unknown (Graeber et al., 1996). Any tumour cell acquiring a p53 lesion may escape from hypoxia-induced p53-mediated apoptosis and can become a predominant clone within the tumour. Additionally the expression of negative angiogenic factors can be dependent on wild type p53, e.g. thrombospondin-1 (TSP-1`; \Dameron, 1994 #1938; Van Meir, 1994 #2087; Volpert, 1995 #2091]. On the other hand, the Ala135→Val mutant of murine p53 is able to upregulate positive angiogenic factors like VEGF (Kieser et al., 1994).

Diagnosis and prognosis. The degree of tumour vascularisation - measured directly as the microvessel density in histologic specimens or indirectly by quantitation of angiogenic proteins such as bFGF and VEGF in body fluids - has been shown to be a good prognostic marker, especially for the estimation of the risk of metastasis and disease outcome in various types of tumours (Weidner et al., 1991; Weidner et al., 1993; Li et al., 1994; Nguyen et al., 1994; Weidner, 1995). The correlation between tumour angiogenesis and the risk of metastasis is obvious, since metastases depend on angiogenesis at two stages of their development:

- Firstly, metastatic cells do not usually escape from a tumour until the tumour becomes vascularised (Liotta et al., 1974). The probability of a tumour cell escaping from the tumour into the circulation is thought to increase with the area of the vascular surface within the tumour (Folkman, 1995a).
- Secondly, upon arrival in the target organ, metastatic cells can develop only into detectable metastases if they undergo neovascularisation.

Primary tumours usually do not kill patients, since most of them can be excised by a surgeon. Patients usually die because tumour metastases occur in critical organs, and consequently disease outcome is closely linked to angiogenesis via the risk of metastasis.

VEGF overexpression by tumours. VEGF overexpression by tumours is frequently seen (Berse et al., 1992; Shweiki et al., 1992), and many human tumours overexpress additionally VEGF receptors. Reports of this include carcinomas of the

- lung (Mattern et al., 1996),
- thyroid (Viglietto et al., 1995),
- breast (Brown et al., 1995a),
- gastrointestinal tract (Brown et al., 1993b; Takahashi et al., 1995; Suzuki et al., 1996),
- kidney and bladder (Brown et al., 1993a),
- ovarian (Olson et al., 1994),
- uterine cervix (Guidi et al., 1995),

as well as:

- angiosarcoma (Hashimoto et al., 1995),
- glioblastoma multiforme (Plate et al., 1992; Berkman et al., 1993; Hatva et al., 1995),
- meningioma (Hatva et al., 1995),
- (oligo)astrocytoma (Hatva et al., 1995),
- capillary haemangioblastoma (Berkman et al., 1993).

VEGF expression is seen usually by tumour cells. The tumour associated vasculature may express FLK1/KDR at elevated levels. These patterns correlate with the degree of vascularisation of the tumour (Berse et al., 1992; Plate et al., 1992; Berkman et al., 1993; Brown et al., 1993a; Hatva et al., 1995; Takahashi et al., 1995; Warren et al., 1995). Generally, VEGF is a paracrine stimulator of endothelial cells in tumours. In angiosarcoma, however, VEGF probably acts as an autocrine factor (Hashimoto et al., 1995). Tumour-infiltrating inflammatory cells have been proposed as an additional source of VEGF (Freeman et al., 1995).

The involvement of VEGF and its receptors in tumour progression has been studied in several animal models:

Implantation of VEGF-expressing tumourigenic cancer cells into nude mice resulted in highly vascularised tumours. The size and vascularisation of the tumours could be significantly reduced by application of anti-VEGF monoclonal antibodies. This therapeutic effect seemed to be a result of inhibited tumour angiogenesis, since the proliferation rate of tumours cells itself was not downregulated (Kim et al., 1993; Warren et al., 1995). Similarly, $VEGF_{121}$-transfected human MCF-7 breast carcinoma cells gave rise to more vascularised and faster growing tumours in comparison with implanted, untransfected MCF-7 cells, although the transfected cells had no growth advantage *in vitro* (Zhang et al., 1995). A direct visualisation of *in vivo* tumour growth blockade by VEGF antibodies was recently presented (Borgström et al., 1996).

The dependence of FLK1 signalling in tumour angiogenesis was demonstrated in nude mice bearing implanted glioblastoma cells. A retrovirally introduced dominant-negative soluble FLK1 mutant (lacking the cytoplasmic domain) successfully inhibited tumour angiogenesis and progression (Millauer et al., 1994). Much less is known about the role of FLT1 in tumour vascularisation. However, FLT1 does not seem to be frequently upregulated in tumours.

Therapy. The growing knowledge about angiogenesis in tumour progression has guided therapeutic approaches to stop tumour progression by inhibiting angiogenesis, some of which were already outlined above. Several of the anti-angiogenic components mentioned in Table 2 are in clinical trials. The ultimate goal is to archive a high selectivity of action, for which a thorough knowledge of all involved players is needed. Different steps of the angiogenic process are targeted, primarily the balance between positive and negative angiogenic regulators and cell-matrix interactions (reviewed in Folkman, 1995b).

2.4.1.7.10. Tumours derived from vascular endothelium

Hemangiomas. Hemangiomas are non-malignant tumours of blood vessels characterised by an extended network of capillary vessels with large lumens. Although they mostly need no treatment, because they regress after an initial proliferating phase, the tissue damage can be serious if they interfere with vital organs. Several angiogenic proteins are overexpressed by hemangiomas, e.g. bFGF. In case corticosteroid therapy fails, anti-angiogenic therapy with interferon-α2a (a bFGF antagonist) is perhaps the best alternative (Folkman, 1995b).

Lymphangiomas. Lymphangiomas are congenital lymphatic malformations characterised by enlarged fluid-filled lymphatic vessels. They are benign in character and surgical excision is the treatment of choice (Zadvinskis et al., 1992). Although several theories have been proposed to explain the pathogenesis of lymphangiomas (Lee, 1980; Philips, 1981; Weingast et al., 1988), the molecular basis of the uncontrolled growth of the lymphatic vascular endothelium is unknown. Interestingly, FLT4 and its ligand might play a role in the pathogenesis (this work).

2.4.1.8. Biological roles of VEGF signalling

Different approaches have been undertaken to resolve what is commonly addressed as the "function" of VEGF. The expression pattern of VEGF and its receptors, its biological activities and the phenotype of transgenic animals convincingly establish VEGF as the major regulator in the development and maintenance of the vascular system.

Mitogenicity/angiogenicity. The mitogenic activity of VEGF has first been demonstrated towards capillary endothelial cells. *In vitro* half-maximal stimulation of bovine capillary endothelial cell growth was obtained at 100-300 pg/ml (2-6 pM) and a maximal stimulation at 1-5 ng/ml (22-110 pM; Ferrara et al., 1989; Plouët et al., 1989). VEGF has no appreciable mitogenic activity on non-vascular endothelial cell types: fibroblasts, smooth muscle cells, epithelial cells or endothelial cells of the lymphatic vascular system do not respond to VEGF (Connolly et al., 1989a; Ferrara et al., 1989; Plouët et al., 1989; Wilting et al., 1996b; Oh et al., 1997). VEGF exerts its mitogenic activity in a paracrine manner (Qu et al., 1995). This model is supported by the co-localisation of producer cells and target cells in both pathological and physiological circumstances and its short half-life of 3 minutes in the circulation (Folkman, 1995a). *In vitro*, however, an autocrine loop has been identified in some cells (Guerrin et al., 1995; Nomura et al., 1995). The *in vivo* angiogenic potential of VEGF has been shown in different assays, e.g. on the chorioallantoic membrane (CAM) assay (Leung et al., 1989; Plouët et al., 1989; Wilting et al., 1991; Wilting et al., 1996b) and the rabbit cornea assay (Phillips et al., 1994).

Induction of vascular permeability. VEGF has both permeability inducing and mitogenic activity (Shibuya, 1995). On a weight-to-weight basis VEGF is 50,000 times more potent than histamine in increasing vascular permeability (Dvorak et al., 1995b), and it does not work via histamine release from mast-cells (Gruber et al., 1995). Instead VEGF triggers directly fenestration of endothelial cells, even of those resistant to classical inflammatory mediators such as platelet activating factor (PAF) or fibrin breakdown products (Roberts et al., 1995). Related effects of VEGF on the cardiovascular system are vasodilatation *in vitro* (Ku et al., 1993; Doi et al., 1996), resulting in tachycardia and hypotension *in vivo* (Yang et al., 1996).

In vivo the mitogenic and permeability-inducing activities of VEGF are closely linked (Dvorak et al., 1995b). Apart from VEGF, no other angiogenic factor is able to induce vascular hyperpermeability, which indicates that VEGF could be a general "immediate" angiogenic agent, which is activated by indirect angiogenic factors, a mechanism that was shown already for e.g. TGF-β (Pertovaara et al., 1994); see Figure 4..

Migration and differentiation. VEGF is able to promote chemotaxis of endothelial cells (Koch et al., 1994), but also some cells of the myeloid lineage (e.g. monocytes and mast cells) and smooth muscle cells respond to VEGF with migration (Clauss et al., 1990; Koch et al., 1994; Gruber et al., 1995; Grosskreutz et al., 1996). Osteoblasts differentiate in response to VEGF (Midy et al., 1994).

Development. The importance of VEGF-mediated growth and differentiation signals in the development of the vasculature has been shown in knock-out mice. In animals lacking either FLT1, FLK1 or VEGF vascular development was arrested during early embryogenesis (Fong et al., 1995; Shalaby et al., 1995). The developmental block occurred at different stages upon loss of the gene, supporting the view that the signals transmitted by FLT1 and FLK1/KDR are distinct, and that besides VEGF additional factors are capable of stimulating FLK1/KDR.

FLK1 knock-out. Consistent with the early expression of FLK1 in endothelial/hematopoietic cell precursors, the differentiation of both endothelial and hematopoietic cell lineages from hemangioblasts was completely blocked in the embryos lacking FLK1 receptors. No endothelial cells nor hematopoietic cells were present in homozygous animals and the embryos died between E8.5 and E9.5 of development (Shalaby et al., 1995).

FLT1 knock-out. FLT1 does not affect the differentiation or proliferation of endothelial or hematopoietic cells, but instead results in the formation of a disorganised vasculature, which leads to death from embryonic day E8.5 onwards. In the yolk sac and in the embryo proper large vascular channels developed instead of organised blood islands. This migrational misbehaviour was assigned to altered cell-cell or cell-matrix interactions (Fong et al., 1995).

VEGF. The phenotype of transgenic animals overexpressing VEGF in specific tissues substantiates the idea of VEGF being a major regulator of vascularisation in embryogenesis and of maintenance of vasculature in certain organs (Flamme et al., 1995b). Vice versa, appropriately timed vascularisation might be crucial for the proper morphogenesis of certain organs like the lung (Zeng et al., 1996). Quail embryos treated with recombinant VEGF during vasculogenesis appeared similar to FLT1 knock-out mice. The organisation of the vasculature of VEGF-treated embryos was severely affected showing numerous extra vessels and enlarged lumens of fused vessels (Drake et al., 1995). The effect of exogenous VEGF was seen already 5 h after injection[10], supporting the conclusion drawn from the FLT1 knock-out studies, that in addition to endothelial cell proliferation VEGF influences migrational patterns of endothelial cells and restricts vascularisation by its paracrine manner of action.

VEGF knock-out. Loss of a single VEGF allele is lethal in the mouse embryo between E10.5 and E12, thus being the only known lethal heterozygous autosomal knock-out. It seems that VEGF levels have to be tightly regulated during vasculogenesis. Most steps of the early vascular and hematopoietic development were impaired, although not abolished and numerous abnormalities occurred in organogenesis connected with the failure of blood-vessel ingrowth, lumen formation, large vessel formation and the establishment of interconnections. In contrast to FLK1 knock-outs, homozygous VEGF-deficient embryos survive until E10.5, implying the possibility of a partial activation of FLK1 by another ligand (Carmeliet et al., 1996; Ferrara et al., 1996), perhaps VEGF-C (Joukov et al., 1996).

Tie/Tek. The development of an intact and properly organised vasculature was also inhibited in mice deficient of Tie or Tek/Tie-2, two other RTKs, that are restricted to endothelial cells (Dumont et al., 1994; Puri et al., 1995; Sato et al., 1995b).

Induction of other genes by VEGF. VEGF increases or reduces the synthesis of several other proteins/peptides in vascular endothelial cells, most of which have been implied in playing a role in angiogenesis: serine proteases, urokinase-type and tissue-type plasminogen activators (uPA and tPA), PA inhibitor 1 (PA-1) and urokinase-type plasminogen activator receptor (uPAR; Pepper et al., 1991; Pepper et al., 1994; Mandriota et al., 1995). VEGF induces synthesis of interstitial collagenase in human umbilical vein endothelial cells but not in dermal fibroblasts (Unemori et al., 1992). The coexpression of PAs, their cognate receptors and collagenase by VEGF is expected to break the basement membrane and to alter the ECM, thus facilitating migration of endothelial cells, a fundamental step in the angiogenesis cascade (Folkman et al., 1987); see 2.3.1.1..

The relaxing effect of VEGF on smooth muscle cells is indirectly mediated via nitric oxide/endothelium-derived relaxing factor (EDRF; Ku et al., 1993)and a downregulation of the synthesis of the C-type natriuretic peptide (CNP) in ECs (Doi et al., 1996).

2.4.1.9. Regulation of VEGF gene expression

The VEGF gene can be induced by phorbol esters like PMA and TPA (Tischer et al., 1989; Garrido et al., 1993; Finkenzeller et al., 1995), PDGF and cAMP analogues, consistent with the presence of binding sites for the transcription factors AP-1 and AP-2, suggesting that VEGF expression is controlled by protein kinase A- and C-mediated signal transduction pathways (Garrido et al., 1993; Finkenzeller et al., 1995).

Hypoxia-inducibility. The most prominent feature of VEGF regulation is the upregulation of VEGF by hypoxia involving a putative oxygen sensitive heme-protein, whose reduced state can be mimicked by cobalt or manganese. Hypoxia occurs in wounds, ischemic organs or around necrotic areas of tumours. VEGF is the only angiogenic factor for which

10. Certain angiogenic factors are able to induce limited neovascularisation without endothelial cell proliferation solely by migrational rearrangements of the existing vasculature (Sholley et al., 1984).

hypoxia-inducibility has been shown[11]. Upregulation of VEGF by hypoxia has been shown both *in vitro* and *in vivo* (Shweiki et al., 1992; Ladoux et al., 1993) and works probably by a mechanism similar to the upregulation of erythropoeitin (Epo) by hypoxia[12] (Goldberg et al., 1994). Glucose deprivation is able to upregulate VEGF expression to a similar extent as hypoxia does, although when both inducers are applied simultaneously, no change of VEGF expression occurs (Shweiki et al., 1995)[13]. The induction of VEGF in response to both hypoxic and hypoglycemic stress requires several hours and new protein synthesis; these parameters distinguish VEGF from protein products of immediate early genes like c-Myc and c-Fos (Stein et al., 1995).

Teleologically speaking, the ultimate aim of VEGF upregulation by hypoxia or hypoglycemia is to eliminate the hypoxic or hypoglycemic condition. In the retina transient hypoxia during development seems to induce physiological vascularisation; normal oxygen levels reached upon sufficient perfusion of the newly vascularised tissue correspondingly seem to downregulate VEGF expression (Alon et al., 1995; Stone et al., 1995).

mRNA stabilisation. Hypoxia and hypoglycemia mainly affect VEGF mRNA stability and to a smaller extent increase the transcription rate (Goldberg et al., 1994; Finkenzeller et al., 1995; Ikeda et al., 1995; Levy et al., 1995; Stein et al., 1995). The mechanisms underlying the prolonged VEGF mRNA half life in hypoxia are not known, but the 3'-UTR of the rat VEGF gene has been found to contain sequence motifs that probably participate in the regulation (Levy et al., 1995).

Transcriptional activation. The transcriptional activation is thought to occur through binding of a hypoxia-inducible factor (HIF-1) to a 28-bp element (hypoxia-induced enhancer), which is present in the VEGF promoter region as well as in the 3'-flanking region in the Epo gene (Levy et al., 1995). Increased promoter activity under hypoxic conditions and binding of hypoxia-regulated factors in an oxygen-regulated manner have been demonstrated (Levy et al., 1995; Liu et al., 1995), but the overall significance of transcriptional activation in hypoxia-induced upregulation of VEGF is still being questioned (Shima et al., 1995b).

The angiogenic activity of nicotinamide and its analogues (Kull et al., 1987) was recently clarified by the finding that adenosine accumulation and the resulting elevation of intracellular cAMP levels are involved in the hypoxic upregulation of VEGF mRNA levels (Hashimoto et al., 1994; Fischer et al., 1995; Takagi et al., in press-b, a). Protein kinase A and C (Claffey et al., 1992; Finkenzeller et al., 1995; Takagi et al., in press-b, a), c-Src tyrosine kinase (Mukhopadhyay et al., 1995) and, at least in transformed cells, Ras (Rak et al., 1995) have been shown to participate in the signalling that induces VEGF.

Upregulation by inflammatory mediators and growth factors. Numerous inflammatory mediators and growth factors are able to upregulate VEGF. Therefore many of them have been shown to be indirect angiogenic factors (compare with Table 1). Synergism in the induction of angiogenesis has been demonstrated between bFGF, TGF-β and VEGF (Pepper et al., 1992; Goto et al., 1993; Pepper et al., 1993; Asahara et al., 1995). Because VEGF and FGF seem to accompany many types of angiogenic tumours, it was assumed that VEGF and FGF complement each other in a yet unknown way to induce tumour angiogenesis. Aside from its direct effect on endothelial cells, bFGF upregulates VEGF and the VEGF receptor FLK1, which might explain its high angiogenic potential (Flamme et al., 1995b). A list of VEGF inducers includes:

- EGF, TGF-α, TGF-β, KGF and TNF-α in human keratinocytes (Detmar et al., 1994; Frank et al., 1995),
- PDGF in smooth muscle cells and fibroblasts (Finkenzeller et al., 1992; Brogi et al., 1994),
- EGF in malignant glioma cells (Goldman et al., 1993),
- Thyroid-stimulating hormone (TSH) in thyroid follicle cells (Sato et al., 1995a),
- Thromboplastin/tissue factor in transfected meth-A sarcoma cells (Zhang et al., 1994),

11. bFGF does not respond to hypoxia (Shima et al., 1995a; Shweiki et al., 1995).
12. Different, but partially overlapping mechanisms for hypoxic upregulation exist, although their relationship to each other is not completely understood. Hypoxic VEGF upregulation can be experimentally distinguished from the upregulation of the cardiac heme oxygenase-1 (Eyssen-Hernandez et al., 1996).
13. VEGF in turn upregulates glucose transport through endothelial cells (Pekala et al., 1990).

- bFGF and IL-1β in smooth muscle cells (Li et al., 1995; Stavri et al., 1995),
- EGF, PDGF-BB, and bFGF in human glioma cell lines (Tsai et al., 1995),
- IL-1α, and TGF-β in fibroblasts (Brogi et al., 1994; Pertovaara et al., 1994; Ben-Av et al., 1995),
- PGE_2 in osteoblasts and fibroblasts (Ben-Av et al., 1995; Harada et al., 1995),
- estradiol in endometrial carcinoma cells (Charnock-Jones et al., 1993),
- Luteotrophic hormone (LH) in ovarian granulosa cells (Garrido et al., 1993),
- IL-6 in several cell lines (Cohen et al., 1996).

2.4.2. Placenta growth factor (PlGF)

The placenta growth factor (PlGF) was cloned from a placental DNA library. Sequence analysis revealed a 53% identity with VEGF in the PDGF homology domain (Maglione et al., 1991). The molecular and genomic structure of PlGF resembles that of VEGF: PlGF is a dimeric glycoprotein, alternative splicing creates two isoforms ($PlGF_{131}$/PlGF-1 and $PlGF_{152}$/PlGF-2[14]), which differ by the basic domain encoded by alternative exon 6. Mature rat PlGF (the equivalent to human $PlGF_{152}$) shows a heterogeneous NH_2-end, due to differential signal peptide cleavage or additional proteolytic processing, resulting in mature peptides of 132 and 135 aminio acids (PlGF131 and PlGF152; DiSalvo et al., 1995). Because of the absence of the heparin-binding domain, $PlGF_{131}$ is a soluble factor similar to $VEGF_{121}$, whereas $PlGF_{152}$ binds to cell surface and ECM HSPGs. PlGF is highly expressed only in placenta (Hauser et al., 1993; Kaipainen et al., 1993; Maglione et al., 1993), but its biological function is unknown. PlGF may have some mitogenic activity towards certain endothelial cells (Maglione et al., 1991; Hauser et al., 1993).

PlGF binds to the FLT1 receptor with high affinity, but fails to elicit a mitogenic response in endothelial cells known to express FLT1. However, heterodimers between PlGF and VEGF do occur naturally and endogenous PlGF/VEGF heterodimers from a rat glioma cell line showed mitogenic activity towards endothelial cells. This activity was only 3 to 7 times weaker than that of VEGF homodimers (Park et al., 1994; DiSalvo et al., 1995). Thus heterodimer formation might be a negative regulatory mechanism for VEGF expression (Cao et al., 1996). In contrast to VEGF, PlGF seems to be moderately downregulated by hypoxia (Gleadle et al., 1995).

2.4.3. VEGF-B[15]

2.4.3.1. Cloning of VEGF-B

A partial murine cDNA for VEGF-B was discovered when searching for new retinoid acid binding proteins from a murine library by the yeast two-hybrid method. Subsequently, the murine full length cDNA was obtained from an adult heart cDNA library. Homologous human sequences were cloned from the fibrosarcoma cDNA library HT-1080 and the whole coding region from an erythroleukemia cell cDNA library. Genomic clones were also obtained for both species.

Independently from the above described discovery, Grimmond et al. used a chromosome 11q13 specific cosmid probe to isolate the human and mouse VEGF related factor (VRF) gene, which then appeared to be identical with VEGF-B (Grimmond et al., 1996).

2.4.3.2. Genomic structure and splice variants of VEGF-B

Human VEGF-B maps to chromosome 11q13 (Paavonen et al., 1996). It contains 7 exons, and at least two different isoforms with unrelated C-terminal domains ($VEGF\text{-}B_{167}$ and $VEGF\text{-}B_{186}$) are generated by alternative splicing of exon 6 due to the use of distinct splice acceptor sites. Unlike in the alternative splicing of the VEGF mRNA, the alternative

14. Due to a wrongly predicted signal peptide cleavage site $PlGF_{131}$ appears also as $PlGF_{129}$, and $PlGF_{152}$ as $PlGF_{149}$ (Hauser et al., 1993; Maglione et al., 1993).
15. If not differently indicated, the presented data is based on the publications from Birgitta Olofsson (Olofsson et al., 1996a; Olofsson et al., 1996b) and on personal communication by Katri Pajusola, Suvi Taira and Birgitta Olofsson.

splice-acceptor site of VEGF-B exon 6 results in a frameshift and thereby in two overlapping reading frames (rarely seen among higher eukaryotes) coding for the distinct non-homologous C-termini of VEGF-B_{186} and VEGF-B_{167}, the C-terminus of VEGF-B_{186} being much more hydrophobic than the C-terminus of VEGF-B_{167}.

The genomic structure of the VEGF-B gene is highly conserved between humans and mice (compare Figures 17 and 18) and further resembles closely the structure of the VEGF gene. The main difference is that VEGF-B lacks a homologue of exon 6 of VEGF. Thus, exon 6B of VEGF-B corresponds to exon 7 of VEGF (Figure 7.). Both code for the heparin-binding domain, of which VEGF-B_{186} is devoid due to the frameshift by alternative splicing.

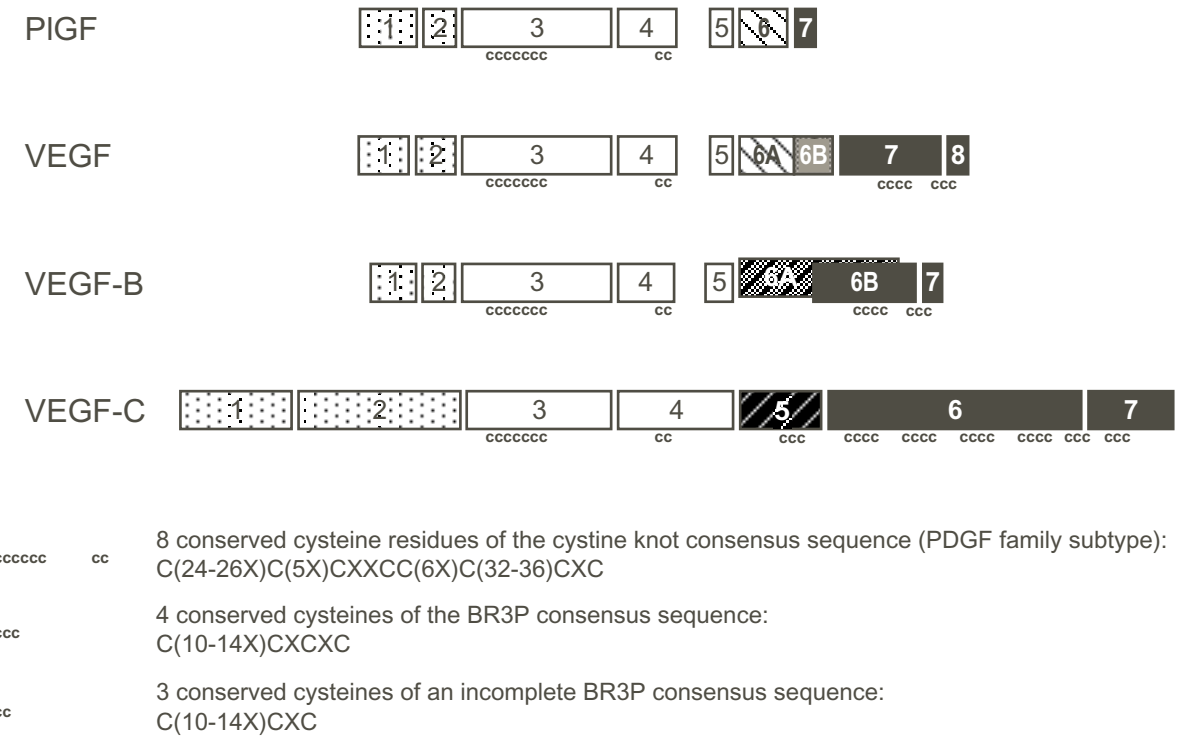

Figure 7. Homologous exons and conserved cysteine residues in the VEGF family

2.4.3.3. Molecular characterisation of VEGF-B

The two secreted murine and human isoforms of VEGF-B are 186 and 167 amino acids long. They show electrophoretic mobilities of 21 and 32 kDa under reducing conditions. Amino acid sequence homology is about 88% between mouse and human VEGF-B with highest divergence in the terminal regions of the protein. The pairwise comparison with other members of the PDGF family revealed that its closest relative is $VEGF_{164/165}$ with approximately 43% amino acid sequence homology. The homology of mVEGF-B_{167} to $PlGF_{131}$ is approximately 30% and to PDGF-A and B approximately 20% (amino acid sequence alignment in Figure 5.).

The cysteine motif defining PDGF family members (PXCVXXXRCXGCC) is present in VEGF-B. VEGF-B forms disulphide-linked homodimers and heterodimers with VEGF. VEGF-B_{167} remains cell surface or ECM-associated after secretion by 293 EBNA cells, but can be released by heparin treatment. VEGF-B_{186} is secreted in a soluble form by Cos-1 cells, probably because it lacks the basic domain encoded by exon 6B.

The glycosylation pattern of VEGF-B is unique in the PDGF family. Unlike other growth factors of this family no N-glycosylation occurs in VEGF-B, whereas VEGF-B_{186} is the only PDGF family member that is O-glycosylated. This is rare among growth factors generally (Hudgins et al., 1992; Lu et al., 1995).

2.4.3.4. VEGF-B receptor(s)

The receptor for VEGF-B remains to be identified. Exon 6B of VEGF-B reveals a high homology with exon 7 of VEGF, which recently has been shown to code for a domain mediating the interaction with a novel class of VEGF receptors

(Soker et al., 1996). However, VEGF-B binding to such receptors has not been reported to date. Two of the three acidic determinant amino acids for FLT1 binding of VEGF (Asp63 and Glu64) are conserved in VEGF-B, suggesting that VEGF-B could bind to FLT1. VEGF-B was shown to induce proliferation of FLT1- and KDR-expressing HUVEC and KDR-expressing bovine capillary endothelial (BCE) cells.

2.4.3.5. VEGF-B expression

VEGF-B is abundantly expressed in cardiac and skeletal muscle. Its expression pattern is partly overlapping with VEGF, thus showing that heterodimerisation between VEGF and VEGF-B might be of biological significance in regulating the release, bioavailability and signalling properties of VEGF. The two isoforms seem to be present in equimolar amounts in various tissues.

VEGF-B gene expression starts early during embryonic development. At day 14 p.c. VEGF-B is expressed in most cells of murine embryos. Spinal cord, cerebral cortex and especially the heart display much higher expression levels than other tissues and around day 17 p.c. significant expression is seen mostly in the heart, brown fat tissue and the spinal cord (Lagercrantz et al., 1996).

2.4.3.6. Biological roles of VEGF-B signalling

VEGF-B is an endothelial cell mitogen as shown with experiments on HUVEC and BCE cells. Its tissue distribution implies a paracrine fashion of action similar to that of many other growth factors. The tight association of VEGF-B_{167} with the cell surface indicates either a requirement for direct cell-cell interactions for stimulation or is a means of regulating the bioavailability of VEGF-B (similar to VEGF). VEGF-B-mediated stimulation through direct cell-cell interactions may guide migrating endothelial cells and organise the correct spatial distribution of the vascular network in the development and maintenance of organs.

