***VEGF-B as a target for cancer therapy – Characterization of Monoclonal Antibodies
towards VEGF-B and Generation of VEGF-B expressing tumour cell lines***

Hanna Ollila

Master's Thesis

University of Jyväskylä

Jyväskylän yliopisto

Preface

The work presented in this Master's thesis was carried out at the Ludwig Institute for Cancer Research, Stockholm Branch, Karolinska Institutet at the laboratory of Professor Ulf Eriksson in June 1st– August 31st, 2004. I want to express my gratitude to Ulf Eriksson and Ralf Pettersson for providing me with an interesting topic to work on as well as Christina Fieber and Christian Oker-Blom for supervision.

University of Jyväskylä   Abstract of Master´s Thesis

Faculty of Mathematics and Science

Author: Hanna Ollila

Title of thesis: VEGF-B as target for cancer therapy – Characterization of Monoclonal Antibodies towards VEGF-B and Generation of VEGF-B expressing tumour cell lines

Finnish title: VEGF-B ja syöpä – monoklonaalisten anti-VEGF-B vasta-aineiden karakterisointi ja VEGF-B:tä ilmentävien syöpäsolulinjojen tuottaminen

Date: 9.4.2007   **Pages:** 48 + 6

Department: Department of Biological and Environmental Science

Chair: Cell Biology

Supervisor(s): Ulf Eriksson, Christina Fieber, Christian Oker-Blom

Abstract: Vascular Endothelial Growth Factor-B (VEGF-B) was found over 10 years ago but its function is still unknown. VEGF-B belongs to a protein family that has been shown to function in tumour angiogenesis, lymphangiogenesis and metastasis. In addition monoclonal antibodies against VEGF-A are used to treat colorectal cancer. We wanted to elucidate the function of VEGF-B in tumour angiogenesis by generating Murine Embryonic Fibroblast (MEF) tumour cell lines expressing VEGF-B isoforms and studied the tumorigenic profile of corresponding MEF-*Vegf-b-/-* cell lines by RT-PCR. In addition we characterized monoclonal antibodies targeted towards VEGF-B using biochemical methods in order to find possible candidates for blocking VEGF-B function. We discovered that knocking down *Vegf-b* did not affect the amount of total RNA produced by the cells. Interestingly, we found one good candidate antibody, Mab3372, that was able to recognize the receptor binding domain of VEGF-B in immunoprecipitation and western blotting and several antibodies that recognized the protein in immunoprecipitation. I suggest that the blocking function of Mab3372 should be studied and cell lines generated injected into mice to further elaborate the function of VEGF-B in tumour angiogenesis.

Keywords: Vascular Endothelial Growht Factor B (VEGF-B), tumour angiogenesis, monoclonal antibody therapy

Jyväskylän yliopisto   Pro gradu -tutkielman tiivistelmä

Matemaattis-luonnontieteellinen tiedekunta

Tekijä: Hanna Ollila

Tutkielman nimi: VEGF-B ja syöpä – Monoklonaalisten anti-VEGF-B vasta-aineiden karakterisointi ja VEGF-B:tä ilmentävien syöpäsolulinjojen tuottaminen

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Päivämäärä: 9.4.2007 **Sivumäärä:** 48 + 6

Laitos: Bio- ja ympäristötieteiden laitos

Oppiaine: Solubiologia

Tutkielman ohjaaja(t): Ulf Eriksson, Christina Fieber, Christian Oker-Blom

Tiivistelmä: VEGF-B (Vascular Endothelial Growth Factor-B) löydettiin jo yli kymmenen vuotta sitten. Silti sen biologisesta toiminnasta tiedetään hyvin vähän. Muilla VEGF-geeniperheen jäsenillä on todettu olevan merkitys syövän verisuonituksessa, imusuonten kasvussa sekä etäispesäkkeiden muodostumisessa. Tarkoituksenamme tässä työssä oli valaista VEGF-B:n merkitystä syövän kehityksessä ja verisuonituksessa tekemällä MEF (Murine Embryonic Fibroblast) syöpäsolulinjoja, jotka ilmentävät VEGF-B:n eri muotoja sekä analysoida MEF- *Vegf-b-/-* solulinjojen verisuonitukseen vaikuttavien geenien profiili käyttäen RT-PCR:ää. Määritimme myös VEGF-B:tä vastaan kehitettyjen yksikotoisten vasta-aineiden sitoutumista VEGF-B:hen käyttäen biokemiallisia menetelmiä ja etsimme sopivia kandidaatteja kokeisiin, jossa määritetään näiden vasta-aineiden VEGF-B:n neutralisointikyky. Saimme selville, että MEF-*Vegf-b-/-* solulinjat eivät merkittävästi poikkea villityypin solulinjoista. Löysimme neutralisointikokeisiin myös yhden hyvän kandidaatin, joka tunnistaa myös VEGF-B:n reseptoriin sitoutuvan osan ja jota tulisi tutkia tarkemmin neutralisoivien ominaisuuksien selvittämiseksi. Tämä tutkimus antaa mahdollisuuden tutkia paremmin VEGF-B:n vaikutuksia syövän kehityksessä injisoimalla tuotetut solulinjat hiiriin ja seuraamalla näiden kasvua mahdollisesti myös neutralisoimalla VEGF-B.

Avainsanat: Vascular Endothelial Growht Factor B (VEGF-B), syövän verisuonitus, vasta-ainelääkkeet

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Abbreviations

Brefeldin A	p4-dihydroxy-2-[6-hydroxy-1-heptenyl]4- cyclopentane-crotonic acid lambda-lactone
COS-1	African green monkey kidney fibroblas
DMEM	Dulbecco's Modified Eagle Medium
DTE	Dithioerythritol
FCS	Fetal Calf Serum
FIGF	Fetal Induced Growth Factor (VEGF-D)
IAA	Iodoacetamide
mRNA	Messenger ribonucleic acid
Nrp	Neuropilin
PDGF	Platelet Derived Growth Factor
PAS	Protein A Sepharose
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PVDF	Polyvinylidene fluoride
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
TBS	Tris Buffered Saline
TCA	Trichloro Acetic Acid
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

1. Introduction

1.1 Angiogenesis and Cancer

Cancer has become the leading cause of death in developed countries. The development of cancer is a multistep process that requires several alterations in the genome of a single cell that give it a growth advantage. The cell must also inactivate control mechanisms and tumour suppressor genes. In addition to these protective mechanisms a tumour cell must overcome other barriers: A solid tumour must be able to induce the growth of new blood vessels (angiogenesis). To be able to metastase to distant organs, the tumour needs to break through the extra cellular matrix surrounding it, move to the site of metastasis and colonialize it in order to survive. In addition, the colonialized cells must endenger angiogenesis at the target tissue (Hanahan and Weinberg, 2000).

Solid tumour mass cannot persist without a supply of oxygen and nutrients from blood vessels. Therefore the maximum size a tumour can grow without a blood supply is 100-200µm from a vessel. Blood vessels also transport metabolic waste products out of tumours (Folkman *et al.*, 1971, Folkman J., 1971, Helmlinger *et al.*, 1997). Blood vessels, as well as lymphatic vessels, are also the route that tumour cells use for metastasis. The patterning of blood vessels within a tumour is often chaotic. The vessels are disorganized and dilated, have excessive branching and shunts and contains incomplete smooth muscle cell (SMC) coverage. Many tumour cells are also in direct contact with the blood vasculature which also advance metastasis (Carmeliet and Jain, 2000, Hashizume *et al.*, 2000).

Normally in adults, angiogenesis is minimal and takes place only in conditions such as wound healing and female reproductive cycle. It is thought that the balance between pro-angiogenic and anti-angiogenic factors and proteins define whether angiogenesis occurs or not. This is named as "angiogenic switch" which is thought to be "on" when angiogenesis occurs (Hanahan and Folkman, 1996).

The proteins that function in normal development but are quiescent at adulthood are thus turned on at tumour angiogenesis. These factors include the VEGF/PDGF (Vascular Endothelial Growth Factor/Placenta Derived Growth Factor) family described below, angiopoietins, that are important in adult vascular remodelling as well as factors that make space for growing blood vessels, such as MMPs (Matrix Metalloproteinases) and collagenases (Suri *et al.*, 1996). New blood vessels are formed by endothelial cells but also other cell types take part in vessel maturation. Also macrophages and smooth muscle cells (SMC) contribute to angiogenesis by secreting angiogenic factors as well as SMC being part of the newly formed vessels (reviewed in Cleaver and Melton, 2003).
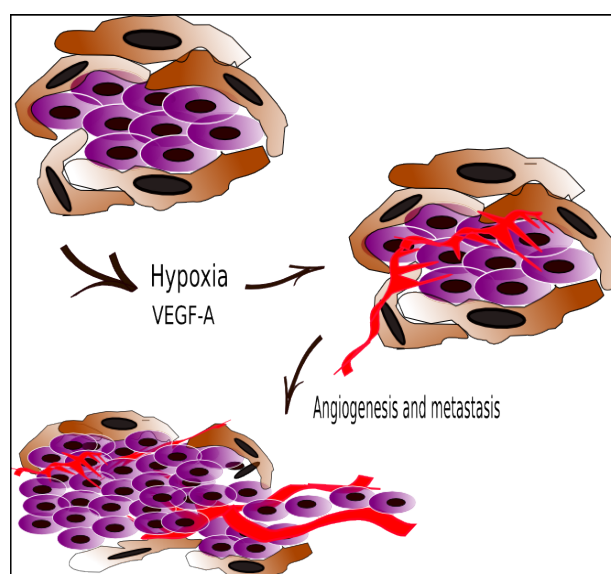

Illustration 1: In order to prosper, tumour needs oxygen and nutrients. When a tumour cell lacks oxygen, it becomes hypoxic and secretes factors that attracts blood vessels. This facilitates metastasis through blood vessels. © Hanna Ollila

