Aus der Universitätsklinik für Anaesthesiologie und Intensivmedizin

Ärztlicher Direktor: Professor Dr. K. Unertl

Role of Semaphorin 7A in Hypoxia

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von:
Daniel Philipp Oliver Napiwotzky,
aus
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2. Berichterstatter: Professor Dr. T. Schroeder

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Abbreviations, Glossary

A549	carcinomic human alveolar basal epithelial cells
AA	amino acid
AB	antibody
ATG	adenin, thymin, guanine a startcodon in the genetic code
BCA	bicinchoninic acid
BM	basolateral membrane
bp	base pair
CaCo-2	human colorectal adenocarcinoma cells
CAM	cell adhesion molecule
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
ChIP	Chromatin Immunoprecipitation
CMV	cytomegalovirus
CO_2	carbon dioxide
DC	dendritic cells
DNA	desoxyribonucleic acid
DNAse	deoxyribonuclease
D-PBS	dulbeccos phosphate buffered saline
EC	endothelial cell
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
G418	geneticin
GM-CSF	granulocyte-macrophage -colony stimulating factor
GPI	glycosylphosphatidylinositol
GTP	guanine triphosphate
HBS	HIF-1 binding site
HBSS	Hanks' balanced salt solution

HIF	hypoxia inducible factor
HIF-1α	hypoxia inducible factor-1α
HMEC-1	human microvascular endothelial cells
HRE	hypoxia responsive element
HRP	horseradish peroxidise
hrs	hours
ICAM	intracellular cell adhesion molecule
IF	immunofluorescence
IgG	immunoglobulin G
IL8	interleukin 8
JMH	John Miller Hagen blood group antigen
ko	knock-out
MAPK	mitogens activated protein kinase
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
NE	neutrophil elastase
NO	nitric oxide
O_2	oxygen
ORF	open reading frame
PCR	polymerase chain reaction
PMN	polymorphonuclear leukocyte
PSI	plexin-semaphorin-integrin
PVDF	polyvinylidenfluorid
RIPA	radio immunoprecipitation assay
RNA	ribonucleic acid
RT	room temperature
Sema	semaphorin (animal)
SEMA	semaphorin (human)
siRNA	small interfering ribonucleic acid

TEM	transendothelial migration
TEMED	N,N,N′,N′-tetramethylethylenediamine
TGF	transforming growth factor
TIG	transcription factors immunoglobin
TSS	transcription start site
VBF	vascular barrier function
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
WB	western blot
WT	wildtype

Table 1: Abbreviations, Glossary

List of Figures and Tables

1 Introduction

1.1 Transmigration of leukocytes in response to hypoxic conditions

Medicine nowadays is often confronted with acute inflammation and hypoxic conditions in various organ tissues. Pathologies like Respiratory Distress Syndrome, inflammation or infarcts are the most lethal diseases and still needed to be researched for a better understanding and to improve existing therapies. Hypoxia is caused by an inadequate supply of oxygen, results in increased vascular permeability and invasion of immune cells similar a process termed hypoxic inflammation.[2] As described in 1901 by Mechnikov leukocytes have to be able to transmigrate to the sites of tissue hypoxia or acute inflammation.[3] A malfunction in this transmigration process can lead to tissue destruction, organ dysfunction or failure.[4]

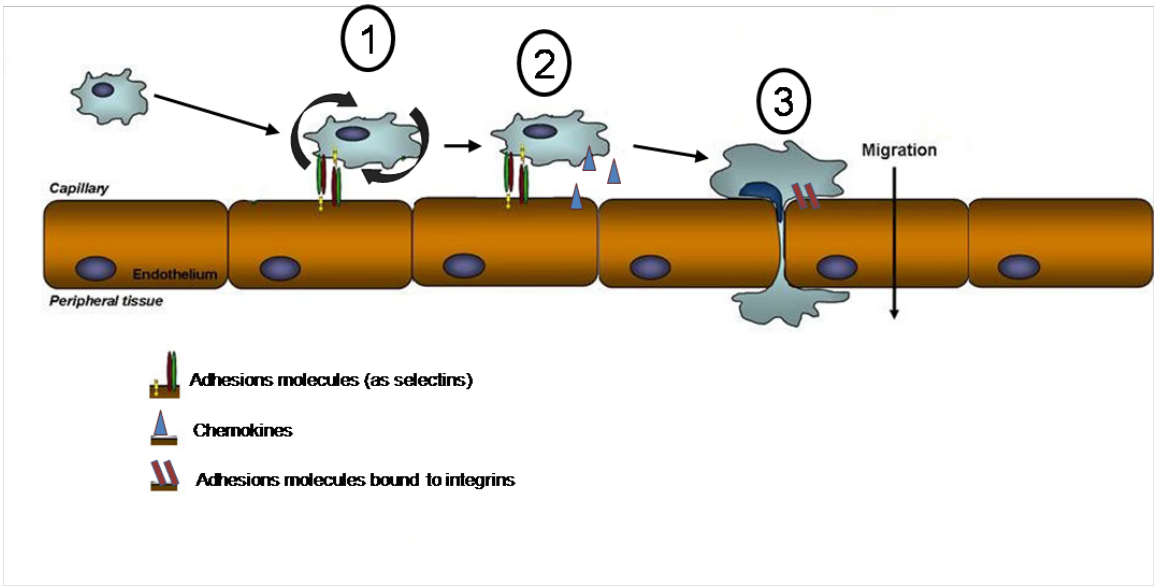

Figure 1: Transmigration process of leukocytes

① Rolling adhesion: leukocytes are slowing down by rolling along the endothelial surface and connect to the EC initiated by selectins, adressins and a stimulus like inflammation or hypoxia

② Chemokine-activation: expression of adhesion factors on leukocytes and the cell surface chemokine binding activates integrin 1α and 1β

③ Consolidated adhesion: cell adhesion molecules on endothelial cells bind to integrin on leukocytes. An outside-in signal of selectins on leukocytes enhances the affinity for integrins, which leads to the connection between intracellular adhesion molecules and vascular cell adhesion molecule. This connection causes the transmigration process through the cell layer.

The transmigration of leukocytes starts with the rolling adhesion①, initiated by selectins, adressins and an stimulus like inflammation or hypoxia.[5] Selectins are glycoproteins in the plasmatic membrane which belong to the cell adhesion molecule (CAM) family (Figure 1). The next step is chemokine-activation② through the expression of adhesions factors on leukocytes and on the EC surface. Chemokine-binding to a G-Protein receptor activates integrin 1α and 1β. After the activation the consolidated adhesion③ occurs; an outside in signal of selectins on leukocytes enhances the affinity for integrins, which leads to the connection between intracellular adhesion molecules (ICAM) and vascular cell adhesion molecules (VCAM). This connection of activated leukocytes inducing integrins leads to transmigration, also known as diapedesis.[6-10]

Chemokines are the important factor for whether or not a leukocyte passes through the vascular barrier.[11-12] Around 50 chemokines have been identified to date. Some chemokines just affect specific subtypes of the leukocytes, for example IL8 mainly affects neutrophils and macrophages and thus influences the early immune response.[13]

Recent studies have demonstrated that other proteins besides chemokines namely some of the neuronal guidance molecules, also can influence immune dependent transmigration processes. These proteins were initially described to guide axon growth cones during neuronal development, cell bound or soluble with an attracting or repulsive effect. Several neuronal guidance molecules were also reported to influence the leukocyte transmigration.[14-17] Five families of these guidance molecules have been described so far: netrin, slit, ephrin, repulsive guidance molecule (RGM) and semaphorin. Some examples for the functions of these neuronal guidance molecules are: Netrin 1 has been shown to attenuate leukocyte traffic in inflammation due hypoxia.[16] Slit 2 has been revealed to be a regulator of T-cell migration.[18] Ephrins coordinate developmental processes in the embryogenesis and also the normal physiology and homeostasis of many adult organs.[19] RGMs functions, ranging from axonal guidance during development (RGMa) to regulation of systemic iron metabolism (RGMc).[20]

1.2 Semaphorins

The name semaphorin is derived from “semaphore”, which means “information transport over a signaling system”.[21] The first described semaphorin, Sema1a, formerly known as fasciclin IV, was found in 1992 in the nervous system of grasshoppers.[22] Only shortly afterwards collapsin, now known as Sema3a, was described as a protein which induces growth cone collapse in chicken brain membrane.[23] Subsequently, an antigen with a high frequency on red human blood cells, the John Milton Hagen (JMH) blood group antigen, which was first described in the 1970s, was discovered to be a glycosylphosphatidylinositol (GPI)-linked semaphorin (SEMA7A; also known as CDw108).[24-27]

Semaphorins are expressed in different organ systems in addition to the central nervous system. For example, they are expressed in the cardiovascular, endocrine, gastrointestinal, hepatic, immune, musculoskeletal, renal, reproductive and respiratory systems. Their impact on neuronal development like neuronal path finding and axon guidance is well described. Semaphorins play an important role controlling which area neurons expand or where the neurons re-grow after injury.[8-9,28-31] Given the common mechanisms vessels and nerves share, semaphorins are also involved in vessel guidance.[32] In this context, semaphorins are thought to have an influence on direct tissue morphogenesis through cellular processes such as adhesion, aggregation, fusion, migration, patterning, process formation, proliferation, viability, and cytoskeletal organization.[33-41] Furthermore, there are studies showing the role of semaphorins in several pathologies, such as epilepsy, retinal degeneration, cancer, Alzheimer’s disease, motor neuron degeneration, schizophrenia, and Parkinson’s disease.[42-45]

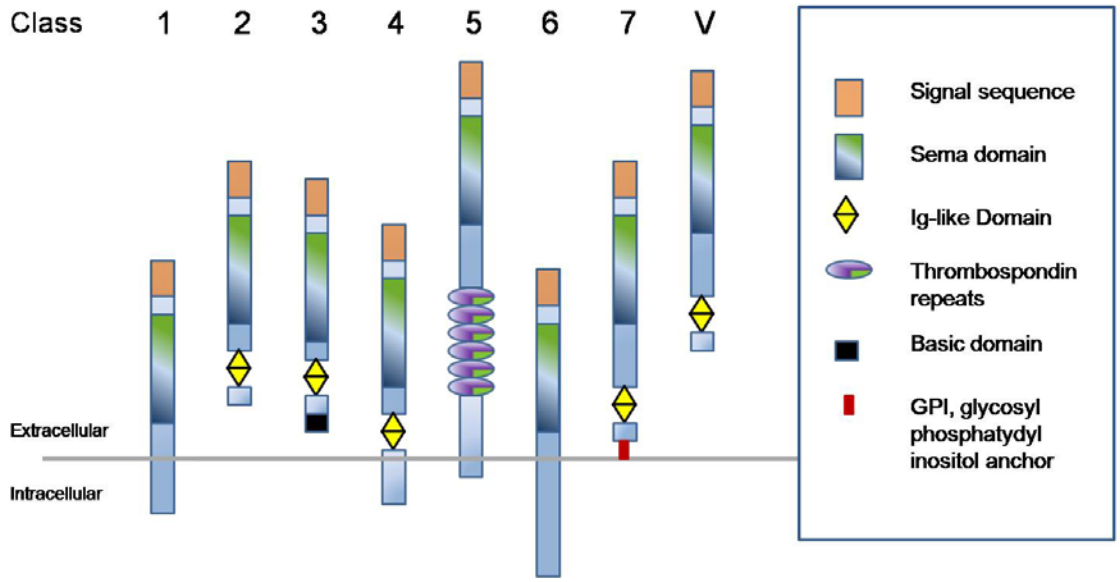

Figure 2: The semaphorin family

8 subclasses of semaphorins are described with a total of 20 proteins. Semaphorins are proteins with a typical amino-terminal, cystein-rich 'Sema' domain. Classes 1 and 2 are expressed in invertebrates, classes 3 - 7 belong to vertebrates and class V is encoded by DNA viruses. An additional distinctive feature is their membrane association: Classes 1, 4-7 are membrane-associated whereas classes 2, 3 and V are secreted.

The vertebrate semaphorin 7A (SEMA7A (human), Sema7a (animal), also known as CDw108 or Sema K1) is a neuronal guidance molecule. It was first described in 1998[46-47] and is a membrane-associated glycosylphosphatidylinositol (GPI) linked protein consisting of 666 amino acids (AA). Sema7a was identified during the search for vertebrate homologues of virally encoded semaphorins.[46-48] The gene locus of SEMA7A is 15q22.3-q23, which includes 2001 base pairs (bp) structured in 14 exons and 13 introns. SEMA7A is a polypeptid which contains a 391 AA 'SEMA' domain[49-50] and a plexin semaphorin integrin (PSI) domain with 38 AA. SEMA7A also has 3 relevant HIF binding sites (see figure 3 and 4).

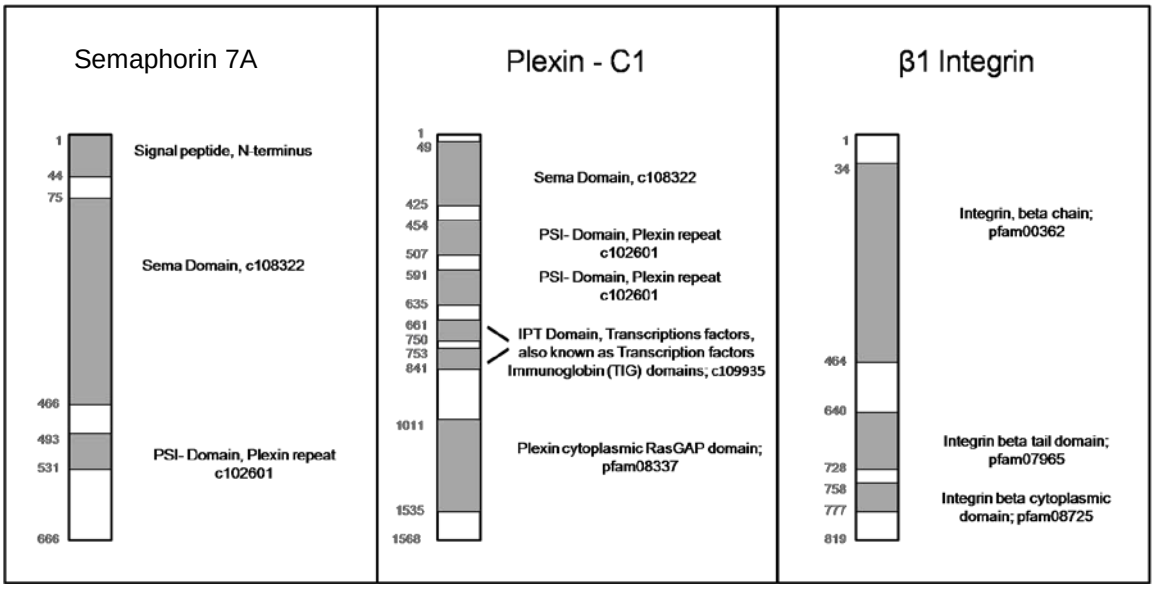

Figure 3: Protein structure of semaphorin 7A and its receptors

The protein structure of transmembrane proteins plexin and integrin show their binding abilities for SEMA7A. Especially the PSI (**P**lexin **S**emaphorin **I**ntegrin) domains show the close connection between these three proteins.

Semaphorin 7A

Figure 4: HIF Binding sites

Semaphorin 7A has three relevant hypoxia inducible factor (HIF) binding sites (HBS). Based on Roland Wenger et al.[1] and the online tool mat inspector, the HBS are located at -314 to -318, -718 to -722 and -914 to -918, relative to major transcription start site (TSS).

