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The Function and Role of the Transcription Factor MEF2C during Angiogenesis

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Mag.rer.nat Caterina Sturtzel

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1.a. Zusammenfassung:

Der Wachstumsfaktor VEGF (vascular endothelial growth factor) ist der primäre Auslöser von Angiogenese, der Neubildung von Blutgefäßen. Im Zuge der Untersuchung der molekularen Mechanismen dieses Vorgangs konnten wir Transkriptionsfaktoren identifizieren, die in Endothelzellen spezifisch von VEGF reguliert werden. Unter den am stärksten induzierten Transkriptionsfaktoren waren MEF2C und HLX. Überraschenderweise zeigen beiden Faktoren inhibierende Wirkung auf die Angiogenese. Zum Beispiel bewirkt eine Überexpression des MADS box Transkriptionsfaktor MEF2C eine Hemmung der angiogenen Sprossung. Analysen von Genmustern in Endothelzellen, die MEF2C überexprimieren, zeigten eine Hochregulierung des alpha-2-Makroglobulin (A2M) Gens. A2M ist bekannt als Serumprotein und kann als Trägerprotein für Wachstumsfaktoren und als universeller Serinproteasenhemmer fungieren. Weiterführende Experimente belegten, dass A2M den antiangiogenen Effekt von MEF2C verursacht. Erst konnte nachgewiesen werden, dass sowohl der Überstand von MEF2C überexprimierenden Zellen als auch aufgereinigtes A2M Protein angiogene Sprossung verringern kann. Weiters kann durch shRNA-vermittelte Reduktion von A2M die antiangiogene Wirkung von MEF2C vermindert werden. In Analogie konnte gezeigt werden, dass der Transkriptionsfaktor HLX die Sprossung durch Regulation des abstoßenden Lenkungsfaktor UNC5B unterdrückt. Um zu untersuchen, warum VEGF-abhängige Transkriptionsfaktoren antiangiogene Proteine induzieren und angiogene Sprossung unterdrücken, wurde der Einfluss von Hypoxie auf MEF2C untersucht. Ähnlich wie bei HLX und UNC5B waren in Sauerstoff-armer Umgebung MEF2C und A2M nicht mehr durch VEGF zu stimulieren. Zusammengefasst vermitteln diese Ergebnisse das Bild, dass Hochregulierung von MEF2C/A2M (wie auch HLX/UNC5B) am stärksten unter Normoxie stattfindet und umgekehrt kaum bei Hypoxie. Diese Mechanismen fungieren also als Bremse für die Angiogenese, die unter Hypoxie gelöst wird oder verstärkt angezogen wird, wenn nach Bildung neuer Gefäße die Sauerstoffkonzentration wieder steigt. Dies dürfte die Sprossungsaktivität an die O₂-Konzentration anpassen.

1.b. Abstract:

Vascular endothelial growth factor (VEGF) is the main trigger of angiogenesis leading to the formation of new blood vessels by sprouting. To gain further insight into the molecular mechanisms of the signalling process induced by VEGF we identified transcription factors specifically upregulated by VEGF in endothelial cells. Among the most strongly induced transcription factors were the MADS box factor MEF2C and the homeobox factor HLX. Surprisingly, in gain-of-function assays both factors exerted inhibitory effects on angiogenesis. Gene profiling of endothelial cells overexpressing MEF2C revealed the alpha-2-macroglobulin (A2M) gene to be a prominent target of this transcription factor. A2M is a large serum protein that functions as a carrier protein for growth factors and as a global protease inhibitor. The further obtained data support that A2M mediates the anti-angiogenic effect of MEF2C. First, conditioned medium from MEF2C overexpressing cells as well as purified A2M protein displayed inhibitory potential for sprout formation. Second, silencing of A2M by means of shRNA diminished the anti-angiogenic effect of MEF2C. In analogy it was demonstrated that HLX upregulates the negative guidance cue UNC5B which can mediate inhibition of sprouting. To dissect why VEGF-dependent transcription factors would induce anti-angiogenic proteins and down-modulate angiogenic sprouting, the influence of hypoxia was investigated. Indeed, under hypoxic conditions VEGF-induction of MEF2C and its target gene A2M is strongly reduced, similar to HLX and UNC5B. Taken together these data suggest that upregulation of MEF2C/A2M (and HLX/UNC5B) by VEGF is most strongly pronounced during normoxia and is inversely correlated with hypoxia. This supports that these mechanisms function as angiogenic breaks which are released when hypoxia increases or are put on when oxygen concentration rises again after new vessel formation to adapt angiogenic sprouting activity to oxygen concentration.

2. Introduction:

2.1. Endothelial Cells:

2.1.1. Endothelial Cells:

Cobblestone-shape is a main histological characteristic of endothelial cells (EC), but they constitute more than static mechano-protective plates. When EC line the inner cell wall of a vessel, they are not at all inert bystander cells (Risau, 1995). EC are central and active parts of two major systems in the body, the immune and the vascular system. Often these systems react in an integrative way, like in wound healing, but there are also conditions where EC engage in distinctive roles in each of them.

As part of the immune system EC not only build a mechanical barrier against intruders but also essentially secrete cytokines and regulate leukocyte extravasation (Vestweber, 2012). The vascular system is basically composed of EC lining the vessels, and of smooth muscle cells or pericytes supporting the vessel structure (Armulik et al., 2011). Strongly adapted to the various tissues the lymphatic and the blood vessel system pervade the whole body. Lymphatic vessels are blind-ended tubes equipped with valves to fulfill efficiently their major task. They collect and drain interstitial fluid leaking out of the blood vessels, and are thereby also a transport route for nutrients or mobile cells (Alitalo, 2011). Blood vessels again exist as capillaries, veins or arteries, covered by supporting cells and have their major tasks in the transport of oxygen, nutrients and immune cells to the different tissues of the body (Potente et al., 2011). Therefore, genesis of the lymphatic system in the developing embryo is triggered by rising fluid pressure, whereas blood vessel vasculogenesis and angiogenesis is driven by hypoxia (Planas-Paz et al., 2012; Semenza, 2003). In embryonic development the first vascular plexus is established by coalescing haemangioblasts and then in the mature body vessels are built up by sprouting of EC, the process defined as angiogenesis (Potente et al., 2011).

ECs isolated from different tissues will exhibit organ specific adaptations and shape adjustments. For example, EC of the central nervous system build up the

blood-brain barrier, uterine EC express estrogen receptors, EC of the high endothelial venules seem to allow for transcellular routes of leukocyte extravasation or EC committed for either arterial or venous vessels show different sprouting capacities (Adams and Alitalo, 2007; Domigan and Iruela-Arispe, 2012).

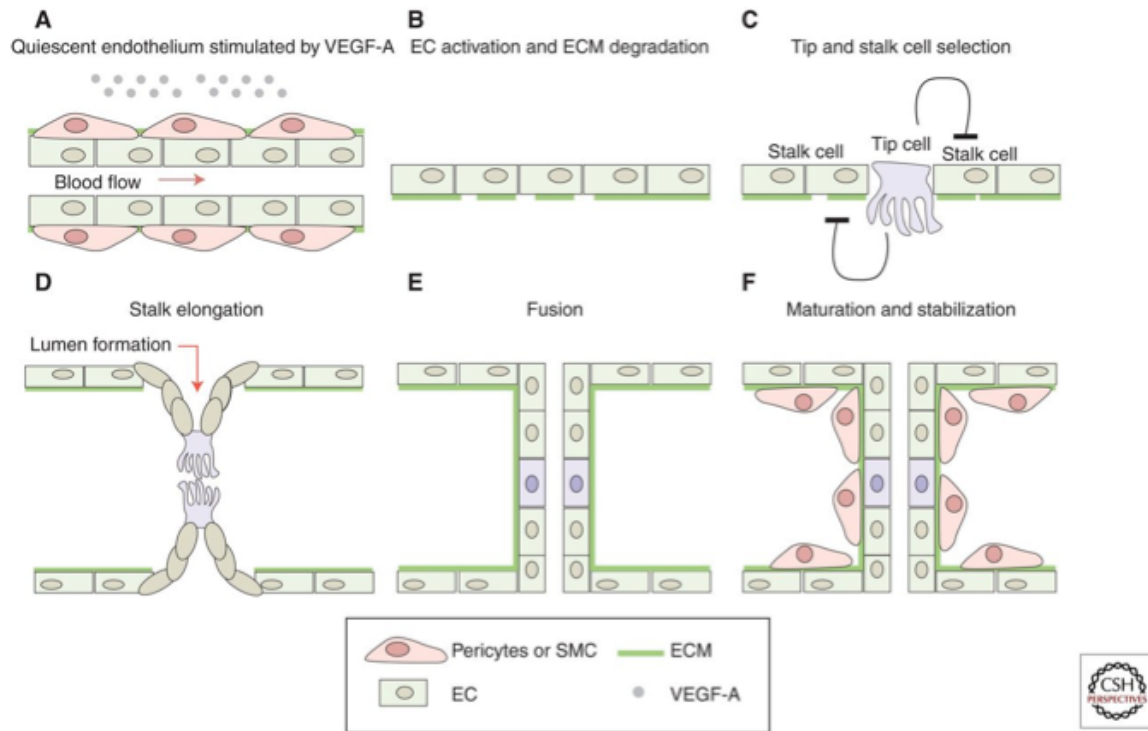


Figure 1: Schematic model of sprout initiation, vessel branching, and maturation.

Angiogenesis is activated in response to local tissue hypoxia. The hypoxic tissue releases endothelial growth factor-A (VEGF-A), which (re)activates the quiescent endothelial cells (ECs). (A) At the cellular level, the angiogenic initiation requires the degradation of the extra cellular matrix (ECM) (B) as well as the specification of the (re)activated ECs into tip and stalk cells (C). ECs proliferate and collectively invade the hypoxic tissue while they remain connected to the original vascular network. (D) In the nascent sprout, the tip cells, characterized by their migratory behaviour and dynamic filopodia, lead the sprout toward the VEGF-A source, whereas the stalk cells proliferate to support sprout elongation. The tip cells connect the new sprouts into a functional vessel loop. (E) The new connection between different sprouts occurs through tip cell fusion (anastomosis). Formation of the vascular lumen initiates blood flow, increases tissue oxygenation, and, in turn, reduces the release of endothelial growth factors, supporting the establishment of quiescence. (F) Vessel maturation and stabilization proceed with the recruitment of mural cells (pericytes) and the deposition of ECM (Blanco and Gerhardt, 2013)

2.1.2. EC in angiogenesis - tip and stalk cells

During angiogenesis EC become activated and single lines of cells start to migrate out of a pre-existing vessel towards a gradient of Vascular Endothelial Growth Factor (VEGF) (Figure 1A), a growth factor produced under hypoxic conditions (Gerhardt, 2008). When a tissue is not supplied with enough oxygen newly formed vessels are demanded to compensate for this. The broadly accepted concept of tip/stalk cells in the growing sprout implies that the leading outgrowing cell, termed tip cell, is highly migratory and protrudes many filopodia, on which VEGF receptors are expressed to sense the gradient. Furthermore, tip cells display upregulation of the marker molecules PDGFB, Unc5B and Dll4. Behind the leading tip cells so-called stalk cells follow. These are highly proliferative and will eventually form a new, lumenized vessel. Dll4, a notch receptor ligand, expressed on the tip cell induces Notch signaling in the neighboring cell, upon which KDR (=VEGF receptor 2) surface expression is downregulated implementing the stalk cell phenotype (Hellstrom et al., 2007; Phng and Gerhardt, 2009) (Figure 1B-C). Several factors might select for a cell to become a tip cell, like random relative overexpression of KDR, metabolic advantages of some cells in terms of elevated glycolysis conferring higher motility capacity or cell arrangements orientating cells into certain directions facilitating migration (Fraisl et al., 2009; Jakobsson et al., 2010). Tip/ stalk cell selection is also a dynamic process, for example when a tip cell encounters a negative guidance molecule and retracts another cell may take over the leading cell position (Adams and Eichmann, 2010). When a sprout encounters another they will anastomose and blood flow can run via a newly formed vessel (Blanco and Gerhardt, 2013; Wacker and Gerhardt, 2011) (Figure 1D-F).

2.1.3. EC Pathology:

Several pathologies are associated with malfunctions of EC. Cardio-vascular diseases and cancer account for two third of deaths in the world with an overt contribution of frail EC, but also for example age-related macular degeneration (AMD), diabetes or dermatitis represent disorders of EC (Carmeliet, 2005). Subsequent to heart infarcts dysfunctional and inflamed ischemic scar tissue spreads, which can prevent recovery (Qayyum et al., 2012). Also in tumor situations balance between pro- and anti-angiogenic stimuli is lost leading to the “angiogenic switch” whereupon the tumor is able to recruit new blood vessels in an unlimited way for nutritional support to its own benefit (Bergers and Hanahan, 2008). Therefore, huge efforts to further understand function and malfunction of EC are made.

In the last decades big hopes were raised that it would be possible to fight tumors by abrogating vessel growth after administration of VEGF blocking antibodies, thereby cutting the tumor off from nutrient and oxygen supply. Nevertheless, rapidly acquired resistances of tumors against such treatment and unexpected side effects showed more detailed knowledge about (tumor) angiogenesis is needed for the design of effective therapeutics (Bergers and Hanahan, 2008).

2.1.4. VEGF and VEGF receptors:

Based on the work of N. Ferrara, P. Carmeliet, K. Alitalo, E. Keshet, L. Claesshon-Welsh and their collaborators, just to name a few, the generally accepted basic knowledge about the vascular endothelial growth factor, its family and receptors can be summarized as follows: originally this factor was described as vascular permeabilization factor referring to one of its obvious physiological effects (Dvorak et al., 1995). Meanwhile, VEGF usually stands for VEGF-A, the classical pro-angiogenic factor of the structurally related family consisting of VEGF-A, VEGF-B, VEGF-C, VEGF-D and Placental Growth Factor PlGF. Different splice forms of VEGF-A result in differently sized proteins, built up by either 121, 145, 165, 189 or 206 amino acids in humans, mainly differing in

capability of binding the extracellular matrix compound heparan sulfate (del Toro et al.), ranging from a soluble VEGF-A121 to a cell surface retained VEGF-A206 (Ferrara, 2010). The binding of VEGF to its receptors and coreceptors, the neuropilins (NRP), is thereby regulated, since for example VEGF-A145 is already captured by HS but hardly interacts with NRP (Goel and Mercurio, 2012).

VEGF-A prototypically sustains EC survival and promotes blood vessels angiogenesis, VEGF-B is typically expressed in energy demanding tissue like the heart where it regulates lipid-uptake of EC, VEGF-C and -D are the principal growth factors involved in lymphangiogenesis but after proteolytic processing VEGF-C might also play a role in tip-stalk cell determination (Bry et al., 2010; Tammela et al., 2011). PIGF seems dispensable during mouse embryonic development but plays an active role during pathologic tumor angiogenesis (Carmeliet et al., 2001).