1.2 Vascular Endothelial Growth Factor Family

1.2.1 VEGF-A

Vascular Endothelial Growth Factor-A (VEGF-A, Vascular Permeability Factor, VPF) was found almost twenty years ago as an angiogenesis stimulating factor and permeability factor (Keck *et al.*, 1989, Leung *et al.*, 1989). Presently it is known as endothelial cell mitogen and it is the most potent angiogenic protein known. VEGF-A is crucial in the formation of blood vessels: Deletion of even one copy of *Vegf-a* leads to embryonic lethality due to incapability to form blood vessels and island (Carmeliet *et al.*, 1996). VEGF-A has eight cysteine residues that are also conserved in other members of the protein family. These cysteines form intermolecular
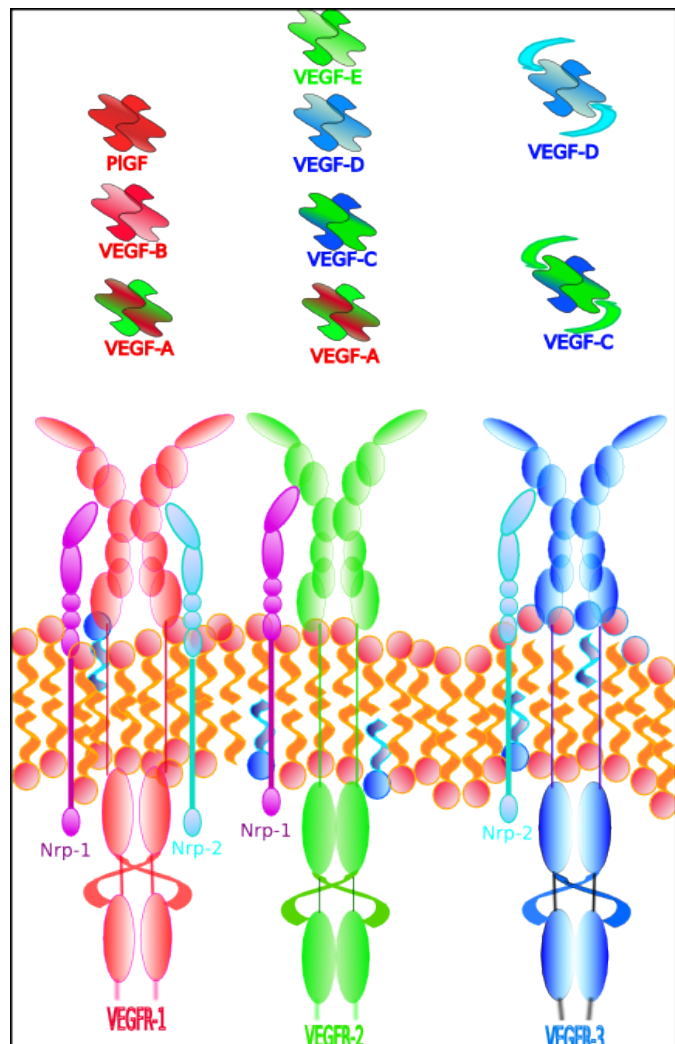

Illustration 2: Binding of VEGFs to Neuropilin co-receptors and to VEGFRs. Binding of a ligand to a receptor induces autophosphorylation of the receptor. © Hanna Ollila

bridges which leads to dimerization of the protein. The VEGF-family members function as dimers (Muller *et al.*, 1997). VEGF-A has nine isoforms that are generated by alternative splicing which form homodimers. (Bates and Harper, 2002, Lange *et al.*, 2003 , Takahashi and Shibuya, 2005). VEGF-A is also required for survival of freshly formed blood vessels and even in adult tissues with little angiogenesis it is needed for fenestrated blood capillary maintenance (Kamba *et al.*, 2006).

VEGF-A signals through VEGFR-1 (VEGF Receptor-1) and VEGFR-2 but it can also bind Neuropilin-1 and Neuropilin-2 that act as co-receptors for VEGFRs (Shibuya *et al.*, 1990, Matthews *et al.*, 1991, Soker *et al.*, 1998, Zoya *et al.*, 2000). The expression of VEGF-A is regulated upon stimuli such as hypoxia and oncogenes, p53 mutation and tumour promoters. The role of VEGF-A as a key regulator in tumour angiogenesis and metastasis has been elucidated in several studies. In addition to tumour mass, also tumour stroma produces VEGF-A that facilitates the growth of new blood vessels into the tumour. VEGF-A secreted by tumour cells also induce the expression of collagenases that degrade the basement membrane and thereby facilitate tumour metastasis. VEGF-A also increases vascular permeability at the site of tumour but also in other pathological conditions as in wounds and in inflammation which further promote tumour metastasis (Fukumura *et al.*, 1998, reviewed in Takahashi and Shibuya, 2005, Kalebic *et al.*, 1983).

1.2.2 VEGF-B

VEGF-B (VRF) was found over ten years ago as a VEGF-related factor, yet the function of VEGF-B has remained largely unknown (Olofsson *et al.*, 1996a, Grimmond *et al.*, 1996). The mouse and human genes are almost identical and the VEGF-B gene is composed of seven exons (Olofsson *et al.*, 1996a). VEGF-B has two isoforms, VEGF-B$_{167}$ and VEGF-B$_{186}$, that differ in their C-terminals. The different isoforms are generated due to alternative splicing in exon 6. VEGF-B$_{167}$ has strong affinity to heparin sulphate which resembles the heparin binding domain of VEGF-A$_{165}$ and it remains cell-associated after secretion. Neither of the isoforms are N-glycosylated unlike the other growth factors in VEGF/PDGF-family but the C-terminus of VEGF-B$_{186}$ high in serine and threonine is O-glycosylated and proteolytically processable. VEGF-B$_{167}$ is one of the rare growth factors that is not glycosylated at all (Olofsson *et al.*, 1996b, Olofsson *et al.*, 1996a). VEGF-B$_{167}$ has molecular mas of 21 kDa whereas VEGF-B$_{186}$ is produced as an unmodified peptide with molecular mas of 26 kDa which is further glycosylated to 32 kDa active protein. At amino acid level VEGF-B is 45% identical with VEGF-A and 30% identical with PlGF (Placental Growth Factor) (Olofsson *et al.*, 1996a).

VEGF-B isoforms are produced as disulfide-linked homodimers. Both isoforms can also form heterodimers when co-expressed with VEGF-A$_{165}$ in cells but it is not known whether heterodimers exist in nature. VEGF-A$_{165}$/VEGF-B$_{167}$ heterodimers remain cell-bound whereas VEGF-A165/VEGF-B$_{186}$ heterodimers are freely secreted. If heterodimers do exist the balance between homo- and heterodimers could affect VEGF-A signalling (Olofsson *et al.*, 1996a).

VEGF-B is also expressed during embryonic development. It is most eminent in developing myocardium but also detected in muscle,

bone, pancreas, adrenal gland and at the smooth muscle layer of larger vessels as well as in spinal cord and cerebral nervous system. However, during embryonic development VEGF-B is not detected in endothelial cells (Aase *et al.*, 1999). In later stages of embryogenesis the expression of VEGF-B is still high in heart but also in brown adipose tissue and in spinal cord (Lagercrantz *et al.*, 1998). The 1.4kb mRNA of VEGF-B is strongly expressed in tissues containing high energy metabolism such as heart, brain, skeletal muscle, kidney and brown adipose tissue. It is also expressed in majority of other tissues but at lower levels (Olofsson *et al.*, 1996b). VEGF-B$_{167}$ is the most abundant form of VEGF-B comprising 80% of the total transcript detected in human and mouse tissues. Furthermore, VEGF-B$_{186}$ is expressed at lower levels and less widely than as VEGF-B$_{167}$ (Li *et al.*, 2001). The expression of VEGF-B is very stable and not upregulated by hypoxia or other factors that affect VEGF-A expression (Enholm *et al.*, 1997). However, VEGF-B is upregulated in human and rodent tumour cell lines. Especially VEGF-B$_{186}$ was upregulated in mouse and human tumour cell lines compared to the normal expression which makes it an potent target for treating cancer (Li *et al.*, 2001).

Both VEGF-B isoforms bind and activate VEGFR-1. VEGF-B also binds to Neuropilin-1 (Olofsson *et al.*, 1998, Makinen *et al.*, 1999). Of the VEGF family members VEGF-B and PlGF are specific for VEGFR-1. VEGF-B has weaker binding activity to VEGFR-1 than VEGF-A even though the tertiary structure of VEGF-B is almost superimposible with the structure of VEGF-A (Iyer *et al.*, 2006). However, the binding of VEGF-B to VEGFR-1 may stimulate the expression and activity of urokinase type plasminogen activator and plasminogen activator inhibitor 1 in endothelial cells (Olofsson *et al.*, 1998).

The role of VEGF-B in physiological and pathological conditions is still unclear. Knock-out studies suggest that VEGF-B may have a role in

heart functioning or in angiogenesis. One group has reported that *Vegf-b -/-* mice have an atrial conduction phenotype. The mice have prolonged PQ-interval in echocardiography. However, the phenotype is controversial. *Vegf-b -/-* mice are viable and fertile. Another group has reported that the mice have smaller hearts, dysfunctional coronary arteries and impaired recovery from induced myocardial ischemia (Bellomo *et al.*, 2000, Aase *et al.*, 2001).

1.2.3 PlGF

PlGF (Placenta Growth Factor) has four splice variants that are generated through alternative splicing: PlGF1 through PlGF-4. PlGF-2 has strong affinity to heparin sulphate proteoglycans whereas the other isoforms do not bind heparin. PlGF binds to VEGFR-1 but not to VEGFR-2. The isoforms PlGF-2 and PlGF-4 also bind neuropilins. PlGF can also form heterodimers with VEGF-A (reviewed in Takahashi and Shibuya, 2005, DiSalvo *et al.*, 1995). The protein is expressed in high levels predominantly in placenta but it is also expressed in lung, heart, thyroid gland and skeletal muscle (Persico *et al.*, 1999). PlGF is also upregulated in many human tumours as hypervascular renal cell carsinoma and its loss impaires cancer and angiogenesis (Carmeliet *et al.*, 2001, Takahashi *et al.*, 1994, Takahashi and Shibuya, 2005).

1.2.4 VEGF-C

VEGF-C (also known as VEGF Related Protein, VRP) does not have splice variants like the factors discussed above but its activity is

regulated by proteolytical processing of the protein which determines its receptor binding. The fully processed form of VEGF-C is cabable of binding both VEGFR-2 and VEGFR-3 whereas the unprocessed or partially processed forms only bind VEGFR-3. VEGF-C prepropeptide consist of aminoterminal and carboxyterminal domains that are cleaved off during processing and a VEGF Homology Domain (VHD). The aminoterminal domain has no homology to other known proteins, whereas the carboxyterminal peptide is rich in cystein and resembles the Balbiani ring 3 domain of silk proteins (Joukov *et al.*, 1996).

VEGF-C induces the growth of lymphatic vessels and blood vessels by activating its receotors VEGFR-2 and VEGFR-3 (Jeltsch *et al.*, 1997). VEGF-C has an important role in lymphangiogenesis: VEGF-C is required for initial sprouting of lymphatic vessels and for the subsequent survival of lymphatic endothelial cells (Karkkainen *et al.*, 2004). Furthermore, *Vegf-c-/-* mice die and heterozygous knock-out mice have defects in lymphatic vascular development. However, VEGF-C is required for angiogenesis in other organisms such as in zebrafish and in *Xenopus* tadpoles (Ober *et al.*, 2004).

Vegf-c gene contains seven exons and the expression of the VEGF-C mRNA is detected in high levels in human placenta, ovary, heart, skeletal muscle and small intestine (Chilov *et al.*, 1997, Joukov *et al.*, 1996). Also other tissues such as kidney, lung, pancreas, prostate and spleen express VEGF-C in moderate levels as well as mesenchymal cells in mouse embryos at regions that undergo lymphatic sprouting (Kukk *et al.*, 1996).

VEGF-C is expressed in vast amount of human tumours. it is especially expressed in breast, cervix, colon, lung, prostate and stomach cancers. The expression of VEGF-C in tumours is associated with metastasis, especially through lymphatic vessels and to lymph nodes, as well as with vascular invasion and thereby with poor patient

survival (reviewed in He *et al.,* 2004).