Semaphorin receptors table

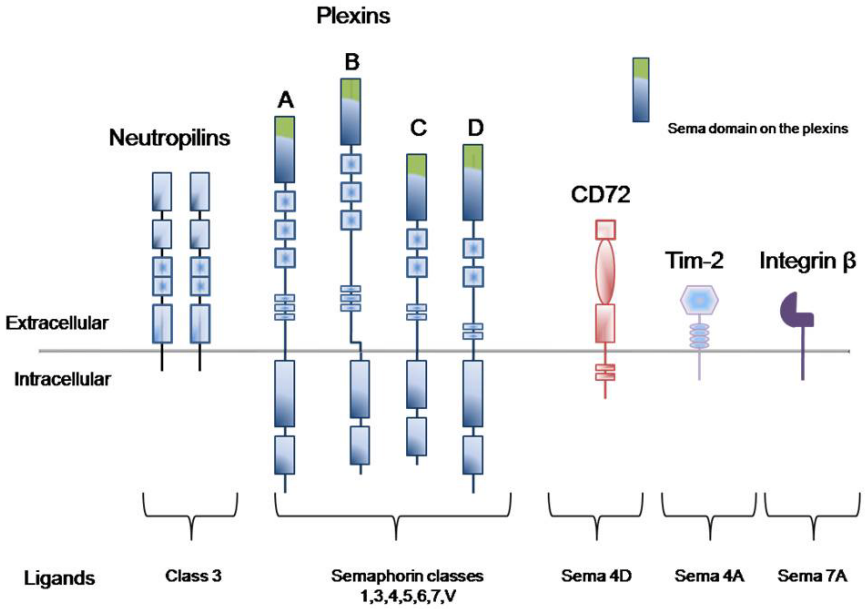

Figure 5: Important semaphorin receptors

SEMA7A	β1 integrin	SEMA7A is expressed on activated T-cells; it stimulates macrophages through α1 β1 integrin to promote inflammatory responses. Also SEMA7A activates integrin β1 and MAPK (mitogen activated protein kinase), resulting in neurite extension, cytokine production, migration of neurites, monocytes and bone cells.[49]
	Plexin C1	Plexin C1 inactivates cofilin, this leads to decreased adhesion and migration. Plexin C1 is also described as a tumor suppressor in melanoma progression through its effects on cofilin. Experiments showed: if Plexin C1 is silenced, melanoma cells present enhanced adhesion and spreading in response to SEMA7A.[51]

Table 2: Important semaphorin 7A receptor table
The important receptors of SEMA7A , and their relevant functions.

During the last years SEMA7A has revealed itself as a crucial molecule taking active part in cell migration, cell spreading, cell fusion, immune responses, neural connectivity, macrophage activation, induction of cytokines production[38] and, as stated before, cell adhesion. Particularly, all these

processes need the interaction of SEMA7A with its specific receptors Plexin C1 and α1β1-integrin.[37,52-53] Activation of semaphorin 7A in the nervous and immune system occurs through specific integrin receptor interactions.[26,42,49,54-55] Integrins are important for the connection between the extracellular matrix and the cytoskeleton, they play a role in cell adhesion and influence transmigration.[52,56-58] The interaction between SEMA7A and β-integrin affects neurite extension, cytokine production and the migration of monocytes.[49,52,54] Furthermore, β-integrin binds type 1 collagen, which is a main part of the bone organic matrix, and is highly expressed on osteoclasts and osteoblasts. Both types of cells are central in bone growth, especially in bone reformation through resorbing and replacing. This implicates that SEMA7A impairs the osteoblast migration and osteoclast fusion.[37]

Plexins inhibit integrins and thus have opposite effects. An association between plexins and semaphorins is obvious because plexins consist of a cystein rich sema domain. Additionally plexins have a PSI domain a plexin – semaphorin - integrin domain which shows the tight association between these three proteins. Plexin C1 (VESPR) has a molecular weight of 200 kDA and is a transmembrane glycoprotein (see also figure 3).[59] Plexin C1 is considered a tumor suppressor protein [51], as silencing of this protein significantly enhances the adhesion and spreading of melanoma cells and leads to higher incidence of this mortal skin cancer. In fact, expression levels of Plexin C1 have been shown to be attenuated in human melanoma cells.[52] In addition, Plexin C1 signals inactivation of cofilin, a protein involved in disassembling actin filaments, which might show its influence on transmigration processes.[60-62]

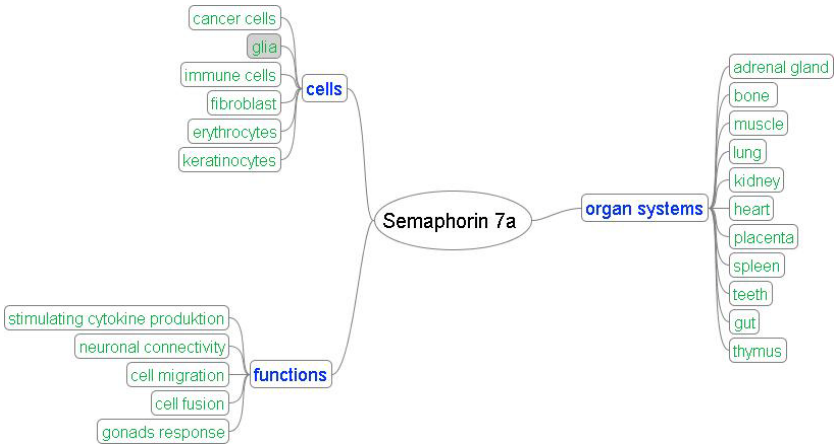

Figure 6: Semaphorin 7a allocation

An idea of the notable importance of semaphorin 7A is given by the fact that it is expressed in many organ systems and cell types of the body:
Adrenal gland[46], bone[37], cancer cells[63], cell fusion[37], cell migration[37,59], erythrocytes[63], fibroblasts [52,64], glia[65], gonads responses [59], stimulating cytokine production[59], gut[46], heart[46], kidney [46], lung [46], neural connectivity[49], lymph nodes[46], immune cells[63], muscle[46], neurons[46], placenta[46], spleen[46], teeth[66], thymus[46], keratinocytes[52].

Semaphorin 7a is expressed in many locations, amongst others lymphoid and myeloid cells[59], especially on activated T cells.[54] Its expression stimulates macrophages to produce proinflammatory cytokines. The inflammation process is also supported by the interaction between T-cells and macrophages through the previously described linkage of α1β1-integrin with Sema7a.[54] Sema7a has also been reported to be a negative regulator of T-cell-mediated inflammatory responses.[67] Further evidence of the role of Sema7a as immunomodulator is given by the fact that Sema7a-defective mice show attenuated cell-mediated immune responses such as contact hypersensitivity and autoimmune disorders.[54] In this matter, Sema7a has been related to several autoimmune inflammatory pathologies like rheumatoid arthritis,[68] encephalomyelitis.[54] Even its JMH antigen has been associated with a clinically benign autoimmune disorder.[49] In addition, other studies have shown soluble SEMA7A acting as a strong chemotractant and stimulator of monocytes.[49,59] A connection between Sema7a and the progression of lung fibrosis, which depends on stimulation by the transforming growth factor, has even been found.[69] Taken together, the role of Sema7a on different inflammatory processes is evident.

1.3 Scientific hypothesis

As mentioned above, semaphorins hold key influence on the transmigration process of cells during inflammation. The aim of this study was to highlight the role of SEMA7A and Sema7a during periods of hypoxia. Several class 3 semaphorins (3E, 3A and 3F) have been described to be induced through hypoxia inducible factor 1 (HIF-1).[70] Given these results, HIF-1α might as well be important in hypoxia-regulated processes of SEMA7A and Sema7a. Also the well described cell adhesion influence of semaphorin leads to the following hypotheses.[49,51] Based on the influence of hypoxia on the barrier function, and given the contribution of Sema7a in cell adhesion, we hypothesized a regulation of SEMA7A expression levels by hypoxia with HIF-1α is an important factor. Additionally we assume a possible role of SEMA7A in regulating barrier function and the extravasation of polymorphonuclear neutrophils (PMN). So the central questions for this thesis are:

1. Is SEMA7A and Sema7a expression influenced by hypoxia?
2. What is the role of HIF-1α binding sites on the hypoxia induced induction?
3. Is up regulated SEMA7A leading to increased transmigration through the membrane barrier?
4. Can the induction of Sema7a be confirmed in a murine model of hypoxia?

2 Materials and Methods

2.1 Materials

2.1.1 Cell culture

Name of product	*Product number*	*Company*	*Registered office*
HMEC-1 human microvascular endothelial cells	-	Gift from F. Candal	Centers for Disease Control, Atlanta, GA, USA
Endothelial Cell Growth Medium MV	C-22020	Promocell	Heidelberg, Germany
Supplement Mix / Endothelial Cell Growth Medium MV	C-39225	Promocell	Heidelberg, Germany
MCDB 131 1x	10372	Invitrogen Corp.-Gibco	Carlsbad, CA, USA
F-12 Nutrient Mixture (Ham)1x	21765	Invitrogen Corp.-Gibco	Carlsbad, CA, USA
L-Glutamine 200mM 100x	25030-024	Invitrogen Corp.-Gibco	Carlsbad, CA, USA
hEGF	354052	Becton Dickinson	Franklin Lakes, NJ, USA
Hydrocortisone	354203	Becton Dickinson	Franklin Lakes, NJ, USA
Accutase	L11-007	PAA Laboratories GmbH	Pasching, Austria
Foetal Bovine Serum GOLD	A15-151	PAA Laboratories GmbH	Pasching, Austria
Antibiotic-Antimycotic Solution	A5955	Sigma-Aldrich Inc.	St.Louis, MO, USA
G418-Bc Sulfate Powder Subst.	A291-25	Biochrom	Berlin, Germany
G-418 disulfate salt solution	G8168	Sigma-Aldrich Inc.	St.Louis, MO, USA

D-PBS 1x *+(CaCl2) +(MgCl2)* *-(CaCl2) -(MgCl2)*	 14040 14190	Invitrogen Corp.-Gibco	Carlsbad, CA, USA
Trypan Blue solution	T8154	Sigma-Aldrich Inc.	St.Louis, MO, USA
Venor GeM-qEP Mycoplasma Detection	11-4100	Minerva Biolabs GmbH	Berlin, Germany
Mycoplasma removal agent (MRA)	BUF035	Serotec Ltd.	Oxford, UK
Tissue Culture Flask	90076	Techno Plastic Products AG	Trasadingen, Switzerland
Cell Culture Plate *6 Well* *24Well* *12Well*	 3506 3512 3527	Corning Inc. Life Sciences	Lowell, MA, USA
Tissue Culture *Dishes* *35x10mm* *60x15mm* *94x16mm*	 627 160 628 160 633 171	Greiner Bio-One GmbH	Frickenhausen, Germany
Cell Lifter *Cell Scraper*	3008 3010	Corning Inc. Life Sciences	Lowell, MA, USA
Diaphragm Vacuum Pump	18807 417	Vacuubrand GmbH + Co. KG	Wertheim, Germany
Microscope DMIL	090-131.001	Leica Mikroskopie & Systeme GmbH	Wetzlar, Germany
Cytoperm 2	51011660	Heraeus Instruments GmbH	Hanau, Germany
LaminAir HB 2472 S GS	50033854	Heraeus Instruments GmbH	Hanau, Germany

Table 3: Cell Culture

2.1.2 RNA-Isolation, reverse transcription, PCR

Name of product	*Product number*	*Company*	*Registered office*
Ultra PURE Distilled Water, DNAse, RNAse Free	10977	Gibco	Gaitherburg, MA, USA
Ethanol absolut	A3678.1000	AppliChem	Darmstadt, Germany
iScript cDNA Synthesis Kit	170-8891	Bio-Rad Laboratories, Inc.	Munich, Germany
iQ™ SYBR® Green Supermix	170-8884	Bio-Rad Laboratories, Inc.	Munich, Germany
NucleoSpin RNA II	740955.250	Macherey & Nagel	Düren, Germany
Primer		Biomers	Ulm, Germany
2-Mercaptoethanol ≥98%	M3148	Sigma-Aldrich, Inc.	St.Louis, MO, USA
rDNAse	740963	Macherey-Nagel GmbH & Co. KG	Düren, Germany
TRIzol reagent	15596-018	Invitrogen Corp.	Carlsbad, CA, USA
Chloroform 99%	C2432	Sigma-Aldrich, Inc.	St.Louis, MO, USA
2-Propanol 99,8%	1.09634.2511	Merck KgaA	Darmstadt, Germany
iCycler iQ Optical Tape	2239444	Bio-Rad Laboratories, Inc.	Hercules, CA, USA
Primer	-	Biomers.net GmbH	Ulm, Germany
0,2ml Rigid PCR Ultra Plate	21970	Sorenson Bioscience, Inc.	West Salt Lake City, Utah, USA
Hettich Zentrifuge EBA 12 R	1002	Andreas Hettich GmbH & Co. KG	Tuttlingen, Germany
SPROUT Centrifuge	3079140	Heathrow Scientific LLC	Illinois, USA
TJ-6RS Centrifuge	340440	Beckman RIIC Ltd.	Glenrothes-Fife, Scotland

C1000 Thermal Cycler	185-1096	Bio-Rad Laboratories, Inc.	Hercules, CA, USA
iCycler Thermal Cycler	170-8740	Bio-Rad Laboratories, Inc.	Hercules, CA, USA

Table 4: RNA-Isolation, reverse transcription, PCR

2.1.3 Western Blot

Name of product	*Product number*	*Company*	*Registered office*
Skim milk	A0830.0500	AppliChem	Darmstadt, Germany
Magic Mark XP Western Standard	LC5602	Invitrogen	Carlsbad, CA, USA
SeeBlue Plus 2, Prestained Standard	LC5925	Invitrogen	Carlsbad, CA, USA
Immun-Blot-PVDF-Membrane	162-0177	Bio-Rad Laboratories, Inc.	Munich, Germany
Restore Western Blot Stripping Buffer	21059	Pierce	Bonn, Germany
Tris Glycine Buffer 10x	161-0771	Bio-Rad Laboratories, Inc.	Munich, Germany
Tris Glycine SDS Buffer 10x	161-0772	Bio-Rad Laboratories, Inc.	Munich, Germany
Methanol	1.06009.2511	Merck	Darmstadt, Germany
Detection Buffer ♦ H_2O_2		Merck	Darmstadt, Germany
Bovine Serum Albumin	A7906-50G	Sigma	Taufkirchen, Germany
ECL detection system:			
♦ *1M Tris-HCl pH 8,0*	A3404	AppliChem GmbH	Darmstadt, Germany
♦ *p-Coumeric-acid*	C9008	Sigma	Taufkirchen, Germany
♦ *Luminol*	09253	Fluka	
Temed	T9281	Sigma	Taufkirchen, Germany

beta-Actin (13E5) Rabbit mAb	4970	Cell Signaling Technology, Inc.	Danvers, MA, USA
Goat Anti-mouse IgG-HRP	sc-2005	Santa Cruz Biotechnology, Inc.	Santa Cruz, CA, USA
Goat anti-rabbit IgG-HRP	sc-2004	Santa Cruz Biotechnology, Inc.	Santa Cruz, CA, USA
Amersham Full Range Rainbow Recombinant Protein Molecular Weight Marker	RPN 800	GE Healthcare UK Ltd	Buckingham-shire, UK
Rotiphorese Gel 30	3029.1	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
Ammonium persulfate	A9164	Sigma-Aldrich, Inc.	St.Louis, MO, USA
D-PBS 1x *+(CaCl2) +(MgCl2)* *-(CaCl2) -(MgCl2)*	 14040 14190	Invitrogen Corp.-Gibco	Carlsbad, CA, USA
BCA protein assay kit	23225	Thermo Fisher Scientific, Inc.	Rockford, IL, USA
Complete - protease inhibitor cocktail tablets	11 836 145 001	Roche Diagnostics Corp.	Indianapolis, IN, USA
TWEEN 20	A4974	AppliChem GmbH	Darmstadt, Germany
Restore western blot stripping buffer	21059	Thermo Fisher Scientific, Inc.	Rockford, IL, USA
SuperSignal West Pico chemiluminescent substrate	34078	Thermo Fisher Scientific, Inc.	Rockford, IL, USA
Ponceau S solution	P7170	Sigma-Aldrich, Inc.	St.Louis, MO, USA
RIPA-buffer:			
♦ *EDTA*	A3145	AppliChem GmbH	Darmstadt, Germany
♦ *Sodium chloride*	567440	Merck KGaA	Darmstadt, Germany

♦ *Tris (→pH7.4)*	A2264	AppliChem GmbH	Darmstadt, Germany
♦ *Igepal CA-630*	I8896	Sigma-Aldrich, Inc.	St. Louis, MO, USA
Loading buffer (4x), non-reducing:			
♦ *1M Tris-HCl (→pH 6,8)*	A3452	AppliChem GmbH	Darmstadt, Germany
♦ *Glycerol*	G5516	Sigma-Aldrich, Inc.	St. Louis, MO, USA
♦ *Sodium dodecyl sulfate*	L4390	Sigma-Aldrich, Inc.	St. Louis, MO, USA
♦ *Bromophenol blue*	A3640	AppliChem GmbH	Darmstadt, Germany
Upper buffer:			
♦ *0,5M Tris*	A2264	AppliChem GmbH	Darmstadt, Germany
♦ *0,4% Sodium dodecyl sulfate (→pH 6,8)*	L4390		
Lower buffer:			
♦ *1,5M Tris*	A2264	AppliChem GmbH	Darmstadt, Germany
♦ *0,4% Sodium dodecyl sulfate (→pH 8,8)*	L4390		
10x Tris/Glycine buffer *10x TGS - Tris/Glycine /SDS buffer*	161-0771 161-0772	Bio-Rad Laboratories, Inc.	Hercules, CA, USA
TBS:			
♦ *1000ml A.d.*			
♦ *24,2g Tris*	A2264	AppliChem GmbH	Darmstadt, Germany
♦ *80g Sodium chloride*	567440	Merck KGaA	Darmstadt, Germany
♦ *Hydrochloric acid (→pH 7,6)*	100319	Merck KGaA	Darmstadt, Germany

