The vascular development is modulated by endostatin and restin

I n a u g u r a l - D i s s e r t a t i o n

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

Annette Schmidt

aus Bergisch Gladbach

(Hundt Druck GmbH, Köln)

2003

Acknowledgements:

First of all, I would like to thank Professor Dr. med. K. Addicks for his attentive guidance and concern for this subject as well as for many helpful discussions.

I would also express my gratitude to Professor Dr. rer. nat. S. Roth for his confidence.

I am deeply grateful to PD Dr. med. W. Bloch for his guidance and valuable advice in the whole process of this research, for innumerable helpful discussions and subject designing and experimental arrangement.

For technical support I would like to thank M. Ghilav, C. Hoffmann and E. Janßen.

I would like to thank my parents for their tireless support.

This study was supported by the Köln Fortune Program of Medicin, University of Cologne (No. 117/2001)

Table of contents

Abbreviations

AP	alkaline Phosphatase
bFGF	basic Fibroblast Growth Factor
bNOS	brain NO-synthase
BPH	benign prostatic hyperplasia
BSA	Bovine Serum Albumin
CD31/34	Cluster Determinant 31/34
°C	degree Celsius
cm	centimetre
cGMP	cyclic Guanosin-mono-phosphate
CO_2	carbon dioxide
C-PAE	Bovine Pulmonary Arterial Cell Line
DAB	Diaminobenzidin
DIG	Digoxigenin
DMEM	Dulbecco`s Modified Eagle Medium
E	embryonic day
EB	Embryoid Body
EBs	Embryoid Bodies
eNOS	endothelial NO-synthase
ERK 1/2	Extracellular Signal-regulated Kinase 1/2
ES-cells	embryonic stem cells
FCS	Foetal Calves Serum
Fig.	figure
FGF	Fibroblast Growth Factor
Flk-1	Foetal Liver Kinase-1
Flt-1	Fms-like tyrosine kinase-1 receptor
GFP	Green Fluorescent Protein
HRP	Horseradish Peroxidase
HSPGs	Heparansulfat-Proteoglycane
iNOS	inducible NO-synthase
kDa	Kilodalton
KDR	Kinase Insert Domain
MACS	Magnetic Associated Cell Sorting

MAPK	Microtubule Associated Protein Kinase
µg	Microgram
µl	Microlitre
µM	Micromolar
mA	Milliampere
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimolar
ms	Millisecond
min.	Minute
M	Molar
MMP	Matrix-Metalloprotease
MW or MG	Molecular weight
NC-1	Non Collagenous-1
ng	Nanogram
nm	Nanometre
NO	nitrogen monoxide
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PBS	Phosphate Buffered Saline, PBS-buffer
PECAM	Platelet Endothelial Cell Adhesion Molecule
peNOS	phosphorylated/activated eNOS
PFA	Paraformaldehyd
PVDF	Polyvinylidene Fluoride
RA	R158/R184/R270A(heparin mutant endostatin, AS R>A)
rpm	Rounds per minute
SDS	Sodium Dodecyl Sulphate
Ser1177	Serin1177
SGC	soluble Guanylate Cyclase
TBS	Tris Buffered Saline, TBS-Puffer
TEMED	N,N,N',N'-tetramethylethylenediamine
U/ml	Units per Millilitre
U/µl	Units per Microlitre
V	Volt

VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

Zusammenfassung

Endostatin und Restin sind proteolytische Spatprodukte von Kollagen XVIII bzw. XV. Sie sind in der Literatur als anti-angiogenetische und aufgrund dessen tumor-inhibierende Proteine beschrieben. Die Verteilung von Endostatin und Restin sowie die Verteilung ihrer Bindungsstellen im Embryoid Body (EB), im Embryo und bei der Wundheilung deutet darauf hin, dass sie eine wichtige Rolle bei der Gefäßentwicklung spielen. Bezogen auf die Gefäßentwicklung differenzierter embryonaler Stammzellen zeigten diese beiden Faktoren sowohl eine angiogenetische als auch eine anti-angiogentische Wirkung, da sie auf für die Gefäßentwicklung und Gefäßneubildung entscheidende Prozesse, wie Proliferation, Migration der Endothelzellen einen steigernden Einfluss nehmen. Andererseits jedoch induzieren sie Apoptose in Endothelzellen. Die Wirkung von Endostatin und Restin auf die Gefäßneubildung kann somit als angio-modulatorisch bezeichnet werden. Endostatin und Restin nehmen jedoch nicht nur Einfluss auf die endotheliale Proliferation, Migration und Apoptose, sondern beeinflussen auch die Morphogenese der Gefäßanlagen in Form einer Kontraktion oder Retraktion, was sowohl mittels vitalmikroskopischer Beobachtung und Dekonvolutionsmikroskopie von embryonalen Gefäßen deutlich wird, als auch bei der elektronenmikroskopischen Betrachtung neugebildeter Gefäße während der Wundheilung ersichtlich ist. Die Beeinflussung der Morphogenese führt zur Frage, welche Signaltransduktionswege einbezogen sind. Insbesondere für die Morphogenese legen Vorbefunde nahe, dass eine die Regulation über den VEGF-Signalwegs oder von Molekülen des VEGF-Signalwegs geschieht. Eine Betrachtung der Endostatin- und Restin-Wirkung auf zellulärer Ebene brachte zum Vorschein, dass diese Faktoren tatsächlich Einfluss auf Signalmoleküle in Endothelzellen nehmen. Und zwar kommt es unter Einfluss von Endostatin und Restin zu einer Dephosphorylierung der aktivierten ERK1/2-Kinase sowie zu einer Runterregulation der löslichen Guanylat Cyclase (sGC) innerhalb eines Zeitraums von 6 Stunden. Diese endostatin-vermittelte Beeinflussung der Signaltransduktion ist über die Protein-Phosphatase 2A (PP2A) induziert, da eine Hemmung der PP2A sowohl eine Dephosphorylierung der aktivierten ERK1/2-Kinase als auch die Runterregulation der löslichen Guanylat Cyclase verhindert. Die Dephosphorylierung der aktivierten ERK1/2-Kinase ist mit Hilfe von zusätzlichem cGMP aufhebbar. Modulatorische Versuche unter Verwendung von cGMP und ODQ legen nahe, dass cGMP unter definierten Bedingungen diese Dephosphorylierung der aktivierten ERK1/2-Kinase stabilisieren kann.

Summary

Endostatin and restin are proteolytic fragments of collagen XVIII and XV. They are described in literature as anti-angiogenic and consequently tumour-inhibitory proteins. The observation of endostatin and restin distribution as well as the distribution of their binding sites in the Embryoid Body (EB), in the embryo and during wound healing, suggests that they have an important role during vessel development. During vessel development of differentiated embryonic stem cells this two factors show both, an angiogenic and an anti-angiogenic effect by increasing endothelial proliferation and migration, processes which play an important role for vessel development and neovascularisation. On the other hand they induce endothelial apoptosis. The effect if endostatin and restin on neovascularisation and vessel development can therefore be described as an angio-modulatory. But endostatin and restin do not only influence endothelial proliferation, migration and apoptosis, they also influence endothelial morphogenesis by inducing a contraction or retraction of vessels. This phenomena was observed by vital-microscopy and deconvolution microscopy of embryonic vessels as well as during wound healing. The influence on morphogenesis leads to the question to which signaling pathways are involved. Especially for the morphogenesis our results imply that these processes are regulated by the VEGF signaling pathway or by molecules of the VEGF-signaling pathway. On cellulary level endostatin and restin show an influence of signaling molecules in endothelial cells. They lead to a dephosphorylation of the activated ERK1/2-kinase as well as to a down-regulation of the solouble Guanylate Cyclase (sGC) in a time course of 6 hours. The modulation of signaltransduction is induced by the protein phosphatase 2A (PP2A) because of the fact that inhibition of PP2A prevents dephosphorylation of activated ERK1/2-kinase and down-regulation of the sGC. The dephosphorylation of the activated ERK1/2-kinase is preventable by additional cGMP. Modulatory experiments by using cGMP and ODQ suggest that cGMP, under definitive conditions is able to stabilise the phosphorylation of the activated ERK1/2-kinase.

1. Introduction

1.1 Vessel Development

1.1.1 Angiogenesis and vasculogenesis

Already in 1508 Leonardo da Vinci speculated that human circulation and vessel system develop in analogy to the botanical example: *the tree,* the heart with *sprouting roots*, the liver capillary network, and a *trunk with major branches,* the aorta and arteries. Later it emerged that the vascular system is determined before the heart starts beating (Risau 1997). Today it is known that the early vascular network is formed from mesoderm by differentiation of angioblasts. Angioblasts form primitive blood vessels. The cell biological mechanism which is responsible for this process is called vasculogenesis. The differentiation of angioblasts from mesoderm and formation of primitive blood vessels by angioblasts are two different steps during early embryonic vasculogenesis (Risau and Flamme 1995). However angioblasts do not only form blood vessels at places of genesis, rather they migrate over long distances, forming a vascular plexus at a distant site (Noden 1991). After a primary vascular network is formed, more endothelial cells are generated, which can form new capillaries by sprouting or by splitting from their vessels of origin in a process termed angiogenesis (Risau 1997). The sprouting angiogenesis occurs mainly in the yolk sac and in the embryo and includes the proteolytic degradation of extra cellular matrix. Afterwards a chemotactic migration and proliferation of endothelial cells, with lumen formation and functional maturation of the endothelium follow (Risau 1997). During non-sprouting angiogenesis vessels arise by splitting pre-existing vessels from transcapillary pillars or posts of extra cellular matrix (Short 1950). *In vivo* non-sprouting angiogenesis can occur by proliferation of endothelial cells inside a vessel, producing a wide lumen that can be split by transcapillary pillars, or fusion and splitting of capillaries (Pardanaud et al. 1989). The emerging vascular plexus is rapidly remodelled to resemble a mature system with larger and smaller vessels. The responsible process is termed pruning (Risau 1997). The maturation of big vessels includes the differentiation of smooth muscle cells and the formation of the lamina elastica interna. Formation of a luminal tubus is the key event during endothelial cell maturation and includes differentiation of polarised and functional different cell surfaces (Risau 1995).

1.1.2 Stimulators of angiogenesis

Processes like angiogenesis and vascular remodelling involve endothelial proliferation as well as regression of endothelial cells and they are thereby determined by different growth factors. For example bFGF (basic fibroblast growth factor) and VEGF (vascular endothelial growth factor) contribute synergistically to the regulation of blood vessel formation (Pepper et al. 1992) and are very important factors regarding neovascularisation and vessel development as well as for endothelial morphogenesis. They are suggested to induce proliferation, migration and differentiation of angioblasts in vitro. BFGF seems to play a major role in the induction of angioblasts whereas VEGF appears to determine the initiation of morphogenesis leading to the initial vascular pattern. Moreover, VEGF has been reported to augment the population of endothelial precursor cells (EPCs) in vivo (Kalka et al. 2000). Kazemi et al. could show that bFGF and VEGF influence survival of angioblasts and that VEGF cause differentiation of angioblasts whereas bFGF does not suggesting a differential role of these factors for vasculogenesis (Kazemi et al. 2002). Capillary endothelial cells of embryonic tissue and organs do not express perceptible amounts of FGF-receptor coding mRNA (Heuer et al. 1990, Wanaka et al. 1991, Peters et al. 1992). Endothelial cells of bigger vessels however express FGF-receptor and react on FGF treatment *in vivo* (Peters et al. 1992, Lindner et al. 1990, Liaw and Schwartz 1993). Flk-1 (VEGFR-2) is the first endothelial receptor to be expressed in angioblast precursors in the primitive mesoderm of the mouse and is required for angioblast differentiation, followed shortly later by Flt-1 (VEGFR-1), which is required for assembly of blood vessels (Breier et al. 1997).

1.1.3 Inhibitors of angiogenesis

Vasculogenesis and angiogenesis underlay a stringent control of stimulatory and inhibitory factors (Hanahan and Folkman 1996). Many tumours are able to produce angiogenic and anti-angiogenic factors (Folkman 1990, Chen et al. 1995, Gately et al. 1996). In the past numerous angiogenesis inhibitors were identified and characterised. Among them the most important: Thrombospondin-1 is an adhesive glycoprotein with a size of 160 kDa and was found in the α –granula of thrombocytes. Presumably it is involved in the stabilisation of thrombocyte aggregates (Phillips et al. 1980). In a series of studies it has been shown that thrombospondin-1 suppress neovascularisation (Good et al. 1990). Also it inhibits angiogenesis and migration of endothelial cells whereas an inhibition of endothelial proliferation could not be shown (Klagsbrun and D'Amore 1991). Thrombospondin-1 is regulated by the tumour suppressor

protein p53 (Good et al. 1990). The complete thrombospondin-1 as well as its specific fragments are able to suppress angiogenesis (Good et al. 1990, Tolsma et al. 1993). On the contrary the potent angiogenesis inhibitors like endostatin and angiostatin are fragments of common extra cellular proteins, which by themselves do not participate in regulation of angiogenesis (Hohenester et al. 1998). Also the angiogenesis inhibitor platelet factor IV was found in the α–granula of thrombocytes and it is released during thrombocytes aggregation. It is a big tetrameric protein with a size of 28 kDa and was initially characterised by its high affinity to heparin (Walz and Hung 1985). Platelet factor IV was characterised as a potent anti-angiogenic factor (Taylor and Folkman 1982). It prevents angiogenesis and endothelial cell proliferation, whereas an inhibition of endothelial migration was not observed (Klagsbrun and D'Amore 1991). Therefore this inhibitor shows a comparable biological action to endostatin. Interferon-γ, a 50 kDa big protein inhibits like endostatin angiogenesis, endothelial migration and proliferation (Klagsbrun and D'Amore 1991). The angiogenesis inhibitor angiostatin is a fragment of plasminogen and has a size of 38 kDa. It suppresses angiogenesis and formation of metastasis (O'Reilly et al. 1994).

1.1.4 Tumour vascularisation

The proliferation of blood vessels is a necessary process for normal tissue growth (Folkman 1971). Vasculogenesis and angiogenesis are subject to a stringent control of stimulatory and inhibitory factors (Hanahan and Folkman 1996). When in normal adult tissue vasculogenesis rests, the balance is lying presumably on the side of inhibitors, however during tumour expansion the balance is disturbed (Chang et al. 1999). In adult organisms angiogenesis appears rarely and it is part of a body internal reparation/regeneration system during healing of wounds or part of the female reproduction cycles (Klagsbrun and D'Amore 1991). Opposite to that an angiogenesis out of balance is often part of a pathological event, like during angiogenesis induced tumour growth (Folkman 1972). Tumour growth is angiogenesis dependent (Folkman 1971). An insufficient tumour vascularisation has the consequence that tumour cells become necrotic and/or apoptotic (Holmgren et al. 1995, Parangi et al. 1996). Following to a tumour genesis every increase in tumour cell population precedes an increase of vessel growth (Folkman 1990). Experiments regarding tumour growth and angiogenesis show that the growth rate of an implanted, subcutan tumour is low before vascularisation, however high and nearly exponential after vascularisation (Algire 1945). In correlation with an increased angiogenesis in tumours it was observed that many tumours are able to produce

angiogenic factors, which leads to an increased tumour expansion (Relf et al. 1997). Some malign tumours also produce angiogenic inhibitors (Chen et al. 1995, Gately et al. 1996). The increased angiogenesis during tumour growth could lead to an increase of metastases because neovascularisation could permit and support spreading of cells of the primary tumour (Liotta et al. 1974). On the other side a decrease of angiogenesis is associated with decreased metastasis (Starkey et al. 1988). The vascularisation of a tumour and the resulting metastasis are demonstrated in the following:

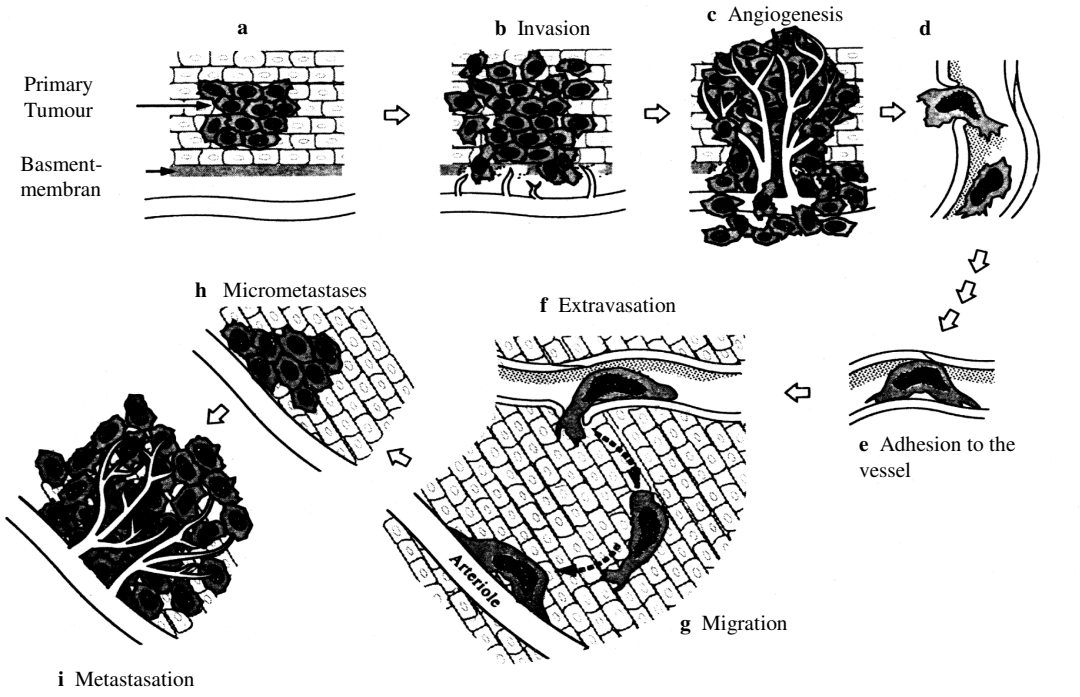


Fig. 1.1: Vascular components of tumour metastasis (Zetter 1998). The steps of metastasis pathway that involve interactions with blood vessels: (a) small primary tumours (<2 mm) remain avascular until they (b) invade the local epithelia basement membrane. If the tumour cells produce angiogenic factors (c) angiogenesis will occur, allowing expansion of the primary tumour. (d) The new blood vessel provides a route of entry into the bloodstream and the tumour cells circulate until they die or (e) attach specifically to endothelial cells in the vessels (usually venules) of downstream organs. (f) The tumour cells extravasate through the vessel wall and the (g) migrate to sites proximal to arterioles where their growth is enhanced. (h) Micrometastases can remain dormant for extended time periods during which angiogenesis is suppressed. (i) Initiation of angiogenesis at the secondary site releases the metastatic colonies from dormancy and allows rapid growth.

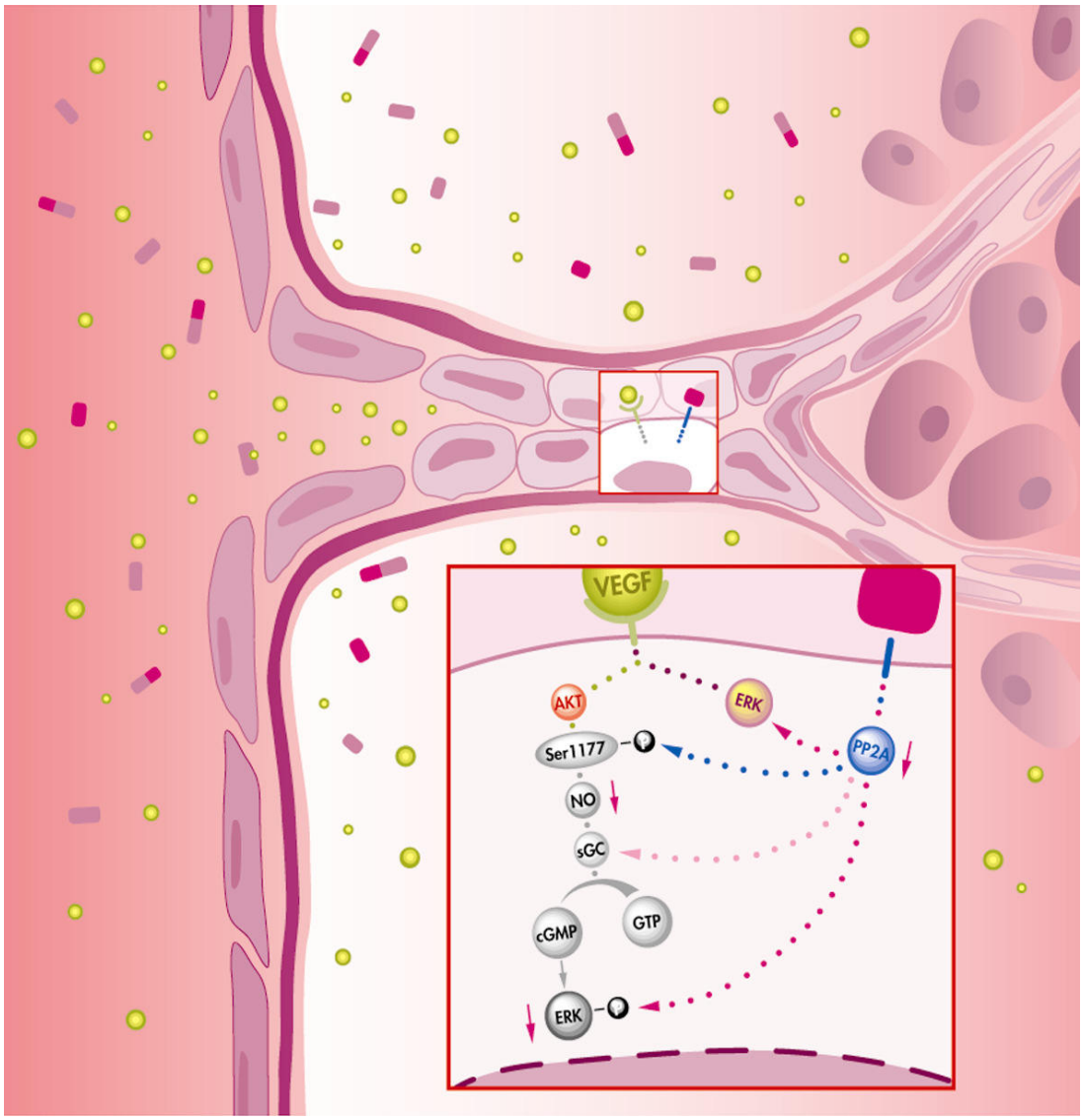
1.1.5 Wound Healing

Wound healing is traditional divided into three phases-the inflammatory phase, the proliferation phase, and the remodelling phase. Injury to adult tissues initiates a series of events including inflammation, new tissue formation, and tissue remodelling (Clark 1996, Martin 1997). The formation of new tissue is initiated by migration and proliferation of keratinocytes at the wound edge within 1 day after injury. New stroma, often called granulation tissue, begins to form after a delay of 2 or 3 days. This tissue consists of inflammatory cells, fibroblasts. Loose connective tissue, and numerous capillaries that endow the neostroma with its granular appearance. The new capillaries are generated by sprouting from pre-existing vessels, in a process called angiogenesis. The later involves migration and proliferation of endothelial cells, lumen formation, and stabilisation of the new vessel by recruitment of associated supporting cells (Risau 1997, Augustin 1998). Wound angiogenesis is essential to support the regenerating tissue with oxygen and nutrition (Clark 1996).

1.2 Signaltransduction

Changes of the vascular system induced by growth factors, are mostly linked to receptor-mediated signalling pathways. An exemplary pathway for such processes, but very important for this work is the VEGF/Flk-1/eNOS/NO/sGC/cGMP/ERK1/2 pathway. Vascular endothelial growth factor (VEGF) is a secreted protein that is a specific growth factor for endothelial cells (Ferrara and Henzel 1989, Keck et al. 1989). It is angiogenic *in vivo* and *in vitro* assays (Breier et al. 1992, Goto et al. 1993) and its physiological importance in vasculogenesis is well documented (Fong et al. 1995, Shalaby et al. 1995). The action of VEGF is regulated by two receptor belonging to the tyrosine kinase family Flt-1 and Flk-1 (De Vries et al. 1992, Terman et al. 1992). Flt-1, which has higher affinity for VEGF than Flk-1, is required for endothelial cell morphogenesis, whereas Flk-1 is involved in primarily mitogenesis (Fong et al. 1995, Shalaby et al. 1995, Waltenberger et al. 1994, Keyt et al. 1996). VEGF effects on permeability (Wu et al. 1996) and vascular tone (Ku et al. 1993) are coupled to nitric oxide (NO) production. Consistent with these findings, in the past it was demonstrated that NO production and cGMP elevation contribute to the angiogenic effect of VEGF (Morbidelli et al. 1996, Ziche et al. 1997). The activation of mitogen-activated protein kinase (MAPK) cascade by VEGF has been recently demonstrated (D'Angelo et al. 1995). MAPKs are important intermediates in signal transduction pathways and they are stimulated

by a variety of agents (Seger and Krebs 1995). The 44- and 42-kDa MAPK (ERK1/2) isoforms are ubiquitously expressed (Blenis 1993, Davis 1993). Finally, Parenti et al. were able to describe the postreceptor signalling pathway underlying VEGF actions on endothelial cells as Flk-1/eNOS/NO/sGC/cGMP/ERK1/2 cascade activated by VEGF (Parenti et al. 1998). An example for the regulation of eNOS and consequently for the regulation of this pathway is the activation of eNOS by phosphorylation of eNOS at Ser1177. This activation leads to an increased NO-production, whereas the phosphorylation of the eNOS at Ser495 decreases the NO-production (Dimmeler et al. 1999).



1.3 Endostatin

In the year 1997 endostatin was identified first by a Harvard group and was described as an endogen inhibitor of angiogenesis and tumour growth (O'Reilly et al. 1997). It is a proteolytic fragment of the C-terminal end of collagen XVIII (α1(XVIII) collagen) with a size of 20-22 kDa (O'Reilly et al. 1997). The α1(XVIII) collagen is a usual collagen, characterised by 10 domains of tripelhelical repetitions which are separated by non-tripelhelical repetitions (Oh et al. 1994A and B, Rhen and Pihlajaniemi 1994). α1(XVIII) collagen contains a non-collagenous domain (NC-1) which consists of 300 amino acids. The angiogenesis inhibitor endostatin is a component of this NC-1 domain and consists of 184 amino acids (Hohenester et al. 1998). By analysing the crystal structure of the proteolytic fragment of the NC1-domain of collagen XVIII, (Hohenester et al. 1998) a heparin-binding site was detected with the presence of an extensive basic patch formed by 11 arginine residues. Therefore Hohenester et al. suspected that endostatin inhibits angiogenesis by a competitive binding to heparan sulphate proteoglycans (HSPGs) to which normally angiogenesis stimulating factors bind. Also the heparin binding capability of endostatin was already used for its isolation from hemangioendothelioma-conditioned medium (O'Reilly et al. 1997). However Chang et al. could show, that binding of endostatin to components of the extracellular matrix, contrary to that of pro-angiogenic factors, is resistant to a treatment with heparinase. Further it has been shown that there is no competition between endostatin and pro-angiogenic factors for binding sites of the extra cellular matrix (Chang et al. 1999). Therefore the anti-angiogenic effect of endostatin could not be a consequence of displacement of pro-angiogenic factors (Chang et al. 1999). The findings regarding heparin-binding capacity of endostatin and heparin necessity were mainly analysed biochemically by solid phase binding (Sasaki et al. 1998/99). This analysis led to the conclusion that the heparin-binding site of endostatin is necessary for its anti-angiogenic activity. On contrary Yamaguchi et al., analysing endothelial migration showed, that heparin binding is not necessary for endostatin's anti-angiogenic effect. It is still unclear, though, whether the heparin binding site is necessary for the anti-angiogenic activity of endostatin (Sasaki et al. 1998, Sasaki et al. 1999, Karumanchi et al. 2001) or not (Chang et al. 1999, Yamaguchi et al. 1999). Further discussed binding sites for endostatin are glypican and tropomyosin, binding sites with a varying affinity (Karumanchi et al. 2001, MacDonalds et al. 2001).

Recombinant endostatin emerged as a specific inhibitor of endothelial cell proliferation in doses dependent manner. O'Reilly et al. could show that a systemic treatment of mice with 0.3mg/kg/day endostatin leads to a prevention of metastasis and simultaneously to an inhibition of lewis-lung-carcinoma growth (O'Reilly et al. 1997), on the contrary endostatin treatment did not influence resting endothelial cells and did not induce drug resistance (Boehm et al. 1997). On the other hand mice deficient in the endostatin precursor collagen XVIII develop normally with limited evidence of disturbed vascular morphogenesis (O'Reilly et al. 1997, Fukai et al. 2002). Thus, collagen XVIII itself does not appear a relevant regulator of vascular development but releases its activity after proteolytic cleavage. Another effect of endostatin is the induction of apoptosis in endothelial cells (Dhanabal et al. 1999A, Dixelius et al. 2000). In endothelial cells a reduction of the anti-apoptotic protein Bcl-2 and Bcl-x_L could be observed while the pro-apoptotic protein Bax remained unchanged (Dhanabal et al. 1999A). Proliferation (Dhanabal et al. 1999B, Yamaguchi et al. 1999) and migration (Dhanabal et al. 1999B and C, Shichiri and Hirata 2001) of growth factor stimulated human umbilical vein endothelial cells (HUVEC), porcine pulmonary artery endothelial cells (PAEC), bovine aortic endothelial cells (BAEC) was also inhibited under influence of endostatin. For endostatin two different binding sites with varying affinity have been described. These binding sites are glypican and tropomyosin (Karumanchi et al. 2001, MacDonalds et al. 2001).

Endostatin influences morphology of endothelial cells and vessels (Dixelius et al. 2000), wound healing (Bloch et al. 2000, Mundhenke et al. 2001), of tumour vessels (Mundhenke et al. 2001, Read et al. 2001) as well as of embryonic stem cell derived endothelial cells and tubes.

Endostatin, which influences signalling molecules in endothelial cells, leads to a down-regulation of MAP-kinase (ERK-1/2) (Knebelmann et al. 1999), influences signalling molecules c-myc and c-fos (Shichiri and Hirata 2001), activates PKA and increases intracellular cAMP (Shichiri and Hirata 2001), induces Shb tyrosine phosphorylation (Dixelius et al. 2000). It increases Ca^{2+}-influx (Jiang et al. 2001) and influences VEGF-receptors as well as phosphorylation of 38 MAKP and 125^{FAK} (Kim et al. 2002). Endostatin has also an effect on the activity of endothelial NO-synthase (eNOS) by influence the

phosphorylation of Ser1177 (Urbich et al. 2002), which probably leads to a reduction of cGMP (Verma et al. 2002). Recently it has been shown that endostatin down-regulated the VEGF-expression in tumour cells (Hajitou et al. 2002).

1.4 Restin

Restin, a proteolytic fragment of collagen XV is described as an anti-angiogenic protein that inhibits tumour growth and endothelial migration (Ramchandran et al. 1999). Ramchendran et al. have shown that restin has 60% homology to endostatin. It decreases tumour volume by 30% and did not bind to heparin. Collagen XV is like collagen XVIII a basement membrane collagen (Sasaki et al. 2002). Both are members of the mulitplexin family (Oh et al. 1994A and B, Sasaki et al. 2002).

1.5 Embryonic Stem Cells (ES-cells)

The embryoid body as *in vitro* system gives the opportunity to observe comprehensively the endothelial differentiation program and it is therefore an adequate system to analyse the molecular mechanisms, which are involved in vasculogenesis and angiogenesis (Vittet et al. 1996). Embryonic stem cells are non-transformed cell lines, which are extracted directly out of pluripotent foundation tissue of mice embryo, the epiblast (Brook and Gardner 1997, Evans and Kaufman 1981, Martin 1981). It has been shown that they have the potential to generate all embryonic cell lineages when they undergo differentiation (Smith 1992). ES-cells can be maintained in their pluripotential state if cultured in the presence of leukaemia inhibition factor (LIF), which inhibits their differentiation. When LIF is removed, ES cells spontaneously differentiate into cyst-like structure, termed embryoid bodies (EBs), which contain derivatives of the three primitive germ layers (Smith 1992). The appearance of blood island-like structures that consist out of immature hematopoietic cells surrounded by endothelial cells and the formation of vascular-like channels have been reported on the surface of cystic EBs, suggesting that ES-cells produce all factors which are necessary for the induction of vasculogenesis (Doetschman et al. 1985, Risau and Lemmon 1988, Wang et al. 1992, Doetschman et al. 1993, Young et al. 1995). The aggregation of ES-cells induces the formation of several cell types (Bain et al. 1995, Okabe et al. 1996, Fraichard et al. 1995, Strubing et al. 1995). To receive EBs of equal size, which therefore are comparable, a defined

amount of ES-cells were taken to perform EBs by a cultivation technique called „hanging drop“ method (Fasseler et al. 1996) (fig. 2.1). After attachment, EBs begun to grow out centrifugal from the central cell accumulation. The area around the point of attachment shows strongest proliferation but low differentiation. Due to the centrifugal outgrowth in the periphery of EBs only a thin cell layer could be found, however with the strongest differentiation. By differentiation of pluripotent ES-cells inside of EBs several different cells types could be found, like endothelial cells (Wang et al. 1992, Keller 1995), muscle cells (Keller 1995, Drab et al. 1997), neuronal cells (Bain et al. 1995, Okabe et al. 1996, Fraichard et al. 1995, Strubing et al. 1995, Keller 1995), cardiac muscle cells (Wobus et al. 1991, Maltsev et al. 1994, Hescheler et al. 1997), keratinocytes (Bagutti et al. 1996), heamatopoetic cells (Keller 1995) and so on. During the formation of vascular structures in EBs several different endothelial specific marker appear, like PECAM-1 (Platelet Endothelial Cell Adhesion Molecule-1) (DeLisser et al. 1994), VE-(vascular endothelial)cadherin (Lampugnani et al. 1992) and growth factor receptors like Flk-1 (Yamaguchi et al. 1993, Oelrichs et al. 1993 and tie-1/tie-2 (Sato et al. 1993, Schnurch and Risau 1993). The expression pattern of endothelial specific markers, found in EBs, seems to be very similar to that of embryonic angio- and vasculogenesis (Baldwin et al. 1994, Yamaguchi et al. 1993, Sato et al. 1993, Breier et al. 1996, Dumont et al. 1995).

1.6 Target of the Work

The target of this work is to analyse the influence of the extracellular matrix derived factors endostatin and restin on the endothelial cell biology during vascular development. This examination should be performed by a model system of embryonic vessel development, the Embryoid Body (EB) as well as by a model system of regenerative neovascularisation, the physiological wound healing. The following questions should be answered:

- Give the existence and distribution of binding sites for endostatin and restin, evidence for an anti-angiogenic influence in human urological tumours?

- Are embryonic vessels accessible for endostatin and restin, do they express and accumulate these molecules and show binding sites for them?

- Do endostatin and restin influence the embryonic vessel development and the regenerative neovascularisation?

- Is the heparin-binding site decisive for the effects of endostatin and restin?

- Is the morphogenesis of vessels influenced by endostatin and restin?

- Do this factors influence the endothelial signaltransduction?