2.4.4. VEGF-C[16]

2.4.4.1. Cloning of VEGF-C

VEGF-C was first purified from the supernatant of PC-3 human prostatic adenocarcinoma cells based on its activity to bind and to stimulate the autophosphorylation of FLT4 expressed in transfected NIH3T3 cells. Its cDNA was cloned subsequently from a PC-3 library, both human and murine genomic sequences were obtained from the EMBL3 library. Independently, a matching cDNA sequence was later reported and named VRP (vascular endothelial growth factor related protein). This cDNA was obtained from a human glioma library by screening with a probe derived from an expressed sequence tag (EST), that showed 36% homology to VEGF (Lee et al., 1996).

2.4.4.2. Genomic structure and splice variants of VEGF-C

The genes for human and mouse VEGF-C are very similar to each other. All exon lengths are conserved except for exon 2, where the human gene has a 12 nucleotide insertion (compare Figure 20. with Figure 21.). Compared to other genes of the VEGF family the VEGF-C gene is much larger and encompasses at least 35 kb. The VEGF-C gene has one exon less than the VEGF gene. It lacks sequences that correspond to the VEGF exon 6 and confer heparin binding to the protein.

Three mRNA splice variants have been reported for the human VEGF-C gene (Lee et al., 1996) and two for the murine gene. These shorter mRNAs are rare species and it is not known whether they are translated.

2.4.4.3. Molecular characterisation of VEGF-C

Biologically active VEGF-C migrates as a 23 kDa band on SDS-PAGE under reducing conditions. The amino acid sequence of VEGF-C shows in its N-terminal half homology to VEGF and related proteins. In the VEGF-homology

16. If not differently indicated, all presented data is based on the publications by Vladimir Joukov (Joukov et al., 1996)and Eola Kukk (Kukk et al., 1996) and on personal communications by Vladimir Joukov, Eola Kukk, Suvi Taira and Dmitry Chilov.

domain (encoded by exon 3 and 4 in both VEGF and VEGF-C) VEGF-C is 30% identical with $VEGF_{165}$, 27% with $VEGF\text{-}B_{167}$, 25% with $PlGF_{131}$ and approximately 22-24% to PDGF-A and PDGF-B. The C-terminal half of VEGF-C is characterised by four complete and three incomplete cysteine repeats, which are encoded by exon 6. Cysteine repeats of this type were first identified from the larval saliva of the midge in the Balbiani ring 3 protein (BR3P), which plays a constitutive role in the formation of the silk-like fibres of the salival secretory proteins. Interestingly, also in the very N-terminal part of VEGF the BR3P motif can be identified (Figure 5. and Figure 7.).

Processing. VEGF-C is synthesised as a prepropeptide and cleaved after removal of the signal peptide at least in two positions. The resulting fragments give rise to multiple forms of monomers and dimers apart from the biologically active 23 kDa form, among which the 32 kDa form is the most prominent one. Depending on the expression system variable relative amounts of the different forms are produced.

2.4.4.4. VEGF-C receptors

VEGF-C, having been isolated as the FLT4 ligand, binds and stimulates FLT4 (fms-like tyrosine kinase 4, also called VEGFR-3). Reports concerning the second putative VEGF-C receptor (KDR/FLK1) are contradictory. Although VEGF-C is probably capable of activating KDR/FLK1, little is known about the significance of this interaction. A partial activation of FLK1 by VEGF-C might be important during early vascular development, and it might be responsible for the differences between the VEGF and FLK1 knockout phenotypes. KDR/FLK1 has been described as a receptor for VEGF under 2.4.1.4.

2.4.4.4.1. Cloning and chromosomal localisation of FLT4

FLT4 was discovered from a human erythroleukemia (HEL) cell cDNA library (Aprelikova et al., 1992; Pajusola et al., 1992), as well as from a placental cDNA library (Galland et al., 1992; Galland et al., 1993). The mouse and quail homologues of FLT4 (mFLT4 and Quek2 = quail endothelial kinase 2) have been cloned from embryonic cDNA libraries (Eichmann et al., 1993; Finnerty et al., 1993). The gene for human FLT4 is located in chromosome 5q35 telomeric to the genes for PDGFR-B and Fms and centromeric to the FGFR4 gene, a region affected by several translocations. In these translocations the FLT4 gene stays intact, whereas the ALK RTK becomes activated (Warrington et al., 1992; Armstrong et al., 1993; Morris et al., 1994).

2.4.4.4.2. Molecular characteristics of FLT4

Having seven extracellular Ig like domains, FLT4 is a member of the FLT subfamily within the PDGF class of receptor tyrosine kinases and thus it shares many features with FLT1 and KDR. FLT4 is about 35% homologous with FLT1 and KDR in its extracellular domain, to about 80% in its kinase domain, but differs greatly in its kinase insert and C-terminus (Pajusola et al., 1992; Finnerty et al., 1993; Pajusola et al., 1994).

In humans (but not in mice; Galland et al., 1993) two isoforms of FLT4 are generated by alternative mRNA polyadenylation and splicing. These isoforms differ mainly in the length of their C-terminal tails (Pajusola et al., 1993). Only the longer isoform (FLT4l) seems to be the biologically active one, probably due to a tyrosine residue (Y1337), which is involved in the receptor autophosphorylation, but which is absent in the shorter form (FLT4s; Fournier et al., 1995).

FLT4l is produced as a 195 kDa glycosylated precursor, which is proteolytically cleaved into fragments of 125 and 70 kDa. The 125 kDa C-terminal fragment spans the cell membrane and contains the tyrosine kinase domain, whereas the 70 kDa N-terminal fragment forms a major part of the extracellular domain. These fragments are held together by disulphide bonding. In reducing SDS-PAGE bands of 195, 175, 125 and 70 kDa can be detected, which correspond to the fully glycosylated unprocessed form[17], the unglycosylated unprocessed forms and the two cleavage products of the mature receptor (Pajusola et al., 1994).

17. Endogenous FLT4 undergoes complete processing and therefore the 195 kDa band is frequently not seen (Pajusola et al., 1994).

2.4.4.5. VEGF-C and VEGF-C receptor expression

2.4.4.5.1. VEGF-C

VEGF-C is expressed both in embryonic and in adult mice. It is strongly expressed on day 7 p.c., before the onset of the expression of its main receptor FLT4. This suggests that VEGF-C interacts also *in vivo* with KDR/FLK1, as proposed (Joukov et al., 1996).

VEGF-C is seen in developing perinephric, mesenterial and jugular regions as well as in the non-neural parts of the cephalic region and becomes - like its main receptor FLT4 - restricted to endothelial structures, from which the lymphatic vessels originate according to the theory of Sabin. In the mesenterium, which is rich in developing lymphatic vessels, VEGF-C is strongly expressed by mesenchymal cells adjacent to endothelia that express its receptor. A paracrine action in the regulation of lymphatic angiogenesis is therefore presumed (Kukk et al., 1996).

2.4.4.5.2. FLT4 and FLK1/KDR

The expression of FLT4 starts on day E8.5 of mouse development, when it can be seen in the angioblasts of the head mesenchyme, in the cardinal vein and extraembryonically in the allantois (Kaipainen et al., 1995). Similarly, the onset of expression of its quail homologue Quek2 occurs later and recedes earlier than that of Quekl, which is homologous to both FLT1 and KDR/FLK1 (Eichmann et al., 1993). Quek2 is expressed in almost all endothelial cells of 2 day-old quail embryos. During further development, the expression pattern of FLT4/Quek2 becomes more restricted. FLT4 is seen in veins on E8.5 and on E12.5, FLT4 expression concentrates in the lymphatic premordia, lymph sacs and sprouting lymphatic vessels (Kaipainen et al., 1995). Later, on days E14.5 and E16.5, the endothelium of the main lymphatic vessel, the thoracic duct, as well as many other smaller vessels devoid of red blood cells were found to express FLT4. From adult human tissues only the lymphatic endothelia and some high endothelial venules maintain FLT4 expression (Kaipainen et al., 1995). Similarly, in differentiated CAM FLT4 expression is restricted to the endothelial cells of lymphatic vessels (Wilting et al., 1996b). FLT4 has not been detected in malignant primary tumours *in vivo*, but increased expression was seen in metastatic lymph nodes and in lymphangiomas (Hatva et al., 1995; Kaipainen et al., 1995).

2.4.4.6. Biological roles of VEGF-C signalling

Little is known about the biological function of VEGF-C. *In vitro* VEGF-C stimulates migration of bovine capillary endothelial cells (Joukov et al., 1996) and mitogenesis of human lung endothelial cells (Lee et al., 1996). Based upon their expression patterns, VEGF-C and its receptor are thought to play a general role in early vascular development and a more specialised role in the development of the venous and in particular the lymphatic vascular system. My own work adds proof to this point (see 5.1.6.) in demonstrating that the biological function of VEGF-C is targeted to the lymphatic system.

Before discovery of its ligand, the intracellular signal transduction pathways following FLT4 activation were studied with receptor chimeras, which could be activated by heterologous ligands (Pajusola et al., 1994; Borg et al., 1995; Fournier et al., 1995). In these studies SHC and GRB2 were shown to interact strongly with FLT4, although SHC interacted probably not via its SH2 domain (Fournier et al., 1995). No interaction was seen with Ras-GAP, the p85 PI3-K subunit or c-Src. In spite of efficient activation by the heterologous ligand CSF-1, the chimeric receptor elicited a mitogenic response only in NIH3T3 cells, whereas no response was seen in transfected endothelial cell lines (Pajusola et al., 1994).

3. Aims of this work

The experiments were conducted to study the biological effects of VEGF-B and VEGF-C *in vivo*. Main objective was the comparison between the biological effects of VEGF-B and VEGF-C, especially their specificities towards their target cells. Two independent experimental approaches were undertaken:

- Transgenic animals overexpressing VEGF-B and VEGF-C
- Direct application of recombinant VEGF-B and VEGF-C

None of the two mentioned projects could be completed, but all are in advanced stage. The phenotype of the transgenic animals resulted in the confirmation of the hypothesis, that VEGF-C is indeed an angiogenic factor specific for lymphatic endothelium. Since experiments with recombinant VEGF-B and VEGF-C are still going on, only the establishment of the expression system and the purification of the protein is described.

4. Experimental procedures

4.1. Transgenic mice overexpressing VEGF-B and VEGF-C

4.1.1. Expression vector constructs

Targeting of transgene expression by the keratin 14 (K14) promoter. The K14 expression cassette was obtained from Elaine Fuchs (described in Vassar et al., 1989). It contains approximately 2 kb of the human K14 promoter/enhancer and 500 bp of 3'-flanking sequence including the K14 polyadenylation signal. Constructs based on this cassette have been shown to target expression of transgenes appropriately to cells of stratified squamous epithelia (Vassar et al., 1989; Vassar et al., 1991), such as epithelial basal cells of the skin, the cornea, eyelids, ears, tongue, oesophagus and forestomach (Nelson et al., 1983). The K14 promoter is also active in hepatic epithelial cells of embryonic day 12 (Germain et al., 1988), driving the expression of keratin 14, which is also known as cytokeratin 52 (CK52) according to its molecular weight of 52 kDa.

The K14 promoter was chosen to target transgene expression because of several reasons:

- Transgenic mice expressing VEGF under the same promoter gave rise to an increased vascularisation of the skin with blood vessels (personal communication by Michael Detmar), which makes a direct comparison between VEGF, VEGF-B and VEGF-C possible.
- Keratinocytes are physiological producers of VEGF (Detmar et al., 1994) and the overexpression of VEGF plays a crucial role in many physiological and pathological processes in the skin, such as wound healing, delayed type hypersensitivity skin reactions (Brown et al., 1992b; Brown et al., 1995c) and psoriasis (Detmar et al., 1994).
- The analysis of the transgene is easy, since the skin is the most exposed organ and a first examination is feasible without exploratory operations.

K14-VEGF-B167. The human cDNA of the 167 amino acid isoform of VEGF-B in pCRII-VEGF-B167 (obtained from Katri Pajusola) was excised with EcoRI and the resulting 595 bp fragment containing the open reading frame (nt 4-570) and the Kozak consensus sequence was isolated by gelelectrophoresis on 1.2% agarose and recovered using the Qiaex II gel extraction kit (Qiagen). The 3'-recessed ends were filled in with the Klenow fragment of DNA polymerase I. The K14 vector was opened with BamHI, blunted as described above and dephosphorylated using calf intestinal phosphatase (CIP) to prevent the vector from self-ligating. The construct was assembled by blunt-end ligating both fragments and the correctness of the whole coding region, including both junctions, was verified by sequencing (Figure 25.).

K14-VEGF-B186. The human cDNA of the 186 amino acid isoform of VEGF-B was excised from pREP7-VEGF-B186 (Katri Pajusola) with BamHI/NheI. The ≈ 780 bp fragment containing the open reading frame (nt 5-628) and the Kozak consensus sequence was isolated and the vector assembled as described above. The EcoRI site within the transcriptional unit (derived from pREP7-VEGF-B186) was destroyed by a partial EcoRI digest, fill-in with the Klenow fragment of DNA polymerase I and a blunt-end backligation (Figure 26.).

K14-VEGF-C-FL. The pCI-neo vector (Promega) containing the human full length cDNA of VEGF-C (Dmitry Chilov) was cut with XhoI/NotI, and the resulting 2027 bp fragment containing the open reading frame (nt 361-1617) as well as approximately 350 bp of 5'- and 400 bp of 3'-UTR region was isolated and ligated to the K14 vector as described above for K14-VEGF-B167. The resulting construct contained the EcoRI site derived from the polylinker of the pCI-neo vector. In order to destroy this site, the construct was opened by a partial EcoRI digest, filled in with the Klenow fragment of DNA polymerase I, blunt-end backligated and the correctness of both junctions was verified by sequencing (Figure 27.).

K14-ΔNΔC-VEGF-C. K14-VEGF-C-FL and pREP7-ΔNK214stop-VEGF-C (Vladimir Joukov) were cut with SacII/ClaI and the ≈1200 bp fragment from pREP7-ΔNK214stop-VEGF-C was ligated to the CIPped K14-VEGF-C-FL. The correctness of the 3'-junction and the whole coding sequence was verified by sequencing (Figure 28.).

Targeting of transgene expression by the α-myosin heavy chain (α-MHC) promoter. The murine α-MHC expression cassette was a gift from Jeffrey Robbins. It consists of the entire intergenic region between the β-MHC gene (upstream) and the α-MHC gene (Gulick et al., 1991) and the human growth hormone polyadenylation signal connected by a short linker with a stop codon in each reading frame. The intergenic region between the β- and the α-MHC gene is sufficient to properly direct expression in a tissue-specific manner (Subramaniam et al., 1991). The α-myosin heavy chain is one of the components of α-myosin, a major constituent of the contractile apparatus of cardiac myocytes, and is expressed very early in embryonic development (Sánchez et al., 1991).

The α-MHC promoter was chosen to target transgene expression because VEGF-B mRNA is present at high levels in the heart, indicating that it could be involved in the maintenance of the heart vasculature.

αMHC-VEGF-B167. The recessed 3'-ends of the EcoRI fragment from VEGF-B167-pCRII (described above in the construction of K14-VEGF-B167) were filled in with the Klenow fragment of DNA polymerase I and ligated to the SalI-opened and Klenow filled-in α-MHC expression cassette. Orientation and sequence of the junctions were checked by sequencing (Figure 29.).

αMHC-VEGF-C-FL. The human full length cDNA VEGF-C fragment (described above in the construction of K14-VEGF-C-FL) was ligated to the SalI-opened and Klenow-filled-in α-MHC expression cassette. Orientation and sequence of the junctions were checked by sequencing (Figure 30.).

Targeting of transgene expression by the insulin I promoter (RIP). The RIP1DipA plasmid was a gift from Gerhard Christofori. The rat insulin I promoter targets gene expression exclusively to the β-islets of the pancreas (Hanahan, 1985). VEGF is expressed at high levels by endothelial cells in islets and RIP-VEGF transgenic mice have been made to study the impact of VEGF on tumour progression in RIP (SV40 smallT antigen x VEGF) transgenic mice (personal communication by Michael Pepper).

RIP-VEGF-B167. The RIP1DipA plasmid was opened with XbaI/HindIII and CIPped. The VEGF-B_{167} cDNA was cut out as a XbaI/HindIII fragment from VEGF-B167-pCRII (Katri Pajusola) and ligated to the opened plasmid. The resulting construct contained the BamHI site derived from the polylinker of the pCRII vector. In order to destroy this site, the construct was opened by a partial BamHI digest, filled in with the Klenow fragment of DNA polymerase I, blunt-end backligated and the correctness of both junctions was verified by sequencing (Figure 31.).

RIP-VEGF-C-FL. The human full length cDNA VEGF-C fragment (described above in the construction of K14-VEGF-C-FL) was ligated to the SalI-opened, Klenow filled-in and CIPped RIP1DipA plasmid. Orientation and sequence of the junctions were checked by sequencing (Figure 32.).

4.1.2. Transgenic mice

Isolation and purification of the full transcriptional unit. The constructs K14-VEGF-B167, K14-VEGF-B186, K14-VEGF-C-FL and K14-ΔNΔC-VEGF-C were digested with EcoRI/HindIII to cut out the full transcriptional unit (FTU) minimising the amount of prokaryotic vector sequence remaining in the transgene construct. The construct K14-VEGF-B186 was additionally treated with DraI/ApaLI and the construct K14-ΔNΔC-VEGF-C with ApaLI to cut the remaining prokaryotic vector sequences into small fragments to ease the isolation procedure ("killing"). The constructs αMHC-VEGF-B167, αMHC-VEGF-C-FL and RIP-VEGF-B167 were similarly digested with BamHI. The construct RIP-VEGF-C-FL was additionally digested with AlwNI to kill the remaining vector fragment into two pieces. The FTUs were isolated by gel electrophoresis in 1-1.5% agarose. Slices from the left and the right sides of the gel were stained in 0.5 mg/ml ethidium bromide and the FTU localised under UV light. The transgene band was cut out blindly from the corresponding region. The DNA was recovered with the Qiaex II gel extraction kit (Qiagen) according to the manufacturer's instructions with the following modifications: the DNA-loaded beads were never resuspended by vortexing but by slow pipetting, and an additional final washing step with 70% ethanol was included. The FTU was released from the beads with the injection buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.4), the concentration was assayed against a known DNA standard (λ HindIII or λ PstI marker) and diluted with the same buffer to 7.5 μg/ml. For the K14 constructs and

αMHC-VEGF-B167 the above mentioned procedure was repeated including the restriction digest, thus minimising prokaryotic vector contamination. Thereafter the DNA was twice spun at 16000g for 40 min, and approximately 75% of the solution was transferred to a new tube after each round, avoiding any floating or sedimented beads and other particles.

Microinjection. The DNA was then microinjected into FVB-NIH fertilised eggs (CBAJB6 in case of the RIP constructs), which were then implanted into the oviducts of pseudopregnant (C57BL/6 x DBA/2J) hybrid females using standard techniques (Hogan et al., 1994).

4.1.3. Screening: PCR, Southern blotting and dot blot analysis

Genomic DNA was isolated of founder mice and F1 mice from positive founders. Mouse tails were taken at 3 weeks of age and processed according to two different standard procedures (Laird et al., 1991; Drews et al., 1994). Controls were set up as follows: control mice were processed simultaneously with the founders and mouse tail DNA was spiked with different amounts of the uncut plasmid, from which the transgene was cut out. The amounts used were equivalent to 0.1, 10 and 100 transgene copies/diploid genome.

PCR. PCR was performed on 50-100 ng of the isolated mouse tail DNA. Primers with numbers greater than 3895 (except of no 5864) were designed and the annealing temperatures of all primers calculated with help of the Primer program (Version 0.5, The Whitehead Institute). The following PCR conditions were used:

K14-VEGF-C-FL/K14-ΔNΔC-VEGF-C

forward primer: `5 -catgtacgaaccgccaG-3` (hVEGF-C CDS, SBL-internal no 3169)
reverse primer: `5 -AATGACCAGAGAGAGGCGAG-3` (hK14 polyadenylation signal, SBL-internal no 3862)
amplification product: 683 bp

primer concentration	1 µM	initial denaturation	4 min; 95ºC
dNTP concentration	200 µM	denaturation	45 sec; 94ºC
Dynazyme DNA poly-		annealing	45 sec; 66ºC, 65ºC, 64ºC, 63ºC, 62ºC, 28 x 61ºC
merase (Finnzymes)	2 units	elongation	45 sec; 72ºC
reaction volume	25 µl	final elongation	7 min; 72ºC

forward primer: `5 -ACAGAGAACAGGCCAACC-3` (hVEGF-C CDS, SBL-internal no 3664)
reverse primer: `5 -GTCTCTTCATCCAGCTCCTT-3` (hVEGF-C CDS, SBL-internal no 3624)
amplification product: 592 bp

primer concentration	1 µM	initial denaturation	4 min; 95ºC
dNTP concentration	200 µM	denaturation	50 sec; 94ºC
Dynazyme DNA poly-		annealing	45 sec; 60ºC, 69ºC, 58ºC, 57ºC, 56ºC, 28 x 55ºC
merase (Finnzymes)	2 units	elongation	35 sec; 72ºC
reaction volume	25 µl	final elongation	7 min; 72ºC

K14-VEGF-C-FL/K14-ΔNΔC-VEGF-C/αMHC-VEGF-C-FL

forward primer: `5 -CGGGAGGTGTGTATAGATGTGGGG-3` (hVEGF-C CDS, SBL-internal no 6049)
reverse primer: `5 -CTGGTTTGGGGCCTTGAGAGAG-3` (hVEGF-C CDS, SBL-internal no 6050)
amplification product: 196 bp

primer concentration	0.5 µM	initial denaturation	5 min; 94ºC
dNTP concentration	200 µM	denaturation	40 sec; 94ºC
Dynazyme DNA poly-		annealing	30 sec; 30 x 60ºC
merase (Finnzymes)	1.6 units	elongation	30 sec; 72ºC
reaction volume	25 µl	final elongation	7 min; 72ºC

K14-VEGF-B167/αMHC-VEGF-B167

forward primer: 5 -TCTCCCAGCCTGATGCCCCT-3 (hVEGF-B CDS, SBL-internal no 2841)
reverse primer: 5 -CAGCTGGGCACCAGCTGTTT-3 (hVEGF-B CDS, SBL-internal no 2723)
amplification product: 158 bp

primer concentration	1 µM	initial denaturation	4 min; 95ºC
dNTP concentration	200 µM	denaturation	45 sec; 94ºC
Dynazyme DNA poly-		annealing	45 sec; 69.5ºC, 69ºC, 68.5ºC, 68ºC, 32 x 67.5ºC
merase (Finnzymes)	2 units	elongation	10 sec; 72ºC
reaction volume	25 µl	final elongation	7 min; 72ºC

forward primer: 5 -TCTCCCAGCCTGATGCCCCT-3 (hVEGF-B CDS, SBL-internal no 2841)
reverse primer: 5 -GGACTTGGTGCTGCCCAGTG-3 (hVEGF-B CDS, SBL-internal no 2843)
amplification product: 225 bp

primer concentration	1 µM	initial denaturation	4 min; 95ºC
dNTP concentration	200 µM	denaturation	50 sec; 94ºC
Dynazyme DNA poly-		annealing	45 sec; 71ºC, 70ºC, 69ºC, 68ºC, 30 x 67ºC
merase (Finnzymes)	2 units	elongation	30 sec; 72ºC
reaction volume	25 µl	final elongation	7 min; 72ºC

forward primer: 5 -TCTCCCAGCCTGATGCCCCT-3 (hVEGF-B CDS, SBL-internal no 2841)
reverse primer: 5 -GCCATGTGTCACCTTCGCAG -3 (hVEGF-B CDS, SBL-internal no 2722)
amplification product: 508 bp

primer concentration	1 µM	initial denaturation	4 min; 95ºC
dNTP concentration	200 µM	denaturation	50 sec; 94ºC
Dynazyme DNA poly-		annealing	45 sec; 71ºC, 70ºC, 69ºC, 68ºC, 32 x 67ºC
merase (Finnzymes)	2.4 units	elongation	30 sec; 72ºC
reaction volume	25 µl	final elongation	7 min; 72ºC

αMHC-VEGF-C-FL

forward primer: 5 -TCTTCCCCAGCCAATGTG-3 (hVEGF-C CDS, SBL-internal no 5864)
reverse primer: 5 -AAATCAGAAGGACAGGGAAGG-3 (hGH polyadenylation signal, SBL-internal no 3852)
amplification product: 1215 bp

primer concentration	1 µM	initial denaturation	4 min; 95ºC
dNTP concentration	200 µM	denaturation	45 sec; 94ºC
Dynazyme DNA poly-		annealing	50 sec; 66ºC, 65ºC, 64ºC, 63ºC, 62ºC, 28 x 61ºC
merase (Finnzymes)	2.4 units	elongation	60 sec; 72ºC
reaction volume	25 µl	final elongation	7 min; 72ºC

Southern blotting. 1 to 10 µg of DNA were digested with 5 to 15 units enzyme/µg DNA. The K14-constructs were digested o/n with EcoRV and the αMHC-constructs with EcoRI thus generating internal fragments of known sizes (Figure 8.). If sufficient DNA was available, approximately 200 ng of the restricted DNA were assayed on a 0.9% agarose gel against uncut DNA to ensure complete digestion. The concentration of DNA was not determined for DNA from founder mice because of possible mosaics. For F1 mice the amount of DNA was determined by measuring the OD_{260nm} of a 1:100 dilution of the DNA sample. Electrophoresis of the DNA was performed o/n on 0.9% agarose in 0.5xTBE applying 1V/cm. That approximately equal amounts of DNA had been electrophoresed, was checked visually after staining with ethidium bromide under UV illumination and documented with a photography including a millimetre-scale. The DNA was then transferred and fixed to Hybond N^+ nylon membrane (Amersham) according to standard techniques (Sambrook et al., 1989) or the manufacturer's protocols for alkali blotting with the following modification: the buffer used for alkaline transfer was not drawn through Whatman 3MM paper from the reservoir to the gel, but instead

the buffer reservoir itself was a brick-shaped sponge saturated with 0.4 NaOH, on which three sheets of Whatman 3MM paper were placed, followed immediately by the agarose gel, that was equilibrated in 0.4 NaOH for 30 min prior to the transfer.

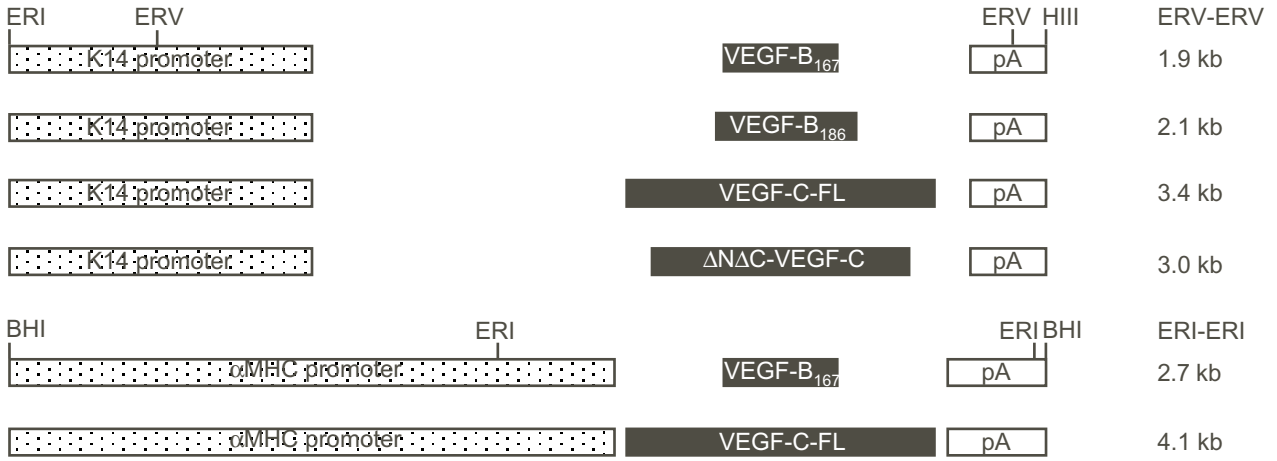

Figure 8. Transgene detection by Southern blotting: EcoRI/EcoRV restriction fragments
Legend: ERI = EcoRI, ERV = EcoRV, HIII = HindIII, BHI = BamHI

Probes. [α-^{32}P]dCTP-labelled DNA probes were generated using the Random Primed DNA Labeling Kit (Boehringer Mannheim). The full transcriptional unit of K14-VEGF-C-FL was used as template to screen for K14 transgenes and the full transcriptional unit of αMHC-VEGF-B167 was used to screen for αMHC transgenes.

Prehybridisation, hybridisation, washes and exposure. Prehybridisations and hybridisations were performed 2-4 hours (prehybridisation) and o/n (hybridisation) at 42ºC with low abundance formamide (pre)hybridisation solution (Sambrook et al., 1989) or (pre)hybridisation solution according to instructions provided with the Hybond N$^+$ nylon membrane. Washes were done according to standard protocols (Sambrook et al., 1989) or according to instructions provided with the Hybond N$^+$ nylon membrane. Blots were exposed either 2 days to 2 weeks to X-ray film or o/n to an imaging plate (Fuji BAS 1500).

Dot blot analysis. Dot blot analysis was performed to identify the mice transgenic for RIP-VEGF-C-FL and RIP-VEGF-B167. 10 µg of each tail DNA were used. The DNA was immobilised to Hybond-N (Amersham) by backing for 2 h, prehybridisation, hybridization, washes and exposure were done as described for the Southern blots. As a probe the SV40 intron was used, labelled with the Megaprime system (Amersham).

