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The role of MEF2C in endothelial cells

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Contents:

I. Introduction:

I.1. Vasculogenesis, Angiogenesis and Lymphangiogenesis:

The intricate body architecture of vertebrates makes high-capacity transport of gases, liquids, nutrients, signalling molecules and circulating cells between tissues and organs necessary. Two highly branched tubular networks constituted by endothelial cells are in duty of mediating this vital metabolic exchange between tissues and blood; the blood and the lymphatic vessels (Adams and Alitalo, 2007). The development of the vascular system is a complicated process governed by the concerted action of many genes (Bi et al., 1999).

Chronologically, vessel morphogenesis occurs in three distinct mechanisms: vasculogenesis, angiogenesis and lymphangiogenesis (Adams and Alitalo, 2007). Carmeliet additionally defines arteriogenesis as the stabilisation of angiogenic sprouts by mural cells, like pericytes for medium-seized and smooth muscle cells for large-seized vessels (Carmeliet, 2004).

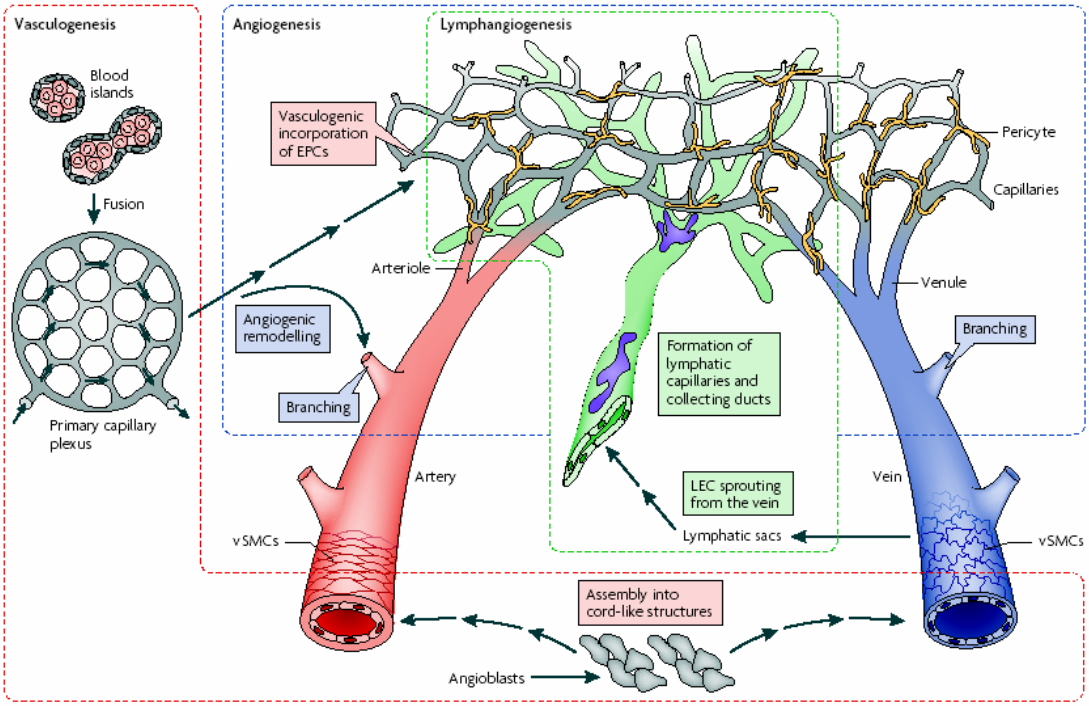


Figure 1: Assembly of the vasculature (Adams and Alitalo, 2007)

During vasculogenesis angioblasts, the precursors of endothelial cells, establish a primitive vascular network through aggregation and successive lumen formation into cord-like structures (Bi et al., 1999). Extracellular matrix (ECM) is produced by mural cells and provides a framework and bed for the dynamic installation processes. Subsequently, based on this starting points, events of matrix remodelling by MMP (matrix metallo proteinases) and of extension of the nascent vessels by sprouting shape a mature vascular network, a process referred to as angiogenesis (Carmeliet, 2004).

Principally, arteries and veins expand through circumferential growth to increase blood transport capacity, whereas capillaries sprout and ramify into complex networks, thereby facilitating exchange of gases like oxygen (Adams and Alitalo, 2007). Notwithstanding, angiogenesis also plays a role in the mature organism, contributing to organ growth and wound healing (Carmeliet, 2005).
Contact face of the two vessel systems is the largest lymphatic vessel, the thoracic duct, where lymph is drained into the blood circulation (Adams and Alitalo, 2007) (Figure 1). Lymph vessels transdifferentiate from veins stimulated by the growth factor VEGF-C (Alitalo et al., 2005).
Arterial or venous fates of angioblasts are sealed very early under the influence of VEGF and are thought to be irreversible. Previous to this determination, endothelial progenitors themselves are very versatile in regard of their potential differentiation capacitiy. In the developing embryo precursors were identified that can either give rise to only endothelial cells (EC), angioblasts, or ECs and blood cells, haemangioblasts (Carmeliet, 2000). In contrast, adult neovascularization depends on circulating not local endothelial progenitor cells, mobilized from the bone marrow. They are incorporated at distant organs in a VEGF dependent way (Rafii and Lyden, 2003).

In the embryo, initially under control of TGF-ß1 and succeedingly PDGF-BB, smooth muscle progenitors which originated from mesenchymal cells, neural crest or epicardial cells are recruited to cover newly branched channels (Carmeliet, 2000). Furthermore, potential common vascular progenitors were found. These VEGFR-2 positive precursors are either sent along the

developmental pathway to become ECs in response to VEGF or forced into becoming smooth muscle cells by PDGF-BB (Yamashita et al., 2000) (Figure 2).

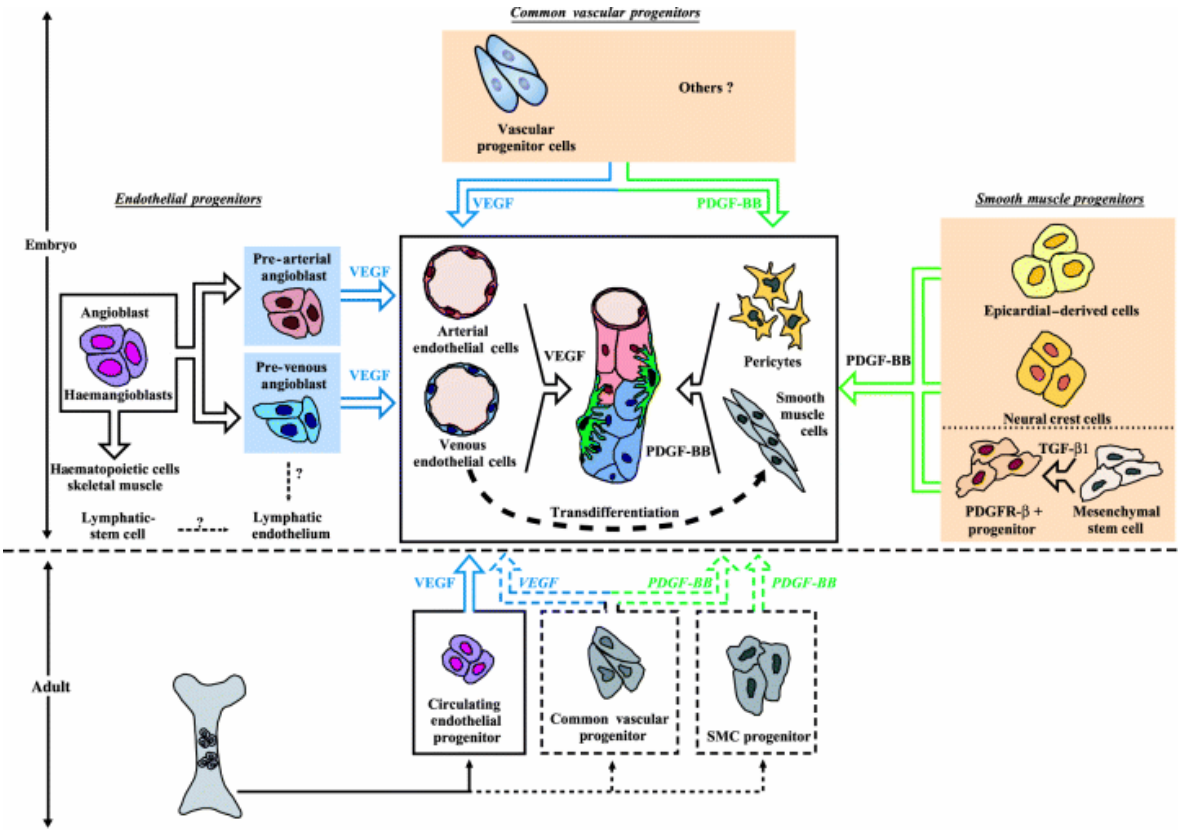


Figure 2: Vascular Progenitors (Carmeliet, 2004)

During vascular delopment in the embryo endothelial, smooth muscle and common vascular progenitor cells interact. In the adult endothelial and smooth muscle progenitors were shown to be derived from the bone marrow.

Moreover, another important mural cell recruiting mechanism is the Ang-1/ Tie2 system. EC receptor tyrosine kinase Tie2 signalling upon Ang1- binding, which is secreted from surrounding tissue, also mediates mature vessel wall formation (Cleaver and Melton, 2003).

I.2. Angiogenesis in disease:

Balanced and well synchronized angiogenesis, as the basis for a functional endothelium, highly contributes to the homoeostasis of an organism. Unfortunately, when this system gets out of control it may also contribute to a variety of diseases (Galley and Webster, 2004). It seems reasonable that excessive vessel growth as well as impaired blood vessel supply leads to a variety of ailments. Prominent disorders are arthritis, psoriasis, retinopathies, angina pectoris, arthrosclerosis and cancer. Indeed, the list is much longer, including hypertension, restenosis, obesity, complications in diabetes, heart and brain ischemia, dermatitis and many more (Carmeliet, 2004). Consequently, an urgent need of therapeutics to cure these diseases prevails. To obtain new therapeutic targets elucidation of the underlying mechanisms of angiogenesis is essential.

Recently, one research focus was on tumour angiogenesis. The first approach to stop tumour growth was to attempt to block initiation of angiogenesis by administration of an antibody against the major angiogenic growth factor VEGF with the goal to achieve starvation of the tumour. Experiments, designed to block VEGF, revealed tumour angiogenesis is a much more complex affair than initially anticipated (Ferrara and Kerbel, 2005).
Tumour vasculature differs significantly from the vasculature of healthy individuals. It is unorganized, has branches of uneven diameters, vessel walls are interrupted and highly fenestrated and they are often incapable of blood transportation (Fukumura and Jain, 2007). It is still controversial whether tumour angiogenesis is cause or consequence of cancer; the question is whether the tumour recruits novel vasculature actively or does uncontrolled upregulation of genes coincidently provoke the “angiogenic switch”? Therefore, distinct choice of either stabilization of tumour vessels for the sake of an enhanced transport of cytotoxic therapeutics to the tumour, or growth inhibition of vessels in order to starve the tumour will individually decide over the outcome of the therapy (Shchors and Evan, 2007).

I.3. Triggering angiogenesis:

Generally, beside NO hypoxia is the classical trigger of angiogenesis. When the vascular system is ruptured, for example in wounded tissue, new blood vessels have to be rebuilt rapidly. Hypoxia, caused by injury or present in the pathologically dys-structured environment of tumours, is sensed by the protein HIF (hypoxia inducible factor).
In oxygenated cells this transcription factor is kept in check by two separate, elaborated mechanisms. Oxygen-dependent hydroxylation marks this protein for ubiquitin-dependent degradation and prevents association with a transcriptional coactivator. Under hypoxic conditions these regulatory mechanisms fail and HIF becomes transcriptionally active, which leads (in addition to several other cellular responses) to the release of angiogenic factors, like bFGF, PlGF or VEGF (Pugh and Ratcliffe, 2003).

I.4. VEGF and its receptors:

VEGF (vascular endothelial growth factor) was discovered and isolated from a solid tumour in the late 80ies and initially named vascular permeability factor, because it was the most potent enhancer of vascular leakage of the skin known so far (Ferrara et al., 2003).
VEGF-A is regarded as the major angiogenic factor. It is important for blood vessel angiogenesis and vasculogenesis. VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E and PlGF belong to a glycoprotein superfamily of growth factors.
VEGF-A particularly contributes to blood vessel growth, whereas VEGF-C and VEGF-D, which have to be especially activated by proteolysis, are the key regulators of lymphangiogenesis (Byrne et al., 2005). As VEGF-$B^{-/-}$ mice are viable and only exhibit a weak heart phenotype, its role remains obscure as well as the function of the receptor VEGFR-1 (Takahashi and Shibuya, 2005). VEGF-E is produced by the human parapox orf virus and has similar biological activity as VEGF-A_{165} (Takahashi and Shibuya, 2005), emphasising the potentially mitogenic capacity of this growth factor. PlGF only binds to

VEGFR-1, thereby exerting an blocking function (Takahashi and Shibuya, 2005).

Five different isoforms of VEGF-A are identified (Byrne et al., 2005). They result from alternative splicing of the eight exons and seven introns, the *VEGFA* gene is separated in. $VEGF_{121}$, $VEGF_{145,}$ $VEGF_{165,}$ $VEGF_{189}$ and $VEGF_{206}$ consist of 121, 145, 165, 189 and 206 amino acids respectively. They are distinguished by their altered capability of heparin binding. Whereas $VEGF_{189}$ and $_{206}$ are nearly completely absorbed and sequestered by the ECM, $VEGF_{121}$ is highly diffusible (Byrne et al., 2005; Ferrara et al., 2003). However, the intermediate 165, which is secreted but remains bound to the cell surface as well, seems to be the predominant form featuring the optimal characteristics of bioavailability (loss of heparin-binding domain renders the protein mitogenically inactive) and biological potency (as only $VEGF_{165}$ knockout seems to be really detrimental to the mice) (Ferrara, 2004). By plasmin cleavage of VEGF189 sequestered in the ECM a biologically active form of VEGF might also be released, thereby contributing to the local availability of VEGF (Figure 3).

During hypoxia binding of the proteins HuR (RNA binding protein) and PAIP2 (Poly-(A)-binding protein) to AU-rich elements in the 5'- and 3'- UTRs confers additional stability of the VEGF mRNA. Also the 5' present IRES of the VEGF mRNA maintain efficient translation during stress conditions (Takahashi and Shibuya, 2005).

VEGF-A binds two highly related receptor tyrosine kinases, VEGFR-1 and VEGFR-2. Another member of the VEGFR family is VEGFR-3, which binds VEGF-C and VEGF-D as ligands. The structure of these kinases encompasses seven extracellular Ig-like domains, a single transmembrane region and a tyrosine kinase sequence. Additionally, VEGF interacts with another family of coreceptors, the neuropilins (Ferrara, 2004).

Although still controversial, VEGFR-1 probably functions as a decoy receptor, thereby preventing and fine tuning the VEGF response, especially the soluble form. Despite the higher binding affinity to VEGF in comparison to VEGFR-2, no ligand-induced mitogenic signalling and only weak autophosphorylation was detected. Nevertheless, the whole spectrum of functions of VEGFR-1,

like governing chemotaxis or migration, can differ due to the developmental stage and the cell type (Ferrara, 2004).

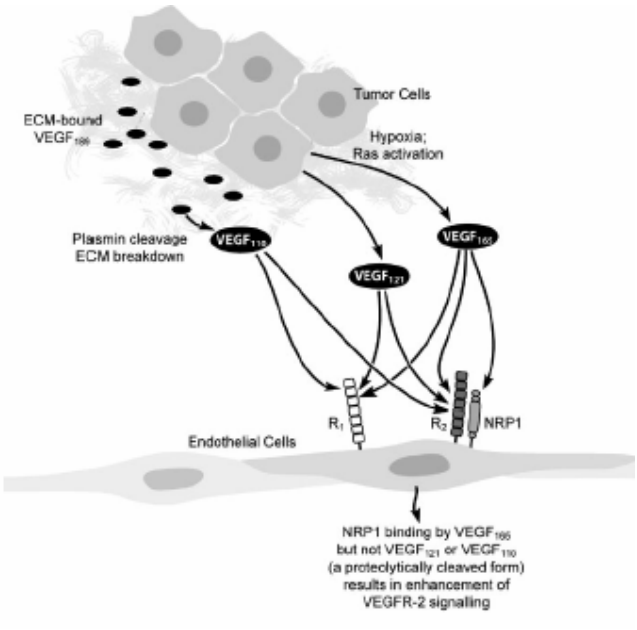


Figure 3: VEGF isoforms and their interaction with VEGFRs (Ferrara, 2004)

Upon VEGF binding VEGFR-2 undergoes dimerization and tyrosine (auto)phosphorylation resulting in a mitogenic, chemotactic and prosurvival signal (Ferrara et al., 2003). Indeed, VEGF rescues endothelial cells from apoptosis during serum starvation (Byrne et al., 2005). Proteins activated by VEGF induced phosphorylation are phospholipase C-γ, PI-3 kinase, Ras GTPase-activating protein, src-family members or Raf (Zachary, 2003). VEGFR-2$^{-/-}$ mice die due to a lack of endothelial cell growth and blood vessel formation as well as extremely poor haematopoiesis (Takahashi and Shibuya, 2005). Nowadays, there is general agreement that VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF (Ferrara, 2004).

Albeit, VEGFR-2 is supported by a molecule, previously found to be involved in neuronal guidance, neuropilin (NP). NP1 binds VEGF, associates with VEGFR-2 and presents VEGF in a way that increases effectiveness of its mitogenic signalling (Ferrara, 2004).

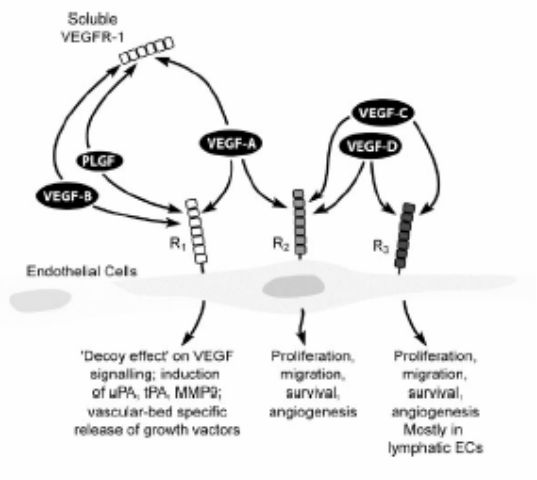


Figure 4: Role of VEGFR tyrosine kinases in endothelial cells (Ferrara, 2004)

I.5. DNA Microarray – VEGF versus EGF versus IL-1

The expression profile of HUVEC induced by VEGF in comparison to the general growth factor EGF was analyzed with a microarray by Mag. Bernhard Schweighofer (Dep. Vascular Biology, Medical University of Vienna). HUVEC were stimulated with 100ng/ml medium VEGF165 and with 100ng/ml EGF at the time points 30 min, 60 min, 150 min and 360 min in order to monitor the early response to the angiogenic growth factor and dismantle potentially regulated genes relevant to sprouting and angiogenesis.

Therefore, whole RNA was isolated from the induced cells and subjected to cRNA synthesis. The obtained cRNA was used as probes for a DNA Microarray (Affymetrix). Subsequently, the resulting expression rates were analyzed on a Microsoft Acces data sheet.

A group of genes was found to be specifically upregulated by the angiogenic VEGF, but not by the general growth factor EGF. They were considered to be primarily relevant for angiogenesis. Furthermore, the microarray analysis included samples derived from HUVEC stimulated with the inflammatory cytokine interleukin-1. Thus, further dissection of genes regulated primarily during angiogenesis (VEGF), general growth (EGF) and inflammation (IL-1) was possible. In summary, from all 60 genes induced by VEGF around 20% were regulated by all three growth factors, 60% were stimulated by interleukin-1 and VEGF and about 20% of the genes were selectively regulated by VEGF alone (Schweighofer et al., 2007).