Detection buffer BCIP/NBT: ♦ *220ml A.d.* ♦ *5ml 5M NaCl* ♦ *25ml 1M Tris-HCl* ♦ *BCIP* ♦ *NBT BioChemica*	 567440 A2264 A1117 A1243	 Merck KGaA AppliChem GmbH	 Darmstadt, Germany Darmstadt, Germany
Gel cassettes 1,5mm	NC2015	Invitrogen Corp.	Carlsbad, CA, USA
F-bottom microplate 96 well	655101	Greiner Bio-One GmbH	Frickenhausen , Germany
Trans-blot cell electro-phoretic transfer cell	36S/2832	Bio-Rad Laboratories, Inc.	Hercules, CA, USA
Ready gel cell	108BR01057	Bio-Rad Laboratories, Inc.	Hercules, CA, USA
XCell SureLock electrophoresis cell	1167482-625	Invitrogen Corp.	Carlsbad, CA, USA
Powerpac 3000	277BR	Bio-Rad Laboratories, Inc.	Hercules, CA, USA
Centrifuge 5417 R	5407	Eppendorf AG	Hamburg, Germany
Incubator	T6060	Heraeus Holding GmbH	Hanau, Germany
Speci-Mix platform mixer	M33120	Thermo Fisher Scientific, Inc.	Rockford, IL, USA
RET basic C magnetic stirrer	3197601	IKA Works, Inc.	Wilmington, NC, USA
Impulse sealer TISH-200	E82163 (S)	TEW Electric Heating Equipment CO., LTD	Taipei, Taiwan
Diana multifunctional darkroom	1006	raytest GmbH	Straubenhardt, Germany

Table 5: Western Blot

2.1.4 Polymorphonuclear transmigration assay

Name of product	*Product number*	*Company*	*Registered office*
Percoll sterile ♦ *63%* ♦ *72%*	17-0891	GE Healthcare Bio-Sciences AB	Uppsala, Sweden
Coulter Isoton II diluent	8448011	Beckman Coulter GmbH	Krefeld, Germany
Coulter Clenz cleaning agent	8417-222	Beckman Coulter GmbH	Krefeld, Germany
Lysis buffer			
♦ *EDTA solution pH.8.0*	A3145	AppliChem GmbH	Darmstadt, Germany
♦ *Sodium Bicarbonate*	S-8875	Sigma-Aldrich, Inc.	St. Louis, MO, USA
♦ *Ammonium chloride*	A0988	AppliChem GmbH	Darmstadt, Germany
HBSS 1x *+($CaCl_2$) +($MgCl_2$)* *- ($CaCl_2$) - ($MgCl_2$)*	 14025 14175	Invitrogen Corp. - Gibco	Carlsbad, CA, USA
N-Formyl-Met-Leu-Phe (Chemotactic peptide; fMLP)	F3506	Sigma-Aldrich, Inc.	St.Louis, MO, USA
PSB 1115 potassium salt hydrate	P0373	Sigma-Aldrich, Inc.	St.Louis, MO, USA
Citric buffer:			
♦ *200 mM NaCitrate*	3580	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
♦ *200 mM Citric Acid*	8.18707	Merck Schuchardt OHG	Hohenbrunn, Germany
Triton 10%			
♦ *Triton X-100*	A4975	AppliChem GmbH	Darmstadt, Germany

ABTS ♦ *Citric buffer* ♦ *A.d.* ♦ *ABTS* ♦ *Hydrogen peroxide solution 30%*	 A1888 1.08597	 Sigma-Aldrich, Inc. Merck KgaA	 St.Louis, MO, USA Darmstadt, Germany
Cellstar TC tube, sterile, 12 ml	163 160	Greiner Bio-One GmbH	Frickenhausen Germany
Transwell permeable supports 3,0μm polyester membrane	3472	Corning Inc.	Corning, NY, USA
Butterfly winged needle infusion set	P293 A05	Hospira Inc.	Lake Forest, IL, USA
Multi-adapter for S-monovette	14.1205.050	Sarstedt AG & Co.	Nümbrecht, Germany
S-monovette	02.1067.001	Sarstedt AG & Co.	Nümbrecht, Germany
Softasept N	3887138	B.Braun Melsungen AG	Melsungen, Germany
Coulter Z2	72	Coulter Electronics LTD.	Luton, England
Megafuge 1.0R	75003060	Heraeus Instruments	Osterode, Germany

Table 6: Polymorphonuclear transmigration assay

2.1.5 Immunofluorescent staining

Name of product	*Product number*	*Company*	*Registered office*
Sema-7A *rabbit polyclonal antibody*	ab23578	abcam	Cambridge, UK
Pecam rat polyclonal antibody	sc101454	Santa Cruz	Santa Cruz, CA, USA
E-cadherin antibody (K20)	sc-31021	Santa Cruz Biotechnologies	Santa Cruz, CA, USA
Goat serum (normal)	X0907	Dako Denmark A/S	Glostrup, Denmark

Negative control rabbit Ig fraction (normal)	X0903	Dako Denmark A/S	Glostrup, Denmark
Alexa Fluor 488 goat anti-rabbit IgG (H+L)	A11008	Invitrogen Corp. – Molecular Probes	Eugene, OR, USA
Alexa Fluor 594 chicken anti rat	A21471	Invitrogen Corp. – Molecular Probes	Eugene, OR, USA
ProLong Gold antifade reagent with DAPI	P36931	Invitrogen Corp. – Molecular Probes	Eugene, OR, USA
PBS tablets	18912-014	Invitrogen Corp.- Gibco	Carlsbad, CA, USA
MBT pap pen	297840010	Micro-Bio-Tec-Brand	Giessen, Germany
Xylene	28975.325	VWR International S.A.S	Briare, France
Acetone	24201	Sigma-Aldrich, Inc.	St.Louis, MO, USA
Formalin solution, neutral buffered, 10%	HT5011-1CS	Sigma-Aldrich, Inc	St.Louis, MO, USA
Disposable scalpel No.11	02.001.30.011	Feather Safety Razor Co. Ltd.	Osaka, Japan
Embedding cassettes	09-0201	R.Langenbrinck Labor- & Medizintechnik	Emmendingen, Germany
SuperFrost plus object plate	03-0060	R. Langenbrinck Labor- und Medizintechnik	Emmendingen, Germany
Roti-plast paraffin pellets	6642.5	Carl Roth GmbH & Co. KG	Karlsruhe, Germany
Confocal laser scanning microscope LSM 510 Meta ^{MK4}	-	Carl Zeiss MicroImaging GmbH	Jena, Germany
Fully-enclosed tissue processor TP1050	04612711R001	Leica Microsystems GmbH	Wetzlar, Germany
Embedding center, dispenser + hot plate EG1160	14038630528	Leica Microsystems GmbH	Wetzlar, Germany

Rotary microtome RM 2235 + *disposable blades type 819*	14050038602 14035838925	Leica Microsystems GmbH	Wetzlar, Germany
Flattening bath HI1210	14041521466	Leica Microsystems GmbH	Wetzlar, Germany
Flattening table HI1220	14042321474	Leica Microsystems GmbH	Wetzlar, Germany

Table 7: Immunofluorescent staining

2.1.6 Evan's blue staining

Name of product	*Product number*	*Company*	*Registered office*
Evan's blue	E2129	Sigma-Aldrich, Inc.	St.Louis, MO, USA
Formamide	F7508	Sigma-Aldrich, Inc.	St.Louis, MO, USA

Table 8: Evan's blue staining

2.1.7 Myeloperoxidase assay

Name of product	*Product number*	*Company*	*Registered office*
ABTS	A1888	Sigma-Aldrich, Inc.	St.Louis, MO, USA
Hydrogen peroxide solution 30%	1.08597	Merck KgaA	Darmstadt, Germany
Citric buffer:			
♦ *200 mM NaCitrate*	3580	Carl Roth GmbH + Co. KG	Karlsruhe, Germany
♦ *200 mM Citric Acid*	8.18707	Merck KgaA	Darmstadt, Germany
Triton X-100	A4975	AppliChem GmbH	Darmstadt, Germany

Table 9: Myeloperoxidase assay

2.1.8 Biotinylation

Name of product	*Product number*	*Company*	*Registered office*
EZ-Link Sulfo-NHS-Biotin	21217	Pierce	Bonn, Germany
Streptavidin-HRP	21126	Pierce	Bonn, Germany
Protein-A/G beads	17-6002-35 17-0469-01 17-0618-01	Amersham Biosciences Pharmacia Biotech GE Healthcare	Buckingham-shire, UK
PBS	Gibco #18912-014	Invitrogen	Carlsbad, CA, USA
RIPA-buffer:			
♦ *EDTA*	A3145	AppliChem GmbH	Darmstadt, Germany
♦ *Sodium chloride*	567440	Merck KGaA	Darmstadt, Germany
♦ *Tris (→pH7.4)*	A2264	AppliChem GmbH	Darmstadt, Germany
♦ *Igepal CA-630*	I8896	Sigma-Aldrich, Inc.	St.Louis, MO, USA
Complete - protease inhibitor cocktail tablets	11 836 145 001	Roche Diagnostics Corp.	Indianapolis, IN, USA
PMSF *Phenylmethansulfonyl-fluorid*	A0999	AppliChem GmbH Biochemica	Darmstadt, Germany
Cell Culture Plate 6 Well	3506	Corning Inc. Life Sciences	Lowell, MA, USA
Pipetboy acu	155000	Integra Bio-sciences GmbH	Fernwald, Germany
Parafilm "M" laboratory film		Pechiney Plastic-Packaging	Chicago, IL, USA
Safe-Lock tubes 0,5ml	0030 121.023	Eppendorf AG	Hamburg, Germany
Safe-Lock tubes 1,5ml	0030 120.086	Eppendorf AG	Hamburg, Germany
Falcon blue max 50 ml	352070	Becton Dickinson	Franklin Lakes, NJ, USA

cell scrapers	541070	Greiner Bio-One GmbH	Frickenhausen, Germany

Table 10: Biotinylation

2.1.9 Antibodies

Name of product	*Product number*	*Company*	*Registered office*
Beta-Actin (13E5) Rabbit mAb	4970	Cell Signaling	Danvers, MA, USA
Goat anti-rabbit IgG-HRP	sc-2004	Santa Cruz Biotechnology, Inc.	Santa Cruz, CA, USA
Monoclonal Anti-human/mouse Semaphorin 7A (CD108) Antibody	ab90242	abcam	Cambridge, UK
SEMA-7A *(MEM-150):* *mouse monoclonal antibody raised against human T cell line HBP-ALL.*	sc-51695	Santa Cruz Biotechnologies	Santa Cruz, CA, USA
Anti-human Semaphorin 7A antibody produced in goats	AF2068	R&D Systems	Minneapolis, MN USA
Semaphorin 7A antibody *Reacts with Ms, Rat* *Tested applications* *ICC/IF, WB*	ab23578	abcam	Cambridge, UK
Semaphorin 7A antibody produced in rabbit polyclonal ICC/IF: Use at a concentration of 1-5 µg/ml. Is unsuitable for WB.	ab31449	R&D Systems	Minneapolis, MN USA
Anti-human Plexin C1 Antibody	AF3887	R&D Systems	Minneapolis, MN USA

Anti-integrin Alpha1, Clone FB12, Azide Free (MAB1973Z)	MAB1973Z	Chemicon International	Billerica, MA USA
Anti-integrin Beta1, Clone P4C10, Azide Free	MAB1987Z	Chemicon International	Billerica, MA USA
Mouse IgG1 Isotype Control	ab27479	abcam	Cambridge, UK
Anti- HIF-1α (Upstate, 07-628)	Upstate, 07-628	Millipore, Chemicon International	Billerica, MA USA
anti-Hypoxia-inducible factor 1 alpha antibody *Purified Mouse Anti-Human*	BD, 610959	BD Transduction Laboratories	San Jose, California USA, 95131
Anti -VEGF (147):	sc-507	Santa Cruz Biotechnologies	Santa Cruz, CA, USA
HIF 1 alpha antibody	NB100-105	Novus Biologicals, Inc	Littleton, CO 80160
HIF 1 alpha antibody	NB100-134	Novus Biologicals, Inc	Littleton, CO 80160
E-cadherin (K-20) Basolateral marker	sc-31021	Santa Cruz Biotechnologies	Santa Cruz, CA, USA
CD34 (MEC 14.7): Endothelial Marker	sc-18917	Santa Cruz Biotechnologies	Santa Cruz, CA, USA
VEGF (147)	sc-507	Santa Cruz Biotechnologies	Santa Cruz, CA, USA
Alexa Fluor 594 chicken anti rat	A-21471	Invitrogen	Carlsbad, CA, USA
Alexa Fluor® 488 goat *anti-rabbit IgG*	A11008	Invitrogen	Carlsbad, CA, USA
PECAM-1	sc-101454	Santa Cruz Biotechnologies	Santa Cruz, CA, USA
CD 31 antibody RM0032-1D12	ab56299	abcam	Cambridge, UK

Table 11: Antibodies

2.1.10 Transfection, Luciferase, Reporter Assay

Name of product	*Product number*	*Company*	*Registered office*
Gene Juice Transfection Reagent	70967-3	Novagen / Merck	Darmstadt, Germany
Luciferase Assay System	E1501	Promega	Madison, WI, USA
Passive Lysis Buffer 5x	E1941	Promega	Madison, WI, USA
Disposable Cuvettes	E2371	Promega	Madison, WI, USA
TD-20/20 Luminometer	2030-000	Turner BioSystems, Inc.	Sunnyvale, CA, USA
Spreadsheet Interface Software V1.0.0	-	Turner BioSystems, Inc.	Sunnyvale, CA, USA

Table 12: Transfection, Luciferase, Reporter Assay

2.1.11 Technical equipment

Name of product	*Product number*	*Company*	*Registered office*
iCycler		Bio-Rad Laboratories, Inc.	Munich, Germany
Gene expression macro	Free download	Bio-Rad Laboratories, Inc.	Munich, Germany
Novex Mini-Cell		Invitrogen	Carlsbad, CA, USA
Mini Trans-Blot Electrophoretic Transfer Cell	170-3930	Bio-Rad Laboratories, Inc.	Munich, Germany
Sterile workbench Lamin Air HB 2472		Haereus Instruments	Hanau, Germany
Photometer Ultrospec 3000 pro		Biochrom	Berlin, Germany
Thermocycler		Bio-Rad Laboratories, Inc.	Munich, Germany
Magellan software	B01760001	Tecan Group Ltd.	Männedorf, Switzerland

TD-20/20 Luminometer		Turner BioSystems, Inc.	Sunnyvale, CA, USA
Spreadsheet interface software			
Diana luminescence imaging system		Raytest	Straubenhardt, Germany
Adobe Photoshop CS 2		Adobe	
Endnote X3		Thomson Reuters	San Francisco CA USA
siRNA Wizard	http://www.sirnawizard.com	InvivoGen	San Diego, California 92121, USA
Mat inspector	http://www.genomatrix.de		

Table 13: Technical equipment

2.1.12 Things of general use

Name of product	*Product number*	*Company*	*Registered office*
Eppendorf reference pipet *0,5-10µl* *10-100µl* *100-1000µl*	 4910 000.018 4910 000.042 4910 000.069	Eppendorf AG	Hamburg, Germany
Eppendorf research pipet 500-5000µl	3111 000.173	Eppendorf AG	Hamburg, Germany
Eppendorf multipette plus	4981 000.019	Eppendorf AG	Hamburg, Germany
Pipetboy acu	155000	Integra Bio-sciences GmbH	Fernwald, Germany
Photometer Ultrospec 3000 pro + *Thermal printer DPU-414-30B*	80-2111-30 -	Biochrom Ltd. Seiko Instruments Inc.	Cambridge, UK Chiba, Japan

GENios microplate reader *+ Magellan software V5.03*	F129004	Tecan Group Ltd.	Männedorf, Switzerland
Graph Pad Prism 5	-	GraphPad Software, Inc.	La Jolla, CA, USA
Vortex genie 2	G-560E	Scientific Industries, Inc.	Bohemia, NY, USA
Tissue homogenizing system MICCRA D-9 + Pico DS-8/P	090000	Art-moderne Labortechnik e.K.	Müllheim, Germany
N811 KN.18 vacuum pump	2.537712	KNF Neuberger	Freiburg, Germany
Analytical balance HR-200	-	A & D Company, Ltd.	Tokyo, Japan
Thermomixer 5436	5436 10471	Eppendorf AG	Hamburg, Germany
Kirsch super refrigerator	8704	Glen Dimplex Deutschland GmbH	Kulmbach, Germany
Scotsman AF10 automatic ice machine	AF10ASE0600	Frimont S.p.a.	Milano, Italy
Rotating plate	AK82	Infors AG	Bottmingen, Switzerland
BD Plastipak Syringe *BD Microlance 3*	300013 302200	Becton Dickinson	Franklin Lakes, NJ, USA
Falcon serological pipet *1ml* *5ml* *10ml* *25ml* *50ml*	 356521 356543 356551 356525 357550	Becton Dickinson	Franklin Lakes, NJ, USA

