From **The Department of Molecular Medicine and Surgery**
Karolinska Institutet, Stockholm, Sweden

# FROM GENE MUTATION TO GENE EXPRESSION:

# STUDIES ON MULTIPLE ENDOCRINE NEOPLASIA TYPE 1 AND VASCULAR ENDOTHELIAL GROWTH FACTORS

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For my children

Research is the act of going up alleys to see if they are blind.

- Plutarch

# ABSTRACT

Multiple Endocrine Neoplasia type 1, MEN1, is an inherited cancer syndrome whose gene was localised to chromosome 11q13 in 1988. A number of candidate genes were characterised before the *MEN1* gene was cloned in 1997. DNA sequencing of *MEN1* to search for mutations in patients is used as a complement to clinical diagnosis. Since 1997, a total of 202 index cases were referred to the Department of Clinical Genetics for mutation screening, but no systematic review of their mutations or clinical characteristics has been performed. By analysing the results of DNA sequencing and deletion detection (using multiplex-ligation-dependent probe amplification, MLPA) on blood samples and correlating mutations to clinical data from the referring physicians, 37 unique mutations were found, of which 19 have not been previously reported. Heredity for MEN1 or hyperparathyroidism, an early age of onset and the presence of multiple tumours greatly enhanced the risk of carrying a *MEN1* mutation. Mutations were spread all over the gene and there was no genotype-phenotype correlation. The results from this study have led to the addition of MLPA as a standard method of mutation detection in MEN1 patients and have identified patient categories which should be tested for *MEN1* mutations. In addition, the compilation of missense mutations and polymorphisms found in the Swedish population will facilitate interpretation of single base pair substitutions in the future.

One of the genes isolated as a *MEN1* candidate gene was a novel gene related to vascular endothelial growth factor A (*VEGF-A*). This gene was called VEGF Related Factor (*VRF*) and later renamed *VEGF-B*. It was expressed in all normal tissues examined and consisted of two splice forms: VEGF-B167 and VEGF-B186. They had completely different carboxyl-terminal ends due to different reading frames. VEGF-A was known as a potent inducer of blood vessel growth (angiogenesis) and can also cause inflammation. To further study the role of VEGF-B, two different strategies were used. The first was to produce recombinant VEGF-B protein and to test its function. VEGF-B167 was successfully produced and purified from retrovirally infected HEK293 cells. However, no detectable effect of VEGF-B was found in cell proliferation or monocyte migration assays.

The second strategy was to study the expression of VEGF-B in concert with other angiogenic factors in models of disease that affected organs with high expression of VEGF-B: the heart (dilated cardiomyopathy, DCM) and central nervous system (multiple sclerosis, MS). VEGF-A (but not VEGF-B) was significantly increased in a mouse model of DCM due to mitochondrial dysfunction, but there was no parallel increase in capillary density. The expression of VEGF-A (but not VEGF-B) was decreased in the spinal cord resident cells in a rat model of MS, but the invading inflammatory cells did express VEGF-A. It was the heparin-binding splice forms that tended to decrease while the soluble VEGF-A120 isoform remained unaltered. These results were corroborated by a decrease in VEGF-A mRNA in mononuclear cells from cerebrospinal fluid (CSF) from MS patients compared to controls.

Thus, the role of VEGF-B remains largely unknown and our data support the idea that VEGF-A functions in concert with other factors and an increase in VEGF-A as a single factor does not always lead to angiogenesis. VEGF-A may function as a neuroprotective and pro-inflammatory factor simultaneously in different cell types in the same tissue, which further complicates the picture. Studies in larger clinical materials are warranted to be able to correlate the net effect of VEGF-A with clinical outcome in MS.

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# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ANF | Atrial natriuretic factor |
| ALS | Amyotrophic lateral sclerosis |
| Ang1 or 2 | Angiopoietin 1 or 2 |
| ARP | Acidic ribosomal protein |
| BBB | Blood brain barrier |
| BSA | Bovine serum albumin |
| cDNA | Complementary DNA |
| CNS | Central nervous system |
| COS | CV-1 simian cells transformed by origin-defective mutant of SV40 |
| COX | Mitochondrial cytochrome-c oxidase |
| CSF | Cerebrospinal fluid |
| DCM | Dilated cardiomyopathy |
| DMEM | Dulbecco's modified eagle medium |
| DTT | 1,4-Dithiothreitol |
| EAE | Experimental autoimmune encephalomyelitis |
| ECL | Enhanced chemiluminescence |
| EPT | Endocrine entero-pancreatic tumour |
| FACS | Flow activated cell sorter |
| FCS | Fetal calf serum |
| FIHP | Familial isolated hyperparathyroidism |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GFAP | Glial fibrillary acidic protein |
| GFP | Green fluorescent protein |
| MBP | Myelin basic protein |
| HEK293 | Human embryonic kidney cells |
| HIF1$\alpha$ | Hypoxia inducible factor 1 alpha |
| HPT | Hyperparathyroidism |
| HRE | HIF1$\alpha$ -responsive elements |
| HRP | Horseradish peroxidase |
| ICM | Ischaemic cardiomyopathy |
| IFN$\gamma$ | Interferon gamma |
| IPTG | Isopropyl-b-D-thiogalactopyranoside |
| IRES | Internal ribosome entry site |
| KS-IMM | Immortalised cell line from Kaposi sarcoma lesion |
| MEN1 | Multiple endocrine neoplasia type 1 |
| MLPA | Multiplex ligation-dependent probe amplification |
| mRNA | Messenger ribonucleic acid |
| MS | Multiple sclerosis |
| mtDNA | Mitochondrial DNA |
| Ni | Nickel |
| NP1 or 2 | Neuropilin 1 or 2 |
| ORF | Open reading frame |
| PBMC | Peripheral blood monocytic cells |
| PBS | Phosphate-buffered saline |
| p.i. | Post immunisation |
| PIT | Pituitary tumour |
| PlGF | Placental growth factor |

| | |
|---|---|
| RT-PCR | Reverse transcriptase PCR |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SSCP | single strand conformation polymorphism |
| Tfam | Mitochondrial transcription factor A |
| Tie-1 or 2 | Tyrosine kinase with immunoglobulin-like and EGF-like domains |
| TSA | Tyramide signal amplification |
| UTR | Untranslated region |
| VEGF | Vascular endothelial growth factor |
| VEGFR | Vascular endothelial growth factor receptor |
| VHL | Von Hippel Lindau |

# 1 INTRODUCTION

## 1.1 THE SEARCH FOR THE *MEN1* GENE

Multiple Endocrine Neoplasia Type 1 (MEN1) is an autosomal dominantly inherited cancer syndrome characterised by tumours in the parathyroid, endocrine pancreas and anterior pituitary glands (OMIM 131100). In addition, some patients develop adrenal tumours, carcinoids or rarely, paragangliomas (Trump et al. 1996). MEN1 occurs in approximately 2-10/100,000 individuals (Marx et al. 1999). Symptoms are caused by an overproduction of hormones from these glands resulting in e.g. hypercalcaemia and depression (parathyroid hormone); hypoglycaemia or gastric ulcus (insulin or gastrin), lactation or acromegaly (prolactin or growth hormone). The age of onset of clinical symptoms varies, but is most common in the third to fourth decade with full penetrance by 70 years of age (Trump et al. 1996). MEN1 patients have a 50% risk of death by the age of 50, usually due to malignant tumour growth (especially gastrinomas and thymic carcinoids) or other sequelae of the disease (Doherty 2005).

Today, the entire human genome is available on computer databases only a mouse click away, but 20 years ago, when the search for the *MEN1* gene began, very little was known about the human genome. By 1980, only a handful of genes for monogenic disorders had been identified through knowledge of the protein defect. The discovery of DNA sequence variations between individuals (markers) enabled searching for disease genes using a positional cloning approach. This entails following the linked inheritance of genetic markers with the studied disease in large families. If a genetic marker is physically close to the disease gene, then it will be more often inherited together with the disease than if it is further away. Using genetic maps that have been constructed in a similar fashion, one can then define which markers follow the disease and thus must be close to the disease gene (linkage analysis). The first genetic markers were often enzyme cleavage sites (restriction fragment length polymorphisms, RFLPs), that could be detected by cleaving the DNA and running a Southern blot. In 1989, microsatellite makers were identified (Weber and May 1989). These consist of di-, tri- or tetranucleotide repeats that vary in number between different individuals and are detected by PCR and separating the resulting fragments on a polyacrylamide gel. Once the chromosomal region that carries the disease gene has been identified, the candidate region can be narrowed down by e.g. looking for crossovers in family members that have inherited the disease. Once a minimal region is identified, the arduous task of finding genes in that area and then testing if they represent the disease-causing gene by looking for mutations begins. The first disease genes discovered this way were the X-linked genes for chronic granulomatous disease and Duchennes muscular dystrophy in 1986. 10 years later over 40 disease genes had been isolated (Collins 1995). Today, the number of known monogenic disease genes exceeds 1900 (OMIM database).

Thus, the search for the *MEN1* gene could begin when the *MEN1* locus was assigned to chromosome 11q13, close to *PYGM* by linkage analysis and tumour deletion mapping in 1988 (Larsson et al. 1988). Tumours demonstrated loss of heterozygosity (loss of the normal allele) at 11q13 (Bystrom et al. 1990; Larsson et al. 1988) indicating that *MEN1* must be a tumour suppressor gene in accordance with Knudson's two hit hypothesis (Knudson 1971). These studies also placed the *MEN1* gene telomeric to *PYGM*.

European *MEN1* research groups in the UK, France, Sweden, Belgium, the Netherlands, Finland and also in Australia united forces in order to increase the chances of finding the *MEN1* gene. Further genetic mapping narrowed down the linkage interval, but it was difficult to adjust this map to the available physical maps due to conflicting alignment of markers. We first searched for candidate genes in the region between *PYGM* and marker D11S457 which was thought to be telomeric to *PYGM* (Wood et al. 1996). We could exclude *PLCβ3* (phospholipase C beta 3), *PNG (*PLC neighbouring gene), *FKBP2* (FK506 binding protein 2) and *VRF/VEGF-B* (vascular endothelial growth factor related factor) as candidate genes for MEN1 (*paper I* and (Grimmond et al. 1995; Lemmens et al. 1997a; Weber et al. 1997). In 1996, improved physical and genetic maps over the 11q13 area placed D11S457 and D11S427 clearly centromeric of *PYGM* and thus outside of the *MEN1* locus (Courseaux et al. 1996). This was later confirmed by several groups (Guru et al. 1997; Lemmens et al. 1997a; Sawicki et al. 1997), (Figure 1). Two key recombinations in MEN1 families narrowed the *MEN1* region down first to 2Mb (Courseaux et al. 1996) and after analysis with two new markers (one in the *VRF* gene and D11S1783) the minimal region was judged to be <900kb. A sequence-ready contig consisting of 26 cosmids, 8 bacterial artificial chromosomes (BACs) and 8 P1 artificial chromosomes (PACs) encompassing this region was constructed. These clones were used in a cDNA selection procedure using a bovine parathyroid cDNA library to identify new genes. In total the contig contained at least 3 ESTs and 19 genes. (Lemmens et al. 1997a). As one somatic deletion (in a parathyroid tumour from a MEN1 patient) had already placed the *MEN1* gene distal to *PYGM* (Byström et al. 1990), we concentrated our search to the five genes located within this 300kb region.

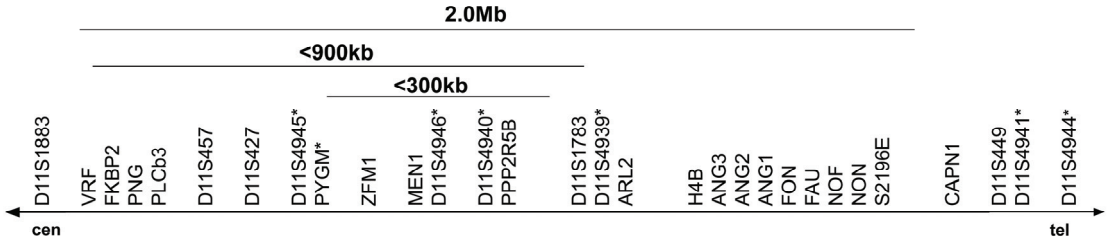


**Figure 1: Genetic map of chromosome 11q13.** VRF was initially though to lie telomeric to PYGM, as D11S457 was falsely thought to be telomeric to PYGM. In 1996, the correct marker order as shown in this figure could be established. The markers with * were used to confirm linkage to 11q13 in MEN1 families where no MEN1 mutation was found in paper II. (Courseaux et al. 1996; Guru et al. 1997; Lemmens et al. 1997a; Sawicki et al. 1997).

How do you know if you have the right gene? The first clue in positional cloning is localisation, as outlined above. The next is to investigate whether the candidate gene is expressed in the tissues of interest (i.e. endocrine glands in MEN1) and if the gene has a function that (in the case of *MEN1*) might convey tumour suppressor properties. If so, one must analyse the gene for mutations that segregate with the disease.

Of the five genes in the *MEN1* region, the Consortium could exclude two. One (*ZFM1, Zinc finger gene in the MEN1 locus*) had been formally excluded as the *MEN1* gene (Lloyd et al. 1997) and one (*PYGM*), glycogen myophosphorylase, was retained in the parathyroid tumour mentioned above and was known to cause McArdle's disease (Tsujino et al. 1993). It was considered an unlikely candidate due to its expression and function in skeletal muscle. The remaining three candidate genes (*SCG1*, *SCG2* and

*PPP2R5B* (B56β subunit of protein phosphatase 2A) were all screened for *MEN1* mutations. *PPP2R5B* was involved in DNA replication and transcription, but was not mutated in MEN1 patients (Forbes et al. 1997). *SCG1* and *2* were ubiquitously expressed and had no known function and were therefore screened with single strand conformation polymorphism (SSCP) for mutations segregating with the disease in MEN1. *SCG1* was subsequently shown to be an alternative splice form of *ZFM1*. Simultaneously, the *MEN1* gene was published and proved to be identical to *SCG2* (Chandrasekharappa et al. 1997), enabling screening of all 10 *SCG2* exons for *MEN1* mutations. In all, 10 distinct mutations were found in our MEN1 families, thus confirming that *SCG2* was indeed the *MEN1* gene (Lemmens et al. 1997b). The *MEN1* gene contained 10 exons that spanned over more than 9kb of genomic DNA and produced a major transcript of 2.9kb. Exons 2-10 encode a 610 amino acid protein termed menin.

## 1.2 SCREENING FOR *MEN1* MUTATIONS

MEN1 patients and their relatives at risk (children have a 50% risk of inheriting the disease) are regularly screened biochemically and radiologically in order to monitor the development of endocrine tumours (Falchetti et al. 2005). Current guidelines recommend biochemical screening from the age of 5 years as insulinomas have been detected in early childhood (Brandi et al. 2001). Prospective clinical screening has been shown to reduce morbidity and mortality due to earlier detection of tumours and increasing the chance of radical treatment (Skogseid et al. 1991). Since 1997, mutation screening by sequencing the entire coding region of the *MEN1* gene (exons 2-10) has been used in clinical practice. Once a family mutation has been identified, sequencing of the affected exon is performed to confirm or exclude the MEN1 diagnosis in relatives to the proband. Clinical screening of a relative with no *MEN1* mutation can then be discontinued as they have no risk of developing the disease.

An extensive analysis of the mutation spectrum and associated phenotypes in Swedish MEN1 patients are presented in *paper II*.

## 1.3 ANGIOGENESIS

One of the *MEN1* candidate genes, *VRF/VEGF-B (paper I)*, was related to a family of endothelial cell growth factors. The role of this gene and its relatives in angiogenesis and inflammatory disease has been studied in paper III and IV.

### 1.3.1 Introduction to angiogenesis

Angiogenesis, or the growth of new blood vessels, is vital for tissue growth and survival. Mammalian cells require oxygen and nutrients and are therefore always located within 0.1-0.2mm from a blood vessel. Whenever tissues grow, they must thus recruit new blood vessels to match their increased metabolic demands. In early embryonic development (and in some instances in the adult) this occurs by the recruitment of endothelial precursor cells to form a primitive vascular network in a process termed vasculogenesis. The cells involved are angioblasts in the embryo and

bone marrow endothelial progenitor cells in the adult (Jussila and Alitalo 2002; Risau 1997).

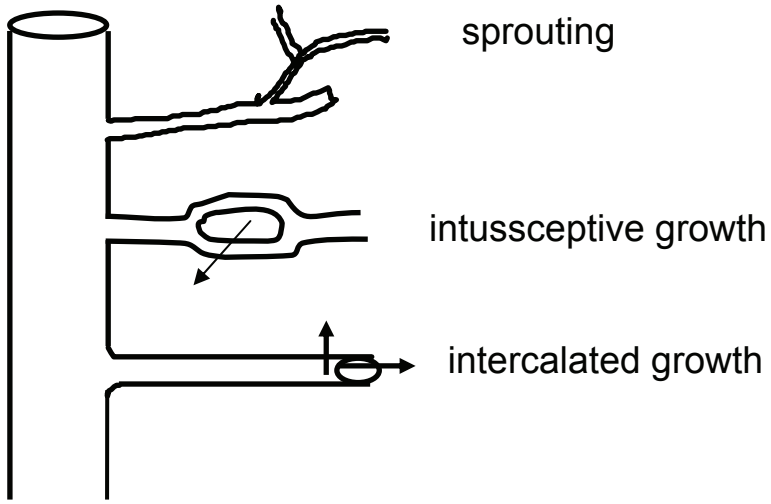


**Figure 2: Angiogenesis occurs through three major mechanisms.** 1) sprouting of new branches, 2) ingrowth of pillars of endothelial and peri-endothelial cells which split the vessel into two (intersection) and 3) endothelial cell proliferation in situ leading to increased vessel diameter and length (intercalated growth).

This network is then remodelled by angiogenesis which entails endothelial cell growth to form new blood vessels. Angiogenesis occurs by three major mechanisms (Figure 2): the sprouting of new branches; ingrowth of endothelial and surrounding cells which split the vessel into two (intussceptive growth) and endothelial cell proliferation leading to an increased diameter and length of the vessel (intercalated growth). The development of lymphatic vessels (lymphangiogenesis) probably occurs in a similar fashion (Jussila and Alitalo 2002; Risau 1997).

Angiogenic sprouting is the most common form of vessel growth and involves a number of well-defined steps. First, the vessels dilate and become permeable, resulting in an extravasation of plasma proteins. Local proteases degrade the extracellular matrix, detaching endothelial and smooth muscle cells which can then migrate along the plasma protein scaffold. The proteases also release sequestered vascular endothelial growth factors which stimulate the proliferation and migration of endothelial cells towards an angiogenic gradient. These cells form new sprouts which are initially leaky, fragile and immature, so-called mother vessels (Pettersson et al. 2000). Mesenchymal cells are recruited and inhibit endothelial cell growth and motility and stimulate the production of extracellular matrix, stabilising the vessels (Bussolino et al. 1997; Carmeliet and Collen 2000; Jussila and Alitalo 2002).

A blood vessel in its simplest form is a capillary, a tube of endothelial cells that create a lumen, surrounded by supporting pericytes within the same basement membrane. Recruitment of additional smooth muscle cells and further differentiation creates veins and arteries (Figure 3). Arteries are formed during development and can increase in size and perfusion in response to tissue damage in a process termed arteriogenesis or collateral growth (Carmeliet 2000).

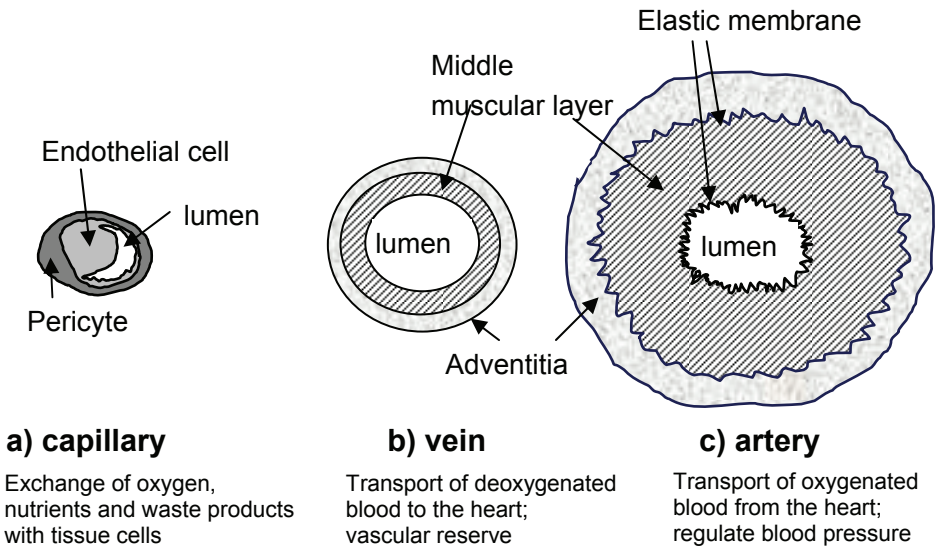


**Figure 3: Stucture of the three basic blood vessel types.**

### 1.3.2 Molecular basis of angiogenesis

Angiogenesis is crucial for tissue development, but uncontrolled vessel growth leads to vascular tumours and oedema. Therefore angiogenesis is tightly controlled by a fine balance between stimulators and inhibitors (Figure 4). Some of these act directly on the endothelial cells; others act indirectly via other cell types or regulate non-endothelial steps in the angiogenic process. Vascular endothelial growth factor A (VEGF-A) was first thought to be an endothelial cell-specific mitogen and is the major mediator of blood vessel growth. The angiopoietins are antagonists that either stabilise or destabilise blood vessels, the latter is required for VEGF-A driven angiogenesis (Holash et al. 1999). VEGF-A, VEGF-B and the angiopoietins have been studied in this thesis.