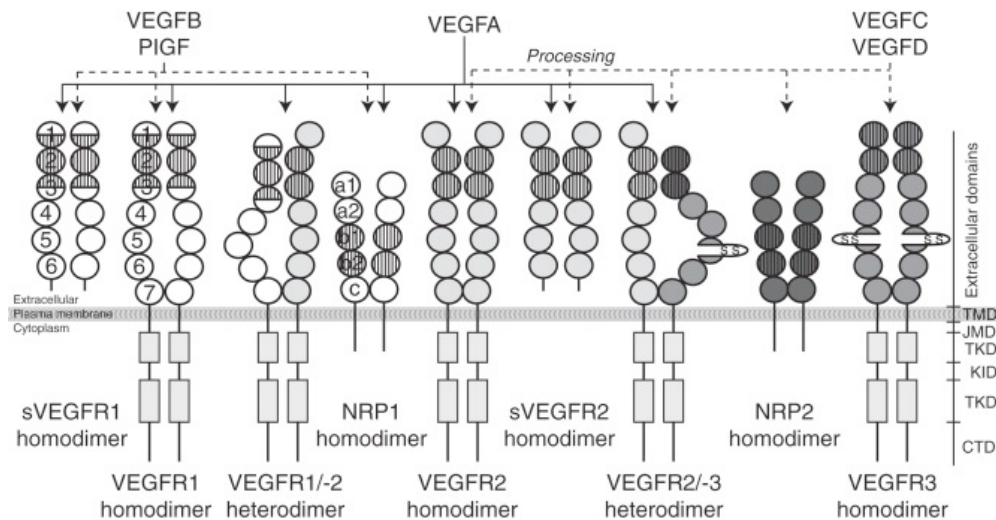


Figure 2. VEGF binding specificities and VEGFR signaling complexes.

Five vascular endothelial growth factors, VEGFA, VEGFB, VEGFC, VEGFD, and PIGF, bind with different affinities to three VEGF receptor tyrosine kinases (VEGFR) and two NRP coreceptors initiating homo- and heterodimer formation. Proteolytic processing of VEGFC and VEGFD allows binding to VEGFR2. Extracellular domains of VEGFRs and NRPs involved in VEGF binding are indicated by hatched circles. PIGF, placenta growth factor; sVEGFR1, soluble VEGFR1; sVEGFR2, soluble VEGFR2; TMD, transmembrane domain; JMD, juxtamembrane domain; TKD1, ATP binding domain; KID, kinase insert domain; TKD2, phosphotransferase domain; CTD, carboxy-terminal domain (Koch et al., 2011) (requested permission for reprint from CSH perspectives)

VEGF proteins are the ligands for the receptor tyrosine kinase family of VEGF receptors. There are three receptors, VEGFR1, 2 and 3, also known as Flt1, Flk1 and Flt4, respectively. The knock-out of flt1, flk1 or flt4 in mice is embryonically lethal at day E9.0, E8.5 and E.10, respectively, indicating the vitally important functions of these genes. VEGFR1 binds VEGF-B, PlGF and VEGF-A, VEGFR2 primarily VEGF-A. However, VEGFR1 binds VEGF-A with higher affinity than VEGFR2, but VEGFR1 is hardly mitogenic and the intrinsic signaling kinase activity is very low (Figure 2). VEGFR1 and also its soluble splice form sFlt1 are therefore regarded as decoy receptors, titrating out abundant VEGF at times of massive angiogenesis, for example during pregnancy, or to maintain avascularity of corneas. PlGF might block the binding of VEGF-A to VEGFR1 thereby promoting VEGF dependent tumor growth (Koch et al., 2011) (Figure 2).

VEGF-A and presumably also cleaved VEGF-C associate with the VEGFR2, which possesses high intrinsic kinase activity. Ligand binding induces receptor dimerization, change of conformation and intrinsic tyrosine kinase activity, upon which defined VEGFR2 tyrosine residues as well as tyrosines of downstream signaling partners become phosphorylated. Consecutive internalization of the receptors is an essential part of signaling transmission. Thereby activated intracellular signaling cascades lead to induction of survival, proliferation and/ or migration pathways. The process of angiogenesis including the outgrowth of tip cells and proliferation of stalk cells is initiated resulting in the build-up of new vessels in three-dimensional structures. Proliferation of ECs is stimulated by VEGF primarily via PLCgamma and PKC leading to RAF/ERK/MAPK pathway signaling. Migration of ECs involves the T-cell specific adaptor molecule TAd, which is necessary for actin-reorganization during cell movement. The pro-survival effect of VEGFA-VEGFR2 signaling is mediated through PI3K/PIP3/PKB/AKT dependent phosphorylation and blocking of pro-apoptotic caspases. Vascular permeability occurs through creation of transcellular endothelial pores and transient opening of paracellular endothelial junctions involving nitric oxide as signaling molecule to regulate VE-cadherin functionality (Koch et al., 2011).

VEGFR3 binds VEGF-C and VEGF-D and was previously believed to be restricted to lymphatic vessels. VEGFR3 and its ligands are indeed the key factors for lymphangiogenesis but are also found at sites of active blood vessel angiogenesis as well as on different other sites of developmental processes (Benedito et al., 2012). VEGFR3 might also form heterodimers with VEGFR2 modulating their signaling output. During lymphangiogenesis pathways similar to blood vessel angiogenesis are stimulated to induce proliferation, migration and survival of the endothelial cells (Adams and Alitalo, 2007).

Two neuropilin homologs, NRP1 and NRP2, are coreceptors for VEGF receptors. NRP1 rather contacts VEGFR1 or VEGFR2, NRP2 contacts VEGFR3. Principally, NRP are receptors for class 3 semaphorins, which are guidance molecules leading the way for a growth cone. Neuropilins exert stabilizing function on the VEGF-VEGFR signaling complexes integrating guidance signaling to an endothelial cell. VEGF might also bridge NRP and VEGFR molecules on neighboring cells for example in tumors (Goel and Mercurio, 2012; Koch et al., 2011)

Seeking for identification of further downstream constituents of VEGF-A signaling gene profiling experiments were undertaken also from our group. Defining transcription factors specifically induced by VEGF, MEF2C (myocyte enhancing factor 2 c) was found to be strongly and selectively inducible by VEGF (Schweighofer et al., 2009)

2.2. MEF2C

2.2.1. MEF2C: a transcription factor

As I have already reviewed in my diploma thesis once, the transcription factor MEF2C belongs to the MADS box containing protein family (Gossett et al., 1989; Sturtzel, 2007). The MADS box domain confers dimerization and DNA binding capabilities. Denotation is derived from the four initially discovered transcription factors possessing this DNA binding domain: MCM1, Agamous, Deficiens and serum response factor. MADS box transcription factors prototypically play a role during developmental processes. The animal and fungal MADS box transcription factors can be categorized into the SRF-like and the MEF-like MADS box proteins (Messenguy and Dubois, 2003). The isoforms MEF2A, B, C and D constitute the human MEF2 family. Next to their very N- terminal MADS box they all share a highly homologous region of 27 amino acids, the MEF2 motif. This motif is important for DNA binding, mediates dimerization and regulates transcriptional activation. Two essential TADs (Trans Activation Domain) are located at the C- terminus of MEF2 proteins (Molkentin et al., 1996). The consensus sequence recognized by MEF2 proteins was identified as C/TTA(A/T)₄TAG/A (Gossett et al., 1989).

The first null MEF2C mutant mice were designed to targetedly delete the MADS- and MEF-domains, which is Exon 2. No homozygous neonates were obtained, although the heterozygotes did not show an apparent phenotype. The embryos died at day E9.5 to 10.5 for which basically the obviously severe heart phenotype resulting from aberrant cardiac morphogenesis was held responsible (Lin et al., 1997). Additionally, intricate vascular defects were observed. Organization of endothelial cells into an ordered pattern of a vascular plexus failed as well as recruitment and differentiation of smooth muscle cells, a typical step during late angiogenesis. In all these pathological appearances similarities to VEGF-A mutant mice as well as VEGF receptor-2 defective models can be detected, implicating a likely association between VEGF and MEF2C signaling 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 and its vessels' rupture, furthermore 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, gene expression patterns were altered particularly in cardiac cells, showing a reduced expression of Ang1 and VEGF, again connecting MEF2C signaling with VEGF in a vascular development background (Bi et al., 1999). The group of Jeffrey Molkentin tried to obtain MEF2 overexpressing transgenic mice, but was only able to generate MEF2A transgenics (Xu et al., 2006).

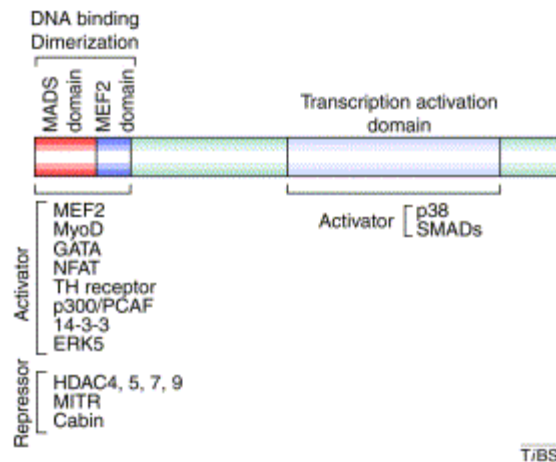


Figure 3. MEF2 structure and protein interaction domains.

All MEF2 proteins possess similar structure, with a MADS domain (present in MCM1, Agamous, Deficiens and serum response factor) at their N-termini that mediates homo- and heterodimerization, and binding to the DNA sequence CTA(A/T)₄TAG/A. Adjacent to the MADS domain is a MEF2-specific domain that influences DNA-binding affinity and cofactor interactions. MEF2 proteins contain C-terminal transcriptional activation domains. Numerous MEF2-interacting proteins have been identified. Some of the cofactors stimulate MEF2 activity (activator) whereas others repress MEF2 function (repressor). (McKinsey et al., 2002) (with permission for reprint from Elsevier)

A major characteristic of MADS box proteins in general including MEF2C is their cooperative dependence on many different co-repressors and -activators or other transcription factors such as class II HDACs and p300, MyoD, NFAT or ERK5 (Messenguy and Dubois, 2003). This broad range of potential regulation partners could be responsible for the contradictory findings implicating MEF2C in different regulation of cell division, differentiation and death only depending on the context or type of the cell (McKinsey et al., 2002).

2.2.2. Regulation of MEF2C:

By default, at least in mature cardiomyocytes, MEF2C is constitutively located in the nucleus and packed with histone deacetylases. All class II HDACs (HDAC4, 5, 7 and 9) were shown to associate via their N-terminus with MEF2 bound to DNA (Chang et al., 2006; Wu et al., 2001). These HDACs repress transcriptional activity of MEF2C in a way that deacetylation of histones increases bound DNA density and thereby reduces accessibility of the promoter region for the transcription machinery (Jenuwein and Allis, 2001). Phosphorylation of HDACs by for example CaMK disrupts the repressive HDAC-MEF2C interaction, as it creates a docking side for the chaperone 14-3-3 (McKinsey et al., 2002) and the conformational change of the HDAC demasks a NES (nuclear export signal) leading to export of the HDACs from the nucleus (Haberland et al., 2007). Additionally, MEF2C becomes then accessible for the binding of the transcription enhancing factor HAT p300. Of notice, p300 not only acetylates histones but MEF proteins as well, thereby additionally elevating their transcriptional activity (Ma et al., 2005).

Repressive regulation of MEF2 by HDAC4 was shown for several aberrant cell maturation processes including heart hypertrophy, which is dependent on Ca²⁺ signaling, or bone chondrocyte hypertrophy (Arnold et al., 2007; Youn et al., 2000b). In endothelial cells HDAC5/MEF2 interaction was found to be regulated by shear stress, which induces release of repression thereby establishing arthereoprotective KLF2 and eNOS expression (Wang et al., 2010). Furthermore, HDAC7 knockout mice display a disturbed vascular integrity due to the loss of MEF2C repression. Metalloproteinase 10 is a direct target of MEF2C and consequently becomes upregulated, leading to an increased degradation of the ECM (extracellular matrix), thereby disrupting the vascular system (Chang et al., 2006). As the promoter of HDAC9 contains a MEF2 binding site its own transcription is inhibited in a negative feedback loop. This is involved in the regulation of gene expression during muscle differentiation altering expression levels in a time course dependent manner (Haberland et al., 2007).

The MAPKs (Mitogen- Activated Protein Kinases) are a family of serine/threonine kinases that play an essential role in cell signal transduction by modulating gene transcription in response to changes in the cellular environment. They encompass the extracellular signal-regulated protein kinases ERK1 and ERK2, the c-Jun N- terminal kinases JNK1-3, p38 and ERK5. All MAPKs were demonstrated to interact with MEF2C in the context of different cell types and activation states (Han et al., 1997; Turjanski et al., 2007; Youn et al., 2000a). Particularly, ERK5 was shown to mediate responses to external stimuli by regulating 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 (Kato et al., 2000). Therefore it is also termed big map kinase 1 (BMK1). Of interest, the BMK1/ERK5 knock-out-mice phenotype is similar to the MEF2C knock-out phenotype. It is embryonic death at day E9.5- 10.5, revealing severe defects during cardio- and vasculogenesis (Hayashi and Lee, 2004). Ablation in an inducible BMK1 knock-out-mouse is lethal within two to four weeks and displays all signs of perturbed vascular integrity (abnormally leaky blood vessels, haemorrhages in multiple organs, lining vessels became round, irregularly aligned and apoptotic). Additionally, conditional knock-out of BMK1 in endothelial cells shows a nearly identical phenotype to the global knock-out. *In vitro* a constitutively active form of MEF2C partially rescued the BMK1 knock-out phenotype (Hayashi et al., 2004). This supports the assumption that ERK5/ BMK1 induced by serum or VEGF mediates pro-survival cell signalling in endothelial cells via MEF2C (Olson, 2004).

2.2.3. MEF2C cell expression pattern

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 involving MEF2C in cardiomyocytes. In reprogramming of induced pluripotent cells MEF2C is transduced to initiate functional cardiomyocytes differentiation (Ieda et al., 2010). Furthermore, MEF2C is also highly expressed in the human brain during fetal development (Leifer et al., 1994). Evidence is mounting that there are parallels between the regulation of muscle and neuron development in regard of MEF2C, however the mechanisms differ in the specific targets activated by MEF2C (Shalizi and Bonni, 2005). In the recent years MEF2 was found to be involved in neuronal survival and to regulate the neuronal plasticity and synapse formation for example during learning. Of interest, some patients suffering from the Rett syndrome bear a MEF2C mutation (Dietrich, 2013).

In the cells of the immune system considerable MEF2C expression was found in B-cells but also in monocytes, what was shown in an inducible knock out model (Swanson et al., 1998). MEF2C exerts again developmental functions in these cells. Monocytes become further activated by a pro-inflammatory stimulus through MEF2C and the factor dictates cell fate in myeloid differentiation. MEF2C was also defined as a strong oncogene for leukemias (Cante-Barrett et al., 2013; Han et al., 1997; Schuler et al., 2008).

In chondrocytes MEF2C, regulated by HDAC4, controls the programme for hypertrophy and accurate bone development (Arnold et al., 2007). Although expression is low, MEF2C was also shown to be essential for the proper development of neural-crest derived melanocytes (Agarwal et al., 2011). And last but not least, as already stated, MEF2C is expressed in endothelial cells (Bi et al., 1999). Generally, MEF2C seems to possess an important function as a regulating switch for development, however this function is not specific for a certain cell type. It appears that the onset of differentiation for a number of specific cell types, for example into mature cardiomyocytes, is not possible without functional MEF2C (Potthoff and Olson, 2007).

3. Aims:

The principal aim of this study was to delineate the role of the transcription factor MEF2C for endothelial cells, especially for VEGF-induced angiogenic sprouting, that displays features of a differentiation process. An important role of MEF2C in this process was indicated by its specific upregulation by VEGF and the vascular phenotype of MEF2C null mice.