4.1.4. Analysis of the mice

Histological and immunohistochemical analysis of the mice is still going on. Among others, electron microscopy, permeability assays and tracer experiments with non-invasive monitoring are performed to characterise in more detail the nature of the vessel-like structures. Figures 11-15 represent therefore preliminary results. A comprehensive description of the analysis and the techniques used will be included in the publication.

4.2. Recombinant VEGF-B and VEGF-C produced with the baculovirus system

The baculovirus system has been efficiently used for the expression of mammalian genes. It combines the high level expression of bacterial expression systems with a post-translational modification similar to mammalian expression systems (Miller, 1988). The baculovirus system was chosen, because in many cases protein modification is crucial to biological activity and because the baculovirus expression system has been recently successfully used to express human cytokines structurally so different as IFN-γ (a 4-a-helical cytokine; Sareneva et al., 1994) and PDGF-B (Giese et al., 1989) or VEGF (Fiebich et al., 1993).

4.2.1. Insect cell lines and culture

The cell line Sf9 is a clonal isolate of Sf21 cells, which are derived from ovary cells of the fall army worm (Spodoptera frigiperda; Vaughn et al., 1977). It was obtained from Ilkka Julkunen (National Public Health Institute, Helsinki) and maintained as adherent culture at 27ºC in TMN-FH completed with fetal bovine serum to a final concentration of 10%. 2.5 µg/ml amphotericin B (Fluka), 100 µg/ml streptomycin and 10 units/ml penicillin were used to minimise the risk of bacterial or fungal contamination. Routine culture was done according to standard procedures (O'Reilly et al., 1992).

HighFive (HF) cells were developed from ovarian cells of the cabbage looper (Trichoplusia ni; Wickham et al., 1993). They were maintained as adherent culture and suspension culture at 27ºC in the serum-free media Ex-Cell 400 (JRH Biosciences) according to the instructions of the supplier (Invitrogen).

4.2.2. Generation of recombinant baculoviruses by transposon-mediated insertion in E.coli

The recombinant baculovirus was produced by site-specific transposon-mediated insertion of the cDNAs for VEGF, VEGF-B and VEGF-C into a baculovirus genome propagated in E.coli. The Bac-to-bac baculovirus expression system (GibcoBRL/LifeTechnologies) was used including modifications of the protocol as described below. The process is schematically described in Figure 9..

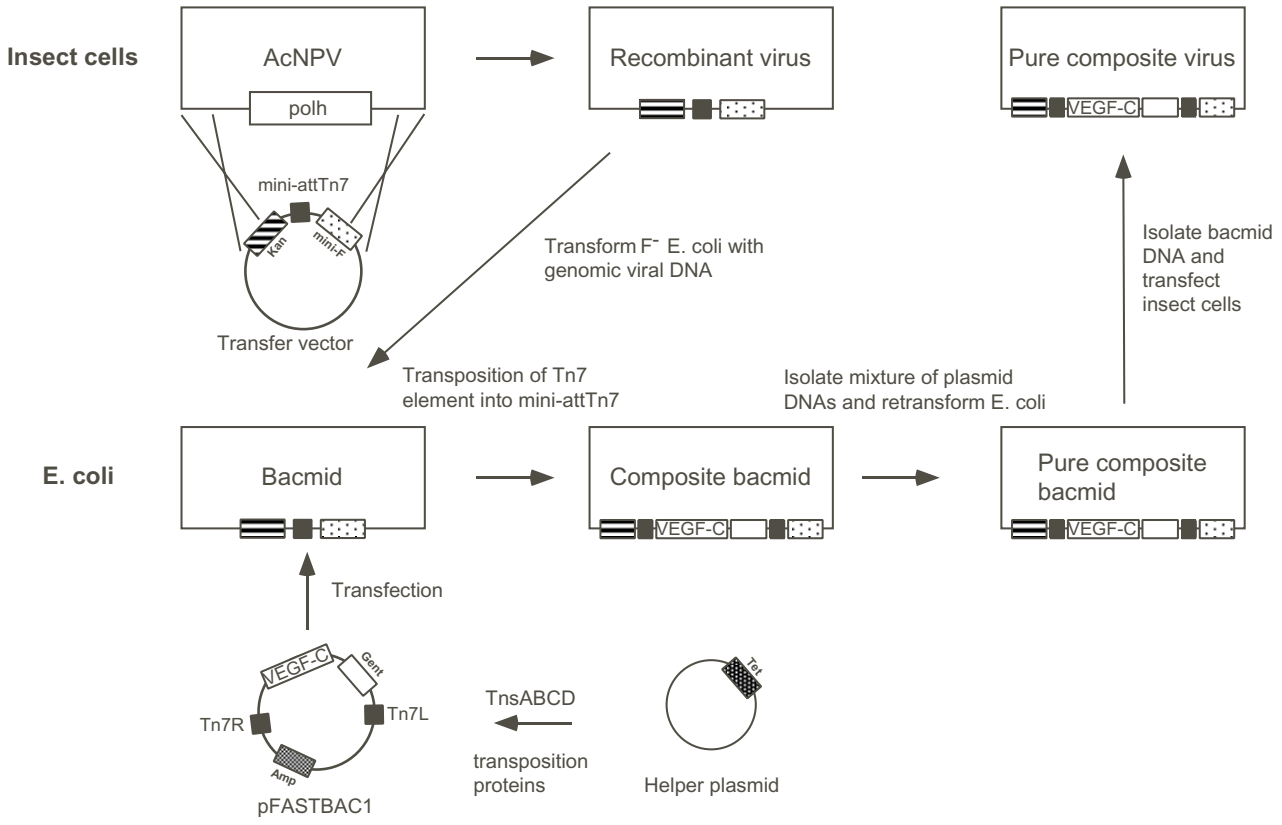

Figure 9. Transposon-mediated insertion of VEGF-C cDNA into a baculovirus genome in E.coli

4.2.2.1. Transfer plasmids and baculovirus shuttle vector

The Transfer plasmid pFASTBAC1 was cut with the appropriate restriction enzyme(s), dephosphorylated and the genes of interest were ligated into the opened vector. The junctions of all transfer plasmids were sequenced. An overview on transfer plasmids based on pFASTBAC1 is given in Table 3; the corresponding plasmid maps (drawn with Plasmid Artist Version 1.13 or Plasmidmap from the GCG package) can be found in the appendix. An overview on the corresponding recombinant proteins is also given in the appendix (Figure 54.).

Table 3 Baculovirus shuttle vectors based on pFASTBAC1

name of transfer plasmid	*parent vector, enzymes used to open*	*insert*	*origin of insert, enzymes used to cut out with*
VEGF-C constructs			
pFB1-VEGF-C (Nina Korsisaari)	pFASTBAC1 (Gibco BRL/LifeTechnologies), EcoRI/NotI	VEGF-C full length cDNA	p1.pcDNAI (Vladimir Joukov), EcoRI/NotI
pFB1-melSP-NΔ–VEGF-C	pFASTBAC1 (Gibco BRL/LifeTechnologies), SnaBI/SstI	polyhedrin promoter, melittin signal sequence fused to the sequence of the mature 23 kDa form of VEGF-C	pVT-Bac-melSP-ΔN-VEGF-C, EcoRV/SstI
pFB1-CΔ-C1HIS-VEGF-C	pFB1-VEGF-C, SphI/HindIII (blunt)	H_6-tagged VEGF-C cDNA (H_6-tag after VEGF homology domain, C1HIS)	273 bp PCR-product[a], SphI/EcoRI (blunt)
pFB1-melSP-NΔCΔ-C1HIS-VEGF-C	pFB1-melSP-NΔ–VEGF-C, SphI/HindIII (blunt)	H_6-tagged VEGF-C cDNA (H_6-tag after VEGF homology domain, C1HIS)	273 bp PCR-product[20], SphI/EcoRI (blunt)
pFB1-CΔ-C2HIS-VEGF-C	pFB1-VEGF-C, AccI (partial)/NotI	H_6-tagged VEGF-C cDNA (H_6-tag after VEGF-C homology domain, C2HIS)	VCΔNΔCHis-pALTER (Vladimir Joukov), AccI/NotI
pFB1-melSP-NΔCΔ-C2HIS-VEGF-C	pFB1-melSP-NΔ-VEGF-C, NruI/NotI	H_6-tagged VEGF-C cDNA (H_6-tag after VEGF-C homology domain, C2HIS)	VCΔNΔCHis-pALTER (Vladimir Joukov), NruI/NotI
pFB1-NΔCΔ-C2HIS-VEGF-C	pFASTBAC1 (Gibco BRL/LifeTechnologies), EcoRI/NotI	H_6-tagged VEGF-C cDNA (H_6-tag after VEGF-C homology domain, C2HIS)	VCΔNΔCHis-pALTER (Vladimir Joukov), EcoRI/NotI
pFB1-C3HIS-VEGF-C	pFASTBAC1 (Gibco BRL/LifeTechnologies), EcoRI/NotI	H_6-tagged VEGF-C cDNA (C-terminal H_6-tag, C3HIS)	VEGF-C-CHis-pALTER (Vladimir Joukov), EcoRI/NotI
pFB1-melSP-C3HIS-VEGF-C	pFB1-melSP-NΔ-VEGF-C, EcoNI/NotI	H_6-tagged VEGF-C cDNA, (C-terminal H_6-tag, C3HIS)	VEGF-C-CHis-pALTER (Vladimir Joukov), EcoNI/NotI
pFB1-NHIS-VEGF-C	pFASTBAC1 (Gibco BRL/LifeTechnologies), EcoRI/NotI	H_6-tagged VEGF-C cDNA (N-terminal HIS, NHIS)	VC-NHIS-pALTER (Vladimir Joukov), EcoRI/NotI
pFB1-VEGF-C (short splice variant)	pFB1-VEGF-C, AccI (partial)/NotI	cDNA of C-terminal part of the short splice variant of VEGF-C	pCRII-VEGF-C [short splice variant] (Eola Kukk), AccI/NotI
pFB1-melSP-VEGF-C (short splice variant)	pFB1-melSP-VEGF-C, AccI/NotI	cDNA of C-terminal part of the short splice variant of VEGF-C	pCRII-VEGF-C [short splice variant] (Eola Kukk), AccI/NotI
VEGF-B constructs			

pFB1-VEGF-B167 (Nina Korsisaari)	pFASTBAC1 (Gibco BRL/LifeTechnologies), XbaI/HindIII	VEGF-B_{167} cDNA	VEGF-B167-pCRII (Katri Pajusola), XbaI/ HindIII
pFB1-melSP-VEGF-B167	pFB1-melSP-NΔ–VEGF-C, BamHI/SpeI	VEGF-B_{167} cDNA (mutated to create a BamHI restriction site after the signal sequence)	VEGF-B167X3-pCRII, BamHI/SpeI
pFB1-melSP-CHIS-VEGF-B186	pFB1-melSP-VEGF-B167, AccI/NotI	C-terminally H_6-tagged VEGF-B186 cDNA (C-terminal part)	pIC9-NHIS-VEGF-B186, clone 17 (Vijay Kumar), AccI/NotI
pFB1-CHIS-VEGF-B186	pFB1-melSP-CHIS-VEGF-B186, Eco47III/EcoRV	(N-terminal part of VEGF-B including polh promoter and vector sequences)	pFB1-VEGF-B167 (Nina Korsisaari), Eco47III/partial EcoRV
VEGF constructs			
pFB1-VEGF165 (Nina Korsisaari)	pFASTBAC1 (Gibco BRL/LifeTechnologies), XbaI/KpnI	$VEGF_{165}$ cDNA	pGEM3Zf(+)VEGF (Daniel Conolly), XbaI/ KpnI

a. 100 ng of p1.pcDNAI (Vladimir Joukov) were submitted to PCR using the forward primer 5'-GGAATTCACAGAAGAGAC-TATAAA-3' (SBL-internal no 4047) and the reverse hybrid primer 5'-G**GAATTC**AATGATGATGATGGTGATGCAGTTTA-GACATGC-3' (tag underlined, EcoRI restriction site in bold characters; SBL-internal no 4048) using the following PCR conditions: primer concentration 1 µM; dNTP concentration 200 µM; Dynazyme DNA polymerase (Finnzymes) 4.0 units; reaction volume 0.1 ml; initial denaturation 4 min, 95ºC; denaturation 30 sec, 94ºC; annealing 35 sec, 50ºC-48ºC-46ºC-44ºC-42ºC-40ºC-39ºC-38ºC-37ºC-36ºC-12 x 35ºC; elongation 25 sec, 72ºC; final elongation 5 min, 72ºC.

4.2.2.2. Transposition and transfection

Transposition of the constructs into the baculovirus genome was performed as described (Luckow et al., 1993). Sf9 cells and HF cells were transfected using liposomes (Cellfectin reagent, Gibco BRL or Insectin, Invitrogen) according to the protocols of the manufacturers. For each construct at least four transfections were done with four independent bacmid preparations. For the transfection procedure of the Sf9 cells Grace's insect medium (Gibco BRL/LifeTechnologies) was used, HF were transfected in Ex cell 400 (JRH Biosciences). The transfection mixture was kept on the cells from 5 h to o/n.

4.2.3. Generation of recombinant baculovirus by allelic replacement in insect cells

The transfer plasmid pVT-Bac (described in Tessier et al., 1991) provides the honey bee melittin signal peptide to facilitate the secretion of expressed gene products. Heterologous gene expression is under the control of the polyhedrin promoter and the recombinant viruses are occlusion-negative. The strategy of allelic replacement in insect cells is schematically described for VEGF-B_{167} in Figure 10. The chosen cloning option led to a two amino acid insertion (Asp-Pro) between the melittin signal sequence and VEGF-B_{167}, VEGF-B_{186} and VEGF-C. The predicted signal peptide cleavage would release the 167 and 186 amino acid forms of VEGF-B and the mature 23 kDa form of VEGF-C respectively, both preceded by the amino acids asparagine and proline. The VEGF-B isoform of 167 amino acids was expected to be releasable from the insect cell surface similar to mammalian cells by heparin treatment, while the 186 amino acid isoform was expected to be freely secreted into the medium (Olofsson et al., 1996a; Olofsson et al., 1996b).

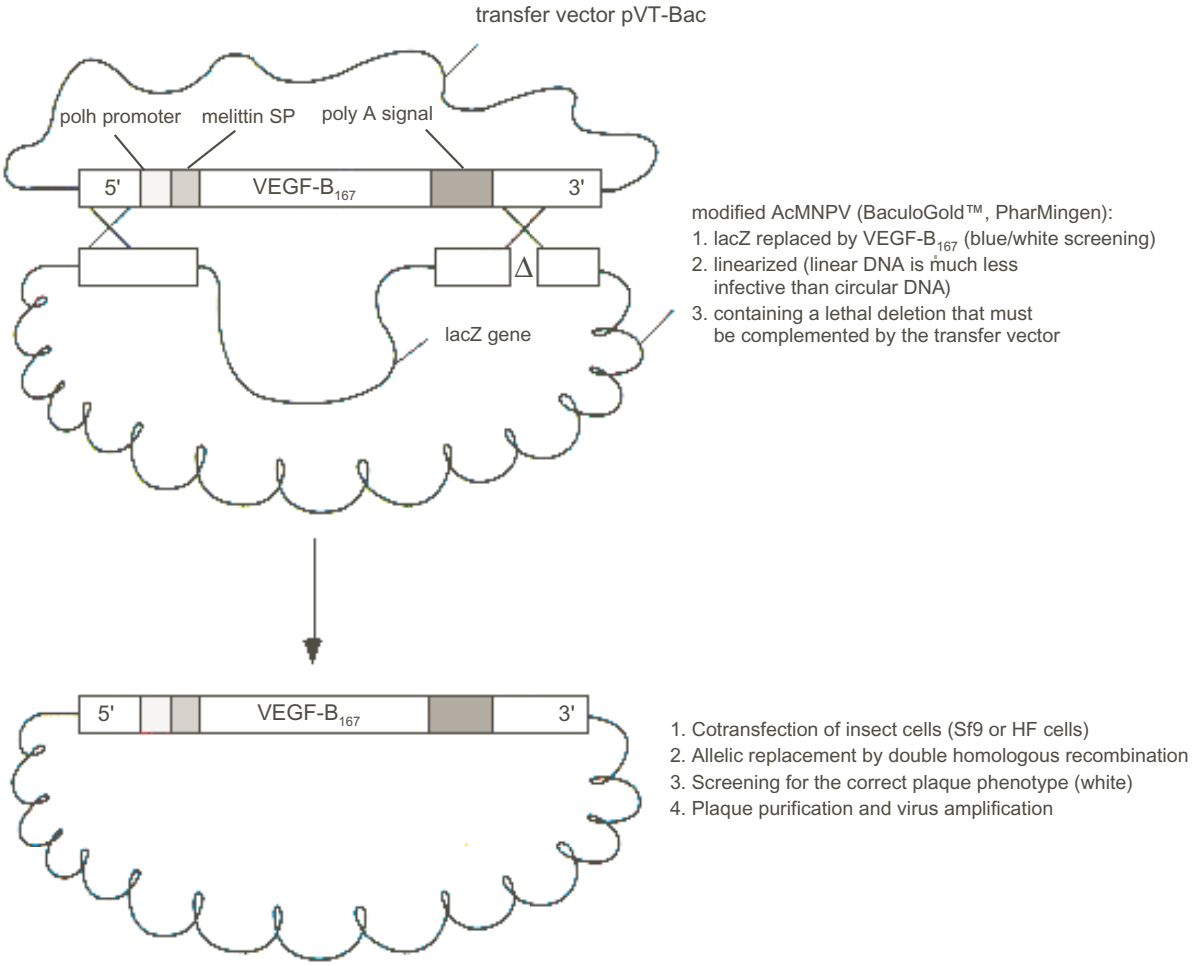

Figure 10. Allelic replacement in insect cells

4.2.3.1. Transfer plasmid and parent virus

An overview on the transfer plasmids based on the vector pVT-Bac is given inTable 4.

pVT-Bac-VEGF-B167. The VEGF-B_{167} cDNA sequence was mutated to generate a BamHI restriction site immediately before the sequence which codes for the mature, secreted protein. This mutated cDNA was excised from the construct VEGF-B167-X3-pCRII[18] with BamHI and non-directionally cloned into the BamHI-opened and CIPped pVT-Bac. Both 3'- and 5'-junction were sequenced to verify orientation and the sequence derived from the PCR.

pVT-Bac-VEGF-C. The mutated VEGF-C sequence, containing a BamHI restriction site immediately in front of the sequence, which corresponds to the mature 23 kDa form of VEGF-C, was excised from the construct VEGF-C-X3-pCI-neo[19] with BamHI/SstI and cloned into the BamHI/SstI-opened and CIPped pVT-Bac. Both 3'- and 5'-junction were sequenced to verify orientation and the sequence derived from the PCR.

18. A 74 bp fragment from the 5'-end of the VEGF-B_{167} CDS was amplified from 200 ng of the vector VEGF-B167-pCRII (Katri Pajusola, whole CDS and translation initiation consensus region verified by sequencing). The forward hybridprimer: 5'-GATAT**GGGCCCGGATCC**TGTCTCCCAGCCTGATGCCCCT-3' (tag underlined, ApaI and BamHI restriction sites in bold characters; SBL-internal no 3355) and the reverse primer: 5'-GTAGCGCGA**GTATAC**ACATCTATC-3' (AccI site in bold characters, SBL-internal no 3356) were used with the following PCR conditions: primer concentration 1 µM; dNTP concentration 200 µM; native Pfu DNA polymerase (Stratagene) 2.5 units; reaction volume 0.1 ml; initial denaturation 1 min, 95ºC; denaturation 60 sec, 95ºC; annealing 40 sec, 56ºC; elongation 30 sec, 74ºC; 18 cycles; final elongation 5 min, 72ºC. The amplified DNA was cut with ApaI and AccI, purified by gelelectrophoresis on 1.8% agarose and isolated using the Wizard PCR preps DNA purification system (Promega) and subsequently cloned into the ApaI/AccI opened VEGF-B167-pCRII (Katri Pajusola).

Table 4 Baculovirus shuttle vectors based on pVT-Bac

name of transfer plasmid	*parent vector, enzymes used to open*	*insert*	*origin of insert, enzymes used to cut out with*
pVT-Bac-melSP-VEGF-B167	pVT-Bac (Tessier et al., 1991), BamHI	VEGF-B_{167} cDNA (mutated to create a BamHI restriction site after the signal sequence)	VEGF-B167-X3-pCRII, BamHI
pVT-Bac-melSP-VEGF-C	pVT-Bac (Tessier et al., 1991), BamHI/SstI	VEGF-C cDNA (mutated to create a BamHI restriction site after the signal sequence)	VEGF-C-X3-pCI-neo, BamHI/SstI

4.2.3.2. Co-transfection

Sf9 cells were co-transfected with the transfer plasmid and linearised baculovirus DNA (BaculoGold, Pharmingen) using insect cell-specific liposomes (Insectin, Invitrogen) according to the instructions of the manufacturer. As positive control for the transfection procedure wt baculovirus DNA was used. The supernatant containing the recombinant virus was harvested 5 to 7 days post co-transfection upon clear signs of infection and cleared from cellular debris by centrifugation at 1500 rpm (400g) for 5 min.

4.2.4. Identification, plaque purification, amplification and titration of recombinant virus

pFASTBAC1-based constructs. Preliminary analysis of viral stocks was done before plaque-purification. When the protein yield was low in spite of a high virus titre, the purity of the recombinant virus stock was checked and from impure virus-stocks pure clonal stocks were generated by plaque purification. The following virus stocks which were obtained from the initial transfection with bacmid DNA were not pure recombinants: pFB1-melSP-VEGF-B167, pFB1-VEGF-C and pFB1-melSP-NΔ-VEGF-C. This was shown by PCR[20] or no yield of recombinant protein in the expression screening of isolated clones. These virus stocks were therefore plaque-purified for two rounds. Sixteen clones were picked for the construct pFB1-VEGF-C, four from each of the four independent transfections. For the constructs pFB1-melSP-VEGF-B167 and pFB1-melSP-NΔ-VEGF-C seven clones each were picked. All other virus stocks obtained from initial transfections were used as such without further purification. VEGF-recombinant virus was produced as a control and its viral stocks were not extensively analysed since purified VEGF-recombinant virus (Fiebich et al., 1993) could obtained from Herbert Weich.

pVT-Bac-based constructs. The co-transfection-derived virus stocks based on the pVT-Bac vector were screened by a plaque-assay and recombinant plaques (lacking the lacZ gene due to allelic replacement by the transfer plasmid and thus being colourless in IPTG/X-Gal agarose overlays) identified under a dissecting microscope. Isolates of the primary screen were replaqued once to generate a pure virus stock.

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19. A 74 bp fragment from the 5'-end of the VEGF-C CDS was amplified from 100 ng of the vector p1.pcDNAI (Vladimir Joukov). The forward hybridprimer: 5'-GATG**CTCGAGGATCC**GACAGAAGAGACTATAAAATTTGC-3' (tag underlined, XhoI and BamHI restriction sites in bold characters; SBL-internal no 3457) and the reverse primer: 5'-CCCACATCT**GTAGAC**GGACACAC-3' (AccI site in bold characters, SBL-internal no 3458) were used with the following PCR conditions: primer concentration 1 µM; dNTP concentration 200 µM; native Pfu DNA polymerase (Stratagene) 2.5 units; reaction volume 0.1 ml; initial denaturation 1 min, 95ºC; denaturation 60 sec, 95ºC; annealing 45 sec, 55ºC; elongation 30 sec, 74ºC; 15 cycles; final elongation 2 min, 72ºC. The amplified DNA was cut with XhoI and AccI, purified by gelelectrophoresis on 1.8% agarose and isolated using the Wizard PCR preps DNA purification system (Promega) and subsequently cloned into the XhoI/AccI-opened VEGF-C-pCI-neo (Dmitry Chilov).
20. PCR was done on 2-5 µl of cell lysate prepared as described under 4.2.5. To confirm the presence of recombinant virus derived from pFB1-VEGF-B167 two different primer combinations were used: 1. T7 primer with the VEGF-B-specific reverse primer: 5'-GCCATGTGTCACCTTCGCAG-3' (SBL-internal no: 2722) and 2. T3 primer with VEGF-B-specific forward primer: 5'-AGCTCAACCCAGACACC-3' (SBL-internal no 3417). The presence of non-recombinant viral genomes in which the lacZ gene was not disrupted by the transposition event could be demonstrated by the short amplification product using T7 primer and T3 primer. PCR conditions: primer concentration 1 µM; dNTP concentration 200 µM; Dynazyme DNA polymerase (Finnzymes) 2 units; reaction volume 0.05 ml; initial denaturation 5 min, 95ºC; denaturation 60 sec, 95ºC; annealing 60 sec, 55ºC; elongation 90 sec, 72ºC; 30 cycles; final elongation 10 min, 72ºC

Titration, plaque purification and amplification. Titration and plaque purification was done following standard protocols (O'Reilly et al., 1992). For titration and the primary screen, 35 mm or 60 mm tissue-culture plates seeded with 0.7-0.85 or 2-2.5 million cells were infected for 1-2 h with 1 or 2 ml of $1:10^{-3}$ to $1:10^{-7}$ dilutions of the virus stock and overlaid with 2 or 5 ml 1% SeaKem LE agarose (FMC). Individual clones were identified under a dissecting microscope 5 to 7 days post infection and counted and/or picked. For replaquing, cells were infected with 1 or 2 ml media containing 50%, 5% and 0.5% of the total primary screen isolate. Amplification of pure virus stocks was done in three steps: 25 cm^2, 60 cm^2 and 175 cm^2 cell culture flasks seeded with 2.5 million, 6 million and 17.5 million cells were infected with 0.1 ml of supernatant from the preceding amplification or purification step and harvested after clear signs of complete infection (3 to 9 days p.i.) and cleared from cellular debris by centrifugation at 1500 rpm (400g) for 5 min.

4.2.5. Harvest and characterisation of the recombinant protein

The supernatant was harvested 72 h post transfection or 48-50 h post infection (p.i.). Floating cells and debris were removed from the supernatant by centrifugation at 1500 rpm (400g) for 5 min. Recombinant viruses obtained with the FastBac system were identified by their recombinant gene product. In some cases this could be accomplished by direct analysis of the recombinant protein from the cell lysates or supernatants of the initial transfection, sometimes subsequent rounds of virus amplification had to be performed to generate high titre stocks that could be used to produce sufficient protein for analysis. Recombinant viruses obtained by allelic replacement in insect cells (pVT-Bac-based vectors) were not further characterised.

4.2.5.1. Immunoprecipitation and Western blotting

Immunoprecipitation (IP). Immunoprecipitation was done on conditioned supernatant and insect cell lysates. Cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed in RIPA buffer containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.03 U/ml aprotinin. The lysates were shortly sonicated and clarified by centrifugation for 20 min at 4°C and 14000 rpm (16000g).

For VEGF-B-IP 2.5 µl of rabbit anti-VEGF-B serum (874) were added to 500 µl lysate (equalling 25 cm^2 confluent cells) and 4 µl to 1 ml conditioned medium. For VEGF-C-IP 4 and 6 µl of rabbit anti-VEGF-C serum (882) were used, and for VEGF 5 and 10 µl AB-293-NA (R&D Systems). Incubation was done with gentle agitation at 4°C o/n. Immunoprecipitates were bound to 50 µl 50% CL-4B protein A sepharose (Pharmacia) for 1-2 h under the above mentioned conditions, washed once with ice-cold lysis buffer and two times with PBS. According to the purpose, 20-30 µl 2x or 5x Laemmli standard or non-reducing buffer was added, the samples were heated to 95°C for 5 min and shortly centrifuged.

SDS-PAGE and Western blotting. SDS-PAGE was either performed on the immunoprecipitates or straight on lysates/conditioned medium. 5 to 30 µl of the supernatant were subjected to SDS-PAGE in a 12.5% or 15% gel. The transfer to Protran nitro-cellulose membrane (Schleicher & Schuell) was done at 100 mA for 1 h by semidry electroblotting.

The transfer was checked by Ponceau staining for 5 min and the stain subsequently removed by several washes in H_2O and TBS-T. Blocking was done in TBS-T containing 2.5-5% BSA for 1 h (at RT) to o/n (at 4°C). Excess protein was washed away with four 15-min-washes in TBS-T.

Detection by enhanced chemical luminescence (ECL). The appropriate primary antibody/antiserum was added, diluted 1:500 - 1:1200 into 5 ml TBS-T or according to the instructions of the manufacturer. Incubation was done in sealed plastic bags on a rocking platform for 2 h (at RT) to o/n (4°C). After washing as above, the appropriate horseradish peroxidase-coupled secondary antibody was applied according to the instructions of the manufacturer. The ECL detection was performed according to the protocol by Amersham. A selection of the results is given in the appendix.

4.2.5.2. Metabolic labelling

36 h after infection 35 mm dishes of confluent Sf9 cells were once washed and incubated for 15 min in methionine-deficient Grace's medium. 8 µCi S^{35}-methionine/ml was added to methionine-deficient Grace's medium and each dish was

incubated with 2 ml for 1 h. The cells were harvested as described above and 5% of the lysate was subjected to SDS-PAGE. The gel was incubated in fluorographic liquid (Amplify, Amersham), vacuum-dried and exposed to X-ray film for one week.