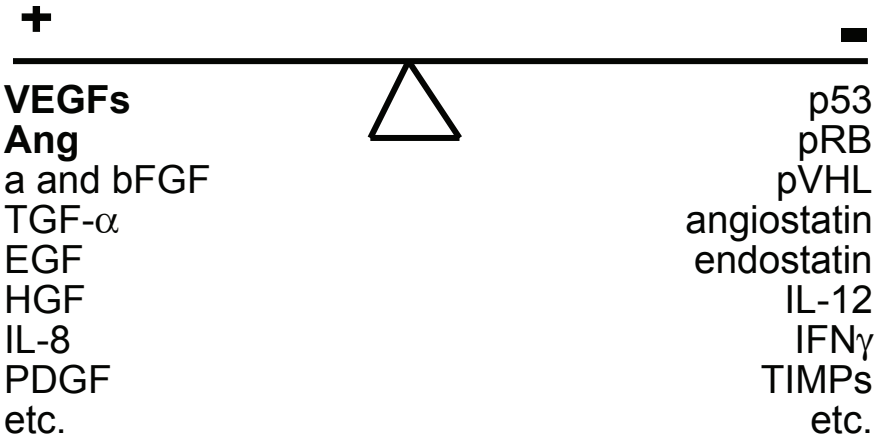


**Figure 4: Angiogenesis is regulated by a number of stimulators and inhibitors.** VEGFs (Vascular endothelial growth factors); Ang (angiopoietins), a and bFGF (acidic and basic fibroblast growth factor), TGFα (transforming growth factor alpha), EGF (epidermal growth factor), HGF (hepatocyte growth factor), IL-8 (interleukin 8), PDGF (platelet-derived growth factor), pRB (protein from the retinoblastoma gene), pVHL (von Hippel-Lindau tumour suppressor gene protein), IL-12 (interleukin 12), IFNγ (interferon gamma), TIMPs (Tissue Inhibitors of Metalloproteinases). Angiostatin and endostatin are endogenous fragments of plasminogen and collagen respectively that inhibit angiogenesis via a direct effect on endothelial cells.(Adapted from (Folkman 1992)).

### 1.3.3 The VEGF family members

One of the genes we isolated during the search for the *MEN1* gene was the VEGF Related Factor gene, *VRF*, later renamed vascular endothelial growth factor B, *VEGF-B (paper I)* and (Olofsson et al. 1996a). This gene was the third member of the vascular endothelial growth factor family to be isolated, after *VEGF-A* in 1989 (Keck et al. 1989; Leung et al. 1989) and placental growth factor (*PlGF*) in 1991 (Maglione et al. 1991)). To date, two additional members (*VEGF-C* (Joukov et al. 1996) and *VEGF-D* (Achen et al. 1998; Rocchigiani et al. 1998) have been identified in mammals) (Figure 5). A sixth VEGF homologue, VEGF-E has been identified in Orf viruses which cause an extensive vascular proliferation in human skin upon infection (Lyttle et al. 1994; Meyer et al. 1999; Ogawa et al. 1998). Several VEGF-related molecules have also been isolated from snake venom, the VEGF-Fs (Takahashi and Shibuya 2005)

The VEGF family of proteins belongs to the cystine-knot superfamily of hormones and extracellular signalling molecules (including the platelet-derived growth factors, PDGFs) and are characterised by eight conserved cysteine residues. Crystal structures of the VEGF proteins show that they consist of two monomers arranged head-to-head in a homodimer with two interchain disulphide bridges with symmetrical receptor binding sites at each pole (Muller et al. 1997; Wiesmann et al. 1997).

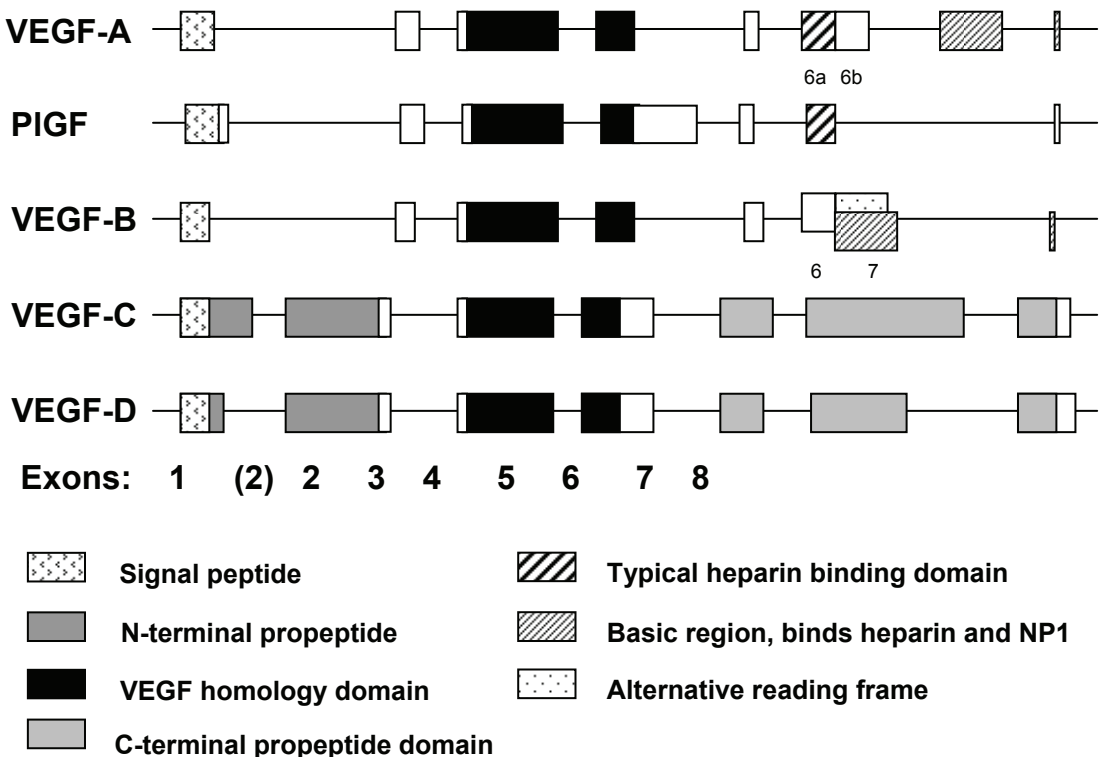


**Figure 5: The vascular endothelial growth factor family.** The five members all contain eight conserved cysteine domains in the VEGF homology domain region (the receptor binding domain). They all have a signal peptide in exon 1 that targets the protein for secretion (and is cleaved off in the process). VEGF-A, PlGF and VEGF-B all have multiple splice forms which have exons 1-4 in common. Some splice forms have heparin binding ability and can also bind the co-receptor, neuropilin-1 (NP1), see below. Both VEGF-C and D are produced as long propeptides that are proteolytically processed. (Figure modified from (Holmes and Zachary 2005)).

### 1.3.4 The VEGF receptors

The VEGFs have three signalling receptors (Figure 6). They are tyrosine kinase membrane bound receptors with six to seven immunoglobulin-like domains and a split tyrosine kinase domain. They dimerise and autophosphorylate upon ligand binding.

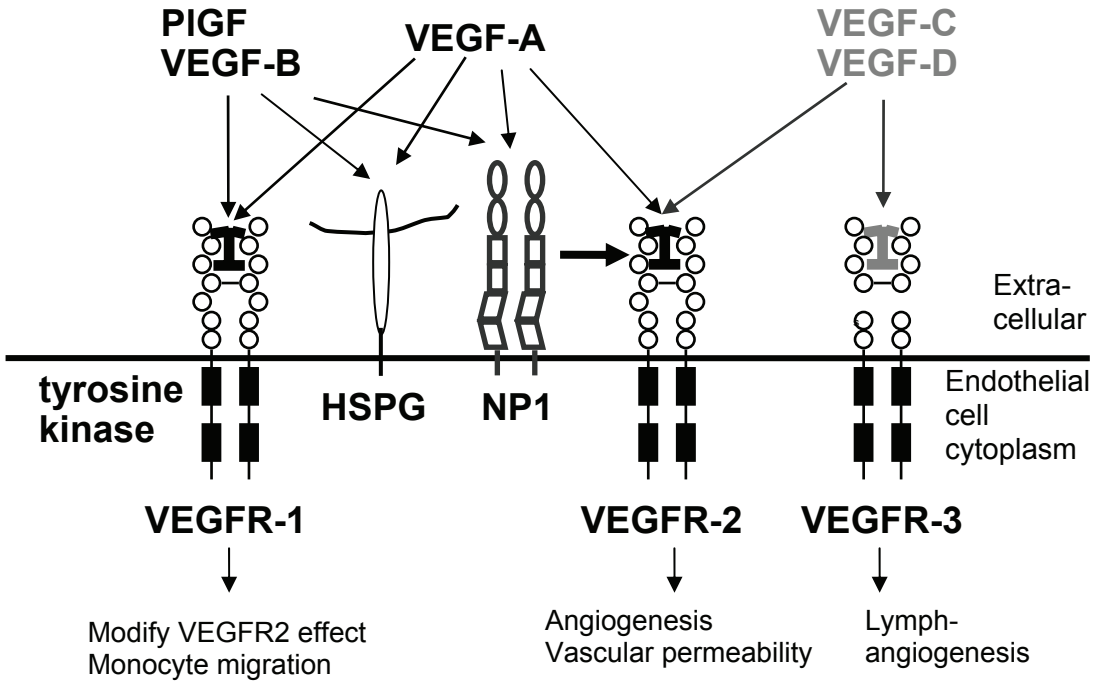


**Figure 6: Vascular endothelial growth factors and their receptors.** Three tyrosine kinase receptors (VEGFR1-3) are shown together with their ligands. They all have six to seven immunoglobulin-like domains (circles) in the extracellular domain and an intracellular split tyrosine kinase domain. Upon ligand binding, the receptors dimerise, are autophosphorylated on tyrosine residues and transmit intracellular signals. In addition, two co-receptors, neuropilin-1 (NP1) and heparan sulphate proteoglycans (HSPG) are shown. They lack intracellular domains and do not transmit their own signals. They bind to selective splice forms of each factor and can enhance or inhibit signalling via VEGFRs. (Figure modified from (Olofsson et al. 1999)).

VEGFR1 (Flt-1) has a high affinity for VEGF-A, VEGF-B, PlGF and VEGF-F (de Vries et al. 1992; Olofsson et al. 1998; Park et al. 1994; Takahashi and Shibuya 2005), but very weak autophosphorylation upon ligand binding (de Vries et al. 1992; Olsson et al. 2006; Waltenberger et al. 1994). Alternative splicing results in a soluble VEGFR1, isolated from placenta that can inhibit VEGF-A driven angiogenesis (Kendall and Thomas 1993; Kendall et al. 1996). VEGFR1 is expressed on endothelial cells as well as many other cell types, (Table 1) (Hattori et al. 2002; Krum and Rosenstein 1998; Shibuya and Claesson-Welsh 2006; Wey et al. 2005; Yamagishi et al. 1999).

VEGFR1 was first thought to be a decoy receptor as knockout mice display disorganised blood vessels and blood islands with an excess of endothelial cells and die by E9.5 (Fong et al. 1995). In addition, knockout of the tyrosine kinase (TK) domain of VEGFR1 resulted in a normal vasculature (Hiratsuka et al. 1998), suggesting that signalling via VEGFR1 was not required for embryonic angiogenesis. This is further supported by the fact that double knockouts of the two VEGFR1-specific ligands, PlGF

and VEGF-B are normal, healthy and fertile (Carmeliet et al. 2001). However, VEGFR1 can induce its own signals (Autiero et al. 2003) and can also interact with VEGFR2 in a synergistic or inhibitory manner (Olsson et al. 2006) (Figure 7).

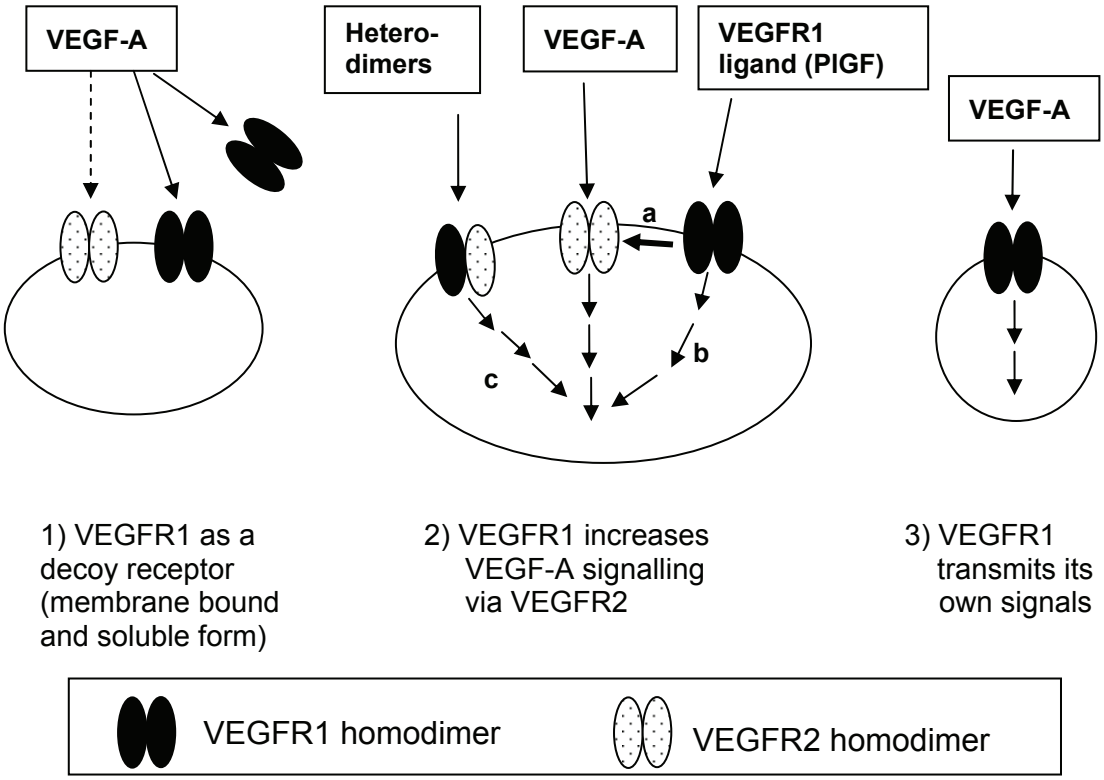


**Figure 7: VEGFR1 can inhibit or synergise with VEGF-A signalling via VEGFR2 or transmit its own signals.** 1) membrane-bound and soluble VEGFR1 can bind VEGF-A, preventing it from binding to VEGFR2, the major receptor involved in angiogenesis. 2) Upon ligand binding (e.g. PlGF), VEGFR1 can (a) transphosphorylate VEGFR2 and further activate it; (b) signal to augment VEGF-A/VEGFR2 activity. In addition, (c) heterodimers of VEGF-A and PlGF or VEGF-B can bind to heterodimerised receptors and signal to augment VEGF-A activity. Finally, VEGFR1 can be autophosphorylated upon ligand binding and transmit its own intracellular signals, leading to effects independent of VEGFR2. (Luttun et al. 2002b; Tjwa et al. 2003)

VEGFR2 is expressed by mainly by endothelial cells, but also by other cell types including some of those in Table 1 (Shibuya and Claesson-Welsh 2006). VEGFR2 (Flk-1/KDR) (Millauer et al. 1993; Quinn et al. 1993; Terman et al. 1992) binds VEGF-A, VEGF-E, VEGF-F and processed VEGF-C and VEGF-D. In addition, VEGFR2 can heterodimerise with either VEGFR1 or VEGFR3 and bind heterodimers of VEGF-A/PlGF or VEGF-A/VEGF-B or full-length/processed VEGF-C or D respectively (Olsson et al. 2006). Alternative splicing also results in a soluble form, of as yet unknown significance (Ebos et al. 2004). VEGFR2 is crucial for vasculogenesis, angiogenesis and hematopoietic stem cell differentiation in the embryo (Shalaby et al. 1995) and for angiogenesis in the adult (Olsson et al. 2006; Shibuya and Claesson-Welsh 2006).

VEGFR3 (Flt-4) is expressed on endothelial and lymphatic endothelial cells and binds VEGF-C and VEGF-D (Achen et al. 1998; Joukov et al. 1997a; Joukov et al. 1996). Alternative splicing results in two isoforms with different C-terminal tails (Hughes 2001). VEGFR3 is required for lymphangiogenesis (Jussila and Alitalo 2002).

**Table 1: Effects of signalling through VEGFR1 and VEGFR2 in different cell types.**

| Cell type | VEGFR1 | VEGFR2 |
|---|---|---|
| Endothelial cells | Production of paracrine growth factors (LeCouter et al. 2003) | Proliferation, migration, survival, tube formation, angiogenesis, vessel permeability, up-regulation of adhesion molecules (Olsson et al. 2006; Shibuya and Claesson-Welsh 2006) |
| Monocytes | Migration and activation (Barleon et al. 1996; Clauss et al. 1996; Hiratsuka et al. 1998; Luttun et al. 2002c). | Do not express VEGFR2 (Usui et al. 2004) |
| Stem cells | Recruitment of hematopoietic stem cells (Hattori et al. 2002; Luttun et al. 2002c) | Recruitment of endothelial progenitor cells (Rafii et al. 2002) |
| Pericytes | Growth and migration (Yamagishi et al. 1999) | No direct effect (Winkler et al. 2004) |
| Cancer cells | Migration and invasion (Fan et al. 2005; Wey et al. 2005) | No direct effect (Takahashi and Shibuya 2005) |
| Astrocytes | Proliferation (Krum et al. 2002) | Do not express VEGFR2 (Storkebaum et al. 2004) |
| Neurons | Proliferation of neuronal precursors (Sun et al. 2006) | Neuronal outgrowth, neuron survival and proliferation of neuronal precursors (Jin et al. 2002; Sondell et al. 2000; Storkebaum et al. 2004) |
| Microglia | Migration and proliferation (Forstreuter et al. 2002) | Do not express VEGFR2 (Forstreuter et al. 2002) |

The VEGFs have two co-receptors, neuropilin 1 and 2 (NP1, NP2) that lack an intracellular domain and do not transduce any signals on their own (Gluzman-Poltorak et al. 2000; Soker et al. 1998). They are important as repellents of nerve growth cones during neuronal development (Chen et al. 1997; He and Tessier-Lavigne 1997; Kolodkin et al. 1997) and play a crucial role in vascular development (Kawasaki et al. 1999; Kitsukawa et al. 1995; Yuan et al. 2002). Neuropilins bind specific splice forms of VEGF-A, PlGF and VEGF-B as well as VEGF-E. NP1 can potentiate binding to and signalling via VEGFR2, (Soker et al. 2002; Whitaker et al. 2001), but can inhibit ligand binding to VEGFR1 (Fuh et al. 2000; Soker et al. 2002).

Heparin sulphate proteoglycans (HSPGs) are also recognised as co-receptors to some VEGF isoforms. HSPGs increase the effect of VEGFs by restoring the VEGFR2-binding ability of oxidised VEGF-A165; by producing a conformational change in the receptor that favours ligand binding; and by increasing the number of binding sites (Ng et al. 2006).

### 1.3.5 The role of the vascular endothelial growth factors

#### *1.3.5.1 VEGF-A*

VEGF-A was initially isolated as a vascular permeability factor from tumour cells, 50,000 times more potent than histamine (Senger et al. 1983). Since then, it has been shown to stimulate all steps in angiogenesis, from vasodilatation and vessel permeability with extravasation of plasma proteins (Connolly et al. 1989b; Dobrogowska et al. 1998), induction of proteases (Lamoreaux et al. 1998; Mandriota et al. 1995; Pepper et al. 1991; Unemori et al. 1992); stimulation of endothelial cell proliferation and migration (e.g. (Connolly et al. 1989a; Ferrara and Henzel 1989; Leung et al. 1989; Plouet et al. 1989; Yoshida et al. 1996) and endothelial cell survival (Alon et al. 1995; Gerber et al. 1998). VEGF-A stimulates the formation of new blood vessels in angiogenesis models such as the cornea assay, the chick chorio-allantoic membrane assay or the aortic explant assay (Connolly et al. 1989a; Leung et al. 1989; Nicosia et al. 1994; Pepper et al. 1992; Plouet et al. 1989). The angiogenic effects of VEGF-A are mediated by VEGFR2 (Cebe-Suarez et al. 2006).

In addition to its effects on endothelial cells, VEGF-A also promotes monocyte chemotaxis (Clauss et al. 1990), recruits endothelial cell precursors from the bone marrow (Rafii et al. 2002) and promotes the survival of haematopoietic stem cells (Gerber et al. 2002). VEGF-A can up-regulate expression of adhesion molecules on endothelial cells and can induce inflammation (Croll et al. 2004; Detmar et al. 1998; Lee et al. 2002; Melder et al. 1996; Proescholdt et al. 1999).

VEGF-A consists of nine different exons (including two alternative exon 6) and has five major splice forms (Figure 8) (Houck et al. 1991; Leung et al. 1989; Poltorak et al. 1997; Tischer et al. 1991). VEGF-A121, 165 and 189 are expressed in most cell types (Bacic et al. 1995), while VEGF-A145 is mostly expressed in reproductive organs (Anthony et al. 1994; Charnock-Jones et al. 1993; Cheung et al. 1995; Poltorak et al. 1997) and VEGF-A206 has been detected in foetal liver, lung and reproductive tissues (Anthony et al. 1994; Burchardt et al. 1999; Houck et al. 1991). All forms contain a signal peptide and are secreted. They are named after the number of amino acids left after cleavage of the signal peptide (Houck et al. 1991; Leung et al. 1989; Tischer et al. 1991). As mouse VEGF-A is one amino acid shorter, the corresponding rodent names are VEGF-A120, 164 etc., The longer splice forms are sequestered by the extracellular matrix and are released only after proteolytic cleavage by plasmin, urokinase or heparin, while VEGF-A165 is partially and VEGF-A121 is completely soluble (Houck et al. 1992; Park et al. 1993; Plouet et al. 1997). VEGF-A165 and VEGF-A145 bind to the co-receptors NP1 and NP2 respectively (Soker 98, Gluzmann-Poltorak 2000). All forms can stimulate endothelial cell proliferation and angiogenesis, although VEGF-A165 has been reported to be the most potent in some studies (Park et al. 1993; Poltorak et al. 1997).

