

Lösliches Endoglin (sCD105)

Diagnostische Wertigkeit bei chronischen Lebererkrankungen und funktionelle Bedeutung für die Signaltransduction von transforming growth factor (TGF)- β

Mohamed Soliman Rizk

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growth factor (TGF)- β

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Mohamed Soliman Rizk

M. B. B. Ch.

M. Sc. in Clinical Biochemistry

aus

El-Menofyia (Ägypten)

Berichter: Herr Universitätsprofessor
Dr. med. Prof. h.c. (RCH) Axel M. Gressner

Herr Privatdozent
Dr. med. Andreas Geier

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Soluble endoglin (sCD105)
Diagnostic validity for chronic liver diseases and functional
relevance in signal transduction of transforming growth factor
(TGF)- β

Faculty of Medicine
AACHEN UNIVERISTY

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To approbate MD degree

Presented by

Mohamed Soliman Rizk

M. B. B. Ch.

M. Sc. in Clinical Biochemistry

From

El-Menofya (Egypt)

To
my parents,
my wife
and my children: Ahmed, Asmaa and Soliman

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List of abbreviations

AFB1, aflatoxin B1	EDTA, ethylene diamine tetraacetic acid
AFP, α -fetoprotein	EGFP, enhanced green fluorescent protein
ALK, Activin receptor like kinase	ELISA, enzyme-linked immunosorbent assay
AVM, arterial venous malformations	et al., et altera
α -SMA, alpha-smooth muscle actin	Et.Br, ethidium bromide
A, absorbance	f, female
Aa, amino acid(s)	FCS, fetal calf serum
ALT, alanine amino transferase	FGF, fibroblast growth factor
AMP, ampicillin	GFP, green fluorescent protein
AST, aspartate aminotransferase	h, hour(s)
AUC, area under the ROC curve	HCC, hepatocellular carcinoma
BSA, bovine serum albumin	HBSS, Hank's balanced salt solution
CD, Cluster of differentiation	HRP, horse radish peroxidase
CD105, endoglin	HHT, human hereditary telangiectasia
CD95 L, also called APO-1L/Fas-L	HSC, hepatic stellate cell(s)
Cf, Compare figure	IB, immunoblotting
CI, confidence interval	(Id), inhibitor of differentiation
CMV, cytomegalovirus	IL, interleukin
Col, collagen	Kbp (kb), kilo base pair(s)
CLD, chronic liver disease	kDa, kilo Daltons
dest. water, distilled water	l or 1L, one litre
dGTP, 2-deoxyguanosine 5'-triphosphate	LB, Luria-Bertani medium
DMEM, Dulbecco's modified Eagle medium	m, male
DMSO, dimethyl sulfoxide	MMP, matrix metalloproteinase
DNA, deoxyribonucleic acid	Mab, monoclonal antibody
dNTP, deoxyribonucleotide triphosphate	MAD1 or Mothers against decapentaplegic homolog 1 (Drosophila –protein)
DOC, sodium deoxycholate	MFB, myofibroblast-like cell(s)
Ds, double stranded	Min, minute(s)
DTT, dithiothreitol	MOPS, 3-[N-Morpholino] propanesulfonic acid
ECD, extracellular domain(s)	NF κ B, nuclear factor kappa B
ECM, extracellular matrix	
Eng, Endoglin	

NP 40, Nonidet P 40	sec, second (time)
NSS, normal saline solution	SCID, severe combined immunodeficient
Nt, nucleotide(s)	SDS, sodium dodecyl sulphate
OD, optical density	SSC, saline-sodium citrate solution
PAGE, polyacrylamide gel electrophoresis	sTβRII, soluble transforming growth factor-β
PAI, Plasminogen activator inhibitor	receptor type II
PBS, phosphate buffered saline	STE buffer, saline-Tris-EDTA buffer
PCR, polymerase chain reaction	sCD105, soluble endoglin
PDGF, platelet-derived growth factor	TBS, Tris buffered saline
PEG, polyethylene glycol	TBST, Tris buffered saline with Tween-20
PIC, protease inhibitors cocktail	⁹⁹ TcM, radiochemical technetium
PLC, primary liver cancer	TCA, trichloroacetic acid
PVP, polyvinyl pyrrolidone	TE, buffer Tris-EDTA buffer
PPabE1, purified polyclonal antibody E1	TGF-β, transforming growth factor-β
p53, phosphoprotein 53	TIMP, tissue inhibitor of metalloproteinase
rh, recombinant human	TY, tryptone-yeast extracted medium
RNA, ribonucleic acid	μ, micro
ROS, reactive oxygen species	V, volt(s)
Rpm, rotation cycle per minute	v, volume
RT, room temperature	v/v, volume per volume
RT-PCR, reverse transcriptase polymerase	w/v, weight per volume
chain reaction	Y, years
SC, Santa Cruz	

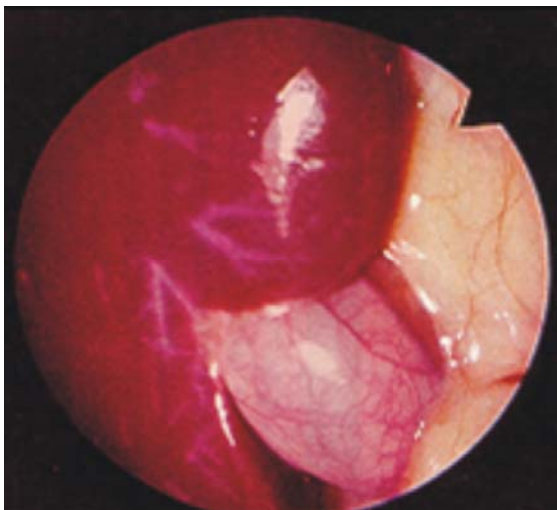
1 Introduction

1.1 Pathogenesis of liver fibrosis

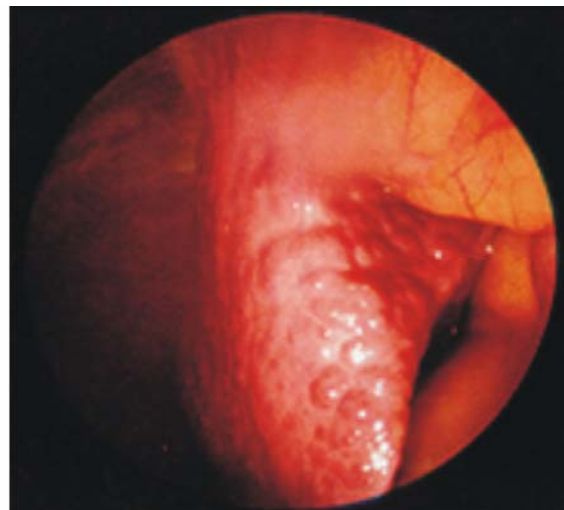
Hepatic fibrosis is the result of an unrestricted wound healing response due to repeated injury of the liver. Acute liver injury (e.g., viral hepatitis) leads to regenerating parenchymal cells replacing the necrotic or apoptotic cells. This process is associated with an inflammatory response and a limited deposition of extracellular matrix (ECM). If the process of hepatic injury persists, then eventually the liver regeneration fails, and hepatocytes are substituted with abundant ECM, including fibrillar collagen. The distribution of this fibrous material depends on the cause of the liver injury. In chronic viral hepatitis and chronic cholestatic disorders, the fibrous tissue is initially located around portal tracts (porto-portal fibrosis), in alcohol-induced liver disease, it locates pericentral and in perisinusoidal areas [Pinzani, 1999]. In schistosomiasis periportal fibrosis and granulomas are found [Silva *et al.*, 2006; Da Silva and Carrilho, 1992; Andrade and Peixoto, 1992]. Liver fibrosis is associated with major alterations in both the quantity and composition of the ECM [Benyon and Iredale, 2000]. In advanced stages, the liver contains approximately 6 times more ECM than normal, including collagens (I, III, and IV), fibronectin, undulin, elastin, laminin, hyaluronan, and proteoglycans. Accumulation of ECM results from both increased synthesis and decreased degradation [Arthur, 2000]. Decreased activity of ECM-removing MMPs is mainly due to an overexpression of their specific inhibitors (TIMPs).

The key pathologic features of cirrhosis reflect irreversible chronic injury to the hepatic parenchyma, resulting in excessive fibrosis and conversion of normal liver architecture into structurally abnormal regenerative nodules (Fig.1A-D).

A



B



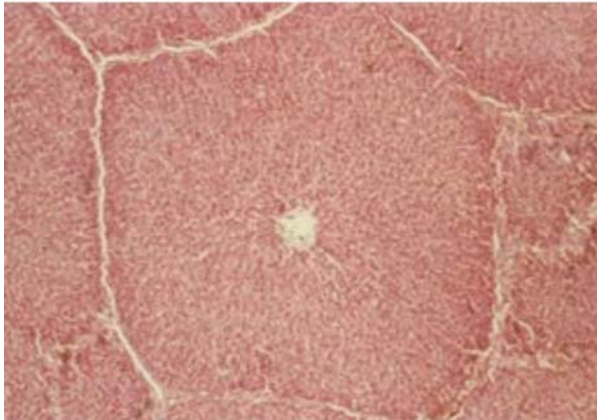
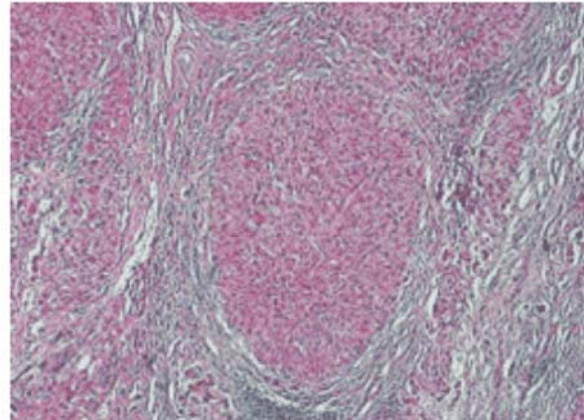
C**healthy liver****D****cirrhotic liver**

Figure 1: Representation of healthy and cirrhotic liver. Depicted is an excised normal human liver (A) and a liver which shows signs of cirrhosis (B) with regenerating nodules and contraction of condensed fibrous tissue later on. A focus on a liver lobule of the normal liver (C) and under the pathologic condition of cirrhosis (D). The massive ECM deposition is stained in grey, surrounding the lobule in the cirrhotic liver. The liver lobule area has decreased and the central vein is strongly compressed (C&D microscpic bild were taken from the website: <http://www.gastroenterologe.de>).

This process is the common, irreversible, and final pathway for a variety of liver diseases. The excessive deposition of ECM components in the space of Disse has an impact on the exchange capacity between sinusoidal lumen and the liver parenchyma due to the loss of fenestration of the sinusoidal endothelial cells. Because of the higher mechanical resistance of the sinusoids the blood pressure is increased leading to portal hypertension [Rockey, 2001] (Fig. 2).

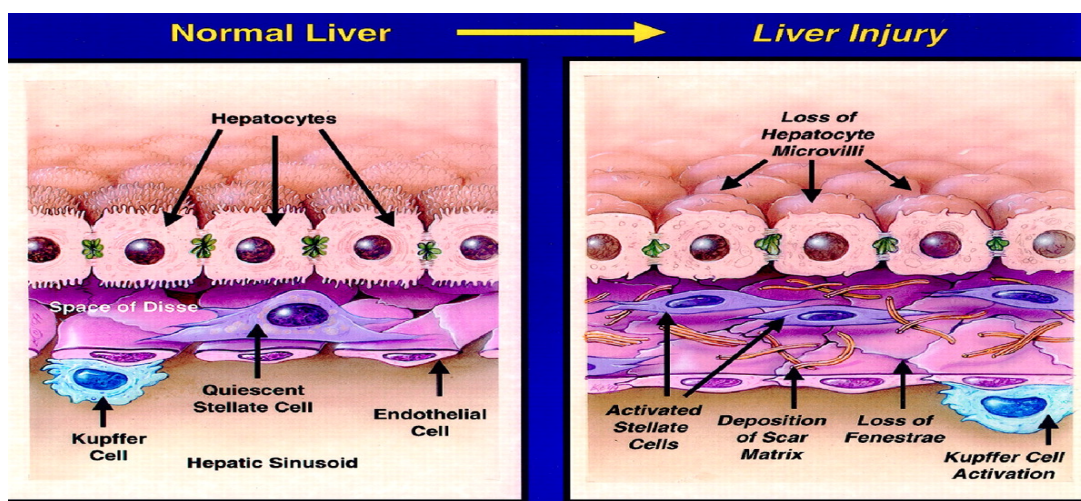


Figure 2: Cellular changes in the hepatic sinusoidal compartment in the course of fibrosis. Following chronic liver injury, inflammatory lymphocytes infiltrate the hepatic parenchyma. Hepatocytes lose their microvilli and some hepatocytes undergo apoptosis. Kupffer cells are activated, releasing fibrogenic mediators. As one of the main events, HSC undergo a dramatic phenotypical activation resulting in the “transdifferentiation” to a myofibroblast-like cell (MFB), showing an increased proliferation rate and secreting

large amounts of extracellular matrix proteins. Sinusoidal endothelial cells lose their fenestrations and the tonic contraction of HSC causes increased resistance to blood flow in the hepatic sinusoid (taken from Friedman 2000).

Unfortunately, cirrhosis is associated with high morbidity and mortality affecting many persons during the most productive years of life. The spectrum of characteristic clinical manifestations and potentially life-threatening consequences that can result from cirrhosis includes hepatic encephalopathy, bleeding from esophageal and gastric varices, ascites, hepatorenal syndrome, hepatocellular carcinoma (HCC), and liver failure [Arthur, 2001]. In most countries, HCC comprises 85–90% of primary liver cancer (PLC) and so the terms are often used interchangeably. PLC is the fifth most common cancer in the world [Parkin, *et al.*, 1999], and the fourth most common cause of cancer mortality [Pisani, 1999]. HCC rates have an extremely wide geographic variation, such that 80% of the cases arise in developing countries, particularly those of southeast Asia and sub-Saharan Africa. Even within a confined geographic area, certain ethnic groups have higher PLC rates than others. In these high-rate populations, chronic infection with hepatitis B virus (HBV), and contamination of foodstuffs with aflatoxin B1 (AFB1) are recognized as major risk factors [Jin *et al.*, 1999]. In developed world the most likely causes are alcohol ingestion and increasingly hepatitis C infection. In this chronic inflammatory liver disease which has the potential to promote the development of HCC, the pathophysiologic process most commonly associated with is cirrhosis, found in up to 80%. It is not known whether cirrhosis itself or the mechanism underlying cirrhosis is responsible for malignant transformation of hepatocytes [Flicker *et al.*, 1997].

1.2 Resolution of liver fibrosis

During recovery from acute human and experimental liver injury, the number of activated HSC decreases as tissue integrity is restored [Iredale 1998]. A pivotal unresolved issue is whether activated HSC can revert (back) to a quiescent state. Regression of activated HSC, clearance of MFB and a diminished availability of soluble, activating cytokines leads to the reconstitution of the normal subendothelial ECM. When HSC are grown on a basement membrane substratum they remain quiescent [Olaso *et al.*, 2001]. The other potential fate of activated HSC is apoptosis. HSC apoptosis associated with reduced TIMP-1 expression has been documented during the recovery phase of experimentally induced liver injury [Iredale, 1998]. HSC also undergo apoptosis during spontaneous activation, paralleled by increased expression of CD95L (Fas ligand), Bcl-2 and p53. HSC are central to the whole process of

liver tissue injury, repair, fibrogenesis, ECM remodeling and resolution [Gressner, 1998]. The process describing this excessive ECM deposition is called fibrogenesis and occurs in the most types of chronic liver diseases. Although fibrosis results from a wide spectrum of insults to the liver, the underlying basis for the excessive overproduction of ECM is common and due to the activation of HSC.

1.3 Molecular basis of fibrogenesis

1.3.1 HSC activation

Insults of the liver regardless of the cause leads in a first step to the damage of hepatocytes constituting the liver parenchyma. The damage of the hepatocytes in turn leads to the release of signals triggering an inflammatory response, which are accompanied by signals released by liver resident Kupffer cells and infiltrating inflammatory cells. The release of a diverse set of cytokines causes finally the activation of hepatic stellate cells (HSC). Activated HSC “transdifferentiate” and acquire a myofibroblast-like phenotype (MFB), which is characterized by an excessive production of extracellular matrix (ECM) proteins [Gabele *et al.*, 2003; Gressner and Weiskirchen, 2006]. HSC reside in the space of Disse at the interface between the fenestrated sinusoidal endothelium and the liver parenchyma, and they are the major storage sites of vitamin A. HSC have been considered as immunomodulatory cells [Sato *et al.*, 2003], as well as mediating neurologic function. Following chronic injury, HSC activate and transdifferentiate into MFB, acquiring contractile, proinflammatory, and fibrogenic properties [Milani *et al.*, 1990 ; Iredale, 2007] (Fig.3).

Activated HSC migrate and accumulate at the sites of tissue injury, where the repair by secreting large amounts of ECM is initiated. Interestingly, HSCs express a number of neuroendocrine markers (e.g., reelin, nestin, neurotrophins, synaptophysin, and glial-fibrillary acidic protein) and bear receptors for neurotransmitters [Geerts, 2001; Sato *et al.*, 2003; Oben *et al.*, 2003].

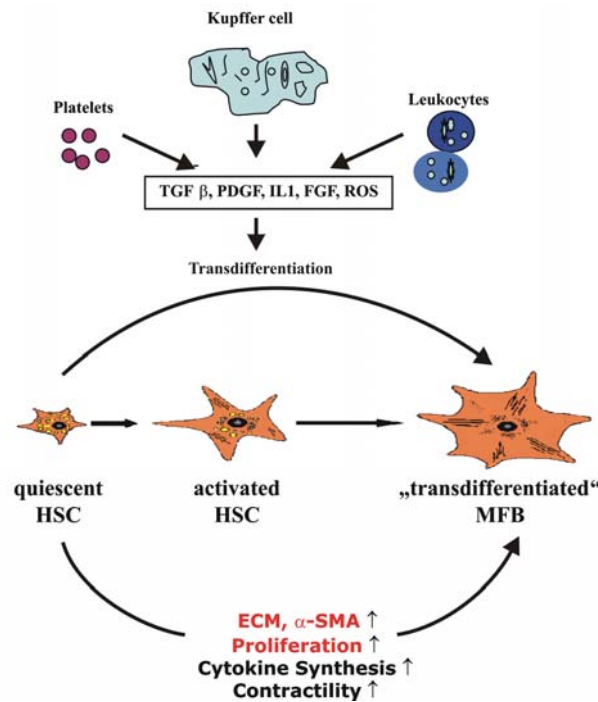


Figure 3: Characterization of the transdifferentiation process. Activation of quiescent HSC to activated HSC and finally to MFB is governed by the release of cytokines and reactive oxygen species (ROS) from leucocytes, Kupffer cells, platelets as well as hepatocytes (not shown). The activation process is accompanied by an increase in ECM synthesis and α -smooth muscle actin (α -SMA) expression. Proliferation takes place and cytokines like TGF- β 1 are actively synthesized. Moreover, MFB acquire contractility (modified from Gressner, 1998).

1.3.2 Fibrogenic signalling of transforming growth factor- β (TGF- β)

Cytokines regulating the inflammatory response to injury modulate hepatic fibrogenesis *in vivo* and *in vitro* [Schwabe *et al.*, 2003]. TGF- β is a member of transforming growth factor- β superfamily of proteins. The biologically active form of TGF- β is a dimer of 25 kDa in which the two subunits are linked by a disulfide-bridge [Roberts and Sporn, 1985]. TGF- β which acts as potent growth inhibitor for many different (preferentially epithelial) cell types, plays a key role in the control of parenchymal apoptosis, and stimulates the production of ECM components, during hepatic fibrogenesis. TGF- β 1 appears to be a key mediator in human fibrogenesis. It has a pivotal role in initiating, promoting, and progression of the transdifferentiation process from HSC into MFB. Concomitant with increased release/activity of TGF- β during fibrogenesis, activated HSC increase the production and deposition of collagen leading to progressive scarring and loss of organ function. Thus, overexpression of TGF- β in the liver induces severe liver fibrosis [Friedman, 2000]. Conditionally regulated expression of TGF- β further reveals that the induction of fibrogenesis is directly linked to the concentration of this ligand [Ueberham *et al.*, 2003]. Conversely, the blockade of TGF- β synthesis or signalling using different experimental strategies prevents ongoing liver fibrosis in various

animal models [George *et al.*, 1999; Qi *et al.*, 1999; Nakamura *et al.*, 2000; Arias *et al.*, 2003].

Signalling by TGF- β isoforms is initiated by binding to the type I activin-receptor-like kinase-5 (ALK-5) or ALK-1 and the type II serine/threonine kinase receptor the so called signaling receptors, which are essential for the TGF- β activity [Massague, 2000]. The TGF- β binding is further modulated by accessory receptors, i.e. betaglycan (T β RIII) and endoglin (CD105) which lack a cytoplasmic kinase domain. CD105 appears to interact not only with TGF- β but also with activin and bone morphogenetic proteins (BMP) in the presence of their respective type I and type II receptors [Barbara *et al.*, 1999; Lopez-Casillas *et al.*, 1993]. The ligand binding to the dimeric type II receptor leads to the recruitment of a type I receptor dimer into the complexes, in which the type II phosphorylates the type I receptor in the GS domain [rich in glycine and serine residues]. The activated type I receptor kinase propagates the signal inside the cell through the phosphorylation of receptor-regulated Smads (R-Smads). The specificity of R-Smad phosphorylation is dictated by the corresponding type I receptor. ALK-5 interacts and activates R-Smad2 and R-Smad3, whereas ALK-1 directs the signal to R-Smad1 and R-Smad5 [Massague, 2000]. Activated R-Smads form heteromeric complexes with the common Smad4. These complexes accumulate in the nucleus, where they control gene expression in a cell-type specific and ligand dose-dependent manner through interactions with transcription factors, coactivators and corepressors [Massague, 2000] (Fig. 4). In case of the accessory receptor CD105, the binding of TGF- β requires the presence the receptor II, because endoglin is not able to bind ligand by itself [Barbara *et al.*, 1999; Torsney *et al.*, 2002]. Upon association, the cytoplasmic domain of CD105 is phosphorylated by the type I and type II receptor resulting in increased phosphorylation of Smad2 but not Smad3. Since Smad2 can interact with a variety of transcription factors, coactivators, and repressors, phosphorylated Smad may act as an integrator of multiple signals to modulate gene transcription [Barbara *et al.*, 1999; Guerrero-Esteo *et al.*, 2002]. Inhibitory Smads (i.e I-Smads; Smad6, Smad7) can associate with activated TGF- β type I receptors and inhibit R-Smad phosphorylation. In primary cultured HSC undergoing transdifferentiation the effects of TGF- β on intracellular signalling is dependent on the cellular activation state. TGF- β causes phosphorylation and nuclear translocation of Smad2 primarily in quiescent HSC and Smad3 in transdifferentiated HSC [Liu *et al.*, 2003]. Furthermore, this cytokine is able to inhibit proliferation of quiescent but not transdifferentiated HSC or MFB [Liu *et al.*, 2003; Dooley *et al.*, 2000]. In addition, phosphorylated nuclear Smad2 but not Smad3 is present

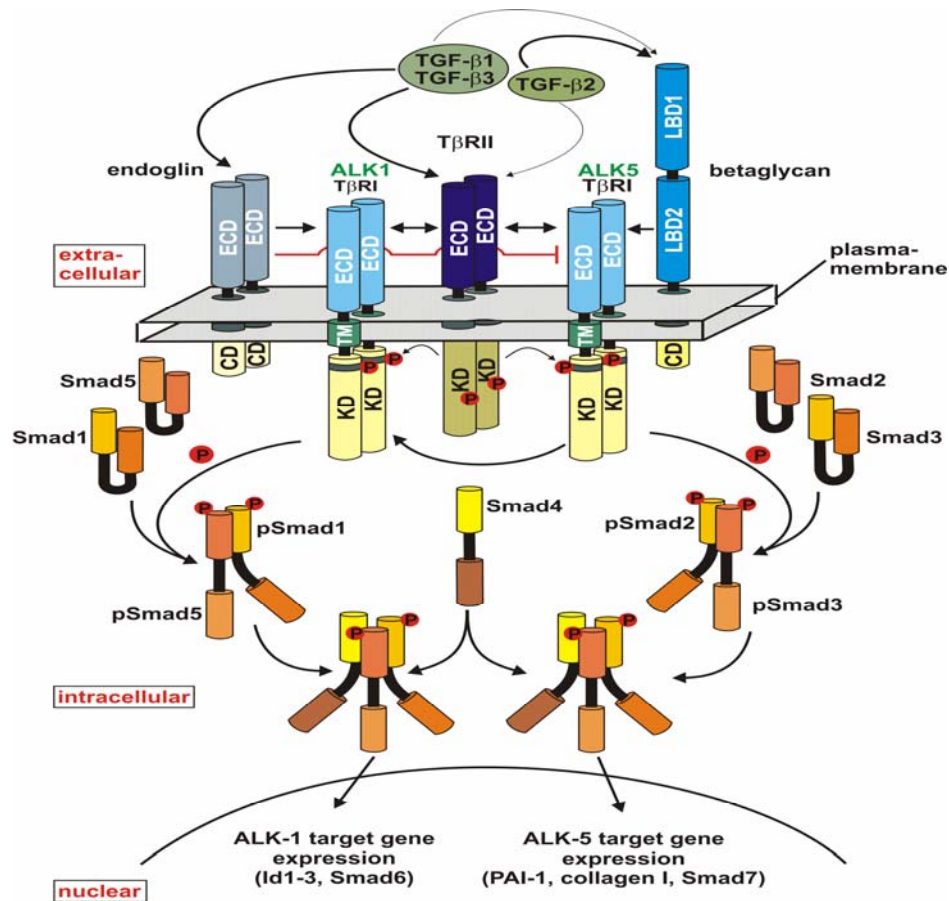


Figure 4: TGF- β signaling cascade in endothelial cells. Depicted is the signaling pathway in response to TGF- β in endothelial cells because this the best analyzed system. Endoglin has been shown to promote ALK-1 signaling (left) and to inhibit ALK-5 (right) signaling. Activation of these two receptors leads to different transcriptional responses. ALK-1 causes induction of Id1 and Smad6 whereas ALK-5 induces PAI-1, collagen I and Smad7. In HSC it was shown that both of these signaling pathways are active, too. The function of endoglin in HSC is currently under investigation. KD; Kinase Domain (For a detailed discussion of the signaling pathways see text.)

constitutively and TGF- β independent in activated HSC [Liu *et al.*, 2003], suggesting that the two R-Smads have distinct roles in the process of cellular activation and transdifferentiation.