The specific aims of this work were:

- 1- To confirm a specific upregulation of MEF2C by VEGF-A via VEGFR-2
- 2- To perform gain- and loss-of-function studies to evaluate the effects of MEF2C on proliferation, migration and angiogenic sprouting of endothelial cells
- 3- To delineate from gene profiling studies of endothelial cells overexpressing MEF2C a major gene/protein responsible for the detected inhibitory activity of MEF2C on migration and sprouting
- 4- To demonstrate the functional role of this gene/protein for the inhibitory activity defined for MEF2C
- 5- To provide and evaluate an hypothesis for the unexpected role of inhibitory transcription factors, such as MEF2C and HLX, as a regulatory mechanism adapting sprouting activity to the oxygen/hypoxia gradient

4. Material & Methods:

4.1. Cell culture and materials:

Primary Human Umbilical Vein Endothelial cells (HUVECs) were isolated as already described elsewhere (Wojta et al., 1989). HUVECs were cultured on 1% gelatine-coated plates in either EGM-2 medium (Lonza, Switzerland) or in M199 Medium (PAA, Austria) supplemented with 20% FCS, 1% antibiotic mix (penicillin, streptomycin, fungizone, PAA), 0.4% ECGS-H (endothelial cells growth supplement with heparin) (Promocell, Germany) and used for experiments from passage 2 to 5.

HEK293 cells (CRL-1573; ATCC) were grown in minimal essential medium alpha (MEMa) completed with 10% Newborn Calf Serum (NCS) and 1% antibiotic mix (all from PAA). 293T cells (CRL-11268; ATCC) were maintained in Dulbecco's modified Eagle medium (DMEM) (Lonza, Switzerland) with 10% FCS (Invitrogen). Cells were cultivated in an incubator at 37°C, in 5% CO₂ and in 21% O₂ during "normoxia" conditions or in 1-5% O₂ during "hypoxia" conditions.

Alpha 2-macroglobulin (Sigma-Aldrich), VEGF-A (vascular endothelial growth factor) and bFGF (basic fibroblast growth factor) (both Immunotools, Germany) were applied in indicated concentrations.

4.2. Viral transduction

4.2.1 Generation of recombinant adenoviruses

The cDNA clone of human MEF2C (IRATp970F1023D; <http://www.imagenes-bio.de/>) was obtained from RZPD, Germany, as described already (Sturtzel, 2007). The coding region was subcloned into the pShuttle-IRES-hrGFP-1 vector plasmid which is part of the AdEasy™ Adenoviral Vector System kit from Stratagene. pShuttle.MEF2C and the pAdEasy-1, which encodes for the adenoviral backbone, were co-transformed into supplied BJ5183 RecA⁺ E.coli for recombination. The successfully recombined vector was linearized with PacI (NEB) and transfected into HEK293 cells using a mammalian transfection kit (Stratagene/ Agilent Genomics, USA) to generate primary adenoviruses. These

were subcloned, amplified and purified by ultracentrifugation over a CsCl gradient. An empty control adenovirus was prepared in parallel. Viral titer was determined using the Adeno-X rapid titer kit (Clontech, USA). The dominant-negative MEF2 encoding adenovirus was a generous gift of Prof. Leon de Windt (University of Maastricht, Maastricht, The Netherlands).

4.2.2. Production of recombinant lentiviruses

Plasmids (Mission shRNA pLKO.1-puro) encoding the corresponding short hairpin RNA (shRNA) sequences targeted against A2M and a scattered sequences control shRNA plasmid were purchased from Sigma-Aldrich. The shRNA plasmids were delivered into 293T cells together with 2 packaging vectors (pMD2.G and psPAX.1) by calcium phosphate transfection. After 8 hours medium was changed to M199 complete. Supernatants were harvested after 24 and 48 hours and filtered through a 0.4µm membrane. Virus preparations for 3 different shRNA sequences against A2M were mixed in a ratio 1:1. For infection supernatants were added to subconfluent ECs mixed with fresh medium in a ratio of 1:1 or 1:2.

4.3. Molecularbiological analyses

4.3.1. RNA preparation and cDNA synthesis:

For growth factor stimulation, HUVECs were grown dense, serum-starved overnight, and then treated for indicated time points. For overexpression experiments HUVECs were infected in sub-confluent state with control (Ad.con), MEF2C encoding (Ad.MEF2C) or dominant-negative MEF2 encoding (Ad.dnMEF2) adenovirus with multiplicity of infections (MOI) of 10 to 30 for indicated time points. For harvesting cells were pretreated with RNAlater (Ambion/ Life technologies) for 1 minute and lysed with QIAzol (Qiagen, Germany). Total RNA was extracted following the manufacturer's protocol. Total RNA (1,5µg) was used to synthesize cDNA with RevertAid™ H Minus Reverse Transcriptase provided with oligo dT18 primers, dNTPs, and RiboLock™ RNase

Inhibitor (all from Fermentas/ Thermo fisher, Germany). Concentrations and PCR schedule followed instructions of Fermentas.

4.3.2. Real-time RT PCR:

mRNA levels were measured using real-time reverse transcription-polymerase chain reaction (RT-PCR) detecting SYBR Green I with a Rotor-Gene Q cyclor (Qiagen). 1:2 dilutions of cDNA were analysed with Rotor Gene SYBR Green kit (Qiagen). Values were normalized to beta 2-microglobulin mRNA levels as internal control. Oligonucleotide primers were designed using Primer3 software (<http://frodo.wi.mit.edu/primer3/>) and listed in Table 4.

4.3.3. Western Blot analysis:

Cells were trypsinized, collected, washed with phosphate-buffered saline (PBS) (PAA), pelleted and lysed in Laemmli buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel (8%) electrophoresis (SDS-PAGE) and transferred to Immobilon-P membranes (Millipore) in a semidry-blot transfer system (Peqlab). The membrane was blocked with 5% skin milk/PBS with 0.1% Tween 20 (PBST) and incubated overnight with primary antibody at 4°C. The membrane was washed and incubated with corresponding peroxidase-conjugated secondary antibody for 1 hour. Antibodies used were rabbit-ant-human-MEF2C (abcam) and secondary horseradish peroxidase-conjugated donkey-anti-rabbit antibodies (GE Healthcare). The membrane was incubated with ECL Plus Reagent (GE Healthcare) following manufacturer's instructions and exposed to x-ray film (Fuji).

4.3.4. ELISA analysis:

To obtain supernatants to be tested for alpha 2Macroglobulin content, sub-confluent HUVECs in 6-well plates were first cultured in EGM2-MV (Lonza), left without virus or infected with Ad.con or Ad.MEF2C with MOI 3-10 for 16 hours and meanwhile they should grow dense. Then Medium was changed to 1ml serum-free optimem® (Invitrogen/ Life technologies) for 48 hours. Supernatants were harvested on ice and 50µl of each sample was immediately applied to the

previously antibody coated ELISA 96 well plate as instructed by the manufacturer. Alpha 2 Macroglobulin Human ELISA kit from Abcam was used to quantify A2M content of the HUVEC supernatants following the manufacturer's suggested protocol.

4.3.5 Flow cytometry analysis:

For determination of cell-surface markers expression by flow cytometry as described (Lehmann et al., 2012) HUVEC were infected with Ad.MEF2C or Ad.con or left without infection for 48 h. Cells were harvested with Accutase (PAA, Austria) and fixed with paraformaldehyde for 15 min at RT. For immunophenotypical staining cells were first incubated with FcR-blocking reagent (Miltenyi Biotec), followed by consecutive incubation with first (mouse anti-hCD62E, R&D) and second antibody (hoat anti-mouseIgG- APC, BD Biosciences) for 30 min on ice or corresponding isotype control (IgG1-APC, Miltenyi Biotec). After washing expression was measured using a FACSCalibur and analysed with CellQuestPRo software (both from BD Biosciences).

4.3.6. Affymetrix microarray analysis:

Subsequent to overexpression for indicated time points HUVECs were harvested as described under 4.3.1. RNA preparation. Extracted RNA was further purified using the RNeasy kit (Qiagen). Total RNA (200 ng) was analysed on genome-wide human Gene Level 1.0 ST GeneChips (Affymetrix, Santa Clara, CA, USA) as decribed in detail in (Tauber et al., 2010). Scannings of the arrays were carried out according to manufacturer's protocols listed at <https://www.affymetrix.com>. RMA Signal extraction, normalization and filtering was performed as described (<http://www.bioconductor.org/>). A variation filter was applied for selecting informative (i.e., significantly varying) genes. The filtering criteria for the exemplary data sets required an interquantile range > 0.5 and at least one sample with expression intensity > 50.

The full gene lists are now available at Gene Expression Omnibus <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46279>

4.4. Cellbiological analyses:

4.4.1. Proliferation Assay:

HUVECs were infected with adenoviruses using a MOI of 10. The cells were trypsinized 24 hours later and seeded into 4 96-well plates (5000 cells/well) in quintuplicates. Proliferation was determined by measuring the total protein content using the sulforhodamine B (SRB) colorimetric assay. Therefore cells were fixed, incubated with SRB (Sigma Aldrich), washed three times with Tris solution, and then SRB was solved in acetic acid, and the solution was measured with fluorescence at OD492.

4.4.2. Wound healing Assay:

HUVECs were infected with indicated adenoviruses using a MOI of 10. The cells were trypsinized 24 hours later, seeded into 24-well plates in triplicates and allowed to grow dense overnight. Then the cell monolayer was scratched with a yellow tip forming a cross. Pictures were taken afterwards and 24 hours of cultivation in full media later with a Nikon Diaphot TMD microscope and a CCD camera (Kappa GmbH). Percentage of refill of the wounded area was assessed using the Image J software (<http://www.uhnres.utoronto.ca/facilities/wcif/imagej/>).

4.4.3. Spheroid Sprouting Assay:

The principles of the spheroid sprouting assay were described (Korff et al., 1998). Here, HUVECs were infected with adenoviruses of MOI 3 to 5 or transduced with lentivirus particles containing 293T cell supernatant. Cells were used 4 hours after infection with adenoviruses or 48 hours after transduction with lentiviruses. HUVECs were suspended in growth medium containing 0.25% (wt/vol) methylcellulose (Sigma-Aldrich) and grown in hanging drops overnight to obtain single spheroids of approximately 450 cells. These were embedded into a gel consisting of 50% rat collagen (self-isolated) and 50% of methylcellulose mixed with FCS (finally 10%). A tenth of volume basal endothelial medium without or containing VEGF-A and bFGF (100ng/ml each final concentration) was layered on top of the polymerized gel. After 24 hours sprouts were quantified by

measuring the total sprout length grown out of each spheroid on pictures taken on the Nikon microscope with ImageJ software. At least 15 Spheroids per well were analysed.

4.4.4. Spheroid-based Sprouting Assay with cell culture supernatants:

HUVEC were infected with adenovirus using a MOI of 5-10 for 24 hours, or transduced with lentivirus particles containing 293T cell supernatant for 24 hours and subsequently infected with adenovirus again for 24 hours. Then medium was removed and fresh opti-mem medium (Invitrogen) was added for 48 hours. The cell supernatants were collected on ice and concentrated with 50 kDa cut-off centrifugal concentrator (Vivaspin 20, Sigma). Samples were mixed directly to the collagen gel, and also the A2M protein was added this way. Full-blown sprouting was stimulated with VEGF and bFGF (100ng/ml each, final concentration) afterwards.

5. Results:

5.1. VEGF-A specifically upregulates four transcription factor genes via VEGFR-2:

Within a previous project it was the aim of our group to identify distinct transcriptional response patterns elicited in ECs upon stimulation towards either the angiogenic or the inflammatory signaling route. For this purpose HUVEC were stimulated with either VEGF-A, the major physiological inducer of angiogenesis, bFGF, another strongly pro-angiogenic factor, or IL-1 alpha, a typical pro-inflammatory cytokine. As a control, the general growth factor EGF was used to principally deduce common responses leading to general cell activation and proliferation as they are triggered by many different growth factors. Genes specifically upregulated by VEGF, but not by IL-1 or EGF, were delineated from transcriptional profiling studies and considered to have a specific function during VEGF-induced angiogenesis (Schweighofer et al., 2009).

Among the upregulated genes/proteins we then focused on specifically VEGF-A-regulated transcription factors with the potential to direct downstream secondary transcriptional responses important for angiogenesis. In this regard NR4A2, EGR3, HLX and MEF2C were further analyzed and confirmed by realtime RT-PCR analysis to be the transcription factor genes most strongly and specifically upregulated by VEGF-A (Figure 4A). From these the MEF2C and the HLX gene were also induced by bFGF supporting their potential role for angiogenesis. To address whether the upregulation of these transcription factor genes is mediated by binding of VEGF-A to VEGFR-2 or VEGFR-1, stimulatory effects of VEGF-E, a viral homologue only binding to VEGFR2, or PIGF, a growth factor specific for VEGFR-1 were additionally tested. VEGF-E induced all four genes to the same extent as VEGF-A, whereas PIGF could not induce them at all (Figure 4B). This demonstrates a VEGFR-2-dependent signaling route for the induction of these transcription factor genes by VEGF-A.

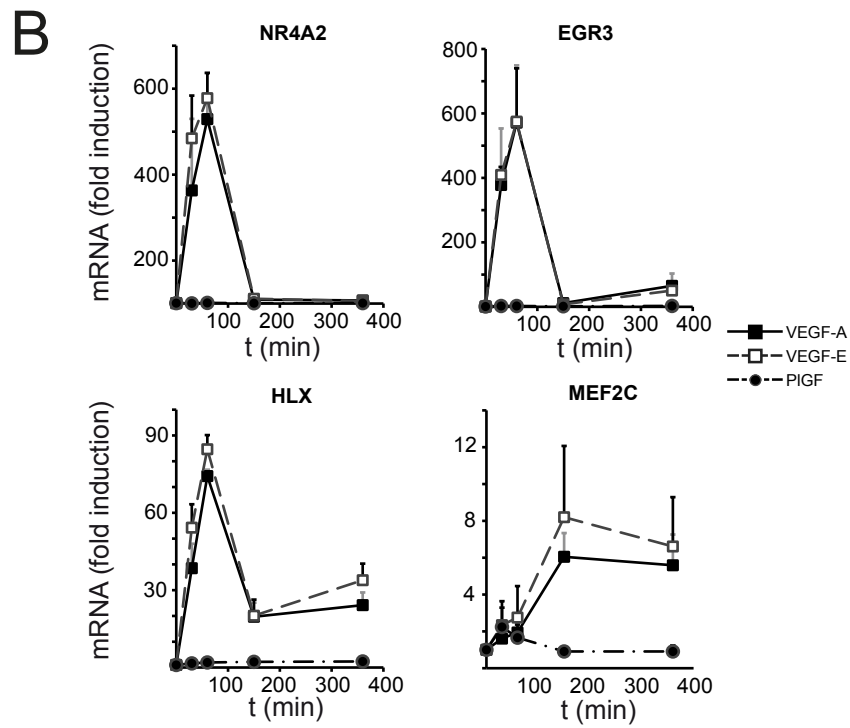
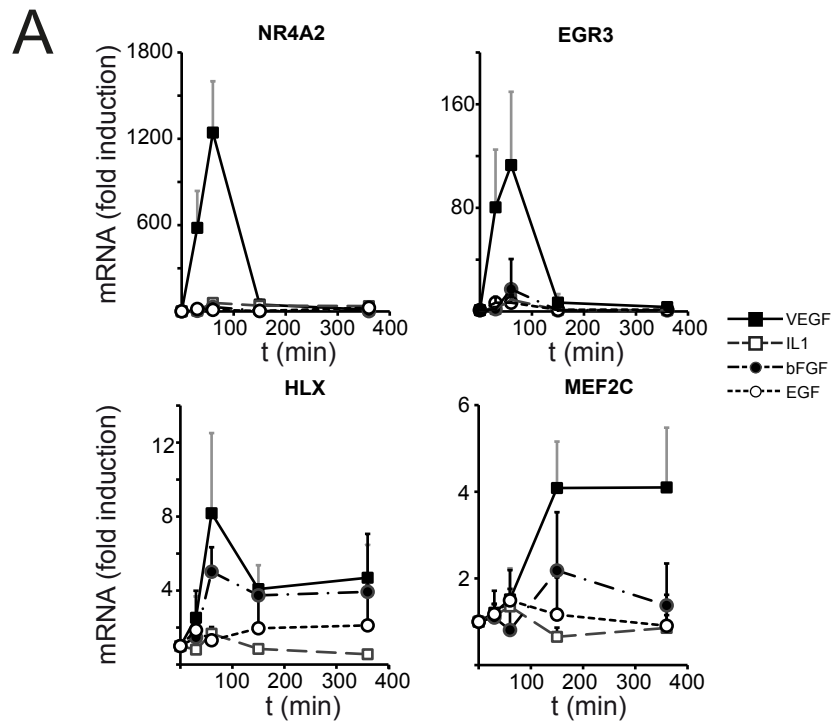


Figure 4: Specific induction of transcription factors by VEGF-A through VEGFR2.