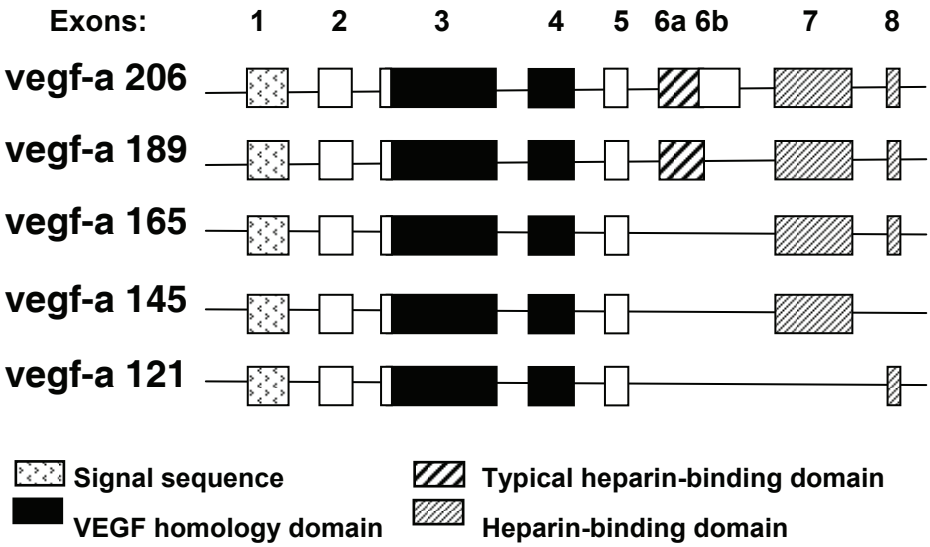


**Figure 8: The VEGF-A splice forms.** All forms contain exons 1-5 (which contains the receptor binding domain). The different C-terminal ends confer different properties to the protein. (Figure modified from (Holmes and Zachary 2005))

VEGF-A levels are tightly regulated during embryonic development. Heterozygous knockout mice and mice with a 2-3-fold over-expression of VEGF-A die before birth due to the disruption of normal blood vessel and cardiac development (Carmeliet et al. 1996; Ferrara et al. 1996; Feucht et al. 1997; Miquerol et al. 2000). Both heparin-binding and soluble isoforms are required for normal angiogenesis. VEGF-A120 is required for an increase in lumen calibre of existing vessels (intercalated growth), while VEGF-A188 bound to the extracellular matrix guides activated endothelial cells to initiate formation of vascular branches (sprouting) and is involved in arterial development. VEGF-A165, which is partially soluble seems to be sufficient for normal blood vessel development (Ruhrberg et al. 2002; Stalmans et al. 2002). However, VEGF-A165 is more potent than VEGF-A120 in inducing inflammation and pathological angiogenesis in the murine eye (Ishida et al. 2003a; Usui et al. 2004).

#### *1.3.5.2 VEGF-B*

VEGF-B was isolated and characterised in 1996 by two independent groups (*paper I* and (Olofsson et al. 1996a). The first studies showed that VEGF-B consisted of two splice forms (*paper I*), both forms bound VEGFR1 but not VEGFR2, that VEGF-B could form heterodimers with VEGF-A and that it might stimulate endothelial cell proliferation and up-regulation of uPA and PAI-1 (Olofsson et al. 1998; Olofsson et al. 1996a; Olofsson et al. 1996b) However, these effects were later attributed to heterodimer formation with VEGF-A and/or could not be reproduced, (Nash et al. 2006). Although it was difficult to show a function for recombinant VEGF-B, both forms had been expressed in mammalian cells and insect cells. VEGF-B167 was found to be a 21kDa monomer and a 46kDa dimer. The 167 isoform was secreted, but remained cell-associated in the absence of heparin (Olofsson et al. 1996a). VEGF-B186 was produced as a 25kDa monomer and was freely secreted. O-linked glycosylation

created a 32kDa monomer in the conditioned medium. In addition cleavage at Arg127 created an active processed form (Makinen et al. 1999; Olofsson et al. 1998). VEGF-B167 and processed VEGF-B186 were found to bind to the co-receptor neuropilin-1 in 1999 (Makinen et al. 1999).

VEGF-B was highly expressed in the heart, brown fat and central nervous system neurons during embryonic development and adulthood where it was also found in the kidney, thymus and testis (Lagercrantz et al. 1998; Lagercrantz et al. 1996). However, the physiological and pathological function of VEGF-B was unknown.

#### *1.3.5.3 VEGF-C and D*

VEGF-C and VEGF-D were first identified in a prostate carcinoma cell line and human lung respectively. VEGF-C and VEGF-D have no known splice variants in humans, but are produced as prepropeptides that bind VEGFR3 (Achen et al. 1998; Joukov et al. 1996; Rocchigiani et al. 1998). Once these precursor proteins have been activated by proteolytic cleavage, they bind more easily to VEGFR2 (Joukov et al. 1997b; Stacker et al. 1999). VEGF-C is expressed in many tissues and is important for lymphatic vessel development during embryogenesis via VEGFR3 (Karkkainen et al. 2004; Karpanen et al. 2001). VEGF-C can also induce endothelial cell proliferation, migration, angiogenesis and vessel permeability, although it is less effective compared to VEGF-A. VEGF-D has similar properties (Jussila and Alitalo 2002), although its role in angiogenesis and lymphangiogenesis is not completely determined.

#### *1.3.5.4 PlGF*

PlGF, like VEGF-B, is a specific VEGFR1 ligand that is not required during embryonic angiogenesis (Carmeliet et al. 2001). PlGF was initially isolated from placenta (Maglione et al. 1991), where it is highly expressed, although low amounts have been detected in most tissues analysed (in the mouse) (DiPalma et al. 1996). PlGF has been reported to be up-regulated in pathological situations such as tumours, atherosclerosis, myocardial infarction and skin wounds (Carmeliet et al. 2001; Luttun et al. 2002c; Parr et al. 2005; Zhang et al. 2005). PlGF itself does not induce angiogenesis in the mouse cornea assay (Eriksson et al. 2002), but can either potentiate or antagonise VEGF-A-driven angiogenesis and permeability (Carmeliet et al. 2001; Eriksson et al. 2002; Luttun et al. 2002a; Oura et al. 2003; Park et al. 1994). PlGF also has effects that are independent of VEGFR2. These include recruitment and activation of monocytes (Clauss et al. 1996) that are required for the arteriogenic effect of PlGF (Luttun et al. 2002c; Pipp et al. 2003; Scholz et al. 2003).

### 1.3.6 Regulation of the vascular endothelial growth factors

Sufficient perfusion of a tissue is crucial for cell survival. Indeed, one of the most powerful regulators of angiogenesis is oxygen, where low oxygen levels (hypoxia) up-regulate angiogenesis.

The major hypoxia regulated factor is hypoxia inducible factor 1 alpha (HIF1$\alpha$) which directs the up regulation of VEGF-A and VEGFR1. During normoxia, HIF1$\alpha$ protein is

rapidly degraded. Upon hypoxia, HIF1α protein is stabilised and rapidly accumulates in the cell cytoplasm. In addition, HIF1α mRNA levels are increased. HIF1α moves to the nucleus, dimerises with HIF1β and transcriptional co-activators, binds to HIF1α - responsive elements (HRE) in target gene promoters and stimulates transcription of genes involved in angiogenesis, erythropoiesis, glycolysis and cell growth (Semenza 1999; Sharp and Bernaudin 2004).

Hypoxia also increases VEGF-A levels in HIF1α-independent ways. VEGF-A mRNA is rapidly degraded in normoxia, but is stabilised up to eight times during hypoxia. This is due to binding of proteins to its specific motifs in its 3' UTR (Levy et al. 1997; Levy et al. 1998; Onesto et al. 2004). During hypoxia, when the normal translation machinery is limited, uncapped VEGF-A can also be transcribed through an alternative transcription start site followed by an internal ribosome entry site (IRES) in its long 5' UTR (Akiri et al. 1998; Stein et al. 1998).

VEGF-A in turn can up-regulate expression of its two receptors (Wilting 96, Barleon B 97). VEGFR1 is also regulated by HIF1α via its HRE, while hypoxic up-regulation of VEGFR2 occurs indirectly via a posttranscriptional mechanism (Gerber et al. 1997; Waltenberger et al. 1996).

VEGF-A mRNA has a half-life of 15-40 minutes (Levy et al. 1998) and is regulated by a number of different growth factors, oncogenes, tumour suppressor genes and cytokines, including FSH, TGFβ, PDGF, H-Ras, p53 and IL-6. Growth factors and cytokines have also been shown to regulate VEGF-C and PlGF (Enholm et al. 1997; Failla et al. 2000; Ferrara and Davis-Smyth 1997; Laitinen et al. 1997; Ristimaki et al. 1998). VEGF-D was discovered as a c-fos responsive gene and is also regulated by other AP-1 transcription factors as well as cell adhesion molecules (McColl et al. 2004). VEGF-B mRNA is stable, with a half-life greater than 8h. The VEGF-B promoter has several SP-1 and AP-2 sites, but no hypoxia response element (Silins et al. 1997) and VEGF-B is not regulated by hypoxia or any of the cytokines or growth factors tested (Enholm et al. 1997; Laitinen et al. 1997; McColl et al. 2004).

### 1.3.7 VEGFs and their role in pathological angiogenesis

In healthy adult individuals, angiogenesis is crucial for female fertility and wound healing. It is now recognised that angiogenesis and vascular endothelial growth factors are also involved in the pathogenesis of many diseases including rheumatoid arthritis, psoriasis, duodenal ulcers, disorders of female reproduction, diabetic retinopathy, cancer and developmental disorders (Folkman 1995).

#### *1.3.7.1 VEGFs and cardiac disease*

VEGF-A and VEGF-B are highly expressed in cardiomyocytes in the heart (Lagercrantz et al. 1998) and (*Paper III)*. VEGF-A is up-regulated in acute ischemia of the heart (Banai et al. 1994; Hashimoto et al. 1994). However, data indicates that mice of older age, with diabetes or with hypercholesterolemia have reduced VEGF-A production and administration of exogenous VEGF-A can increase angiogenesis and improve clinical parameters after myocardial infarction (Couffinhal et al. 1999; Rivard et al. 1999a;

Rivard et al. 1999b; Yoon et al. 2005). A number of clinical trials on administration of VEGF-A to patients with coronary artery disease have been performed with some positive results, although there has been a lively debate on the risk of stimulating cancer growth (cancer patients have been excluded from the clinical trials) and whether or not VEGF-A is the best therapeutic factor considering its potential to cause hypotension, tortuous and leaky vessels and inflammation (Yoon et al. 2004). PlGF, angiopoietin-1 and VEGF-B have been suggested as more attractive candidates due to their ability to stimulate arteriogenesis and/or lack of effect on quiescent vessels (Luttun et al. 2002b; Siddiqui et al. 2003; Takahashi et al. 2003).

VEGF-A has been shown to be elevated in correlation with an increased capillary density in heart failure due to ischaemic heart disease, although it was decreased in non-ischemic cardiac failure (Abraham et al. 2000). VEGF-B protein was not altered in this study and has not been extensively studied in cardiac pathology, although VEGF-B knockout mice were reported to have discrete cardiac defects, suggesting that VEGF-B might play a homeostatic role in the heart (Aase et al. 2001; Bellomo et al. 2000).

#### *1.3.7.2 VEGFs and inflammation*

VEGF-A has been implicated in the pathology of inflammatory conditions as well. VEGF-A is produced by leukocytes, which also express VEGFRs, and can induce inflammation in the skin (Detmar et al. 1998; Kunstfeld et al. 2004). Expression of VEGF-A is increased in inflammatory conditions such as asthma, neointimal formation and allograft rejection (Takahashi and Shibuya 2005). VEGF-A, PlGF and VEGF-B and their receptors are expressed in synovial tissues in rheumatoid arthritis (RA) (Bottomley et al. 2000; Ikeda et al. 2000; Luttun et al. 2002c; Mould et al. 2003). Knockout of VEGF-B or inhibition of VEGFR1 significantly decreased inflammation, angiogenesis and clinical symptoms of RA in mouse models of the disease (Luttun et al. 2002c; Mould et al. 2003), demonstrating that VEGFR1 and its ligands can induce inflammatory-mediated pathology.

The VEGF-A165 isoform has been shown to be more inflammatory than VEGF-A121 *in vivo* (Usui et al. 2004) and is crucial for the leukocyte-associated pathological angiogenesis, but not normal physiological vessel growth in the eye (Ishida et al. 2003b). On the other hand, the presence of monocytes seems to be required for arteriogenesis and collateral growth stimulated by PlGF via VEGFR1 (Luttun et al. 2002b; Pipp et al. 2003; Scholz et al. 2003). Thus, in some instances, leukocyte infiltration is beneficial and in others, it contributes to the disease phenotype.

#### *1.3.7.3 VEGFs and brain disease*

Expression of VEGF-A increases within 3 hours after ischemic injury (stroke) to the rodent brain, with a peak at 12-48 hours (Hayashi et al. 1997; Plate et al. 1999; Wang and Zhu 2002). Administration of VEGF-A to ischaemic rodent brain can lead to increased tissue damage associated with increased oedema and inflammation (if administered directly after the ischaemic insult, at a high dose or if there was additional CNS pathology) (Kaya et al. 2005; Manoonkitiwongsa et al. 2006; Proescholdt et al. 1999; Proescholdt et al. 2002; Shen et al. 2006; Zhang et al. 2000). On the other hand, several studies have shown that VEGF-A can protect from ischaemic damage by

decreasing inflammation and oedema. In some cases, VEGF-A induced an angiogenic response, in others it did not, thus it seems as if the protective effect was independent of neovascularisation (Harrigan et al. 2003; Kaya et al. 2005; Shen et al. 2006; Sun et al. 2003). Even when VEGF-A was administered to normal rodent brain, the result was tortuous, leaky vessels in the adult rat, but functional vascular networks in newborn or foetal rat brain (Rosenstein et al. 1998). Thus it seems that the dose, timing, administration route and local environment determine whether or not VEGF-A165 can diminish cerebral injury.

VEGF-A is expressed by neurons and glia in the CNS and its receptors have been detected on endothelial cells as well as neurons (VEGFR2) and astrocytes (VEGFR1) (Krum et al. 2002). *In vitro* and *in vivo* experiments have shown that VEGF-A stimulates neuronal outgrowth, proliferation and migration of neuronal precursors via VEGFR2. It is also mitogenic for astrocytes and microglia and can induce microglial migration, probably via VEGFR1 (Forstreuter et al. 2002; Krum et al. 2002; Storkebaum et al. 2004). Ischaemia up-regulates VEGF-A expression in the CNS and VEGF-A promotes neuron survival in response to hypoxia. If this mechanism is perturbed by deletion of the HRE in the VEGF-A promoter in mice, the mice develop motor neuron degeneration similar to human amyotrophic lateral sclerosis (ALS) (Oosthuyse et al. 2001). ALS is an adult neurodegenerative disease with progressive degeneration of upper and lower motor neurons resulting in increasing paralysis and normally leads to death within five years of onset. 10% of the cases are familial and 20% of these are caused by mutations in the Cu/Zn superoxide dismutase (*SOD1*) gene. There is no effective treatment. However, administration of VEGF-A to a mice with mutant *SOD1* delayed onset of symptoms and increased survival, representing the largest therapeutic effects ever in animal models of this disease (Azzouz et al. 2004; Storkebaum et al. 2004; Storkebaum et al. 2005) Furthermore, specific variations in the VEGF-A gene that lead to decreased transcription and impaired IRES-dependent translation (important during hypoxia) have been identified. These variations result in lower levels of VEGF-A in the circulation and are associated with an increased risk of ALS in humans (Lambrechts et al. 2003). Variations in the VEGF-A gene are also associated with an increased risk of Alzheimer's disease, although the mechanisms behind this effect are not yet clear (Del Bo et al. 2005).

All in all, there is convincing evidence that VEGF-A is a neuroprotective factor that may be implicated in several types of neurological disease. However, VEGF-A is also pro-inflammatory and in diseases (including cerebral ischaemia or multiple sclerosis, see below) where inflammation is present, the net effects of VEGF-A are more difficult to determine.

VEGF-B is highly expressed in normal brain (Lagercrantz et al. 1996), but has not been reported to increase after cerebral hypoxia (although it was up-regulated after cerebral cold injury (Nag et al. 2002). VEGF-B can have pro-inflammatory effects (Mould et al. 2003), but can also protect from ischaemic brain damage and can stimulate neurogenesis, suggesting that VEGF-B can have a neuroprotective effect (Sun et al. 2004, 2006). As for VEGF-A, the role of VEGF-B in brain pathology is far from clear.

### 1.3.8 Angiopoietins

The angiopoietins are important auxiliary factors in VEGF-mediated angiogenesis and were analysed in *paper III*.

Angiopoietin-1 is constitutively expressed by peri-endothelial cells in most tissues and acts in a paracrine manner, binding to and activating Tie-2 receptors on endothelial cells (Davis et al. 1996). Knockout mice and expression studies have provided strong evidence that Ang-1 recruits pericytes (Stoeltzing et al. 2003) and is important for vessel stability in mature, quiescent vasculature (Dumont et al. 1994; Goede et al. 1998; Maisonpierre et al. 1997; Sato et al. 1995; Suri et al. 1996).

Recent data has demonstrated a role for Ang-1 in inhibition of vessel leakage (Suri et al. 1998; Thurston et al. 1999) and suppression of inflammation (Gamble et al. 2000; Hughes et al. 2003; Kim et al. 2001; Kim et al. 2002; Pizurki et al. 2003). Ang-1 can counteract VEGF-A induced permeability, angiogenesis and inflammation (Kim et al. 2001; Nambu et al. 2005; Thurston et al. 2000; Visconti et al. 2002). However, Ang-1 also has an angiogenic function and can stimulate endothelial cell migration, tube formation and sprouting as well as survival *in vitro*. Ang-1 can also synergise with the angiogenic effect of VEGF-A (Saito et al. 2003; Shyu et al. 2003; Zhu et al. 2002). Over-expression of Ang-1 *in vivo* results primarily in enlarged vessel size without oedema (Thurston et al. 1999; Thurston et al. 2005) . In addition, Ang-1 may signal via Tie-1 (Saharinen et al. 2005), a related endothelial cell receptor with a role in vascular integrity and endothelial quiescence (Patan 1998; Puri et al. 1995; Sato et al. 1995).

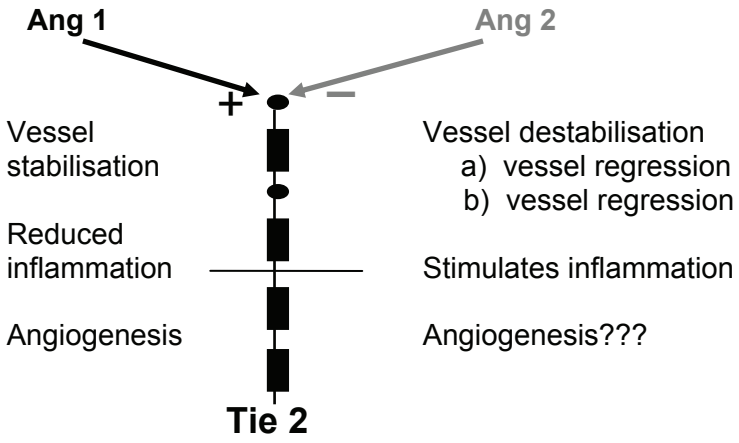


**Figure 9: The antagonising roles of the angiopoietins.** Ang-1 activates the endothelial cell receptor, Tie-2, leading to blood vessel stabilisation and reducing inflammation. Ang-1 can also stimulate angiogenesis in synergy with VEGF-A. Ang-2 binds to Tie-2 without activating it. Ang-2 destabilises the blood vessels, and in the absence of growth factors, the vessels regress. If VEGF-A is present, the vessels grow. Ang-2 is pro-inflammatory and may also induce angiogenesis in some instances.

Angiopoietin-2 was found to be an antagonist of Ang-1 (Figure 9). This notion is supported by the fact that mice over-expressing Ang-2 display a similar phenotype to those with an Ang-1 or Tie-2 knockout (Dumont et al. 1994; Maisonpierre et al. 1997; Sato et al. 1995; Suri et al. 1996). Ang-2 binds to Tie-2 with similar affinity as Ang-1, but does not activate the receptor (Maisonpierre et al. 1997). In contrast to Ang-1, Ang-2 is not constitutively expressed, but is up-regulated in endothelial cells upon hypoxia or

other stimuli (Brindle et al. 2006). Ang-2 destabilises mature blood vessels. If VEGF-A is present, the destabilised vessels initiate angiogenesis, if not, the vessels regress (Holash et al. 1999; Maisonpierre et al. 1997; Yancopoulos et al. 2000). Recent data have also found that Ang-2 can stimulate inflammation (Fiedler et al. 2006). In addition to its role as an antagonist, it is becoming increasingly clear that in some instances, Ang-2 can act as an agonist to Ang-1 and also induce endothelial cell migration, tube formation and sprouting angiogenesis via Tie-2 (Eklund and Olsen 2006; Gale et al. 2002).

Two additional members (orthologues) of the angiopoietin family have been identified in mice and humans: Ang-3 and 4 (Kim et al. 1999; Nishimura et al. 1999; Valenzuela et al. 1999), but to date, not much is known about their function.

## 1.4 DETERMINING THE FUNCTION OF A NOVEL GENE

We isolated a novel gene, VEGF-B with no known function. How do we proceed? There are many complementary ways to determine the function of a new protein. To determine the role of a protein in the growth and development of an entire organism, animal studies where the gene is either knocked out or over-expressed are vital. In order to study the function of a protein on the cellular level, one must produce recombinant protein and then test its function in a number of different assays *in vitro* and later confirm the results *in vivo*. As VEGF-B is related to VEGF-A, assays measuring functions in inflammation and angiogenesis are of primary interest (Auerbach et al. 2003). In addition, indirect studies of expression in disease models can lead to clues to protein function and open an opportunity to intervene in the disease course by blocking or administering the protein of interest.

The knockout/over-expression approach was pursued by several labs (Aase et al. 2001; Bellomo et al. 2000), although the resulting phenotypes were very discrete. (See discussion for more details). We chose to produce recombinant VEGF-B and also to study the expression pattern of VEGF-B and other angiogenesis molecules in disease models.