2. Material and Methods

2.1 Used chemicals and materials

The following chemicals were obtained from Gibco BRL Gaithersburg:

Dulbecco`s Modified Eagle Medium (DMEM)
Foetal Calves Serum (FCS)
L-Glutamin
MEM (non-essential amino acids)
Penicillin
Streptomycin

The following chemicals were obtained from Merck, Darmstadt, Germany:

Boron acid (crystal/H_3BO_3)
Di-Sodium-carbonate (Na_2CO_3)
Di-Sodiumhydrogenphosphate-di-hydrate (Na_2HPO_4)
EDTA (Ethylendinitroilotetraaceticacid/$C_{10}H_{14}N_2Na_2O_8$)
HEPES (N-[2-Hydroxyethyl]-piperazineN‘-(2-ethansulfonacid/$C_8H_{18}N_2O_4S$)
Potassium-Chloride (KCl)
Magnesium-Chloride ($MgCl_2$)
Sodium-Chloride (NaCl)
Sodium-di-hydrogenphosphatemonohydrate (NaH_2PO_4)
Sodium-hydrogen-carbonate ($NaHCO_3$)
Paraformaldehyd ($(CH_2O)_n$)
Proteinase K
Saccharine ($C_{12}H_{22}O_{11}$)
Tri-Sodium-Citrate ($C_6H_5Na_3O_7$)
Tris (Trishydroxymethylaminomethan/$C_4H_{11}NO_3$)
Tween 20

Chloroform ($CHCl_3$)

Acetic acid ($C_2H_4O_2$)

Methanol (CH_4O)

The following materials were obtained from Sigma Aldrich, Taufkirchen, Germany:

Acrylamid (C_3H_5NO)

Ammoniumchlorid (NH_4Cl)

Amoniumpersulfat ($(NH_4)_2S_2O_8$)

Bovine Serum Albumin (BSA)

DAB (3-3‘Diaminobenzidin)

DMSO (Dimethylsulfoxide/C_2H_6OS)

DTT (Dithiothreitol)

Gelatine

Glycerin ($C_3H_8O_3$)

N’N‘-Methylenbisacrylamid ($C_7H_{10}N_2O_2$)

Pipes (Piperazine-N,N‘-bis[2-ethanesulfonicacid]/$C_8H_{18}N_2O_6S_2$)

PMSF (Phenylmethylsulfonyl Fluoride/$C_7H_7FO_2S$)

Salmon Sperm DNA

SDS (Sodium Dodecyl Sulfat/$C_{12}H_{25}O_4SNa$)

TEMED (N,N,N‘,N‘-Tetramethylenediamine/$C_6H_{16}N_2$)

Trypsin

Yeast tRNA

The following materials were obtained from Serva, Heidelberg, Germany:

Bromphenolblue

Mitomycin C

PG 6000 (Polyethylen Glycol)

Triton X-100

Glycin ($C_2H_5NO_2$)	Fulka Chemie AG, Buchs, Switzerland
Formamid (CH_3NO)	Fulka Chemie AG, Buchs, Switzerland

Coomassie Brilliant Blue R 250	BioRad Laboratories Munich, Germany
Powdered milk (fatty free):	Glücksklee, Nestle, Germany
Paraffin	Sherwood Medical (Paraplast Plus), Ireland
Ethanol	Firm Hofmann, Düsseldorf, Germany

The following materials were obtained from Falcon (Becton Dickinson), Heidelberg, Germany:

Bacteriological tissue culture dishes (6 cm, 10 cm)
Multiwellplates
Centrifugation tubes

The following equipment was obtained from Heraeus Instruments, Hanau, Germany:

CO_2-Incubator
Lamina

The following materials were obtained from BioRad Laboratories, Munich, Germany:

Gel chamber
PowerPac 300

The following materials were obtained from B. Braun Biotech International, Melsungen, Germany:

Micro-Dismembranator U
Teflon-container
Wolfram carbide-bead

The following equipment was obtained from Zeiss, Oberkochen, Germany:

Deconvolution-Microscope, Axiovert

Deconvolution-Software KS 400 3.0

Fluorescence-Microscope, Axiophot

Glass-Teflon-Homogenisator (Potter), IKA Laborwerke, Hannover, Germany

Micro pipettes/Variopipettes Eppendorf, Hamburg, Germany

All aqueous solutions were prepared with distilled water from our own distiller Milli-RX 20 from Millipore.

Humane kidney, bladder and prostate tissue was obtained from „Urologischen Poliklinik“ University cologne.

2.2 Technical Basics

2.2.1 Cell Culture

For culturing cells were kept in a CO_2-incubator surrounded by 5% CO_2, 37°C and 95% humidity. All steps were taking place under sterile conditions, working under laminar air with sterilised materials and media.

2.2.1.1 Stem Cell Culturing - the D3 cell line

Mouse blastocyst-derived embryonic stem cells of the D3 cell line were maintained and cultured as described by Doetscham et al. 1985. The adherent grown cells were passaged all 2-3 days to prevent a high cell density. A high density would have induced cell differentiation of the pluripotent embryonic stem cells. For passaging cells were washed wit 0.1 M PBS. Then cells were removed from the bottom by using the synthetic enzyme accutase, which has both characteristics of collagenase and trypsin, but in a mild manner. Cell suspension was centrifuged (5 min., 800 rpm, Heraeus Instruments, Labofuge 400, Hanau). After rejecting of supernatant the cell pellet was resuspended in DMEM-medium containing 15% FCS. After counting, 10^6 cells were plated in a 6 cm dish containing an inactivated feeder layer. The feeder layer provides the possibility to attach but more important the feeder cells produce leukaemia inhibition factor (LIF), which prevents the differentiation of embryonic stem cells. Stem cells medium was changed every day.

0.1 M PBS:	81mM Di-Sodiumhydrogenphosphate-di-hydrate
	19 mM Sodium-di-hydrogenphosphatmonohydrate
	150 mM Sodium chloride, pH 7.4

2.2.1.2 Preparation of mouse embryos to gain feeder cells

To gain feeder cells 14-16 days pregnant mice were required. After killing, the uterus was cut out incubated in a petridish containing 0.1 M PBS. Then the embryos were preparated out of uterus and amniotic sac and incubated in fresh PBS. The thorax was opened; organs and the head were removed. The embryos were chopped up with scalpels in a dish with 0.1% trypsin and 0.02% EDTA. After homogenisation the cell suspension were filtered by using a sieve and distributed in petridishes (10 cm) containing DMEM-medium (10% FCS).

2.2.1.3 Culturing of feeder cells

Regularly feeder cells were passaged in the same way as described for the embryonic stem cells. Because of a lower metabolism rate, feeder cell passage and medium exchange was effected once a week. Feeder cells were cultured in DMEM-medium containing 10% FCS.

2.2.1.4 Inactivation of feeder cells

A layer of feeder cells constitutes the basis for embryonic stem cell growth. But to prevent their proliferation they were inactivated using the cell toxin mitomycin C. Adherent cells were treated for 2 hours (37°C) to prevent mitosis activity with a concentration of 10 µg/ml. After inactivation cells were washed three times with PBS and stored in DMEM-medium containing 10% FCS till use.

2.2.1.5 Preparation of hanging drops

Embryonic stem cells were cultured in hanging drops (fig. 2.1) as described by Fassler et al 1996. Therefore adherent cells were removed and pelleted before counting them. The volume of a hanging drop should grasp 20 µl. Every drop should contain 600 cells. After dilution of the cell suspension in an appropriate manner (DMEM+20% FCS) 50-60 drops were prepared on the inside of the lid. The bottom of this bacteriological petridish was filled with 10 ml PBS to prevent dry out of hanging drops. Then the lid was turned over and placed on the bottom of the dish. Within the following two days the single cells join together to form aggregates.

2.2.1.6 Culturing of aggregates in suspension

Two days after preparing hanging drops, the aggregates (embryoid bodies) were rinsed and cultured in suspension (10 ml DMEM-medium plus 15% FCS) for the next three days. For this purpose bacteriological (non-adhesive) petridishes were used. During these three days cells proliferate but do not differentiate.

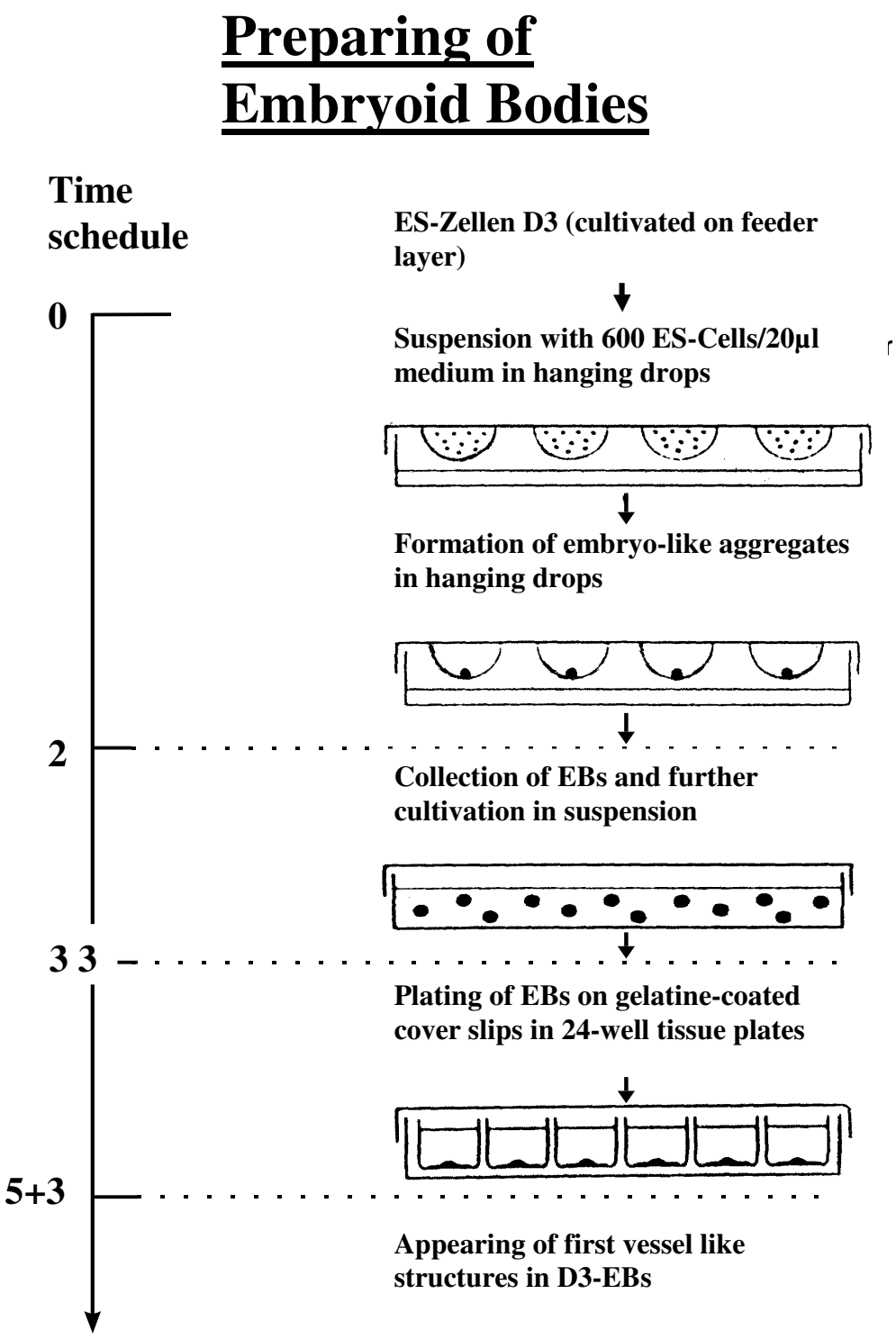


Fig. 2.1 Culturing of embryonic stem cells.

2.2.1.7 Gelatine coating and plating of embryoid bodies

Differentiation of the embryoid bodies (EBs) was induced by plating them on gelatine-coated cover slips. Therefore cover slips (12 mm diameter) were placed in every well of a 24-multi well plate and covered with 0.1% gelatine in 0.1 M PBS. Cover slips were incubated in gelatine solution over night. Before plating, the gelatine solution was removed, cover slips were air-dried and every well was filled with 1 ml medium (DMEM+15% FCS). Each embryoid body was taken out of suspension and placed in the middle of a gelatine-coated cover slip. Medium was changed the first time after three days to let EBs attach.

2.2.1.8 Components of DMEM-medium

DMEM-medium:	DMEM (Dulbecco's Modified Eagle Medium)
	50 U/ml Penicillin
	50 U/ml Streptomycin
	200 µM L-Glutanine
	100 µM β–mercaptoethanol
	1% MEM (non-essential amino acids)
	15% or 20% Foetal Calves Serum (FCS, v/v)

Before usage the foetal calves serum was heat inactivated for 30 min. at 56°C.

2.2.2 Tissue Processing

2.2.2.1 Fixation of Tissue

Cells and tissue, received fresh from operation room, were chemically fixed by using 4% paraformaldehyde (4% PFA) in 0.1 M PBS. During fixation the NH_2-groups of cellular and membrane proteins get connected by aldehyde bindings. The duration of fixation procedure depended on tissue size, due to the fact that PFA saturate tissue with a speed of 0.5 mm per hour. The tissue was then washed several times with 0.1 M PBS. For electron microscopy tissue was fixed in 2% paraformaldehyde/2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 over night. After washing in cacodylate buffer tissue was treated with 1% uranyl acetate.

4% PFA	4% parformaldehyde, 0.1 M PBS, pH 7.4

0.1 M PBS:	81mM Di-Sodiumhydrogenphosphate-di-hydrate 19 mM Sodium-di-hydrogenphosphatmonohydrate 150 mM Sodium chloride, pH 7.4
0.1 M Cacodylate-buffer	0.1 M Dimethylarsinicacid sodium salt trihydrate

2.2.2.2 Tissue embedding for electron microscopy

For the preparation of thin and ultrathin sections, tissues were fixed as described above. The sections were then incubated in 70% ethanol for 8 hours. Thereafter dehydration was performed in a series of graded ethanol, and specimens were then embedded in araldite. Thin sections of plastic embedded tissue were cut with a glass knife on a Reichert ultramicrotome (Reichert, Bensheim, Germany) and stained with methylene blue. Ultrathin sections (30-60 nm) for electron microscopic observation were processed on the same microtome with a diamond knife and placed on copper grids. The transmission electron microscopy was performed with a Zeiss 902A electron microscope (Zeiss, Oberkochem, Germany).

2.2.2.3 Tissue embedding in paraffin

For paraffin embedding tissue must be dehydrated. Therefore tissue was incubated for 24 hours in 30%, 50%, 70%, 90%, 96% ethanol and two times in 100% isopropanol. As inter medium chloroform was used for 24 hours. Thereafter tissue was incubated two times for 2-3 hours and following over night in paraffin (60°C). At the end tissue was lined up in a block, filled with fresh paraffin before cooled down to solidify. For optimal cutting conditions the paraffin block was cooled.

2.2.2.4 Tissue embedding for cryostat sections

To perform cryostat sections the tissue was fixed and washed as described above. Thereafter tissue was incubated in tissue freezing medium (Jung, Nussloch, Germany) covered by tin foil and store in –80°C. Before cutting tissue was stored for 30 min. at –30°C. The cryostat temperature during cutting was –30°C.

2.2.2.5 Shock freezing of tissue

Tissue for SDS-PAGE and western blotting received freshly from operation room was directly incubated in fluid nitrogen and stored in –80°C.

2.2.3 Immunohistochemistry

During the procedure of immunohistochemistry proteins and enzymes were specifically marked and detected by using corresponding antibodies. Immunohistochemistry with the following course is a standard procedure, however here only the principal will be described. The specific antibody choice and arrangement will be detailed individually for each experiment.

2.2.3.1 Procedure of immunohistochemistry

The permeabilisation of cells is an important point to facilitate the antibody binding to inner cell proteins. Therefore tissue sections and cells were incubated 10 min. with 0.25% Triton-X-100 and 0.5 M ammonium chloride in 0.05 M TBS. The detergent Triton-X-100 permeabilises the cell membrane while ammonium chloride reacts with free aldehyde groups to prevent an unspecific binding of the antibody. Following the cells were washed with TBS (3 x 10 min.). To prevent unspecific bindings due to loading differences, sections and cells were incubated with 5% BSA in 0.05 M TBS. The following incubation with the primary antibody occurred over night at 4°C. After several washings the incubation with the corresponding secondary antibody lasted 1 hour at room temperature. If the secondary antibody was conjugated with biotin instead of the fluorochrom CY2 or CY3, an incubation with streptavidin-conjugated CY2 or CY3 followed. For light microscopic observation a development based on an enzymatic reaction was performed by using DAB-solution. Therefore a streptavidin horseradish-peroxidase-complex was used after incubation with the biotin-conjugated secondary antibody. The development with DAB was controlled under the light microscope.

0.05 M TBS-buffer	50 mM Trishydroxymethylaminoethan (tris) 150 mM Sodium chloride, pH 7.6
DAB-solution:	0.1 M PB, 1% DAB, 0.75% H_2O_2
0.1 M PB:	81 mM Di-Sodiumhydrogenphosphate-di-hydrate, 19 mM Sodium-di-hydrogenphosphatemonohydrate, pH 7.4

2.3 Bladder, Kidney and Prostate Tissues

Malignant and benign bladder tissue specimens were obtained from 12 patients. Eight patients underwent transurethral resection and four patients underwent radical cystectomy for bladder cancer (mean age 67.9 years, median 69 years). In final histological staging, there were two patients with stage pTaG1, four with pT1G2, two with pT1G3, three with pT2G2, and one with pT3G3. In this study, prostate samples from 24 patients were tested. Prostate biopsies were collected from 12 patients with prostate cancer (mean age 69.7 years, median 70 years) before they underwent external beam radiation and HDR brachytherapy. In this prostate cancer group there were three pT2G1 cases, eight pT2G2 cases and one pT2G3 case. Normal benign prostate tissue specimens from 12 further patients with benign disease (mean age 69.7 years, median 70 years) served as controls. None of the prostate cancer patients in the first group had received preoperative hormonal therapy, radiation, or chemotherapy. The 21 patients benign prostatic hyperplasia underwent suprapubic prostatectomy.

Kidney tissues (malignant and benign) were obtained from twelve patients (average 63.8 years, median 64 years). All patients underwent nephrectomy for kidney tumours. In final histological staging, there were three patients with stage pT2G2, four with pT1G2, two with pT2G3, and three with pT3G2.

2.4 Animals

Balb/c mice and B2D2F2 mice were obtained from the animal care facility of the Max-Planck-Institute of Biochemistry, or from RCC (Füllinsdorf, Switzerland). They were housed and fed according to federal guidelines, and all procedures were approved by local authorities.

2.5 Wounding and preparation of wound tissue

Wounding and preparation of wound tissue was performed by Sabine Werner (Institute for cell biology, ETH-Zurich, Switzerland). Mice were anaesthetised with a single intreperitoneal injection of ketamine/xylazine. The hair on the animals' back was cut and the skin was wiped with 70% ethanol. Four full-thickness excisional wounds (6 mm diameter, 3-4 mm apart, two

wounds on each side of the spinal cord) were generated on the back of each animal by excising skin and *panniculus carnous* as described by Munz et al. 1999. The wounds were allowed to dry to form a scab. Animals were killed at various time points after injury. Prepared wound tissue was fixed as described above.

2.6 Different kinds of endostatin

For the following experiments three different kinds of endostatin were used. Mouse-endostatin M-ES(4), human endostatin (human ES(2)), restin, a proteolytic fragment of collagen XV as well as R158/R184/R270A (RA) a heparin binding mutant form of endostatin (A kindly gift of Dr. R. Timpl, MPI for biochemistry, Martinsried). This heparin binding mutant form of endostatin was described in detail before (Sasaki et al. 1999). The purification of recombinant mouse endostatin from transfected human kidney cells and a radioimmunoinhibition assay for quantitation have been described before (Sasaki et al. 1998). The extraction of tissue was performed as recently described (Miosge et al. 1999)

2.7 Endostatin treatment of cells and mice

Embryoid bodies (EBs) were treated with 50 ng/ml and 200 ng/ml endostatin, restin as well as the heparin mutant form of endostatin R158/R184/R270A (RA).
Female Balb/c mice or B2D2F2 mice (12 weeks of age) were injected with 0.3 mg/kg s.c. murine endostatin, restin and R158/R184/R270A in 100 µl 0.9% saline/0.02M ammonium acetate pH 6.8. Control animals were injected with solvent alone. Injection was performed daily at 11:00 a.m. for 2 days before wounding, immediately after injury and for 5 consecutive days. Mice were killed at day 5 after wounding.

2.8 Binding-Assay

2.8.1 Biotinylation of endostatin

The biotinylation of endostatin was performed as described previously by Friedl et al. 1997. Briefly, the protein endostatin, restin as well as the heparin mutant form of endostatin (R158/R184/R270A) were diluted in boron buffer (pH 8.8) up to a concentration of 1 mg/ml.

Biotin-X-NHS (MW 454.5, Calbiochem, Schwalbach, Germany) was dissolved in DMSO (1 mg/ml). 12.5% (v/v) of the biotin solutions were incubated with the endostatin solution at 4°C for 4 hours. Subsequently, surplus biotin was removed by filtration against boron buffer. The efficiency of the biotinylation procedure was analysed by detecting endostatin and biotin-conjugated endostatin through western blotting.

Boron buffer:	10 mM Boron acid (crystal 150 mM Sodium Chloride, pH 8.8

2.8.2 Fluoresceinisothiocyanat-(FITC-)conjugation of endostatin

FITC (Isomer I, on Celite, Sigma, Chemical Company, St. Louis, MO, USA) mixed with silica gel (N-HR, Macherey Nagel Düren, Germany) 1:10 (w/w). The FITC/ silica gel-mixture was mixed with the endostatin solution 1:3 (w/v) and incubated for 25 min. at room temperature. Then the solution was centrifuged to pellet the silica gel (5 min. at 2000 rpm, Heraeus Instruments, Labofuge 400, Hanau, Germany). The supernatant was dialysed against sodium-carbonate/bicarbonate-buffer (pH 9.5) to remove surplus FITC.

Sodium-carbonate/bicarbonate-buffer:	1 M sodium-hydrogen-carbonate 1 M Di-sodium-carbonate, pH 9.5

2.8.3 Western -Blot to examine the biotin-conjugation

In order to examine the biotinylation of endostatin a western-blot was performed as described below. The slots were filled either with biotin-conjugated endostatin or non-conjugated endostatin. One half of the blot was treated with a rabbit-anti-endostatin-antibody (polyclonal, 1:2000, a kind gift of Dr. R. Timpl MPI for biochemistry, Martinsried), the other half was treated with streptavidin-biotinylated-horseradish-peroxidase-complex (Amersham, LIFE SCIENCE, Little Chalfont, Buckinghamshire, England, 1:200). Streptavidin binds to biotin. Afterward the blot was developed with DAB-solution.

DAB-solution:	0.1 M PB, 1% DAB, 0.75% H_2O_2
0.1 M PB:	81 mM Di-Sodiumhydrogenphosphate-di-hydrate,

19 mM Sodium-di-hydrogenphosphatemono hydrate, pH 7.4

2.8.4 Incubation with biotin-conjugated endostatin

Embryoid bodies and tissue slices were incubated over night at 4°C with 50 ng/ml biotinylated endostatin, biotinylated restin and biotinylated heparin mutant endostatin respectively in 0.05 M TBS. After several washings bounded biotinylated endostatin was detected by using streptavidin conjugated CY3. Following an immunohistochemical detection of endothelial cells and vessels was performed as described below.

2.8.5 Ultrastructural Cytochemistry

Tumour tissues were fixed immediately after surgical removal in a fixative, containing freshly prepared 4% paraformaldehyde, in 0.1 M phosphate buffered saline (PBS, pH 7.4). After 4 hours of immersion fixation, tissue sections of 50-70 µm thickness were prepared on a vibratome (Leica VT 1000) and washed carefully in PBS. The endostatin-biotin binding was conducted for 12 hours at 4°C in 0.05 M tris buffered saline (TBS) followed by five washing cycles in TBS. Finally, the biotin-detection was carried out with Extravidin, coupled to 15 nm colloidal gold (Sigma-Aldrich, Taufkirchen, Germany) in TBS containing 0.8% bovine serum albumin (BSA) overnight at 4°C. After rinsing in TBS, the incubated tissue sections were stabilised with 2% glutaraldehyde in cacodylate buffer and post-fixed with osmiumtetroxide. After „en bloc„ counter staining with 1% uranylic acetate, the samples were dehydrated with ethanol and embedded flat in Araldite epoxy resin, using propyleneoxide as intermedium. Thin sections of silver-grey interference colour were cut on an ultramicrotome (Reichert Ultracut UCT) and collected on 150 mesh formvar coated copper grids, which were not further stained in order to intercept the gold signal in the electron microscopy. Also thin sections were collected on 100 mesh formvar coated nickel grids for preparing a pre-immunogold staining. Sections were washed several times in TBS, permeabelized with 5% HCl, blocked with 1% powdered milk and 0.1% Tween-20 in TBS for 30 min. at room temperature and incubated with Extravidin coupled with 15 nm colloidal gold (1:20, Zymed Laboratories, INC., San Francisco, CA, USA) for 2h at room temperature. Gold staining, to detect bound, biotinylated endostatin was stabilised with 2% glutaraldehyde for 10 min. and counter stained with uranylic acetate for 20 min. The sections were evaluated with a Zeiss EM 902A electron microscope at 80 kV.

2.9 Detection of endothelial cells and vessels

The immunohistochemical procedure was performed as described above. To mark endothelial cells and vessel like structures the antibody rat anti-mouse-PECAM-1 (CD31) was used (1:800, mAb, Pharmingen, San Diego, CA, USA). The relevant secondary antibody was sheep anti-rat Ig biotinylated (1:400, Amersham, LIFE SCIENCE, Little Chalfont, Buckinghamshire, England). This biotinylated antibody was detected with Extravidin conjugated CY 3 (1:1000, Sigma Chemicals, St. Louis, MO, USA; absorption 552 nm, emission 565 nm) or Alexa Fluor™ conjugated 488 (Mo Bi Tec, Göttingen), a fluorochrom that absorbs blue light with a wavelength of 495 nm and emit green light with a wavelength of 519 nm. For evaluation every vessel-like, PECAM-positive structure of at least six EBs from endostatin treated as well as untreated were counted.

2.10 Proliferation-Assay

In order to analyse the influence of endostatin on the proliferation an immunohistochemistry was performed with an antibody against Ki67 (Kosco-Vilbois et al. 1997, Traut et al. 1998, Heidebrecht et al. 1996). Ki67 is active in all cell cycles phases (G_1, S, G_2 and mitosis). In resting cells (G_0-phase) Ki67 lacks. After identifying the endothelial cells as described, proliferating cells were detected with the primary antibody rabbit anti-mouse-Ki67 (1:150, pAb, Dianova, Hamburg, Germany). The relevant secondary antibody was goat anti-rabbit conjugated CY2 (1:200, Dianova, Hamburg, Germany; absorption 495 nm, emission 519 nm). For utilisation proliferating endothelial cells of vessel-like structures were counted (n=6) with an Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany) by using a fluorescent double filter.

2.11 BrdU cell proliferation

Proliferation of EBs cultured with and without endostatin, restin as well as the heparin mutant form (R158/R184/R270A) were analysed by using a BrdU-Assay (n=4) (for detail see Bloch et al. 1997).

2.12 Apoptosis-Assay

The apoptotic effect of endostatin (O'Reilly et al. 1997, Dhanabal et al. 1999A) was analysed immunohistochemically focusing on caspase-3 and anti-Poly(ADP-Ribose)Polymeras(PARP)p85-fragment (Ellis et al. 1991, Steller 1995, Burek and Oppenheim 1996, Johnson et al. 1996, Cohen et al. 1992, Arends et al. 1990, Trucco et al. 1998, Smulson et al. 1998, Duriez and Shah 1997, Simbulan-Rosenthal et al. 1998, Saldeen and Welsh 1998, Patel et al. 1996, Alnemri et al. 1996, Fujita and Tsuruo 1998). Apoptotic cells were detected with a rabbit anti-active-caspase 3 antibody (1:500, pAb, Pharmingen, San Diego, CA, USA) or a rabbit anti-Poly(ADP-Ribose)Polymeras (PARP) antibody p85 fragment (1:250, pAb, Promega, Madison, WI, USA). The secondary antibody goat anti-rabbit conjugated CY 2 was used and the number of apoptotic endothelial cells were counted under a fluorescence microscope (n=6).

2.13 Migration-Assay

Migration-Assays were performed in a modified Boyden-Chamber by using a 24-well HTS Fluoro Blok ™ insert system (fig. 2.2) (Falcon Becton Dickinson GmbH, Heidelberg, Germany). This insert is made out of a polyethylene membrane with 8.0 µm pores and it blocks >99% of the light transmission in a wavelength region from 490 till 700 nm. Inserts were coated with 0.1% gelatine solution (in 0.1 M PBS) for 1 hour. EBs (5+12) were dissociated with Accutase (PAA Laboratories GmbH, Linz, Austria). Afterwards 10^4 single cells were put on top of an insert and incubated for 8 hours in DMEM with 15% FCS for control or additionally with endostatin, restin as well as the heparin mutant form (R158/R184/R270A) in a concentration of 50 and 200 ng/ml. Then, cells were fixed and immunohistochemically stained with PECAM-1 antibody. Afterwards, the membrane was cut out of the insert and covered in DAPI mounting medium (4´,6-diamidino-2-phenyl-indole, Vectashield, Vector Laboratories, Burlingame, CA, USA; absorption 360 nm, emission 460 nm) between two thin cover slips. The total number of migrated cells was counted as well as the number of migrated endothelial cells (n=6). It was not possible to perform a chemotaxis chamber with different gradient of endostatin as described by Dhanabal et al. 1999B and C

and Yamaguchi et al. 1999 because of its high diffusion rate trough the chamber. Difference in concentration would be balanced over a period of a few minutes.

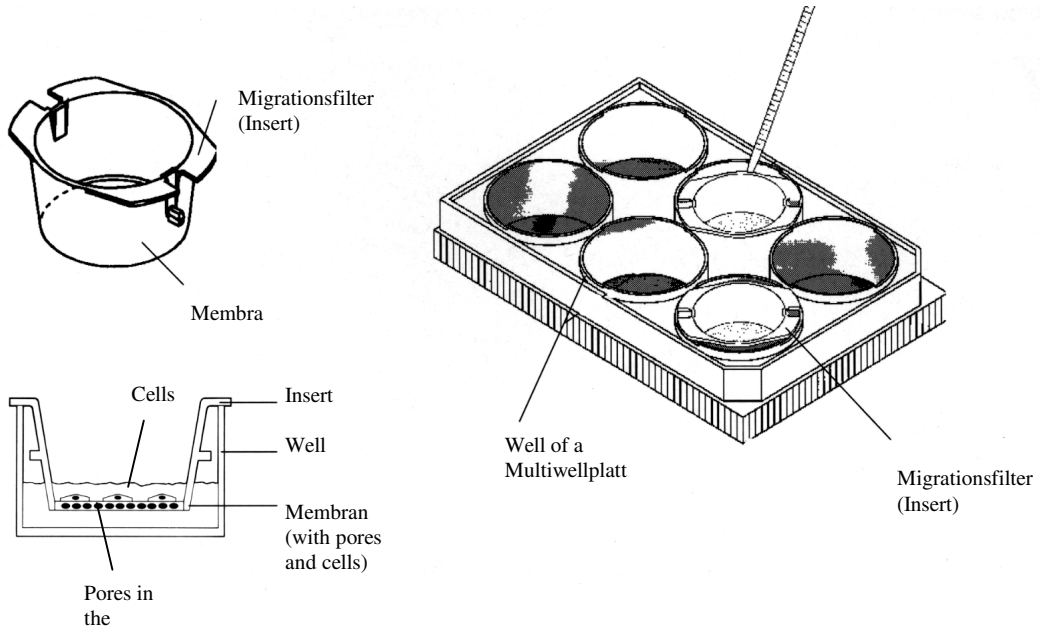


Fig. 2.2 Migration. Schematic depiction of a modified Boyden chamber

2.14 Signaltransduction

2.14.1Treatments

Embryoid bodies (EBs) were treated with 8bromo-cGMP (5 x 10^{-4}M, Sigma, Deisenhofen, Germany) for immunohistochemical analysis and for vital microscopy as well as with ODQ (10^{-6}M, Alexis, Grünberg, Germany), NONOate (10^{-5}M, Alexis, Grünberg, Germany), PD98059 (5 x 10^{-4}M, Calbiochem, Darmstadt, Germany) and with PP2A inhibitor, ocadaic acid (10^{-10}M, Calbiochem, Schwalbach, Germany) for immunohistochemical analysis.

2.14.2 Immunohistochemistry

The primary antibodies anti-active ERK1/2-kinase (1:400, m mAb, Sigma, Sant Louis, USA), anti sGC (1:1000, rb pAb, Alexis Biochemicals, Grünberg, Germany), anti-active AKT-kinase (1:500, rb pAb, upstate, Hamburg, Germany). anti-ERK1/2-kinase (1:400,rb, pAb, upstate, Hamburg, Germany), anti-AKT-kinase (1:500, rb, pAb, upstate, Hamburg, Germany), anti-cGMP (1:600, rb, pAb, Biogenesis, Poole, England), anti-iNOS (1:1000, rb, pAb, Biomol,

Hamburg, Germany), anti-bNOS (1:1000, mouse, mAb, Transduction, Lexington USA), anti-Nitrotyrosine (1:300, mouse, mAb, Calbiochem, Darmstadt, Germany), anti-eNOS (1:1500, rb, pAb, Biomol, Hamburg, Germany), anti-Flk-1 (1:200, rb, pAb, Santa Cruze, California, USA), anti-Flt-1 (1:200, rb, pAb, Santa Cruze, California, USA), anti-VEGF (1:200, rb, pAb, Santa Cruze, California, USA). Secondary antibodies, goat-anti-rabbit and goat-anti-mouse conjugated with biotin (1:400, Dako, Hamburg, Germany) were used in a dilution of 1:400 in 0.8% BSA for slices. Sheep-anti-rat Ig biotinylated (1:400, Amersham, LIFE SCIENCE, Little Chalfont, Buckinghamshire, England), goat-anti-rabbit Ig conjugated CY2, goat-anti-mouse Ig conjugated CY2 (1:200, Dianova, Hamburg, Germany, absorption 495 nm, emission 519 nm) as well as streptavidin-conjugated CY3 (1:1500, Amersham, LIFE SCIENCE, Little Chalfont, Buckinghamshire, England, absorption 552 nm, emission 565 nm) were used for cells. For utilisation an Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany) was employed for light and fluorescence (filter: CY3: absorb.: 552 nm and emiss.: 565 nm; CY2 absorb.: 495 nm and emiss.: 519 nm).

2.15 Magnet associated cell sorting (MACS)

Differentiated embryonic stem cells, cultured as described above, in the age of 5+7 EBs were dissociated with Accutase (PAA Laboratories GmbH, Linz, Austria). The single cell solution was washed with medium and incubated with the endothelial specific marker rat anti-mouse-PECAM-1 (CD31) (1:800, mAb, Pharmingen, San Diego, CA, USA) for 30 minutes at 37°C, 5% CO_2. Cells were washed with MACS-buffer (0.5% BSA and 2 mM EDTA in 0.1 M PBS) and incubated with the secondary antibody, which was conjugated with goat anti-rat IgG micro beads (1:4, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) for 15 minutes at 6-8°C. After washing cells were MACS sorted by using a mini-MACS system with MS separation columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The positive fraction gets cultured on gelatine-coated dishes. After 2-3 weeks the endothelial cells get passaged for the first time and afterwards once till twice a week. Cells were used till passage 8. MACS sorted endothelial cells (passage 3) were cultured with and without endostatin in a concentration of 50 ng/ml and immunohistochemical stained as described above.