(A) Comparison of inducibility of mRNAs of NR4A2, EGR3, HLX and MEF2C by VEGF-A, IL-1, EGF and bFGF. HUVEC were seeded in six-well plates, grown to confluence and stimulated in parallel with VEGF-A (100 ng/ml), IL-1 α (100 ng/ml), EGF (50 ng/ml) or bFGF (100 ng/ml) for 0.5, 1, 2.5 and 6 hours. RNA was isolated and analysed by real-time RT-PCR as described in the methods section real-time RT-PCR analysis. All values were normalised to β 2-microglobulin. The mean values $\pm$ SD calculated from three independent experiments are shown. (B) Comparison of inducibility by VEGF-A, VEGF-E and PlGF. HUVEC were stimulated by VEGF-A (100 ng/ml), VEGF-E (100 ng/ml) or PlGF (100 ng/ml) and analysed as described above. (adapted from Figure 3 in Schweighofer et al. with permission of reprint of Schattauer Verlag)

5.2. MEF2C protein is upregulated by VEGF-A concomitant with the induction of angiogenic sprouting

From the four transcription factor genes most strongly and selectively upregulated by VEGF-A, HLX (J. Testori, PhD thesis 2011; Testori et al., 2011) and MEF2C were chosen for further detailed analyses because of their inducibility by a second angiogenic growth factor, bFGF, and since they have not been implicated in angiogenesis previously.

First a time kinetic for the induction of MEF2C by VEGF-A was established. HUVEC were induced by VEGF-A for 4 to 24 h and subjected to Western blot analysis. Indeed, as suggested by the realtime RT-PCR data (Figure 4A), MEF2C protein was upregulated displaying a peak value at 8 h followed by a slow decrease until 24 h post VEGF-A addition (Figure 5A). This confirms that following upregulation of MEF2C mRNA by VEGF-A, which occurs with peak values at about 2 to 4 h, the MEF2C mRNA is translated leading to a significant increase in the MEF2C transcription factor following the mRNA induction kinetic with a corresponding delay.

This induction kinetic for MEF2C occurs with a time kinetic corresponding to the initial phase of angiogenic sprout formation. In a standard cellular angiogenesis assay, the spheroid sprouting assay, such angiogenic sprouting of endothelial cells can be studied upon addition of stimulants such as VEGF-A *in vitro*. Principally, HUVECs are allowed to form spheroids in hanging drops in a full medium/methocel mix, which are then seeded into a collagen matrix and stimulated. Cumulative sprout length gives a read out for angiogenic sprouting capacity. When spheroids of HUVECs are stimulated with VEGF-A sprout

formation starts at about 4 h leading to sprouts consisting of several consecutive EC over a period of 20 h (Figure 5B and C).

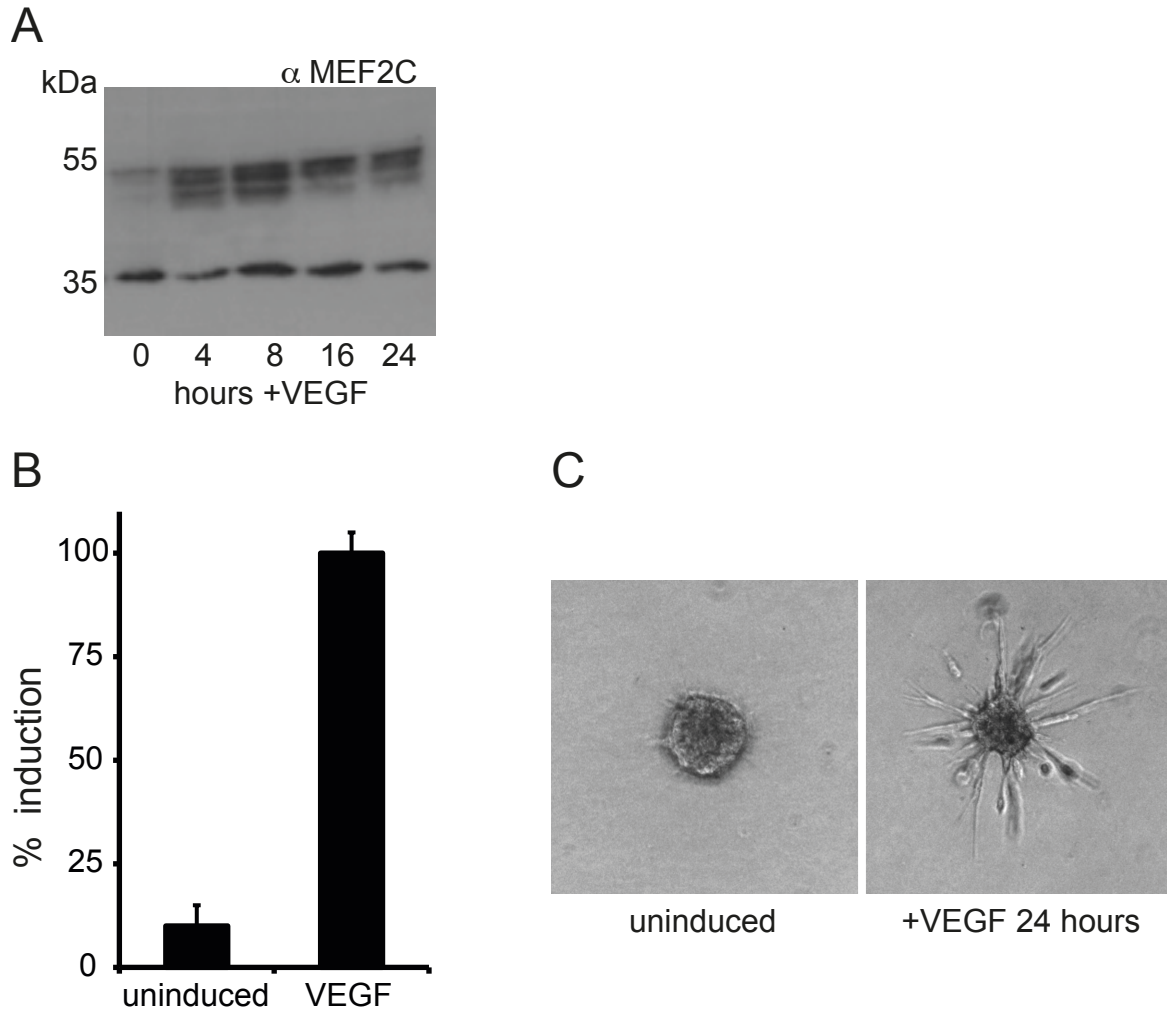


Figure 5: VEGF-A induces MEF2C protein concomitant with angiogenic sprouting.

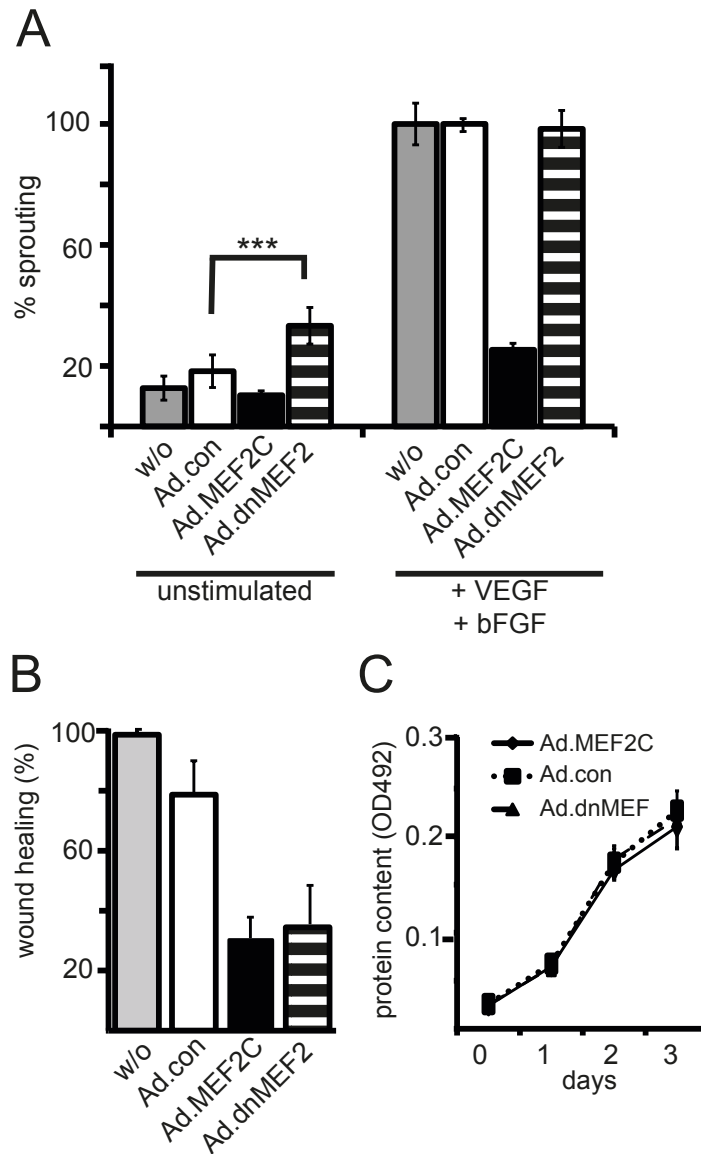
(A) Western Blot analysis: HUVEC were seeded in six-well plates, grown to confluence and stimulated with VEGF-A (100 ng/ml) for 4, 8, 16 or 24 hours. Cells were harvested in Lämmli sample buffer, the lysates were subjected to polyacrylamide gel electrophoresis, the separated proteins blotted and detected after incubation with polyclonal rabbit anti-MEF2C antibodies. (B) Angiogenic sprouting assay: HUVEC spheroids were generated, embedded into collagen gels and stimulated with VEGF (50ng/ml) or cultured without cytokine treatment as described in the Materials and Methods section. Sprouts were monitored for 24 hours, then pictures were taken for quantitative analyses. Total sprout length per spheroid was assessed using ImageJ software, 15 spheroids per condition were analyzed. Sprout formation induced by VEGF was set to 100%, The data shown were calculated from 1 representative experiment, displayed as mean values $\pm$ SD. (C) Representative spheroids without or after induction with VEGF-A are depicted for the time point 24 h.

5.3. MEF2C acts as an inhibitor of angiogenic sprouting of ECs:

To elucidate a potential role of MEF2C for angiogenesis, gain- and loss-of-function experiments were performed with endothelial cells. Adenoviral overexpression of MEF2C and of a dominant negative MEF2 (dnMEF2) form was tested in the spheroid sprouting assay. HUVEC were infected with Ad.MEF2C, Ad.dnMEF2 or the corresponding control virus Ad.con and then spheroids were allowed to form. These were seeded into the collagen matrix and stimulated with VEGF and bFGF overnight. Surprisingly, overexpression of MEF2C strongly inhibited VEGF-induced as well as basal sprouting, whereas the dominant negative form of MEF2 induced sprouting activity in the absence of VEGF-A and bFGF. This indicated that MEF2C, in contrast to our initial assumption, exerts an inhibitive function on sprouting angiogenesis (Figure 6A).

As angiogenesis involves migration and proliferation of endothelial cells these processes were further evaluated separately in a wound healing and a proliferation assay. In the wound healing assay the refill of a scratched cell-free area in an EC monolayer is mainly accomplished by migration of the EC. In this assay, adenoviral overexpression of MEF2C again compromised the migratory capacity of HUVECs in comparison to cells infected with control adenoviruses (Figure 6B). However, in an SRB proliferation assay no differences in proliferation rates were observed for Ad.MEF2C, Ad.dnMEF2 or Ad.con infected cells. This supports that MEF2C affects migratory mechanisms and does not influence proliferation (Figure 6C).

Figure 6: MEF2C strongly inhibits angiogenic sprouting and migration of endothelial cells but does not affect proliferation. HUVEC were infected with Ad.MEF2C, Ad.DNMEF2 or Ad.con using MOIs of 3 to 10 or left without infection. The capacity of the EC to sprout, migrate or proliferate was evaluated (A) Angiogenic sprouting assay: Spheroids were generated, embedded into collagen gels and stimulated with VEGF and bFGF (50ng/ml) or cultured without treatment. Sprouts were allowed to form for 24h, subsequently pictures were taken for analyses. Total sprout lengths per spheroid was assessed using ImageJ software. Sprout formation of uninfected HUVEC spheroids and induced with VEGF and bFGF was arbitrarily set to 100%, 15 spheroids



were analyzed per condition and experiment. Shown data were calculated from 7 independent experiments and displayed as mean values $\pm$ SEM. * $P < 0.01$, *** $P < 0.001$, t-test. (B) Wounding assay: For the wound healing assay HUVECs were re-seeded in EGM-2 MV medium and grown to complete density. The monolayer was scratched with a plastic pipette tip in cross form. Photographs were taken immediately after scratching and 24 hours later. Relative refill of the wounded area was measured with ImageJ software. Values obtained with HUVEC without infection were arbitrarily set to 100 %. The shown results were calculated from 3 independent experiments performed in triplicates and are displayed as mean values $\pm$ SEM. C) Proliferation assay: To assess proliferation rate the SRB assay was performed as described in the Methods section. Total protein content was measured after 0, 1, 2 and 3 days of culturing HUVECs in full growth medium in 96 well plates. Data for proliferation of HUVEC infected with Ad.con ($\bullet$), Ad.MEF2C ($\blacklozenge$) or Ad.dnMEF2C were calculated from 2 independent experiments performed in quintuplicates and displayed as mean values $\pm$ SEM.

5.4. The inhibitive effect of MEF2C on sprouting is not closely related to Notch signaling or directly regulated by HDACs:

In ECs Notch signaling is the central pathway to regulate the tip versus stalk cell phenotype during sprouting (Phng and Gerhardt, 2009). In this regard it seemed relevant that it had been reported that MEF2C binds to Notch and interacts with Notch signaling in muscle cells (Wilson-Rawls et al., 1999). To test whether the inhibitive effect of MEF2C overexpression on sprouting of EC results from augmented Notch signaling and consecutive arrest of cells in the stalk cell phenotype, the gamma secretase inhibitor DAPT was tested in the spheroid assay. DAPT specifically interrupts Notch signaling by interfering with the cleavage of the Notch receptors by gamma secretase. As expected, interruption of Notch signaling promoted basal sprouting, but DAPT did not change the inhibitive capacity of MEF2C on stimulated sprout formation (Figure 7). It seems therefore unlikely that Notch receptors are involved in the sprouting inhibition observed. Furthermore no upregulation of Notch receptors could be detected in a gene profiling experiment (see Table 2).

Moreover, it is well characterized that MEF2C is often repressed by class II HDAC proteins (Karamboulas et al., 2006). In these situations HDACs could switch off the pro-transcriptional activities of MEF2C for certain genes. Since HDACs were also reported to have a regulating function during angiogenesis (Rossig et al., 2002), the class II HDAC inhibitor Trichostatin A was employed to test for a possible connection of HDAC binding with the inhibitory effect of MEF2C on sprouting. Treatment with TriA slightly dampened sprout formation in all conditions, but no significant alteration of the inhibitory effect of MEF2C on sprouting could be observed in the presence of TriA. It therefore appears that HDACs do not play a significant role in establishing the inhibitory activity of MEF2C (Figure 7).