### 1.4.1 Producing recombinant protein

It is possible to use cell systems such as bacteria, yeast, insect cells or mammalian cells to produce recombinant protein. In addition, some proteins can be produced in cell-free systems. Each method has their advantages and disadvantages (Table 2). Mammalian cells give small yields, but contain all the necessary co-factors and enzymes needed for protein modifications and correct tertiary structure of the recombinant protein. Production of protein in bacteria is cheap, fast and gives high yields, however, they lack many of the protein modification systems that eukaryotic cells have and often fail to produce correctly folded protein. This results in the formation of dense aggregates of misfolded protein and RNA, inclusion bodies, in the cell cytoplasm. Although it is very easy to wash away most of the contaminating cytoplasmic proteins, it is often very difficult to refold the aggregated protein and thus recovery of biological function can be difficult to achieve. In order to increase the solubility of the recombinant protein, it is possible to try using different bacterial strains, to lower the temperature during protein

production, to lower the expression levels or to produce recombinant protein as a fusion protein with a highly soluble bacterial protein, such as thioredoxin, that can confer solubility to the linked heterologous protein (Ausubel et al.; Coligan et al. 1997)

**Table 2: Different cell systems for production of recombinant protein.**

| | **Bacteria** | **Yeast** | **Insect cells** | **Mammalian cells** | **Cell-free*** |
|---|---|---|---|---|---|
| Cell growth | Rapid (30min) | Rapid (90min) | Slow (18-24h) | Slow (24h) | - |
| Yield | High | Moderate | Moderate | Low | Can be high |
| Cost | Low | Low | High | High | High |
| Protein modifications | Refolding usually required | Refolding usually required | Proper folding | Proper folding, all cofactors and chaperones present | Refolding usually required |
| Expression | Intracellular or periplasmic | Intracellular or secretion to medium | Intracellular or secretion to medium | Intracellular or secretion to medium | Usually high |
| N-linked glycosylation | No | Intermediate | Simple | Complex | varies |
| O-linked glycosylation | No | yes | yes | yes | varies |
| Phosphorylation | No | yes | yes | yes | varies |
| Acetylation | No | yes | yes | yes | varies |
| Acylation | No | yes | yes | yes | varies |
| Carboxylation | No | No | no | yes | varies |

*Cell-free systems are usually based on bacterial, wheat germ or rabbit cell extracts and thus have different post-translational modification capacities. Modified from www.embl-hamburg.de

Most expression vectors for production of recombinant protein in bacteria use inducible systems such as the lac operon system of transcription control. This system enables bacteria to turn off their lactose metabolism genes when their preferred substrate, glucose is present. If glucose levels decline, but lactose is plentiful, lactose will inactivate the lac repressor, enabling transcription of the lac genes from the lac promoter. For protein production in bacteria, the lac genes are replaced by VEGF-B and addition of a lactose analogue, IPTG, will enable transcription of the inserted gene (Figure 10).

An additional level of transcriptional control used in bacterial protein production vectors is to utilise the bacteriophage T7 promoter and the *lac* operator to direct transcription of the recombinant gene. As *E. coli* RNA polymerase does not recognise the T7 promoter there is no background expression of the target gene and the cloning step is therefore uncoupled from expression which may simplify stable cloning. After successful cloning, the vector is transformed into a host with the T7 RNA polymerase gene under the control of the *lac* promoter, such as the *E. coli* strain BL21 Gold (a

λDE3 lysogen). Expression of both T7 RNA polymerase and the target gene (VEGF-B) are induced by the addition of IPTG, resulting in very high protein yields.

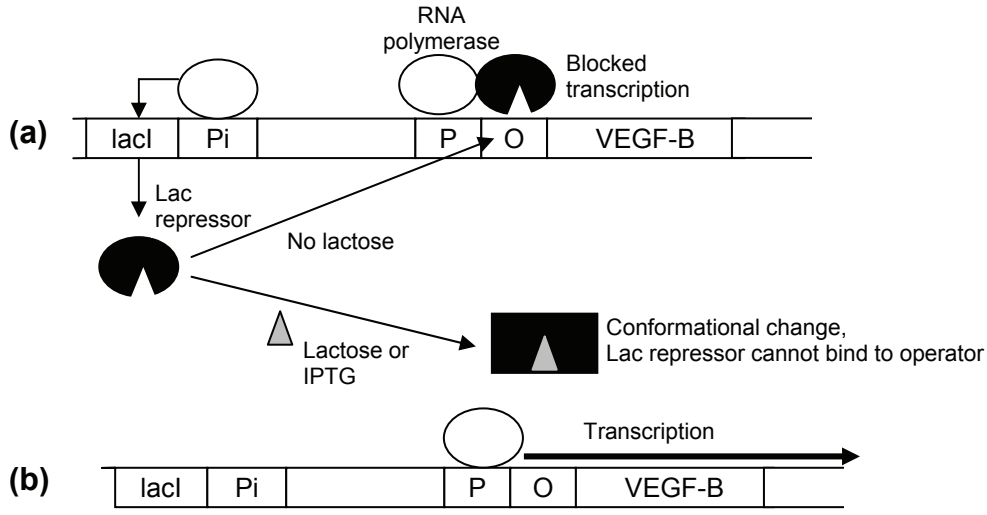


**Figure 10: The lac operon is used to regulate recombinant protein transcription in bacteria.** In the absence of lactose (a), the lac repressor (lacI) binds to the operator (O) and inhibits RNA polymerase from initiating transcription from the lac promoter (P). When lactose or a lactose analogue, IPTG is added (b), the lac repressor changes conformation and can no longer bind to the operator, enabling transcription of lac genes, or the inserted gene, in this case, VEGF-B. (Modified from http://web.mit.edu/esgbio/www/pge/lac.html).

For proper folding of recombinant proteins, production in mammalian cells is usually better, although more expensive. Most vectors for high expression have origins of replication that allow extra-chromosomal replication as episomes in the transfected cell, giving larger yields. Cells can be transiently transfected, but with each cell division, a non-replicating vector and its insert will be diluted. If retrovirus is used to infect the cells, the virus and its insert integrate into the cellular genome and will replicate in every cell division and enable permanent production of protein.

Previous attempts to produce recombinant VEGF-B from baculovirus-infected cells had not resulted in protein dimers (Weber G and Tvrdik T, unpublished data). We therefore focused our efforts towards the production of large quantities of VEGF-B in bacteria and refolding of the protein, and alternatively in the production of smaller amounts from mammalian cell lines.

Proteins can be purified in many different ways and several steps of purification may be necessary to obtain a single protein species. The first step may be to separate the protein into specific compartments such as an inclusion body in bacterial cells or secreted into the medium of mammalian cells.

Subsequent purification steps are based on different forms of chromatography. Chromatography is a means of separating proteins using two phases: a stationary phase bed and a mobile phase, which percolates through the stationary bed. There are several types of chromatography that separate proteins according to their biochemical properties such as size (gel filtration), isoelectric point (chromatofocusing), net charge (cation or

anion exchange), hydrophobicity (reversed-phase chromatography) or affinity to various molecules (affinity chromatography).

Affinity chromatography makes use of a protein's ability to bind to an antibody, ligand etc. In other words, the ligand is immobilised on the stationary phase and binds to the protein of interest. After washing off non-bound proteins, the protein of interest is released into the mobile phase by modifying its binding capacity to the ligand by e.g. altering the pH or adding a competitor in increasing concentrations. Affinity chromatography is often very effective and can give pure protein in just one purification step.

One special type of affinity chromatography makes use of the very strong affinity of nickel ions (Ni) for histidine. Proteins with a stretch of six histidines bind to Ni-NTA (NTA, nitrilotriacetic acid as chelator) and are immobilised on the stationary phase, (metal beads or sepharose). Imidazole, which resembles histidine in structure, can also bind to nickel. At low concentrations, imidazole will prevent the binding of cellular histidine-rich proteins and at high concentrations will win over the His-tagged recombinant protein in the competition for Ni-binding sites, resulting in elution of tagged protein. Addition of detergents, glycerol, salt and β–mercaptoethanol to the binding and washing buffers will reduce the risk of co-purifying associated proteins by reducing nonspecific protein interactions. This method is reported to result in >95% protein homogeneity (Janknecht et al. 1991; Petty 2003).

### 1.4.2 Expression studies

The second strategy that was pursued to determine the function of VEGF-B was to analyse its expression in disease models. As VEGF-B was most highly expressed in the the heart and central nervous system (CNS), these organs were selected for further study. The expression of VEGF-B in context to related genes was studied in dilated cardiomyopathy and the inflammatory CNS disease, multiple sclerosis.

#### *1.4.2.1 Dilated cardiomyopathy*

Cardiac failure occurs when the heart cannot pump oxygenated blood at a rate sufficient to meet the demands of the metabolising tissues. It can be caused either by intrinsic heart muscle disease or by increased tissue oxygen demands due to e.g. sepsis. It affects 2% of the US population and has a 5-year mortality rate of 50%, due to arrhythmias, thromboembolism or due to insufficient perfusion of other organs leading to respiratory, renal or liver failure (Zevitz 2006). Blood fills the ventricles of the heart during diastole and is then pumped out into the pulmonary or systemic circulation during systole (contraction of the ventricles). Both of these functions can be perturbed in cardiac muscle failure.

Heart failure due to cardiac muscle disease, cardiomyopathy, can be divided into four structural types: dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM) and arrhythmogenic right ventricular cardiomyopathy (ARVCM) (Richardson et al. 1996). HCM is characterised by growth of the left and/or right ventricle often leading to a reduced ventricular volume. Most cases are familial diseases with mutations in sarcomeric contractile proteins. RCM is

characterised by a reduced filling of the ventricles during diastole with a normal systolic function. It is either associated with other disease such as amyloidosis or is idiopathic. ARVCM is characterised by different types of arrhythmias and patients rarely develop cardiac failure, although there is progressive replacement of right ventricular muscle tissue with fatty and fibrous tissue. It is most often due to monogenic disease (OMIM 107970).

Dilated cardiomyopathy is the most common of the cardiomyopathies and is characterised by dilatation and impaired contraction of the left or both ventricles. It is the most common reason for heart transplantation among Swedish patients (Sigurdardóttir and Bergh 2006). The most common cause of DCM is coronary artery disease, which is often referred to as a separate entity termed ischaemic cardiomyopathy, ICM. Many other etiological factors of DCM have been determined including virus infection, autoimmunity, toxic compounds (including alcohol) and genetic defects (including mutations in cytoskeletal and mitochondrial genes). A large number of DCM cases have no known cause and are referred to as idiopathic DCM (Richardson et al. 1996).

Included among the genetic causes of DCM are mitochondrial defects that often cause symptoms from skeletal and heart muscle and the central nervous system due to the high energy requirements of these tissues (DiMauro and Hirano 1998). 71 of the proteins involved in mitochondrial function are nuclear-encoded. Defects in these genes follow Mendelian inheritance. 13 mitochondrial proteins (including components of the respiratory chain) have genes in the mitochondrial DNA. Defects associated with cardiomyopathy include mtDNA deletions and duplications and point mutations (DiMauro and Hirano 1998). In addition, a substantial proportion of the idiopathic cases of DCM have mitochondrial disturbances (Arbustini et al. 1998).

There is accumulating evidence that angiogenesis factors may be involved in cardiomyopathy. Mice who lack the two major cardiac isoforms of VEGF-A (VEGFA-164 and VEGFA-188) develop DCM and die shortly after birth (Carmeliet et al. 1999). Likewise, a cre-loxP mediated knockout of VEGF-A in collagen2a1-expressing cells in the mouse (eye, epidermis, myocardium, endoderm and chondrogenic tissues) can cause DCM (Haigh et al. 2000). In addition, 21 transplant patients with DCM had decreased levels of VEGF-A165 and VEGFR1 mRNA and protein as well as a lower capillary density. VEGFA-165 and VEGF-C were increased in the 20 patients with ICM, who also had increased capillary density (Abraham et al. 2000). No studies on the expression of angiogenic factors in mitochondrial DCM had been published.

In *paper III* mice with mitochondrial DCM were created via a *cre-loxP* conditional knockout of *Tfam*, a nucleus-encoded factor that is required for transcription of mitochondrial DNA. Cre-recombinase under the control of the creatinine kinase promoter causes post-natal excision of the lox-P flanked alleles specifically in myocytes. As a result, these cells cannot transcribe mitochondrial genes including those involved in electron transport such as the mitochondrial cytochrome-c oxidase subunit (*COX*). The mutant mice develop DCM, manifest heart failure and atrioventricular conduction blocks and die at 2-4 weeks of age (Wang et al. 1999). Histopathologically, the murine hearts are characterised by dilated left ventricles, with no evidence for

fibrosis, necrosis or inflammation. The cardiomyocytes show a mosaic pattern of respiratory chain deficiency and increased apoptosis (Wang et al. 2001).

#### *1.4.2.2 Multiple sclerosis*

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS). In a majority of cases, MS at disease onset is characterized by a relapsing-remitting disease course, with discrete clinical disease attacks followed by a period of improvement (remission). MS can also be progressive, either from the start of disease as in primary progressive MS, or after years of a relapsing-remitting disease course, secondary progressive MS. Due to the effects of inflammatory lesions throughout the CNS, MS can give rise to a large variety of CNS symptoms, where sensory and visual disturbances, balance problems and muscle weakness (paresis) are the most common. It most often affects young adults (20 to 45 years of age) and women twice as often as men. MS is common in northern Europe and USA and has an incidence of 5/100.000/year in Sweden (Fredrikson and Årman 2003). MS is diagnosed by clinical and/or MRI evidence for at least two demyelinating lesions affecting different areas of the brain and spinal cord and separated in time (Compston and Coles 2002; Polman et al. 2005).

The exact aetiology of MS is unknown, but is thought to depend on a complex interaction between multiple genetic and environmental factors. Whole-genome scans have identified a number of possible MS loci, but the HLA region on chromosome 6p21 is the only established genetic susceptibility factor so far. However, it is clear that as yet unknown environmental factors are required for disease development and infectious agents are likely candidates (Dyment et al. 2004; Lutton et al. 2004; Noseworthy et al. 2000; Sotgiu et al. 2004). There is a large body of evidence supporting that MS is an autoimmune disease, although it has been questioned if autoimmunity alone can account for the entire spectrum of different forms of MS (Chaudhuri and Behan 2004; Weiner 2004). Indeed, MS might in fact be the end result of a number of different disease processes (Ludwin 2006).

MS is characterised histopathologically by the breakdown of the blood brain barrier (BBB) and perivascular inflammation with T-lymphocytes and monocytes. B cells are probably also involved as most MS patients have enhanced antibody production in the cerebrospinal fluid (CSF) visible as oligoclonal bands upon CSF protein electrophoresis. The myelin sheath surrounding neuron axons is damaged, disrupting saltatory axonal conduction and later leading to axon loss. In areas of inflammation and demyelination reactive gliosis develops creating the typical sclerotic plaques in the brain and spinal cord that has given the disease its name (Compston and Coles 2002).

There are no spontaneous animal models of MS. However, a MS-like disease can be induced in rats or mice by immunisation with CNS white matter proteins or the transfer of T cell clones specific for myelin proteins. Common CNS proteins used include myelin basic protein (MLP), proteolipid protein (PLP) or myelin oligodendroglial glycoprotein (MOG). The resulting models are named experimental autoimmune (or allergic) encephalomyelitis (EAE). Many of them cause acute illness that is resolved over time, which reflect mainly the inflammatory aspects of MS and closely resemble

the acute relapses of MS patients. Other EAE models result in a chronic, progressive form of EAE with episodes of inflammation that lead to permanent disabilities, mirroring the demyelination, neurodegeneration and glial scar formation that also occurs in MS patients (Steinman 1999; Wekerle et al. 1994).

There is some evidence in support of a role of neovascularisation and/or endothelial-related growth factors, such as VEGF, in the pathogenesis of MS. Some histopathological features of the MS lesions resemble hypoxic damage, perhaps due to metabolic disturbances, and also MS patients display increased expression of HIF1α, a transcription factor for VEGF-A (Lassmann 2003). An elevated expression of VEGF-A has been detected in EAE and MS (Graumann et al. 2003; Kirk and Karlik 2003; Proescholdt et al. 2002; Su et al. 2006). In addition, endothelial cell proliferation and increased blood vessel density have been documented in acute MS lesions (Ludwin 2001) and EAE (Kirk and Karlik 2003). However, so far no studies on the expression of the VEGF-A splice forms or on VEGF-B and the VEGF receptors in MS or EAE have been published.

In *paper IV*, the expression of angiogenic factors were studied in a well characterised model of acute monophasic EAE. This model of EAE is induced by immunisation of Lewis rats with an encephalitogenic peptide of guinea pig MBP (gpMBP63-88). The MBP-immunised animals develop an ascending inflammation in the spinal cord with predominantly tail and hind limb paralysis. Clinical symptoms appear at around day 10-11 post-immunisation (p.i) and reach a peak at day 12. By 19 days p.i. animals are in full remission. The main features of the disease mechanisms can be summarized as follows: At initial stages adhesion molecules on CNS microvessels become up-regulated (by e.g. T cell derived IFNγ) and encephalitogenic T cells are extravasated. Subsequently, macrophages and microglia are recruited to the area of inflammation mainly around vessels (denoted perivascular infiltrates). These cells release additional inflammatory mediators, such as nitric oxide, TNF and clotting system products that contribute to BBB disrupture and oedema. Inflammatory mediators and oedema block or disturb normal nerve conduction leading to clinical symptoms in the form of paralysis. Later on, regulatory T cell activity is increased in parallel with increased local tissue expression of immunomodulatory substances, such as TGF-β. This down-regulates the proinflammatory cytokines and also induce anergy/apoptosis of disease-driving encephalitogenic Th1-T cells, which leads to resolution of the inflammation (Swanborg 2001).

# 2 AIMS OF THE STUDY

The work in this thesis stems from work on isolating candidate genes for Multiple Endocrine Neoplasia type 1 (MEN1) on chromosome 11q13. One of the isolated candidate genes was vascular endothelial growth factor B (*VRF*/*VEGF-B*), which was closely related to vascular endothelial growth factor A, but its function was unknown. Later, the *MEN1* gene was identified and screening for mutations in the *MEN1* gene is now part of clinical routine.

## SPECIFIC AIMS

1. To characterise the gene structure and expression of *VRF*/*VEGF-B* as a putative candidate gene for MEN1
2. To analyse the mutation spectrum in the *MEN1* gene and the associated clinical phenotypes in the Swedish population
3. To produce recombinant VEGF-B protein and study its function *in vitro*
4. To study the expression of VEGF-B and other angiogenesis factors in diseases that affect organs with high levels of VEGF-B such as the heart and CNS
    a. In a mouse model of heart failure
    b. In both a rat model of and patients with multiple sclerosis

# 3 MATERIAL AND METHODS

## 3.1 ANALYSING DNA

The structure of the *VRF* gene was analysed in *paper I* and methods to detect mutations in genomic DNA of the *MEN1* gene were used in *paper II*.

### 3.1.1 Characterising the structure of the *VRF/VEGF-B* gene

As part of our search for the *MEN1* gene, we had created a contig of genomic clones that spanned the *MEN1* candidate region on chromosome 11q13. A genomic cosmid clone (CLGW4) from that contig was used to screen cDNA libraries for expressed genes. The expressed genes (cDNAs) were cloned and sequenced and could be arranged in a composite sequence of the entire open reading frame of a single gene. Our gene was compared to other DNA sequences by BLAST and peptide homology searches to detect related genes and conserved protein domains. Our novel gene displayed homology to VEGF-A and was called VEGF-related factor, *VRF*.

The exon/intron borders were mapped by sequencing CLGW4 using oligonucleotide primers from the *VRF* cDNA sequence determined above. These sequences were then compared to the whole genome sequence of cCLGW4 to define the location of the exon/intron boundary. Intron sizes were determined by PCR on the genomic clone using flanking exon primers. The sizes of the amplified products (and thus the introns) were determined by electrophoresis on high percentage agarose gels. In addition, the sizes of the smaller introns were confirmed by DNA sequencing.

VEGF-A had multiple 3' splice variants. To search for similar isoforms in *VRF*, RT-PCR on cDNA from a panel of normal human tissues using primers in the VEGF homology domain and at the 3' end of the *VRF* gene was performed. PCR products were size separated by electrophoresis on a 3% agarose gel and visualised by ethidium bromide staining. An additional band that was 101bp smaller than the original *VRF* mRNA was detected and sequenced.

The structure of the *VRF/VEGF-B* gene is presented in paper I.

### 3.1.2 Mutation detection of the *MEN1* gene

Once the *MEN1* gene was isolated, mutation testing, using DNA sequencing of the coding region, could be used as a complement to clinical diagnosis. The *MEN1* gene contains 10 exons, of which exon 2-10 encode the protein, menin. Exons 2, 3 and 10 are rather large and are divided into two PCR fragments in order to obtain robust DNA sequences of the entire exon and adjacent intron sequences. DNA sequencing was performed on clinical samples referred to Clinical Genetics for mutation screening from all over Sweden. DNA was extracted from peripheral blood leukocytes. All exons were sequenced bidirectionally using fluorescently labelled terminators (or initially, labelled primers).

However, DNA sequencing has not detected mutations in all MEN1 families. For instance, larger deletions cannot be identified by DNA sequencing. A relatively new, PCR-based method for detecting larger deletions in the *MEN1* gene has therefore been used since 2006 on our clinical samples. The method, multiplex ligation-dependent probe amplification (MLPA) can detect deletions (or duplications) of one or more exons in a single allele. The principle of MLPA is shown in FIG). Two probes that each bind to exons 1, 2, 3, 7, 8, 9 and 10 of the MEN1 gene have been designed by MRC Holland. If the exon sequence complementary to the probe is present in the sample (i.e. not deleted), the two probes will hybridise, be ligated and will be amplified in the following PCR reaction. The PCR products are then size separated and analysed by capillary electrophoresis. Each PCR product has a unique size thanks to the different linkers attached to each exon-specific probe. If an exon is deleted in one allele, the total amount of PCR product from that exon will decrease by 35-50%. If only one exon is deleted, point mutations or polymorphisms in the probe binding sites must be excluded by DNA sequencing (always performed on our MEN1 patients) **(**Figure 11).