Injekt single-use syringe *5 ml* *10 ml* *20ml*	 4606051V 4606108V 4606205V	B.Braun Melsungen AG	Melsungen, Germany
Nunc 1,8 ml cryotube vials	368632	Nunc A/S	Roskilde, Denmark
Parafilm "M" laboratory film		Pechiney Plastic-Packaging	Chicago, IL, USA
Latex examination gloves	PFC4303972	Ansell LTD	Bangkok, Thailand
SafeSeal-tips premium 10ml	692150	Biozym Diagnostik GmbH	Hess. Oldendorf, Germany
SafeSeal-tips premium 100ml	692066	Biozym Diagnostik GmbH	Hess. Oldendorf, Germany
SafeSeal-tips premium 1000ml	692079	Biozym Diagnostik GmbH	Hess. Oldendorf, Germany
Eppendorf *combitips plus 2,5 ml* *combitips plus 0,5 ml*	 0030 069.242 0030 069.420	Eppendorf AG	Hamburg, Germany
Safe-Lock tubes 0,5ml	0030 121.023	Eppendorf AG	Hamburg, Germany
Safe-Lock tubes 1,5ml	0030 120.086	Eppendorf AG	Hamburg, Germany
Falcon blue max 50 ml	352070	Becton Dickinson	Franklin Lakes, NJ, USA
Cellstar PP-test tubes 15 ml	188 271	Greiner Bio-One GmbH	Frickenhausen, Germany

Table 14: Things of general use

2.2 Methods

2.2.1 Cell culture

Human microvascular endothelial cells (HMEC-1) were grown in MCDB-131 medium containing 10% FCS, 0,01µg/ml EGF, 1µg/ml hydrocortisone, 10mM L-glutamine, 1% antibiotic / antimycotic solution. The Medium of the HIF-ko (knockout) cells additionally contains the antibiotic G418 (500mg/ml). These HMEC-1 cells are immortalized but they have same functionalities as regular HMEC cells. The cells, which were in use for these experiments were gifted by J. Candal from the Centers for Disease Control, Atlanta, GA, USA for further use. Before culturing, the flasks were examined with an inverted contrasted microscope to ensure that the growth rate, the confluence and the cell morphology are comparable. Additionally the cells were checked for infectious and fungal decay signs. When the adherent endothelial cells were forming a monolayer the cells were harvested from the culture flasks by adding 4 ml of Accutase and incubated for 20 minutes at 37°C. Before adding the Accutase, cells were washed with warmed D-PBS (- $MgCl_2$ and - $CaCl_2$) to remove the cell detritus and FCS.

Detached cells were centrifuged to remove the cell culture media. The cell pellet was dissolved in a defined amount of media and counted by using Trypan blue. Now the cells were ready for further use; some for experiments and some for inoculation of new culture flasks. Final concentration in a new flask was 1 x 10^5 cells/ml and confluence was reached mostly after 3 days. Mycoplasma testing was performed in existing cell cultures on a monthly basis and before cryopreservation using the Minerva Biolabs Mycoplasma Detection Kit which is highly specific and sensitive through its PCR technology.

Cryopreservation was performed using a special freezing medium containing 20% foetal bovine serum, 10% DMSO and no antibiotic-antimycotic suspension. For this purpose cells were rinsed and detached as described above, then centrifuged and resuspended in freezing medium. The cell tubes were placed in a cryofreezing container surrounded by isopropyl alcohol, put in a mechanical freezer at -80°C for 4 hours and then placed in a long-term liquid

nitrogen storage freezer. Frozen cells were thawed by rapid agitation in a 37°C water bath, washed in normal growth medium and put in 75cm^2 tissue culture flasks. Cells were maintained at 37°C, 20% O_2 and 5% CO_2.

2.2.2 Polymerase chain reaction (PCR) and Transcriptional studies

Transcriptional studies were performed using real-time RT-PCR (iCycler; Bio-Rad Laboratories Inc) to examine SEMA7A expression in human microvascular endothelial cell (HMEC-1). Confluent grown HMEC-1 cells were exposed to normobaric hypoxia (2% O_2, 98% N_2) over indicated time periods. RNA was extracted according to the manufacturer's instruction of the Macherey&Nagel NucleoSpin RNA II kit. Primer sets contained 10pM of the sense primer 5'-ACA GGG GCA CTA TCC ACA AG-3' and the antisense primer 5'-CTC AGC ATC CAG CGA CAT-3' for analysis of human semaphorin 7A. The primer set was amplified using increasing numbers of cycles of 95°C for 4 min, 63°C for 45 sec, 72°C for 45 sec, and a final extension of 72°C for 7 min. Samples were controlled for human ß-Actin using following primers: sense primer, 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3'; and antisense primer, 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3'.

Transcriptional analysis of murine semaphorin 7a was performed using the sense primer 5'-CGT GTA TTC GCT TGG TGA CAT-3' and the antisense primer 5'-GTG GGT ATG GGC TGC TTT TT-3'. Samples were controlled for murine ß-Actin using following primers: sense 5'-TCA CGC ACG ATT TCC CTC TCA G-3', antisense 5'-CTC TCC CTC ACG CCA TCC TG-3'. As additional control of hypoxia, Vascular Endothelial Growth Factor (VEGF), a well known hypoxia sensible protein was used with these primers: for human using the sense primer 5'-TTG CCT TGC TCT ACC TC-3' and the antisense primer 5'-AGC TGC GCT GAT AGA CAT CC-3'; for mouse using the sense: 5´-CAG CTA TTG CCG TCC GAT TGA GA-3´ and the antisense: 5´-TGC TGG CTT TGG TGA GGT TTG AT-3´.

2.2.3 Silencing of the HIF-1α gene

Stable silencing of the HIF-1α gene was achieved by the introduction of small interfering RNA in HMEC-1 cells. With the siRNA Wizard (http://www.sirnawizard.com) a sequence was chosen within the coding region of the gene of interest. The chosen hairpin primer with the sequence 5`-ACC TCG CTG ACC AGT TAT GAT TGT GAT CAA GAG TCA CAA TCA TAA CTG GTC AGC TT-3´ and 5'-CAA AAA GCT GAC CAG TTA TGA TTG TGA CTC TTG ATC ACA ATC ATA ACT GGT CAG CG-3' correspond to Position 2666 of the HIF-1α gene. Primers were annealed for 2 minutes at 80°C to create the hairpin structure and ligated into the Bbs1/Bbs1 digested psiRNA-hH1neo G2 vector. After transformation using the Lyocomp GT116 *E. coli* strain cells were spread on a Kan X gal agar plate with the advantage of white/blue selection. A recombinant white clone was grown, DNA was extracted and HMEC-1 cells were transfected using an electroporation procedure. Two days after transfection the appropriate concentration (1mg/ml) of G418 was added. Stable transfectants were individualized after 2-3 weeks. As control a psiRNA-hH1 neo-scr plasmid was used.

2.2.4 Gain of HIF-1α function

HIF-1α-overexpressing (ΔODD) cells were gained through a lentiviral vector design, with production and transduction in HMEC-1 cells. The HIV-1 lentiviral vector used was based on a vector previously described in detail.[71] In short, the _ODD variant of HIF-1α (containing a proline 3 alanine substitution at position 564) [72] was cloned into the Bam H1/*Cla*I sites of the lentiviral vector. The lentivirus was produced and stable cell lines were generated as described before.[73] (Characterization in Fig.14)

2.2.5 Cell Transfection

For 60mm dishes the following cocktail was used: 150 µl Media without Serum and Antibiotics and 7.5µl Gene Juice per dish. After vortexing, the mix was incubated for 5 min at room temperature. Depending on the number of

constructs to be transfected the mix was divided in different tubes and the appropriate construct was added and incubated for 10-15 min at RT. The mixture was added to each dish drop wise and further incubated for 3-6 hrs at 37 °C. Later on, the medium was removed and replaced with complete (+FCS and Antibiotics) medium. Referring to the transfection, cells were used to investigate induction of hypoxia or the cells were incubated at 37 °C for 24-72h. Transfected products were siRNA, pCMV vectors and constructs of SEMA7A.

2.2.5.1 Silencing of SEMA7A

Transient silencing of SEMA7A was achieved via a specific siRNA against human semaphorin 7A. The specific siRNA was purchased from Ambion. (Characterization in Fig.15)

2.2.5.2 Forced overexpression of SEMA7A

Following the protocol as described above, HMEC-1 cells were transfected with a plasmid containing the promoter of the cytomegalovirus (CMV) followed by the sequence corresponding to the SEMA7A gene. Cells were transfected with SEMA7A-pCMV6 vector (RC210966; OriGene, Rockville, MD, USA) or control plasmid pCMV6_XL5 (OriGene) using the Gene Juice protocol for transcription, according to standard protocol. (Characterization in Fig.16)

2.2.6 Transmigration

2.2.6.1 Isolation of human polymorphonuclear (PMN) cells and transendothelial migration assay (TEM)

Peripheral blood was taken by venepuncture from healthy donors into a Sarstedt Monevette containing Sodium citrate (Sarstedt, Nümbrecht, Germany). PMNs were isolated by density gradient centrifugation at 500 x g for 30 min at room temperature. The neutrophils were collected from the middle interface and erythrocytes were removed by hypotonic lysis with 150 mMol NH_4Cl, 10 mMol $NaHCO_3$ and 1µMol EDTA in cold deionized water. After centrifugation PMNs were washed and resuspended in HBSS- . Number and viability of the PMNs was determined in a Neubauer chamber by using Trypanblue.

2.2.6.2 Transendothelial Migration Assay (TEM)

HMEC-1 cells were grown on transwell inserts (3µm pore size) until confluency was achieved (7-12 days after seeding). The expression of semaphorin 7A was then either silenced through transfection of specific siRNA for semaphorin 7A (or scrambled control) or enhanced through transfection of a CMV-semaphorin 7A containing plasmid (or control), as detailed before. Monolayers were washed and placed in HBSS-, containing the lower compartment formyl-met-leu-phe (fMLP 10ng/ml) to induce chemotaxis. Next, 1×10^6 PMNs were placed on the apical side of monolayers, which were then incubated for 45 min at 37°C on a rotating platform (30 rotations/min). Alternatively, the PMNs were incubated under rotation 30 min at RT with AB against Plexin (15µg/ml), α1β1 integrin, or isotype control, prior to be added to the apical side of non-transfected HMEC-1 monolayers. To control for non specific protein effect, bovine serum albumin (BSA) was used for transmigration assay. The next step was to remove the inserts to stop the further transmigration. To prevent agglutination and to perform cell lysis 50 µl 10% Triton X and 50 µl citrate buffers were added and the plates were placed for 20 min on a rocking shaker. Afterwards, the samples were pipetted in a 96-well microplate. A serial dilution was made with the original PMN solution to be used as standard curve. Transmigration was assessed by measuring MPO with a microplate reader to quantify transmigrated PMNs in the lower compartment.

2.2.7 Western blotting

HMEC-1 cells were grown to confluence on 60-mm dishes and exposed to indicated experimental conditions. The monolayers were lysed for 10 min with 300 µl lysis buffer (150 mM NaCl, 25 mM Tris, pH 8.0, 5 mM EDTA, 2% Triton X-100 and protease inhibitor cocktail), scraped and collected into microfuge tubes. After spinning at 12,000 *g* to remove cell debris, the supernatant was removed and protein content was measured using BCA Protein Kit™ from Pierce. The same procedure was used for mouse tissue. Proteins were solubilised in non-reducing Laemmli sample buffers and heated to 90°C for 10

min and then placed on ice. The samples were resolved on a 12% polyacrylamide gel and transferred to a PVDF membrane. The membrane was blocked overnight at 4 °C in TBS supplemented with 0.1% Tween 20, 3% skim milk and 3% BSA. The membrane was then incubated in 5 µg/ml polyclonal goat anti-semaphorin-7a antibody (Abcam af2068) for 1h at room temperature, followed by three 15 min washes in PBS-T. Alternatively, anti-semaphorin-7a (Abcam ab23578) was used for murine samples. The membrane was incubated in 1:1000 goat anti-rabbit IgG-HRP antibody (Santa Cruz SC2004) for 1 hrs at room temperature. Labeled proteins were detected by enhanced chemiluminescence, utilising the Raytest DIANA densitometer software. To control for protein loading, blots were restored by using a stripping buffer for about 15 min at room temperature and then reprobed for ß-Actin using a rabbit monoclonal anti–human ß-Actin antibody.

Also we detected HIF-1α and VEGF using the same protocol for western blotting with these antibodies:

- Anti-Hypoxia-inducible factor 1 alpha antibody NB100-134 (Novus Biologicals Inc.)
- Anti -VEGF (147) sc-507 (Santa Cruz)

2.2.8 SEMA7A in medium

The protocol for SiM (Sema in medium) consisted of exposing confluent HMEC-1 cells to normoxia or to different time periods of hypoxia. The supernatant was collected and centrifuged to remove cell debris. To avoid potentially different evaporation the volume of all samples was equalized among the Petri dishes. Finally, the maximum possible volume was submitted to electrophoresis and Western blot to detect released-soluble semaphorin 7A.

2.2.9 Biotinylation

HMEC- 1 human endothelial cells were grown on a permeable support system and either the apical or the basolateral side of the cells was labeled with biotin, subsequently lysed and cell debris was removed by centrifugation. Lysates were precleared with 50 µl preequilibrated protein G-Sepharose.

Immunoprecipitation was performed with polyclonal anti-semaphorin-7A antibodies (R&D Systems af2068 or Abcam ab23578) followed by addition of 50 µl preequilibrated protein G-Sepharose and overnight incubation. Washed immunoprecipitates were boiled in reducing sample buffer (2.5% SDS, 0.38 M Tris, pH 6.8, 20% glycerol, and 0.1% bromophenol blue), separated by SDS-PAGE, transferred to nitrocellulose, and blocked overnight in blocking buffer. Biotinylated proteins were labelled with streptavidin-peroxidase and visualized by enhanced chemiluminescence.

2.2.10 Reporter assays (Luciferase)

Plasmids expressing sequences corresponding to full length SEM7A (-988 to +28) have been previously characterized by GeneArt. As a control for hypoxia, cells were transfected with a PGL4.17-based HRE plasmid, containing four tandem HIF-1 enhancer sequences from the 3'-region of the erythropoetin gene.[74] Constructs were transfected into HMEC-1 cells. For transfection, the Gene Juice protocol for transcription was used as described above. Next, cells were subjected to hypoxia or normoxia for 48 hours. Luciferase activity was assessed (Turner Designs, Sunnyvale, CA) utilizing a luciferase assay kit (Promega). All firefly luciferase activity was normalized with respect to the protein content and the empty PGL4.17 vector. Three different SEMA7A full-length constructs containing site-directed mutations in one of the three identified HBS (HIF-1 binding site), respectively, were purchased from GeneArt (Germany) and assayed for luciferase activity as described before.

2.2.11 Animal experiments

2.2.11.1 *In vivo* hypoxia model

C57BL/6 mice were matched according to sex, age, and weight. Semaphorin 7a deficient mice were purchased from Charles River Laboratories. Animals were exposed to normobaric hypoxia (8% O_2, 92% N_2) or ambient air for 4 hours (n=6 animals per condition). After hypoxia/normoxia exposure, the lungs, heart, brain and liver were collected and the levels of Sema7a were

determined. In subset of experiments, the lungs were fixed by instillation of 0,6ml formalin solution (3.7 % formaldehyde) through the trachea and then embedded in paraffin for further immunohistochemistry.

2.2.11.2 Vascular Barrier Function

2.2.11.2.1 Collecting samples with Evan's blue

The WT and Sema7a -/- mice were anesthetized with ether and 200µl 0.5% Evan's blue was injected into the vein. Then the mice were exposed to hypoxic or normoxic conditions for 4 hours. Afterwards the mice were anesthetized with Pentobarbital, the hearts were washed with 10ml PBS and the lungs, heart, liver, kidney, colon and brain were collected and stored at -80°C.