4.2.5.3. Stimulation of receptor phosphorylation by recombinant VEGF-C

Confluent NIH3T3 cells or PAE cells, stably transfected for FLT4 or KDR, were starved for 12 to 24 h in DMEM or Ham's F12 media, respectively, containing 0.2% BSA or 0.1% FCS. They were incubated 5 min at RT with the appropriate dilutions of the sample to analyse (1:2 - 1:500 dilutions of conditioned media, 1:100 - 1:10,0000,000 dilutions of the purified protein). As positive control cells were incubated for 10-20 min with 100 µM Na_3VO_4 and 7 mM H_2O_2. Cells were treated as described in 4.2.5.1., additionally the wash buffer contained 100 µM Na_3VO_4, and the lysis buffer 1 mM Na_3VO_4. The lysates were processed, subjected to IP, 7.5% SDS-PAGE, Western blotting and ECL as described above. Tyrosine phosphorylation was detected using horseradish peroxidase-coupled PY20 phosphotyrosine-specific monoclonal antibodies (Transduction Laboratories), and the receptors were monitored using 1:200 dilutions of FLT4- or KDR-specific antiserum. A selection of the results is given in the appendix.

4.2.6. Large scale production and affinity purification of recombinant VEGF-C

For large scale protein production adherent insect cell cultures in 650 ml flasks were infected at a MOI of >10. Two constructs were used for large scale production: pFB1-melSP-NΔCΔ-VEGF-C and pFB1-melSP-NΔCΔ-C2HIS-VEGF-C. The concentration of these two forms of VEGF-C in the supernatant was roughly determined by silver and Coomassie Brilliant Blue staining as 2-5 µg/ml. Between 2 and 40 flasks of 80-100% confluent HF cells were infected to obtain VEGF-C conditioned media or 0.25-5 mg of purified VEGF-C.

Untagged VEGF-C. Serumfree media from HF cells conditioned with untagged VEGF-C was used without further purification for experiments. The following controls were used: conditioned media of non-infected HF cells or of HF cells that were infected with wt, VEGF- or VEGF-B_{167}-recombinant baculovirus. A purification protocol for untagged VEGF-C remains to be established.

Affinity purification of histidine-tagged VEGF-C and VEGF-B_{186}. Histidine-tagged protein was affinity-bound to Ni^{2+} chelate resin and eluted with imidazole. Prior to loading of the resin, the harvested supernatant was cleared once more (4000 rpm [2750g] for 5 min.). Depending on the purpose BSA was facultatively included during all purification steps to reduce the observed unspecific binding of VEGF-C to various surfaces (0.1 µg BSA/ml of supernatant). A two-fold concentration and a 90% removal of histidine[21] was accomplished by ultrafiltration (C-10 Centriprep, Amicon). The loading buffer for the affinity purification was modified by adding 5 mM imidazole and 10% glycerol.

The Ni^{2+} NTA Superflow resin (Qiagen) was used for batch purification according to the instructions of the manufacturer. 2.5 µl of resin were used for each ml of supernatant.

The Ni^{2+} resin was prewashed with 2% BSA and 50% FCS followed by an elution with 200 mM imidazole prior to use. After adding sample, the resin was incubated with agitation for 12-24h at RT. The resin was washed 8 times for 1 h with 1 ml modified washing buffer (10 mM imidazole, 10% glycerol). The affinity-bound protein was eluted with each 800 µl 125 mM, 250 mM, 375 mM and 500 mM imidazole. The eluate was dialysed for 3 days against 1-2xPBS changing the buffer 6 times. Alternatively, the imidazole elution buffer was exchanged in a Sephadex G-25 column against 100 mM ammonium acetate or 2xPBS. To obtain saltfree protein, the ammonium acetate was evaporated in a SpeedVac at RT for 12-24h.

Quantity and quality of the protein was assayed on a silver- and Coomassie Brilliant Blue-stained 15% SDS-PAGE (see appendix) and its biological activity tested as described under 4.2.5.3..

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21. from the insect cell culture medium

5. Results and discussion

5.1. Transgenic mice

5.1.1. αMHC-VEGF-C-FL

55 potential founders were obtained for the αMHC-VEGF-C-FL construct. Both PCR and Southerns were repeatedly negative. A probable explanation would be that VEGF-C overexpression in the heart is lethal in utero. It was considered to determine time point and cause of the potential developmental arrest by examining the embryos, but the project was not pursued due to capacity reasons.

5.1.2. αMHC-VEGF-B167

42 potential founders were obtained for the αMHC-VEGF-B167 construct, out of which 6 were positive. Four of the 6 founders were positive in both Southern blotting and PCR, one founder was positive only in PCR and another only in Southern blotting analysis. It became clear that the PCR-positive, but Southern-negative founder had undergone a rearrangement in the transgene, which resulted in the loss of the αMHC promoter for which the Southern probe was specific. A possible reason, why one other potential founder was Southern positive, but PCR negative, could have been a minor deletion resulting in the loss of one primer annealing site. However, this was not confirmed (a pedigree of the mice is included in the appendix, Figure 78.).

All Southern-positive founders were mated and the pups were screened as described for the transgene. One mouse did not transmit the transgene, whereas the others transmitted to 37.5 - 53% of the pups. The copy number was not exactly determined, but ranged between 2 and 25 copies/diploid genome, as judged by the Southerns.

Positive pups from all founders and their nontransgenic littermates were sacrificed for histological and in situ hybridisation analysis. Especially the heart was subject of examination, where the transgene was expected to be expressed. Surprisingly, no transgene expression could be detected by in situ hybridisation. In agreement with this, the histological findings are normal, but analysis is still going on. In this respect it is noteworthy, that much more founders were positive in nested PCR, but semiquantitative analysis revealed apparent copy numbers significantly lower than 0.1/diploid genome. These mice might have been either mosaics or false positives.

5.1.3. RIP-VEGF-B167

Of 32 potential founders one contained an high copy number (35-50/diploid genome) of the transgene, two a copy number between 5-10/diploid genome, and one a copy number near to the sensitivity border of the dot blot assay. The mouse with high copy number was reduced in size, had faint cranial malformations and seemed to be blind on the right eye. It was killed at four weeks of age to avoid natural death and loss of material. Its pancreas was histologically analysed by both hematoxylin-eosin staining and immunostaining for insulin, glucagon, pancreatic polypeptide and somatostatin. The islets appeared to be normal. Whether the transgene was expressed at all, remains to be determined. Instead, the observed phenotype might result from the disruption of an unrelated gene by the insertion of the transgene. If the transgene is transmitted, the F2 generation of the other two founders (both females, which appear completely normal) will be subject of more detailed analysis.

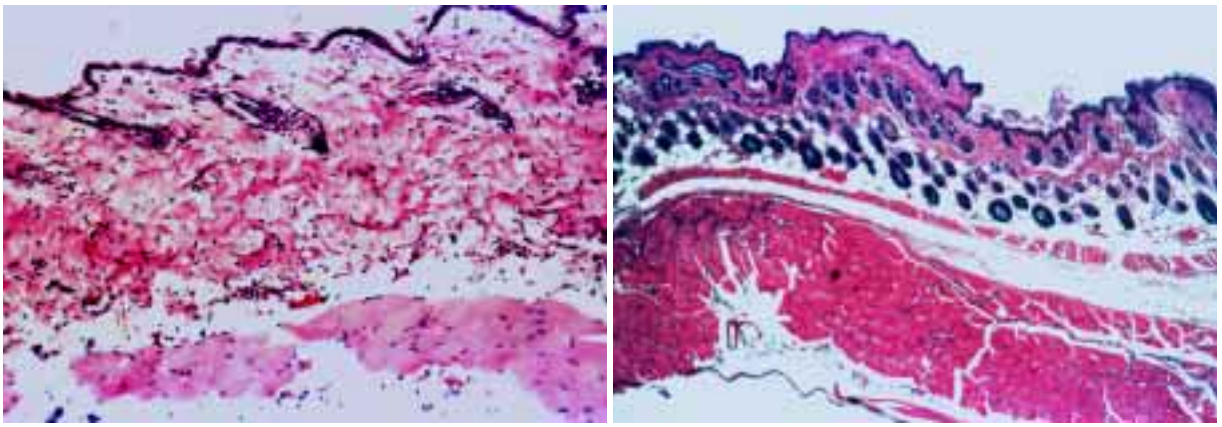
5.1.4. RIP-VEGF-C-FL

Two positive mice were identified out of 46 potential founders, both with approximate copy numbers between 5-10/diploid genome. Similarly to the RIP-VEGF-B167 transgenic mice, they appear normal, but detailed analysis awaits the F2 generation.

5.1.5. K14-VEGF-B167

50 potential founders were obtained, one of which died shortly after birth and was not analysed. 5 of them had unmistakably more then 1 copy of the transgene in their genomes, although signals approximately equivalent to the 0.1 copy number control were obtained by PCR for 7 other potential founders, probably mosaics. For the five undoubted founders the copy numbers were determined as approximately 2-5 (in two cases), 10-20 and 30-50 (in two cases). All of them were mated, but three of them failed repeatedly to transmit the transgene (altogether 17, 16 and 6 pups were born, respectively). The founder which had 2-5 copies in its genome transmitted to 5 out of 13 pups (38%). The other founder which transmitted (to 3 out of 21 pups, 14%) had 30-50 copies in its genome.

Positive pups and negative littermates were sacrificed for analysis. Preliminary data shows a distinct phenotype in both mice, which is difficult to interpret and which requires further investigation. The most striking in a first comparison to the negative control was the reduced number of hair follicles in the dermis, which was itself thickened due to an increase in extracellular matrix forming collagen. The reduction of hair follicles was frequently reported from mice overexpressing cytokines under the keratin 14 promoter, e.g. TGF-α, PTHrP and KGF (Vassar et al., 1991; Guo et al., 1993; Wysolmerski et al., 1994) and might be therefore unspecific, perhaps due to cytotoxic effects. However, in contrast to VEGF-B_{167} overexpressing mice, in these mice no increased collagenisation was observed. The possibility was considered, whether VEGF-B would only be active in the presence of both isoforms, probably as heterodimer, since recent unpublished data indicates a constant 1:1 ratio of these isoforms. Consequently, we decided to direct the expression of a VEGF-B_{186} cDNA under the same keratin 14 promoter. Since the genomic structure was meanwhile sufficiently explored, a genomic fragment capable of generating the two different mRNAs was considered as well.



A **B**

Figure 11. Hematoxylin-eosin stained sections of K14-VEGF-B167 transgenic mouse and control littermate
A K14-VEGF-B167 transgenic mouse; **B** transgene-negative littermate

5.1.6. K14-VEGF-C-FL

Out of 27 founder mice analysed at 3 weeks of age, 3 were positive, having approximately 40-50, 20 and 4-6 copies of the transgene in their genome.

The mouse with the high copy number transgene was small and developed slower than its littermates. Further examination showed a swollen, red snout, poor fur and abnormally long incisors. It had difficulties in breast feeding and breathing. Although nourished with special liquid diet, it suffered from oedema of the upper respiratory and digestive tracts and died eight weeks after birth. It was immediately processed for histology, immunohistochemistry and in situ hybridisation.

The other two positive mice, both males, exhibited a similar, although milder phenotype and in spite of initial difficulties they were mated successfully. One transmitted the gene to 6 out of 11 pups (55%) before it died at the age of 4 months; the other mouse transmitted the gene to 1 out of 17 pups (6%), although two mice were born dead and not included in the analysis. A pedigree of the mice is given in the appendix, Figure 79..

Histological examination showed that, in comparison with the skin of littermates, the dermis of K14-VEGF-C-FL transgenic mice was atrophic and the connective tissue of the skin was replaced by large lacunae devoid of red cells, but lined with an attenuated endothelial cell layer. At the walls of the distended vessel-like structures remnants of valves were identified, a distinguishing feature between initial lymphatics and blood vessels in the upper dermis (Ryan et al., 1986). The vessel-like structures resembled those seen in human lymphangiomas and were most remarkable in the snout and dorsal region.

In situ hybridisations of skin samples show that the major VEGF-C receptor FLT4 is highly upregulated in these transgenic mice, and that this overexpression is confined to the endothelial lining of the vessel-like structures. Notably, FLT4 is the most promising nominee as a marker for lymphatic vascular endothelial cells in adults (Kaipainen et al., 1995). It is interesting that the effect of VEGF-C was completely confined to the skin. Probably VEGF-C sticks not only *in vitro*, but also *in vivo* to various surfaces. This quality might be mediated through its silk homology domain and is probably significant in the spatial limitation of VEGF-C activity.

Thus it appears, that VEGF-C induces selective angiogenesis of the lymphatic vessels *in vivo*. Few entirely specific lymphatic endothelial cell markers have been established to date. Antibodies against collagen type XVIII gave weak or no staining of the vessels, while the basement membrane of blood vessels was stained prominently. The expression of desmoplakin I and II and the less developed basement membranes compared to blood capillaries strongly suggest that the abnormal vessels were derived from lymphatic vessels (Casley-Smith, 1980; Schmelz et al., 1994). Platelet-endothelial cell adhesion molecule (PECAM-1) was used as a general marker for vascular endothelium (Dejana et al., 1995).

The lymphatic structures were mainly increased in size, but lymphatic sprouting was absent or subordinate. According to preliminary data (measuring cell proliferation of *in vitro* explants by BrdU incorporation), the observed lymphatic vascular endothelium is proliferating and the increased size of the vessels might be therefore due to an absent sprouting signal. An alternatively explanation is that the oversized capillary network in the skin cannot be drained efficiently by the larger lymphatic vessels, which are normally sized. In this case the enlarged vessel lumen would be a secondary effect due to congestion of lymphatic fluid. Accordingly, the reduced number of skin adnexal organs and hair follicles might be merely due to a physical displacement of the dermal connective tissue. The first explanation would suggest that at least two distinct signals are required for the establishment of a lymphatic network: one signal that would stimulate migration and sprouting and another, distinct signal, that would stimulate the proliferation of the involved endothelium.

As a conclusion, VEGF-C delivers a growth signal rather to lymphatic endothelial cells than to non-lymphatic endothelial cells, thus being distinct from VEGF (Wilting et al., 1996a). The VEGF subfamily of growth factors is still expanding, and the present work tried to address the question, why different VEGFs have emerged during evolution and to what degree their functions are still redundant.

Figure 12. K14-VEGF-C-FL transgenic mouse with littermate

5.1.6.1. Histological analysis

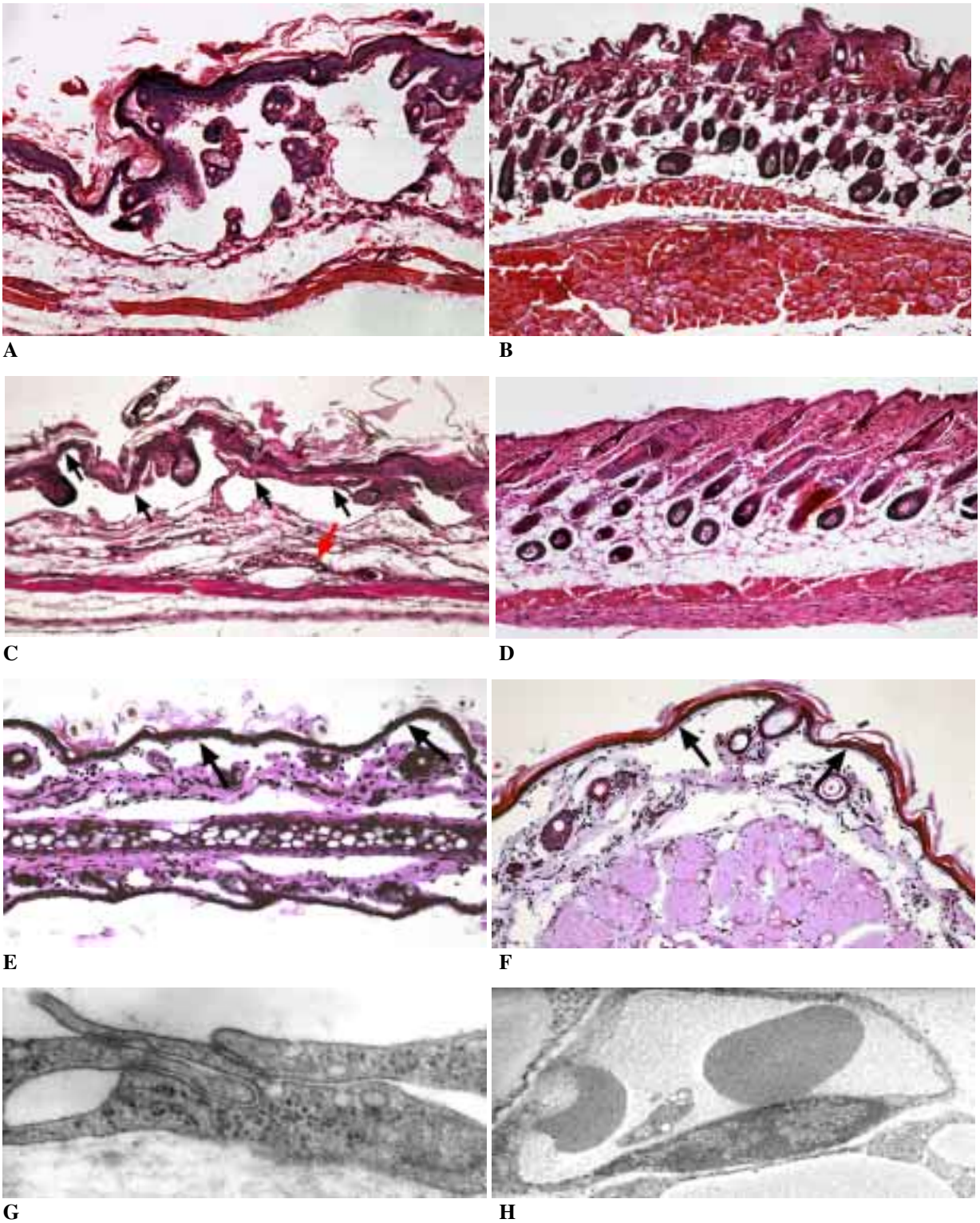


Figure 13. Histological sections and electron microscopy of K14-VEGF-C-FL transgenic mouse skin

Hematoxylin-eosin stained skin sections of a VEGF-C-FL transgenic mouse (**A**, **C**, **E**) and of a control littermate (**B**, **D**, **F**). Note a hyperkeratotic epidermis with underlying vessel spaces lined with an endothelium (black arrows), but devoid of red cells (compare with the dermal vein shown with a red arrow). The dermis is atrophic in comparison with the littermate skin and the muscular layer is also reduced.

Electron microscopic pictures of the endothelial junctions of the abnormal vessels (**G**) and of a normal capillary (**H**) beneath.

5.1.6.2. In situ hybridisation analysis

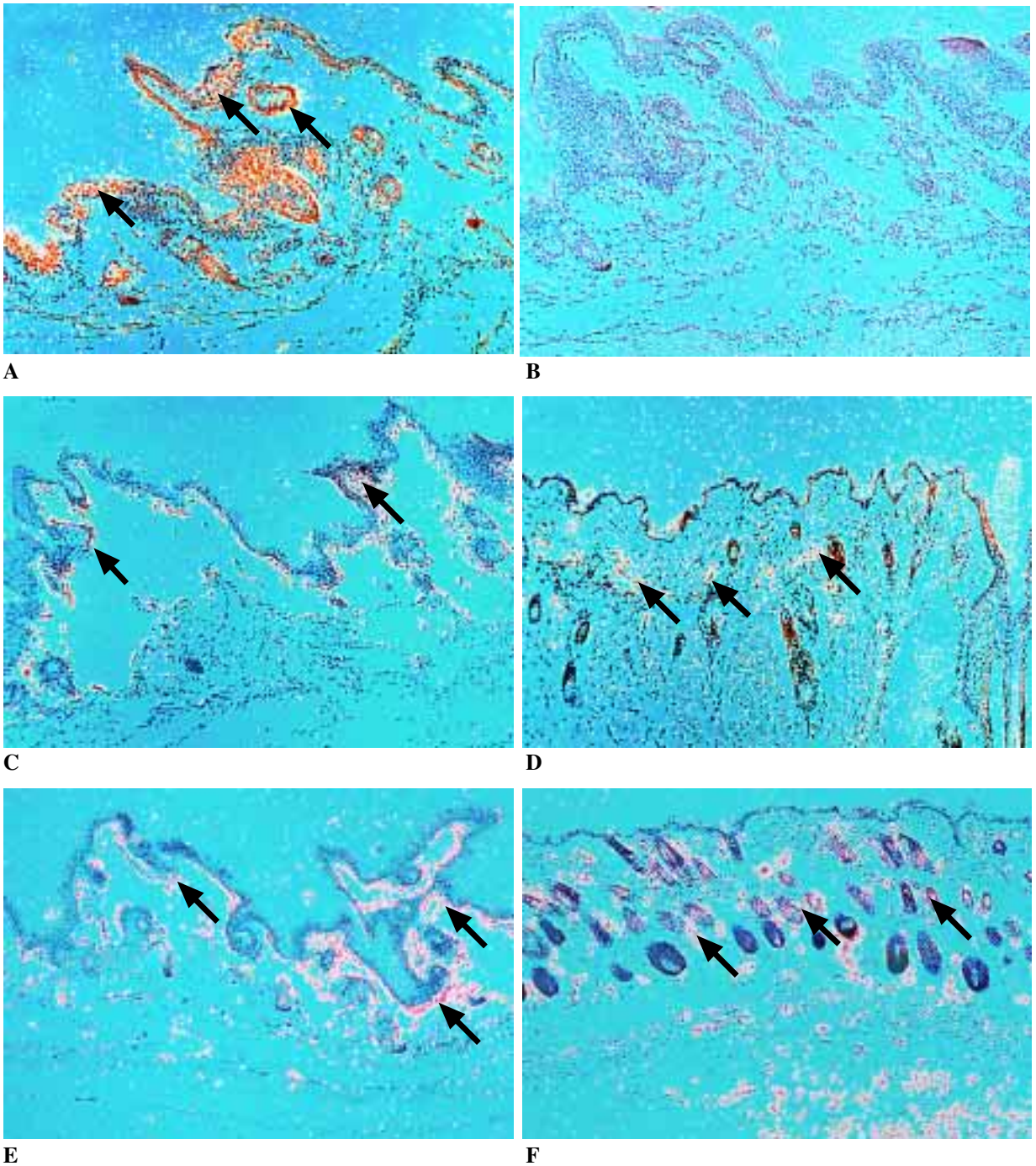


Figure 14. VEGF-C, FLT4 and FLK1 in situ hybridisations of K14-VEGF-C-FL transgenic mouse skin

VEGF-C antisense probe**:** VEGF-C-FL transgenic mouse (**A**)

VEGF-C sense probe: VEGF-C-FL transgenic mouse (**B**)

FLT4 antisense probes: VEGF-C-FL transgenic mouse (**C**), control littermate (**D**)

FLK1 antisense probes: VEGF-C-FL transgenic mouse (**E**), control littermate (**F**)

Abundant VEGF-C mRNA was detected in the epidermis and hair follicles of the transgenic mice (black arrows in Figure **A**). mRNAs encoding the VEGF-C receptors FLT4 and FLK1 were expressed in endothelial cells lining the abnormal vessels (black arrows in Figures **C** and **E**). In the skin of littermate control animals, FLT4 was detected only in the superficial subpapillary layer of lymphatic vessels (black arrows in Figure **D**), while FLK1 was found in all endothelia (Figure **F**)

5.1.6.3. Immunohistochemical analysis

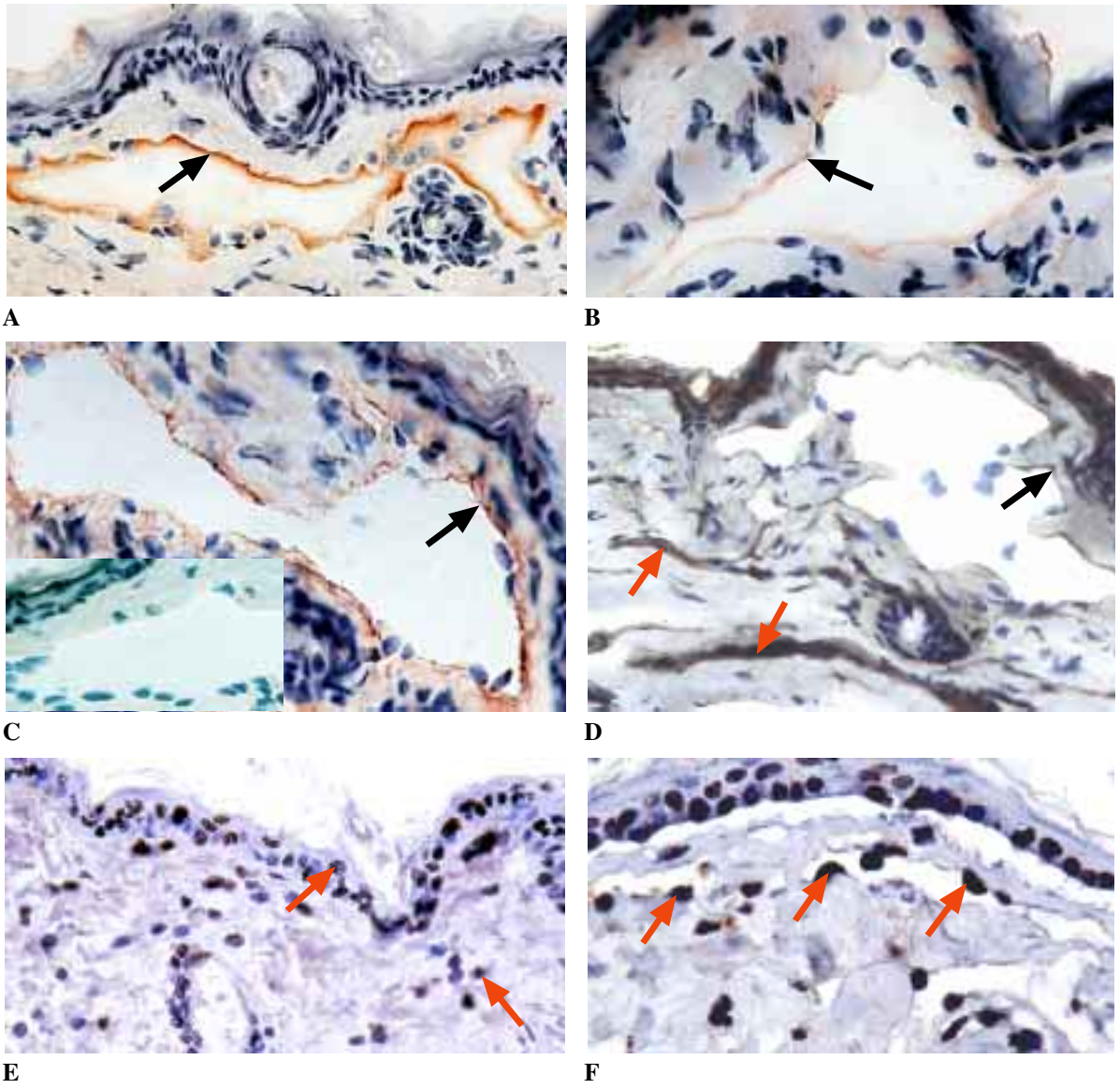


Figure 15. Immunohistochemical analysis of K14-VEGF-C-FL transgenic mouse skin

Sections of were stained with: **A** anti-rat PECAM IgG, **B** anti-mouse desmoplakin I and II IgG, **C** human anti-FLT4 antiserum, **D** anti-rabbit collagen XVIII IgG; Black arrows point to a positive reaction in lymphatic endothelium. In **D** the basal lamina is stained in veins (red arrows), but not in the lymphatic endothelium (black arrow). The negative control in **C** shows staining with FLT4 preimmune serum. In **E** and **F** cells are detected which are in the S phase of the cell cycle, using *in vitro* BrdU incorporation into DNA. In control littermates mainly nucleae of keratinocytes of the epidermis and some dermal cells are stained with anti-BrdU (arrows in **E**), whereas sections of transgenic mice show nuclear staining in many endothelial cells of the lymphatic vessels as well as in the keratinocytes (arrows in **F**).

5.1.7. K14-ΔNΔC-VEGF-C, K14-VEGF-B186

No data is available yet.

5.2. Recombinant VEGF-B and VEGF-C

Although the full length cDNA led to the expression of biologically active VEGF-C, total amount, processing and secretion seemed not to be optimal. Different constructs were generated to meet the different purposes (see Table 3 and Table 4). Firstly, the melittin signal peptide was used instead of the endogenous one to enhance secretion. Secondly, the protein was trimmed at both N- and C-termini to circumvent the proteolytical processing, which was inefficiently done in insect cells, but which is inevitable for biological activity. Unlike most other members of the VEGF family VEGF-C shows no affinity towards heparin, on which the purification procedures could have been based similar to VEGF (Ferrara

et al., 1991). Therefore a histidine tag was incorporated for affinity purification. Since the protein was secreted into serum-free media a one-step affinity purification was enough to obtain VEGF-C clean enough for most applications. melSP-ΔNΔC-C1HIS-VEGF-C mostly seems to be secreted as a non-covalently linked dimer or monomer, although some of it migrates even in reducing SDS-PAGE as a dimer.

Interestingly, from all four tested locations, the N-terminal histidine tag seemed to interfere with receptor activation. The C-terminal histidine-tag was designed to purify the C-terminal proteolytical cleavage product and the uncleaved propeptide. Both histidine tags that were placed behind the VEGF homology domain functioned in the affinity purification using Ni^{2+}-NTA Superflow resin (Qiagen)[22], although the more N-terminally located histidine-tag (C1HIS) seemed to be slightly superior.

The short splice variant of VEGF-C was produced much less efficiently than all other forms. In the used concentrations it seems neither to stimulate FLT4 nor KDR phosphorylation. Inhibition experiments were also negative; the short splice variant seems not to interfere with receptor activation by biologically active VEGF-C.