A more recent study demonstrated that the overexpression of Smad3 was sufficient to exhibit increased deposition of fibronectin and type 1 collagen and increased α -SMA organization in stress fibers [Uemura *et al.*, 2005]. These characteristics were not noticed when Smad2 was overexpressed supporting the notion that Smad3 plays the more important role in transmitting the morphological and functional maturation of activated HSC and that Smad3 is a direct mediator of matrix production in activated HSC. Interestingly, MFB which produce high amounts of matrix are insensitive towards TGF- β and devoid of phosphorylated Smad3 [Dooley *et al.*, 2001]. Hence, given the prominent role of Smad3 in mediating the

pathobiology of fibrotic diseases, inhibition of Smad3 signalling could be a prime target for intervention in fibrotic conditions [Uemura *et al.*, 2005; Schnabl *et al.*, 2001].

1.3.3. Further cytokines involved in hepatic fibrosis

Beside TGF- β 1, several other members of the TGF- β -superfamily have been shown to be involved in the process of fibrosis. Platelet-derived growth factor (PDGF) is the most potent mitogen for HSC, mainly produced by Kupffer cells and is up-regulated in the fibrotic liver [Pinzani *et al.*, 1989]. PDGF inhibition attenuates experimental liver fibrogenesis [Borkham-Kamphorst *et al.*, 2004]. Adipokines, which are cytokines mainly derived from the adipose tissue are also involved in the regulation liver fibrogenesis. Leptin is required for HSC activation and fibrosis development [Marra, 2007; Ikejima *et al.*, 2002; Saxena *et al.*, 2004]. In contrast, adiponectin markedly inhibits liver fibrogenesis in vitro and in vivo [Kamada *et al.*, 2003]. The actions of these cytokines may explain why obesity influences fibrosis development in patients with chronic hepatitis C [Ortiz *et al.*, 2002]. Angiotensin II is the effector peptide of the renin-angiotensin system, which is a major regulator of arterial pressure homeostasis in humans. Key components of this system are locally expressed in chronically injured livers, and activated HSC de novo generate angiotensin II [Paizis *et al.*, 2002; Bataller *et al.*, 2003a]. Importantly, pharmacological and/or genetic ablation of the renin-angiotensin system markedly attenuates experimental liver fibrosis [Kanno *et al.*, 2003; Jonsson *et al.*, 2001; Paizis *et al.*, 2001; Ramalho *et al.*, 2002; Wei *et al.*, 2000]. Angiotensin II induces hepatic inflammation and stimulates an array of fibrogenic actions in activated HSC, including cell proliferation, cell migration, secretion of proinflammatory cytokines, and collagen synthesis [Bataller *et al.*, 2000; Bataller *et al.*, 2003b; Bataller *et al.*, 2003c]. In addition it is critical to note, that the endoglin expression in endothelial cells and in cardiac fibroblasts is upregulated by angiotensin II [Chen *et al.*, 2004].

1.4 The auxiliary TGF- β receptor endoglin (CD105)

1.4.1 Structure of the transmembrane receptor endoglin

CD105 is expressed on the cell surface as a 180 kDa homodimeric transmembranal protein, first isolated by immunoaffinity purification from HOON pre-B leukemic cell line using the monoclonal antibody 44G4 [Gougos and Letarte, 1988a]. CD105 was purified from human

placenta as a glycoprotein since it binds to *ricinus communis* agglutinin, wheat germ agglutinin [Gougos and Letarte, 1990]. It has a disulfide-linked extracellular region and a short, constitutively phosphorylated cytoplasmic tail [Yamashita *et al.*, 1994; Koleva *et al.*, 2006]. The extracellular domain binds TGF- β 1 and 3 isoforms with high affinity [Cheifetz *et al.*, 1992; Barbara *et al.*, 1999]. There have been two endoglin splice variants (S and L) identified. The predominantly expressed L-form consists of an extracellular domain of 561 amino acid (aa) residues, a single transmembrane domain of 25 aa and a short intracellular domain of 47 aa, while the S-endoglin comprises a 14 aa cytoplasmic region [Bellon *et al.*, 1993] (Fig 5). The S-form is expressed in a variety of mouse tissues and has in contrast to the L-form an antiangiogenic function [Perez-Gomez *et al.*, 2005]. Whereas the extracellular ligand binding domain of endoglin shows only low homology to betaglycan, the cytoplasmic domain of endoglin is 70% homologous to betaglycan, leading to the interaction with common binding partners like β -arrestin 2 by this domain [Cheifetz *et al.*, 1992; Chen *et al.*, 2003; Meurer *et al.*, 2005; Lee and Blobel, 2007]. Endoglin and betaglycan differ widely in their cellular distribution and functionally they appear to have opposing effects [Letamendia *et al.*, 1998]. The human endoglin sequence contains an RGD tripeptide, which is missing in the mouse and rat orthologues [Ge and Butcher, 1994; Meurer *et al.*, 2005]. The presence of RGD, a key recognition structure in cellular adhesion, suggests a critical role for endoglin in the binding of endothelial cells to integrins and/or other RGD receptors [Gougos and Letarte, 1990].

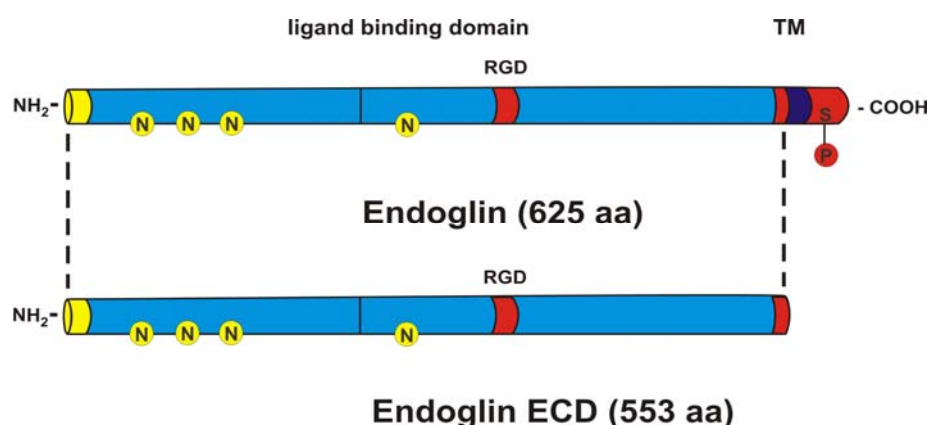


Figure 5: Schematic representation of the full length- and soluble endoglin structure. The human full length endoglin receptor comprises 625 amino acids (aa) including a extracellular ligand binding domain (blue), a transmembranal domain (purple) and an intracellular domain (red). In the extracellular domain of the human ortholog there is a RGD tripeptide located, which is not present in the rat and the mouse endoglin. Putative N-glycosylation sites are indicated by yellow circles. In the C-terminal domain a serine is indicated which is phosphorylated by the TGF- β receptor II. The soluble endoglin comprises only the extracellular domain and is shedded by a specific yet unknown protease in a membrane proximal part. It consists of 553 aa.

The most conserved regions of CD105 are the transmembrane and cytoplasmic domain, with 95% identity between human and murine forms, which strongly suggests that the cytoplasmic domain of CD105 interacts with other intracellular proteins and is likely to be involved in the transduction of signals associated with the TGF- β receptor system [Ge and Butcher, 1994; St.-Jacques *et al.*, 1994]. In addition to these genomically encoded variants of endoglin it has been detected a soluble form of endoglin with a high diagnostic potential in preeclampsia [Lopez-Novoa, 2007] (Fig. 5). The soluble endoglin is released from the cells by posttranslational modification of the membrane receptor by proteolytical cleavage of the extracellular domain [Llorca *et al.*, 2007] facilitated by a yet unknown protease.

1.4.2 Expression of endoglin

CD105 was discovered by Letarte's group during staining of a variety of human tissue sections (lymph node, tonsil, spleen, thymus, kidney, lung, and liver) using mab 44G4, which was produced against a human pre-B leukemic cell line. The antibody was strongly reactive with vascular endothelium [Gougos and Letarte, 1988a; Gougos and Letarte, 1988b] while the other cell types observed in these tissues were unreactive. Cells from some patients with non-T cell acute lymphoblastic leukaemia reacted weakly with this antibody, most of T cell acute lymphocytic leukaemia were negative [Gougos and Letarte, 1988a]. In contrast to HIVEC (Human Iliac Vein Endothelial Cells), murine platelets do not express endoglin. In addition, murine platelets do not express betaglycan as revealed by TGF- β 1 crosslinking studies [Hoying *et al.*, 1999]. Endoglin is expressed in stromal cells *in vivo* [St.-Jacques *et al.*, 1994; Balza *et al.*, 2001], in syncytiotrophoblasts [Kruse *et al.*, 1999], developing bone marrow [Rokhlin *et al.*, 1995; Barry *et al.*, 1999]. Moreover, expression of endoglin was detected in basal epidermal keratinocytes [Quintanilla *et al.*, 2003], in monocytes/macrophages from different tissue compartments and at different stages of differentiation [Lastres *et al.*, 1992], vascular smooth muscle cells [Adam *et al.*, 1998] and human as well as rat mesangial cells [Rodriguez-Barbero *et al.*, 2001]. In the liver, expression of endoglin is confined to HSC, Kupffer cells and sinusoidal endothelial cells, but is not expressed in hepatocytes [Meurer *et al.*, 2005, and manuscript in preparation].

1.4.3 Regulation of endoglin expression

Hypoxia is a common feature of ischemic tissues and tumors. It has been shown that hypoxia is a potent stimulator of CD105 gene expression in vascular endothelial cells and this effect is

potentiated in the presence of TGF- β 1 [Sanchez-Elsner *et al.*, 2002]. It was found that the CD105 promoter is 2.6 kb in length but does not contain TATA or CAAT transcription initiation boxes. However, it has two GC-rich regions, consensus motifs for Sp1, ETS, GATA, Ap-2, NF κ B, and Mad, as well as TGF- β response elements [Rius *et al.*, 1998; Botella *et al.*, 2001]. The basal level of transcription appears to require ETS and Sp1 sites, but the relative restriction of expression, to endothelial cells, appears to involve multiple regulatory regions [Graulich *et al.*, 1999]. CD105 is up-regulated by TGF- β requiring a critical Sp1 site upstream of SBE sites binding Smads 3 and/or Smads4 which are activated by TGF- β signalling [Botella *et al.*, 2001]. As mentioned before, endoglin expression is also upregulated by angiotensin II [Chen *et al.*, 2004]

1.4.4 Endoglin function and its association with vascular dysfunction

1.4.4.1 Endoglin function in TGF- β signaling

Most of the knowledge about the physiological role of endoglin has been derived from the human disease hereditary haemorrhagic telangiectasia (HHT). The clinical symptoms of HHT include bleeding from the nose, mouth, tongue, gastrointestinal tract, lungs, skin, and, less frequently, other organs. Other symptoms of HHT include shortness of breath, fatigue, strokes, transient ischemic attacks (TIAs), brain abscesses, migraine headaches, and chronic anemia. Currently, it is estimated that liver involvement occurs in 8% to 31% of patients affected by HHT. Thirty percent to 50% of those remain asymptomatic. Thus, the true prevalence of the disease is unknown [Larson, 2003; Gallione *et al.*, 2004]. There are four diagnostic criteria [Larson, 2003]. If three or four are met, a patient has definite HHT, while the appearance of two gives a possible diagnosis:

1. Spontaneous recurrent epistaxis
2. Multiple telangiectasias on typical locations
3. Proven visceral arteriovenous malformation (AVM) in lung, liver and brain
4. First-degree family member with HHT

It has been recognized that HHT-1 patients suffer from haploinsufficiency of endoglin due to defects in the corresponding gene [McAllister *et al.*, 1994]. The finding that HHT-2 patients, having mutations in the ALK-1 gene [Johnson *et al.*, 1996], and display a similar phenotype compared to the HHT-1 patients, led to the hypothesis that both receptors act in the same signaling pathway. The molecular nature of these defects have been derived from the corresponding endoglin and ALK-1 deficient mouse models. The Eng-null mice die at mid gestation from defective angiogenesis and severe cardiovascular abnormalities, while the

heterozygous mice have normal life spans and are predisposed to develop HHT-1-like vascular abnormalities [Srinivasan *et al.*, 2003; Bourdeau *et al.*, 1999]. By analysis of these models it was demonstrated that endoglin is a component of the TGF- β signaling pathway [Fernandez *et al.*, 2006]. It was shown that endoglin promotes the ALK-1 pathway and inhibits the ALK-5/Smad3 dependent signaling in endothelial cells [Lebrin *et al.*, 2004]. This inhibitory function of endoglin on the ALK-5/Smad3 mediated responses to TGF- β have now been established in other systems, including fibroblasts and L₆E₉ cells as a model system for TGF- β signaling [Letamendia *et al.*, 1998; Chen *et al.*, 2004; Obreo *et al.*, 2004; Scherner *et al.*, 2007].

1.4.4.2 Endoglin function in angiogenesis

A study with an antisense approach has demonstrated that suppression of CD105 expression in human umbilical vein endothelial cells (HUVEC) resulted in marked inhibition of *in vitro* angiogenesis in the presence of TGF- β . Thereby, it was suggested that CD105 is a proangiogenic component in endothelial cells [Fernandez *et al.*, 2006]. Moreover, as mentioned above CD105 knock out mice exhibit multiple vascular and cardiac defects in the form of failure of primitive vascular plexus of the yolk sac to remodel into mature vessels, causing vascular channel dilation, rupture, and haemorrhage, failed endocardial cushion formation, pericardial edema, leading finally to death at an early embryonic stage [Bourdeau *et al.*, 1999; Fernandez *et al.*, 2006]. Owing to the observations that CD105 is strongly up-regulated in the endothelium of various tumour tissues compared with that in the normal tissues, it was proposed that endoglin may be associated with tumour angiogenesis [Burrows *et al.*, 1995; Takahashi *et al.*, 2001a]. The discrepancies in intensity of immunological tissue staining between different monoclonal antibodies (mabs) could be explained in several ways, such as the criteria for grading, the negative and positive control used, the specific epitopes recognised by the mabs, and the definition of normal tissue. In general, these mabs revealed an increased expression of CD105 in tumour endothelium than corresponding normal tissues [Kumar *et al.*, 1999; Fonsatti *et al.*, 2001].

1.4.4.3 Endoglin as a biomarker for clinical conditions

1.4.4.3.1 Endoglin and anti-angiogenic strategies

Due to these characteristics, CD105 represents an ideal target for antiangiogenic tumour therapies. However, it is difficult to test these using in vivo animal tumour models, because most anti-CD105 antibodies do not react with the vasculature in engrafts of human tumours, due to the fact that endothelial cells in the transplanted tumours are derived from the host's blood vessels. Seon's group has partially overcome this problem by developing a series of anti-CD105 antibodies that cross-react weakly with murine CD105 [Matsuno *et al.*, 1999; Tabata *et al.*, 1999; Takahashi *et al.*, 2001b]. Their studies have demonstrated long-lasting complete abrogation of human breast tumors in SCID mice using mab to CD105 conjugated to immunotoxins [Tabata *et al.*, 1999] and growth suppression of human solid tumors using a radiolabeled mab to CD105 [Seon *et al.*, 1997; Tabata *et al.*, 1999]. They have observed synergism between naked anti-CD105 antibodies and conventional chemotherapeutic schedules in a human skin/SCID mouse chimera model [Seon *et al.*, 1997]. By using ^{99}Tcm -labeled mab E9, they have shown specific localization of the labeled antibody in the tumour vasculature of the kidneys [Duff *et al.*, 2003] (Fig. 6).

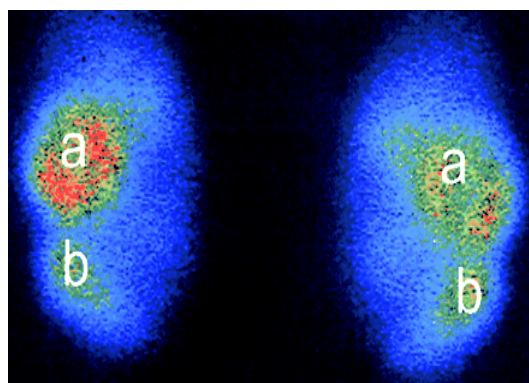


Figure 6: Tumour imaging using labeled mab to CD105. After perfusion of a kidney from a patient with renal cell carcinoma using ^{99}Tcm mab E9, immunoscintigraphy clearly shows two hot spots of radioactivity. Although a pre-surgery MRI scan found only one tumor mass (a), the histopathology report identified two tumors—one corresponding to “a” and another, a well-vascularized 0.75 cm diameter tumor “b” (b). These findings strongly indicate that ^{99}Tcm -labeled mab E9 should be useful for detection of occult metastatic disease that conventional imaging modalities are unable to locate [taken from Duff *et al.*, 2003].

1.4.4.3.2 CD105 as a marker for tumor imaging and tumor prognosis

1.4.4.3.2.1 Transmembrane endoglin

Early studies suggest that the use of radiolabeled antibodies against CD105 is a feasible approach for immunoscintigraphy of tumors. The overexpression of CD105 in the

proliferating endothelium and its location on the luminal surface make it an ideal target for tumor detection by an antibody and thereby selecting the patients who would have a benefit from the antiangiogenic therapy. Pharmacokinetics demonstrated that 97% of the injected dose of anti-CD105 was removed from the circulation within 15 min and the blood half-life was less than 1 min. Optimization of imaging an endothelial target requires that the background blood pool activity and ligand extravasation is minimized. This can be achieved by using small doses. Autoradiography and immunohistology showed that anti-CD105 accumulated at the tumor edge (Fig. 6), where the highest density of blood vessels was found [Bredow *et al.*, 2000].

1.4.4.3.2 Soluble circulating endoglin

Circulating sCD105 was marked elevated in plasma samples of breast cancer patients compared with healthy controls, and sCD105 was significantly increased in those patients who subsequently developed distant metastasis [Burrows *et al.*, 1995; Kumar *et al.*, 1999]. In blood samples from patients with colorectal, breast, lung and other cancers with metastasis, sCD105 had significantly elevated levels in comparison with metastasis-negative individuals and healthy controls. Chemotherapy significantly reduced the level of CD105 [Kumar *et al.*, 1999].

1.5 Diagnosis of liver fibrosis

In general hepatic fibrosis can be estimated by imaging techniques. Ultrasonography, computed tomography, and MRI can detect changes in the hepatic parenchyma due to moderate to severe fibrosis [Hirata *et al.*, 2001]. Due to its low cost, ultrasonography is an appealing technique. It is able to detect liver cirrhosis based on changes in liver echogenicity and nodularity as well as signs of portal hypertension. However, ultrasound is highly operator-dependent, and the presence of increased liver echogenicity does not reliably differentiate hepatic steatosis from fibrosis.

Liver biopsy is considered now the gold-standard method for the assessment of liver fibrosis [Afdhal and Nunes, 2004]. Histologic examination is useful in identifying the underlying cause of liver disease and assessing the necroinflammatory grade and the stage of fibrosis. Fibrosis stage is assessed by using scales such as Metavir (stages I–IV) and Ishak score (stages I–V) [Bedossa and Poynard, 1996; Ishak *et al.*, 1995]. Specific staining of ECM proteins (e.g., with Sirius red) can be used to quantify the degree of fibrosis, using computer-

guided morphometric analysis. Liver biopsy is an invasive procedure, with pain and major complications (as bleeding) that may occur in 40% and 0.5% of patients, respectively [Thampanitchawong and Piratvisuth, 1999]. Sampling error can occur, especially when small biopsies are analyzed. Histologic examination is prone to intra- and interobserver variation and does not predict disease progression [Regev *et al.*, 2002; Guido *et al.*, 2004]. Therefore, there is a need for reliable, simple, and non-invasive methods that include routine laboratory tests, such as platelet count, aminotransferase serum levels, prothrombin time, and serum levels of acute phase proteins for assessing liver fibrosis scores have been proposed [Imbert-Bismut *et al.*, 2001; Forns *et al.*, 2002]. Serum levels of proteins directly related to the hepatic fibrogenic process including N-terminal propeptide of type III collagen, hyaluronic acid, tissue inhibitor of metalloproteinase type 1 (TIMP-1) could be used as markers of liver fibrosis [Fontana and Lok, 2002]. On the other hand, Fibrosis-specific markers may reflect fibrogenesis in other organs (i.e., pancreatic fibrosis in alcoholic patients). Although these scores are useful in detecting advanced fibrosis (cirrhosis) in patients, as well as minimal or no fibrosis, they are not effective for differentiating intermediate grades of fibrosis. These tests give the physician an alternative before going straight to a liver biopsy, and are sensitive to changes associated with disease progression and/or response to therapy. They provide an effective means for more frequent monitoring as compared to a biopsy. Efforts are made to find the suitable new blood protein marker.

1.5.1 α -fetoprotein and chronic liver disease

α -fetoprotein (AFP) is a fetal protein produced during the embryonic period by the visceral endoderm of the gestational sac and, later on, by the liver. Its re-expression in patients with HCC has been described first over 40 years ago. Some studies have demonstrated that the presence of elevated levels of AFP in patients with liver cirrhosis is a risk factor for the development of HCC [Harada *et al.*, 1980; Arrieta *et al.*, 2006] thus suggesting that increased AFP-production in patients with liver cirrhosis might reflect, largely and abnormal or altered liver cell regeneration.

Although screening for HCC in patients with liver cirrhosis includes assessing AFP, the use of ultrasound and a variety of other radiological techniques and at present, assessment of AFP levels in conjunction with ultrasound is the “gold standard” for screening of high-risk patients [Pateron *et al.*, 1994]. It was found that AFP is produced by only 40%-75% of HCCs and AFP serum concentrations are also elevated during pregnancy, in germ-cell tumours, in diseases

such as ataxia telangiectasia, hereditary tyrosinaemia and teratocarcinoma. Elevation in benign hepatic diseases, such as acute or chronic hepatitis, is usually transient [Zhou *et al.*, 2006; Tong *et al.*, 2001]. Therefore, the clinical application of HCC diagnosis by AFP assessment is limited, especially for the early cancer stages [Sherman, 2001; Yuen and Lai, 2005]. The reported diagnostic value of AFP for detecting HCC varies widely (sensitivity of 39-97%, specificity of 76-95%, and a positive predictive value of 9-32%) when used for screening of high-risk populations [Sherman *et al.*, 1995; Gebo *et al.*, 2002]. Thus, early identification of HCC in high-risk patients who are at a potentially curable stage is still an unresolved clinical problem.

1.6 Aim of the study

It is known that liver cirrhosis is considered as a premalignant state for developing a HCC, since about 80% of HCC is associated with liver cirrhosis. Identification of HCC in high-risk patients is still an unresolved clinical problem. Therefore the aim of this study was focused on two topics :

1. To analyse the diagnostic potential of soluble endoglin in serum of patients with liver cirrhosis at a high-risk to develop a hepatocellular carcinoma. Therefore, the sCD105 should be measured in patients without disease, with fibrosis, cirrhosis, cirrhosis in combination with HCC or HCC as the sole pathologic event. Because of the low abundance of soluble endoglin, being present in nanomolar concentrations, the measurement will be performed with a commercially available ELISA for the detection of human endoglin.
2. To determine the functional relevance of the soluble endoglin in the serum, which is currently under debate. It should be clarified, if the soluble domain of endoglin indeed is able to influence TGF- β signalling. For this purpose the extracellular domain of rat endoglin should be cloned and heterologously overexpressed. If the secretion of this extracellular domain is accomplished, the respective construct will be used in TGF- β sensitive cells to evaluate the functional impact of soluble endoglin on Smad3 dependent TGF- β signaling.

2 Material and Methods

2.1 Material

2.1.1 Human serum samples

2.1.1.1 Patients

Blood samples were taken from 121 patients with chronic liver diseases [CLD]. With respect to the gender there were 79 males and 42 females. Patients were subdivided into liver fibrosis subgroup (19 patients); liver cirrhosis subgroup (57 patients); liver cirrhosis plus HCC subgroup (45 patients) and 9 patients with hepatocellular carcinoma (HCC) without suffering from liver cirrhosis. The patients were admitted to the Department of Medicine III and Gastroenterology at the University Hospitals in Aachen and Regensburg (Germany). The serum was separated from the samples, and was stored in aliquots at -80°C . Patients with liver fibrosis had persisting high aspartate-aminotransferase and alanine-aminotransferase levels for more than 6 months but were negative for markers of viral hepatitis. Patients were diagnosed using ultrasound-guided liver biopsy. Liver specimens were analysed in a blinded fashion, by an experienced pathologist according to the score proposed by Batts and Ludwig, 1995. The diagnosis of liver cirrhosis was based on clinical findings, regular laboratory and ultrasound analysis. The patients did not show any ultrasound and radiological signs of HCC. Computed tomography (CT) scans were performed in 35 patients with liver cirrhosis. Informed consent was obtained from the participants in the study.

2.1.1.2 Control group

The control group consisted of 70 blood donors from the Department of Transfusion Medicine at the University Hospital Aachen. The healthy volunteers had normal levels of aminotransferases, normal blood counts and negative markers for viral hepatitis and HIV.

Another control group consisted of 94 non-liver-diseased patients. The five most frequent diagnoses in this group were non-malignant, cardiovascular, pulmonary, gastropancreatic and bowel diseases. Such an in-hospital control population was selected, as recommended, to minimize bias [Schultz and Grimes, 2002a; 2002b].