2.15.1 Purity of MACS-sorted endothelial cells

The purity of cells was tested with dil-Ac-LDL (Paesel+Lorei GmbH & Co, Hanau, Germany). The MACS-sorted endothelial cells were tested for several endothelial specific markers. They were positive for PECAM-1, flk-1, flt-1, VEGF, Lectin, VE-Cadherin, eNOS and occasionally positive for CD34 and CD133. After staining with dil-Ac-LDL as well as PECAM-1/Hoechst Dey (Sigma Chemicals, St. Louis, MO, USA) the purity of the MACS-sorted endothelial cells was determined and amounted 89% (data not shown).

2.16 The live reporter gene EGFP under control of the PECAM-1 promoter

2.16.1 Vectors

The plasmid GL3-4500/+196 containing the human PECAM-1 promoter fragment was kindly provided by Dr. P.J. Newman (Gumina et al. 1997). The pEGFP vector construct (BD-Biosciences-Clontech, Heidelberg, Germany), which lacks eukaryotic promoter and enhancer sequences, was used as backbone expression vector. To generate the PECAM-1-promoter/EGFP construct, GL3-4500/+196 –was digested with PstI, and the resulting 4.6-kb PstI fragment of the human PECAM-1 promoter (-4500/+196) was subcloned into EGFP. The resulting vector GL3-4500/+196-(PECAM-1)-EGFP was used for electroporation of ES cells of the D3 line (Doetschman et al. 1995, Kolossov et al. 1998, Wobus et al. 1991).

2.16.2 Electroporation and selection procedure

GL3-4500/+196-(PECAM-1)-EGFP was digested by Eco47III restrictase in order to linearize the vector. 30µg of the Eco47III linearized vector was used for electroporation (240V/500µF) of 5 x 10^6 ES cells. Then, G418 selection was performed according to standard protocols (Kolossov et al. 1998). Embryoid bodies were generated using the hanging drop technique (Fasseler et al. 1996). Hanging drops were established by plating a suspension of ES cells in final concentration of 2-2.5 x 10^4 cells/ml in 20 µl (400-500 cells) of DMEM+20% of foetal calf serum on the lids of bacterial dishes. After incubation at 37°C, 5% CO_2 for 2 days the growing embryoid bodies were washed with 10 ml DMEM + 20% FCS into bacterial dishes and incubated for further 3 days. Then, embryoid bodies were plated separately on gelatine pre-treated 24 well plates. During all stages of development growing embryoid bodies were monitored under the fluorescent microscope using a FITC filter set (Zeiss, Jena, Germany). Consistent EGFP expression restricted to vessel-like structures was detected in 7 clones out of 20 clones investigated. For the present study were used preferentially two of these clones.

2.17 Vital microscopy or Time-lapse microscopy

In order to *live* monitor early stages of vasculogenesis, ES-cell derived vessel like structures were continuously observed up to 4 days (Kazemi et al. 2002). For that purpose an inverted microscope (Axiovert 100, Zeiss, Jena, Germany) was equipped with a computer controlled motorised stage (Merzhaeuser, Nikon, Germany), and a temperature/CO_2 controlled chamber (Buehler, Nikon). Using alternate transmission- and fluorescent pictures (conventional 100 W halogen lamp) were recorded at 30 min intervals using a FITC filter set as well as an oil-immersion objective 40x (Plan-Neofluar, Zeiss). Images were acquired using a colour video camera (Sony, DXC 950P, AVT Horn, Tuebingen, Germany) digitised on-line via a video frame grabber card (Matrox Corona, Nikon) and the Lucia software (Nikon). Enhanced fluorescence was achieved by the employment of a video-based buffer device (Sony MPU-F100P, AVT Horn) and the integration mode of the camera (integration of 5 to 10 pictures, respectively). The development of embryonic stem cell derived endothelial cells and vessels cultured in DMEM medium (20% FCS) was compared to differentiation influenced by endostatin (100 ng/ml).

2.18 Deconvolution

2.18.1 General description of the deconvolution method

2.18.1.1 The 3D-image stack

The prerequisite for a 3-dimensional analysis of an object is the data recording of each of the three dimensions (x, y, z). This is possible recording 2-dimensional pictures with a simultaneous focussing through the object. For this purpose every focused layer have a constant distance. The result is a stack of 2-dimensional pictures (Fig. 2.3), which contain the whole 3-dimensional information. For this a motorised focus mechanism is required.

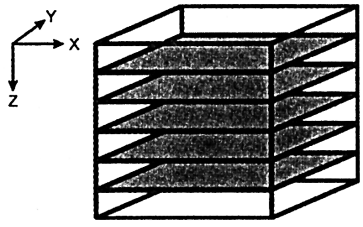


Fig. 2.3 Schematic depiction of a stack of pictures

2.18.1.2 Physical Background

In case of a fluorescent microscopic observation the object is fluorescent in a defined scale. During observation the fluorescent signal of the object passes the „optical path“ of the microscope while a line of optical components, like lenses, mirrors and prism appears before it reaches the corresponding sensor, like the retina or the camera. When the objective light signal passes the microscope it changes depending on different parameters with the result that the received representation of the object does not correspond to the original. This is not only linked to the „optical path“. The fact that fluorescent light is a poly-chromatical light and that each component of this poly-chromatical light is positioned on another focus level, leads to the fenomenous of light scattering. The result is a blurred representation of the original structure. In physical description, this means that during the process of representation the light signal gets „convoluted“ by the „Point-Spread-Function“ of the optical system. This „convolution“ generates relatively low problems in the case of a 2-dimensional representation of an object during lateral (xy-)observation. However upon 3-dimensional representation of an object the axial (z-)observation results in significant changes in comparison with the original form. The effect of this „convolution“ can be represented for objects with a known form, for which usually fluorescent beads with a known diameter were used. In lateral xy-sectional plane the representation shows a round but slight distorted form (fig. 2.4 A). However in axial xz-sectional plane it is difficult to recognise the form of a bead (fig. 2.4 B). The representation shows that the bead is strongly distorted in z-plane, what makes clear that artefacts were generated during a 3D-representation of an object what results in a form, which does not correspond to the original form.

2.18.1.3 Inversion of the representation process

With the help of mathematical methods the process, which leads to degeneration of the representation, can be calculated. The result is an approximately reconstruction of the original object. The documented stack gets „deconvoluted“ by employing the known „Point-Spread-Function“. The result of the „deconvolution“ is a new stack that shows the object in its original form (fig. 2.4 C and D). This process is complex and demands theoretical

assumptions about the conditions of the optical systems. As theoretical assumptions represent an approach, the 3D-deconvolution is an approximation to the real form of the object.

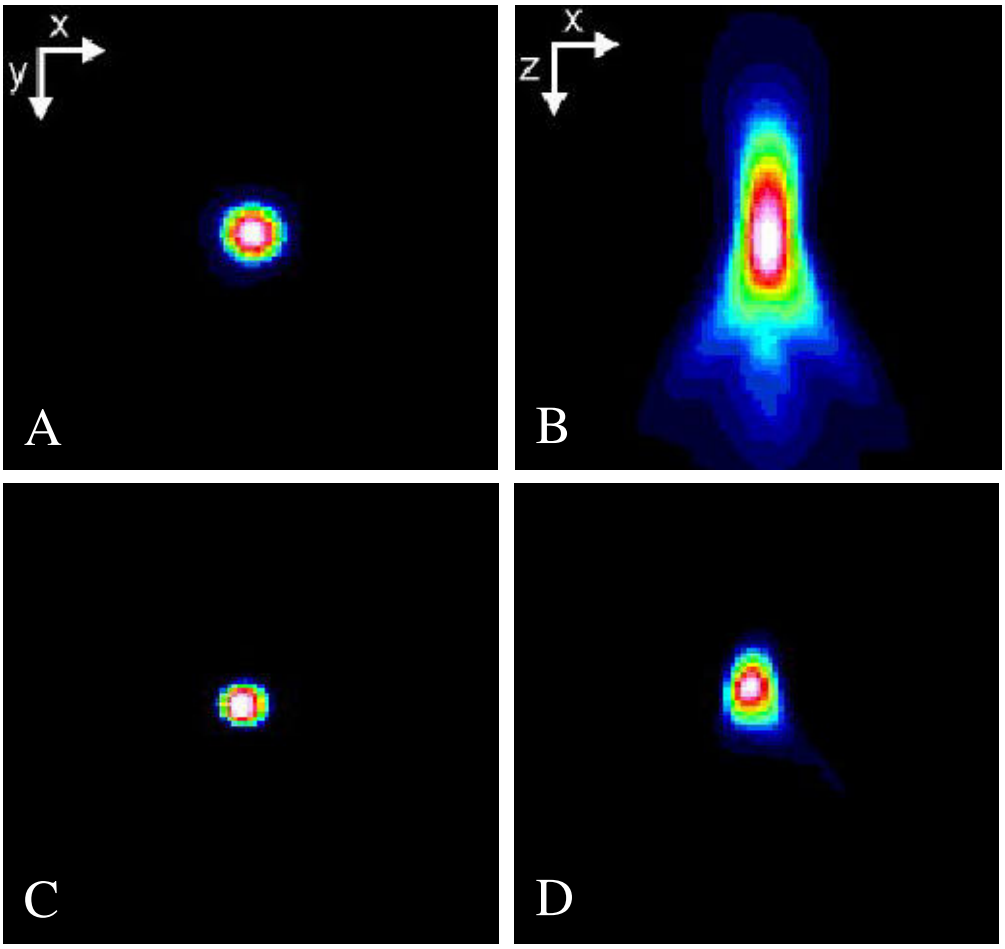


Fig. 2.4: Fluorescent bead to represent and calculate the „Point-Spread-Function“ of an optical system. (A) Fluorescent bead before deconvolution is represented. The presentation occurs in xy-orientation. The round form of this bead is observable. (B) Fluorescent bead before deconvolution in xz-orientation. The beat is strongly distorted. (C) The fluorescent bead after deconvolution in xy-orientation shows a clear round form. (D) After deconvolution the bead is rather less distorted in xz-orientation.

2.18.2 3D-Deconvolution of PECAM-positive vessels

Deconvolution microscopy was used as a method to prove if there are morphological differences between PECAM-positive vessel like structures in Embryoid bodies when treated with and without endostatin. This method was prepared as described by Bohle et al. 2001. An inverted Zeiss Axiovert 100 M with a Zeiss 63 Planapochromat oil immersion-objective was used. Bandpass filter sets for CY 2 and CY 3 (AHF) without overlap in the fluorescence emission spectrum as well as the commercial software package KS 400 from Zeiss were used. Each specimen was acquired as an image stack with 64 planes. The distance of the image planes were set to 100 nm to assure proper oversampling of the optical resolution for the subsequent iterative deconvolution process. Both, theoretical point-spread functions based on

microscope and specimen data and measured point-spread functions (PSF) calculated from fluorescent beads with a diameter of 1µm (Molecular Probes, T-14792) were used. The conjugated gradient maximum likelihood deconvolution algorithm with 60 iterations was performed on the image stacks. Output intensities were scaled between grey value 0 and 255. Generally theoretical and measured point-spread functions generated comparable results with slightly higher resolution in the resulting images by using the measured PSFs. Afterwards a 3-dimensional reconstruction of the object was performed by cutting the vessel like structure in diagonal direction. This facilitates to look into the vessel lumen.

2.19 Immuno-Blot (Western-Blot)

2.19.1 Homogenisation of tissue

For homogenisation urological tissues as well as mouse embryos in the age of E 10.5, E 13.5, E 15.5 and E 18.5 were incubated in fluid nitrogen directly after preparation. The frozen state of the tissue facilitated to smash bigger tissue pieces to small ones. The small tissue fragments were crushed afterwards with a wolfram carbide bead (5 mm) in a Teflon container by using a micro-dismembranator (1600 beats/min.) for 2 min. During this procedure the tissue pieces were crushed to powder. Following the powder was added with protease inhibitor containing preparation buffer and potted to a homogeny mass. After addition of freezing buffer, the homogeny probes were centrifuged 20 min. at 8000 rpm (4°C); the supernatant was used for a Bradford assay (Bradford 1976).

Preparation buffer:	240 mM Saccharine, 1 mM PMSF, 20 mM PIPES, 10 mM EDTA, 50 mM Sodiumhydrogenphosphate, pH 7.4
Freezing buffer:	400 mM Saccharine, 5 mM HEPES, 5 mM Tris, 10 mM EDTA, 50 mM Sodiumhydrogenphosphate, pH 7.2

2.19.2 Bradford-Assay

The determination of the protein concentration was performed according to Bradford with Coomassie Brilliant Blue. After a dilution line with bovine serum albumin (BSA) a calibration curve was drawn up. After adding Bradford reagent (BioRad Laboratories GmbH, Munich,

Germany) probes were measured in a spectrophotometer DU 650 (Beckman, Munich, Germany) with a wavelength of 595 nm.

2.19.3 SDS-PAGE

The electrophoresis was prepared under denaturing conditions in a modified way according to Laemmli 1970. Gels with a size of 7x10 cm and 0.5 mm thickness include stacking gel and running gel. Gels were vertically fit in a flat gel chamber. The protein containing homogenate was used in a total concentration of 40 µg/ml, taken in SDS-probe buffer, denatured 5 min. at 95°C and centrifuged (6400 rpm, neo Lab-Micro-Zentrifuge, neo Lab Heidelberg, Germany). The protein mixture were pipetted with a Hamilton syringe (Hamilton Bonaduz AG, Switzerland) in the slots. A protein standard of Sigma (Sigma, St. Louis, MO, USA; see below) was used as molecular weight marker. During electrophoresis 70 V / 16 mA were used per gel until the running front (Bromphenolblue) reached the running gel, than 100 V / 24 mA. Following the gel was stained with Coomassie-Blue or used for immunoblot (Western-Blot).

Assembly of gels:

	running gel (13.5% Acrylamid)	stacking gel
1.5 M Tris/HCl pH 8.8	1.5 ml	
0.5 M Tris/HCl pH 6.8		0.5 ml
Acrylamid solution	2.7 ml	0.34 ml
10 % SDS	60 µl	20 µl
10 % Amonium persulfat	40 µl	40 µl
TEMED	3 µl	2 µl
H_2O	1.7 ml	1.1 ml

Electrode buffer: 25 mM Tris (pH 8.3), 192 mM Glycin, 0.1% SDS

SDS-Probe buffer: 200 mM Tris/HCl, 50 % Glycerin, 6.4% SDS,
4% β-Mercaptoethanol, 0.0052% Bromphenolblue, (pH 7.2)

Acrylamid solution: 30% Acrylamid, 0.75% N'N'-Methylenbisacrylamid

Molecular weight marker (Sigma):	HMW-marker proteins	molecular weight (kDa)
	Myosin	205
	β-Galactosidase	116
	Phosphorylase b	97.4
	Albumin Bovine	66
	Albumin Egg	45
	Carbonic Anhydrase	29

2.19.4 Coomassie-blue-(CB-)staining of protein gels

The coomassie staining was prepared in a modified way according to Meyer and Lamberts 1965 in which methanol was replaced by ethanol. After electrophoresis the gel was incubated for 30 min. in CB-staining solution and following incubated in CB-destaining solution until the background was colourless. The gels were scanned for documentation.

CB-destain solution :	25% ethanol, 5% acetic acid, 70% H_2O
CB-staining solution:	0.2% Coomasie Brilliant Blue R 250 in CB-destain solution

2.19.5 Transfer of proteins on a PVDF-membrane

The transfer of electrophoretic separated proteins on a PVDF-membrane (BioRad, 0.2 micron) was carried out in a semi-dry-procedure with a „SemyDryBlotter II„ (KEMENTEC, Copenhagen, Denmark). The SDS-gel was equilibrated after electrophoresis for 15 min. in cathode buffer. Four layers of filter paper were equilibrated in cathode buffer, two layers in anode buffer I and one layer in anode buffer II. The membrane was washed and activated in methanol and equilibrated in anode buffer II. Afterwards the following transfer unity was built up between the two electrodes:

Cathode

four layers filter paper in cathode buffer

SDS-gel (equilibrated in Cathode buffer)

membrane (in equilibrated in anode buffer II)

one layers filter paper in anode buffer II

two layers filter paper in anode buffer I

Anode

A voltage 170 mA was used for 1 hour for blotting. Afterwards the blot-gel was stained with Coomassie to control the transfer.

Cathode buffer:	25 mM Tris, 40 mM Glycin, 20% methanol, pH 9.4
Anode buffer I:	300 mM Tris, 20% methanol, pH 10.4
Anode buffer II:	25 mM Tris, 20% methanol, pH 10.4

2.19.6 Immunological Detection

After the electron transfer of proteins, the PVDF-membrane was blocked for 1 hour at room temperature or over night at 4°C in washing buffer I containing 1% powdered milk and following washed three times for 15 min. in washing buffer I. Thereafter the incubation with the primary antibody rabbit-anti-endostatin (polyclonal, 1:2000, a kind gift of R. Timpl MPI for biochemistry, Martinsried) in washing buffer I followed (over night at 4°C). After washing (three times for 15 min.) with wash buffer I the incubation followed with the secondary antibody swine-anti-rabbit-conjugated-horseradish-peroxidase (Dako, Glostrup, Denmark, 1:200) in washing buffer I with 0.1% powdered milk for 1 hour at room temperature. The membrane was washed three times for 15 min. with washing buffer II and incubated for detection in DAB-solution (3-3‘Diaminobenzidin).

Washing buffer I:	25 mM Tris/HCl, 137 mM Sodium Chloride, 3 mM Potassium Chloride, 0.5% Tween 20, (pH 7.8)
Washing buffer II:	correspond washing buffer I with 0.05% Tween 20

DAB-solution: 0.1 M PB, 1% DAB, 0.75% H_2O_2

0.1 M PB: 81 mM Di-Sodiumhydrogenphosphate-di-hydrate,
19 mM Sodium-di-hydrogenphosphatemonohydrate, pH 7.4

2.20 In situ-Hybridisation

The usage of in situ-Hybridisation should give an inside in the expression pattern of endostatin in urological tumours and mouse embryos as well as regeneration during physiological wound healing.

2.20.1 Preparation of the endostatin cDNA-sonde

The vector pUC18 containing the sequence for establishing an endostatin cDNA-sonde (fig. 2.5) was a kind gift of Dr. R. Timpl MPI for biochemistry Martinsried.

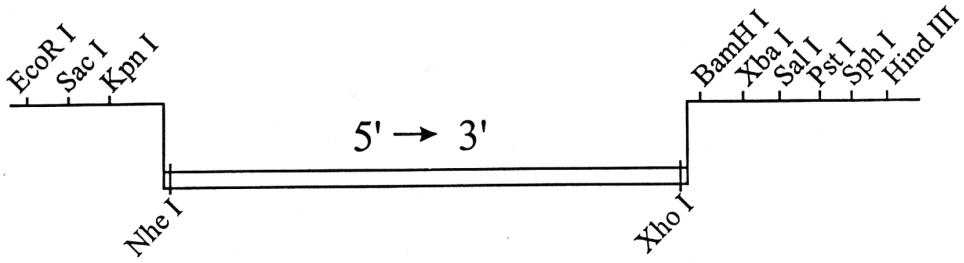


Fig. 2.5: Human α1(XVIII)ES/pUC18. The PCR-fragment with a size of 0.55 kb contained a NheI- and a XhoI-restriction side and was added in the with SamI cut pUC18-vector.

The necessary sequence was cut with the restriction enzymes Xho I and Nhe I (Pharmacia Biotech, CA, USA) with the following assembly:

15 µl DNA (1,5µg/µl, α1-human-endostatin in pUC-vector)
3 µl 10x buffer
2 µl Nhe I (8 U/µl)
2 µl Xho I (10 U/µl)
8 µl H_2O
30 µl

This solution was incubated over night at 37°C. After separation on a 1% agarose gel a 550 bp big fragment was received. The concentration of DNA was determined by using λ-DNA (New England Biolabs, Schwalbach/Taunus, Germany) in a further electrophoresis. The received DNA-band was cut out of the gel. Isolation of DNA occurred with a kit as described in manufacturers' instructions (QIAEX II Gel Extraction Kit, Qiagen, Hilden, Germany). By using a DIG-DNA-Labelling-Kit digoxigenin was bound via alkali labile ester binding to the nucleotides. The digoxigenin labelling of DNA was executed also as described by manufacturers' instructions (DIG-DNA-Labeling-Kit, Boehringer Mannheim, Germany). The DNA-sonde was stored at -20°C.

2.20.2 ProteinaseK-Treatment and Pre-hybridisation

After deparaffinisation, slices were washed in PBS/$MgCl_2$ buffer. The proteinaseK-buffer was warmed up to 37°C and was mixed with proteinaseK (10 µg/ml). The tissue was incubated for 10 min. at 37 °C. The reaction was stopped by washing in PBS/$MgCl_2$ (5 min., room temperature) before the probes were equilibrated in Glycin/Tris-Puffer (10 min., RT). For pre-hybridisation the pre-hybridisation solution was warmed up to 55°C and the tissue was incubated for 1 hour at 55°C.

PBS/$MgCl_2$ buffer: PBS, 1 mM Mg/Cl_2

ProteinaseK-buffer: 0.5 M Tris/HCl, 0.1 M EDTA, 0.1 M Sodium Chloride

Glycin/Tris-buffer: 0.1 M Glycin, 0.2 M Tris/HCl, pH 7.4

Pre-hybridisation solution: 2 x SSC (0.3 M Sodium Chloride, 30 mM tri-Sodium-Citrate, pH 7.0), 50 % Formamid, 10 µg/ml Salmon Sperm DNA

2.20.3 Preparation of cDNA-sonde and Hybridisation

For preparation of the sonde 5% of cDNA (endostatin-sonde, v/v), 45% hybridisation solution (v/v) and 50 % formamid (v/v) were denatured for 5-15 min. at 95°C and incubated on ice for

3 min. The tissue was incubated in this mixture over night at 55°C. After washing at 55°C the tissue was incubated for 1 min. in buffer I.

Hybridisation solution: PEG 6000, 5 M Sodium Chloride, 1M $MgCl_2$, 1 M Pipes, 0.2 M EDTA, 1 M DTT, 10 mg/ml Salmon Sperm DNA, 10x Denhardt, 5 mg/ml Yeast tRNA (10:12:1:1:0.5:1:1:1:0.5)

Wash buffer: 2 X SSC (0.3 M Sodium Chloride, 30 mM tri-Sodium-Citrate pH 7.0), 50 % Formamid

Buffer I: 0.1 M Tris/HCl, 0.15 M Sodium Chloride

2.20.4 Blocking and Detection

For blocking a Boehringer-Blocking-Mix (10 mg/ml, Boehringer Mannheim, Germany) was used together with FCS and buffer I (30 min. at room temperature). Thereafter the incubation with an anti-DIG-antibody-conjugate-AP (1:100, in buffer I + FCS, Boehringer Mannheim, Germany) lasted for 30 min. at room temperature. After three washing steps with buffer I slices were incubated in buffer III (2 min., room temperature). For detection 0.35% 5-Bromo-4-Chloro-3-Indolyl-Phosphate and 0.45% 4-Nitro-blue-tetrasodium-chloride (Boehringer Mannheim, Germany) was used. The detection was controlled under the microscope and stopped with Tris/EDTA-buffer.

Buffer III: 0.1 M Tris/HCl, 0.1 M Sodium Chloride, 50 mM $MgCl_2$

Tris/EDTA-buffer: 8 mM Tris/HCl, 0.8 mM EDTA

2.21 RT in situ-PCR

After fixation with 4% PFA (in 0.1 M PBS, pH 7.4, 30 min., room temperature), cells were washed with PBS/$MgCl_2$. Endogene peroxidase was blocked with 80% MeOH/2% H_2O_2 for 20 min. For digestion 10 mg/ml Proteinase-K was used in Proteinase-K-buffer (10 min., 37°C). To stop the reaction, cells were washed with PBS/$MgCl_2$ and equilibrated in 0.1 Glycin/0.2 M Tris-HCl, pH 7.4. For amplification a „C. therm Polymerase: One Step-RT-PCR

system“ (Roche Diagnostics, Mannheim, Germany) was used as described by the manufacturer. For amplification (thermocycler: MWG-Biotech AG, Ebersberg, Germany) of sGCalpha1 the following sequences were used: biotin 5’>CTC CAC CTT GTA GAC ATC CA <3’ / 5’>TGC ACT TCA GAG AAC CTT G<3’ (43.5°C annealing temperature) and for sGCbeta1: biotin 5’>CCC TTC CTT GCT TCT CAG TAG<3’ / 5’>GAC ACC ATG TAC GGT TTC GT (49°C annealing temperature). After amplification cells were washed several times and incubated with horseradish-peroxidase-complex (1:150, 45 min., room temperature). The detection of cells was prepared with DAB solution.

Proteinase-K-buffer	0.5 M Tris-HCl, pH 7.5; 0.5 M EDTA; 0.05 M Sodium Chloride
DAB-solution	0.1 M PB, 1% DAB, 0.75% H_2O_2
0.1 M PB:	81 mM Di-Sodiumhydrogenphosphate-di-hydrate, 19 mM Sodium-di-hydrogenphosphatemonohydrate, pH 7.4

2.22 Statistical analysation

The amount of endostatin binding to vessels was collected on randomly selected endostatin-labeled tissue sections and is presented as mean ± standard deviation. For each patient 50 vessels were examined with an Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany) using 400-fold magnification (CY2: absorb.: 552 nm and emiss.: 565 nm; CY3 absorb.: 495 nm and emiss.: 516 nm). 50 vessels per tumour slice and normal tissue slice were analysed for each patient. Binding intensity was evaluated by comparison of the fluorescence intensity derived from PECAM-1 and endostatin-binding staining. Distinct binding was described if the fluorescence intensity derived by endostatin-binding was comparable or higher then the fluorescence intensity derived from PECAM-1 staining. In case of recognizable but lower fluorescence intensity of binding staining compared to PECAM-fluorescence staining, a weak binding was described. Statistical analysis was performed using the adjusted t test with p-values corrected by the Bonferroni method ($p=<0.05$).

All data are presented as mean ± SD. Data analysis are performed using analysis of variance with Bonferroni post hoc test and/or Student`s test for unpaired data. Significance was considered at a P value < 0.05.

3. Results

3.1 Endostatin Binding to Benign and Malignant Bladder, Prostate and Kidney Tissues

From western-blot, immunohistochemistry and in situ-hybridisation analysation, we observed a distribution and expression of endostatin in both benign and malign bladder, prostate and kidney tissue (diploma work), concluding that binding sites for endostatin must be available. In order to investigate if a comparable binding pattern could be observed *in vivo*, we incubated cryosections of fixed and sucrose-embedded human bladder, prostate and kidney tissue with biotinylated endostatin. The tissue was derived from normal bladder, prostate and kidney tissue as well as from bladder, prostate and kidney tumours and benign prostatic hyperplasia (BPH). With regard to the bladder tumour tissue, distinct endostatin binding was observed in 94.2±3.01% of vessels of various diameters (Tab. 1, Fig. 1A and B), while weak endostatin binding was detectable in only 2.0±1.48% of normal bladder tissue vessels (data not shown). Significant differences in both the number of vessels showing endostatin binding and the binding intensity were observed when comparing benign and malignant prostate tissues ($p<0.005$). While distinct endostatin binding was found in 73.8±19.47% of prostate tumour vessels, a weak binding, was seen in 1.7±1.67% of normal prostate vessels (Tab. 1).

tissue type	endostatin binding vessels [%]
malignant bladder	94.2 +/- 3.01%
benign bladder	2.0 +/- 1.48%
malignant prostate	73.8 +/- 19.47%
benign prostate	1.7 +/- 1.67%
malignant kidney	11.32 +/- 3.85%
benign kidney	1.5 +/- 1.73%

Table 1: Distribution of endostatin binding in benign and malignant bladder, prostate and kidney tissues.

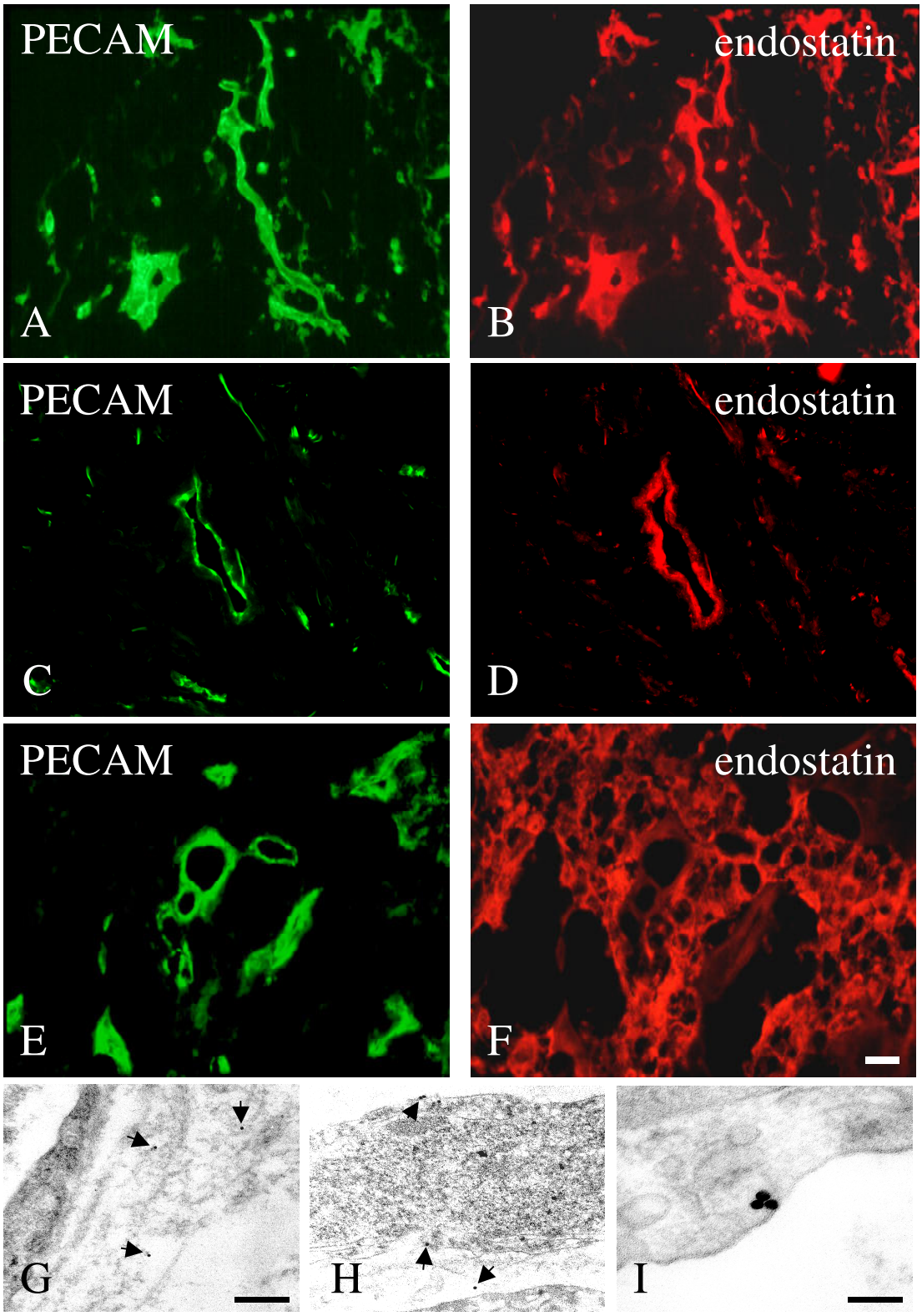


Figure 1: (A, C, F) Immunohistochemical detection of endothelial cells and vessels in bladder, prostate and kidney tumour with an anti-human-PECAM-1 antibody. (B) Binding of endostatin to vessels in bladder tumour. A distinct binding of endostatin to the PECAM-positive tumour vessels can be seen. (D) Binding of biotinylated endostatin to human prostate tumour vessels could be observed. (F) The PECAM-positive kidney tumour vessels do not show any endostatin binding. On the other site malignant kidney tumour cells show strong endostatin binding. Bar = 10 µm. (G, H, I) Ultrastructural detection of bound endostatin with streptavidin-gold. (G) Bar = 1 µm, (I) Bar = 70 nm.

In contrast to vessels in malignant bladder (Fig. 1A and B) and prostate tissue (Fig. 1C and D), only 11.32±3.85% of vessels in malignant kidney tissue (Fig.1E and F) show a weak or no detectable endostatin binding (Fig. 1F). The endostatin binding to vessels in benign kidney tissue was also weak (data not shown) and restricted to 1.5±1.73% of the vessels (Tab. 1). A distinct endostatin binding was observed for BPH in 89.12%±10.72% vessels of various diameters (Fig. 2).

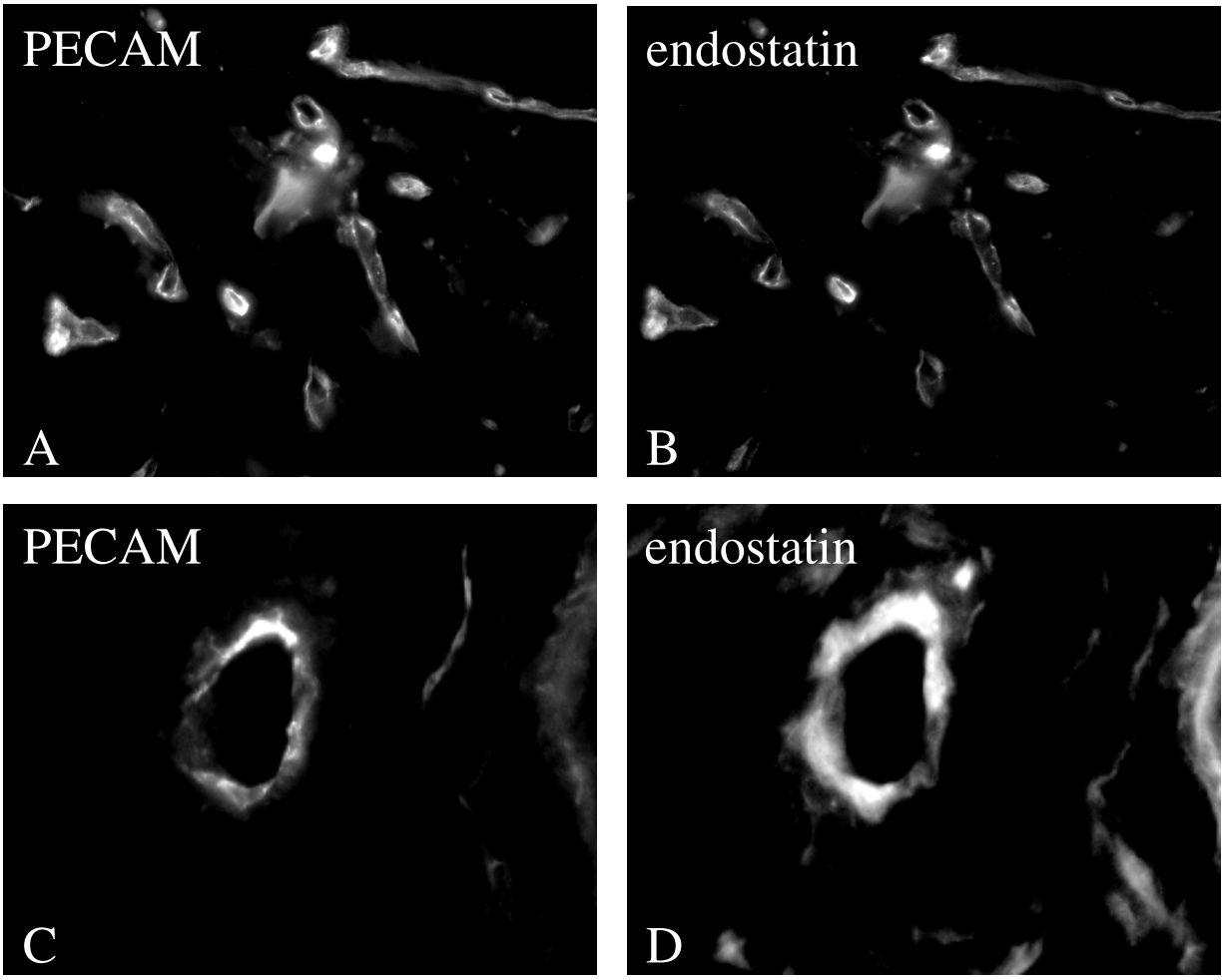


Figure 2: A-D: Endostatin binding to PECAM-positive vessels of prostatic hyperplasia. A and C show an immunohistochemical staining with PECAM-1 to marks endothelial cells and vessels. B and D show the detection of biotinylated endostatin boundet to these vessels.