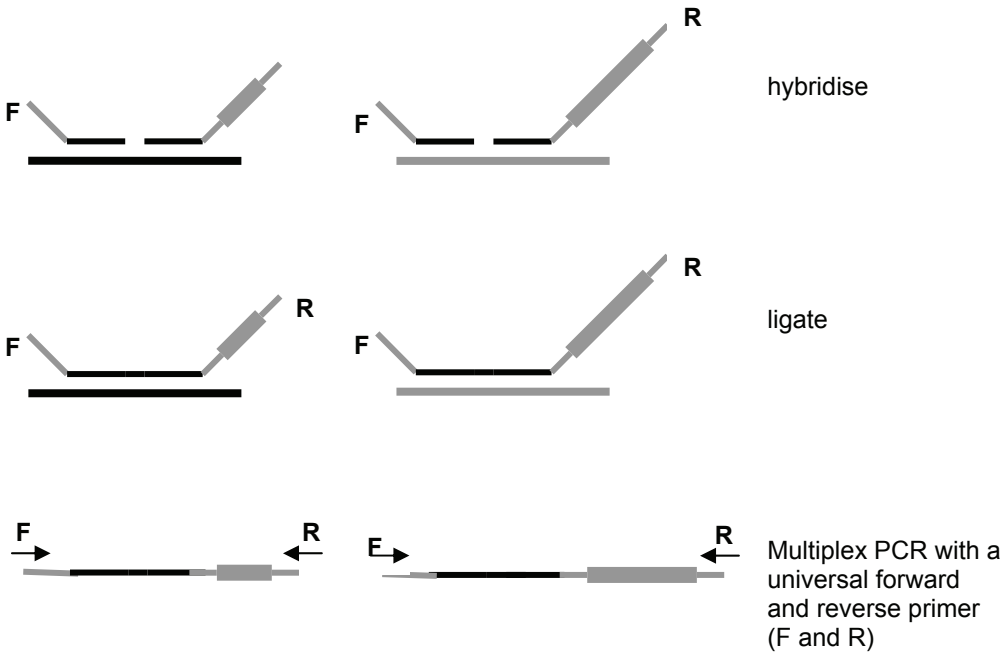


**Figure 11: MLPA**. Probes containing both unique and common primer sequences and a linker of varying length are hybridised to total DNA and will bind specifically to the region of interest. Bound probes are ligated by DNA ligase. Multiplex PCR is then performed. PCR products from each exon are of a unique length (130-480bp) and are size separated by capillary electrophoresis. The resulting bands are shown as peaks and the area under the curve is calculated. A >20% reduction in area represents a deletion of one allele. (Figure modified from MRC Holland)

### 3.1.3 Allele analysis

Patients with no known blood relationship who shared the same *MEN1* mutation may derive from a common founder. If so, they will share both the mutation and common sequence variations in the vicinity. Therefore the number of nucleotide repeats in the *MEN1* intragenic marker D11S4946 and the nearby D11S4940 were analysed in all individuals with shared mutations using PCR amplification and sequence gel electrophoresis to determine allele sizes.

## 3.2 TISSUES AND CELLS USED IN EXPRESSION STUDIES

(1) Hearts from mice with dilated cardiomyopathy (paper III). Hearts from *Tfam*-knockout mice at 3 weeks of age and from their healthy littermates were snap-frozen and then either cryosectioned or used for protein extraction.

(2) Spinal cords from rats with EAE (paper IV): Spinal cord from rats sacrificed 8, 12, 15 and 19 days after immunisation with MBP or CFA controls were removed, freed from meninges and nerve roots, and snap-frozen. RNA was isolated from the L3 segment and stored at -70°C. The L4-5 segments were cryosectioned.

(3) Blood and cerebrospinal fluid from MS patients (paper IV): Peripheral blood and cerebrospinal fluid (CSF) samples from MS patients, patients with other neurological disease (OND) and healthy controls (HC) were collected and centrifuged. The supernatant was stored at -70°C and cell pellets were lysed and total RNA was extracted. RNA samples were frozen at -80°C within three hours of sampling.

## 3.3 RNA EXPRESSION IN TISSUES

The RNA expression of the VRF/VEGF-B gene in normal tissues was analysed by Northern blot in *paper I*. The mRNA levels of VEGF-A, VEGF-B and related genes in rodent heart and spinal cord and CSF and blood samples from human patients (*paper III and IV*) were studied using both quantitative (real-time PCR) and qualitative methods (mRNA *in situ* hybridisation).

### 3.3.1 Northern blot

Northern blot detects the transcript sizes of expressed RNA in whole tissue extracts. Total mRNA was isolated from normal adrenal, pancreas, thyroid, parathyroid, kidney, fibroblasts, lymphoblastoid cell lines as well as parathyroid and pancreatic endocrine tumours, adrenocortical and medullary thyroid carcinomas. The RNA was run on formaldehyde denaturing agarose gels and blotted onto nylon membranes which were then hybridised using a radioactively labelled *VRF* cDNA clone or a control *GAPDH* probe, washed and exposed to autoradiographic film. Commercially available multiple tissue Northern blots were also used (Weber et al. 1994).

### 3.3.2 Quantitative real time PCR

Real-time PCR can be used to quantify mRNA levels of specific genes and isoforms in tissue or cell extracts. Regular PCR is not quantitative as the accumulated PCR products are measured after termination of the PCR reaction, when most products have already reached a plateau level. Real-time PCR measures the amount of fluorescent PCR product continuously throughout the PCR reaction, enabling quantification during the linear phase. The mRNA of interest is amplified with specific primers together with a

fluorescent probe which can be either specific for the gene (e.g. Taqman) or a nonspecific DNA-binding dye (SYBR Green).

The Taqman probe is designed to have a reporter fluorochrome at the 5' end and a quencher at the 3' end. As long as the probe is intact, the quencher dye extinguishes the fluorescence from the reporter by fluorescence resonance energy transfer (FRET). When the probe binds to the target sequence, downstream of the PCR primer, the reporter is cleaved off by the 5' nuclease activity of the Taq DNA polymerase as it extends the primer. The reporter is then separated from the quencher and emits its fluorescent signal. The rest of the probe is digested in a similar manner so that the PCR primer is extended to the end of the template strand. The reporter dyes are cleaved from their probes in each PCR cycle resulting in an increase in fluorescent signal that is proportional to the amount of PCR product produced. The specificity of the Taqman PCR reaction is very high as three different unique sequences need to bind to the target in order for fluorescence to be detected.

SYBR Green I binds immediately to all double stranded DNA and emits a fluorescent signal without obstructing the PCR reaction. Again, the increase in fluorescent signal is proportionate to the amount of double stranded product generated in each PCR cycle. As SYBR Green is a general dye, it is easier to optimise and use for many different genes at a lower cost. However, it is crucial that there is no contaminating DNA in the sample (can be eliminated by e.g. DNase digestion of the cDNA sample prior to use). Also, it is of utmost importance that the PCR primers are designed to be highly specific.

After termination of the PCR reaction, the amplification plot is analysed. The baseline is chosen to remove background and the threshold where all PCR products are in the linear phase is chosen (Figure 12a). The cycle number at the threshold level (Ct value) of each sample (run in duplicate or triplicate) is determined. This Ct value is then translated into amount of starting mRNA using the relative quantification method. In short, serial dilutions of a standard mRNA sample that expresses all the genes studied (in our case, concanavalin-A stimulated peripheral blood monocytic cells, PBMCs) are PCR:d on the same plate as the samples. The Ct values of the standard are plotted against the dilution factor to create a straight line, the standard curve (black dots in Figure 12b). The Ct values of the samples are then plotted onto the same curve (red dots in Figure 12b) and the relative amount of RNA in each sample can be determined using the equation of the line. If SYBR Green is used, then the specificity of the fluorescent signal is checked by analysing the dissociation curve of the fluorescent products. The dissociation of a full-size PCR fragment will occur at a higher temperature (70-80ºC) than shorter products or primer dimer (Figure 12c).

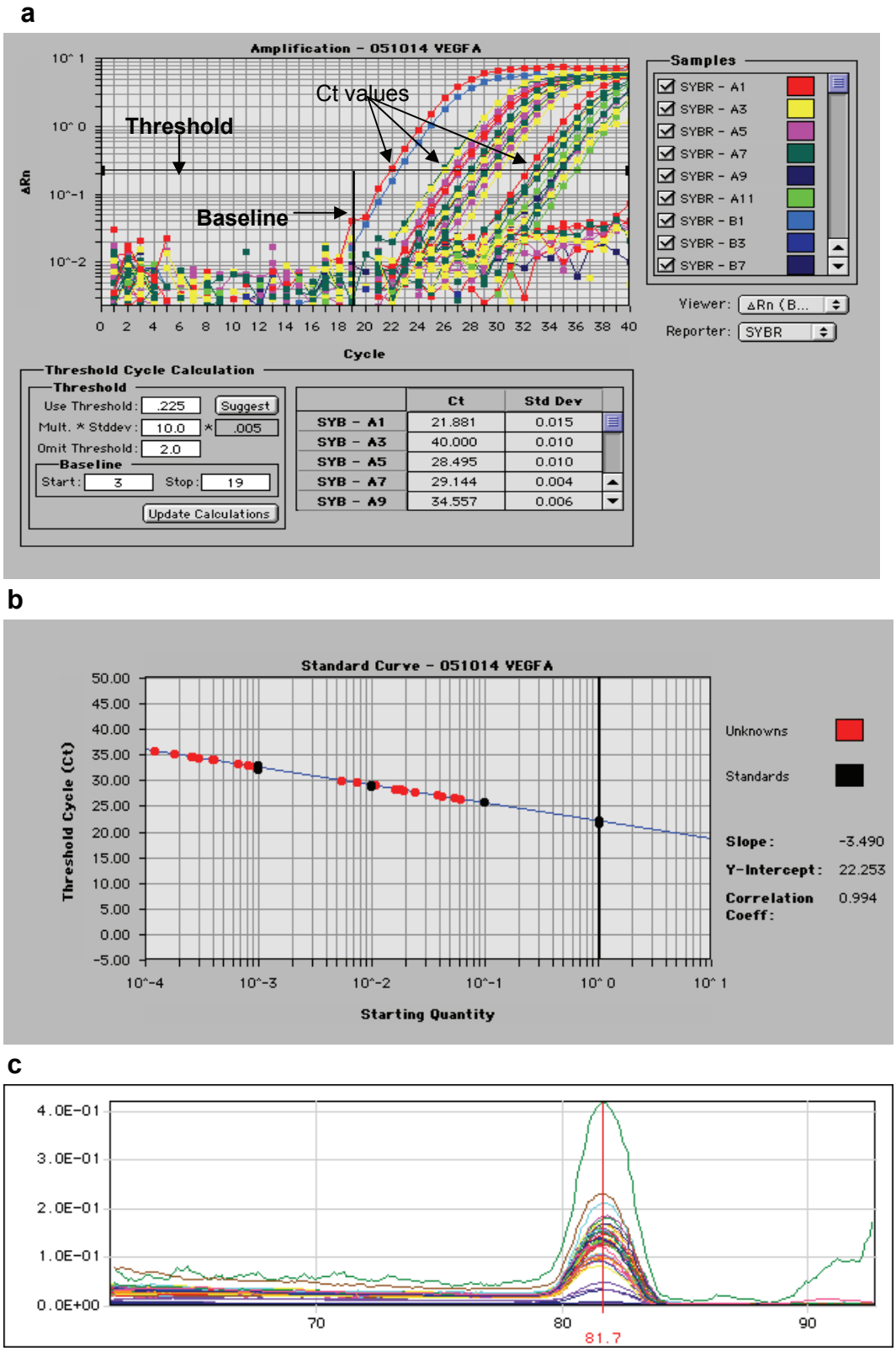


**Figure 12: Real time PCR of VEGF-A.** (a) Amplification plot of SYBR Green PCR on VEGF-A. The threshold was chosen so that all samples were in the linear phase of amplification. The baseline was set 3 cycles before the samples passed the chosen threshold. (b) The relative standard curve showing the Ct values of the serially diluted standard (in this case human concanavalin-A-stimulated PBMCs) as black dots and the Ct values of the samples as red dots. The amount of VEGF-A relative to the amount of standard can be deduced using the equation of the line. (c) For SYBR Green PCR products, the dissociation curves were determined to exclude contamination of e.g. primer dimer. All PCR products had a melting point of 81.7ºC, confirming that they were full-size VEGF-A products. The well with an increase in signal above 90ºC (green line) was excluded from the analysis.

### 3.3.3 mRNA *in situ* hybridisation

mRNA *in situ* hybridisation (ISH) is a technique that allows detection of transcripts in individual cells in a tissue and can thus give valuable information on cell-specific expression patterns. ISH can be performed using in principle, four different kinds of probes: oligonucleotides (40bp); RNA probes/riboprobes (generated by *in vitro* transcription); single stranded DNA probes (200-500bp); or double stranded DNA probes, although the first two are the most common. Oligonucleotide probes are cheap to produce, stable and single-stranded. Their G/C content can be standardised, enabling one protocol for many different probes. As they are small, approximately 40 nucleotides, they can easily penetrate into the tissue. However, oligo probes are end-labelled and thus fewer labelled nucleotides are incorporated per probe. Therefore oligo probes are less sensitive than longer nucleic acid probes and do not work well on paraffin-embedded tissues. Riboprobes have the advantage that RNA:RNA hybrids are resistant to digestion by RNAses that can therefore be used to remove non-hybridised probes that would otherwise give background signal. However, riboprobes are more difficult to produce and the sensitivity of RNA to RNAses during synthesis, makes them difficult to work with. Riboprobes can be labelled along the length of the entire probe and give a much stronger signal than oligo probes and have been used successfully on paraffin-embedded tissues. However, if they are too large, their tissue penetration is reduced and different steps to increase penetration (such as protease digestion) must be employed. Single-stranded DNA probes require expensive, time-consuming preparation and are rarely used. Double-stranded DNA probes are also rarely used today due to the tendency of the two DNA strands to re-hybridise to each other (Wilkinson 1998; www.genedetect.com).

Oligonucleotide ISH was performed on tissues from mouse heart (*paper III*) and rat spinal cord (*paper IV*). Gene-specific, 40 nt long oligonucleotide probes were designed and tested for specificity *in silico* and on a panel of normal mouse tissues (Figure 13). As each oligo has a similar GC content and a similar length, their unique binding pattern constitutes a control for non-specific binding (Figure 13). The rodent tissues were frozen at -70°C, sectioned at -20°C and fixed in acetic anhydride in acetone (acetylates the tissue and decreases background). The oligonucleotide probes were radioactively end-labelled with $^{35}$S-dATP using terminal deoxynucleotidyl transferase (TdT). The hybridisation mix contained blocking agents (e.g. yeast tRNA, salmon sperm DNA), volume contractors (e.g. polyvinyl-pyrrolidone and dextran sulphate) and agents to reduce background (e.g. dithiothreitol, DTT). Hybridisation was performed at 42°C overnight. The slides were then washed in 1x SSC buffer at 60°C, dehydrated and then exposed to autoradiographic film before being dipped in autoradiographic emulsion. The radioactivity causes the transformation of silver halide to metallic silver grains closely scattered around the radioactive source. After developing and fixing the slides, the silver grains can be visualised by bright field (grains appearing black) or dark field (grains appearing bright) microscopy. In order to semi-quantitate the number of silver grains (*paper III*), we analysed the slides using the NIH Image 1.55VDM programme that measures the pixel intensity of the silver grains. This measurement correlates well to the true number of silver grains (Piehl et al. 1995).

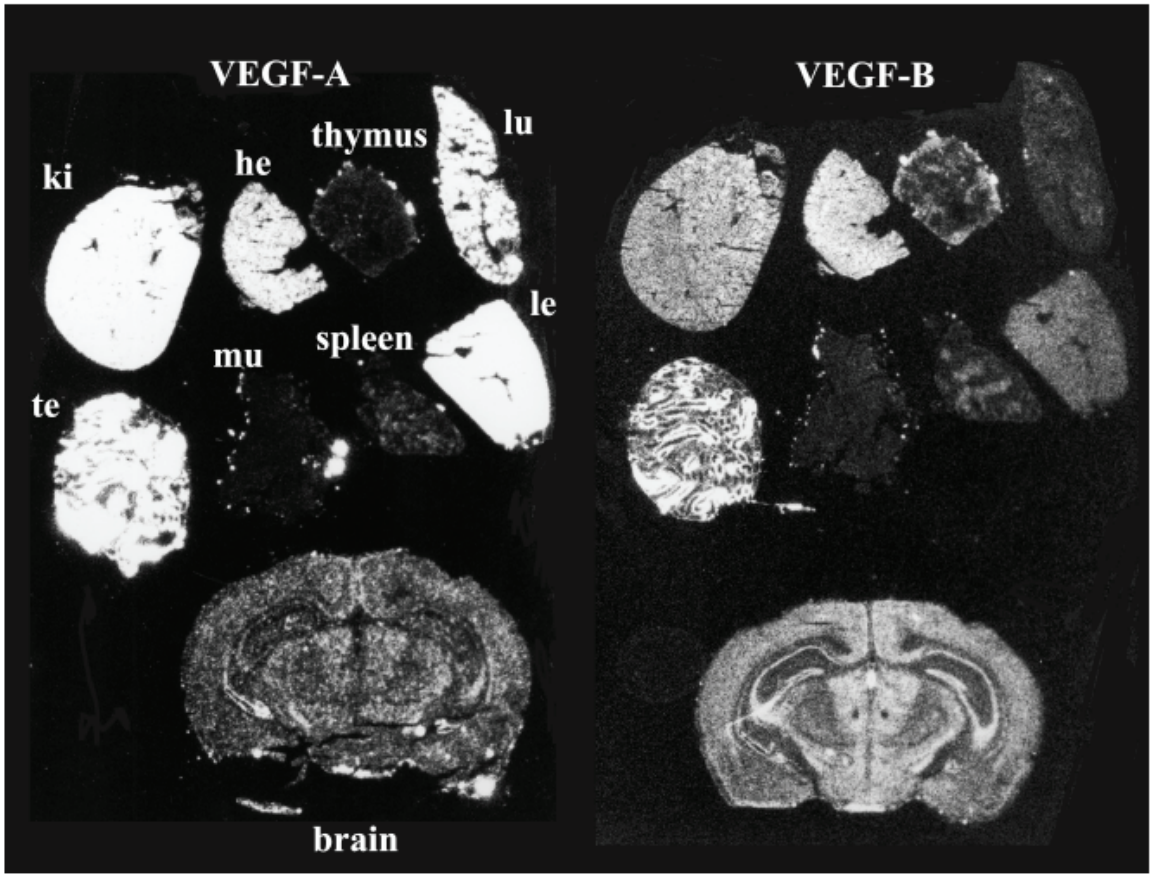


**Figure 13: A photograph of an autoradiographic film showing mRNA in situ hybridisation on a panel of normal mouse tissues using an oligonucleotide against VEGF-A or VEGF-B.** Each oligonucleotide probe had a specific labelling pattern in mouse tissues and they could therefore function as internal controls for binding specificity, due to their similarities in size and GC-content. Ki=kidney, he=heart, lu=lung, te=testis, mu=skeletal muscle, le = liver. VEGF-A was highly expressed (white signal) in kidney, heart, lung, testis and liver, while VEGF-B was highly expressed in heart, testis and brain.

## 3.4 PROTEIN EXPRESSION IN TISSUES

The expression of VEGF-A and VEGF-B protein was studied in rodent heart and spinal cord in *paper III and IV*. In addition, we also used Western blot to analyse recombinant VEGF-B produced *in vitro* (see section on Recombinant protein).

### 3.4.1 Western blot

Western blot detects the sizes of different protein species in whole tissue and can be used to semi-quantify them. Tissue protein was extracted from COS-1 or HEK293 cells in cell culture (*unpublished data*) or mouse heart (*paper III*), heated and loaded onto a SDS-PAGE gel in a buffer containing SDS and DTT (or without DTT for non-reducing conditions). After size separation, the proteins were electro-blotted onto a nitrocellulose membrane. The membrane was stained using a general protein dye, Ponceau S and scanned into the computer to use as a loading control. The membrane was then blocked in non-fat milk and incubated with primary antibody against the protein of interest (VEGF-B or VEGF-A) overnight. The secondary antibody, labelled with horseradish

peroxidase (HRP) was incubated at room temperature for 2h. After washing, the proteins were detected by adding a solution of hydrogen peroxide and luminol to the blot. The latter is oxidised by HRP and the excess energy is released as chemiluminescence in a process termed enhanced chemiluminescence (ECL). The resulting signal was measured by a CCD camera and could be quantified using Image Gauge software. Either the ratio of ECL and amount of protein present as determined by ponceau S staining was calculated. Alternatively, to quantify purified recombinant VEGF-B (rVEGF-B) which could not be visualised by ponceau staining, the ECL signal generated by known concentrations of VEGF-B from baculovirus cell extracts (Weber G and Tvrdik T, unpublished data) was compared to that from rVEGF-B loaded on the same gel. For quantitative analysis, the detection of chemiluminescence by a CCD camera is by far superior to autoradiography. Still, ECL is an enzymatic process and as such only linear over a limited range, by our experience at less than a magnitude of difference.

### 3.4.2 ELISA

Another method for detection of whole tissue protein is ELISA, Enzyme-Linked Immunosorbent Assay. ELISA provides a robust method of specific protein quantification. ELISA was used to detect VEGF-A in cerebrospinal fluid in MS patients (paper *IV*). The principle is as follows: a monoclonal antibody against VEGF-A is pre-coated onto a 96-well plate. Samples and serial dilutions of known concentrations of recombinant VEGF-A protein (the standard) are added to the wells and bind to the immobilised antibody. Unbound substances are washed away and then a polyclonal, HRP-conjugated antibody specific for VEGF-A is added. After binding and washing, a substrate solution of hydrogen peroxide and tetramethylbenzidine is added and the buffer turns blue in proportion to the amount of VEGF-A bound in the initial step. The colour development is stopped and the optical density (OD) is measured. The amount of VEGF-A protein in the samples can be deduced by comparing their OD measurements to those of serial dilutions of the standard. ELISA is a very sensitive method and can detect as little as 5pg/ml of VEGF-A protein.

### 3.4.3 Immunohistochemistry

Immunohistochemistry detects cell-specific expression of protein and also allows for double staining experiments to confirm the co-localisation of two proteins. Frozen rodent tissue sections (*paper III and IV*) were fixed in acetone and methanol or paraformaldehyde. The tissues slides may be incubated in blocking agents (serum, bovine serum albumin, BSA or blocking agent from NEN Life Sciences) to block non-specific binding of the antibodies. The slides are then incubated overnight at 4°C with the primary antibody (e.g. goat anti-VEGF-) in phosphate-buffered saline (PBS) with or without blocking agents and then with the secondary antibody (e.g. donkey anti-goat) for 45 minutes at 37°C or two hours at room temperature. The secondary antibodies can either be detected directly or further amplified (Figure 14). Secondary antibodies that were directly labelled with a fluorophore (Figure 14a) are detected by fluorescent microscopy. In ABC amplification (Figure 14b), the secondary antibody is biotinylated and then binds a pre-formed avidin:biotinylated HRP complex (ABC), resulting in multiple copies of HRP in the vicinity of the antibody. Each HRP molecule catalyses the precipitation of diaminobenzidine tetrahydrochloride (DAB) resulting in a brown

product. In tyramide signal amplification (TSA) (Figure 14c), the secondary antibody is labelled with HRP which oxidises tyramide derivatives which then bind covalently to nucleophilic residues in the proteins in the immediate vicinity of the antibody. The tyramide can be labelled with a fluorophore allowing immediate visualisation of the signal. As each HRP can oxidise many tyramide molecules, the detection signal is amplified up to 1000 times.