1.2.5 VEGF-D

VEGF-D is also produced as preproprotein that undergoes proteolytical processing in a similar manner as VEGF-C generating the mature protein. In humans the protein is cabable of binding both VEGFR-2 and VEGFR-3 whereas in mice it can only bind VEGFR-3 (Stacker *et al.,* 1999). Even though the receptor binding of VEGF-D resembles that of VEGF-C, the *Vegf-d -/-* mice are viable and display normal lymphangiogenesis and functional lymphatic vessels during development and adulthood (Baldwin *et al.,* 2005). The *Vegf-d* gene is located at X-chromosome and contains seven exons (Rocchigiani *et al.,* 1998, Yamada *et al.,* 1997). The expression of VEGF-D is detected in colon, heart, lung, skeletal muscle and small intestine. Also ovary, testes prostate and spleen express small amount of VEGF-D (Achen *et al.,* 1998). VEGF-D can also promote tumour metastasis in lymph nodes and it is expressed in human melanoma in vessels near tumour cells (Achen *et al.,* 2001, Stacker *et al.,* 2001).

1.2.6 VEGF-E

VEGF-A homologues carried by several viruses are called VEGF-E. They are ligands for VEGFR-2 and Neuropilin-1. VEGF-E:s stimulate endothelial cell proliferation and vessel permeability as VEGF-A (Lyttle *et al.,* 1994).

1.3 Vascular Endothelial Growth Factor Receptors and Neuropilins

1.3.1 VEGFR-1

VEGFR-,1 also known as Flt-1 (Fms-like tyrosine kinase-1), binds VEGF-A, VEGF-B and PlGF (see illustration 1). VEGFR-1 exists also in soluble form and it has been postulated that VEGFR-1 functions as a trap for VEGF-A which is a very strong mitogen for endothelial cells and which binds VEGFR-1 at 10-fold affinity compared to its affinity towards VEGFR-2. However, VEGFR-1 signalling activates MAP-kinase only weakly and none of the ligands can induce the proliferation of endothelial cells by activating VEGFR-1 in culture. The downstream signalling isn't though thoroughIy understood. (Kendall *et al.*, 1996, Waltenberger *et al.*, 1994, Seetharam *et al.*, 1995, for review see Takahashi and Shibuya, 2005). VEGFR-1 knock-out studies further support the idea of controlling function of VEGFR-1. The mice die due to excessive proliferation of endothelial cells and disorganized and overmuch vasculature and not due to lack of blood vessels. Furthermore, if only the tyrosine kinase signalling domain is knocked-out (VEGFR-1TK-/-mice) the mice are born and show only a mild phenotype on the migration of macrophages towards VEGF-A, which suggests that the ligand binding property of VEGFR-1 is important for its function by trapping VEGF-A (Fong *et al.*, 1995, Hiratsuka *et al.*, 1998).

Interestingly, VEGFR-1 has been shown to have a role in pathological conditions such as cancer and atherosclerosis. It has been observed that tumours in mice expressing only the tyrosine kinase

deficient VEGFR-1 do not metastase into lungs as well as tumours in the control mice. Also, if mice are treated with monoclonal antibody blocking VEGFR-1 function, the premetastatic cells move to the site of metastasis poorly. Further, the amount of Matrix Metalloproteinase-9 (MMP-9) is lower in the lungs of VEGFR-1TK-/- mice. MMP-9 is an important factor in cancer metastasis and it speeds metastasis by degrading extracellular matrix (Hiratsuka *et al.*, 2001, Hiratsuka *et al.*, 2002, Kaplan *et al.*, 2005).

1.3.2 VEGFR-2

VEGFR-2 also called Flk1 (fetal liver kinase 1), or KDR (kinase insert domain containing receptor) is a receptor for VEGF-A, VEGF-C, VEGF-D and VEGF-E. A soluble form of VEGFR-2 also exists but it has a weaker affinity towards VEGF-A, the main ligand, than the membrane bound receptor (Ebos *et al.*, 2004). VEGFR-2 is the main receptor for angiogenesis. VEGFR-2 is also the earliest marker for endothelial cell development and it stimulates endothelial cells to migrate in adulthood and embryogenesis. VEGFR-2 knock-out mice die from lack of endothelial and hematopoietic cells. (Hiratsuka *et al.*, 2005, Shalaby *et al.*, 1995, Kabrun *et al.*, 1997). VEGFR-2 is a key player in angiogenesis and it is expression is upregulated in several human tumours, which makes VEGFR-2 also an interesting object for drug targeting. (Kaliberov *et al.*, 2005, for review see Cross *et al.*, 2003).

1.3.3 VEGFR-3

VEGFR-3, also called Flt-4 (Fms-like tyrosine kinase 4), binds VEGF-C and VEGF-D. In humans VEGFR-3 exists in two splice isoforms that differ in their C-terminals (Hughes, 2001). VEGFR-3 has a role both in blood vessel and in lymphatic vessel development. VEGFR-3 deficient mice die at E9.5 of abnormal remodelling of primary vascular plexus (Dumont *et al.*, 1998). VEGFR-3 mediates the sprouting and migration of differentiated lymphatic endothelial cells from the veins. It is also required for the survival and maintenance of the lymphatic vessels during embryogenesis (Mäkinen *et al.*, 2001, Karkkainen *et al.*, 2004).VEGFR-3 has a role in tumour angiogenesis since it is upregulated at the blood vessels of many tumours even though it normally is expressed only in lymphatic vessels (Partanen *et al.*, 1999). As described above, VEGF-C promotes tumour metastasis and treatment with soluble VEGFR-3-Ig protein inhibits tumour metastasis to lymph nodes and tumour angiogenesis (He *et al.*, 2002).

1.3.4 Neuropilins

Neuropilin-1 (Nrp-1) and Nrp-2 are co-receptors for Clas III Semaphorins and VEGFs with limited signalling capacity. At least Nrp-1 can induce endothelial cell migration but not proliferation (Wang *et al.*, 2003). Nrp-1 is expressed at endothelium of arteries whereas Nrp-2 is found in veins and lymphatic vessels (Herzog *et al.*, 2001, Yuan *et al.*, 2002). Nrp-1 knock-out mice die at embryonic state from vascular and neuronal defects whereas Nrp-2 mutant mice are viable but lack small lymphatic vessels and capillaries and have moderate neural defects (Kitsukawa *et al.*, 1997, Chen *et al.*, 2000, Yuan *et al.*, 2002). At least

Nrp-1 has been detected in tumours as an important receptor for VEGF-A (Marcus *et al.,* 2005).

1.4 Treatment of cancer with anti-angiogenic drugs

Since tumours are dependent on proper vasculature, treatment with anti-angiogenic drugs is a potent and promising tool for treating cancer. Treating tumours with anti-angiogenic drugs seems very promising since angiogenesis is common to many tumour types and thus would not be restricted to one cancer type only. One can attack tumour vasculature and the angiogenic factors by several ways: By blocking growth factor function, by blocking receptor function or by blocking transcription (see Figure 2). All these methods aim for the same result which is halting the growth and metastasis of the tumour and prolonging patient survival time. Presently, there are a growing number of drugs at the market that are used alone or in combination with more traditional treatments at clinics. The most famous one of these is probably Bevacizumab - a monoclonal antibody inhibiting VEGF-A function. It was approved by FDA year 2004 and is used for treating metastatic colorectal cancer in combination with chemotherapy (for review see Fernando and Hurwitz, 2003 ,Hurwitz, 2004).

Another way of warding off cancer is to use tyrosine kinase inhibitors (for review see, Shawver *et al.*, 2002). Instead of blocking the ligand these pharmaceuticals block the receptor function by inhibiting its signalling. The most famous tyrosine kinase inhibitors are herceptin, gleevec and iressa. Hercepcin is a monoclonal antibody towards HER2

(Human Epidermal growth factor Receptor-type 2) developed by Genentech and it is used in treatment of breast cancers overexpressing HER2/*neu* receptor. Also these drugs are most effective when used in combination with traditional treatments, such as chemotherapy.
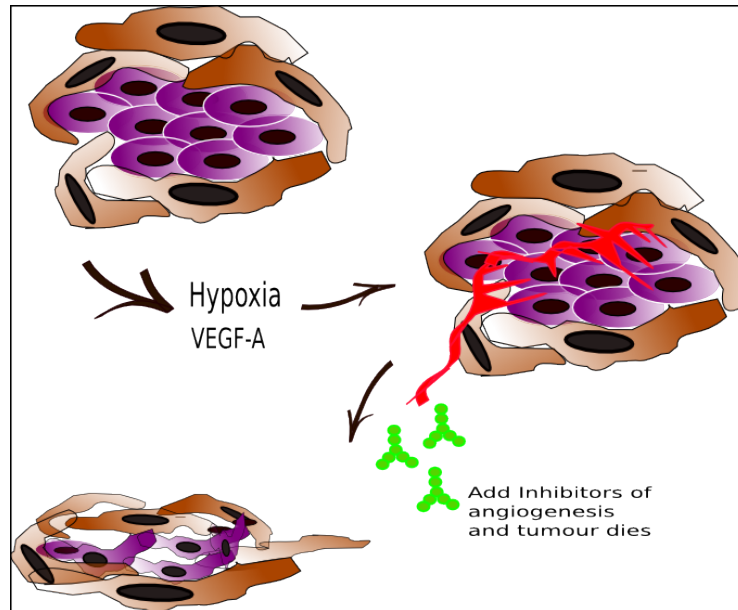

Illustration 3: Hypoxia and angiogenic growth factors make blood vessels grow. Adding anti-angiogenic drugs kills the tumour. © Hanna Ollila

2. Aims of the study

The aims of this study were to elucidate the role of VEGF-B in tumorigenesis and find tools for studying VEGF-B function by

1. Characterizing monoclonal antibodies targeted towards VEGF-B for their binding capacity using different biochemical methods in order to find candidates for blocking VEGF-B function

2. Comparizing of two *Vegf-b* deficient Murine Embryonic Fibroblast (MEF) cell lines to the wild type MEF cell lines of their angiogenic profile

3. Creating transformed MEF cell lines expressing VEGF-B$_{167}$ and VEGF-B$_{186}$ to elucidate the function of VEGF-B in tumour progression

3. Materials and Methods

Antibodies

The antibodies Mab337, Mab3371, Mab3372, Mab751 and Mab767 were obtained from RandD Systems. The antibodies are produced by hybridoma cell lines derived from mice immunized with purified *E. Coli*-derived human VEGF-B$_{167}$ or human VEGF-B$_{186}$, except for the Mab3372 which was obtained from *SF21*-derived purified human VEGF-B$_{186}$. The IgG fraction of the tissue culture supernatant has been purified by Protein G affinity chromatography. The antiserum K595 was previously produced by immunizing a rabbit with mouse VEGF-B and by collecting the serum, which was purified with protein G affinity chromatography.

Recombinant VEGF-B proteins

All recombinant proteins used in this study (VEGF-B$_{167}$, VEGF-B$_{186}$ and VEGF-B$_{10\text{-}108}$) were produced in *E. Coli (*Amrad, Australia).

Blocking Buffer

4% non-fat milk powder, 0.1% Tween-20 in PBS

Cell Culture

The cells were maintained at 37 °C in a humidified athmosphere containing 5% CO_2.

The cells were cultured in DMEM supplemented with 10% FCS, 2 mM L-Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. For transfections medium without FCS (Fetal Claf Serum) was used.

