Aus dem Institut für Klinische Chemie der
Ludwig-Maximilians-Universität München

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Investigations on endothelial maturation and anticoagulant properties

Dissertation
zum Erwerb des Doktorgrades in Humanbiologie
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

Vorgelegt von
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aus
Sliven, Bulgaria

2005

Mit Genehmigung der Medizinischen Fakultät
der Universität München

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Tag der mündlichen Prüfung: 04.08.2005

I. Introduction

Endothelial cells (ECs) line the inner wall of blood vessels in every organ and regulate the flow of nutrient substances, diverse biologically active molecules, and the blood cells themselves. The gate-keeping role of endothelium is influenced by the presence of membrane-bound receptors for numerous molecules including proteins (e.g. growth factors, procoagulant and anticoagulant proteins), lipid transporting particles (e.g. low-density lipoprotein [LDL]), metabolites (e.g. nitric oxide and serotonin), and hormones (e.g. endothelin-1), as well as through specific junctional proteins that govern cell-cell and cell-matrix interactions. Thus, the endothelium represents a dynamic, heterogeneous structure, a widely disseminated organ that possesses vital secretory, synthetic, metabolic, and immunologic functions.

The endothelial cells are highly heterogeneous among and within the tissues. Variations in the morphology of the capillaries in different organs have long been recognized and differences in function have been recently postulated. For example, the brain and retina are lined by continuous endothelial cells (ECs) connected by tight junctions that help to maintain the blood-brain barrier. The liver, spleen, and the bone marrow sinusoids are lined by discontinuous ECs that allow cellular trafficking between intercellular gaps, while the intestinal villi, endocrine glands, and kidneys are lined by fenestrated ECs that facilitate selective permeability required for efficient absorption, secretion, and filtering (Dejana 1996). ECs from diverse tissues are also heterogeneous with respect to their surface phenotype and protein expression. For example, von Willebrand factor (vWF), used commonly as a marker for ECs, is not expressed uniformly in all types of vessels (Kumar et al. 1987), the expression of tissue type plasminogen activator is limited in vivo to approximately 3% of vascular ECs (Levin et el. 1997), and the constitutive expression of u-PA is reportedly confined to renal ECs (Wojta et al 1989; Louise et al 1994). The induction of Tissue Factor (TF) after infusion of cytokines or endotoxin is similarly restricted to specific vessels (Drake et al. 1993).

One of the clearest examples for EC heterogeneity lies in the expression of homing receptors involved in cell trafficking. In the mouse, Lu-ECAM-1 (lung-specific EC adhesion molecule) is exclusively expressed by pulmonary post capillary ECs and some splenic venules (Zhu et al. 1991), whereas Mad-CAM-1 (mucosal addressin cell adhesion molecule-1) is expressed primarily on high endothelial venules in Payer's patches of the small intestine (Butcher et al. 1996). Microvascular ECs derived from the bone marrow bind to megakaryocytes and $CD34^+$ progenitor cells, and constitutively secrete hematopoietic stimulating factors such as Kit-ligand, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and interleukin-6 (IL-6), that contribute to control cell trafficking, proliferation, and hematopoietic lineage-specific differentiation (Rafii et al. 1995).

I.1 Endothelial progenitor cells (EPCs)

Mature endothelial cells are terminally differentiated cells with a low proliferative potential, and their capacity to substitute damaged endothelium is limited. Therefore, the endothelial repair may need the support from other cell types. Accumulating evidence in the last years indicates that the peripheral blood of mammalian organisms contains a unique subtype of circulating, bone marrow-derived cells with properties similar to those of embryonal angioblasts (Asahara et al. 1997). These cells, which have the potential to proliferate and differentiate into mature endothelial cells, were termed endothelial progenitor cells (EPCs). Recent studies in animals and humans suggest the ability of EPCs to ameliorate the function of ischemic organs, apparently indicated by both induction and modulation of vasculogenesis and angiogenesis in areas with reduced oxygen supply and/or by stimulating reendothelialization of injured blood vessels (Hristov et al. 2003). EPCs may have different origins, depending on the time of isolation and the source of isolation. There are bone marrow-derived EPCs characterized by markers like $CD34^+$, $CD133^+$, and vascular endothelial growth factor receptor-2 (VEGFR-2) known as KDR or FLK-1 (Peichev et al. 2000; Gehling et al. 2000). CD 34 is the main marker for progenitor cells; it is a cell surface glycoprotein involved in cell-cell adhesion and mediates the attachment of stem cells to bone marrow extracellular matrix or directly to stromal cells. CD133 (termed originally AC133) is a 120 kDa transmembrane polypeptide, expressed on hematopoietic stem and progenitor cells from human bone marrow, fetal liver and peripheral blood (Yin et al. 1997). A possible mix of both, early progenitor and endothelial phenotype is a $CD133^+$/ $CD34^+$/ VEGFR-2^+ cell, which does not express vascular endothelial (VE) cadherin and von Willebrand factor (vWF). Cells with these characteristics are localized predominantly in the bone marrow (Quirici et al. 2001). In the peripheral circulation of adults, more EPCs are found which have lost CD133, but are positive for CD34 and VEGFR-2. Mature ECs show a high expression of VEGFR-2, VE-cadherin, and von Willebrand factor. It is unclear at which time point the EPCs begin to lose their CD133, during the transmigration from bone marrow into the systemic circulation or later during their circulation. Circulating EPCs express with different intensity a variety of markers, typical for the endothelial lineage. These markers include platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31), CD146, VE-cadherin, vWF, endothelial NO synthase, and, after stimulation, E-selectin (Quirici et al. 2002; Kaushal et al. 2001). The release of EPCs from the bone marrow is regulated by a variety of growth factors, enzymes, ligands, and surface receptors. Activation of matrix metalloproteinase-9 (MMP-9), which promotes the transformation of membrane bound Kit ligand to a soluble Kit ligand and the subsequent migration of c-Kit-positive stem and progenitor cells to the vascular zone of the bone marrow microenvironment are initials steps in this process (Heissig et al. 2002).

I.2. Embryonic endothelial progenitor cells (eEPCs)

EPCs first appear at the early stages of vasculogenesis and angiogenesis during embryonic vascular development (embryonic EPCs or eEPCs). Hatzopoulos et al 1998, were the first to establish a method for isolation of cells from murine embryos at E7.5, with characteristic properties of endothelial progenitors by using a combination of an appropriate stromal cell layer and growth conditions. The isolated embryonic cells displayed unlimited stem-cell-like growth potential and a stable phenotype in culture. Analyses on the RNA level revealed that these cells express the endothelial specific genes Tie-2 and Thrombomodulin (TM), as well as the early mesodermal marker Fgf-3. These cells possess the potential for in vitro differentiation with retinoic acid and cAMP treatment, leading to the induction of a set of genes, including Flk-1, vWF, TM, GATA-4, and GATA-6. Electron microscopy reveals that in vitro differentiation is associated with increased amounts of rER and Golgi, and a dramatic increase in secretory vesicles packed with vWF. When these embryonic cells are cultured in Matrigel, they assume the characteristic endothelial cobblestone morphology and form tubes (Hatzopoulos et al 1998). Injection into chicken embryos shows incorporation of the cells in the endocardium and the brain vasculature. The expression of TM, Tie-2, GATA-4 and GATA-6 suggests that the eEPCs are derived from the proximal lateral mesoderm, where the pre-endocardial tubes form. These eEPC are a powerful tool for analyzing regulation of early vascular development. The circulating eEPCs appear to have a specific homing into tumor microvasculature, extravasate into the interstitium, form multicellular clusters, and incorporate into functional vascular networks. Expression analysis and in vivo blocking experiments provide evidence that the initial cell arrest of eEPC homing is mediated by E- and P-selectins and by P-selectin glycoprotein ligand 1 (Vajkoczy et al. 2003). Even more, genetically manipulated eEPCs armed with suicide genes like the yeast cytosine deaminase (CD) fused to uracil phosphoribosyl transferase (UPRT) converting the harmless 5-fluorocytosine (5-FC) to the cytotoxic compound 5-fluorouracil, have been used successfully to reduce lung metastases (Wei et al. 2004).

I.3. Endothelium and the MAPK signaling pathways

ECs are actively responding to a large variety of stimuli incoming from the surrounding environment. Such a response needs to be quick, specific, spatially and temporary coordinated with the signals received from other cells or tissues, in order to induce an appropriate response to the challenge. The intracellular signaling includes the mitogen-activated protein kinase (MAPK) signaling cascades. Activation of MAPK represents an early mechanism by which the cells transduce intracellular signals in response to extracellular stimuli. MAPK are activated via phosphorylation catalysed by distinct upstream kinases on both tyrosine and threonine residues within their catalytic domains. Since MAPK activations are vital for the cells, a high degree of homology exists between different species. The core component of a MAPK module is a set of three sequentially acting kinases. In the ECs, like in most mammalian cell types three MAPK modules can be distinguished: ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase) and the p38/MAPK pathways. These pathways regulate a variety of responses including cells growth, differentiation, and adaptation to the environment as well as apoptosis (Robinson 1997; Ip 1998). The MAPKs also control numerous regulatory processes during development and homeostasis (Schaeffer et al. 1999; Stronach et al. 1999).

The mammalian ERK module, also known as the *classic* mitogen cascade, consists of the serine/threonine kinase Raf, MEK, and ERK. For MEK and ERK, two isoforms have been described, while three isoforms for Raf, A-, B-, and C- are known. The Raf/MEK/ERK signaling cascade becomes strongly activated by mitogenic stimuli such as growth factors.

Binding of a growth factor [e.g. epidermal growth factor (EGF)] to the corresponding receptor tyrosine kinase (RTK) leads to its oligomerisation and subsequent autophosphorylation. Phosphorylated tyrosines on the intracellular part of the receptor serve as docking sites for the SH2-domain (*Src homology)* of adapter protein Grb2, which itself binds via the SH3-domain to the proline rich motif of the GDP-GTP exchange factor Sos (son of sevenless). Sos interacts with Ras and activates the exchange of GDP to GTP (Bonfini et al. 1992; Koretzky et al. 1997; Schlessinger et al. 1993). GTP loaded Ras leads to the recruitment to the cell membrane of the cytosolic serine/threonine kinase Raf (Rapp et al. 1983; Avruch et al. 1994; Stokoe et al. 1994). This translocation results in the activation of the membrane associated Raf. Ras-Raf interaction alone is not sufficient to activate Raf completely (Dent et al. 1995; Haffner et al. 1995). Important as scaffold proteins for Raf functions are also the 14-3-3 proteins, which modulate Raf activities (Hagemann et al. 1999).

The regulation of Raf is complex involving protein-protein interactions, phosphorylation of tyrosine, threonine, and serine residues and cellular relocalisation. Activation mediated by multiple kinases like Src, PKC (Protein-kinase C) and PAK (p21 (Rac/Cdc42)-activated protein kinase) leads to the phosphorylation and positive regulation of Raf (Morrison et al. 1997; Kolch et al. 1993). The down regulation of the Raf activity is achieved by PKA (protein kinase A) (Graves et al. 1993).

The first characterised downstream partner of Raf isoenzymes was the MEK kinase phosphorylated at Serine 218 and 222 (Macdonald et al. 1993; Papin et al. 1995). Once activated, MEK transduces the signals through phosphorylation on T-E-Y motifs in the MAP-kinases ERK1 (p44) and ERK2 (p42) (Boulton et al. 1991; Thomas et al. 1992). ERK phosphorylation increases its catalytic activity, mediates oligomerisation and transmits the signals into the nucleus. ERKs are nuclear shuttle kinases and have several described substrates in contrast to its upstream activators Raf and MEK. Their target proteins are e.g. serine/threonine kinases like 3pK, ribosomal S6 kinase ($RSK1^{p90}$), RNA-polymerase II, phospholipase A2, and several transcription factors like, Elk-1 and c-Jun (Sithanandam et al. 1996; Blenis et al. 1993; Ludwig et al. 1996; Hill et al. 1995). Expression of constitutively active components of the pathway causes cell transformation. All the kinases of the ERK pathway participate in tumour formation. It is important to note that decision to proliferate or differentiate depends on the length of time that MAPK pathway is activated (Lawlor at al. 2000).

I.4. Raf family proteins in the MAPK signaling

Raf comprises a family of three kinases, A-Raf, B-Raf and Raf-1 (also named C-Raf or C-Raf-1). C-Raf is expressed in all cell types and tissues but mostly in striated muscle, cerebellum, and fetal brain. A-Raf and B-Raf exhibit a rather restricted distribution. A-Raf is mostly expressed in kidneys, ovaries, epididymis and at low levels in other tissues (Storm et al.1990). B-Raf is predominantly expressed in neuronal tissue, testes, endothelium and in mammalian hematopoietic cells (Eychene et al. 1995). The genes for A-, B-, and C-Raf proteins are located on X chromosome at position Xp11.2, chromosome 7p34, on 3p25 (Naumann et al. 1996).

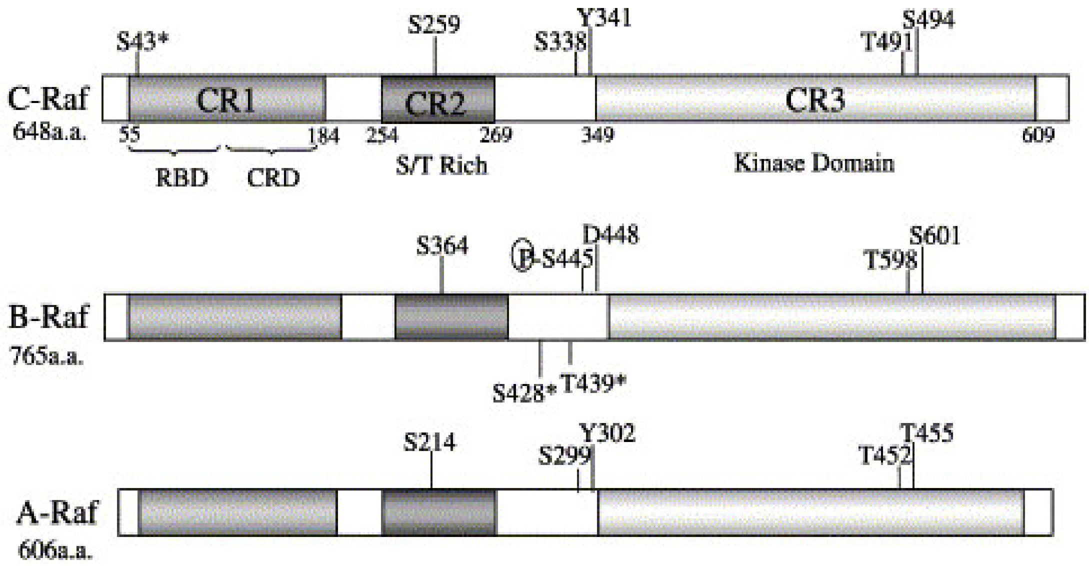


Figure 1-1 Raf family proteins domain structure

(From Chong et al. 2001)

B-Raf has an open reading frame of 2.3 kb, A-Raf transcript is 2.6 kb, and C-Raf is 3.4 kb. Molecular weight of A-Raf is 68 kDa; C-Raf has a calculated molecular weight of 74 kDa and B-Raf of 95 kDa. B-Raf has several splice variants, which are specific for the different tissues and cell types. For example in mouse brain extracts splice products were detected ranging from 65-70 kDa to 95-105 kDa (Moodie et al. 1994). In the PC12 cell line, B-Raf proteins were detected either as a single 95 kDa band (Stephens et al. 1992) or as two proteins at 67 and 95 kDa (Oshima et al.1991). The three proteins from the Raf family have a similar type of organization and high level of homology in between. They consist of three conserved regions CR1, CR2, and CR3. In between them there are linker areas. B-Raf shows 52% homology to A-Raf and 54% to C-Raf (Sithanandam et al. 1990) (Fig.1-1). The molecules in the Raf family contain two functional domains. The amino-terminal regulatory domain (containing the conserved regions CR1 and CR2), and the carboxy-terminal kinase domain (constituting the third conserved region, CR3) (Daum et al. 1994).

Caenorhabditis and *Drosophila* express a gene similar to mammalian Rafs. In bony fish, amphibians and birds only C- and B-Raf isoforms have been identified, but not A-Raf, while mammals have all three Raf genes (Calogeraki et al. 1993).

I.4.1. Regulation of Raf activities

The process of Raf activation, best analyzed for C-Raf, is still not completely understood. It is known to require protein-protein interactions, phosphorylation, and presumably lipid binding (Daum et al. 1994; Slupsky et al. 1998). It was proposed that Raf dimerization also might be involved in activation (Luo et al.1996; Masson et al. 1999; Farrar et al. 1996). There is increasing evidence that individual Raf isozymes are differentially regulated by these factors.

I.4.1.1. Small G Proteins

The small G protein Ras is the direct upstream activator of Raf, which in its GTP-bound activated form recruits the inactive cytoplasmic Raf to the plasma membrane for activation (Slupsky et al. 1998). There are differences in the ability of the of the different Ras isoforms to activate Raf proteins. K-Ras is more efficient in recruiting C-Raf to the plasma membrane than N-Ras and H-Ras, and N-Ras is more effective than H-Ras (Yan et al. 1998; Voice et al.1999). B-Raf is stimulated by K-Ras in a phosphatidylserine-dependent fashion, whereas H-Ras is able to increase B-Raf activity independent of this phospholipid (Kuroda et al. 1996).

The differences in Raf-isoform-specific binding of Ras-family members may be due to different binding affinities to the RBD of the Raf isozymes (Weber et al. 1999). The binding affinities of isolated A-Raf-RBD for H-Ras, TC21, and Rap1A were markedly diminished compared to those of C-Raf and B-Raf-RBDs. The interacting amino acids in Ras/Raf interface are identical for C-Raf and B-Raf, but there is a change from arginine to lysine in the A-Raf-RBD at the residue corresponding to position 59 in C-Raf. These data suggest that C-Raf and B-Raf act as preferential effectors for Ras, and that there may be different activators for A-Raf.

Ras-binding does not activate Raf directly, but seems to serve for translocation of Raf from the cytosol to the membrane where subsequent activation events occur (Dhillon and Kolch 2002). These comprise a complex series of events, starting with dephosphorylation of S259, which allows phosphorylation of S338 and possibly Y341 as well as two sites in the activation loop. These modifications work together not only to determine the quantity of signaling output, but also regulate its quality (Dhillon et al.2003)

The Rho family of small GTPases has been implicated in C-Raf activation. The Rho family which consists of Rho, Rac and Cdc42, regulates cytoskeletal structures such as stress fibers, lamellipodia and filopodia, respectively. However, these small G-proteins do not bind Raf directly but signal via activation of downstream kinases such as PKN1, PRK2 and ROCK1, where Rac and Cdc42 can signal through p21 activated kinase (PAK). For C-Raf activation the phosphorylation of S338 by PAK is crucial (King et al. 1998; Chaudhary et al.

2000). Consistent with the role of PAK, the upstream activators Rac and Cdc42 cooperate with Ras to activate C-Raf (Li et al.2001). The Rho family of small GTPases is a critical mediator which contributes indirectly to Ras-induced activation of Raf.

TC21 is another member of the family of the small G-proteins which binds B-Raf and at least the cysteine rich region of C-Raf involved in C-Raf activation (Rosario et al. 1999).

I.4.1.2. PKA and cAMP

The cAMP dependent Protein kinase A (PKA) differently regulates the three Raf isoforms. It is able to phosphorylate serine 43 of C-Raf, which inhibits the C-Raf kinase activation (Schramm et al 1994, Wu et al 1993). Additionally, Rap1 interacts with the RBD and CRD of Raf in competition with Ras binding (Hu et al. 1997) and antagonizes the Raf activation by Ras (Kitayama et al. 1989; Sacoda et al. 1992). In PC12 cells C-Raf activity is blocked by PKA, while B-Raf is activated and the differentiation of PC12 cells is promoted (Vossler et al. 1997; Ohtsuka et al. 1996; York et al. 1998). In neurons cAMP activates Erk in a Rap1/B-Raf dependent manner, while in astrocytes cAMP decreases Erk activity by inhibition of C-Raf. Thus B-Raf appears to be the molecular switch, which causes differential regulation of Erk in neurons versus astrocytes in response to cAMP (Dugan et al. 1999)

I.4.1.3. 14-3-3 proteins

14-3-3 proteins are expressed in most tissues and are highly conserved during the evolution. They form homo- and heterodimers in which each monomer is able to bind proteins implicated in signal transduction resulting in tetrameric complexes. Different 14-3-3 isoforms (e.g. β, ζ, ε, η, θ) interact with various Raf family members and are necessary for the stability of the inactive as well as the fully active conformation of Raf (Tzivion et al. 2002; Papin et al. 1996).

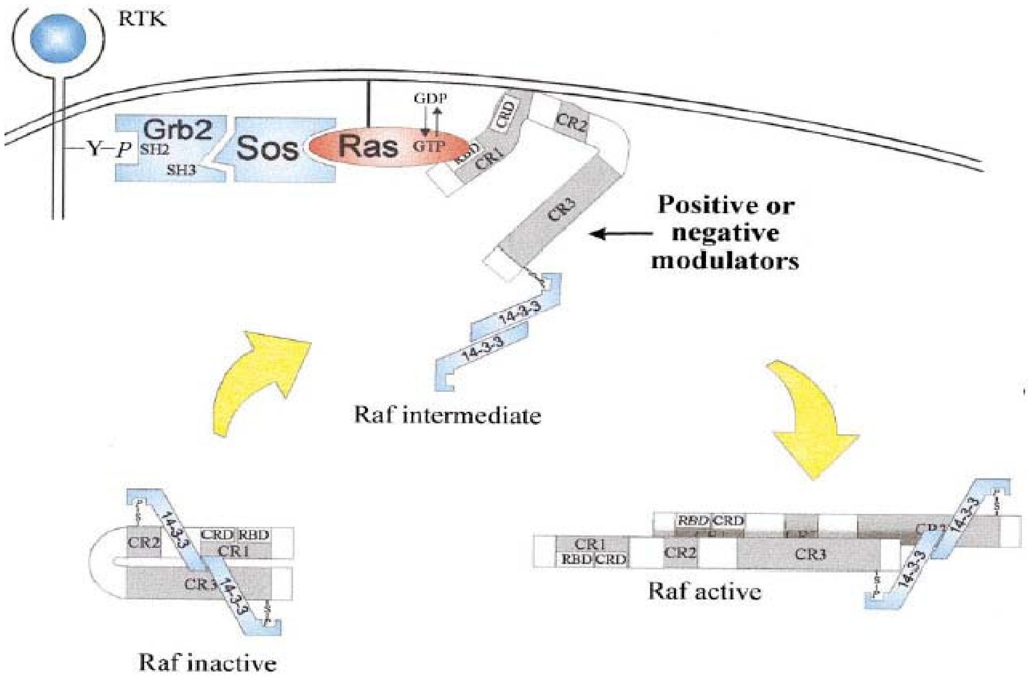


Figure 1-2 Raf regulatory mechanisms (adapted from Hagemann and Rapp 1999)

In the cytoplasmic conformation the N-terminal regulatory half of the Raf protein probably interacts with its own kinase domain (Daum et al. 1994, Morrison et al. 1997). This structure is presumably stabilized by binding of the 14-3-3 dimer to the phosphoserines 259 and 621 (C-Raf) (Tzivion et al. 2002) (Fig.1-2). Binding of active Ras to the RBD and the cysteine-rich region of Raf displaces the 14-3-3 proteins at least from serine 259 (Rommel et al. 1996), possibly from both binding sites (Roy et al. 1998). This leads to opening of the protein structure and causes a basal activation. Then the active Raf is able to dissociate from Ras and to transmit the signal to its substrate MEK in the cytoplasm.