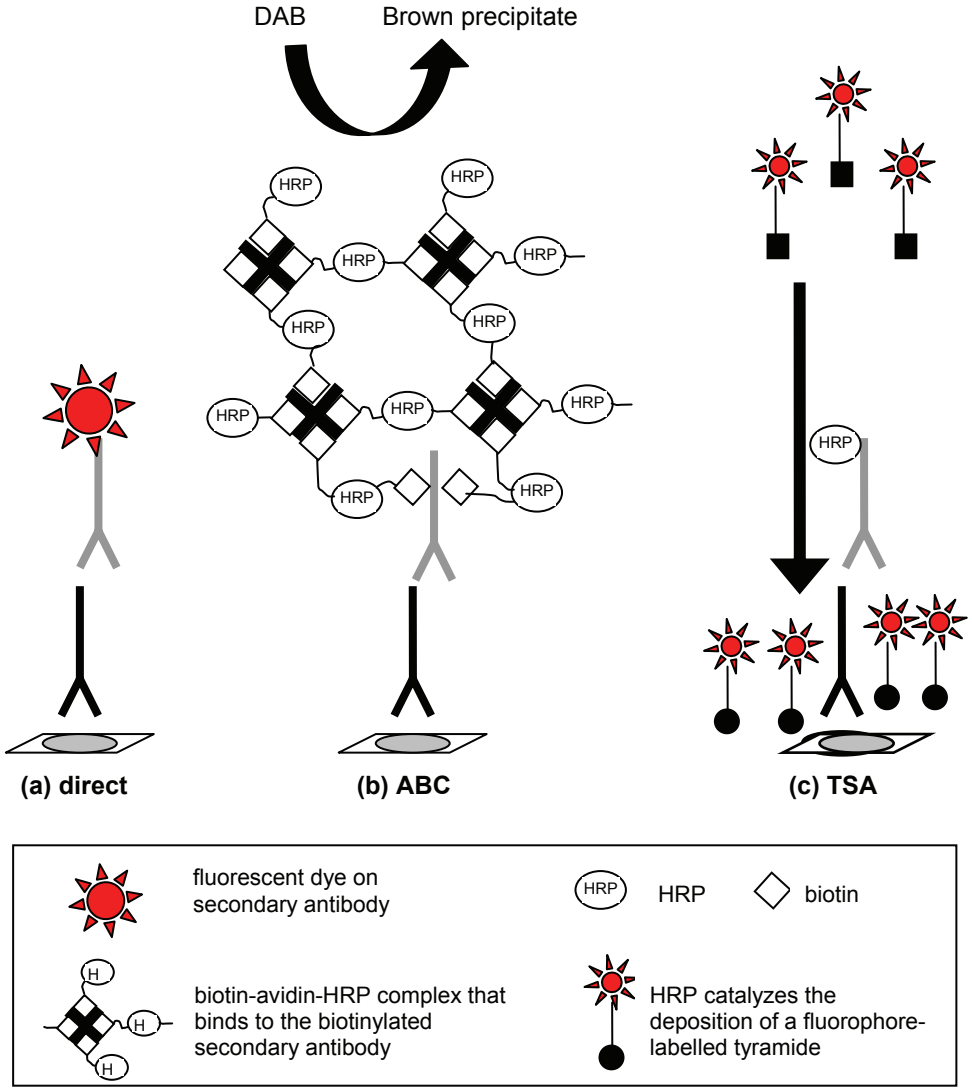


**Figure 14: Different immunohistochemical techniques.** (a) Primary antibody (black) binds to the protein of interest on tissue slide. Secondary antibody (grey) is labelled with a fluorescent dye and signal is detected in a fluorescent microscope. (b) ABC technique. The secondary antibody is labelled with biotin. A preformed complex of avidin, biotin and HRP is then added. Each secondary antibody is thus labelled with many HRP molecules. HRP then catalyses the precipitation of DAB. (c) TSA technique. The secondary antibody is labelled with HRP that catalyses the deposition of multiple tyramide-fluorophore complexes around the antibody.
(Figure adapted from http://probes.invitrogen.com/handbook/figures/1536.html and http://www.piercenet.com: Protein Detection > Immunohistochemistry)

## 3.5 RECOMBINANT PROTEIN

In order to study the function of the newly isolated *VEGF-B* gene, two different cell systems (bacteria and mammalian cells) were used to produce recombinant VEGF-B protein. The aim was to produce active VEGF-B dimers and then to test their function in angiogenesis and inflammation assays.

### 3.5.1 Prokaryotic systems (bacterial cells)

#### *3.5.1.1 Materials*

##### *3.5.1.1.1 Vectors and cells*

In order to express VEGF-B in bacteria, the gene must be cloned into an appropriate expression vector. Two different constructs, one for each splice form were used. They had been previously created by *in vitro* mutagenesis for baculovirus cell expression (Weber G and Tvrdik T, unpublished data). Two vectors for prokaryotic cell expression were used. One created a fusion protein of VEGF-B and thioredoxin with the aim of creating a soluble protein (pthioHis, Invitrogen, NV Leek, The Netherlands). This thioredoxin contained a His-patch in its tertiary structure and could therefore be used for purification with metal affinity chromatography. Later six extra histidine residues (a His-tag) were added to increase the protein's affinity for nickel in the purification process (see below). In addition, a vector with a signal sequence for secretion to the periplasmic space was used, with the aim of simplifying the purification procedure (pET21b, Novagen, Merck KGaA, Darmstadt, Germany). This vector also has lower basal production of recombinant protein and thus may prevent unwanted degradation or inclusion body formation.

Two bacterial strains recommended for use in production of recombinant protein were used: JM109 (Stratagene, La Jolla, CA) and BL21 Gold (Novagen). They lack endonucleases (gives improved plasmid yield) and *hsd* restriction enzymes that may cleave recombinant plasmid. BL21 Gold also lacks two proteases that might degrade eukaryotic proteins expressed in the cell and transcription of the inserted gene is under the rigid control of IPTG-stimulated T7 RNA polymerase.

#### *3.5.1.2 Methods*

##### *3.5.1.2.1 Cloning*

The bacteria (JM109 or BL21 Gold) were transformed by electroporation or heat shock respectively and cultured first in tubes and then on agar plates with appropriate antibiotics for selection. Individual clones were picked, DNA was extracted and checked for the presence of inserted VEGF-B by restriction digestion and DNA sequencing. Positive clones were expanded in culture.

##### *3.5.1.2.2 Production of recombinant protein*

Production of recombinant protein by the bacteria was induced by IPTG (isopropyl-b-D-thiogalactopyranoside) which removes the lac-repressor from the lac operon enabling transcription of the inserted gene. The bacteria were harvested by centrifuging and the cell pellet was lysed. If the recombinant protein was soluble in the bacterial cytoplasm, cell lysis using lysozyme and homogenisation (native conditions) was sufficient. If

however, the protein was found in insoluble inclusion bodies, then cell lysis under denaturing conditions (e.g. 8M urea) was required to solubilise the protein (Figure 15). In an attempt to increase the soluble fraction of VEGF-B we decreased the culture temperature to 30°, lowered the IPTG concentration and tested various induction times in JM109 and BL21 bacteria.

*3.5.1.2.3 Purification of recombinant protein*

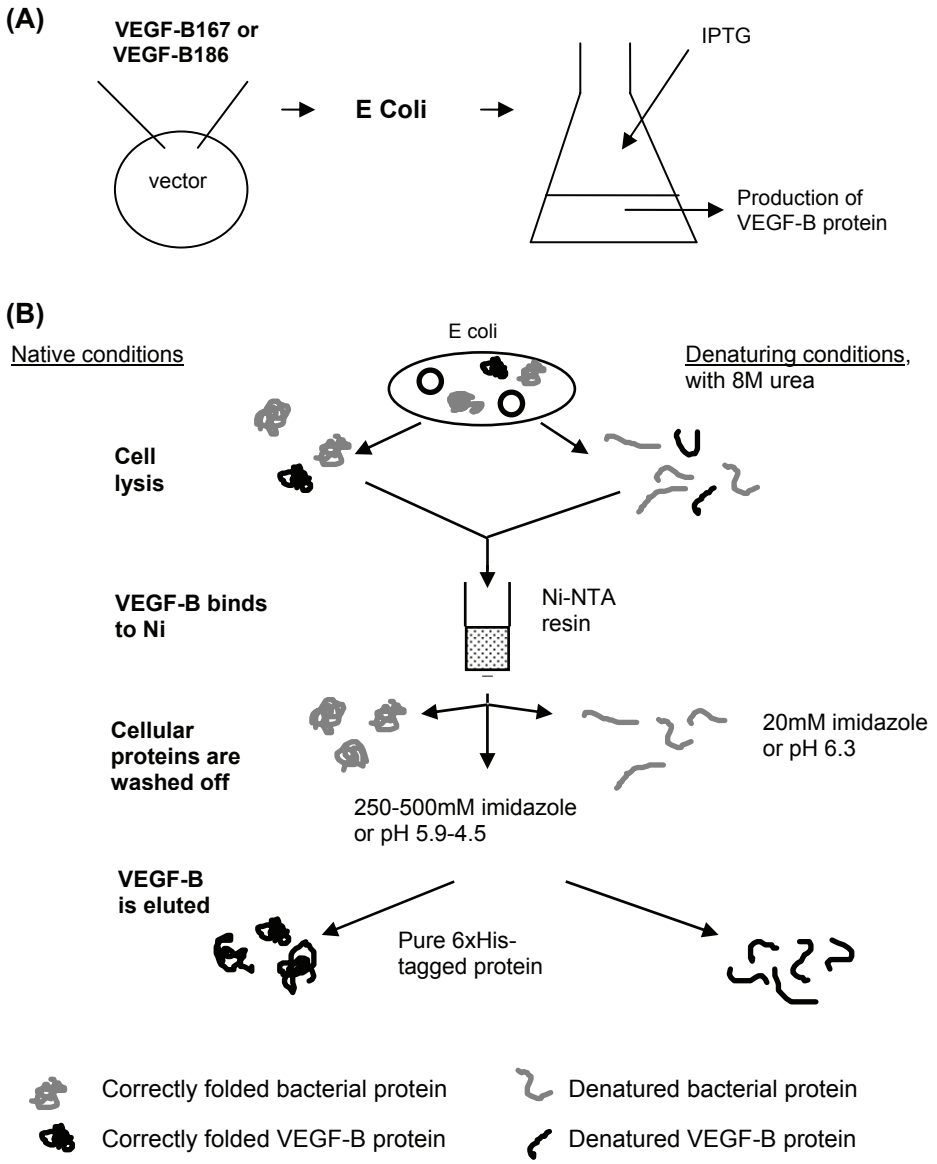


**Figure 15: Expression and purification of protein from prokaryotic systems. (**A) VEGF-B is cloned into an expression vector, bacteria are transformed and cultured. IPTG is then used to induce production of VEGF-B. (B) Protein purification: The bacterial cells are lysed using either native conditions, left, or, if the protein is found in inclusion bodies, denaturing conditions, right. The protein extracts bind to the nickel-NTA affinity columns, bacterial proteins are washed off and VEGF-B is eluted using increasing concentrations of imidazole or decreasing pH.
(Modified from the QIAexpressionist handbook).

Nickel-affinity chromatography was chosen as the first purification step (Qiagen, Hilden, Germany). Nickel (Ni) binds to stretches of histidine residues (his-tag) in the protein, immobilising it on the column. The column is washed and then the protein is

eluted using either increasing concentrations of imidazole, which competes for Ni binding or decreasing the pH which protonates His-residues and prevents them from binding to Ni (Figure 15).

VEGF-B functions as a homodimer, which the bacteria could not produce. Several protocols for *in vitro* dimerisation were tested. Dimerisation requires a folding buffer containing various amounts of salt, buffer, stabilising agents (e.g. glycerol or HEPES) and solubilising agents (detergents, glycine). As VEGF-B contains disulphide bonds, reducing and oxidising substances such as glutathione were also included. There are two schools of thought regarding dimerisation: either the dilution into the folding buffer should be performed rapidly or slowly (i.e. dropwise or by dialysis) both methods were employed.

##### *3.5.1.2.4 Detection of recombinant VEGF-B protein*

In order to detect VEGF-B protein, Western blotting was performed as described above. The following antibodies were used: anti-VEGF-B; initially C-19 and N-19 both from Santa Cruz, (Santa Cruz, CA), later anti-VEGFB167/186 from R&D Systems (Oxford, UK). Anti-VEGF-A; A147, and A20, both from Santa Cruz were used to check for contaminating VEGF-A production. The SDS-PAGE gels were also stained by silver staining for detection of total protein in elution fractions (BioRad, Hercules, CA). Protein concentrations were determined using the Bradford method of protein concentration with BSA as a standard (Biorad).

### 3.5.2 Eukaryotic (mammalian) systems

Recombinant VEGF-B was also produced in two different vector systems using both transient transfection and permanent infection of two different mammalian cell lines.

#### *3.5.2.1 Materials*

##### *3.5.2.1.1 Cell lines*

Two different mammalian cell lines were used: (1) COS-1, derived from an African green monkey kidney cell line. These cells were established from CV-1 Simian cells (*Cercopithecus aethiops*) which were transformed by an origin-defective mutant of SV-40 (European Collection of Cell Cultures, ECACC). Thus these cells support the extrachromosomal propagation of vectors with SV40 origins of replication. (2) HEK293 cells which are human embryonic kidney cells transformed with sheared human adenovirus 5 DNA (ECACC).

##### *3.5.2.1.2 Vectors*

VEGF-B167 and VEGF-B186 were cloned with a eukaryotic translation initiation site (ACCATGA**)** and a STOP codon in addition to a His-tag into two different vectors for transient expression in COS-1 cells: (1) pREP8 (Invitrogen) – with an Epstein-Barr Virus origin of replication and nuclear antigen, enabling extrachromosomal replication in a wide variety of mammalian cell types and the Rous Sarcoma Virus long terminal repeat enhancer/promoter for high level transcription of recombinant genes; (2) pcDNA3.1 (Invitrogen) – with extrachromosomal replication in cells that express SV40 large T antigen such as COS-1 cells and human Cytomegalovirus immediate early

promoter for high expression of recombinant genes. In addition, VEGF-B clones in pEF-BOS (provided by N. Hayward, Queensland Institute of Medical Research, Australia) were also used. pEF-BOS also has a SV40 origin of replication, but uses the human polypeptide chain elongation factor 1α promoter for high levels of recombinant gene expression (Mizushima and Nagata 1990).

#### *3.5.2.1.3 Retrovirus in HEK293 cells*

A retrovirus vector was used for permanent expression of VEGF-B in HEK293 cells. The retroviral vector, pCMMP-GFP, contained VEGF-B167 (cloned by Filip Farnebo, Department of Cell and Molecular Biology, Karolinska Institutet). The virus is a derivative of the Moloney murine leukaemia virus. All virus genes required for replication and infection have been replaced by the insert, thus this virus can only infect once and can not propagate itself unless additional genes are provided by a special packaging cell line. pCMMP can infect actively dividing cells, is reverse transcribed and incorporated into the cell's genome where it makes use of cellular genes to express its inserts: VEGF-B167 and green fluorescent protein, GFP, expressed from the same transcript with an internal ribosomal entry site, IRES, in front of GFP (Ory et al. 1996).

### *3.5.2.2 Methods*

#### *3.5.2.2.1 Transfection, infection and protein production*

Transfection by electroporation was compared with three different cationic lipid-based transfection methods, reported to be relatively easy to use and able to transfect a variety of cell types. Fugene 6 (Roche Diagnostics, Basel, Switzerland) is a multi-lipid mix in 80% ethanol reported to be non-toxic and easy to use. Superfect (Qiagen) is a sphere of activated-dendrimer molecules that radiate from a central core and terminate at charged amino groups which can then interact with negatively charged phosphate groups of nucleic acids. Lipofectin (Invitrogen) is a liposome formation of two lipids in water. COS-1 cells were transfected using these four methods with a β-galactosidase-expressing vector, were stained blue using Xgal and counted.

The most efficient method (Fugene) was then used to transfect COS-1 cells with VEGF-B in pREP8, pcDNA and pEF-BOS. Transfected COS-1 cells were cultured in Dulbecco's modified eagle medium (DMEM) in 10% foetal calf serum (FCS). Before harvesting medium samples, they were incubated in DMEM with either 1% FCS or 1% BSA. In some cases, we also added heparin to the culture medium at final concentrations of 1-100μg/ml.

Human embryonic kidney cells (HEK293) were infected with our retroviral construct and polybrene (Sigma-Aldrich, St Louis, MO) while in active cell division (50-60% confluence). The infected cells become green fluorescent due to the simultaneous production of VEGF-B and GFP from the same transcript. GFP-expressing cells could therefore be selected using a flow activated cell sorter (FACS). To further increase the protein yield, various culture media (DMEM, Optimem, SFM-media) were tested and the surface area of the culture flasks was increased using Cytodex-3 beads (Amersham Biosciences, Piscataway, NJ).

#### *3.5.2.2.2 Protein purification, detection and quantification*

VEGF-B protein was purified by metal-affinity chromatography as outlined above. For COS cell protein, heparin sepharose affinity chromatography (HiTrap, Amersham Biosciences) was also used. Conditioned serum-free medium containing VEGF- protein was adjusted to appropriate salt concentrations and 10mM β–mercaptoethanol (a reducing agent) was added before applying the samples to the heparin sepharose column. VEGF-B was eluted with increasing NaCl concentrations.

Retroviral VEGF-B167 was purified on nickel beads, with the addition of up to 2mM vitamin C and 10μg/ml reduced glutathione in the culture medium and purification buffers. Excess imidazole and salt was removed from nickel-bead elution fractions using G-25 sephadex columns (Amersham Biosciences) and VEGF-B167 was eluted in PBS with 1-2mM vitamin C for use in proliferation or migration assays.

#### *3.5.2.2.3 Testing the function of recombinant protein*

The function of purified VEGF-B167 produced by pCMMP-infected HEK293 cells was tested in both proliferation and migration assays.

##### *3.5.2.2.4 Proliferation assays*

Kaposi's sarcoma (KS) is an angiogenic proliferative lesion that derives from endothelial cell precursors and often develops in immunosuppressed individuals (e.g. AIDS patients). KS is associated with infection by human herpes virus 8 (HHV8) (Antman and Chang 2000). HHV8 infected endothelial cells and KS lesions express VEGFR1 (Carroll et al. 2004; Masood et al. 1997; Masood et al. 2002; Naranatt et al. 2004). For proliferation assays, an immortal cell line from a KS lesion (KS-IMM) (Albini et al. 1997) was used. In collaboration with Sergiu Bogdan-Catrina, (Department of Molecular Medicine and Surgery, Endocrinology unit, Karolinska Institutet), the effect of VEGF-B167 on proliferation of KS-IMM cells was assayed using both thymidine incorporation and the MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) which measure DNA synthesis and cell viability respectively.

##### *3.5.2.2.5 Migration assays*

To test the effect of VEGF-B on cell migration, an acute monocytic leukaemia cell line, THP-1 (ECACC, Salisbury, UK), which has been shown to be activated via VEGFR1 (Selvaraj et al. 2003) was used. Migration assays were based on a modified Boyden chamber assay with two cell culture compartments separated by a membrane. On one side of the membrane, the cells of interest (THP-1) were placed in serum-free medium. On the other side, the factor of interest was placed. This was either VEGF-B; a positive control, VEGF-A or FCS; or a negative control, GFP or unconditioned medium. The cells were left to migrate for 3h at 37°C. Then the inner chambers with the remaining cells were removed and the number of cells in the lower chamber was manually counted (Figure 16). Similar experiments were performed in a pilot experiment on primary monocytes in collaboration with Gezahegn Gorfu, Department of Odontology, Karolinska Institutet).

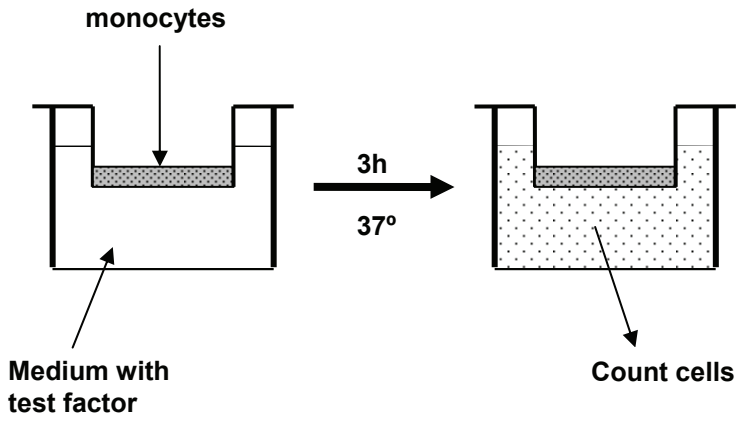


**Figure 16: Monocyte migration assay.** Monocytes are placed in the upper chamber (the transwell). Various concentrations of VEGF-B167, GFP, VEGF-A, FCS or no factor at all were added to the lower chamber. The cells were incubated for 3h at 37ºC. The transwell was removed and the cells in the lower chamber were counted.

# 4 RESULTS AND DISCUSSION

## 4.1 CLONING THE *VRF/VEGF-B* GENE

### Cloning and characterisation of a novel human gene related to vascular endothelial growth factor (Paper I)

With the aim of finding additional candidate genes for MEN1 on chromosome 11q13, a cosmid clone, CLGW4, located in the candidate region, was used to screen cDNA libraries for expressed sequences. A novel MEN1 candidate gene was discovered and it received the name Vascular endothelial growth factor- Related Factor, *VRF*, later renamed *VEGF-B*. The cDNA clones were sequenced revealed a 621bp open reading frame (ORF), 412bp of the 3' untranslated region (UTR) and 60bp of the 5' UTR. RT-PCR on cDNA from human foetal brain and renal cell carcinoma detected a splice form that was 101bp shorter and introduced a frameshift within the ORF resulting in a completely different 3' end. The two splice forms were named *VRF*-167 and 186, in keeping with the standard VEGF nomenclature (Houck et al. 1991; Tischer et al. 1991). *VRF*167 comprised exons 1-5 and 7-8 with a stop codon at position 189 in exon 8. *VRF*186 comprised exons 1-5 and 6-8 with a different reading frame and a stop codon at position 208 in exon 7 (Figure 17). VRF167 protein was 48% identical and 69% similar to *VEGF-A*, while *VRF*186 with its new C-terminus only had 32% identity and 49% similarity to *VEGF-A*. The exon/intron borders were in the same location as *VEGF-A*, except for exon 6 which is contiguous with exon 7 and probably represents an incorporated intronic sequence.