2.1.2 Chemicals

Chemicals	Providers
Albumin (Cat N. 17-0442A) [as a standard protein for FPLC]	Amersham Pharmacia-Biotec, Uppsala, Sweden
Acetic acid (glacial)	Merck, Darmstadt, Germany
Aceton	Merck, Germany
Agarose	Sigma, Taufkirchen, Germany
Albumin (BSA)	Sigma, Taufkirchen, Germany
Ammonium acetate	Merck, Hohenbrunn, Germany
Ampicillin	Sigma, Taufkirchen, Germany
Bacto TM Agar	Becton & Dickinson, USA
Bacto TM Peptone	Becton & Dickinson, USA
Bacto TM Yeast Extract	Becton & Dickinson, USA
Bio-Rad protein assay	Bio-Rad laboratories, Hercules, CA, USA
Bicin	AppliChem, Germany
Bis-Tris	AppliChem, Germany
Blue Dextran 2000 (Cat N17-0441E)	Amersham Pharmacia-Biotec, Uppsala, Sweden
BM blue POD	Roche, Mannheim, Germany
Boric acid	Merck, Hohenbrunn, Germany
Bromphenol blue	Serva, Heidelberg, Germany
BSA	Sigma, Germany
Calcium chloride dehydrate	Merck, Hohenbrunn, Germany
Catalase (Cat N. 17-0441)	Amersham Pharmacia-Biotec, Uppsala, Sweden
Chlorobutanol	Fluka, Swiss
Chloroform J. T.	Baker, Deventer, The Netherlands
Collagen type I	Becton & Dickinson, USA
Collagenase	Roche, Germany
Complete Protease Inhibitor Cocktail	Roche, Germany
Coomassie Brilliant Blue	Serva, Heidelberg, Germany
Dimethylsulfoxide (DMSO)	Fluka, Swiss
Deoxycholate (DOC)	Merck, Darmstadt, Germany
di-Sodium hydrogen phosphate	Merck, Darmstadt, Germany
Dithiotreitol (DTT)	Carl Roth GmbH, Karlsruhe, Germany
EDTA	Merck, Darmstadt, Germany
Ethanol	Merck, Darmstadt, Germany
Ethidium bromide	Sigma, Taufkirchen, Germany
Ferritin (Cat N. 17-0441)	Amersham Pharmacia-Biotec, Uppsala, Sweden
Ficoll 400	Pharmacia, Bromma, Sweden
Fugene6 TM	Roche, Germany
Glucose	Sigma, Taufkirchen, Germany
Glycerol	Merck, Darmstadt, Germany
Glycine	Merck, Darmstadt, Germany
guanidine hydrochloride	Sigma, Taufkirchen, Germany
Guanidine thiocyanate	Sigma, Taufkirchen, Germany
Gel filtration standard (Nr.151-1901) (composed of Thyroglobulin; Ig G;Ovalalbumin; Myoglobulin;& Cynacoblamin(Vit B12)	Bio-Rad laboratories, Hercules, CA, USA

Hydrochloric acid 32%	Merck, Darmstadt, Germany
Isoamylalcohol	Merck, Germany
Isopropanol	Merck, Germany
Lithium chloride	Sigma, Taufkirchen, Germany
Lipofectamine	Invitrogen, USA
L-Tyrosine (Cat. N8371)	Merck, Germany
Magnesium chloride hexahydrate	Merck, Darmstadt, Germany
Magnesium sulfate heptahydrate	Merck, Darmstadt, Germany
Magnesium chloride	Merck, Darmstadt, Germany
Methanol	RWTH, Aachen, Germany
Milk powder (non fat)	Roth, Germany
MOPS	Sigma, Taufkirchen, Germany
2-Mercaptoethanol	Fluka Biochemika, Germany
NP-40	Roche, Germany
Nycodenz®	Nycomed Pharma AS, Oslo, Norway
Paraffin oil	Caesar & Loretz, Hilden, Germany
PBS Dulbecco without Ca ²⁺ /Mg ²⁺	Biochrom AG, Berlin, Germany
Phenol	Sigma, Germany
Phosphatase Inhibitor Cocktail 2	Fluka, Swiss
Protein-G Plus agarose	SantaCruz, USA
Polyethylenglycol	Sigma, Taufkirchen, Germany
Polyvinyl pyrrolidone	Sigma, Taufkirchen, Germany
Ponceau-S	Sigma, Taufkirchen, Germany
Potassium acetate	Merck, Darmstadt, Germany
Potassium chloride	Merck, Darmstadt, Germany
Potassiumdihydrogenphosphate	Merck, Germany
Dipotassiumhydrogenphosphate	Merck, Germany
Pronase E	Merck; Darmstadt, Germany
Roti-Phenol	Carl Roth GmbH & Co Karlsruhe, Germany
Rubidium chloride	Sigma, Taufkirchen, Germany
Sodium acetate	Merck, Darmstadt, Germany
Sodium azide	Merck, Germany
Sodium hydrogencarbonate	Merck, Darmstadt, Germany
Sodium chloride	Merck, Darmstadt, Germany
Sodium dihydrogen phosphate	Merck, Darmstadt, Germany
Sodium dodecyl sulfate (SDS)	Bio-Rad Laboratories, Hercules, CA, USA
Sodium hydroxide 50% solution	Merck, Darmstadt, Germany
Sodium hydroxide pellets	Merck, Darmstadt, Germany
Sulfuric acid	Merck, Darmstadt, Germany
Supersignal Chemiluminescent Substrate	Pierce, Rockford, IL, USA
Trichloroacetic acid (TCA)	Carl Roth GmbH & Co Karlsruhe, Germany
tri-Sodiumcitrate-dihydrate	Merck, Darmstadt, Germany
Triton X-100	Fluka Biochemika, Neu-Ulm, Germany
TRIZMA®	Base Sigma, Taufkirchen, Germany
Tryptone phosphate broth	DIFCO, USA
Tween 20	Merck, Hohenbrunn, Germany
Xylene	Merck, Darmstadt, Germany

2.1.3 Disposable material

Products	Providers
Autoclave bags	Roth, Germany
Centri-Sep columns for sequencing	Princeton Separations, Adelphia, NJ, USA
Cryo tubes	Nunc, Denmark
Culture plates: 6-well	Nunc, Germany
Cuvetts for nucleic acid	Carl Roth GmbH, Karlsruhe, Germany
Cuvetts for protein and bacteria (6774)	Sarstedt, Nuembrecht, Germany
Dialysis bags (Visking® 44101)	Serva, Heidelberg, Germany
Dialysis clips	Spectrum Medical Industries.USA
ELISA 96-well plates (C8 Maxisorp 445101)	Nunc, Wiesbaden, Germany
Falcon centrifuge tube (15,50 ml)	Greiner bio-one, Frickenhausen, Germany
Filter units	Sartorius AG, Göttingen, Germany
F16 BlackMaxisorp 96 Wells	Nunc, Denmark
Plastic wrap, SARAN WRAP	SARAN, USA
Gauze bandage	Fuhrmann Verbandstoffe, Deutschland
Gloves, semper med	Semperit technical products, Austria
GSA beaker	Beckman, Munich, Germany
Low protein binding tubes	Eppendorf, Germany
Microreaction tube (1.5 ml)	Sarstedt, Nuembrecht Germany
Microreaction tube (2 ml)	Sarstedt, Germany
Microvette® (Monovette for new born)	Sarstedt, Nuembrecht, Germany
Microwell black 96-wells plates (475515)	Nunc, Nidderau, Germany
Nunc Cryotube 1.8 ml (363401)	Nunc, Intermed, Denmark
Pasteur pipettes	Faust, Germany
Peleusball	Faust , Germany
Petri dishes (for bacterial culture, 821473)	Sarstedt, Nuembrecht, Germany
Petri dishes (for cell culture, 831802)	Sarstedt, Nuembrecht, Germany
Pipette 1 ml, 2 ml, 5 ml,10 ml, 25 ml	Falcon, Germany
Pipette Tipps, 1-20 µl, 200 µl, 1000 µl	Sarstedt, Nuembrecht, Germany
PP-tubes, 15 ml, 50 ml	Greiner, Germany
Polyallomer Centrifuge Tubes (328874)	Beckman, Munich, Germany
Protran® membrane (0.22 µm)	Schleicher & Schuell, Dassel, Germany
Scalpel	Feather Safety Razor Co, Japan
Whatman 3MM chromatography paper	Whatman, Kent, UK
µMACS beads ProteinG (130.071.101)	Miltenyi Biotec, Bergisch Gladbach, Germany
MACs multistand (130-42-602)	Miltenyi Biotec, Bergisch Gladbach, Germany
µMACS colums (130.042.701)	

2.1.4 Kits for assays

System	Provider
Bigdye Terminator Cycle Sequencing	Applied Biosystems, USA
Ready reaction kit	
Bradford Protein assay	Bio-Rad, USA
DC Protein Assay	Bio-Rad, USA
Human Endoglin ELISA	R&D Systems, USA

BCA Protein assay	Pierce
QIAquick Gel Extraction kit	Qiagen, Germany
RNeasy Mini Kit	Qiagen, Germany
Steady-Glo-Luciferase Assay System	Promega, USA
SuperScript Reverse Transcriptase AMV Kit	Roche, Germany
High Fidelity PCR System	Roche, Germany
NuPage Bis-Tris precast gels (10 %, 4-12 %)	Invitrogen, USA
NuPage [®] Tris-Acetate precast gels (3-8 %)	Invitrogen, USA
NuPage [®] LDS-sample buffer	Invitrogen, USA
NuPage [®] transfer buffer	Invitrogen, USA

2.1.5 Technical equipment

System	Provider
ABI 310 Genetic Analyzer	Applied Biosystems, USA
Agarosegel-Electrophoresissystem DNA Pocket Bloc-UV	Biozym, Germany
Beckmann Avanti [™] J-15 centrifuge	Beckman Coulter, USA
Beckman Optima L-70k Ultracentrifuge	Beckman Coulter, USA
Cell culture lamina flow Hera safe, HS 12 and HSP 12	Heraeus, Germany
Gel jet imager (computer device)	Lot-Oriel , Germany
INTAS digital graphic printer UP-D895	INTAS, Germany
INTAS GDS software	INTAS, Germany
Transilluminator CN-UV/WL Vilbert-Lourmat	MWG-Biotech
Ice facility	Ziegra, Germany
Heraeus Biofuge 15R table top centrifuge	Heraeus, Germany
Heraeus Biofuge primo table top centrifuge	Heraeus, Germany
Lumi-Imager [™] ECL-Documentationsystem	Boehringer, Germany
LumiAnalyst 3.0 software	Boehringer, Germany
Microbeta 1450 Jet Liquid Scintillation & Luminescence Counter	PerkinElmer, USA
Microscope Axiovert 135 M	Zeiss, Germany
Microskoce Leica DMLB	Leica, Germany
MilliQ water destillation system	Millipore, France
PCR-Automat Biometra T3 (Thermocycler)	Biometra, Germany
pH-Meter	WTW, Germany
Pipette 10 µl, 20 µl, 200 µl, 1000 µl	Eppendorf, Germany
Sartorius 1212MP scale	Sartorius, Germany
Sartorius 1364MP scale	Sartorius, Germany
Shaker Biometra WT16	Biometra, Germany
Speed Vac concentrator	Fa. Savant
Powersupply PAC 300	Bio-RAD, USA
Spectrophotometer Cary 50 Probe UV-Visible	Varian, USA
Thermomixer 5436	Eppendorf, Germany
Overhead tumbler	Heidolph, Germany
Victor [™] 1420 Multilabel Counter	Turku, Finland
Vortexer Vortex Genie2	Fisher Scientific, USA
XCell Blot Module	Invitrogen, USA
XCell SureLock [™] Mini-Cell	Invitrogen, USA
FPLC(Äkta Purifier)	Amersham
	Pharmacia Biotec, Sweden

2.1.6 Biochemical material

2.1.6.1 Enzymes

Restriction enzymes	Providers
<i>EcoR</i> I	Roche, Germany
<i>Not</i> I	Roche, Germany

Other enzymes	Providers
T4-DNA-Ligase	Roche, Germany
Expand high fidelity PCR system	Roche, Germany
Klenow enzyme	Roche, Germany
Lysozyme	Sigma, Germany
RNase A	Roche, Germany
Taq DNA-polymerase	Roche, Germany
Trypsin	Roche, Germany

Enzyme buffers	Providers
Restriction enzyme buffers:	
A-Buffer (10x)	Roche, Germany
B-Buffer (10x)	Roche, Germany
H-Buffer (10x)	Roche, Germany
L-Buffer (10x)	Roche, Germany
M-Buffer (10x)	Roche, Germany

2.1.6.2 Antibodies

Primary antibodies were detected with the corresponding secondary antibody conjugated to HRP. Blots were developed by using the Supersignal chemiluminescence detection system or alternatively by using the chromogen Fast Red stain.

2.1.6.2.1 Primary antibodies

antibody	species	provider	epitope	clonality
MAB1097	mouse	R&D systems	human CD105	monoclonal
MAB10972	mouse	R&D systems	human CD105	monoclonal
sc-6199	goat	SantaCruz	human Betaglycan	polyclonal
PPabE1	rabbit	Davids Biotechnology	rat CD105	polyclonal
sc-1060	goat	SantaCruz	human HSP70	polyclonal
A5441	mouse	Sigma	β -actin	monoclonal

2.1.6.2.2 Secondary antibodies

antibody	species	provider	epitope	conjugate
SC-2055	goat	SantaCruz	mouse	HRP
SC-2056	donkey	SantaCruz	goat	HRP
SC-2007	goat	SantaCruz	rabbit	HRP
BA-9200	goat	Vector Laboratories	mouse	Biotin
P 0397	Streptavidin	DAKO	Biotin	HRP

2.1.6.3 Cytokines

Cytokine	Provider
rhTGF-β1	R&D systems, USA

2.1.6.4 Protein Size Marker

Material	Provider
SeeBlue Plus2 Pre-stained Standard	Invitrogen, USA

2.1.7 Molecular biological material

2.1.7.1 Primers

Gene	Acc. No.	Primer	Provider	MWG Biotech AG, Ebersberg, Germany
rendoglin	AY562420	forward	SM012	5'-d(CTC CCG GGT GGA CAG C)-3'
rendoglin	AY562420	reverse	SM091	5'-d(GCG GCC GCT CAG CCA GAC AGG TCA GGA C)-3'
rendoglin	AY562420	Forward	SM010	5'-d(CAG GCA TCC AAC ACC ATA G)-3'
rendoglin	AY562420	Reverse	SM011	5'-d(CGA TGC TGT GGT TGG TAC)-3'
rendoglin	AY562420	Forward	SM014	5'-d(CCA AGG CTG CCA CTT GG)-3'
CMV Promoter	-	Forward	MA001	5'-d(AAT GTC GTA ACA ACT CCG CC)-3'
M13/pUC	-	Forward	For	5'-d(GTT TTC CCA GTC ACG AC)-3'
M13/pUC	-	Reverse	Rev	5'-d(AAC AGC TAT GAC CAT G)-3'

2.1.7.2 Vectors

Vector	reference
(CAGA) ₁₂ -MLP-Luc (Appendix 7.6)	Dennler <i>et al.</i> , 1998
pcDNA3.1 (Appendix 7.4)	Invitrogen, USA
pGEM [®] -T Easy (Appendix 7.3)	Promega, USA
pcDNA3.1(+)-endoglin (Appendix 7.1)	Meurer <i>et al.</i> , 2005

2.1.7.3 Molecular Size Markers

Marker	Provider
GeneRuler 100 bp DNA Ladder Plus	Fermentas, Germany
Lambda λ Hind III Ladder	NEB, USA

2.1.8 Cell culture

2.1.8.1 Prokaryotic cell culture

2.1.8.1.1 Media for prokaryotic cell culture

Medium	component
LB-Medium	1000 ml: 10 g Tryptone (Bacto), 5 g Yeast extract and 10 g NaCl dissolved in 950 ml H ₂ O and adjusted to a pH 7.4 using 1 N NaOH. The solution was adjusted to a volume of 1 l and autoclaved. For the agar medium, 15 g of agar was added, and proper antibiotics when required.
2 x TY-Medium	1000 ml : 16 g Tryptone (Bacto), 10 g Yeast extrakt and 5 g NaCl dissolved in 1 l H ₂ O and autoclaved.

2.1.8.1.2 Bacterial strain

Strain	genotype
JM105	<i>F'</i> traD36 lacI ^q Δ (lacZ)M15 proA ⁺ B ⁺ /thi rpsL(Str ^r endA sbcB15 sbCC hsdR4 (<i>r</i> ⁻ _{Km⁺}) Δ (lac-proAB)

2.1.8.2 Eukaryotic cell culture

2.1.8.2.1 Media for eukaryotic cell culture

Articles	Providers
DMEM	Bio-Whittaker Europe, Verviers, Belgium
DMSO	EGA-Chemie, Steinheim, Germany
FCS	Biochrom KG, Berlin, Germany
HBSS	PAA Laboratories, Austria
Hepatozome	Gibco, USA
L-Glutamin	PAA Laboratories, Austria
Penicillin/Streptomycin	PAA Laboratories, Austria
Trypsin solution (10x)	PAA Laboratories, Austria

Media	compositions
Freezing medium (2x)	20 ml: 11 ml DMEM, 5 ml FCS and 4 ml DMSO were mixed.
Growth medium	10 % FCS, 2 % L-Glut und 1 % Pen/Strep
Starvation medium	0.5 % FCS, 2 % L-Glut und 1% Pen/Strep
Stimulation medium	0.2 % FCS, 2% L-Glut und 1% Pen/Strep

2.1.8.2.2 Cells

Celltype	Origin
COS-7	SV-40 transformed Fibroblast cell line from kidney (monkey, African green) ATCC® Number: CRL-1651™ <i>Cercopithecus aethiops</i>
CHO	<i>Cricetulus griseus</i> (hamster, Chinese) ATCC® Number CCL-61
L ₆ E ₉	Myoblast cell line from thigh muscle of neonate rats
rHEP	primary rat hepatocytes

2.1.9 Buffers and Solutions

All buffers and solutions were prepared using autoclaved Millipore™-water.

Solution	composition
Ammonium acetate (10 M)	20 ml: 15.42 g Ammonium acetate were dissolved in 20 ml H ₂ O and sterilized by filtration.
Ampicillin (100 mg/ml)	50 ml: 5 g Ampicillin were dissolved in 50 ml H ₂ O, sterilized by filtration and stored in 1 ml aliquots at –20°C.
Bromophenol blue solution 0.5% (w/v)	10 ml: 50 mg Bromophenol blue were dissolved in 10 ml H ₂ O and stored at 4°C.
CaCl₂ (1 M)	100 ml: 14.7 g Ca Cl ₂ x 2 H ₂ O were dissolved in 100 ml H ₂ O.
Collagen solution (coating)	50 ml: 50 µl Acetic acid (100%) dissolved in 50 ml H ₂ O plus 1.25 ml of a 2 mg/ml concentrated Collagen-Stock solution.
Coomassie Brilliant Blue stain	500 ml: 2.5 g Coomassie Brilliant Blue R-250 were dissolved in the solution of 250 ml methanol, 50 ml acetic acid, and 200 ml distilled water.
Decoloration buffer (for Coomassie gel)	500 ml: 150 ml Methanol, 50 ml acetic acid and 300 ml dest H ₂ O were mixed.

Glucose (1 M)	100 ml: 19.8 g Glucose x H ₂ O were dissolved in 100 sterile H ₂ O, sterilized by filtration and stored at 4°C.
Glycerol	Glycerol was autoclaved before use.
HCl (1 M)	200 ml: 19.64 ml 32% HCL was dissolved in 200 ml H ₂ O.
Immunoprecipitation fixing buffer	(25% 2-Propanol, 10% Acetic acid) 1 l: 250 ml 2-Propanol and 100 ml acetic acid were added to 650 ml dest H ₂ O.
Immunoprecipitation washing buffer I	(0.05 M Tris-HCl, 0.15 M NaCl, 0.01 M EDTA, 1% Triton X-100, 0.1% SDS, 1% Deoxycholate) 1 l: 6.05 g Tris, 8.766 g NaCl, 3.72 g EDTA, 1 g SDS and 10 g deoxycholate were dissolved in 800 ml dest H ₂ O. A pH of 7.5 was established with HCl and the solution was filled up to 1 L with dest H ₂ O.
Immunoprecipitation washing buffer II	(0.02 M Tris-HCl, 0.5 M NaCl, 1% Triton X-100) 1 L: 2.42 g Tris, 29.22 g NaCl and 10 ml Triton X-100 were dissolved in 800 ml dest H ₂ O, a pH of 7.5 was established with HCL and the solution was filled up to 1 L with dest H ₂ O.
Potassium acetate (5 M)	100 ml: 49.07 g Potassium acetate were dissolved in 100 ml H ₂ O.
Lithium chloride (5 M)	100 ml: 21.2 g LiCL was dissolved in 100 ml H ₂ O.
Lysozyme solution (10 mg/ml)	5 ml: 50 mg Lysozyme were dissolved in 5 ml 0.01 M Tris-HCL (pH 8.0). The solution was applied immediately upon preparation.
MgCl₂ (1 M)	100 ml: 20.33 g MgCl ₂ x 6 H ₂ O were dissolved in 100 ml H ₂ O and autoclaved.
MgSO₄ (1 M)	100 ml: 24.6 g MgSO ₄ x 7 H ₂ O were dissolved in 100 ml H ₂ O and autoclaved.

MOPS (1 M)	100 ml: 20.93 g MOPS were dissolved in 60 ml H ₂ O. A pH of 7.0 was established with 5 M NaOH and the solution was filled up to 100 ml with sterile H ₂ O, further sterilized by filtration.
MOPS (20 X)	(1 M MOPS, 1 M TrisBase, 69.3 mM SDS und 20.5 mM EDTA) 1000 ml: 209.2 g MOPS, 121.12 g TrisBase, 20 g SDS und 6 g EDTA were dissolved in H ₂ O ad 1000 ml.
MnCl₂ (1 M)	20 ml: 3.96 g MnCl ₂ x 4 H ₂ O were dissolved in 20 ml H ₂ O, sterilized by filtration and stored at – 20°C.
Net-G buffer (immunoprecipitation)	(Tris-HCl 50 mM, NaCl 150 mM, NP40 0.1 % (v/v), EDTA 1 mM, 0.25% (v/v) Gelatine; pH 7.4) 1000 ml: 6.07 g Tris, 8.7 g NaCl, 0.4 g EDTA, 1 ml NP-40 und 1 ml Gelatine were dissolved in 1000 ml H ₂ O. A pH of 7.4 was established by HCl.
PBS (10 x)	1 L: 11.45 g Na ₂ HPO ₄ , 2 g KH ₂ PO ₄ , 2 g KCl and 80.0 g NaCl were dissolved in 800 ml H ₂ O, the pH was approximately 7.2 without adjusting and filled up with H ₂ O to 1 l. The solution was autoclaved or filtered sterile and stored at RT.
Ponceau-S staining	(0.2% (w/v) Ponceau-S, 3% Trichloroacetic acid) 500 ml: 1 g Ponceau-S and 15 g TCA were dissolved in 500 ml H ₂ O.
Potassium acetate (5 M)	500 ml: 245.38 g CH ₃ COOH was dissolved in 500 ml H ₂ O.
Protein lysis buffer	(0.05 M Tris-HCL, 0.25 M NaCl, 2% (v/v) NP40, 0.0025 M EDTA, 0.1% (w/v) SDS, 0.5% (w/v) deoxycholate) 1 l: 6.05 g Tris, 14.61 g NaCl and 0.93 g EDTA were dissolved in 800 ml dest H ₂ O, 20 ml NP40, 1 g SDS and 5 g deoxycholate were added. A pH of 8.0 was established with

	HCl and the solution was filled up to 1 L with dest H ₂ O.
Protein lysis buffer + proteases inhibitor cocktail (PIC)	1 tablet PIC Complete TM was dissolved in 50 ml protein lysis buffer. Aliquots of 5 ml were stored at –20°C, remaining stable for up to 3 month.
RIPA buffer	(0.02 M Tris, 0.15 M NaCl, 2% NP40, 0.1% SDS, 0.5% Sodium deoxycholate, pH 7.2) 1 l: 2.42 g Tris, 8.766 g NaCl, 1 g SDS and 5 g deoxycholate were dissolved in 800 ml dest H ₂ O. After that 20 ml NP40 were added. A pH of 7.2 was established with HCL and the solution filled up to 1 l with dest H ₂ O.
RNase A solution (10 mg/ml)	5 ml: 50 mg RNase A were dissolved in 5 ml sterile H ₂ O, cooked 10 min and stored in 1 ml aliquots at –20°C.
Rubidium chloride (4 M)	20 ml: 9.67 g RbCl were dissolved in 20 ml sterile H ₂ O and sterilized by filtration.
SDS 20% (Sodium dodecyl sulfate)	100 ml: 20 g SDS were dissolved in 80 ml sterile H ₂ O, filled up to 100 ml.
Sodium acetate (3 M) pH 6.0 (RNA precipitation)	100 ml: 24.6 g Sodium acetate were dissolved in 60 ml sterile H ₂ O. A pH of 6.0 was adjusted with acetic acid (glacial) and the solution was filled up to 100 ml with sterile H ₂ O.
Sodium acetate (0.3 M) pH 6.0 (RNA resuspension)	100 ml: 10 ml 3 M Sodium acetate were mixed with 90 ml sterile H ₂ O and stored at 4°C.
Sodium acetate (3 M) pH 4.5 (DNA sequencing)	100 ml: 24.6 g Sodium acetate were dissolved in 60 ml sterile H ₂ O. A pH of 4.5 was adjusted with acetic acid (glacial) and the solution was filled up to 100 ml with sterile H ₂ O.
Sodium acetate (3 M) pH 6.8 (DNA precipitation)	100 ml: 24.6 g Sodium acetate were dissolved in 60ml sterile H ₂ O. A pH of 6.8 was adjusted with acetic acid (glacial) and the solution was filled up to 100 ml with sterile H ₂ O.
Sodium chloride (5 M)	200 ml: 58.44 g of NaCl were dissolved in

	200 ml H ₂ O.
Sodium chloride (1.6 M)/PEG 20% (w/v) (Plasmid isolation)	100 ml: 20 g PEG and 9.35 g NaCl were dissolved in 200 ml sterile H ₂ O.
Sodium hydroxide (2 M)	100 ml: 8 g NaOH pellets were dissolved in 100 ml sterile H ₂ O and stored in a polyethylene bottle.
Sodium hydroxide (5 M)	100 ml: 20 g NaOH pellets were dissolved in 100 ml sterile H ₂ O and stored in a polyethylene bottle.
Sodium dihydrogen phosphate, monohydrate (1 M)	100 ml: 13.79 g NaH ₂ PO ₄ x H ₂ O were dissolved in 100 ml sterile H ₂ O and sterilized by filtration.
Di-sodium hydrogen phosphate, dihydrate (1 M)	100 ml: 17.79 g Na ₂ HPO ₄ x 2 H ₂ O were dissolved in 100 ml sterile H ₂ O and sterilized by filtration.
Sodium phosphate buffer (1 M)	1 M Sodium dihydrogen phosphate monohydrate and 1 M disodium hydrogen phosphate dihydrate were mixed under pH-control until a pH of 7.0 was obtained.
Solution I (Plasmid preparation)	(0.05 M Glucose; 0.01 M EDTA; 0.025 M Tris-HCl; pH 8.0) 100 ml: 5 ml 1 M Glucose, 2 ml 500 mM EDTA (pH 8.0), 2.5 ml Tris-HCl (pH 8.0) and 90.5 ml sterile H ₂ O were mixed and stored at 4°C.
Solution II (Plasmid preparation)	(1% SDS; 0.2 M NaOH) 100 ml: In 85 ml sterile H ₂ O were dissolved 10 ml 2 M NaOH and 5 ml 20% SDS (w/v). The solution was prepared and applied immediately.
Solution III (Plasmid preparation)	200 ml: 120 ml 5 M Potassium acetate, 23 ml acetic acid (glacial) and 57 ml sterile H ₂ O were mixed and stored at 4°C.
STE buffer	(0.1 M NaCl ; 0.001 M EDTA; 0.01 M Tris-HCl; pH 8.0) 1 L: 20 ml 5 M NaCl, 2 ml 0.5 M EDTA and 10 ml 1 M Tris-HCl pH 8.0 were dissolved in 968 ml sterile H ₂ O.