14-3-3 proteins are able to serve as adapter proteins. 14-3-3 proteins are highly helical proteins. They interact via their N-termini with each other and form a large negatively charged channel which is responsible for recognition of target proteins (Petosa et al. 1998). For example the serine/threonine kinase Bcr forms a complex with 14-3-3 and associates with membrane bound C-Raf.

I.5. The role of Raf proteins in vivo

Mice with a targeted genetic disruption of the A-Raf gene revealed neurological and intestinal abnormalities, abnormal movement, tremor and rigidity in musculature. The mice died from extensive bowel distension between day 7 and 21 postpartum (Pritchard et al. 1996). However, the lack of A-Raf did not affect the regulation of ERK in mouse embryonic fibroblasts or the transformation of these cells, suggesting that these functions are compensated for by other Raf family members (Mercer et al. 2002).

B-Raf knockout mice die *in utero* around day 12.5 (Wojnowski et al. 1997). They have increased number of embryonic endothelial progenitor cells, increased apoptosis of differentiated endothelial cells, and dramatically enlarged and ruptured vessels leading to massive hemorrhage into major body cavities. They show subtle anatomical abnormalities in neuronal tissue with disturbed growth and differentiation of the neuroepithelium.

The phenotype of the C-Raf knockouts is dependent on the genetic background. Target disruption of the C-Raf gene in an inbred background results in a lethal phenotype between E10.5 and 12.5, although the mice still express low amounts of a N-terminal truncated form of C-Raf with reduced kinase activity. In the outbred background 129/OLA the mice develop to term, are born, and die within hours after birth, because their lungs fail to inflate. They show general growth retardation and defects in placenta, skin, and lungs (Wojnowski et al. 1998)

These findings indicate that the Raf proteins play essential roles during embryonal development and organogenesis. While A-Raf is apparently important for the intestinal and neurological development, B-Raf regulates vascularization and suppresses programmed cell death, and C-Raf is involved in growth control and skin, lungs, and placental development. The dependence of A- and C-Raf phenotypes on the genetic background implies that other genetic modifiers or the other Raf isoforms can partially compensate for the lack of one Raf isozyme. The phenotype of C-Raf and B-Raf mutants is exacerbated in the absence of functional alleles of the other gene (Wojnowski et al. 1998). When a C-Raf mutant is crossed into a B-Raf mutant background, the loss of one additional Raf allele (C-Raf$^{-/-}$ /B-Raf$^{-/+}$ or C-Raf$^{-/+}$ /B-Raf$^{-/-}$) increases dramatically the abnormalities. On the molecular level it results in complete loss of ERK activation and severely reduced expression of downstream transcriptional targets c-Fos, Egr-1 (O`Neil and Kolch 2004). The phenotype is characterized with underdevelopment of brain and head structures, defects in heart and limbs. All this leads to the death of 90% of the embryonic lineages, but had no effect on cell proliferation and implantation of the embryo. Embryos developed to small clumps of cells, divided into two distinct lineages closely resembling the inner cell mass and the trophoblast cells of the mouse blastocyst (Wojnowski et al. 1999; Mikula et al. 2001; Huser et al. 2001).

The results suggest that in addition to their unique functions, B-Raf and C-Raf may cooperate during embryonic development. The molecular mechanism of the cooperation between B-Raf and C-Raf is not well understood. It could be related to the ability of C-Raf to heterodimerise with B-Raf. The heterodimer, conceivably, could have different signaling properties than either of the individual proteins alone (O`Neil and Kolch 2004).

I.6. Anticoagulant properties of the endothelium

A crucial physiologic function of the endothelium is to facilitate blood flow by providing an antithrombotic surface that inhibits platelets adhesion and clotting. When the endothelium is perturbed by physical forces or by specific chemical factors, the cells undergo programmatic biochemical changes that culminate in their transformation to a prothrombotic surface. A dynamic equilibrium exists between these two states, modulated both at the level of gene transcription and at the level of the intact cell that often permits the injured endothelium to return to its unperturbed state once the procoagulant stimuli has dissipated (Fig. 1-3).

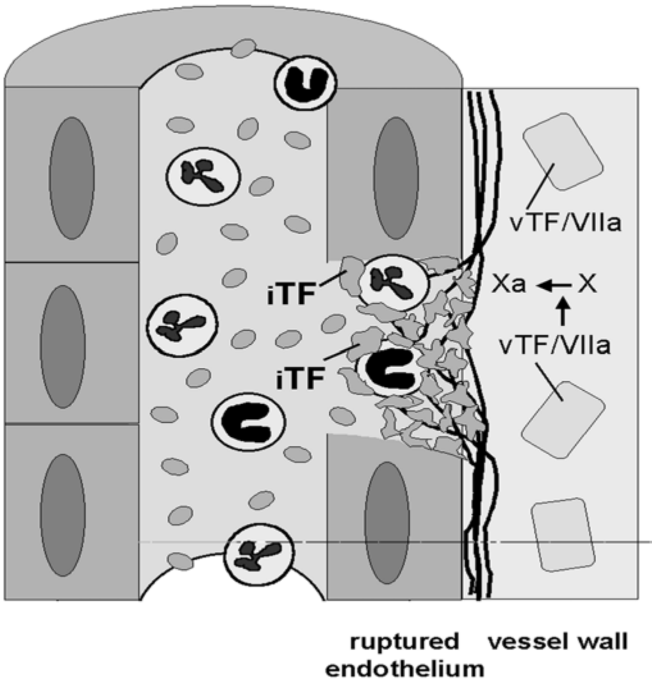


Figure 1-3 Model of activation of intravascular coagulation, where the main participants are the injured endothelium and the factors from the coagulation cascade released by the activated blood cells.
(From Engelmann et al. 2003)

Control of thrombin generation is a pivotal step in the balance between the natural antithrombotic and the induced procoagulant activities of the endothelium. Thrombin, a serine protease, serves diverse functions in coagulation, including the activation of platelets and of several coagulation enzymes. Several highly regulated pathways have evolved to constrain the generation and activity of thrombin (Rosenberg and Rosenberg 1984). The matrix surrounding the endothelium contains heparan sulfate and related glycosaminoglycans (GAGs) that promote the activity of the cell/matrix associated antithrombin III (AT-III) (Marcum and Rosenberg 1984). The endothelium also supports the anticoagulant influence of thrombin by expression of thrombomodulin (TM). Binding of thrombin to TM facilitates the enzyme's ability to activate the anticoagulant protein C. In turn the activity of activated protein C (APC) is enhanced by its cofactor protein S, which is synthesized by ECs, among other cell types (Fair et al. 1986). ECs express also receptors for APC (Fukodome et al.1996) that regulate the activity of this pathway.

All these mechanisms were already known as anticoagulant mechanisms prior to 1983. Today, tissue factor (TF) is considered as the main and basic inducer of the coagulation cascade. Although there were earlier indications that an inhibitor of TF/VIIa complex may be present in the serum, compelling evidence for such an inhibitory mechanism was not discovered until 1983 in Rapaport's laboratory.

The Rapaport's laboratory found that the VIIa/TF inhibitor is a trivalent Kunitz-type inhibitor, previously referred to as the extrinsic pathway inhibitor (EPI) or the lipoprotein associated coagulation inhibitor (LACI), and now termed tissue factor pathway inhibitor (TFPI) (Sanders et al. 1985; Broze et al. 1990). The endothelium is generally recognized as the main source of TFPI.

I.6.1. TFPI gene structure and regulation

The TFPI gene has been mapped to chromosome 2 at region 2q31-2q31.1 (Girard et al.1991; van der Logt et al. 1991). It spans approximately 70 kb and consists of nine exons and eight introns (Fig. 1-4).

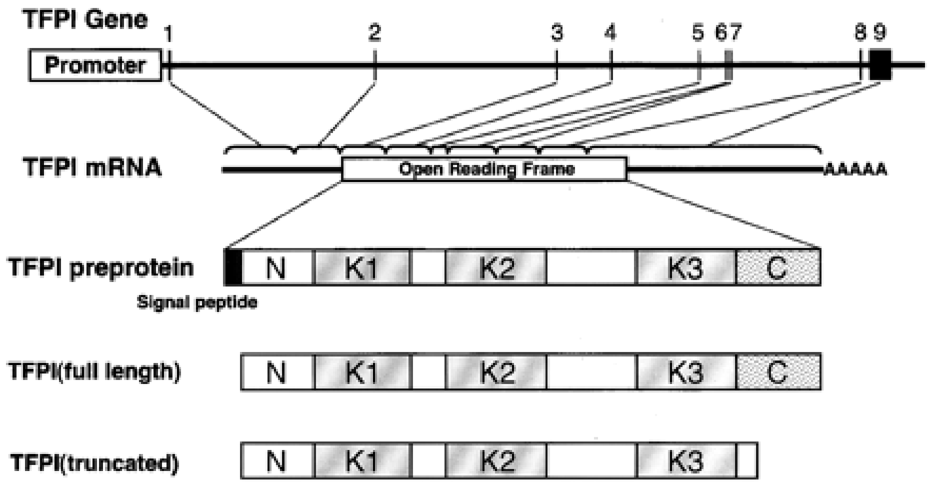


Figure 1-4 Expression of the TFPI protein from the TFPI gene. TFPI mRNA is transcribed from the TFPI gene, which consists of 9 exons. After the signal peptide is released from the TFPI preprotein, full-length TFPI is expressed. By the action of various proteases, a truncated form of TFPI is produced. N indicates N-terminal portion; K1, K2, and K3, Kunitz domains 1, 2, and 3, respectively; and C, C-terminal portion

(From Kato H. 2002)

The entire exons 1 and 2 make up the 5' untranslated region of the TFPI mRNA, exon 3 codes for the signal peptide and the NH_2-terminal of the TFPI protein. Exons 4, 6 and 8 code for the three Kunitz-type domains, and exons 5 and 7 code for the linker regions. Exon 9 codes for the C-terminal of TFPI, as well as the entire 3' untranslated region. The TFPI mRNA found in the endothelium and various other cell types exist as a 4 kb and 1.4 kb species (Bajaj et al. 1990; Girard et al.1989). The difference between the two mRNA sizes is attributed to the presence of ~2.6 kb additional untranslated sequences in the 4 kb transcript. TFPI has two isoforms known as alpha and beta that result from alternative splicing. The beta isoform is the shorter one with 251 amino acids translation product, and the alpha isoform has 304 amino acids, where 28 are signal peptide (Zhang J et al. 2003).

A unique transcription initiation site for TFPI mRNA synthesis has not been mapped; neither a consensus TATA box nor a CCAAT sequence. Instead two major transcription initiation sites have been identified in the 5' flanking region at cytosines 508 and 521 (Girard et al.1991; van der Logt et al. 1991). Two NF-1 (TGF-β- like response elements) and two AP-1 sites (phorbol ester response elements) with imperfect sequence homology have been reported (Tyson et al. 1993) Additionally, four perfect and one imperfect GATA motifs have been identified, which act as cis-regulatory elements, three of them closely flanking the major transcription initiation sites. GATA motifs in TFPI gene may bind the GATA-2 transcription factor, which is also expressed in endothelium, and thereby regulate TFPI gene expression (Bajaj et al 1994; Ameri et al. 1992). Also one SP-1 site, known for its role in the transcription activation of TATA-less promoters, and two c-Myc sequences are believed to play a role in the tissue specific expression of TFPI. Notably, no sites for OCT-1, OCT-2, NFκB, AP-2, CREB or CRE have been identified in the sequenced regions (Petit et al. 1999).

Recently, a second related gene named TFPI-2 has been identified on chromosome 7 into the human genome. Its product is also a Kunitz type inhibitor, but less potent than TFPI and more engaged as a blocker of other serine proteases (Chand et al. 2004).

I.6.2. TFPI synthesis and distribution

TFPI is synthesized primarily by the vascular endothelium under normal physiologic conditions (Bajaj et al. 1990), and restricted to the microvascular endothelium and megakaryocytes (Werling et al. 1993). The expression of the TFPI gene by the endothelium of different organs appears to vary, probably based on the physiologic demands of the tissue. Lungs and heart express the highest quantities of TFPI mRNA in human and mice (Bajaj et al. 1999). Importantly, cells that do not synthesize TFPI under physiologic conditions in adults are hepatocytes, neutrophils and lymphocytes (Osterud et al. 1995). In human fetus TFPI is expressed in the endothelium, liver, as well as in the epithelium of the lung, kidney, intestine, and it is abundantly present in placental syncytiotrophoblasts and cytotrophoblasts (Edstrom et al. 2000), where it helps to maintain the blood in a fluid state.

TFPI expression can be modulated in response to various stimuli. Shear stress applied to human endothelial cells increases TFPI expression at around two-fold (Grabowski et al. 2001). Thrombin may release cellular TFPI from the vascular endothelium and contribute to an increase in plasma TFPI with a simultaneous increase in endothelial surface TFPI (Lupu et al. 1995). Higher TFPI levels are observed when heparin is administrated intravenously in part because of the release the endothelial glucosaminoglycan-bound TFPI. These observations may explain some of the anticoagulant effects of heparin (Sandset et al. 1988).

TFPI is distributed in three pools in vivo; ~80-85% is associated with the endothelial surface, ~10% circulates in plasma in association with lipoproteins and a small amount in free form, and ~ 5% to 10% is found in platelets (Broze et al. 1994). TFPI associated with plasma lipoproteins is C-terminally truncated, and is a less efficient anticoagulant compared with the full length TFPI (Wesselschmidt et al.1992).

I.6.3. TFPI protein structure and function

The translated amino acid sequence from the human TFPI cDNA consists of 276 amino acids with 18 cysteines and three potential N-linked glycosylation sites. The molecular weight of the protein varies depending on the complexes it takes part and the level of glycosylation. The translated protein is around 34 kDa, the plasma lipoprotein associated form (LDL, HDL, VLDL) is between 35-45 kDa, and the platelets glycosylated form is at around 45-47 kDa. TFPI contains a highly negatively charged amino terminus, three repeated Kunitz-type domains in tandem with intervening linker regions, and a highly positively charged carboxyl terminus (Wun et al. 1988).

The domain 1 (K1) of TFPI binds to factor VIIa and domain 2 (K2) binds to factor Xa. The precise function of the third Kunitz-type domain is not fully understood, but it has no inhibitory properties (Girard et al. 1989). The mechanism of inhibition of TFPI involves first inactivation of factor Xa, followed by binding to the VIIa/TF complex (Baugh et al. 1998) (Fig.1-5). Importantly, the Xa-TFPI complex is a much more potent inhibitor of VIIa-TF than TFPI by itself.

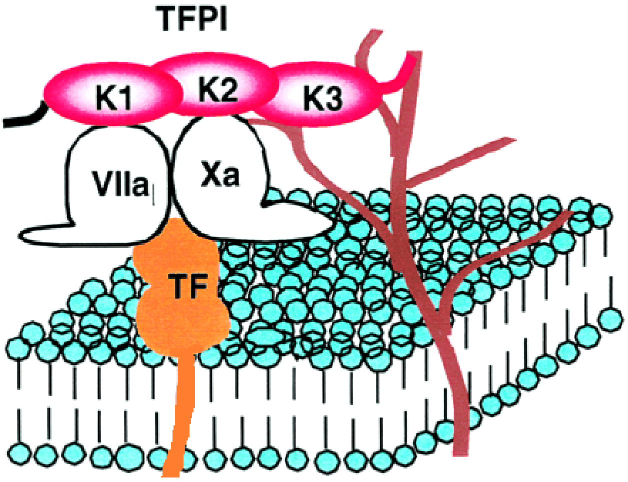


Figure 1-5 Inhibitory mechanisms of the initial reaction of blood coagulation by TFPI on cell surfaces. TFPI binds to factors VIIa and Xa via the K1 and K2 domains and to proteoglycans via K3 and C-terminal domains. (Adapted from Kato H. 2002)

Although a number of additional targets for attachment are reported, the C-terminal basic region appears to play an important role in binding TFPI to cell surfaces. TFPI has an important function in embryonic development because TFPI homozygote gene knockout mice die around day 9.5-11.5 characterized by retardation of embryonic growth and hemorrhages in the central nervous system, tail and the yolk sac. The heterozygote TFPI knock outs are viable and reproductive; they have around 50% reduced amounts of TFPI in the plasma and increased risk of atherosclerosis and thrombosis (Huang et al. 1997; Westrick et al. 2001)

I.6.3.1. Proteolytic digestion of TFPI

In many inflammatory conditions the activated leukocytes release several types of proteases, and TFPI is one of their substrates. TFPI is indeed cleaved by serine proteases, including elastase (Petersen et al. 1992; Higuchi et al. 1992), cathepsin G (Petersen et al. 1992), as well as by different matrix-metalloproteinases (Belaaouaj et al. 2000; Cunningham et al. 2002). Proteases included in the blood coagulation also take part in the TFPI degradation, such as thrombin (Ohkura et al. 1997), factor Xa (Salemink et al.1998), and plasmin (Stalboerger et al. 2001). The sites of cleavage in the TFPI molecule are denoted on Fig.1-6. Proteolytic degradation of TFPI increases the activity of its antagonist tissue factor, and therefore prolongs the initiation process of the blood coagulation.

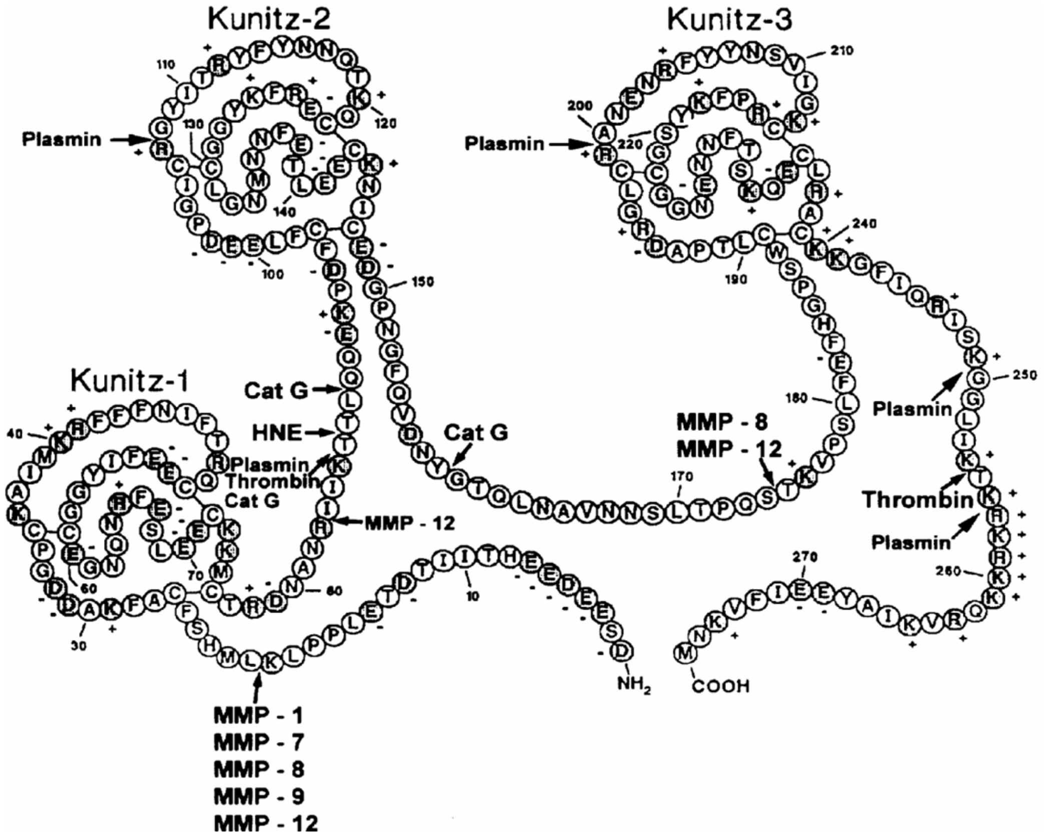


Figure 1-6 Sites within TFPI susceptible to proteolytic degradation. Sites of cleavage by MMP-8 and other proteinases, including MMP-1, -7, -9, -12, human neutrophil elastase (HNE), thrombin, plasmin and cathepsin G (Cat G) are denoted by arrows on the diagram of the primary amino acid sequence and Kunitz domain structure. (From Cunningham et al. 2002)

I.7. Aims of the investigation

During the last years, increasing evidence has indicated an important role for the endothelial cells and their precursors in vasculogenesis, angiogenesis, and anticoagulant processes. The Raf family proteins are part of the main signal transduction pathways regulating the homeostasis of the endothelial cells. It was therefore of interest to explore the functions of the Raf proteins in differentiation and proliferation of the eEPCs. To this purpose, we prepared gene constructs allowing us to activate or inhibit the downstream signaling of B-Raf and C-Raf. To further delineate the participation of the endothelium in coagulation, the role of native TFPI and its mutated forms in intravascular fibrin formation was analyzed. Particular attention was given to mutants being resistant towards cleavage by leukocyte proteases that might inactivate TFPI under physiological and pathophysiological conditions. In the present study, we addressed the following questions:

1. What is the role of B-Raf and C-Raf in the differentiation and proliferation of the embryonic endothelial progenitor cells?
2. How do these cells respond to a regulatable over expression of the kinase domains of B-Raf and C-Raf?
3. How are the functions of the eEPCs affected by the “knock down” of both B-Raf and C-Raf?
4. What is the role of the native TFPI and its different mutated forms for the coagulation start within the blood vessel?
5. Do alterations of proteolytic cleavage sites modify the anticoagulant behaviour of TFPI?