The expression of VEGF-B in insect cells appeared to be intrinsically difficult. Differently from mammalian cells, VEGF-B_{167} is seemingly not secreted from Sf9 and HF cells. The efficiency of secretion could not be altered by exchanging the endogenous signal peptide against the honey bee melittin signal peptide. Alternatively, VEGF-B_{167} might be very strongly bound to the cell surface unable to be released by heparin concentrations ≤20 µg/ml.

VEGF-B_{186} is secreted, but N-terminally histidine-tagged VEGF-B_{186} tends to aggregate (personal communication by Birgitta Olofsson). The C-terminally histidine-tagged VEGF-B_{186} was secreted into the medium, probably because the histidine-tag compensated for the otherwise hydrophobic C-terminus. It showed on SDS-PAGE the same size and dimerisation as the mammalian protein. Apparently the O-glycosylation is performed by insect cells. Sf9 cells infected with the same virus produced a protein with a much lower molecular weight. This protein cannot be affinity-purified, probably due to proteolytical cleavage which removes the histidine-tag.

Opposite to VEGF-C, the VEGF-B receptor(s) is/are unknown and hence no efficient way exists to confirm the biological activity and specificity of modified versions of VEGF-B (Aase et al. 1999).

22. Very inefficient affinity purification was seen, when the Talon™ metal affinity resin (Clontech) was used instead.

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7. Appendix

7.1. EMBL/GenBank™ accession numbers (nucleotide sequence)

PDGF

hPDGF-A, genomic	S51624 (part one)
hPDGF-A, genomic	S50869 (part two)
hPDGF-B, cDNA	M12783; M16288

VEGF

$hVEGF_{165}$, cDNA	M32977
hVEGF, genomic	M63971 (exon 1)
hVEGF, genomic	M63972 (exon 2)
hVEGF, genomic	M63973 (exon 3)
hVEGF, genomic	M63974 (exon 4)
hVEGF, genomic	M63975 (exon 5)
hVEGF, genomic	M63976 (exon 6)
hVEGF, genomic	M63977 (exon 7)
hVEGF, genomic	M63978 (exon 8)
mVEGF, genomic	S64321 (alternatively spliced exon 6)
mVEGF promoter	U41383

PlGF

$hPlGF_{149}$, cDNA	X54936
$rPlGF_{132/135}$, cDNA	L40030

VEGF-B

$hVEGF\text{-}B_{167}$, cDNA	U48801
$hVEGF\text{-}B_{186}$, cDNA	U52819
$mVEGF\text{-}B_{186}$, cDNA	U52820
$hVRF_{186}$, cDNA	U43368
$hVRF_{167}$, cDNA	U43369
$mVRF_{186}$, cDNA	U43836
$mVRF_{167}$, cDNA	U43837

VEGF-C

hVEGF-C, cDNA	X94216
mVEGF-C, cDNA	U73620
hVRP, cDNA	U43142

FLT1

hFLT1, cDNA	X51602
sFLT1, cDNA	U01134

KDR/FLK1

FLK1, cDNA	X59397
hKDR, cDNA	L04947

FLT4

hFLT4s, cDNA	U43143
hFLT4l, cDNA	X68203; X69878; S59182
hFLT4l, cDNA	S66407 (alternatively spliced 3'-end)
mFLT4, cDNA	L07296

7.2. Genomic structures and mRNA splice variants of VEGF, VEGF-B and VEGF-C

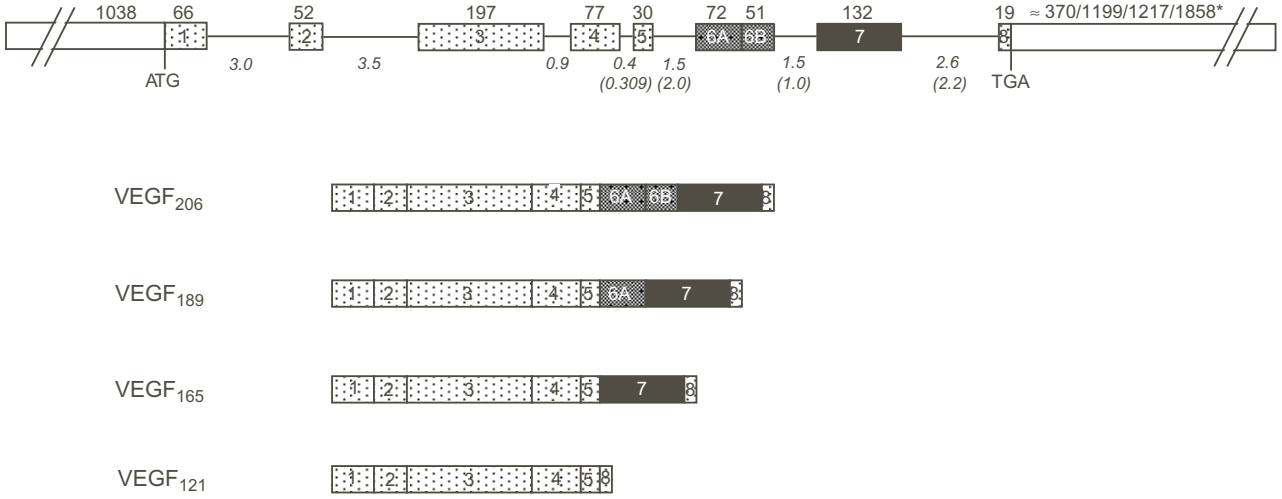


Figure 16. Genomic organisation of the human VEGF gene and mRNA splice variants

Legend: squares: exons (empty = untranslated sequence, filled = translated sequence); lines: introns; numbers above exons: exon length (bp); numbers under introns in italics: intron length (kb) according to Tischer (Tischer et al., 1991), in brackets according to Houck (Houck et al., 1991); scale: 1 cm exon = 83 bp, 1 cm intron = 2 kb.

* The lengths given for the 3'-UTR refer to the polyadenylation signals identified in the rat VEGF gene (Levy et al., 1995), the length of the major transcript in both human and rat (approximately 3.7 kb; Goldberg et al., 1994) suggests for the major human mRNA a 3'-UTR of approximately 2.1 kb.

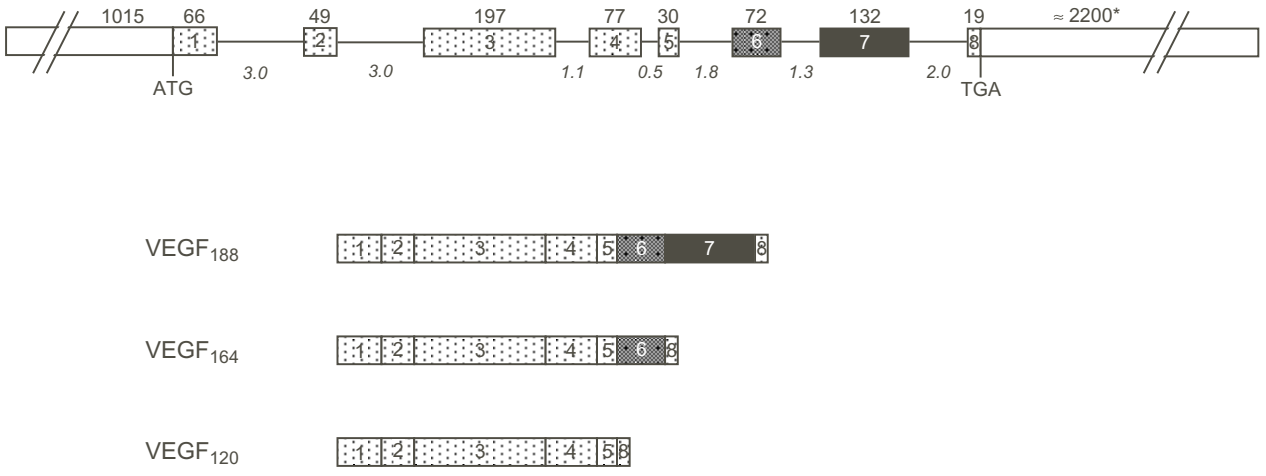


Figure 17. Genomic organisation of the murine VEGF gene and mRNA splice variants

Legend: squares: exons (empty = untranslated sequence, filled = translated sequence); lines: introns; numbers above exons: exon length (bp) according to Breier (Breier et al., 1992); numbers under introns: intron length (kb) according to Shima (Shima et al., 1996); scale: 1 cm exon = 83 bp, 1 cm intron = 2 kb.

* The length given for the 3'-UTR refers to one identified site of transcription termination (Shima et al., 1996); however mRNA transcripts vary in length between 2.7 kb and 4 kb, indicating that upstream at least one alternative termination site is present.

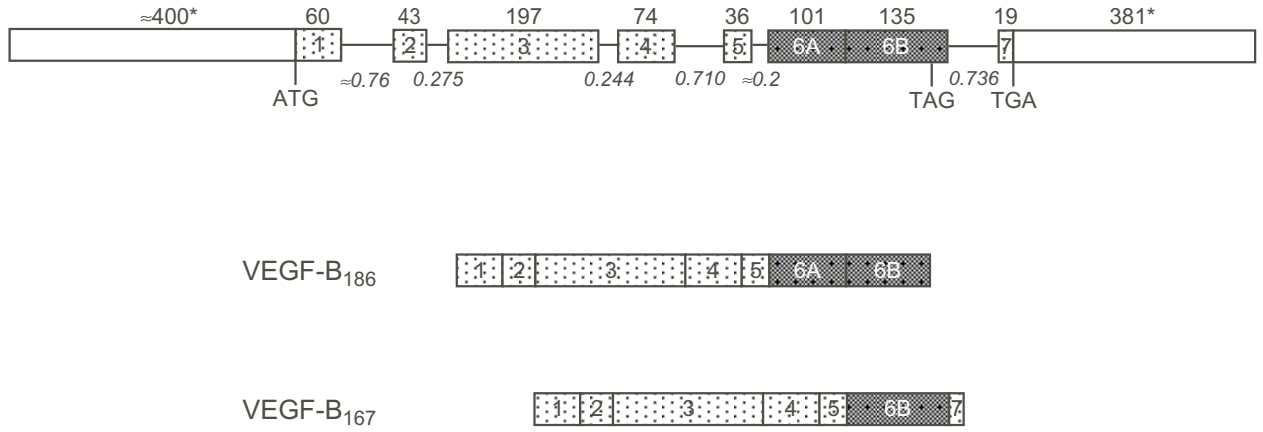


Figure 18. Genomic organisation of the human VEGF-B gene and mRNA splice variants

Legend: squares: exons (empty = untranslated sequence, filled = translated sequence); lines: introns; numbers above exons: exon length (bp); numbers under introns in italics: intron length (kb); scale: 1 cm exon = 83 bp, 1 cm intron = 1 kb (data according to Olofsson et al., 1996b).

* The 3'-UTR comprises 381 bp (personal communication by Suvi Taira) and based on the length of the mature mRNA for VEGF-B186 (approximately 1.4 kb; Olofsson et al., 1996b) the transcriptional start site can be expected about 400 bp upstream of the translation initiation ATG.

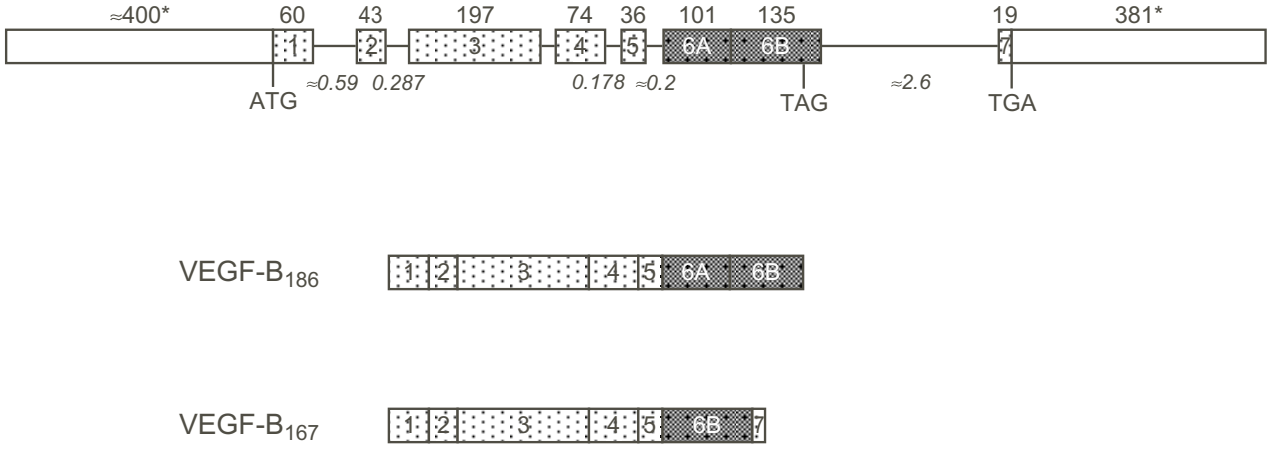


Figure 19. Genomic organisation of the murine VEGF-B gene and mRNA splice variants

Legend: squares: exons (empty = untranslated sequence, filled = translated sequence); lines: introns; numbers above exons: exon length (bp); numbers under introns in italics: intron length (kb); scale: 1 cm exon = 100 bp, 1 cm intron = 1 kb (data according to Olofsson et al., 1996b). *Length of 3'-UTR and the transcriptional start site seem to be similar for the murine and human gene (personal communication by Suvi Taira) and the lengths refer to the human gene.

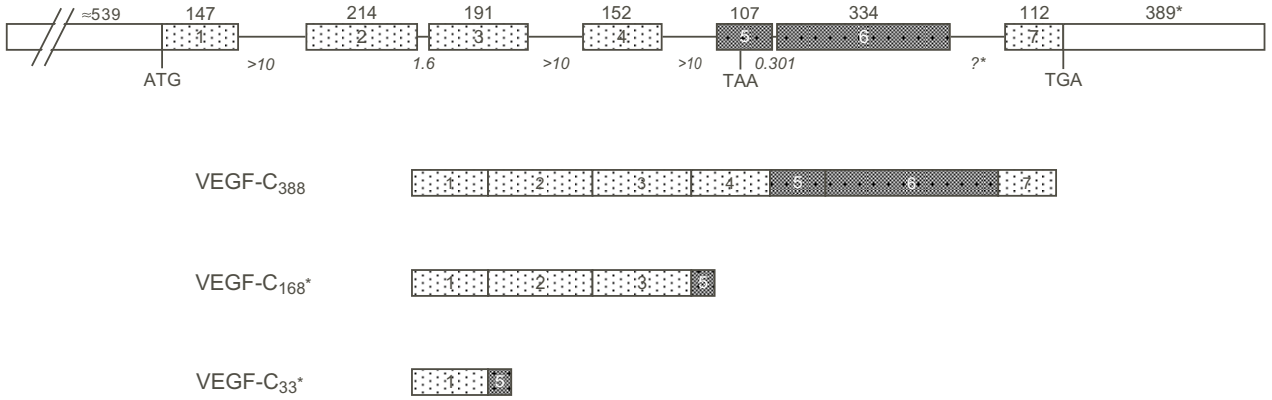


Figure 20. Genomic organisation of the human VEGF-C gene and mRNA splice variants

Legend: squares: exons (empty = untranslated sequence, filled = translated sequence); lines: introns; numbers above exons: exon length (bp) according to Dmitry Chilov (personal communication); numbers under introns in italics: intron length (kb) according to Dmitry Chilov (personal communication); scale: 1 cm exon = 100 bp, 1 cm intron = 1 kb, if the intron size was not or only approximately known, the drawing was based on the size of the corresponding mouse gene intron according to Suvi Taira (personal communication).

* The lengths given for the 3'-UTR refers to the polyadenylation signal present in the first VEGF-C cDNA clone (Joukov et al., 1996). The two shorter forms of VEGF-C are hypothetical, mRNA species of corresponding lengths are described (Lee et al., 1996), but it is unknown whether they are translated.

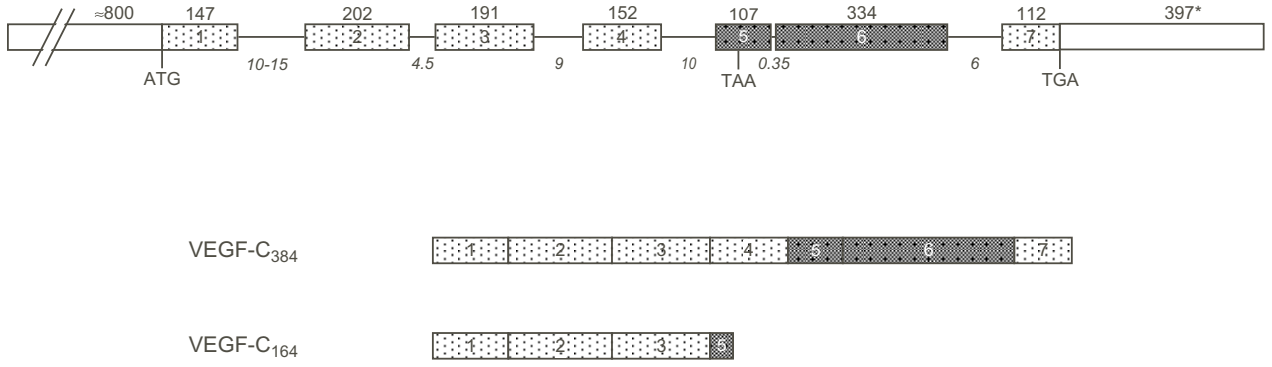


Figure 21. Genomic organisation of the murine VEGF-C gene and mRNA splice variants

Legend: squares: exons (empty = untranslated sequence, filled = translated sequence); lines: introns; numbers above exons: exon length (bp) according to Suvi Taira (personal communication); numbers under introns in italics: intron length (kb) according to Suvi Taira (personal communication); scale: 1 cm exon = 100 bp, 1 cm intron = 1 kb.

* The length given for the 3'-UTR refers to the known genomic clone covering the 3'-flanking region with the polyadenylation signal, the length of the 5'-UTR is deduced on basis of the major murine transcript (approximately 2.4 kb; Suvi Taira, personal communication).

7.3. Exon/intron borders of VEGF, VEGF-B and VEGF-C genes

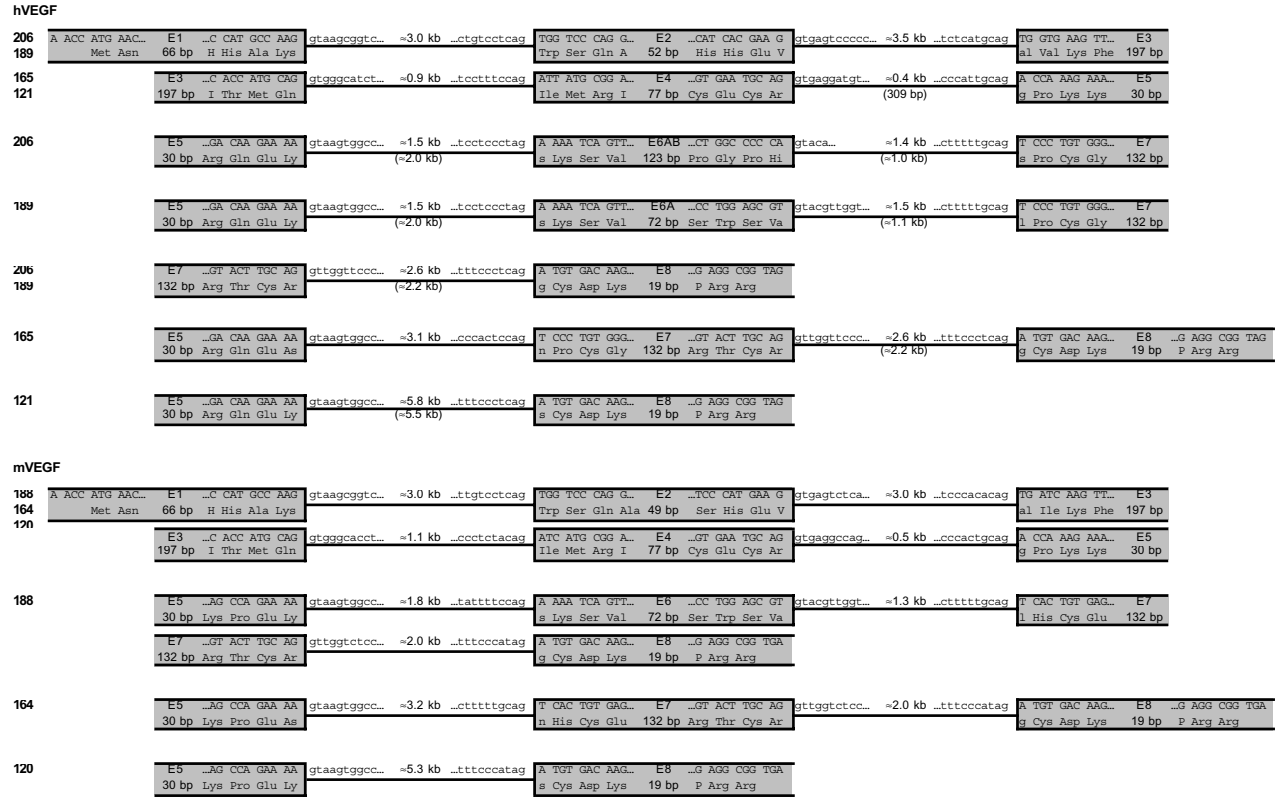


Figure 22. Exon/intron borders for human and murine VEGF

E = exon; upper case characters = exonic sequence; lower case characters = intronic sequence; the length of the translated parts of the exons is indicated; intronic sequence length in brackets refers to the intron length given by Houck, which differs for the introns 4, 5, 6 and 7 from the data given by Tischer (Sources: Houck et al., 1991; Tischer et al., 1991; Breier et al., 1992; Shima et al., 1996).

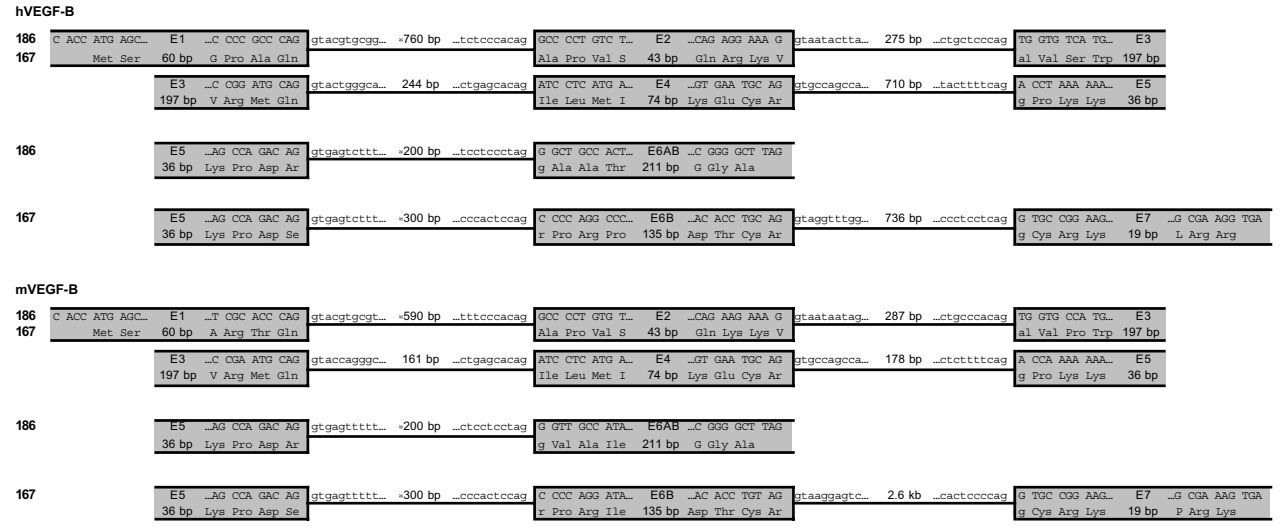


Figure 23. Exon/intron borders for human and murine VEGF-B

E = exon; upper case characters = exonic sequence; lower case characters = intronic sequence; the length of the translated parts of the exons is indicated (Source: Olofsson et al., 1996b).

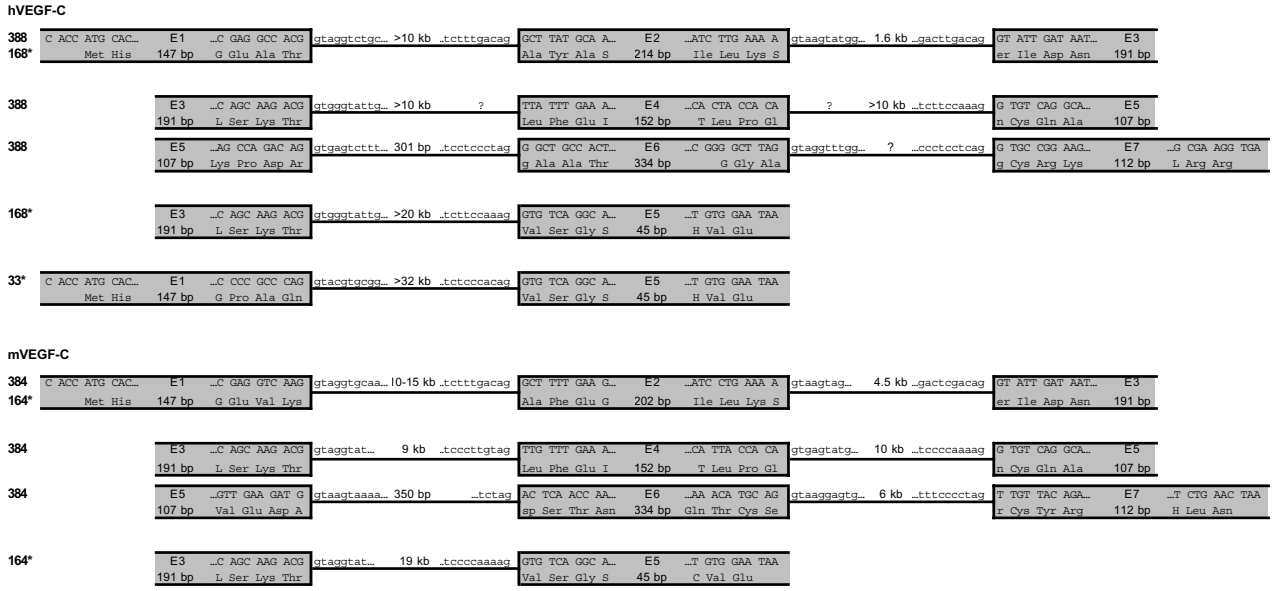


Figure 24. Exon/intron borders for human and murine VEGF-C

E = exon; upper case characters = exonic sequence; lower case characters = intronic sequence; the length of the translated parts of the exons is indicated. *The shorter forms of VEGF-C are hypothetical, mRNA species of corresponding lengths are described for humans (Sources: Eola Kukk, personal communication; Lee et al., 1996).