I.6. MEF2C:

I.6.1 The pedigree of MEF2C:

MEF2C (myocyte enhancing factor) is member of the MADS box containing transcriptions factor family (Gossett et al., 1989). The MADS box domain confers dimerization and DNA binding capability. This name is drawn from the four initially discovered transcription factors possessing this DNA binding domain: MCM1, Agamous, Deficiens and serum response factor. All MADS box transcription factors throughout all eukaryotic kingdoms have in common that they play a role in developmental processes. The animal and fungal MADS box transcription factors are subdivided into two categories: the SRF-like and the MEF-like MADS box proteins (Messenguy and Dubois, 2003).

The human MEF2 family comprises MEF2A, B, C and D. Adjacent to their very N- terminal MADS box they all share a highly homologous MEF2 motif; a region of 27 amino acids. This motif partly influences DNA binding affinity, indirectly dimerization and independently transcriptional activation. The C-terminus of MEF2 proteins confers transcriptional activity via two particular TADs (Trans Activation Domain) (Molkentin et al., 1996). MEF2 proteins bind the consensus sequence C/TTA(A/T)$_4$TAG/A via the alpha helical part of the MADS box (Gossett et al., 1989). A contiguous beta- sheet mediates with its protruding hydrophobic interface dimerization to another MEF protein conferring DNA sequence specifity (Molkentin et al., 1996).

A characteristic feature of MADS box proteins is their vast cooperation with a plethora of co-repressors and activators or transcription factors of other families (Messenguy and Dubois, 2003).

Among the proteins reported to cooperate with MEF2C are: HDAC4, 5, 7, and 9, MITR, Cabin and p300, MyoD, NFAT or ERK5 (Figure 5).

This broad range of binding-partners accounts for the seemingly contradicting cellular processes MEF2C was shown to orchestrate. MEF2C regulates cell division, differentiation and death solely depending on the context or type of the cell (McKinsey et al., 2002).

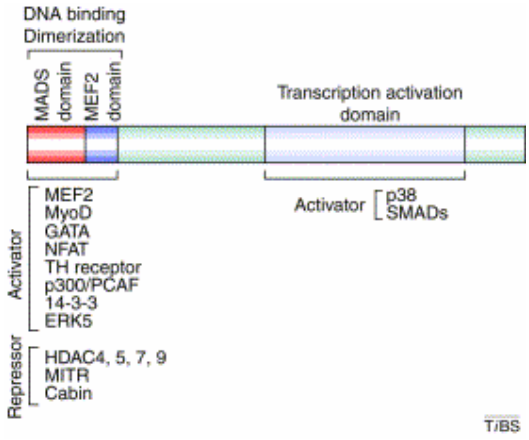


Figure 5: Interaction partners of MEF2C (McKinsey et al., 2002)

I.6.2. MEF2 regulation by HDACs:

Chromatin histone modification is a widely accepted mode of transcriptional modification (Jenuwein and Allis, 2001). Accessibility of the promoter region for the transcription machinery vitally affects an expression rate of genes. Acetylations by HATs (Histone Acetyl Transferases) of positively charged lysines of histone proteins evoke repulsion of the negatively charged DNA. Consequently, local chromatin adopts a less constrained and therefore a more open conformation. However, when HDACs (HistoneDeACetylase) neutralize this transcription enhancing effect, chromatin becomes confined again and less accessible.

All class II HDACs (HDAC4, 5, 7 and 9) were shown to constitutively associate via their N-terminus to DNA bound MEF2 (Chang et al., 2006; Wu et al., 2001). Thereby, they act as repressors on MEF2.

For example, HDAC7 knockout mice reveal a disturbed vascular integrity. This is the result of the loss of MEF2C repression by HDAC7, because metalloproteinase 10 (MMP10) is a direct target of MEF2C and consequently becomes upregulated. This leads to an increased degradation of the ECM, thereby resulting in an interrupted vascular system (Chang et al., 2006). Furthermore, the existence of a negative- feedback loop involving HDAC9 and MEF2C was identified. The promoter of HDAC9 contains a MEF2 binding site whereby its own transcription is inhibited. During muscle differentiation this mechanism regulates gene expression by altering time course dependent expression levels (Haberland et al., 2007).

I.6.3. Ca^{2+} dependent MEF2 regulation:

The Ca^{2+} binding adaptor protein calmodulin is the central platform, where the different Ca^{2+} currents-triggered signalling pathways are integrated. Several anabranches of Ca^{2+} signalling contribute to MEF2 regulation at various levels in diverse forms.

The Ca^{2+} /Calmodulin dependent kinase CaMKI's capability to phosphorylate MEF2D could be demonstrated, but an active phosphorylation of MEF2A and C failed to be detected. However, CaMKI seems to influence MEF2C indirectly, because CaMKI dependent phosphorylation of HDACs disrupts the repressive HDAC-MEF2C interaction, as it creates a docking side for the chaperone 14-3-3 (McKinsey et al., 2002). Upon binding, a conformational change of the HADC demasks a NES (nuclear export signal) (Haberland et al., 2007). Moreover, MEF2C is thus accessible for the binding of the transcription enhancing factor HAT p300. Interestingly, p300 not only acetylates histones but MEF proteins as well, thereby auxiliary increasing their transcriptional activity (Ma et al., 2005).

The Ca^{2+} /Calmodulin dependent phosphatase CaN (CalciNeurin) also has an impact on MEF2 signalling (McKinsey et al., 2002). Principally, one of the main targets of CaN is the transcription factor NFAT, which translocates into the nucleus upon dephosphorylation (Hogan et al., 2003). There, it is a potential coactivator of MEF2, binding cooperatively to a binding site proximate to the MEF2 consensus sequence. Thereby MEF2 activity increases. For MEF2D a further, protecting mechanism accomplished by the CaN was proposed. Hypophosphorylated MEF2D was less prone to caspase activity and subsequent proteolytic removal of MEF2D's TAD (transactivation domain) (McKinsey et al., 2002). Whether this mechanism applies for all MEF2s and cell type remains unclear.

The importance of Ca^{2+} signalling for MEF2C, at least in myocytes, demonstrates the finding of a positive feedback loop governed by the protein Calreticulin (Lynch et al., 2005). Calreticulin is a Ca^{2+} buffering protein, controlling its efflux from the endoplasmatic reticulum. It was shown, that CaN dependent dephosphorylation of MEF2C's NLS containing C- terminus facilitates its nuclear import. The calreticulin gene is a direct target of MEF2C.

Thereby, a constant supply of Ca^{2+} and subsequently nuclear MEF2C is maintained.

I.6.4. MAPK dependent MEF2 regulation:

The MAPKs (Mitogen- Activated Protein Kinsases) are a family of serine/ threonine kinases that play an essential role in signal transduction by modulating gene transcription in response to changes in the cellular environment. They include the extracellular signal-regulated protein kinases ERK1 and ERK2, the c-Jun N- terminal kinases JNK1-3, p38 and ERK5 (Turjanski et al., 2007). All MAPKs were shown to interact with MEF2C under a range of circumstances.

p38 is the mammalian orthologoue of the yeast HOG Kinase, a cell stress sensor (Ren et al., 2007). Han et al. found MEF2C to be specifically phosphorylated by p38 upon LPS stimulation of monocytes, demonstrating MEF2C is involved in pro-inflammatory processes in leukocytes (Han et al., 1997). Also in B-cells MEF2C is expressed (Swanson et al., 1998), whereas in T-cells MEF2D plays a regulatory role stimulated by Ca^{2+} signalling enhanced by p300 (Youn et al., 2000). In cardiac myocytes the p38/MEF2C pathway is induced by all-trans retinoic acids, thereby stimulating developmental processes in the embryonic heart (Ren et al., 2007). These findings stand in one line with other investigations, suggesting MEF2C is a key regulator of myocardial development. Additionally for example, it was demonstrated, that ROS-induced cardiovascular differentiation via the p38/MEF2C pathway. This study suggested that the p38/MEF2C pathway is involved in cardiogenesis but not in angiogenesis (Schmelter et al., 2006).

ERK5 was shown to be responsive to stimuli and to regulate early gene expression through MEF2C (Kato et al., 1997). The structure of ERK5 is unique and permits distinct biological functions and mechanisms of regulation. Aside from its kinase activity ERK5 possesses an additional domain, which is capable to recruit the transcription machinery. Thereby ERK5 enhances MEF2C activity in a dual manner. Because of this extra domain, the size of

ERK5 is considerably increased, hence the synonym big MAP kinase 1 (BMK1) (Kato et al., 2000).

BMK1 knock-out-mice phenotype resembles the MEF2C knock- out phenotype. It causes embryonic death at day E9.5- 10.5, displaying severe defects during cardio- and vasculogenesis (Hayashi and Lee, 2004). Ablation in an inducible BMK1 knock-out- mouse leads to lethality within two to four weeks and to all signs of perturbed vascular integrity (abnormally leaky blood vessels, haemorrhages in multiple organs, lining vessels became round and irregularly aligned and apoptotic). Surprisingly, mice with a conditional knock out in smooth muscle cells remained viable to term. In contrast, conditional BMK1 knock- out- mice in endothelial cells show an indistinguishable phenotype to the global knock- out. Interestingly, *in vitro* a constitutively active form of MEF2C partially rescued the BMK1 knock- out phenotype (Hayashi et al., 2004). This undermines the hypothesis serum or VEGF induced ERK5/ BMK1 mediates pro- survival cell signalling in endothelial cells via MEF2C (Olson, 2004).

I.6.5. Posttranslational modifications:

The functional mode of a transcription factor is regulated by posttranslational modifications of the protein. In summary, MEF2C was reported to be phosphorylated, acetylated, sumoylated and ubiquitinated. Phosphorylation has been shown to alter the function of MEF2C. For example, p38 (Thr293, Thr300 and Ser387) or ERK5 (Ser387) mediated phosphorylation was shown to be activating (Kato et al., 1997; Ren et al., 2007) whereas other phosporylations (Ser396) probably mediated by CDK1 kinase seemed to facilitate sumoylation and consequently inhibition of MEF2C (Kang et al., 2006). Additionally, association of MEF2C with class II HDACs potentiated sumoylation of MEF2C, which further regulates MEF2C negatively (Gregoire and Yang, 2005). Acetylation was proved to enhance DNA binding and therefore activate transcription (Ma et al., 2005). Nevertheless, ubiquitination of MEF2C seems to be part of the normal metabolic protein turn-over.

I.6.6. MEF2C cell expression pattern:

Generally, the effects of MEF2C's signalling depend on the cellular context. During embryogenesis MEF2C is the first of the four MEF2 family members to be expressed and here especially in embryonic muscle cells. As MEF2C has long been proven to be the key regulator of muscle differentiation, most investigations are focused on muscle cells (Olson et al., 1995). Because of the missing heart morphogenesis in MEF2C null mice, researchers expect to gain insights into pathologies like heart failure when elucidating mechanisms in cardiomyocytes involving MEF2C.

Nevertheless, MEF2C is also highly expressed in the human brain during fetal development (Leifer et al., 1994). Only recently, data on the role of MEF2C during neurogenesis emerge. Remarkably, there seem to be parallels between regulation of muscle and neuron development, merely differing in the specific targets reached by MEF2C (Shalizi and Bonni, 2005).

A minor requirement for MEF2C is exhibited by leucocytes (Swanson et al., 1998) and chondrocytes. It exerts again developmental functions in these cells. Monocytes become further activated by a pro-inflammatory stimulus through MEF2C (Han et al., 1997). In chondrocytes MEF2C, regulated by HDAC4, controls the programme for hypertrophy and accurate bone development (Arnold et al., 2007).

I.6.7. MEF2C in angiogenesis:

In regard of angiogenesis and endothelial cell differentiation and function results from the MEF2C knockout mouse are very promising.

The first null MEF2C mutant mice were created by targeted deletion of the MADS- and MEF-domains. No homozygous neonates were obtained, although the heterozygotes did not show a discernible phenotype. The embryos succumbed at day 9.5 to 10.5 postcoitum for which primarily the obviously severe heart phenotype resulting from aberrant cardiac morphogenesis was held responsible (Lin et al., 1997). Additionally, intricate vascular defects occurred. Organisation of endothelial cells into an ordered

pattern of a vascular plexus failed as well as recruitment and differentiation of smooth muscle cells, a characteristic step during late angiogenesis. All these pathological appearances are reminiscent of VEGF-A mutant mice as well as VEGF receptor-2 defective models, implicating a likely association between VEGF and MEF2C signalling in endothelial cells during vasculo- and angiogenesis (Lin et al., 1998).

Bi et al dissected the vascular phenotype of MEF2C-null mice to even more detail: they describe vascular malformations including the arrest of yolk sac vascular development as a capillary structure and its vessels' rupture, vessels were enlarged, but exhibited a simpler branching whereas veins were constricted and endothelial cells were of round shape and abnormal orientation (Bi et al., 1999). Remarkably, expression patterns were altered particularly in cardiac cells, showing a reduced expression of Ang1 and VEGF, again connecting MEF2C signalling with VEGF in a vascular development background (Bi et al., 1999).

The group of Jeffrey Molkentin struggled to obtain MEF2 overexpressing transgenic mice, but unfortunately, they only acquired MEF2A transgenics. The only viable offspring of the MEF2C transgenic line showed a detectable MEF2C overexpression of only 1.6-fold, indicating a barely tolerable impact of the overexpression on the animals' homoeostasis (Xu et al., 2006). On cellular level, overexpression of MEF2C by adenovirus in cardiomyocytes revealed upregulation of three functional groups of genes detected on a microarray. These groups include metabolism related genes, genes involved in remodelling of the ECM and genes concerned with ion handling (Xu et al., 2006). All these genes could also play a reasonable role in the process of angiogenesis.

I.7. Adenovirus:

For the purpose of gene delivery into a cell several ways can be chosen. On the one hand DNA might be transfected into a cell by means of chemical manipulation like PEI mediated transfection. On the other hand a vector-containing virus like adeno- or retroviruses might carry a gene of interest into a cell. It is also endeavoured to optimize adenoviruses in a way that they can be administered to patients as gene therapy. Cancer could be treated by gene therapy by introducing a growth inhibiting gene for example (Yang et al., 2007).

Concerning laboratory use, transfections are comparably easily accomplished considering expenditure of time to prepare a suitable expression vector as well as the relatively cheap reagents, but for HUVECs efficiency ranges only from 30-50%, which is too less for physiological assays. Additionally, the chemical manipulation of the cells harms their integrity, which is counterproductive for testing physiologic cell behaviour such as angiogenesis assays.

Hence, for the purpose of angiogenesis assays the adoption of an adenoviral vector to introduce a gene of interest into cells seems reasonable; a suitable multiplicity of infection warrants 100% target gene delivery efficiency and the cells are not physically harmed by the replication-deficient Adenovirus.

Adenoviruses are non-enveloped linear DNA- viruses. 49 subtypes subdivided into 6 subgroups are described. In humans they can cause respiratory, gastrointestinal and ocular diseases. Commonly, adenoviruses of type 2 and 5 are employed in molecularbiology. Principally, adenoviruses code for five early (E1A-E4), two intermediate and five late transcription elements. The latter are encoding structural proteins for the capsid. Inverted Terminal Repeats (ITRs) positioned at the viral chromosome's end serve as replication origins. E1A+B are necessary for starting the replication of the viral chromosome and have cell transforming properties. Therefore, E1 deficient viruses are rendered replication deficient. These viruses can be propagated in E1-transcomplementing cell lines. Consequently, deletion of these genes provides space for inserting a gene-of-interest as well as it prevents

unintentional, potentially harmful virus propagation outside of complementing cell lines.

A further benefit of adenoviruses encompasses their capability of infecting non-dividing as well as dividing cells. The classical way of invasion into a cell for adenoviruses proceeds via the CAR receptor. The size of the obtained, available cloning space of adenoviral vectors arises from the elimination but trans-complementation of the E1/E3 genes and from the fact, that it tolerates 105% of the natural viral genome packaging. Altogether 8.1–8.2 kb are the size limit for the introduced foreign fragment. Finally, it is noteworthy, that the viral genome remains episomal in contrast to for example retroviruses. Therefore, undesirable recombination events do not take place (Mizuguchi et al., 2001).

Several different methods to generate recombinant adenoviruses were established and optimized during the last decades. First, there is the in vitro ligation method, where the gene of interest is ligated to the adenoviral DNA and transfected into 293 cells. Second, there is the homologous recombination method in 293 cells. A plasmid encoding the gene of interest and a plasmid harbouring the adenoviral genome are co-transfected into 293 cells, which are supposed to recombine at the ITRs, but these events occur very rarely in mammalian cells and often randomly. Third, a continuation of the recombination idea is the homologous recombination method in bacteria. The bacterial homologous recombination system is highly efficient and reliable. Additionally, the co-transformation efficiency of the two plasmids into bacteria is very high. One plasmid contains the gene of interest under the control of a suitable promoter and optionally a reporter gene. The other plasmid encodes the adenoviral backbone. Several companies provide a kit for this method; for example the AdEasy™ Adenoviral Vector System kit from Stratagene®, which is explained to further detail in Materials&Methods. Subsequently, the large scale amplification of the recombinant virus becomes necessary for efficient use in angiogenesis assays. Here, purification of the viral particles is the crucial step. On the one hand abundant loss of viral particles has to be avoided and on the other hand, one has to be aware, that not all produced particles are viable and functional. The last question can be addressed by a titre determination, because only viable particles are

infectious. Titres can be tested with antibody- based staining kits. For the viral particle purification from a vast amount of cell lysate still the established technique of caesium chloride density centrifugation with subsequent glycerol buffer dialysis seems to be the method of choice. Alternatively, newly developed filtering kits based on chromatographically separation of the particles are available. Moreover, caesium chloride density centrifugation is even capable to separate complete particles from incomplete like empty capsids, aberrant particles or clustered junk material when adequately equipped centrifuges are available (Berkowitz and Philo, 2007; Mizuguchi et al., 2001).

II. Materials and Methods:

II.1. Vectors and Plasmids:

The cDNA of the gene of interest (MEF2C) was cloned in the high-copy plasmid pBluescriptR. For expression in mammalian cells and in order of adenovirus production MEF2C was subcloned into the pShuttle-IRES-hrGFP-1 Vector, containing a CMV promoter and recombination sites homolog to the recombination sites in the pAdEasy vector, which was providing the adenoviral construct, resulting in a MEF2C expressing adenovirus after bacterial recombination subsequent to cotransfection into E.coli.

Plasmid/ Vector	**Application**	**Source**	**Size**
pBluescriptR-IRATp970F1023D	Full length cDNA clone of MEF2C (amp^R)	RZPD	4.1 kb
pShuttle-IRES-hrGFP-1	Shuttle vector for bacterial recombination for adenovirus production; CMV promoter containing expression vector for mammalian cells (kan^R)	Stratagene	8.9 kb
pAdEasy-1	E1/E3 deleted adenoviral backbone containing vector for bacterial recombination for adenovirus production	Stratagene	33.5kb

The restriction maps of the vectors are shown in the appendix.

II.2. Primers:

For Primer design see II.2.3.5. All primers were purchased from Invitrogen.