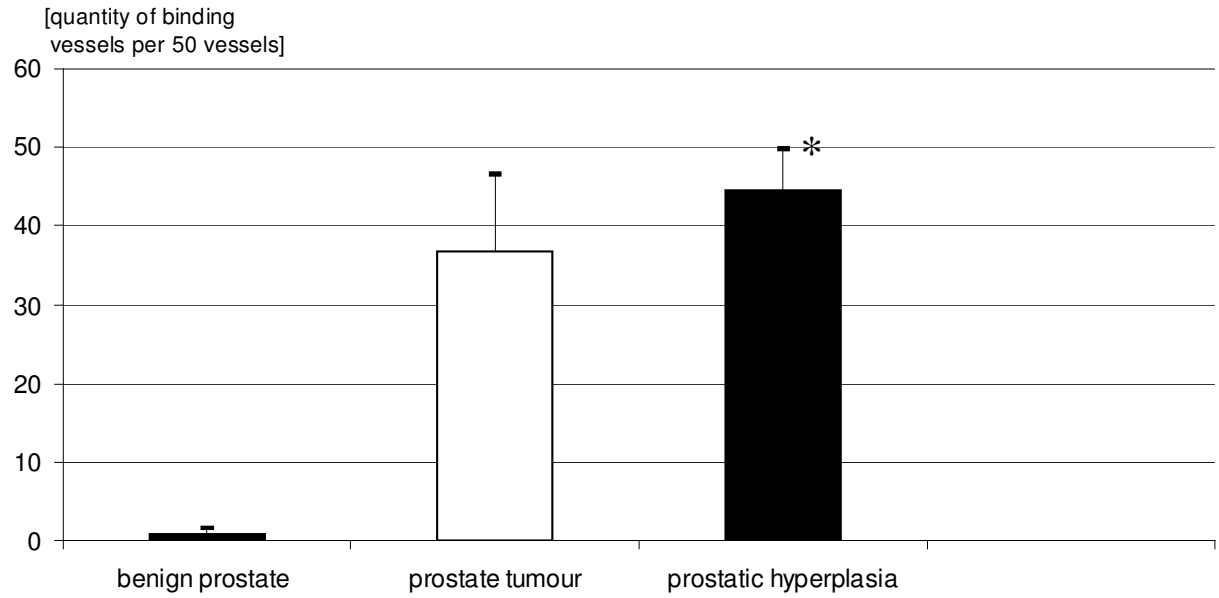


Figure 2: E: Statistical analysation of endostatin binding to vessels of prostate tissue, prostate tumour and BPH. Only few vessels bind endostatin in prostate tissue but significant more vessels bind endostatin in prostate tumour. Compared to this significant more vessels bind endostatin in BPH.

Control experiments, which were performed without biotinylated endostatin, did not show a fluorescence signal for any of the tissues (data not shown). Pre-incubation with a 100-fold higher concentration of endostatin prevents binding of biotinylated endostatin. Comparable results were obtained by using biotinylated restin and heparin mutant endostatin (data not shown). On ultrastructural level using extravidin gold to detect the bound biotinylated endostatin, the binding could be localised in the perivascular matrix including the endothelial basement membrane (Fig. 1G and H) and directly on the endothelial cell membrane as well as in endothelial cytoplasm (Fig. 1G and H). Abluminal and luminal membrane invaginations, also called caveolea, are preferred binding sites of endostatin (Fig. 1I).

3.2 Expression pattern of collagen XVIII/endostatin in the embryo

An important role for endostatin and restin during tumour development was described in literature. By western-blot, immunohistochemistry, in situ-hybridisation and distribution of binding sites we also concluded an important role and speculated about their role during embryonic vessel development which in terms is comparable with the tumour vasculatisation. In order to gain insight into the role of endostatin on embryonic vessel development we first analysed its distribution and expression in mouse embryos and EBs. In mouse embryos of different age we found positively stained blood islands (Fig. 3A) as well as a strong signal for

collagen XVIII/endostatin in vessels of mesenchymal tissue (Fig. 3B). This was corroborated by in situ-hybridisation (Fig. 3C, D) showing high expression of collagen XVIII/endostatin in blood islands (Fig. 3C) and mesenchymal cells (Fig. 3D) of mouse embryo, suggesting that the site of expression must not coincide with accumulation and site of activity. These results indicate that endostatin could play an important role during embryonic angio- and vasculogenesis, in case endostatin was cleaved from collagen XVIII, throughout the whole embryo during all embryonic stages.

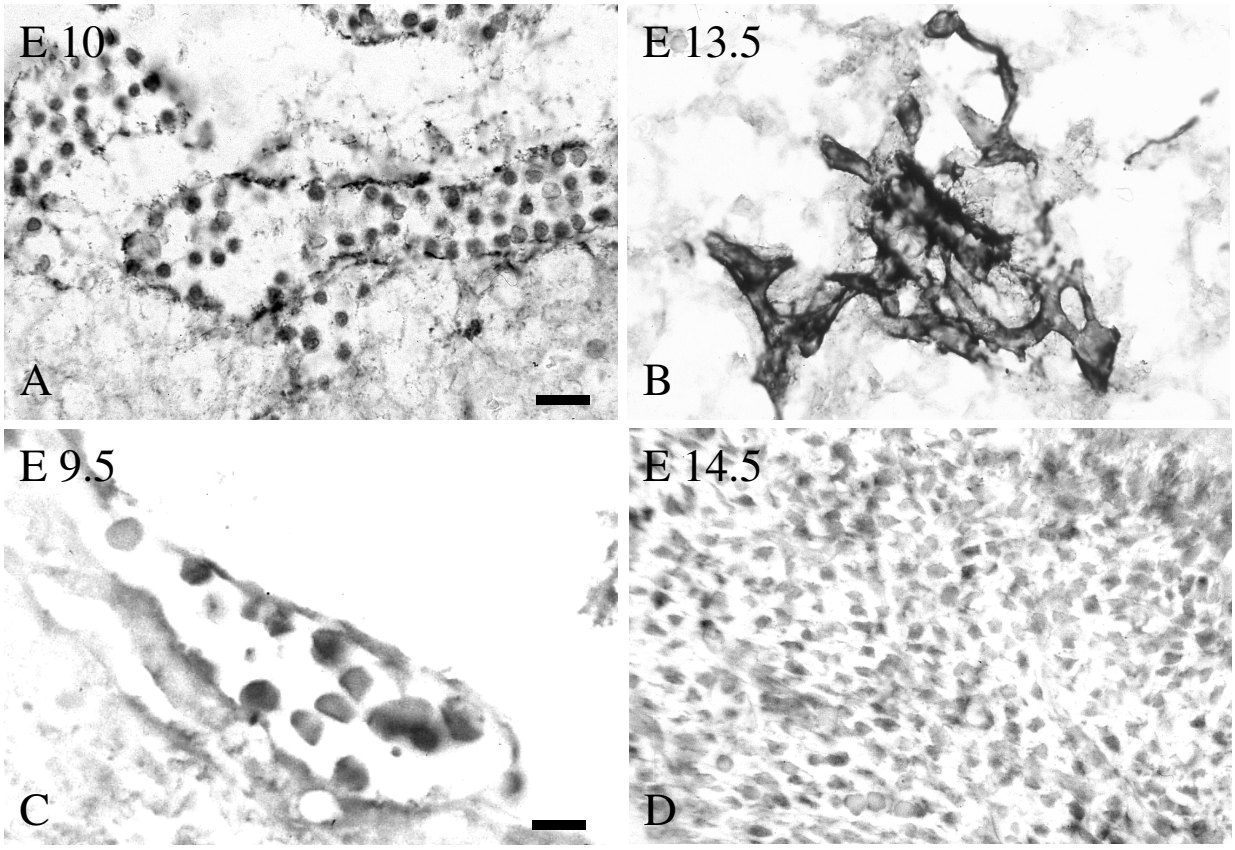


Figure 3: Immunohistological analysis and in situ hybridisation of endostatin/collagen XVIII in mouse embryos. For the immunohistochemical analysis of mouse embryos (A and B) an anti-endostatin antibody was used. In E10 mouse embryos strong staining of blood islands for collagen XVIII/endostatin (A) and of vessels in E13.5 mesenchymal tissue (B) was observed. For in situ hybridisation (C and D) a cDNA probe complementary to endostatin mRNA was employed. Strong staining of blood islands in E9.5 embryos (C) and expression of collagen XVIII/endostatin in E14.5 mesenchymal tissue was observed (D). Bar B = 25 µm. Bar D = 10 µm

The immunohistochemical detection does not allow differentiating between collagen XVIII and his proteolytic fragments e.g. endostatin. Therefore we addressed by Western Blotting whether the activated molecule endostatin is formed in murine embryonic tissue. In fact, we obtained bands of different sizes at E 10.5, E 13.5, E 15.5 and E 18.5 (Fig. 4). The 180-kDa band represents collagen XVIII, the 38 kDa band represents the NC1-fragment of spliced collagen XVIII and the 22 kDa band represents endostatin, the C-terminal fragment of the NC1-domain of collagen XVIII. The band for collagen XVIII is very weak whereas the bands

for the NC1-fragment and endostatin are clearly stronger; no visible differences in band intensities at the different time points of embryonic development were noticed. Thus, the major part of immunohistochemically detected protein in embryos is endostatin.

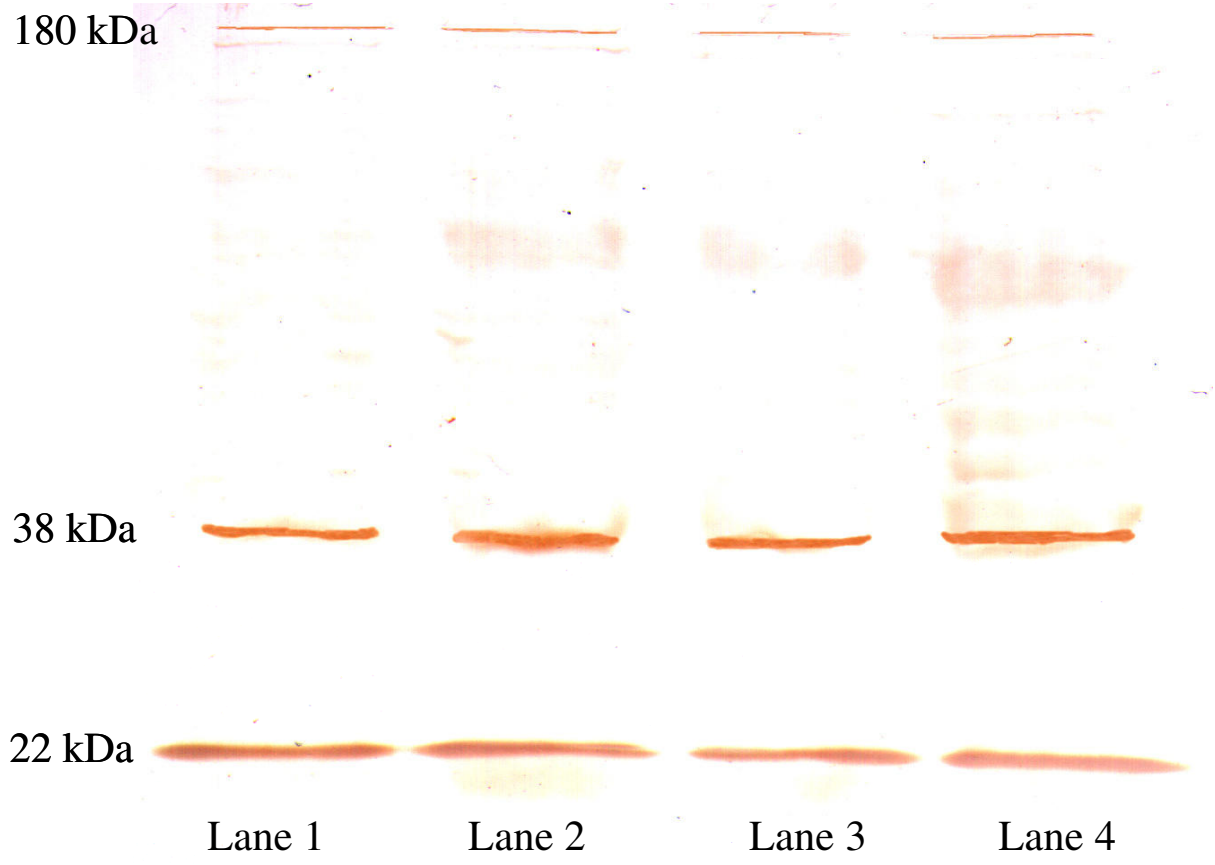


Figure 4: Western-Blot analysis of endostatin/collagen XVIII in mouse embryos of different age. Lane 1: E 10.5, lane 2: E 13.5, lane 3: E 15.5 and lane 4: E 18.5. The 180 kDa band was detected at all stages and demonstrates collagen VXIII; similarly the 38 kDa protein, the NC1- fragment of collagen XVIII was also seen all embryonic stages. The 22 kDa protein represents endostatin, which is the activated proteolytic fragment of collagen XVIII.

We also analysed the immunohistological distribution of collagenXVIII/endostatin in embryonic stem cell-derived vessels (Fig. 5) to compare the distribution of the collagen XVIII/endostatin in mouse embryo and EBs. For this purpose we stained angioblasts and endothelial tubes with a mouse specific PECAM-1 (CD31) antibody (Fig. 5A, C). A double immunfluorescence with PECAM-1 and collagen XVIII/endostatin enabled to determine its distribution in angioblasts and endothelial tubes. Endostatin is accumulated in endothelial tubes of embryoid bodies, in sprouting regions as well as in angioblasts (Fig. 5B, D). These results suggest that endostatin plays a similar role in EBs and in the embryo for vasculo- and angiogenesis.

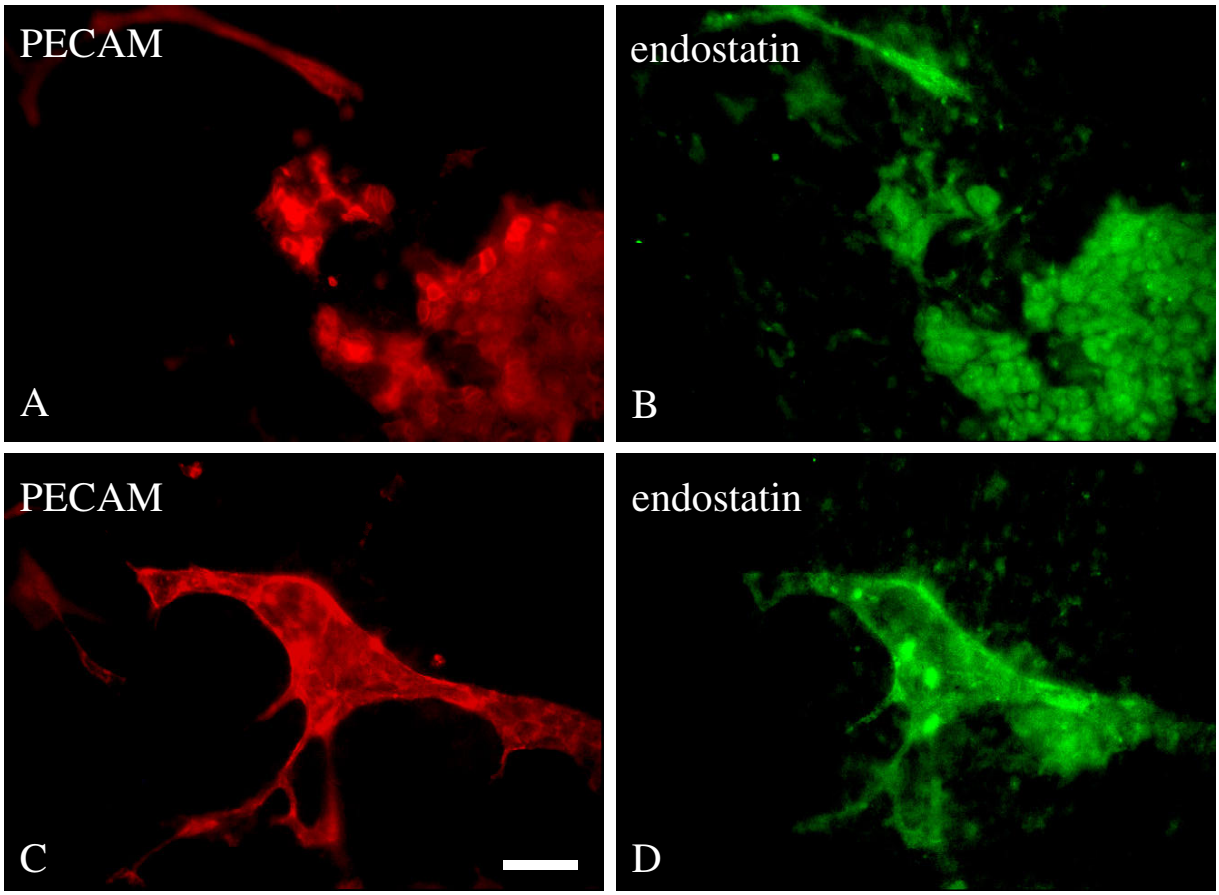


Figure 5: Immunohistological analysis of embryonic stem cell derived endothelial cells and vessels. In an double immunfluorescence vessels and endothelial cells were stained with an anti-PECAM-1 antibody (A and C) as well as an anti-endostatin antibody (B and D). Endostatin is distributed in angioblasts and vessels of embryonic stem cell derived endothelial cells as well as in sprouting areas of vessel like structures. Bar = 10 µM

3.3 Embryonic stem cell-derived vessels bind endostatin

From the distribution of the protein endostatin in embryos and EBs we were interested if also binding sites for endostatin are existence, which makes these tissue accessible for endostatin. In order to analyse the occurrence and the distribution of binding sites for endostatin, which is an important determinant for its cell biological action, we biotinylated endostatin. After detection of bound biotinylated endostatin, we observed nearly exclusive binding of endostatin (Fig. 6A and B), restin (data not shown) as well as of the heparin mutant form of endostatin (Fig. 6C and D) to all embryonic stem cell-derived angioblasts, endothelial cells and tubes detected by PECAM-1 immunostaining. The binding of biotinylated endostatin was abolished by pre-treating EBs with a 100-time overdose of unlabelled endostatin. These results were also obtained by using a FITC-conjugated endostatin (data not shown).

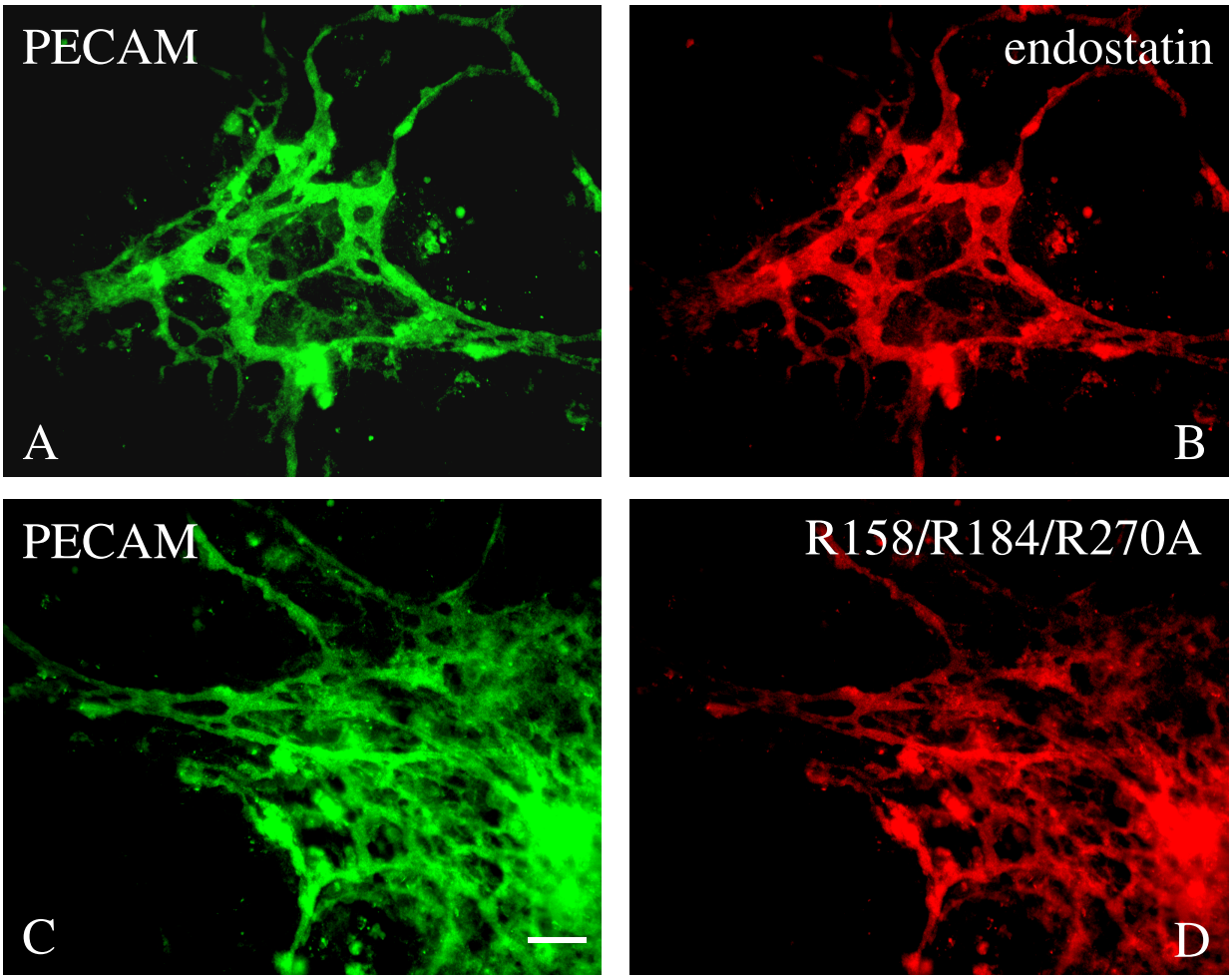


Figure 6: Binding of biotinylated endostatin and heparin mutant endostatin (R158/R184/R270A) to embryonic stem cell derived endothelial cells and vessels. A strong binding of endostatin (B) and the heparin mutant endostatin (D) was observed for PECAM-positive vessels (A and C). Bar = 10 µM

3.4 Role of endostatin during embryonic vessel growth

Since endostatin and its binding sites are distributed in endothelial cells and vessels we wondered whether endostatin inhibits embryonic vessel development. This was investigated in embryonic stem cell-derived endothelial cells, by examining the number of endothelial tubes and the proliferation, apoptosis and migration of endothelial cells in presence of endostatin, restin or the heparin mutant.

Opposite to the described anti-angiogenic effect (O'Reilly et al. 1997) of endostatin we observed a dose dependent statistically significant increase in the amount of PECAM-1 stained endothelial tubes in endostatin treated EBs (50ng/ml: 551.3±95.6; 200ng/ml: 571.3±183.8 vessels per EB) compared to untreated EBs (343.8±65.3 vessels per EB; Fig. 7 and 8A, B). Interestingly, also the heparin mutant form of endostatin (Fig. 8B) caused a dose

dependent increase in the number of vessels (control: 279±110.7; 50ng/ml: 403.3±139.7; 200ng/ml: 442±184.2 vessels per EB). To exclude that other cell types within the EBs influence the endothelial cells and underlie the endostatin effect, we isolated endothelial cells using MACS. We observed also for MACS-sorted endothelial cells a significant increase in the amount of endothelial cells after treatment with 50 ng/ml endostatin (control: 1090.25±89,05 cells; 50ng/ml: 1548.75±254 cells; Fig. 8C). Results for restin were nearly the same (data not shown).

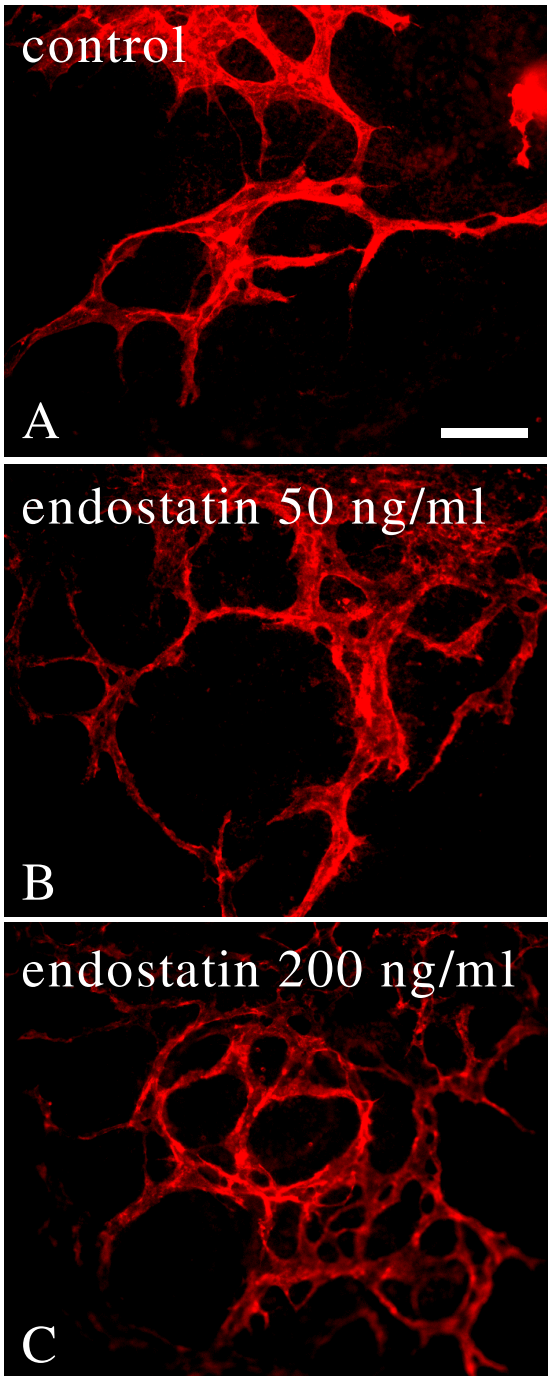


Figure 7: Influence of endostatin on the number of vessels. In vitro differentiated embryonic stem (ES) cells were treated with 50 ng/ml and 200 ng/ml endostatin or without (control). After12 days PECAM-1 antibody was performed to estimate the number of endothelial cells and vessels.

Endostatin evoked an increase in number of vessels (B and C) when compared to control (A). Bar = 10 µM.

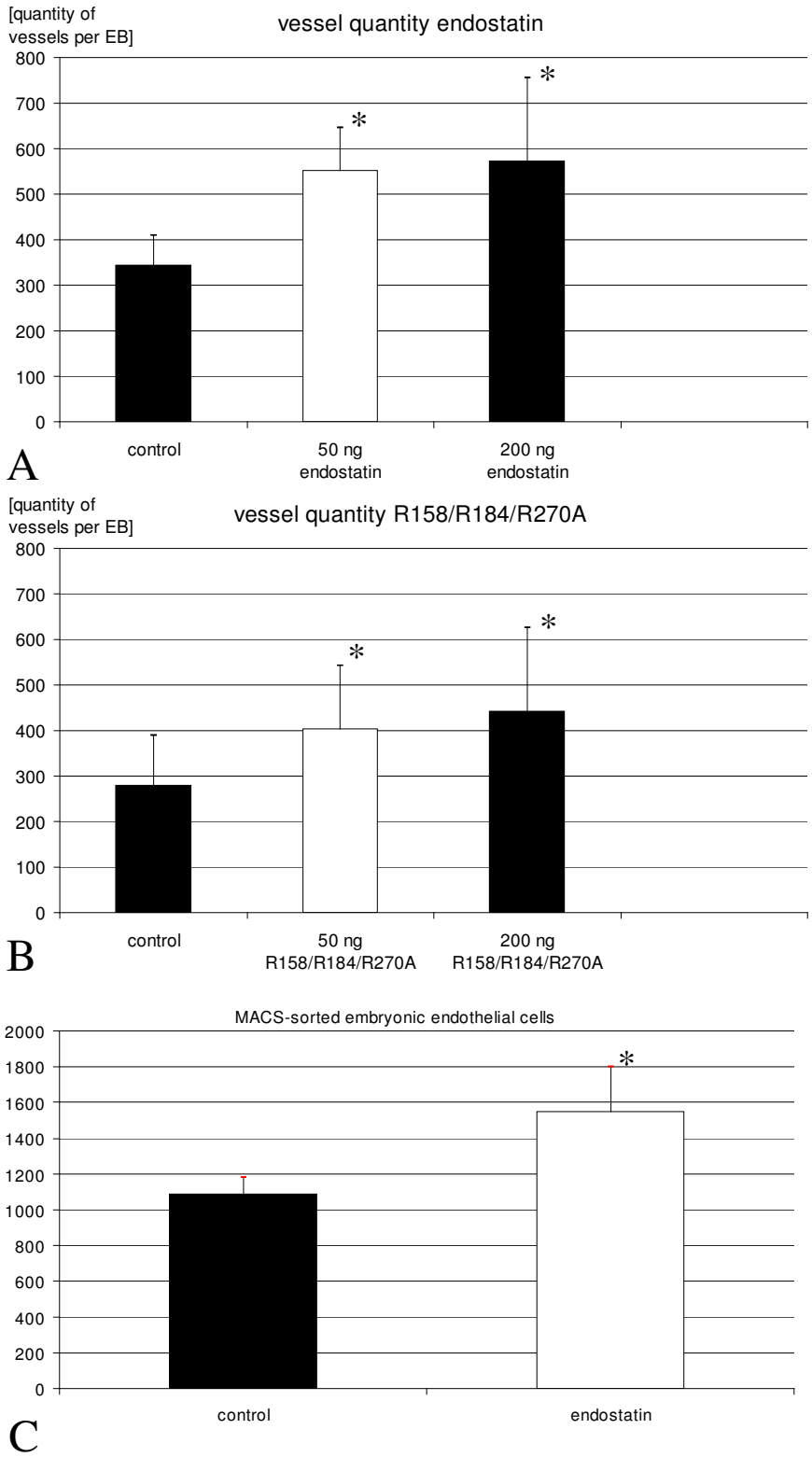


Figure 8: Statistics of the number of vessels in presence of endostatin (A) and the heparin mutant endostatin (R158/R184/R270A; (B)). In vitro differentiated ES cells were treated with 50 ng/ml and 200 ng/ml endostatin and heparin mutant endostatin as well as without endostatin. After immunostaining with PECAM the number of vessels in 6 Embryoid Bodies (EBs) were counted for every condition. MACS-sorted endothelial cells were also treated with/without 50 ng/ml endostatin and statistically analysed (C). Mean ± SD, $p < 0.05$.

Next, we investigated how endostatin lead to increased endothelial cell generation by determining proliferation. In contrast to previous reports (Dhanabal et al. 1999B, Yamaguchi et al. 1999) we detected a significant increase in ES cell-derived endothelial cells after endostatin treatment (Fig. 9 and 10) and treatment with restin and heparin mutant endostatin. The amount of Ki67-positive nuclei in endothelial tubes was significantly higher after treatment with 50 ng/ml and 200 ng/ml endostatin compared to control.

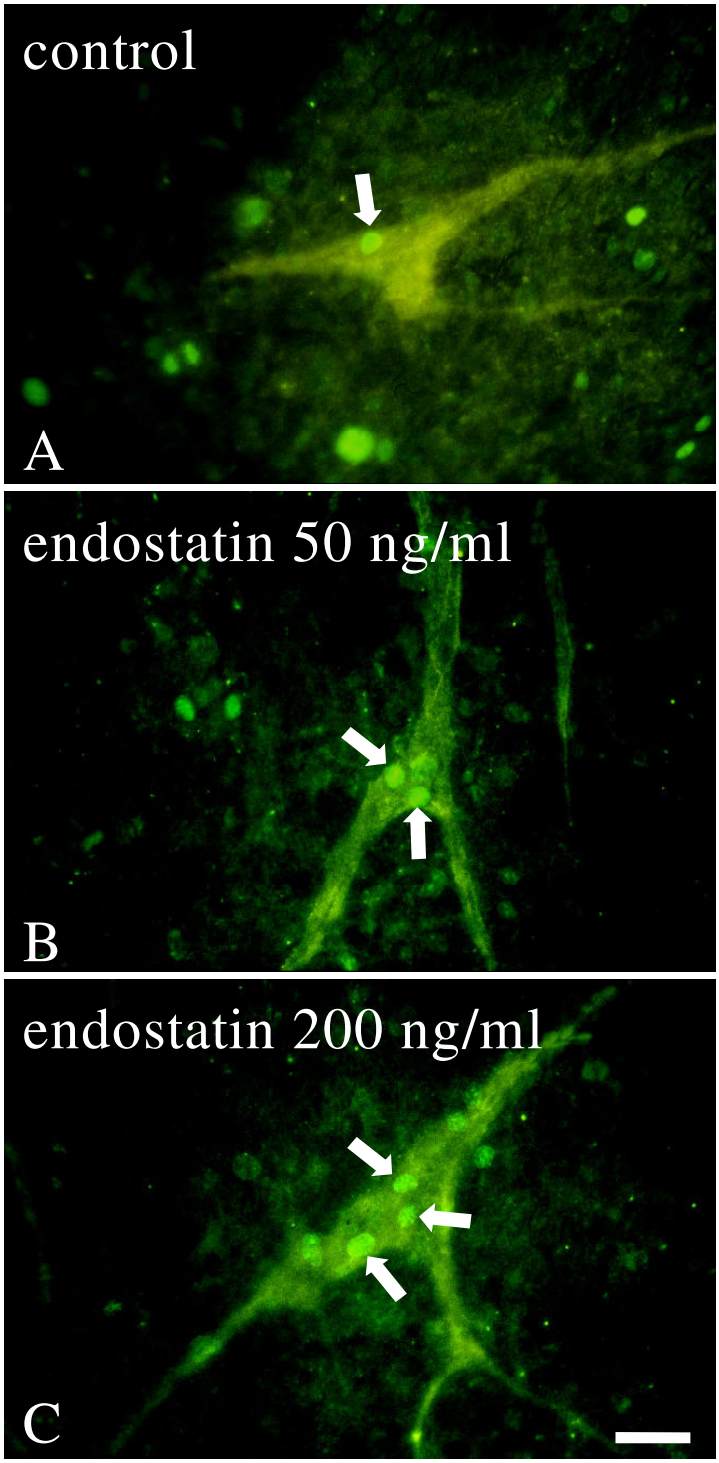


Figure 9: Proliferation of ES cell-derived endothelial cells under influence of endostatin. In vitro differentiated ES cells were treated with 50 ng/ml and 200 ng/ml endostatin and without. Double fluorescence staining with

PECAM- and Ki67 allowed the identification of proliferating endothelial cells and vesselsUnder influence of endostatin an increase of Ki67-positive cores in PECAM-positive vessels (B and C) compared to control (A) was detected. Bar = 10µM

In Fig. 9 double staining for the endothelial specific marker PECAM-1 and for the proliferation marker Ki67 is depicted. Quantification of the data demonstrates, that a significant increase (control: 20.5±3.4; 50ng/ml: 162.2±7.5; 200ng/ml: 188.8±11.8 Ki67-positive nuclei per 50 vessels) of proliferating endothelial cells (Fig. 10A) is detected after exposure to endostatin as well as to the heparin mutant form of endostatin (control: 23.2±10.3; 50ng/ml: 72.5±12.9; 200ng/ml: 99.2±28.9 Ki67-positive nuclei per 50 vessels; Fig. 10B). Results for restin were comparable (data not shown). Having used the Ki67-antibody we could not completely exclude a G1-arrest (Ki67 is mainly expressed during G1-phase), which was recently shown for endostatin in endothelial cells (Dhanabal et al. 1999A,B,C, Hanai et al. 2002). We have therefore analysed the proliferation of endothelial cells using BrdU (Fig. 10C, D). Similar to our findings with Ki-67, we also observed with BrdU a significant increase of endothelial cell proliferation in presence of endostatin (control: 57±2.4; 50ng/ml: 167.8±23; 200ng/ml: 180.8±14 BrdU-positive nuclei per 50 vessels) and of the heparin mutant endostatin (control: 56±20.01; 50ng/ml: 125±48.8; 200ng/ml: 138±52.1 BrdU-positive nuclei per 50 vessels). A G1-arrest was further excluded by investigating the increase of MACS sorted endothelial cells. Taking together these data (Fig. 8C) supported an augmented proliferation.