II. Materials and Methods

II.1. Materials

II.1.1. Instruments

Items	**Companies, Type**
Bacterial incubator	**Heraeus B 6200**
Bacterial shaker	New Brunswick Scientific innova 4330
Cell culture incubator	Köttermann
Cell culture microscope	Carl Zeiss
Chromatography columns (0.8 x 4 cm)	Bio Rad
Coulter counter	Becton Dickinson
Culture Hood	HLB2472, BIO-FLOW Technik
Developing machine	AGFA
Electrophoresis power supply EPS600,	Pharmacia
Electrophoresis unit, small	Bio-Rad Mini-Protean II
ELISA Reader Dynatech MR7000	Dynatech Laboratories
Fine scale	Scaltec SBC 21
Gel dryer	Bio-Rad Gel Dryer 583
Heat block	Liebisch, Type 2099-DA
Horizontal electrophoresis gel	MWG Biotech
Mega centrifuge	J-6B,Beckman; Megafuge 1.0 R, Heraeus; RC 5B plus, Sorval
Mini centrifuge	5417R, Eppendorf
pH meter	Microprocessor, WTW
Shakers	Heidolph, Unimax 2010, Edmund Bühler
Scale	BP2100S, BP310S, Sartorius
Spectrophotometer	U-2000, Hitachi
Thermocycler	Byozim
Thrombelastograph RoTEG	Dynabyte
Vortex	Scientific Industries Genie-2
Water bath	GFL 1083, Amersham-Buchler

II.1.2. Reagents and general materials

Items	**Companies**
1 kb DNA ladder	Sigma
100 bp DNA ladder	New England Biolabs
Acetyl-CoenzymA	Sigma
Acrylamide (30%)/Bisacrylamide (0,8%)	Roth
Adenosin-5'Triphosphate (ATP)	Sigma
Agarose, ultra pure	Life Technologies, Inc.
Ammonium peroxydisulfate (APS)	Sigma
Ampicillin	Sigma
Anhydrotetracyline	IBA
Aprotinin	Roth
Bacto-Agar	Roth
Beriplex PN 500	Behring
Bovine serum albumin (BSA)	Sigma
Bradford-reagent	Biorad
Bromphenolblue	Sigma
Calciumchloride ($CaCl_2$)	Sigma
Dimethylsulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
dNTP	Roche
Ethylenediaminetetraacetic acid (EDTA)	Sigma
EGTA	Sigma
Ethanol	Roth
Ethidiumbromide	Life Technologies, Inc.
Glutathion-sepharose	Pharmacia
Glycerol	Sigma
Glycine	Roth
Hydrochloride (HCl)	Roth
4-hydroxyazobenzene-2-carboxylic acid-HABA	IBA
HEPES	Roth
Isopropanol	Merck

Kanamycin	Sigma
Leupeptin	Sigma
Magnesiumchloride	Sigma
Nitrocellulose membrane	Schleicher & Schüll
Pefablock	Roth
PMSF	Roth
Ponceau S	Sigma
Potassium acetate (KAc)	Sigma
Potassiumchloride (KCl)	Sigma
Potassiumdihydrophosphate (KH_2PO_4)	Merck
Protein A-agarose	Roche
Protein marker (SDS-7B)	Sigma
SDS ultra pure	Roth
Sodiumdihydrophosphate (NaH_2PO_4)	Merck
Sodiumhydrophosphate ($NaHPO_4$)	Merck
Sodiumhydroxide (NaOH)	Sigma
Sodium orthovanadate	Sigma
Strep-Tactin II	IBA
S2222	Haemochrom diagnostica
TEMED	Roth
Tris-(hydroxymethyl)-aminomethane (Tris)	Roth
Triton-X100	Sigma
Whatman 3MM Papier	Schleicher & Schüll
X-ray film	Amersham-Buchler
Xylencyanol	Roth
Yeast extract	Life Technologies, Inc.

II.1.3. Cell culture materials

Items	**Companies**
Lypofectamin 2000 TM	Life Technologies, Inc.
Oligofectamine	Life Technologies, Inc.
Fetal bovine serum (FBS)	GIBCO
L-Glutamine	Life Technologies, Inc.
G-418 (antibiotic)	Invitrogen
Penicillin /Streptomycin	Life Technologies, Inc.
DMEM	Life Technologies, Inc.
Trypanblue	Sigma

II.1.4. Enzymes

Items	**Companies**
Calf Intestinal Phosphatase (CIP)	New England Biolabs
DNase	Qiagen
Pfu Taq Polymerase	Stratagene
Pfx Platinum Polymerase	Invitrogen
Reverse transcriptase	Invitrogen
RNase	Roche
T4 DNA Ligase	New England Biolabs
Vent Polymerase	New England Biolabs

General restriction enzymes are purchased from Fermentas and New England Biolabs

II.1.5. Antibody conjugates

Antibodies	**Antigens**	**References**
anti-ER (C-14)	ER	Santa Cruz (sc-154)
anti-B-Raf (C-19)	B-Raf	Santa Cruz (sc-166)
anti-C-Raf (C-12)	C-Raf	Santa Cruz (sc-133)
anti-B-Raf	B-Raf	Upstate (07-453)
anti-C-Raf	C-Raf	Upstate (07-396)
anti-MEK1/2	MEK1/2	Cell signaling technology (9122)
anti-Phospho-MEK1/2	Phospho-MEK1/2	Cell signaling technology (9121)
anti-TFPI (C-20)	TFPI	Santa Cruz (sc-18713)
anti-Mouse IgG conjugated peroxidase (POD)		Dianova
anti-Rabbit IgG conjugated peroxidase (POD)		Dianova
anti-Goat IgG conjugated peroxidase (POD)		Dianova

II.1.6. Kits

Items	**Companies**
ECL Western blotting detection reagents	Amersham
Gene Tailor^{Tm} Site-Directed Mutagenesis Kit	Invitrogen
Macs magnetic microbeads cell isolation Kit	Milltenyi Biotech
QIAGEN Gel Extraction Kit	Qiagen
QIAGEN Plasmid Kit (Midi, Maxi)	Qiagen
QIAquick PCR purification Kit	Qiagen
Silencer^{Tm} siRNA Cocktail Kit (RNaseIII)	Ambion

II.1.7. Plasmids

pPGK ß-gal pA	Hatzopoulos A.K.
pPGK ß-geo pA	Hatzopoulos A.K.
pPGK neo pA	Hatzopoulos A.K.
pPGK EGFP pA	Hatzopoulos A.K.
pPGK ER-B-Raf pA	in this work

pPGK ER-C-Raf pA	in this work
pBK-CMV	Clontech
pBK-CMV-B-Raf	in this work
pBK-CMV-C-Raf	in this work
pASK-IBA3	IBA
pASK-IBA3-TFPI	in this work (wt + mutants)

II.1.8. PCR-primers

All the primers were synthesised by MWG-Biotech AG

II.1.8.1. Cloning primers

Primer	*Restriction site*	*Sequence*
ER forward	(BamHI)	5'-ATTCCACCAT**GGATCC**ATCTGCTG-3'
ER reverse	(EcoRI)	5'-GAATGTAGGCAT**GAATTC**GACTGTGG-3'
B-Raf forward	(EcoRI)	5'-G**GAATTC**GTGAAAGCCTTACAGAAATCTCCAG-3'
B-Raf reverse	(XhoI)	5'-CCG**CTCGAG**GGTGTTTCAGTGGACTGG-3'
C-Raf forward	(EcoRI)	5'-G**GAATTC**AGTCCAACAGGCTGGTCACAG-3'
C-Raf reverse	(SalI)	5'-ACGC**GTCGAC**CTAGAAGACTGGTAGCCTTG-3'
TFPI forward	(BamHI)	5'-CG**GGATCC**GCTGATTCTGAGGAAG-3'
TFPI reverse	(EcoRI)	5'-G**GAATTC**GCTGATTC TGAGGAAG-3'

II.1.8.2. Site directed mutagenesis primers

Primer	*Sequence*
TFPI L21A forward	5'-GAGTTGCCACCACTGAAA**GCT**ATGCATTCATTTTG-3'
TFPI L21A reverse	5'-CAGTGGTGGCAACTCCGTATCTGTGATAATTG-3'
TFPI T87F forward	5'-CAAACAGGATTATAAAG**TTT**ACATTGCAACAAG-3'
TFPI T87F reverse	5'-CTTTATAATCCTGTTAGCAATATCTCTTG-3'
TFPI L89A forward	5'-GGATTATAAAGTTTACA**GCG**CAACAAGAAAAGC-3'
TFPI L89A reverse	5'-TGTAAACTTTATAATCCTGTTTGCATTATCTC -3'
TFPI Y159A forward	5'-GGTTTCCAGGTGGATAAT**GCT**GGAACCCAG-3'
TFPI Y159A reverse	5'-ATTATCCACCTGGAAACCATTCGGACCATC-3'

II.1.9. Bacteria strains and cell lines

Cell line	*Species*	*Classification*	*Reference*
DH5α	E.coli	bacterial cells	Invitrogene
BL21			
eEPCs	M. Musculus	embryonic endothelial cells progenitor cells	Hatzopoulos et al. 1998

II.1.10. Bacterial and cell culture media

D 7.5 media (embryonic endothelial progenitor cells)

77% DMEM 25 mM Hepes

(Without Sodium Pyruvate, with 4500 mg/l Glucose, with Pypidoxine)

20% Fetal Bovine Serum

1% L-Glutamine

1% Pen/Strep

1% non essential amino acids

ß-Mercaptoethanol 3,5 µl per 500 ml

Freeze Medium for eEPCs

90% Fetal Bovine Serum

10% DMSO

LB (Luria-Bertani) medium

1% Bacto-tryptone

1% NaCl,

0.5% Bacto-yeast extract

Adjust pH to 7.5 with NaOH and total volume to 1 L with H_2O

For plates, add 15 g Bacto-agar

2x TY medium

16 g Bacto-tryptone

10 g Bacto-yeast extract

5 g NaCl

Adjust pH to 7.4 with NaOH and total volume to 1 liter with H_2O

For plates, add 15 g Bacto-agar

II.1.11 Solutions

Antibody buffer

0.13% EDTA

0.15% bovine serum albumin

In phosphate-buffered saline (PBS)

Blotting Buffer (for transferring the proteins to the nitrocellulose membrane)

150 mM Glycine

20 mM Tris

0.1% SDS

20% Methanol

Blocking Buffer

5% (w/v) of nonfat dry milk in TBST

Buffer HSB (electroporation)

25 mM Hepes pH 7.1

134 mM NaCl

5 mM KCl

0.7 mM Na_2HPO_4

Buffer for IP (lysis and wash)

20 mM Tris, pH 7.5

5 mM $MgCl_2$

10 mM EGTA

1% Trion X-100

50 mM NaF

0.7 mM $Na_4P_2O_7$

0.7 mM Na_2VO_4

1 mM PMSF*

10 µg/ml aprotinin before use*

10 µg/ml leupeptin before use*

*- directly before use just for the lysis.

Buffer NX 10x (reverse transcription)

0.4 M KCl

0.1 M Tris-HCl, pH 8.4

20 mM $MgCl_2$

2% Tween 20

Buffer P1 (Resuspension buffer)

50 mM Tris-HCl, pH 8.0

10 mM EDTA

10 mg/ml Rnase A

Buffer P2 (Lysis buffer)

10% SDS

200 mM NaOH

Buffer P3 (Neutralization buffer)

3 M potassium acetate, pH5.5

Buffer QBT (Equilibration buffer)

15% ethanol

0.15% Triton X-100

Buffer QC (Wash buffer)

2.0 M NaCl

50 mM MOPS, pH7.0

15% ethanol

Buffer QF (Elution buffer)

1.25 mM NaCl

50 mM Tris-HCl, pH 8.5

15% ethanol

Set Qiagen RNA isolation buffers

Buffer RLT- cat Nr. 79216

Buffer RW1- cat Nr. 79216

Buffer RPE -cat Nr. 79216

1x CIP Buffer

50 mM NaCl

10 mM Tris-HCl

10 mM $MgCl_2$

1 mM dithiothreitol , pH7.9

10x DNA Gel Loading Buffer

40% (w/v) saccharose

0.25% bromphenolblue

0.25% xylencyanol, use as 1x solution

Lysis Buffer (for cell culture)

50 mM Na-MES, pH 7.8

50 mM Tris-HCl, pH 7.8

10 mM DTT

2% Trion X-100

Phospate-Buffered Saline (PBS)

136 mM NaCl

2.6 mM KCl

10 mM Na_2HPO_4,

1.5 mM KH_2PO_4, pH7.4

Running Buffer (for SDS-PAGE)

25 mM Tris

250 mM Glycine

0.1 % SDS

5x SDS-loading buffer (for SDS-PAGE)

31 mM Tris HCl, pH6.8

1% SDS

5 % Glycerin

2.5 % Mercaptoethanol

0.05 % Bromphenoblue, work at 1x solution

Sodium Tris-EDTA buffer (STE)

100 mM NaCl

10 mM Tris-HCl, pH 8.0

1 mM EDTA

TLB buffer

20 mM Tris, pH 7.4

50 mM Sodium glycerophosphate

20 mM Sodium pyrophosphated

500 mM NaCl

10 % (v/v) glycerol

0.1% Triton X-100

2 mM EDTA

1 mM pefablock

1 mM sodium orthovanadate

5 mM benzamidine

5 µg/ml aprotinin before use

5 µg/ml leupeptin before use

1x Tris-Acetate-EDTA (TAE)

40 mM Tris-HCl,

40 mM acidic acid,

2 mM EDTA; pH7.8

10x Tris-Borate-EDTA (TBE)

108 g Tris base

55 g boric acid

20 ml 0.5 M EDTA, pH 8.0

Adjust the volume to 1 liter with H2O

1x Tris-Buffered Saline (TBS)

10 mM Tris-HCl,

137 mM NaCl

TBST

1x TBS + 0.1% Tween

Washing buffer (protein isolation)

100 mM Tris/HCl, pH 8.0,

150 mM NaCl,

1.0 mM EDTA,

0.02% w/v NaN_3

Elution buffer (protein isolation)

100 mM Tris/HCl, pH 8.0,

150 mM NaCl,

1.0 mM EDTA,

0.02% w/v NaN_3,

2.5 mM desthiobiotin

Regeneration buffer (protein isolation)

100 mM Tris/HCl, pH 8.0,

150 mM NaCl,

2 mM EDTA,

0.02% (w/v) NaN_3,

5 mM of 4-hydroxyazobenzene-2-carboxylic acid (HABA)

II.2. Methods

II.2.1. Cell cultures

II.2.1.1. Bacterial cultures

Plasmid transformed bacteria were selected on LB plates with ampicillin (100 µg/ml) or kanamycin (50 µg/ml) for 24 hr. For overnight mini cultures, 1 colony was picked and inoculated in LB medium with appropriate antibiotics and shaken overnight at 37°C. The culture was then used for preparing frozen glycerine cultures, plasmid DNA or fusion protein purification. For storage of bacteria, a glycerol stock culture was prepared by growing bacteria in culture medium and the OD was measured with a photometer at a wavelength of 600 nm. When the OD reached 0.8, the bacterial culture was added to 500 µl 80% glycerine and then mixed thoroughly in a small 1.5 ml tube. This stock solution was subsequently frozen at –80°C. To start an overnight culture again, bacteria were held at room temperature (RT) until the surface was thawed. A small amount of cells was picked and mixed into 2-5 ml culture medium and left to grow for several hours at 37°C in a bacterial culture shaker. The frozen stock was immediately returned to storage at –80°C.

II.2.1.2. Preparation of competent cells ($CaCl_2$ method)

An overnight preculture was started from a single colony on a petri dish in 2 ml LB media by incubation at 37°C and was shaken for oxygenation. The second day, 1 ml of the preculture was inoculated in 100 ml fresh media and the culture was grown at 37°C until the OD at the wavelength of 600 nm reached 0.2 to 0.4. The culture was cooled down on ice for at least 15 min. The following steps were done at 4°C in pre-cooled sterile tubes. The cells were harvested by a 5-min centrifugation at 5000 g, and the supernatant was discarded. The bacterial pellets were thoroughly resuspended in a small volume of ice-cold 100 mM $CaCl_2$. The suspension was diluted with the $CaCl_2$ solution to a final volume of 30-40 ml, and left on ice for 25 min with occasionally shaking. The cells were span down as before, the supernatant was carefully discarded and the pellets were resuspended in 5 ml glycerol/$CaCl_2$. The suspension was aliquoted in 100 to 400 µl and stored at –70°C. The transformation efficiency of the bacteria prepared by this method should reach at least 10^6 cells.

II.2.1.3. Transformation of competent bacteria

The competent bacteria were thawed on ice. At around 40ng ligated-DNA or purified plasmid-DNA was added to 100 µl competent cells in a cold 1.5 ml microfuge tube, then mixed carefully and kept on ice for 20 min or longer. The bacteria were heat-shocked at 42°C for 90 sec, 1 ml antibiotic-free LB medium was added, and oxygenated at 37°C for 30 min. Selection of transformed bacteria was done by plating 100 µl of the bacterial suspension on the antibiotic-containing agar plates. Only bacteria that have taken up the desired plasmids, which normally contain antibiotic resistance cassette, could grow on the agar antibiotic plates. Some of the colonies that were grown on the plate were expanded in LB medium and used for DNA preparation.

II.2.1.4. Maintaining of cell culture

The mouse embryonic endothelial progenitor cell line (eEPC) was maintained in a special medium as described below. The cells were cultured until reaching 90% confluence and than split to obtain adequate density. Important for the eEPCs was the coating of the plates with 0.1 % gelatine for at least 10 min at 37° C for sufficient cell adhesion.

To freeze the cells, cell cultures were centrifuged at 1100 rpm for 5 min and resuspended into freeze medium. Aliquotes were set into a styropore box and left to freeze gradually in the -80°C freezer. DMSO was added to prevent ice crystal formation and to allow maintenance of the integrity of the cells. After 24 hr or later, the cells were transfered to liquid nitrogen where they were kept for longer periods of time.

II.2.1.5. Transfection of eEPCs

II.2.1.5.1. Electroporation

For each electroporation 1.5-2x10^6 cells were used. The cells were trypsinized and it was stoped with 5 ml medium, and then resuspended in 20 ml HSB buffer. After spinning them down, they were again resuspended in 10 ml HSB buffer. After a new centrifugation step they were resuspended to a concentration of 1.5-2x10^6 cells in 0.8 ml HSB and transferred to the electroporation cuvette. 30 µg linearised plasmid was added to the cells, mixed well and kept 10 min on ice. Then the cells were electroporated at 350 V, 500 microfarrads. The times were recorded concomitantly. After the electroporation the cells were kept 10 min on ice and transferred into 24 ml medium. The cells were plated in 2x10 cm plates (gelatinised) in order to let them recover over night. The next day the selection was started with 200-400 µg/ml G418 (active conc.). The medium was exchanged daily. The first colonies were usually formed within 1-2 weeks.

II.2.1.5.2. Lipofectamine transfection

1. The day before transfection, the cells were trypsinized, counted and plated at 1-$3x10^5$ cells per well so that they were 80-85% confluent on the day of transfection. Cells were plated in their normal growth medium containing serum, but in the absence of ANY antibiotics.

2. For each well of cells to be transfected, 0.8 to 1.0 µg of DNA was diluted into 50 µl of OPTIMEM without antibiotics.

3. For each well of cells, 1-3 µl of LF2000 Reagent was diluted into 50 µl OPTIMEM (as indicated above) and incubated for 5 min at room temperature.

4. The diluted DNA (from step 2) with the diluted LF2000 Reagent (from step 3) were combined and incubated at room temperature for 20 min to allow DNA-LF2000 Reagent complexes to form.

5. DNA-LF2000 Reagent complexes (100 µl) were added directly to each well and mixed gently by rocking the plate back and forth.

6. The cells were incubated at 37°C in a CO_2 incubator for a total of 24-48 h until they were ready to assay for transgene expression. It was not necessary to remove the complexes or to change the medium. Alternatively, growth medium was replaced after 4-6 h without loss in transfection activity.

II.2.2. DNA techniques

II.2.2.1. Electrophoresis of DNA on agarose gel

Double stranded DNA fragments with lengths between 0.1 kb and 10 kb can be separated according to their lengths on agarose gels. Agarose was added to 1x TAE to obtain a final concentration between 0.7-2%. The suspension was boiled in the microwave until the agarose was completely solubilised. The agarose was cooled down to around 50°C, before the ethidium bromide was added up to 0.5µg/ml and poured into the gel apparatus. DNA gel loading buffer was added to the DNA sample and applied on the gel. Electrophoresis was performed in 1x TAE buffer at 3-8 volts/cm. The DNA fragments were visualised under UV-light.

II.2.2.2. Isolation of plasmid DNA from Agarose (QIAGEN gel extraction kit)

This protocol is designed for the extraction of DNA fragments from 0.7-2% standard agarose gels in TAE or TBE buffer. DNA molecules were adsorbed to QIAGEN silica columns. All non-nucleic acid impurities such as agarose, proteins, salts, and ethidium bromide were removed during the washing steps. The desired DNA band was excised from the agarose gel under the UV light. The gel slice was weighed and 5 volumes of Buffer QG were added to 1 volume of gel for DNA fragments 100-bp 4 kb; for DNA fragments of > 4 kb, 2 volumes of QG plus 2 volumes of H_2O were added, and then incubated at 50°C for 10 min to solubilise the agarose. The solubilised agarose was resuspended by vortexing and the sample was added to the QIAGEN silica columns to bind the DNA. The sample was centrifuged for 30 sec. The column was washed with 500 µl of Buffer QG and then twice with Buffer PE. Thereafter it was centrifuged for additional 30 sec to remove residual alcohol from the buffers. The column was span for 1 min to elute the DNA in 30-50 µl 10 mM Tris-HCl or H_2O.

II.2.2.3. Purification of plasmid DNA (QIAquick PCR purification kit)

This protocol is designed to purify single- or double-stranded PCR products or DNA plasmids ranging from 100 bp to 10 kb. DNA adsorbs to the silica-membrane in the presence of high salt while contaminants pass through the column. The impurities were washed away and the pure DNA was eluted with Tris buffer or H_2O. 5 vol of buffer PB was added to 1 vol of the contaminants and mixed. A QIAquick spin column was placed in a 2 ml collection tube. The mixed sample was added to the QIAquick column and centrifuged for 30-60 sec. The flow-through was discarded and the QIAquick column was placed back into the same collection tube. 0.75 ml washing buffer (PE) was added to the column and centrifuged for 30-60 sec. The flow-through was discarded and the QIAquick column was placed back into the same collection tube. The column was centrifuged for an additional 1 min at maximum speed, and placed in a clean 1.5 ml microfuge tube, and then 30-50 µl elution buffer (EB) or H_2O was added to the centre of the QIAquick column and centrifuged for 1 min. The purified DNA was stored at – 20°C.

II.2.2.4. Ligation of DNA fragments

Calf-intestinal-phosphatase (CIP) reaction (5' phosphorylation)

Alkaline phosphatase catalyses the removal of 5' phosphate groups from DNA, RNA and ribo as well as deoxyribonucleoside triphosphates. For the blunt end ligation, the 5' phosphate group of the vector must be removed by the CIP reaction. The same reaction is also used to prevent the religation of the vectors. 2.5 µg of DNA fragments were phosphorylated at 37µC for 30 min in 100 µl of reaction volumes, consisting of 1x CIP buffer and 1µl of phosphatase. 5 mM EDTA was then added and the mixture incubated for 15 min at 65°C to inactivate the enzyme. The DNA fragments were purified by phenol and ethanol precipitation before the ligation reaction.

II.2.2.5. Cohesive-end ligation

The plasmid DNA or DNA fragment were prepared by cutting with suitable restriction enzymes, followed by their purification. 1:3 molar ratios of vector: insert DNA fragments were incubated together with 1 µl (1 U/µl stock) of T4 ligase in 1x ligation buffer in a total volume of 20 µl for 4 hr at room temperature or overnight at 16°C. The mixture was heated at 65°C for 10 min to inactivate the enzyme.