2.2.11.2.2 Processing and measurement

The collected organs were weighed (50-70µg) and homogenized in PBS with 5 µl PBS/mg of tissue. Evan's blue was extracted from tissue as described in the work of Barone, et al.[75] Therefore, we added two volumes of formamide to the samples (for example, 100 µl formamide to 50 µl homogenized tissue). The mixes were incubated 12-18 hours (overnight) at 60 °C, followed by centrifugation for 10 min at 12.000 g and the supernatant was removed. Absorption of Evan's blue was measured in a 96 well plate with 100 µl of each mix. The concentration was measured with wavelength 620nm and 720nm, using the GENios microplate reader with Magellan software.

2.2.11.2.3 Migration of leukocytes

WT and semaphorin 7A deficient mice were exposed to hypoxic or normoxic conditions. The hearts were washed with 10 ml PBS and the heart, lungs, liver, kidney, colon and brain were collected and stored at -80°C. Afterwards, the tissues were weighed and 3-5 µl 10% Triton X-100 (stock-solution at 4°C) per mg of tissue (3µl for intestine, 5µl for the others) was added. The tissues were homogenized using the homogenizing system MICCRA D-9 and centrifuged at 12000g for 15 minutes at 4°C. 100 µl of the supernatant was taken and mixed with 100 µl of Citrate buffer stock solution at 4°C (Citrate buffer: 500ml 1M Citric Acid, 500ml 1M Sodium Citrate pH 4.2).

Then 200µl ABTS solution was added (ABTS solution: 28mg ABTS, 5ml 1M Citrate Buffer, 45ml ddH_2 O, 50µl 30% H_2O_2 added prior to use). This mix was incubated for 10-20 min at 37°C and afterwards centrifuged at 12000g 4°C 15min. 100 µl of the supernatant was taken to evaluate the MPO activity with the ELISA reader (405 – 450nm absorption).

2.2.12 Microscopy

Immunohistochemistry

To examine the influence of hypoxia on the expression of semaphorin 7A, wild-type C57BL/6 mice were submitted to normoxia or ambient hypoxia (8% oxygen) for 4 hours. Mice were afterwards euthanized, the lungs collected and kept in 3.7 % formalin before being embedded in paraffin. Paraffin embedded sections were dewaxed and washed in phosphate buffered saline (PBS; pH7.4). Epitope retrieval was performed by a standard microwave oven method, using 0.1 M citrate buffer (pH 6.0) for 20 min, cooled and washed in PBS (5 min x2).

The sections were incubated with 5% skimmed milk in TBS containing 0,1% Triton-X-100 for 30 min followed by primary antibody incubation overnight at 4°C. The following primary antibody was used: Sema7a rabbit polyclonal antibody (Abcam ab23578). Sections were washed three times in TBS before being incubated with appropriate Alexa-Fluor-488 conjugated goat anti-rabbit secondary antibody (1:200 dilution). DAPI (4', 6-Diamidino-2-phenylindol) was used for nuclear counter staining. Fluorescence was visualized with a confocal laser scanning microscope (Leica, Germany).

2.2.13 Data analysis

Data were compared by one-factor analysis of variance or by Student's *t*-test, where appropriate. Values are expressed as the mean ± SD from at least three separate experiments. A $p<0.05$ was considered to be statistically significant.

3 Results

3.1 Regulation of SEMA7A *in vitro*

The first experiments of this thesis should show how hypoxia influences semaphorin 7A *in vitro*.

3.1.1 Timecourse in Hypoxia

To show the regulation of semaphorin 7A by hypoxia, endothelial HMEC-1 cells were seeded like described above. Once confluent, the cells were put in a hypoxic chamber, with new pre-incubated hypoxic medium. After 4, 6, 12, 24 and 48 hrs the dishes were collected for further use, some for western blotting and some for real time PCR.

3.1.1.1 Western Blot

The western blots revealed a significant upregulation of semaphorin 7A by hypoxia in a time-dependent manner. The analysis of the densitometry of 3 different western blots corroborated the upregulation of semaphorin 7A in hypoxia compared to β-Actin.

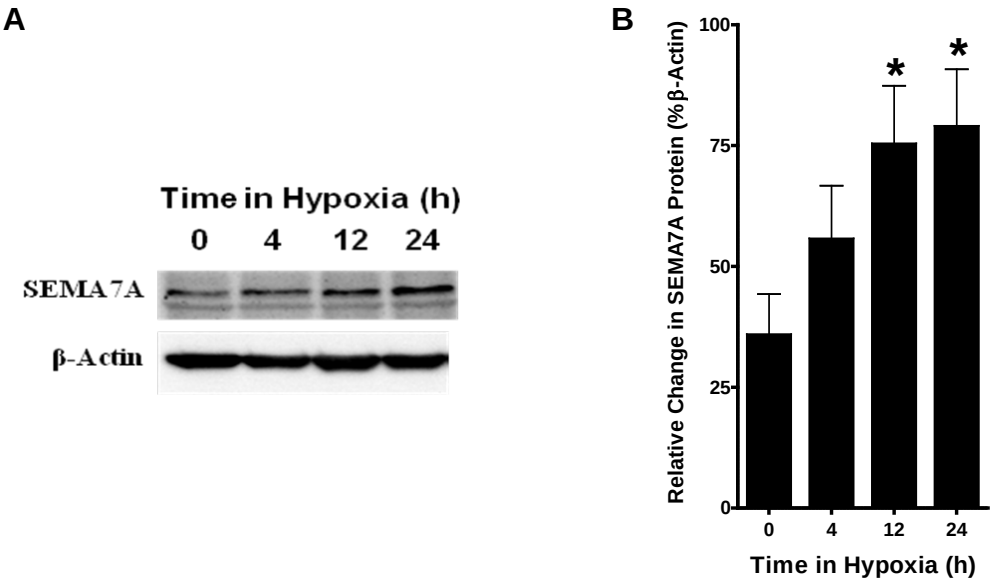

Figure 7: Regulation of Semaphorin 7A during hypoxia *in vitro*.

(A) Western blot analysis of HMEC-1 cells at different time points of hypoxia exposure.

(B) Densitometry of western blots. Data are expressed as relative change in SEMA7A Protein to β-Actin. (*P < .05)

3.1.1.2 Semaphorin 7A in medium

The protocol for Sema in medium (SiM) consisted of exposing confluent HMEC-1 cells to normoxia or to different time periods of hypoxia. The supernatant was collected and used for measuring the soluble fraction of SEMA7A.

We tried to make sure that the medium type did not influence the experiment using either HBSS or normal HMEC medium.

The results show an increase in the content of semaphorin 7A in the medium that is time-dependent up to 48hrs. The results for HBSS and HMEC medium are nearly the same. After 48hrs of hypoxia, the cells in HBSS (Fig. 8A) started to die due to the lack of nutrients, resulting in the attenuation of the signal.

Figure 8: Semaphorin 7A in medium

(A) Western-blot analysis of soluble SEMA7A in HBSS medium in a timecourse from 0 - 72 hours.
(B) Western-blot analysis of soluble SEMA7A in HMEC medium in a timecourse from 0 - 72 hours.

3.1.1.3 mRNA (RT-PCR) + VEGF

The polymerase chain reaction also showed an upregulation of semaphorin 7A in hypoxia. VEGF was used as control for hypoxia.[76] The collected data show that semaphorin 7A is even higher expressed than VEGF in hypoxia.

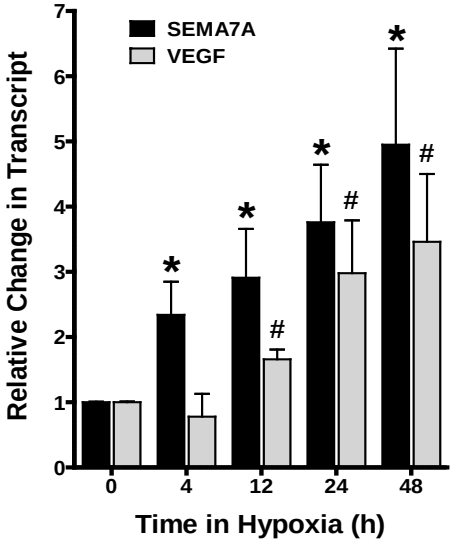

Figure 9: RT-PCR of SEMA7A in hypoxia

SEMA7A mRNA expression in HMEC-1 cells after exposure to hypoxic conditions in different timesteps. VEGF was used as a control for hypoxia. Data are expressed as relative change in transcript. (*P < .001; #P < .01)

3.1.2 Biotinylation

Biotinylation should reveal on which side of the cell membrane semaphorin 7A is expressed more under conditions of hypoxia. For these experiments, HMEC-1 cells were grown on a permeable support system and either the apical or the basolateral aspects of the cells were labelled with biotin. After immunoprecipitation with specific antibodies anti-SEMA7A, biotinylated SEMA7A was incubated with streptavidin-peroxidase and visualized by enhanced chemiluminescence. These experiments unveiled an upregulation of SEMA7A on apical parts of cell layers in hypoxia.

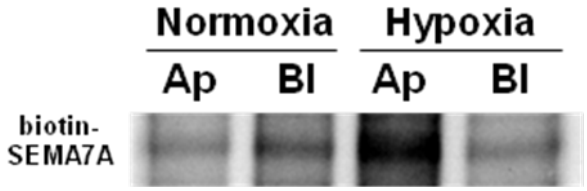

Figure 10: Biotinylation
Western-blot analysis of HMEC-1 WT cells in hypoxic and normoxic conditions after labelling apical (Ap) oder basolateral (Bl) membranes with biotin.

3.2 Promoter studies

In order to gain specific insight into the mechanisms of SEMA7A upregulation during hypoxia, we sought to identify some of the transcription factors involved on this process. Luciferase experiments revealed the responsible HREs (hypoxia responsible elements) on that response. Furthermore, some additional experiments showed the critical role of HIF (hypoxia inducible factor) in the upregulation of semaphorin 7A.

3.2.1 SEMA7A promoter activity during hypoxia

For the luciferase experiments a full length SEMA7A promoter was used.

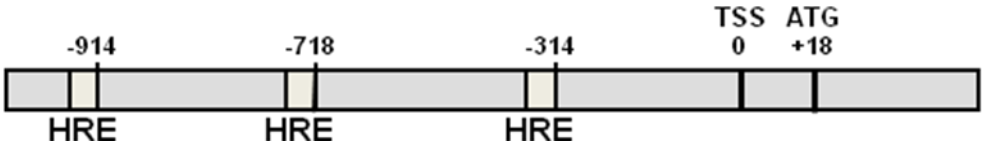

Figure 11: SEMA7A HIF binding sites full length. HREs, transcription start site (TSS) and start codon (ATG) are depicted.

As a control for hypoxia, cells were transfected with a PGL4.17-based HRE plasmid containing four tandem HIF-1 enhancer sequences from the 3'-

region of the erythropoietin gene[74] and as second control to normalize an empty vector PGL4 was used. Three additional constructs containing mutations in one of the three HREs detected were also employed. After transfection into HMEC-1 cells, they were subjected to hypoxia or normoxia for 48hrs. All firefly luciferase activity was normalized with respect to the protein content and the empty PGL4.17 vector. As shown in Fig.12, luciferase activity was induced by hypoxia in the full-length promoter as well as in the constructs Δ-314 and Δ-718. The lack of regulation in the construct Δ-914 suggests this HRE to be responsible for the regulation of SEMA7A during hypoxia.

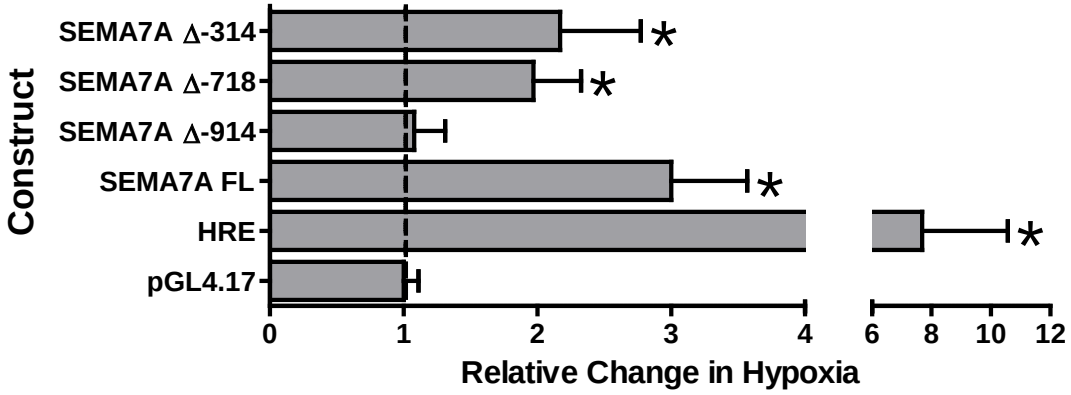

Figure 12: Luciferase results

Graph shows semaphorin (full length and mutated SEMA7A constructs) upregulation in hypoxia compared to an empty PGL4 vector and to an HRE (hypoxia responsive element). (*P < .001 indicates differences between constructs and empty pGL4.17)

3.2.2 Role of gain and loss of HIF function

To ascertain the role of HIF-1α in the regulation of semaphorin 7A during hypoxia, we carried out experiments of gain and loss of HIF-1α function. To this purpose, we made use of HMEC cell lines either stable overexpressing HIF-1α or with siRNA directed against HIF-1α.[77-78] As shown in Figure 14 and 15, the significantly high levels of SEMA7A even in normoxic conditions in the HIF-1α overexpressing cells, together with the lack of SEMA7A regulation in the HIF-1α knockdown cells during hypoxia, strongly support our hypothesis that HIF-1α plays a role in the transcriptional induction of SEMA7A by hypoxia.

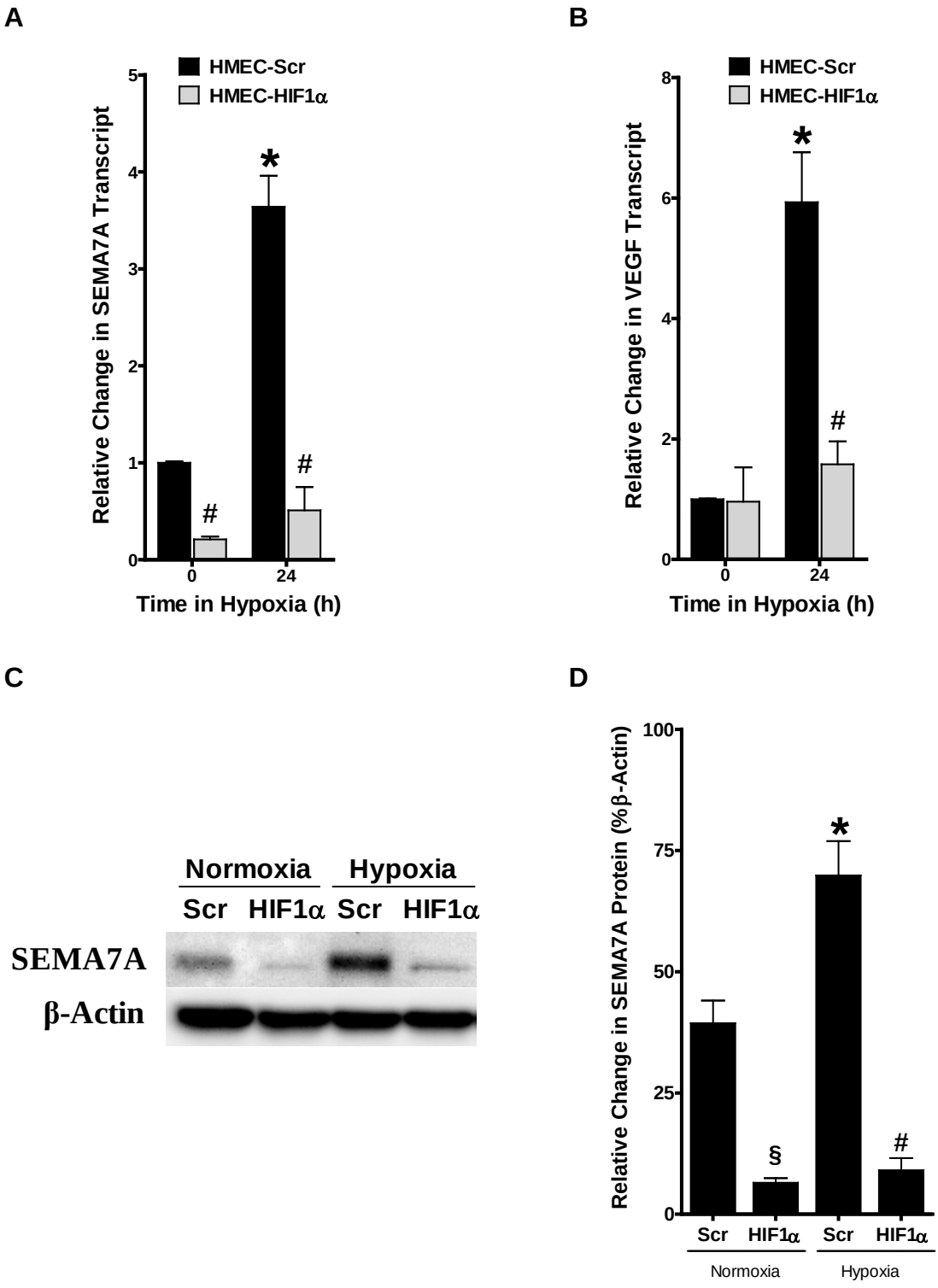

Figure 13: Loss of HIF-1α function

Relative change in SEMA7A (A) and VEGF (B) mRNA expression levels in scrambled (control) and HIF1α-knockdown HMEC cells, after exposure to normoxia or hypoxia (*P < .001 indicates differences between normoxia and hypoxia; #P < .001 between different cell types). (C) Western-blot of the same cells and (D) densitometry analysis of 3 different blots. Data are expressed as relative change in SEMA7A protein respecting to β-actin (*P < .01 indicates differences between normoxia and hypoxia; #P < .001 and §P .01 between different cell types).