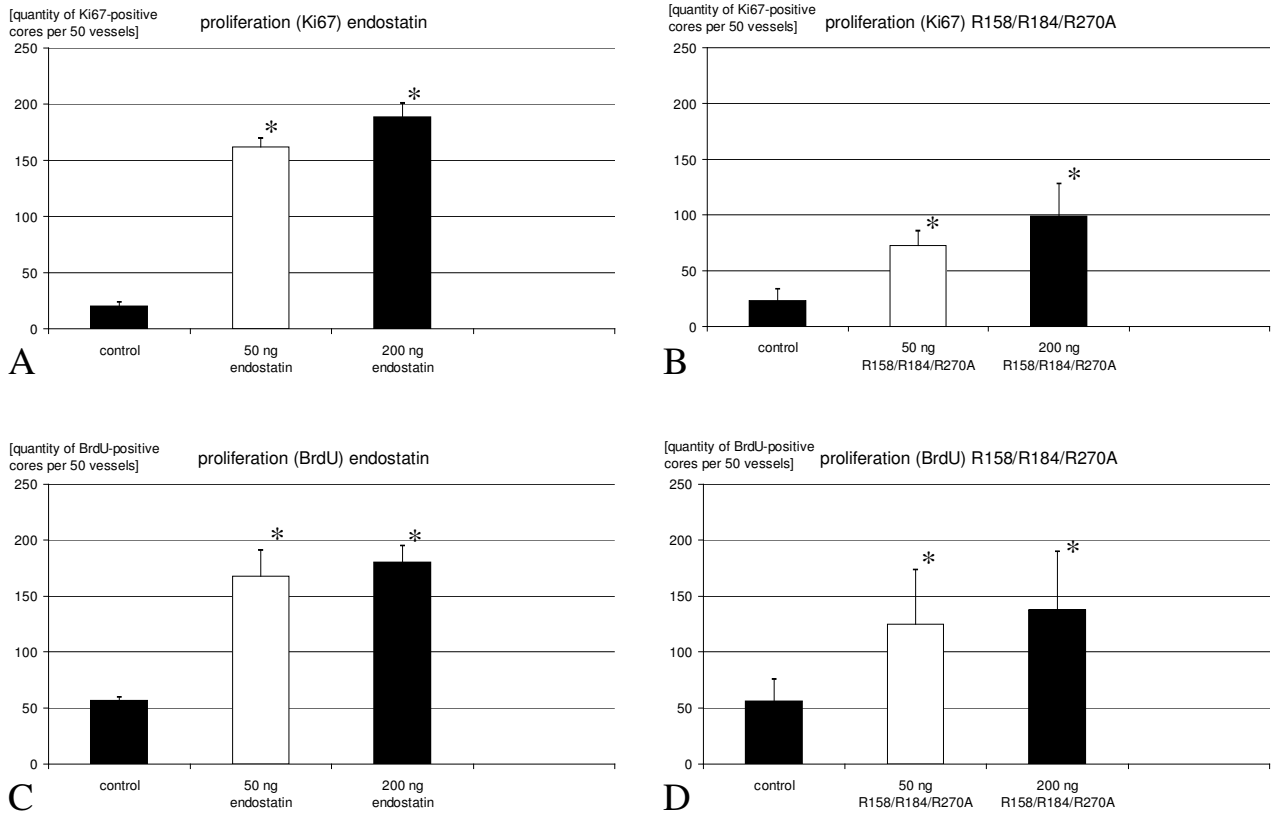


Figure 10: Statistics of the proliferation of endothelial cells under influence of endostatin as well as heparin mutant endostatin (R158/R184/R270A). Ki67-positive cores of 50 vessels from 6 EBs per condition were counted (A and B). In the same way BrdU-positive cores of 50 PECAM-positive vessels from 6 EBs per condition were counted (C and D). Mean ± SD, $p < 0.05$.

Due to the fact that the very high proliferation rate is not in accord with the increase of vessels we wondered whether endostatin influences the cell cycle of embryonic endothelial cells in the direction of apoptosis. For this purpose we used as a model the embryonic stem cell-derived endothelial cells and performed via double immunohistochemical staining (Fig. 11A, B) with the endothelial cell marker PECAM-1 and the apoptosis markers activated caspase-3 or Poly(ADP-Ribose)-Polymerase, PARP, (p85-fragment). As described before (Dhanabal et al. 1999A, Dixelius et al. 2000) we could detect an increased endothelial apoptosis under the influence of endostatin (Fig. 11B, C) compared with control (Fig. 11A, C); in fact apoptotic cell cores (Fig. 11A, B arrows) are visible in PECAM-positive vessels. The statistical data corroborate a significant increase of the apoptosis rate in endothelial cells for endostatin (control: 17.5±8.1; 50ng/ml: 100.8±23.8; 200ng/ml: 114±30.2 PARP-positive cores per 50 vessels; Fig. 11C) as well as for the heparin mutant form of endostatin (control: 16±5.7; 50ng/ml: 70.7±23.3; 200ng/ml: 85.3±25.5 PARP-positive cores per 50 vessels; Fig. 11 D). Results for restin were comparable (data not shown).

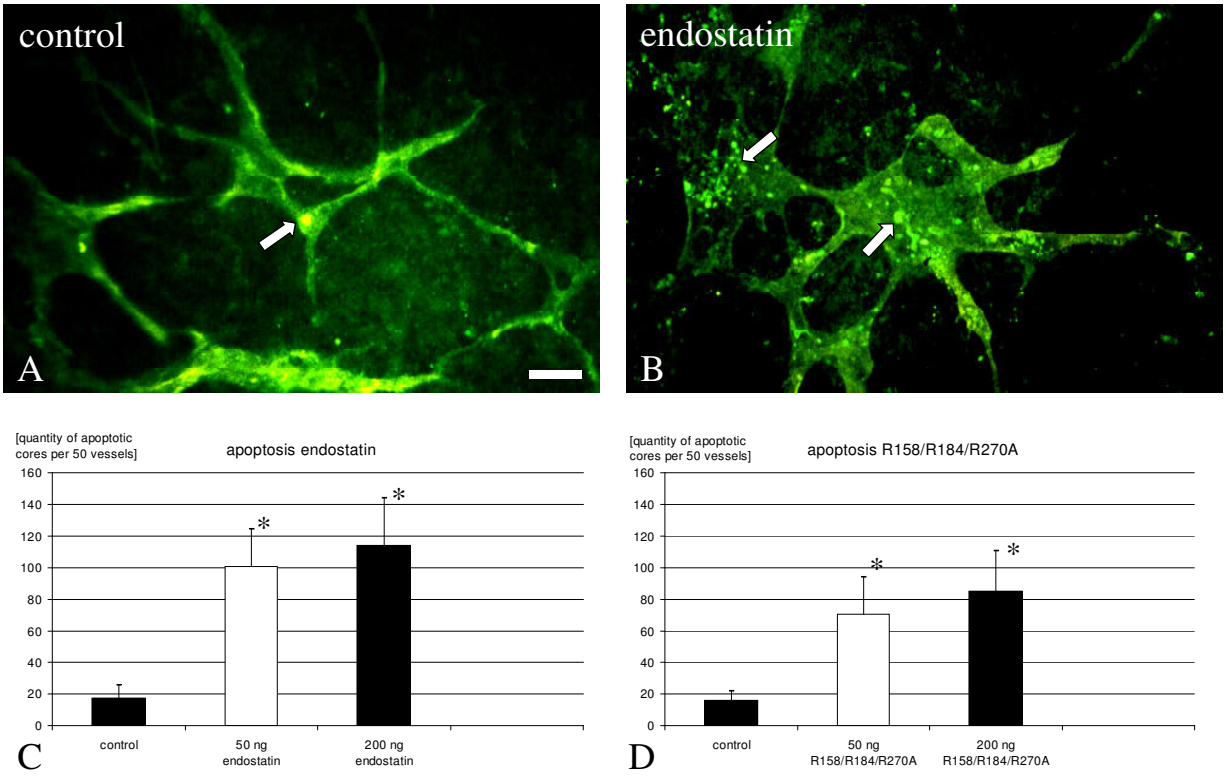


Figure 11: Apoptosis of ES cell-derived endothelial cells under influence of endostatin. After double immunfluorescence with anti-PECAM-1 antibody and anti-activated caspase-3 antibody or anti-PARP (p85 fragment) apoptotic cores (A and B, arrows) and vessels could be seen. For the statistical analysis of endothelial apoptosis (C and D) apoptotic core of 50 vessels from 6 EBs per condition were counted. Apoptosis were measured under influence of endostatin (C) as well as under influence of heparin mutant endostatin (D). Mean ± SD, $p < 0.05$. Bar = 10 µM

The migration of endothelial cells is an important cell biological aspect for vasculo- and angiogenesis. Therefore we analysed next the endostatin effect on migration of ES cell-derived endothelial cells (Fig. 12) in a Boyden-chamber (Dhanabal et al. 1999B and C, Yamaguchi et al. 1999). Under the influence of endostatin (50 ng/ml: 662±236 cells; 200 ng/ml: 702±14.7 cells) and the heparin mutant endostatin (50 ng/ml: 599±7.2 cells; 200 ng/ml: 603±21.2 cells) we noticed a significantly enhanced endothelial cell migration as compared to control (437±88 cells; Fig. 12 A and B). In order to exclude that this effect was due to increased proliferation instead of migration we chose a migration time of 8 hours. This excluded proliferation as potential mechanism since the minimum cell division time averaged 10 to 38 hours (Smith 1982, Edmunds and Adams 1981, Gallwitz 1981).

No difference between control and the treatment with control peptide M2BPI H2 could be observed for all analysed angiogenic aspects (data not shown).

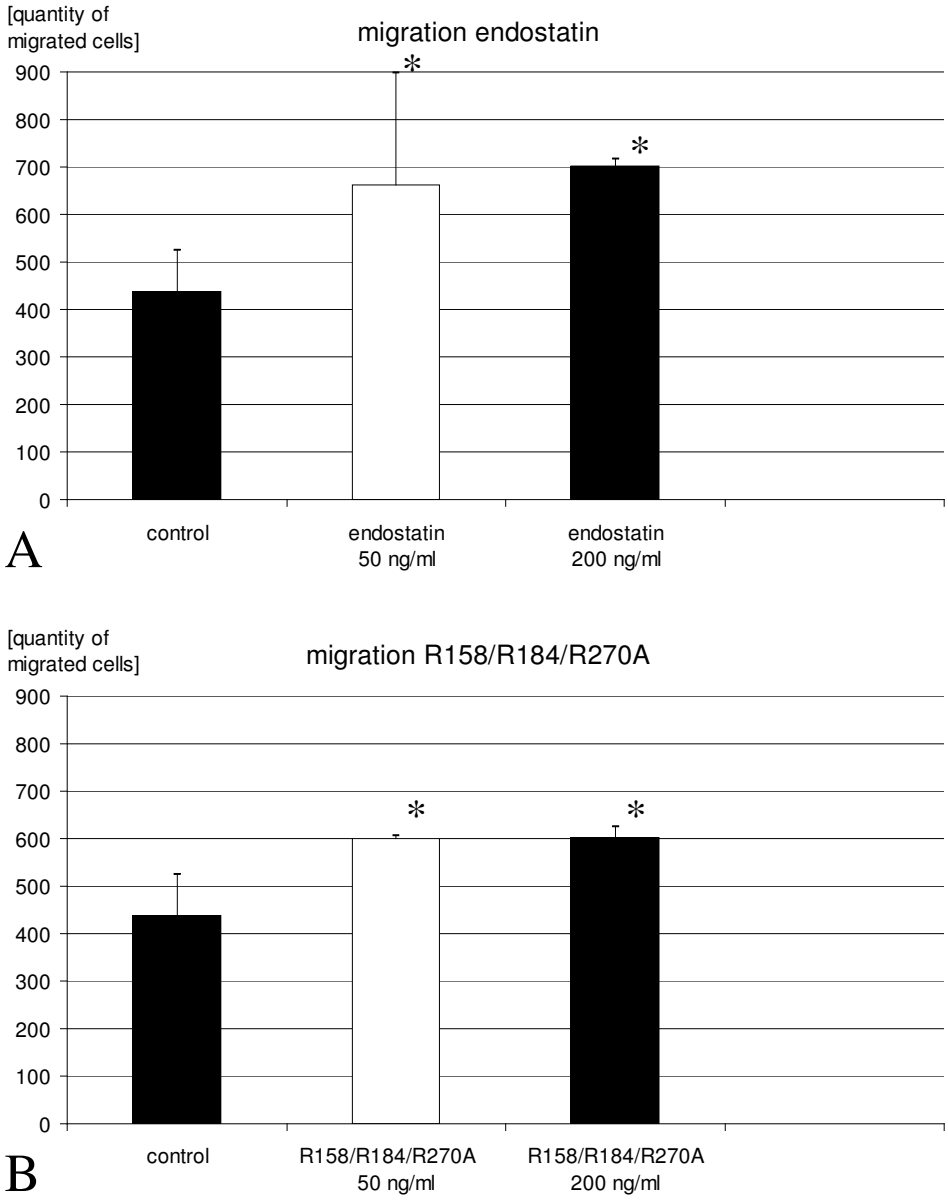


Figure 12: Statistical data of endothelial migration under influence of endostatin as well as heparin mutant endostatin. After preparing a migration assay in a modified Boyden chamber under influence of 50 ng/ml and 200 ng/ml endostatin as well as without endostatin, the cells were counted which migrated from the top of the membrane to the bottom within 8 h. The migration assay was prepared for endostatin (A) as well as heparin mutant endostatin (B).

3.5 Endostatin influences the morphology of endothelial cells and tube formation

We hypothesised that endostatin influences the morphology of embryonic stem cell-derived endothelial cells as described for other models before (Bloch et al. 2000, Dixelius et al. 2000, Mundhenke et al. 2001, Read et al. 2001). To analyse this we used high-resolution deconvolution microscopy with 3D-reconstruction (Fig. 13). After deconvolution microscopy we observed for vessels of EBs, flat PECAM-positive bands and after three-dimensional reconstruction (Fig. 13A) we could see that these vessels form flat bowls or grooves. However, for endothelial tubes of EBs we observed after three-dimensional reconstruction a modulatory effect of endostatin on ES-cell derived endothelial cells (Fig. 13B) in form of contraction. To investigate this in detail we analysed the effect of endostatin on the morphology of endothelial cells and endothelial tubes with vital microscopy.

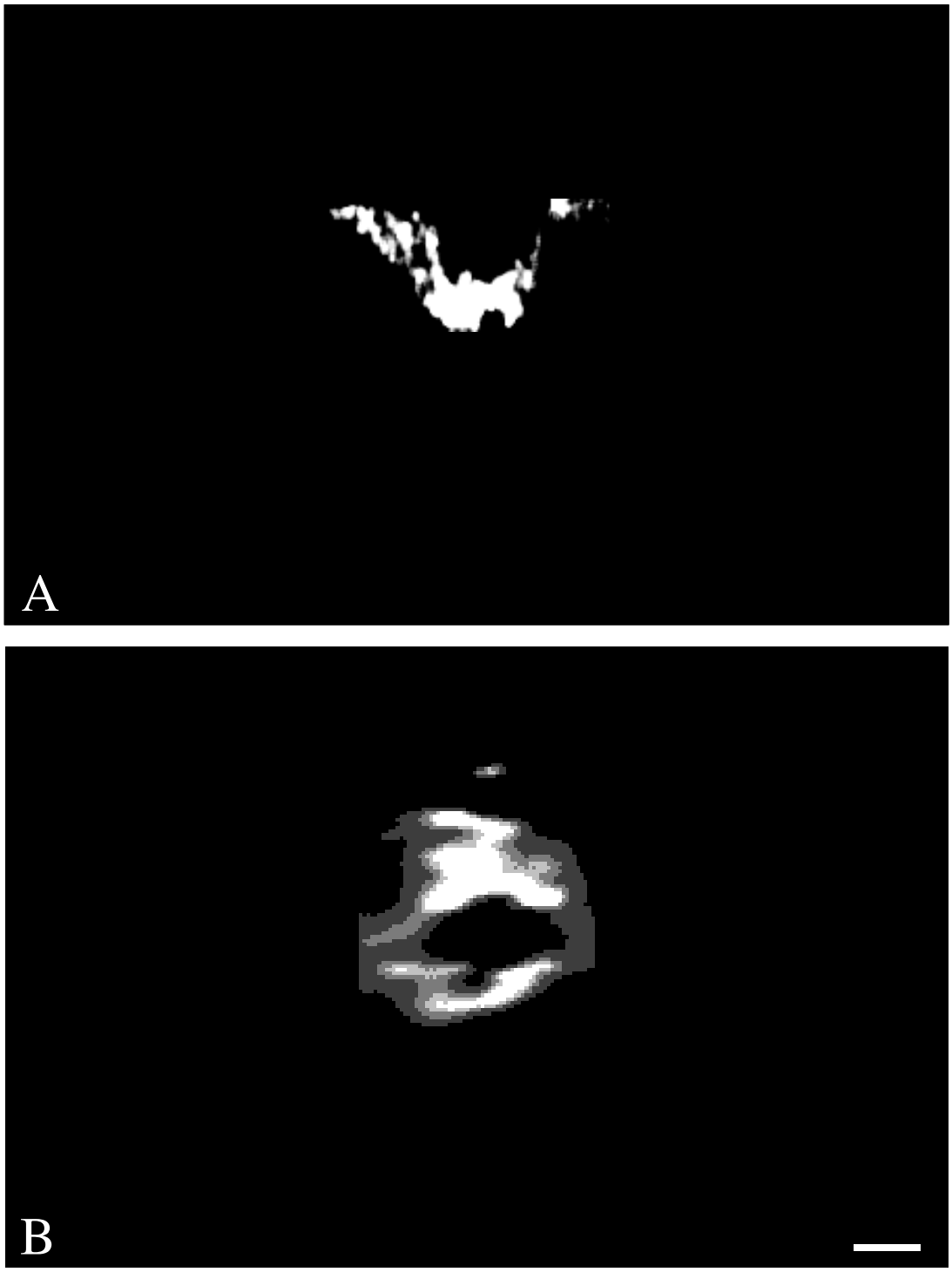


Figure 13: Morphological analysis by using high resolution deconvolution microscopy. The influence of endostatin on the morphology of in vitro differentiated ES cell- derived endothelial cells and vessels were analysed by high resolution deconvolution microscopy with 3D-reconstruction under influence of endostatin (B) as well as without endostatin (A). Without endostatin PECAM-positive vessels resemble grooves or bowls (A) whereas after treatment of endostatin vessels appear to be angiogenic-modulated in form of contraction (B). Bar = 1.4 µm

To visualize endothelial tubes within EBs we employed transgenic ES cell lines where the live reporter EGFP is driven by the PECAM-1 promoter (Kazemi et al. 2002). During observation

(Fig. 14) we detected continuous rebuilding events (Fig. 14 A and B) but after addition of endostatin (Fig. 14 C-F) sudden endothelial contractions occurred, leading to retraction of the endothelial tubes and cells. Thus, endostatin induces immediate changes in the morphology of endothelial tubes.

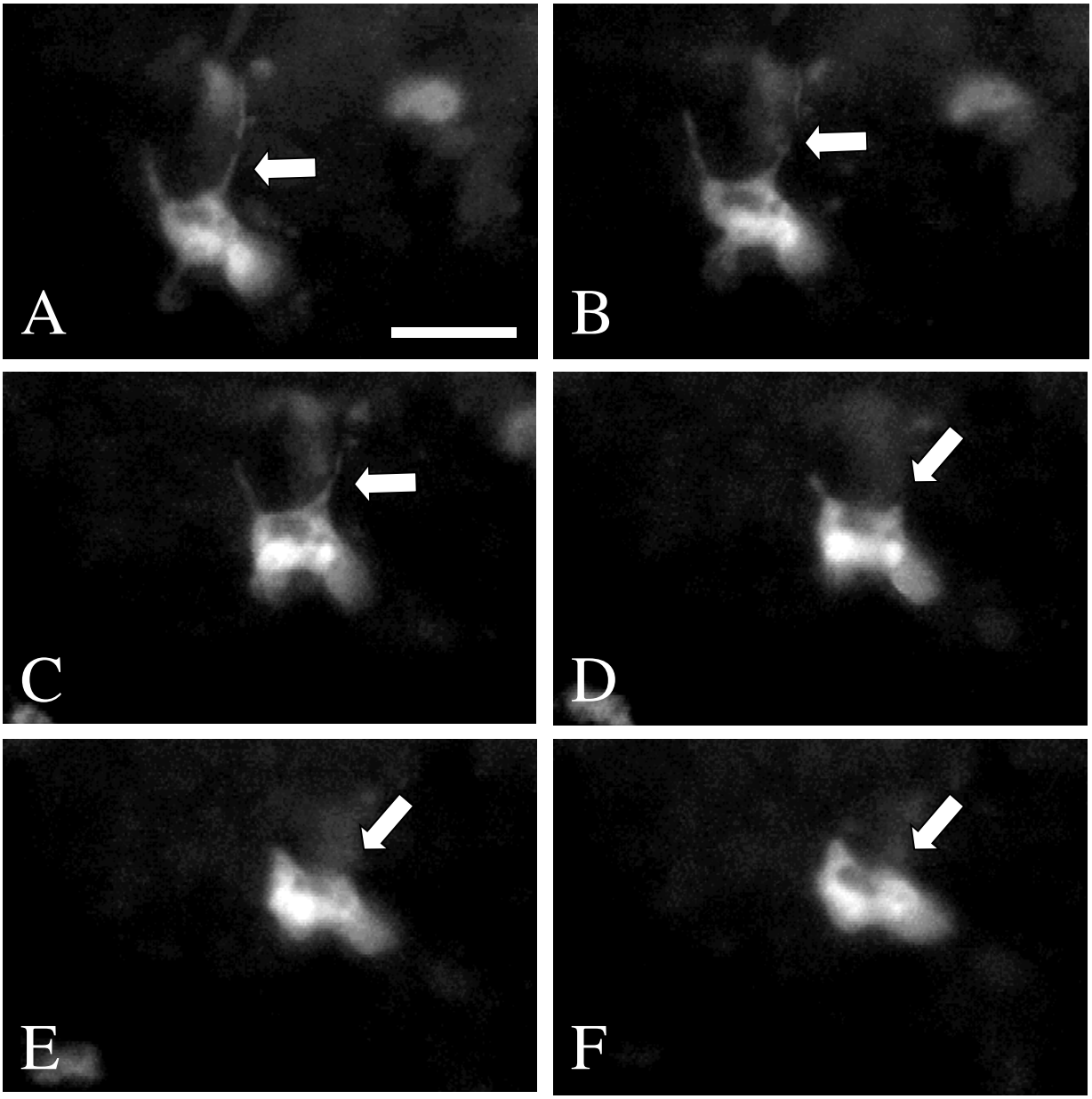


Figure 14: Vital microscopic analysis of vessel morphology under influence of endostatin in a period of 6 h. Under in vitro conditions ES cell-derived endothelial cells form vessel like structures (arrows, A and B). In presence of endostatin (C-F) contraction as well as a retraction of endothelial cells and vessel like structures were observed (arrows). The time period between pictures is 2-3 h. Bar = 20 µm

3.6 Role of endostatin, restin and the heparin mutant form of endostatin during wound healing

The vital microscopic observation is an example for physiological vessel development what also the regenerative neovascularisation of wound healing is a model system for. From endostatin expression in hyperproliferative epithelium and in endothelial cells of microvessels during wound healing (Bloch et al. 2000) we were able to evidence an endostatin-mediated effect. Therfore we analysed vessel quantity, proliferation and apoptosis during regenerative neovascularisation of physiological wound healing. We further investigated the mechanism the anti-angiogenic protein, restin and the heparin binding mutant form of endostatin. After treatment of wounded mice with endostatin we found an increase in vessel quantity (520±20 vessels/mm²) compared to control (315±114 vessels/mm²) (Fig. 15A and B, Fig. 16A). This was also observed after treatment with restin (496±41 vessels/mm²) and R158/R184/R270A (525±41 vessels/mm²) (Fig. 15A-D, Fig. 16A). Therefore we asked for proliferation rate and observed an increase in endothelial proliferation in wound areas after treatment with endostatin (2.8±0.4 Ki67-positive vessels/mm²), restin (2.5±1 Ki67-positive vessels/mm²) (Fig. 15F, Fig. 16B) as well as R158/R184/R270A (1.4±1.1 Ki67-positive vessels/mm²) compared to control (0.5±0.8 Ki67-positive vessels/mm²) (Fig. 15E, Fig. 16B). From the fact that the increase in endothelial proliferation was not in accord with the increase in vessel amount, we conclude that endostatin influence cell cycle during wound healing in direction of apoptosis. What we observed in endothelial cells of wounded areas was increased apoptosis after treatment with endostatin (2.6±0.8 apoptotic vessels/mm²), restin (2.2±0.8 apoptotic vessels/mm²) (Fig. 15H, Fig. 16C) and the heparin mutant form of endostatin (R158/R184/R270A) (1.6±1.1 apoptotic vessels/mm²) compared to control (1±0.9 apoptotic vessels/mm²) (Fig. 15G, Fig. 16C).

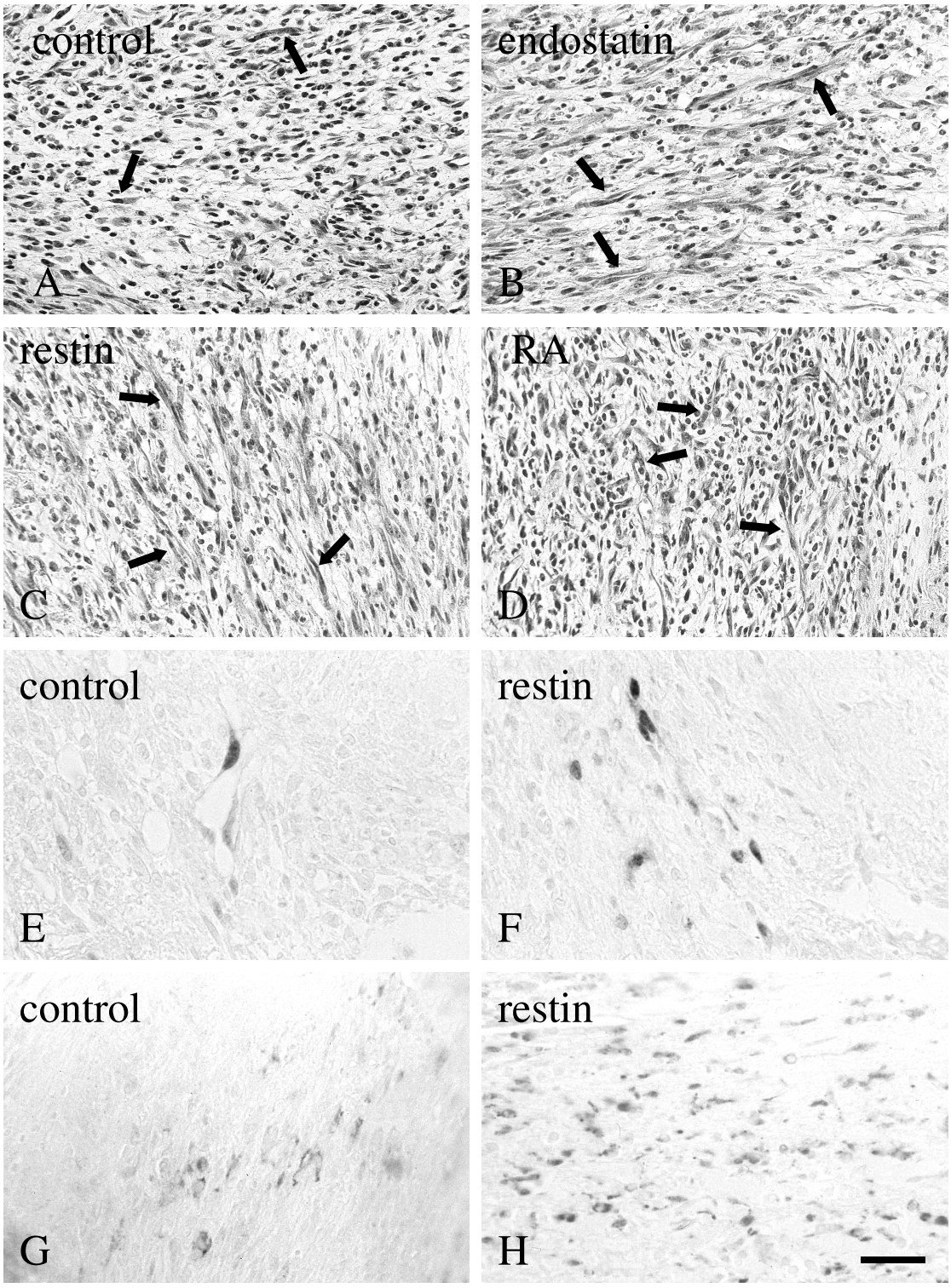


Figure 15: Influence of endostatin, restin and the heparin mutant form of endostatin on vessel quantity, endothelial proliferation and apoptosis during wound healing. Wound healing under influence of endostatin (B), restin (C) and the heparin mutant form of endostatin (D) lead to an increase in vessel quantity in 5d old wounds compared to control (A). This proteins also lead to an increased endothelial proliferation here shown for restin (F) compared to control (E) as well as to an increased apoptosis, as shown for restin (H) compared to control (G).

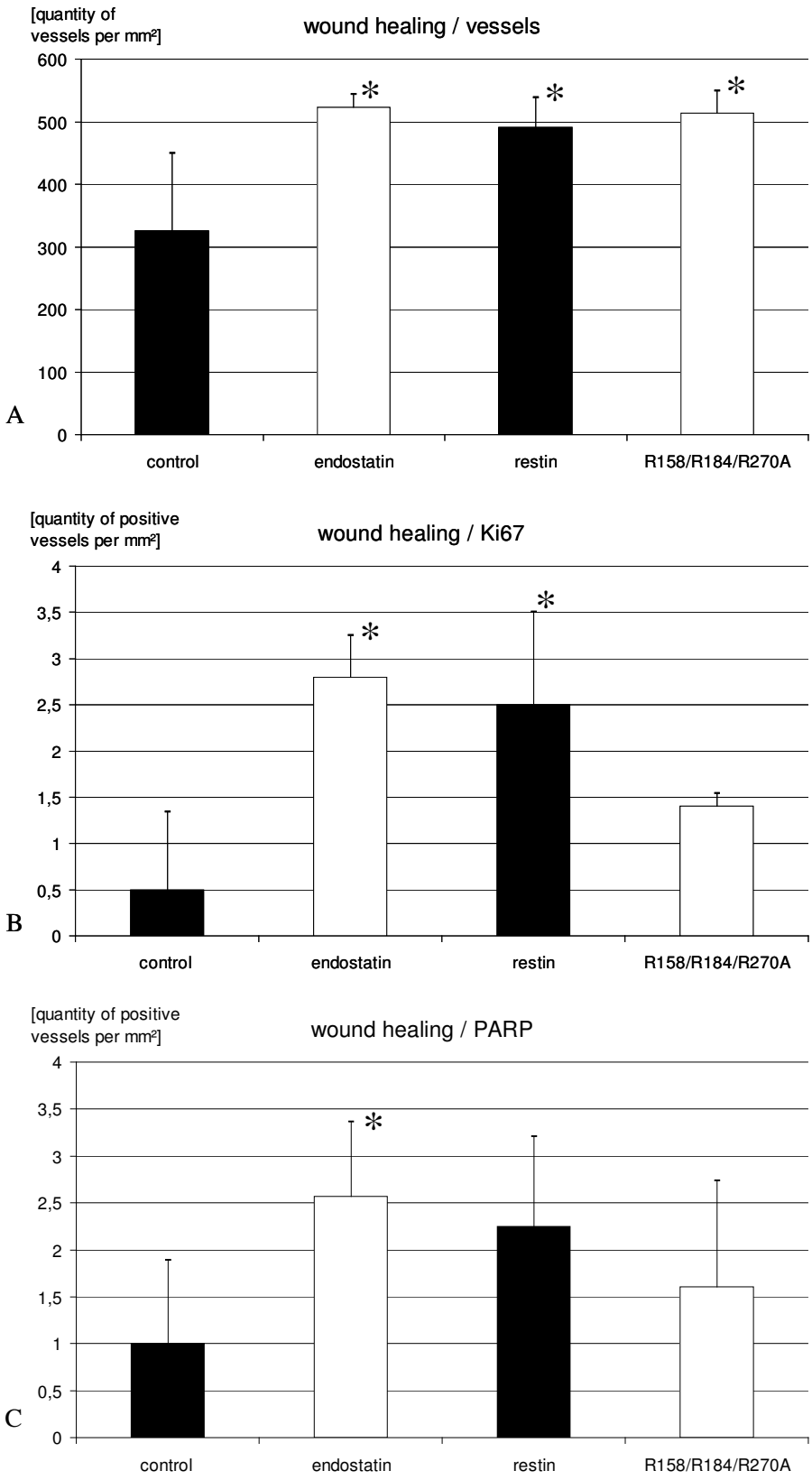


Figure 16: Statistical analysation of the amounts of vessel, the endothelial proliferation and apoptosis. (A) Vessels under influence of endostatin, restin and the heparin mutant form of endostatin show an increased amount of endothelial cells in wounded areas. (B) Also the proliferation and (C) the apoptosis was increase under influence of these three proteins.