Gene	Sequence (5'-NNN-3')	Application
pShuttle-IRES-hrGFP1_fw	ctcacggggatttccaagtc	sequencing
pShuttle-IRES-hrGFP1_rev	atgcagtcgaggaattg	sequencing
MEF2C_472-446	cgaggattatggatgaacgt	sequencing
MEF2C_1078-1097	tggtctcacctggtaacttg	sequencing
M13 fw 18-mer	tgtaaaacgacggccagt	sequencing
M13 rev 17-mer	caggaaacagctatgac	sequencing
MEF2A_fw	ccgactgcctacaacactga	realtime PCR
MEF2A_rev	cctgagataactgccctcca	realtime PCR
SELE_fw	ggaagctaccaaaagccttc	realtime PCR
SELE_rev	ccagagacccgaggagagtt	realtime PCR
MEF2C_fw	catccactgccaccatctgc	realtime PCR
MEF2C_rev	cgtgtgttgtgggtatctcg	realtime PCR
EGR3_fw*	gacaatctgtaccccgagga	realtime PCR
EGR3_rev*	tccaagtaggtcaggtct	realtime PCR
DSCR1_fw*	tagctccctgattgcctgt	realtime PCR
DSCR1_rev*	ggagaaggggttgctgaagt	realtime PCR
NR4A2_fw*	tttctgccttctcctgcatt	realtime PCR
NR4A2_rev*	gtggcaccaagtcttccaat	realtime PCR
HLX1_fw*	ctccaacctgcagaggaaag	realtime PCR
HLX1_rev*	ggttctggaaccacaccttc	realtime PCR
MEF2C_endogen_fw	ggcagaagccaagagacctt	realtime PCR
MEF2C_endogen_rev	aggcaggctagcatccttta	realtime PCR
MMP10_fw	tgagcctaaggttgatgctg	realtime PCR
MMP10_rev	ccctatctcgcctagcaatg	realtime PCR
ß2M_fw	gatgagtatgcctgccgtgtg	realtime PCR
ß2M_rev	caatccaaatgcggcatct	realtime PCR

* Asterix marked primers were kindly provided from Mag. Bernhard Schweighofer

II.3. Restriction endonucleases and DNA modifying enzymes:

For subcloning of the fragment coding for MEF2C the pIRAT plasmid and pShuttle-IRES-hrGFP-1 were cut with *Not*I and *Spe*I (Roche).
T4 DNA Ligase (New England Biolabs) was employed for sticky end ligation.
For sequencing PCR fragment amplifaction was performed with Pfu polymerase (Biotherm).
Linearization of the pShuttle-IRES-hrGFP-1 vector was done with *Pme*I (New England Biolabs).
Subsequent dephosphorylation was conducted with alkaline Phosphatase (Roche).
Control restriction of positive recombination after cotransfection was performed with *Pac*I. (Roche)

II.4. Antibodies (Western blot):

Application	Name	Source
Primary Antibody	goat-anti-MEF2C	Santa Cruz Biotechnologies
Secondary Antibody	HRP-donkey-anti-goat-IgG	Santa Cruz Biotechnologies
development reagents	Lumiglo+ peroxide	Cell Signalling Biotechnologies

II.5. Cells:

II.5.1 HUVEC:

HUVECs are human umbilical vein endothelial cells, directly extracted from umbilical cords after collagenase treatment. They are biosafety level 1, primary cells, usually used up to passage number 6. Splitting in a ratio of 1:3 or medium change every two to three days is recommendable. They are adherent cells and require 1% gelatine coated culture vessels.

HUVEC are kept in M199 medium (Lonza), completed with 20% EC-FCS (endothelial fetal calf serum), 1% antibiotic mix (Penicillin, Streptomycin, Funghizon and Glutamin), 0.4% ECGS (endothelial growth supplement) and 0.026% heparin to prevent fibroblasts growth and sterile filtered through a 0.2µm pore membrane (Milipore) at 37° and 5% CO_2. Protocol for subculturing is

- Remove and discard old culture medium.
- Briefly rinse the cell layer with 1x PBS solution to remove all traces of serum which contains trypsin inhibitor.
- Add 3.0 ml of Trypsin-EDTA solution to a 175 cm² flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
- (Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.)
- Add 9 ml of complete growth medium and aspirate cells by gently pipetting.
- Add appropriate aliquots of the cell suspension to freshly coated new culture vessels and fill up with complete medium to 25ml.

For stimulation with growth factors HUVEC were serum starved meaning not fed for 3 days.

100ng VEGF- A_{165}, bFGF and IL1 and 25ng EGF diluted in serum- free medium were added to the starved HUVEC cells of a 6- well covered with exactly 1ml medium.

II.5.2 293 cells:

293 cells are human embryonic kidney (epithelium) cells. They originate from a cell line. Immortalization was obtained by transformation with Adenovirus 5. An insert consisting of ~4.5 kilobases from the left arm of the viral genome became incorporated into human chromosome 19, rendering them also useful for adenoviral titration.

293 cells are maintained in Mem-alpha medium (Invitrogen), supplemented with 10% NCS (neonatal calf serum), 1% Penicillin/ Streptomycin mix and 1% L-Glutamin (200mM) and sterile filtered.
They do not urgently need gelatinized plates but coating supports their accommodation. 293 cells should not be grown to full density to prevent dividing stop, can be splitted in ratios 1:4 up to 1:10 and are used up to passage number 25. For subculturing the protocol for HUVECs applies likewise. (see II.1.5.1.)

II.6. Bacterial Cultures:

All *E. coli* strains (DH10B, XL Gold and BJ5183 Rec^+) used were grown in LB (Luria- Broth) medium and plated on selective agarose dishes.
Therefore, a ready-to-use LB medium mix (Invitrogen) containing 1% Trypton, 0.5% yeast extract and 1% NaCl was solved in bidest water and autoclaved for sterilization.
Antibiotic stocks were prepared with concentrations of 50 mg/ml of Ampicillin in water and 10 mg/ml of Kanamycin in water. For selective plates final working concentrations were 50µg/ml antibiotics in LB medium.
Principally, for the preparation of bacteria culture plates, 4 g Bacto-Agar (BectonDickinson) added to 200 ml sterile LB medium were heated until the solution was homogenous and solved completely. After subsequent cooling down to ~50°C, antibiotics stocks were added in adjusting concentrations. Immediately afterwards, the LB- Agarose solution was casted into approximately ten to fifteen round dishes (10cm), allowed to solidify and eventually stored at 4°C.

II.7. Agarose gel electrophoresis:

For separation of DNA fragments agarose gel electrophoresis was performed with 1% agarose gels for fragments up to 15 kb or 0.8% agarose gels for fragments >15 kb using a multipurpose agarose (Roche). For preparative DNA separation a low-melting agarose was employed to obviate potential enzyme- inhibitive contamination with agarose of subsequently extracted DNA.

Principally, agarose was added to 30 ml of 1x TAE buffer and the solution was heated in the microwave ensuring complete resolving of the agarose. Subsequent to cooling, 1µl of ethidium bromide solution, which is very carcinogenic and tends to sublimate, was added. Finally, the gel was casted into the chamber followed by insertion of the comb of size, appropriate for the amount of DNA desired to be loaded.

After polymerization of the gel, samples were mixed with a 6x loading buffer (fermentas) and loaded into the gel slots. Additionally, to enable determination of the DNA fragment size either a 1kb, a 123bp or a lambda DNA BstII ladder (fermentas) was loaded as a marker. The gel ran at 70 to 80 Volts in the 1x TAE buffer filled chamber for approximately 45 minutes or until the marker was separated satisfyingly.

II.8. Protein analysis:

II.8.1. SDS- Polyacrylamid Gel separation:

For electrophoresis the PeqLab system was used. A standard gel was set up employing either 0,75mm or 1,5mm spacers, a 12 teeth comb and 10x10cm glass plates. The protein MEF2C has 44kDa and therefore a 12% resolving gel was prepared.

1.6ml dH_2O, 2ml acrylamide mix, 1.3ml 1.5M Tris (pH 8.8), 50µl 10%SDS, 50µl freshly prepared 10% APS and 2µl TEMED are mixed in a disposable tube, poured into the ready assembled casting frame and allowed to polymerize. To ensure an even gel edge the mixture was overlayed with

isopropanol. After polymerization, the isopropanol was removed thoroughly. For a 5% stacking gel 2.1ml dH_2O, 0.5ml 30% acrylamide mix, 0.38ml 1M Tris (pH 6.8), 30µl 10% SDS, 30µl 10% APS and 3µl TEMED were mixed and filled onto the top of the resolving gel. The comb was inserted instantly to ensure a regular polymerization. It was crucial to avoid air bubbles to warrant an uninterrupted sample run. After polymerization the comb was removed and the slots were washed with electrophoresis buffer (25mM Tris, 250mM glycine and 0.1% SDS).

Samples were resolved in 100µl/ 6-well cells sample- "Lämmli"- buffer (0.0625M Tris (pH 6.8), 10% glycerol, 2%SDS, 5% mercaptoethanol, 0.05% bromphenolblue in water). Previous to loading they were heated at 95°C for 10 minutes. Samples were loaded into the slots with loading tips as well as the prestained protein marker (fermentas). The tank was filled up with electrophoresis buffer. For power supply the 500/ 1000 Biorad power supplier was used. The gel was run at 150V and 20mA until loading buffer front reached the bottom.

II.8.2. Western blot:

Immuno-blotting using the semi-dry blotter from PeqLab followed.

Thus, 3mm Whatman Paper and the PVDF membrane (Millipore) were cut to the right size. The membrane was soaked in methanol for 15 seconds, in water for 2 minutes and in blotting buffer (25mM Tris, 192 mM glycine and 20% methanol) for 2 minutes. The Whatman Paper was soaked in blotting buffer. The blotting sandwich was built up starting from bottom with 3 pieces Whatman Paper, the membrane, the gel and finally 3 pieces Whatman Paper again on the top. To ascertain unhindered current flow air, bubbles within the sandwich were removed and the lower blotter plate was dried around the sandwich area. The top cover of the semi-dry blotter should not be closed too tightly to avoid drying of the gel. For power supply the 200/0.2 Biorad supplier was employed. In case of one small gel, blotting was allowed for 1 hour at maximally 200mA according to the manuals.

II.8.3 Evaluation of Blot:

After blotting, the membrane was removed, washed in methanol and left in the flue to dry completely. Afterwards, the membrane was blocked with 3ml 5%BSA in 0.1%Tween 20 in PBS for 30 minutes. Subsequently, the first antibody was added in recommended concentration and allowed to bind at 4°C overnight. The membrane was washed three times for 5 minutes with 0.1% Tween 20 in PBS. Incubation for one hour at room-temperature with HRP-conjugated second antibody followed. Finally, the membrane was washed again three times for 5 minutes with 0.1% Tween 20 in PBS.

Afterwards, the membrane was incubated with 125µl solution A and 125µl solution B (Cell Signalling) diluted with 2.25ml dH_2O. After one minute a film (Kodiak) was exposed to the membrane and succeedingly developed in the machine Curix60/ Agfa.

II.9. Real Time Reverse Transcription Polymerase Chain Reaction:

II.9.1. Total RNA Isolation:

Prerequisite for isolating total RNA is to work with adequate material. This includes RNAse- free pipettes washed with RNAaway, aerosol resistant tips and solutions, like sterile or DEPC treated water.
Cells were fixed with 300µl/ 6- well RNAlater (Ambion) for 1 minute, washed with 1ml/ 6-well sterile water and lysed with 1ml/ 6-well TRIzol (Invitrogen). The thoroughly mashed cell-lysate was transferred to a prepared 1.5ml reaction tube. At this point, the resuspension might either be stored at –20°C or incubated at room- temperature for 5 minutes.
Succeeding, 200µl chloroform were added, mixed intensely for 15 seconds and subsequently incubated for 2-3 minutes, followed by centrifugation for 15 minutes at maximum speed. RNA should only be in the aqueous, top phase and transferred to a new reaction tube. Attention should be paid, not to touch the intermediated phase. 500µl isopropanol were added, mixed, incubated for 10 minutes at RT and centrifuged for 10 minutes at 12000g. The pelleted RNA was washed twice with ice- cold 75% RNAse free Ethanol. The RNA was dissolved in 22µl DEPC treated water (Invitrogen) and incubated at 55°C for 5 minutes.

II.9.2. cDNA Synthesis:

For a preparation of 20µl total 10µl of the obtained RNA Isolation, 1µl Oligo$(dT)_{12\text{-}18}$ (500µg/ml; Invitrogen) and 1µl dNTP Mix (10mM; Invitrogen) were incubated at 65°C for 5 minutes, cooled on ice and spinned down and complemented with 4µl 5x FirstStrandBuffer, 2µl DDT (100mM) and 1µl RNAse OUT (40U/µl) (Invitrogen), mixed and incubated at 42°C for 2 minutes. Finally 1µl Superscript enzyme (Invitrogen) was added.
Superscript was allowed to work for 50 minutes at 42°C and inactivated for 15 minutes at 70°C.

II.9.4. Quantitative Real Time PCR:

The obtained cDNA had to be diluted with 30µl water.
Initially, the Light Cylcler Mastermix (enzyme and cybergreen) was prepared according to the manufactor's instructions (Le Roche). For a total of 10µl 1µl cDNA, 1µl of each 5mM Primer (see design), 1µl Light Cylcer mastermix and 6µl water were mixed. This is a quantitative analysis. Therefore precise pipettes had to be used. The reaction mix is light sensitive. Therefore it demanded diligent pipetting and reaction tubes were kept cooled and light protected. The reaction mix was transferred to according capillaries and these were inserted into the light cycler in correct order.

The amplification and detection process ran on the following conditions:

Temperature	**Hold time**
Denaturation	Cycles: 1
95°C	600 sec
Amplification	Cycles: 54
95°C	5 sec
63°C (dep. on primer)	5 sec
72°C	15 sec
Melting	Cycles: 1
95°C	10 sec
70°C	30 sec
95°C	0 sec
Cooling	Cycles: 1
40°C	10 sec

Recording of the results is automated. When PCR reaction was finished melting curves were examined on the computer for the correct course of action.
Examples for amplification and melting curves for the gene MEF2C are shown in the appendix.

To calculate the fold induction values of unstimulated control cells the means of the turning points (cycles) for ß2Microglobulin and the analysed gene of the unstimulated control cells had to be determined beforehand. The fold

induction normalized with ß2Microglobulin was calculated with the following formula:

$$F = a^{(Mx - x)} * b^{(y - My)}$$

"F" is the result of the calculated fold induction based on the values obtained from the control cells. "a" and "b" describe the efficiency of the employed primers for the tested gene and the internal standard ß2Microglobulin respectively. The efficiency of the ß2Microglobulin primers was presumed to be 2.2 and the efficiency of the primers for the tested gene was presumed to be 2. "Mx" represents the mean value of the turning points of the analysed gene of the unstimulated cells and "x" is a particular value of turning point of the tested gene. Similarly, "My" represents the mean value of the turning points of ß2Microglobulin (internal standard) of the unstimulated cells and "y" is a particular value of turning point of the internal standard. Afterwards, the mean value and the resulting standard deviations of the calculated fold induction of three samples were determined.

II.9.5. Primer design:

Primers were designed with the program "Prime3". For light cycling PCR primers had to meet certain demands. The PCR product must not be longer than 200bp, ideally 100- 150bp. The amplified sequence should include an intron- exon- border to ensure the amplification of cDNA instead of an eventual chromosomal DNA contamination. Additionally, 3' end seated sequences should be preferred, because it may happen that mRNA transcription by Superscript was incomplete.

II.10. Plasmid DNA assignments:

For plasmid DNA amplification plasmids are transfected into either chemical or electro competent E.coli.
Standard procedure steps of transfecting **chemical competent cells** are thawing of a 100µl aliquot of bacteria on ice, addition of 0.2µg DNA in 1µl dH_2O and incubation for 20 minutes on ice, transfer into a 14 ml cooled, round bottom tube, heat shock at 42°C for 40 seconds, instant reanimation with 1ml 37°C prewarmed LB medium and shaking for one hour at 37°C at 200rpm. Aliquots are plated on selective plates and incubated at 37° overnight subsequently.

When higher transfection efficiency was needed or when two plasmids had to be cotransfected, **electro competent** bacteria were used. For transfection of electro competent E.coli, electroporation cuvettes were prechilled. Vials with 40µl aliquots of electro-competent cells were allowed to thaw on ice. They were gently mixed with the added 1µg/µl DNA. Amount of water and salt purity of the sample are critical in this procedure. The bacteria were transferred to the ice-cold cuvette, which was inserted into the electroporator. (BioRad) The parameter adjustment for an electroporation impulse of bacteria was 2.1 kV, 25 µF, 200Ω and 4.2 milliseconds. Afterwards, cells were reanimated immediately with 400µl prewarmed LB medium (37°C). The cells were incubated at 37°C and 200rpm for one hour. Subsequently, they were plated on selective LB plates and incubated at 37° overnight.

Positively resistant colonies were picked under sterile conditions, inoculated into 5ml LB media and incubated at 37° overnight. This was used as either a starting culture for large scale amplification in 300ml LB media, which was incubated overnight again, or for plasmid preparation employing the **Mini Plasmid Purification** kit (Qiagen) subsequent to 15 minutes centrifugation at 3000 rpm. Hereby obtained DNA was eluted with 50- 100µl dH_2O. Concentration and protein contamination purity was measured with the UV/ visible Spectrophotometer (Pharmacia Biotechnologies) at 260 nm/ 280 nm.

The **Midi Plasmid Purification** kit (Qiagen) was used to acquire a higher amount of DNA out of the obtained 300ml bacteria suspension.

To ensure whether the correct plasmid has been amplified, a small DNA aliquot was subjected to restriction enzyme digestion and subsequent 1% agarose DNA separation showing potentially appropriate fragments.

For directed DNA cutting **restriction enzymes** are employed.

Considerations about special requests of an enzyme regarding optimal working temperature and particularly buffer conditions are important when two different enzymes are used for a double digestion. In a standard course of action a 10 fold excess of enzyme activity is prepared, meaning for 1µg DNA to be digested, 10 Units enzyme are added.

Double digestion preparations of pBluescriptR-IRATp970F1023D and pShuttle-IRES-hrGFP-1 plasmids with *Not*I and *Spe*I serve as an example:

4µl pBluescriptR-MEF2C (0.5µg/ µl)	1µl pShuttle-IRES-hrGFP-1 (1µg/ µl)
1µl 10x buffer H (Roche)	1µl 10x buffer H (Roche)
1µl dH_2O	7µl dH_2O
4µl *Spe*I (10U/ µl) (Roche)	1µl *Spe*I (10U/ µl) (Roche)
or	or
4µl *Not*I (10U/ µl) (Roche)	1µl *Not*I (10U/ µl) (Roche)
Total: 10µl at 37°C for 90 minutes	Total: 10µl at 37°C for 90 minutes
(2 times)	(2 times)

The first digestion was controlled on an agarose gel to ascertain a complete digestion. Save of that, the reciprocal enzyme was added and incubated for 90 minutes at 37°C again.

In order to obtain a plasmid fragment supposed to be subcloned, the whole volume of the enzyme digestion preparation was loaded onto a 1% low-melt agarose gel and separated. Under UV light the desired fragment of the right size was cut out of the gel with a scalpel. DNA was retrieved from the agarose piece with the **Gel Purification** kit (Qiagen) and eluted with water. This kit can also be used as an **Enzyme Reaction Purification** kit.

To reassemble the fragment and the target plasmid or any cut DNA pieces a T4 ligase mediated **ligation** was performed. Crucial for this reaction was the molar insert to vector ratio, which should range from 1:4 to 1:10. Total amount of DNA is (as in all enzyme reactions) also critical.
Ligation of the MEF2C cDNA containing fragment and pShuttle-IRES-hrGFP-1 vetcor serve as an example.

0.5µl pShuttle-IRES-hrGFP-1 (100ng DNA) } 1: 9 molar ratio
2µl insert (225ng DNA)
1µl ATP (10mM) (Invitrogen)
1µl 10x buffer
1µl T4 Ligase (400U/ µl) (Fermentas)
Total: 10µl at 16°C overnight

1µl of the ligation had to be retransformed into chemical competent cells. DNA from positive colonies on the according selective plate was tested by enzyme restriction and control agarose gel separation to possess the correct fragment insertion.