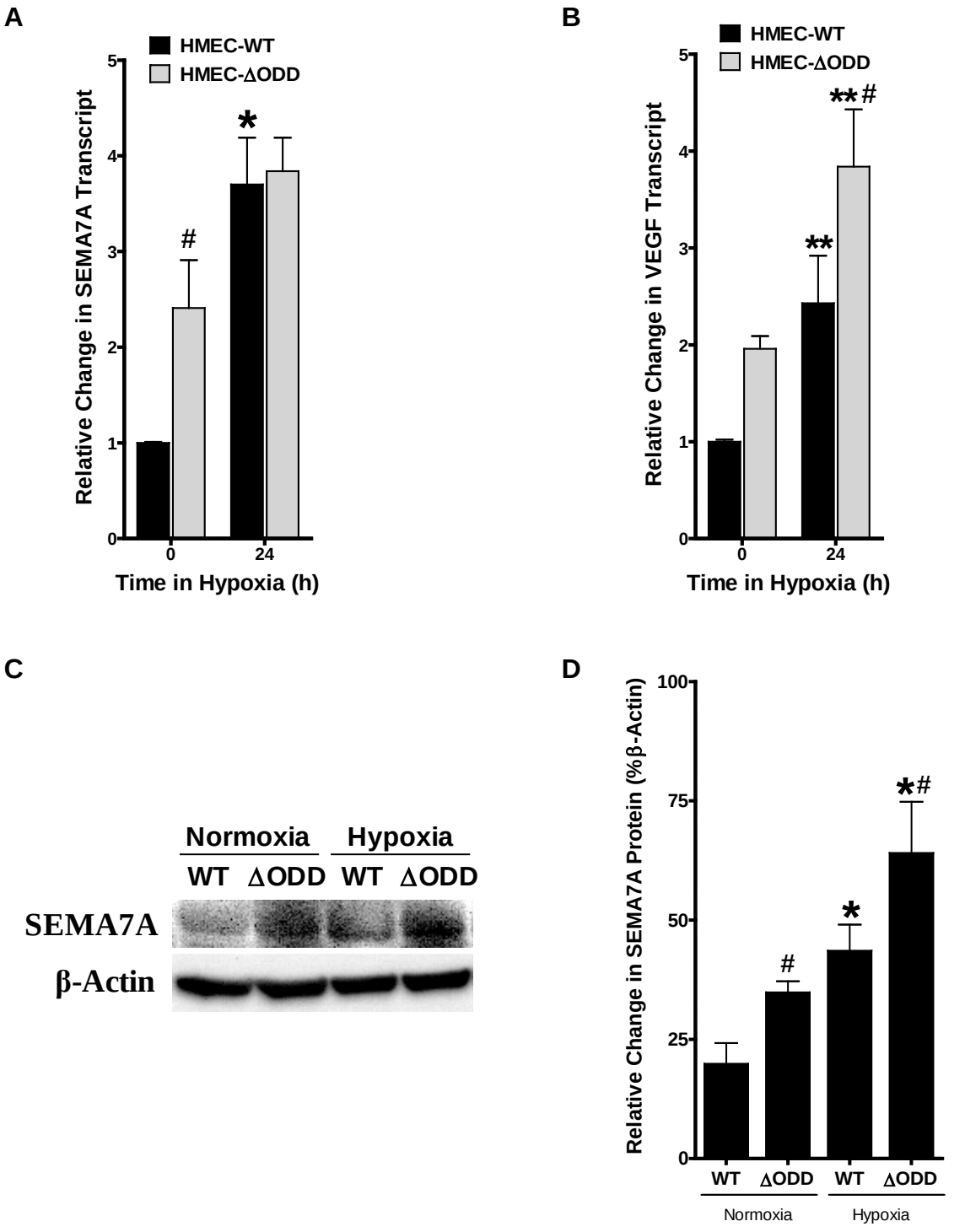

Figure 14: Gain of HIF-1α function

Relative change in SEMA7A (A) and VEGF (B) mRNA expression levels in control and HIF-1α-overexpressing (ΔODD) HMEC cells, after exposure to normoxia or hypoxia (*P < .001 and **P < .05 indicate differences between normoxia and hypoxia; #P < .05 between different cell types). (C) Western-blot of the same cells and (D) densitometry analysis of 3 different blots. Data are expressed as relative change in SEMA7A protein respecting to β-actin (*P < .05 indicates differences between normoxia and hypoxia; #P < .05 between different cell types).

3.3 Silencing and overexpression of SEMA7A

3.3.1.1 Characterization of Scr-/siRNA-SEMA7A

These experiments should show that specific siRNA against human SEMA7A successfully attenuates the expression of semaphorin 7A and can be used for further functional experiments such as transmigration. As shown in Figure 15, SEMA7A siRNA resulted in optimal SEMA7A repression at a concentration of 100nM.

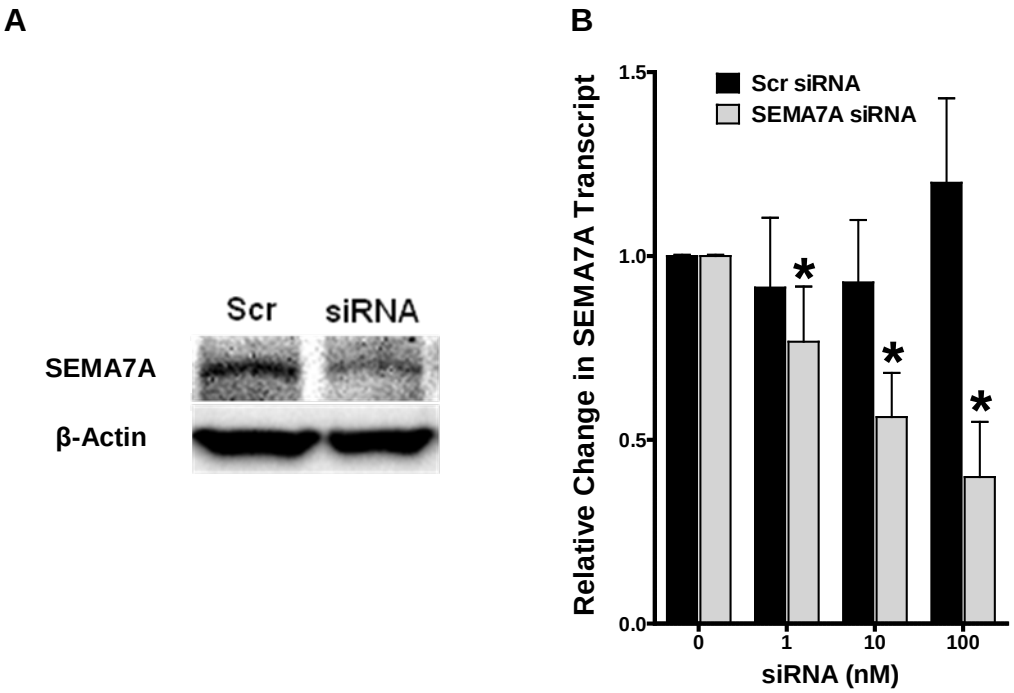

Figure 15: Characterization of Scr/siRNA-SEMA7A

(A) Western-blot analysis of SEMA7A in scrambled and SEMA7A siRNA treated HMEC-1 cells

(B) SEMA7A mRNA expression in scrambled and SEMA7A siRNA treated HMEC-1 cells with different concentration of siRNA. Data are expressed as relative change in SEMA7A transcript (*P < .01)

3.3.1.2 Characterization of Control/CMV- SEMA7A

These experiments should demonstrate that transfection with CMV-SEMA7A enhances expression of human semaphorin 7A and can be used for further functional experiments. Through western blots and RT-PCR we proved

that cells treated with CMV-SEMA7A express a significantly higher amount of semaphorin 7A (Figure 16).

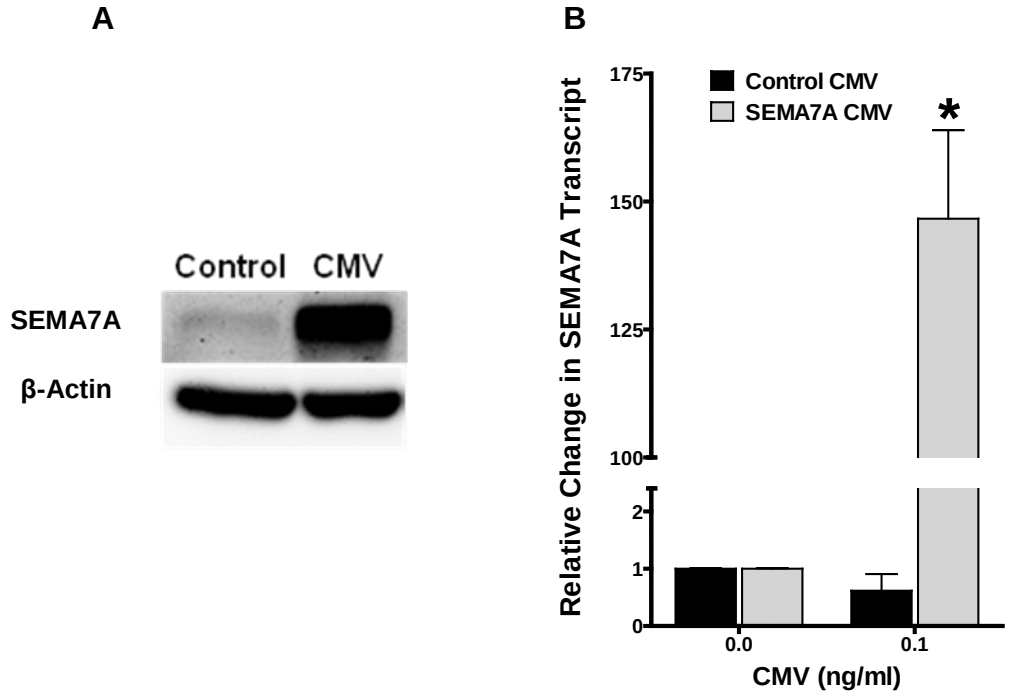

Figure 16: Characterization of Control/CMV- SEMA7A

(A) Western-blot analysis of SEMA7A in empty CMV cells (KCMV) and SEMA7A CMV treated HMEC-1 cells.

(B) SEMA7A mRNA expression in empty CMV cells (KCMV) and SEMA7A CMV (0,1 ng/ml) treated HMEC-1 cells. Data are expressed as relative change in SEMA7A transcript (*P < .001)

Barrier function

3.4.1 Possible role of SEMA7A in adhesion and migration

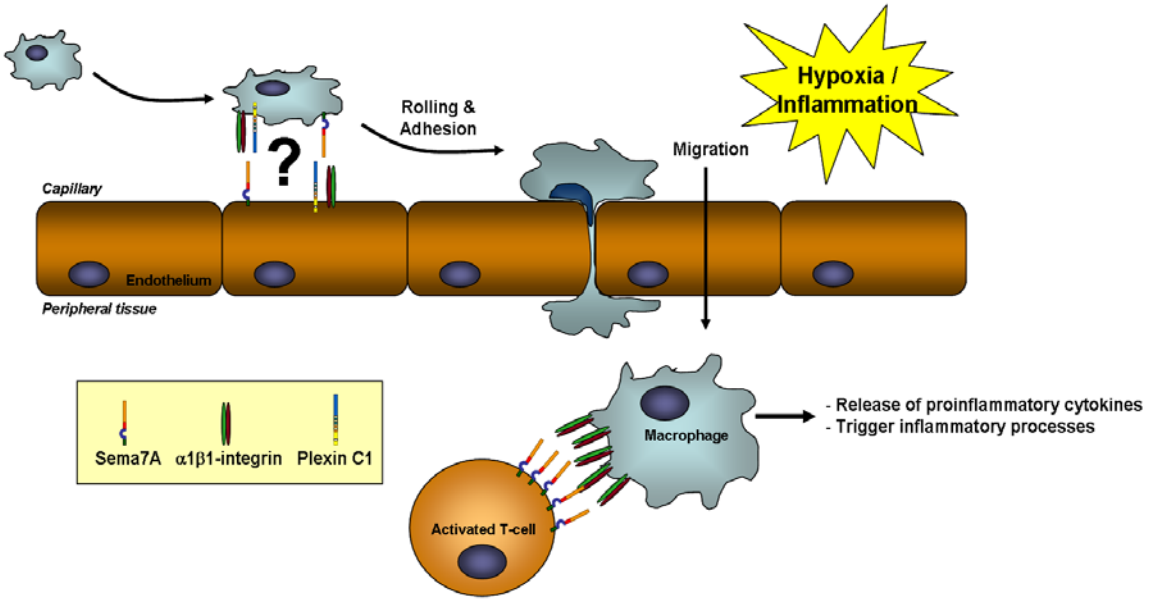

Figure 17: Role for SEMA7A in adhesion and migration of inflammatory cells during hypoxia/inflammation.

Based on our findings, this figure gives an overview about how and where SEMA7A could promote the adhesion and migration of inflammatory cells into the peripheral tissue through interaction with Plexin C1 and α1β1-integrin receptors. The transmigrated cells are then activated by T-cells by means of SEMA7A–α1β1-integrin, and trigger inflammation through the release of a variety of cytokines.[54] The following results give a better understanding of the semaphorin-dependent mechanisms of transmigration.

3.4.2 Transmigration assay

The purpose of the transmigration assays was to define the role of SEMA7A in migration of leukocytes through endothelia. To this purpose we used cells wherein the expression of SEMA7A was silenced using siRNA technology, cells wherein the expression was enhanced by using the promoter of the cytomegalovirus, and control cells. These enhanced and silenced cells

were mentioned and tested above (see 3.3). Isolated human polymorphonuclear cells (PMNs) were loaded on the apical side of the inserts, and after 45 min the transmigration of PMNs was evaluated by measuring myeloperoxidase (MPO) activity on the basal compartment. As seen in Figure 19, transmigration of PMNs was significantly attenuated by silencing of SEMA7A, whereas it was notably increased through the SEMA7A-overexpressing cells. We hypothesize that the lack of SEMA7A mitigates the adhesion of leukocytes to the endothelia and their subsequent migration, with the opposite effect being induced by the overexpression of SEMA7A. To find more evidence, we also added different concentrations of exogenous SEMA7A to the medium with the aim of exploring the possibility of exogenous SEMA7A blocking SEMA7A receptors and therefore the transmigration. The result revealed a notable attenuation in the migration of the leukocytes as the concentration of SEMA7A increased (Fig. 18), thus supporting our hypothesis.