II.2.2.6. Oligonucleotide primers

All oligonucleotide primers were synthesized from MWG-Biotech (Germany) and delivered in lyophilised form. The oligonucleotides were dissolved in sterile water to obtain a 100 pmol solution. From this primer solutions were prepared at the desired concentration, for conventional PCR, sequencing and site directed mutagenesis. Oligonucleotide primers used for the estrogen receptor (ER), B-Raf, C-Raf and TFPI cloning (bold letters show the restriction sites), for site directed mutagenesis of TFPI (bold letters indicate the mutated bases) are listed in the Materials section.

II.2.2.7. Polymerase chain reaction (PCR)

The coding regions of the kinase domains of B-Raf and C-Raf were amplified by PCR from mouse embryo cDNA. The hormon binding site of ER was amplified from a vector available in the laboratory, and the full length of TFPI was amplified by PCR from total carotid plaque cDNA. The reactions were performed with 3µl total cDNA, 0.2 µl/reaction dNTP 20 mM mix, 5µM primer mix 2 µl/reaction, 5 U/µl DNA polymerase with proof reading activity (Vent, Pfu) 0.5 µl/reaction, 2 µl 10X PCR buffer (in a total volume of 20 µl).

The PCR amplification was performed with the following protocol by varying the annealing temperatures and the elongation times:

Step 1 Initial denaturation: 94°C for 2 min

Step 2 Denaturation: 94°C for 20 sec

Step 3 Annealing: 52-68°C for 30 sec

Step 4 Elongation: 68°C 1 min per kilobase

Step 5 Closing the cycle and back to step 2

Step 6 Final elongation: 68°C for 5 min

Step 7 End, keep at 4°C

The conventional quantitative PCR was performed with 3µl total cDNA, 20 mM mix dNTP 0.2 µl/reaction, 5µM primers mix 2 µl/reaction, 5 U/µl Taq DNA polymerase 0.5 µl/reaction, 2 µl 10X PCR buffer (in total volume of 20 µl). The PCR amplification was performed with the following protocol by varying the annealing temperatures and the elongation times:

Step 1 Initial denaturation: 94°C for 2 min

Step 2 Denaturation: 94°C for 20 sec

Step 3 Annealing: 52-68°C for 30 sec

Step 4 Elongation: 72°C 1 min per kilobase

Step 5 Closing the cycle and back to step 2

Step 6 Final elongation: 72°C for 5 min

Step 7 End, keep at 4°C

II.2.2.8. Mini-preparation of plasmid DNA

3 ml overnight cultures were grown in LB media with 100 µg/ml ampicillin (or kanamycin, 50 µg/ml) at 37°C overnight. The cells were pelleted at 14,000 rpm for 1 min, the supernatant was removed, and the cells resuspended in 100 µl Buffer. 200 µl Buffer P2 (Lysis Buffer) was added and incubated at RT for 5 min, then 200 µl ice-cold 3 M acidic KOAc (Neutralisation Buffer) was added and mixed by inverting the tubes for 6-7 times. The lysates were incubated on ice for 5 min and centrifuged at 15,000 rpm for 3 min. The supernatant was transferred to a fresh eppendorf tube and 900 µl of pre-cooled 100% ethanol was added for precipitation at -70°C for 10 min. The pellet was centrifuged at 15,000 rpm for 10 min. Subsequently it was washed with 200 µl 70% ethanol and air-dried for a few min. Then it was resusspended in 30-50 µl of 10 mM Tris-HCl, (pH 7.8).

II.2.2.9. Maxi-preparation of plasmid DNA

Bacterial cultures containing plasmids or recombinant plasmids were grown in 50 ml LB media overnight in a 37°C-incubator with shaking at 190 rpm. The bacteria were collected and DNA plasmids were isolated by using a Quiagen Plasmid Maxi Kit. This extraction method is based on Birnboim's alkali lysis principle. The bacterial pellet was resuspended in 10 ml of buffer P1. 10 ml of buffer P2 were added and mixed gently. Then the lysate was incubated at RT for 5 min, 10 ml of chilled Buffer P3 was added and mixed immediately, and further incubated on ice for 20 min. The suspension was centrifuged at 4,000 rpm for 30 min at 4°C and the supernatant was filtered over a prewetted folded filter. The supernatant was applied to an equilibrated QIAGEN-tip 500 and the resin was allowed to enter by gravity flow. The QIAGEN-tip was washed twice with buffer QC. The DNA was eluted with 15 ml buffer QF. These processes resulted in the isolation of a DNA-salt pellet, which was precipitated by 0.7 volumes (10.5 ml) of isopropanol, and centrifuged further at 4000 rpm for 30 min. The resulting pellet was washed twice with 70% ethanol and air-dried at RT. The pellet was then carefully resuspended in TE buffer and quantified.

II.2.2.10. Restriction digests

Digestion of DNA with restriction enzymes was performed according to the manufacturer's instructions using recommended buffer systems and the appropriate reaction temperatures. Generally, 1 U of enzyme was needed per 1 µg DNA. The plasmid DNA was usually digested for 1-2 h. The completion of the reaction was monitored by agarose gel electrophoresis.

II.2.2.11. Measurement of DNA concentration

The DNA concentration was determined by using an UV spectrophotometer at a wavelength of 260 nm. Absorption of 1.0 at 260 nm corresponds to a concentration of 50 µg/ml double stranded DNA. Identity, integrity, and possible purity of the DNA was subsequently analysed on an agarose gel. The ratio in absorption at 260/280 nm which is determining pure DNA is over 1.8.

II.2.2.12. DNA Site directed mutagenesis

Site directed mutagenesis was performed using the **Gene Tailor^{Tm} Site-Directed Mutagenesis System-** Invitrogen. The method includes in a variety of different techniques such as primer design, PCR, transformation of E.coli, mini and maxi preparation of plasmid

DNA. The method is used to exchange a single amino acid within a polypeptide chain, or to remove or insert up to 5 amino acids in one reaction. First it was performed methylation of the plasmid containing the gene of interest to mutate. The methylated parental plasmid will be degraded by the bacterial strain used for transformation; this will reduce the background of unmutagenised DNA, and will increase the efficiency. The primers were designed to contain the mutated bases (the TFPI primers used are listed in the Materials section). The primers must be overlapping and containing protruding ends to enable the recirculization of the vector. For the PCR of the methylated template, Platinum^R Pfx polymerase was employed, which has proof reading activity. After amplifying the whole construct, the transformation into the bacterial strain was made as supplied from Invitrogen. Detection of the transforming activity was done by PCR and by restriction assay (the new formed sequence could potentially form or destroy the restriction sites). Subsequently, sequencing was performed in order to check for the vector and of the mutations in the gene of interest.

II.2.2.13. DNA Sequencing

All sequencing reactions were performed in **SeqLab-**Göttingen, Germany. The evaluation of all results was done with the PC program Chromas.

II.2.3. RNA techniques

II.2.3.1. Total RNA isolation from animal cells

The protocol is designed to isolate total RNA from animal cells with silica Rneasy columns (Qiagen). The cells grown in monolayer can be lysed directly on the plate. Depending on the cell number, 350 µl RLT lysis buffer containing ß-Mercaptoethanol (ß-ME) (10 µl per 1 ml RLT) were added to $5x10^6$ cells (6 cm plate), or 600 µl was added to $5x10^6$-$1x10^7$ cells (10 cm plate). The lysed cells were pipetted onto a QIAshredder column and centrifuged for 2 min at maximum speed to be homogenized. One volume of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. The sample was applied on the RNeasy spin column, and centrifuged for 15 sec at 10000 rpm, then 700 µl buffer RW1 was pipetted onto the column and centrifuged for 15 sec. DNAse (RNAse free) was applied on the column to degrade the genomic DNA; it was incubated for approx. 20 min at room temperature. The column was washed with new 700 µl buffer RW1. The column was transferred into a new 2 ml tube, 500 µl of RPE buffer were loaded onto and centrifuged for

15 sec at 10000 rpm. The last step had to be repeated once. Then the flow-through was discarded and centrifuged for 1 min at full speed to ensure that there was no alcohol left from the last washing step. The RNeasy column was transferred into a new tube and the total RNA was eluted with 30-50 µl of RNase free water, then quantified and finally stored at –80° C.

II.2.3.2. Measurement of RNA concentration

The RNA concentration was determined by using an UV spectrophotometer at a wavelength of 260 nm. The absorption of 1.0 at 260 nm corresponds to a concentration of 40 µg/ml total RNA. Identity, integrity, and purity of the RNA were subsequently analysed on an agarose gel. The ratio in absorption at 260/280 nm determining pure (low amount of proteins) RNA was over 1.8.

II.2.3.3. Reverse transcriptase reaction

1 µg of RNA was incubated with oligo dT primers (1.25 µl of 17 µM conc.; 80 ng/µl) at 65°C for 5 min in order to destroy secondary structures of the RNA and to make accessible most of the poly A tags of the mRNA. The mix containing the reverse transcriptase (polymerase, 0.5 µl 200 U/µl), dNTPs 0.5 µl 20 mM , ß-mercaptoethanol 0.5 µl, 0.1 M and RNAsin (protection from RNAses) 0.25 µl, 40 U/µl, NX buffer 10x 1.5 µl was added and incubated at 37°C for 55 min. Then the mixture was incubated for 5 min at 95°C to inactivate the enzyme, and 90 µl H_2O were added to the reaction. From the cDNA prepared, 2-3µl were used as template for the PCR reaction.

II.2.3.4. RNA interference

RNA interference was performed with **Silencer^{Tm} siRNA Cocktail Kit (RNaseIII) -** Ambion. The purpose of our study was to knock down the B- and C-Raf genes by specifically degrading their mRNA. We designed primers and searched for a PCR spanning area, of around 400-500 bp, from the desired gene. The forward and reverse primers must contain tags of T7 promoters to be able to perform in vitro transcription (2-4 hrs) and produce dsRNA on the DNA template. After purification of the transcribed RNA it was digested with DNases (1 hr at 37°C), and again purified. The dsRNA was then digested with RNase III (1 hr at 37°C), which produces the siRNAs. Thereafter we performed a new purification and quantified the yields of the siRNAs. For the transfection of the cells we used siRNA of a final concentration of 100 nM.

II.2.4. Protein analyses

II.2.4.1. Immunoprecipitation

For immunoprecipitation of cellular proteins, 20 µl sepharose was incubated with 0.5-4 µg of antibody, 500-2000µg lysate and 300 µl IP buffer. Depending on the type of the experiment, lysates were additionally precleared with beads if background problems arose. Samples were incubated on a rotator for at least 2 hr at 4°C, and then washed with different wash buffers, depending on the stringency required. The washing buffer should insure low background and maximum preservation of complexed proteins. For example, when interactions between two proteins already known to have high interactions were analyzed, a strong wash buffer such as RIPA was used to reduce the background. When proteins with unknown interaction were evaluated, a mild washing buffer such as TLB Buffer was used in order to insure that interactions between the proteins of interest were not destroyed. The immunoprecipitated proteins were boiled in Laemmli buffer and subjected to SDS PAGE and Western blotting.

II.2.4.2. Western blot

Proteins separated on SDS-PAGE gels were transferred to a PVDF membrane using a wet transfer system with all components soaked beforehand in transfer buffer. The gel was placed on top of a piece of Whatman 3MM filter paper and sponge, overlaid with PVDF membrane, pre-washed in methanol, and equilibrated in transfer buffer. The blotting sandwich was completed by the addition of a second layer of filter paper and a sponge. The transfer in cassette assembly was carried out at 150 mA for 90 min. Following the transfer, the PVDF membranes were washed repeatedly in 1 x TBS/Tween-20 to remove any gel remnants. The remaining protein binding capacity of the gel was neutralised by incubation in blocking buffer for >1 h, under shaking. Excess blocking reagent was removed by washing 3 x with the appropriate washing buffer, before the membrane was incubated with the primary antibody diluted to the appropriate concentration in washing buffer containing 5 % BSA for 1 h at RT. The membranes were washed 3 x with washing buffer to remove the excess of the primary antibody. A peroxidase-conjugated, secondary antibody, specific for the isotype of the primary antibody used, was applied to the membrane at its correct working concentration, diluted in washing buffer containing 5 % BSA for 1 h at RT. Excess antibody was removed by washing 3 x with washing buffer. Protein-antibody complexes were detected on X-ray film using a chemoluminesence reaction catalysed by the antibody-conjugated peroxidase. The ECL solution was used as substrate for this reaction, following the manufacturer's protocol.

II.2.4.3. Recombinant protein expression and purification

II.2.4.3.1. Culture conditions and expression of TFPI protein

Luria–Bertani (LB) broth medium was used to produce TFPI wild type and the mutant forms of the protein. 500 ml of LB^{amp} (100µg/ml amp.) medium was inoculated with 10 ml of BL21 *E. coli* cells from an overnight culture. The mixture was shaken in an incubator at 37 °C and 190 rpm. When the culture medium reached an OD_{600} of 0.4, 50 µl of anhydrotetracyline was added to the cell culture to a final concentration of 2.0 µg/ml, in order to induce the expression of the recombinant protein. The cell culture was then incubated for additional 2 h at 25 °C for the synthesis of the protein.

II.2.4.3.2. Purification of TFPI

After incubation, the cell culture was centrifuged at 5000 rpm for 20 min at 4 °C to pellet the cells. The pellets were resuspended in 10 ml of pre-cooled buffer W, and sonicated under ice-cold conditions until lysis was complete. The suspension was then centrifuged at 6,000 rpm for 15 min at 4 °C to remove the cell debris; the supernatant was additionally centrifuged in an ultracentrifuge at 35,000 g for 30 min at 4 °C to reduce the amount of total protein. After the centrifugation, the supernatant containing the TFPI protein was collected in a 50 ml Falcon tube. A chromatography column (0.8 x 4 cm) containing 2 ml Strep-Tactin II was initially washed with 10 ml of washing buffer at a flow rate of 0.5 ml/min. Then 10 ml of the supernatant containing the TFPI protein were loaded onto the column. Washing buffer (30 ml) was passed through the column to remove impurities. To elute the protein from the column, 7 x 500 µl of elution buffer was passed through the column. The eluent was collected (0.5 ml/tube). Thirty microliters of each fraction was analysed using a 1.5 mm 12% SDS–polyacrylamide gel. The amount of protein was also quantified using UV spectrophotometry. To regenerate the column, it was first washed with 10 ml of washing buffer, followed by 30 ml of regeneration buffer. Finally, 40 ml of washing buffer was run through the column to wash away the HABA.

II.2.4.4. Measurement of protein concentration (Bio-Rad protein assay)

The Bio-Rad Protein Assay is based on an absorbency shift when Coomassie Brilliant Blue G-250 binds to the protein. Cell lysate containing 1-20 µg of protein was added to dilute Dye Reagent (1:5 dilution of Dye Reagent Concentrate in ddH2O). Mixing for 5-60 min the absorption was measure at a wavelength of 595 nm versus reagent blank (containing buffer only).

II.2.4.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

Cells were collected, washed with ice-cold phosphate-buffered saline (PBS) and lysed in 50 µl of lysis buffer. The crude cell lysates were cleared by centrifugation. 5 µl 5x SDS-loading buffer containing SDS was used to denature 20 µl of precleared cell extracts at 95° C for 5 min. SDS is an anionic detergent that disrupts nearly all noncovalent interactions in native proteins. ß-Mercaptoethanol is also included in the sample buffer to reduce disulphide bonds. The SDS complexes with the denatured proteins were then electrophoresed on a polyacrylamide gel in the form of a thin vertical slab. Vertical gels were set in between 2 glass plates with an internal thickness of 1.5 mm between the two plates. In this chamber, the acrylamide mix was poured and left to polymerise for at least 30 min at RT. The gels are composed of two layers: a 6-15% separating gel (pH 8.8) that separates the proteins according to size; and a lower percentage (5%) stacking gel (pH 6.8) that insures the proteins simultaneous entry into the separating gel at the same height. The separating and the stacking gels consisted of the following components:

	Separating gel	**Stacking gel**
Tris pH 8.8	2.5 ml.	1.25 ml
Acrylamide/bisacrylamide 29:1 (30%)	2.0-5.0 ml	1.7 ml
10% SDS	0.1 ml	0.1 ml
ddH2O	5.4-2.4 ml	6.8 ml
10% APS	0.1 ml	0.1 ml

The separating gel was poured in between the two glass plates; a space of about 1cm plus the length of the teeth of the comb was left and isopropanol was added to the surface of the gel. After the separating gel was polymerised, the isopropanol was removed. The stacking gel was poured on top of the separating gel and a comb was inserted. The gel was left to polymerise. Then the samples were loaded in the wells of the slab and running buffer was added in the chamber. A cover was then placed over the gel chamber and 200 volts were applied. The negatively charged SDS-proteins complexes migrate in the direction of the anode at the bottom of the gel. Proteins that differ in mass by about 2% can be distinguished with this method.

II.2.4.6. Functional assays

II.2.4.6.1. Xa formation

The functional activity of TF can be measured by the formation of factor Xa. The TF-VIIa cleaves factor X either directly or indirectly via factor IX activation. The formed factor Xa cleaves the substrate S2222; thereby a product is formed that absorbs at 405 nm and can be measured photometrically. The blood coagulation factors were added in the form of the commercially available Beriplex that, besides the coagulation factors VII, X, IX and II, also contains the anticoagulant proteases antithrombin and protein C. Therefore, similar to the conditions in the blood not only the procoagulant, but also the anticoagulant components are included. For the measurement of the procoagulant activity the isolated blood cells (neutrophils, platelets) were incubated for 10 min at 37° C.

Afterwards, the samples were put in a 96 well plate to measure the OD in U/ml. In each measurement standard curves were prepared from recombinant TF. Subsequently to each well 50 µl 8 mM $CaCl_2$ solution and 100 µl mixture of resuspension buffer, substrate S2222, and Beriplex (3.375 ml resuspension buffer, 400 µl S2222, 225 µl Beriplex) were added to the cell samples and the OD values were measured photometrically during 30 min in a ELISA reader.

II.2.4.6.1.1. Platelets isolation

Platelets were isolated from blood of healthy donors. The blood was drawn into sodium citrate (0.38% final concentration) and subsequently centrifuged at 130 x *g* for 15 min. The upper two thirds of the supernatant were supplemented with apyrase (0.2 U/ml). The suspension was centrifuged at 330 *g* for 10 minutes, and the platelet pellet was washed once with the platelet washing buffer, followed by an additional centrifugation at 330 *g* for 10 min and resuspended in the buffer of choice.

II.2.4.6.1.2 Neutrophils isolation

Human neutrophils were prepared by incubation of freshly obtained buffy coats with microbeads coupled to anti-CD15 antibodies for 15 minutes at 8°C. The suspensions were thereafter applied on a selective column and washed 3 times with 2 column vol of antibody buffer. Then the column was transferred to a 15 ml falcon tube and the neutrophils were eluted with 1 column vol of antibody buffer.

II.2.4.6.2. Thrombelastography

Currently, thrombelastography (TEG) is the most adequate method to measure the fibrin formation rate in whole blood and therefore allows functional examination of the blood coagulation system under conditions that are close to the in vivo situation. The principle of TEG depends on a stamp that is moving slowly forward and backwards in a small tube containing the blood. If a stimulus induces the formation of fibrin polymers, the movement of the stamp is reduced by the formed clot, which is registrated by a detection system and indicated in a graphic form. The fibrin formation rate represents the coagulation time (CT). Further parameters of the measure are thrombus growth rate, or clot formation time (CFT).

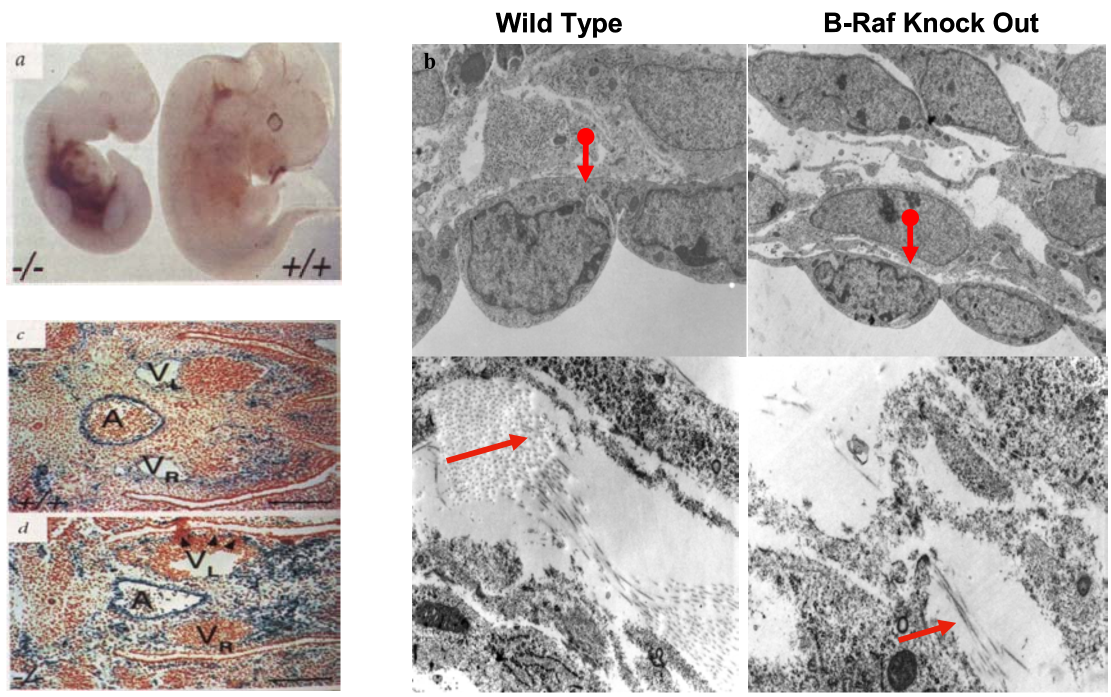
Blood was collected in a syringe with citrate (1/10 of the blood volume) and corn trypsin inhibitor was added (CTI 32 µg/ml, inhibitor of the intrinsic coagulation pathway). Then, 300 µl of blood were pipetted into a TEG tube and collagen (12 µg/ml) and the different TFPI variants were added. By addition of 60 µl of a 100 mM $CaCl_2$/10 mM HEPES solution, the coagulation process was started. The addition of activators or inhibitors was always performed before the stimulation with collagen.

III. Results

III.A. Raf proteins and their role in endothelial maturation

III.1. B-Raf is critical for vascular development

A large number of molecules regulate distinct steps in vascular development such as vasculogenesis, angiogenesis, remodeling and maturation of the blood vessels. Wojnowski et al. (1997) found that the serine-threonine kinase B-Raf is critical for the proper formation of blood vessels. B-Raf knock out embryos die between d 10-12, suffering from extensive hemorrhages due to enlarged and ruptured vessels (Fig. 3-1 panels a, c & d). Further analysis revealed that knock out mice had apoptotic mature endothelial cells and higher number of progenitor cells embedded within the embryonic tissues. Studying this phenotype in more detail and comparing electron microscopy pictures of knock out and wild type embryos, Hatzopoulos et al. (unpublished data) found differences within the structure of the vessel wall, characterized by reduced collagen fibers and malformed peri-vascular structures (Fig. 3-1 right panels b). These findings prompted us to investigate the role of B-Raf in the differentiation of endothelial progenitor cells to better understand the molecular basis of this phenotype.