To prevent religation and to ensure persistent linearization of a cut vector, DNA was subjected to **alkaline Phosphatase** (1U/ µl) (Roche) in supplemented 10x buffer at 37°C for 60 minutes; 1 unit alkaline phosphatase dephosphorylates 1pmol DNA in 60 minutes at 37°C.

For exact single base pair verification of DNA samples, **sequencing** was necessary. Therefore, Ethanol precipitated DNA (+ 1/10x Volume NaAcetat pH 5.2, + 2.5x Volume 100% EtOH for 20 minutes at RT and 25 minutes centrifugation at maximum speed at 4°C) was sent to the company MWG biotechnologies. Standard primers were provided and individual primers had to be delivered. Transmitted sequence was analyzed with the programme McVector.

For sequencing of a small fragment of a large vector or any reliable qualitative amplification, the fragment had to be amplified by **PCR using *Pfu*** polymerase, which contains a proofreading function.

32µ dH_2O
2µl DNA
5µl Primer (10nM) forward
5µ Primer (10nM) reverse
5µl 10x buffer
0.5µl dNTPs (10mM) (Invitrogen)
0.5 µl *Pfu* Polymerase
Total: 50µl for touch down PCR schedule

PCR program ran on PerkinElmer machine:

Time	Temperature	
3’	94°C	
30’’	94°C	35 cycles, -0.3°C
30’’	65°C	
3’30’’	72°C	
7’	72°C	
	4°C	

II.11. Production of a recombinant adenovirus:

Recombinant adenovirus was produced with the help of the AdEasy™ Adenoviral Vector System kit from Stratagene®.
Here, the gene of interest is cloned into the supplied “shuttle vector” into its MCS under the control of a CMV promoter and a SV40- polyadenylation site. This plasmid pShuttle-IRES-hrGFP-1 also encodes a humanized GPF protein driven by an IRES element and provides the option of a 3xFLAG tag labelling of the protein of interest. The kit also provides the pAdEasy-1 vector, which encodes the E1/E3 deleted adenoviral genome and the homologous recombination sites. These plasmids are co-transformed into the supplied BJ5183 $RecA^+$ E.coli bacteria. The expectantly recombined plasmid is re-transformed into the also provided $RecA^-$ XL-Gold E.coli bacteria and amplified. Taking advantage of the rare cutter *Pac*I, the linearized, new adenoviral genome is transfected into E1-trans complementing HEK 293 cells for virus production.

Using standard cloning techniques as described in chapter II.2.4., the gene of interest was integrated into an adenoviral genome stepwise, following the schematic steps depicted in Figure 6.

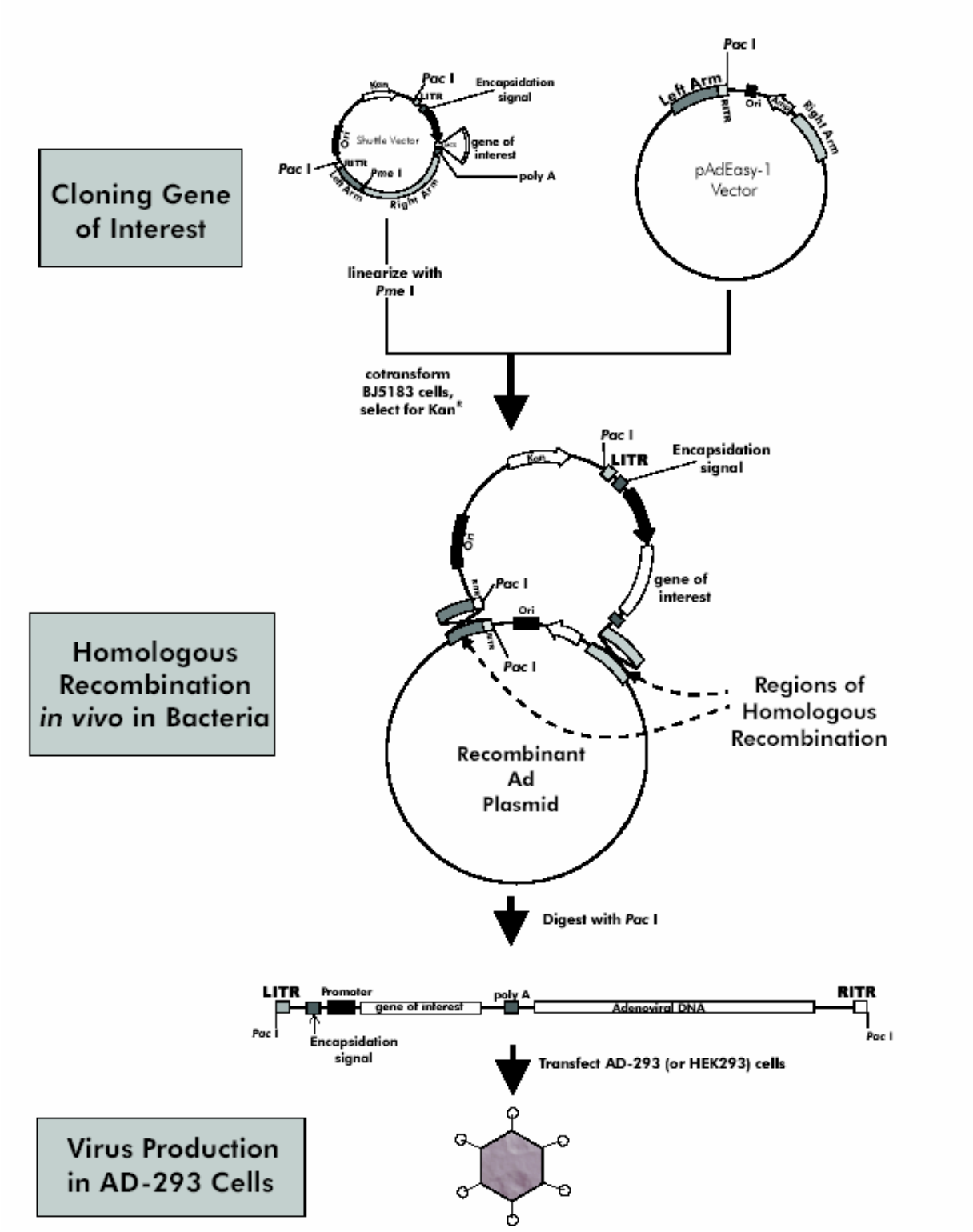


Figure 6: Steps of adenovirus generation

(adapted from pAdeasy kit, Stratagene, manual)

II.11.2. Expansion of recombinant adenovirus:

After confirmed *Pac*I linearization of the sequence verified plasmid, containing the target gene recombined to the adenoviral backbone, 293 cells of a 70% confluent 6-well were transfected employing the Stratagene **mammalian transfection** kit.
Therefore, 4 µg DNA was solved in 90µl water. 10µl Solution 1 and 100µl solution 2 were very well mixed and added to the DNA under continuous mixing. The combined solutions were incubated at RT for 15 minutes and subsequently added to the cells drop wise. Cells were incubated at 37°C for 20 hours and then medium was changed. From this step on all following steps have to be made under precautions and working conditions adapted to handling security level two viruses.

The progressions of the transfection and possibly propagating infections, which were visible by either a GFP reporter or plaque formation, had to be examined under the microscope daily. Medium of the cells should be changed considering the accommodation of the producing cells on one hand and an infection feasible virus-concentration on the other hand.

To propagate the infection and to expand the number of virus particles, a high number of 293 cells had to be infected. Therefore, a big flask of non-confluent 293 cells was infected with primary virus lysate. This was prepared from the transfected cells, respectively, by a **freeze-and-thaw procedure** when first plaques appeared. The cells were harvested, collected by centrifugation, resuspended in HE- buffer (10mM HEPES, 1mM EDTA) and lysed by 3 cycles of repetitive freezing in liquid nitrogen and warming to 37°C in a water bath. The lysate was centrifuged at RT at 11000rpm for 10 minutes. The viral particles were in the supernatant. The suspension has to be stored at –80°C.
For infection of a big 175cm² flask of 293 cells approximately a tenth of the primary lysate was used depending on its infection grade. When this big flask was infected thoroughly but before it would have been lysed completely, the cells were harvested and treated like the primarily infected cells.
Consequently, the resulting lysate was used to infect 40 big tissue plates of non-confluent 293 cells for the final expansion step. The crucial point of this step is to keep the balance between a sufficiently high infection rate to prevent the cells from growing dense and a consequently metabolic shut down on one hand and an

abundant lysis of the cells due to substantially high infection on the other hand. Ideally, 293 cells of the 40 infected plates were allowed to produce viral particles 3-5 days and were then lysed by 4 cycles of freeze-and-thawing consecutively.

II.11.3. CsCl purification:

As the virus will be used for infection of for example primary cells, the viral particles of this big prep lysate were purified by Cesiumchloride-density-centrifugation. Therefore, a cesiumchlorid density gradient of 1,33 and 1,45 in HE-buffer- had to be established in a 50ml ultracentrifugation tube to underlay the lysate with. It was made sure that there were no air bubbles potentially destroying the gradient. The tube was sealed cautiously and centrifuged for 3 hours at RT at 45000 rpm in the VTi 50 rotor of the ultracentrifuge. A virus particle containing, dark band should be visible and extracted with an injection needle. This suspension was reloaded into a 5ml ultracentrifugation tube, which was filled up with 1,33 CsCl-solution air-bubble free, and centrifuged overnight at RT at 50000 rpm in the VTi 65 rotor. Hereafter, again a dark band should be formed. The virus band was retrieved with a thin needle and purified from the salt solution by dialysis.

The virus solution was filled directly into the slid-A-lizer cassette (Pierce) with the syringe and needle used for taking the band. The cassette was allowed to swim in 500ml dialysis buffer; for 4 hours at 4°C first and left overnight in fresh dialysis buffer subsequently. Finally, the purified virus suspension was aliquoted into 2ml cryo-tubes and stored at –80°C.

II.11.4. Titration of the virus:

The titer of the virus was determined in doubles with the Adeno-X Rapid Titer kit from Clontech according to the manufacturer's instructions. In short, a well determined number of cells were infected with different dilutions of the virus. After 48 hours amount of produced shell proteins was detected with a visible antibody reaction. Thereby, titer could be calculated.

II.11.5. Sequencing of the virus:

Virus-DNA was isolated with the Blood-Mini-kit (Qiagen) following the manufacturer's protocol. Here, the cell lysate of a big flask was used to ensure that the sequence of the target gene in the viral particles has not changed unintentionally during virus expansion. On the DNA (40kb fragments) standard PCR was exerted with the pShuttle-IRES-hrGFP1 primers recommended by Stratagene. The fragment of the correct size was isolated from a low-melt-agarose-gel as described and sent in for sequencing.

II.12. FACS analysis:

In order to detect the reporter-GFP produced in pShuttle-MEF2C-IRES-hrGFP-Adenovirus virally infected cells, classical staining or labelling became unnecessary and was therefore omitted here. As infected HUVEcells were in a bad shape and sensitive, cells were not scraped but trypsinized for harvesting. The reaction was stopped with medium. Cells were washed with 1xPBS and centrifuged in lock-cap tubes to avoid virus spreading. Therefore, cells were fixed with 0,5ml 4% paraformaldehyde per 6-well for 15 minutes on ice as well. Cells were pelleted for 0,5 minutes at 8000rpm and subsequently resuspended in 200µl ice cold 1x PBS.
After transfer of the samples into FACS tubes, cells were analysed in the FACSCalibur machine.

II.13. Matrigel- Assay:

Matrigel (BectonDickinson) was thawed on ice in the refrigerator overnight. The matrigel was diluted with serum-free M199 medium and aliquoted in order to obviate repeated freeze-thawing. All materials used like pipettes, plates, tubes, needles or syringes have to be pre-cooled, preparations have to be done on ice or in a cold room. For a standard experiment 100µl of liquid Matrigel were cautiously dropped into a 48-well to avoid air bubbles. For gelatinizing, the Matrigel was incubated at 37°C for at least 30 minutes.
In the mean time 16 hours adenovirus-infected HUVECs of two 6-wells were trypsinized, counted and seeded in 500µl M199 medium complete at a concentration of $2,5*10^5$ cells per well. HUVECs were allowed to form a network for 16 hours at 37°C. Progress of tube formation was documented by photography.

II.14. Sprouting Assay:

For the purpose of a sprouting assay spheroids of 400 HUVECs per 30µl drop were aspired. When adenovirus-infected cells are to be tested, they are infected 16 hours before harvesting for spheroid formation. Cells were infected with MOI 15.

Principally, cells were trypsinized and counted. 150000 cells were resuspended in 80% EGM complete medium and 20% methocel. Methocel had to be centrifuged previously for 2 hours at 4000rpm and only the supernatant was used. With a multi-channel pipette drops of 30µl of the cells containing medium-methocel suspension were dispersed over the cover of a 150mm plate. The drops were incubated at 37° overnight hanging up-side-down.

To collect the formed spheroids, they were washed with a 10% FBS-EZ containing PBS buffer and harvested carefully. The spheroids were centrifuged at 700 rpm for 5 minutes. The supernatant was removed very cautiously.

Two kinds of media were prepared for the following steps. Medium 1 contained 80% methocel and 20% FBS-EZ. For 1 gel 0.5ml of this medium was necessary. Medium 2 consisted of rat-tail collagen diluted in 0.1% acidic acid and 10x Medium M199. Moreover, 90µl 1M HEPES buffer were added and 500µl 0.2N NaOH for neutralization. Amounts were adjusted for 8 gels, which will principally contain 0.5ml Medium 2 each. Note, after neutralization collagen started immediately to polymerize and had to be processed instantly.

The collagen gels were casted into a 24-well-plate for suspension cells (Greiner). Previously, the surrounding wells were filled with 1ml PBS. This should avoid subsequent drying of the gels in the inner wells.

The spheroids pellet was resuspended gently in Medium 1. This suspension is mixed in a 1:1 ratio with Medium 2. 1ml of this mix is filled quickly into an inner empty well, altogether 8 times. The gels were allowed to polymerize for 1 hour at 37°C. Finally, spheroids were stimulated. Therefore, 100µl basal medium containing VEGF for a final concentration of 50 ng/ ml were added. On the following day pictures were taken to record the sprouting behaviour of the spheroids.

III. Aims:

The aim of this work was to investigate the role of the VEGF-induced MADS box transcription factor MEF2C in endothelial cells and angiogenesis. Therefore, expression and consequences of overexpression of MEF2C in endothelial cells had to be analysed with appropriate tools which had to be generated.
As VEGF is a key regulator and main trigger of early angiogenesis, comprehension of the functions of VEGF-dependent proteins is of special interest to perceive the procedures involved in formation of new blood vessels.
The particular aims were as follows:

- Verify the MEF2C mRNA induction by VEGF in HUVEC by real time RT-PCR measurements
- Generate recombinant adenoviruses for MEF2C overexpression studies
- Evaluate the overexpression of MEF2C by the recombinant adenovirus and analyse the influence of MEF2C on VEGF-induction for certain genes in HUVEC by real time RT-PCR
- Perform *in vitro* angiogenesis assays (“Matrigel assay” and “Sprouting assay”) during overexpression of MEF2C by the recombinant adenovirus.

This work should contribute to the knowledge about the VEGF-regulated transcription factor MEF2C, provide the basis to study the expression patterns governed by MEF2C and give insights into the influence of MEF2C on angiogenesis on a cell physiological level.

IV. Results:

The objective of this work was to elucidate the role of MEF2C in EC during angiogenesis. Therefore, the intended tasks were: i) to verify the VEGF dependent upregulation of MEF2C by real-time RT-PCR, ii) to construct a MEF2C-expressing and a corresponding control adenovirus, iii) to test these viruses in a matrigel assay and a sprouting assay and iv) to search for genes, which are modulated in their VEGF-inducibility by MEF2C.

IV.1. Verification of MEF2C induction by VEGF in HUVEC:

MEF2C was selected as a gene selectively upregulated by VEGF, but not by EGF and IL-1 from microarray data obtained by B. Schweighofer (PhD thesis in preparation). To verify this data, which indicated a significant upregulation of MEF2C at the 150 minutes time point, cDNA was produced for real-time RT-PCR. In short, HUVEC were stimulated with VEGF, bFGF, EGF and IL1 for 30 minutes, 60 minutes, 150 minutes and 360 minutes. The RNA from these cells was isolated and served as a template for cDNA synthesis. cDNA was analysed by real-time PCR using purpose-built primers for MEF2C and ß2-microglobulin for normalization. The used methods are described in detail in chapter II.9.

Results shown in Figure 7A-D represent data obtained from 3 independently generated cDNA series, which were evaluated by real-time PCR twice.

On average, MEF2C was induced by VEGF 3.7-fold at the 150 minutes time-point and persisted at this elevated level for at least 120 minutes. Upon bFGF stimulation MEF2C mRNA expression increased 1.9-fold already at 60 minutes, peaked at 150 minutes with a 2.7-fold induction and declined afterwards to its basal level. EGF dependent MEF2C induction constantly remained on a very low level below a 2-fold induction. IL1 did not stimulate MEF2C mRNA expression at any time point.

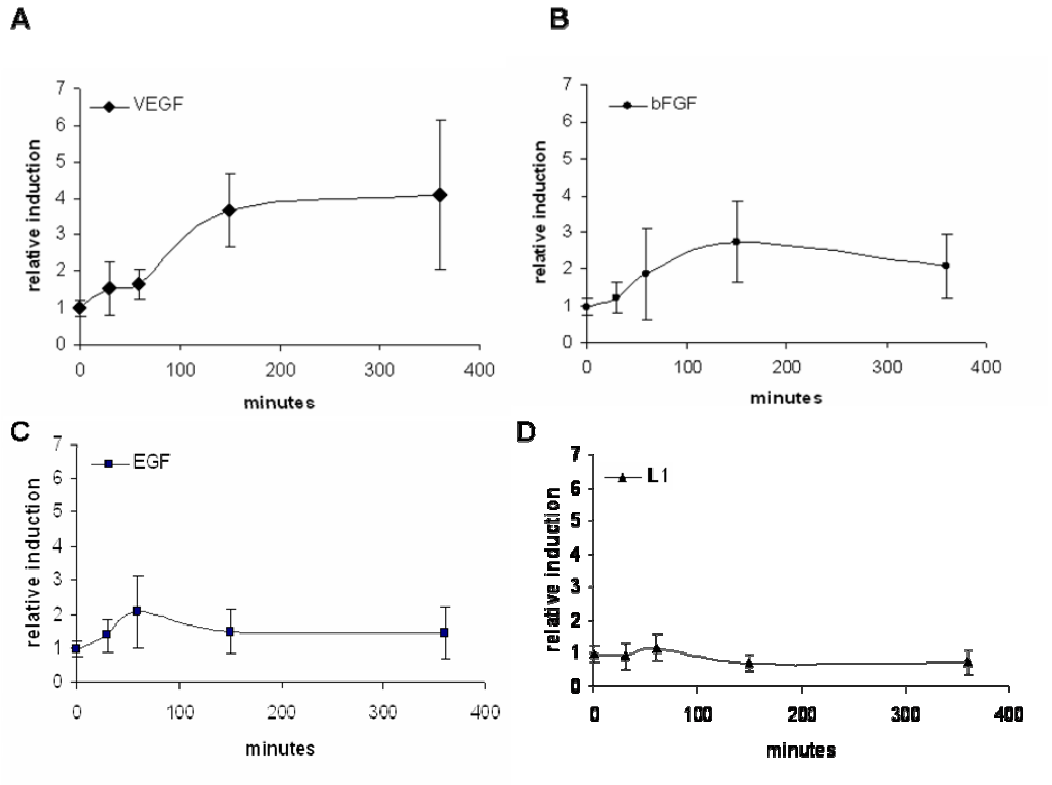


Figure 7: VEGF specific induction of MEF2C

Serum starved HUVEC in 6-well plates were stimulated for 30 min, 60 min, 150 min and 360min with 50µg/ ml VEGF, bFGF or IL-1 or 25µg/ ml EGF. Then cDNA was synthesized. MEF2C expression was measured with MEF2C primers encompassing the exon-intron boarders 8-9. For calculation of the relative induction the expression of the housekeeping gene ß2-microglobulin was also determined. All values were normalized to ß2M. Results are displayed as fold induction, relative to the basal expression level of untreated cells. The standard deviation was calculated from data obtained from 3 series of cDNA.