Gel Running Buffer

192 mM Glysine, 0,1% SDS, 25 mM Tris

4x Laemli Loading Buffer

4% Sucrose, 8% SDS in 250 mM Tris pH 6.8

PBS

8% NaCl, 0.2g of KCl, 1.44% Na_2HPO_4, 0.24% KH_2PO in H_2O, pH 7.4

Plasmids

The plasmids used for transfections were pcDNA3.1VEGF-B167 clones 8 and 9 and pcDNA3.1VEGF-B186 containing human *Vegf-b* cDNAs for isoforms VEGF-B$_{167}$ and VEGF-B$_{186}$ under Zeocin selection. We also used empty pcDNA3.1 vector with Zeocin selection marker as well as pREP7VEGF-B167 and pREP7VEGF-B186 as positive control plasmids. Also these plasmids have human *Vegf-b* cDNAs.

50xTAE

2M Tris-acetate and 100mM Na2EDTA in H_2O pH 8

TBS

8% NaCl, 0,2% KCl, 1M Tris-HCl in H_2O pH 7.4

Transfer Buffer

10% Methanol, 192 mM Glysine, 25 mM Tris in H_2O

Western Blotting and protein detection

The recombinant VEGF-B proteins were loaded into 12% 50 µl 10-well-SDS-PAGE gels (Biorad), 50 ng of recombinant protein was loaded into each well. Rainbow marker (Amersham) was used to define the protein weight of the samples. The gels were run with 25 mA per gel untill the marker reached the bottom of the gel. Polyvinyl fluoride membranes (PVDF; Millipore) were activated in methanol for 1 minute at room temperature. The proteins were transfered to the PVDF-membranes 100 V 1 hour at 4°C after which the membranes were blocked in Blocking Buffer overnight at 4°C.

The primary antibody was diluted to 1:1000 (0.5µg/mL) in Blocking Buffer. The PVDF- membrane was incubated for 2 h with the primary antibody solution at room temperature. After this the membrane was washed five times with 10ml PBS-0.1%-Tween-20. The membrane was then incubated with the solution containing Horseradish peroxidase conjugated anti-mouse-IgG-HRP secondary antibody, diluted 1:10 000 in Blocking Buffer. The bound antibodies were detected using Enhanced Chemiluminescence Plus reagent (ECL+, Amersham) according to manufacturer's protocol. The blot was imaged using Luminescent Image Analyzer CCD-Camera (LAS-1000 CH, FUJIFILM).

Transfection

In order to find out which of the antibodies work in immunostaining, we decided to test the affinity of the RandD antibodies towards endogenously produced mouse and human VEGF-B isoforms, VEGF-B$_{167}$ and VEGF-B$_{186}$. We also wanted to know whether these antibodies work at environment that is near physiological conditions. We also wanted to know which antibodies would be applicable in the future tumour tests to block VEGF-B binding to VEGFR-1.

COS-1 cells were seeded in 6-well-formats in 50% confluency. The transfection was done with Lipofectamin-PLUS-reagent (Invitrogen) according to the manufacturers protocol using serum free DMEM. After the transfections, the media was aspired and 1ml DMEM with appropriate antibiotics and L-Glutamine and FCS and were mixed to final concentration of 10% FCS. Brefeldin A, was dissolved into the media to a final concentration of 12,5%. In order to visualize the proteins inside the cells, we used Brefeldin A (Sigma), a chemical that prevents the secretion of the proteins out of the cells.

Immunostaining of transfected cells

Staining was continued 24 hours after transfection. The medium was aspired and the cells were washed once with 2ml PBS for four minutes. The cells were incubated for ten minutes with 4% paraformaldehyde in PBS. This was continued with two washes with PBS four minutes each. Then the cells were permeabilized for 30 minutes with PBS-0.1% Triton-X100. The cells were washed three times with PBS and the unspecific binding sites were blocked with 5% FCS-

PBS for 30 minutes. The primary antibodies were added onto the cells in a final concentration of 0.5µg/ml (1:1000) in 5% FCS-PBS. The non-bound primary antibodies was washed away two times four minutes with PBS. The cells were incubated with the secondary Alexa 594-conjugated rabbit anti-human antibody, 1:3000 in 5% FCS-PBS. The cells were washed three times with PBS and mounted. Images were obtained with fluorescent microscope with 40x magnification.

Immunoprecipitation

A solution of 100 ng of recombinant VEGF-B protein, 1 µg monoclonal antibody or Mouse Isotype Control and 50 µl 50% protein G-sepharose slurry in 500µl 0.5% BSA 1 mM phenylmethylsulphonylfluodire in PBS. The tubes were incubated for two hours rotating at 4°C.

The procedure was continued by aspiring the supernatant and washing three times with 500µl 0.05% Tween-20 in PBS including centrifugation for 30 seconds 1500rpm in 4°C between the washes. The washes were followed by additional rinsing with 500µl PBS in order to remove Tween20. Finally the pellets were resuspended in 50µl 2xSDS Laemli loading buffer containing 0,1M (dithioerythritol) DTE and 0,1M IAA (iodoacetamide) as reducing agents. A gel was run as described before and the blotted for human and mouse VEGF-B proteins with antibodies Ab3372 and K959, respectively.

RT-Reaction

The total RNA of the cells was transcribed using Oligo(d)T as a primer and Reverse Transcriptase (Invitrogen) according to manufacturer's protocol. The final total cDNA product was diluted into 200µl H_2O and stored at –80 ˚C.

GAPDH-PCR

For the PCR, 5µl of cDNA was amplified in reaction mix containing 13µl H_2O, 2.5µl 10x PCR–Buffer

Table 1: PRC reaction conditions for GAPDH PCR

	Time	Temperature ˚C	
Heat start	1 minute	94	
Denaturation	30 seconds	94	
Annealing	30 seconds	58	x 19
Elongation	30 seconds	72	
	4 minutes	72	

(200mM Tris-HCl, pH 8.4, 500mM Kcl, Invitrogen), 2.5µl 10x dNTPs (final concentration 2.5mM), 0.75µl $MgCl_2$ (50mM stock, final concentration 1.5mM), 0.5µl forward primer (10pmol/µl), 0.5µl reverse primer (10pmol/µl) and 0.25µl Taq-DNA-polymerase (5U/µl , Invitrogen). The PCR conditions are shown at the table below.

PCR of angiogenic factors

The expression level of angiogenic factors in the cell lines of interest were studied using PCR analysis. The PCR conditions for angiogenic factors were first optimized by temperature and cycle number. The final conditions are presented in Table 2 and Table 3.

Table 2: PCR conditions

	Time	Temperature °C	Cycle numbers
Heat start	1 minute	94	
Denaturation	30 seconds	94	
Annealing	unique	unique	unique
Elongation	30 seconds	72	
	4 minutes	72	

Table 3: PCR conditions for angiogenic factors: the appropriate cycle numbers for each factor,, annealing temperatures and sequences of the corresponding primers.

Name of Primer used	Number of cycles	Annealing temperature °C	Forward Primer sequence	Reverse Primer sequence
mouse VEGF-A	31	58	GAT CAT GCG GAT CAA ACC TCA CC	CTC CGG ACC CAA AGT GCT CCT C
mouse VEGF-B	30	58	CTC TGA GCA TGG AAC TCA TGG	GAG TCT GGA AAG CAG CTT GTC
mouse VEGF-C	36	55	CAT TAC CAC AGT GTC AGG CAG C	CAC GGT CTT CTG TAA CAA CTG C
mouse VEGF-D	35	60	CAA CCA ACA CAT TCT TCA AGC C	GTT CCT GGT AAG AGT GG
mouse PlGF	36	55	CCC ACA CCC AGC TCA CGT ATT TA	TCC CCT CTA CAT GCC TTC AAT GC
mouse PDGF-A	42	56	CTT GGA GAT AGA CTC CGT AG	ACA TGC ACA CTC CAG GTG TT
mouse PDGF-B	40	58	GTA TGA AAT GCT GAG CGA CC	CAA TCT TTC TCA CCT GGA CC
mouse PDGF-C	40	56	TTT GGG CTG GAA GAT CCA GA	CTG TCC TCT TTA GCT CTT CC
mouse PDGF-D	40	56	CTG GGA ATC AGT CAC AAG CT	GGA CTT CCA GTT GAC AGT TC

After PCR, 2,5 µl (1/10 of final volume) of Loading dye (Ready Load) was added to the reaction mixes from PCR reactions. The DNA was pipetted into 1.8% agarose-TAE gel and run with 70 mA with 1kb Plus DNA Ladder marker (Ready Load) till the bands were separated.

Stable transfection of MEF-SV40-hras VEGF-B -/- cells with VEGF-B$_{167}$, VEGF-B$_{186}$ or VEGF-B$_{167}$ and VEGF-B$_{186}$

The cells were seeded in 6 well plates at a 50% confluency. The transfections were done using Lipofectamin-PLUS-reagent (Invitrogen) according to the manufacturers protocoll in serum-free DMEM. After the transfection, 1ml DMEM and 1.5ml 25% FCS-DMEM was added to a final concentration 10% FCS DMEM. The next day the cells were split 1:10 into 10cm cell culture plates using 10ml medium. One day later the selection was started using 150µg/ml Zeocin (Invitrogen). The medium with Zeocin was changed twice a week. After ten days selection eight colonies per plate were selected for cell line generation. The media was aspired, each colony was trypsinized individually and replated on 24-well-plates. After the colonies had grown nearly confluent they were plated into 6-well-plates. After this the cells were grown in 15ml flasks. The Zeocin selection was continued throughout the expansion. We also kept the original colonies in 10cm cell culture plates under Zeocin selection splitting them three times a week. In addition, we kept the transfected pools further under selection and maintenance.

TCA precipitation of expressed proteins

The expression of VEGF-B by the transfected cells was defined by TCA-precipitating the proteins and immuno blotting. The cell supernatant, 400 µl, was precipitated by adding 100 µl 20% TCA (trichloro acetic acid) and incubated for 45 minutes on ice. The samples were centrifuged for 15 minutes at 4°˚C 13 000rpm on a table centrifuge. The supernatant was soaked off and the pellets were washed with 500µl 80% ethanol at -20˚C. The samples were centrifuged 15 minutes at 4˚C 13000 rpm. The pellets were dried in room temperature and 30µl 4 times concentrated Laemli loading buffer was added onto each sample. The precipitated proteins, 25 µl, were run in 12% SDS-PAGE gels and detected by immuno blotting as described above with Mab3372 antibody.

Freezing of the cells

The cells were frozen down for maintenance. Cryovials were cooled at -20˚C. The media was aspired from the plates. The cells were trypsinized and resuspended in 6ml growth medium. After this the cells were centrifuged 3 minutes in 1200 rpm after which the cells were resuspended in 2ml FCS. Into each cryovial 0.5ml FCS-Cell-suspension and 0.5ml 20% DMSO in FCS was added. The cells were kept at -80 ˚C freezer for three days after which they were moved to -135 ˚C freezer for permanent maintenance.

4. Results

4.1 Antibody Mab3372 recognizes VEGF-B$_{167}$ and VEGF-B$_{186}$ in Western Blotting, Immunostaining and in Immunoprecipitation

Even though VEGF-B was found over ten years ago, there are still only a few antibodies targeted against it. We wanted to test five commercially available antibodies by RnD Systems and find out whether they can be used in Western Blotting, immunostaining and in immunoprecipitation.