Stop-mix (5 x) (Gel electrophoresis)	(0.02 M EDTA; 30% (v/v) Glycerol; 0.5% SDS (w/v); 0.1% (w/v) Bromophenol blue) 5 ml: 0.2 ml 0.5 M EDTA (pH 8.0), 1 ml bromophenol blue solution, 1.7 ml glycerol and 0.125 ml 20% (w/v) SDS-solution were mixed and sterile H ₂ O was added up to 5 ml. 1: 200 g Trichloroacetic acid were dissolved in 1 l H ₂ O and stored at 4°C.
TBE (10 x)	(0.89 M Tris-HCl; 0.89 M Boric Acid; 20 mM EDTA) 1 l: 108 g TrisBase, 55 g boric acid and 7.44 g Tritiplex III were dissolved in 1 l H ₂ O and autoclaved.
TBS	(0.01 M Tris-HCl pH 7.6; 0.15 M NaCl) 1 l: 1.21 g Tris and 8.766 g NaCl were dissolved in 800 ml dest H ₂ O. A pH of 7.6 was adjusted with HCl and the solution filled up to 1 l with dest H ₂ O.
TBST	(0.01 M Tris-HCl pH 7.6, 0.15 M NaCl, 0.1% Tween 20) 1 l: 1.21 g Tris, 8.766 g NaCl and 1 ml Tween 20 were dissolved in 800 ml dest H ₂ O. A pH of 7.6 was adjusted with HCl and the solution filled up to 1 l with dest H ₂ O.
TE (pH 8.0)	(0.01 M Tris-HCl of pH 8.0 and 0.001 M EDTA) 100 ml: 1 ml 1 M Tris-HCl pH 8.0 and 0.2 ml 0.5 M EDTA pH 8.0 were dissolved in 98.8 ml sterile H ₂ O.
TFB I	(0.1 M RbCl; 0.05 M MnCl ₂ ; 0.01 M CaCl ₂ , 0.03 M Potassium Acetate pH 6.0; 15% (v/v) Glycerol) 100 ml: 2.5 ml 4 M RbCl, 5 ml 1 M MnCl ₂ , 1 ml 1 M CaCl ₂ , 3 ml 1 M potassium acetate pH 6.0, 15 ml Glycerol and 73.5 ml sterile H ₂ O were mixed. The solution was prepared and freshly used.
TFB II	(0.01 M MOPS pH 7.0; 0.075 M CaCl ₂ , 0.01 M NaCl, 15% (v/v) Glycerol) 100 ml: 1 ml 1 M MOPS pH 7.0, 7.5 ml 1 M CaCl ₂ , 1 ml 1 M NaCl,

	15 ml glycerine and 75.5 ml sterile H ₂ O were mixed and the solution used freshly.
Tris-HCl (1 M) pH 7.5; pH 8.0; pH 8.4	1 L: 121.1 g Tris was dissolved in 800 ml H ₂ O. The required pH was adjusted with HCl and H ₂ O was added up to 1 l and autoclaved.
Tris/NP40 buffer	(10 mM Tris-HCl, NP40 0.1 % (v/v); pH 7.5) 1000 ml: 1.2 g Tris und 1 ml NP40 were dissolved in 1000 ml H ₂ O. A pH of pH 7,5 was established with HCl.
Western blot blocking buffer	(5% non-fat milk in TBST (0.02 M Tris-HCl pH 7.5; 0.15 M NaCl) 100 ml: 5 g non-fat milk were dissolved with constant agitation in 90 ml TBST for 2 h and the solution filled up to 100 ml and filtered.
Western blot running buffer	NuPage Mops SDS running buffer (20 x): 0.5 l: 104.6 g MOPS(1M),;60.6 g Tris base(1M),69.3 g SDS (1mM), and 3 g EDTA (20.5 mM) were dissolved in distilled H ₂ O to a final volume of 500 ml.
Western blot transfer buffer	NuPage transfer buffer (20x): 0.5 L: 40.8 g Bicine (500 mM), 52.24 g Bis-Tris (500mM), 3 g EDTA (20.5 mM), and chlorobutanol 1 m M dissolved in distilled H ₂ O to have 500 ml. Methanol is added before preparation to be 10%.

2.2 Methods

2.2.1 Biochemical Methods

2.2.1.1 Protein isolation from cell monolayers

To prepare protein lysate from monolayer cell cultures, the culture medium was removed and cells were rinsed three times with ice-cold PBS. The ice-cold RIPA protein lysis buffer (denaturing but not reducing) supplemented with the protease inhibitor cocktail (1 tablet per 50 ml, Roche) was added, the adherent cells were detached from the culture dish with a cell

scraper and the cell-lysate transferred into a microfuge tube. The lysate was passed through a 21 G needle to shear the DNA, incubated on ice for 60 min, and centrifuged (10.000 rpm, 4 °C, 10 min). The supernatant containing the solubilized proteins was transferred to a new microfuge tube, conserved on ice for protein quantification and stored at –20 °C for further analysis.

2.2.1.2 Protein enrichment by TCA precipitation

To concentrate proteins from culture supernatants, proteins were precipitated using Trichloroacetate (TCA). Supernatants were first centrifuged (2000 rpm, 5 min, 4 °C). to remove floating cells. This was necessary to discriminate between secreted and cytosolic (not secreted) soluble endoglin. To 1 ml of the supernatant 100 µl TCA (72 %) and 150 µl DOC (1 mg/ml) were added, the sample was mixed by vortexing and incubated for 30 min at 4 °C. Thereafter precipitated proteins were pelleted by centrifugation (13.000 rpm, 15 min, 4 °C). The pellet was washed two times with 500 µl acetone and centrifuged as before. The acetone was aspirated and proteins were resolved in 30 µl RIPA buffer.

2.2.1.3 Protein measurement by using the Bio-Rad DC protein assay

The Bio-Rad protein assay is a dye-binding assay in which a differential color change of dye occurs in response to various concentrations of protein. This assay was compatible with the buffer formulations of the standard protein lysates and with luciferase samples in 1x luciferase buffer. As a standard a linear range of BSA was used. 5 µl of each standard or the samples were pipetted into the cavities of a microtiterplate. Thereafter, 25 µl of reagent A' and 200 µl of reagent B were added and the samples in the cavities were mixed by gentle agitation. The samples were incubated for 15 min at room temperature and the absorbance was measured at a wavelength of 750 nm in a Victor 1420 Multilabel Counter using the multicalc program.

Composition of reagent A'

1 ml reagent A

20 ml reagent S

2.2.1.4 Analysis of proteins by denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

The negatively charged detergent SDS (sodium dodecylsulfate) binds in a constant amount per unit weight of protein (1.4 g of SDS per g of protein). As a result, the proteins behave as if they had a uniform shape (because SDS denatures the proteins) and identical charge-to-mass ratio. Equal amounts of proteins were supplemented with 5 x LDS-Loading buffer and boiled 10 min at 75 °C. Samples were centrifuged and run on precasted 4-12 % Bis-Tris gradient gels using MOPS-running buffer. The samples were run at a constant voltage of 150 V until the protein standard was sufficiently resolved. After the run gels were processed for Coomassie Brilliant Blue staining or blotted onto nitrocellulose membrane for Western blot analysis.

The gel with the resolved proteins in the SDS-PAGE was removed from the pre-cast gelcassette and placed in NuPage-transfer buffer for 10 min to equilibrate. Whatman-papers and sponges used for blotting were also equilibrated in NuPage transfer buffer. The nitrocellulose membrane was activated in 30 % methanol prior to equilibration in NuPage transfer buffer. The transfer stack was prepared as depicted in Fig. 7. The transfer was performed in an XCell Blot Module at a constant voltage of 35 V for 2 – 3 hrs. The whole blotting apparatus was placed in an ice box to cool the facility during the transfer.

2.2.1.5 Detection of proteins by immunological methods

2.2.1.5.1 Western Blot

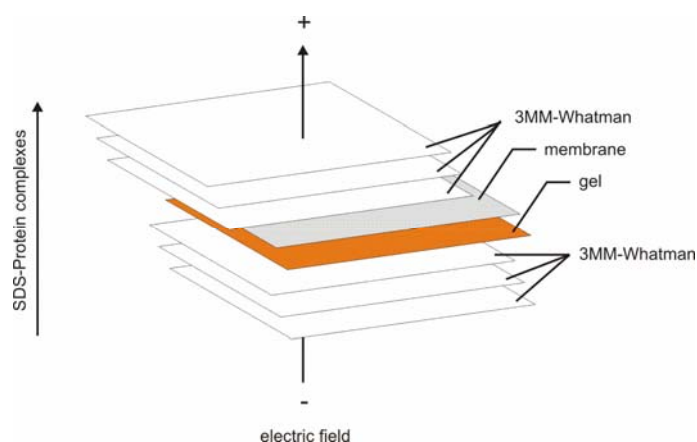


Figure 7: Preparation of a Western blot stack for the transfer of proteins from a polyacrylamide gel onto a nitrocellulose membrane. When an electric field is applied the proteins, which are soluble by dodecylsulfate and thereby achieve an overall negative charge, migrate to the anode.

After the transfer the membrane was soaked in TBST for 5 minutes. To monitor equal loading, the membrane was incubated in Ponceau S which binds reversible and unspecific to the transferred proteins. The membrane was scanned and Ponceau S was removed by washing in TBST. Thereafter the membrane (free binding sites) was blocked by incubation in 5 % non fat milk powder for 2 hrs at room temperature. Primary antibodies were applied with the indicated concentrations over night in 2.5 % at 4 °C. The unbound antibodies or antibodies that unspecifically bound to proteins were removed by 4 washing steps in TBST (each 5 min). The corresponding secondary antibody was diluted 1: 5000 and applied for 2 hrs at room temperature. Both, primary and secondary antibodies were diluted in 2.5 % non fat milk powder including 0.1 % (v/v) Tween 20. Again, the unspecific bound and unbound secondary antibodies were removed by washing in TBST (4 times, each 5 min) and TBS (2 times, each 5 min). The secondary antibody was detected by using the Supersignal West Dura Extended substrate. The membrane was pre-incubated for 5 min prior to analysis with the Lumi-ImagerTM ECL-Documentationsystem.

2.2.1.5.2 ELISA measurement

For the determination of serum CD105 concentrations in patient and control groups a quantitative sandwich enzyme linked immunoassay (ELISA, R&D Systems, Minneapolis, MN, USA) was carried out. For the measurement 50 µl of standards and 50 µl of the samples were diluted with 100 µl assay diluent and were pipetted into the wells of a microtiterplate which was pre-coated with a monoclonal antibody specific for human CD105. After incubation for 2 hrs and washing 4 times with washing buffer, 200 µl horseradish-peroxidase-conjugated monoclonal antibody specific for human CD105 was added to the wells, incubated 2 hrs and washed 4 times again with washing buffer. For the detection of the immunocomplexes 200 µl substrate solution (100 µl solution A + 100 µl solution B) was added to the wells. After an incubation time of 30 min, 50 µl stop solution was added. The colour intensity was measured as specified by the manufacturer using a (VictorTM 1420 Multilabel Counter, Turku, Finland).

AFP was determined using an automated AxSYM analyzer (AxSYM Abbott Laboratories, Abbott Park, IL, USA) by microparticle enzyme immunoassay (MEIA) with an alkaline-phosphatase-conjugated monoclonal anti-AFP antibody, according to the manufacturer's instructions.

2.2.1.5.3 Immunoturbidimetric measurement

This method was used for invitro quantitative determination of C-reactive protein (CRP).

The general principle for this method that antibodies coupled to latex microparticles react with antigen in the sample to form antigen/antibody complex. Following agglutination, this is measured turbidimetrically.

2.2.2 Molecular biological methods

2.2.2.1 Preparation of competent bacteria (Hanahan, 1985)

Under normal conditions most bacteria take up only limited amounts of foreign DNA. In order to increase transformation efficiency, bacteria are made “competent” by chemical treatment that enhances their ability to take up DNA. Competent cells are capable of incorporating exogenous DNA that is initially adsorbed in a double stranded form. All steps have been performed at 4 °C. An overnight culture of JM105 bacteria was inoculated 1:100 in 2 x TY-Medium (+1/100 VT 1 M KCl, +1/50 VT 1 M MgSO₄) and incubated at 37°C and 225 rpm until an absorbance of OD₆₀₀= 0.5, corresponding to the mid-exponential growth phase. The bacteria suspension was conserved on ice and transferred into sterile 50 ml PP tubes, followed by centrifugation (3000 rpm, 10 min, 4 °C). The bacterial pellet was resuspended gently in 50 ml of ice cold TFB I solution per initial 100 ml culture and incubated 10 min on ice. The suspension was centrifuged again (3000 rpm, 10 min, 4 °C), and the pellet was re-suspended with 4 ml/100 ml culture of TFB II solution. Aliquots of 200 µl were stored at –80 °C.

2.2.2.2 Transformation of competent bacteria

The uptake of the DNA by competent cells is stimulated by briefly raising the temperature to 42 °C. For this purpose, a 200 µl aliquot of competent bacteria was thawed on ice (approximately 15 min) and 10 µl of ligation mix was added and incubated for 30 min on ice. A heat shock was performed for 2 min at 42 °C, followed by 2 min on ice. After adding 600 µl of LB Medium, the bacterial suspension was incubated 1 h at 37 °C with 225 rpm agitation. Different dilutions of the bacterial suspension were plated on an agar plate including the appropriate selection marker and cultured overnight at 37°C.

2.2.2.3 Preparation of plasmid-DNA from bacteria

2.2.2.3.1 Plasmid small scale preparation (Birnboim and Doly, 1979)

The small-scale preparation (miniprep) is used to extract plasmid-DNA for analytic purposes in a quick manner which is especially useful to identify recombinant DNA. This rapid isolation technique is based on the alkaline lysis of cells. The solution is neutralized with a mixture of KOAc and SDS. SDS complexes proteins and material from the bacterial wall, to which the bacterial chromosomal-DNA is bound. The KDS-complexes are insoluble and could be precipitated by centrifugation, whereas the plasmid DNA is recovered from the supernatant. Supercoiled plasmid DNA thus can be easily purified by centrifugation and used directly. A single bacterial colony was inoculated into 5 ml LB medium containing the appropriate antibiotic in a 15-ml Greiner tube and incubated overnight at 37 °C under vigorous agitation. Then 1.5 ml of bacterial culture was transferred into a microfuge tube and centrifuged (13.000 rpm, 30 sec, RT). The medium was removed by aspiration and the bacterial pellet was resuspended in 100 µl solution I under vigorous vortexing. Thereafter, 200 µl of solution II was added, mixed (inverted not vortexed!) and incubated for 5 min on ice until the solution cleared up. The solution was neutralized with 150 µl solution III and mixed (inverted). After addition of 100 µl Chloroform, the solution was vortexed, followed by centrifugation (13.000 rpm, 10 min, RT). The supernatant was transferred to a fresh 1.5 ml reaction tube and the DNA was precipitated with 1 ml of ethanol for 15 min on ice, followed by centrifugation (13.000 rpm, 15 min, 4 °C). The pellet was washed with 150 µl ice cold 70 % ethanol and centrifuged again (13.000 rpm, 5 min, 4 °C). The supernatant was removed by aspiration. The DNA pellet was dried at 37 °C and dissolved in 20 µl TE.

2.2.2.3.2 Plasmid large scale preparation

The DNA produced by this protocol was used for preparative purposes (e.g., cloning, DNA-fragment isolation, sequencing). For large-scale preparation of plasmid DNA, 250 ml LB medium in a 1-liter flask containing the appropriate antibiotic was inoculated with 200 µl of overnight bacterial culture or 10 µl of glycerol stock, and incubated overnight at 37 °C under vigorous agitation. The bacterial suspension was transferred into a GSA-beaker and centrifuged (3000 rpm, 4°C, 15 min) using a JA13-rotor. The medium was aspirated and the bacterial pellet was re-suspended in 100 ml STE buffer and centrifuged again (3000 rpm, 4°C, 15 min). The resulting bacterial pellet was re-suspended in 10 ml of solution I, and 1 ml of a

of lysozyme solution (10 mg/ml) was added. The solution was incubated for 10 min on ice. After addition of 20 ml solution II the sample was incubated for 10 min at RT. Thereafter, 15 ml of solution III were added and the sample incubated for 10 min on ice, followed by centrifugation (6000 rpm, 15 min, 4°C). The supernatant was filtered into a fresh GSA-beaker through four layers of gauze bandage. 25 ml of isopropanol were added, the solution was mixed, and after an incubation of 10 min at RT centrifuged (6000 rpm, 15 min, 4°C). The resulting DNA/RNA-pellet was washed with 15 ml 70 % ethanol and centrifuged (6000 rpm, 5 min, 4°C). The DNA/RNA-pellet was dissolved in 3 ml of TE pH 8.0, 3 ml of 5 M LiCl were added, mixed and centrifuged (10.000 rpm, 10 min, 4°C). The pellet containing the RNA was discarded and the DNA in the supernatant was precipitated with 6 ml isopropanol by centrifugation (10.000 rpm, 10 min, 4°C) after in incubation of 10 min at RT. The DNA-pellet was washed again with 70 % ethanol, dissolved in 500 µl of TE pH 8.0 and incubated with 20 µl RNase A at 37°C for 30 min. Then 520 µl 1.6 M NaCl/13% PEG solution was added, mixed, and centrifuged (13000 rpm, 10 min, 4°C). The pellet was dissolved in 400 µl of TE pH 8.0. The proteins were removed by extraction with phenol, then with phenol/chloroform (1:1) and finally with chloroform. The aqueous phase (400 µl) was transferred to a fresh 1.5 ml reaction tube and the DNA precipitated by addition of 100 µl 10 M ammonium acetate and 1 ml of ethanol. The solution was incubated for 10 min at RT and centrifuged (10.000 rpm, 4°C, 10 min). The DNA-pellet was washed in 200 µl of 70 % ethanol. The DNA-pellet was dissolved in 500 µl of TE pH 8.0. The DNA was quantified by spectrophotometry and the quality of purified DNA was analyzed by gel electrophoresis.

2.2.2.4 Purification and analysis of nucleic acids

2.2.2.4.1 Phenol/Chloroform extraction

To remove protein and lipid contaminations from DNA-solutions a Phenol extraction was performed. For this purpose the Phenol solution was saturated with TE pH 8.0. DNA-solution were first extracted with an equal volume of Phenol, followed by a Phenol/Chloroform mixture (1:1 v/v) and finally Chloroform. The sample was centrifuged (13.000 rpm, 2 min, RT) to separate organic and aqueous phases. The DNA in the upper aqueous phase was precipitated with ethanol.

2.2.2.4.2 Ethanol precipitation

To concentrate DNA solutions or to exchange the working buffer (e.g., different enzymatic reactions) the DNA was precipitated by addition of 1/10 (v/v) 3 M sodium acetate and 2.5 volumes of ethanol, followed by centrifugation (13.000 rpm, 15 min, 4 °C). The supernatant was aspirated, 70 % ethanol was added and the sample centrifuged (13000 rpm, 5 min, 4 °C). The DNA was dissolved in TE pH 8.0.

2.2.2.4.3 Quantification of nucleic acids (Spectrophotometry)

Nucleic acids like DNA and RNA show a maximal absorption at a wavelength of 260 nm. Therefore the determination of the optical density (OD) of nucleic acids solution at the indicated wavelength is a measure for its concentration in solution. Reference values are:

nucleic acid	OD ₂₆₀	concentration (µg/µl)
Double stranded DNA	1	50
RNA	1	40
Oligonucleotides	1	33

In contrast, proteins have a maximal absorption at a wavelength of 280 nm. To evaluate the purity of a nucleic acid solution the quotient (OD₂₆₀/OD₂₈₀) is observed. Reference values are:

nucleic acid	quotient
RNA solution	1.9 – 2
DNA solution	1.6 – 1.8

2.2.2.4.4 Analysis of DNA by agarose DNA-gel electrophoresis

Under neutral or alkaline pH conditions, the phosphate groups of DNA chains (phosphate groups) become ionized into polyanions. As a consequence, DNA fragments placed within a porous matrix (agarose gel) and subjected to a difference in electric potential travel toward the anode. Under these conditions, small linear fragments or tightly packed supercoiled circular DNA molecules migrate faster through the pores of the gel relative to their larger or more loosely packed (open circle DNA, nicked plasmids) counterparts. In this respect, the porous matrix acts as a molecular sieve. By using this technique, known as gel electrophoresis, it

becomes possible to separate DNA molecules of different size or conformation. The main factor affecting the mobility of a DNA fragment is the viscosity of the matrix in which it is placed. 0.5 % (w/v) agarose gel is used for molecules of the size ~6 kbp, at the other end of the scale 2 % (w/v) agarose gel is used to separate much smaller DNA molecules in the range of 50 to 2000 bp. Plasmid DNA was cleaved with restriction enzymes and subjected to electrophoresis using 1 x TBE running buffer. To visualize the nucleic acids, Ethidium bromide was added directly to the agarose gel matrix at a concentration of 0.5 µg/ml. After running, the gel was photographed under 254 nm UV light on a transilluminator.

2.2.2.4.5 Purification of DNA from Agarose gel slices

2.2.2.4.5.1 DNA preparation by electroelution

DNA-fragments separated by electrophoresis were cut out from the agarose gel with a sharp scalpel. The gel slice was transferred to a dialysis bag filled with 0.1 x TBE and sealed at the ends with dialysis clips. The bag was immersed in a shallow layer of 0.1 x TBE in an electrophoresis chamber and the DNA was electroeluted out of the gel slice for 1 h at 200 V. The process was monitored with a hand-held ultraviolet lamp. Before DNA recovery, the polarity was inversed and the same voltage applied for 2-3 min in order to liberate the DNA stuck to the inner side of the dialysis bag. The buffer content of the dialysis bag was transferred to a fresh 1.5 ml microfuge tube and the DNA precipitated by adding 0.1 v of 3 M sodium acetate and 2.5 v of ethanol, followed by incubation at -80 °C for 30 min. After centrifugation at 12.000 g for 15 min, the DNA was washed with 70 % ethanol, centrifugated once again, and dried in a *Speed-Vac*. The amount and quality of the DNA fragments were checked by gel electrophoresis.

2.2.2.4.5.2 DNA preparation using the QIAEX II system (Roche)

The separated DNA-fragment was cut out from the gel with a sharp scalpel. The gel slice was transferred in a 1.5 ml reaction tube and weighed. Three volumes of buffer QX1 was added to one volume of gel (e.g. 100 mg => 300 µl). The sample was incubated at 50 °C (~5 min) to dissolve the gel slice. Thereafter, 10 µl of the QIAEX II binding matrix was added and the sample was incubated at 50 °C for further 10 min with gentle agitation. The matrix (with the bound DNA) was pelleted (30 sec, 13.000 rpm), the elution buffer aspirated and the matrix resuspended in 500 µl washing buffer QX1. The sample was centrifuged as before, the

supernatant aspirated and the matrix resuspended in 500 µl buffer PE. After centrifugation this step was repeated and thereafter, the matrix was air-dried for 15 min. To elute the DNA, the matrix was resuspended in 20 µl 10 mM Tris/HCl pH 8.5 and incubated for 5 min at room temperature. The suspension was centrifuged (30 sec, 13.000 rpm) and the DNA containing supernatant transferred into a new 1.5 ml reaction tube. The quality of DNA was determined by gel electrophoresis .

2.2.2.5 Manipulation of nucleic acids

2.2.2.5.1 Restriction of plasmid-DNA

With restriction enzymes it is possible to cleave the DNA double strand in a site specific manner. All restriction enzymes were purchased from Roche and used at the optimal temperature with the recommended 1 x restriction enzyme buffer (supplied as 10 x). If possible double digests were performed at one time. The maximal enzyme volume used per sample was 1/10 (v/v) and an activity of 3 U/µg of DNA. The restriction reaction was analyzed by gelelectrophoresis and fragments used for further cloning eluted .

2.2.2.5.2 Preparation of blunt-end DNA fragments

If there were no compatible restriction sites available for a cloning step using *sticky* ends, the generated protruding ends had to be blunted for cloning (*blunt* ends) by a fill in reaction with free nucleotides. Therefore, fragments Phenol/Chloroform extracted and ethanol precipitated. The fragments were resolved in 30 µl sterile H₂O. 4 µl M-restriction buffer, 4 µl nucleotide mix III and 2 µl Klenow fragment of DNA-polymerase I (5 U/µl) were added and the sample incubated for 30 min at 37 °C. Free nucleotides were removed by ethanol precipitation or gel electrophoresis.

Molecular cloning of DNA fragments by ligation

The stable storage of DNA-fragments for further analysis and/or cloning is achieved by inserting the fragments into an appropriate plasmid vector by ligation of the free fragment ends with the free plasmid-vector ends. This reaction is mediated by the enzymatic action of a DNA ligase. This enzyme catalyzes the formation of a phosphodiester bond between the 3'-hydroxyl and 5'-phosphate groups of the nucleotides located on the separate DNA fragments (in trans) in order to yield a continuous molecule. Ligation was performed in a total volume of 20 μ l in sterile microfuge tube containing 2 μ l of 10 x ligase buffer and 2 μ l (1U/ μ l) of T4 DNA ligase. The ratio of 3 times molecular excess of the insert over the vector was maintained (using ~100 ng of the vector), and incubated for a minimum of 12 hrs at 16 °C (blunt ends). For cloning steps using sticky ends incubation times of up to 5 hrs at room temperature were sufficient.