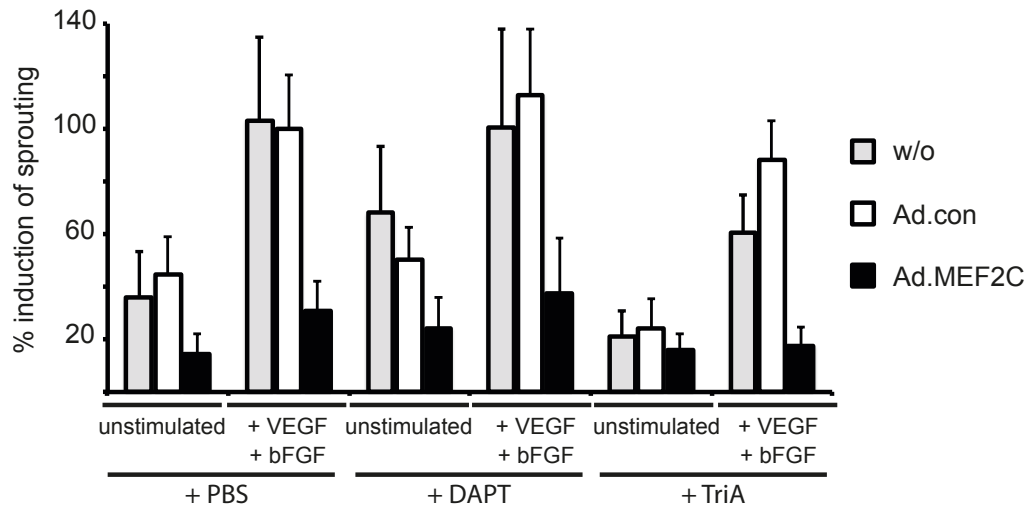


Figure 7: DAPT and TriA did not revert the repressive function of MEF2C on angiogenic sprouting. HUVEC were infected with Ad.MEF2C or Ad.con using a MOI of 10 or left without infection. Then they were used in a standard spheroid sprouting assay as described in the Methods section „Spheroid-based in vitro angiogenesis assay“. Spheroids were allowed to form sprouts for 24 hours unstimulated or stimulated by VEGF and bFGF (50ng/ml). Where indicated additional treatment with either DAPT (40μM) or TriA (100μg/ml) was employed. To display results the mean sprout formation of spheroids containing Ad.con infected HUVEC which were induced with VEGF and bFGF was arbitrarily set to 100%. Shown data were calculated from 1 representative experiment out of 2 independent experiments performed and displayed as mean values $\pm$ SD.

5.5. Identification of target genes of MEF2C in ECs:

It was further inquired which might be the potential target genes, that could be responsible for the observed inhibitive activity of MEF2C on angiogenic sprouting. We therefore proceeded to transcriptional profiling. Total RNA was isolated from uninfected HUVEC or cells infected with Ad.MEF2C or Ad.con for 8, 16 and 32 hours and subjected to microarray analysis. The basic data obtained are displayed in Table 2. As infection with control adenoviruses per se can affect the expression of particular genes, results were further displayed as fold upregulation triggered by MEF2C overexpression in comparison to control adenovirus infected cells. Of notice, only a handful of genes were specifically upregulated more than 2.5 fold suggesting a limited capacity of MEF2C on its own to regulate genes in EC. This is in contrast to a corresponding analysis performed in the lab for the

transcription factor HLX (Testori et al., 2011), which was able to upregulate a larger gene repertoire. For MEF2C the alpha-2-macroglobulin (A2M) and E-selectin (SELE) genes were identified as the most strongly induced by MEF2C (Table 1).

mRNA Accession	Gene Symbol	Gene Description	Ad.MEF2C/Ad.con		
			8h	16h	32h
NM_002397	MEF2C	myocyte enhancer factor 2C	12.42	21.96	29.05
NM_000014	A2M	alpha-2-macroglobulin	0.90	2.97	11.80
NM_000450	SELE	selectin E (endothelial adhesion molecule 1)	1.08	2.96	5.64

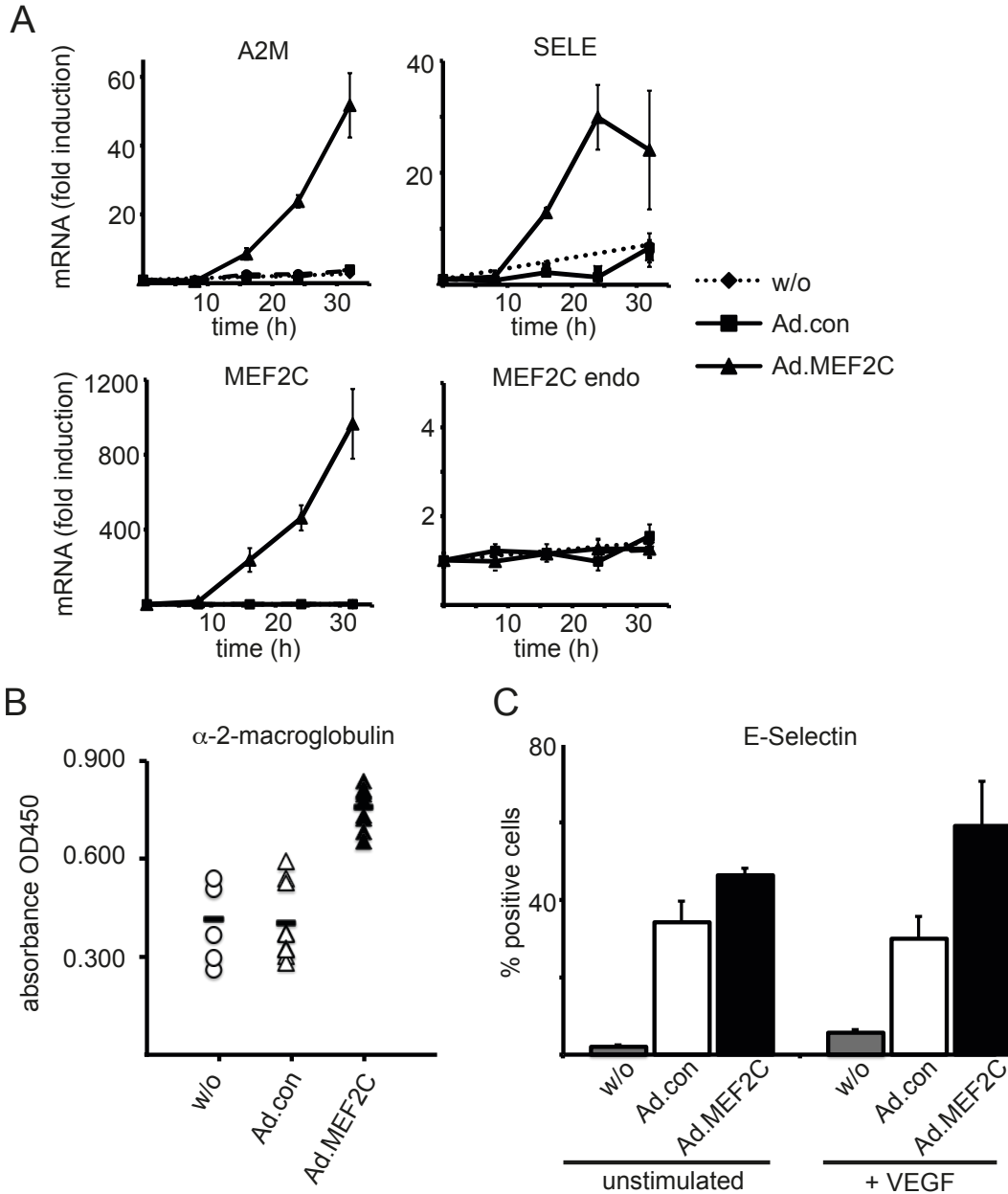
Table 1: Transcriptional profiling reveals alpha-2-macroglobulin and E-selectin as the genes most strongly upregulated by MEF2C overexpression in EC.

Total RNA was isolated from HUVECs after infection with MEF2C-encoding (Ad.MEF2C) and control adenoviruses (Ad.con) for 8, 16 and 32 hours. RNA was subjected to microarray analysis using Affymetrix Human Gene Level 1.0 ST GeneChips. Changes in gene expression induced in Ad.MEF2C relative to Ad.con-infected cultures are displayed as fold-induction at the indicated timepoints.

These results obtained from the microarray analysis were positively confirmed by realtime RT-PCR demonstrating that A2M and SELE mRNAs were upregulated by MEF2C in a double-digit range (Figure 8A). In these experiments MEF2C mRNA was increased several hundred-fold following infection with Ad.MEF2C, whereas endogenous MEF2C measured using primers for the 3'-untranslated region not present in the virally expressed MEF2C mRNA did not change.

Figure 8: Alpha-2-macroglobulin and E-selectin are upregulated by MEF2C in HUVECs on mRNA and protein level.

(A) Realtime RT-PCR analysis of alpha-2-macroglobulin and E-selectin mRNAs: HUVECs were transduced for 8, 16, 24 and 32 hours with recombinant adenoviruses encoding MEF2C (Ad.MEF2C) or control viruses (Ad.con) using a MOI of 10. Then total RNA was isolated, cDNA synthesized and subjected to realtime RT-PCR analysis as described in detail in the methods section "real-time RT-PCR analysis". Obtained ct-values were normalized to β 2-microglobulin mRNA values as internal standard. Fold induction levels compare corresponding mRNA levels after infection with Ad.MEF2C (—▲—), or Ad.con (—■—) with non-infected (···◆···) cells at time point 0. Mean values $\pm$ SD were calculated from triplicates of one representative experiment out of three independent experiments performed.



(B) ELISA analysis of secreted α -2-macroglobulin: HUVECs were infected with Ad.MEF2C, Ad.con or left without infection for 8 hours, then medium was changed to serum-free optimum medium. Supernatants were harvested after 48 hours and secreted α -2-macroglobulin determined with the help of a commercial A2M ELISA kit as described in detail in the methods section "ELISA". Mean values $\pm$ SD are shown for fold A2M increase in MEF2C overexpressing cells relative to uninfected cells and were calculated from three independent experiments.

(C) FACS analysis of surface E-selectin levels: HUVECs were infected with Ad.MEF2C, Ad.con or left without infection for 48 hours. Then the cells were stimulated with VEGF (50 ng/ml) for 4 hours or remained uninduced. Detection of surface E-Selectin protein was performed by flow cytometry using PFA-fixed cells as described in detail in the methods section "FACS Analysis". Results are depicted as percentage of positive cells, mean values $\pm$ SD were calculated from triplicates of 1 representative experiment out of 3 independently performed.

5.6. Alpha-2-macroglobulin and E-selectin protein levels are increased by MEF2C:

Based on the finding that MEF2C upregulated A2M and SELE mRNAs we tested whether this would result in increased secretion of A2M and higher levels of E-selectin on the cell surface.

Indeed, MEF2C overexpression stimulated the production of A2M in HUVEC (Figure 8B). Alpha-2-macroglobulin is known as big serum-protein of more than 700kDa comprised of 4 homodimers. As it serves as a docking site for growth factors or cytokines and is a global protease inhibitor, it may sequester growth factors such as VEGF-A and proteases necessary for sprout invasion and block their normal function.

When we further evaluated the expression of E-Selectin by flow cytometry we found that MEF2C was also able to increase the presence of E-selectin on the cell surface (Figure 8C). E-selectin is a molecule specific for mature endothelial cells, which usually highly upregulate this protein upon inflammatory activation. It is essentially involved in leucocyte rolling and extravasation. As the data supported that MEF2C could upregulate E-selectin at the mRNA and also measurably at the protein level, a potential role of MEF2C in mediating the previously observed partial induction of E-selectin by VEGF-A (Schweighofer et al., 2009) could be supposed. However, advancing analysis of this phenomenon was complicated by the fact that already the control virus led to a variable, but frequently strong increase of the surface exposure of E-selectin, although a comparable effect has not been observed for the mRNA induction. Further work was therefore restricted to the analysis of the effects of MEF2C on A2M production.

5.7. Alpha-2-macroglobulin secreted from MEF2C overexpressing endothelial cells mediates inhibition of sprouting

To evaluate the hypothesis that secreted A2M protein would cause the inhibitive effects of MEF2C, conditioned supernatants obtained from Ad.MEF2C-infected cells as well as purified A2M were tested in the angiogenic spheroid sprouting

assay. For this purpose HUVEC were infected with Ad.MEF2C or Ad.con and further cultivated in serum-free Opti-mem media for 2 days. Then supernatants were collected and concentrated by diafiltration to be tested in the spheroid sprouting assay. Indeed, the supernatants of MEF2C overexpressing cells strongly reduced sprouting when compared to supernatants from control virus infected cells (Figure 9A). To further show that A2M can inhibit sprouting, commercially available A2M protein isolated from human serum was tested and found to inhibit sprouting displaying half-maximal inhibition at about 4.5×10^{-6} M (Figure 9B).

To finally confirm that A2M mediates, at least to a significant extent, the observed inhibitory effects of MEF2C, the consequences of knock-down of A2M by shRNA expressing lentiviruses were analysed. Indeed, when the increased expression of A2M in cells infected with Ad.MEF2C was prevented by A2M-specific shRNA the inhibitory effect of MEF2C on sprouting was completely abrogated (Figure 9C). Furthermore, when conditioned supernatants were prepared from these cells also the inhibitive activity of these supernatants was largely ameliorated (Figure 9D). These findings strongly support that MEF2C functions in EC to upregulate A2M secretion which causes inhibition of angiogenic sprouting.

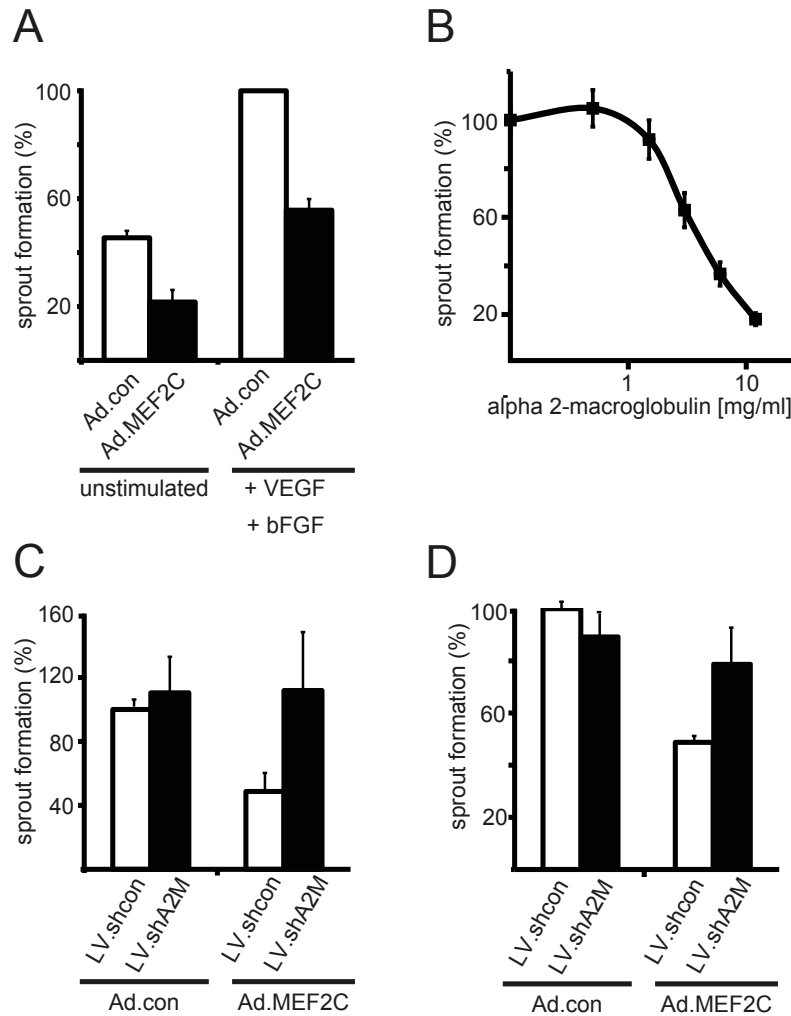


Figure 9: A2M mediates inhibition of sprout formation by MEF2C.