7.4. Plasmid maps

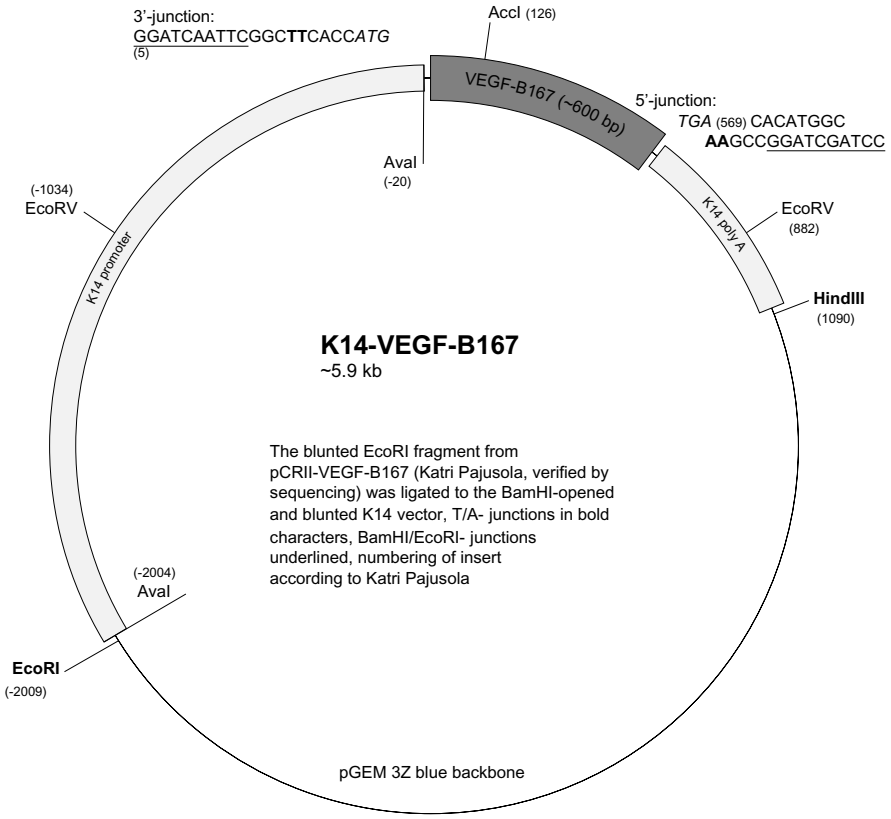


Figure 25. K14-VEGF-B167

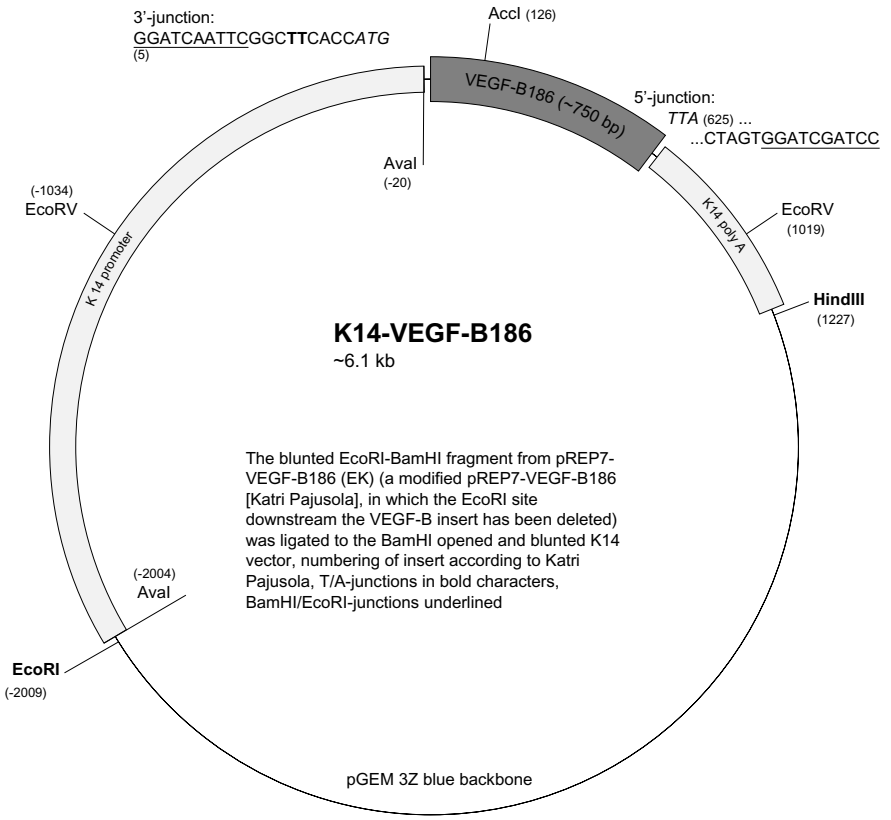


Figure 26. K14-VEGF-B186

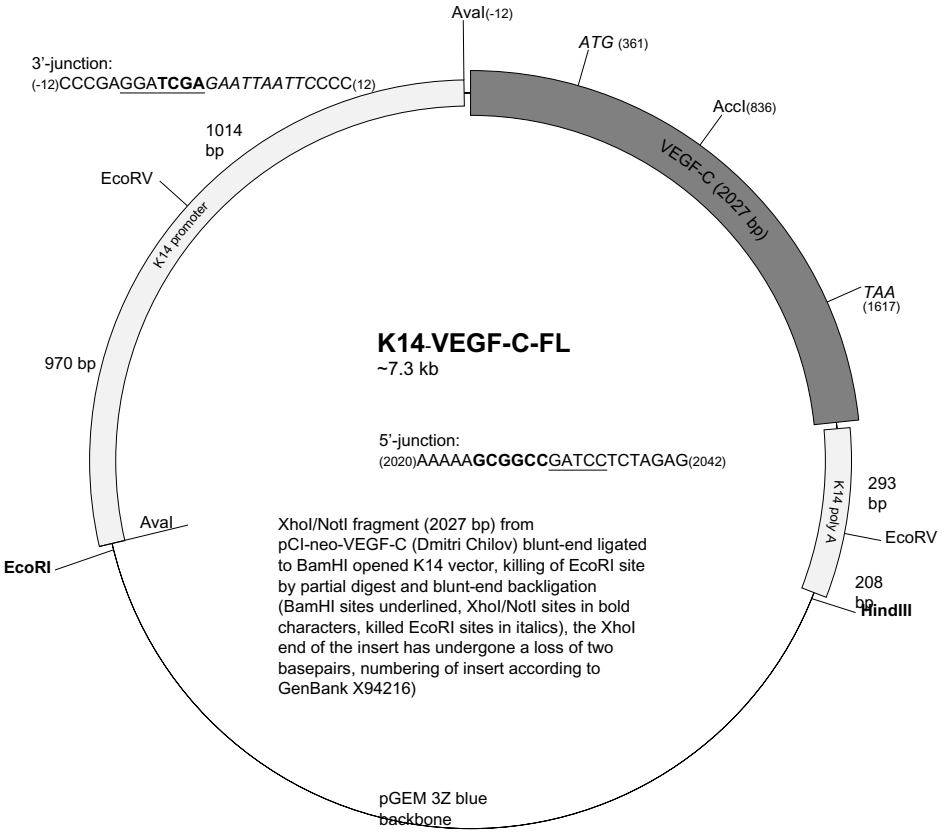


Figure 27. K14-VEGF-C-FL

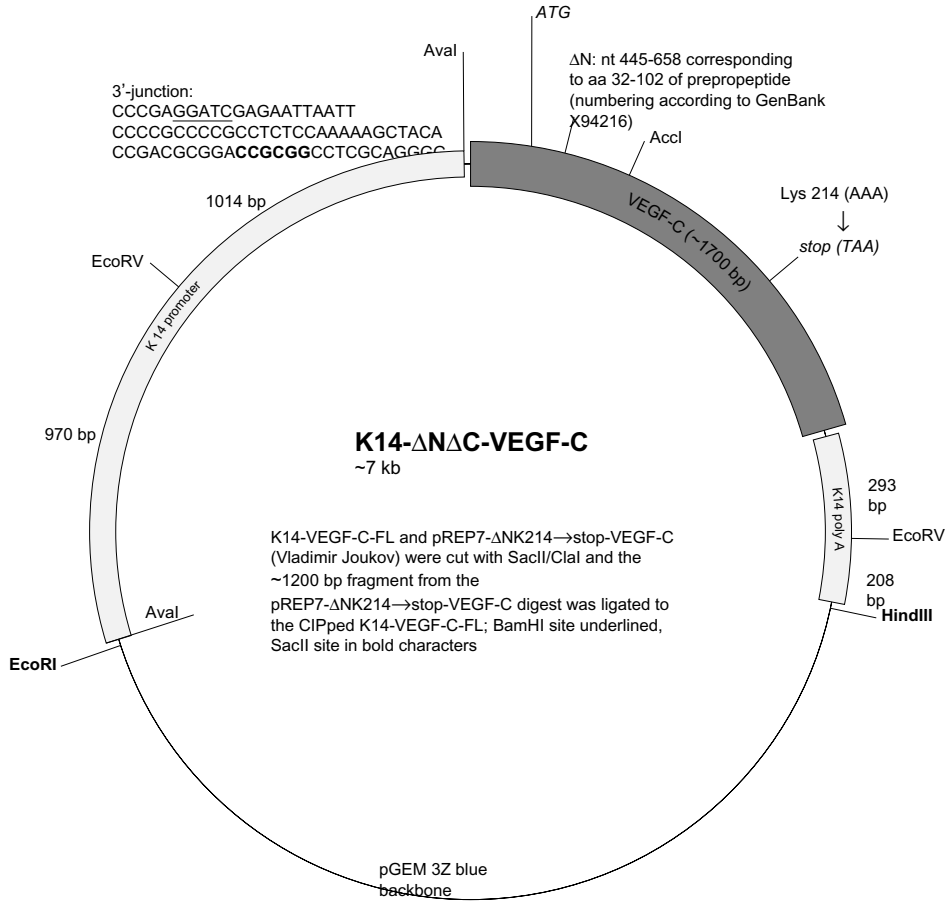


Figure 28. K14-ΔNΔC-VEGF-C-FL

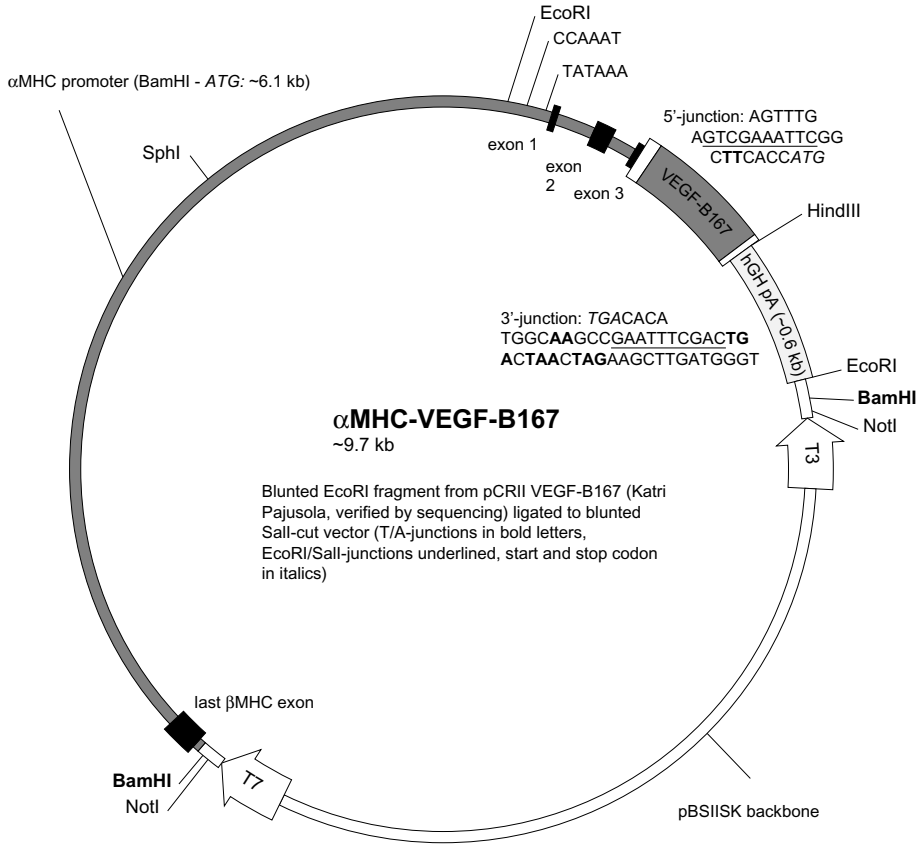


Figure 29. αMHC-VEGF-B167

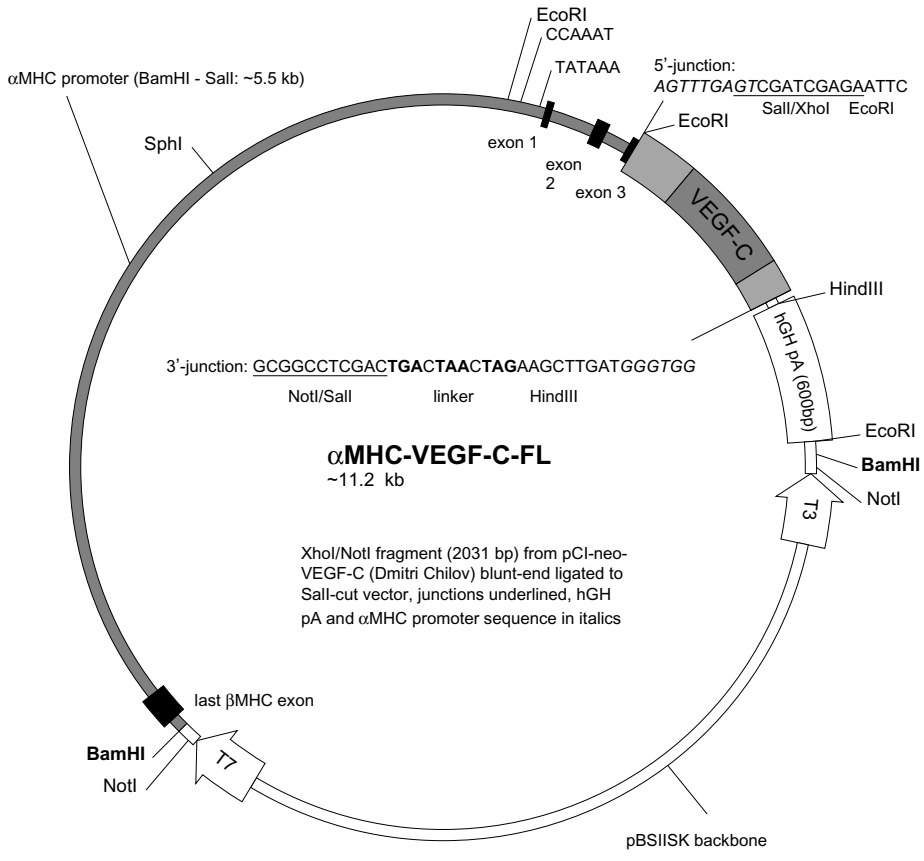


Figure 30. αMHC-VEGF-C-FL

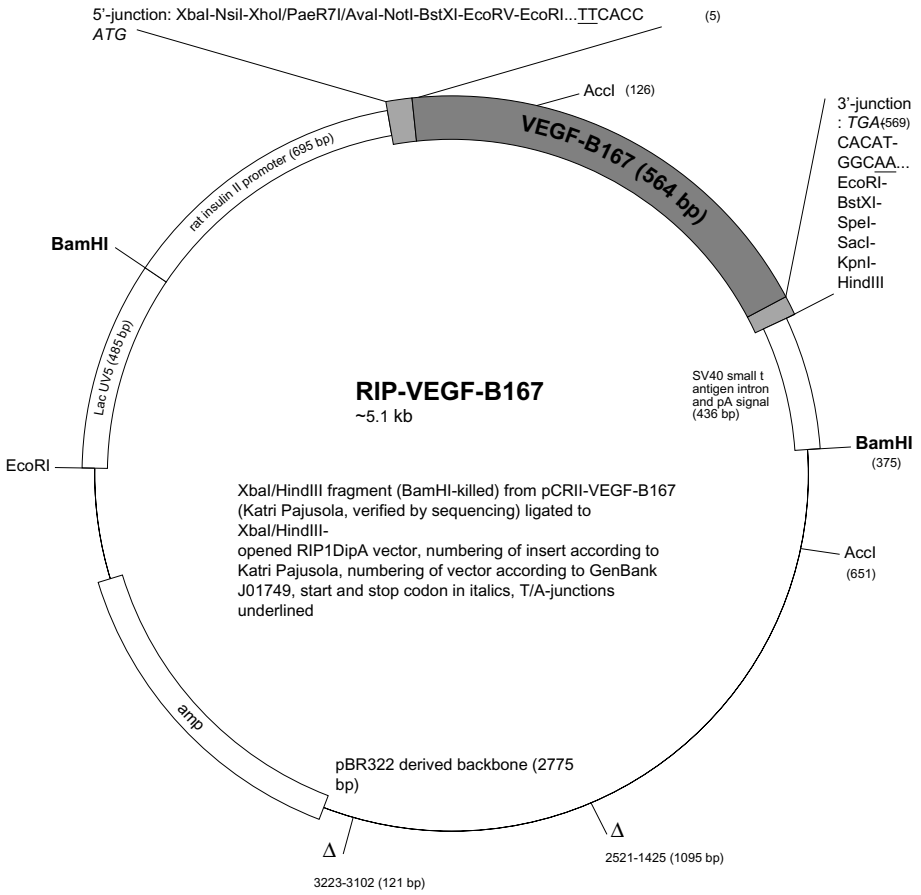


Figure 31. RIP-VEGF-B167

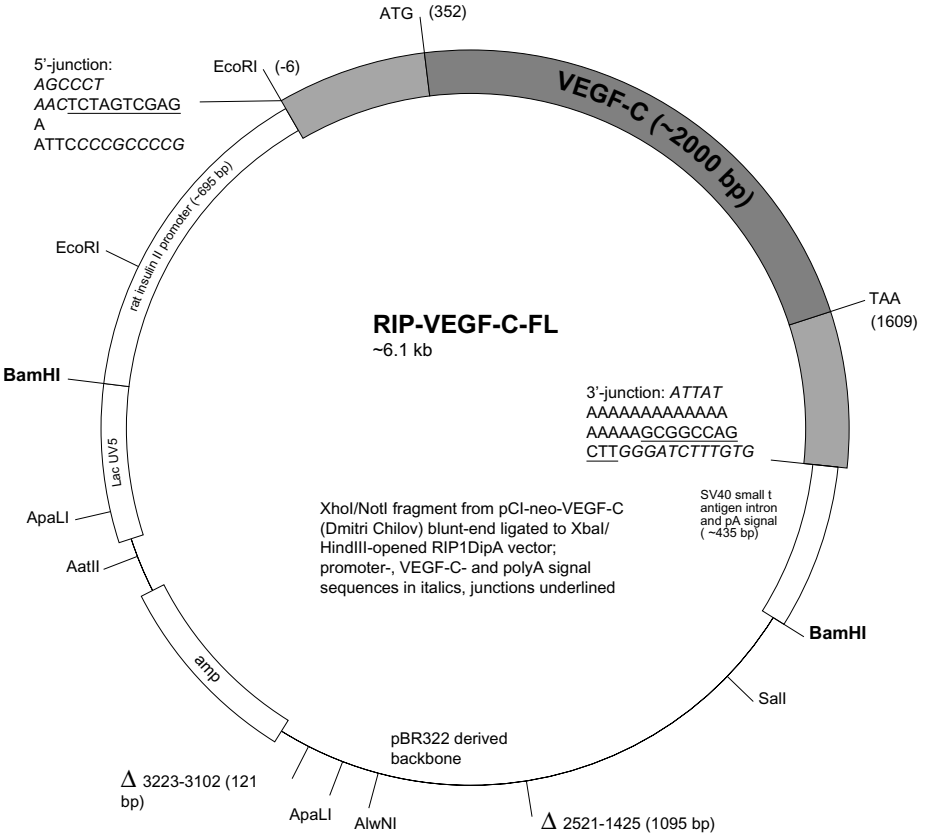


Figure 32. RIP-VEGF-C-FL

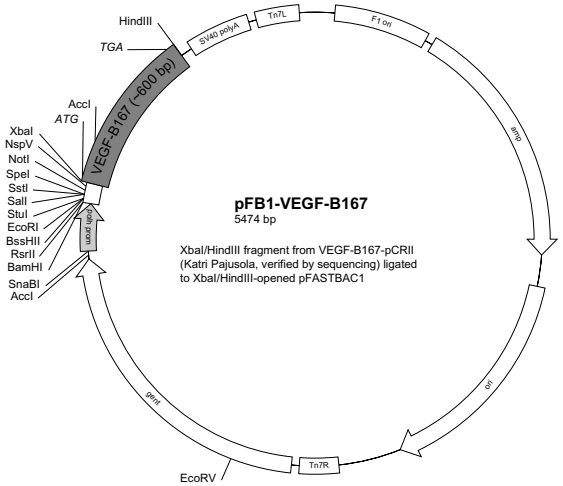


Figure 33. pFB1-VEGF-B167

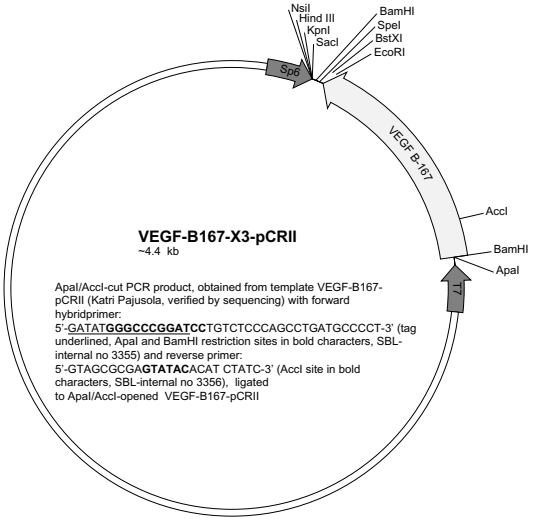


Figure 34. VEGF-B167-X3-pCRII

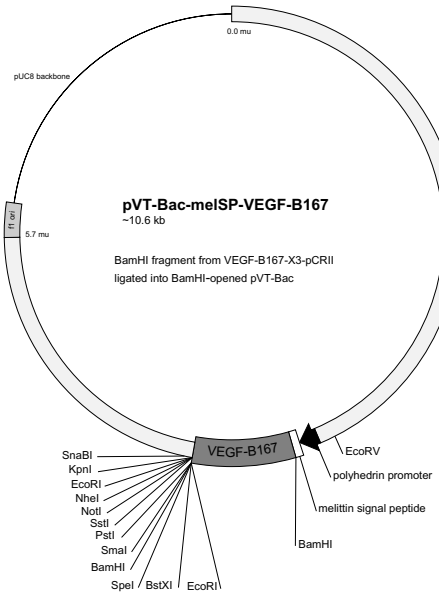


Figure 35. pVT-Bac-melSP-VEGF-B167

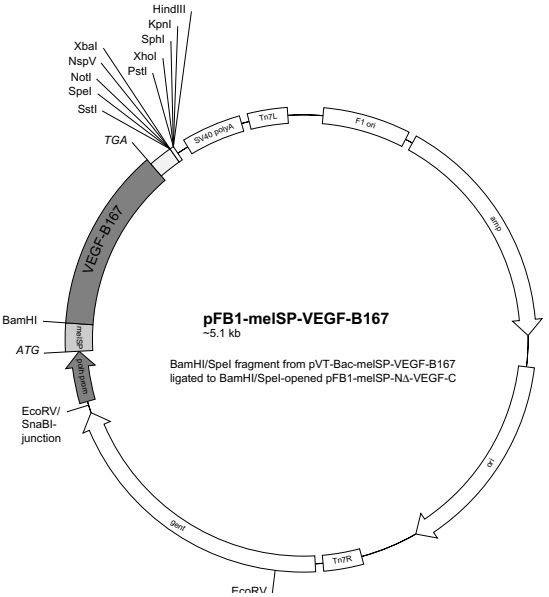


Figure 36. pFB1-melSP-VEGF-B167

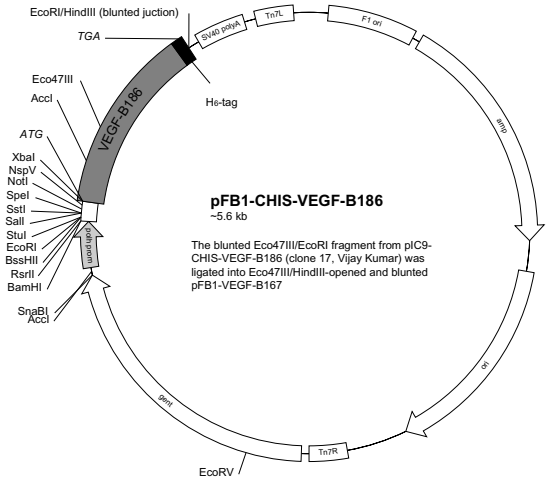


Figure 37. pFB1-CHIS-VEGF-B186

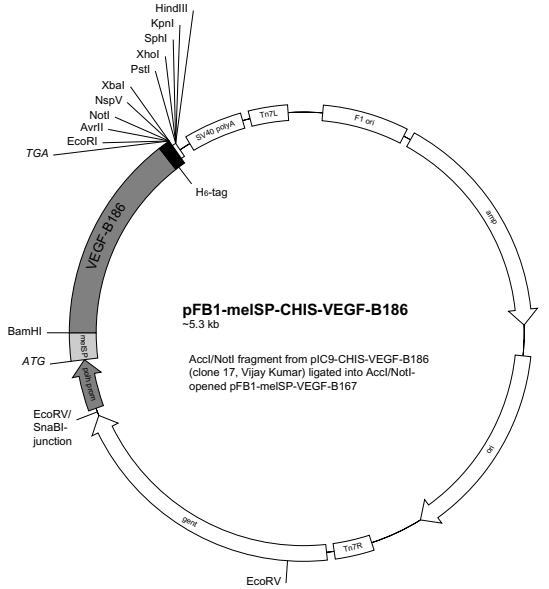


Figure 38. pFB1-melSP-CHIS-VEGF-B186

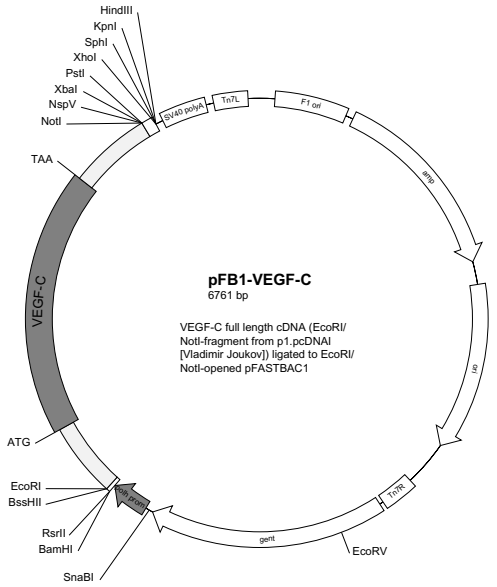


Figure 39. pFB1-VEGF-C

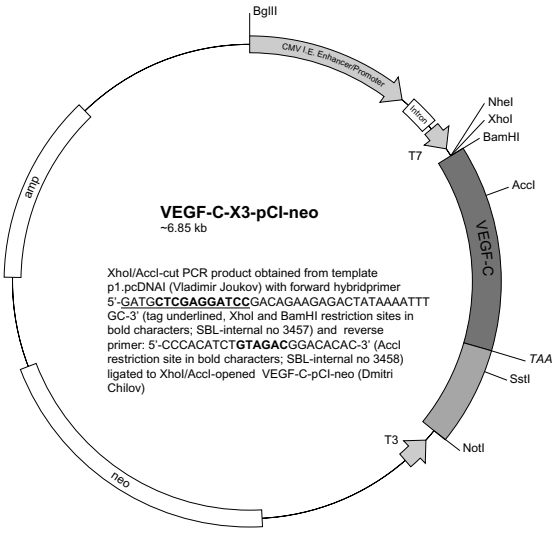


Figure 40. VEGF-C-X3-pCI-neo

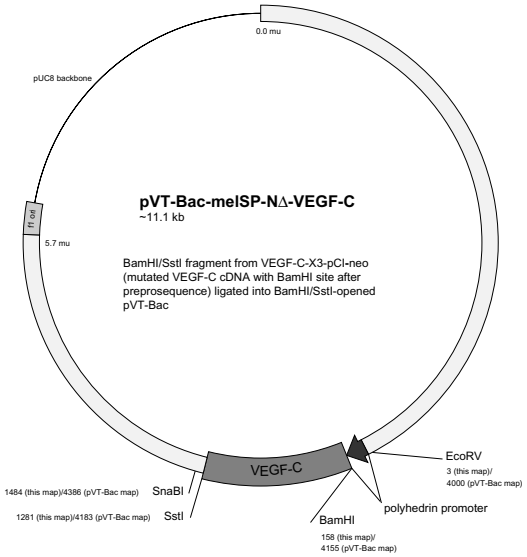


Figure 41. pVT-melSP-NΔ-VEGF-C

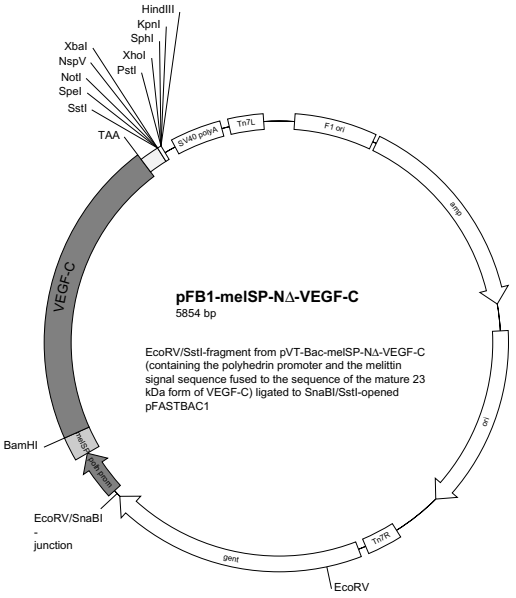


Figure 42. pFB1-melSP-NΔ-VEGF-C

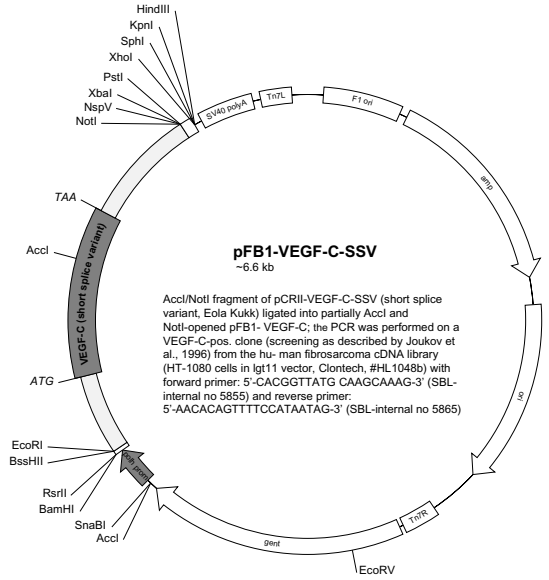


Figure 43. pFB1-VEGF-C-SSV (short splice variant)