2.2.2.6 Isolation of total RNA from cells (RNeasy[®] Mini Kit)

Preparation of RNA was performed by using the RNeasy[®] Mini Kit (Qiagen). Confluent cell layers were washed three times with PBS and cells were resuspended in 500 μ l RLT lysis buffer supplemented with 5 μ l β -Mercaptoethanol. The lysate was transferred onto a QIAshredder[®] column and centrifuged (13.000 rpm, 2 min) and sheared three times through a 20-gauge needle (0.5 diameter) for further mechanical lysis. One volume of 70 % ethanol was added and the sample mixed by pipetting. The sample was transferred to the RNeasy[®] mini column and centrifuged (13.000 rpm, 15 sec). The bound RNA was washed in two steps with 700 μ l RW1 buffer followed by 500 μ l RPE buffer and centrifuged each time as above. The bound RNA was eluted with 50 μ l RNase-free water and the RNeasy[®] mini column centrifuged (13.000 rpm, 1 min). The concentration of the RNA was determined photometrically.

2.2.2.7 Preparation of first strand cDNA

To detect specific mRNA by PCR, the purified RNA had to be reverse transcribed into complementary DNA. To accomplish this, a RNA-dependent DNA-polymerase (SuperScript[™] II RT, Invitrogen) from M-MLV (Moloney Murine Leukemia Virus) was used.

sample composition	condition	time
1 µg RNA		
1 µl random primer (250 ng)		
2 µl dNTP-mix (5 mM)		
add 13 µl H ₂ O		
	65 °C	5 min
		transfer on ice
4 µl first strand buffer (FSB, 5 x)		
2 µl DTT (0.1 M)		
	42 °C	2 min
1 µl SuperScript TM II RT		
	25 °C	10 min
	42 °C	1 h
	70 °C	15 min

The cDNA was stored at –20 °C and 1 µl of the cDNA was used per PCR reaction.

2.2.2.8 RT-PCR analysis (Polymerase chain reaction, Mullis *et al.*, 1986)]

PCR permits the selective *in vitro* amplification of a particular DNA region by mimicking the phenomenon of *in vivo* DNA replication. The standard amplification protocol contains 3 steps which are repeated for the appropriate times. In the first step the two DNA strands are separated at 94 °C (Denaturation). In the second step primers (2.1.7) are permitted to bind to their targets (Annealing). The temperature of this step is calculated according to the lowest melting temperature of the used primers:

$$T_m \text{ (melting temperature): } (G/C) \times 4 \text{ °C} + (A/T) \times 2 \text{ °C} - \text{mismatches} \times 4 \text{ °C}$$

In the third step the heat stable DNA polymerase (Taq-Polymerase, Roche) synthesizes the complementary strands of the templates by elongation of the annealed primers at their 3' ends for the indicated times (depending on the length of the fragment to be amplified) at 72 °C (Elongation). The three steps are repeated until a sufficient amount of the desired fragment is amplified. All PCR reactions were performed in a T3 Thermocycler (Biometra, Göttingen, Germany). The reaction components were mixed in a sterile 0.5 ml microfuge tube. PCR products for expression analysis as in the current thesis were amplified by a mix of Tag/Pwo

polymerases using the Expand high fidelity PCR system (Roche, Mannheim, Germany) since Pwo DNA Polymerase synthesizes DNA 10 times more accurately than Tag DNA Polymerase due to its inherent 3'-5' exonuclease proofreading activity. For qualitative analysis 1/10 of the PCR reaction was checked by gel electrophoresis.

PCR-sample (25 µl)	Cycling programm		
1 µl (50 ng of pcDNA 3.1 full length endoglin)	1) 94 °C	5 min	x 35
3 µl forward primer (SM012, 300 ng)	2) 94 °C	15 sec	
3 µl reverse primer (SM091, 300 ng)	3) 54 °C	30 sec	
2.5 µl 10x PCR buffer (Roche)	4) 72 °C	2 min	
0.5 µl 10 mM dNTP mix	5) 72 °C	10 min	
0.75 µl High fidelity polymerase (Roche)	6) 4°C	-	
14.25 µl sterile H ₂ O			

2.2.2.9 Purification of PCR-products

For cloning of the DNA-fragment in pGEM[®]-T Easy the whole PCR sample was run on a gel and the corresponding DNA-fragment was purified and used for ligation .

2.2.2.10 DNA-sequencing

All primers were obtained from MWG-Biotech AG (2.1.7) and sequence reactions were performed with the ABI Prism BigDye[®] termination reaction mix.

sample (vol: 20 µl)

8 µl of BigDye[®] termination reaction mix
 40 ng primer
 500 ng DNA
 add 20 µl H₂O

cycle temperature	time	repeat
95 °C	5 min	50
95 °C	30 sec	
55 °C	20 sec	
60 °C	4 min	
4 °C	-	

The sequencing reaction products were purified on Centri-Sep spin columns, dried in a *Speed Vacuum* system, re-suspended in 20 µl template suppression reagent (TSR), denatured at 95 °C for 2 min and then separated on the ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems) automatic sequencer.

2.2.3 Heterologous gene expression in eukaryotic cells

2.2.3.1 Collagen coating of culture dishes

Primary hepatocytes have to be plated on a collagen substrate for optimal culture conditions. Therefore, culture dishes were coated with collagen type I isolated from rat tail (Becton & Dickinson). The collagen stock solution (2 mg/ml) was diluted 1 :40 in 0.1 % acetic acid and 5 ml was applied to each culture dish for 2 hrs. Subsequently the supernatant was aspirated, the dishes were washed two times with PBS and stored at 4 °C.

2.2.3.2 Eukaryotic cell culture

For the experiments shown in this thesis the following celllines have been used: **COS-7** (ATCC No. CRL-1651), **L₆E₉** (Cristina Cardoso, Max Delbrück Center for Molecular Medicin) and **CHO** (ATCC® Number CCL-61) In addition primary rat hepatocytes were used. Hepatocytes were isolated from rat liver according to method introduced by Seglen, 1976. All cells were cultured in a humidified incubator at a temperature of 37 °C in the presence of 5 % CO₂.

L₆E₉ cells were cultured in DMEM containing 20 % FCS whereas COS-7 cells were cultured in DMEM containing 10 % FCS. Hepatocytes were plated in Hepatozym on collagen coated dishes. 1 h after plating the medium was changed with new Hepatozym. CHO cells were cultured in RPMI medium containing 10 % FCS.

The cells were plated in sufficient amounts under the indicated conditions. 24 hrs after plating medium was renewed. A medium change was performed every second day using 2 ml (6 well plate) or 10 ml (culture dish, 10 cm).

2.2.3.3 Subculture of cells

Cultures (COS-7, L₆E₉, CHO) which reached a confluency of 90 – 100 % were subcultured. To do this properly it is necessary to obtain a single cell suspension first, by using proteolytic enzymes, such as trypsin. The medium was removed by aspiration, cells washed with 3 ml PBS/1 x trypsin. Thereafter cells were incubated in PBS/1 x trypsin for 4-7 min at 37 °C until the cells were detached from the dish. The cells were examined microscopically to make sure that rounding had started. Culture medium was added to inactivate the proteolytic enzyme. The cells were dispersed by gently pipetting a few times and an aliquot was removed for cell counting in a Neubauer chamber. Appropriate numbers of cells were then transferred onto new culture dishes.

2.2.3.4 Cryopreservation and storage of cell lines

This procedure allows the storage of cell lines by cryopreservation. In order to avoid crystal formation which can produce irreversible damage to the cell structure, a cryopreservative such as 10 % dimethyl sulfoxide (DMSO) must be added. The medium was removed by aspiration, cells were washed with 3 ml PBS/1 x trypsin and incubated with 2 ml PBS/1 x trypsin for 4-7 min at 37 °C. The cells were examined microscopically to make sure that rounding of the cells had commenced. Once the cells were detached culture medium was added, the cells gently resuspended, cell suspensions pooled together, counted and centrifuged at 1000 rpm and 4°C for 5 min. The cell pellet was resuspended at a density of $1-2 \times 10^6$ cells/ml in medium. To this suspension an equal volume of freezing medium (2 x) [2.1.8.2.1] was added and the suspension dispensed 1 ml aliquots into sterile cryotubes, frozen and stored in liquid nitrogen or at -80°C.

2.2.3.5 Thawing of cryopreserved cells

An aliquot of frozen cells was immediately thawed, plated in 10 ml culture medium on a 10 cm culture dish and incubated at 37 °C, 5 % CO₂ in humidified atmosphere. 24 hrs later the medium was exchanged to remove residual cytotoxic DMSO.

2.2.3.6 Transient transfection of eukaryotic cells

Transient transfection was performed using two different transfection media which are both commercially available. COS-7 cells, CHO cells and hepatocytes were transfected using the FuGENETM reagent. In case of L₆E₉ cells the lipofectamine reagent in combination with the plus reagent was used.

Transient transfection with the FuGENETM6 reagent was performed one day after suculture at 50-80 % confluency (cell lines) or one day after seeding (hepatocytes). In general, the cells were seeded in 6-well plates at a density of $2-3 \times 10^5$ cells per well in 2 ml medium. 1 h before the transfection the medium was exchanged. In case of hepatocytes the medium was exchanged from Hepatozym to the standard DMEM medium. CHO cells were transfected in standard RPMI medium. Cells were transfected with 2 µg of expression plasmids or 1 µg of the (CAGA)₁₂-MLP-Luc reporter and 3 µl of FuGENETM6 per 1 µg DNA. The medium and FuGENETM6 were mixed in the first microfuge tube, while the DNA was pipetted into the second microfuge tube. After the appropriate incubation time the content of microfuge tube 1 was transferred dropwise into the microfuge tube 2. following a second incubation time the suspension was transferred dropwise onto the cells.

Transfection schedule:

component	time
100 µl DMEM/RPMI medium	
3x Y µl FuGENE TM 6	
	10 min (room temperature)
Y µg DNA	
+ DMEM/FuGENE TM 6	
	15 min (room temperature)

24 hrs after transfection the medium was exchanged (DMEM or RPMI) in the morning. In the afternoon the medium was exchanged again and the serum content was lowered to 0.2 % FCS. 48 hrs later the cells were either harvested for western blot analysis, supernatants were collected and TCA precipitated for western blot analysis. In case of luciferase experiments the cells were induced after 24 hrs for a time period of 16 hrs.

Transient transfection with the Lipofectamine reagent was performed one day after subculture at 50 - 80 % confluency. In general the cells were seeded in 6-well plates at a density of 2×10^5 cells per well in 2 ml medium. 1 h before the transfection the medium was exchanged to 1 ml DMEM without supplements. Cells were transfected with 2 μ g of expression plasmids or 1 μ g of the (CAGA)₁₂-MLP-Luc reporter and 3 μ l Lipofectamine reagent and 4 μ l Plus reagent per 1 μ g DNA. The DMEM, DNA and Plus reagent were mixed in the first microfuge tube, while DMEM and Lipofectamine reagent were pipetted into the second microfuge tube. After the appropriate incubation time the content of microfuge tube 1 was transferred into the microfuge tube 2. following a second incubation time the suspension was transferred dropwise onto the cells.

Transfection schedule:

component	time
100 μ l DMEM	
4x Y μ l Plus reagent	
Y μ g DNA	
	15 min (room temperature)
100 μ l DMEM	
3x Y μ l Lipofectamine	
+ DMEM/Plus reagent	
	15 min (room temperature)

6 hrs after transfection the medium was exchanged (DMEM including 10 % FCS). After 24 hrs the medium was exchanged. Medium was exchanged again and the serum content was lowered to 0.2 % FCS. 24 hrs later the cells were induced for luciferase experiments for 16 hrs.

Luciferase measurement

For performing the luciferase measurements, samples stimulated with TGF- β 1 for 6 – 16 hrs were washed three times with PBS. The cells were resuspended and lysed in 350 μ l 1 x Cell Culture Lysis Reagent (Promega). The cell lysate was incubated 5 min on ice and afterwards centrifuged (10.000 rpm, 10 min, 4 °C). The cleared supernatant was stored at –20 °C or directly utilized to determine the luciferase activity. The measurement was executed using the Steady-Glo-Luciferase Assay Kit (Promega). For each sample 100 μ l LAR substrate buffer and 20 μ l of the cell lysate were mixed in a 96 well microtiterplate and samples were measured for a 2 sec time interval in a Microbeta 1450 Jet Liquid Scintillation & Luminescence Counter. In order to standardize the samples the protein concentration of the samples was determined by using the DC-method. Luciferase activities were normalized to the protein content of each sample.

2.2.4 FPLC fractionation of serum proteins

To characterize the serum CD105 a molecular-weight fractionation of serum proteins was carried out using the fast performance liquid chromatography (FPLC) method by gel filtration. A size exclusion superdex 200HR 10/30 column (Äkta Purifier, Amersham Pharmacia-Biotech, Uppsala, Sweden) was used and calibrated by a run of known proteins covering different molecular weights included in a standards [as Blue dextran 2000, thyroglobulin, gamma globulin, albumin, ovalbumin (chicken), myoglobin (equine), vitamin B12, L-tyrosine, ferritin and catalase]. The K_{av} was calculated from the peak fractions of each standard protein and from the peak fraction of each sample and by application of the following equation:

$$K_{av} = (V_e - V_0) / (V_t - V_0)$$

(V_0 = void volume, V_t = total gel bed volume, V_e = elution volume of protein of interest, K_{av} = value of protein of interest)

As an internal standard, the recombinant ECD of human CD105 was used (Cat. No. 1097-EN, R&D Systems). Serum samples (300 μ l) of healthy controls and patients with liver cirrhosis were analyzed by FPLC. Elution of proteins was performed using PBS–Dulbecco buffer (Biochrom KG, Berlin, Germany) with a flow rate of 0.3 ml/min. The endoglin elution profile was determined by ELISA.

2.2.5 Statistical analysis

Data are given as minimum, maximum, and median values. Separately for each of the study groups investigated 95% confidence intervals for the median values of the CD105 serum levels measured were computed for each patient group. The degree of association between CD105 and AFP was assessed by calculation using the Spearman rank correlation coefficient for each of the following three subgroups: liver cirrhosis, HCC and HCC with liver cirrhosis. The non-parametric Wilcoxon rank sum test was used for pair-wise comparison of CD105 values between different patient groups.

ROC analysis was carried out to establish the diagnostic sensitivity and specificity of CD105 and AFP concentrations. The accuracy criterion was chosen for selection of an “optimal” cut-off value for serum CD105 as well as AFP (dividing positive from negative test results) – that is, the chosen cut-off values maximize the accuracy of the corresponding diagnostic test. ROC curves were plotted for various situations; Moreover, the areas under the ROC curves (AUCs) were calculated, as well as for sensitivities and the specificities that were, reached with the optimal cut-off value. Parallel diagnostic testing was performed in all situations in order to judge whether the diagnostic values (sensitivity and specificity) of the two single markers (CD105 and AFP), that were reached using the optimal cut-off values calculated in the ROC analyses, could be improved if the combination of the two markers were used. In this parallel diagnostic testing situation, test positives were considered to be all patients that had a positive test result for at least one of the two markers, whereas test negatives were those patients who had negative results for both markers (assessed using the optimal cut-off values for both markers, CD105 and AFP, calculated in the previous ROC analyses).

As the aim of this study was to generate new hypotheses rather than to confirm hypotheses obtained from the literature or one's own research, an explorative study design was used. Therefore, all statistical analyses were conducted in an explorative manner – that is, an α -adjustment was not carried out. Therefore, P values of $P \leq 0.05$ could be considered to be statistically significant (with respect only to the study population investigated).

All statistical analyses were performed using MedCalc Version 7.3.0.1 (MedCalc software; Mariakere, Belgium), S-Plus 6.1 (Insightful Corporation, Seattle, WA, USA) and SigmaPlot 9.0 (Systat Software, Inc., Point Richmond, CA, USA).

3 Results

The results are presented in two parts according to the topics raised in the aim of the study. In the first part of the results (3.1) Soluble endoglin as biomarker for the diagnosis of cirrhosis and developing HCC was evaluated. For this purpose, the serum endoglin concentrations were measured by ELISA in the patients groups with chronic liver disease and compared the results to a control population, respectively. In addition the value of serum endoglin was compared with this of established common tumour marker α -fetoprotein (AFP)

In the second part (3.2), the function of the soluble endoglin and whether this soluble fragment of the membrane receptor could have an effect on TGF- β 1/Smad3 signaling were analysed. In order to solve this question, the rat soluble extracellular domain of endoglin was cloned and heterologously expressed in different cellular systems. The functional aspects were examined with regard to the role of endoglin on the profibrogenic TGF- β 1/Smad3 signaling pathway using a Smad3 specific luciferase reporter system.

3.1 Soluble endoglin as biomarker for the diagnosis of cirrhosis and developing HCC

3.1.1 Clinical and laboratory data of the analyzed patients and control groups

The clinical and laboratory data for every patient in different subgroups and controls are collected in the reference Table [from R1a-R1f in Appendix]. The median of age for the healthy controls (n = 70; 54 m. and 16 f.) was 50.5 years. The respective median of age in the fibrosis subgroup (n = 19; 9 m and 10 f) was 47 years, in the cirrhotic subgroup (n = 57; 39 m. and 18 f.) the median of age was 60 years. HCC associated with cirrhotic liver subgroup (n = 45; 31 m. and 14 f.) the median of age was 57 years, whereas in HCC patients group (n = 9; 6 m. and 3 f.) the median of age was 63 years. All of the data were summarised in Table 1.

The most common underlying etiologies for liver cirrhosis without HCC were alcohol abuse (n = 19; 33%), virus hepatitis (chronic viral hepatitis B and/or C infection, n = 17; 29%), or cryptogenic (n = 14; 24.5%). The associated etiologies in liver cirrhosis with HCC were viral hepatitis (n = 32; 71%), alcohol (n = 6; 13%) or both factors (n = 7; 16%), while in HCC without cirrhosis the associated etiology was mainly cryptogenic (n = 5; 55.5%), viral hepatitis (n = 3; 33.5%) and chronic alcohol abuse with viral hepatitis (n = 1; 11%). The corresponding data set is summarized in Table 2.

Table 1: Characteristics of the individuals in the subgroups included in this study. Age (range and median value) and gender of the individuals in the subgroups are indicated.

Data and subgroups	healthy controls	non-liver-diseased	Liver fibrosis	liver cirrhosis	HCC Plus liver cirrhosis	HCC
(n. of cases)	70	94	19	57	45	9
age (years)	50.5	60	47	60	57	63
Range (Y)	(36-67)	(21-85)	(21-72)	(38-82)	(38-78)	(54-74)
gender (m/f)	54/16	43/51	9/10	39/18	31/14	6/3

Table 2: Etiologic factors in each patient subgroup. In the columns the subgroups of patients are splitted in the patient numbers (and percentages with respect to the total subgroup) belonging to the different etiologies.

Patients subgroups and etiology	total number (m/f)	Hepatitis n. (%)	Alcoholic n. (%)	Hepatitis and alcoholic n. (%)	Cryptogenic
Liver fibrosis	19 (9/10)	2 (10%)	0	0	17 (90%)
Liver cirrhosis	57 (39/18)	17 (30%)	19 (33.5%)	7 (12%)	14 (24.5%)
HCC with cirrhosis	45 (31/14)	32 (71%)	6 (13%)	7 (16%)	0
HCC	9 (6/3)	3 (33.5%)	0	1 (11%)	5 (55.5%)

3.1.2 Measurement of serum sCD105 concentrations

For the measurement of the sCD105 concentration in the serum samples of the described collective, we used a commercially available ELISA kit (R&D systems). This ELISA kit is specific for the detection of human sCD105 including monoclonal antibodies for capturing and detection of sCD105. To establish this ELISA kit in our laboratory for the “routine” measurement of the afore mentioned samples, the variation coefficient of the assay was determined. To evaluate the intra-assay variation one control sample was measured in multiple repeats in duplicate manner with the same ELISA kit. The calculated variation coefficient for the intra-assay precision was 3.0 %. The inter-assay precision was determined by including the same control sample (which was stored in aliquots) in each measurement with a new ELISA kit in parallel to the used patient samples. Thereby, the calculated inter-assay precision was 6.5 %. Here it is important to note, that the used control sample for the precision analysis was stored in aliquots at – 80°C, so that the samples used in the control measurements had to be thawed on ice just one time before usage. The analysis of the sCD105 concentrations showed that the median concentration of sCD105 in the serum

samples of healthy controls was 3.7 µg/l, and 95 % confidence interval (CI) was between 3.4 and 4.0 (µg/l). In the serum of the non-liver-diseased and non-cancer-diseased control patients the median for sCD105 concentration was 3.9 µg/l, and the 95 % CI was 3.7-4.2 (µg/l) as in Table 3. There was no significant difference in sCD105 concentrations between the two control groups. Patients with liver cirrhosis plus HCC subgroup had the highest sCD105 concentrations with a median of 7.4 µg/l and the 95 % CI was between 6.4-8.8 (µg/l). sCD105 concentrations in the fibrotic liver subgroup had a median of 3.5 µg/l and the 95 % confidence interval was between 3.3-4.1 µg/l. While in serum samples of patients with liver cirrhosis the median for sCD105 concentration was 5.8 µg/l, and 95 % CI was between 5.0 and 6.3 µg/l. HCC patients without cirrhosis had a median 5 µg/l, and a 95 % CI was 3.8 -7.5 µg/l. All these data are summarized in Table 3.

The comparison between the patients subgroups showed that there was a significant elevation for sCD105 concentration in the HCC plus cirrhosis subgroup with respect to the other groups [to the fibrosis subgroup the P-value was 0.0001, the cirrhosis subgroup (P = 0.0006) and HCC only subgroup (P = 0.0134). In the fibrotic liver subgroup (n= 19 cases , without history of viral hepatitis), sCD105 was not significantly elevated in the serum if compared to the controls groups (for healthy control P= 0.8218; non liver non cancer disease P = 0.2479) as in Table (4), figure (8).

Table 3: Median value, range and 95 % CI of sCD105 in every subgroup.

	healthy controls	non-liver-diseased	liver fibrosis	liver cirrhosis	HCC plus liver cirrhosis	HCC
(n)	70	94	19	57	45	9
Median endoglin (µg/l)	3.7	3.9	3.5	5.8	7.4	5.0
Range(µg/l)	2.5 - 7.8	2.4 - 15.1	2.8 - 7.4	2.8 - 12.6	2.4-12.0	3.3 - 8.5
95%-CI (µg/l)	3.4 - 4.0	3.7 - 4.2	3.3 - 4.1	5.0 - 6.3	6.4 - 8.8	3.8 - 7.5

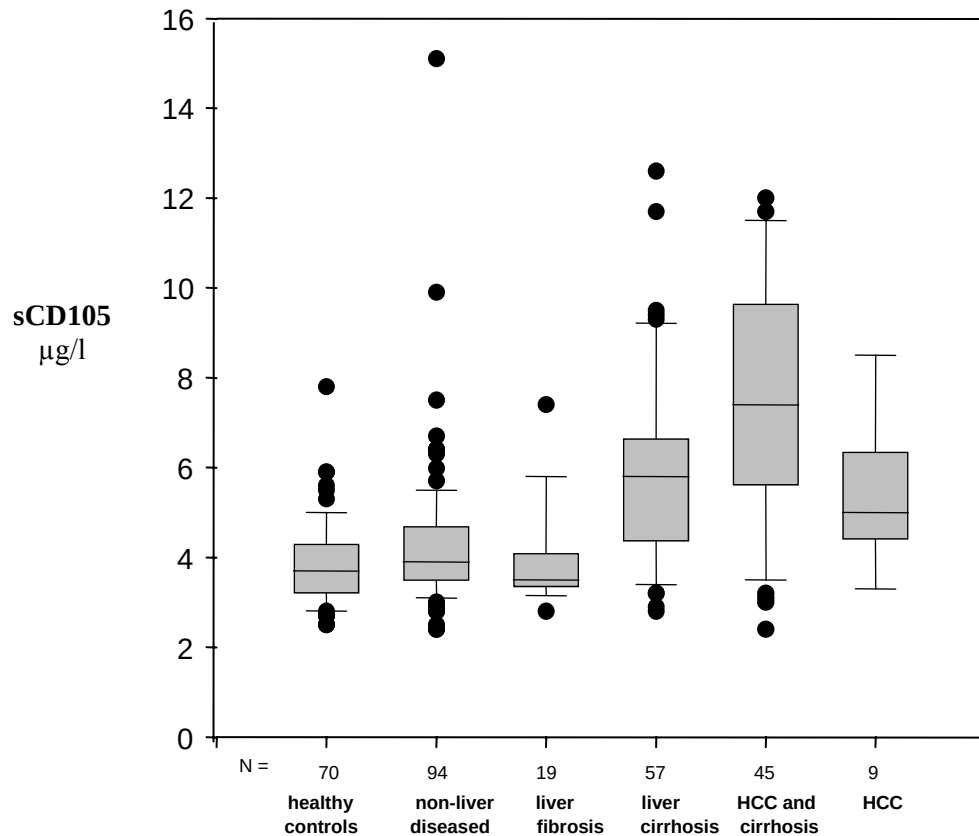


Figure 8: Statistical summary of sCD105 concentration in the subgroups by box-and-whiskers plots. Comparisons between subgroups are illustrated using Box-and-Whiskers-plots, where the line in the box indicates the median per group, the box represents 50% of the values, and horizontal lines show minimum and maximum values of the calculated non-outlier values; dots indicate outlier values.