Wojnowski et al., Nat.Genetics 1997 **Hatzopoulos et al.**

Figure 3-1 The B-Raf knock out is lethal at day 10.5 of the embryonic development of the mice and has a vascular phenotype characterized with reduced amounts of collagen in the vessel wall, enlarged and ruptured vessels leading to hemorrhages in the body cavities.

Based on a previously established method (Hatzopoulos et al. 1998), we isolated embryonic endothelial progenitor cells (eEPCs) at d 7.5 from the preendocardial tubes of mouse embryos. It was earlier found that these cells exhibit stem-cell like unlimited proliferation and are able to differentiate into a mature endothelial phenotype upon treatment with retinoic acid and cAMP (Hatzopoulos et al. 1998). We analyzed the expression of a set of genes potentially induced upon differentiation of the eEPCs. These include early endothelial markers such as Tie-2, thrombomodulin and VGFR-1, which characterize these cells as part of the endothelial lineage.

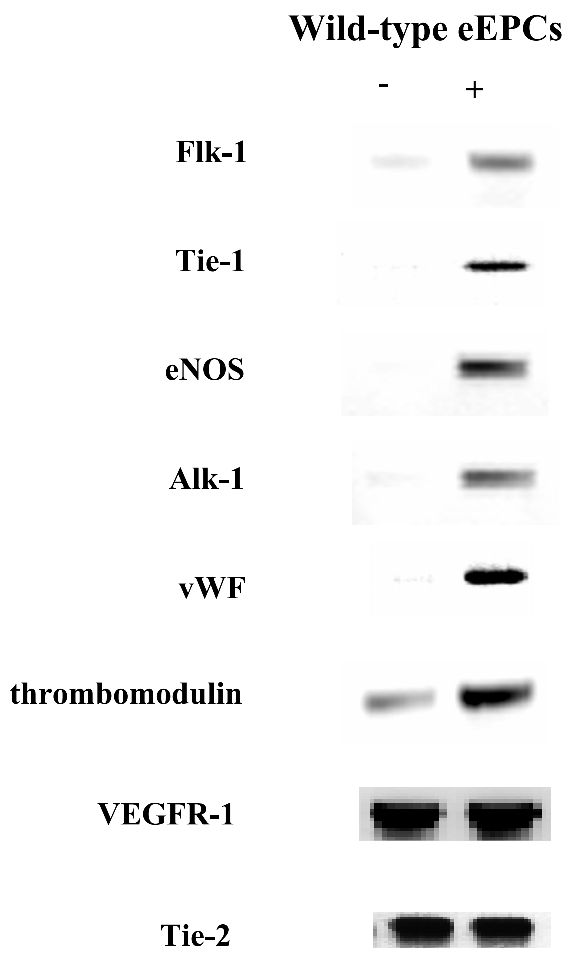


Figure 3-2 cAMP leads to differentiation of the eEPCs, influencing specific genes involved in the process. RT-PCR analysis using gene-specific primers reveals that a subset of endothelial markers are expressed in eEPCs (e.g., Tie-2, thrombomodulin, VEGFR-1), while others are induced upon differentiation (e.g., vWF, eNOS, Tie-1, VEGFR-2

III.2. Gain of function of B- and C-Raf in embryonic endothelial progenitor cells

III.2.1. Preparation of constructs for induction of constitutively active Raf proteins

We created constructs containing the hormone binding site of the estrogen receptor fused to the kinase domain of the B-Raf or C-Raf proteins using standard molecular biology techniques. By applying or removing estrogen from the culture medium, the fusion molecule was expressed, it was bound by heat shock proteins (HSP90) and kept in an inactive state in the cytoplasm of the cells, until an estrogen molecule associates with the hormone binding site of the estrogen receptor. This type of constructs gave us the opportunity for switching on and off the activity of both kinases. Estrogen receptor binding changes the conformation of the fusion molecule, the HSP90 molecules are released, allowing the functional activity of the B- and C-Raf kinase domains. Figure 3-3 shows a schematic drawing of the B- and C-Raf fusion constructs and their mode of activation. Both constructs were cloned in vectors containing a PGK (phosphoglycerol kinase) promoter, known to be highly active in the eEPCs.

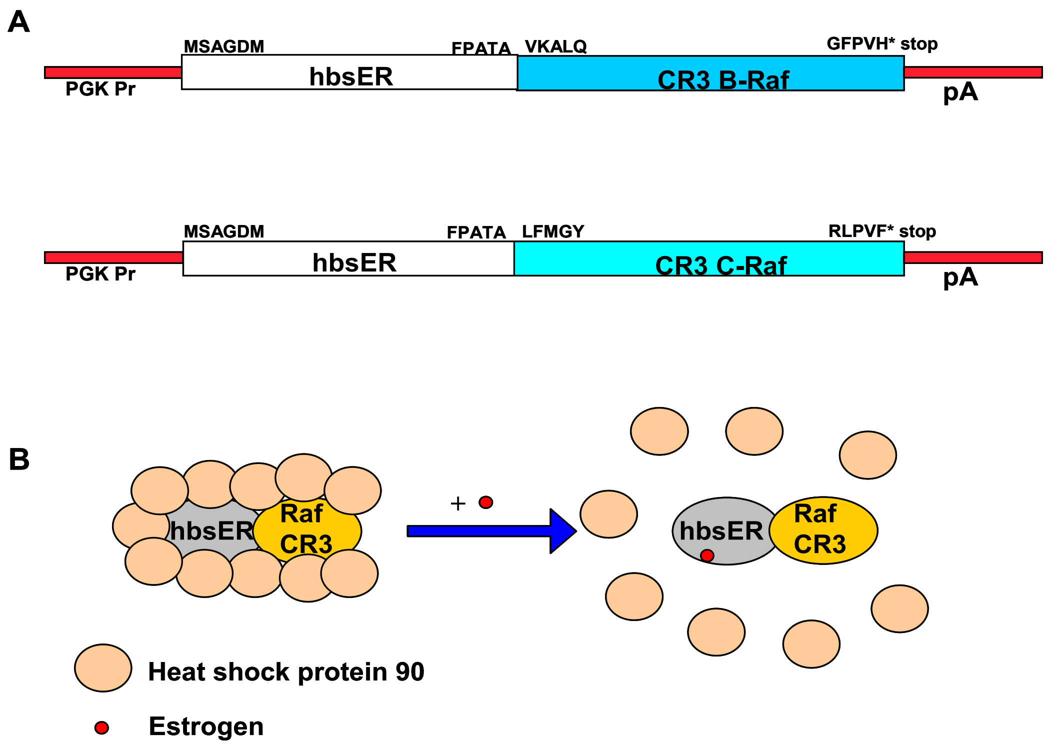


Figure 3-3 Constructs for B-Raf and C-Raf fused to the hormone binding domain of the estrogen receptor. Panel **A** represents the structure of the constructs under the PGK promoter and containing a polyA site in the 3' ends. The start and the terminal amino acids in the hormone binding site of the estrogen receptor and the kinase domains of B- and C-Rafs are marked with letters. Panel **B** depicts a schematic drawing of the Raf domain activation upon estrogen stimulation.

The transfection efficiency of the eEPCs was high, with values of over 90%. That allowed us to work with approximately homogeneous cell lines in every experiment. Figure 3-4 shows a typical transfection experiment of eEPCs with the reporter EGFP gene under the control of the PGK promoter. The intensity and the amount of the EGFP indicates that the experimental system is suitable for the projected experiments.

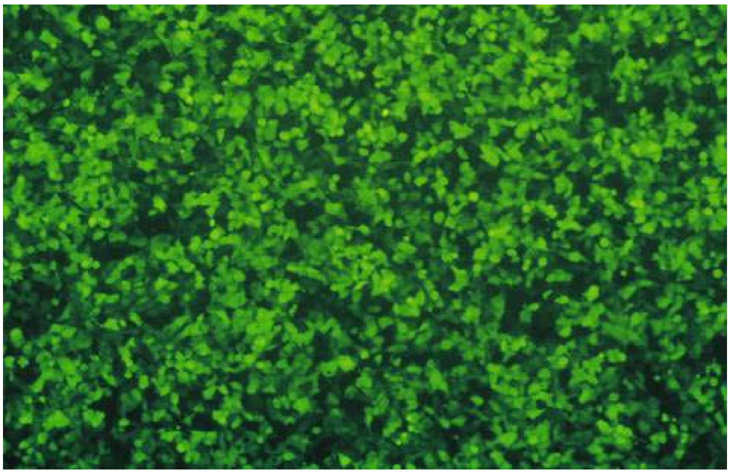


Figure 3-4. The eEPCs exhibit a high transfection efficiency (>90%). The PGK promoter is highly active as indicated by the strong expression of the reporter EGFP gene (the cells were ~80% confluent, in green colour are the transfected eEPCs).

III.2.2. Establishment of stable eEPC lines with constitutively active B- and C-Raf

To obtain stable lines expressing the B- and C-Raf fusion constructs, we used electroporation as the transfection method and employed the neomycin antibiotic G418 for selection. The constructs were shown to be integrated into the genomic DNA (Figure 3-5a panel A) and expressed as mRNA (Figure 3-5a panel B). The PCR primer pairs used spanned a unique region of the ER and the kinase domains of B- and C-Raf. Then single positive colonies were expanded to establish stable cell lines.

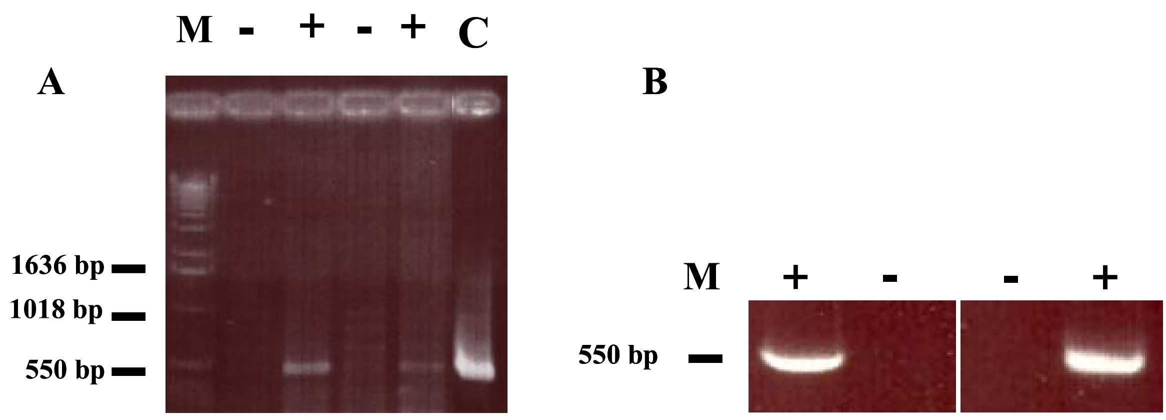


Figure 3-5a. Verification of DNA integration (panel **A**) and mRNA expression (panel **B**) of the fusion constructs between the hormone binding site of the estrogen receptor and the kinase domains of Raf proteins. With – and + are indicated the non transfected (mock) and transfected cells. M is the marker, and C represents the positive control, where the plasmid with the construct was used a as template for the PCR.

To prove the expression of the fusion molecules on the protein level, we used immunoprecipitation (IP) and western blot techniques. Using different antibodies directed against the estrogen receptor and the carboxyl terminus of Raf proteins, we indeed verified the expression of the 61.5 kDa proteins (Fig. 3-5b).

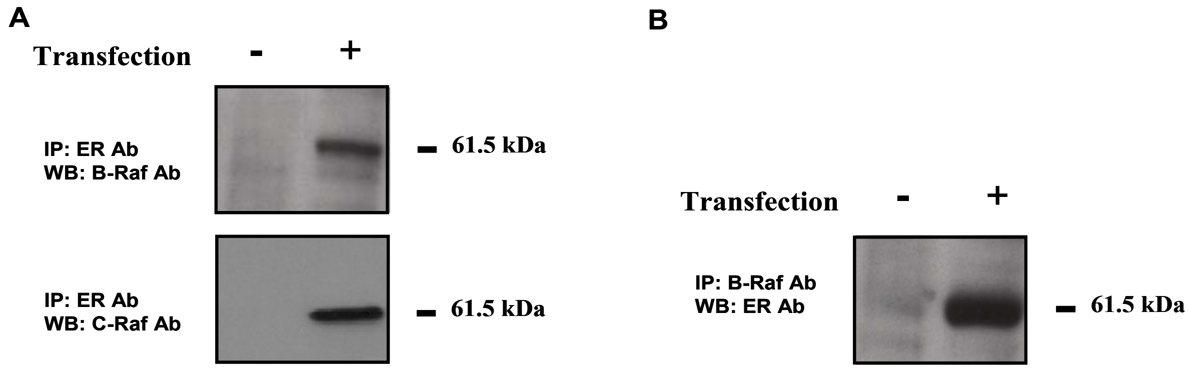


Figure 3-5b.Fusion proteins are properly expressed in eEPCs. B- and C-Raf parts of the molecules were detected by an IP with an anti-ER antibody followed by western blot with antibodies that specifically recognize the kinase domains of B- and C-Raf. In complementary fashion, the same proteins were detected by IP with the B- or C-.Raf antibodies followed by western blotting with the anti-ER antibody.

The first downstream partners in the signaling pathway of the Raf proteins are MEK1/2, which in turn phosphorylate the ERKs, capable of translocating to the nucleus and to influence the transcriptional activation of target genes. To test if the fused proteins were functional, we examined the phosphorylation of MEKs after estrogen stimulation. eEPCs were transiently transfected with the constructs and the empty vector (mock transfection) using Lipofectamine2000. Some of the cells were grown for 12 hours in nutridoma (a starvation medium without any mitogens to reduce MEK phosphorylation) after the transfection (Fig. 3-6 lanes 3, 4, 7, 8) and then induced with estrogen (Fig. 3-6 lanes 1, 3, 5, 7). Using MEK antibodies, we found expression of the MEK's in all samples with some variations (Lanes 1 to 8 in panel A). However, using specific phospho-MEK antibodies recognizing only the phosphorylated forms, we detected them only in the estrogen-induced samples transfected with the Raf constructs. Mock transfected, starved and estrogen induced cells were negative, demonstrating that the fusion constructs were active only after estrogen stimulation (Fig. 3-6, compare lane 3 with lane 7).

Based on these results we went ahead to study the effect of Raf activation in the eEPCs.

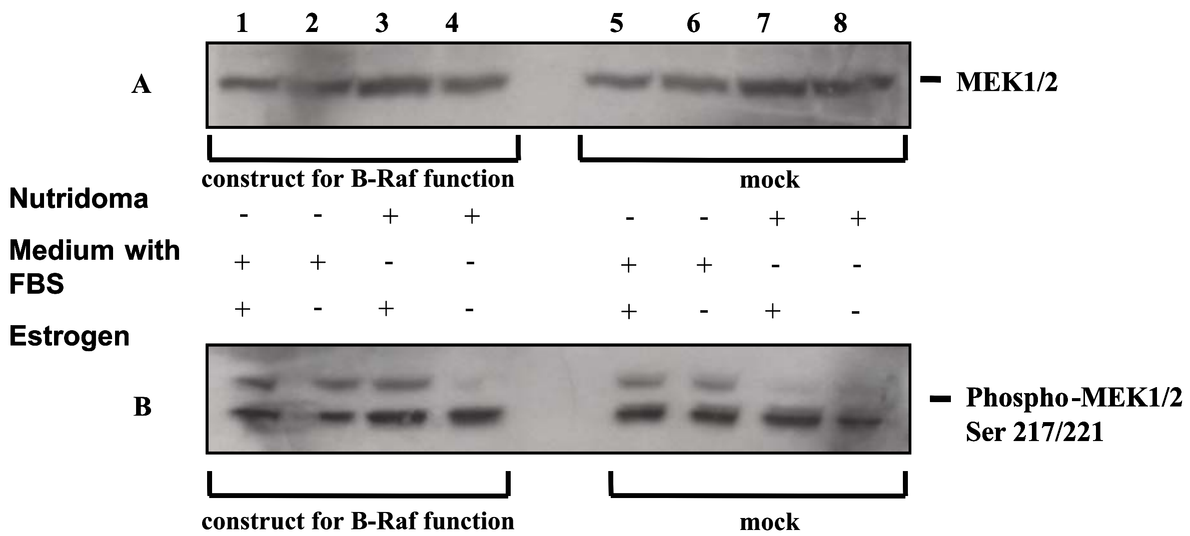


Figure 3-6 Functional kinase assays document that the constructs are transmitting the signal to their downstream partners MEK only after estrogen stimulation. Panel **A** western blot with MEK antibody shows the expression levels of MEKs in the eEPCs. Panel **B** western blot with phospho MEK antibody presents the phosphorylated forms of the MEKs. The lower molecular weight band represents a non-specific recognized protein used as a loading control.

III.2.3. Effects of inducible B- and C-Raf activation in eEPCs

III.2.3.1. Effect on eEPC proliferation

B-Raf and C-Raf are engaged in the regulation of proliferation in many cell types (Smalley et al. 2004). To identify their functional effects, we performed proliferation assays in eEPCs transfected with B-Raf and C-Raf inducible constructs and EGFP constructs as negative controls. The samples were transfected and part of them induced for 8 hours with estrogen. The cells were then allowed to grow for 48 hours and counted. From the results presented in Fig. 3-7, it can be deduced that in the C-Raf transfected and estrogen induced cells, the cell numbers were almost doubled compared to the non-estrogen treated samples. B-Raf also slightly increased the cell numbers. EGFP-transfected cells with or without estrogen served as control, verifying that estrogen application alone did not influence cell proliferation.

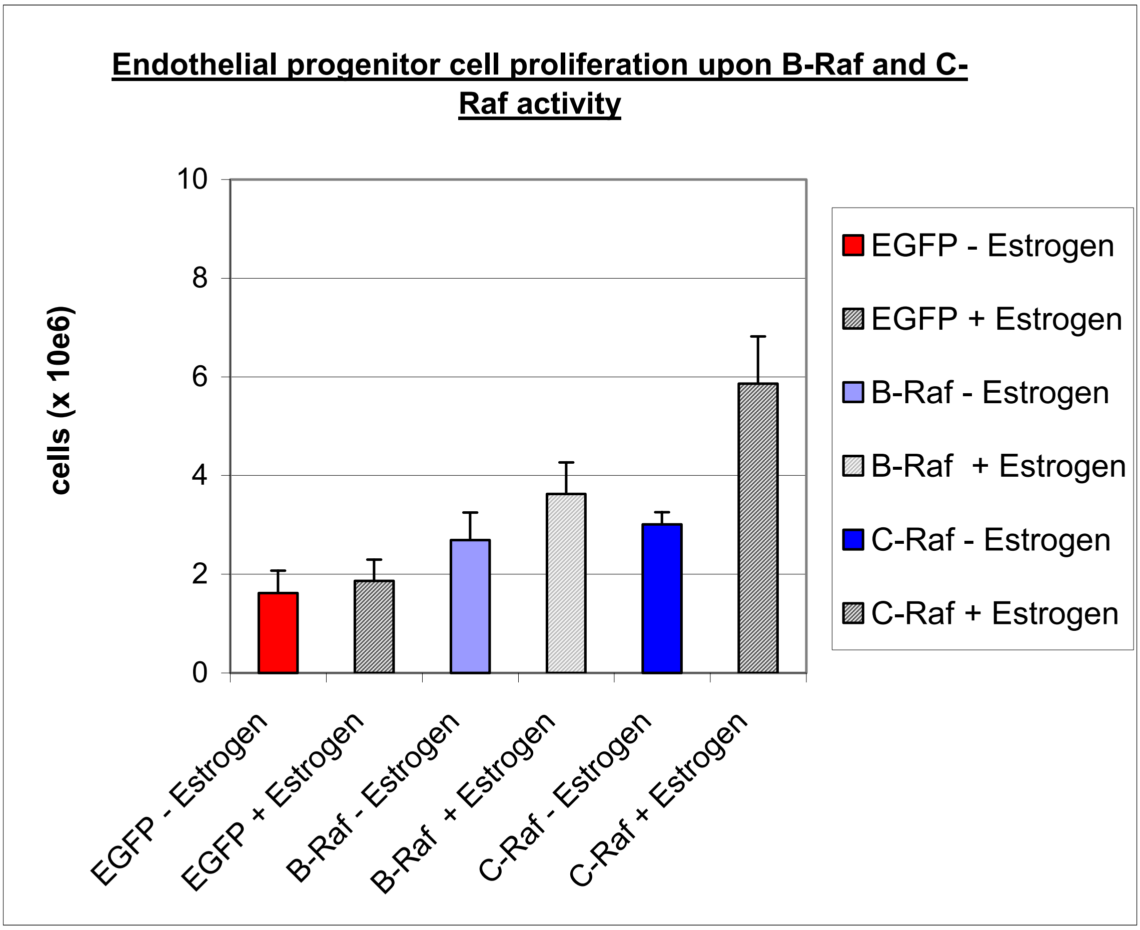


Figure 3-7. C-Raf markedly increases proliferation of eEPCs while B-Raf has a moderate effect. Data are mean ±SD, n=3.

III.2.3.2. Effect of Raf kinases on the differentiation of eEPCs

To further explore the effects of B-Raf and C-Raf on the differentiation of eEPCs, the cells were transfected with the constructs as described above. After 8 hours induction we isolated RNA and prepared cDNA. The cDNAs were then used as templates for the PCR analysis, using a set of gene-specific primers. The genes analyzed included the house keeping aldolase gene as control, and genes expressed and induced in eEPCs upon differentiation with cAMP, such as Flk-1, vWF, thrombomodulin, P-selectin, and M-CSF. Upon transfection with both B- and C-Raf constructs, the expression of all of the mentioned differentiation markers was elicited (Fig. 3-8, lane 7, 11) at the same level as by cAMP treatment. From these experiments, we concluded that the kinase domains of B-and C-Raf are different in stimulating the proliferation, but can substitute for each other in the induction of the differentiation of the eEPCs.