3.7 Do restin and heparin mutant endostatin influence the morphology of endothelial cells and vessels?

Endostatin influences the morphology of embryonic stem cell derived endothelial tubes (as described above), of vessels during wound healing (Bloch et al. 2000, Mundhenke et al. 2001) as well as of vessels in tumour tissue (Mundhenke et al. 2001, Read et al. 2001). But till now it was not analysed whether restin and the heparin mutant form of endostatin have also an effect on vessel morphology. In semi-thin sections of wound areas we observed a comparable morphological change for endothelial cells and vessels after treatment with restin and R158/R184/R270A (Fig. 17C and D) as observed for endostatin (Fig. 17B + Bloch et al. 2000) compared to control (Fig. 17A). Vessels were narrowed or closed and irregular in shape, thereby restricting the integration in the circulation system. Thus a significant reduction in number of functional vessels was observed in wounds of endostatin treated mice. On the contrary vessels of buffer injected animals were round, open and filled with blood cells. Data were further confirmed by ultrastructural analyse (Fig. 17E-H). Also in this case, we also observed narrowed or closed vessels because of either a contraction of vessels in wound areas or a lack of lumen building after treatment with endostatin, restin and R158/R184/R270A (Fig. 17F-H) compared to control (Fig. 17E).

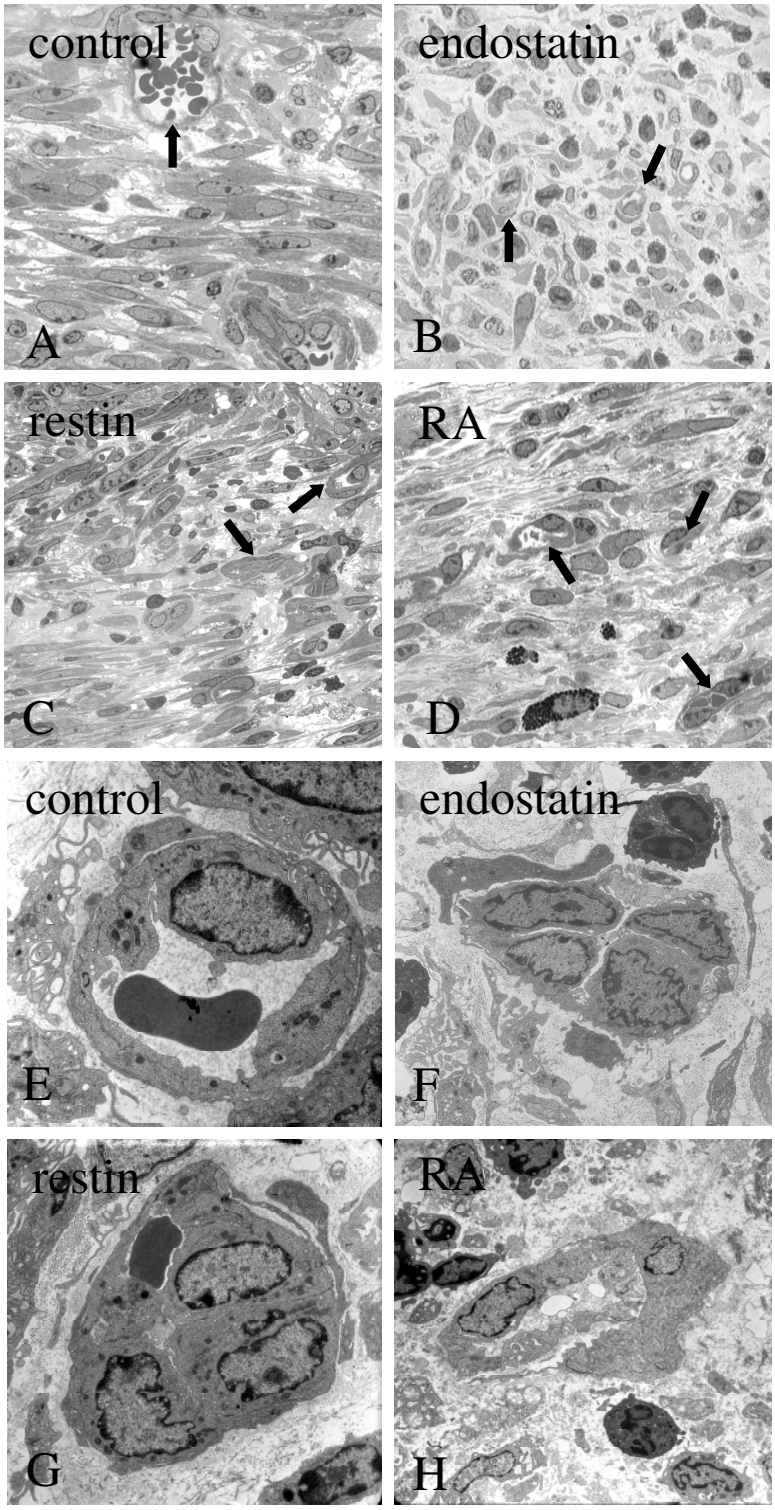


Figure 17: Vessel morphology during wound healing under influence of endostatin, restin and the heparin mutant form of endostatin, analysed on semi-thin and ultra structural level. On semi-thin level (A-D) we observed under influence of endostatin (B), restin (C) and the heparin mutant form of endostatin (D) a modulated vessel morphology in form of a contraction. Vessels were narrowed or closed compared to control (A). Also on ultra structural level we observed under influence of endostatin (F), restin (G) and the heparin mutant form of endostatin (H) narrowed or closed vessels compared to control (E).

To answer the question if endostatin-mediated morphological changes of vessels are a consequence of a lacked lumen building or of a contraction, we analysed this morphological changes under influence of endostatin, restin and R158/R184/R270A in form of a dynamic process, by performing vital microscopic examination. For this purpose we transfected embryonic stem cells with the vital reporter gene GFP under an endothelial-specific promotor (Kazemi et al. 2002) which facilitates us to analyse the influence of this three factors on the development of embryonic stem cell derived endothelial tubes. During the vital microscopic analysation of GFP-positive tubes (Fig. 18), we observed continuous rebuilding events (Fig. 18 A, D and G). But from the supply of endostatin we observed, with a temporal delay -as described before- fast contraction and retraction of endothelial cells and tubes (Fig. 18B and C), which lead to a loss of the integrity. This finding was also seen for restin (Fig. 18D-F) and the heparin mutant form of endostatin (Fig. 18G-I). All three factors modulate vessel morphology in wounded areas as well as during vital microscopic examinations in a comparable way. This described modulation of vascular morphology was limited on vessels and endothelial tubes of a defined differentiation degree. The defined differentiation degree means endothelial tubes and vessels, which have started to mature and started to build up a lumen. Comparable morphological changes of endothelial tubes were also observed under influence of the MAP-kinase inhibitor PD98059 (data not shown).

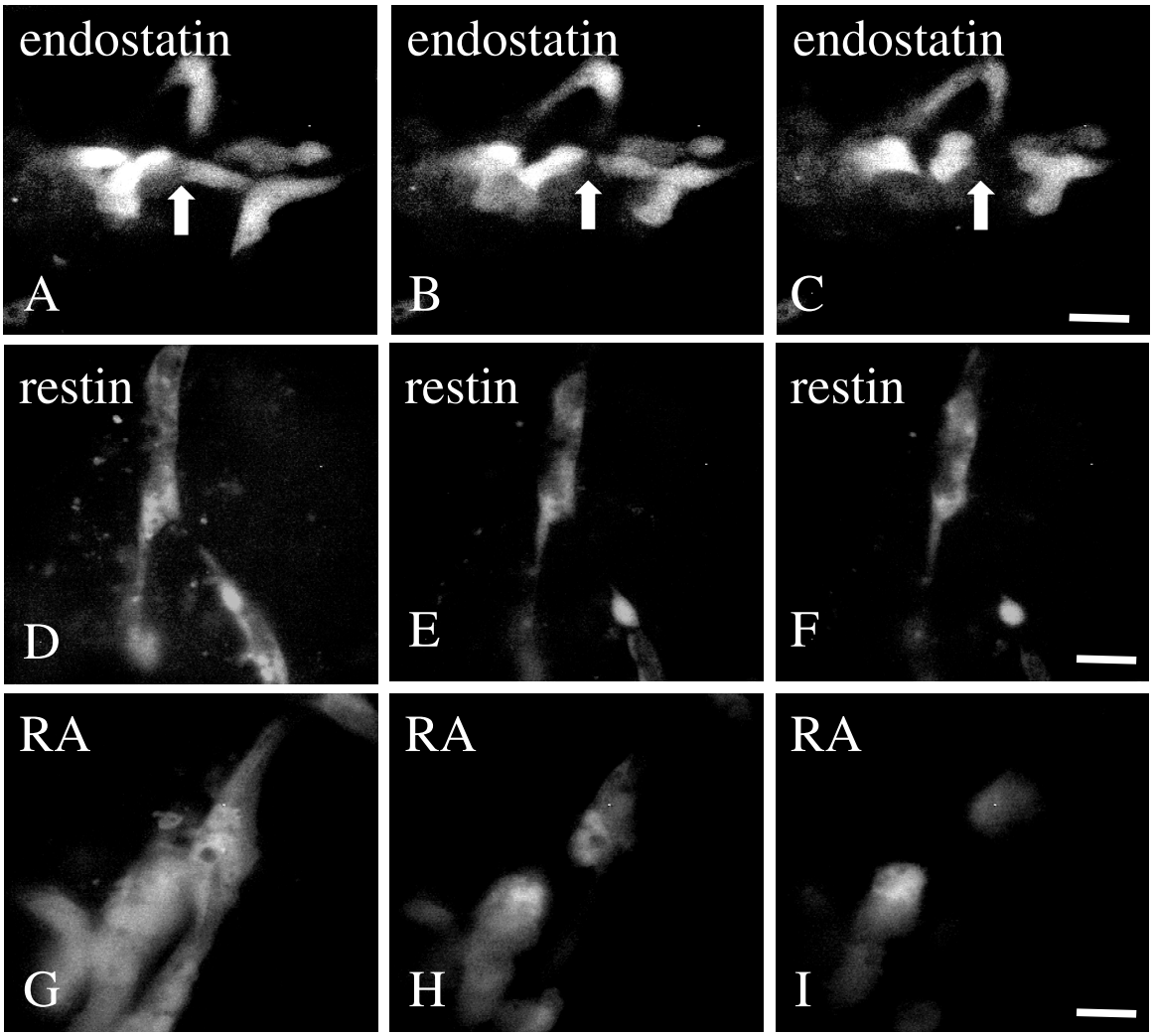


Figure 18: Vital microscopic analysation of GFP-positive endothelial tubes under influence of endostatin, restin and the heparin mutant form of endostatin. Endothelial tubes of in vitro differentiated embryonic stem cells were visualised by the vital reporter gene GFP under an endothelial specific promotor. By vital observation of endothelial tubes under influence of endostatin (A-C), restin (D-F) and the heparin mutant form of endostatin (G-I) over a period of 6h we observed a modulation of endothelial morphology in form of a contraction and retraction. The sequence of pictures makes the pull together, the retraction visible.

3.8 Do endostatin, restin and heparin mutant endostatin influence signaltransduction?

Morphological changes of vessels mainly underlie the influence of angiogenic factors like VEGF. For morphological changes of vessels VEGF is an important factor. Therefore we checked if endostatin, restin and R158/R184/R270A influence the VEGF signaling pathway as well as components of this pathway like VEGF, Flk-1, eNOS, sGC, cGMP and ERK1/2-kinase as well as AKT-kinase. For two of this components (sGC and activated ERK1/2-

kinase) we observed changes of expression and phosphorylation respectively after treatment with endostatin, restin as well as R158/R184/R270A (Fig. 19). Treatment of mice during wound healing leads to a dephosphorylation of the activated ERK1/2-kinase (Fig. 19A and B) as well as to a down-regulation of the soluble guanlyate cyclase (sGC) in vessels (Fig. 19C and D). Whereas no changes for the activated AKT-kinase after treatment with endostatin, restin and R158/R184/R270A (Fig. 19E and F) were observed. Also for VEGF, Flk-1 and eNOS we did not observed any changes in vessels after treatment (data not shown). Under influence of endostatin, restin and R158/R184/R270A there is a significant reduction of vessels which are positive for phosphorylated ERK1/2-kinase (9.4 ± 3.2 / 9.56 ± 5.3 / 6.3 ± 2.1 ERK-positive vessels/mm²) as well as for sGC (9.2 ± 3.2 / 14.96 ± 7.1 / 10.36 ± 5.6 sGC-positive vessels/mm²) (Fig. 20A and B) in wounded areas, whereas the amount of positive vessels for the phosphorylated AKT-kinase (84.7 ± 29 / 102.52 ± 15.1 / 98.8 ± 18.1 AKT-positive vessels/mm²) did not changed (Fig. 20E and F).

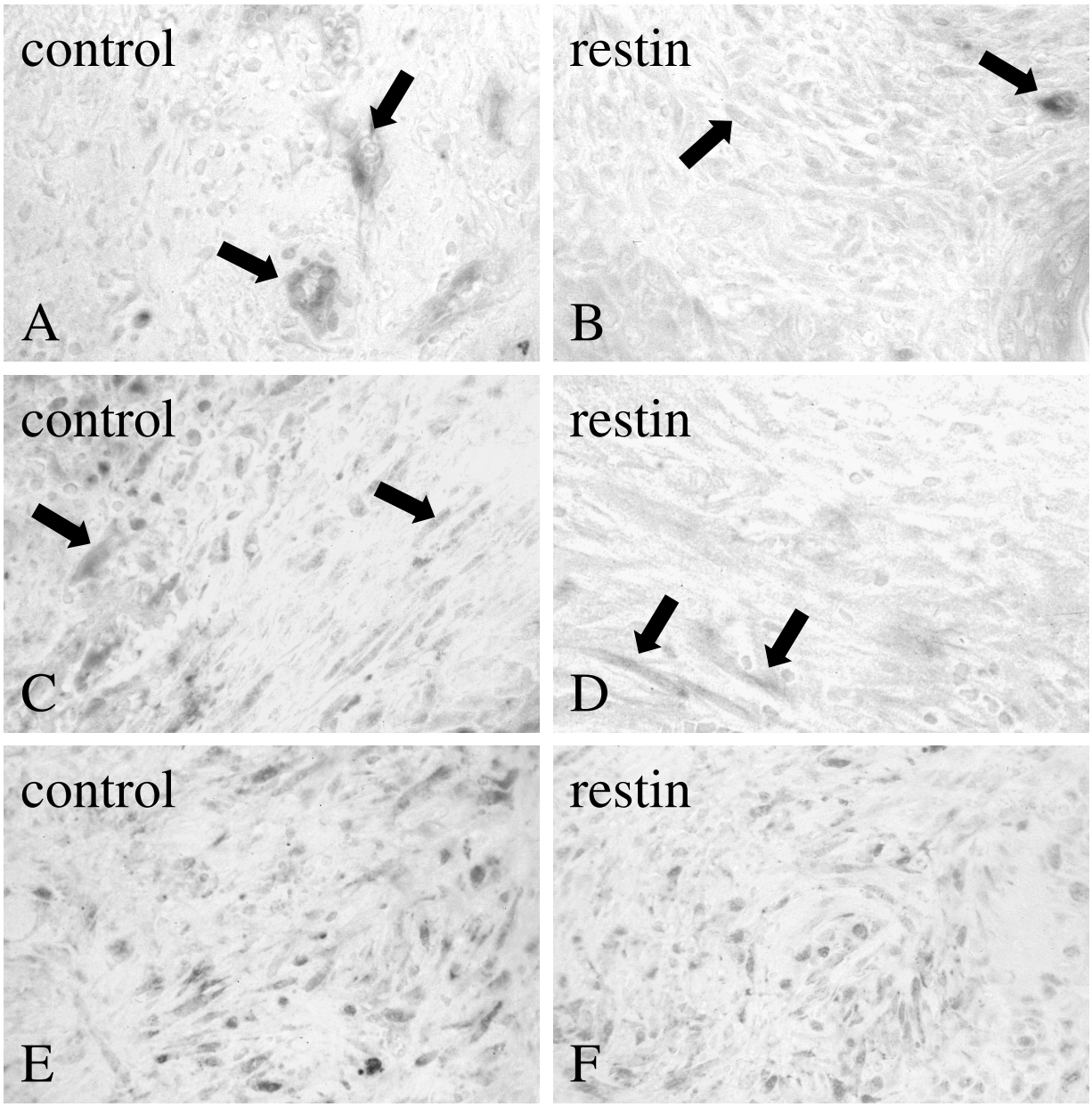


Figure 19: Signal transduction in endothelial cells of wounded areas under influence of endostatin, restin and the heparin mutant form of endostatin. Under influence of this three proteins we observed a dephosphorylation of ERK1/2-kinase in endothelial cells and vessels here shown for restin (B) compared to control (A). Also we observed a down-regulation of sGC in endothelial cells and vessels of wounded areas under influence of this three protein as shown for restin (D) compared to control (C). Whereas activated AKT-kinase was not influences in endothelial cells and vessels under influence of endostatin, restin (F) and the heparin mutant form of endostatin compared to control (E).

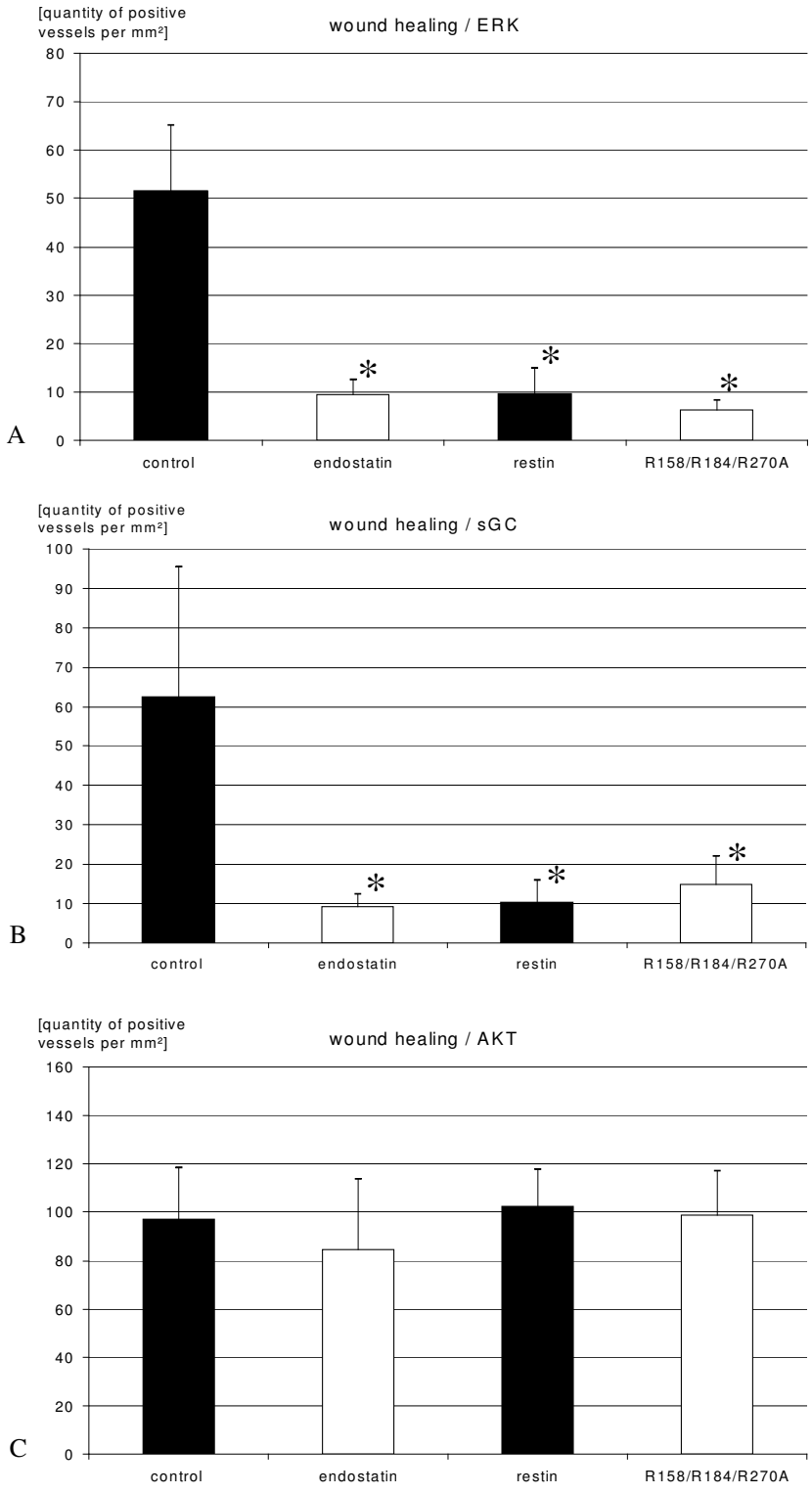


Figure 20: Statistical analysation of the signalling molecules activated ERK1/2-kinase, sGC and activated AKT-kinase in vessels under influence of endostatin, restin and the heparin mutant form of endostatin in wounded areas. Vessels positive for the activated ERK1/2-kinase (A) were reduced under influence of endostatin, restin and the heparin mutant form of endostatin. SGC-positive vessels (B) were also reduced under influence of these three proteins, whereas the activated AKT-kinase (C) was not reduced in endothelial cells. Mean ± SD $p<0.05$

In order to control if the described influence on components of the VEGF signaling pathway is also visible for embryonic stem cell derived endothelial tubes in EBs, a model which allowed us to analyses directly the influence of the altered signaling pathway on morphology of endothelial tubes, we treated embryoid bodies (EBs) with endostatin, restin, and R158/R184/R270A for 12 days. After immunohistochemistry we observed a dephosphorylation of the activated ERK1/2-kinase in PECAM-positive tubes (Fig. 21C and D) whereas a strong expression was observed for control (Fig. 21A and B). We observed also a down-regulation of sGC in PECAM-positive endothelial tubes of EBs (Fig. 21G and H) compared to control (Fig. 21E and F).

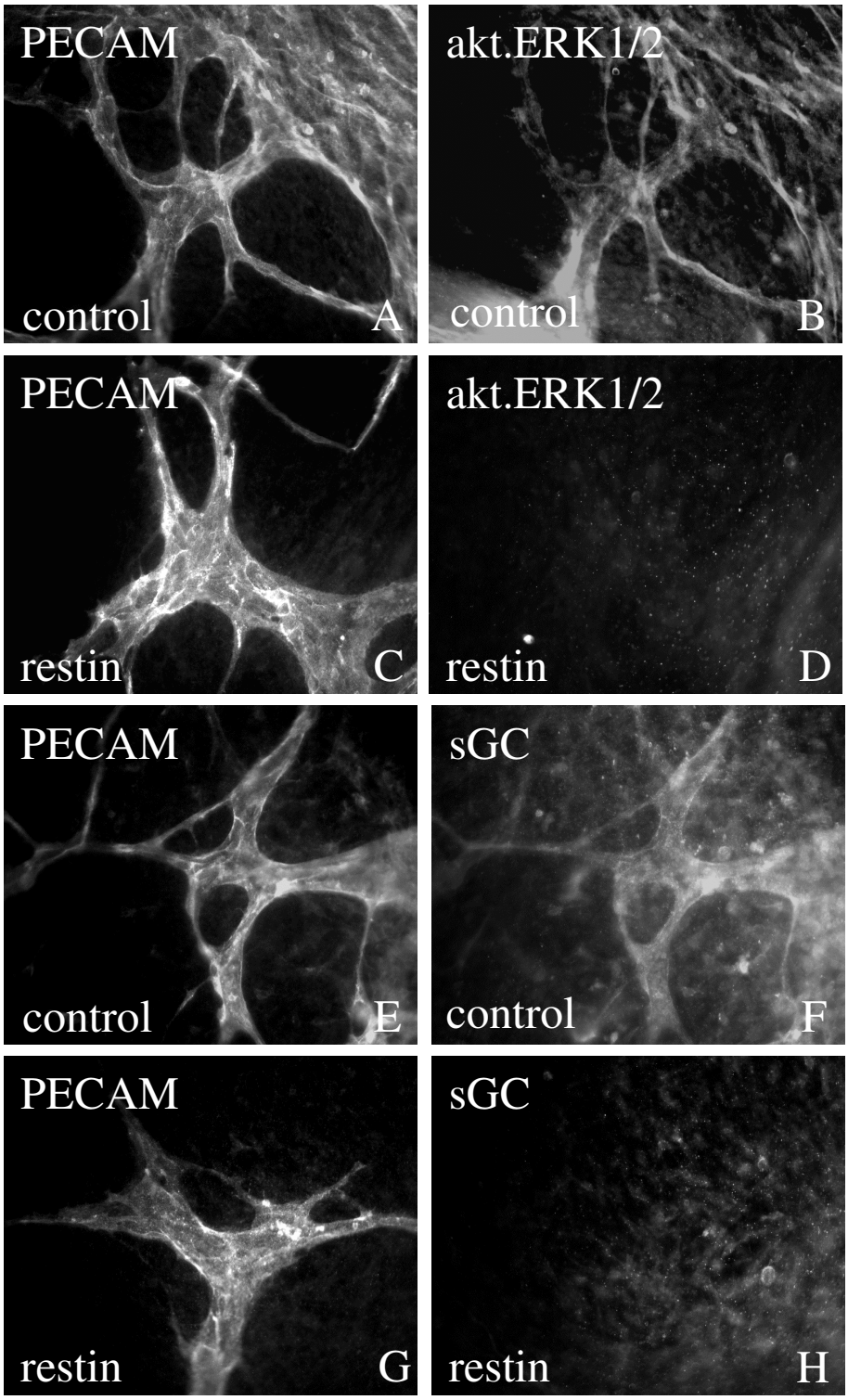


Figure 21: Signal transduction in endothelial tubes of in vitro differentiated stem cells. Under influence of endostatin, restin and the heparin mutant form of endostatin we observed a dephosphorylation of ERK1/2-kinase in PECAM-positive endothelial tubes as shown for restin (C and D) compared to control (A and B). Also we observed a down-regulation of sGC under influence of this three proteins in endothelial tube, as shown for restin (G and H) compared to control (E and F).

Questioning for the period of time in which down-regulation of this signaling molecules takes place we observed no reduction in endothelial tubes positive for the activated ERK1/2-kinase within 1 hour of treatment (Fig. 22B) but a beginning of down-regulation after 3 hours (Fig.

22C). After 6 hours we observed a nearly complete dephosphorylation of ERK1/2-kinase in vessels of EBs (Fig. 22D). For the soluble guanylate cyclase (sGC) any down-regulation was observed within a period of 3 hours (Fig. 22C) but a nearly complete down-regulation after 6 hours (Fig. 22D).

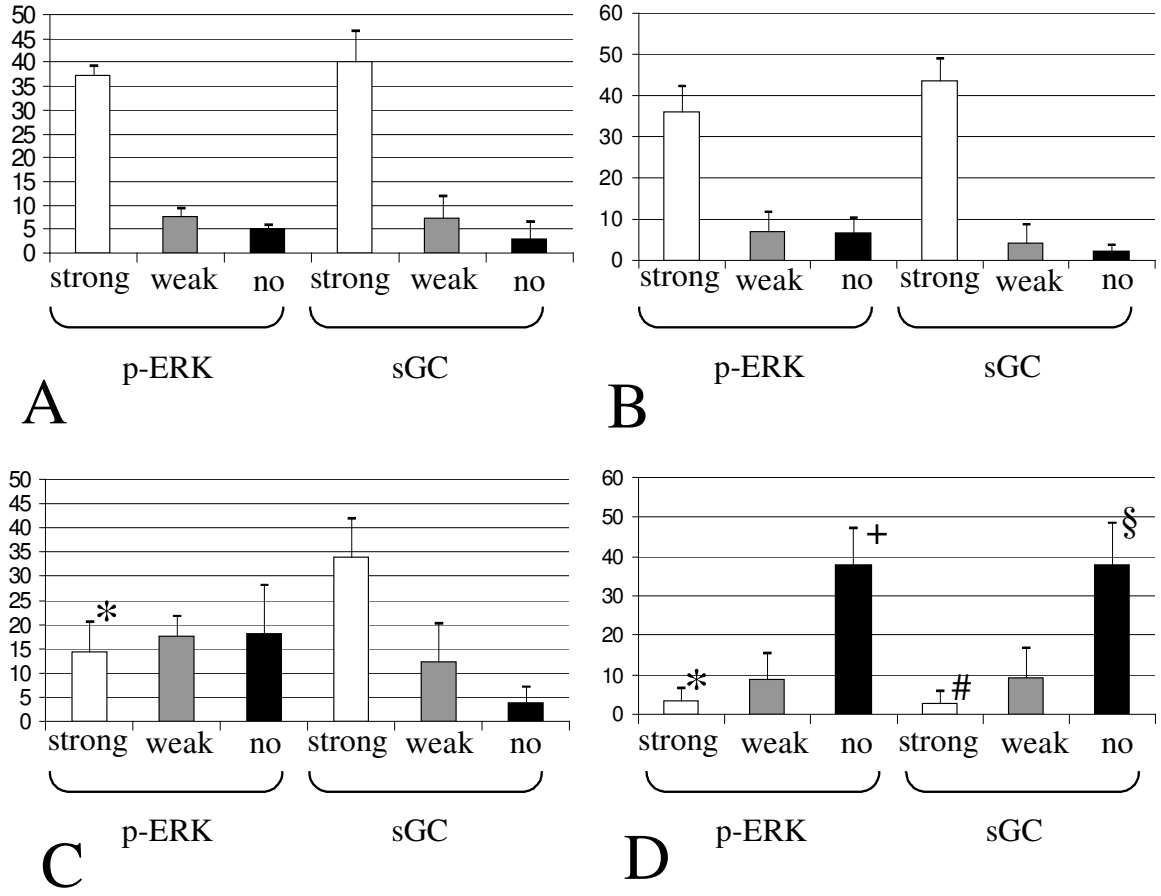


Figure 22: Statistical analysis of the number of endothelial tubes displaying ERK1/2-kinase dephosphorylation and sGC down-regulation under the influence of endostatin over a period of 6 hours (A-D). This effect was analyzed after 1 hour (B), 3 hours (C), 6 hours (D) as well as without endostatin incubation (0 hour, A). At each time point the number of endothelial tubes with strong, weak or no signal were counted. Mean ± SD $p<0.05$.

3.9 Is the effect of endostatin NO/cGMP dependent?

Endostatin mediates the dephosphorylation of the activated ERK1/2-kinase and down-regulation of sGC, both molecules of the VEGF/Flk-1/eNOS/sGC/cGMP/ERK signaling pathway (Parenti et al. 1998, Kroll and Waltenberger 1997). Further it was shown that endostatin inhibits the activity of eNOS (Urbich et al. 2002), which lays upstream of sGC and activated ERK1/2-kinase. As a consequence of less eNOS activity there is a down-regulation of cGMP (Verma et al. 2002). To answer the question if endostatin's effects on sGC and activated ERK1/2-kinase could be the consequence of its influence on upstream signaling molecules, we treated EBs with 8bromo-cGMP a cell permeable derivat of cGMP. From immunohistochemical analysis we observed that 8bromo-cGMP, which did not influence the activated ERK1/2-kinase by itself (Fig. 23A and B), prevents endostatin-meditated dephosphorylation of the activated ERK1/2-kinase (Fig. 23C and D) after preincubation with endostatin in a period of 30 min. Simultaneously by vital microscopy we observed a prevented endostatin-mediated retraction of endothelial tubes after treatment with 8bromo-cGMP when preincubation with endostatin was in a period of 30 min. (Fig. 24A-C). Because of the fact that endostatin-mediated retraction of endothelial tubes as well as the dephosphorylation of the activated ERK1/2-kinase takes place with a temporal delay we wondered if an initiated dephosphorylation, as well as an introduced retraction of vessels is also preventable by 8bromo-cGMP. To answer this question, we treated EBs for 1 hours and more with endostatin before we treated them with 8bromo-cGMP. After immunohistochemistry we observed the described endostatin-mediated dephosphorylation of the activated ERK1/2-kinase (Fig. 23E and F). An initiated endostatin-mediated dephosphorylation of ERK1/2-kinase could not be blocked by 8bromo-cGMP and was not reversible. By vital microscopy we observed that preincubation with endostatin for 1 hour and more before treatment with 8bromo-cGMP leads to the described endostatin-mediated retraction of endothelial cells (Fig. 24D-F). An initiated endostatin-mediated retraction of endothelial tubes was not preventable by 8bromo-cGMP. Treatment with 8bromo-cGMP could not influence the endostatin-mediated down-regulation of the sGC (data not shown).

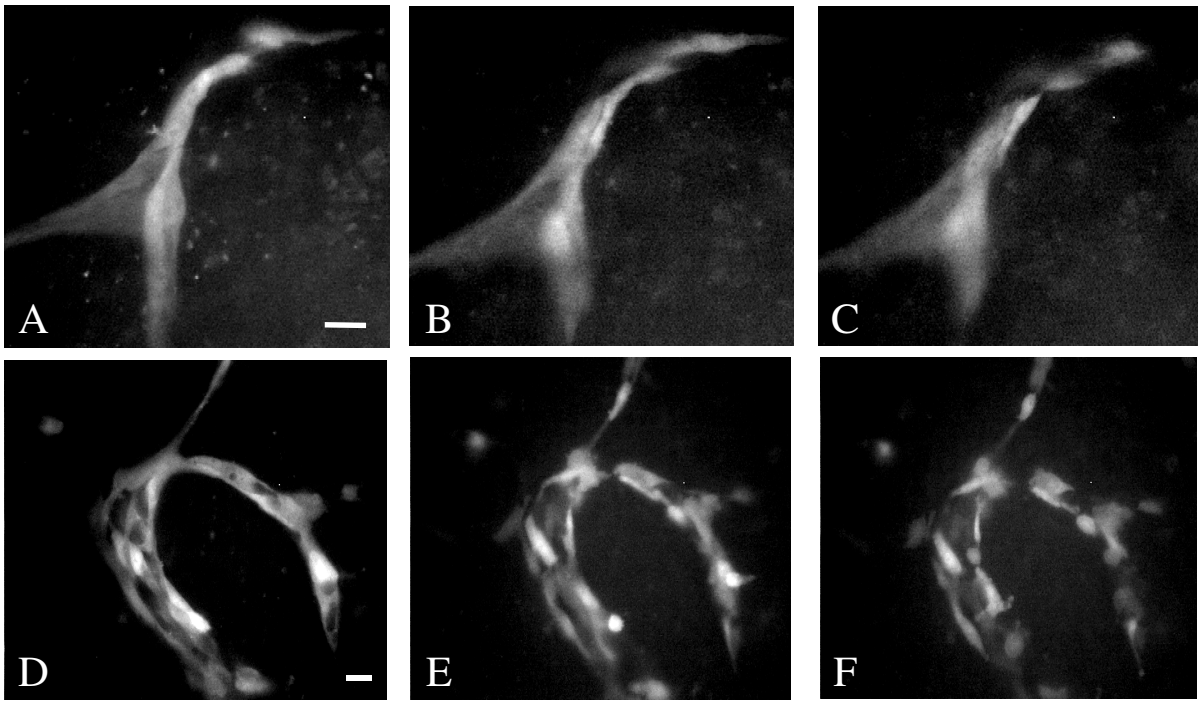


Figure 24: Vital microscopic observation of endostatin-mediated modulation of endothelial tube morphology under influence of 8bromo-cGMP. For vital observation stem cell derived endothelial tubes where pre-incubated for 30 min. with endostatin and then treated with 8bromocGMP (A-C). 8bromo-cGMP was able to block endostatin-mediated retraction of endothelial tubes (A-C). By pre-incubation of 1h and more with endostatin, 8bromo-cGMP was not able to block endostatin-mediated retraction of endothelial tubes (D-F).