IV.2. Generation of a MEF2C adenovirus:

A fragment encompassing the coding region for MEF2C was cut out of a cDNA clone obtained from the RZPD with the help of the restriction enzymes *Not*I and *Spe*I. The fragment was subcloned into the shuttle vector pShuttle-IRES-hrGFP-1, which is part of the AdEasy™ Adenoviral Vector System kit from Stratagene®. To confirm the correct insertion the pShuttle-MEF2C-IRES-hrGFP-1 was sequenced with primers recommended by Stratagene. Afterwards, the plasmid was linearized with *Pme*I and dephosphorylated. In this form the pShuttle-MEF2C-IRES-hrGFP-1 was co-

transfected with the pAdEasy vector into E.Coli of the strain BJ5183 RecA$^+$ by electroporation to obtain a recombinant adenovirus genome by bacterial homologous recombination. Successful recombination at either the ITRs or at the origins could be identified by *Pac*I control restriction, which revealed a 3kb or a 4.5kb fragment, respectively, in combination with a 30kb fragment. In Figure 8 both types of positive recombination are depicted in comparison to an un-recombined pShuttle-MEF2C-IRES-hrGFP-1 to distinguish unambiguously the sizes of the bigger fragments. A schematic representation of the recombination of the shuttle vector with the adenovirus plasmid is given in Materials and Methods and of the vectors in the Appendix.

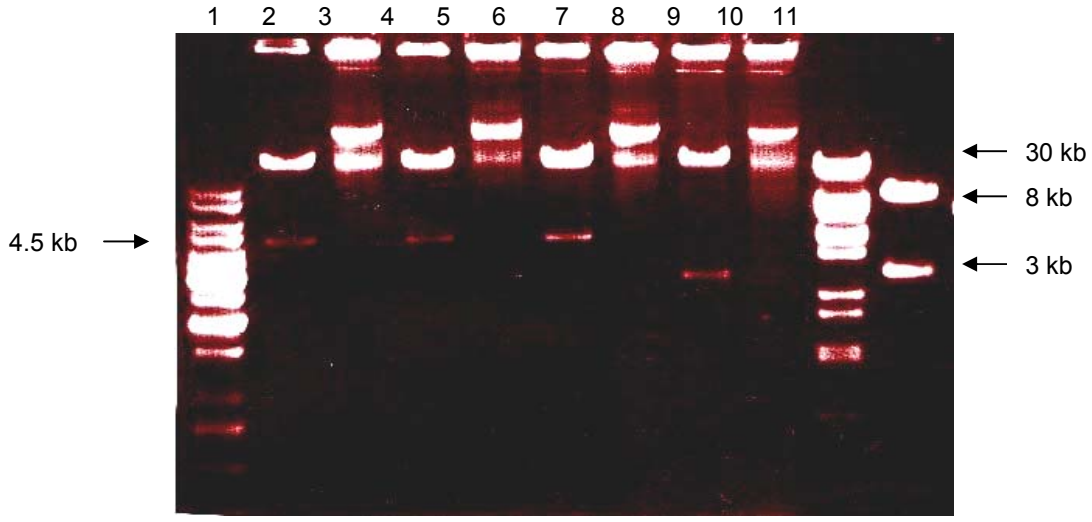


Figure 8: Identification of positively recombined clones

After co-transfection of the pShuttle vector and the pAdEasy vector Kanamycin resistant clones were selected, grown, prepped and subsequently digested with *Pac*I to select a positively recombined clone

0,8% Agarose Gel for analysis of large-sized DNA

Lane 1: 1kb Marker (see Appendix)

Lane 2, 4 and 6: *Pac*I digested individual plasmid clones pAdE.hrGFP.MEF2C-7 to 9 obtained after cotransfection of pAdEasy plasmid and pShuttle-MEF2C-IRES-hrGFP-1 into electro-competent E.Coli; bands sizes of 4.5kb corresponds to recombination at the origins

Lane 3, 5 and 7: uncut plasmid preparation of clones pAdE.hrGFP.MEF2C-7 to 9 respectively

Lane 8: *Pac*I digested plasmid clone pAdE.hrGFP.MEF2C-10; positively recombined at the inverted terminal repeats corresponding to the band size 3kb

Lane 9: uncut plasmid pAdE.hrGFP.MEF2C-10

Lane 10: *Bst*I lambda Marker (see Appendix)

Lane 11: *Pac*I digested pShuttle-MEF2C-IRES-hrGFP-1 plasmid preparation

After successful recombination the pAdE.hrGFP.MEF2C-7 DNA was sequenced again for control. Then the recombined large plasmid was isolated from a large scale preparation using the plasmid maxi purification kit from Quiagen. The plasmid was digested with *Pac*I to expose the ITRs, which function as replication origins of the adenovirus in mammalian cells. Following linearization, the DNA was transfected into 293 cells seeded in a 6-well-plate using the mammalian transfection kit from Stratagene®. Medium was changed partially every three days to keep the cells viable. Eleven days after transfection the first plaque was detected (Figure 9).
Subsequently, when many plaques appeared, cells were scraped from the plate and lysed by freeze-thawing cycles. The viral particles isolated in this process were used for infection of 293 cells in bigger plates to amplify the virus. The final adenovirus containing cell lysate was purified by CsCl density gradient centrifugation.

For the generation of the control adenovirus, all steps were performed similarly to the MEF2C expressing virus except the very first subcloning step of the gene of interest. The empty pShuttle-IRES-hrGFP-1 vector was recombined with the pAdEasy vector.

The titres of both big viral preparations were determined with the AdenoRapidTitre kit from Clontech®. A titre of 6.5×10^8 pfu (plaque forming units)/ ml for the AdE.hrGFP.MEF2C and a titre of 5.0×10^9 pfu/ ml for the AdE.hrGFP.ctrl were assessed.
All mentioned procedures and methods are described under II.11. in detail.

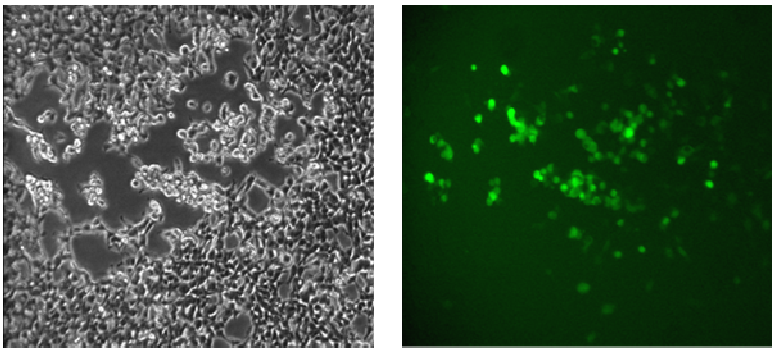


Figure 9: Plaque formation

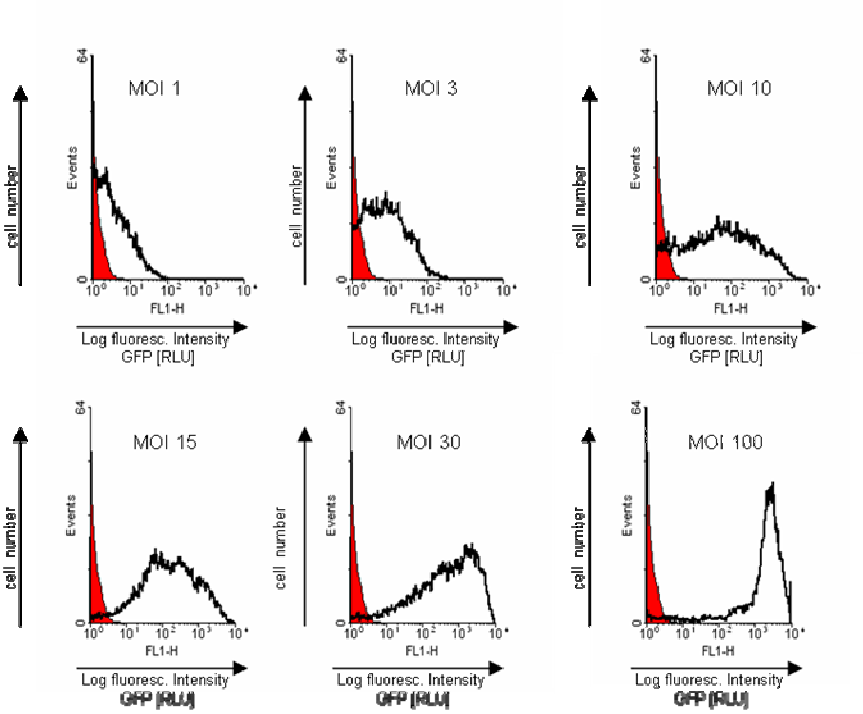
293 cells were seeded into 6-well plates and grown to 70% confluence. They were transfected with a positively recombined pAdEasy-MEF2C-IRES-hrGFP-1 clone with the mammalian tranfection kit from Stratagene. Under the phase contrast microscope the first plaque appeared after eleven days (1). Under the fluorescence microscope transfected and infected cells were traced via the green fluorescence reporter protein (2).

IV.3. Determination of optimal MOI (Multiplicity of Infection):

Investigations of cell signalling are very sensitive to experimental conditions. When cells are stressed unintentionally, the stress response can mask normal cell responses or activated signalling pathways. In comparison to standard transfection procedures infections with replication defective adenoviruses do not involve harsh treatment of the cells, but still can provoke inflammatory anti-viral responses ("virus effect"). Therefore, the exact MOI has to be defined, at which all cells are infected, but multiple infections are avoided to prevent excess viral effects.

HUVEC were infected with AdE.hrGFP.ctrl with MOI of 1, 3, 10, 15, 30 and 100 for 32 hours. Then the cells were analysed by FACS analysis as described in chapter II.12.. Figure 10A shows that already with MOI 10 nearly all cells were infected. With MOI 15 clearly 100% infection rates were achieved.

Corresponding to an increasing MOI the mean fluorescence per cell also increased as it can be seen in Figure 10B. After the infection rate reached 100% saturation at MOI 10 to 15, the mean fluorescence per cell continued to increase further with higher MOIs and reached saturation level at about MOI 100.



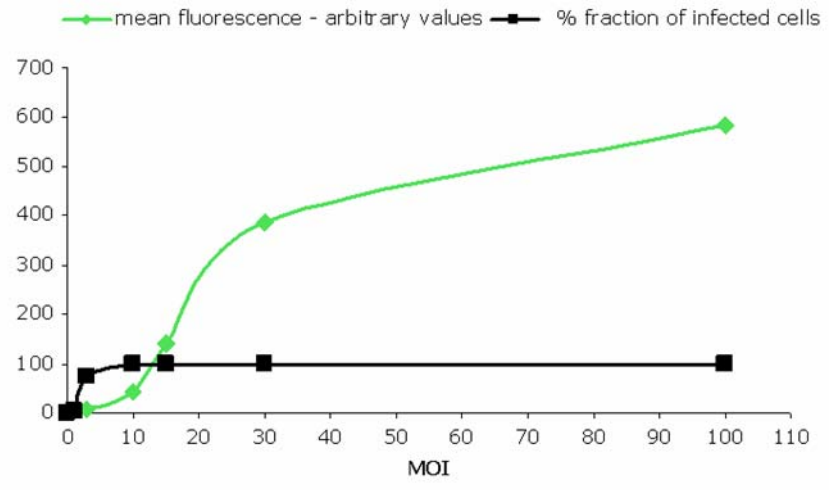


Figure 10: Identification of Adenovirus Infection

A. 10^6 HUVEC were counted and infected with control adenovirus pAdEasy-IRES-hrGFP-1 with MOI 1, 3, 10, 15, 30 and 100 for 32 hours. They were harvested, fixed and evaluated by FACS analysis.

B: The fraction of infected cells corresponds to the percentage of fluorescent cells of a sample. The mean fluorescence of the samples was recorded and charted automatically by the FACS machine and are expressed and displayed as relative values.

IV.4. Overexpression of MEF2C:

To test the function of AdE.hrGFP.MEF2C the production of MEF2C mRNA and protein during infection was determined. To test whether the MEF2C virus generated the correct protein and when the protein starts to emerge, HUVEC were infected with MEF2 virus with MOI 15 for 4 hours, 8 hours, 16 hours, 32 hours and 48 hours, lysed with Lämmli buffer afterwards and subsequently subjected to Western blotting.
As shown in Figure 11, after 4 and even 8 hours of infection hardly any MEF2C was noticeable. At the 16 hours time point the beginning of substantial translation of MEF2C became visible. After 32 and 48 hours a massive accumulation of MEF2C was detectable.

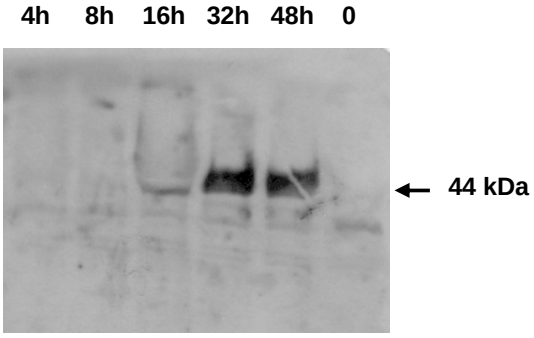


Figure 11: Time course of MEF2C protein (44kDa) overexpression
HUVEC were seeded into 12 well-plates and infected with AdE.hrGFP.MEF2C with MOI 15 for 4, 8, 16, 32 and 48 hours. Then cells were lysed and resuspended in Lämmli buffer. These samples were subjected to SDS-PAGE and Western blotting and analysed with a polyclonal goat-anti-MEF2C-antibody.

To prove mRNA overexpression by the AdE.hrGFP.MEF2C HUVEC were infected with AdE.hrGFP.MEF2C and AdE.hrGFP.ctrl with MOI 15 for 16 hours and subsequently stimulated with VEGF for 30 min, 60 min and 150 min. Total RNA was isolated and subjected to cDNA synthesis. cDNA was evaluated by real time RT-PCR.
Evaluation of the cDNA revealed a typical induction pattern of MEF2C of around 3 fold at 150 minutes after VEGF stimulation in HUVEC, which were not infected or infected with the AdE.hrGFP.ctrl. In comparison, cells that had been infected with the AdE.hrGFP.MEF2C showed levels of MEF2C mRNA about 1000-fold over the

endogenous levels without VEGF identification and lead not to any further induction by VEGF (Figure 12).

The results confirm the MEF2C virus is able to overexpress MEF2C efficiently.

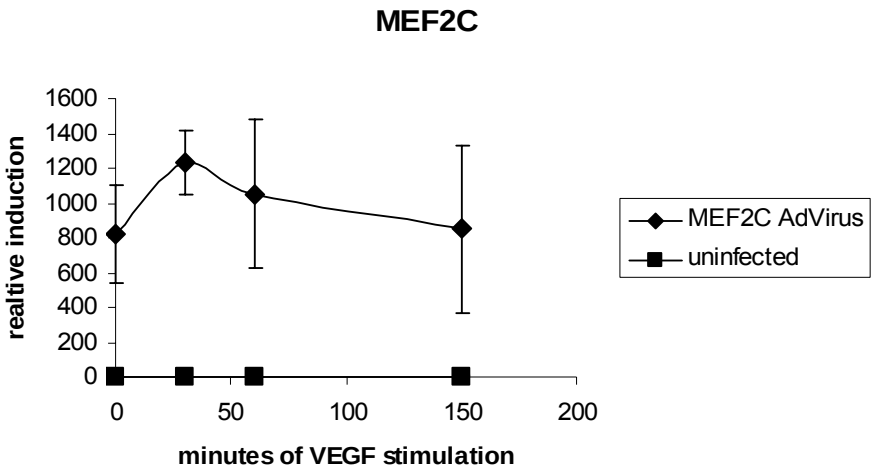


Figure 12: Testing of MEF2C mRNA overexpression

Serum starved HUVEC seeded in 6-well plates were infected for 16 hours with MOI15 of control or MEF2C virus. Cells were induced with 50ng/ ml VEGF for 0 min, 30 min, 60 min or 150 min. The total RNA was isolated and subjected to cDNA synthesis. Real time RT-PCR was performed using the primers MEF2C_fw and MEF2C_rev for the quantification of MEF2C mRNA. Obtained real time RT-PCR data were normalized to ß2-microglobulin as internal standard using the primers ß2M_fw and ß2_rev. Data were calculated from two series of experiments performed with single wells or duplicates wells, respectively.

IV.5. Influence of MEF2C on VEGF-mediated gene induction in HUVEC

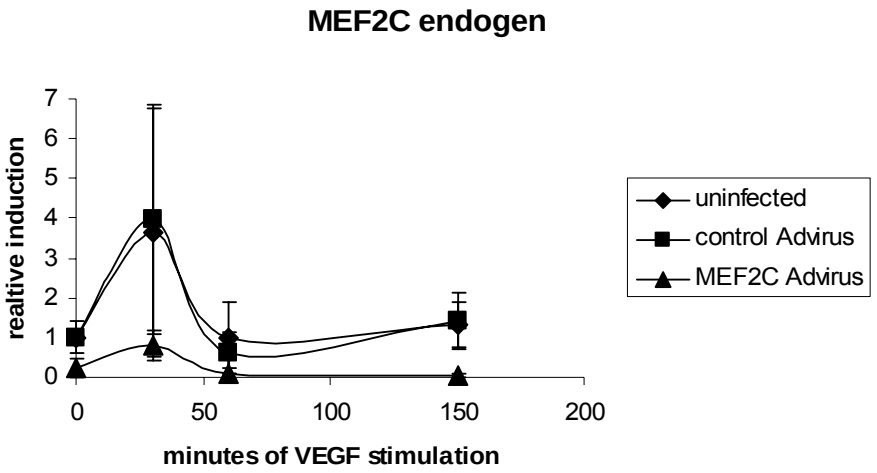
As MEF2C is a transcription factor, it was suggested that MEF2C overexpression might modulate the gene expression pattern of AdE.hrGFP.MEF2C infected cells. For studying angiogenesis it is of particular interest whether MEF2C might influence VEGF-stimulated gene induction in HUVEC.
Therefore, serum starved HUVEC were infected with MEF2C virus and control virus with MOI 15 for 16 hours and subsequently stimulated with VEGF up to 150 min. Total RNA was isolated and served as template for cDNA synthesis. The cDNA was tested for several VEGF-regulated genes by real time PCR, using ß2Microglobulin expression for normalization.
Many transcription factors downregulate their own expression in a negative feed-back loop. This also seems to be the case with MEF2C. When the VEGF-dependent induction of the endogenous MEF2C was evaluated by real time PCR with the primer pair MEF2C_endo_fw and MEF2C_endo_rev, the result was very clear (Figure 13A). Endogenous MEF2C showed the usual expression pattern of 3- to 4-fold induction upon VEGF stimulation in the uninfected and in the control virus infected cells. In contrast, in the AdE.hrGFP.MEF2C infected cells endogenous MEF2C was already down-regulated in non-induced cells. Following VEGF treatment only little upregulation of MEF2C could be observed and MEF2C levels always remained below the basal level (set to 1) of uninfected and unstimulated cells.