4.1.2 Antibody Mab337, Mab3371 and Mab3372 recognize VEGF-B in Western Blotting

We tested the binding of the antibodies towards the two isoforms of VEGF-B, VEGF-B$_{167}$ and VEGF-B$_{186}$, as well as to a recombinant protein VEGF-B$_{10\text{-}108}$ comprising the receptor binding domain. In western blotting, three of the five tested monoclonal antibodies detect at least one form of VEGF-B, Mab337, Mab3371 and Mab3372. The isoform best recognized was clearly VEGF-B$_{186}$ that was recognized by all these three antibodies. Antibodies Mab337 and Mab3371 could recognize the isoform VEGF-B$_{186}$ (Figures 1-3).
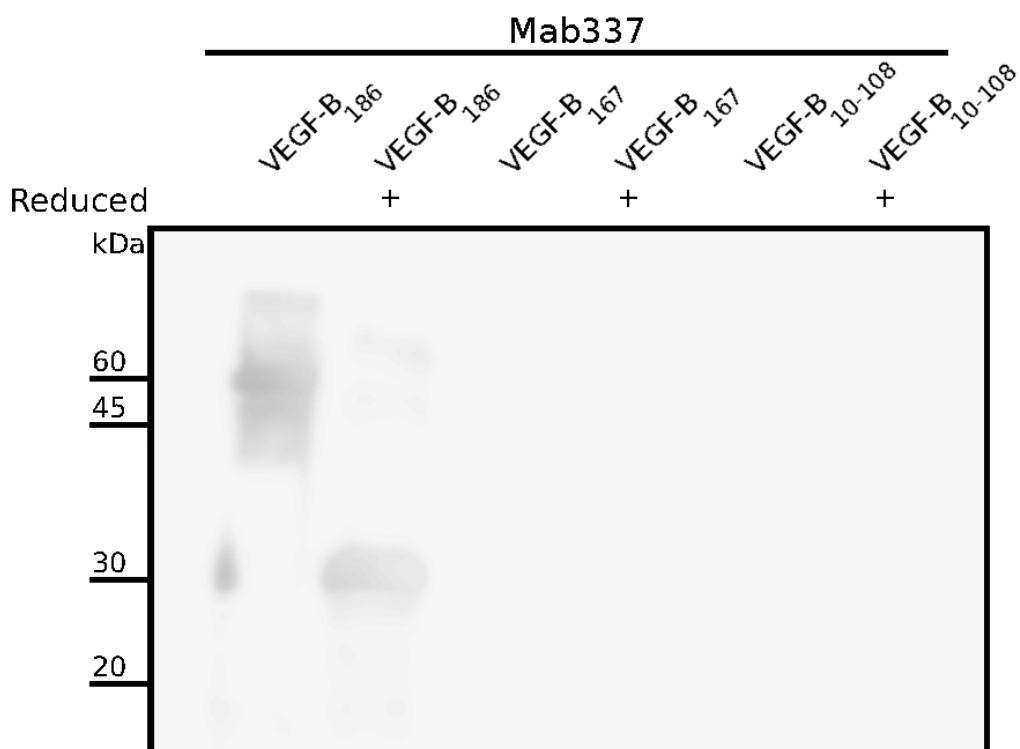
Figure 1: **AntibodyMab337 recognizes recombinant VEGF-B$_{186}$ non-reduced and reduced forms but not VEGF-B$_{167}$ non-reduced or reduced forms.** The binding of antibody Mab337 to recombinant VEGF-B proteins and to a peptide comprising of VEGF-B amino acids 10-108 was analysed in 12% PAGE under reduced, containing ß-mercaptoethanol, and non-reduced conditions. Reduced lanes marked with a plus. Bands corresponding VEGF-B$_{186}$ are monomeric form at 30 kDa and dimeric form 60 kDa.
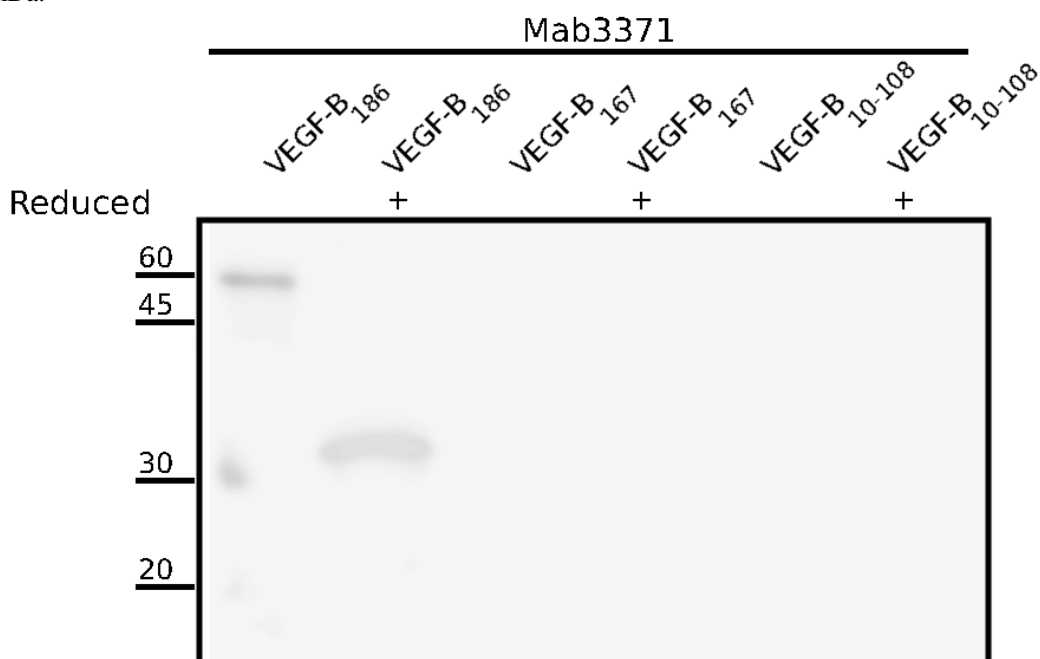

Figure 2: **Antibody Mab3371 recognizes VEGF-B$_{186}$ non-reduced and reduced proteins but not VEGF-B$_{167}$.** The binding of antibody Mab3371 to recombinant VEGF-B proteins and to a peptide comprising of VEGF-B amino acids 10-108 was analysed in 12% PAGE under reduced, containing ß-mercaptoethanol, and non-reduced conditions. Reduced lanes marked with a plus. Bands corresponding VEGF-B$_{186}$ are monomeric form at 30 kDa and dimeric form 60 kDa.

The isoforms VEGF-B$_{167}$ and the recombinant protein VEGF-B$_{10\text{-}108}$ comprising the receptor binding region of VEGF-B were detected by antibody, Mab3372. However, there are differences in how well antibody Mab3372 detects the different isoforms in Western blot. It seems that it best recognizes VEGF-B its reduced, monomeric form. Furthermore, VEGF-B$_{186}$ is recognized better than VEGF-B$_{167}$ the peptide comprising the receptor binging region VEGF-B$_{10\text{-}108}$. Antibodies Mab767 and Mab751 did not recognize any of the recombinant proteins tested (Figure 4).
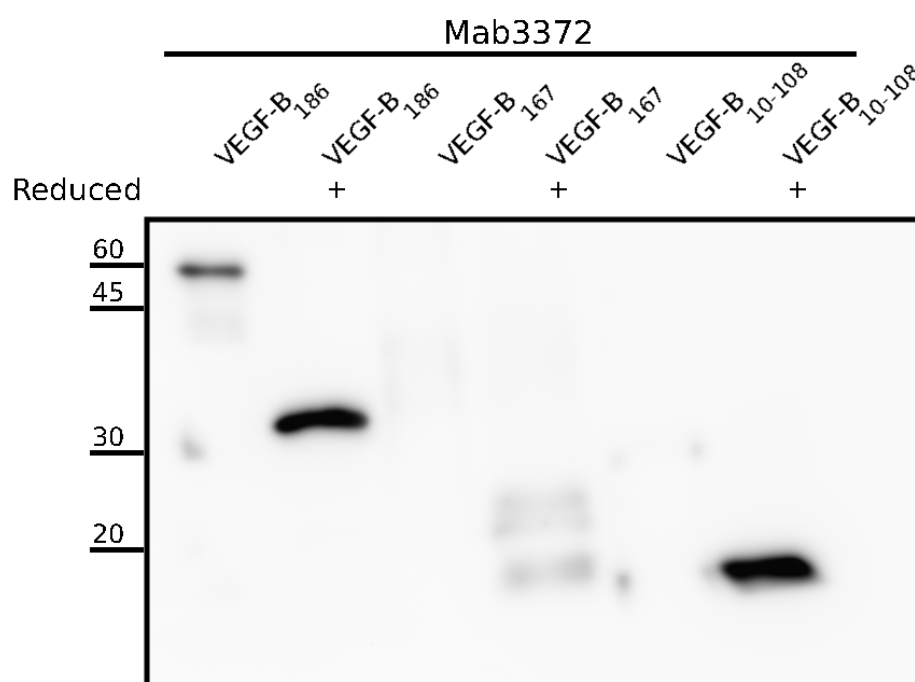
Figure 3: **Mab3372 recognizes both non-reduced and reduced VEGF-B$_{186}$, reduced VEGF-B$_{167}$ and reduced VEGF-B$_{10\text{-}108}$, which comprises the receptor binding region of VEGF-B.** The binding of antibody Mab3372 to recombinant VEGF-B proteins and to a peptide comprising of VEGF-B amino acids 10-108 was analysed in 12% PAGE under reduced, containing ß-mercaptoethanol, and non-reduced conditions. Reduced lanes marked with a plus. Bands corresponding VEGF-B$_{186}$ are monomeric form at 30 kDa and dimeric form 60 kDa. VEGF-B$_{167}$ at 21 kDa and VEGF-B$_{10\text{-}108}$ at 14 kDa.
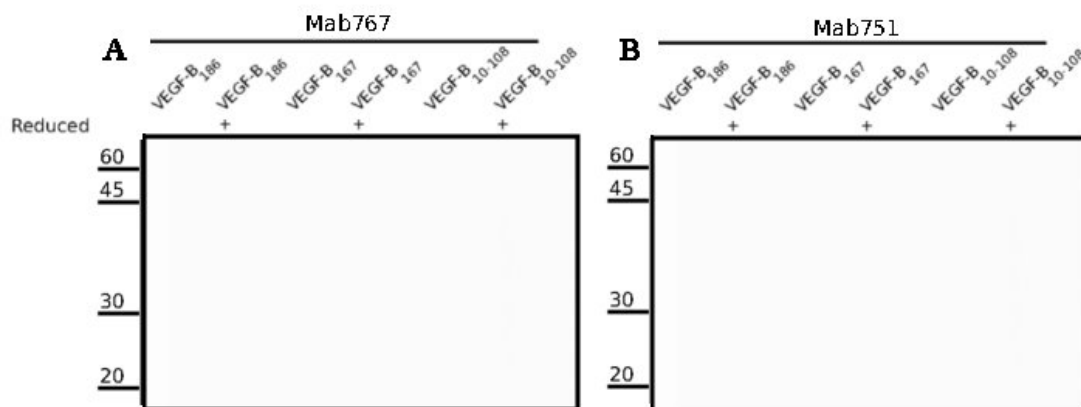
Figure 4: **Antibody Mab767 and antibody Mab751 do not bind VEGF-B in western blotting.**The binding of antibodies Mab767 (A) and Mab751 (B) to recombinant VEGF-B proteins and to a peptide comprising of VEGF-B amino acids 10-108 was analysed in 12% PAGE under reduced, containing ß-mercaptoethanol, and non-reduced conditions. Reduced lanes marked with a plus. Antibodies Mab767 and Mab751 did not recognize VEGF-B in Western Blotting.