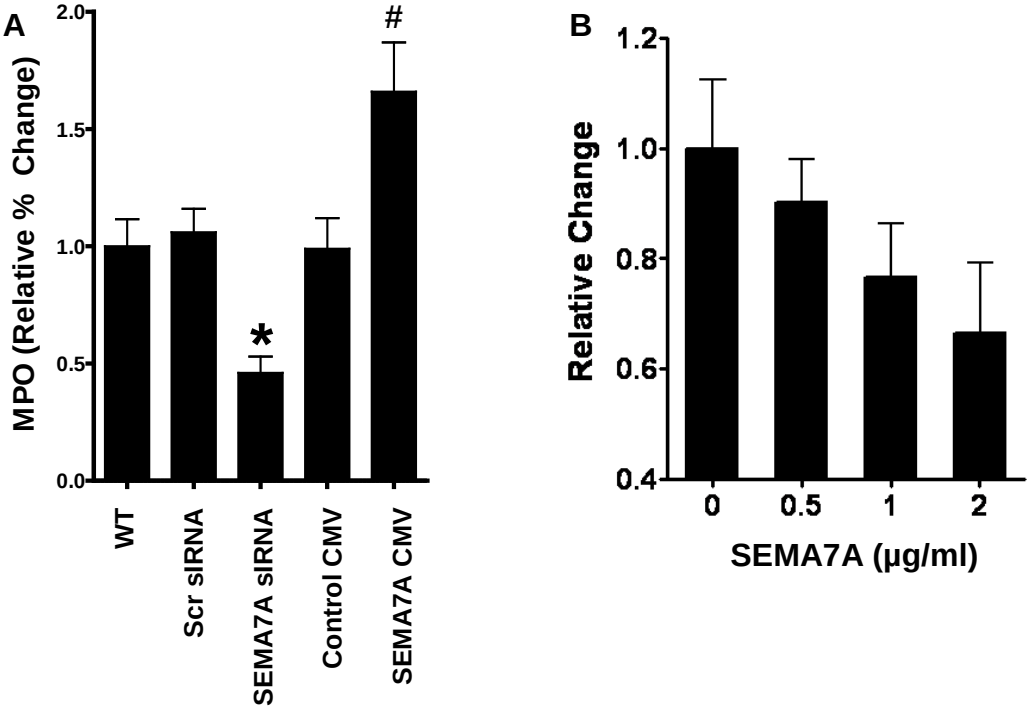

Figure 18: Role of semaphorin 7A in transmigration *in vitro*

(A) Confluent HMEC-1 monolayers were transfected with siRNA for SEMA7A or control (SCR), or with a plasmid containing the promoter of cytomegalovirus (CMV) right before the SEMA7A gene or control. (*P < .001; #P < .01)

(B) Confluent HMEC-1 monolayers were incubated with indicated concentrations of exogenous SEMA7A 30 min before the PMNs were added. The migration process was then evaluated as described before.

Transendothelial migration was evaluated by measuring MPO activity in the basal compartment. Data are expressed as relative change of MPO activity to the control cells ± SD.

3.4.3 Plexin C1 and α1β1-integrin role in transmigration

To test the influence of Plexin C1 and α1β1-integrin on the barrier function, we incubated PMNs with AB against Plexin, α1β1-integrin or isotype control under rotation 30 min at RT, prior to being added to the apical side of non-transfected HMEC-1 monolayers. The results of this experiment showed attenuated transmigration after blocking of Plexin C1 or α1β1-integrin, therefore suggesting a role for both receptors on migration of PMNs through endothelia *in vitro*.

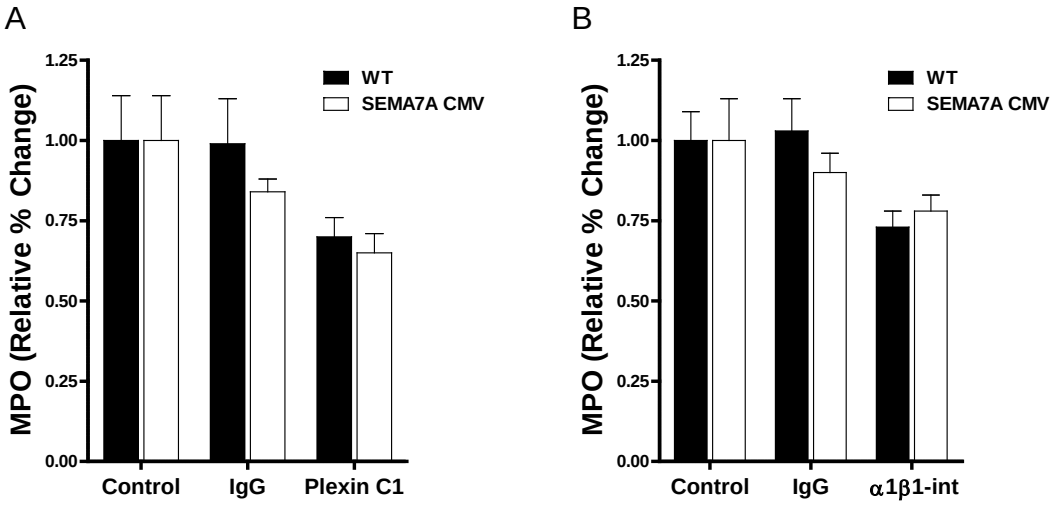

Figure 19: Transmigration results for Plexin and α1β1-integrin

Confluent HMEC-1 monolayers were transfected with a vector containing the promoter of cytomegalovirus (CMV) right before the SEMA7A gene, or control. Plexin C1 (A) or α1β1-integrin (B) receptors were blocked by adding specific antibodies, or IgG control. Transendothelial migration was evaluated by measuring MPO activity in the basal compartment. Data are expressed as relative change of MPO activity to the control cells ± SD.

3.5 Regulation of Sema7a *in vivo*

In order to verify all the above findings *in vivo*, we made use of a murine model for hypoxia (8% O_2 for 4 hours) and investigated regulation of semaphorin 7a during limited oxygen availability. After exposure to normoxia or hypoxia, mice were euthanized and the organs were perfused via the right ventricle with 5 ml of PBS. The lungs and other organs were subsequently collected for RT-PCR or western blot analysis. Alternatively, the lungs were

embedded in paraffin for immunofluorescence imaging with a confocal laser scanning microscope (Leica, Germany).

3.5.1 RT-PCR

Real time PCR demonstrated regulation of semaphorin 7a during hypoxia in heart, liver and especially in the lungs, where it is expressed almost 4 times more than in the control tissue. The highest regulation found in lungs and liver which made us focus on these organs for further experiments.

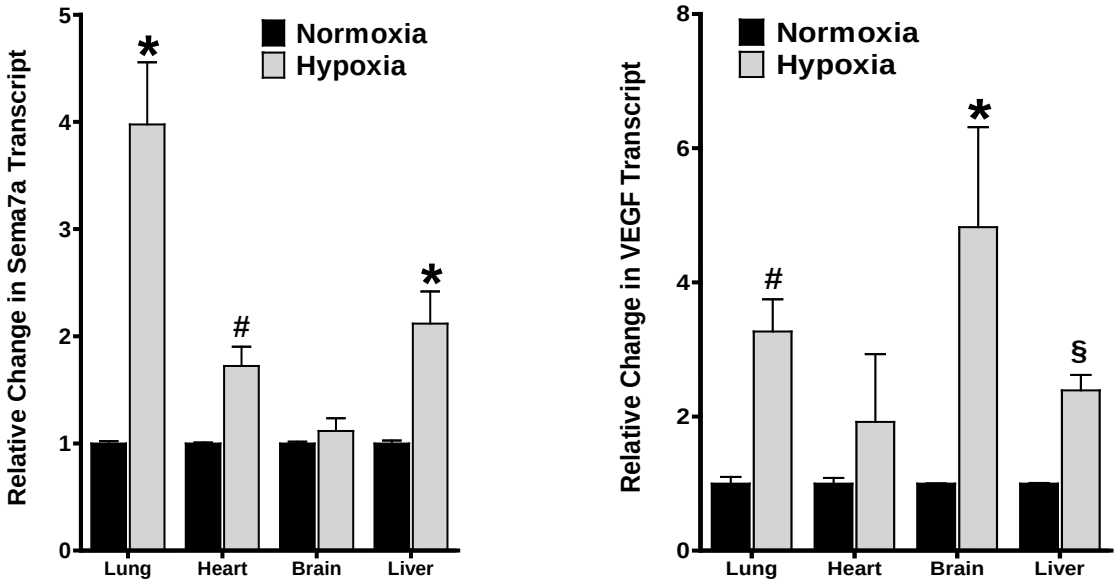

Figure 20: *in vivo* mRNA PCR results normoxia hypoxia

Sema7a mRNA expression in wild-type animals after exposure to normoxia or hypoxia (2% O2) for 4 hours. VEGF mRNA expression as a control for hypoxia.

Data expressed as relative change to normoxia ±; n=6

(*P < .001, #P < .01 and §P < .05 indicate differences between normoxia and hypoxia)

3.5.2 Western blot

Western blot analysis of lungs and liver corroborated upregulation of semaphorin 7a at the protein level during hypoxia. Different settings were initially used for the blots, ending up with pooling of 6 normoxic and 6 hypoxic organs to see the best result.

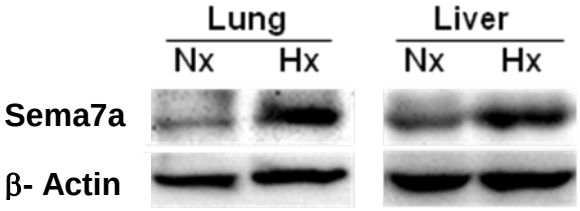

Figure 21: *in vivo* Western blots

Western blot analysis of murine Sema7a from pooled samples of lungs and liver, after exposure to normoxia (Nx) or hypoxia (Hx).

3.5.3 Immunohistochemistry

Regulation of Sema7a during hypoxic conditions *in vivo* was verified by confocal laser scanning microscopy. Specific staining of Sema7a in sections of lungs from mice exposed to normoxia or hypoxia demonstrated increased levels of Sema7a protein after hypoxia exposure (Figure 22). These studies confirmed hypoxia-dependent induction of pulmonary Sema7a *in vivo*.

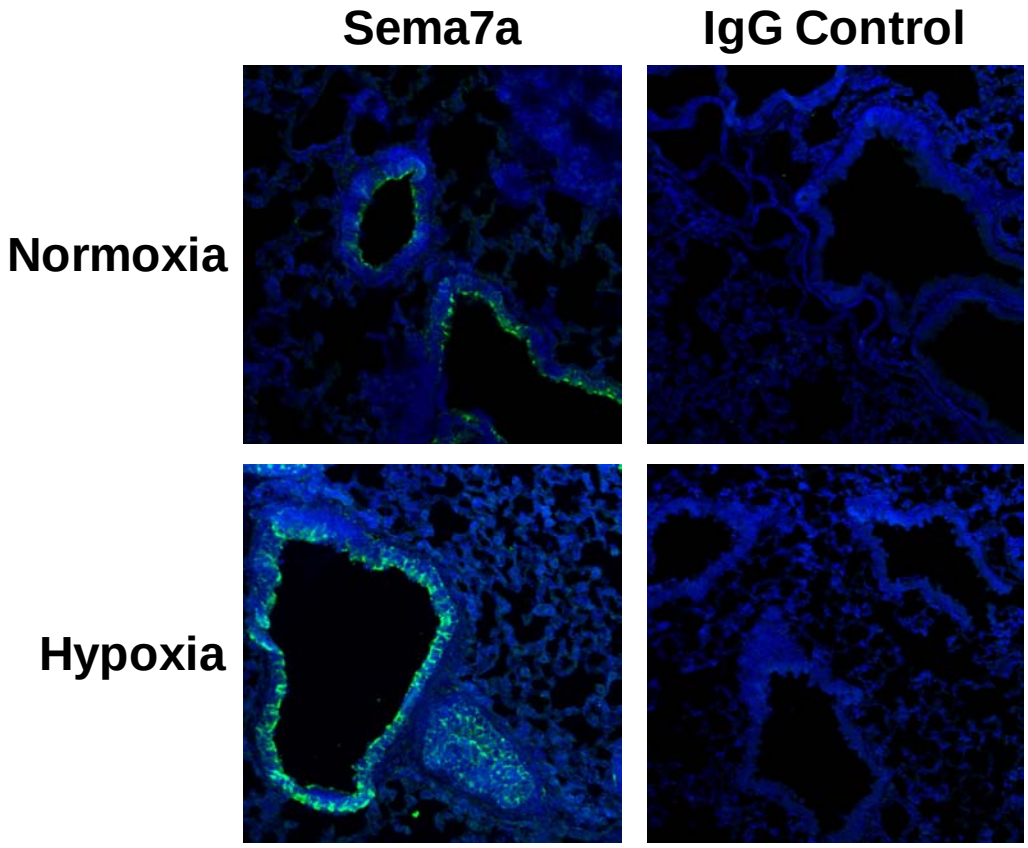

Figure 22: Pulmonary immunohistochemistry for Sema7a during hypoxia

Lungs from mice exposed to normobaric hypoxia (8% oxygen) or normoxia for 4 hours were harvested. Sections were stained with specific antibodies against murine Sema7a (green), or isotype control. Nuclear counterstain performed with DAPI (blue); magnification x630.

3.5.4 Role of Sema7a during hypoxia *in vivo*

3.5.4.1 Vascular barrier function

VBF was evaluated by injection of Evan´s blue, which is a marker of albumin. The WT and Sema7a$^{-/-}$ mice were anesthetized with ether and injected i.v. with 200µl 0.5% Evan's blue. Then the animals were exposed to hypoxic or normoxic conditions for 4 hours before collecting the organs for assessment of Evan´s blue content.

As expected[77], hypoxia induces a systemic vascular leakage that is reflected by the extravasation of Evan´s blue (Fig. 23) and by the myeloperoxidase activity (MPO activity assay) (Fig. 24). However, the extension of the injury was significantly mitigated in the Sema7a knockout mice, revealing a crucial role of Sema7a in regulating vascular permeability.

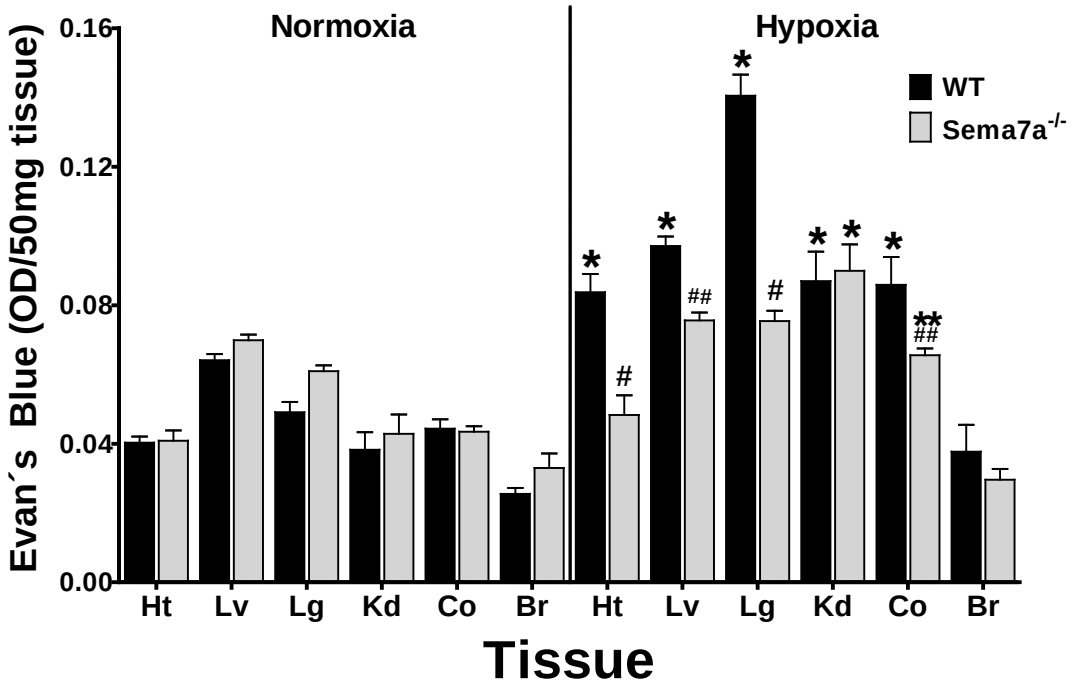

Figure 23: Role of Sema7a in vascular permeability *in vivo*.

WT and Sema7a-deficient (Sema7a$^{-/-}$) mice were injected with the albumin marker Evan´s blue (1µg/mouse, i.v.) and then exposed to normoxia or hypoxia (8% O2) for 4 hours. Afterwards the organs were collected and assessed for Evan´s blue concentration. Ht=Heart; Lv=Liver; Lg =Lung; Kd=kidney; Co=colon; Br=brain.

(**P* < .001 and ***P* < .01 indicate differences between normoxia and hypoxia; #*P* < .001 and ##*P* < .05 indicate differences between wild-type and Sema7a$^{-/-}$ organs)

3.5.4.2 Migration of leukocytes

Myeloperoxidase activity evaluates the amount of inflammatory cells transmigrating through the membrane layer. As mentioned above in the Evan´s blue experiments, the myeloperoxidase activity experiment also confirms the role of Sema7a in the inflammatory transmigration process. Whereas in the WT-mice the amount of infiltrated cells is notably increased by hypoxia, the migration is significantly attenuated in lungs and hearts of Sema7a-deficient mice. By implication, this means Sema7a plays an important role in the transmigration of inflammatory cells under hypoxic conditions *in vivo*.