MMP10 has previously been reported to be a direct target of MEF2C. Therefore, expression of MMP10 during MEF2C overexpression was analysed. As shown in Figure 13B no VEGF-mediated induction was detected in any of the tested cells; the uninfected, the AdE.hrGFP.ctrl and the AdE.hrGFP.MEF2C infected cells. However, it was noticed that MMP10 exhibited significantly lower basal expression levels in the AdE.hrGFP.MEF2C infected cells in comparison to the control cells.

DSCR1 and NR4A2 are among the most strongly induced genes by VEGF in HUVEC, which was shown by microarrays (B.Schweighofer, PhD thesis in preparation). Both proteins are upregulated in response to Ca^{2+}-signalling and MEF2C was also reported to affect Ca^{2+}-signalling. As Ca^{2+}-signalling generally plays an import role in regulation of angiogenesis, influence of MEF2C overexpression on DSCR1 and NR4A2 was tested (Figure 13 C+D).

DSCR1 was induced by VEGF in uninfected cells at 30 minutes 22- to 30-fold. The control virus infected cells exhibited a virus effect as VEGF induction was decreased in comparison to uninfected cells, but cells still were inducible up to 10-fold.In contrast, VEGF could not mediate significant DSCR1 induction in AdE.hrGFP.MEF2C infected cells, DSCR1 mRNA expression remained on the basal level all the time.
NR4A2 was strongly induced by VEGF in uninfected as well as in control virus infected HUVEC up to a 300-fold at 30 minutes, whereas in MEF2C overexpressing cells VEGF induction was strongly diminished to a much lower induction of 16-fold.

A



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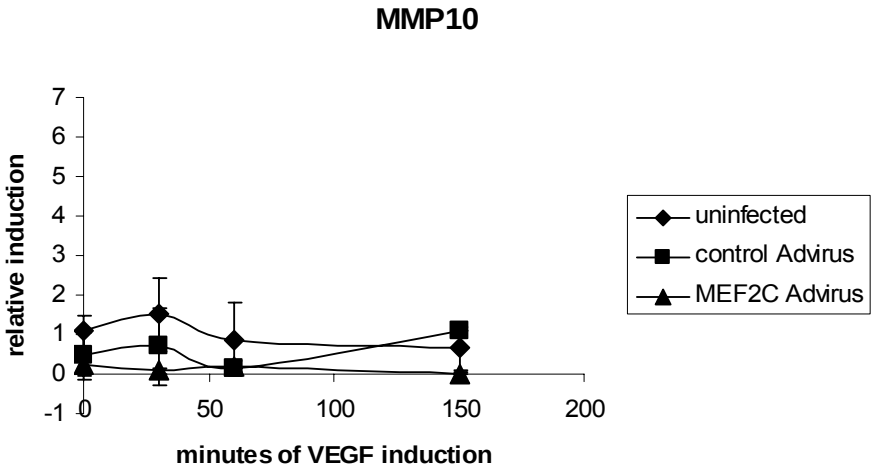

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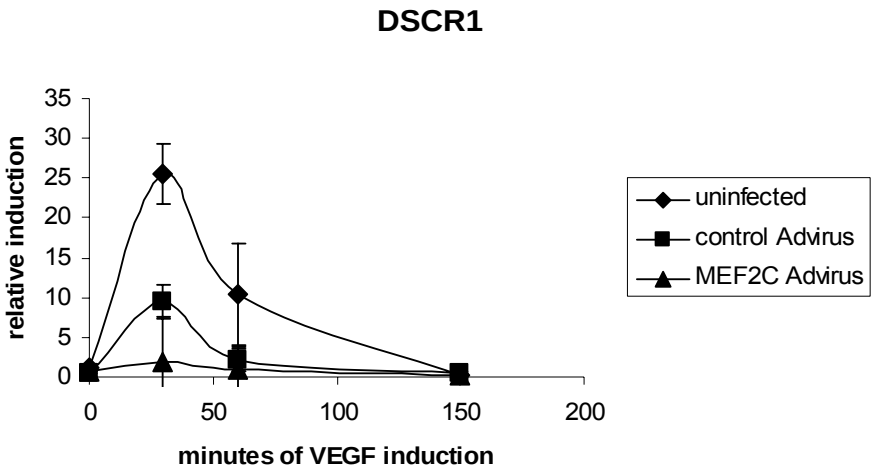

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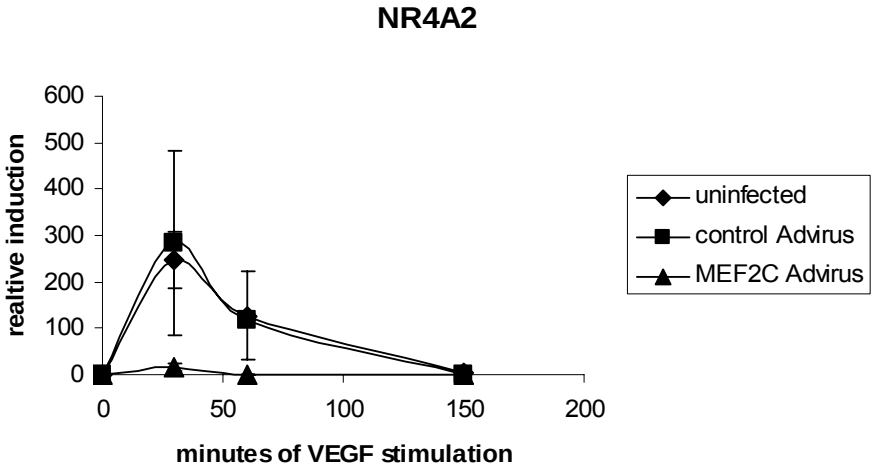


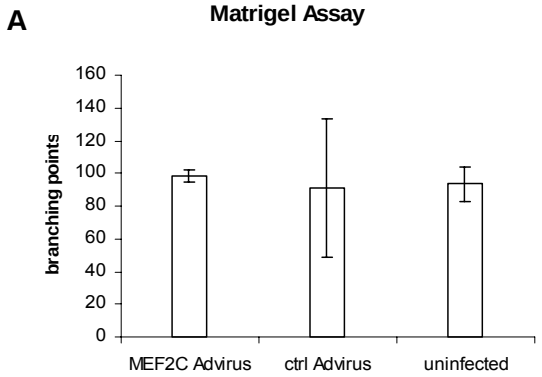
Figure 13 A-D: Modulation of endogenous MEF2C, MMP10, DSCR1 and NR4A2

Serum starved HUVEC seeded in 6-well plates were infected for 16 hours with MOI15 of control or MEF2C virus. Cells were induced with 50ng/ ml VEGF for 0 min, 30 min, 60 min or 150 min. The total RNA was isolated and subjected to cDNA synthesis. Real time RT-PCR was performed using the primers MEF2C_endo_fw and MEF2C_endo_rec for the quantification of endogenous MEF2C (A), MMP10_fw and MMP10_rev for MMP10 (B), DSCR1_fw and DSCR1_rev for DSCR1 (C) and NR4A2_fw and NR4A2_rev for NR4A2 (D). All obtained real time RT-PCR data were normalized to ß2Micoglobulin as internal standard using the primers ß2M_fw and ß2_rev.

IV.6. In vitro Angiogenesis Assays:

To study the mechanisms of angiogenesis and to elucidate the function of the proteins involved, several angiogenesis assays provide a good opportunity to monitor endothelial cell behaviour *in vitro* under defined conditions. In the Matrigel assay the capacity of two dimensional tube formation can be tested. On Matrigel, an ECM-rich gel, endothelial cells spontaneously form networks. This can be modulated by angiogenesis enhancers like VEGF or potential inhibitors. When HUVEC are infected by adenoviruses overexpressing a certain gene the influence of this gene on angiogenesis can be analysed.
In order to test the effects of MEF2C overexpression, HUVEC were infected with MEF2C or control virus for 16 hours, then harvested carefully and subsequently seeded onto Matrigel. Cell behaviour was monitored under the microscope for eight hours. At four hours the network seemed to be formed.
As shown in Figure 14 no significant difference between the tube formation of uninfected, control virus infected or MEF2C virus infected cells could be observed.

Since angiogenesis is defined as the formation of new vessels by sprouting from existent vessels the influence of a potential modulator on sprouting is important. The *in vitro* spheroid assay takes advantage of the phenomenon that endothelial cells form spheroids by attaching to each other when they are devoid of an adherent surface. When these spheroids are embedded into a collagen gel the endothelial cells on the surface of the spheroid spontaneously form sprouts. This can be strongly enhanced by angiogenic growth factors like VEGF. With this system angiogenesis regulating substances or genes can be tested.



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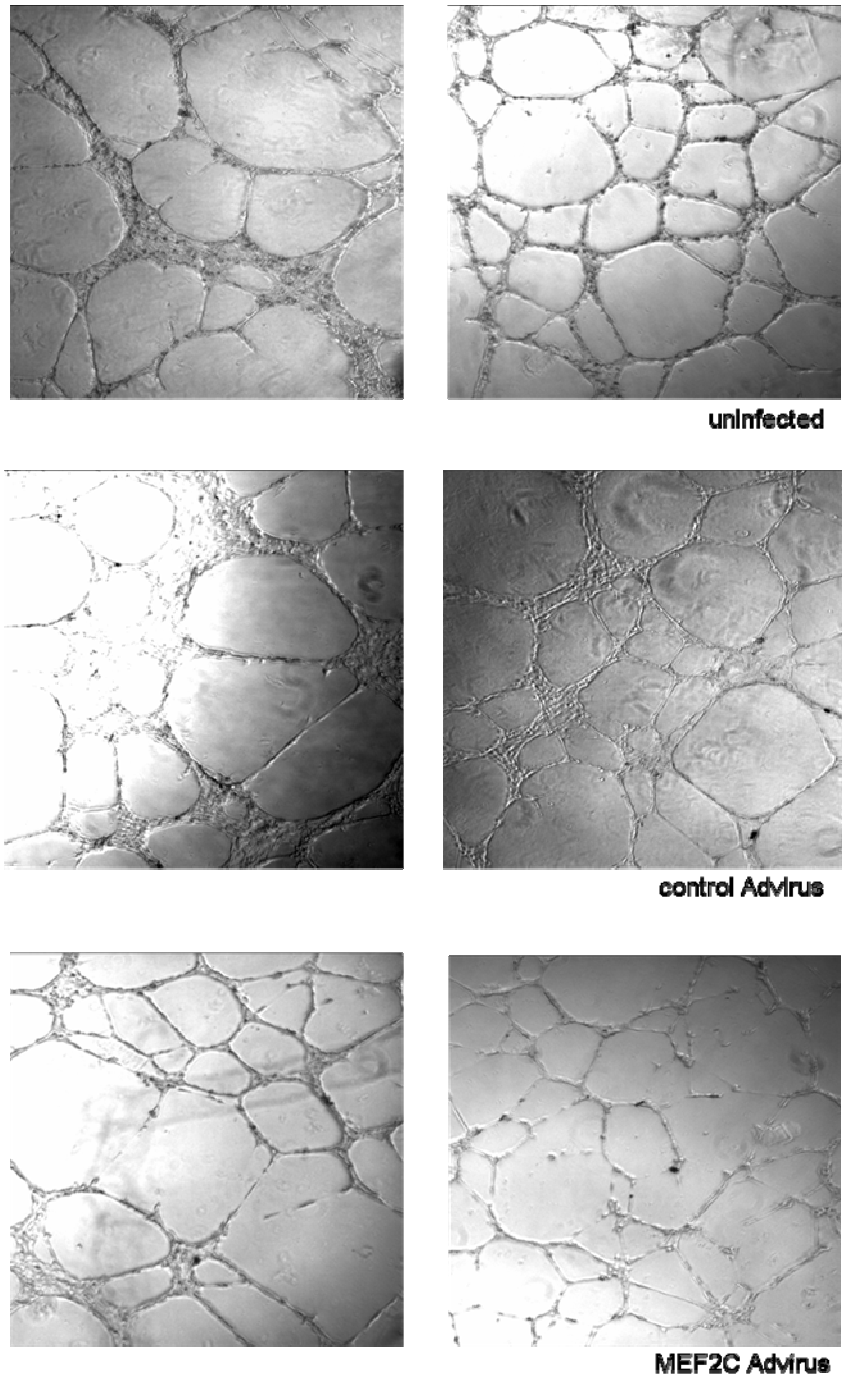

Figure 14: Matrigel Assay

HUVEC were infected with MOI 15 of control or MEF2C virus for 16 hours, harvested and counted. Matrigel was thawed on ice, transferred into 48 wells a 4°C and subsequently allowed to gelatinize at 37°C. HUVEC were seeded onto the matrigel and allowed to form a network for eight hours.

A: Numbers of total branching points counted in triplicate wells

B: Network formation documented under the phase contrast microscope.

To elucidate the influence of MEF2C overexpression, HUVEC were infected with control or MEF2C virus and subsequently allowed to form spheroids by the hanging drop method. These spheroids were transferred into a matrix consisting of rat tail collagen for stabilization and medium solved methocel as a scaffold and nourishment supply. The spheroids were stimulated with VEGF overnight and then examined under the microscope. As example one spheroid of every condition is documented in Figure 15.

VEGF stimulation positively enhanced sprout formation in the uninfected and also in the control virus infected cells as it is shown in Figure 15. A small reduction of sprout formation in the control virus infected cells is visible, but the adenovirus effect seems not to be critical. Strikingly, MEF2C virus infected cells do not exhibit any sprout formation, neither in the unstimulated samples nor in the VEGF stimulated samples. This inhibition of sprout formation was observed in every MEF2C overexpressing spheroid.

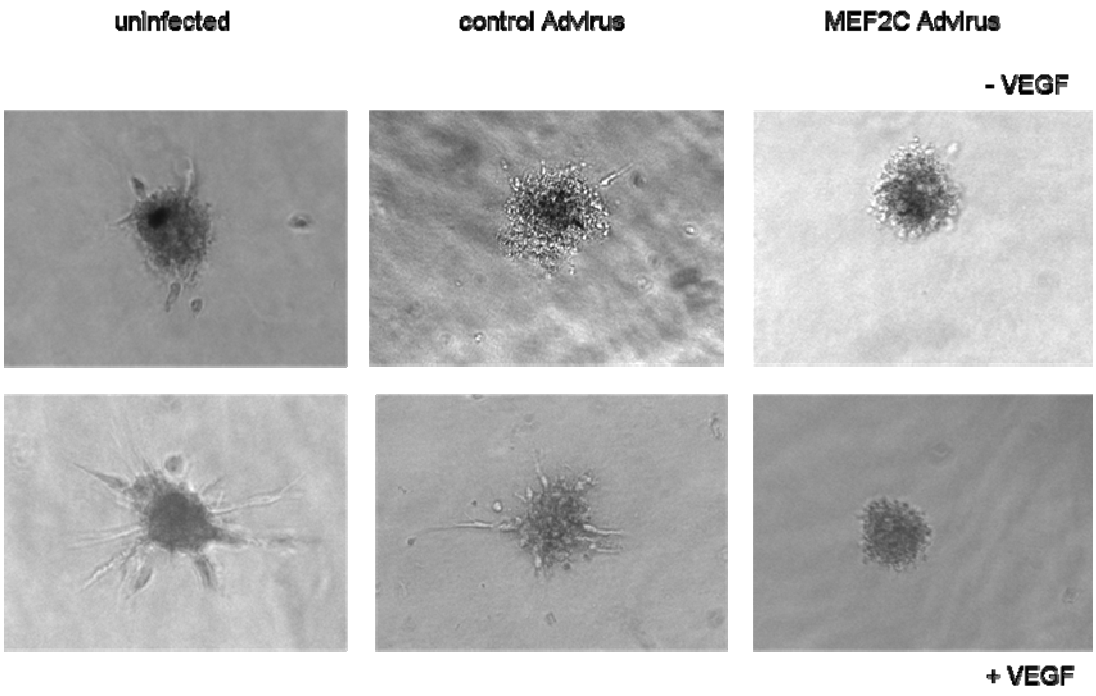

Figure 15: Spheroid Assay

HUVEC cells were infected with control virus or MEF2C virus for 16 hours, harvested and subsequently allowed to form spheroids by the hanging drop method. Therefore, the cells were resolved in 20% methocel and 80% medium. 30µl drops of the cell suspension were put on the lid of a dish and incubated overnight. The thereby formed spheroids were harvest carefully and embedded into a 1:1 matrix of rat tail collagen and methocel. They were stimulated with 50ng/ ml VEGF overnight.

V. Discussion:

This work was focused on elucidating the role of the transcription factor MEF2C in endothelial cells during angiogenesis. MEF2C is a gene specifically induced by VEGF in endothelial cells and therefore assumed to be involved in angiogenesis. Angiogenesis is the formation of new (blood or lymph) vessels by sprouting from pre-existing vessels. As many diseases like ischemic heart diseases or cancer are associated with pathological angiogenesis the processes involved in angiogenesis are topics of disease-related investigations. It is important to understand the molecular basis of the associated cellular regulation systems to be able to develop alternative therapies in the future. Since MEF2C is implicated to be a key player during development in other cell types, it seemed possible, that MEF2C plays a role in endothelial cells during angiogenesis. To support this hypothesis, specific upregulation of MEF2C upon VEGF induction had to be verified by real time RT-PCR. Moreover, an adenovirus, which expresses MEF2C, had to be constructed as a basis to identify genes that are modulated by this transcription factor and to study its influence on angiogenesis assays.

V.1. MEF2C is specifically upregulated by VEGF:

In a microarray gene profiles of HUVEC were compared after treatment with the angiogenic growth factor VEGF, or the general growth factor EGF or the inflammatory cytokine IL-1. The hypothesis was that genes which are specifically upregulated by the angiogenic growth factor VEGF are potentially important for angiogenesis. When some of these genes in addition fulfil the criteria not to be induced by the general growth factor EGF or the inflammatory cytokine IL-1, they are likely neither part of the regular growth process nor the inflammatory response, but will rather accomplish a VEGF specific angiogenic function. Therefore it was concluded that these genes are potential candidates to be angiogenesis-specific genes. As we are interested especially into the initial process of sprouting, early time points of induction (30 min, 60 min, 150 min and 360 min) were selected for analysis. MEF2C is an example of a gene, which is specifically upregulated by VEGF with peak values at 150 min.

From microarrays broad information can be obtained, but exact quantification is limited. Therefore, potentially interesting results have to be verified quantitatively with

another reliable method; here with real time RT-PCR. The obtained results for MEF2C confirmed that the microarray results were correct, because a VEGF-dependet 3.7-fold upregulation at 150 min of stimulation was determined (Figure 7). MEF2C seems not to be an immediate early gene, which usually peak at 30-60 minutes, but to belong to a group of second wave genes revealing peak values between 2-4 hours. Real time RT-PCR also revealed that MEF2C was slightly induced by bFGF, which is also an angiogenic growth factor. This substantiated the notion that MEF2C is involved in angiogenesis.

V.2. The experimental tool adenovirus:

Standard methods of transfections of plasmid DNA into mammalian cells do not work efficiently for HUVEC. Additionally, such procedures can violate the cells' constitutions to an unacceptable extent for physiologic assays.

Therefore, a MEF2C encoding adenovirus was constructed. Thereby MEF2C could be overexpressed in HUVEC which were used for angiogenesis assays subsequently. An appropriately high infection rate ascertains that 100% of the cells can be transduced by the recombinant virus. The use of a reporter gene like that for the green fluorescent protein facilitates to control the infection efficiency. It was therefore decided to generate an adenovirus which encodes a GFP under the control of an IRES following the gene-of-interest (MEF2C) in the viral construct. This avoids potential problems which could arise from a directly GFP labelled fusion protein of interest when the label disturbs its function. Likewise also an his-tag was abandoned which would have been provided on the vector pShuttle-IRES-hrGFP-1 from Stratgene®. This shuttle vector is part of the pAdEasy kit. With the help of this kit a complete adenovirus can be generated (Figure 6).