4.1.2 Antibody Mab3372 recognizes human VEGF-B in immunostaining

We next tested whether the different antibodies can detect VEGF-B in immunostaining of VEGF-B$_{167}$ or VEGF-B$_{186}$ expressing COS-1 cells. We wanted to test the antibodies in immunostaining since this would provide us with information of how the proteins produced in mammalian cells, instead of recombinat proteins produced in bacterial cells, are recognized by the antibodies.

We found out that antibody Mab3372 detects both human VEGF-B isoforms but not the corresponding mouse isoforms. Antibody Mab3372 was also the only antibody that recognized any of the proteins produced by COS-1 cells (Figure 5).
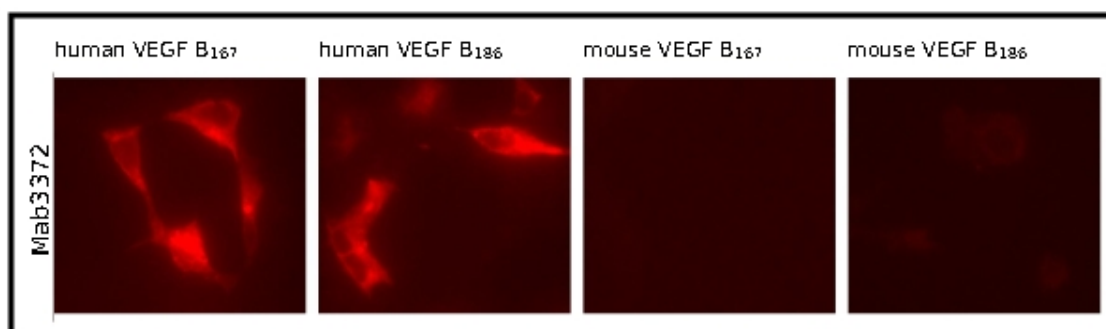
Figure 5: **Antibody Mab3372 recognizes human VEGF-B$_{167}$ and human VEGF-B$_{186}$ isoforms.** The binding of Mab3372 to VEGF-B produced in COS-1 cells was analysed by transiently transfecting COS-1 cells with human and mouse VEGF-B and staining for VEGF-B with antibody Mab3372 using Alexa594 conjugated secondary antibody. The visualization is at x40 magnification.

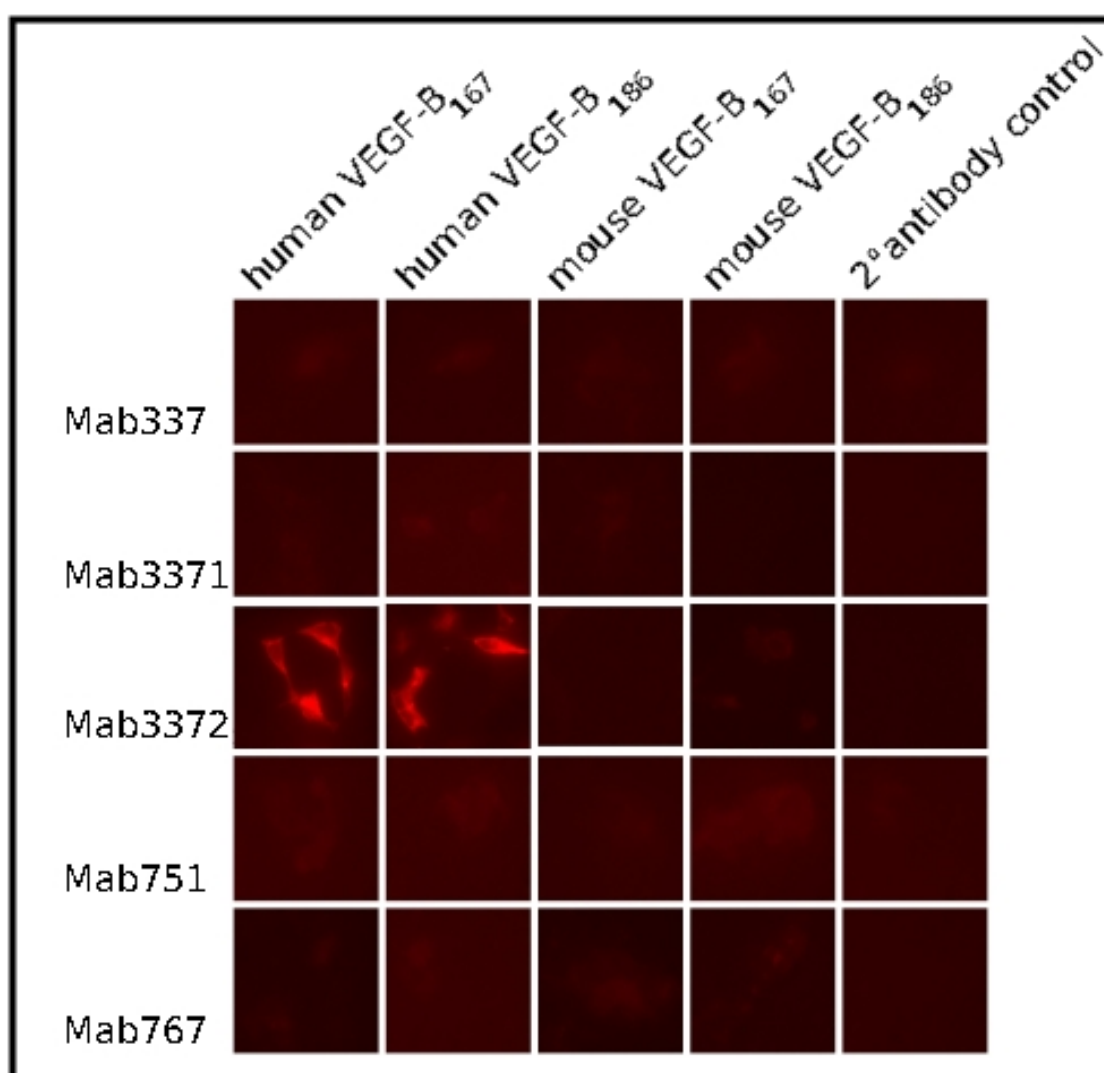The other antibodies did not recognize mouse or human VEGF-B isoforms.

Figure 6: **Antibody Mab3372 recognizes human VEGF-B$_{167}$ and human VEGF-B$_{186}$ isoforms.** The binding of antibodies Mab337, Mab3371, Mab3372, Mab751 and Mab767 to VEGF-B produced in COS-1 cells was analysed by transiently transfecting COS-1 cells with human and mouse VEGF-B and staining for VEGF-B with the antibodies using Alexa594 conjugated secondary antibody. Antibody Mab3372 recognized human VEGF-B isoforms whereas the other antibodies did not recognize the human or mouse isoforms. Visualization at x40 magnification.

4.1.3 Immunoprecipitation

Last, we tested the different monoclonal antibodies in immunoprecipitation. After the immunostaining studies we wanted to further characterize the binding properties of the antibodies with a sensitive and natural system that could distinguish between minor differences in binding of the antibodies to the recombinant proteins.
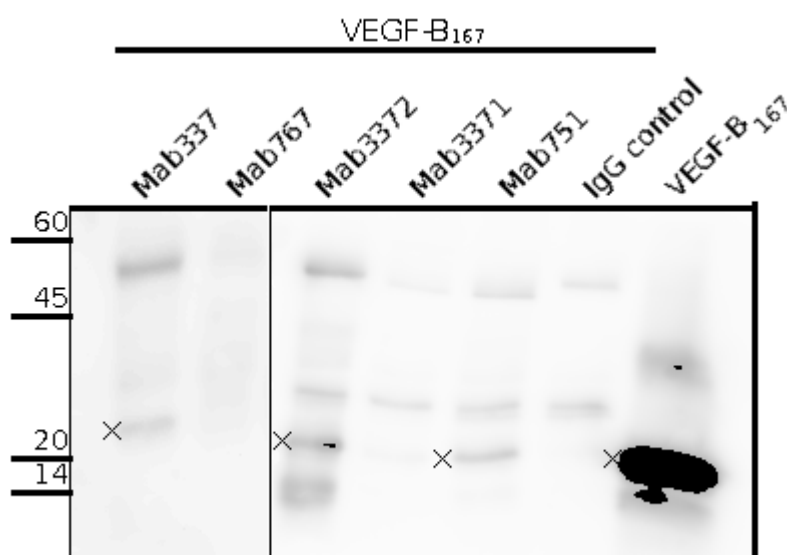

Figure 7: **Antibodies Mab337, mAB3371, Mab3372 and Mab751 recognize VEGF-$_{B167}$ in immunoprecipitation.** Testing of the antibodies for their ability to immunoprecipitate VEGF-B$_{167}$. Specific binding marked with asterisk. A IgG control for unspecific binding was used and recombinant VEGF-B$_{167}$ was loaded on the gel for positive control. VEGF-B$_{167}$ was precipitated with the antibodies and Protein A sepharose. The precipitated samples were run on 12% PAGE gels and blotted with Mab3372.The antibodies Mab3372, Mab751 and Mab337 have stronger specificity whereas the antibody Mab3371 binds only weakly. The antibody Mab767 does not recognize the protein.

We were especially interested to see whether the antibodies would bind the recombinant protein representing the receptor binding site, VEGF-B$_{10-108}$. In order to find this out, we decided to use immunoprecipitation. This would allow us to detect the binding even when it was low. We also could benefit from our results from Western

Blotting studies and use Ab3372 for blotting.