(A) Supernatants of Ad.MEF2C infected cells inhibit sprouting: Supernatants of HUVECs infected with Ad.MEF2C or Ad.con for 48 h were collected, concentrated by diafiltration and added to the collagen solution together with spheroids of uninfected HUVECs immediately before plating and gelation as described in the Methods section. Spheroids were allowed to form sprouts for 24 h either in the presence or absence of VEGF-A and bFGF. (B) Purified A2M inhibits sprouting: A2M protein solved in PBS was added in the indicated concentrations to the collagen solution together with spheroids of uninfected HUVECs immediately before plating and gelation. Samples were stimulated with VEGF and bFGF and allowed to form sprouts for 24 hours. (C) Lentiviral expression of A2M shRNA prevents the inhibitory effects of Ad.MEF2C: HUVECs were first transduced with LV.shA2M or LV.shcon for 24 h and then infected with Ad.MEF2C or Ad.con for 6 h before they were used to form spheroids by the hanging drop method. After embedding into the collagen matrix samples were induced with VEGF and bFGF or left uninduced. Samples were assessed for sprouting after 24 hours. (D) Lentiviral expression of A2M shRNA diminishes the inhibitory activity of supernatants from Ad.MEF2C infected cells: HUVECs were first transduced with LV.shA2M or LV.shneg for 24 h and then infected with Ad.MEF2C or Ad.con for further 24 h. Then incubated for 48 hours in serum-free medium (Opti-mem), then supernatants were collected. Concentrated supernatants were added to the collagen solution together with spheroids of uninfected HUVECs immediately before plating and gelation. Samples were induced with VEGF and bFG or left uninduced and spheroids were allowed to form sprouts for 24 h before analysis.

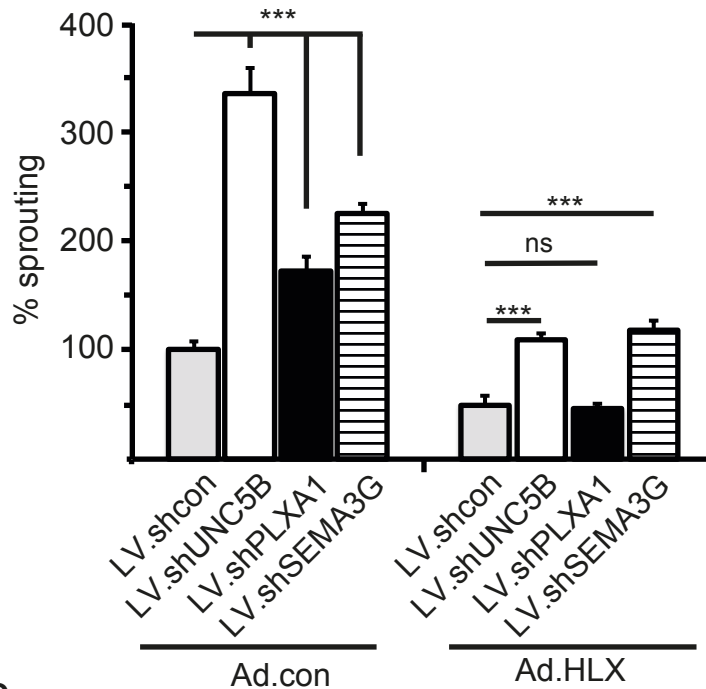
For analysis photographs were taken and analyzed with ImageJ software. 15 spheroids were evaluated per sample. To display the data, for each series the respective mean total sprout length obtained for Ad.con infected cells stimulated with VEGF and bFGF (50ng/mL) was arbitrarily set to 100%. All results display mean values $\pm$ SEM calculated from 3 independent experiments.

5.8. The transcription factor HLX causes inhibition of sprouting via upregulation of several guidance cues

A second example of a VEGF-A-induced transcription factor analyzed in our group, that also downmodulates angiogenic sprouting, is the homeobox factor HLX (J. Testori, PhD thesis 2011; Testori et al., 2011). Similar to MEF2C, HLX was selected as a transcription factor specifically induced in EC by VEGF-A and not by IL-1 or EGF. When overexpressed it exerts a strong inhibitory effect on angiogenic sprouting. In the case of HLX, gene profiling indicated that the repellent guidance molecules UNC5B, PLXA1 and SEMA3G could be the potential mediators of this inhibition. In collaboration with Mag. J. Testori initially the question was addressed whether the guidance receptor UNC5B could be involved in the observed inhibition. shRNA mediated knock-down of UNC5B indeed counteracted the inhibitory effects of overexpression of HLX supporting that in the case of HLX the guidance receptor UNC5B, at least in part, mediated the inhibition (Testori et al., 2011, Figure 10A). Furthermore, I extended these studies to examine whether shRNA mediated knock-down of PLXA1 and SEMA3G would also have similar effects. The obtained results reveal that downmodulation of SEMA3G leads also to a significant rescue of inhibition, whereas downmodulation of PLXA1 had no detectable effect. Of notice, we further observed that downmodulation of UNC5B not only rescued HLX-mediated inhibition of sprouting, but also significantly promoted VEGF-A-induced as well as basal sprouting activity. Therefore, I further analyzed to which degree also the two others guidance cues regulated by HLX, the guidance receptor PLXA1 or the secreted SEMA3G, are involved in reducing sprouting activity. The data demonstrate that downmodulation of all three guidance molecules leads to increased basal as well as VEGF/ bFGF-induced sprouting (Figure 10B). This indicates on the one hand that low expression of UNC5B, PLXA1 and SEMA3G,

triggered presumably by a basal HLX level also in the VEGF/ bFGF uninduced state, already limits basal sprouting activity and on the other hand that UNC5B, PLXA1 and SEMA3G usually become upregulated by VEGF-A/ bFGF via HLX, probably in a modulating negative feed-back loop.

A



B

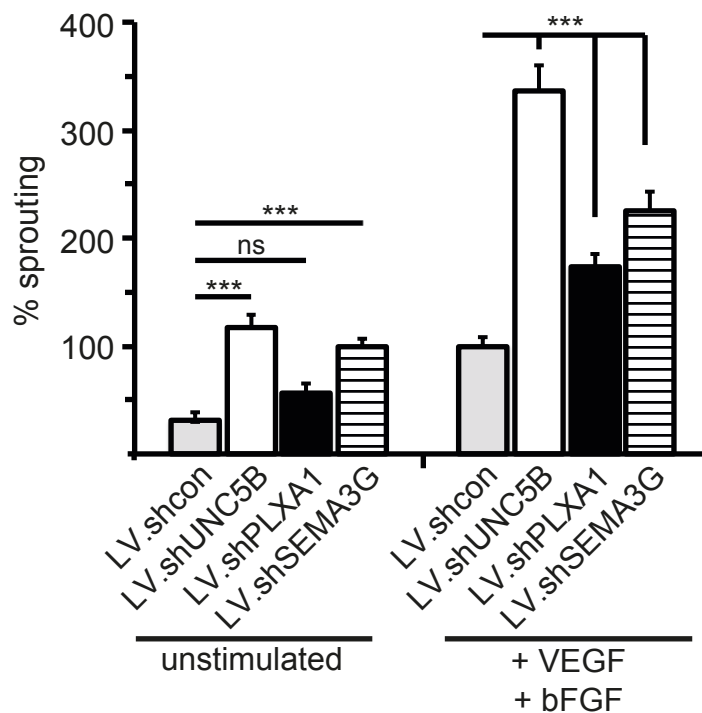


Figure 10: Effects of downmodulation of UNC5B, PLXA1 and SEMA3G on sprouting activities.

(A) Rescue of HLX-mediated inhibition of sprouting: HUVECs were first transduced with LV.shUNC5B or LV.shcon for 24 h and then infected with Ad.HLX or Ad.con for 24 h before they were used to form spheroids by the hanging drop method. Spheroid sprouting assays were then performed following the Methods section “Spheroid-based in vitro angiogenesis assay”. Samples were stimulated with VEGF and bFGF at the time when they were seeded into the collagen matrix. Sprouting was assessed after 24 h.

(B) Increased basal and VEGF/bFGF-induced sprouting: HUVEC were infected with LV.shUNC5B, LV.shPLXA1, LV.shSEMA3G for 48 h and then tested in the spheroid sprouting assay in the presence or absence of stimulation VEGF and bFGF.

To display the results the mean total sprout length obtained for LV.shcon infected samples stimulated with VEGF and bFGF (50ng/mL) were arbitrarily set to 100%. All shown results were calculated from 1 representative experiment of 3 independent experiments performed, displaying mean values $\pm$ SEM.

5.9. Under hypoxic conditions the expression of MEF2C and HLX as well as their target genes is strongly reduced:

Pondering on the puzzling observation that two VEGF-A induced transcription factors would inhibit and not activate angiogenic sprouting, we reasoned that a potential explanation might be found in distinct endothelial cell response during normoxic in contrast to hypoxic conditions, since the latter actually apply in tissues when angiogenesis has to be initiated. To examine this hypothesis we tested how MEF2C and consecutively A2M would be regulated during hypoxia. We found that upregulation of MEF2C by VEGF-A was less pronounced under hypoxic condition and the increase of A2M mRNA was even more strongly reduced (Figure 11A). Presumably as a consequence the effect of MEF2C on sprouting is ameliorated under hypoxic conditions, i.e. MEF2C causes relatively less sprouting reduction (Figure 11B). Comparable results have been obtained when induction of HLX and UNC5B by VEGF-A was tested under hypoxic conditions. HLX induction was reduced and the upregulation of UNC5B was nearly abrogated (Testori et al., 2011). Hypoxia also mitigated the inhibitory effect of HLX on VEGF-A-induced sprouting, to a large extent due to the decreased upregulation of UNC5B. These parallels in the inhibitory activities of MEF2C and HLX might point at their joint involvement in negative feedback mechanisms, which would avoid overshooting angiogenesis. These mechanisms will prevent initiation of inappropriate endothelial sprouting during normoxic conditions and will turn-off angiogenic sprouting once oxygen supply is restored.

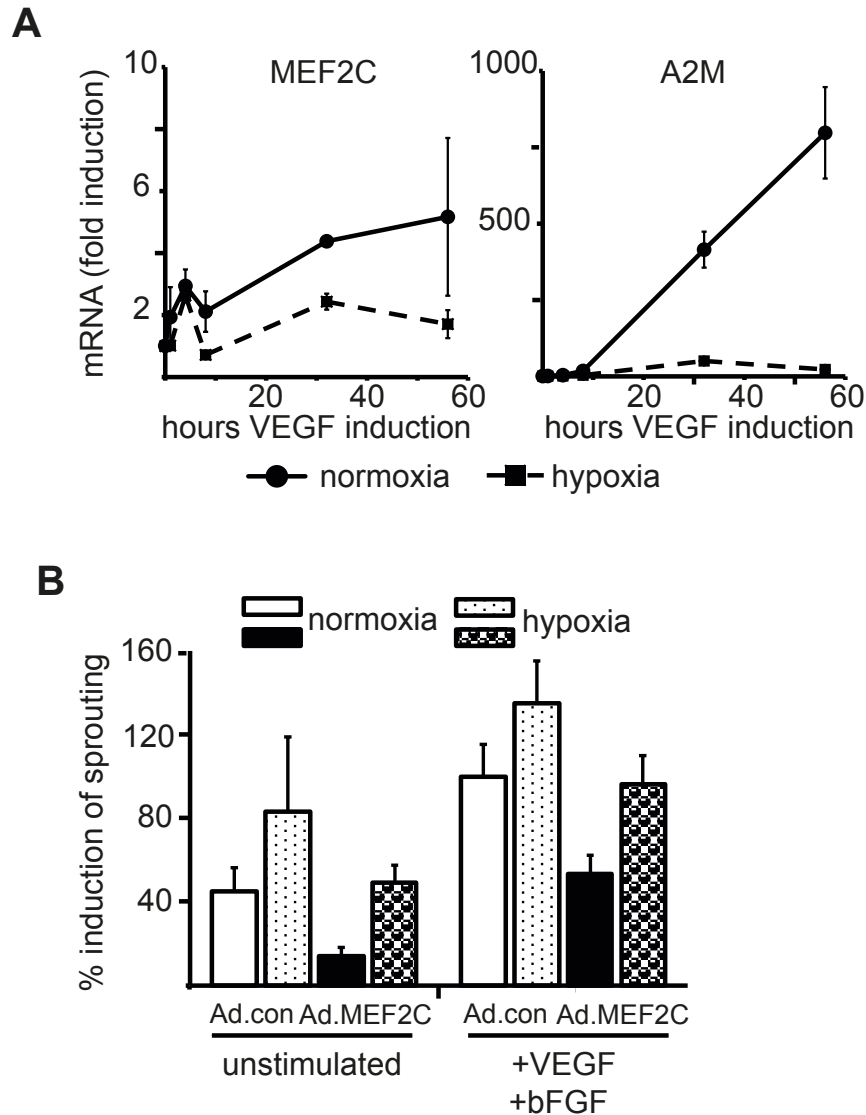


Figure 11: Under hypoxia VEGF-dependent induction of MEF2C and A2M is reduced and inhibition of sprouting by MEF2C is ameliorated.

(A) Reduction of VEGF-mediated upregulation of MEF2C and A2M mRNAs during hypoxia: Differences in VEGF-A-mediated regulation of MEF2C and A2M mRNA under hypoxic or normoxic conditions were analyzed by "realtime RT-PCR". Starved HUVEC were stimulated with VEGF-A and cultured under either normoxic or hypoxic conditions for 4, 8, 32 and 56 h. Total RNA was isolated, subjected to cDNA synthesis and analysed by realtime RT-PCR as described in the Methods section. One representative experiment of 3 performed in triplicates is shown. The values depict the mean of triplicates $\pm$ SD. (B) MEF2C-mediated inhibition of sprouting under normoxic and hypoxic conditions: To compare the role of MEF2C in angiogenesis under normoxic (21% O₂) or hypoxic conditions (0.5- 2% O₂) HUVEC were infected with Ad.MEF2C or Ad.con using a MOI of 3 to 10 and tested in a standard spheroid sprouting assay as described in the Methods section "Spheroid-based in vitro angiogenesis assay". Spheroids were allowed to form sprouts for 24 h either under standard conditions or in a hypoxia chamber. 15 spheroids per condition were analyzed. Shown data were calculated from 4 independent experiments and are displayed as mean values $\pm$ SEM.

6. Discussion:

Angiogenesis is an essential process for example during embryonic development to adapt the vessel system to the growing organism and in the adult to achieve functional revascularization during wound healing. However, it also plays an important role in many pathological conditions such as cancer. Vessel formation by angiogenic sprouting is a complex process involving different cellular tasks such as restrained proliferation and oriented migration triggered by growth factors of the VEGF family (Carmeliet, 2005). Despite significant knowledge on the VEGF's, their receptors and downstream signaling cascades, the transcription factors consequently induced and controlling transcriptional programs during angiogenesis are still incompletely understood.