The central mechanism of this system is the homologous recombination (for schematic depiction of the reaction see Materials and Methods) in bacteria of a shuttle vector, which contains the coding sequence of MEF2C (for schematic depiction of the construct see Appendix), and the vector encoding the adenoviral genome after co-transfection. The coding sequence of MEF2C's cDNA (for sequence see Appendix), including the START and STOP codon, was subcloned into the MCS of the pShuttle vector. Thereby it came under the control of the very strong CMV promoter. At the 3' side it is followed by the IRES, the GFP cDNA and a SV40

polyadenylation site. These conditions ensured an efficient mRNA production subsequently.

The crucial step in the generation of the adenovirus was the selection of positively recombined clones. The two plasmids can recombine at two regions: the origins of replication or the ITRs (Figure 6). Two patterns of DNA fragments are obtained from clones after *Pac*I digestion. Along with a 30kb fragment either a 4.5kb (ori) or a 3kb (ITRs) fragment emerges. But a 3kb fragment can also derive from a non-recombined clone along with an 8kb fragment (pShuttle vector backbone). To ensure to select a positively recombined construct, clone pAdE-MEF2C-IRES-hrGFP-7, which showed a 4.5kb fragment, was sequenced (Figure 8). This was necessary, since recombination events are error prone and could produce mutations.

The MEF2C coding sequence was found to be correct. Then clone pAdE-MEF2C-IRES-hrGFP-7 was transfected into 293 cells, which can be transfected efficiently. The DNA had to be digested with *Pac*I to expose the ITRs. Otherwise the circular adenoviral genome would not be replicated in the host cells. After transfection only a small number of viral particles form. When they finally lyse the cells they re-infect the local neighbour cells. Thus a plaque, a hole in an otherwise dense cell layer, forms. From clone pAdE-MEF2C-IRES-hrGFP-7 a first plaque was formed 11 days after transfection (Figure 9). Subsequently, when cells were infected with a high virus concentration all cells died concomitantly and plaques were not observed. Adenovirus particles were amplified and subsequently purified. Finally, titres of $6.5x10^8$ pfu/ml for the AdE.hrGFP.MEF2C and $5.0x10^9$ pfu/ml for the AdE.hrGFP.ctrl were obtained.

In parallel to the AdE.hrGFP.MEF2C virus a control virus was generated. It encoded GFP, but did not contain the MEF2C. A control virus is necessary for experiments to be able to exclude virus effects. Also the overexpressed GFP might cause effects, which can be dissected by the control virus.

Whether the adenovirus AdE.hrGFP.MEF2C overexpresses MEF2C mRNA efficiently in HUVEC and the correct protein, was tested by real time RT-PCR and Western blotting, respectively. MEF2C mRNA accumulated in AdE.hrGFP.MEF2C infected cells to levels about 1000-fold higher than in uninfected cells.

A time-course experiment monitored accumulation of MEF2C protein in AdE.hrGFP.MEF2C infected HUVEC. As the anti-MEF2C antibody detected a protein

of the correct size, the protein was regarded to be expressed correctly (Figure 11). The experiment also revealed that a significant amount of protein can already be detected after 16 hours of infection while hardly any protein was measured in the first 8 hours of infection. After 32 hours very high amounts of protein were present in the cells; likewise after 48 hours. Consequently, we had to consider the appropriate protein-dose which would meet the demanded experimental conditions. On the one hand, too much of one protein could cause a cell to behave differently and atypically alone because of the overload. On the other hand, at the beginning of a steeply increasing expression rate a small variation in time-points leads to big differences in protein concentrations. This could influence the results of experiments also. Therefore, it was decided to infect HUVEC for 16 hours before they were used for experiments.

As HUVEC do not trans-complement E1/E3 deficient adenoviruses like 293 cells, adenoviruses cannot replicate. Thus, HUVEC are not lysed but only express the introduced gene-of-interest. Nevertheless, these HUVEC suffer from an infection. The viral particles can elicit an inflammatory response. Moreover, massive overexpression of a protein itself can disturb a cell in its normal function. Therefore, it is necessary to find a balance between a 100% infection rate and prevention of vast multiple infections. Especially these can induce a viral effect, which can lead for example to a preliminarily changed expression profile for many genes in the tested cells. When signalling pathways are under investigation this effect can simulate or mask induction.

Therefore, in this work different MOI (1, 3, 10, 15, 30 and 100) were tested on resulting infection rates. Infection rate was determined in a FACS analysis via the GFP protein encoded by the adenovirus. It became evident that already with MOI 10 nearly all and with MOI 15 100% of the cells were infected (Figure 10). In many publications MOI 100 is employed. In our experiments cells exhibited a mean fluorescence at MOI 100 10 times higher than at MOI 10 reflecting multiple infections per cell. These numbers suggest that delivery of one viral expression unit into HUVEC requires at least 10 viral particles per cell; 10 pfu established in 293 cells.

V.3. Influence of MEF2C on VEGF induced genes:

Angiogenesis comprises several cellular processes which are supposed to be tightly regulated. MEF2C is a transcription factor which is specifically upregulated upon VEGF stimulation. The pattern of genes which are modulated by MEF2C could therefore provide evidence about the role of MEF2C in the orchestration of angiogenesis.

While many transcription factors activate the transcription of other genes they frequently down-regulate their own transcription in a negative feed-back loop. Here it was shown by real time RT-PCR that MEF2C exhibited a similar behaviour. The endogenous MEF2C was diminished below the control basal level of untreated cells in all AdE.hrGFP.MEF2C infected HUVEC (Figure 13A). Additionally, endogenous MEF2C was not VEGF inducible anymore whereas untreated and control infected cells still displayed the usual induction of 3- to 4-fold upon VEGF stimulation. It is likely that the MEF2C gene is repressed by a protein upregulated by MEF2C overexpression.

To test a target of MEF2C described for SMC, MMP10 was analysed during MEF2C overexpression. As MMPs play an important role during the remodelling processes of angiogenesis, it would be interesting to know whether MEF2C regulates such genes in EC. It has been described previously, that MMP10 is activated by MEF2C in SMC when it is not repressed by HDAC7 (Chang et al., 2006). Therefore, it seemed possible that the high amount of MEF2C in infected cells might titre out the repressing HDAC7 and MMP10 would be upregulated. However, in our experiments MMP10 was not induced by VEGF in HUVEC (Figure 13B) and it was remarkable that MMP10 levels in AdE.hrGFP.MEF2C infected cells were persistently reduced compared to the uninfected or the control virus infected cells. The potential response induced by MEF2C to repress further MEF2C expression in a negative feed-back loop, might also inhibit expression of some other genes such as MMP10.

DSCR1 and NR4A2 are prominent examples for very early specifically VEGF induced genes. Their VEGF-dependent inducibility under the influence of MEF2C was tested. Similar to data from the microarray of B. Schweighofer (PhD thesis in preparation) DSCR1 and NR4A2 were strongly induced upon 30 minutes of VEGF

stimulation in uninfected or control virus infected cells. DSCR1 induction was sensitive to virus infection because it was induced only 10-fold in the control virus infected cells but 25-fold in uninfected cells. This was not the case for NR4A2, which was highly upregulated 300-fold and also control virus infected cells exhibited an equal activation. Strikingly, neither DSCR1 nor NR4A2 remained VEGF inducible in the AdE.hrGFP.MEF2C infected cells (Figure 13C+D). MEF2C overexpression specifically silenced DSCR1 and NR4A2 induction.

A possible interconnection of MEF2C, DSCR1 and NR4A2 is the Ca^{2+} signalling as described in several publications: DSCR1 is the negative regulator of the important transcription factor NFAT, because it blocks calcineurin which activates NFAT by dephosphorylation (Hogan et al., 2003). NFAT is a very important regulator of endothelial cells. As an integrative part of a negative feed-back loop the DSCR1 promoter contains NFAT binding sites. DSCR1 is upregulated by NFAT and in turn inhibits further NFAT activation by calcineurin.

MEF2C was described to have an influence on intracellular Ca^{2+} release by regulating the Ca^{2+} chaperon calreticulin in the ER in a positive feed-back loop. It activates calreticulin transcription, which can further lead to sustained Ca^{2+} supply and in turn (due to a thereby exposed NLS) to extended MEF2C nuclear translocation (Lynch et al., 2005), where it might further enhance calreticulin transcription.

Albeit, Ca^{2+} seems to be a powerful regulator of MEF2C via other direct as well as indirect mechanisms; for example HDACs are phosphorylated by Ca^{2+}/Calmodulin dependent kinases, and thereby loose their repressing influence on MEF2C when they are consecutively exported from the nucleus (McKinsey et al., 2002). Moreover, MEF2 proteins were reported to cooperate with the Ca^{2+} dependent NFAT, thereby enhancing a signal (Youn et al., 2000). For example MEF2 proteins can activate the NR4A proteins also in co-operation with NFAT (Martinez-Gonzalez and Badimon, 2005).

The strongly VEGF-inducible NR4A2 is member of the orphan receptor family NR4A. The highly homologous proteins NR4A1-3 are probably active without a ligand. There are substantial indications that these transcription factors are involved in different developmental processes, but their explicit targets are unknown so far (Maxwell and Muscat, 2006). Recent reports connect NR4A proteins with vascular diseases empirically (Pols et al., 2007). NR4A1 and NR4A3 were studied in different cell types

but especially NR4A2 remains to be further investigated in endothelial cells to elucidate the molecular basis of these incidences.
Although no fully coherent scheme of potential interactions between DSCR1, MEF2C and NR4A2 can be conceived yet, the specificity of the inhibition by MEF2C is substantial and therefore suggestion of a connection might be tantalizing.

V.4. Angiogenesis assays:

Several methods are established to test angiogenesis (Auerbach et al., 2003). There are *in vitro* assays as well as *in vivo* assays. *In vivo* assays provide physiologic conditions but are more labor-intensive. Therefore, for preliminary experiments *in vitro* assays are chosen. In the Matrigel assay tube formation capacity of endothelial cells can be tested, because they spontaneously form networks on this ECM-rich matrix. The spheroid assay provides three dimensional conditions to test the sprout formation capacity of endothelial cells (Korff and Augustin, 1998).

On Matrigel it was tested whether MEF2C has an influence on the network-forming capacity of HUVEC cells to find out whether MEF2C plays an enhancing or an inhibitive role in angiogenesis. However, no significant differences could be observed between the control cells or the MEF2C expressing cells (Figure 14B). Both formed similar numbers of branches in the network (Figure 14A). It seems that MEF2C overexpression does not significantly affect tube formation on Matrigel.

It was further tried to show whether MEF2C inhibits or stimulates sprout formation in a spheroid assay. The formation of sprouts protruding from an endothelial cell spheroid can be promoted by VEGF. The control cells (uninfected and AdE.hrGFP.ctrl infected) produced more sprouts when they were induced with VEGF (Figure 15). In contrast, the AdE.hrGFP.MEF2C infected cells displayed diminished sprout formation and no stimulation by VEGF was detected. Sprout formation seemed to be inhibited in AdE.hrGFP.MEF2C infected cells.

The results from the spheroid assay are in accordance with the results of the real time RT-PCR displaying reduced MMP10 and NR4A2 induction after infection with AdE.hrGFP.MEF2C and suggest that MEF2C, when overexpressed, might rather be an angiogenesis inhibitor.

We can conceive several potential reasons for this finding. Angiogenesis is a process involving cell activation and proliferation and remodelling of the extracellular vicinity. Usually, negative feed-back loops mechanism should prevent hazardous overreactions of such processes. For example similar to inflammation, a process also inducing cell activation, it is suggested, that concomitantly with the initiation of angiogenesis also the subsequent termination of the process is already primed. Frequently, some of the genes of the second wave of induction upon an activating stimulus are inhibitors, which will limit the reaction in time. Therefore it seems possible that the angiogenic growth factor VEGF also induces inhibitory genes and MEF2C might be part of a negative feed-back loop. This is in accordance with MEF2C being induced at the rather late time points of 150 minutes until at least 360 minutes after stimulation.

Another aspect might be that angiogenesis is such a complex and intricate process that testing of a single player by overexpression may perturb the system. It is also possible, that necessary interaction partners of MEF2C are missing or not expressed in appropriate amounts and thereby overexpressing MEF2C may not score the normal functions. To singly overexpress one distinctive component of a usually coordinated functional network of proteins in a cell might cause severe problems in the execution of the normal temporal and spatial order of events. Due to the overexpression MEF2C might not be fully integrated into a probably important nuclear shuttling system or reaction partners might allocate at the wrong time. However, it seems that MEF2C overexpression is not toxic to the cells or would induce apoptosis, since no effect on tube formation on Matrigel was observed. Furthermore, it has to be considered that inhibitors might be required for the fine-tuning of angiogenesis. For example, for correct sprout formation in a vessel EC have to ascertain that only the leading cell on the growing sprout is committed to become a tip cell, whereas additional sprouting of the cells in the stem region needs to be suppressed. In this regard it was shown for another VEGF-inducible gene, HLX1, that it specifically induces repulsive receptors in EC and therefore blocks

sprouting (Mag. Julia Schultes, unpublished data). A similar mechanism is also conceivable for MEF2C.

Nevertheless, in the case of MEF2C it remains to be confirmed that the factor primarily has inhibitory function and this is not consequence of overexpression. Additional evidence should be obtained by testing downmodulation of MEF2C, which then should give complimentary results, i.e. stronger stimulation of gene transcription and sprouting. Knock-down by means of RNA interference might be the preferable method. Another possibility would be the use of a dominant-negative protein to block endogenous MEF2C function. These experiments should show that the inhibition not simply reflects a general functional shut down due to protein overload by the overexpressed MEF2C. Although relative specificity of the repression through MEF2C was observed in the real time RT-PCR experiments, where this inhibition was specific for certain genes, further data on cellular processes such as proliferation, apoptosis or migration will be required under conditions of MEF2C overexpression and knock-down. Moreover, a microarray could provide additional information about genes which are directly upregulated (or downregulated) by MEF2C.

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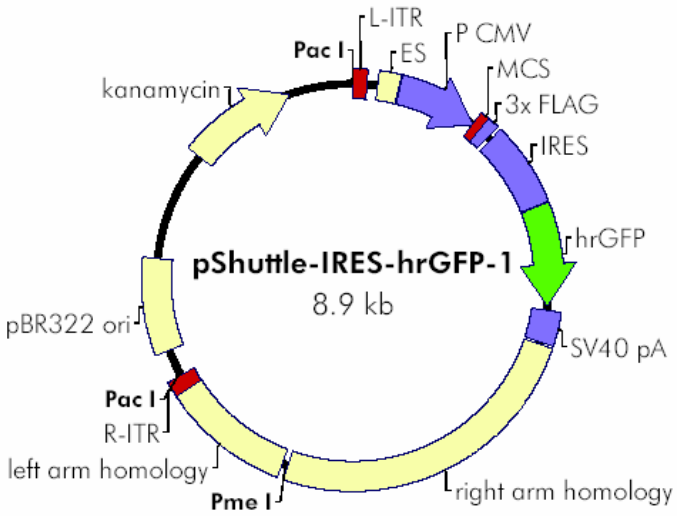
VII. Appendix:

VII.1. Vectors:

VII.1.1. pShuttle-IRES-hrGFP-1

pShuttle-IRES-hrGFP-1 Vector Map



pShuttle-IRES-hrGFP-1 Multiple Cloning Site Region (sequence shown 933–1005)

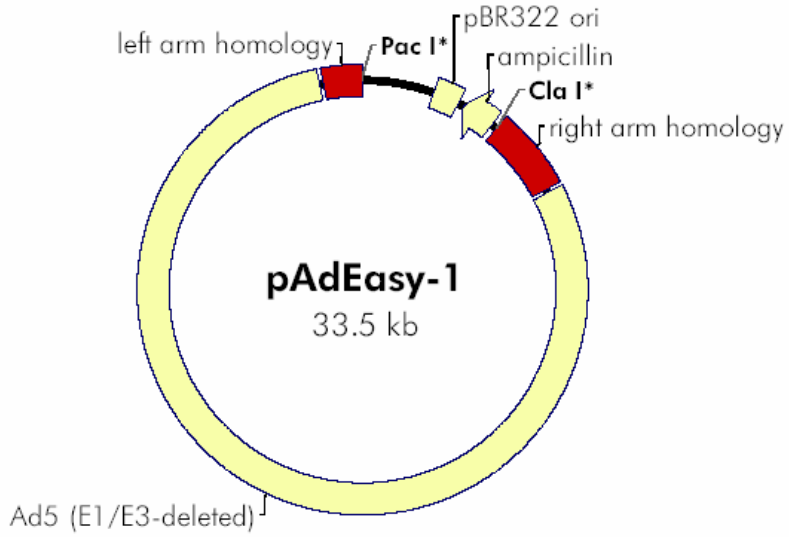
Bgl II*	Not I*	Sca I*	Nhe I*	Spe I*	EcoR V	Pvu I	Sal I	Srf I	Xho I			
A GAT CTG	CGG CCG	CAG TAC	TGC TAG	CAC TAG	TGA TAT	CCG ATC	GGT CGA	CGC CCG	GGC CTC	GAG GAC	TAC AAG	
			STOP*	STOP*	STOP*						D Y K	
											start of 3× FLAG	

*The presence of stop codons in-frame with the 3× FLAG tag must be considered when inserting genes into the MCS. Do not use the *Bgl* II, *Not* I, *Sca* I, *Nhe* I or *Spe* I sites for cloning unless the cloning strategy removes the stop codons by double digestion using one of the upstream sites plus a site downstream of the stop codons.