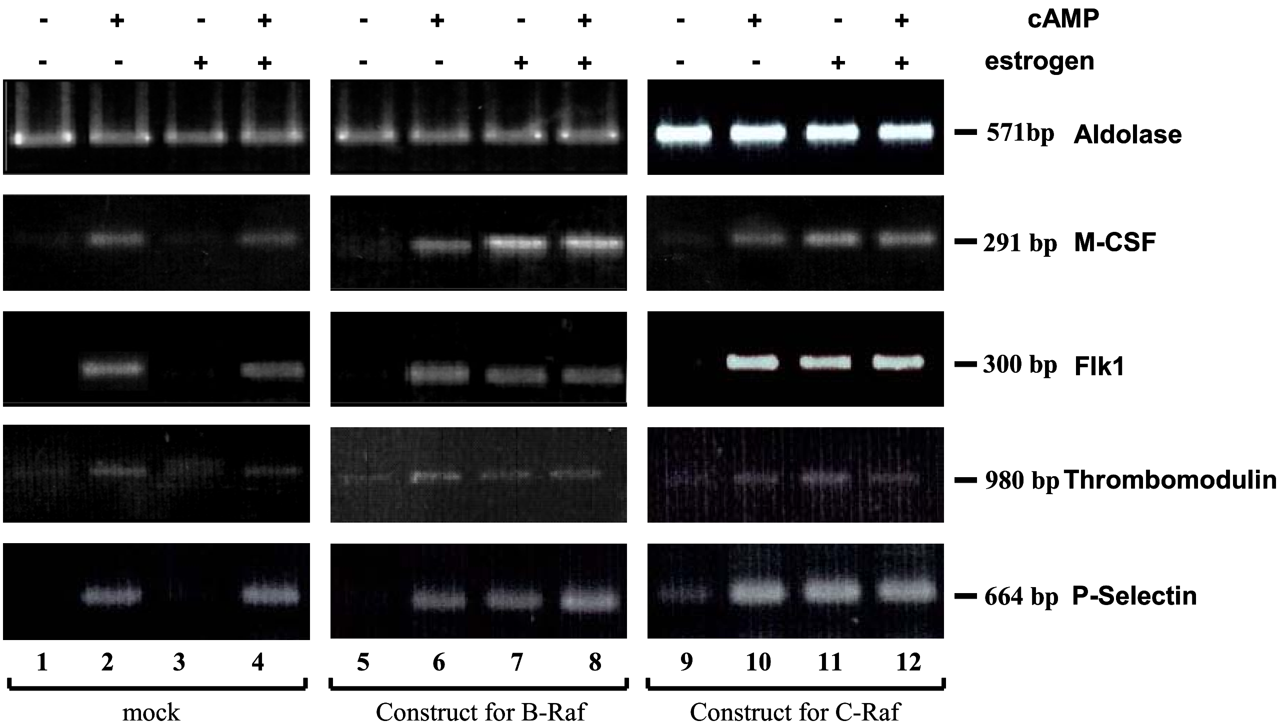


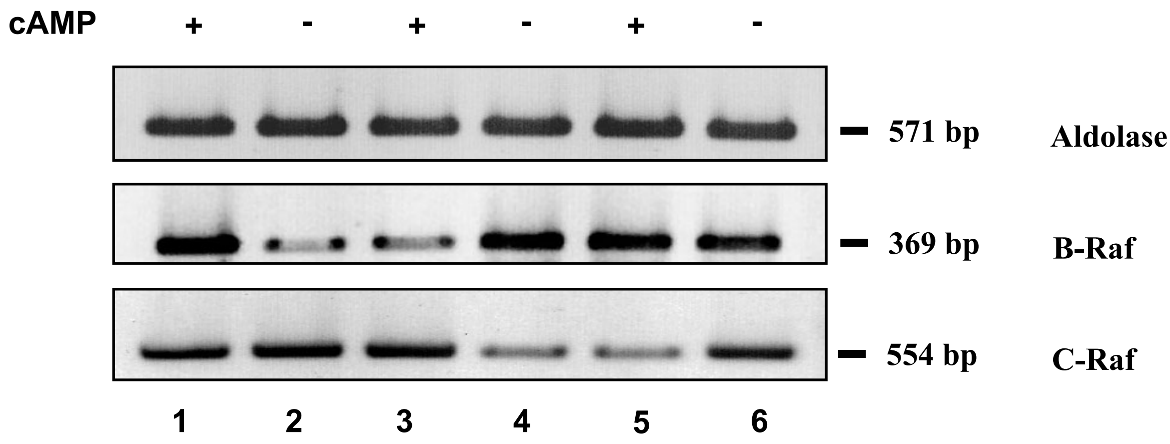
Figure 3-8. B-Raf and C-Raf kinase domains have similar effect on the differentiation of the eEPCs. RT-PCR analysis using gene-specific primers reveals that a subset of endothelial markers including Flk-1, Thrombomodulin, P-selectin, M-CSF are upregulated under B- and C-Raf activation with estrogen. Lane 7 and 11 show the stimulation of the differentiation after the induction of B- and C-Raf respectively, aldolase being used as a loading control.

III.3. Loss-of function experiments with B- and C-Raf in eEPCs

To further elucidate the differential function of B-Raf and C-Raf in eEPCs, we investigated the effects of B-Raf and C-Raf deficiency on the proliferation and differentiation of the eEPCs. To this purpose, we knocked down the expression of both molecules in eEPCs, using the recently developed technique of RNA silencing by double stranded small inhibitory RNAs. The **Silencer^{Tm} siRNA Cocktail Kit (RNaseIII) -** Ambion was employed in all experiments. Because of the high structural similarity between the two Raf family proteins, it was important to use siRNAs prepared from areas of no or very low homology. Alignment of B-Raf and C-Raf gene sequences indicated that the 5´untranslated areas of the two genes exhibit substantial differences. Therefore, we designed primers for this part of the gene and the PCR-amplified products were used as templates to prepare siRNA cocktails specific for murine B- and C-Raf.

With the optimised dsRNAs we performed transfection of eEPCs using oligofectamine as vehicle for 48 hours. Then we tested the specificity of our RNA mixtures by isolating total RNA. The RNA samples were used to synthesize cDNA and analysed by PCR with specific primer pairs for B-Raf and C-Raf. The results in figure 3-9a show that each cocktail specifically reduced the mRNA levels of its gene of origin, but had no effect on the expression level of the other Raf gene. Aldolase was used to control for equal loading.

We also observed that cAMP differentiation did not interfere with the silencing process (compare line 2 to 3 and line 4 to 5 in Fig. 3-9a).



1. Oligofectamine treated cells

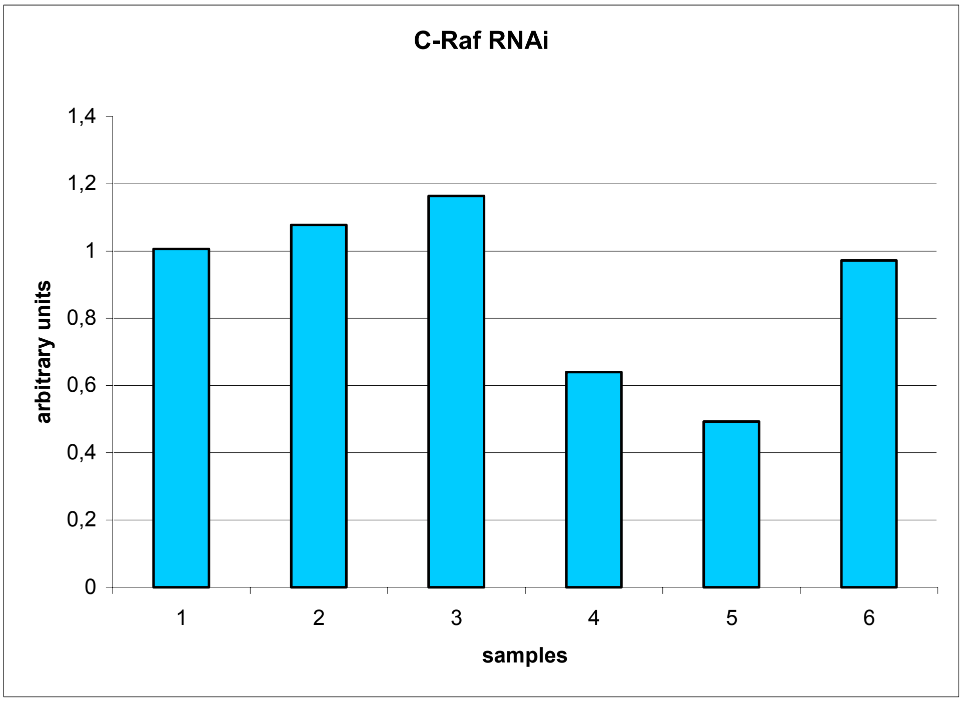
2. } 3. B-Raf si RNA treated cells

4. } 5. C-Raf si RNA treated cells

6. Untreated cells

Figure 3-9a. Silencing of the mRNA expression for B-Raf and C-Raf by RNAi was successfully performed in eEPCs, since there is no interference of the B-Raf siRNA with the C-Raf expression and viceversa.

Using a Scicon densitometric program, we quantified the differences in the mRNA expression levels before and after the B- and C-Raf knock-down. Figure 3-9b shows that for B-Raf we achieved a 3-fold reduction in the RNA levels, while for C-Raf the reduction rate was around 2-fold.



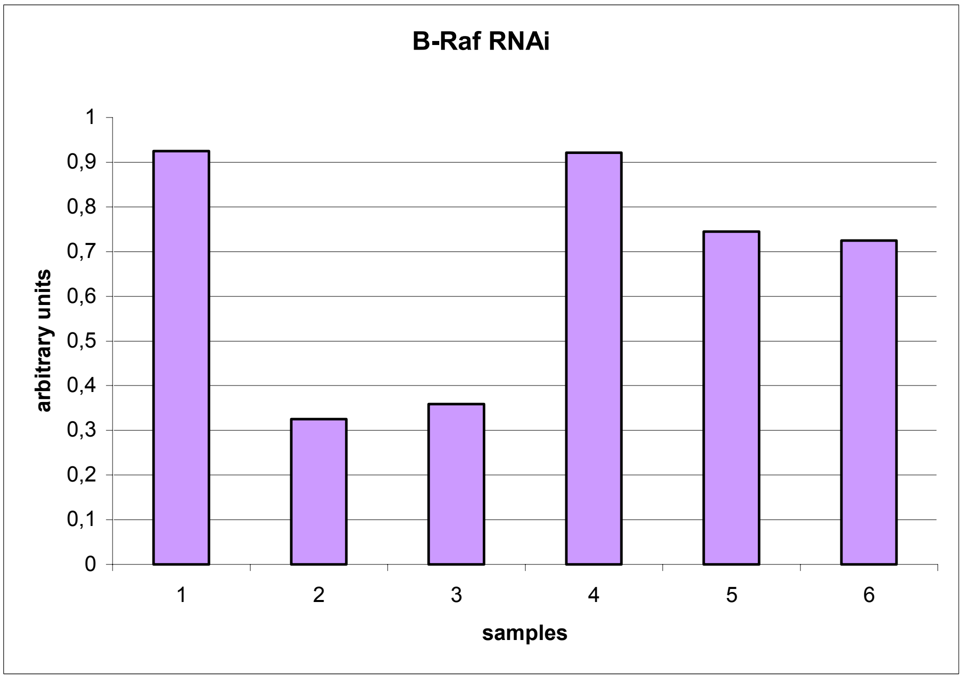


Figure 3-9b. Densitometric quantification of the mRNA expression levels after RNAi treatment indicates ~3-fold reduction of B-Raf mRNA levels and ~2 -fold lowering for C-Raf. All results were equalized using aldolase mRNA levels. Column 1-Oligofectamine treated cells; 2 and 3-B-Raf si RNA treated cells; 4 and 5-C-Raf si RNA treated cells; 6-Untreated cells.

In a next step, we checked the effects of the RNAi induced silencing on the expression of the B-Raf and C-Raf proteins. To this purpose, we performed experiments using eEPCs transfected for 72 hours with oligos for B-Raf siRNA and C-Raf siRNA in two repeats each, and then made immunoprecipitations (IP) and western blots with specific antibodies, recognising the B-Raf isoforms and C-Raf. The results are presented in figure 3-9c. B-Raf protein is present with two splice variants in eEPCs, with molecular weights of 94 kDa and 68 kDa. Panel A of the figure shows that both B-Raf isoforms were significantly reduced after treatment with the B-Raf siRNAs, while at the same time the C-Raf-specific siRNAs had no effect on B-Raf, further supporting their specificity. The stronger reducing effect of the RNAi at the protein level compared to the mRNA expression may be due to the fact that RNA interference not only leads to mRNA degradation, but also blocks mRNA translation.

We performed parallel experiments using the C-Raf siRNAs, followed by IP/Western blot for detection of the C-Raf protein. The extend of reduction was also pronounced and specific for C-Raf, since the B-Raf siRNA oligos did not interfere with the C-Raf expression.

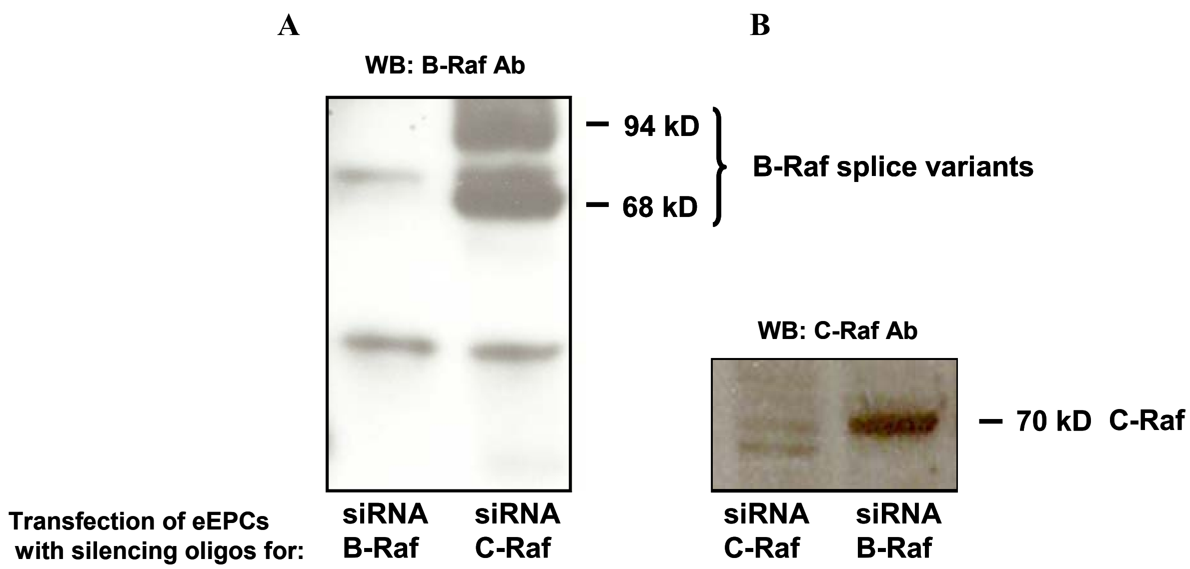


Figure 3-9c. The B- and C-Raf proteins were efficiently down-regulated with RNAi in eEPCs, as proved by western blotting using specific B- and C-Raf antibodies. The unspecific lower molecular weight band in panel A served as loading control.

III. 4 Silencing of B-Raf and C-Raf has different functional effects in eEPCs

To explore the effects of the RNAi on the proliferation and differentiation of eEPCs, we performed similar experiments as with the above mentioned gain-of-function approach using the inducible constructs for B- and C-Raf (III.2).

First, we examined the effects of B- or C-Raf knock-down on the proliferation rate of the eEPCs. For this purpose, we split the cells in two equal amounts each in three repeats, and transfected them with RNAi oligos for B- and C-Raf for 72 hours. The controls were just treated with oligofectamine for the same time period. Then, we detached the cells with EDTA and counted them using a Coulter counter. The results are presented in Fig. 3-10. We found that RNAi treatment for both B- and C-Raf impaired the growth of the cells. The effect was somewhat stronger in cells transfected with siRNA oligos for C-Raf. This was consistent with the observations of the gain-of-function experiments using the inducible constructs, where C-Raf was more efficient in inducing eEPC proliferation.

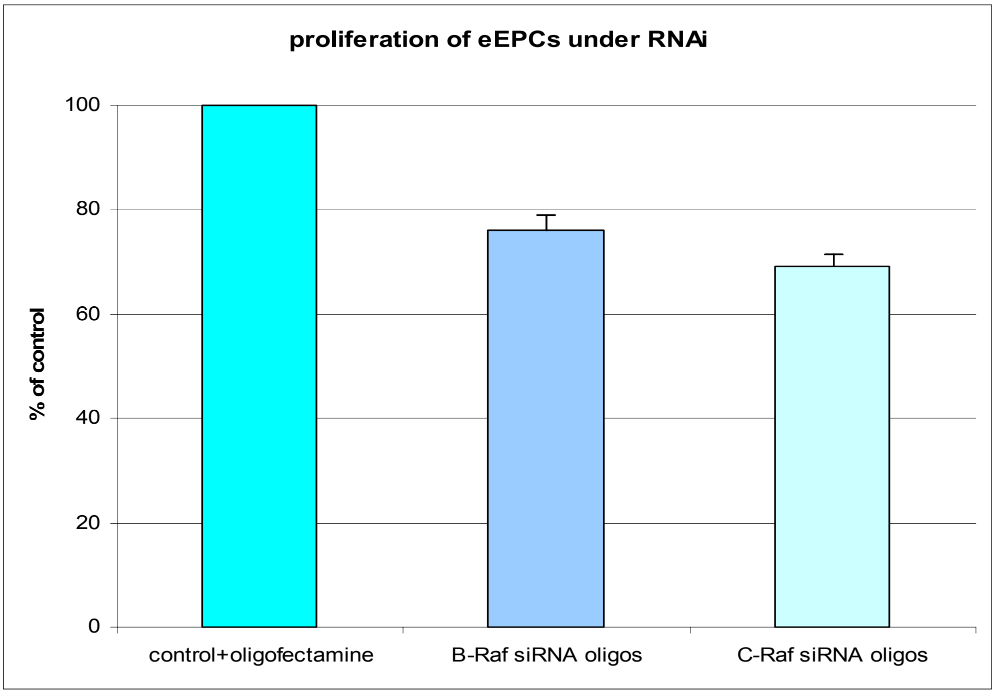


Figure 3-10. C-Raf knock-down suppresses the proliferation of eEPCs. Specific reduction of B-Raf mRNA has a similar, but more moderate effect. Data are mean ±SD, n=3.

We then investigated whether the RNAi for B- and C-Raf had an influence on the differentiation of the eEPCs. In Fig. 3-11, the results are presented from a representative experiment, where we transfected eEPCs with siRNAs for B-Raf, C-Raf, or oligofectamine treated cells for 72 hours. During the last 8 hours, one of the two samples was induced by cAMP and at the end of the experiment, RNA was prepared by standard isolation protocols and processed for cDNA preparation. Using the same primers for the set of genes we analyzed above in the gain-of function approach, we performed PCR to evaluate whether the reduced levels of the B- and C-Raf proteins would influence the differentiation of the eEPCs. In figure 3-11 results are shown in which cAMP was employed to induce the differentiation process in the oligofectamine control treated and C-Raf transfected with siRNA (lanes 2 and 6 Fig.3-11). Differentiation was indeed abrogated in the B-Raf siRNA oligos transfected cells (Fig. 3-11 lane 4). This indicates that B-Raf and not C-Raf is required for the cAMP-mediated eEPC-differentiation. When comdined, the gain- and loss-of-function experiments indicate that B-Raf and C-Raf kinase domains can compensate for each other in their kinase activities. Moreover B-Raf appears to be more engaged in the process of differentiation. In addition, while both B- and C-Raf stimulate proliferation of the eEPCs, C-Raf is a more potent activator of this process.

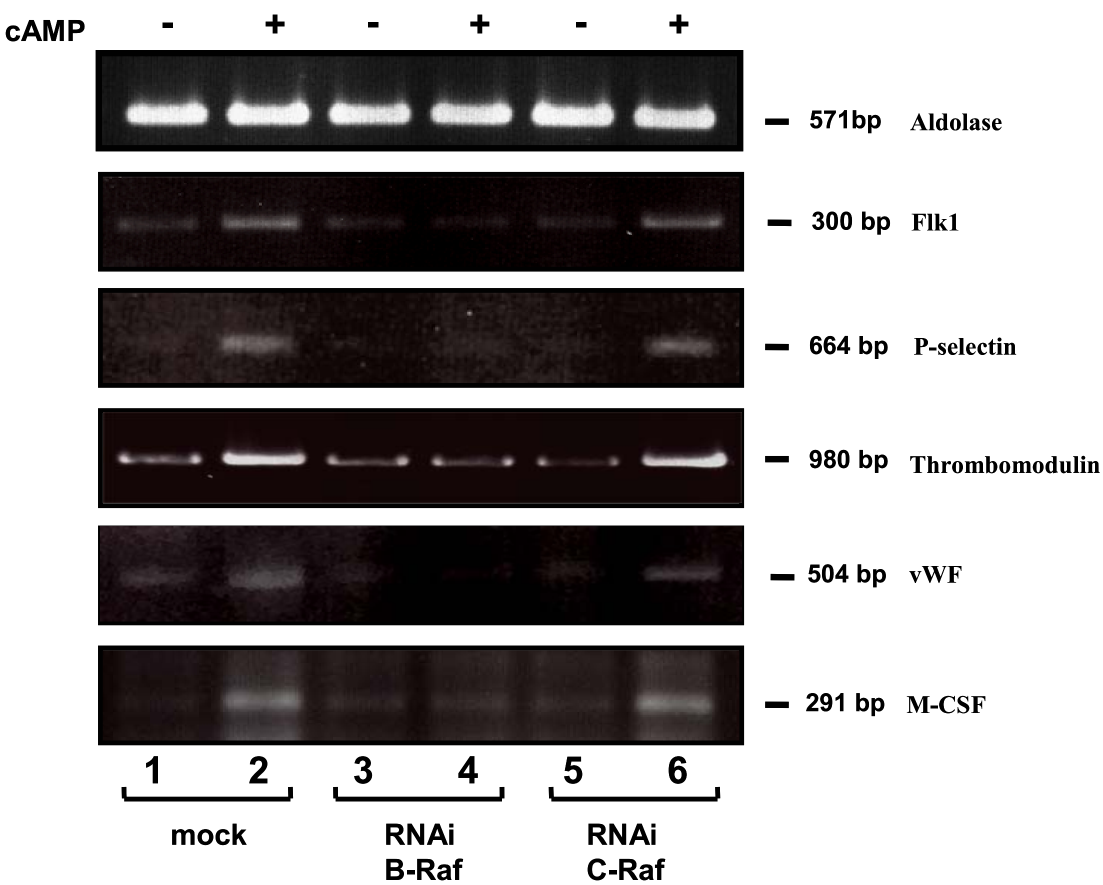


Figure 3-11. B-Raf knock-down ablates cAMP-mediated eEPC differentiation, while silencing of C-Raf has no effect on this process. RT-PCR analysis using gene-specific primers reveals that the endothelial genes Flk-1, Thrombomodulin, P-selectin, M-CSF, vWV are differentially regulated under conditions of B- and C-Raf knock down. Aldolase was used as a loading control.

To further corroborate this conclusion, we isolated eEPCs from mouse embryos, deficient for B-Raf. B-Raf knockout mice are lethal at around d 10-12, therefore isolation of eEPCs at d 7.5 was feasible, as determined by genotyping of the respective eEPC clones. We then performed the differentiation experiment with cAMP and retinoic acid and compared the results between wild type and B-Raf null cells. RT-PCR confirmed the data described above. Importantly, B-Raf null cells failed to differentiate, corroborating the key role of B-Raf in the process of differentiation of the eEPCs (Fig. 3-12).