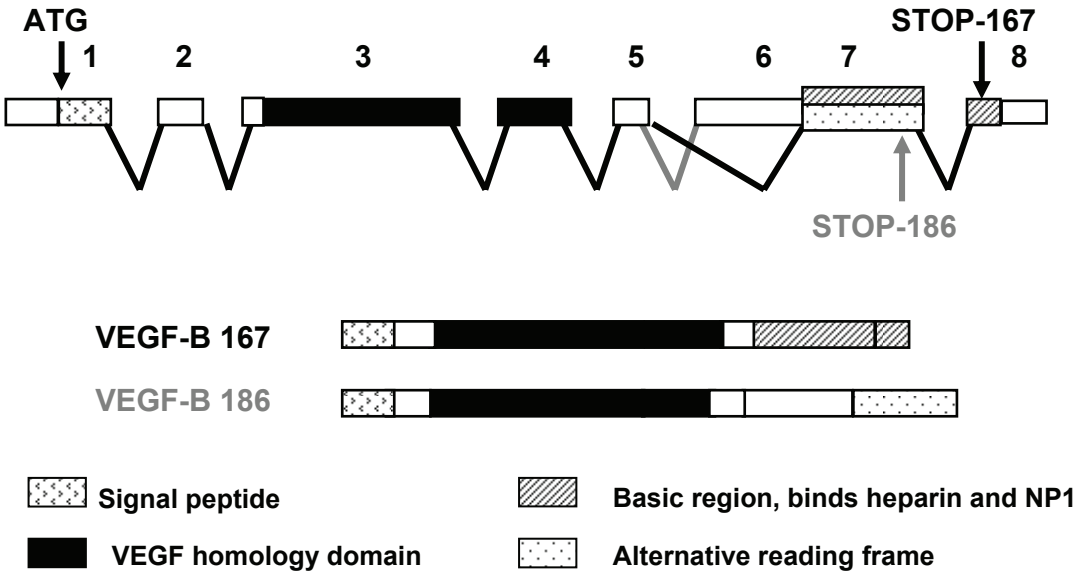


**Figure 17: The two splice forms of VEGF-B.** VEGF-B167 is encoded by exons 1-5 and 7-8. VEGF-B186 is created by alternative splicing which changes the open reading frame, resulting in a completely different C-terminal.

Both *VRF* isoforms contained the eight cysteines conserved among *VEGFs* and platelet-derived growth factors (in the VEGF homology domain), while VRF167 had an additional eight cysteine residues also found in the C-terminal end of *VEGF-A*, suggesting that the tertiary structure of all these proteins is very similar. The heparin binding domain in exon 6a of *VEGF-A* (Tischer et al. 1991) was not conserved, but VRF167 did contain a region similar to the basic domain in VEGF-A exon 7. *VRF* was ubiquitously expressed in 20 normal human tissues on Northern blot and both splice forms were present in all tissues examined by RT-PCR. *VRF* was also expressed at normal levels in non-endocrine tumours on Northern blot and was decreased by 50% in the two endocrine tumours that had loss of one chromosome 11.

In conclusion a new member of the VEGF family with two splice forms and unknown function has been isolated. Although *VRF/VEGF-B* was subsequently excluded as the *MEN1* gene due to changes in the genetic map over the region, we were interested in studying its function as a novel angiogenic factor.

## 4.2 TESTING FOR *MEN1* MUTATIONS IN SWEDEN.

### Clinical testing for mutations in the multiple endocrine neoplasia type 1 gene in Sweden, a report on 202 unrelated cases (Paper II)

The *MEN1* gene was cloned in 1997 (Chandrasekharappa et al. 1997; Lemmens et al. 1997b). Since then, a total of 370 blood samples have been sent to the Department of Clinical Genetics at Karolinska University Hospital for mutation testing, but the results of these tests have not been systematically analysed. In this paper, the mutation spectrum in the *MEN1* gene and the associated clinical symptoms of the Swedish MEN1 patients are studied. Mutation analysis was performed on DNA from peripheral blood leukocytes and clinical data was provided by the referring physician.

Of the 370 patients, there were 202 index cases. Forty-five of them shared thirty-seven independent mutations. 70% of the mutations resulted in a truncated or absent protein. Two altered a splice site, two were complex (one double missense and one a nucleotide substitution and deletion). Ten were missense mutations. Nineteen of the 37 mutations have not been previously reported.

As fifteen probands had seven mutations in common, allele analysis using two microsatellite markers within and adjacent to the *MEN1* gene was performed. Eight probands shared alleles suggesting a common founder, while the remaining probands did not share alleles and thus represent independent mutations (two nonsense, one frameshift deletion and one frameshift insertion). If the individuals with shared alleles are considered to be members of the same extended families, then in all, 41 unrelated index cases shared 37 different mutations.

Twenty-two index cases belonged to MEN1 families with at least two first degree relatives with clinically diagnosed MEN1 or *MEN1* gene mutations. Of these, 18 had a mutation detected by DNA sequencing of the coding region of *MEN1*. The four remaining families were linked to the *MEN1* locus and two had large deletions detected

by MLPA (=5% of all mutations). However, mutations have still not been found in two MEN1 families (9%). Five of eight families with familial isolated hyperparathyroidism (FIHP) had *MEN1* mutations. Additional typical MEN1 lesions later developed in two of these families, suggesting that FIHP may be a variant of MEN1 with reduced penetrance of lesions other than hyperparathyroidism (Pannett et al. 2003).

MEN1 is defined clinically by the existence of tumours in at least two of the following glands: parathyroid (HPT), endocrine pancreas and duodenum (EPT) or anterior pituitary (PIT). Of the patients who fulfilled these criteria, 68% of those with a first degree relative with MEN1 or HPT, but only 13% of the sporadic cases had *MEN1* mutations. These frequencies are similar to those found in other clinical screening reports (Cardinal et al. 2005; Ellard et al. 2005; Klein et al. 2005). All patients with a detected mutation had 1-3 typical MEN1 lesions in combination with a first degree relative with MEN1 or hyperparathyroidism, and/or an age of onset before 30 years and/or multiple tumours, in other words, fulfilled the criteria for hereditary cancer. A number of patients screened for mutations did not fulfil these criteria and had no detectable *MEN1* mutations.

Nineteen percent of the presymptomatic relatives to MEN1 probands had *MEN1* mutations, which is lower than the expected 50% as many of those who had inherited the mutation had already developed symptoms. Some of these symptomatic relatives were also tested and 100% of those with at least one confirmed MEN1 typical lesion had *MEN1* mutations.

Ninety-nine percent of the patients with MEN1 had hyperparathyroidism, 49% had entero-pancreatic tumours, 36% had pituitary tumours, 14% had carcinoids and 7% had reported adrenal gland enlargement. The youngest affected individual (an index case) was operated for insulinoma at 13 years of age, which lends support to the current consensus screening recommendations to initiate biochemical screening from the age of five years (Brandi et al. 2001). The oldest patient found to have a *MEN1* mutation was operated for EPT at 71 years, thus the clinical penetrance was 100% by the seventh decade, although prospective screening studies have demonstrated biochemical abnormalities in all *MEN1* mutation carriers before the age of 30 years (Skogseid et al. 1991).

The patients with clinical MEN1 were divided into two groups, those with detected *MEN1* mutations and those without (excluding the two families who were linked to the *MEN1* locus). The patients with mutations were younger at their first operation, had a higher frequency of EPT and more often glucagonoma. They also had a higher incidence of prolactinoma. Conversely, the patients without a *MEN1* mutation were more likely to have growth hormone (GH)-producing pituitary tumours. In fact the random combination of hyperparathyroidism and GH-producing pituitary tumours, has been shown to occur as a phenocopy to MEN1 (Burgess et al. 2000; Hai et al. 2000; Stock et al. 1997).

A few of the mutations seemed to be associated with a higher frequency of glucagonoma, bronchial or thymic carcinoid or paraganglioma. However, loss of the entire *MEN1* gene did not result in a different phenotype compared to single missense

mutations and there was no correlation between three-organ or malignant disease and mutation types or location as expected from previous studies (Wautot et al. 2002). This may be because both missense and truncating mutations result in protein instability and rapid degradation of menin (Wautot et al. 2000; Yaguchi et al. 2004).

In conclusion, a total of 37 different mutations (19 novel) were identified in 202 index cases. 5% of all *MEN1* mutations found were large deletions and MLPA is now included in the standard clinical mutation screening procedure. 27% of the mutations were missense mutations and a list of these and all published polymorphisms has been compiled in order to facilitate future interpretation of single base pair substitutions. Mutations were detected in 91% of all cases with confirmed familial MEN1. The sporadic cases were most likely to have *MEN1* mutations if they had multiple tumours in one endocrine organ and/or an age of onset before 30 years, suggesting that these patient categories should be tested for *MEN1* mutations. As almost all *MEN1* mutations are unique, it was not possible to draw specific genotype-phenotype conclusions. There was however, no correlation between severe disease and mutation type.

### 4.3 PRODUCING RECOMBINANT VEGF-B PROTEIN

## Studies on recombinant VEGF-B (unpublished data)

To further study the novel *VEGF-B* gene, recombinant protein was produced in order to test its function in *in vitro* assays. To this end, three different production systems were used: bacteria, transient expression in mammalian cells and permanent expression in mammalian cells. Each splice form was expressed in a separate construct so that only one splice form was produced from each vector.

Initially, an *E. coli* system was used. With the aim of producing soluble VEGF-B, a fusion protein with thioredoxin was created. The resulting protein was, however, not soluble, but found in inclusion bodies in the bacteria. Solubilisation of the inclusion bodies abolished the ability of the thioredoxin his-patch to bind to the nickel-affinity columns used to purify VEGF-B. An inserted extra His-tag did not improve the binding. After testing various induction protocols and bacterial strains, thioredoxin-VEGF-B fusion protein could be produced as soluble monomers using JM109 bacteria and induction with 0.4mM IPTG for 3h at 30° (Figure 18).

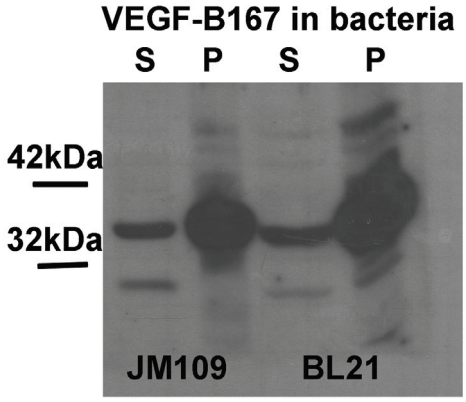


**Figure 18: Western blot of soluble (S) and insoluble pellets (P) of VEGF-B167 protein.** Different bacterial strains, induction times and temperatures and IPTG concentrations were tested. Shown are the conditions with the most soluble protein (0.4mM IPTG, 3h at 30°C) in JM109 and BL21 bacteria. VEGF-B167 is produced as a fusion protein with thioredoxin of approximately 35kDa in size.

However, purification using nickel-affinity columns gave unsatisfactory results. In addition, *in vitro* dimerisation using several different buffers and methods failed and resulted in aggregated protein, but no dimers. Attempts to produce secreted protein using pET21b vectors failed and we only obtained inclusion body monomers.

COS-1 cells were chosen as an alternative system in the hope that they would be able to refold recombinant VEGF-B correctly. VEGF-B with a His tag was cloned into two different vectors: pcDNA3.1 and pREP8. VEGF-B previously cloned into pEF-BOS was also used.

Of the four different transfection methods tested, Fugene gave the best results (30-40% transfection) and was used to transfect VEGF-B into COS-1 cells. The yield of VEGF-B from pEF-BOS was much higher than that from pcDNA or pREP8. Both splice forms were produced by the cells, but only VEGF-B186 was secreted into the medium (and therefore probably correctly processed and active). VEGF-B186 was secreted as a species of 32-36kDa and a 60kDa dimer (Olofsson et al. 1996b) as well as larger aggregates (Figure 19). All of these bands were present on non-reducing and reducing SDS-PAGE gels, the latter using 100mM DTT and heating to 75°C. Therefore it was not possible to differentiate between the glycosylated monomer of 32kDa (Olofsson et al. 1996b) and the processed 34kDa dimer (Olofsson et al. 1998). Addition of up to 100µg/ml heparin surprisingly, did not release VEGF-B167 into the medium, but did slightly enhance the amount of secreted VEGF-B186 contrary to previous reports (Olofsson et al. 1996b). Western blot analysis on cell extracts and conditioned medium from COS-1 cells demonstrated no endogenous VEGF-A or VEGF-B production.

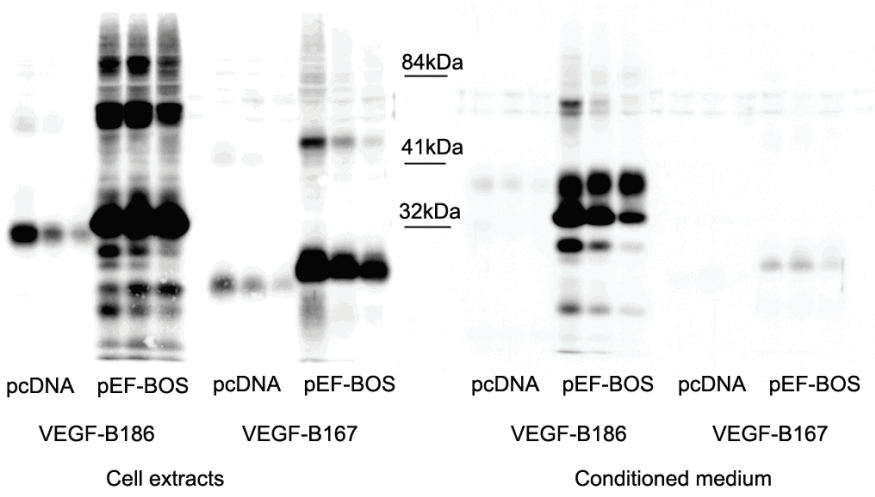


**Figure 19: Western blots showing VEGF-B186 and VEGF-B167 produced from two different expression vectors, pcDNA3.1 and pEF-BOS.** Three samples of each construct were loaded. They were sampled after (loaded from left to right) 90, 60 and 30h of incubation in culture medium with 1% BSA. On the right is cell extract protein, on the left is conditioned medium. Only the 186 isoform was efficiently secreted from pEF-BOS. VEGF-B186 is produced as a 30kDa monomer and a 60kDa dimer. Multiple monomer forms of secreted VEGF-B186 are present in the medium, probably due to glycosylation (see text for further discussion). VEGF-B167 was produced as 20kDa monomers and 40kDa dimers in cell extracts and very little was secreted. The amounts of VEGF-B produced by pcDNA3.1 vectors were much lower.

As VEGF-B186 was properly secreted, we concentrated our efforts on production of this isoform from pEF-BOS. As the pEF-BOS construct did not contain a His-tag,

nickel columns could not be used to purify the protein. Since VEGF-B186 seemed to bind to heparin, purification on heparin-sepharose columns was attempted. Both monomer and dimer forms could be eluted with 300mM NaCl. Subsequent transfections gave much lower yields of VEGFB-186 and we could not purify or further characterise the protein.

Finally, a retroviral construct containing a single insert with VEGF-B167 and GFP, the latter translated from an IRES was used to produce recombinant VEGF-B. Infected HEK293 cells were selected for expression of GFP by FACS analysis. Before selection, 73% of the VEGF-B167 –GFP infected cells were green fluorescent, while the GFP-only construct consistently had lower infection levels of approximately 48%. After selection for GFP expression in the FACS, over 95% of all cells were green for both constructs.

The culture flask surface area was increased using Cytodex beads and the HEK293 cell density and thus yield of VEGF-B was significantly increased. VEGF-B167 was secreted into the medium as a monomer of 20kDa and dimer of 40kDa as expected (Olofsson et al. 1996a), with the highest yield after incubation with Optimem containing 1% FCS or 0.1%BSA for at least 66h. HEK293 cells did not produce endogenous VGEF-B, but multiple isoforms of VEGF-A in HEK293 cell extracts were detected using two different antibodies on Western blot. However, secreted VEGF-A was not detected in conditioned medium. VEGF-B167 was stable and could be stored at 4º, -20º or -70ºC for at least 10 days without degradation, but was very sensitive to freeze-thawing and tended to aggregate or be degraded after one such cycle.

Protein purification on nickel columns was further optimised. By quantifying the VEGF-B ECL signal on Western blot and comparing it to known concentrations of VEGF-B167 from baculovirus extracts, the concentration of purified and eluted VEGF-B167 was determined to be approximately 5ng/µl. Purified VEGF-B167 was desalted and concentrated on G-25 sephadex and tested in proliferation and migration assays. Recombinant VEGF-B167 did not have any detectable effect compared to GFP controls in these assays, although commercially available VEGF-A165 did (Figure 20). Addition of vitamin C, reduced glutathione and/or β-mercaptoethanol in an attempt to prevent aggregate formation did not increase the activity of VEGF-B protein in proliferation assays.

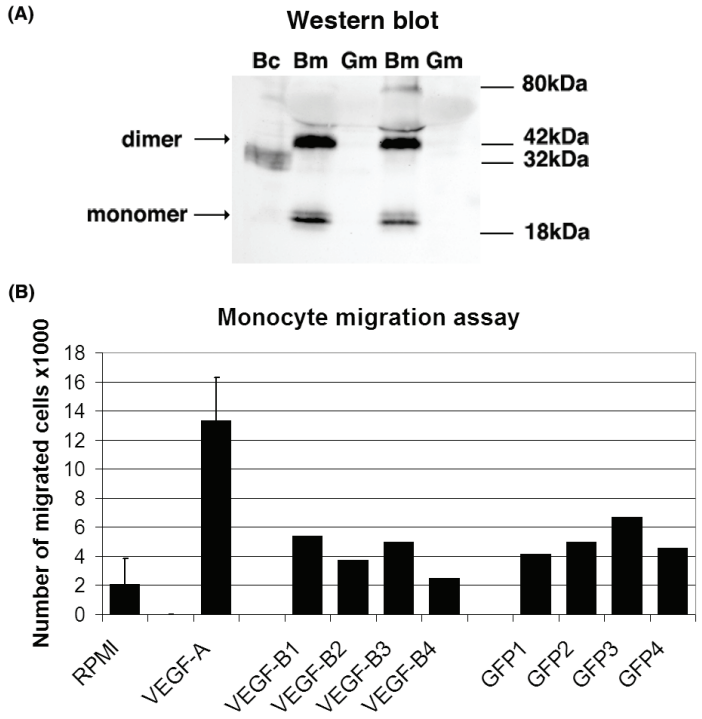


**Figure 20**: **Production of recombinant VEGF-B167 from retrovirus constructs in HEK293 cells and its effect on monocyte migration.** (A) Upon Western blot, VEGF-B167 was visible as a 30kDa species in the cell extracts (Bc), but was secreted into the medium at the expected sizes of 20kDa monomer and a 40kDa dimmer (Bm). Conditioned medium from GFP-expressing cells (Gm) did not contain any VEGF-B protein.
(B) VEGF-B167 and GFP conditioned medium was purified on nickel beads and desalted on G-25 sephadex and the resulting VEGF-B protein (primarily dimer) was tested for effect on monocyte migration of THP-1 cells. RPMI: medium only (negative control), VEGF-A: 200ng/ml recombinant human VEGF-A165 from R&D Systems (positive control). Previous experiments on VEGF-A concentrations of 20-200ng/ml showed a similar effect. VEGF-B1-4: A dilution series of purified VEGF-B protein with approximate concentrations of 125, 25, 5, and 2.5ng/ml. GFP1-4: A dilution series of purified medium from GFP-expressing cells, diluted in the same way as the VEGF-B samples. The standard deviation of the replicates of RPMI and VEGF-A are shown as error bars.

In conclusion, bacterial cells were unable to produce correctly folded VEGF-B and *in vitro* dimerisation attempts failed. Secreted (and therefore probably active) VEGF-B186 was produced by COS-1 cells. It could be partially purified from serum-free medium using heparin sepharose, but the yield was not sufficient for functional assays. Larger yields were obtained from permanently infected HEK293 cells and secreted VEGF-B167 dimers could be successfully purified using metal affinity chromatography. However, VEGF-B167 did not display any detectable effect on cell migration or proliferation in our assays. There are two possible reasons for this, either VEGF-B has no effect on these cell systems or VEGF-B167 was prevented from exerting its biological effect, e.g. due to formation of aggregates.

## 4.4 UPREGULATION OF VEGF-A IN CARDIOMYOPATHY

### Up-regulation of VEGF-A without angiogenesis in a mouse model of dilated cardiomyopathy caused by mitochondrial dysfunction (Paper III)

VEGF-B is expressed at high levels in the heart (Lagercrantz et al. 1996), but has not been extensively studied in cardiac disease models. In order to shed further light on the role of VEGF-B, a comprehensive study of the expression of VEGF-A, VEGF-B, VEGF-C and their receptors as well as the auxiliary factors, the angiopoietins and the hypoxic regulator HIF1α in mitochondrial DCM was carried out. These mice had cardiac mitochondrial dysfunction due to a lack of the transcription factor for mitochondrial DNA, *Tfam*.

As expected the mRNA levels of atrial natriuretic factor (*ANF*), a marker of heart failure increased, while there was no detectable mitochondrial cytochrome-c oxidase (*COX)* in the *Tfam* knockout hearts. *VEGF-A* mRNA was expressed in a subset of cardiomyocytes and the levels of *VEGF-A* increased by 170% (±44%) overall and by 360% (±45%) per cell area in the knockout hearts. *VEGF-B* and *C* were not altered in this model of DCM. VEGFR2 mRNA was decreased, while VEGFR1 was slightly increased. The mRNA of the transcription factor *HIF1α* also increased by 259% (±30%). Four of its target genes were elevated (*VEGF-A, VEGFR1*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and the glucose transporter type 1 (*Glut-1*)), suggesting that the activity of *HIF1α* was up-regulated. The angiopoietins and their receptors were weakly expressed on the blood vessels and were all down-regulated in DCM. 18S ribosomal RNA levels remained constant.