The result of binding of the antibodies to the human two VEGF-B isoforms and to the receptor binding region VEGF-B$_{10-108}$ are displayed in the three figures below. In these pictures also the heavy and light chains of the tested antibodies can be seen. The heavy chain at 55kDa and the light at 25kDa.
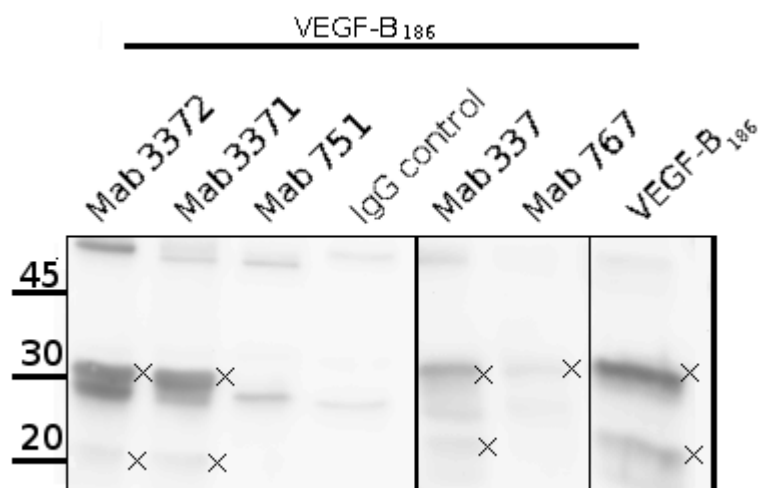

Figure 8: **The antibodies Mab3372, Mab751 and Mab337 and Mab767 recognize VEGF-B$_{186}$.** Testing of the antibodies for their ability to immunoprecipitate VEGF-B$_{167}$. Recombinant VEGF-B$_{186}$ was precipitated with the antibodies and Protein A sepharose. The precipitated samples were run on 12% PAGE gels and blotted with Mab3372. Specific binding marked with asterisk. A IgG control for unspecific binding was used and recombinant VEGF-B$_{167}$ was loaded on the gel for positive control. The size of the recombinant protein is only slightly more than the size of IgG light chain. The antibodies Mab3372, Mab751 and Mab337 and Mab767 recognize VEGF-B$_{186}$.
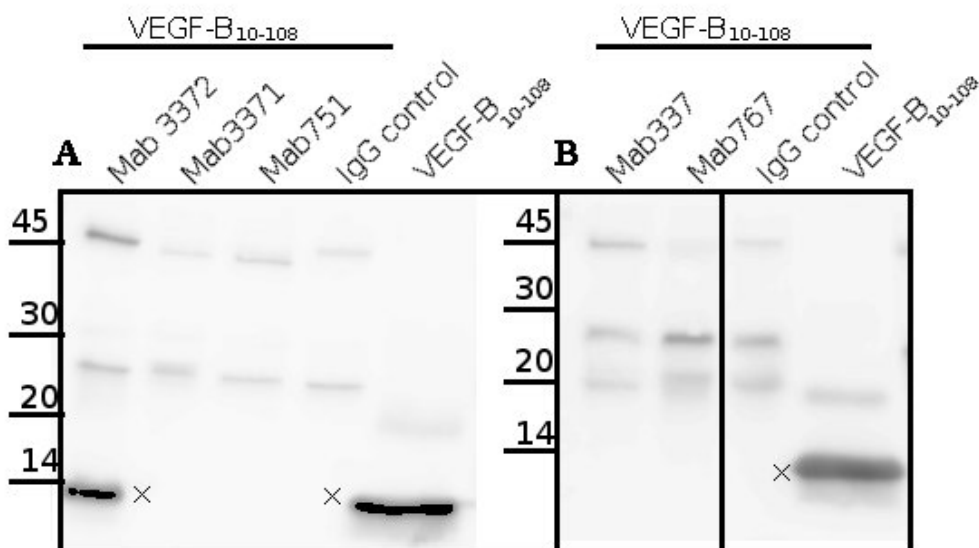
Figure 9: **Antibody Mab3372 precipitates VEGF-B$_{10-108}$.** VEGF-B$_{10-108}$ was precipitated with Protein A sepharose and the antibodies Mab767 (A) and antibody Mab751 (B). The precipitated samples were run on 12% PAGE gels and blotted with Mab3372. The binding of the antibodies towards the VEGF-B derived peptide containing amino acids responsible for receptor binding. Mab3372 recognizes the peptide. Specific binding marked with asterisk. A IgG control for unspecific binding was used and recombinant VEGF-B$_{167}$ was loaded on the gel for positive control. Specific bands marked with asterisk.

The antibodies recognized both the mature VEGF-B$_{186}$ O-glycosylated form at 32 kDa as well as the proteolytically processed protein around 20 kDa. The size of the recombinant protein is only slightly bigger than the size of antibody light chain. The antibodies Mab3372, Mab3371, Mab337, Mab767 recognized the protein (Figure 8).

We also tested how the antibodies recognize the recombinant peptide comprising amino acids from 10 to 108 that are responsible for receptor binding. Antibody Mab3372 was able to recognize this peptide in immunoprecipitation whereas the other antibodies did not recognize this peptide.

4.2 The angiogenic profile of MEF-Vegf-b-/- cells is not largely affected compared to the wild type cells

We wanted to further study the role of VEGF-B in Murine Embryonic Fibroblast (MEF) cell line by using a line where VEGF-B was knocked out and compare that to the respective wild type cell line. These cell lines had been transformed with Simian virus 40 (SV40) and human RAS resulting in immortalized cell lines that could also grow tumours in mice. We wanted to know whether knocking down *Vegf-b,* would affect the expression of other growth factors or receptors.

In order to find out the transcriptional activity of angiogenesis-related genes of interest we decided to measure the amount of total RNA in the SV40 human RAS oncogene transformed MEF-*Vegf-b-/-* cell lines using RT-PCR and compare that to the wild type SV40 human RAS oncogene transformed MEF-*Vegf-b+/+*-cell lines. We decided to test the expression level of VEGF/PDFG family members and VEGFR-1 (Flt-1) using RT-PCR (Figure 10).

GAPDH-PCR of the cell lines was performed in order to evaluate the success of RT-PCR. We also wanted to see whether the preparation of cDNA was equally successful in all cell lines tested (Figure 10).

The two VEGF-B knock-out cell lines were compared to the respective wild type cell lines. No major changes were found in the amount of
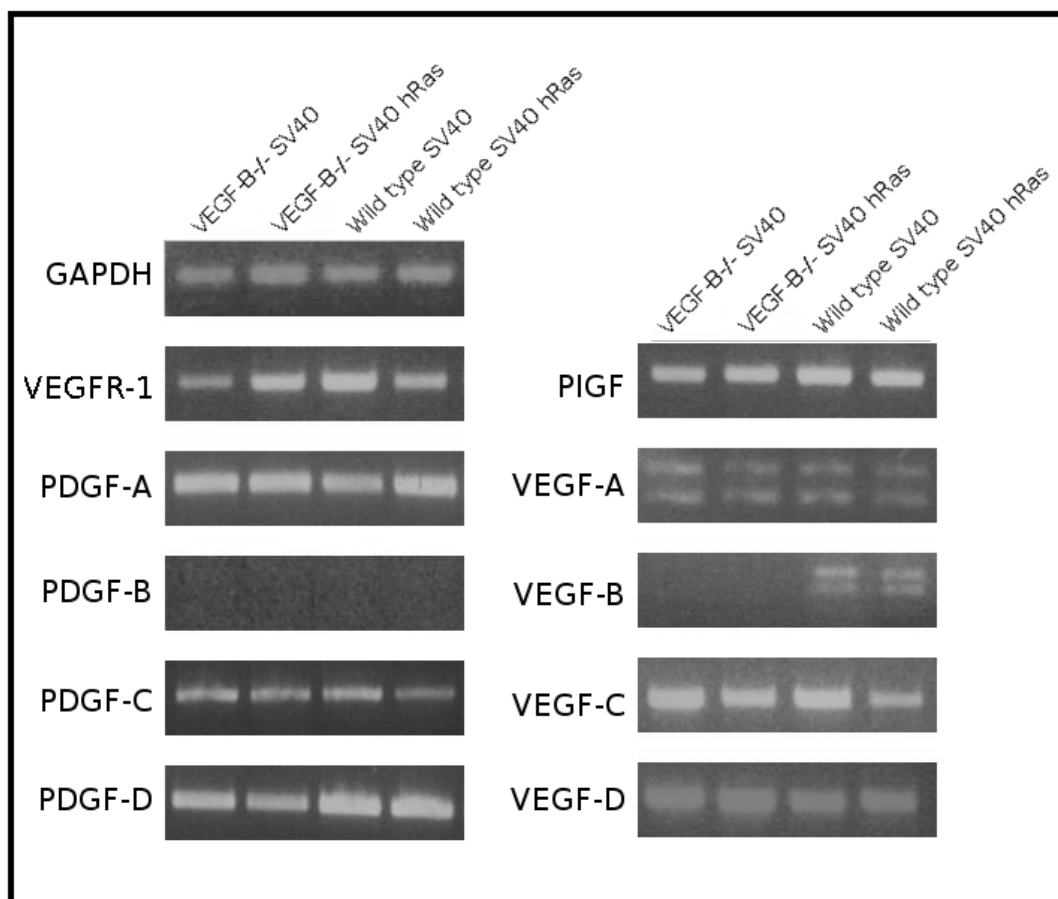

Figure 10: **The expression of angiogenic genes and receptors is not largely affected by knocking down** *Vegf-b.* Comparison of the angiogenic profiles of *Vegf-b* knock-out MEF cell lines and the respective Wild Type cell lines transformed with SV40 and human RAS. The total RNA of the cell lines was extracted and reverse transcribed to cDNA. Expression level of the genes of interest was tested using PCR.

angiogenic factors even though some of the factors tested were noticed to be slightly up or down regulated in the transformed knock-out cell lines. Interestingly, the amount of VEGFR-1 was slightly downregulated in the SV40 transformed MEF-*Vegf-b-/-* cell line and upregulated in MEF *Vegf-b-/-* SV40 and hRas transformed cell line. Also, the amount of PDGF-D transcript is diminished in the knock-out cell lines. Minor upregulation of the amount of VEGF-C and VEGF-D can also be seen in the knock-out cell lines.

4.3 Generation of Cell lines expressing VEGF-B$_{167}$, VEGF-B$_{186}$ or both isoforms

We next wanted to use the MEF-*Vegf-b* SV40 hRAS cell lines for generating tumour cell lines expressing VEGF-B$_{167}$, VEGF-B$_{186}$ or both isoforms. Since the cell lines used for generating the tumorigenic cell lines would not express VEGF-B, we could be certain of which isoform of VEGF-B IS responsible for which effect seen in the tumour cell lines.

We decided to use human VEGF-B isoforms in pcDNA3.1 vectors for transfection under Zeocin selection. We started with testing the constructs by transiently tansfecting COS-1 cells with human VEGF-B$_{167}$ and human VEGF-B$_{186}$ in pcDNA3.1 (pcDNA3.1VEGF-B167 and
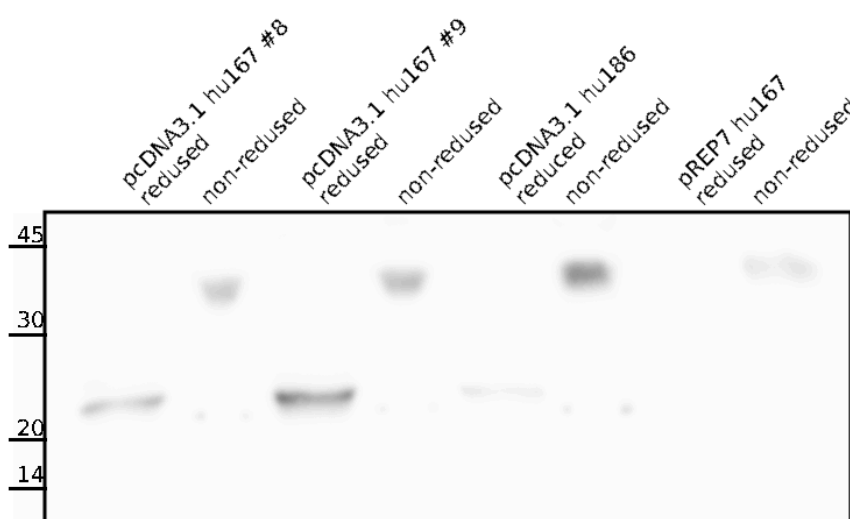

Figure 11: **Pilot transfection with different VEGF-B constructs**. VEGF-B$_{167}$ and VEGF-B$_{186}$ coding plasmids, pcDNA3.1VEGF-B167 clone 8 and pcDNAVEGF-B167 clone 9 used for pilot transfection of VEGF-B$_{167}$ and pcDNA3.1VEGF-B186 for VEGF-B$_{186}$. Human VEGF-B$_{167}$ indicated with hu167 and human VEGF-B$_{186}$ with hu186. Protein can be detected but the different isoforms are of the same size and not as expected VEGF-B$_{167}$ 21 kDa and VEGF-B$_{186}$ 32 kDa. The cells were lyced and TCA-precipitated. The precipitated proteins were run on reducing12% PAGE gels 25 mA and Western Blotted and detected with antibody Mab3372.

pcDNA3.1VEGF-B86) using VEGF-B$_{167}$ in pREP7 as positive control. We expected to see the isoforms VEGF-B$_{167}$ at 20 kDa and VEGF-B$_{186}$ 32

kDa. However, the size of VEGF-B$_{186}$ was not as expected but we saw a band of 21 kDa corresponding the size of VEGF-B$_{167}$ (Figure 11). We decided to sequence the plasmids used for transfections. The sequences of the plasmids were correct.