Table 4: Statistical summary of sCD105 measurements by box-and-whiskers plots. P-values of nonparametric Wilcoxon rank sum tests between different subgroups of the study are given in the Table (bold number = significant)

	non-liver diseased	liver fibrosis	liver cirrhosis	HCC plus liver cirrhosis	HCC
healthy controls	0.0564	0.8218	<0.0001	<0.0001	0.0013
non-liver diseased		0.2479	<0.0001	<0.0001	0.0097
liver fibrosis			0.0001	<0.0001	0.013
liver cirrhosis				0.0006	0.4662
HCC and liver cirrhosis					0.0134

Measurement of the serum α -fetoprotein concentration

To evaluate the potential of sCD105 as a marker for patients at high risk to develop a hepatocellular carcinoma, sCD105 should be thoroughly analysed and compared with a clinically established marker. Therefore serum concentration of AFP was measured using the AxSYM analyser used in our routine laboratory. The median of AFP concentrations for the different subgroups are listed in Table (5). The median was 4.0 $\mu\text{g/l}$ and 95% CI was between 3.4 and 4.9 in patients with liver cirrhosis. In HCC patients with concomitant liver cirrhosis the median of alpha fetoprotein was 5.2 $\mu\text{g/l}$, and 95% CI was 3.3 - 11.3 $\mu\text{g/l}$.

As depicted in Table (5), the median AFP concentration of the patient subgroups and healthy control seemed to be normal (i.e. up to 10 $\mu\text{g/l}$). There was a wide range values for α -fetoprotein in the different patients subgroups.

Table 5 Median of the serum alpha feto-protein concentration, range and 95 % confidence interval in the subgroups.

	healthy controls	non-liver-diseased	liver fibrosis	liver cirrhosis	HCC plus liver cirrhosis	HCC
(n)	70	94	19	57	45	9
Median AFP ($\mu\text{g/l}$)	3.1	3	2.9	4	5.2	5.6
Range ($\mu\text{g/l}$)	1.2 - 10.1	0.7 - 11.2	1.0 - 8.5	0.8 - 153.9	2.4 - 460000	2.0 - 763.0
95%-CI ($\mu\text{g/l}$)	2.6 - 3.6	2.6 - 3.6	1.8 - 4.8	3.4 - 4.9	3.3 - 11.3	2.3 - 388.0

3.1.4 Correlation of sCD105 and AFP

By using the Spearman rank correlation analysis it was shown, that sCD105 serum levels were mild to moderate positively correlated with AFP in both patients with liver cirrhosis ($r = 0.358$, $P = 0.0073$), and positively correlated in patients with liver cirrhosis and HCC ($r = 0.479$, $P = 0.0015$) [Figure 9 a, b]. In Table (6) the results of the multiple logistic regression investigating the impact of two markers (sCD105, AFP) and two covariates (age, gender) on the risk for development of HCC in cirrhotic patients. From this regression analysis it is obvious that serum sCD105 has the most significant impact on the risk for development of HCC in cirrhotic patients when compared to AFP.

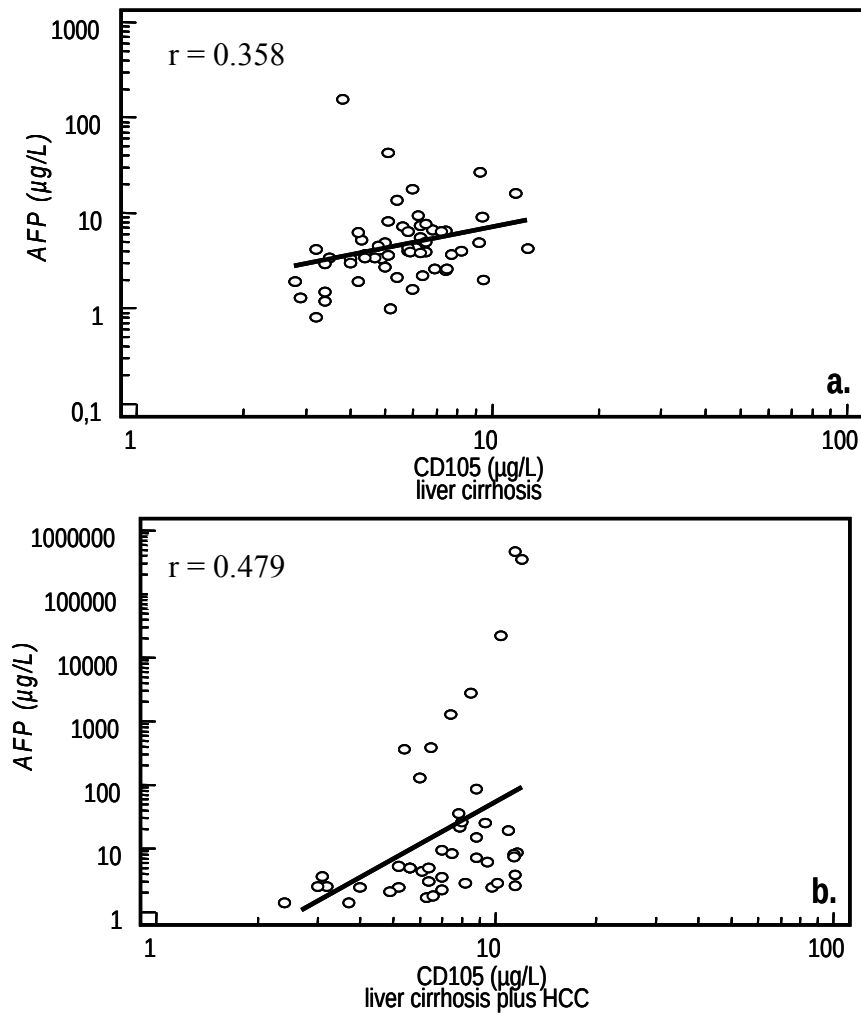


Figure 9 (a and b): Spearman rank correlation analysis of sCD105 and AFP.

The degree of association between sCD105 and AFP in the two liver cirrhosis groups (without or with HCC) was assessed. (a) calculation of the logarithmic transformed Spearman rank correlation coefficient (r) was 0.358 between sCD105 and AFP in the liver cirrhosis subgroup. (b) r value was 0.479 in the liver cirrhosis plus HCC subgroup.

Table 6: Multiple logistic regression investigating the impact of two markers (sCD105, AFP) and two covariates (age, gender) on the risk for development of HCC in cirrhotic patients. Results are given as estimated regression coefficients and standard errors, P-values of the Wald chi-square-test, odds ratios and corresponding 95% confidence intervals for each of the markers and covariates included in the logistic regression model.

variable	regression coefficient	standard error	P value	odds ratio	95% CI
intercept	-0.510	1.335	0.7025	-	-
CD105	0.254	0.095	0.0077	1.289	[1.070; 1.553]
AFP	0.002	0.002	0.3522	1.002	[0.998; 1.007]
Age	-0.027	0.020	0.1781	0.973	[0.936; 1.012]
Gender	0.009	0.240	0.9695	1.019	[0.398; 2.609]

3.1.5 Diagnostic sensitivity and specificity of sCD105 and AFP

To determine the sensitivity and the specificity of sCD105 and AFP we performed a ROC analysis (Fig. 10a, b). The optimum cut-off values reached and the corresponding area AUC for sCD105, according to different subgroups, are shown in figure 10a. The sensitivity was tested for sCD105 between the different subgroups. It varied from 57.8%-84.4%, while the specificity of sCD105 for HCC in the liver cirrhosis subgroup versus the healthy controls was highest (91.4%) with respect to the other studied groups. The specificities of sCD105 in the other studied subgroups varied between 77.8% and 89.9%. The ROC analysis for AFP concentrations in these subgroups showed sensitivities, ranging from 42.2% to 57.8%, whereas the specificity was around 84% (Figure 10b). Sensitivity and specificity values of sCD105 could not be further improved by the use of the combination of sCD105 and AFP at these cut-off values as it is depicted in Table (7).

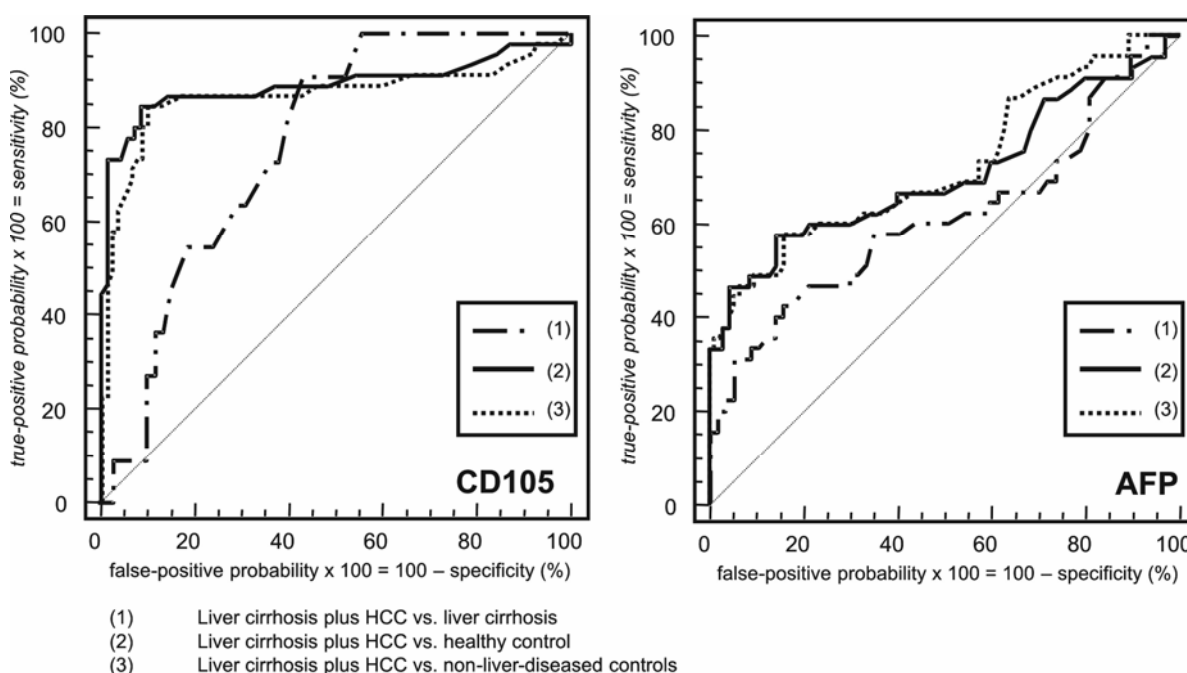


Figure 10: ROC analysis of CD105 and AFP. Various ROC analysis for serum sCD105 and AFP. The areas under the ROC curves (AUCs) were calculated with the corresponding standard errors and 95% confidence intervals. An AUC= 1 (100%) is characteristic for an ideal test, whereas an AUC= 0.5 (50%) or less indicates a test of no value.

Table 7: ROC analysis of sCD105 and AFP to determine the sensitivity and specificity of both markers. This Table contains the AUC, sensitivity (%) and specificity (%) for both AFP and sCD105 at certain cut-off values between liver patient subgroups and controls.

	area under the curve	standard error	95% confidence interval	Sensitivity (%)	95% confidence interval	Specificity (%)	95% confidence interval	optimal cut-off (µg/l)
sCD105								
(1)	0.698	0.053	0.599; 0.785	57.8	0.422; 0.723	78.9	0.661; 0.886	6.9
(2)	0.890	0.034	0.818; 0.941	84.4	0.705; 0.935	91.4	0.823; 0.968	5.0
(3)	0.859	0.038	0.790; 0.912	84.4	0.705; 0.935	89.4	0.813; 0.948	5.1
AFP								
(1)	0.614	0.057	0.512; 0.708	42.2	0.277; 0.578	84.2	0.721; 0.925	7.7
(2)	0.704	0.051	0.612; 0.786	57.8	0.422; 0.723	85.7	0.753; 0.929	4.8
(3)	0.722	0.049	0.640; 0.794	57.8	0.422; 0.723	84.0	0.705; 0.908	4.8

3.1.6. C-reactive protein in the liver fibrotic and liver cirrhotic subgroups:

The median for the two subgroups fibrotic, and cirrhotic were 5 mg/l and 14 mg/l, respectively. These two subgroups, which are associated with inflammation, did not reveal any significant correlation between serum sCD105 level and C-reactive protein concentration (Spearman rank correlation coefficient $r = 0.118$ and $r = 0.207$ respectively).

3.2 Functional analysis of soluble endoglin

Having shown that the soluble endoglin concentration in the serum is elevated in the case of cirrhosis and even more in patients which suffer from cirrhosis accompanied by HCC, we next asked if this circulating endoglin could have any functional consequence for TGF- β signaling especially in liver cells contributing functionally to the progression of cirrhosis to HCC. To solve this question we analyzed the function of the soluble endoglin on TGF- β signaling in an established cellular assay, based on the TGF- β /Smad3 dependent (CAGA)₁₂-MLP Luc reporter. For this analysis we decided to heterologously express the extracellular domain (ECD) of rat endoglin. The data published so far concerning the role of the membrane inserted as well as the soluble endoglin all rely on assays in which the human orthologue of endoglin has been used irrespective of the origin of the cellular system used for the analysis [Letamendia *et al.*, 1998; Obreo *et al.*, 2004 ; Venkatesha *et al.*, 2006]. Although these data are accepted and produce comparable results we thought of using the rat soluble receptor because the cells we were going to use for the functional analysis, i.e. L₆E₉ myoblast cells and

primary hepatocytes, were of rat origin. As a basis for this analysis the full length rat endoglin has been cloned for the first time in our laboratory. With respect to the functional analysis of the soluble endoglin there has been only one study published currently. In this study the commercially available soluble recombinant ECD of human endoglin (R&D) was used in cell culture experiments of human umbilical vein endothelial (HUVEC) cells. As a standard this protein was included in our initial functional experiments.

3.2.1 Cloning of the ECD of rat endoglin

For the cloning of the extracellular domain of rat endoglin, structural characteristics of the receptor protein had to be taken into account. These include the putative protease cleavage site, the antibody epitopes and the domain structure of the full length receptor as shown in Fig. 11. The soluble endoglin that naturally occurs in the serum is most likely a proteolytic product of the membrane receptor. As has been described for many other membrane receptors a portion of the extracellular domain (ligand binding domain) is shedded by a protease to generate a soluble part, i.e. soluble endoglin, and a remnant part (transmembranal domain and C-terminal domain) residing in the membrane. The putative protease cleavage site is located 147 amino acids before the beginning of the transmembrane domain [Raab *et al.*, 1999; Llorca *et al.*, 2007] (Fig. 11). In order to detect the heterologously expressed soluble endoglin (s-Eng) in western blot experiments by

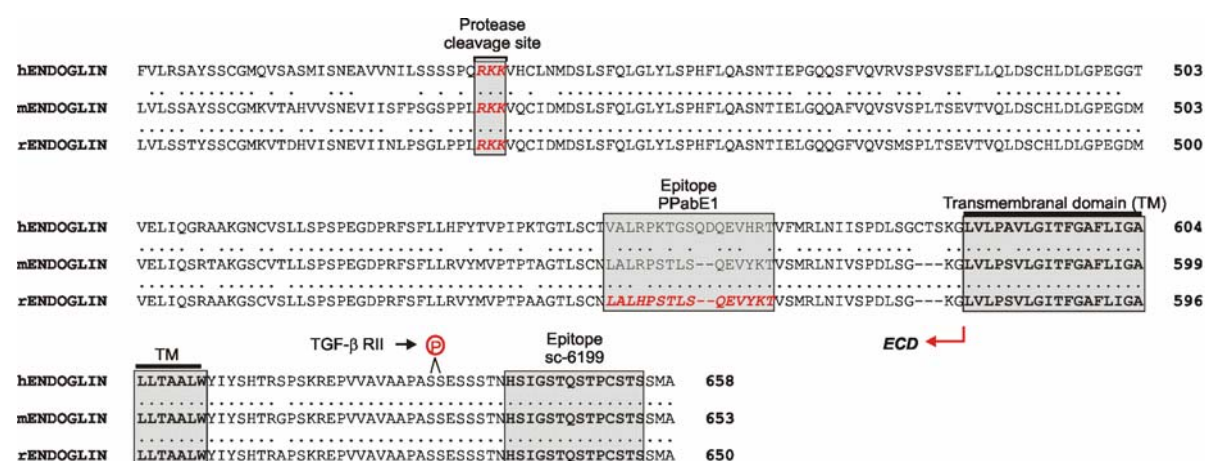


Figure 11: Structural and functional characteristics of the sequences surrounding the transmembranal domain. Alignment of the juxtamembrane part of the ECD, transmembranal domain and C-terminus of human, mouse and rat endoglin. Indicated is the putative protease cleavage site (upper part, red letters, grey box), the epitope of the rat specific anti endoglin antibody PPAbE1 (middle part, red letters italics, grey box), the transmembranal domain (middle part, lower part, grey box and line on the top), the epitope of the commercially available antibody sc-6199 (lower part, grey box) and the TGF-β RII phosphorylation site in the ICD (serine 626 and 627). The boundary between the extracellular domain and the transmembranal domain is indicated below the middle panel (red arrow, ECD).

a specific antibody, we had to preserve the epitope of the PPabE1 antibody (Fig. 11) in our expressed protein. Since the epitope is located C-terminally from the putative protease cleavage site, at which the shedding of the endogenous endoglin is facilitated, the antibody PPabE1 will detect only the heterologously expressed s-Eng, but not the endogenous, shedded s-Eng (Fig. 11). To be a functionally active receptor, most of the TGF- β receptors have to dimerize. To achieve this dimerization in case of a soluble receptor lacking the intracellular domain, some soluble receptor domains have been fused to the Fc portion of the human IgG, which is a disulfide linked dimer. The interaction of the IgG domains forces in addition the receptor domains to associate leading to a functional receptor (Komesli *et al.*, 1998). In case of endoglin it has been previously shown that the extracellular protein moiety comprises cysteins which mediate disulfide bounding and thereby dimerization of the monomeric soluble protein entities. Therefore no fusion to an dimerization inducing protein domain is necessary.

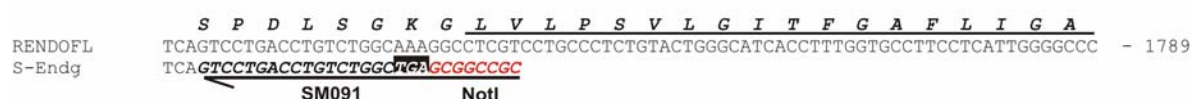


Figure 12: Design of the 3' primer to generate soluble Endoglin. Depicted is the boundary of the extracellular domain (ending with Glycine-Lysine-Glycine) and the transmembranal domain (amino acids underlined, starting with leucine-valine-leucine). By means of RT-PCR a stop codon was introduced (white type on black) substituting the AAA codon (lysine) in the full length endoglin sequence. In addition, a *Not* I restriction site was introduced (red type) for further subcloning steps.

To generate the cDNA encoding the ECD-Endoglin we decided to amplify the part coding for the extracellular domain using the cDNA encoding the full length endoglin by RT-PCR (Appendix Fig. 31). A RT-PCR amplification step instead of restriction enzyme usage was necessary because obviously no appropriate restriction sites were available for further cloning steps. The 5'-primer for the amplification was chosen according to the published sequence (Meurer *et al.*, 2005) and is not depicted in Figure 12. The corresponding 3'-primer contained in addition to the endoglin sequence two more features. As shown in Figure 12 we introduced a stop codon at the end of the coding region for the extracellular domain. This stop codon is followed by a sequence representing a *Not* I restriction site, which is suited for the cloning into the expression vector pcDNA3.1 (see Fig.12). Using the expression clone of endoglin as a template, we amplified the coding region for the extracellular domain of ECD-Eng (Fig. 13A).

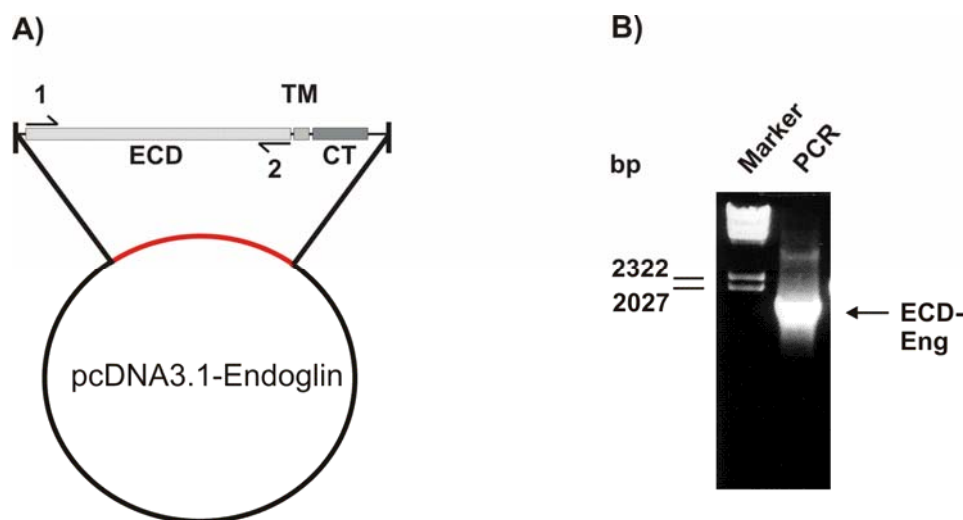


Figure 13: PCR amplification of the extracellular domain of rat endoglin (A) To amplify the extracellular domain of endoglin (ECD-Eng) we designed two specific primers. Primer 1 binds to the initiation region (5' to the start ATG codon). Primer 2 is designed to bind to a region at the boundary between the ECD and TM domain of full length endoglin. In addition primer 2 is used to introduce a stop codon and this is followed by a *Not* I restriction site, for successive cloning into the expression vector pcDNA. (B) Result of PCR for the amplification of the ECD of endoglin. [ECD, extracellular domain; TM, transmembrane domain; CT, C-terminal domain]

The corresponding PCR-fragment was eluted from the gel slice (Fig. 13B) and cloned into the cloning vector pGEM[®]-T Easy (Invitrogen) without further modifications. This initial cloning step was performed because sequence analysis of the PCR product in the pGEM[®]-T Easy vector is easier to handle, because the vector offers primer binding sites which can be used in addition to the endoglin internal primers. The vector primers are essential to check the sequence at the ends of the fragment, because this information is not available using the internal primers only. After ligation of the corresponding PCR-fragment into pGEM[®]-T Easy (appendix Fig. 32), Competent bacteria were transformed with the ligated recombinant DNA and the bacteria were plated onto growth plates containing the appropriate selection marker (ampicillin). Single resistant colonies were used to inoculate small scale cultures consisting of 5 ml growth medium including the selection marker. To determine if the clones contained recombinant vector-DNA, the DNA was prepared and analysed by restriction analysis (Fig. 14). For convenience, the pGEM[®]-T Easy vector offers two *Eco*R I restriction sites in the corresponding multiple cloning region which enclose the inserted fragment on both sides. Therefore, when using this enzyme the inserted fragment is released from the vector (Fig. 14A). A representative restriction analysis of a recombinant clone obtained in these experiments is depicted in Fig. 14B. The upper fragment represents the vector (pGEM[®]-T Easy, 3015 bp) and the lower band the fragment encoding soluble endoglin (ECD-Eng, 1737 bp), respectively (Fig. 14B).

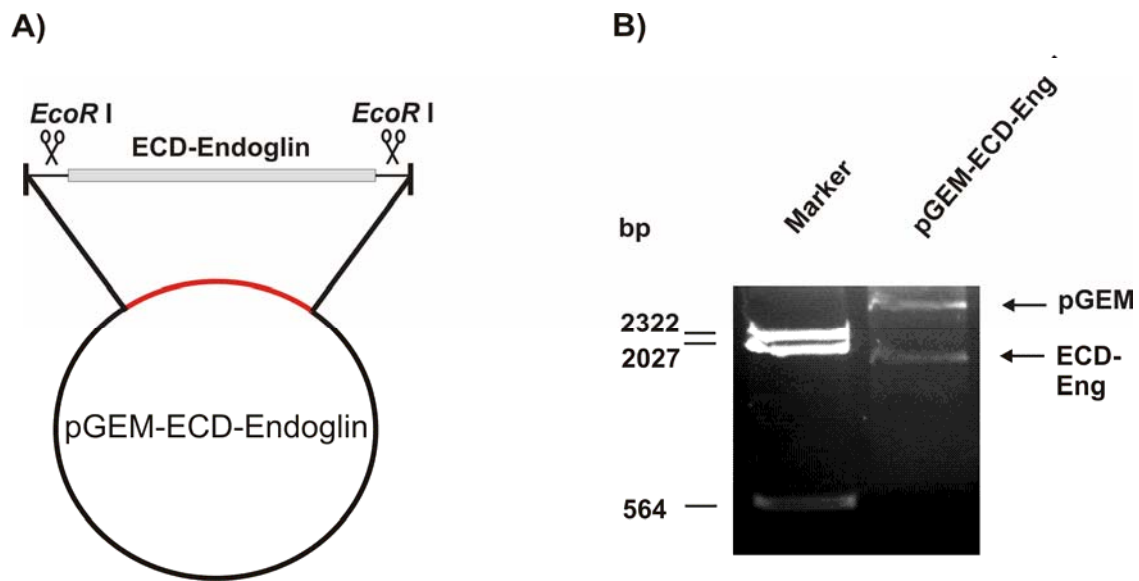


Figure 14: Generation and analysis of the vector pGEM-ECD-endoglin. (A) Scheme to display the construction of the pGEM-ECD of endoglin. To recover recombinant plasmids, small scale LB medium cultures were inoculated with single colonies. (B) DNA was prepared and cut with *EcoRI*, which cuts at both sides of the inserted fragment in the multiple cloning region.

The DNA of the corresponding clone was verified by sequence analysis, whereby it was shown that the sequence contained no mistakes. Because the pGEM[®]-T Easy vector (Appendix Fig. 33) is only suited for cloning and amplification of recombinant DNA the insert encoding the soluble endoglin had to be transferred into an appropriate vector for expression of proteins in eukaryotic cells. For this purpose, we used the pcDNA3.1 vector, which has been already used for expression of the full length endoglin (Meurer *et al.*, 2005). This vector (pcDNA3.1) contains the constitutive active CMV (cytomegalovirus) promoter leading to high expression of foreign proteins in a variety of cellular systems (Appendix 34). To prepare a pcDNA3.1 vector with compatible ends, the pcDNA-HA-T β RIII (encoding betaglycan; Wang *et al.*, 1992) was cut with the restriction enzymes *EcoR* I and *Not* I (Fig. 15B). The pGEM-ECD-endoglin vector was also cut with the enzymes *EcoR* I (multiple cloning site) and *Not* I (introduced with the 3'-primer; Fig. 15A). The compatible cut vector pcDNA and the endoglin ECD-Eng encoding fragments (Fig. 15C) were eluted from the gel slice and used for a ligation reaction. The resulting products of this reaction were used to transform competent bacteria, which were plated in the presence of an appropriate selection marker, i.e. ampicillin.