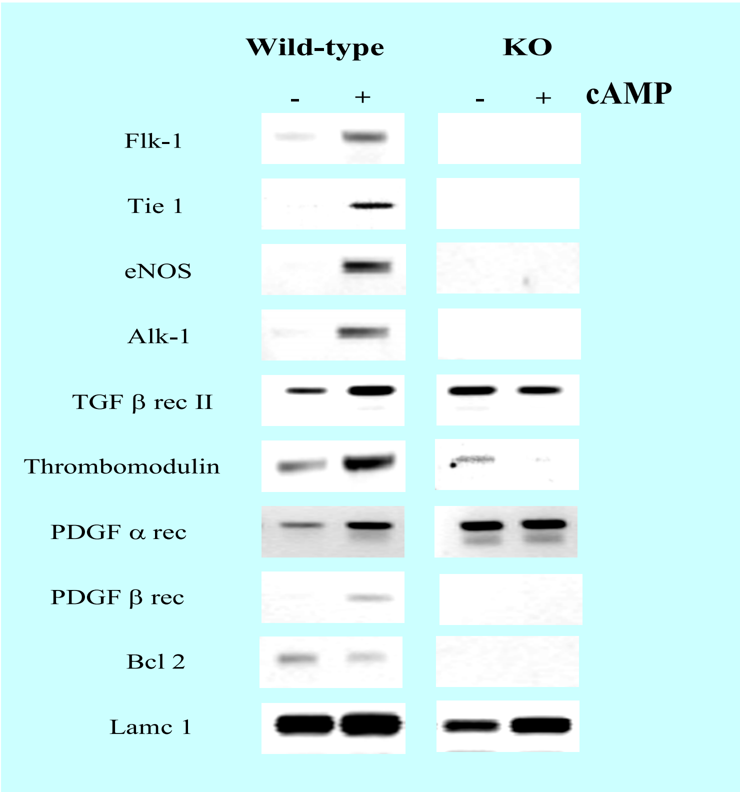


Figure 3-12. B-Raf knock out eEPCs do not differentiate, as indicated by the inability of cAMP to induce the mRNA expression of the marker genes in cells deficient for B-Raf.

III.B. Role of the endothelium in the coagulation start

TFPI which is mainly expressed in endothelial cells is the major anticoagulant molecule in the context of the initiation of blood coagulation. TFPI is known to be degraded by different types of proteases, such as HNE, Cathepsin G, different types of MMPs, and others. The main goal of our study was to analyze weather the proteolytic cleavage of TFPI was of relevance for his anticoagulant properties.

III.5. Expression and purification of human TFPI

Using standard molecular biology techniques, we cloned TFPI in the pASK-IBA3 vector, which allowed us to express the recombinant protein in sufficient amounts and to purify it using a Strep-tag system. This system is known to provide a high purity level of the recombinant protein. The different elution fractions of the expressed TFPI were detected by western blotting, using a specific antibody recognizing the C-terminal part of the protein (Fig. 3-13).

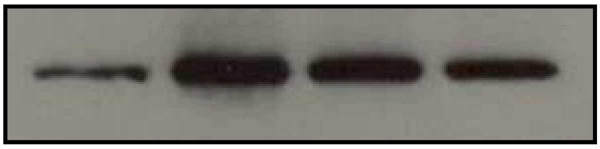


Figure 3-13. Recombinant TFPI was expressed and purified using the Strep-Tag system. An anti-TFPI antibody against the C-terminus was used to verify the expression.

III.6. Site directed mutagenesis of human TFPI

In order to modify the cleavage site of TFPI for HNE, Threonine (T) in position 87 was substituted by Phenylalanine (F). HNE is expressed in high amounts on the surface of the neutrophils, which get in contact with the endothelium upon vascular injury**.** In a next step, this mutant was employed to change two further proteolytic sites. The leucine (L) in position 21 recognized by MMP9, was substituted by alanine (A). In addition, leucine (L) in position 89 recognized by Cathepsin G, was also changed into alanine (A) (Fig 3-14). We then obtained three mutated TFPI forms (T87F, T87F/L21A, and T87F/L89A), together with wild type TFPI.

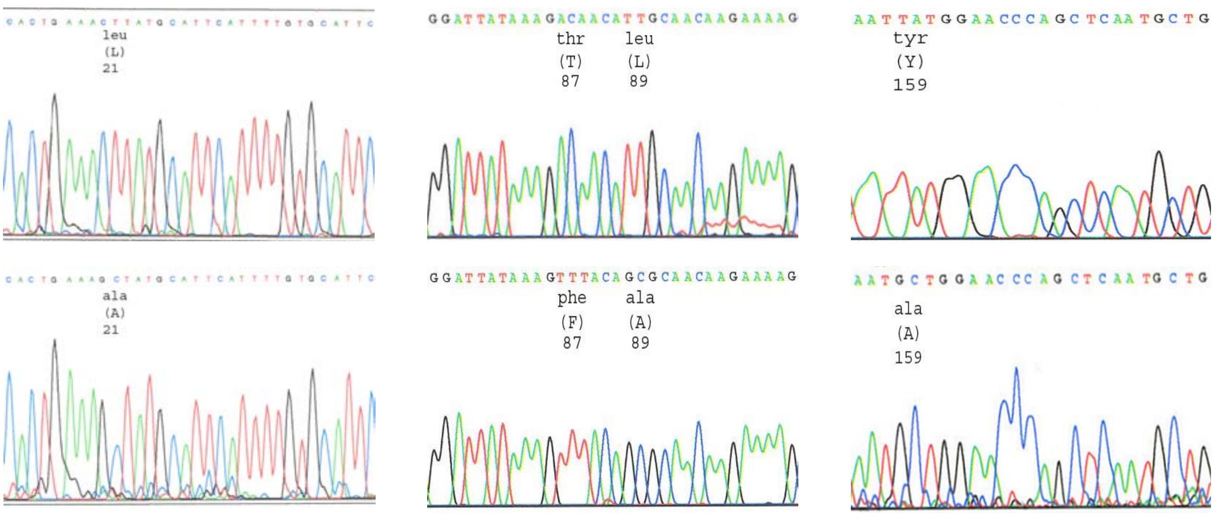


Figure 3-14. DNA sequences analyses verify the correct structure of the TFPI mutants prepared.

III.7. Functional tests with the recombinant human TFPI and its variants

To check the functionality of the recombinant proteins prepared, we performed functional tests including limited proteolytic digestion experiments, formation of factor Xa (procoagulant activities), and thrombelastography. We quantified and equalized all the isolated proteins and then started the experiments.

III.7.1. Limited proteolytic digestion in vitro

We first analyzed whether the different mutated TFPI molecules were indeed resistant against the proteolytic digestion by the different proteases. The recombinant native protein and the mutated forms were digested with the serine proteases HNE, MMP-9, and Cathepsin G. The reactions were performed at 37°C for five to ten minutes and assayed on the western blot using a polyclonal TFPI antibody. All the mutated TFPI forms showed a delay in the fragmentation compared to the native TFPI (Fig. 3-15).

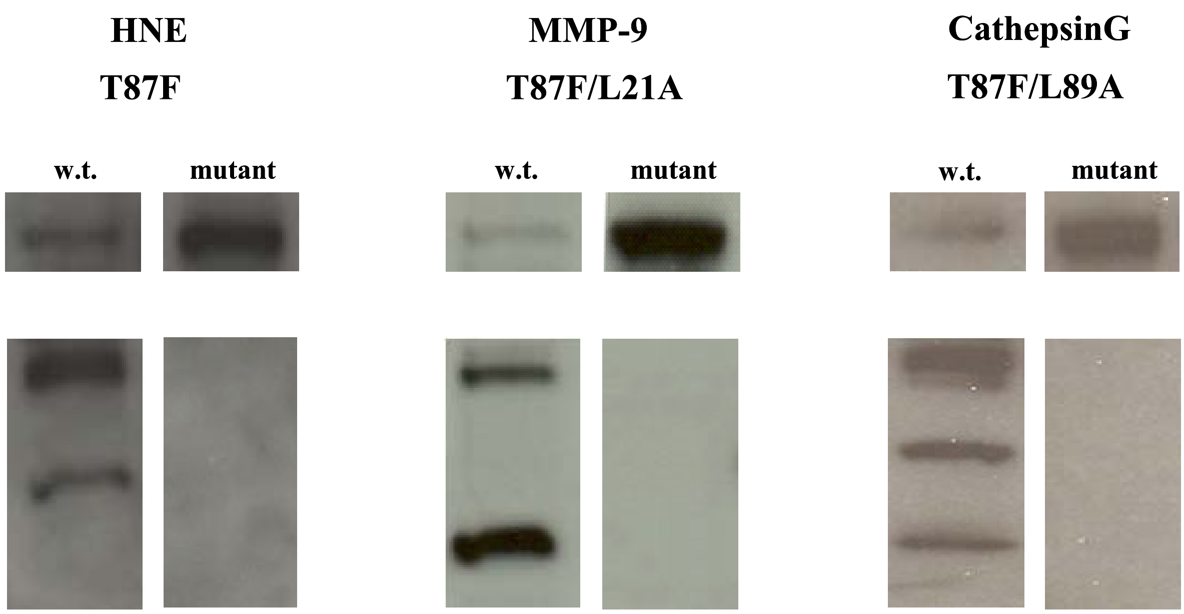


Figure 3-15. The indicated TFPI mutants (5 µg), and the wild type TFPI (5 µg), were digested with serine proteases and with MMP-9 (at concentrations of 10 nM for HNE, 50 nM for CathepsinG and 200 nM for MMP-9). Compared to the native protein, TFPI degradation is delayed after specific substitution of the amino acids at the proteolytic cleavage sites (w.t.- wild type).

III.7.2. Procoagulant activity

To evaluate the functional behaviour of the different mutated forms, we determined the formation of factor Xa in the presence of platelet/neutrophil suspensions. When incubated with neutrophils, platelets rapidly cleave factor X. It has been hypothesized that this is due to proteolytic inactivation of TFPI (Engelmann et al., 2003).

We therefore added the native TFPI and the mutated forms of the protein to suspensions consisting of platelets and neutrophils. The results are presented in Fig 3-16. Native TFPI reduces the procoagulant activity by around 30%, while the mutated forms lower the factor Xa formation by 40% to 60%. The strongest effect was observed with the double mutated TFPI (T87F/L89A), which suppressed the formation of factor Xa by 65 % (Fig. 3-16).

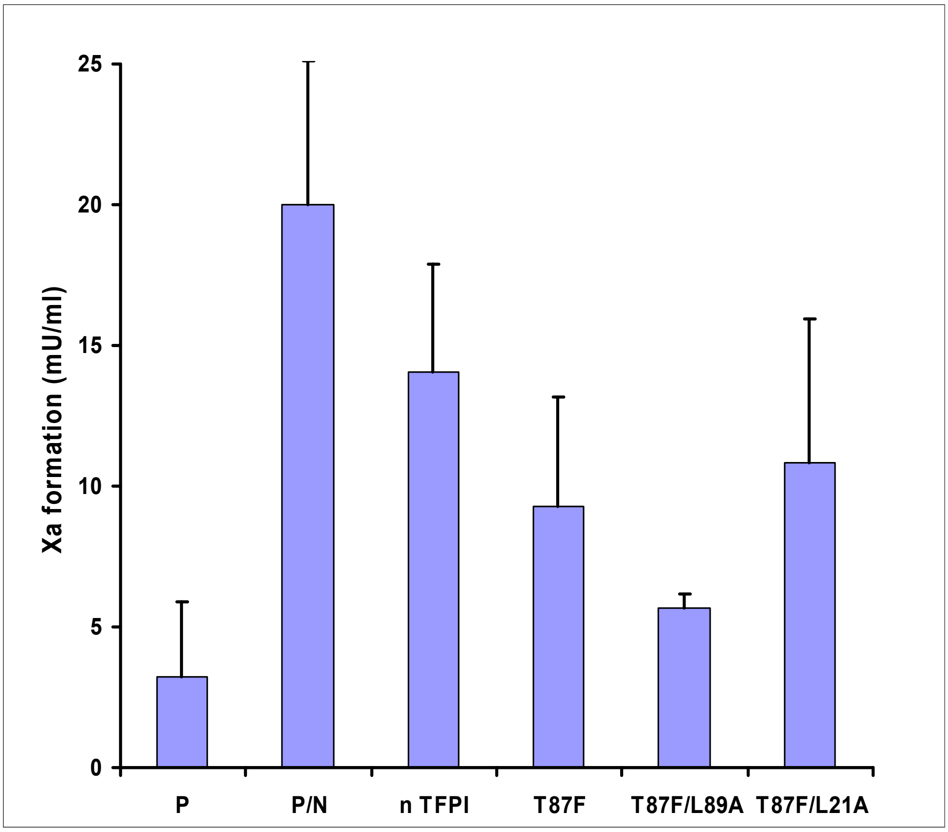


Figure 3-16. Factor Xa formation experiments in suspensions of platelets and neutrophils indicate that the initiation of coagulation is delayed by mutations of the proteolytic cleavage sites of TFPI. P-platelets, P/N-platelets-neutrophils suspension, nTFPI- native tissue factor pathway inhibitor and its mutant forms were incubated in platelets-neutrophil suspension. The recombinant TFPI proteins were used at 2.5 nM (end concentrations). Data are mean ±SD, n=5.

III.7.3. Thrombelastography

To address whether the TFPI mutants affected the formation of fibrin was assessed whole blood. As a control, we used total blood without collagen (Fig 3-17, panel I). In panel II, blood was activated by collagen (12µg/ml) in the absence of TFPI. In panel III native TFPI was included into the blood, while in panel IV the T87F/L89A mutant was added. The results indicate that the inhibition of fibrin formation elicited by the native TFPI is strongly enhanced (by about 3-fold) when the HNE and Cathepsin G cleavage sites are mutated, preventing the inactivation of the proteins.

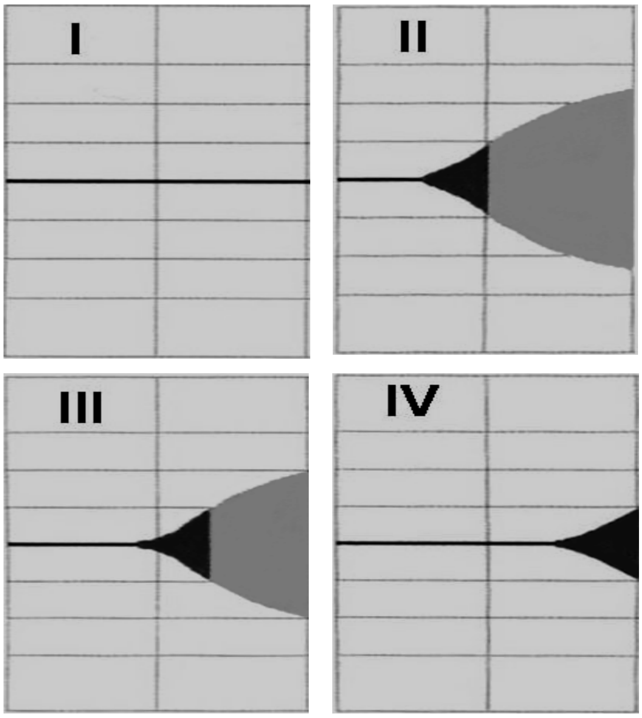


Figure 3-17. Determination of fibrin formation in anticoagulated blood system revealed that the T87F/L89A TFPI mutant delays fibrin formation more efficiently, than the native TFPI. The distance between the starting point and the broadening of the amplitude represents the fibrin formation.The horizontal line of the rectangle corresponds to 600 sec. I-total blood without collagen, II-blood activated by collagen (12µg/ml), III-native TFPI added into collagen activated blood, IV-mutated TFPI added into collagen activated blood. All the recombinant proteins were used at 2.5 nM end concentration.

IV. Discussion

IV.1. B- and C-Raf in endothelial maturation

During the last few years, considerable progress has been made towards elucidating the mechanisms indicating the transition of the endothelial progenitor cells to mature endothelial cells. Moreover, the functions and the possible applications of EPCs as therapeutic agents have been described. Knowing in more detail the signal transduction pathways modulating gene transcription and the ensuing endothelial progenitor cell response will give us an opportunity to influence endothelial cell development and activation.

Until now, little is known about the role and the regulation of the MAPK kinase signal transduction pathway in endothelial progenitor cells. One reason is that adult EPCs are difficult to isolate and maintain in cell culture. Hatzopoulos et al. (1998) established a stable cell line from mouse embryonic endothelial progenitor cells (eEPCs) that arise on E 7.5 at the onset of vascular development and formation of the cardiovascular system. Working with mouse eEPCs could be of relevance and also a good model system for styding how the process of maturation is occuring in human embryonic endothelial progenitor cells, which from an ethical point can not be researched. The eEPCs possess specific features of immature endothelial cells, including expression of endothelial-cell specific markers such as Thrombomodulin and Tie-2. Under in vitro conditions, the cells can be induced to differentiate with cAMP. This process results in the activation of a set of genes specific to the endothelial lineage, i.e., Flk-1, vWF, P-selectin, M-CSF, and others, proving a novel system to study the transition of the eEPCs to the mature endothelium. This differentiation process is probably regulated by a variety of signaling mechanisms. In this work, we have shown that one of the key components in the regulation of the maturation process is the B-Raf kinase, a member of a larger family comprising A-Raf, B-Raf and C-Raf. The role of these proteins in the embryonic development was addressed by the work of Wojnowski et al. (1997), preparing B- and C-Raf knock out mice. The B-Raf KO mice are subjected to embryonic lethality around d 10-12. One of the most relevant structures affected by the absence of the B-Raf protein are the blood vessels and the endothelial cells. C-Raf KO mice die within hours after birth, because their lungs fail to inflate. They show general growth retardation and defects in placenta, skin, and lungs.

The Raf family proteins are crucial units in the "normal" cell life of every cell and even single mutations in their structures disorganize and transform cellular function. For example, B-Raf mutations were identified in human lung adenocarcinoma (K438T), lung small cell carcinoma (T439P), malignant melanoma (K438Q), colorectal tumors (V599E) and others. (Mercer et al.2003; Davies, Bignell, Cox et al. 2002). The Raf family proteins are relatively well studied components of the cell signaling pathways in different cell types and species (Hagemann and Rapp 2000, O'Neil and Kolch 2004). In PC12 cells all three forms of the Raf family are expressed. These cells proliferate under EGF stimulation, but differentiate into a neuronal like phenotype after treatment with NGF. Both growth factors are able to activate Raf isoforms. Wixler et al. (1996) investigated whether there are differences in the activation kinetics of A-Raf, B-Raf, and C-Raf in response to EGF and NGF treatment. An initial activation of all three kinases was detected, but only A- and B-Raf showed sustained activation by NGF, which was not seen after EGF treatment.

The process of regulation of the Raf family member requires protein-protein interactions, phosphorylation, and presumably lipid binding (Slupsky et al. 1998). It was proposed that Raf dimerization also might be involved in the activation (Masson et al. 1999). The initial Raf activation requires the small G-protein Ras, but the following sustained activity of B-Raf is mediated by another small G-protein, Rap1 (York et al. 1998) (Fig. 4-1).

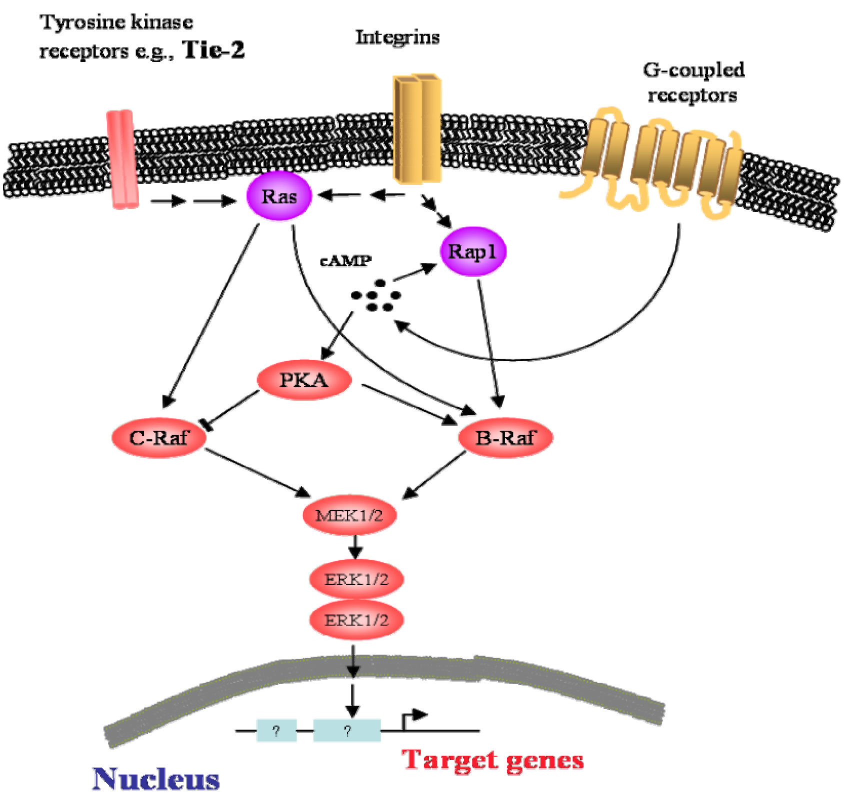


Figure 4-1. Schematic drawing of the Ras/Raf/Erk signal transduction pathway in eEPCs.

In PC12 cells comparison of cAMP sensitivity of C-Raf and B-Raf showed functional differences between the two isoforms (Erhardt et al. 1995). While C-Raf activity is blocked by cAMP, B-Raf is activated (Ohtsuka et al. 1996). cAMP-induced PKA phosphorylates Rap1, which not only blocks C-Raf activation, but binds to B-Raf, resulting in sustained activation of this kinase and concomitant differentiation of PC12 cells (Vossler et al. 1997) (Fig 4-1). This regulatory system has also implications for differential responses of cell types in the brain (astrocytes and neuronal cells) to cAMP. Central nervous system-derived neurons, but not astrocytes, express B-Raf in addition to C-Raf. In neurons cAMP activates Erk in a Rap1/B-Raf dependent manner, while in astrocytes cAMP decreases Erk activity by inhibition of C-Raf. Thus, B-Raf appears to be the molecular switch, which causes differential regulation of Erk in neurons versus astrocytes in response to cAMP (Dugan et al. 1999). This pathway is driven in an opposite way in renal epithelial cells, where the cAMP regulated proliferation is depending on B-Raf, but not on C-Raf (Yamaguchi et al. 2004).

IV.1.1 B- and C-Raf function upon regulated overexpression

Based on the observations in neuronal and other cell types, and the vascular-specific phenotype of the B-Raf KO mice, we investigated the influence of the B-Raf and C-Raf proteins on the proliferation and differentiation of eEPCs. To accomplish this goal, we prepared molecular tools for gain- and loss-of-function experiments. The gain-of-function constructs for B-Raf and C-Raf function were designed to allow an inducible system, where we could obtain regulated activation of the kinase domains of either protein by estrogen treatment. The influence of estrogen itself on the eEPCs was checked before the start of the experiments, confirming that the hormone alone had no influence on the outcome of our experimental procedures.

A second major goal of our work was to reveal the link between cAMP stimulation and the functions of the Raf proteins in the eEPCs. The high transfection level of the eEPCs was an important precondition for performing all our experiments successfully. In optimizing the conditions, we found that the promoter which drives expression in eEPCs is of great importance. With the use of the standard CMV promoter, no satisfying amounts of proteins were obtained. For this reason we chose to use vectors containing the PGK (phosphoglycerol kinase) promoter, which has found to be strong enough for the production of the protein variants in the eEPCs.