In order to answer the question if the endostatin-induced dephosphorylation of ERK1/2-kinase is directly mediated by cGMP reduction, we analysed if the sGC-inhibitor ODQ was also able to induce these endostatin-mediated effects. Therefore we treated EBs with ODQ. After immunohistochemistry we neither observed dephosphorylation of ERK1/2-kinase (Fig. 26A and B) nor down-regulation of sGC in PECAM-positive vessels (Fig. 26C and D). Additionally we asked if the endostatin-mediated dephosphorylation of ERK1/2-kinase as well as the down-regulation of sGC are preventable by an increase of NO. To answer this question, we treated EBs simultaneously with the NO-donor NONOate and endostatin. After immunohistochemical detection we observed activated ERK1/2-kinase in PECAM-positive endothelial tubes. An increasing level of NO was able to block the endostatin-mediated dephosphorylation of ERK1/2-kinase (Fig. 25C and D) whereas NONOate alone did not influence ERK1/2-kinase phosphorylation (Fig. 25A and B).

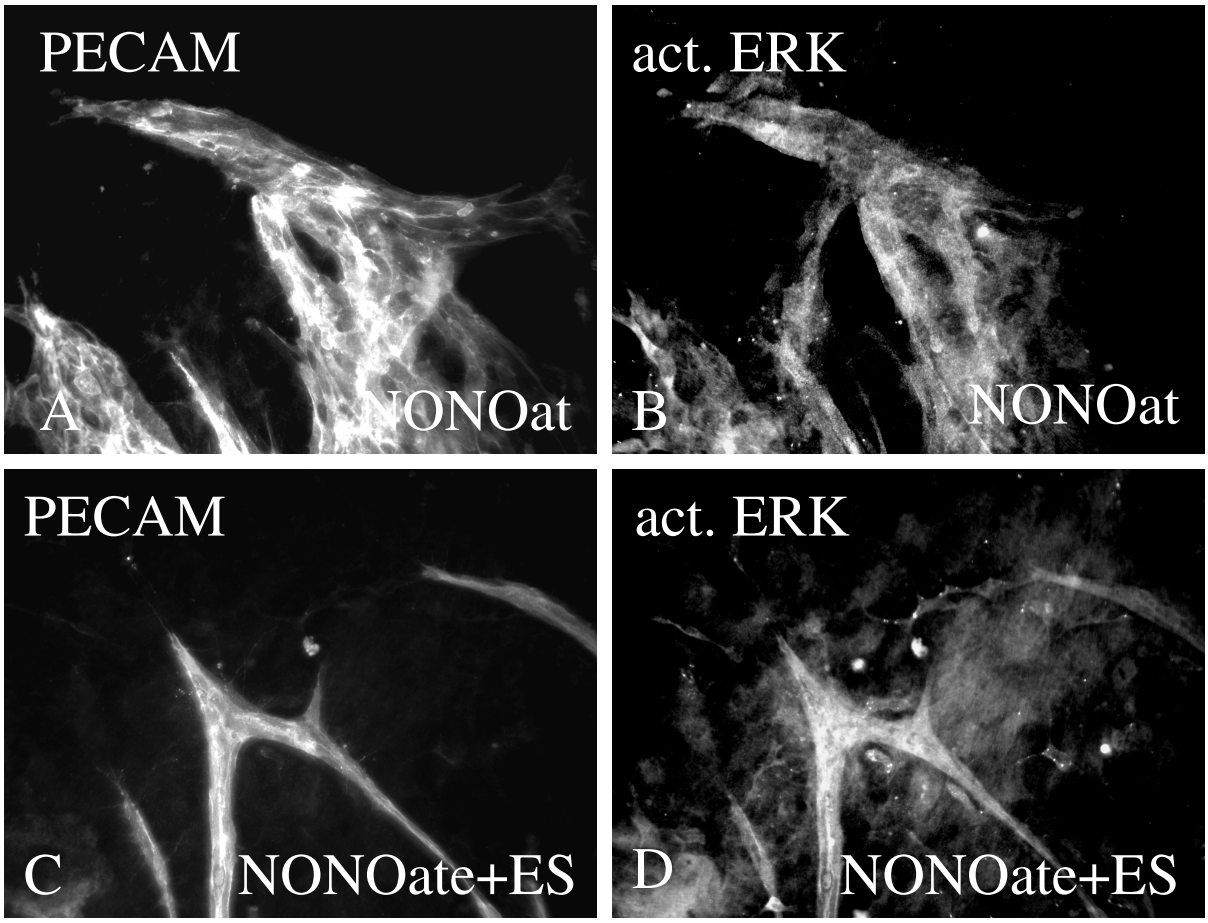


Figure 25: Influence of the NO-donor NONOate on the endostatin-mediated dephosphorylation of the activated ERK1/2-kinase in endothelial tubes of in vitro differentiated stem cells. The NO-donor by itself did not influence the activated ERK1/2-kinase (B) in PECAM-positive endothelial tubes (C).

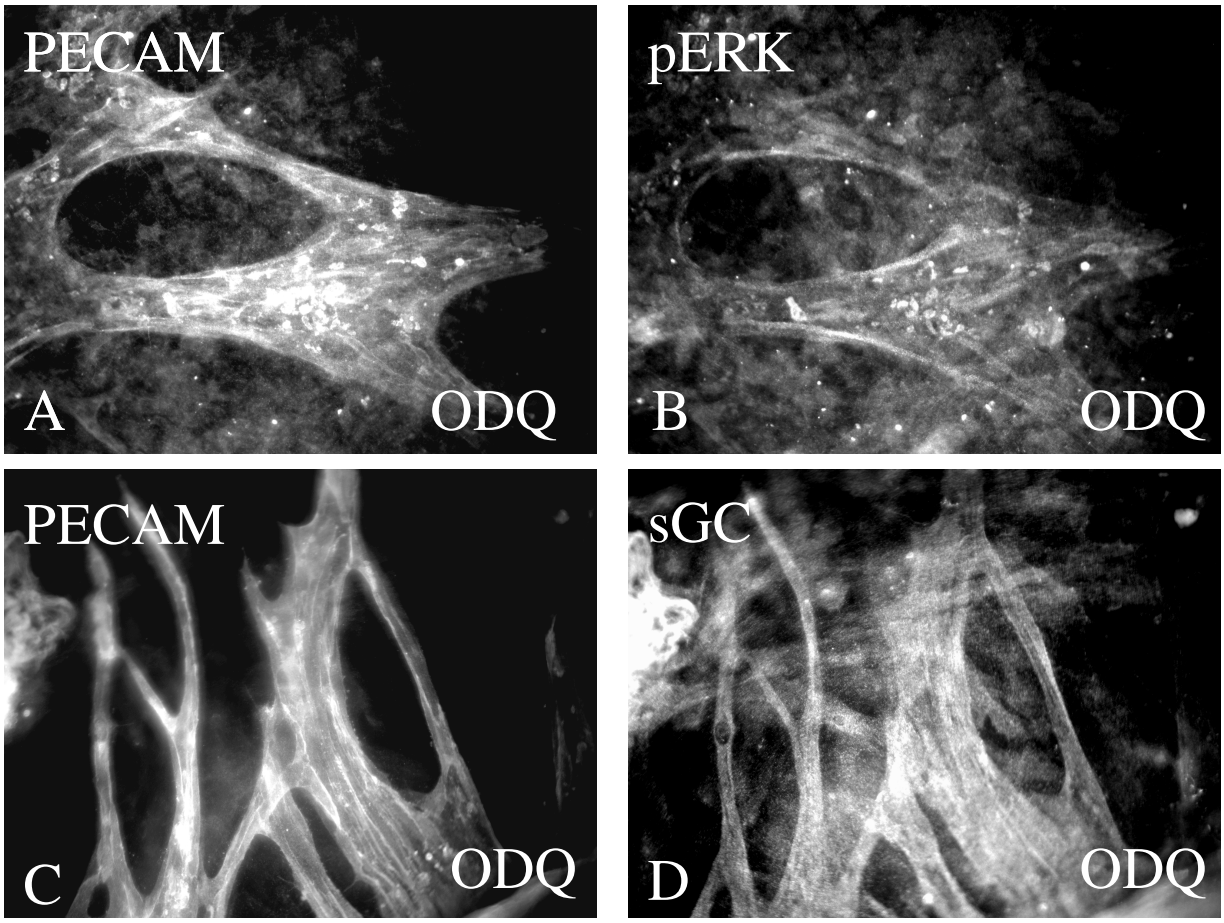


Figure 26: Analysation of ODQ effect on the activated ERK1/2-kinase and sGC in endothelial tubes of in vitro differentiated stem cells. The sGC-inhibitor ODQ did not influence the phosphorylation of the activated ERK1/2-kinase (B) in PECAM-positive endothelial tubes (A). Also under influence of ODQ we did not observed a down-regulation of sGC (D) in PECAM-positive endothelial tubes (C).

3.10 Are further pathways concerned?

As described by Urbich et al. endostatin increase significantly the activity of the Ser/Thr protein phosphatase PP2A (Urbich et al. 2002). To answer the question if PP2A also is responsible for dephosphorylation of ERK1/2-kinase and down-regulation of sGC we treated EBs simultaneous with the PP2A-inhibitor ocadaic acid and endostatin. After immunohistochemistry neither an endostatin-mediated dephosphorylation of ERK1/2-kinase (Fig. 27C and D) nor a down-regulation of the sGC (Fig. 27G and H) was observed in endothelial tubes whereas PP2A alone did not influence phosphorylated ERK1/2-kinase (Fig. 27A and B) or sGC (Fig. 27E and F). By inhibition of PP2A we were able to block endostatin-mediated dephosphorylation of ERK1/2-kinase and down-regulation of sGC. Consequently endostatin-mediated effects are induced by PP2A.

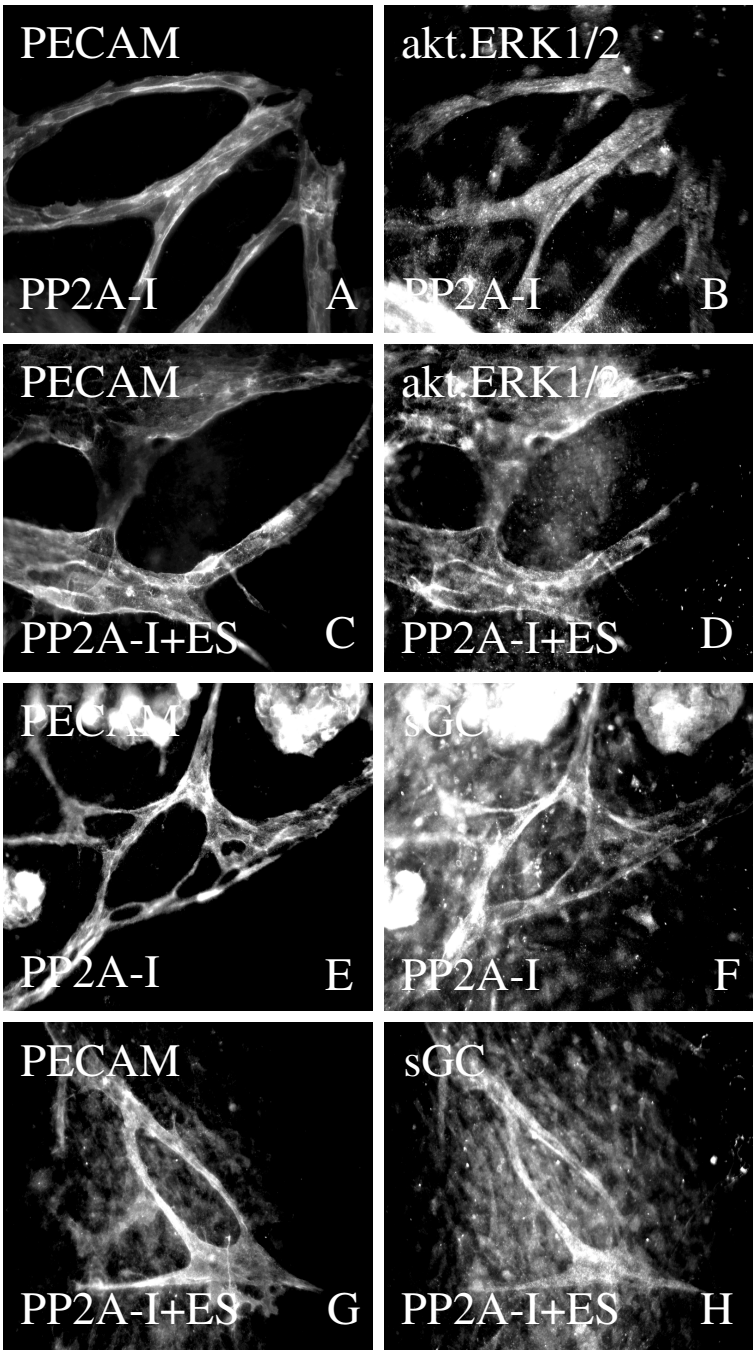


Figure 27: Influence of PP2A-inhibitor, ocadaic acid on the endostatin-mediated dephosphorylation of ERK1/2-kinase and down-regulation of sGC in endothelial tubes of in vitro differentiated embryonic stem cells. PP2A-inhibition by itself did not influenced ERK1/2-kinase phosphorylation (B) in PECAM-positive endothelial tubes (A) but after pre-incubation with endostatin, PP2A-inhibition blocked the endostatin-mediated dephosphorylation of the ERK1/2-kinase (D) in PECAM-positive endothelial tubes (C). PP2A-inhibition by itself also did not influence sGC (F) in PECAM-positive endothelial tubes (E) but after pre-incubation with endostatin PP2A-inhibitor ocadaic acid was able to block endostatin-mediated down-regulation of sGC (H) in endothelial tubes (G).

3.11 Is the reduction of sGC a consequence of transcriptional modification?

Regarding endostatins' influence it was clear, that the observed down-regulation of the activated ERK1/2-kinase in endothelial cells and vessel like structures was the consequence of a dephosphorylation because of an unchanged level of the common ERK1/2-kinase. But for the down-regulation of sGC it was unclear if the down-regulation takes place on the transcriptional level or on posttranscriptional level. In order to get an inside we performed RT in situ PCR and examined if the level of sGC mRNA was changed under influence of endostatin. But the level of sGCalpha1 mRNA as well as sGCbeta1 mRNA was unchanged in MACS-sorted endothelial cells after endostatin treatment, compared to control (data not shown).

4. Discussion

4. 1 The heparin-binding site is not necessary for endostatin action

In literature contrasting views exist on the importance of the endostatin heparin-binding site. Whereas Hohenester et al. 1998, Sasaki et al. 1998/1999 and Karumanchi et al. 2001 conclude that the heparin binding site of endostatin is necessary for its anti-angiogenic activity, Chang et al. 1999 and Yamaguchi et al. 1999 suggest that the anti-angiogenic effect of endostatin is independent from its heparin binding site. In this work we have found that the heparin mutant form of endostatin (R158/R184/R270A) has nearly the same effects as normal recombinant endostatin. This indicates that the heparin-binding site of endostatin is not necessary for its biological action. However the effects of the heparin mutant form shows a weakening compared to endostatin. A reason therefore could be a changed or reduced binding to the binding sites. Also a changed folding of the protein after mutagenesis could be a reason for.

4.2 Reason for contrasting results regarding the endostatin-mediated effects

It is noteworthy, though, that the endostatin concentrations used were often very high and could potentially cause the contrasting results. In fact the groups of Feraud et al. 2001 and Eriksson et al. 2003 report an inhibition of the density and sprouting of endothelial cells in EBs under the influence of endostatin. These contrasting results were obtained employing 60-300 fold higher endostatin concentrations as the present study and other differentiation protocols.

4.3 Binding of endostatin, restin and heparin mutant endostatin to tumour and embryonic vessels

Researchers have discovered that endostatin is a potent inhibitor of angiogenesis and tumour cell growth, while having no effect on a resting vascular system (O'Reilly et al. 1997, Bloch et al. 2000). In order to understand which vessels might be influenced by endostatin, we analysed the binding behaviour of endostatin with benign and malignant tissue. Altogether, our results show that endostatin exhibits different vascular binding properties for benign and

malignant tissue. Bladder and prostate tumours expressed binding sites for endostatin on the vessels. The significantly lower degree of endostatin binding to kidney tumour vessels indicates that the binding characteristics of the vessels of different types of tumours are not uniform.

The assumption that there are differences among various endothelial cell systems has recently been discussed. It has been postulated that endothelial cells from benign and malignant tissues are dissimilar on the molecular level and it has been demonstrated that endothelium derived from tumours is qualitatively different from that obtained from benign tissue (StCroix et al. 2000). These differences between endothelial cells in benign and malignant tissues could explain the diverse endostatin binding patterns seen in the vessels of the tissues investigated. Eberhard et al. give evidence that vessels in tumour tissues can be selectively targeted without affecting the quiescent organ vascularity (Eberhard et al. 2000). This fact is explained by the different molecular phenotypes of immature angiogenic blood vessels, which are distinct from resting blood vessels (StCroix et al. 2000). Subsequently, many animal studies have shown the feasibility of selectively targeting activated proliferating vascularity, without affecting normal organ vasculature (Bloch et al. 2000, Friedlander et al. 1995, Mauceri et al. 1998, Ferrara et al. 1998). Therefore the tumour vascular bed is indicated as a possible therapeutic target for cancer treatment (Tosetti et al. 2002).

A different form of functional immaturity could also explain the variations in endostatin's binding behaviour, seen when comparing bladder tumour vessels with those in kidney tumours. There may be discrepancies in the angiogenic potency of various kinds of endothelial cells, as has been described by other research groups, who found both anatomical and angiogenically-active vessels (Kumar et al. 1999, Ding et al. 2001).

Thus, endostatin binding is seen in most vascular endothelial cells in malignant bladder and prostate tissues, but it is significantly lower in endothelial cells from malignant kidney tumours. It could be speculated that endostatin causes a stable phenotype in superficial bladder tumour vessels, which leads to a low incidence of hematogenous metastasis in these malignant bladder lesions (Messing and Catalona 1998). The same reasoning can also be used to explain the similar low incidence of hematogenous metastasis in the vessels of cancerous prostate tissue (Oefelin et al. 1999, Moreno et al. 1992, Ablin 1993). Furthermore, the weak or absent binding of endostatin to vascular endothelial cells in malignant kidney tissues could explain the relatively high incidence of hematogenous metastasis observed in these tumours (Messing and Catalona 1998). As mentioned above, possible variations in endothelial

phenotypes may explain this characteristic and may subsequently lead to an aggressive growth pattern for malignant kidney tumours as compared to that of superficial bladder tumours. Because of a high amount of endostatin binding sites, malignant bladder and prostate tissue could be a target for a therapy based on endostatin treatment. Also the BPH, which shows high amounts of binding sites for endostatin compared to normal prostate tissue, makes this tissue accessible for an endostatin treatment. A strong binding of endostatin to vessels of bladder and prostate carcinoma as well as BPH give hint for a therapeutic benefit by angiogenesis inhibition.

A lot of binding sites for endostatin could also be observed in vessel-like structures of differentiated embryonic stem cell cultures. This strong binding to such structures suggests an angio-modulatory role of endostatin and restin during embryonic vessel development. The above mentioned differences in endostatin binding to different kinds of tumours but a strong binding to embryonic vessels could be possibly explained by the existence of different binding sites for endostatin i.e. glypican and tropomyosin with varying affinity for the ligand (Karumanchi et al. 2001, MacDonald et al. 2001). This result is supported by the fact that endostatin binds to embryonic stem cell-derived endothelial cells and vessels with high affinity but not to endothelial cell lines like PAEC and HUVEC (data not shown). We therefore assume, that differences in endothelial cell biology like maturation, differentiation and origin can influence the distribution of binding sites and consequently the ability to bind endostatin, which entails differences in its biological effects.

Because of these different binding sites signalling through more than one pathway is likely. In fact, a large variety of different signaling pathways, regulated by endostatin, have been described so far (Dixelius et al. 2000, Jiang et al. 2001, Shichiri and Hirata 2001). Furthermore the interaction with VEGF-R2 and α5ß1 or αvß3 integrin as targets for endostatin leading to alteration of angiogenic effects have also been reported (Furumatsu et al. 2002; Kim et al. 2002).

4.4 Cell biological aspects during embryonic vessel growth under influence of endostatin

We here show that endostatin is expressed in embryonic vessels and blood islands. Moreover, the demonstration of endostatin binding sites suggests an angiogenic regulatory role for endostatin during embryonic development. The proposed function of endostatin is rather

angio-modulatory than exclusively anti-angiogenic because of both, pro- and anti-angiogenic effects during embryonic vascular development. In fact the anti-angiogenic effects in form of increased apoptosis and altered morphogenesis balance the pro-angiogenic effects in form of endothelial cell proliferation and migration.

The modulation of angiogenesis is one of the most attractive approaches to regulate tissue growth in pathological and physiological processes such as neoplasia, ischemic and inflammatory diseases and wound healing. It also plays an important role for the embryonic development. Angiogenesis is a complex process, comprising activation, proliferation and migration of endothelial cells, alteration of the extracellular matrix, formation of endothelial tubes and networks, and their linkage to pre-existing vascular networks. This complicated mechanism is modulated by several angiogenic and anti-angiogenic molecules (Hanahan and Folkman 1996, Risau 1997, Carmeliet and Jain 2000).

The investigation of angiogenesis during development could help to understand better general and tissue specific mechanisms of regulation by angiogenic and anti-angiogenic factors. One of these factors is endostatin, which constitutes one of the most promising anti-angiogenic molecules currently tested in clinical trials (Ryan et al. 2002), characterized by a variety of effects and in particular by its heterogenous anti-angiogenic potential (Ramchandran et al. 2002). Our results on the role of endostatin during embryonic development demonstrate a variety of different effects on vasculo- and angiogenesis. These novel findings allow to compare the role of endostatin during embryonic development with earlier results for wound healing (Berger et al. 2000, Bloch et al. 2000, Mundhenke et al. 2001) and tumour growth (O`Reilly et al. 1997) in order to better understand the complex role of this molecule in the regulation of physiological and pathological angiogenesis.

Experiments for wound healing show that endostatin does not cause inhibition of regenerative vessel development (Berger et al. 2000, Bloch et al. 2000, Mundhenke et al. 2001), but plays a role for the morphogenesis of new formed vessels and the damage of endothelial cells (Bloch et al. 2000, Mundhenke et al. 2001). Our observations in EBs suggest that endostatin plays a similar role during embryonic vessel growth. We demonstrate that endostatin leads to contraction or lack of expansion of endothelial cell forming vessels and retraction of endothelial sprouting as well as apoptosis of endothelial cells. The morphogenic findings can explain the mechanism whereby the vessel diameter is reduced in tumours after endostatin treatment. In accordance to previous reports (Dhanabal et al. 1999A, Dixelius et al. 2000) we

also provide evidence that endostatin works as negative regulator of embryonic angio- and vasculogenesis by stimulating the apoptosis of endothelial precursors.
The complex mode of action of this molecule is further underlined by the observation that besides the anti-angiogenic effects of endostatin also pro-angiogenic actions such as increased proliferation and migration of ES cell-derived endothelial cells are noted.
Similar to endothelial cell migration Boye et al. 2001 could show that the migration of hemangioma-derived endothelial cells isolated from infants was stimulated after endostatin treatment. These findings imply that endothelial cells with a basal high angiogenic activity display different migratory characteristics to endothelial cell lines like HUVEC without basal angiogenic activity. In these cell types migration is inhibited after endostatin treatment (Dhanabal et al. 1999 B and C, Yamaguchi et al. 1999). We therefore propose that endothelial cell migration by endostatin depends on the origin and /or differentiation stage of cells.
The endostatin-mediated increase of the vessel number during embryonic development could be explained by the increase of endothelial proliferation in presence of endostatin in EBs. However, the amount of vessels does not increase in proportion to the high proliferation rate, which is explained by the counter-acting increased apoptotic endothelial cell death.
For proliferation several recent studies using VEGF or FGF stimulated micro- and macrovascular endothelial cell from primarily angiogenic quiescent vessels, which showed in opposite anti-proliferative effect of endostatin, supporting our assumption of endothelial cell type specific regulation.

4.5 Endothelial cell biological aspects during wound healing under influence of endostatin

The tumour-inhibitory protein endostatin did not concern neovascularisation during wound healing as demonstrated during this study as well as by others (Berger et al. 2000, Bloch et al. 2000, Mundhenke et al. 2001). The conclusion that neovascularisation of tumour tissue and during wound healing are different processes was confirmed by the findings that tumour endothelium, in general, is different from the surrounding normal tissue (St. Croix et al. 2000).
During the present story it has been observed in the present of the anti-angiogenic proteins endostatin, restin and the heparin binding mutant form of endostatin an influenced but not an

inhibited neovascularisation during wound healing. These factors increase vessel quantity and this could be explained by a shift of the balance between proliferation and apoptosis.
The fact that endostatin, restin and the heparin mutant form of endostatin lead to an increased vessel quantity during wound healing was confirmed by the experiments with embryonic stem cell derived endothelial cells and vessels and it agrees with the observation of an increased proliferation after treatment with these factors during wound healing as well as demonstrated for embryonic stem cell derived endothelial tubes. Comparable with these results Lai et al. could show that patients with high level of plasma endostatin show an increased angiogenesis in tumours (Lai et al. 2002). The demonstrated increase in endothelial apoptosis during wound healing after treatment with endostatin, restin and the heparin mutant form of endostatin agrees with our previous results as well with previous studies regarding endostatin's effect (Dhanabal et al. 1999A, Dixelius et al. 2000).

4.6 Endostatin, restin and heparin mutant endostatin influences endothelial morphology

Concerning endothelium and vessel morphology we observed under influence of endostatin, restin and heparin mutant endostatin a contraction and retraction of endothelium and endothelial tubes using vital microscopic observation of *in vitro* differentiated embryonic stem cells and of new build vessels in wounded areas by ultra structural electron microscopy. During these observations only endothelial tubes and vessels of a defined differentiation degree were concerned. This finding was confirmed recently by a group of researchers who analyse vessel development in the eye of collagen XVIII knockout mice. It was noticed that collagen XVIII/endostatin is critical for normal blood vessel formation in the eye for a defined age or differentiation degree. Collagen XVIII/endostatin was not critical before birth but at postnatal day 4-8 (Fukai et al 2002).
Under influence of endostatin, restin and the heparin mutant form of endostatin was observed a modulation of vascular morphology during wound healing as well as during vital microscopy of embryonic stem cell derived endothelial tubes in form of a contraction and vessel retraction. Morphological changes in form of a contraction were also observed by Read et al. 2001 after treatment with endostatin. They noticed that the contraction was not as an active process as we did by vital microscopy but in form of a reduces vessel diameter of tumour vessels. Also Bloch et al. 2000 observed narrowed and closed vessels on

ultrastructural level during wound healing after treatment with endostatin. Morphological changes under influence of endostatin could be explained by its above mentioned binding to tropomyosin (MacDonald et al. 2001). Tropomyosin, an endostatin binding protein plays an important role in contraction and morphogenesis of cells by influence on the regulation of the actin cytoskeleton (MacDonald et al. 2001). Additionally Mundhenke et al. 2001 detected changes of vessel morphology during wound healing after treatment with endostatin. But till now morphological changes in form of a dynamic process, the contraction of endothelial cells as well as the retraction of endothelial tubes has not been demonstrated. Also morphological changes under influence of restin and the heparin-binding mutant of endostatin were not described in the past. Because of the fact that inhibition of VEGF signaltransduction influences endothelial morphology and because of the down-regulation of the activated ERK-1/2-kinase by the MAP-kinase inhibitor PD98059 lead to a comparable modulation of endothelial morphology, we concluded that endostatin-mediated effects on signaltransduction cause the observed retraction of endothelial cells and tubes.

The effect of endostatin on vessel morphology could be a consequence of modulating signaltransduction and more precisely by influencing the VEGF signaltransduction. VEGF plays an important role regarding the morphogenesis of vasculo- and angiogenesis in the EB-model (Kazemi et al. 2002) and in the embryo (Poole et al. 2001). Recently it was demonstrated that endostatin influences the VEGF signaling pathway by inhibition of the KDR/Flk-1 phoshorylation (Kim et al. 2002) and the phosphorylation of endothelial nitric oxide (Urbich et al. 2002).

By vital microscopic analysis it was possible to observe that cGMP additionally was also able to inhibit the endostatin-mediated retraction of endothelial tubes.

4.7 Signaltransduction under influence of endostatin, restin and heparin mutant endostatin

It has been described for endostatin an effect on several signaling molecules is. Endostatin influences c-myc, c-fos, MAPK1 and 2 (Shichiri and Hirata 2001), activates PKA and increases intracellular cAMP (Shichire and Hirata 2001), increases Shb tyrosine phosphorylation (Dixelius et al. 2000) and increases Ca^{2+} influx (Jiang et al. 2001). Urbich et al. described an endostatin-mediated influence on eNOS-activity by modulation of the phosphorylation site at Ser1177 (Urbich et al. 2002). Shichiri and Hirata observed an

endostatin-mediated influence on MAPK 1 and 2 expression level (Shichiri and Hirata 2001) whereas we observed an endostatin-mediated influence on the activity of ERK1/2-kinase in form of a dephosphorylation of ERK1/2-kinase in endothelial cells and vessels during wound healing as well as for in vitro differentiated embryonic stem cells after treatment with endostatin. The down-regulation of sGC was demonstrated for the first time. The same effect could also be demonstrated for restin and the heparin mutant form of endostatin. Because of its influence on ERK1/2-kinase and sGC combined with the fact that vascular morphology is affected, which VEGF could be the responsible for, we concluded that this three proteins influence the VEGF/Flk-1/eNOS/sGC/cGMP/ERK pathway, which was described by Parenti et al. Also the fact that we were able to block endostatin-mediated influence of the ERK1/2-kinase with additional cGMP and to block endostatin-mediated ERK1/2-kinase dephosphorylation with additional NO confirms these findings. The supposition that endostatin influences the VEGF signaling pathway described by Parenti is also confirmed by considering that cGMP plays an important role by the relaxation of vessels (Fullerton et al. 1995, Brunner et al. 1996, Sheridan et al. 1997) whereas contraction of vessels under influence of endostatin was observed. The fact that inhibition of PP2A was able to block endostatin-mediated effects shows that these effects are induced by PP2A. To answer the question if the PP2A-induced dephosphorylation of ERK1/2-kinase is directly mediated by cGMP-reduction, we analysed if inhibition by ODQ is also able to dephosphorylate ERK1/2-kinase. This was not the case. But we conclude that endostatin-mediated reduction of cGMP by deactivation of eNOS via PP2A only open up the possibility for PP2A to dephosphorylate ERK1/2-kinase. The fact that additional cGMP was able to block dephosphorylation of the activated ERK1/2-kinase but not the down-regulation of sGC and that inhibition of PP2A was able to block both endostatin-mediated effects we concluded that further pathways and mechanisms of regulation are involved in this complex regulation.

Here we report about an endostatin-mediated dephosphorylation of ERK1/2-kinase and not about the absence of phosphorylation, which is related to our results concerning distribution of common ERK1/2-kinase and activated/phosporylated ERK1/2-kinase. Common ERK1/2-kinase was not reduced after treatment with endostatin whereas the activated form was. This makes clear that the endostatin-mediated effect concerning ERK1/2-kinase is a dephosphorylation. Concerning sGC we observed a down-regulation within 6 hours after treatment with endostatin. This period of time was also observed for sGC down-regulation under influence of 17ß-estradiol (E2) in rat uterus (Krumenacker et al. 2001). Krumenacker et

al. concluded from this period of time that decrease in sGC protein level occurs as a result of the reduction of mRNA level. Therefore we analysed by using RT in situ PCR if the mRNA level of sGCalpha1 and sGCbeta1 was changed after treatment with endostatin, what was not the case. Therefore the down-regulation of sGC under influence of endostatin could presumably be a consequence of a modification on protein level.
The angiogenesis inhibitor thrombospondin also influence signaling molecules and lead to phosphorylation of ERK1/2-kinase (Orr et al. 2002), whereas angiostatin described as anti-angiogenic and tumour inhibitory diminishes ERK1/2-phosphorylation (Redlitz et al. 1999)

5. Conclusion

The proteolytic fragments of collagen XVIII and XV, endostatin and restin, play an important role for embryonic vasculo- and angiogenesis as well as during regenerativ neovascularisation of physiological wound healing. It could be shown that it influences ES-cell derived endothelial cells and neovasculatur of wounded tissue in a pro- as well as anti-angiogenic fashion, suggesting an angio-modulatory role. Endostatin increases proliferation, the amount of vessels and migration but also apoptosis, contraction and morphological changes.
By inhibiting VEGF signaltransduction and especially by dephosphorylate ERK1/2-kinase via PP2A, endostatin presumably influences endothelial morphology. Summarising our observations, we conclude that the endostatin-mediated down-regulation of cGMP by reduction of eNOS activity via PP2A can only open up the possibility for the PP2A effect of ERK1/2-kinase dephosphorylation. The PP2A-mediated dephosphorylation of ERK1/2 in endothelial cells presumably is cGMP-dependent, but not directly cGMP-mediated.
The present study exemplifies that endostatin, restin and other endothelium influencing factors must be examined taking into account differentiation stage, signaltransduction and the origin of cells. These various factors may strongly determine the biological outcome.
An important fact regarding tumour therapies is that different tumours do not express binding sites in an amount, which is sufficient to address this kind of tumours by a treatment with endostatin and restin. From that we can learn, that a tumour therapy with such substances cannot be a uniform treatment, it must be individually adjusted to the type of tumour and the origin of tumour.

6. Outlook

An unclear but important question rises during this work: “Is the down-regulation of sGC under influence of endostatin a consequence of a modification on protein level?“ Answering this question was planed, but unfortunately till now not practicable. From personal comments (NO-Forum, 2002, in Frankfurt, abstract) it was known, that HSP90 stabilizes sGC protein in endothelial cells. Therefore we are interested to see if inhibition of HSP90 using geldanamycin also leads to a down-regulation of sGC in a comparable time course, which would imply prevention or disturbing of HSP90. Another important point is the role of protein modification by proteasoma. In order to examine this problem we are interested if the proteasoma inhibitor MG132, which reduces the degradation of ubiquitin-conjugated proteins, is able to block the endostatin-mediated down-regulation of sGC. This would show us that the endostatin-mediated down-regulation is a consequence of modification on protein level.

Raf-1 is influenced by endostatin by binding to alpha5beta1 integrin (Sudhaker et al. 2003). PP2A is a positive regulator of Raf-1 activation (Abraham et al. 2000). It would be interesting to analyse if there are connections or overlaps between these regulatory ways.

Another interesting point would be to examine the role for the protein phosphatase Shp-1 on endostatin-mediated effects. Shp-1 is also influenced by endostatin and is standing in connection with both, sGC and the activated ERK1/2-kinase. Information’s about this regulation could help us to verify if the described signaling pathway is the only involved, or if other pathways are implicated.