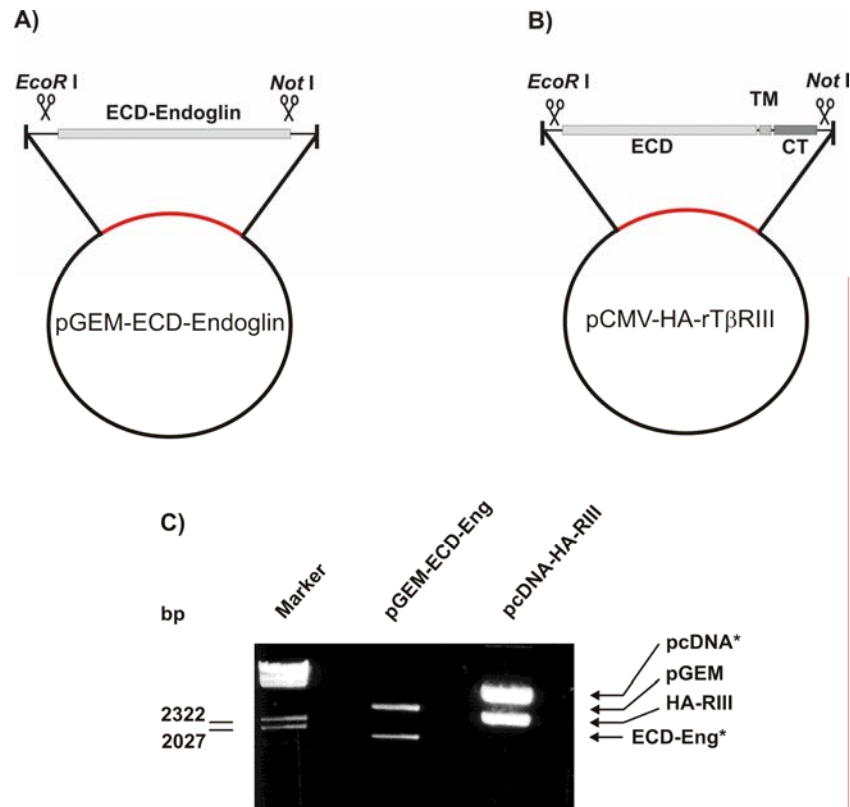


Figure 15: Preparation of the expression vector pcDNA and the coding region of ECD-endoglin. To generate a recombinant expression clone for the analysis of the effects of the ECD-endoglin on TGF- β signaling, the ECD-Eng fragment had to be transferred into an eukaryotic expression vector. We chose pCMV-HA-TβRIII including betaglycan, removed the betaglycan coding fragment by cutting the DNA with the enzymes *EcoR* I and *Not* I. The cutted empty vector was gel purified. To prepare the fragment encoding the ECD of endoglin, the recombinant clone pGEM-ECD-endoglin was cut with *EcoR* I and *Not* I and ECD coding fragment was gel purified. Fragments to be eluted are marked by an asterisk (*).

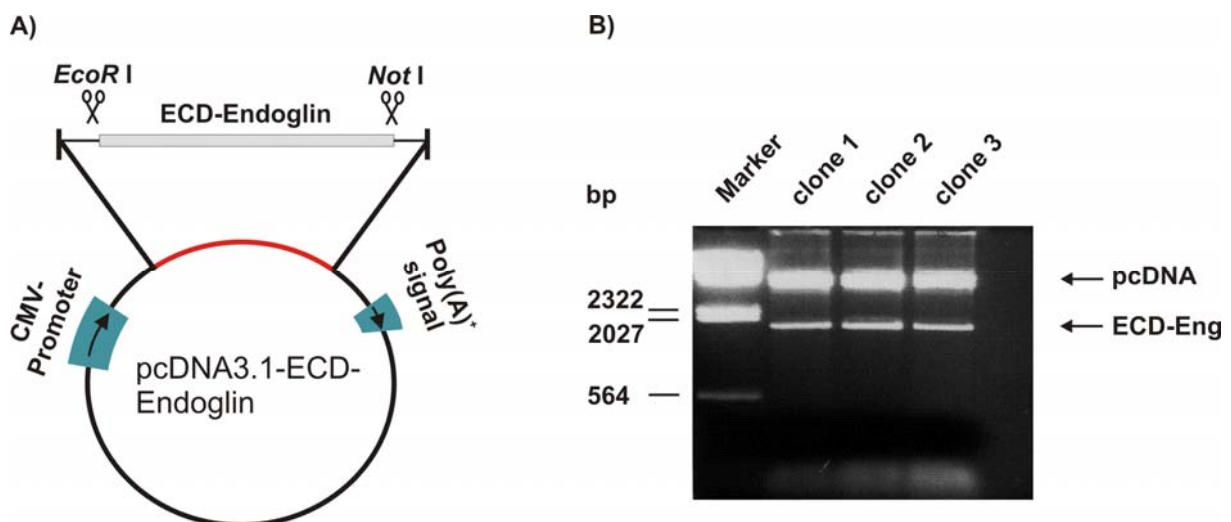


Figure 16: Restriction analysis of recombinant pcDNA-ECD-Eng clones. The purified receptor and vector fragments were used for a ligation reaction using T4-Ligase. JM105 bacteria were transformed and plated on LB dishes in the presence of ampicillin. Several single colonies were used to inoculate small scale cultures (5 ml). DNA was prepared from the bacteria and was cut with *EcoR* I and *Not* I in a double digest. One of the recombinant clones was used to inoculate a large scale culture. A) Representation of the recombinant vector. B) Restriction of recombinant clones with the restriction enzymes *EcoR* I/*Not* I. A molecular weight standard is indicated in the left margin in all gel figures.

Single, resistant colonies were used to inoculate LB-medium cultures, from which DNA was prepared and used for restriction analysis with the corresponding enzymes used for cloning, i.e. *EcoR* I/*Not* I. A representative analysis of three recombinant clones is shown in Figure 16. To obtain pure DNA of high quality for the transient transfection experiments in order to

express the ECD of endoglin, DNA of the three recombinant clones was used to inoculate large scale cultures (50-100 ml). The prepared DNA was checked as described before by restriction analysis as well as sequence analysis to characterize the boundary between the CMV-promoter and the ECD-endoglin coding region (Fig. 17). The test restriction clearly showed that the recombinant pcDNA-ECD-Eng clones migrate at a higher molecular weight compared to the non-recombinant empty vector pcDNA (Fig. 17B). This finding was verified in addition by sequence analysis confirming that the transfer was successful (Figure 17C).

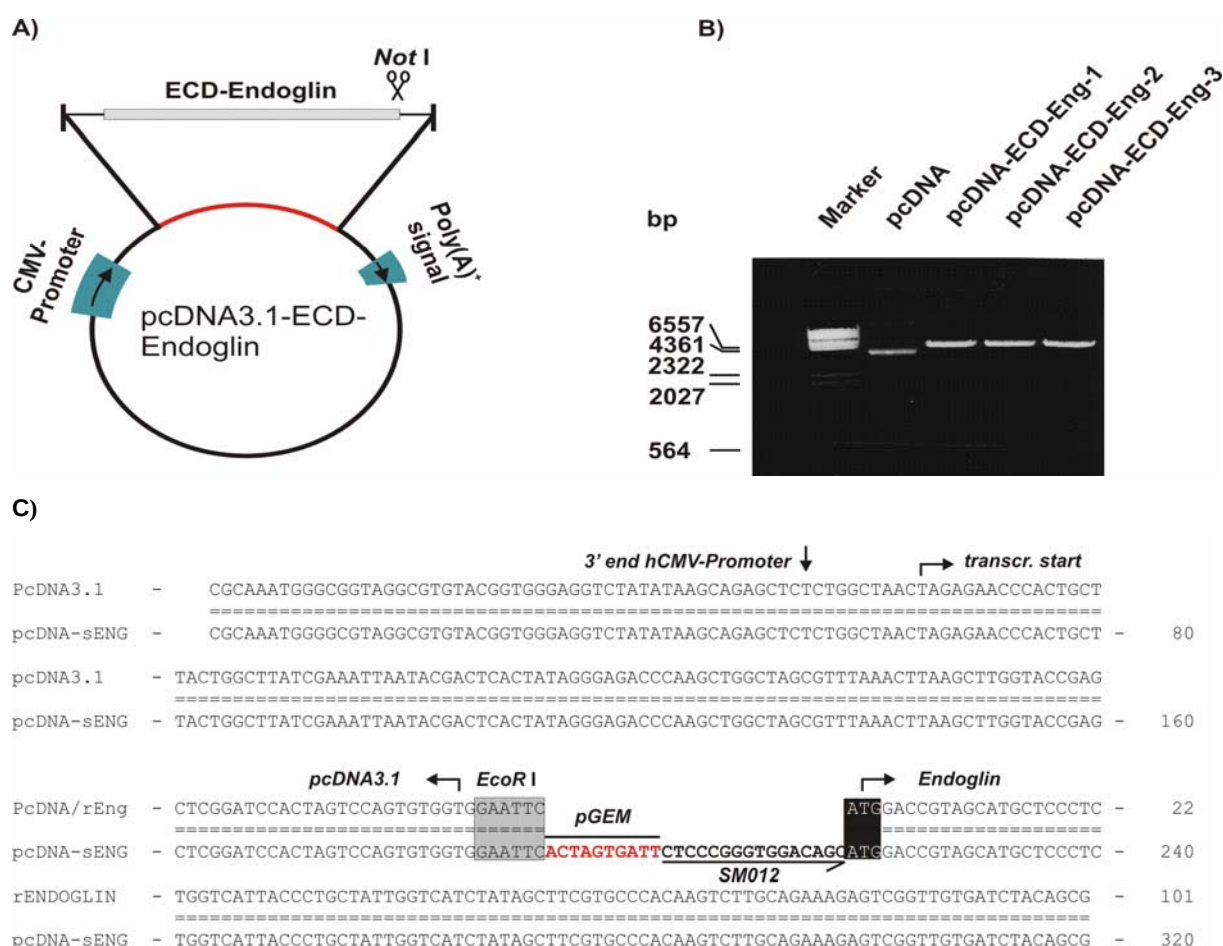


Figure 17: Characterization of the pcDNA-ECD-Eng expression construct. A) Schematic representation of the recombinant expression clone B) The DNA of the large scale preparations was restricted with *Not* I. The molecular weight of the linearized DNA of recombinant clones was compared to the molecular weight of the linearized (*Not* I) DNA of the empty vector. C) The boundary between CMV-promoter and the ECD-Eng coding region was characterized by sequence analysis showing that the fragment is correct inserted. *transcr. start*, putative transcriptional start.

3.2.2 Expression of the soluble endoglin in COS-7 and CHO cells

To test if the cloned expression vector leads to the expression and secretion of the ECD-endoglin in sufficient and detectable amounts, we employed a cellular system, i.e. COS-7 cells, which show a fibroblast morphology (Fig. 18) and are commonly used to overexpress recombinant proteins. In COS-7 cells the full length endoglin could be easily expressed in high amounts (Meurer *et al.*, 2005) making it a promising system to express the ECD of endoglin. The ECD-endoglin has no membrane anchor, therefore the domain should be secreted into the medium. The standard culture conditions in the presence of 10 % fetal calf serum makes it difficult to analyze secreted proteins because of the high amount of proteins present in the serum (5-7.5 mg/ml protein in 1 ml DMEM including 10 % FCS).

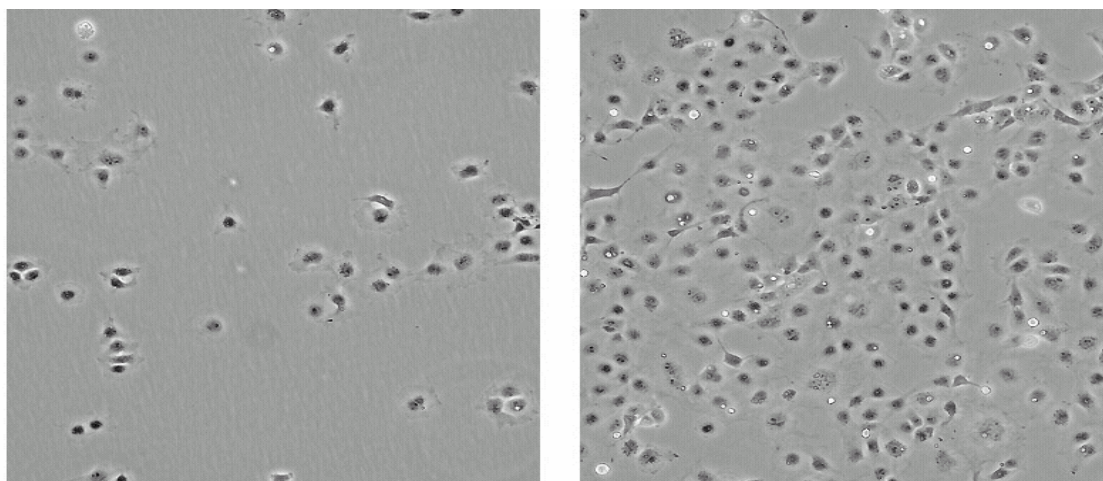


Figure 18: Cultured COS-7 cells. COS-7 cells were cultured in DMEM including 10 % FCS. Cell morphology is depicted under low (left) and high (right) plating density. (taken from the American type culture collection, ATCC) Magnification: 200x

Due to the fact that the medium has to be concentrated for analysis, serum proteins (e.g. albumin) which are already present at very high concentrations, are enriched in parallel. This causes an overload of proteins in the single lanes, an underrepresentation of proteins of interest, and a low quality analysis. Therefore we made a compromise between maximal protein expression (using 10 % FCS) and the best possible analysis (using 0.2 % FCS). COS-7 cells were transfected with three different recombinant pcDNA-ECD-endoglin clones and cultured in the presence of 0.2 % FCS. The medium was collected and cells were harvested after the appropriate time. The proteins present in the medium were precipitated with TCA and resuspended in loading buffer. Equal amounts of proteins of the supernatant and the cell

lysates were resolved in Western blot analysis using 4-12 % Bis/Tris gels under non-reducing conditions, to preserve the dimeric structure of ECD-Eng (Fig. 19). In contrast to the expected result, ECD-endoglin was detected in the supernatant only in negligible amounts (Fig. 19, clones 1-3). On the other hand, in the cell lysates there were proteins detected corresponding in molecular weight to the ECD-endoglin monomer (Fig. 19, Endoglin ECD monomer) and a weaker signal with the apparent molecular weight of the ECD-endoglin dimer (Fig. 19, Endoglin ECD dimer). This results implied that the construct itself is expressed, but most of the heterologously expressed protein is present in the monomeric form and is not secreted.

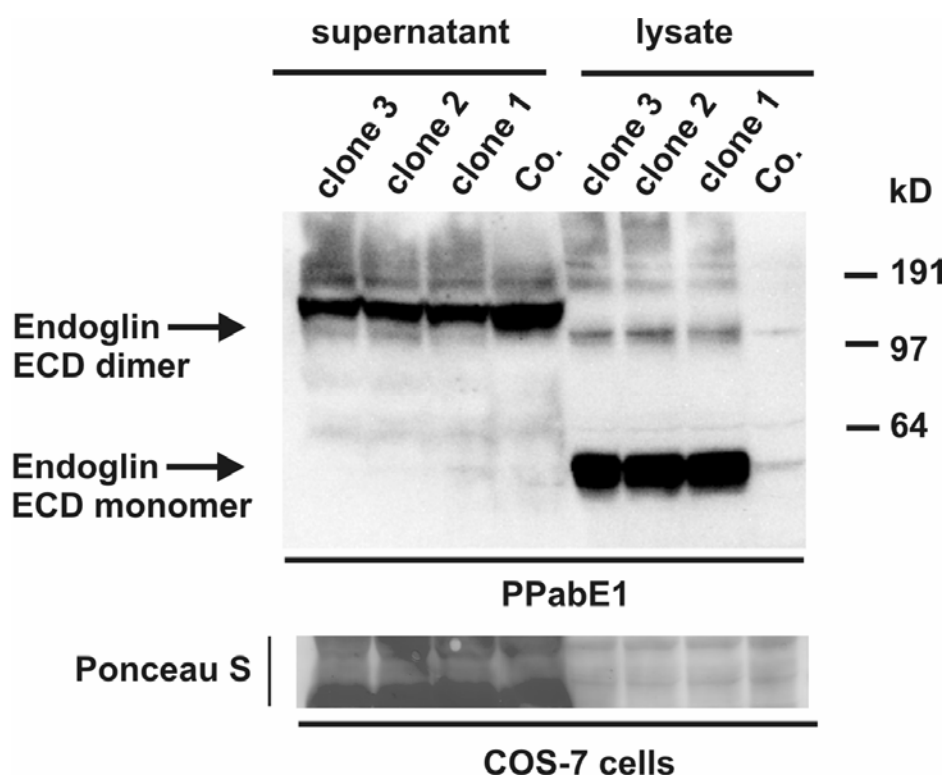


Figure 19: Heterologous expression of ECD-endoglin in COS-7 cells. COS-7 cells were transiently transfected with the Fugene6™ transfection reagent using all three recombinant pcDNA-ECD-endoglin clones (clones 1-3) or pcDNA empty vector (Co.). The supernatants were collected, cell lysates prepared and equal amounts of proteins from cell lysates and precipitated supernatants were analyzed by Western blot using 4-12 % Bis/Tris gels and MOPS running buffer. The blot membrane was developed using the rat endoglin specific antibody PPabE1. Equal protein loading is documented in Ponceau S stain. The molecular weight standard is indicated in the right margin.

From the above shown experiments, it might be difficult to evaluate the functional expression of ECD-endoglin. To circumvent any problem arising from the insufficiency of COS-7 cells to efficiently secrete proteins, we switched to an alternative expression system, i.e. CHO cells (Fig. 20). These cells display an epithelial morphology and are easily transfectable by standard transfection reagents similar to COS-7 cells. In this cell type a lot of studies have been performed where especially secreted proteins were expressed (Saharinen *et al.*, 1996). As described for the cultivation of COS-7 cells we used a reduced serum content (0.2 %) as a

prerequisite for a proper western blot analysis. CHO cells were transiently transfected using a standard procedure and the Fugene6™ transfection reagent.

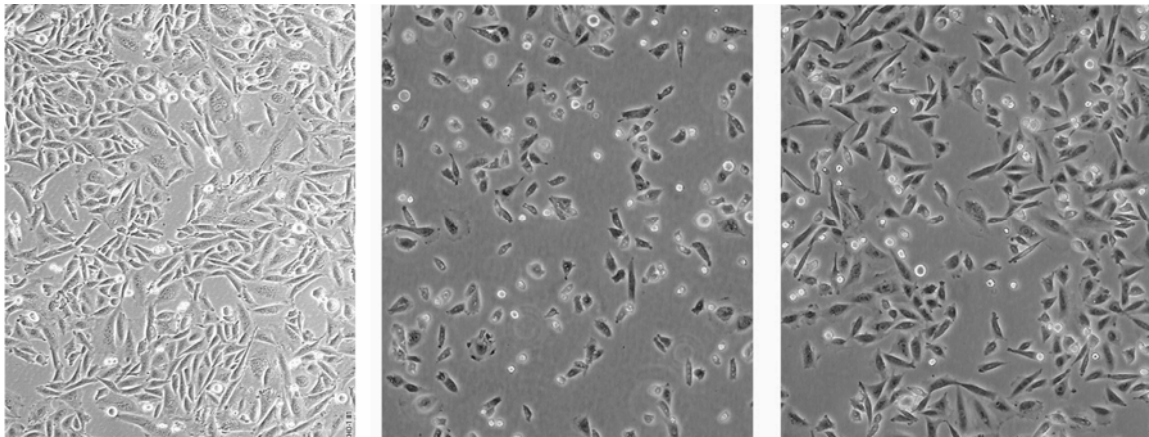


Figure 20: Cultured CHO cells. Depicted are CHO cells cultured in RPMI medium containing 10 % FCS. Cells were either cultured at a high density (left and right panel) or low density (middle panel). (taken from the American type culture collection, ATCC). Magnification: 200x

For transfection in CHO cells, it was used only the recombinant pcDNA-ECD-endoglin clone 2, which gave the “best” expression results in COS-7 cells. After harvesting the cells, the supernatants were collected and precipitated using TCA. The protein content of the cell lysates was measured and the amount of supernatant used of the single samples was calculated according to the protein content of the corresponding cell lysate so that the results of the supernatants are comparable between the different samples.

Using this approach, ECD-endoglin was expressed in a high degree and a large amount of the protein is secreted into the supernatant. In contrast to the result obtained with COS-7 cells, in CHO cells no ECD-endoglin was detected in the cell lysate itself (Fig. 21). In addition, the comparison of the samples transiently transfected with ECD-endoglin and full length endoglin showed that the ECD-endoglin has a higher migration rate (Fig. 21, lane 1) than the full length endoglin (Fig. 21, lane 2) which fits with the fact, that the ECD of endoglin is missing the transmembrane and the intracellular domains. A further validation of the truncation is the finding that the antibody sc-6199 does not detect the ECD-endoglin, due to the absence of the epitope located in the intracellular domain of endoglin (Fig. 22). The expression analysis in CHO cells demonstrated that transient transfection using the recombinant clone 2 of ECD-endoglin leads to the expression and secretion of the dimeric protein into the supernatant. Therefore, in the next step we performed functional experiments in a cell line which is highly sensitive towards TGF- β 1, the cytokine that has a strong impact on processes involving fibrosis and cirrhosis by activating one of the key intracellular mediators of ECM component synthesis, i.e Smad3.

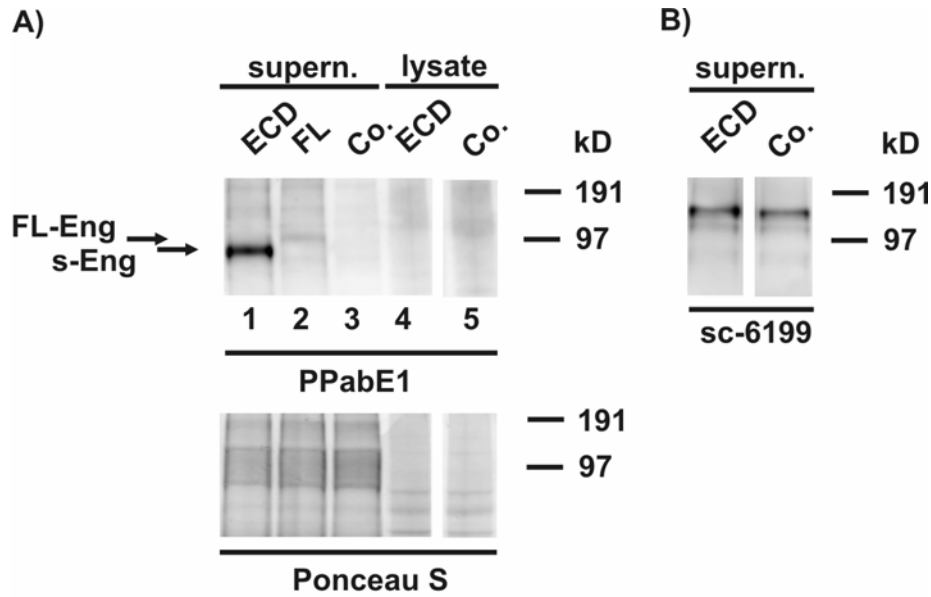


Figure 21: Expression of ECD-endoglin in CHO cells. A and B, CHO cells were transiently transfected with either ECD-endoglin (ECD, clone 2), full length endoglin (FL) or with the pcDNA empty vector (Co.) using the Fugene6™ transfection reagent. Cell lysates and proteins from precipitated supernatants (supern.) were resolved in 4-12 % Bis/Tris gels and analyzed by immuno blot using the rat endoglin specific antibody PPabE1 (A) or the endoglin cross reactive antibody sc-6199 (B). Equal loading of proteins was demonstrated by Ponceau S stain (A) lower panel. Molecular weight standards were indicated in the right margins. Note, ECD-endoglin (s-Eng) is only recognized in supernatants of transfected cells using PPabE1 (A, lane 1) and has a higher migration rate than full length endoglin (A, compare lanes 1 and 2).

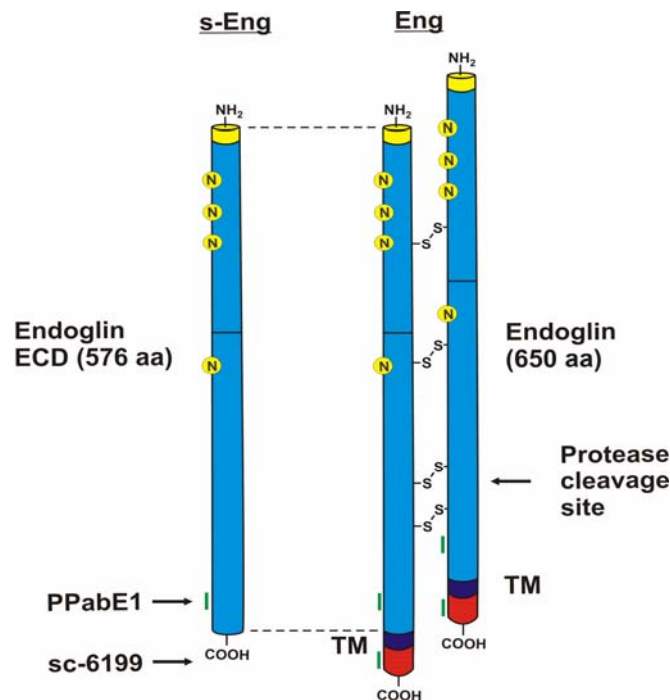


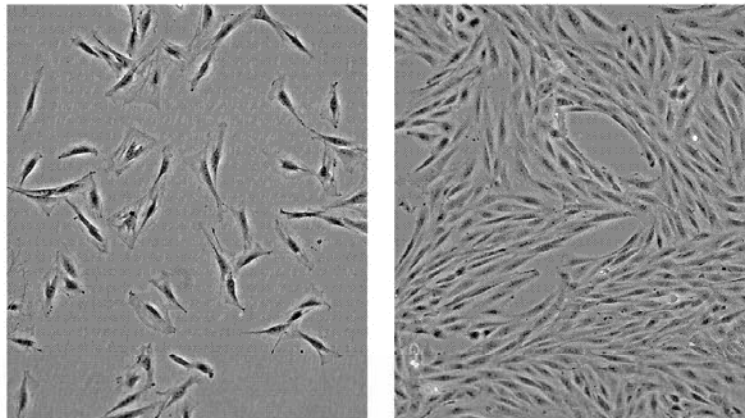
Figure 22: Structure of the full length and the soluble rat endoglin. Depicted are the structural features of endoglin including the signal peptide (yellow), the extracellular (ECD) ligand binding domain (blue), putative N-glycosylation sites (yellow circles) and disulfide bridges in the extracellular domain that were indicated by linked S-letters. The C-terminal domain is indicated in red and is joined to the ECD via the transmembranal domain (purple). The length of the amino acid chains is shown beside the receptors. The putative protease cleavage site is indicated on the right side. The epitopes of the two antibodies used in this study are localized by green lines. Note that the epitope of the antibody sc-6199 is only present in the full length receptor.