6.1. Identification of MEF2C as a transcription factor specifically induced by VEGF-A via VEGFR2:

To gain further insight into the transcriptional mechanisms controlling angiogenesis we have therefore undertaken a gene profiling analysis by filtering for transcription factors specifically induced by VEGF-A in EC (Schweighofer et al., 2009). This approach included a comparison of genes upregulated by the primary angiogenic trigger VEGF-A, the general growth factor EGF and the pro-inflammatory cytokine IL-1 alpha. This was done under the premise that genes and transcriptions factors upregulated by VEGF-A, but not by EGF or IL-1 alpha, would have a high probability to fulfill a specific function in VEGF-A-mediated angiogenic sprouting. In these experiments we discovered that NR4A2, EGR3, HLX and the MADS-box transcription factor MEF2C are most strongly and specifically induced by VEGF, but not by EGF or IL-1 alpha in EC (Liu et al., 2003; Schweighofer et al., 2009). Other groups have in the meantime reported that the transcription factors NR4A2 and EGR3 are important for angiogenic sprouting, since their silencing inhibited VEGF-mediated endothelial cell proliferation, migration and tubulogenesis (Liu et al., 2008, Zhao, 2011 #42; Zhao

et al., 2011). This supports the validity of our approach and that the factors selected possess important functions for angiogenesis.

Our group focused on the homeobox transcription factor HLX and the MADS-box transcription factor MEF2C for the following reasons: i) both factors were also partially induced by another angiogenic trigger, bFGF, additionally supporting their involvement in angiogenesis; ii) some reported data displaying a reduction in organ growth for HLX knock-out mice (Bates et al., 2006) or, aside a prominent heart phenotype, vascular defects for MEF2C knock-out mice (Bi et al., 1999); iii) some more recent evidence that both factors are preferentially expressed in growing sprouts (del Toro et al., 2010). Indeed, the data gained on HLX quickly led to the interesting observation that HLX controls the expression of guidance cues supporting an important role during sprout formation (Testori et al., 2011).

To clarify the roles of these transcription factors I collaborated in part on certain aspects of the analysis of HLX, but focused my main investigation on MEF2C. Initially I confirmed that the MADS box transcription factor MEF2C is specifically inducible by VEGF-A in EC not only at the mRNA but also at the protein level. Furthermore, I was able to demonstrate that MEF2C, as well as HLX, is upregulated specifically via the VEGFR2 and not via VEGFR1 further implicating a role in initiation of angiogenesis (Figure 4) (Maiti et al., 2008; Schweighofer et al., 2009).

6.2. The VEGF-A inducible MEF2C is part of a negative regulatory feedback mechanism for endothelial sprouting:

As MEF2C protein expression rises with VEGF stimulation in chronology with the onset of sprout formation (Figure 5A) we initially assumed a driving role of MEF2C for angiogenesis. However, adenoviral overexpression of MEF2C in EC surprisingly revealed a strong inhibitive effect particularly on sprouting (Figure 6A). This effect was observed in the spheroid sprouting assay, which is a method to study angiogenic behavior of EC *in vitro*. In this assay conditions are comparatively easy defined and manipulated. EC are initially forced to form

spheroids, which are then seeded into a collagen matrix, which has to be invaded by concerted migration and proliferation (Korff and Augustin, 1998). To distinguish whether the invasive, migratory and/or proliferative components are affected, we analyzed in addition migration and proliferation in separate assays. Migration was comparably inhibited whereas proliferation was unaffected (Figure 6B and C). This indicated that MEF2C expression controls sprouting mechanisms related to migration and likely also invasion.

6.3. The inhibitive mechanisms induced by MEF2C do not involve Notch receptors or HDACs:

As Notch signalling is a central mechanism to regulate the stalk versus tip cell phenotype during angiogenesis, I initially considered that MEF2C might regulate the Notch pathway. In addition, reports describing that MEF2C directly binds to Notch in muscle cells indicated Notch receptors as potential mediators of the MEF2C effects (Pallavi et al., 2012; Wilson-Rawls et al., 1999). It was therefore conceivable that MEF2C, when overexpressed, would either increase Notch transcription or could, by binding to Notch, amplify transcriptional responses triggered by Notch. This in turn would lead to a repression of tip cells and induction of stalk cells which could explain the inhibitive phenotype induced by MEF2C. However, abrogation of Notch signalling by the gamma-secretase inhibitor DAPT did not alter the inhibitive effects of MEF2C in the spheroid sprouting assay, although an inducing effect of DAPT on sprouting was observed as expected for a Notch inhibitor (Figure 7). It is therefore unlikely that Notch receptors mediate the MEF2C effects.

Additionally I considered an involvement of HDACs, as class II HDACs are the most prominent co-factors of MEF2C. They normally repress transcriptional activity of MEF2C, which constitutively binds to DNA, by condensing DNA helical structures. Release of this repression happens by export of the HDACs from the nucleus upon phosphorylation. The inhibitor TrichostatinA (TriA) blocks deacetylation activity of HDACs. When a potentially relevant repression of

MEF2C by HDACs has to be overcome for angiogenesis activation, inhibition of HDACs might be a reasonable way to test this. However, application of TriA did not change the inhibitive phenotype of MEF2C in the spheroid sprouting assay. So it appears that the inhibitive effect of MEF2C overexpression can not be converted to an inducing effect by inhibition of deacetylation and it seems therefore not to be connected to histone deacetylation. This is supported by recent data of the Dimmeler group, when after silencing of HDAC5 a co-signalling role of MEF2C was not related to the impact on angiogenesis (Urbich et al., 2009).

As we could not find indications for a direct involvement of Notch or HDACs in the MEF2C inhibitive function, we undertook a systematic gene profiling analysis to delineate potential candidate genes upregulated by MEF2C, which would mediate the inhibitive effects.

6.4. MEF2C upregulates the alpha-2-macroglobulin (A2M) and E-Selectin (SELE) genes:

In order to delineate target genes of MEF2C, which could cause the inhibitive effect, microarray analyses were performed at different time points following overexpression of MEF2C. Usually our group was able to define relevant genes upregulated using this method. For instance from microarray analyses of the overexpressed transcription factors HLX or EGR-1 fifty to more than hundred target genes upregulated more than three times were retrieved, respectively (Lucerna et al., 2006; Testori et al., 2011).

In comparison, overexpression of MEF2C led to a more restricted response (Table 1). Two genes could be found to be reproducibly upregulated in ECs by MEF2C more than 2-fold, A2M and SELE (Table 1, Figure 8A). This suggests that MEF2C either exerts a very limited and specific response in EC or that the MEF2C activity may be rather related to a repressive effect on gene transcription. We therefore also analyzed the downregulation of genes by MEF2C overexpression (Table 3). However, only few genes were detected to be

significantly downregulated slightly more than 2-fold and these genes did not include obvious candidates for mediating the inhibitive effect. By far the most prominent and reproducible result of overexpression of MEF2C was the upregulation of the A2M gene which was found to be about 50-fold by realtime RT-PCR analysis (Figure 8).

We can currently not exclude that a possible reason for the restricted response is a cofactor for transcriptional activation needed together with MEF2C, which is missing or present in limiting amounts, or a mainly repressive function of MEF2C together with e.g. excess amounts of HDACs. However, as reasoned before HDAC inhibition was unable to relieve the inhibitive effects of MEF2C. Furthermore, it can be assumed that VEGF present in the medium should induce phosphorylation of HDAC leading to a partial release of HDAC binding. The latter possibility seems therefore unlikely (Urbich et al., 2009).

I therefore primarily further evaluated the two genes most strongly upregulated by MEF2C in ECs, A2M and SELE (Table 1, Figure 8A) as this effect appeared to be the most prominent and reproducible. A2M is known as a big serum protein, which forms a homotetramer complex of about 700 kDa, and is constitutively produced in large amounts in the liver. It has been shown to function as a global serine proteases inhibitor as well as a carrier protein for different growth factors including VEGF (Bhattacharjee et al., 2000; Mathew et al., 2003; Mehl et al., 1964). Since angiogenesis is induced and promoted by VEGF and includes as an essential step the synthesis of matrix metalloproteases (MMP) to degrade ECM for sprout invasion, we hypothesized that A2M could conceivably be involved in the downmodulation of angiogenesis observed. Here we were indeed able to show for the first time that MEF2C could upregulate the synthesis and secretion of A2M in EC (Figure 8B) pointing to a novel cellular source and role of A2M in the context of control of angiogenesis (see below).

The second gene detected to be reproducibly upregulated at the mRNA level was SELE. E-Selectin is normally upregulated on the cell surface by inflammatory mediators only in fully mature EC and is well-known for its function as adhesion protein for leucocyte rolling (Bevilacqua et al., 1989). This also

raised our interest, as it could be proposed that upregulation of E-selectin could reflect a possible function of MEF2C in promoting final differentiation of ECs to fully mature cells which are primarily reactive to inflammatory activation but less so to angiogenic activation. When tested at the mRNA level by realtime RT-PCR, E-selectin mRNA was reproducibly upregulated 30- to 40-fold by MEF2C overexpression, which compares to less than 10 % of the effect of inflammatory activation. Usually IL-1 alpha causes upregulation in the order of up to thousand fold (Figure 8A) (Schweighofer et al., 2009). As IL-1 alpha does not upregulate MEF2C, inflammatory induction of E-selectin should not be mediated via an increase in MEF2C. Nevertheless, it is still conceivable that MEF2C is a necessary constitutively present cofactor for the upregulation of the E-selectin gene.

However, when we tested E-selectin protein upregulation in response to MEF2C at the cell surface a second level of regulation became apparent. Whereas adenoviral overexpression of MEF2C strongly stimulated E-Selectin surface expression comparable to mRNA induction, already control virus infection led to a strong exposure of E-selectin on the cell surface (Figure 8C). Thus it appears that viral infection per se could upregulate E-selectin at protein level blurring the results obtained.

Comparing these effects of MEF2C on A2M and SELE expression, A2M seemed to be the functionally more relevant for these studies and therefore further focus was put on investigation of this major target gene of MEF2C.

6.5. Alpha-2-macroglobulin (A2M) is a major mediator of the inhibitive effect of MEF2C on sprouting:

Considering A2M is a secreted protein and might be a critical mediator of the inhibition of angiogenic sprout formation by MEF2C, this inhibitory activity should be also revealed by testing the supernatants of MEF2C overexpressing cells. When we evaluated supernatants concentrated by diafiltration using a cut-off at 50 kDa, a process also eliminating potentially interfering small signaling

molecules, indeed inhibition of sprout formation was observed selectively for supernatants from MEF2C overexpressing cells. This demonstrated that a large secreted protein produced from MEF2C overexpressing cells is responsible for the observed sprouting suppression (Figure 9A). Furthermore, when commercial A2M protein isolated from human serum was added to the 3D collagen matrix of the spheroid assay, sprouting was inhibited in a concentration-dependent manner displaying half-maximal inhibition at 4.5×10^{-6} M/l (Figure 9B), which is well in the order of many low affinity inhibitory interactions. Of notice, similar concentrations persist in the blood serum (2-4 mg/ml). However, as VEGF-A is generated in hypoxic tissue areas and diffuses towards adjacent blood vessels, it is conceivable that apical secretion of A2M from endothelial cells might fulfill a dampening effect on angiogenic sprouting.

To directly confirm that A2M mediates the sprouting suppression caused by MEF2C we used silencing of A2M expression by transduction of HUVECs with a lentivirus encoding shRNA against A2M. If the key downstream mediator of inhibition is knocked down in the cells they should present a rescued phenotype. When MEF2C was overexpressed in HUVECs silenced for A2M indeed the inhibition was abrogated, revealing that, at least to a large extent, A2M mediates the observed suppressive effects of MEF2C during angiogenesis (Figure 9C). Similarly, the supernatants from HUVECs silenced for A2M and subsequently infected with Ad.MEF2C displayed reduced inhibitory potential (Figure 9D). Taken together, these data demonstrate that secreted A2M is an integral mediator of the inhibitive effect of MEF2C. It seems therefore likely that VEGF-dependent induction of MEF2C turns on a negative feedback system that contributes to resolving potentially overshooting mechanisms of angiogenesis by upregulation of A2M. It is also possible that this mechanisms works to dampen initiation of inappropriate sprouting.

6.6. In analogy to A2M/MEF2C the guidance cues UNC5B and SEMA3G mediate the inhibitive effects of HLX

Control of angiogenesis by negative feedback seems to be a crucial mechanism, since in a parallel analysis to MEF2C we could show that the transcription factor HLX, which is specifically induced by VEGF with earlier kinetics than MEF2C, also inhibits angiogenic sprout formation when overexpressed in ECs (Testori et al., 2011). In a comparable way to MEF2C, also for HLX target genes were defined by gene profiling, and especially a functional group of guidance molecules was found to be prominently induced. This comprised UNC5B, PlexinA1 and SEMA3G, which are known to be inhibitive guidance molecules mediating retraction of angiogenic sprouts upon ligand binding. These proteins provide guidance to the growing endothelial sprout in a way analogous to the guidance of the neuronal growth cone by isomeric forms of these molecules (Eichmann et al., 2005). Generally, it is now an established concept that the blood vessel system is wired in the body in parallel with the nervous system using analogous developmental strategies. The finding of upregulated repulsive guidance cues outright suggested an explanation for the inhibitive effect of HLX. To demonstrate a direct connection between upregulation of this inhibitive guidance cues und the anti-angiogenic effects of HLX overexpression, HUVECs were transduced with lentiviruses to individually silence the target genes of interest using shRNA strategies as described above for MEF2C. Silencing of UNC5B caused attenuation to baseline of the inhibitive effect of HLX overexpression on sprouting, demonstrating that UNC5B at least in part mediates the inhibitive effects of HLX. Furthermore, also ablation of SEMA3G partially rescued HLX-dependent inhibition of sprouting. In contrast, knock-down of PlexinA1 alone could not significantly compensate the inhibitive effects of HLX overexpression indicating that UNC5B and SEMA3G are the dominant inhibitive factors mediating HLX effects (Figure 10A). However, all three molecules, UNC5B, PlexinA1 and SEMA3G, seem to be involved in slowing down basal and fine-tune VEGF-induced sprouting in the absence of HLX overexpression, since silencing of each boosted basal and VEGF-stimulated sprout formation (Figure

10B). It appears therefore that also in the case of HLX we have a prominent example of a negative feedback inhibition of importance for dampening excessive sprouting.

6.7. Counteracting roles of different transcription factors in the control of angiogenic sprouting of endothelial cells:

It seemed puzzling that from two VEGF-induced transcription factors chosen for analysis both are involved in negative regulation of sprouting. This is however not to be regarded as a general phenomenon as the data of others suggest that two additional factors selectively induced by VEGF-A, NR4A2 and EGR-3, were reported to be positively required for sprouting angiogenesis (Liu et al., 2008, Zhao et al., 2011). Furthermore, to reassure the specificity of the inhibition exerted by MEF2C and HLX in our assays we included another transcription factor, FOXF1, in parallel in our assays. In a collaborative analysis, which I performed with Dr. K. Lipnik, this factor turned out to be a paradigm for a transcription factor with stimulatory activity on sprouting (Lipnik, K., Sturtzel, C. et al., in preparation). We initially identified FOXF1 in a screening for genes differently expressed in endothelial progenitor cells (late outgrowth endothelial colony forming cells, ECFCs) compared to mature HUVECs. FOXF1 is higher expressed in ECFCs and we assume it mediates the increased sprouting capabilities of these cells in comparison to HUVEC. Principally, for ECFCs, currently a role is discussed that they integrate at sites of hypoxia or wounded vessels to stimulate the neighbouring resident endothelial cells (Asahara et al., 1997; Critser et al., 2011)

When FOXF1 is adenovirally overexpressed this transcription factor increases sprout formation of ECs. This FOXF1-induced sprouting was delineated to the upregulation of Notch2. On the one hand FOXF1 as well as Notch2 overexpression induced sprouting to a similar extent, on the other hand when Notch2 expression was silenced, FOXF1-induced sprouting was ameliorated. This supports that FOXF1 via upregulation of Notch2 is a major mediator of

sprouting capabilities (Lipnik K, Sturtzel C. et al, in preparation).