The increase of VEGF-A protein could be confirmed on Western blot, where four of five hearts had a greater than 250% up-regulation of VEGF-A (while VEGF-B protein levels remained constant). VEGF-A protein was also detected by immunohistochemistry where it was expressed both by the cardiomyocytes and by the blood vessels (which were negative on the VEGF-A *in situ* hybridisation) indicating that the protein was secreted and bound to its endothelial cell receptors. However, there was no increase in blood vessel density as determined by counting the CD31/PECAM-1 positive vessels in cross-sectioned hearts.

In conclusion, this is the first comprehensive study of angiogenic factors in dilated cardiomyopathy. *HIF1α* and four of its target genes including *VEGF-A* were up-regulated in this mitochondrial model of DCM. However, there was no concomitant increase in blood vessel density.

To date, more data on the role of angiogenic factors in heart failure has been published. One study on the progression of heart failure showed that the initial phase of coupled cardiac and blood vessel growth developed into a phase of insufficient angiogenesis leading to contractile dysfunction and DCM which could be precipitated by VEGF-A inhibitors (Shiojima et al. 2005). Several studies have shown that some animal models of heart failure and human patients with DCM have a reduced capillary density (Abraham et al. 2000; De Boer et al. 2003). In addition, decreased VEGF-A has been

reported in hearts from DCM patients (Abraham et al. 2000; De Boer et al. 2002). Administration of hepatocyte growth factor, an angiogenic factor, to a hereditary hamster model of DCM increased angiogenesis and cardiac function (Taniyama et al. 2002). All these studies suggest that DCM is associated with a lack of VEGF-A and reduced capillary growth as part of the pathogenesis of heart failure.

On the other hand, VEGF-A is increased in plasma from patients with heart failure (both ICM and DCM, although no difference between the groups was reported) and Ang-2 is decreased (Chin et al. 2002, Chong, 2004 #1299; Ohtsuka et al. 2005; Valgimigli et al. 2004). VEGF-A was also elevated in the myocardium of ICM patients where it was associated with an increased capillary density (Abraham et al. 2000).

It is likely that this model of DCM with impaired respiratory chain function and increased HIF1α, mirrors the hypoxic effects of ischaemic heart disease more than other causes of DCM and therefore VEGF-A mRNA and protein is increased. However, there was no concomitant increase in capillary density. This might be due to a non-permissive environment (Schmidt and Flamme 1998), including decreased angiopoietins and VEGFR2 and perhaps a reduced sensitivity to VEGF-A.

## 4.5 DECREASED VEGF-A IN EAE AND MS

### Decreased expression of VEGF-A in rat experimental autoimmune encephalomyelitis and in cerebrospinal fluid mononuclear cells from patients with multiple sclerosis (Paper IV)

As VEGF-B is highly expressed in the CNS (Lagercrantz et al. 1996) and may be involved in the pathogenesis of inflammatory disease (Mould et al. 2003), the expression of VEGF-B, VEGF-A and their receptors was studied in a well characterised rat model of multiple sclerosis. All MBP-immunised rats developed experimental autoimmune encephalomyelitis (EAE) in the spinal cord with tail and hind limb paralysis by day 12 post immunisation (p.i.). They all displayed monocytic and lymphocytic infiltrates with an up-regulation of interferon-γ as expected (Gielen et al. 2005; Muhallab et al. 2002; Weissert et al. 1998).

The longer VEGF-A transcripts (VEGF-A188, 164 and 144) showed a tendency to decrease in the spinal cord of EAE rats compared to controls upon real-time PCR. There was no alteration in levels of VEGF-B or the VEGF receptors. These results were confirmed by mRNA *in situ* hybridisation. In normal spinal cord, VEGF-A was highly expressed in glia and neurons, but was greatly down-regulated in glia in EAE, while the invading leukocytes expressed VEGF-A. VEGF-B showed no major alterations in endogenous spinal cord expression, but was also expressed by the inflammatory cells. Immunohistochemistry demonstrated VEGF-A and B protein in the infiltrates and also on normal blood vessels. As the latter did not express VEGF mRNA, this suggests that the vascular endothelial growth factors had been secreted and bound to their endothelial cell receptors. VEGF receptor mRNAs and protein were expressed in endothelial cells and were not altered in the EAE rats. In EAE rats the number of blood vessels, remained

constant, however, the endothelial cell marker, CD31 was down-regulated in the infiltrated vessels.

The expression of VEGF-A mRNA in mononuclear cells (MC) from cerebrospinal fluid (CSF) and peripheral blood from MS patients was also quantified. VEGF-A was significantly down-regulated in the CSF-MC in MS patients compared to controls. We attempted to corroborate our results on VEGF-A protein in CSF using both Western blot and the more sensitive ELISA technique. However, the levels of VEGF-A were below the detection limit. VEGF-A is an unstable protein that degrades rapidly in clinical samples not specifically collected for growth factor analysis. Thus, degradation may have occurred in our samples.

In contrast to previous reports on up-regulation of VEGF-A in EAE where no VEGF-A was present in control rats (Kirk and Karlik 2003; Proescholdt et al. 2002), we had robust expression of VEGF-A in CFA-immunised and naïve rat spinal cord using three different methods (real-time PCR, in situ hybridisation and immunohistochemistry). We can therefore conclude that VEGF-A is down-regulated in CNS resident cells in EAE rats and in CSF-MC from MS patients, while the expression of VEGF-B and their receptors remained unaltered. The blood vessel density was also unchanged in this model of EAE. VEGF-A can be both neuroprotective (Hayashi et al. 1998; Krum and Khaibullina 2003; Manoonkitiwongsa et al. 2006; Widenfalk et al. 2003; Zhang et al. 2002) and neurotoxic/proinflammatory (Benton and Whittemore 2003; Manoonkitiwongsa et al. 2004; Proescholdt et al. 2002; van Bruggen et al. 1999; Zhang et al. 2002) and it is not yet possible to determine which role VEGF-A plays in MS. Further studies on clinical material and interventional studies in animal models are warranted. In order to shed more light on the role of VEGF-A in MS, we hope to be able to correlate expression of VEGF-A with disease phenotype in a larger group of MS patients.

## 4.6 WHAT IS THE FUNCTION OF VEGF-B? – AN UPDATE -

Several groups have tried for many years to produce active VEGF-B, without success. The preliminary reports on effects of recombinant VEGF-B on endothelial cell proliferation (Olofsson et al. 1996a) and up-regulation of uPA and PAI-1 (Olofsson et al. 1998), were subsequently attributed to heterodimer formation with VEGF-A or could not be reproduced (Nash et al. 2006). The purification of recombinant VEGF-B167 from *E coli* inclusion bodies was published in 2000. It bound to VEGFR1 but no biological effects were reported (Scrofani et al. 2000). In 2002, VEGF-B186 and a VEGF-B fragment containing amino acids 10-108 were purified from *E coli*. They both bound to VEGFR1, but only VEGF-$B_{10\text{-}108}$ and VEGF-B167 stimulated a chimeric VEGFR1 (Nash et al. 2006; Scotney et al. 2002). In 2003, the first biological activities of recombinant VEGF-B protein were reported as VEGF-B167 was shown to induce endothelial cell infiltration into matrigel implanted in mice (Silvestre et al. 2003).

Studies on VEGF-B manipulation in mice have shown that VEGF-B is not required during embryogenesis (Aase et al. 2001; Bellomo et al. 2000; Carmeliet et al. 2001; Malik et al. 2006) and the mice do not up-regulate their levels of VEGF-A or PlGF

mRNA to compensate for the loss of VEGF-B (Aase et al. 2001). Over-expression or inhibition of VEGF-B does not affect growth or angiogenesis in normal adult tissues (Malik et al. 2006; Mould et al. 2005), suggesting that VEGF-B cannot initiate angiogenesis. However, excessive levels of VEGF-B may be detrimental during embryonic development as no viable offspring with ubiquitous over-expression of VEGF-B survived and only one individual with endothelial cell specific over-expression was born for each isoform (Mould et al. 2005). Not much is known about the possible role of VEGF-B in embryogenesis, although studies in quail embryos suggest that VEGF-B may involved in the formation of coronary arteries (Tomanek et al. 2006).

To date a number of reports have indicated that VEGF-B is angiogenic. Enhanced expression of VEGF-B can potentiate pathological angiogenesis in the presence of other angiogenic factors (e.g. in skin wounds, and bFGF infused matrigel) (Mould et al. 2005) and can up-regulate VEGFR1 and induce limited angiogenesis in normal blood vessel adventitia (Bhardwaj et al. 2003). Administration of plasmids with VEGF-B186 or VEGF-B167 to animals with hind leg ischemia resulted in an increased angiographic score, increased perfusion and an increased number of blood vessels. These effects were blocked by anti-VEGFR1 or an eNOS inhibitor (Silvestre et al. 2003), see also (Nash et al. 2006; Wright 2002). However, adenoviral VEGF-B167 had no effect on atherosclerosis or neovascularisation in non-ischaemic hind limb muscles (Leppanen et al. 2005; Rissanen et al. 2003). Nor does VEGF-B induce vascular permeability (Mould et al. 2005). In addition, there have been no publications on the effect of VEGF-B in traditional angiogenesis assays such as the mouse cornea assay or the chick chorio-allantoic membrane.

VEGF-B is expressed at high levels in the heart (*paper III* and (Lagercrantz et al. 1996). VEGF-B seems to be required for recovery of blood flow and reducing tissue damage after ischaemia in the heart (Bellomo et al. 2000). However, no studies have shown up-regulation of VEGF-B after myocardial ischaemia (although VEGF-B186 was increased in hind limb ischaemia, (Silvestre et al. 2003)) and the expression of VEGF-B remained constant in our model of DCM due to mitochondrial dysfunction.

The role of VEGF-B in inflammation is unclear. VEGF-B did not stimulate macrophage invasion in the matrigel or skin wound assay (Mould et al. 2005) and did not induce monocyte migration *in vitro* in our assays. However, it did increase monocytic invasion in blood vessel adventitia after adenoviral transfer (Bhardwaj et al. 2003). VEGF-B knockout mice had reduced clinical inflammation and vascularisation as well as ameliorated symptoms in models of rheumatoid arthritis (Mould et al. 2003). In addition, VEGF-B was expressed by the infiltrating leukocytes in spinal cord of EAE rats *(paper IV)*.

Now recombinant human (rh) VEGF-B167 and mouse (rm) VEGF-B186 are commercially available (R&D Systems) and additional studies have indicated that VEGF-B might be important in other processes. RhVEGF-B167 has been shown to stimulate migration and matrigel invasion (but not proliferation) of pancreatic and colorectal cancer cell lines (Fan et al. 2005; Wey et al. 2005), although so far VEGF-B has not been correlated to adverse outcome or metastasis in cancer (Gunningham et al. 2001; McColl et al. 2004).

VEGF-B is highly expressed in the CNS (paper IV and (Lagercrantz et al. 1996). VEGF-B167 can increase neuronal precursor cell proliferation and partially protect neurons from hypoxic cell death *in vitro* and *in vivo* (Sun et al. 2004, 2006). However, in contrast to VEGF-A, VEGF-B could not stimulate neurite outgrowth (Jin et al. 2006). VEGF-B is up-regulated after cortical cold injury in rat brain (Nag et al. 2002), but is not regulated by hypoxia (Enholm et al. 1997; Simpson et al. 1999) and expression of VEGF-B is not altered in EAE.

The specific roles of the two splice forms also remain unknown. The two isoforms have completely different carboxyl-terminal ends and different chemical properties (*paper I* and (Olofsson et al. 1996a; Olofsson et al. 1996b). To date, there are no published reports on the activity of recombinant VEGFB-186 protein, and the gene transfer studies have not reported any difference between the isoforms (Silvestre et al. 2003). VEGF-B167 represents up to 80% of all VEGF-B in normal tissues, however in tumours the VEGF-B186 isoform is up-regulated, suggesting that they can exert distinct functions (Li et al. 2001).

In conclusion, VEGF-B is very stable and is not regulated by hypoxia or other agents studied, suggesting that it plays a homeostatic role in the adult. VEGF-B does not seem to be required at all during embryogenesis. Inappropriate expression of VEGF-B can potentiate angiogenesis and may be involved in the recruitment of monocytes, however, none of these functions are unique for VEGF-B. Thus a lot of work remains before we understand why we have a VEGF-B gene and what its highly expressed protein does.

# 5 CONCLUDING REMARKS

The work in this thesis began by searching for a disease gene and studying disease mutations and led to work on expression and function of a protein family.

The search for the MEN1 gene was a fascinating journey that few need to go through today, since the human genome is now fully sequenced and available to all (Lander et al. 2001; Venter et al. 2001). There are currently over 28,900 genes listed in the NCBI Human Genome Map Viewer, although the largest part of the complexity of our genome lies in the large number of alternative splice forms (Takeda et al. 2006). So far over 2000 monogenic disease genes are known (OMIM). Once a disease gene has been identified, molecular studies on gene function can be carried out. Although almost ten years have passed since menin was identified, it is still unknown why tumours develop specifically in endocrine organs when the gene is ubiquitously expressed and no therapeutic novelties have been discovered (Agarwal et al. 2005; Chandrasekharappa and Teh 2003). More immediately, information on a disease-causing gene can be used to perform mutation screening to facilitate clinical diagnosis and to detect presymptomatic gene carriers, as in the case for MEN1. However, a number of questions regarding gene mutation detection still remain. For instance, no mutations have been detected in two of our established MEN1 families, indicating that mutations in regulatory regions of the gene also result in MEN1. In addition, it is difficult to interpret novel base pair substitutions in a single index case. Proof that they are a mutation can only be obtained if they are found to segregate with disease in the family.

Studying gene expression and protein function is even more complex. Not only are there often different isoforms to take into consideration, but the chemical properties of proteins may make them difficult to work with *in vitro*, as was the case with VEGF-B. Studying the expression of a gene can lead to valuable clues to its function. However, once the horizon is expanded and several factors are studied simultaneously, the picture immediately becomes more complicated. What is the net result of an increase in e.g. VEGF-A accompanied by a decrease in its angiogenic receptor VEGFR2 (in DCM)? If both the antagonist and the agonist decrease simultaneously (as the angiopoietins in DCM), how is VEGF-A signalling affected? If the longer splice forms of VEGF-A are decreased in a subset of cells in a tissue that also expresses VEGF-A in other cell populations (e.g. the invading leukocytes in rat spinal cord EAE), what will the impact be? In which situations do the pro-inflammatory and oedematic functions of VEGF-A outweigh its neuroprotective functions? Is this the case in EAE/MS?

It will be an exciting challenge to address these issues in the future. It is clear that the multitude of factors involved in angiogenesis and inflammation act in concert. Therefore therapy with one single growth factor or one single inhibitor and thus perturbing the body's own homeostasis may not give the desired results. Not only the choice of factor, but also the dose, timing, administration route and isoform are of utmost importance for the final effect and much remains to be studied before these therapies can become reality, be it for inflammatory disease such as RA or MS or for ischaemic heart disease.

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# 8 SAMMANFATTNING

Denna avhandling har sitt ursprung i sökandet efter genen för MEN1. MEN1 är en ärftlig form av cancer som drabbar körtlar såsom bisköldkörteln, bukspottskörteln eller hypofysen. Detta resulterar ofta i en överproduktion av hormoner som ger symtom såsom depression och skeletturkalkning från bisköldkörteln, magsår eller lågt blodsocker från bukspottskörteln eller mjölkproduktion från bröstkörteln p.g.a. hypofystumör. För att tidigt upptäcka nya tumörer kontrolleras en patient med misstänkt MEN1 med årliga blodprover och röntgenundersökningar. MEN1 beror på mutationer i en enda gen och ärvs dominant. Detta innebär att i genomsnitt hälften av alla barn till MEN1-patienter ärver sjukdomen, och därför inkluderas också de i de årliga kontrollerna. Efter att *MEN1*-genen identifierades 1997, kan man läsa av gensekvensen hos patienter och därmed hitta sjukdomsframkallande förändringar, s.k. mutationer. Släktingar som har ärvt samma mutation måste då även fortsättningsvis kontrolleras varje år, medan de som inte har mutationen inte behöver följas upp.

Sedan 1997 har 202 obesläktade patienter remitterats till avdelningen för Klinisk Genetik för *MEN1*-mutationstestning. Syftet med *artikel II* i avhandlingen var att göra en sammanställning av fynden bland de svenska MEN1-patienterna. *MEN1*-mutationer hittades hos 45 av de 202. Alla med en mutation hade antingen nära släktingar med MEN1, en tumördebut före 30-års åldern och/eller multipla tumörer. Därför bör i första hand dessa patientkategorier testas för *MEN1*-mutationer. Det finns ett nytt test (MLPA) som kan upptäcka större förluster inom *MEN1*-genen (som man inte ser när man läser av gensekvensen). Denna metod har utvärderats på patienter med säkerställd MEN1 och kunde upptäcka förluster hos två patienter (motsvarande 5% av alla mutationer). Därför kommer nu MLPA att användas rutinmässigt för MEN1-mutationsscreening.

Idag finns hela människans arvsmassa (DNA) i databaser, men i slutet av 1980-talet kände man inte till många av sjukdomsgenerna och fick leta efter *MEN1*-genen. Det tog 10 år att identifiera *MEN1*-genen och under den tiden hittade man flera tidigare okända gener som låg nära *MEN1*-genen. En av dessa liknade en tillväxtfaktor för endotelcellerna i blodkärl (vascular endothelial growth factor A, VEGF-A) och fick därför namnet VEGF-B. Syftet med resterande delar av avhandlingen är att beskriva hur *VEGF-B*-genen ser ut (*artikel I*) och att försöka påvisa vad VEGF-B gör i kroppen genom (a) att producera VEGF-B-protein och testa dess funktion (*ej publicerade data*) och (b) att studera uttrycket av VEGF-A, VEGF-B och besläktade proteiner i sjukdoms-modeller för att indirekt kunna påvisa en roll för VEGF-B (*artiklarna III och IV*).

Alla kroppens celler innehåller exakt likadana DNA-uppsättningar. DNA-koden översätts först till budbärar-RNA (mRNA). Detta är en mellanform som oftast inte har någon egen funktion och översätts till ett biologiskt aktivt protein (dvs. genen uttrycks). Det som skiljer t.ex. en hudcell från en vit blodkropp är alltså inte DNA:t i sig, utan vilka av våra 29.000 gener som uttrycks i cellen. Varje gen kan dessutom översättas till flera olika proteinformer. Exempelvis fann vi att VEGF-B har två olika proteinformer och att båda uttrycks i de flesta celltyperna.

Ett sätt att studera funktionen av en ny gen är att producera proteinet i provrör och sedan testa dess effekt på utvalda celler. För att göra detta kan man utnyttja naturens eget maskineri i form av bakterier eller djurceller som odlas i petriskålar i näringslösning i 37°C. *VEGF-B*-genen tillfördes dessa celler och efter ett par timmar eller dagar kunde VEGF-B-protein skördas. Aktivt VEGF-B består av två enheter som binds ihop. Det visade sig att bakterierna inte kunde tillverka aktivt VEGF-B, däremot kunde djurcellerna. VEGF-B-proteinet påverkade dock inte celltillväxt eller cellvandring, två basala mekanismer som behövs både för tillväxt av blodkärl och för den inflammation som VEGF-B misstänks påverka.

Celler behöver syre och näringsämnen för att överleva. Dessa transporteras till cellerna via blodkärlen. Om det uppstår syrebrist (t.ex. p.g.a. kärlförträngningar), signalerar cellerna att de behöver mer syretillförsel; de uppreglerar bl.a. VEGF-A som stimulerar tillväxt av nya blodkärl in i det syrefattiga området. Syrebrist i hjärtat kan ge hjärtinfarkt eller hjärtsvikt. En annan vanlig orsak till hjärtsvikt är hjärtmuskelsjukdomar. Vissa av dessa beror på mutationer i mitokondrierna, som ansvarar för en stor del av cellens energiförsörjning. Möss med mitokondriell hjärtsvikt hade normala VEGF-B-nivåer, men en ökning av VEGF-A, vilket kanske beror på att energibrist liknar syrebrist. Däremot ledde inte ökningen av VEGF-A till nya blodkärl, kanske p.g.a. en avsaknad av nödvändiga hjälpfaktorer.

Det fanns indikationer på att både VEGF-B och VEGF-A kan skydda nervceller från skador. Dessutom har båda två visat sig vara viktiga för uppkomsten av vissa inflammatoriska sjukdomar där vita blodkroppar invaderar och orsakar vävnadsskada. Därför har jag studerat uttrycket av VEGF-B och VEGF-A i en djurmodell av multipel skleros. Multipel skleros (MS) är en nervsjukdom som oftast drabbar yngre vuxna och som leder till förlust av funktioner av olika slag, såsom synen, balansen och muskelstyrkan. Orsakerna till MS är delvis okända ärftliga faktorer och delvis miljöfaktorer såsom virusinfektioner. Tillsammans sätter de igång en felaktig aktivering av immunsystemet med en invasion av vita blodkroppar in i hjärnan och ryggmärgen där de skadar nervbanorna. I djurmodeller av MS, producerades både VEGF-A och VEGF-B av de invaderande vita blodkropparna vilket skulle kunna påverka aktiveringen av immunsystemet. VEGF-B visade inga förändringar i nervsystemets celler i MS. Däremot fanns det en minskning av VEGF-A mRNA och protein i dessa celler hos råttor med MS. Dessutom har MS-patienter lägre halter av VEGF-A i ryggmärgsvätskan jämfört med friska. Det går ännu inte att avgöra om VEGF-A:s effekt på MS är skyddande eller inflammatorisk, det får studier på större patientgrupper utvisa.

Flera grupper har arbetat med att producera VEGF-B-protein sedan 1996, men det var först 2003 som VEGF-B visade sig ha en effekt på blodkärlstillväxten. Fortfarande vet man väldigt lite om dess roll. Däremot visar mina (och andras) resultat att VEGF-B ofta uttrycks tillsammans med flera andra liknande faktorer i olika celltyper och att det är svårt att avgöra vad nettoeffekten på en viss sjukdom blir. Detsamma gäller för behandling med dessa faktorer, t.ex. pågår försök att ge VEGF-A till hjärtinfarktspatienter med målet att stimulera nybildningen av blodkärl och därmed läkningen. Men vad blir nettoeffekten när man bara ger VEGF-A efter en hjärtinfarkt när många faktorer som kan påverka blodkärlstillväxten uttrycks i hjärtat? Det får framtiden utvisa.