We continued with transfecting the pcDNA3.1VEGF-B167, pcDNA3.1VEGF-B86 or both plasmids into MEF-*Vegf-b-/-* SV40 hRAS cell lines in orger to create VEGF-B$_{167}$, VEGF-B$_{186}$ or both isoforms expressing cell lines. Unfortunatelly none of the cell lines tested expressed the protein at detectable levels (Figure 13 and Figure 14).
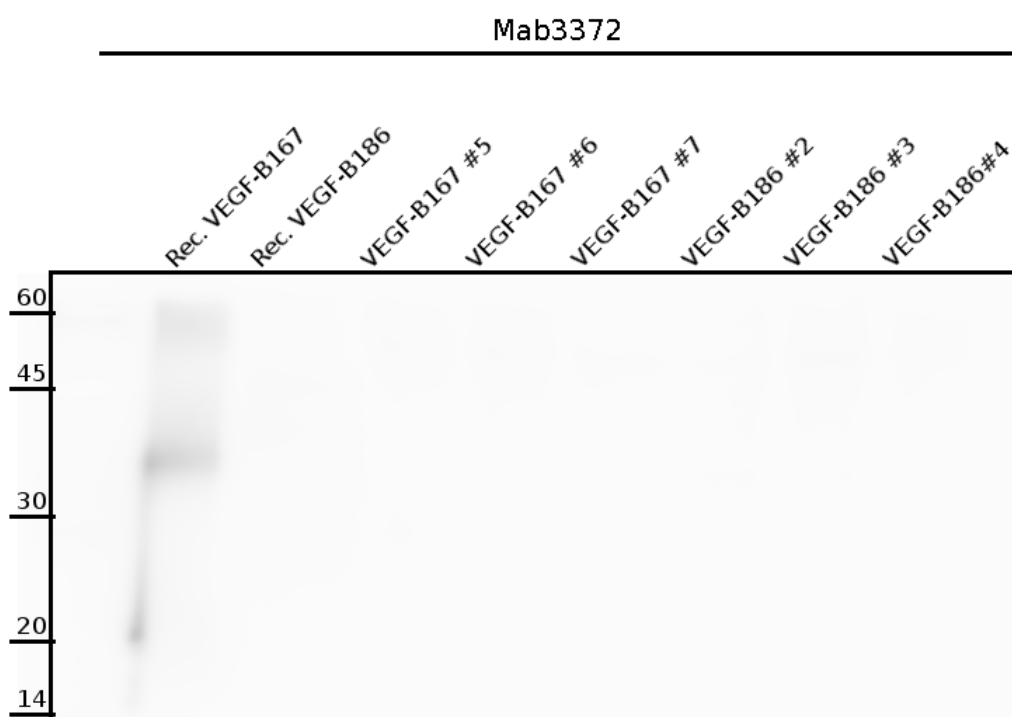

Figure 12: **The clones tested did not express VEGF-B at detectable levels.** The expression of MEF-VEGF-B-/- SV40-hRascells transfected with VEGF-B$_{167}$ or VEGF-B$_{186}$ isoforms was tested by lycing the cells and TCA precipitating the proteins. The precipitated proteins were run on 12% PAGE gels 25 mA and Western Blotted and detected with antibody Mab3372. The clones did not express VEGF-B.
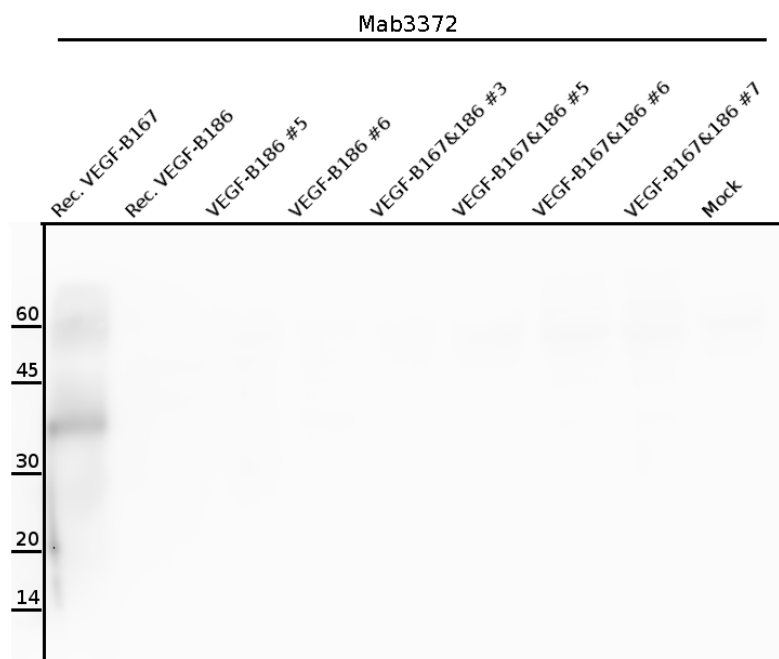
Figure 13: **The clones tested did not express VEGF-B at detectable levels.** The expression of MEF-VEGF-B-/- SV40-hRas cells transfected with VEGF-B$_{167}$ or VEGF-B$_{186}$ isoforms was tested by lycing the cells and TCA precipitating the proteins. The precipitated proteins were run on 12% PAGE gels 25 mA and Western Blotted and detected with antibody Mab3372. The clones did not express VEGF-B.

5. Discussion

In this study we have elucidated the capacity of available antibodies towards VEGF-B for their binding to the protein in Western Blotting, immunostaining and in immunoprecipitation.

One of the antibodies that we tested, Mab3372, was able to bind VEGF-B in Western blotting, immunostaining and in immunoprecipitation recognizing both human VEGF-B isoforms as well as VEGF-B$_{10\text{-}108}$ comrising of the receptor binding site. This makes it a useful tool for further studying VEGF-B function since it is capable of binding VEGF-B in different conditions.

In Western blotting, the proteins that we used were recombinant proteins produced in bacterial cells and thus different from the proteins that would be produced in mammalian cells. I think we could have included mammalian produced proteins in western blotting, for example by transfecting mammalian cells with the same plasmids we used for generation of new cell lines and then collecting the media from the cells and running that on the gel, or by TCA-precipitating the proteins as done when generating the new cell lines. This would have given us information on the binding of the antibodies to the mammalian produced proteins.

It would have made sence also to do the immunoprecipitations with these cell supernatants in addition to purified proteins. However, using purified protein gives more information of the affinity of the antibody, since one can use exactly the same amount of protein in the analysis whereas when doing the experiment with cell supernatants the amount of protein produced by the cells differs from transfection to another as well as between different constructs.

It is also interesting to speculate why immunostaining worked

only with one antibody. This was a protocol that could be further optimized by using different dilutions to test the antibodies and not only the ones recommended at the product sheet of the provider. I would also try staining the live cells by adding the antibody to the growth medium instead of permeabilized cells before fixing and fix and permeabilize after primary antibody incubation.

We also tried to generate new cell lines which did not express the protein. Since we only tested the blots with one antibody, Mab3372, which we previously showed to work in western blotting it might be that for some reason the detection did not work since the positive controls did not work either. I would test the blot with another antibody that's known to recognize VEGF-B for example antibody Mab337 or Mab3371 even though they only recognize one isoform of the protein. We should have also tested whether the original pools expressed the protein.

I also think that for the cell line expressing both VEGF-B$_{186}$ and VEGF-B$_{167}$, it would have been more reasonable to use two selection markers in these two plasmids which would have made it more probable that same cell expresses both VEGF-B$_{186}$ and VEGF-B$_{167}$ which would give us a homogenic population of cells expressing VEGF-B in stead of heterogenous population of cell expressing only one isoform.

It would be interesting to continue this study by elucidating whether these antibodies can block VEGF-B functioning and binding to VEGFR-1 or Nrp-1. We found a good candidate for these further studies, antibody Mab3372. It would be important to determine these antibodies really are blocking. I would suggest testing the antibodies also in VEGFR-1 phosphorylation assays and in BAF/3 VEGFR-1/EPO-assay in order to find out more about their blocking function.

In BAF3-assay the BAF3 cells contain a chimeric receptor

consisting of VEGFR-1 extracellular domain and Erythropoietin (EPO) intracellular domain. In this assay cell proliferation is measured by adding both VEGF-B which should stimulate the cells to grow and then adding the blocking antibody which should stop cell proliferation since EPO signal is missing. This would give direct information of whether the antibody is blocking the binding of VEGF-B to its receptor VEGFR-1. However, it does not tell about the receptor phosphorylation why I would suggest also doing the phosphorylation assay before injecting the tumours into animals.

In all, it is clear that Mab3372 is the most potent of these antibodies to study and maybe even block VEGF-B function, since it is also the only one that could detect the receptor binding region of the recombinant proteins. One way to block VEGF-B function is to inhibit its receptor binding which makes Mab3372 a good candidate for further studies since it could bind the receptor binding site of the protein.

The angiogenic profiles of the transformed knock-out cell lines compared to the transformed wild type cell lines do not differ significantly, which implies that at least knocking down VEGF-B in vitro does not affect angiogenic profile of these cells. I find it however interesting, that the expression of VEGFR-1 was downregulated in the MEF- *Vegf-b-/-* cell lines which would implicate that there is a correlation between the expresion of VEGF-B and its receptor VEGFR-1.

However, I think we should have considered the genes to be tested a bit more in detail. Even though I think that including all VEGFs and PDGFs in the list of tested genes is biased. I think we should have also included the Nrp1 and Nrp-2 co-receptors as well as other VEGFRs even though VEGF-B binds only to VEGFR-1. This would make the angiogenic profile more complete. One solution would have been to

make affymetrix instead of direct RT-PCR of all the cell lines and then testing for genes that seem upregulated in the affymetrix data in addition to VEGFs and their receptors.

In my opinion, the most important experiments for this study are still to be made. It is very interesting that Mab3372 succeeded to recognize VEGF-B in all of these methods we used and it would make sence to continue studying its blocking function. It will also be interesting to see, how VEGF-B expressing tumours would grow in mice, is there a difference in their angiogenic profile and how they differ from wild type tumours and whether VEGF-B function can be blocked with the tested antibodies *in vivo.*

Acknowledgements

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