Taken together these data suggest that angiogenic sprouting is controlled by a complex overlay of several opposing transcriptional regulatory circuits.

6.8. Potential roles of MEF2C/A2M and HLX/UNC5B/SEMA3G in adapting of angiogenic sprouting to the oxygen gradient

Hypoxia is the major trigger to initiate neovascularization via sprouting angiogenesis. Generally when a tissue is not supplied with enough oxygen it will produce and release VEGF to stimulate blood vessel growth (Hickey and Simon, 2006). Therefore, endothelial cells are highly adapted to function under a metabolic stress condition like hypoxia. When we cultured them under hypoxia (1-5% oxygen), they still presented a very regular morphology and proliferated without visible impairment.

Given the surprising finding that two of four specifically VEGF-induced transcription factors inhibited sprouting angiogenesis we reasoned that this might be related to a modulation of transcriptional regulation exerted by oxygen availability. Since our gene profiling studies were performed under normoxic conditions we considered the possibility that under hypoxic conditions, as they prevail when angiogenesis takes place, these inhibitory mechanisms would be less pronounced. Indeed, we found that under hypoxic conditions the VEGF-mediated induction of MEF2C and HLX is strongly reduced. Even more prominently, the upregulation of their target genes, A2M and UNC5B/SEMA3G, respectively, was nearly abrogated (Figure 11A and Testori et al., 2011). This correlated with the observation that also the inhibitory effects of MEF2C (Figure 11B) as well as of HLX (Testori et al., 2011) on sprouting is ameliorated.

Recapitulating, this presents a picture, where MEF2C/A2M and HLX/UNC5B/SEMA3G function as negative feedback regulatory mechanisms in EC to adapt sprouting activities to the oxygen gradient. They seem to work like brakes on angiogenesis, which are released when oxygen concentration is low and put on again when oxygen concentration rises. This will ensure on the one

hand that angiogenesis is turned off again when new vessels have been formed and oxygen concentration is restored. On the other hand it will reduce and prevent inappropriate sprouting activity of endothelial cells in areas with sufficient oxygen even in the presence of VEGF.

Thus we propose that angiogenesis is not only regulated positively by VEGF-A, which may be produced in excess in response to hypoxia, but also negatively by reducing and adapting the sprouting capabilities of EC when oxygen levels are increasing, a function which is mediated by the upregulation of the transcription factors MEF2C and HLX and their target genes.

6.9. Additional evidence for a role of MEF2C and A2M in EC

There is further accumulating evidence that MEF2C may be important for EC from several findings. MEF2C, similar to HLX, was described to be present in ECs in the moment of active sprouting. Actually both factors were shown by gene profiling to be overrepresented at the mRNA level in tip cell-enriched (DII4+/-) murine cornea tissue samples (del Toro et al., 2010). Although this can be interpreted at the first glance to suggest a positive role for sprouting, it is also conceivable that tip cells (or fresh sprouts) are just prepared to get inhibited as soon as they reach tissue areas with increasing oxygen. In regard of HLX the best in vivo evidence for its importance is currently available from the Zebrafish model. In this model HLX turned out to be essential for correct intersegmental vessel formation and to be rather a marker for stalk cells in these vessels. So far for MEF2C no similar data in the Zebrafish are yet available, as primarily the strong cardiac phenotype of MEF2C downmodulation has been analyzed (Herbert et al., 2012; Hinitz et al., 2012)

Furthermore, it was demonstrated that after induction/ phosphorylation by BMK1/ERK5 MEF2C in cooperation with Klf2 installs cell quiescence and VEGF resistance, typical characteristics of the phalanx cells (del Toro et al., 2010; Kanoh et al., 2012; Wang et al., 2010; Wu et al., 2010). In this context it was shown that mice bearing an EC-specific knock out of BMK1 exhibited a similar

phenotype to the MEF2C global knock-out mice. Importantly, it has been shown before that the global MEF2C knock-out displays embryonic lethality at day E10.5 with primarily cardiac, but also vascular defects (Hayashi et al., 2004). Recently, MEF2C was eventually specifically ablated in EC, but these mice, in the absence of any challenge, were found to be viable and not to exhibit major defects in the vascular development. However, when these mice were subjected to oxygen-induced retinopathy, which results in blindness subsequent to massive vessel growth in the retina, it was discovered that ablation of MEF2C stimulated this pathological vessel growth. This observation of an anti-angiogenic function of MEF2C there for example in retinal endothelial cells (Xu et al., 2012) modulated by oxygen concentrations goes in line with our findings.

In this context it may be of relevance that all tissue specific MEF2C knock out mice constructed so far are more or less viable. This holds also true for the cardiomyocyte specific ablation of MEF2C, although an important role of MEF2C for differentiation of cardiomyocytes is well established (Vong et al., 2005). It may therefore be that redundancy among the different MEF2 isoforms can compensate for more detrimental effects. The lethality of the global knock out might represent an additive effect of several, individually mild, developmental impairments, all together not tolerable. Furthermore, as we have shown for the negative feedback inhibition exerted by MEF2C as well as HLX in the presence of oxygen, both factors seem to work in a redundant way to limit sprouting. It could therefore be that results obtained with individual factors could be blurred for this reason and a combination of EC-specific HLX and MEF2C knock-outs should be analyzed.

In regard of A2M additional data support the concept that the protein can be involved in prevention of tissue damage. For example, pathologies such as COPD (chronic obstructive pulmonary disease), rheumatoid arthritis and even cancer were reported to correlate with A2M deficiency. This results in excess activity of metalloproteases leading to tissue injuries or metastatic tumor spreading (Kano et al., 2012; Mocchegiani et al., 2011; Tchetverikov et al., 2003). It is therefore conceivable that A2M has a similar role during angiogenesis

where EC release considerable amounts of MMPs to degrade the ECM to allow the invasive migration of the EC sprout. Moreover, in addition to reducing metalloprotease activity, A2M binds VEGF competing with heparin and may deplete excessive VEGF as well as reduce its systemic spread via the blood stream (Soker et al., 1993).

7.Appendix:

Downmodulation of A2M by shRNA:

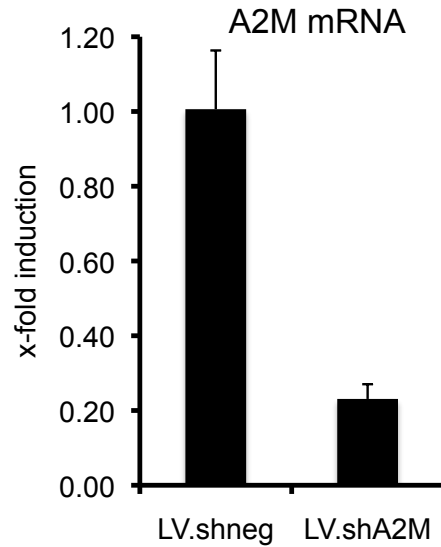


Figure 12: Transduction with LV.shA2M downmodulates A2M mRNA expression: Subconfluent HUVEC were infected with LV.shneg or LV.shA2M viral particles containing supernatant for 48 h, then harvested in TRIzol, total RNA was isolated and subjected to cDNA synthesis. β Actin served as internal control for realtime RT-PCR. 1 representative result from 3 is depicted.

Genes regulated by MEF2C- lists of microarray results

Gene Symbol	0 h	AdV.con			AdV.MEF2C		
		8 h	16 h	32 h	8 h	16 h	32 h
MEF2C	558.47	1.01	0.95	0.77	12.42	21.96	29.05
A2M	85.37	1.12	0.92	1.08	0.90	2.97	11.80
RAD51AP1	213.24	1.01	0.48	0.06	0.53	0.75	6.24
SELE	27.82	1.22	1.34	2.69	1.08	2.96	5.64
STC1	38.96	1.10	1.06	1.09	0.89	1.30	3.81
CDRT1	60.68	0.89	0.71	0.65	0.74	1.78	3.69
LOC642533	116.47	1.20	0.92	0.48	0.82	0.76	3.56
ADAMTS9	606.31	1.14	0.64	0.59	0.40	1.46	3.18
CDC6	485.05	0.78	0.38	0.08	0.49	0.80	2.84
HIST1H3B	641.10	1.16	0.81	0.08	0.57	0.61	2.75
POSTN	48.45	0.81	2.19	1.95	4.43	1.08	2.66
CD34	109.20	0.99	0.97	1.34	1.20	1.30	2.65
SPC25	175.90	1.03	0.56	0.09	0.50	0.65	2.51

Table 2 List of genes upregulated more than 2.5-fold by MEF2C: Total RNA was isolated from HUVECs after infection with MEF2C-encoding (AdV.MEF2C) and control adenoviruses (AdV.con) for 8, 16 and 32 hours. RNA was subjected to microarray analysis using Affymetrix Human Gene Level 1.0 ST GeneChips. Changes in gene expression induced in AdV.MEF2C relative to AdV.con-infected cultures are displayed as fold-induction at the indicated timepoints. Values at timepoint 0h represent random expression intensities measured in the microarray analysis, values below 25 were excluded.

Gene Symbol	uninf	AdV.con			AdV.MEF2C		
		8 h	16 h	32 h	8 h	16 h	32h
HSPA6	74.55	0.96	0.99	24.86	1.14	4.93	0.20
CROT	106.45	1.21	3.94	13.97	3.50	0.54	0.33
HSPA6	214.41	1.31	0.95	4.60	0.88	2.00	0.36
INHBA	213.67	0.93	0.85	1.40	0.99	0.63	0.38
ART4	221.43	1.17	1.97	2.87	2.30	0.66	0.40
ST8SIA6	63.08	1.57	4.38	14.48	3.19	0.56	0.42
IGSF9B	53.72	1.13	1.42	1.05	1.13	0.72	0.43
DKK2	41.89	1.04	0.90	3.29	0.97	0.93	0.43
SPINK5	36.65	0.94	1.24	2.88	1.02	0.83	0.43
ESM1	789.59	1.01	0.51	0.35	0.46	0.81	0.43
ANKRD1	4908.85	0.94	0.84	0.88	0.95	0.77	0.44
IL7R	45.12	1.28	1.47	2.80	0.70	0.90	0.44
SNAI1	72.99	0.93	0.97	1.32	0.99	0.70	0.44
C8orf4	48.39	1.46	3.68	6.37	1.96	0.59	0.44

Table 3 List of genes downregulated below 0.45-fold expression by MEF2C: Total RNA was isolated from HUVECs after infection with MEF2C-encoding (AdV.MEF2C) and control adenoviruses (AdV.con) for 8, 16 and 32 hours. RNA was subjected to microarray analysis using Affymetrix Human Gene Level 1.0 ST GeneChips. Changes in gene expression induced in AdV.MEF2C relative to AdV.con-infected cultures are displayed as fold-induction at the indicated timepoints. Values at timepoint 0h represent random expression intensities measured in the microarray analysis, values below 25 were excluded.

List of Primer Sequences:

A2M	fw 5' ATGTGAGCCGGACAGAAGTC 3' rev 5' CTGGGACATCTTGCAGAACC 3'
SELE	fw 5' GGAAGCTACCAAAAGCCTTC 3' rev 5' CCAGAGACCCGAGGAGAGTT 3'
MEF2C	fw 5' CATCCACTGCCACCATCTGC 3' rev 5' CGTGTGTTGTGGGTATCTCG 3'
β2M	fw 5' GATGAGTATGCCTGCCGTGTG 3' rev 5' CAATCCAAATGCGGCATCT 3'

Table 4: List of Primer Sequences used in realtime RT-PCR

List of Abbreviations:

A2M	alpha-2-macroglobulin
bFGF	basic fibroblast growth factor
bp	Base pair
cDNA	complementary DNA
d	days
DNA	deoxyribonucleic acid
EC	endothelial cells
ECM	extracellular matrix
EGF	epidermal growth factor
EGM	endothelial growth medium
FCS	fetal calf serum
GFP	green fluorescent protein
h	hours
HLX	homeobox like transcription factor
HUVEC	human umbilical vein endothelial cells
IL-1	interleukin 1
MAPK	mitogen activated protein kinases
MEF2C	myocyte enhancing factor
min	minutes
MOI	multiplicity of infection
PBS	phosphate buffered saline
PIGF	placental growth factor
realtime RT-PCR	realtime reverse transcription polymerase chain reaction
RT	room temperature
SDS	sodium dodecylsulphate
sec	seconds
shRNA	short hairpin RNA
SMC	smooth muscle cells
VEGF	vascular endothelial growth factor

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Curriculum Vitae

Name: Caterina Sturtzel

Education:

January 2008- present	PhD studies in Molecularbiology, University of Vienna and Medical University of Vienna Title of Thesis: Function and Role of the Transcription Factor MEF2C during Angiogenesis
October 2001- November 2007	Diploma studies in Molecularbiology, University of Vienna, main subjects: Biochemistry, Immunology and Bioorganic Chemistry, passed with distinction
January 2004- February 2006:	laboratory technician for buildup and maintance of RNAi-drosophila library, IMBA Vienna, Group Barry Dickson
September 1993- May 2001	High School, Albertus-Magnus Gymnasium, 1180 Vienna, main subjects: Mathematics, Chemistry, Biology, passed with extinction

Additional Training:

EBC*L Europäischer Wirtschaftsführerschein Stufe A und Stufe B
Introductive course into Good Clinical Practice

List of Publications:

Sattler S, Reiche D, Sturtzel C, Karas I, Richter S, Kalb ML, Gregor W, Hofer E., The human C-type lectin-like receptor CLEC-1 is upregulated by TGF- β and primarily localized in the endoplasmic membrane compartment. Scand J Immunol. 2012 Mar;75(3):282-92.

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Lehmann D, Spanholtz J, Tordoir M, Sturtzel C, Schlechta B, Hofer E. IL-12 directs further maturation of ex vivo differentiated NK cells with improved therapeutic potential (submitted to Stem Cells Dev)

Sturtzel C, Testori J, Schweighofer B., Bilban M, Hofer E. The transcription factor MEF2C negatively controls angiogenic sprouting of endothelial cells depending on the oxygen gradient (in preparation)

Lipnik K, Sturtzel C, Strasser B, Testori J, Hofer-Warbinek R, Bilban M, Jandrositz A, Preisegger KH, Gunsilius E, Hofer E. FOXF1 mediates endothelial progenitor functions and regulates vascular sprouting via the Notch pathway (in preparation)

Oral Presentations:

1st Vascular Biology Meeting, Vienna, 2013, "VEGF-A-mediated transcriptional responses in endothelial cells display striking differences between normoxia and hypoxia"

Poster Presentations:

Gordon Research Conference, Ventura CA, 2013, "Vascular Cell Biology"

17th International Vascular Biology Meeting, Wiesbaden DE, 2012

Joint Meeting of the European Society for Microcirculation, München DE, 2011

The EMBO Meeting, Vienna AT, 2011

8th International Symposium on the Biology of Endothelial Cells, Zürich SW, 2011

4th International Meeting on Angiogenesis, Amsterdam NL, 2011

7th International Symposium on the Biology of Endothelial Cells, Vienna AT, 2009

Grants and Awards:

Leistungsstipendium, 2006, granted by the University of Vienna

DOC-fORTE Fellowship, 2009-2011 granted by the Austrian Academy of Sciences,

Immunotools Award 2012 for PhD Students, Immunotools GmbH, Germany