Feature	Nucleotide Position
left Ad5 inverted terminal repeat (ITR)	1–103
encapsidation signal (ES)	183–331
CMV promoter	345–932
forward primer binding site	749–768
multiple cloning site	933–996
3× FLAG tag	997–1068
reverse primer binding site	1075–1094
internal ribosome entry site (IRES)	1104–1678
hrGFP ORF	1688–2404
SV40 polyA signal	2443–2670
Ad5 right arm homology	2693–4918
Ad5 left arm homology	4966–5845
right Ad5 inverted terminal repeat (ITR)	5846–5948
pBR322 origin of replication	6152–6819
kanamycin resistance ORF	7628–8419

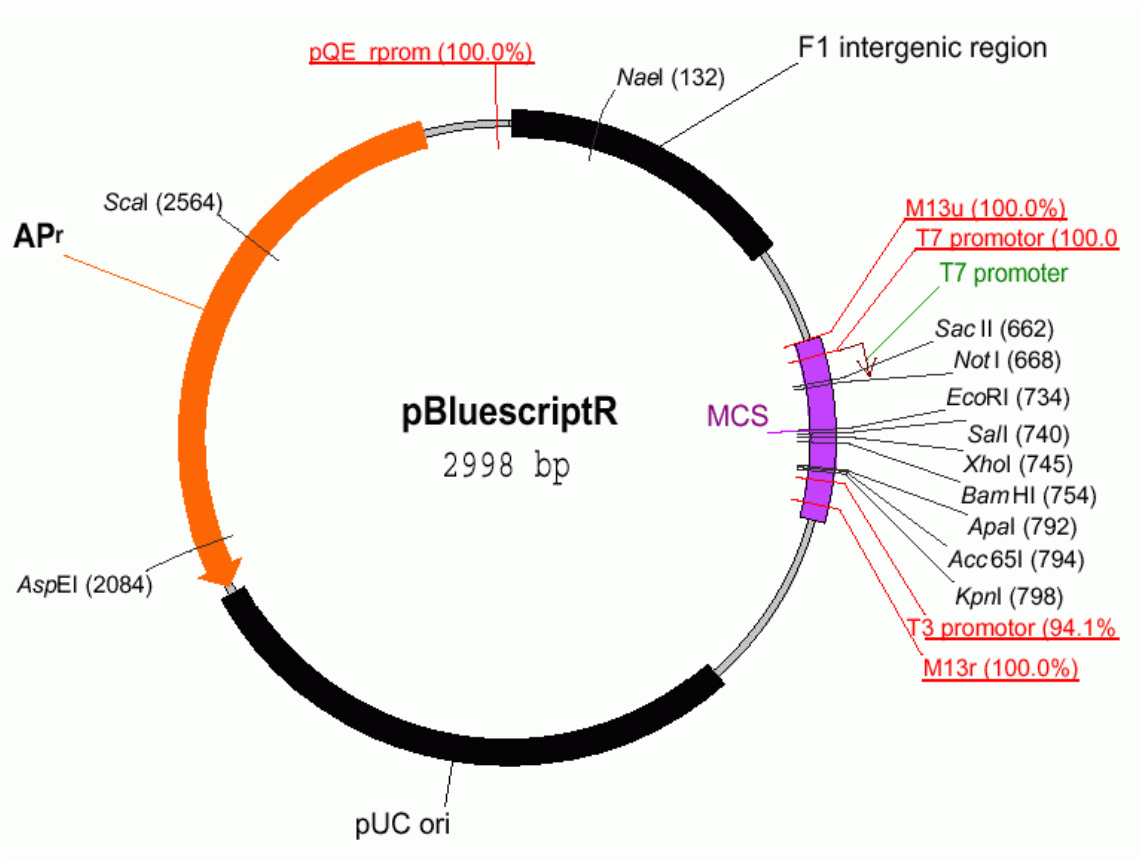
VII.1.2. pAdEasy-1:

pAdEasy-1 Vector Map

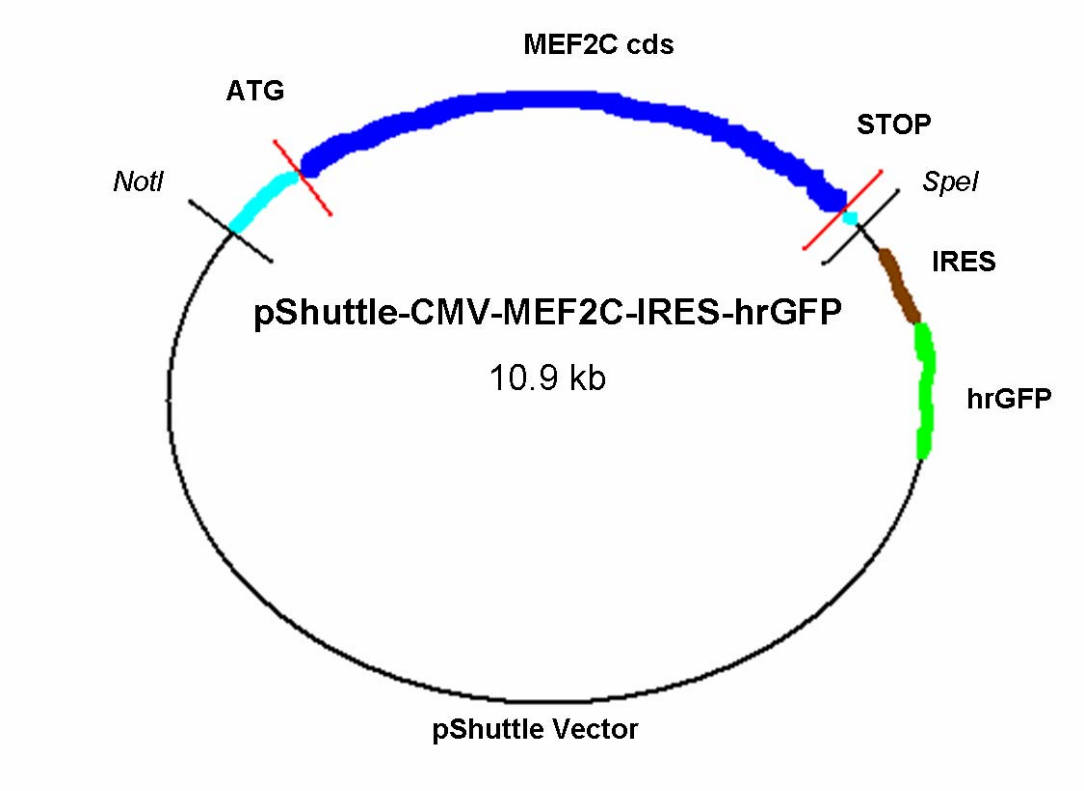


Feature	Nucleotide Position
pBR322 origin of replication	1854–2521
ampicillin resistance (*bla*) ORF	2669–3529
Ad5 right arm homology	3716–5721
Ad5 left arm homology	32483–33471

VII.1.3. pBluescriptR-IRATp970F1023D:



VII.1.4. pShuttle-CMV-MEF2C-IRES-hrGFP:



The MEF2C coding sequence (2kb) was inserted into the pShuttle-IRES-hrGFP MCS vector after *Not*I and *Spe*I digestion.

VII.2. Sequences:

VII.2.1. Amino Acid sequence of MEF2C:

```
     MGRKKIQITR IMDERNRQVT FTKRKFGLMK KAYELSVLCD CEIALIIFNS TNKLFQYAST   60
     DMDKVLLKYT EYNEQHESRT NSDIVETLRK KGLNGCDSPD PDADDSVGHS PESEDKYRKI  120
     NEDIDLMISR QRLCAVPPPN FEMPVSIPVS SHNSLVYSNP VSSLGNPNLL PLAHPSLQRN  180
     SMSPGVTHRP PSAGNTGGLM GGDLTSGAGT SAGNGYGNPR NSPGLLVSPG NLNKNMQAKS  240
     PPPMNLGMNN RKPDLRVLIP PGSKNTMPSV SEDVDLLLNQ RINNSQSAQS LATPVVSVAT  300
     PTLPGQGMGG YPSAISTTYG TEYSLSSADL SSLSGFNTAS ALHLGSVTGW QQQHLHNMQP  360
     SALSQLGACT STHLSQSSNL SLPSTQSLNI KSEPVSPPRD RTTTPSRYPQ HTRHEAGRSP  420
     VDSLSSCSSS YDGSDREDHR NEFHSPIGLT RPSPDERESP SVKRMRLSE              469
//
```

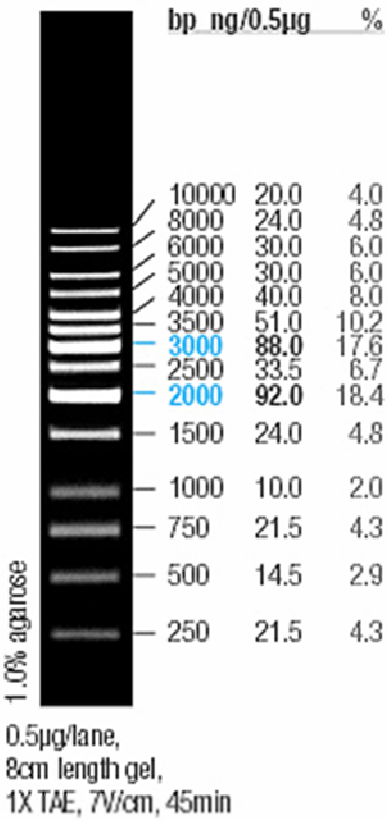
VII.2.2 Sequence of the MEF2C virus construct:

```
tggagctcca ccgcggtggc ggccgcataa cttcgtatag catacattat acgaagttat       60
ggatcaggcc aaatcggccg agctcgaatt cgtcgagagc gggctcccat cgcgcgcaca      120
cacgcacaca tcgtctccag ctctctgctc gctctgctcg cagtcacaga cacttgagca      180
cacgcgtaca cccagacatc ttcgggctgc tattggattg actttgaagg ttctgtgtgg      240
gtcgccgtgg ctgcatgttt gaatcaggtg gagaagcact tcaacgctgg acgaagtaaa      300
gattattgtt gttatttttt ttttctctct ctctctctct taagaaagga aaatatccca      360
aggactaatc tgatcgggtc ttccttcatc aggaacgaat gcaggaattt gggaactgag      420
ctgtgcaagt gctgaagaag gagatttgtt tggaggaaac aggaaagaga aagaaaagga      480
aggaaaaaat acataatttc agggacgaga gagagaagaa aaacggggac tatgggagga      540
aaaaagattc agattacgag gattatggat gaacgtaaca gacaggtgac atttacaaag      600
aggaaatttg ggttgatgaa gaaggcttat gagctgagcg tgctgtgtga ctgtgagatt      660
gcgctgatca tcttcaacag caccaacaag ctgttccagt atgccagcac cgacatggac      720
aaagtgcttc tcaagtacac ggagtacaac gagccgcatg agagccggac aaactcagac      780
atcgtggaga cgttgagaaa gaagggcctt aatggctgtg acagcccaga ccccgatgcg      840
gacgattccg taggtcacag ccctgagtct gaggacaagt acaggaaaat taacgaagat      900
attgatctaa tgatcagcag gcaaagattg tgtgctgttc cacctcccaa cttcgagatg      960
ccagtctcca tcccagtgtc cagccacaac agtttggtgt acagcaaccc tgtcagctca     1020
ctgggaaacc ccaacctatt gccactggct cacccttctc tgcagaggaa tagtatgtct     1080
cctggtgtaa cacatcgacc tccaagtgca ggtaacacag gtggtctgat gggtggagac     1140
ctcacgtctg gtgcaggcac cagtgcaggg aacgggtatg gcaatccccg aaactcacca     1200
ggtctgctgg tctcacctgg taacttgaac aagaatatgc aagcaaaatc tcctccccca     1260
atgaatttag gaatgaataa ccgtaaacca gatctccgag ttcttattcc accaggcagc     1320
aagaatacga tgccatcagt gtctgaggat gtcgacctgc ttttgaatca aaggataaat     1380
aactcccagt cggctcagtc attggctacc ccagtggttt ccgtagcaac tcctacttta     1440
ccaggacaag gaatgggagg atatccatca gccatttcaa caacatatgg taccgagtac     1500
tctctgagta gtgcagacct gtcatctctg tctgggttta acaccgccag cgctcttcac     1560
cttggttcag taactggctg gcaacagcaa cacctacata acatgccacc atctgccctc     1620
agtcagttgg gagcttgcac tagcactcat ttatctcaga gttcaaatct ctccctgcct     1680
tctactcaaa gcctcaacat caagtcagaa cctgtttctc ctcctagaga ccgtaccacc     1740
acccccttcga gatacccaca acacacgcgc cacgaggcgg ggagatctcc tgttgacagc     1800
ttgagcagct gtagcagttc gtacgacggg agcgaccgag aggatcaccg gaacgaattc     1860
cactccccca ttggactcac cagaccttcg ccggacgaaa gggaaagtcc ctcagtcaag     1920
cgcatgcgac tttctgaagg atgggcaaca tgatcagatt attacttact agttttttt      1980
```

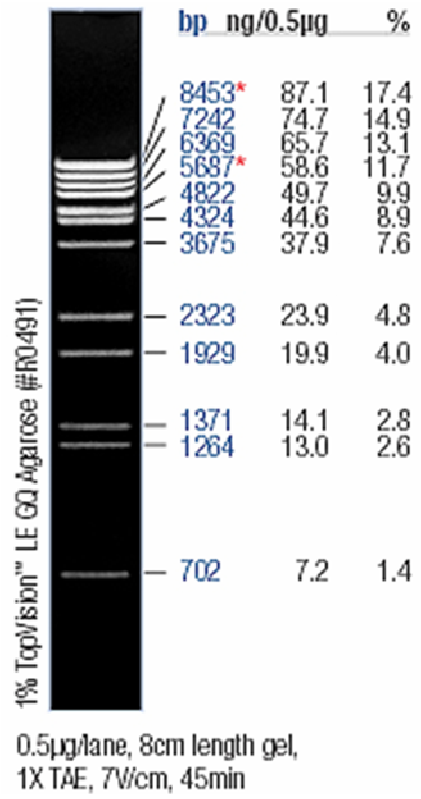
The restriction sites *Not*I (gc^ggcc^gc) and *Spe*I (a^ctag^t) are depicted in green. Start- and Stop codon, atg and tga respectively, of the coding sequence are coloured in red. The sequence segment derived from the multiple cloning site of the MEF2C expressing pBluescriptR vector is marked in italic letters.

VII.3. Markers:

VII.3.1. DNA Markers: 1kb DNA ladder and Lambda DNA/Eco91I (BstEII) digest

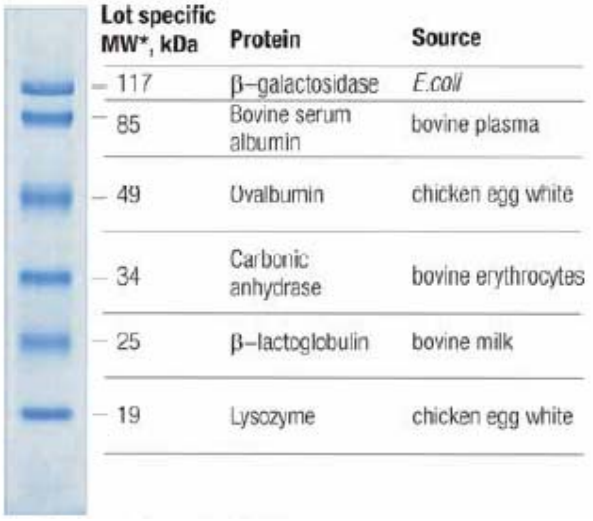


0.5µg/lane,
8cm length gel,
1X TAE, 7V/cm, 45min



0.5µg/lane, 8cm length gel,
1X TAE, 7V/cm, 45min

VII.3.2 Protein Marker:



Lot specific MW*, kDa	Protein	Source
117	β-galactosidase	*E.coli*
85	Bovine serum albumin	bovine plasma
49	Ovalbumin	chicken egg white
34	Carbonic anhydrase	bovine erythrocytes
25	β-lactoglobulin	bovine milk
19	Lysozyme	chicken egg white

VII.4. Abbreviations:

Ang1	Angiopoetin 1
bFGF	basic fibroblast growth factor
bp	base pair
cDNA	complementary DNA
CsCl	cesium chloride
DNA	Desoxyribonucleic acid
DSCR1	Down Syndrom critical region 1
EC	endothelial cells
ECM	extracellular matrix
E. coli	Escherichia coli
EGF	epidermal growth factor
FCS	Fetal calf serum
GFP	green fluorescent protein
HUVEC	human umbilical vein endothelial cells
IL-1	interleukin 1
IRES	internal ribosomal entry site
ITR	inverted terminal repeat
LPS	lipopolysaccharides
MAPK	mitogen activated protein kinases
MCS	multiple cloning site
min	minutes
MMP10	metallo matrix protease 10
mRNA	messenger ribonucleic acid
NES	nuclear export signal
NR4A2	orphan nuclear receptor subgroup 4
PBS	phosphate buffered saline
PDGF-BB	platelet derived growth factor
pfu	plaque forming unit
real-time RT-PCR	real-time reverse transcription-polymerase chain reaction
rpm	revolutions per minute
ROS	reactive oxygen species
RT	room temperature
SDS	Sodium Dodecylsulphat
sec	seconds
SMC	smooth muscles cells
TGFß-1	tumour growth factor beta 1
VEGF	vascular endothelial growth factor

VIII. Table of Figures:

„ Curriculum vitae “

Name: Caterina Sturtzel

Geburtsdatum: 14.08.1983

Geburtsort: Wien

Familienstand: ledig

Staatsbürgerschaft: Österreich

Ausbildung:

Sept. 1989- Juni 1993 Besuch der Volksschule Knollgasse 6, 1170 Wien

Sept. 1993- Mai 2001 Besuch des Albertus Magnus Gymnasiums, 1180 Wien
ab der 9. Schulstufe den Zweig „Realgymnasium mit Naturwissenschaftlichem Schwerpunkt“ belegt
Abschluss der AHS durch die Matura, mit Auszeichnung bestanden

Okt. 2001- Mai 2006 Studium der Molekularbiogie an der Fakultät für Lebenswissenschaften der Universität Wien

Juli 2006- Nov.2007 Diplomarbeit am Institut für Gefäßbiologie und Thromboseforschung, Medizinische Universität Wien
Gruppe: Prof. Erhard Hofer

Summary:

VEGF (Vascular endothelial growth factor) is the major regulator of the formation of new vessels by sprouting from pre-existing ones, a process termed angiogenesis. MEF2C (Myocyte Enhancing Factor 2) is a transcription factor, which was shown by microarray analysis to be specifically upregulated by VEGF in endothelial cells. This work generated the necessary experimental tools and investigated the regulation and potential role of MEF2C in endothelial cells during angiogenesis.

In a first step it was confirmed by real time RT-PCR that MEF2C is selectively upregulated 3- to 5-fold by VEGF, but not by the general growth factor EGF or the inflammatory cytokine IL-1. Then a recombinant adenovirus was engineered to achieve overexpression of MEF2C in human umbilical vein endothelial cells (HUVEC). Using this adenovirus the influence of MEF2C on VEGF-mediated upregulation of several genes was analysed by real time RT-PCR. It was found that MEF2C not only suppressed the expression of the endogenous MEF2C, but also inhibited the upregulation of the strongly VEGF-inducible DSCR1 and NR4A2 genes. In accordance with the inhibitory action on major VEGF-inducible gene, MEF2C inhibited sprouting in the *in vitro* spheroid sprout formation assay. Tube formation in the *in vitro* Matrigel assay however seemed unaffected.

These data suggest that MEF2C may function to downmodulate gene expression and sprout formation after VEGF treatment in a negative feed-back loop.

Zusammenfassung:

VEGF (Vascular endothelial growth factor) ist der Hauptregulator für die Ausbildung neuer Gefäße aus bereits bestehenden durch Sprossung. Dieser Prozess wird Angiogenese genannt. MEF2C (Myocyte Enhancing Factor 2C) ist ein Transkriptionsfaktor, von dem mittels Microarray Analyse gezeigt wurde, dass er in Endothelzellen speziell durch VEGF hoch reguliert wird. Im Zuge dieser Arbeit wurden die notwendigen Konstrukte hergestellt und die Regulierung und mögliche Rolle von MEF2C in Endothelzellen während der Angiogenese untersucht.

Zuerst wurde mittels real time RT-PCR bestätigt, dass MEF2C speziell von VEGF 3- bis 5-fach hochreguliert wird, aber nicht vom allgemeinen Wachstumsfaktor EGF oder dem Entzündungsmediator IL-1. Danach wurde ein rekombinanter Adenovirus produziert, um eine Überexpression von MEF2C in HUVEC (menschliche Nabelschnur Endothelzellen) zu erreichen. Unter Verwendung dieses Adenovirus wurde der Einfluss von MEF2C auf die VEGF-vermittelte Hochregulierung einer Auswahl an Genen mittels real time RT-PCR analysiert.Die Ergebnisse zeigen, dass MEF2C nicht nur die Expression des endogenen MEF2C unterdrückt, sondern auch die Hochregulierung der stark VEGF-induzierbaren Gene DSCR1 und NR4A2 inhibiert. In Übereinstimmung mit der inhibitiven Wirkung auf wichtige VEGF-induzierbare Gene, hemmte MEF2C auch die Aussprossung im *in vitro* „spheroid sprout formation assay“. Die Netzwerkbildung im *in vitro* „Matrigel assay“ blieb durch MEF2C aber unverändert .

Diese Daten indizieren, dass MEF2C im Rahmen einer negativen Rückkopplung die Genexpression und die Ausbildung von Aussprossungen nach VEGF Behandlung reprimiert.