We not only proved that the expression levels of the proteins were sufficiently high, but also showed that the produced proteins were functional and capable of phosphorylating their downstream partners in the signaling pathways. We performed kinase assays after starvation in media without FBS (the main source of growth factors and inducers of the MAPK pathway) thus depleting the cells from any extra cellular signals activating the MAPK pathway. Indeed, the fusion proteins successfully phosphorylated their immediate downstream partners MEK1/2 after estrogen application. By using antibodies that specifically recognized phospho MEK, we documented that activation of the MEKs depends solely on the treatment with estrogen.

Then, we analyzed the functional responses of the eEPCs after triggering the B- and C-Raf activities. The already established knowledge about cAMP differentiation was the starting point for our experiments. We could show that the activation of the kinase domain of B- and C-Raf leads to the upregulation of genes involved in the differentiation of the eEPCs, as for example vWF, thrombomodulin, M-CSF, P-selectin, Tie-1, which are not expressed in the progenitor cells. The effect of this activation led us to conclude that both B- and C-Raf kinase activities are sufficient to reconstitute the cAMP -induced differentiation of eEPCs. In contrast, loss of function experiments (discussed below) revealed that the B-Raf protein alone is necessary for the differentiation of the EPCs. We conclude that the regulatory CR1 and CR2 domains of the Raf proteins are necessary to achieve specificity and that either kinase domain of B- or C-Raf can substitute for each other.

A further scope of the experiments was to investigate the effects of Raf proteins on the proliferation potential of the eEPCs. For this purpose, we used the B- and C-Raf constructs (and control EGFP-containing construct), which were transfected into eEPCs and induced. The results showed that C-Raf is a more potent activator of the proliferation of the eEPCs than B-Raf. This result is consistent with previous results observed in neuronal cell lines, where C-Raf was linked to proliferation and B-Raf to NGF-mediated differentiation (Kao et al. 2001). It is known that just C-Raf but not B-Raf is interacting with Cdc25 phosphatases which play a key role in cell-cycle progression by triggering activation through the removal of inhibitory phosphate groups of the cyclin-dependent kinases (CDK) (Nilsson et al. 2000). The phosphorylation of Cdc25 by C-Raf may serve as a possible link between mitogenic signaling and cell-cycle regulation.

IV.1.2 B- and C-Raf roles into eEPCs in condition of knock down

The technique of RNA interference has recently been developed for the reversible down regulation of gene expression. Using this method, we investigated the role of both B- and C-Raf from another aspect, namely knock down or loss-of-function of Raf genes in eEPCs. The three proteins of the human Raf family have a similar type of organization and high levels of homology in between each other. They consist of three conserved regions, namely CR1, CR2, and CR3, connected between each other by linker areas. The amino-terminal regulatory domain contains the conserved regions CR1 and CR2, while the carboxy-terminal kinase domain constitutes the third conserved region, CR3. B-Raf shows a 75% homology to A-Raf and a 65% homology to C-Raf in the CR1 region, where the RBD (Ras binding domain) and CRD (cysteine-rich domain) are located. The serine/threonine rich CR2 region has a 47.6% homology with both A-Raf and C-Raf. In the CR3 region, the homology is higher, accounting to 76.4% with A-Raf, and 79% with C-Raf. The first 30-40 amino acids of the amino terminus and the last 20 amino acids of the carboxy terminus differ considerably among the various isotypes. Overall B-Raf shows 52% homology to A-Raf and 54% homology to C-Raf (Sithanandam et al. 1990). The Raf family proteins can be generally divided into two functional domains. When projecting the RNAi experiments in order to obtain isoform specific effects, it was important to find well distinguishable non-homologous areas in between the three proteins. This was done successfully, as the results showed specific down regulation of the respective mRNA and protein levels. The siRNA oligonucleotides chosen, corresponded to regions encompassing the 5' ends of the molecules including parts of the untranslated and coding regions, which ensured complete degradation, precluding the formation of any truncated forms through differential splicing. Using the RNAi kit we obtained strong reductions of the mRNA levels of both B-Raf and C-Raf. Indeed, compared to mock transfected cells, an approximately 100% suppression of the B-Raf protein levels and around 80% of the C-Raf contents was achieved. The results can be explained by the canonical mechanism of RNA interference (degradation of mRNA) and by the additional effect of the siRNAs on promoting the process of mRNA degradation and simultaneously blocking mRNA translation. The knock down levels of any protein cannot be really predicted and the success in the method depends mainly on the specificity of the cell type, the efficacy of the siRNA cocktail and the particular mRNA features (secondary structure, stability), also of the protein turnover in the cell. Once having established appropriate siRNA tools, we analyzed the proliferation rates and the cAMP-induced differentiation on the eEPCs. Our results indicated differential roles for the B-Raf and C-Raf proteins in the processes of differentiation and proliferation. While cAMP treatment of eEPCs turns on differentiation under control conditions, in the case of the B-Raf knock down the process was blocked. When we tested the same set of genes already analyzed for the evaluation of the kinase domain functions of the two proteins, however, none of the genes was induced. The knock down of C-Raf in the eEPCs had no effect on the differentiation, and showed induction levels comparable to the mock-transfected cells. These results were confirmed in B-Raf null eEPC clones established from the corresponding KO mice. It is noteworthy that the differential role of the B- and C-Raf kinases in the eEPC proliferation revealed in the gain-of-function studies was also confirmed in the loss-of-function approaches, since knock-down of C-Raf had a more pronounced effect on eEPC proliferation than B-Raf.

The results thus allow a first insight into the molecular mechanisms that drive growth and differentiation of eEPCs. It is known that cAMP has a dual and opposite effect on C-Raf versus B-Raf, influencing both molecules through protein kinase A (PKA). PKA phosphorylates the small G protein Rap1, which in turn blocks C-Raf, which at the same time can act as a sustained activator of B-Raf. In parallel, PKA phosphorylates B-Raf on its own, which has an activation effect as well. Our data are consistent with this scenario, since we find that B-Raf is necessary and sufficient to transmit the cAMP-mediated differentiation signal, while C-Raf appears to be dispensable.

The findings in eEPCs might have broader implications in the overall field of vascular biology. The genes specifically induced by the B-Raf kinase in eEPCs have also important roles under pathophysiological conditions. For example, while thrombomodulin and vWF are involved in coagulation, P-selectin is critical for the homing of cells of the immune system at sites of inflammation, and Flk-1 participates in angiogenesis. Controlling the expression of these genes via modulation of B-Raf could open new ways to manipulate their function.

As discussed above, C-Raf has an important role in eEPC proliferation. The results showed that growth rate reduction was stronger in cells transfected with C-Raf siRNA oligos as compared to mock transfected and B-Raf siRNA transfected cells. These results confirmed data obtained with the inducible expression of B-Raf and C-Raf. We conclude from the proliferation experiments that the eEPCs proliferation is considerably more dependent on C-Raf compared to B-Raf. This finding could assist in finding new tools to preferentially modify endothelial cell growth (a critical process in pathological angiogenesis), while at the same time preserving the ability for stimulation of the mature endothelial cells.

Figure 4-2 presents a schematic drawing of the roles of B-Raf and C-Raf in the processes of differentiation and proliferation of the eEPCs. The decision which pathway is activated depends on the incoming extracellular mitogenic stimuli and the strength of activation. The proteins may be considered as signal collectors that further propagate the incoming signals to their downstream partners (MEKs and ERKs). Thereby, the activities of nuclear transcription factors are directly modulated and a specific cell response is evoked.

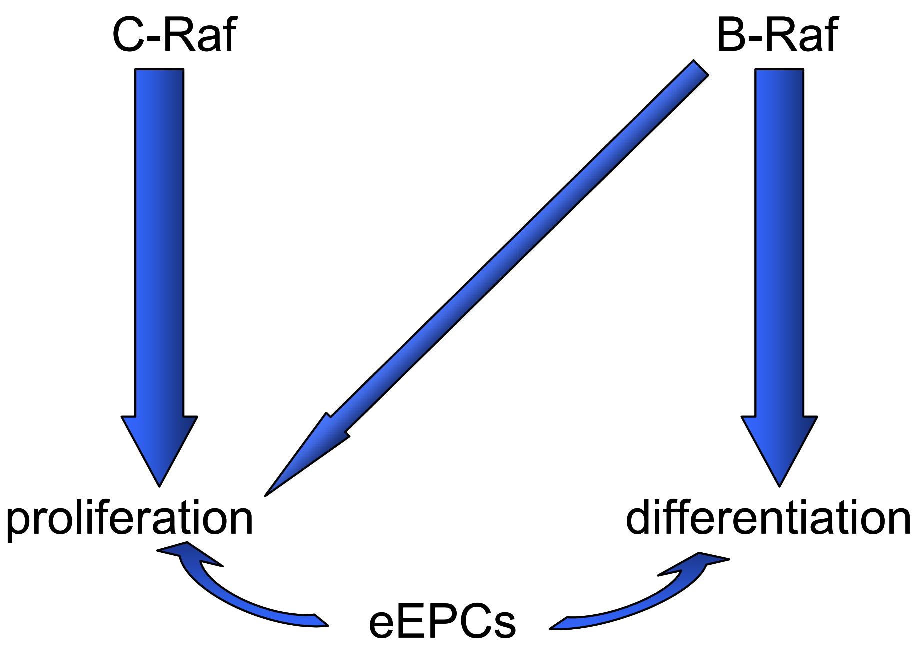


Figure 4-2. The roles of B-Raf and C-Raf in the proliferation and differentiation of eEPCs as revealed by the findings of the present investigation.

IV.2. Anticoagulant properties of the endothelium during the coagulation start

A better understanding of the blood coagulation system is of great importance for elucidating the pathogenesis of a large variety of diseases, in which the prime cause is the clot formation or the absence of it (e.g. arterial and venous thrombosis, hemophilia). Blood coagulation is a complex process, ultimately resulting in the formation of fibrin. Several molecules are involved as positive and negative regulators of the fibrin generation, modulating its velocity and intensity. Currently, TFPI-1 is believed to be the main inhibitor of the initiation of coagulation. The microvascular endothelium is the major cell type of the human organism that synthesizes TFPI-1. Hence, this cell type might be considered as the main anticoagulant unit in the human body. Indeed, any dysfunction in TFPI synthesis in embryonic development or in the adult organism results in severe coagulation abnormalities (Huang et al. 1997; Mestrick et al. 2001). TFPI is distributed in three pools in vivo; ~80-85% is associated with the endothelial surface, representing a strong anticoagulant surface in the vessel wall. ~10% circulates in plasma in association with lipoproteins, a truncated form of the protein that has reduced inhibitory capacity. ~10% is found in platelets, being a storage pool for the protein originally synthesized in the megakaryocytes. In our lab, a model of intravascular coagulation has been developed (Engelmann et al. 2003), suggesting that the TFPI originating from the activated platelets might be degraded by serine proteases such as HNE and Cathepsin G which are known to be exposed on the surface of the neutrophils (Fig. 4-3). Thereby, neutrophils might be able to regulate the coagulation start in complexes with activated platelets.

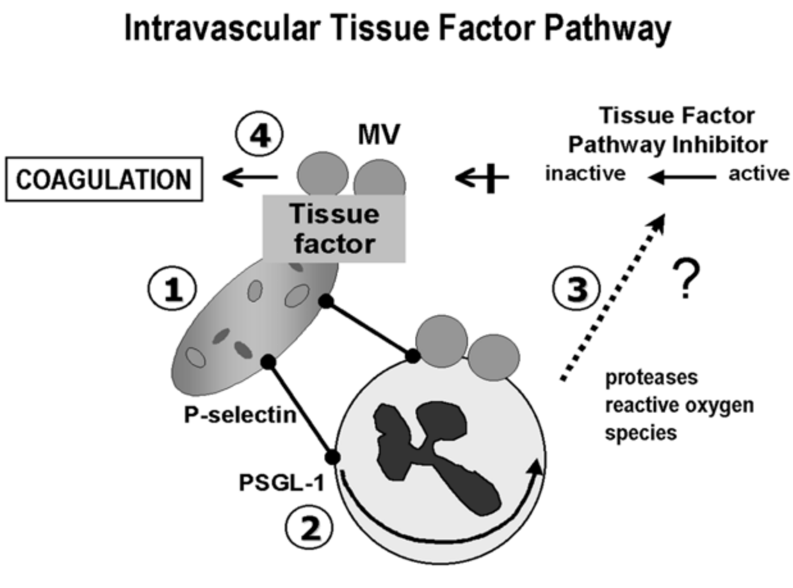


Figure 4-3. Initiation of intravascular coagulation as promoted by platelet-neutrophil interactions.
(From Engelmann et al. 2003)

IV.2.1. The role of the platelet-neutrophil system in the initiation of the coagulation

Neutrophils are the major component of the acute inflammatory response and their local activation leads to the release of the serine protease HNE. In term, HNE can proteolytically inactivate several inhibitors of the coagulation process including antithrombin III, heparin cofactor II and TFPI. It recognizes similar protein motifs in all of them (Val-|-Xaa > Ala-|-Xaa). The degradation of these inhibitors may result in local progression of the coagulation at the site of inflammation (Jochum et al. 1981, Jordan et al. 1989, Higuchi et al. 1992). Platelets are a significant source of TFPI, and ample work has shown that activated platelets form conjugates with neutrophils under a variety of different conditions (Palabrica et al 1992). The interactions are supported by the leukocyte adhesion receptors PSGL-1 and CD11/CD18, as well as by P-selectin and ICAMs from the platelet site. We therefore suppose a possible link between the stability of platelets secreted TFPI and the serine proteases exposed by the neutrophils. Thus, it is feasible that in the local microenvironment of the platelet-neutrophil conjugate TFPI is proteolytically degraded by the surface proteases. Therefore preventing TFPI from fragmentation could be of prime importance to stabilize the anticoagulant function of TFPI for longer time periods. In this work we show that exchanging some of the amino acids involved in the proteolytic cleavage of TFPI leads to a delay in the blood coagulation in vitro. The TFPI molecule is a trivalent Kunitz-type inhibitor which interacts with its K1 domain with factor VIIa, and binds factor Xa with the K2 domain. Thereby, a quaternary complex is formed, which blocks the coagulation cascade, because it prevents the formation of the activated factor X, capable of activating the sequential substrates in the cascade. The linker regions in between the Kunitz domains are the areas that are preferentially attacked by most of the proteases. Exchanging amino acids in the linker areas saves the charge balance of the entire protein and prevents conformational changes in the Kunitz domains, of importance for the overall functional features of the molecule. The first site we exchanged (T87F) was the only one known for HNE (Higuchi et al. 1992). The substitution of the nucleophilic threonine by the aromatic phenylalanine delayed the fragmentation of the molecule, when compared to the native TFPI under the same conditions of limited proteolytic digestion with HNE. In the consequential functional experiments we observed that in suspensions of isolated platelets and neutrophils as well as in total human blood, the activation of coagulation was significantly reduced compared to the effect seen with the wild type TFPI. We assumed that the aromatic phenylalanine had hampered the access of HNE to the cleavage site. Because of these first promising results, we decided to modulate some other digestion sites for proteases.

Exchanging leucine 21 by alanine (L21A), a common proteolytic site for MMPs and especially for MMP-9, improved the resistance of the L21A variant molecule in a limited proteolytic assay with MMP-9. However, the inhibiting effect of the L21A/T87F mutant was only slightly higher than the effect induced by the T87F variant in the factor Xa formation and fibrin generation assays. Since we achieved a delay in the limited proteolytic digestion, this indicates that the substitution by its own led to the desired effect, but was of poor functional relevance. This suggests that there are proteases which attack TFPI with higher affinity. This was confirmed when we substituted leucine in position 89 by alanine (L89A), which is a proteolytic site for Cathepsin G. Also this mutation was introduced in the T87F variant. The results showed a marked delay in the Cathepsin G mediated degradation of the protein. Although Cathepsin G has more than one site for proteolytic digestion of the TFPI (Petersen et al. 1992), degradation of the T87F/L89A variant was delayed, compared to the wild type TFPI, indicating that the mutated site at position L89A into the molecule was of primary relevance for its stability. Moreover, we found that the T87F/L89A double mutant resulted in an approx two fold reduction of the coagulation activity compared to the wild type TFPI. Indeed, this mutant exhibited the strongest inhibition effect of all variants investigated. The exchange of L89A in the vicinity of the already mutated T87F may explain the peculiar stability of the double mutated TFPI molecule. Concentrating mutated sites outside of the Kunitz domains does not interfere with the functionality of the molecule as coagulation inhibitor, but in the same time prevents fragmentation, the main reason for the deactivation of TFPI. The protease-resistant type of recombinant TFPI investigated may have beneficial antithrombotic effect. This may be of relevance under inflammatory conditions, in which the most abundant serine proteases such as HNE, Cathepsin G and MMP-9 are secreted by the activated neutrophils at the site of injury. On the other hand, constructing a molecule which is exclusively modified at many sites of proteolytic cleavage could not be a solution; such a type of recombinant protein may lead to severe immune reactions and other complications. We thus show that it is possible to modify the structure of TFPI in a way that the function of the Kunitz domains remains unchanged, while the stability is largely improved compared to the wild type TFPI.

V.1 Summary

Mature endothelial cells are terminally differentiated cells with a low proliferative potential and their capacity to substitute damaged endothelium is limited. Accumulating evidence in the last years indicates that mammalian organisms contain a unique subtype of circulating, bone marrow-derived cells with properties similar to those of embryonal angioblasts. These cells were called endothelial progenitor cells (EPCs). In the present work, we have studied the role of B-Raf and C-Raf, two members of a central intracellular signalling pathway, for the proliferation and differentiation of mouse embryonic EPCs. A further purpose of the study was to evaluate the anticoagulant properties of the mature endothelium and in particular the role of Tissue factor pathway inhibitor (TFPI).

We prepared gene constructs allowing us to activate or inhibit the downstream signalling of B-Raf and C-Raf, and on the other side we have used RNA interference to knock down these proteins. We found that both B-Raf and C-Raf are engaged in the proliferation of the eEPCs. However, B-Raf is mostly responsible for the differentiation, and cAMP is activating the differentiation through B-Raf, but not through C-Raf.

To delineate the participation of the endothelium in coagulation, the role of native TFPI and its mutated forms in intravascular fibrin formation was analyzed. Particular attention was given to TFPI mutants being resistant towards cleavage by leukocyte proteases that might inactivate TFPI under physiological and pathophysiological conditions. The novel insights on the differentiation and proliferation of the endothelial progenitor cells obtained in the present work, may give us the opportunity to regulate their functionality in certain cases, and eventually using them as therapeutic agents in some kind of diseases (e.g. myocardial infarction, stroke).

V.2 Zusammenfassung

Reife Endothelzellen sind bereits fertig differenzierte Zellen mit einem niedrigen Proliferationspotential, die nur begrenzt dazu in der Lage sind, beschädigtes Endothel zu ersetzen. In den letzten Jahren stellte sich jedoch heraus, dass der Säugetierorganismus über eine Subgruppe von im Blut zirkulierenden, aus dem Knochenmark stammenden Zellen verfügt, die den embryonalen Angioblasten ähneln und ein sehr hohes Differenzierungs-potential besitzen. Diese Zellen werden als „Endotheliale Progenitor Zellen" (EPZs) bezeichnet. In der vorliegenden Arbeit untersuchten wir einen für das Wachstum von Zellen wichtigen Signaltransduktionsweg in embryonalen endothelialen Maus-Progenitorzellen. Dabei interessierten uns insbesondere die beiden Signaltransduktionsproteine B-Raf und C-Raf und ihre Bedeutung für die Proliferation und Differenzierung der Zellen. Um mehr über ihre Funktion zu erfahren, wurden Domänen der Proteine überexprimiert oder die Synthese der Proteine unterdrückt.

Wir stellten Genkonstrukte her, die den Signalweg über B-Raf und C-Raf flexibel aktivieren oder unterbrechen und nutzten die Methoden der RNA-Interferenz um die Expression der Proteine zu inhibieren. Wir fanden, dass beide Proteine, B-Raf und C-Raf, eine wichtige Rolle bei der Proliferation spielen, wobei C-Raf eine herausragendere Rolle zukommt. B-Raf hingegen erwies sich als wichtiger für die Diffenzierung der EPZs. Dies bedeutet, dass cAMP die Differenzierung der Zellen durch B-Raf fördert, nicht aber durch C-Raf.

Außerdem untersuchten wir die antikoagulatorische Wirkung des reifen Endothels und dabei insbesonders den Einfluss des antikoagulatorischen Proteins „Tissue factor pathway inhibitor"(TFPI). Um die Rolle des Endothels bei der intravaskulären Gerinnung näher zu erforschen, wurde die Funktion von nativem TFPI und seiner Mutanten bei der intra-vaskulären Bildung von Fibrin analysiert. Eine TFPI Mutante (T87F/L89A), die resistent gegenüber der Spaltung und damit Inaktivierung durch leukozytäre Elastase und Cathepsin G, erwies sich als besonders effizienter Inhibitor des Gerinnungsstartes.

Die in der vorliegenden Arbeit gewonnenen Erkenntnisse zur Differenzierung, Proliferation und antikoagulatorische Wirksamkeit endothelialer Proteine könnten die Basis für eine bessere Behandlung vaskulärer Erkrankungen erbringen (u.a. Myokardinfarkt, Schlaganfall).

VI. References

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VII. Acknowledgements

First of all, I would like to express my appreciation and thanks to my supervisor Prof. Dr. med. Bernd Engelmann for giving me the opportunity to become a member of his group, for his support and his critical reviews of my work, and guiding me to complete this thesis.

I also wish to thank Dr. Antonis Hatzopoulos the chief of the “Vascular genetics group” in GSF, where the “signal transduction” part of my doctoral work was performed to a greater proportion, for supporting me with his experienced advises all the time.

I wish to express my gratitude to Prof. Dr. med. Wolfgang Siess and GRK for the three years support and the helpful discussions we had.

I would like to thank to Prof. Dr.med. Dr.h.c. D. Seidel for giving me the opportunity to perform my work in the Institut for Clinical Chemistry at the University of Munich.

I also want to say thanks Pia Lohse and Dr. Peter Lohse for their help and support with a lot of stuff since the very first moment we came here.

I say my thanks to all the past and present members of both labs, especially to Susane and Ingrid helping me a lot for my fast adaptation here in Germany and solving numerous problems, to Markus for his honest friendship and the nice football matches we had, to Matthias Semisch and Mathias Lamparter helping me in need.

I would like to say thanks to my present colleagues and friends in the lab: Berid, Christoph, Lenka, Silke and Tini, especially for creating a pleasant working environment, the nice moments we had in all the “pub visiting” evenings, and for their help in the difficult German language.

Finally, I like to thank my parents and brother for their moral support throughout the years and, especially, my wife Olya and daughter Lilly, who shared all doubts and hopes with me, for all their understanding and love without which this work would have never succeeded.

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Hiermit erkläre ich, die vorliegende Arbeit eigenständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt zu haben.

München im November 2004,

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