7. Literature

Ablin RJ.Circulating prostate specific antigen-positive cells correlate with metastatic prostate cancer. Br J Urol. 1993; 71:761-762

Abraham D, Podar K, Pacher M, Kubicek M, Welzel N, Hemmings BA, Dilworth SM, Mischak H, Kolch W, Baccarini M. Raf-1-associated protein phosphatase 2A as a positive regulator of kinase activation. J Biol Chem. 2000; 275:22300-22304.

Algire GH. Vascular reactions of normal and malignant tumor in vivo. Vascular reactions of mice to wounds and to normal and neoplastic transplants. J. Natl. Cancer Inst. 1945; 6:73-85.

Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J. Human ICE/CED-3 protease nomenclature. Cell. 1996; 87:171.

Arends MJ, Morris RG, Wyllie AH. Apoptosis. The role of the endonuclease. Am J Pathol. 1990; 136:593-608.

Augustin HG. Antiangiogenic tumour therapy: will it work? Trends Pharmacol Sci. 1998; 19:216-22.

Bagutti C, Wobus AM, Fassler R, Watt FM. Differentiation of embryonal stem cells into keratinocytes: comparison of wild-type and beta 1 integrin-deficient cells. Dev Biol. 1996; 179:184-196.

Bain G, Kitchens D, Yao M, Huettner JE, Gottlieb DI. Embryonic stem cells express neuronal properties in vitro. Dev Biol. 1995; 168:342-357.

Baldwin HS, Shen HM, Yan HC, DeLisser HM, Chung A, Mickanin C, Trask T, Kirschbaum NE, Newman PJ, Albelda SM. Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31): alternatively spliced, functionally distinct isoforms expressed during mammalian cardiovascular development. Development. 1994; 120:2539-2553.

Berger AC, Feldman AL, Gnant MF, Kruger EA, Sim BK, Hewitt S, Figg WD, Alexander HR, Libutti SK. The angiogenesis inhibitor, endostatin, does not affect murine cutaneous wound healing. J Surg Res. 2000; 91:26-31.

Blenis J. Signal transduction via the MAP kinases: proceed at your own RSK. Proc Natl Acad Sci U S A. 1993; 90:5889-5892.

Bloch W, Forsberg E, Lentini S, Brakebusch C, Martin K, Krell HW, Weidle UH, Addicks K, Fassler R. Beta 1 integrin is essential for teratoma growth and angiogenesis. J Cell Biol. 1997; 139:265-278.

Bloch W, Huggel K, Sasaki T, Grose R, Bugnon P, Addicks K, Timpl R, Werner S. The angiogenesis inhibitor endostatin impairs blood vessel maturation during wound healing. FASEB J. 2000; 14:2373-2376.

Boehm T, Folkman J, Browder T, O'Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. Nature. 1997; 390:404-407.

Bohle T, Brandt MC, Henn N, Schmidt A, Bloch W, Beuckelmann DJ. Identification of the cardiac ryanodine receptor channel in membrane blebs of sarcoplasmic reticulum. FEBS Lett. 2001; 505:419-425.

Boye E, Yu Y, Paranya G, Mulliken JB, Olsen BR, Bischoff J. Clonality and altered behavior of endothelial cells from hemangiomas. J Clin Invest. 2001; 107:745-752.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal Biochem. 1976; 72:248-254.

Breier G, Albrecht U, Sterrer S, Risau W. Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. Development. 1992; 114:521-532.

Breier G, Breviario F, Caveda L, Berthier R, Schnurch H, Gotsch U, Vestweber D, Risau W, Dejana E. Molecular cloning and expression of murine vascular endothelial-cadherin in early stage development of cardiovascular system. Blood. 1996; 87:630-641.

Breier G, Damert A, Plate KH, Risau W. Angiogenesis in embryos and ischemic diseases. Thromb Haemost. 1997; 78:678-683.

Brook FA, Gardner RL. The origin and efficient derivation of embryonic stem cells in the mouse. Proc Natl Acad Sci U S A. 1997; 94:5709-5712.

Brunner F, Schmidt K, Nielsen EB, Mayer B. Novel guanylyl cyclase inhibitor potently inhibits cyclic GMP accumulation in endothelial cells and relaxation of bovine pulmonary artery. J Pharmacol Exp Ther. 1996; 277:48-53.

Burek MJ, Oppenheim RW. Programmed cell death in the developing nervous system. Brain Pathol. 1996; 6:427-446.

Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature. 2000; 407:249-257.

Chang Z, Choon A, Friedl A. Endostatin binds to blood vessels in situ independent of heparan sulfate and does not compete for fibroblast growth factor-2 binding. Am J Pathol. 1999; 155:71-76.

Chen C, Parangi S, Tolentino MJ, Folkman J. A strategy to discover circulating angiogenesis inhibitors generated by human tumors. Cancer Res. 1995; 55:4230-4233.

Clark RAF. Woundrepair: overview and general considerations. The molecular Biology of wound repair 1996; Plenum, New York. pp. 195-248

Cohen JJ, Duke RC, Fadok VA, Sellins KS. Apoptosis and programmed cell death in immunity. Annu Rev Immunol. 1992; 10:267-293.

D'Angelo G, Struman I, Martial J, Weiner RI. Activation of mitogen-activated protein kinases by vascular endothelial growth factor and basic fibroblast growth factor in capillary endothelial cells is inhibited by the antiangiogenic factor 16- kDa N-terminal fragment of prolactin. Proc Natl Acad Sci U S A. 1995; 92:6374-6378.

Davis RJ. The mitogen-activated protein kinase signal transduction pathway. J Biol Chem. 1993; 268:14553-14556.

DeLisser HM, Newman PJ, Albelda SM. Molecular and functional aspects of PECAM-1/CD31. Immunol Today. 1994; 15:490-495.

De Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science. 1992; 255:989-991.

Dhanabal M, Ramchandran R, Waterman MJ, Lu H, Knebelmann B, Segal M, Sukhatme VP. Endostatin induces endothelial cell apoptosis. J Biol Chem. 1999; 274:11721-11726. (A)

Dhanabal M, Volk R, Ramchandran R, Simons M, Sukhatme VP. Cloning, expression, and in vitro activity of human endostatin. Biochem Biophys Res Commun. 1999; 258:345-352. (B)

Dhanabal M, Ramchandran R, Volk R, Stillman IE, Lombardo M, Iruela-Arispe ML, Simons M, Sukhatme VP. Endostatin: yeast production, mutants, and antitumor effect in renal cell carcinoma. Cancer Res. 1999; 59:189-197. (C)

Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature. 1999; 399:601-605.

Ding I, Sun JZ, Fenton B, Liu WM, Kimsely P, Okunieff P, Min W. Intratumoral administration of endostatin plasmid inhibits vascular growth and perfusion in Mca-4 murine mammary carcinoma. Cancer Res. 2001; 61:526-531.

Dixelius J, Larsson H, Sasaki T, Holmqvist K, Lu L, Engstrom A, Timpl R, Welsh M, Claesson-Welsh L. Endostatin-induced tyrosine kinase signalling through the Shb adaptor protein regulates endothelial cell apoptosis. Blood. 2000; 95:3403-3411.

Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. J Embryol Exp Morphol. 1985; 87:27-45.

Doetschman T, Shull M, Kier A, Coffin JD. Embryonic stem cell model systems for vascular morphogenesis and cardiac disorders. Hypertension. 1993; 22:618-629.

Drab M, Haller H, Bychkov R, Erdmann B, Lindschau C, Haase H, Morano I, Luft FC, Wobus AM. From totipotent embryonic stem cells to spontaneously contracting smooth muscle cells: a retinoic acid and db-cAMP in vitro differentiation model. FASEB J. 1997; 11:905-915.

Dumont DJ, Fong GH, Puri MC, Gradwohl G, Alitalo K, Breitman ML. Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. Dev Dyn. 1995; 203:80-92.

Duriez PJ, Shah GM. Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death. Biochem Cell Biol. 1997; 75:337-349.

Eberhard A, Kahlert S, Goede V, Hemmerlein B, Plate KH, Augustin HG. Heterogeneity of angiogensis and blood vessel maturation in human tumors: implication for antiangiogenic tumor therapies. Cancer Res. 2000; 60:1388-1393.

Edmunds LN, Adams KJ. Clocked cell cycle clocks. Science. 1981; 211:1002-1013.

Ellis RE, Yuan JY, Horvitz HR. Mechanisms and functions of cell death. Annu Rev Cell Biol. 1991; 7:663-698.

Eriksson K, Magnusson P, Dixelius, Claesson-Welsh L, Cross J: Angiostatin and endostatin inhibit endothelial migration in response to FGF and VEGF without interfering with specific intracellular signal transduction pathways. FEBS Letters. 2003; 536:19-24

Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981; 292:154-156.

Fassler R, Rohwedel J, Maltsev V, Bloch W, Lentini S, Guan K, Gullberg D, Hescheler J, Addicks K, Wobus AM: Differentiation and integrity of cardiac muscle cells are impaired in the absence of ß1 integrin. J Cell Sci. 1996; 109:2989-2999.

Feraud O, Cao Y, Vittet D. Embryonic stem cell-derived embryoid bodies development in collagen gels recapitulates sprouting angiogenesis. Lab Invest. 2001; 81:1669-1681.

Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun. 1989; 161:851-858.

Ferrara N, Chen H, Davis-Smyth T, Gerber HP, Nguyen TN, Peers D, Chisholm V, Hillan KJ, Schwall RH. Vascular endothelial growth factor is essential for corpus luteum angiogenesis. Nat Med. 1998; 4:336-4340.

Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med. 1971; 285:1182-1186.

Folkman J. Anti-angiogenesis: new concept for therapy of solid tumors. Ann Surg. 1972; 175:409-416.

Folkman J. What is the evidence that tumors are angiogenesis dependent? J Natl Cancer Inst. 1990; 82:4-6.

Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature. 1995; 376:66-70.

Fraichard A, Chassande O, Bilbaut G, Dehay C, Savatier P, Samarut J. In vitro differentiation of embryonic stem cells into glial cells and functional neurons. J Cell Sci. 1995; 108:3181-3188.

Friedl A, Chang Z, Tierney A, Rapraeger AC. Differential binding of fibroblast growth factor-2 and -7 to basement membrane heparan sulfate: comparison of normal and abnormal human tissues. Am J Pathol. 1997; 150:1443-1455

Friedlander M, Brooks PC, Shaffer RW, Kincaid CM, Varner JA, Cheresh DA. Definition of two angiogenic pathways by distinct alpha v integrins. Science. 1995; 270:1500-1502.

Fujita N, Tsuruo T. Involvement of Bcl-2 cleavage in the acceleration of VP-16-induced U937 cell apoptosis. Biochem Biophys Res Commun. 1998; 246:484-488.

Fukai N, Eklund L, Marneros AG, Oh SP, Keene DR, Tamarkin L, Niemela M, Ilves M, Li E, Pihlajaniemi T, Olsen BR. Lack of collagen XVIII/endostatin results in eye abnormalities. EMBO J. 2002; 21:1535-1544.

Fullerton DA, McIntyre RC Jr, Hahn AR, Agrafojo J, Koike K, Meng X, Banerjee A, Harken AH. Dysfunction of cGMP-mediated pulmonary vasorelaxation in endotoxin-induced acute lung injury. Am J Physiol. 1995; 268:L1029-1035.

Furumatsu T, Yamaguchi N, Nishida K, Kawai A, Kunisada T, Namba M, Inoue H, Ninomiya Y. Endostatin inhibits adhesion of endothelial cells to collagen I via a2ß1 integrin, a possible cause of prevention of chondrosarcoma growth. J Biochem. 2002; 131:619-626.

Gallwitz D. Zellteilung und Zellzyklus. Naturwissenschaften. 1981; 68:163-169.

Gately S, Twardowski P, Stack MS, Patrick M, Boggio L, Cundiff DL, Schnaper HW, Madison L, Volpert O, Bouck N, Enghild J, Kwaan HC, Soff GA. Human prostate carcinoma cells express enzymatic activity that converts human plasminogen to the angiogenesis inhibitor, angiostatin.Cancer Res. 1996; 56:4887-4890.

Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA, Bouck NP. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc Natl Acad Sci U S A. 1990; 87:6624-6628.

Goto F, Goto K, Weindel K, Folkman J. Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor on the proliferation and cord formation of bovine capillary endothelial cells within collagen gels. Lab Invest. 1993; 69:508-517.

Gumina RJ, Kirschbaum NE, Piotrowski K, Newman PJ: Characterization of the human platelet/endothelial cell adhesion molecule-1 promoter: identification of a GATA-2 binding element required for optimal transcriptional activity. Blood. 1997; 89:1260-1269.

Hajitou A, Grignet-Debrus C, Devy L, Berndt S, Blacher S, Deroanne CF, Bajou K, Fong T, Chiang Y, Foidart J, Noel A. The antitumoral effect of endostatin and angiostatin is associated with a down-regulation of vascular endothelial growth factor expression in tumor cells. FASEB J. 2002; 16:1802-1804

Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell. 1996; 86:353-364.

Hanai Ji J, Dhanabal M, Karumanchi SA, Albanese C, Waterman M, Chan B, Ramchandran R, Pestell R, Sukhatme VP. Endostatin causes G1 arrest of endothelial cells through inhibition of cyclin D1. J Biol Chem. 2002; 277:16464-16469.

Heidebrecht HJ, Buck F, Haas K, Wacker HH, Parwaresch R. Monoclonal antibodies Ki-S3 and Ki-S5 yield new data on the 'Ki-67' proteins. Cell Prolif. 1996; 29:413-425.

Hescheler J, Fleischmann BK, Lentini S, Maltsev VA, Rohwedel J, Wobus AM, Addicks K. Embryonic stem cells: a model to study structural and functional properties in cardiomyogenesis. Cardiovasc Res. 1997; 36:149-162.

Heuer JG, von Bartheld CS, Kinoshita Y, Evers PC, Bothwell M. Alternating phases of FGF receptor and NGF receptor expression in the developing chicken nervous system. Neuron. 1990; 5:283-296.

Hohenester E, Sasaki T, Olsen BR, Timpl R. Crystal structure of the angiogenesis inhibitor endostatin at 1.5 A resolution. EMBO J. 1998; 17:1656-1664.

Holmgren L, O'Reilly MS, Folkman J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nat Med. 1995; 1:149-153.

Jiang L, Jha V, Dhanabal M, Sukhatme VP, Alper SL. Intracellular Ca(2+) signalling in endothelial cells by the angiogenesis inhibitors endostatin and angiostatin. Am J Physiol Cell Physiol. 2001; 280:C1140-1150.

Johnson EM Jr, Deckwerth TL, Deshmukh M. Neuronal death in developmental models: possible implications in neuropathology. Brain Pathol. 1996; 6:397-409.

Kalka C, Masuda H, Takahashi T, Gordon R, Tepper O, Gravereaux E, Pieczek A, Iwaguro H, Hayashi SI, Isner JM, Asahara T: Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects. Circ Res. 2000; 86:1198-1202.

Karumanchi SA, Jha V, Ramchandran R, Karihaloo A, Tsiokas L, Chan B, Dhanabal M, Hanai JI, Venkataraman G, Shriver Z, Keiser N, Kalluri R, Zeng H, Mukhopadhyay D, Chen RL, Lander AD, Hagihara K, Yamaguchi Y, Sasisekharan R, Cantley L, SukhatmeVP. Cell surface glypicans are low-affinity endostatin receptors. Mol Cell. 2001; 7:811-822.

Kazemi S, Wenzel D, Kolossov E, Lenka N, Raible A, Sasse P, Hescheler J, Addicks K, Fleischmann BK, Bloch W. Differential role of bFGF and VEGF for vasculogenesis. Cell Physiol Biochem. 2002; 12:55-62.

Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT. Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science. 1989; 246:1309-1312.

Keller GM. In vitro differentiation of embryonic stem cells. Curr Opin Cell Biol. 1995; 7:862-869.

Keyt BA, Berleau LT, Nguyen HV, Chen H, Heinsohn H, Vandlen R, Ferrara N. The carboxyl-terminal domain (111-165) of vascular endothelial growth factor is critical for its mitogenic potency. J Biol Chem. 1996; 271:7788-7795.

Kim YM, Hwang S, Kim YM, Pyun BJ, Kim TY, Lee ST, Gho YS, Kwon YG. Endostatin blocks VEGF-mediated signalling via direct interaction with KDR/Flk-1. J Biol Chem. 2002; 277:27872-27879

Klagsbrun M, D'Amore PA. Regulators of angiogenesis. Annu Rev Physiol. 1991; 53:217-239.

Knebelmann B, Dhanabal M, Ramchandran R, Waterman M, Lu H, Sukhatme YP. Proceeding of the American Association for Cancer Research. 1999; 40:2737.

Kolossov E, Fleischmann BK, Liu Q, Bloch W, Viatchenko-Karpinski S, Manze O, Ji GJ, Bohlen H, Addicks K, Hescheler J: Functional characteristics of ES Cell-derived cardiac precursor cells identified by tissue-specific expression of the green fluorescent protein. J Cell Biol 1998; 143:2045-2056.

Kosco-Vilbois MH, Zentgraf H, Gerdes J, Bonnefoy JY. To 'B' or not to 'B' a germinal center? Immunol Today. 1997; 18:225-230.

Kroll J, Waltenberger J. The vascular endothelial growth factor receptor KDR activates multiple signal transduction pathways in porcine aortic endothelial cells. J Biol Chem. 1997; 272:32521-32527.

Krumenacker JS, Hyder SM, Murad F. Estradiol rapidly inhibits soluble guanylyl cyclase expression in rat uterus. Proc Natl Acad Sci U S A. 2001; 98:717-722.

Ku DD, Zaleski JK, Liu S, Brock TA. Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries. Am J Physiol. 1993; 265:H586-592.

Kumar S, Ghellal A, Li C, Byrne G, Haboubi N, Wang JM, Bundred N. Breast Carcinoma. Vascular density determined using CD105 antibody correlates with tumour prognosis. Cancer Res. 1999; 59:856-861.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227:680-685.

Lai R, Estey E, Shen Y, Despa S, Kantarjian H, Beran M, Maushouri T, Quackenbuch RC, Keating M, Albitar M. Clinical significance of plasma endostatin in acute myeloid leukemia/myelodysplastic syndrome. Cancer. 2002; 94:14-17.

Lampugnani MG, Resnati M, Raiteri M, Pigott R, Pisacane A, Houen G, Ruco LP, Dejana E. A novel endothelial-specific membrane protein is a marker of cell-cell contacts. J Cell Biol. 1992; 118:1511-1522.

Liaw L, Schwartz SM. Comparison of gene expression in bovine aortic endothelium in vivo versus in vitro. Differences in growth regulatory molecules. Arterioscler Thromb. 1993; 13:985-993.

Lindner V, Majack RA, Reidy MA. Basic fibroblast growth factor stimulates endothelial regrowth and proliferation in denuded arteries. J Clin Invest. 1990; 85:2004-2008.

Liotta LA, Kleinerman J, Saidel GM. Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. Cancer Res. 1974; 34:997-1004.

MacDonald NJ, Shivers WY, Narum DL, Plum SM, Wingard JN, Fuhrmann SR, Liang H, Holland-Linn J, Chen DH, Sim BK. Endostatin binds tropomyosin. A potential modulator of the antitumor activity of endostatin. J Biol Chem. 2001; 276:25190-25196.

Maltsev VA, Wobus AM, Rohwedel J, Bader M, Hescheler J. Cardiomyocytes differentiated in vitro from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. Circ Res. 1994; 75:233-244.

Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A. 1981; 78:7634-7638.

Martin P. Wound healing--aiming for perfect skin regeneration. Science. 1997; 276:75-81.

Mauceri HJ, Hanna NN, Beckett MA, Gorski DH, Staba MJ, Stellato KA, Bigelow K, Heimann R, Gately S, Dhanabal M, Soff GA, Sukhatme VP, Kufe DW, Weichselbaum RR. Combined effects of angiostatin and ionising radiation in anti-tumour therapy. Nature 1998; 394:287-291.

Messing EM and Catalona W. Urothelial tumours of the urinary tract. In Walsh PC, Retik AB, Vaughan ED, Wein AJ eds, Cambell`s Urology. Philadelphia, London, Toronto: Saunders Company, 1998; 2329-2361.

Meyer TS, Lamberts BL. Use of coomassie brilliant blue R250 for the electrophoresis of microgram quantities of parotid saliva proteins on acrylamide-gel strips. Biochim Biophys Acta. 1965; 107:144-145.

Miosge N, Sasaki T, Timpl R. Angiogenesis inhibitor endostatin is a distinct component of elastic fibers in vessel walls. FASEB J. 1999; 13:1743-1750.

Morbidelli L, Chang CH, Douglas JG, Granger HJ, Ledda F, Ziche M. Nitric oxide mediates mitogenic effect of VEGF on coronary venular endothelium. Am J Physiol. 1996; 270:H411-415.

Moreno JG, Croce CM, Fischer R, Monne M, Vihko P, Mulholland SG, Gomella LG. Detection of hematogenous micrometastasis in patients with prostate cancer. Cancer Res. 1992; 52:6110-6112.

Mundhenke C, Thomas JP, Wilding G, Lee FT, Kelzc F, Chappell R, Neider R, Sebree LA, Friedl A. Tissue examination to monitor antiangiogenic therapy: a phase I clinical trial with endostatin. Clin Cancer Res. 2001; 7:3366-3374.

Munz B, Smola H, Engelhardt F, Bleuel K, Brauchle M, Lein I, Evans LW, Huylebroeck D, Balling R, Werner S. Overexpression of activin A in the skin of transgenic mice reveals new activities of activin in epidermal morphogenesis, dermal fibrosis and wound repair. EMBO J. 1999; 18:5205-5215.

Noden DM. Development of craniofacial blood vessel. The Dev. Vasc. Syst. 1991; 1-24.

Oefelin MG, Ignatoff JM, Clemens JQ, Watkin W, Kaul KL. Clinical and molecular follow up after radical retropubic prostatectomy. J Urol. 1999; 162:307-310.

Oelrichs RB, Reid HH, Bernard O, Ziemiecki A, Wilks AF. NYK/FLK-1: a putative receptor protein tyrosine kinase isolated from E10 embryonic neuroepithelium is expressed in endothelial cells of. Oncogene. 1993; 8:11-18.

Oh SP, Kamagata Y, Muragaki Y, Timmons S, Ooshima A, Olsen BR. Isolation and sequencing of cDNAs for proteins with multiple domains of Gly-Xaa-Yaa repeats identify a distinct family of collagenous proteins. Proc Natl Acad Sci U S A. 1994; 91:4229-4233. (A)

Oh SP, Warman ML, Seldin MF, Cheng SD, Knoll JH, Timmons S, Olsen BR. Cloning of cDNA and genomic DNA encoding human type XVIII collagen and localization of the alpha 1(XVIII) collagen gene to mouse chromosome 10 and human chromosome 21. Genomics. 1994; 19:494-9. (B)

Okabe S, Forsberg-Nilsson K, Spiro AC, Segal M, McKay RD. Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. Mech Dev. 1996; 59:89-102.

O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao YSage EH, Folkman J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell. 1994; 79:315-328.

O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell. 1997; 88:277-285.

Orr AW, Pallero MA, Murphy-Ullrich JE. Thrombospondin stimulates focal adhesion disassembly through Gi- and phosphoinositide 3- kinase-dependent ERK activation. J Biol Chem. 2002; 277:20453-20460.

Parangi S, O'Reilly M, Christofori G, Holmgren L, Grosfeld J, Folkman J, Hanahan D. Antiangiogenic therapy of transgenic mice impairs de novo tumor growth. Proc Natl Acad Sci U S A. 1996; 93:2002-2007.

Pardanaud L, Yassine F, Dieterlen-Lievre F. Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny. Development. 1989; 105:473-485.

Parenti A, Morbidelli L, Cui XL, Douglas JG, Hood JD, Granger HJ, Ledda F, Ziche M. Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase1/2 activation in postcapillary endothelium. J Biol Chem. 1998; 273:4220-4226.

Patel T, Gores GJ, Kaufmann SH. The role of proteases during apoptosis. FASEB J. 1996; 10:587-597.

Pepper MS, Ferrara N, Orci L, Montesano R: Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. Biochem Biophys Res Commun. 1992; 189:824-831.

Peters KG, Werner S, Chen G, Williams LT. Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. Development. 1992; 114:233-243.

Phillips DR, Jennings LK, Prasanna HR. Ca2+-mediated association of glycoprotein G (thrombinsensitive protein, thrombospondin) with human platelets. J Biol Chem. 1980; 255:11629-11632.

Poole TJ, Finkelstein EB, Cox CM. The role of FGF and VEGF in angioblast induction and migration during vascular development. Developmental Dynamics 2001; 220:1-17.

Ramchandran R, Dhanabal M, Volk R, Waterman MJ, Segal M, Lu H, Knebelmann B, Sukhatme VP. Antiangiogenic activity of restin, NC10 domain of human collagen XV: comparison to endostatin. Biochem Biophys Res Commun. 1999; 255:735-739.

Ramchandran R, Karumanchi SA, Hanai J, Alper SL, Sukhatme VP. Cellular actions and signaling by endostatin. Crit Rev Eukaryot Gene Expr. 2002; 12:175-191.

Read TA, Farhadi M, Bjerkvig R, Olsen BR, Rokstad AM, Huszthy PC, Vajkoczy P. Intravital microscopy reveals novel antivascular and antitumor effects of endostatin delivered locally by alginate-encapsulated cells. Cancer Res. 2001; 61:6830-6837.

Redlitz A, Daum G, Sage EH. Angiostatin diminishes activation of the mitogen-activated protein kinases ERK-1 and ERK-2 in human dermal microvascular endothelial cells. J Vasc Res. 1999; 36:28-34.

Rehn M, Pihlajaniemi T. Alpha 1(XVIII), a collagen chain with frequent interruptions in the collagenous sequence, a distinct tissue distribution, and homology with type XV collagen. Proc Natl Acad Sci U S A. 1994; 91:4234-4238.

Relf M, LeJeune S, Scott PA, Fox S, Smith K, Leek R, Moghaddam A, Whitehouse R, Bicknell R, Harris AL. Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and ist relation to angiogenesis. Cancer Res. 1997; 57:963-969.

Risau W, Lemmon V. Changes in the vascular extracellular matrix during embryonic vasculogenesis and angiogenesis. Dev Biol. 1988; 125:441-50.

Risau W, Flamme I. Vasculogenesis. Annu Rev Cell Dev Biol. 1995; 11:73-91.

Risau W. Differentiation of endothelium. FASEB J. 1995; 9:926-933.

Risau W. Mechanisms of angiogenesis. Nautre. 1997; 386:671-674.

Ryan DP, Penson RT, Ahmed S, Chabner BA, Lynch TJ. Reality testing in cancer treatment. The phase I trial of endostatin. The Oncologist. 2002; 7:58-65.

Saldeen J, Welsh N. Nicotinamide-induced apoptosis in insulin producing cells is associated with cleavage of poly(ADP-ribose) polymerase. Mol Cell Endocrinol. 1998; 139:99-107.

Sasaki T, Fukai N, Mann K, Gohring W, Olsen BR, Timpl R. Structure, function and tissue forms of the C-terminal globular domain of collagen XVIII containing the angiogenesis inhibitor endostatin. EMBO J. 1998; 17:4249-4256.

Sasaki T, Larsson H, Kreuger J, Salmivirta M, Claesson-Welsh L, Lindahl U, Hohenester E, Timpl R. Structural basis and potential role of heparin/heparan sulfate binding to the angiogenesis inhibitor endostatin. EMBO J. 1999; 18:6240-6248.

Sasaki T, Hohenester E, Timpl R. Structure and function of collagen-derived endostatin inhibitors of angiogenesis. IUBMB Life. 2002; 53:77-84.

Sato TN, Qin Y, Kozak CA, Audus KL. Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. Proc Natl Acad Sci U S A. 1993; 90:9355-9358.

Schnurch H, Risau W. Expression of tie-2, a member of a novel family of receptor tyrosine kinases, in the endothelial cell lineage. Development. 1993; 119:957-968.

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 1995; 376:62-66.

Seger R, Krebs EG. The MAPK signaling cascade. FASEB J. 1995; 9:726-735.

Sheridan BC, McIntyre RC, Meldrum DR, Meng X, Fullerton DA. Endotoxin stuns cGMP-mediated pulmonary vasorelaxation. Shock. 1997; 8:207-212.

Shichiri M, Hirata Y. Antiangiogenesis signals by endostatin. FASEB J. 2001; 15:1044-1053.

Short R. Avleolar epithelium in relation to growth of the lung. Phil. Trans. R. Soc. London. 1950; B235:35-87

Simbulan-Rosenthal CM, Rosenthal DS, Iyer S, Boulares AH, Smulson ME. Transient poly(ADP-ribosyl)ation of nuclear proteins and role of poly(ADP-ribose) polymerase in the early stages of apoptosis. J Biol Chem. 1998; 273:13703-13712.

Smith JA. The cell cycle and related concepts in cell proliferation. J Pathol. 1982; 136:149-166.

Smith AG. Mouse embryo stem cells: their identification, propagation and manipulation. Semin Cell Biol. 1992; 3:385-399.

Smulson ME, Pang D, Jung M, Dimtchev A, Chasovskikh S, Spoonde A, Simbulan-Rosenthal C, Rosenthal D, Yakovlev A, Dritschilo A. Irreversible binding of poly(ADP)ribose

polymerase cleavage product to DNA ends revealed by atomic force microscopy: possible role in apoptosis. Cancer Res. 1998; 58:3495-3498.

Starkey JR, Crowle PK, Taubenberger S. Mast-cell-deficient W/Wv mice exhibit a decreased rate of tumor angiogenesis. Int J Cancer. 1988; 42:48-52.

StCroix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, Kinzler KW. Genes expressed in human tumor endothelium. Science. 2000; 289:1197-1202.

Steller H. Mechanisms and genes of cellular suicide. Science. 1995; 267:1445-1449.

Strubing C, Ahnert-Hilger G, Shan J, Wiedenmann B, Hescheler J, Wobus AM. Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons. Mech Dev. 1995; 53:275-287.

Sudhakar A, Sugimoto H, Yang C, Lively J, Zeisberg M, Kalluri R. Human tumstatin and human endostatin exhibit distinct antiangiogenic activities mediated by alpha v beta 3 and alpha 5 beta 1 integrins. Proc Natl Acad Sci U S A. 2003; 100:4766-4771.

Taylor S, Folkman J. Protamine is an inhibitor of angiogenesis. Nature. 1982; 297:307-312.

Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Bohlen P. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem Biophys Res Commun. 1992; 187:1579-1586.

Tosetti F, Ferrari N, De Flora S, Albini A. Angioprevention: angiogenesis is a common and key target for cancer chemopreventive agents. FASEB J. 2002; 16:2-14.

Traut W, Scholzen T, Winking H, Kubbutat MH, Gerdes J. Assignment1 of the murine Ki-67 gene (Mki67) to chromosome band 7F3-F5 by in situ hybridization. Cytogenet Cell Genet. 1998; 83:12-13.

Trucco C, Oliver FJ, de Murcia G, Menissier-de Murcia J. DNA repair defect in poly(ADP-ribose) polymerase-deficient cell lines. Nucleic Acids Res. 1998; 26:2644-2649.

Urbich C, Reissner A, Chavakis E, Dernbach E, Haendeler J, Fleming I, Zeiher AM, Kaszkin M, Dimmeler S. Dephosphorylation of endothelial nitric oxide synthase contributes to the anti-angiogenic effects of endostatin. FASEB J. 2002; 16:706-708.

Verma S, Wang CH, Li SH, Dumont AS, Fedak PW, Badiwala MV, Dhillon B, Weisel RD, Li RK, Mickle DA, Stewart DJ. A self-fulfilling prophecy: C-reactive protein attenuates nitric oxide production and inhibits angiogenesis. Circulation. 2002; 106:913-919.

Vittet D, Prandini MH, Berthier R, Schweitzer A, Martin-Sisteron H, Uzan G, Dejana E. Embryonic stem cells differentiate in vitro to endothelial cells through successive maturation steps. Blood. 1996; 88:3424-3431.

Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J Biol Chem. 1994; 269:26988-26995.

Walz DA, Hung GL. In vivo studies on the binding of heparin and its fractions with platelet factor 4. Semin Thromb Hemost. 1985; 11:40-47.

Wanaka A, Milbrandt J, Johnson EM Jr. Expression of FGF receptor gene in rat development. Development. 1991; 111:455-468.

Wang R, Clark R, Bautch VL. Embryonic stem cell-derived cystic embryoid bodies form vascular channels: an in vitro model of blood vessel development. Development. 1992; 114:303-4316.

Wobus AM, Wallukat G, Hescheler J: Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotrophic responses to adrenergic and cholinergic agents and Ca^{2+} channel blockers. Differentiation 1991; 48:173-182.

Wu HM, Huang Q, Yuan Y, Granger HJ. VEGF induces NO-dependent hyperpermeability in coronary venules. Am J Physiol. 1996; 271:H2735-2739.

Yamaguchi TP, Dumont DJ, Conlon RA, Breitman ML, Rossant J. Flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. Development. 1993; 118:489-498.

Yamaguchi N, Anand-Apte B, Lee M, Sasaki T, Fukai N, Shapiro R, Que I, Lowik C, Timpl R, Olsen BR. Endostatin inhibits VEGF-induced endothelial cell migration and tumor growth independently of zinc binding. EMBO J. 1999; 18:4414-4423.

Young PE, Baumhueter S, Lasky LA. The sialomucin CD34 is expressed on hematopoietic cells and blood vessels during murine development. Blood. 1995; 85:96-105.

Zetter BR. Angiogenesis and tumor metastasis. Annu Rev Med. 1998; 49:407-424.

Ziche M, Morbidelli L, Choudhuri R, Zhang HT, Donnini S, Granger HJ, Bicknell R. Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. J Clin Invest. 1997; 99:2625-2634.

1. Berichterstatter: Professor Dr. med. K. Addicks

2. Berichterstatter: Professor Dr. rer. nat. S. Roth

8. Explanation (Erklärung)

„Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit -einschließlich Tabellen, Karten und Abbildungen-, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät der Universität zur Prüfung vorgelegen hat; dass sie -abgesehen von unten angegebenen Teilpublikationen- noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotion sind mir bekannt. Die von mir vorgelegte Dissertation ist von Universitätsprofessor Dr. med. K. Addicks betreut worden.“

Cologne, June the 24th 2003

9. Curriculum vitea

Name:	Annette Schmidt
Date of birth:	5th March 1974
Birth place:	Bergisch Gladbach
Place of residence:	Trotzenburger Weg 8 51465 Bergisch Gladbach
Marital status:	single
School education:	1980-1984 Gemeinschafts Grundschule Moitzfeld
	1984-1990 Städtische Realschule Herkenrath
	1990-1993 Städtisches Gymnasium Herkenrath 1993 School-leaving exam 1993-2000 Study of biology (diploma)
	Theme of diploma work: Angiogenesis and Vasculogenesis under influence of Endostatin.
	Since July 2000 PhD
	From January 2001 till December 2002 stipendium by Köln Fortune. Since January 2003 scientific employee of Institute I for Anatomy, University of Cologne

Cologne, June the 24th 2003

Berichterstatter: Prof. Dr. med. K. Addicks

Prof. Dr. rer. nat. S. Roth

Tag der mündlichen Prüfung: 03. November 2003