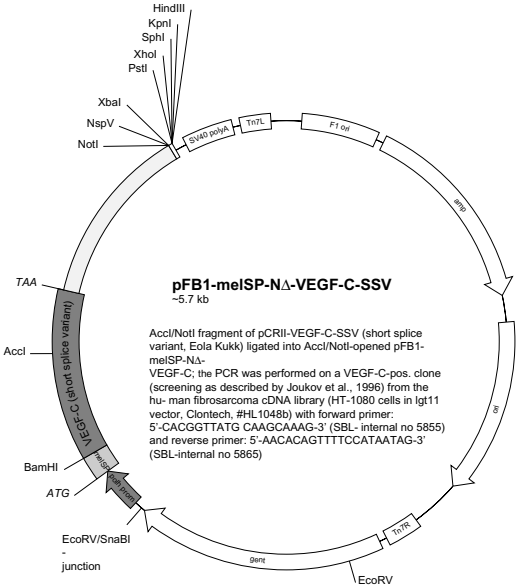


Figure 44. pFB1-melSP-NΔ-VEGF-C-SSV (short splice

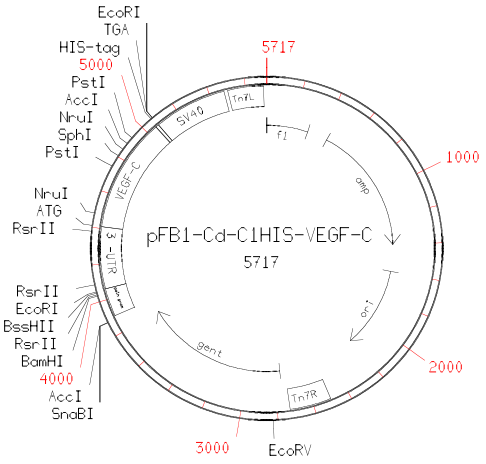


Figure 45. pFB1-CΔ-C1HIS-VEGF-C

VEGF-C with C-term. H6-tag (CMSKLHHHHHH* = C1HIS), C-term. deletion (D = nt 997 - end, corresponding D = aa 216 - end of prepropeptide) in pFASTBAC1, blunted and SphI-cut PCR product (forward hybridprimer: 5'-GGAATTCACAGAAGA GACTATAAA-3' [SBL-internal no 4047] and re-verse hybridprimer: 5'-GGAATTCAATGAT-GAT GATGGTGATGCAGTTTAGACATGC-3' [SBL-internal no 4048] applied to p1.pcDNAI [Vladimir Joukov]) ligated to HindIII(blunt)/SphI-opened pFB1-VEGF-C

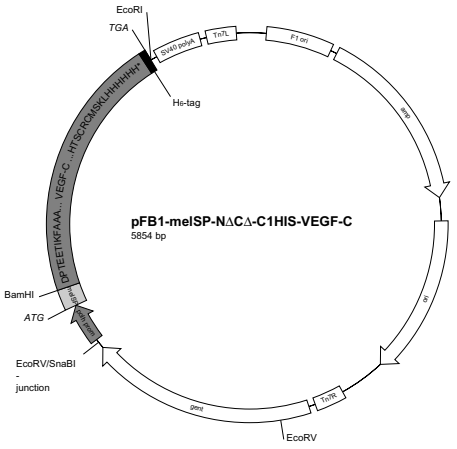


Figure 46. pFB1-melSP-NΔCΔ-C1HIS-VEGF-C

VEGF-C with C-term. H6-tag (CMSKLHHHHHH* = C1HIS), C- & N-term. deletions (Δ = start - nt 657, corresponding Δ = start - aa 102 of pre-propeptide and Δ = nt 997 - end, corresponding Δ = aa 216 -end of pre-propeptide) and melittin signal peptide in pFASTBAC1, T4 polymerase-blunted and SphI-cut PCR-fragment (as described for pFB1-CΔ-C1HIS-VEGF-C) ligated to HindIII-opened, blunted and subsequently SphI-cut pFB1-melSP-NΔ-VEGF-C

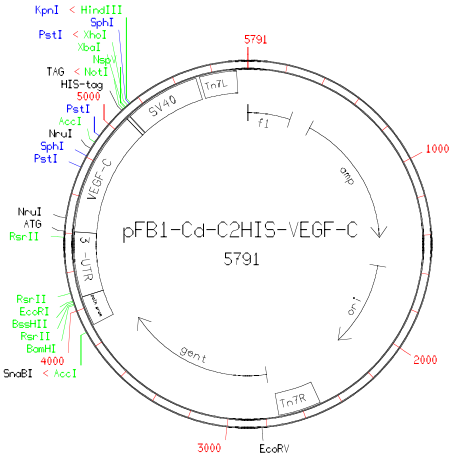


Figure 47. pFB1-CΔ-C2HIS-VEGF-C

VEGF-C with C-term. H_6-Tag (VHSIIHHHHHH* = C2HIS) and C-term. deletion (Δ = nt 1027 - end, corresponding Δ = aa 226 - end of pre-propeptide), AccI/NotI-fragment from VCΔNΔCHIS-pALTER (Vladimir Joukov) ligated to partial AccI/NotI-opened pFB1-VEGF-C

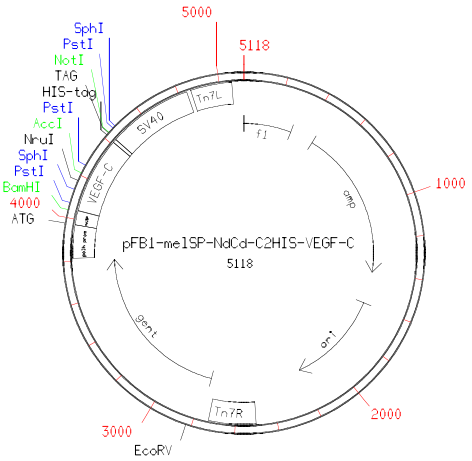


Figure 48. pFB1-melSP-NΔCΔ-C2HIS-VEGF-C

VEGF-C with C-term. H6-Tag (VHSIIHHHHHH* = C2HIS), C- & N-term. deletion (D = start - nt 657, corresponding D = start - aa 102 and D = nt 1027 - end, corresponding D = aa 226 - end of pre-propeptide) and melittin signal peptide in pFASTBAC1, NotI/NruI-fragment from VCD-NDCHIS-pALTER (Vladimir Joukov) cloned into NotI/NruI-opened pFB1-melSP-ND-VEGF-C

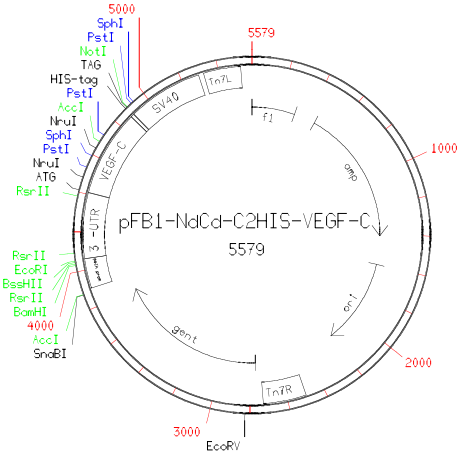


Figure 49. pFB1-NΔCΔ-C2HIS-VEGF-C

VEGF-C with C-term. H_6-Tag (VHSIIHHHHHH* = C2HIS), N- & C-term. deletion (Δ = nt 445 - 658, corresponding Δ = aa 32 - 102 of pre-propeptide), NotI/EcoRI-fragment from VCΔNΔCHIS-pALTER (Vladimir Joukov) cloned into NotI/EcoRI-opened pFASTBAC1

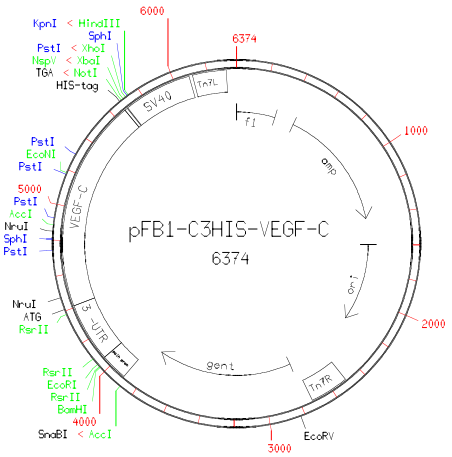


Figure 50. pFB1-C3HIS-VEGF-C

VEGF-C with C-term. H_6-tag (KRPQMSHHHHHH*), NotI/EcoRI-fragment from VEGF-C-CHIS-pALTER (Vladimir Joukov) ligated to NotI/EcoRI-opened pFASTBAC1

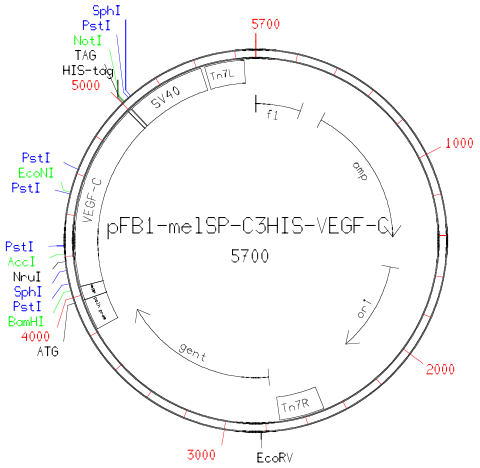


Figure 51. pFB1-melSP-C3HIS-VEGF-C

VEGF-C with C-term. H_6-tag (KRPQMSHHHHHH*), NotI/EcoNI-fragment from VEGF-C-CHIS-pALTER (Vladimir Joukov) ligated to NotI/EcoNI-opened pFB1-melSP-NΔ-VEGF-C

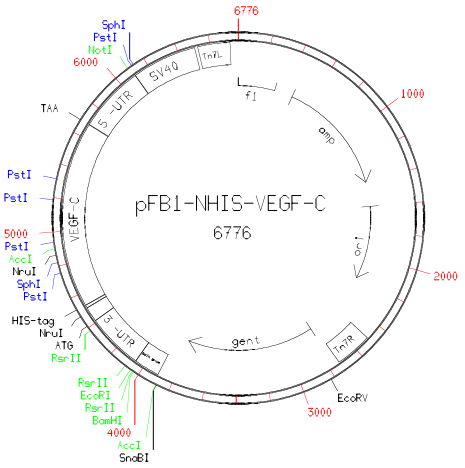


Figure 52. pFB1-NHIS-VEGF-C

VEGF-C with N-term. H_6-tag (PAAAAAHHHHH HESGLDL) and one aa-deletion (Δ = nt 445 - 447, corresponding Δ = aa 32 of prepropeptide), EcoRI/ NotI-fragment from VC-NHIS-pALTER (Vladimir Joukov) ligated to EcoRI/NotI-opened pFASTBAC1

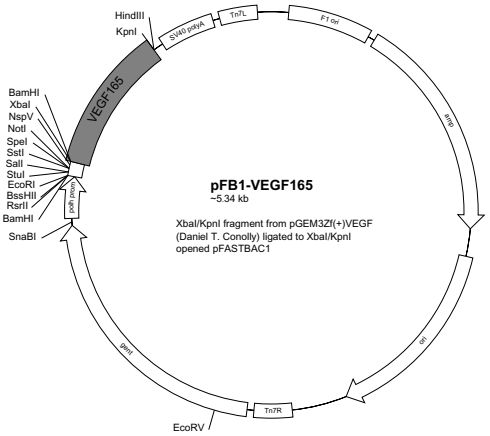


Figure 53. pFB1-VEGF-A165

7.5. Characterisation of recombinant proteins

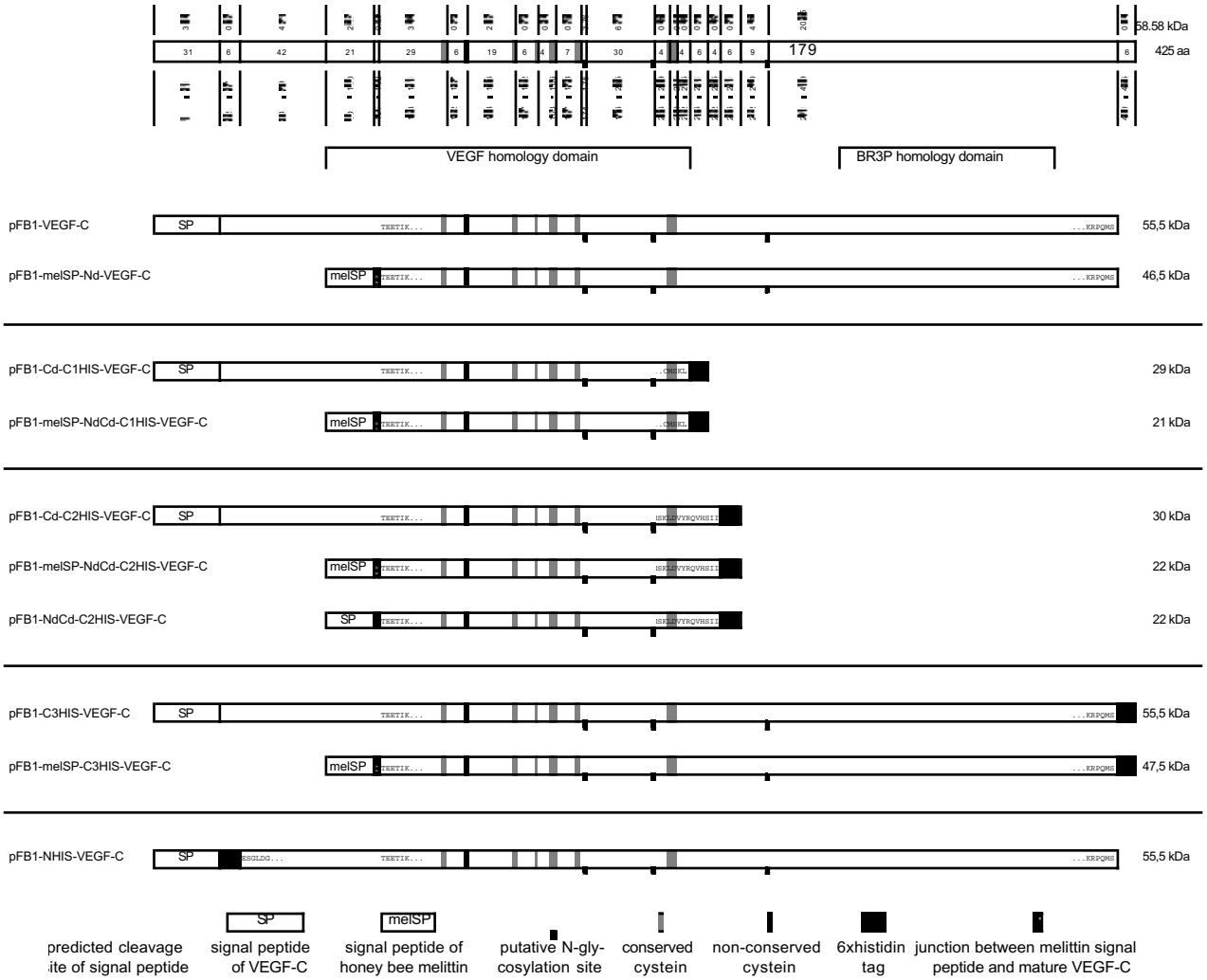


Figure 54. Schematic representation of recombinant baculoviral VEGF-C proteins (selection)

The upper panel shows, how different parts of the protein contribute to its molecular weight, the calculated molecular weight of each protein is given for the secreted propeptide (including N-glycosylation at all three potential sites)

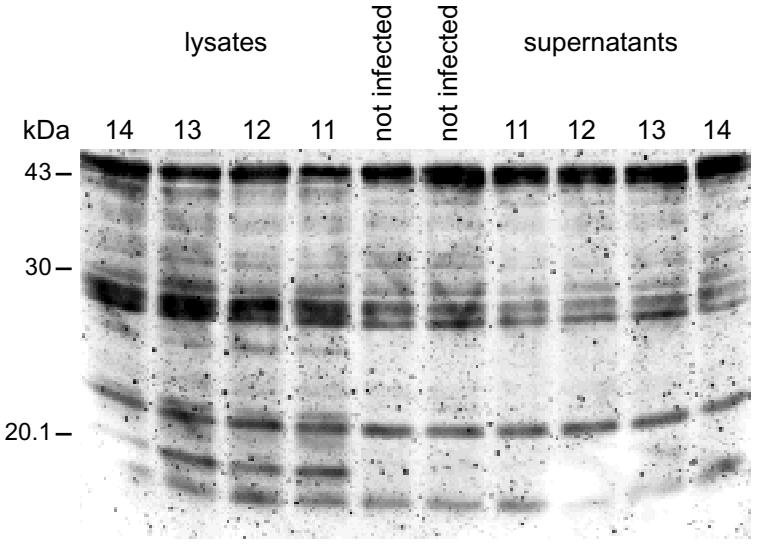


Figure 55. VEGF165

IP/Western of HF cell transfection supernatants and lysates, clones 11-14

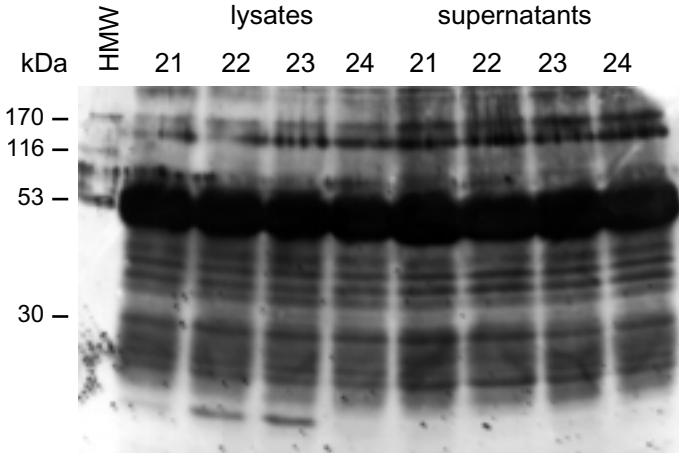


Figure 56. VEGF-B167

IP/Western of Sf9 cell transfection supernatants and lysates, clones 21-24

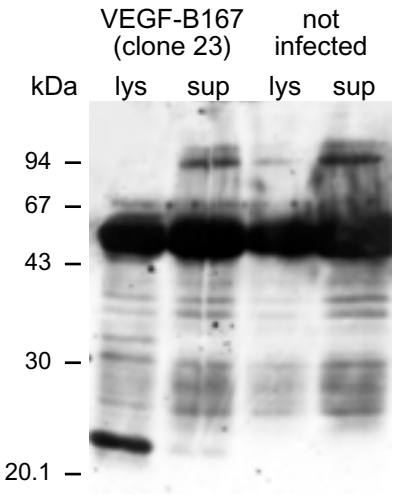


Figure 57. VEGF-B167 (clone 23)

IP/Western of Sf9 cell lysates and conditioned medium after 3. amplification

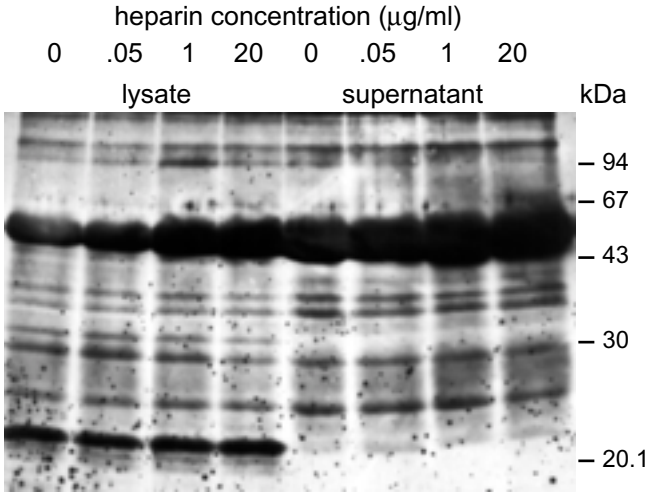


Figure 58. No release of VEGF-B167 upon heparin treatment

IP/Western, Sf9 cells, clone 23

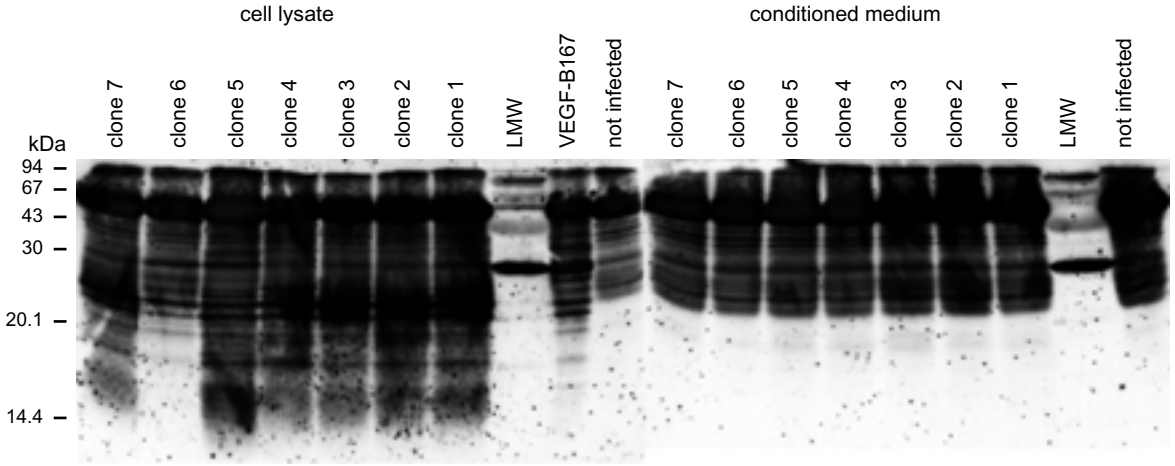


Figure 59. melSP-VEGF-B167

IP/Western of HF cell transfection supernatants and lysates, clones 1-7

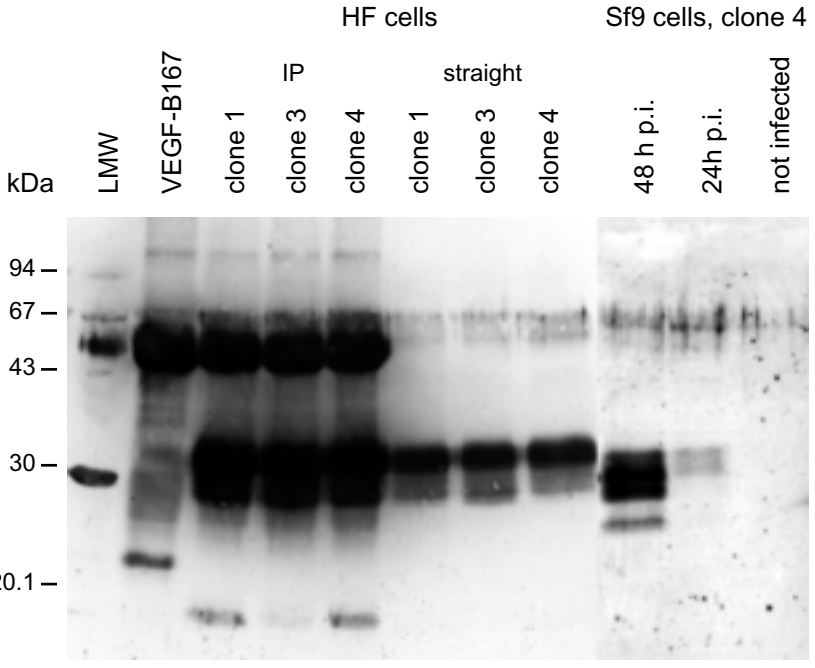


Figure 60. melSP-CHIS-VEGF-B186

IP- and straight Western of medium conditioned by HF or Sf9 cells during 1. amplification

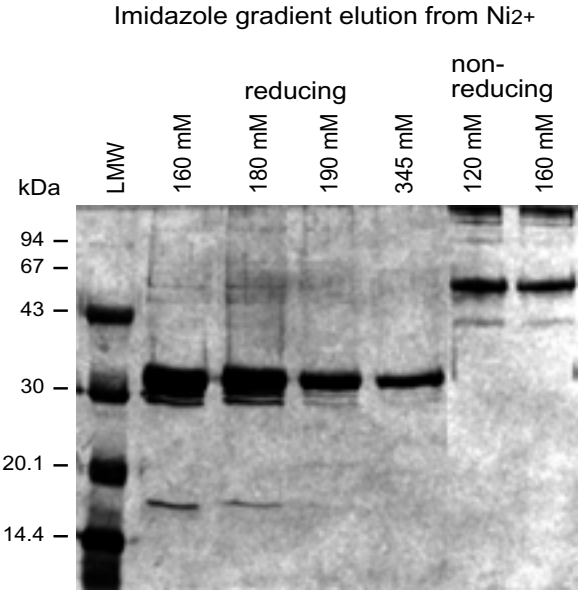


Figure 61. Affinity purification of melSP-CHIS-VEGF-B186 from medium conditioned by HF cells

Silver staining of 15% SDS-PAGE, clone 3

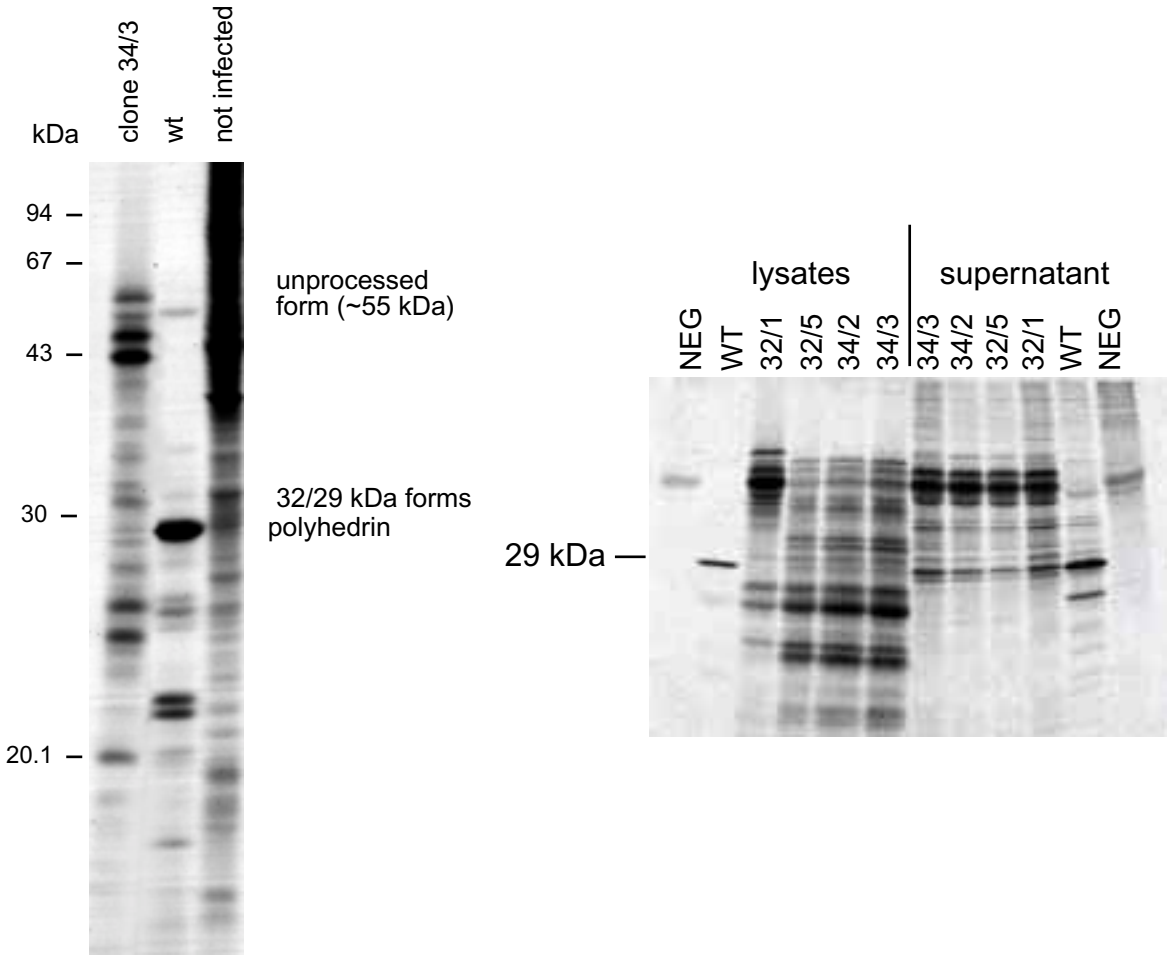


Figure 62. Metabolic labelling of VEGF-C

Sf9 cells, lysate, clone 34/3 after 3. amplification

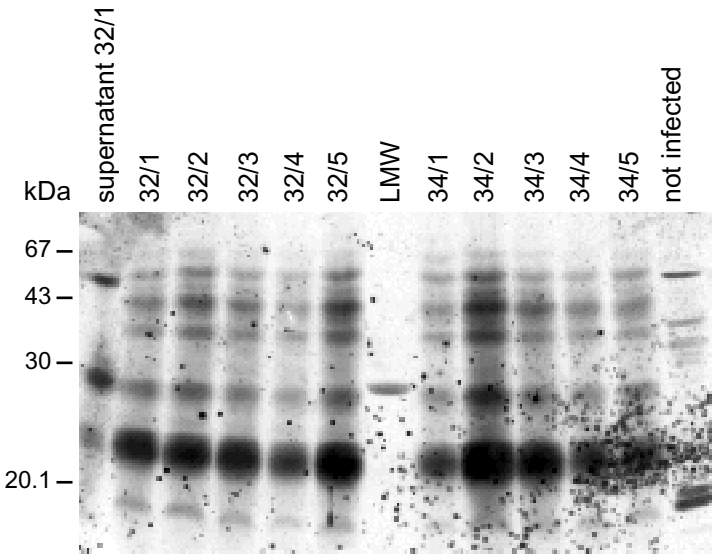


Figure 63. VEGF-C harvested 7 d p.i.