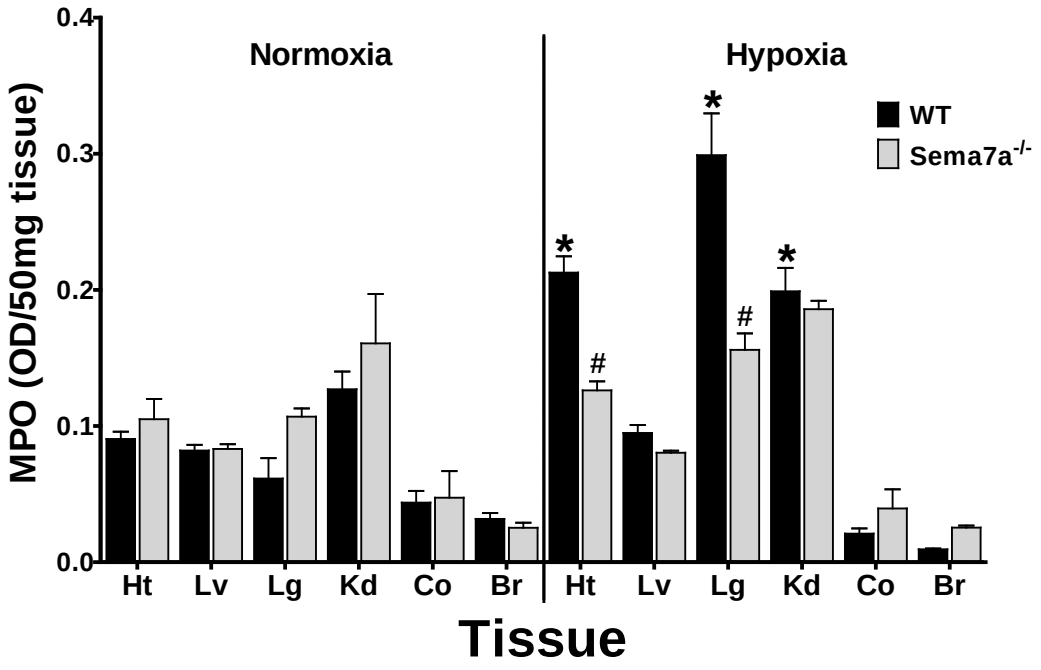

Figure 24: Myeloperoxidase activity

WT and Sema7a -deficient (Sema7A$^{-/-}$) mice were exposed to normoxia or hypoxia (8% O2) for 4 hours. Afterwards the organs were collected and assessed for Myeloperoxidase activity. Ht=Heart; Lv=Liver; Lg =Lung; Kd=kidney; Co=colon; Br=brain.

(*P < .001 indicates differences between normoxia and hypoxia; #P < .001 indicates differences between wild-type and Sema7a$^{-/-}$ organs)

4 Discussion

To date, semaphorin 7A is a protein to be further explored. There are just a few publications, and these publications were promising and led us to take a deeper look at this protein. In the end, current publications clarified that for semaphorin 7A many pieces of basic information are missing. Therefore, we started with some fundamental research experiments aiming at semaphorin 7A. Semaphorin 7A revealed itself as a hypoxia induced transmigration protein. This has been demonstrated in this thesis *in vivo* and *in vitro*. The first steps were to ascertain the influence of hypoxia on semaphorin 7A. In every experiment the upregulation of SEMA7A driven by hypoxia was clearly apparent. Guided by a possible role of an impact of semaphorin 7A on migration processes we continued to employ experiments of transmigration assays. To get a more precise understanding whether semaphorin 7A of inflammatory cells or the semaphorin on the endothelia is more important for the migration, we performed forced induction and repression of SEMA7A. Additionally, we wanted to identify whether Plexin C1 is the most reasonable receptor responsible for the observed process. Furthermore, we tried to combine the first steps, the role of hypoxia with the second steps, the transmigration influence and started with biotinylation and immunofluorescence experiments. These experiments were intended to show in which position on a circulating cell or a membrane layer semaphorin 7A is more upregulated in hypoxia. Seeing promising results we started to move the experiments from the *in vitro* to the *in vivo* stage.

Thus, the conclusions of the presented data are the following:

4.1 Regulation of SEMA7A during hypoxia

In a study by Moriya J et al. (2010) the inhibition of semaphorin was described as an innovative strategy for therapeutic angiogenesis. In this work one of the results was that Sema3E is upregulated in hypoxia.[79] Additionally, the impact of hypoxia-inducible factor-1 on semaphorin was adressed in several publications, for example in a paper by Potiron VA et al.[80] or in a research from Sun Q et al.[81] In this study, the authors discovered a connection of Sema4D

and Sema3F respectively to the hypoxia-inducible factor-1. Our current thesis lays its focus on semaphorin 7A. To the best of our knowledge, an induction of SEMA7A during hypoxia has not been described before. In reading data about other semaphorins we discovered which processes we had to start our experiments with. We deduced that one of the key questions in this thesis had to be which impact hypoxia had on the expression of semaphorin 7A. Western blots and polymerase chain reactions showed a significant upregulation *in vitro* depending on the time in hypoxia. The upregulation of semaphorin 7A through hypoxia revealed itself on HMEC-1 cells as well as in mice. Especially in lung and liver a hypoxia induced upregulation was clearly apparent.

We could also show the dependency of semaphorin 7A upregulation on the hypoxia-inducible factor HIF-1α. To display the dependency of semaphorin on HIF-1α, we used HIF knockdown cells and HIF overexpressing cells. To knockdown or to overexpress HIF, we transfected cells with HIF siRNA or with a HIF plasmid containing a promoter from the cytomegalovirus (CMV) for overexpression, respectively. These experiments showed a significant influence of HIF to the semaphorin 7A expression. These results should point out, that the rising HIF-1α levels caused by hypoxia lead to an upregulation of SEMA7A expression. This knowledge could provide a basis for further *in vivo* studies and result in a better understanding of the role of semaphorin in inflammation and hypoxic-induced processes.

4.2 Role of SEMA7A on migration

Migration of immune competent cells to sites of hypoxia or acute inflammation is an important pathophysiological response to control underlying tissue injury. Influence on this system can on the one hand be physiological and necessary or on the other hand be pathophysiological and result in illness. The influence of semaphorins on cellular transmigration has been described by several investigators. Derlorme, G et al. (2005) reported in his paper “Expression and function of semaphorin 7A in bone cells”, that SEMA7A is capable of increasing the migration of MC3T3 cells.[37] Holmes et al. (2002) showed that semaphorin 7A is able to enhance human monocyte migration *in*

vitro and increases the production of granulocyte-macrophage colony stimulating factor (GM-CSF).[59] Given the fact that a lot of papers are published about these processes we started our experiments with a closer focus. In this thesis we wanted to highlight semaphorin 7A's influence on the migration of PMNs polymorphonuclear leukocytes.

We proved by biotinylation and immunofluorescence that under hypoxic conditions semaphorin 7A is induced in the apical parts of the endothelial membrane layer. Transmigration experiments with endothelial HMEC-1 monolayers either with silenced or enhanced expression of semaphorin 7A resulted in an increased transmigration of PMNs through the cell layer. In addition, the Evan's blue and the MPO activity during *in vivo* experiments corroborate the role of Sema7a in the transmigration of leukocytes during hypoxia. This opens the possibility of blocking semaphorin 7A as a potential therapeutic strategy for dampening the inflammatory response induced by hypoxia. Further studies have to clarify which semaphorin, the soluble or the membrane bound, has more impact on transmigration.

4.3 Effects of Plexin C1 and α1β1 integrin on SEMA7A and the transmigration process.

We do not know through which pathway SEMA7A exerts its function. We can hypothesize that it happens through Plexin C1/ integrin interactions, as it is reported[38,82] and apparent in our work. The role of Plexin C1 and α1β1 integrin in cytoskeleton formation is also an indication of influence on transmigration processes.[55,83] However, further studies will be needed to clarify this point.

The relationship between semaphorins and plexins has been well described in the past. In 1999, Tamagnone et al. concluded that plexins are receptors for multiple classes of semaphorins.[84] Containing a Sema domain in the genomic structure of plexins shows how close semaphorins and plexins are to each other.[38] Plexin C1 (VESPR) has a weight of 200 kDA and is a transmembrane glycoprotein.[59] Integrins are transmembrane proteins linking the extracellular matrix with the cytoskeleton. Integrins are important for cell adhesion, migration, cytokine production and neurite extension.[49,54]

Experiments showed that when α1β1 integrin is repressed, effects of SEMA7A on adhesion and dendrite formation are nullified.[52] Plexin C1 signals inactivate cofilin, a protein involved in disassembling actin filaments.[60-62] Additionally, Plexin C1 is considered to be a tumor suppressor protein [51], as silencing of this protein significantly enhances the adhesion and spreading of melanoma cells.

This research shows that significantly fewer cells can transmigrate through a cell membrane layer if Plexin C1 or α1β1 integrin is repressed. Further experiments have to show that Plexin C1 and α1β1 integrin enhance the transmigration even though these proteins regularly have opposing effects. Plexin C1 leads to decreased adhesion and α1β1 integrin leads to enhanced adhesion.[55] To see why switching off one of these proteins leads to repressed transmigration either way, more studies to understand the precise interaction between both proteins and semaphorin 7A need to be done. In addition, the question of whether soluble or membrane bound SEMA7A has more impact on the transmigration process is not clear right now. We know that Plexins bind on secreted as well as on membrane bound semaphorins[85] and in our experiments we saw no significant difference in the transmigration assay using soluble or membrane bound semaphorin.

4.4 Problems and limitations of this study

For the *in vitro* experiments HMEC cells were used. Studies with human microvascular endothelial cells are complicated. Although HMEC cells have a limited lifespan and are difficult to isolate and fastidious in their *in vitro* growth requirements,[86] they were used for our *in vitro* experiments. These cells were transfected with a plasmid containing the coding region for the simian virus 40 A gene product that has immortalized them. These HMEC-1 cells retain the morphologic, phenotypic, and functional characteristics of normal human microvascular endothelial cells but they live longer, grow faster in higher densities and need a less stringent growth medium.[86]

Preliminary tests showed us the best time intervals for the hypoxia experiments. We determined the ideal time frame to be 12 hours preincubation with a culture medium with subsequent exposure of the HMEC-1 dishes to the

hypoxia chamber. We then collected the cells after 4h, 6h, 12h, 24h, and 48h for protein and mRNA isolation.

Determining the right protocol for the transfection method was a challenging part as well. Initially, we used many different protocols and transfection reagents, such as Gene Juice, Lipofectamin, Fugene HD and Polyfect. In the end, we utilized Gene Juice and the modified protocol described in the Materials and Methods part. With this protocol we got the best cell-confluence and the best results. Another important point was the addition of culture medium without antibiotics and fetal calf serum, because the transfected cells were very vulnerable at this point. After a growing time of about 6 hours, the culture medium could be replaced by normal medium with serum and antibiotics.

There were many difficulties with immunostaining of organ slides, because there was no scientifically proven concept to show which semaphorin 7a, the basolateral or the apical, would be more upregulated in hypoxia. The biotinylation experiments suggested that apical parts of the membrane layer produce a lot more semaphorin 7A in hypoxia, but there is still no clear evidence to answer this question, so additional experiments need to be done.

4.5 Perspectives

In summary, it still remains unclear whether the soluble or the membrane bound semaphorin has a higher impact on transmigration. There are indications that the semaphorin 7A on the membrane layer plays the important part, but there is still data needed to prove this. This thesis shows in detail what needs to be done and under what conditions measures need to be tackled. In addition, we cannot demonstrate right now which of the receptor proteins - α1β1 integrin or Plexin C1 - has more impact on the transmigration.

Many experiments still need to be done to characterize semaphorin 7A in his whole scope. Many animal experiments are in process but not finished and the results are looking promising but much work is yet to be done. Experiments for tissue specificity through bone marrow transplantation with WT and Sema knockout mice as well as injection of soluble semaphorin 7A are in process and

will be part of further studies. Semaphorin 7A may be a auspicious target of immunomodulatory therapies and should be implicated in upcoming studies. Semaphorin 7A is a promising protein and this thesis will be the beginning of novel insights into this subject in the future.

5 Summary (Abstract)

Cellular hypoxia is the pathophysiological correlate of a variety of clinically important conditions such as shock, sepsis and acute inflammation. Hypoxia attenuates the epithelial and endothelial barrier function, leading to edema formation and to the extravasation of inflammatory cells. This is associated with the release of a variety of cytokines. Semaphorin (SEMA) 7A has the ability to modulate neurite extension and to alter the production of cytokines through activation of macrophages. SEMA7A actions are exerted through interaction with the α1β1-integrin and the plexin C1 receptor. Given this, we hypothesized that SEMA7A might hold significant importance during hypoxic inflammation. In initial *in vitro* studies with endothelial HMEC-1 cells we found that hypoxia induces the expression of SEMA7A, mainly in the apical membrane. Moreover, experiments using promoter constructs, and hypoxia inducible factor-1α (HIF-1α) loss and gain of function revealed a crucial role of this transcription factor in regulating SEMA7A expression during hypoxia. Forced induction with a CMV based plasmid or siRNA targeted repression of SEMA7A reflected the importance of SEMA7A on the transmigration of polymorphonuclear cells (PMNs) through endothelial monolayers, respectively. In addition, using a murine model of hypoxia we were able to corroborate an *in vivo* induction of Sema7a in lungs, heart and liver during normobaric hypoxia. Further experiments with Sema7a-deficient mice (*Sema7a-/-*) demonstrated that vascular leakage and transmigration of PMNs during hypoxia is attenuated in *Sema7a-/-* mice compared to wild type controls. Taken together, our data demonstrate a novel role for semaphorin 7A in regulating the inflammatory response during hypoxia.

Zusammenfassung

Zelluläre Hypoxie ist das pathophysiologische Korrelat einer Vielzahl von klinisch relevanten Zuständen wie Schock, Sepsis und akuten Entzündungen. Hypoxie schwächt die epitheliale und endotheliale Barrierefunktion, was zu Ödembildung und Extravasation von Entzündungszellen führt und mit der Freisetzung einer Vielzahl von Zytokinen verbunden ist. Semaphorin (SEMA) 7A hat die Fähigkeit, das Neuronenwachstum und die Produktion von Zytokinen durch eine Aktivierung der Makrophagen zu beeinflussen. Die Wirkung von SEMA7A wird über die Interaktion mit α1β1-Integrin und dem Plexin C1-Rezeptor vermittelt. Basierend auf diesem Wissen vermuteten wir, dass SEMA7A eine erhebliche Bedeutung im hypoxischen Entzündungsgeschehen zukommen könnte. In ersten *in vitro* Studien mit Endothelzellen (HMEC-1-Zellen) fanden wir heraus, dass Hypoxie die Expression von endothelialem SEMA7A, vor allem an der apikalen Fläche dieser Zellen induziert. Darüber hinaus ergaben Experimente mit Promotor-Konstrukten und Hypoxie-induzierbaren Faktor-1α (HIF-1α) in Über- oder Unterexpression eine entscheidende Rolle dieses Transkriptionsfaktors bei der Regulierung der SEMA7A Expression während Hypoxie. Eine gezielte Induktion oder Repression von SEMA7A durch ein CMV-basiertes Plasmid beziehungsweise durch siRNA, zeigte den Einfluss von SEMA7A auf die Transmigration von polymorphkernigen Zellen (PMN) durch endotheliale Monolayer. Ferner konnten wir in einem Mausmodell bestätigen, dass es unter normobarer Hypoxie zu einer *in vivo* Induktion von Sema7a in Lunge, Herz und Leber kommt. Weitere Experimente mit Sema7a-defizienten Mäusen (Sema7a-/-) zeigten während Hypoxie einen verminderten vaskulären Ausstrom und eine Abnahme der Transmigration von PMNs im Vergleich zu den Wildtyp-Kontrollen. Zusammenfassend betrachtet belegt unser Datenmaterial eine neue Rolle von Semaphorin 7A bei der Regulierung von entzündlichen Reaktionen unter hypoxischen Bedingungen.

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8 Appendix

8.1 Statement of contribution

The concept of this thesis, the analysis of data and the interpretation of these were under continuous supervision of Prof. Dr. Peter Rosenberger and Dr. rer. nat. Julio Morote Garcia. Some of the experiments were not only produced by the author. They were rather a collaboration with some members of the workgroup “Zentrum für Hypoxie und Inflammation Tübingen”. The animal experiments were mainly supported by Dr. rer. nat. Julio Morote Garcia and Dr. rer. nat. David Köhler (figure 24, 25). The immunofluorescence experiments were also partly provided by Marion Faigle and Michaela Hoch-Gutbrod (figure 23). The constructs for the Luciferase assays were purchased from GENEART AG (Regensburg, Germany).

Parts of this study will be published beyond this doctoral thesis.