Straight Western of Sf9 cell lysates after 2. amplification, clones 32/1-32/5 and 34/1-34/5

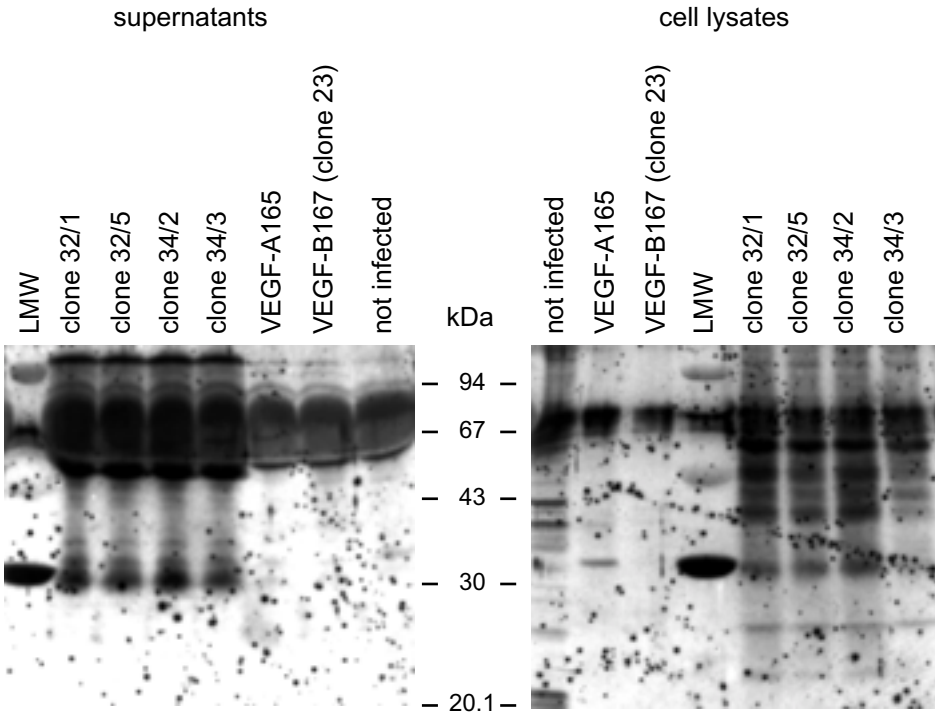


Figure 64. VEGF-C produced by Sf9 cells

Straight Western of cell lysates and conditioned medium; clones 32/1, 32/5, 34/2 and 34/3 after 3. amplification

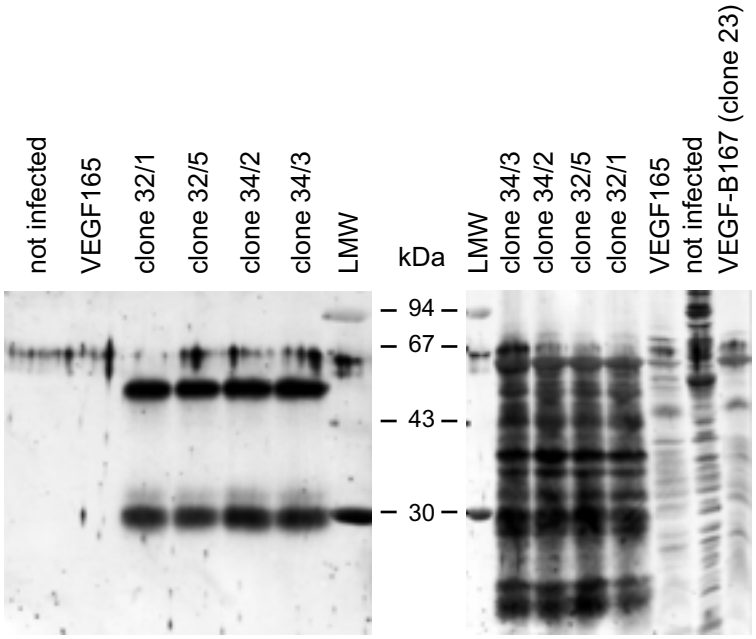


Figure 65. VEGF-C produced by HF cells

Straight Western of cell lysates and conditioned medium; clones 32/1, 32/5, 34/2 and 34/3 after 3. amplification

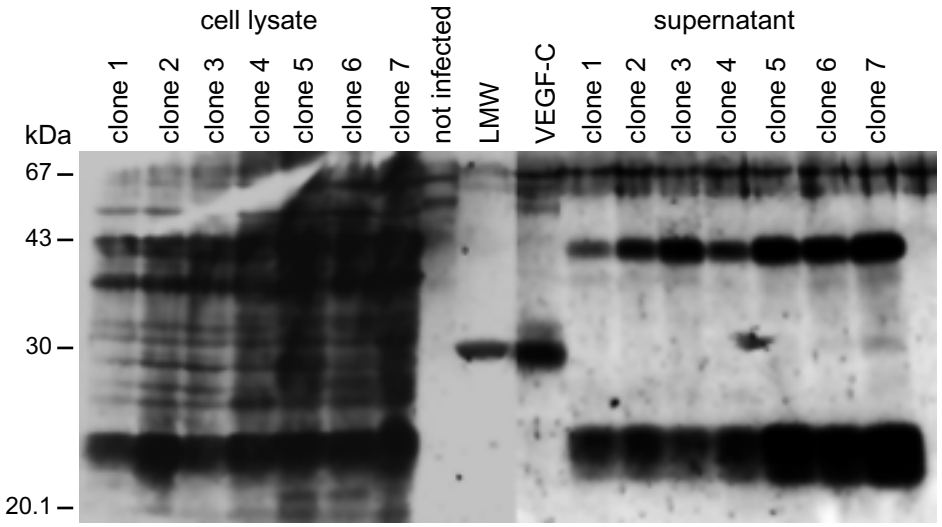


Figure 66. melSP-ΔN-VEGF-C

Straight Western of HF cell lysates and conditioned medium, clones 1-7 after 2. amplification

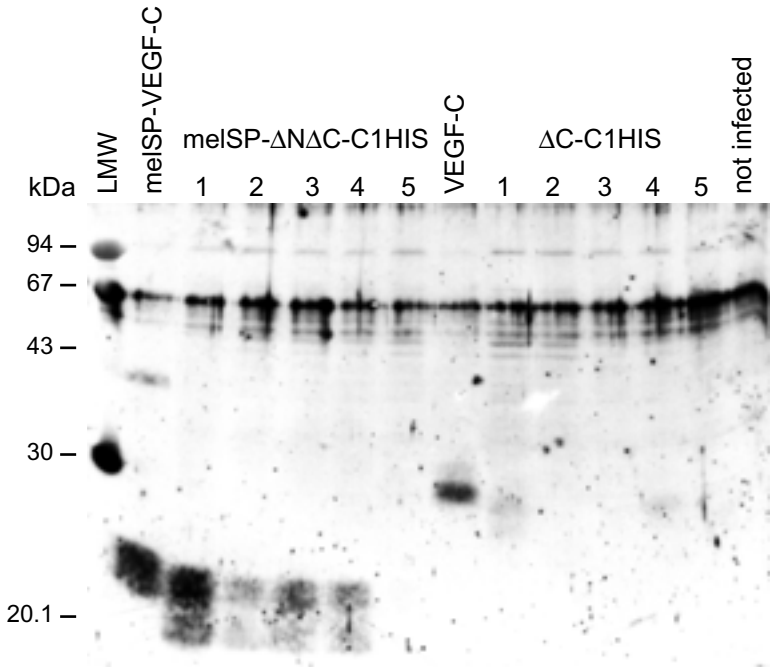


Figure 67. melSP-ΔNΔC-C1HIS-VEGF-C and ΔC-C1HIS-VEGF-C

Straight Western of HF cell transfection supernatant, clones 1-5

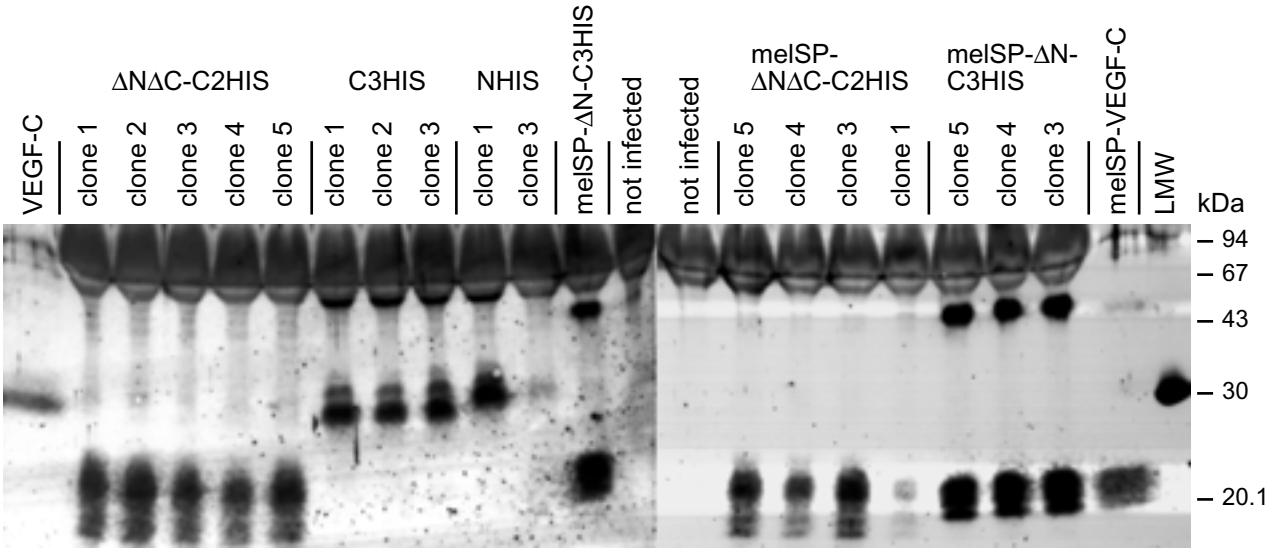


Figure 68. Differently H_6-tagged VEGF-C, reducing SDS-PAGE

Straight Western of Sf9 cell transfection supernatant

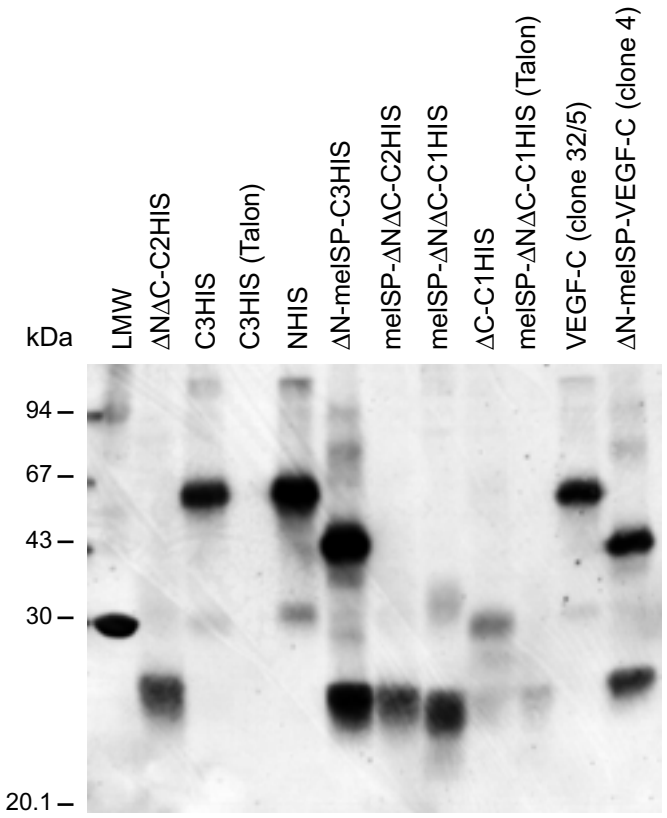


Figure 69. Differently H_6-tagged VEGF-C, non-reducing SDS-PAGE

Straight Western of HF cell conditioned medium, after 3. amplification

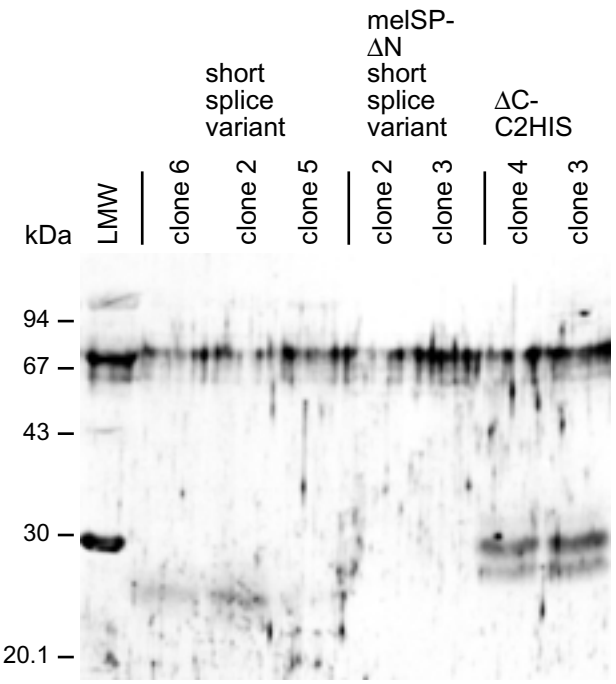


Figure 70. Short splice variant of VEGF-C and melSP-ΔN-VEGF-C

Straight Western of medium conditioned by HF cells, after 2. amplification

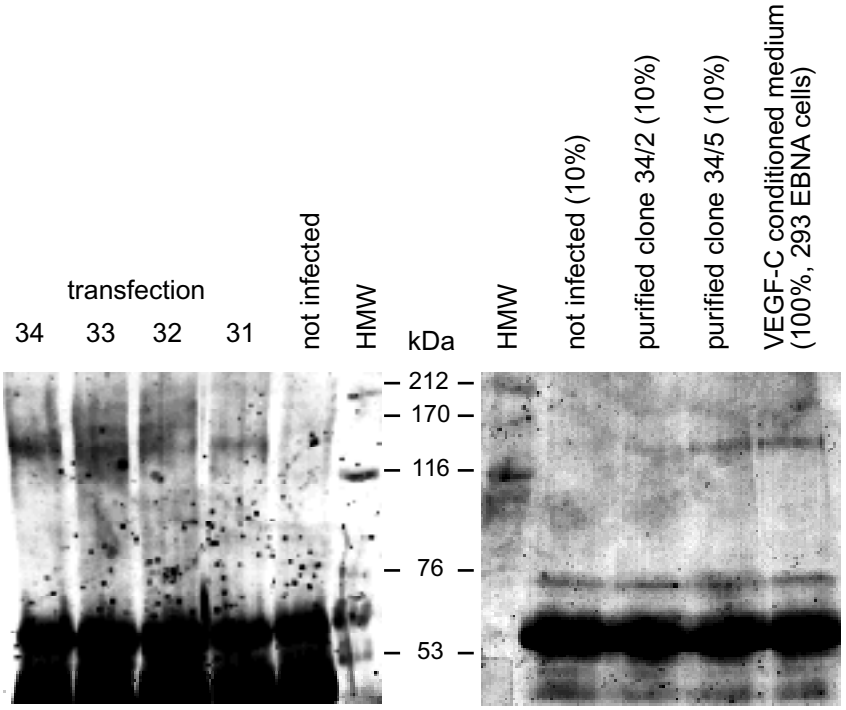


Figure 71. FLT4 stimulation with transfection supernatant

Stimulation with 1:10 dilutions of Sf9 cell transfection supernatants and medium conditioned by Sf9 cells infected with plaque-purified clones 34/2 and 34/5, VEGF-C

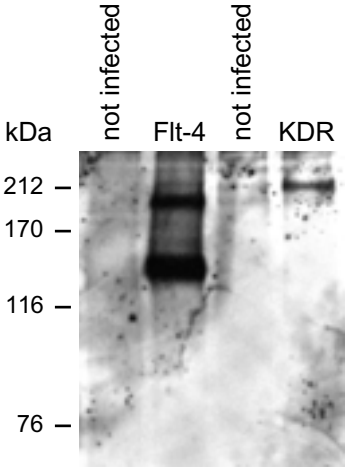


Figure 72. FLT4 and KDR stimulation with melSP-ΔNΔC-C1HIS-VEGF-C

Stimulation with 1:2 dilutions of medium conditioned by HF cells, clone 4

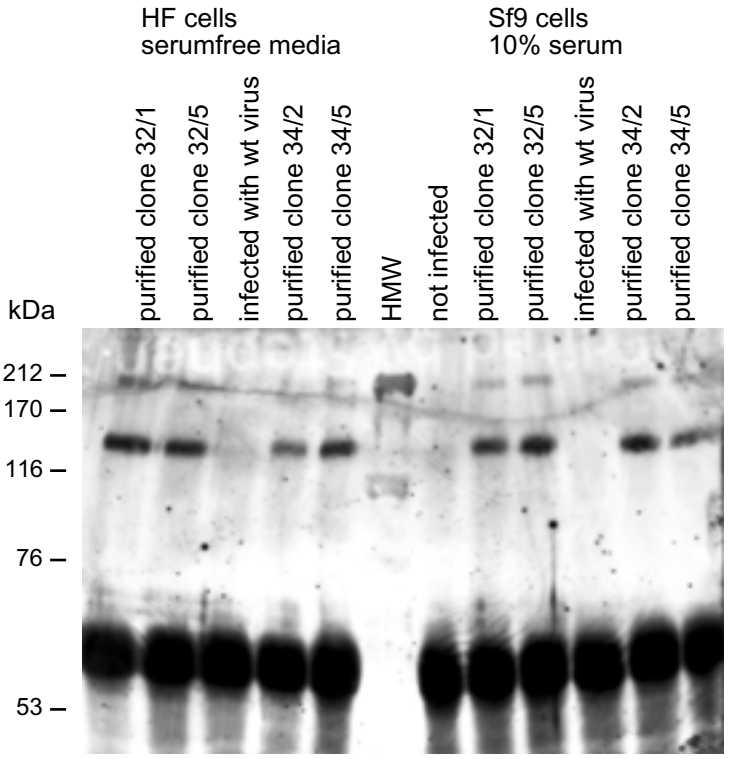


Figure 73. Stimulation of FLT4, comparison between Sf9 and HF cells

Stimulation with 1:5 dilutions of conditioned medium

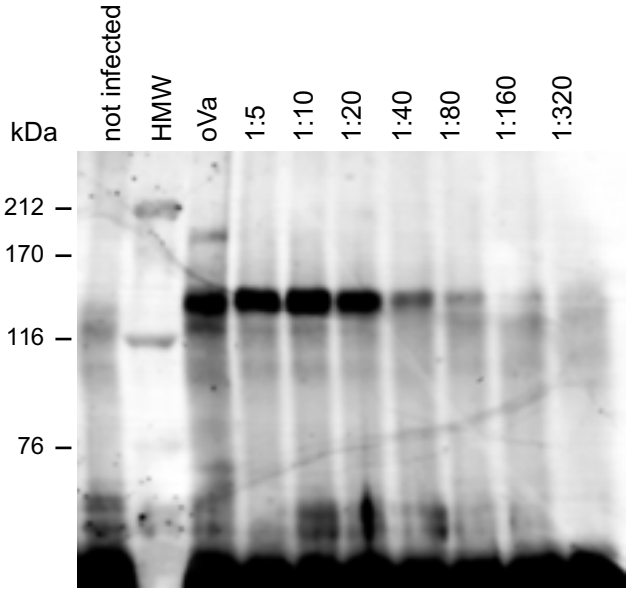


Figure 74. Titration of FLT4 stimulation with melSP-ΔNΔC-VEGF-C

Stimulation with medium conditioned by Sf9 cells, clone 4

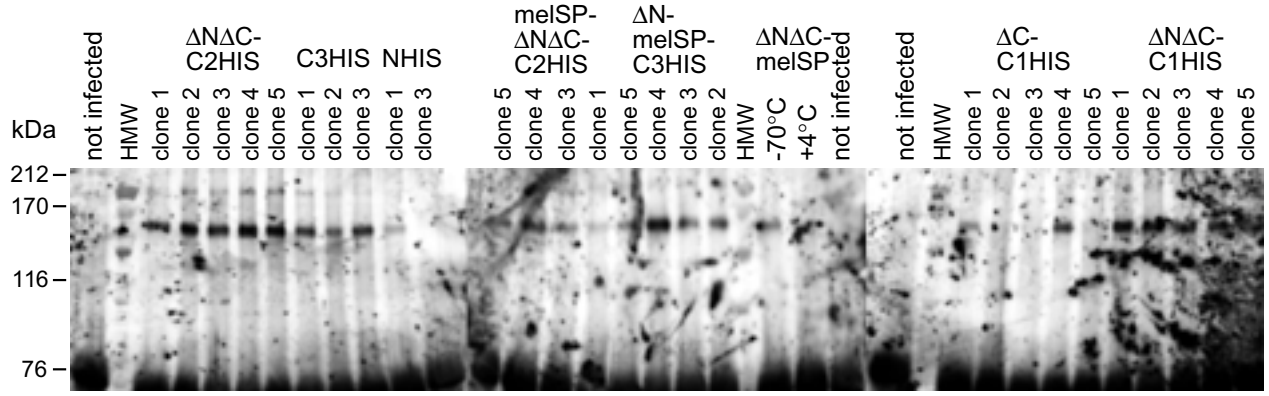


Figure 75. FLT4 stimulation with differently H_6-tagged VEGF-C

Stimulation with 1:2 dilutions of Sf9 cell transfection supernatant

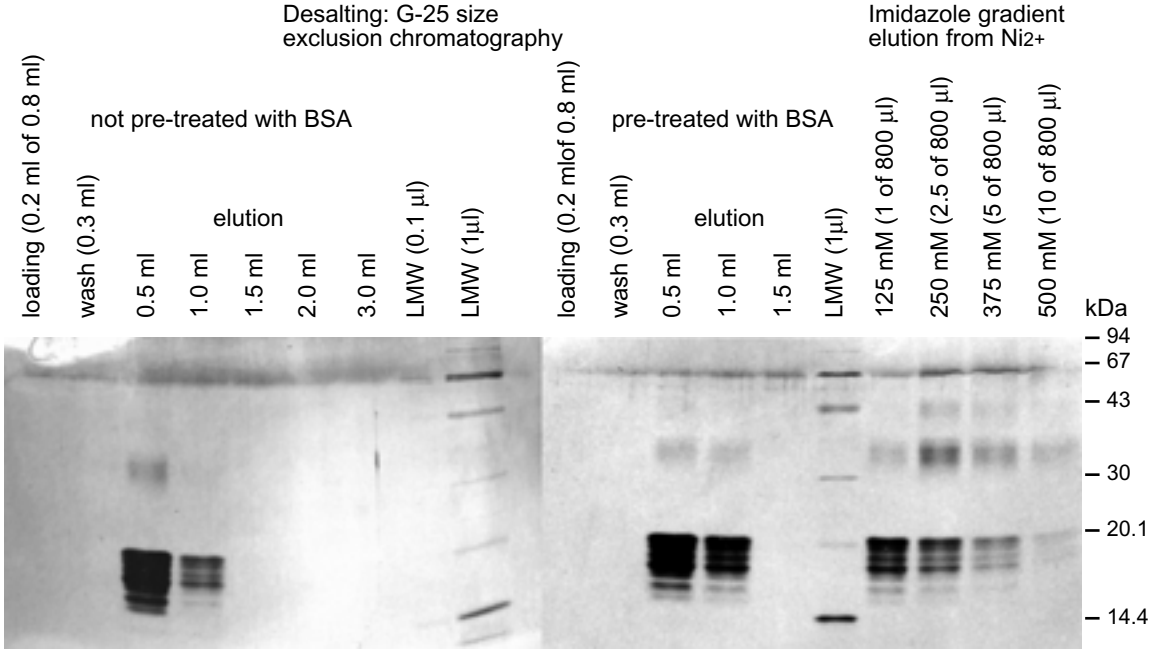


Figure 76. Affinity purification of melSP-ΔNΔC-C1HIS-VEGF-C

Silver staining of 15% SDS-PAGE, purification from medium conditioned by HF cells, clone 1

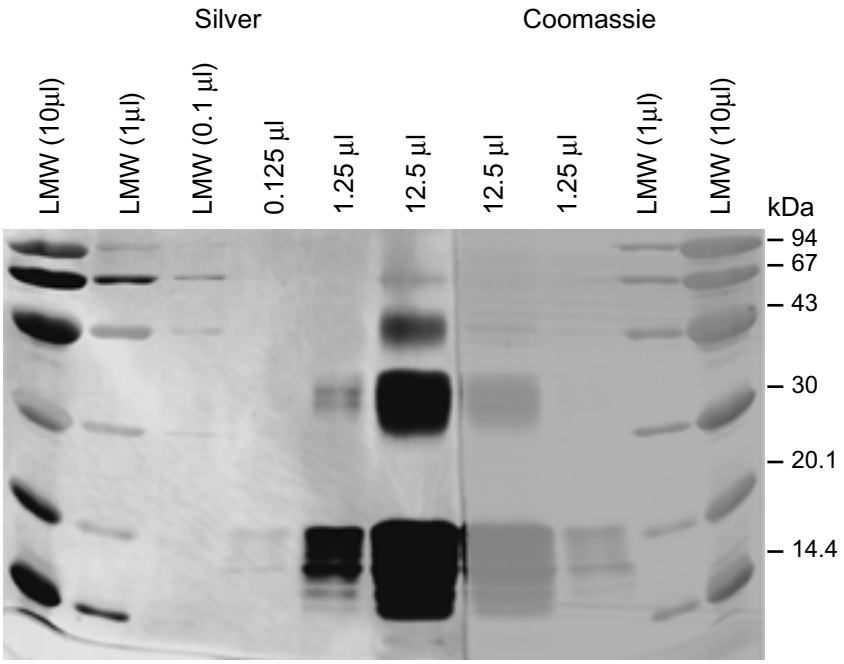


Figure 77. Comparison between Silver and Coomassie Brilliant Blue staining

15% SDS-PAGE, melSP-ΔNΔC-C1HIS-VEGF-C, clone 1

7.6. Pedigrees of transgenic mice

Figure 78. αMHC-VEGF-B167 transgenic mice

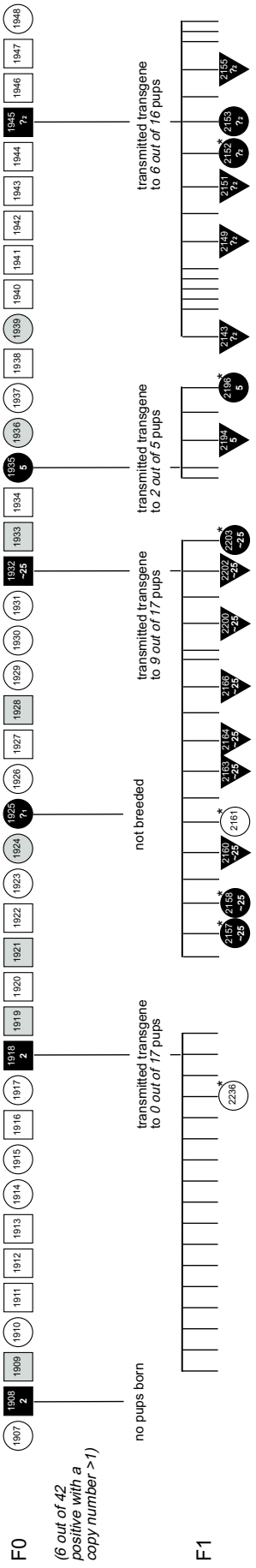
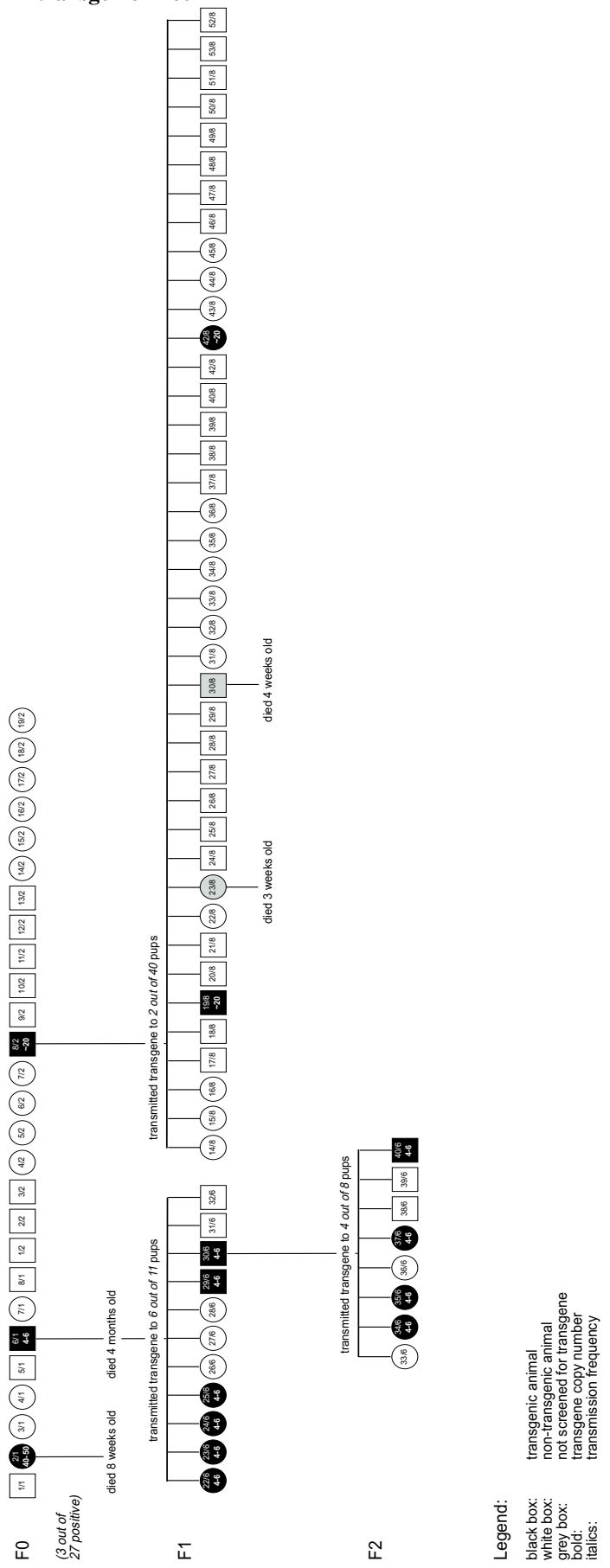


Figure 79. K14-VEGF-C-FL transgenic mice



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