3.2.3 Functional characterization of soluble Endoglin

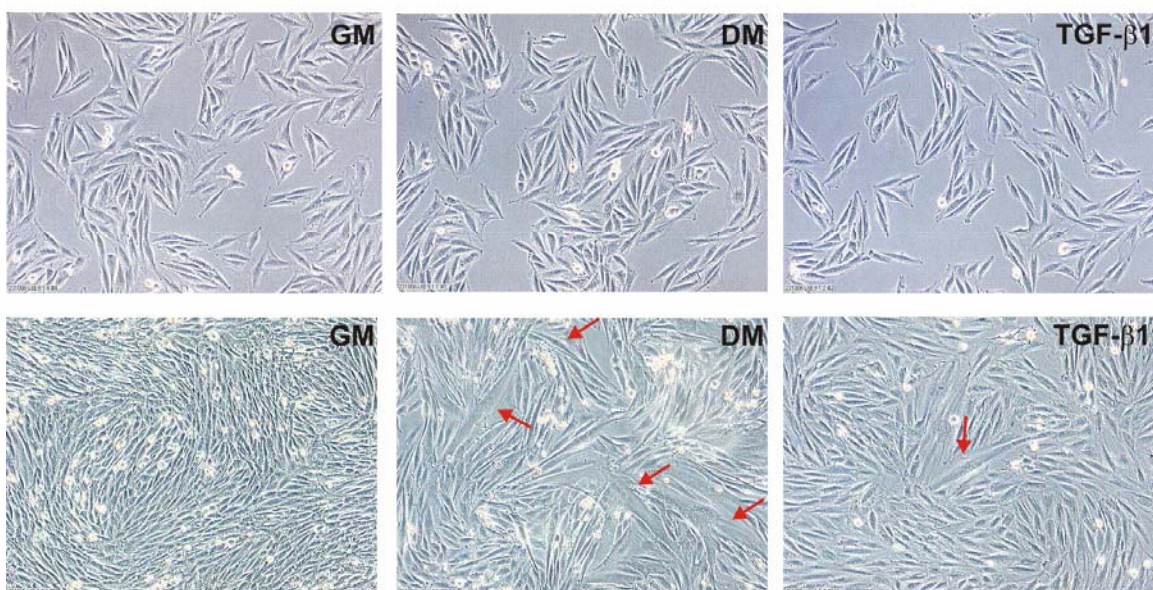
3.2.3.1 Functional analysis of soluble endoglin sCD105 in L₆E₉ cell line

The cell line L₆E₉ is derived from the L₆ myoblast cell line and is a model system to analyze TGF- β signaling and especially the role of endoglin which is not expressed by L₆E₉ cells (Schermer *et al.*, 2007). L₆E₉ cells are highly sensitive towards TGF- β and express the corresponding signaling receptor type I (ALK5) and TGF- β RII necessary for efficient signal transduction (Schermer *et al.*, 2007). In addition it was shown that these cells do not express the modular type III receptors betaglycan and endoglin (CD105) (Schermer *et al.*, 2007).

A)



B)



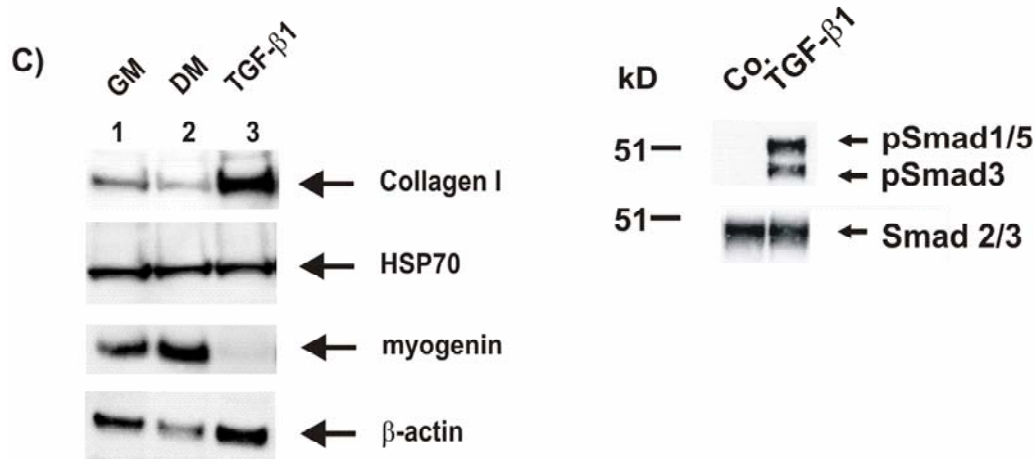


Figure 23: L₆E₉ cell morphology, marker gene expression and activation of signaling intermediates. A), Morphology of L₆E₉ cells plated at different densities. B), L₆E₉ cells cultured in growth medium (GM, left panel), differentiation medium (DM, middle panel) or in DM in the presence of TGF-β1 (TGF-β1, right panel). Cells were monitored directly after plating (upper panel) and after three days of culture (lower panel). Note, prominent fusion of cells (myotubes) occurs only in DM (red arrows). C), Expression of matrix genes (collagen I) or muscle specific marker genes (myogenin) is nearly exclusive under the different culture conditions (left panel). The key profibrogenic mediator Smad3 is potently phosphorylated in response to TGF-β1 (right panel). To monitor equal protein loading the corresponding membranes were reprobbed with antibodies against HSP70 and β-actin (left panel) or total Smad2/3 (right panel). A molecular weight standard is depicted in the left margin of the right figure. Magnification : 200 X

Cultivation of L₆E₉ cells in growth medium (GM, 20 % FCS), leads to a myoblast, single cell appearance (Fig. 23B, left panel). When the cells are shifted to differentiation medium (DM, 0.5 % FCS), the differentiation process is initiated. The cells align and fuse to form multinucleated myotubes (Fig. 23B, middle panel) (Nadal-Ginard, 1978; Blau *et al.*, 1983). As a prerequisite the cellular transcriptional program is changed. Instead of matrix genes, which are downregulated, muscle specific genes are expressed which are characteristic for this myogenic differentiation (Fig. 23C, left panel). In the presence of TGF-β, the differentiation of L₆E₉ cells under this conditions is blocked. This is monitored by an inhibition of fusion, down-regulation of myogenic genes and up-regulation of matrix genes like collagen I (Massague *et al.*, 1986; Heino & Massague, 1990; Zentella & Massague, 1992; Fig. 23B, right panel). One of the key regulators of ECM gene expression switched on by TGF-β is the transcription factor Smad3, which is phosphorylated by ALK5 upon TGF-β1 stimulation (Fig. 23C, right panel). To examine the effect of ECD-Eng on TGF-β signaling we analyzed the activation of Smad3 in the presence or absence of ECD-Eng using a Smad3 specific luciferase-reporter (CAGA)₁₂-MLP Luc (Dennler *et al.*, 1998) (Appendix Fig. 35). This reporter construct is composed of a repeated oligonucleotide (12 repeats). The respective oligonucleotide was originally characterized in the plasminogen activator inhibitor 1 (PAI 1) promoter. Phosphorylated Smad3 in complex with Smad4 binds to this oligonucleotide and

activates the transcription of the reporter luciferase-gene (Fig. 24). As has been tested before (Schermer *et al.*, 2007) transient transfection of L₆E₉ cells with high efficiency is not easy to achieve. Even when using the best suited transfection reagent “Lipofectamine” in combination with the Plus reagent transfection rates never exceed 10 %. Because of this limitation we could not detect the heterologously expressed ECD-Eng in concentrated medium. Nevertheless, the results obtained in the functional assay clearly confirmed the functional expression of the construct (Fig. 26). To analyze the Smad3 activation in L₆E₉ cells and the respective influence of ECD-Eng on this event, we transiently transfected L₆E₉ cells with the CAGA-luciferase read out system. In addition, cells were either transfected with the empty expression vector (pcDNA), the full length endoglin expression vector (FL-Eng) as a control or the ECD-Eng expression vector (Fig. 25 and Fig. 26).

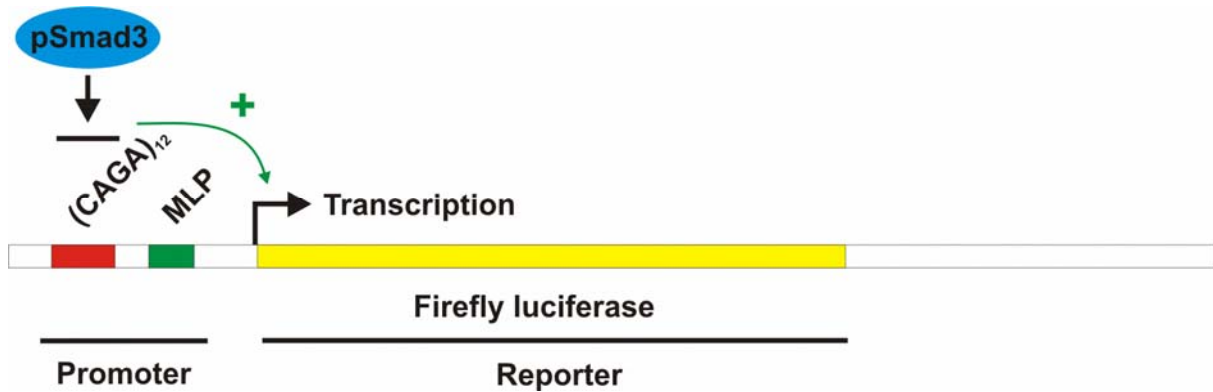


Figure 24: Schematic representation of the (CAGA)₁₂-MLP Luciferase reporter (Dennler *et al.*, 1998). Transcription of this artificial reporter is governed by binding of phosphorylated Smad3 to the CAGA-repeat element. In the presence of a minimal late promoter (MLP), luciferase is expressed. The amount of expressed luciferase is measured by determination of the luciferase activity in the samples using a standard protocol as supplied by the manufacturer. The luciferase activity indirectly reflects the extend of Smad3 activation.

To exclude a very high background signal due to the FCS medium content, the serum concentration was adjusted to DMEM containing 0.2 % FCS in the stimulation experiments. Therefore, the cells had to be cultured under this conditions at least two days before the stimulation experiment, to accumulate as much as possible of the ECD-Eng domain in the supernatant. In the presence of this low FCS conditions the cells initiate the myogenic differentiation (Fig. 25).

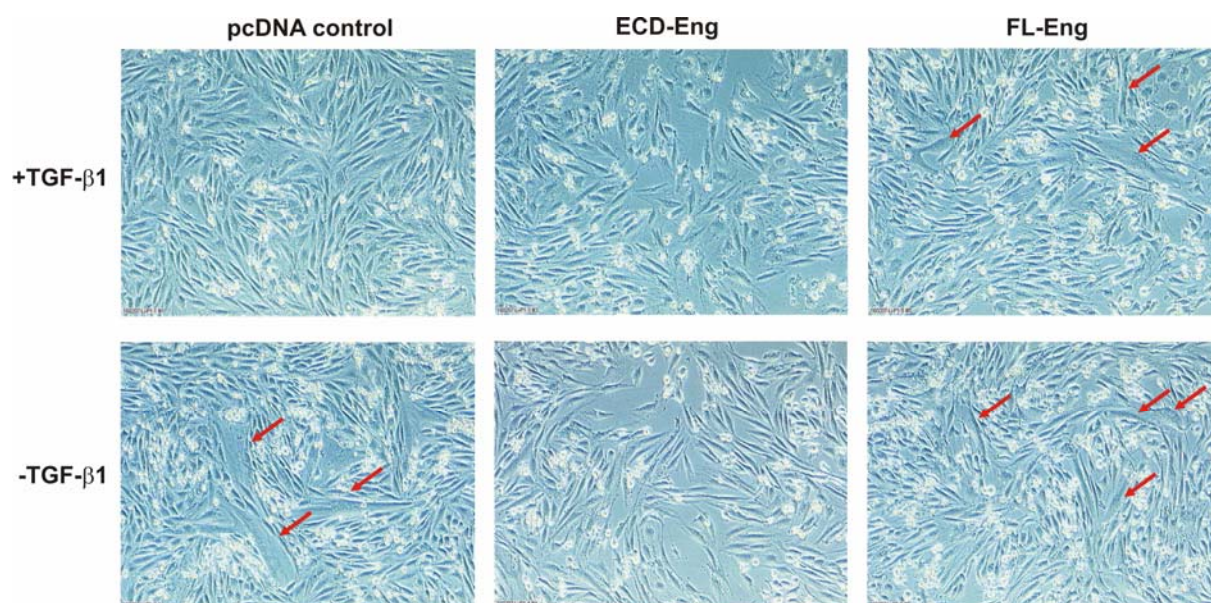


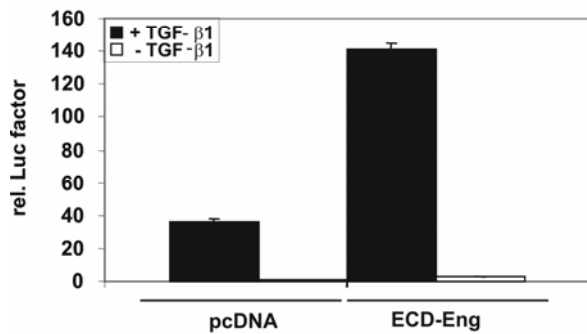
Figure 25: Morphology of L₆E₉ cells transiently transfected with empty vector, the extracellular domain of endoglin or full length endoglin and incubated or not with TGF- β 1. L₆E₉ cells were transiently transfected with the CAGA-luciferase reporter in combination with empty vector (pcDNA control, left panel), extracellular domain of endoglin (ECD-Eng, middle panel) or full length endoglin (FL-Eng, right panel). For luciferase measurement cells were stimulated with TGF- β 1 (upper panel) or left untreated (lower panel). Myotubes formed during cultivation are indicated by red arrows. Magnification: 200x

For the luciferase measurement L₆E₉ cells were co-transfected with the CAGA-luciferase reporter and empty vector (pcDNA control) as a control, the extracellular domain of endoglin (ECD-Eng) or the full length endoglin receptor (FL-Eng). To induce the reporter construct, cells were stimulated with TGF- β 1 (1 ng/ml; 24 hrs). Prior to harvesting the cells were photographed (Fig. 25). As expected control cells formed only myotubes in the absence of TGF- β 1 (Fig. 25, left panel, lower row) whereas in the presence of TGF- β 1 no myotubes were visible (Fig. 25, left panel, upper row). In ECD-Eng transfected cells obviously no myotubes were formed irrespective if TGF- β 1 was present or not (Fig. 25, middle panels). On the other hand transfection of the full length endoglin in both instances with or without TGF- β 1 leads to formation of myotubes (Fig. 25, right panels).

In the next step TGF- β 1 induced luciferase activity was determined (Fig. 26). Stimulation of L₆E₉ cells transiently with the control vector transfected with TGF- β 1 leads to a nearly 40 fold induction of the luciferase activity when compared to the untreated sample (Fig. 26A, B, pcDNA). In contrast, when stimulating L₆E₉ cells in the presence of the soluble Endoglin the luciferase activity was strongly enhanced (Fig. 26A, B, ECD-Eng, black column).

Even in cells, which were not stimulated with TGF- β 1, there was a slight up-regulation of luciferase activity (Fig. 26A, B, ECD-Eng, white column).

A)



B)

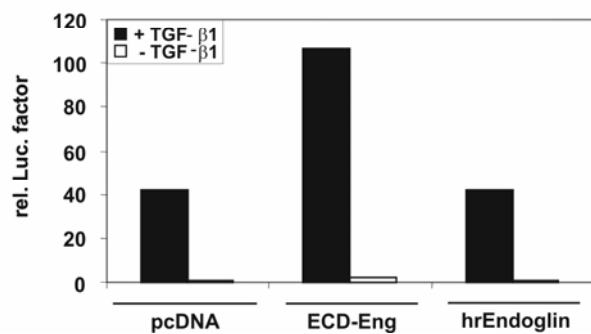


Figure 26: Influence of ECD-Eng on CAGA-luciferase reporter activity. A and B, L₆E₉ cells were transiently co-transfected with the (CAGA)₁₂-MLP Luc reporter in combination with empty vector (pcDNA, control), ECD-Eng (ECD-Eng) or incubated with the commercially available human recombinant extracellular domain of endoglin (B), rhEndoglin). Cells were incubated with or without TGF-β1 and cell lysates analyzed for luciferase activity. B, One experiment out of several is shown in which the recombinant human endoglin has been used under diverse conditions. In all experiments this domain had no effect on Smad3 activation. rel. Luc factor, relative factor of luciferase induction compared to the unstimulated control.

Because the commercially available extracellular domain of human endoglin decreased the CAGA-luciferase activity in human umbilical vein endothelial cells (HUVEC, Venkatesha *et al.*, 2006) we also included this protein in our studies. As shown for one condition in Fig. 26B, (rhEndoglin) the usage of this domain under several conditions did not modify the luciferase read out and therefore the Smad3 phosphorylation in our experiments.

Table 8: Dataset of Figure 26. All cells were transiently transfected with the CAGA reporter construct (Reporter). The cells were co-transfected with empty vector (pcDNA) or soluble endoglin (sEng). Each sample was incubated with (+TGF- β 1) or without (-TGF- β 1) ligand. The rows represent data corresponding to each experiment. A) The relative induction factors (rel. Factor) are calculated from the normalized luciferase counts per second (LCPS/Prot). In the diagram the mean rel. Factors of three representative experiments are shown (Sum) as well as the corresponding standard deviation (SD). B) The cells were treated as described in (A) except that the cells of wells 5 and 6 were incubated in parallel with the human recombinant endoglin extracellular domain.

A) Data L₆E₉CAGA-luciferase measurements

Reporter	Ligand	Construct	well	row 1		Prot mean	Prot Factor	row 1		LCPS Sum	LCPS/Prot	rel. Factor
CAGA 110706	-	pcDNA	1,000	0,180		0,180	1,000	26,330		26,330	26,330	1,000
	TGF-β1		2,000	0,180		0,180	1,000	1002,600		1002,600	1002,600	38,078
	-		3,000	0,190		0,190	0,947	67,200		67,200	63,663	2,418
	TGF-β1	sEng	4,000	0,220		0,220	0,818	4446,000		4446,000	3637,636	138,156
Reporter	Ligand	Construct	well	row 1		Prot mean	Prot Factor	row 1		LCPS Sum	LCPS/Prot	rel. Factor
CAGA 090906	-	pcDNA	1,000	0,128		0,128	0,969	8,900		8,900	8,622	1,000
	TGF-β1		2,000	0,124		0,124	1,000	270,000		270,000	270,000	31,315
	-		3,000	0,156		0,156	0,795	26,700		26,700	21,223	2,462
	TGF-β1	sEng	4,000	0,154		0,154	0,805	1430,400		1430,400	1151,751	133,583
Reporter	Ligand	Construct	well	row 1	row 2	Prot mean	Prot Factor	row 1	row 2	LCPS Sum	LCPS/Prot	rel. Factor
CAGA 241106	-	pcDNA	1,000	0,265	0,251	0,258	0,957	4,200	2,600	3,400	3,255	1,000
	TGF-β1		2,000	0,252	0,242	0,247	1,000	154,100	145,700	149,900	149,900	46,052
	-		3,000	0,256	0,255	0,256	0,967	7,900	6,300	7,100	6,864	2,109
	TGF-β1	sEng	4,000	0,248	0,252	0,250	0,988	397,900	348,800	373,350	368,870	113,324
Construct	V. 11.07.06	V. 09.09.06	V. 24.11.06	Sum	Standarddev.							
pcDNA (-)	1,000	1,000	1,000	1,000	0							
pcDNA (+)	38,078	31,315	46,052	38,482	6,023116957							
s-Eng (-)	2,418	2,462	2,109	2,329	0,157049741							
s-Eng (+)	138,156	133,583	113,324	128,354	10,79058554							

B) Data L₆E₉CAGA-luciferase measurements including hr Endoglin

Reporter	Ligand	Construct	well	row 1	row 2	Prot mean	Prot Factor	row 1	row 2	LCPS Sum	LCPS/Prot	rel. Factor
CAGA	TGF- β 1	pcDNA	1	0,265	0,251	0,258	0,957	154,100	145,700	149,900	143,509	42,209
	-		2	0,252	0,242	0,247	1,000	4,200	2,600	3,400	3,400	1,000
	TGF- β 1		3	0,256	0,255	0,256	0,967	397,900	348,800	373,350	360,929	106,156
	-	sEng	4	0,248	0,252	0,250	0,988	7,900	6,300	7,100	7,015	2,063
	TGF- β 1		5	0,268	0,273	0,271	0,913	157,400	152,900	155,150	141,671	41,668
	-		6	0,263	0,253	0,258	0,957	3,800	2,800	3,300	3,159	0,929

3.2.3.2 Functional analysis of soluble endoglin in primary rat hepatocytes.

The previously outlined experiments clearly showed that the expressed soluble domain of rat endoglin has the potential to modulate TGF- β 1/Smad3 signaling by increasing the Smad3 activity in L₆E₉ myoblast cells. Therefore, we next asked if this protein also affects the signaling in hepatocytes, the cell type which upon transformation gives rise to the hepatocellular carcinoma. For this study we analyzed isolated, cultured primary hepatocytes (Fig. 27), which do not express endoglin endogenously (S. Meurer, unpublished). The cells were plated onto collagen I coated plates and cultured in Hepatozyme medium for at least 24 hrs. The cells display an epithelial morphology and grow in connected groups of cells. Hereafter, the medium was exchanged to DMEM including 10 % FCS. Transient transfections and other treatments were performed in DMEM based medium with varying serum content. In the first step we tried to confirm the expression of ECD-Eng. In contrast to L₆E₉ cells, in primary rat hepatocytes high rates of heterologous protein expression are achieved by standard transfection methods, e.g. using the Fugene6™ transfection reagent.

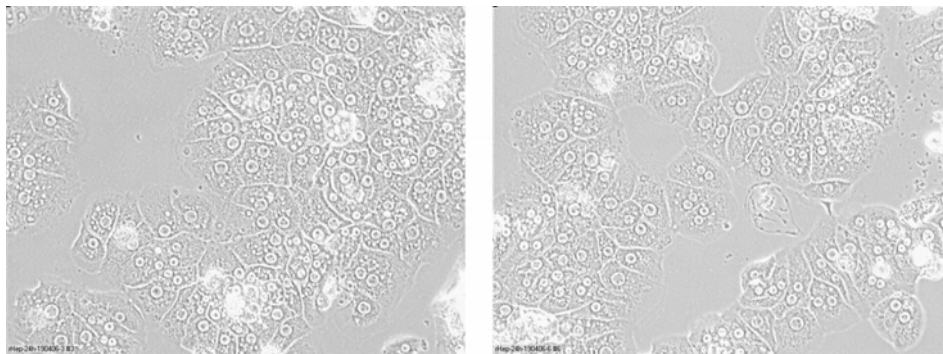


Figure 27: Morphology of cultured primary rat hepatocytes. Rat hepatocytes were isolated according to the collagenase method (Seglen, 1976). Cells were plated on collagen I support in Hepatozyme for 24 hrs. Magnification: 200x.

In order to confirm the functional expression in hepatocytes we collected in addition to the cell lysate (for luciferase measurement) the cell culture supernatant for the detection of the secreted ECD-Eng. To enhance the sensitivity, the proteins of the supernatants were precipitated with TCA and resuspended directly in sample buffer.

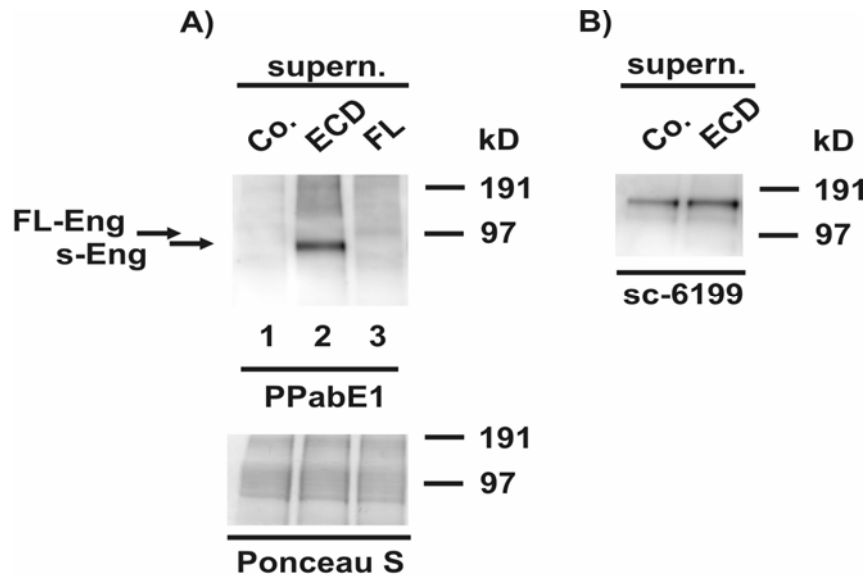


Figure 28: Expression analysis of ECD-Eng in supernatants of primary hepatocytes. A, Primary hepatocytes were transiently transfected with empty vector (lane 1, Co.), ECD-Eng (lane 2, ECD) and full length endoglin (lane 3, FL) using the Fugene6™ transfection method. Supernatants of transfected cells were harvested and proteins were precipitated using TCA and resolved in 4-12% Bis/Tris gels under non-reducing conditions. After blotting the membrane was probed with the antibody PPabE1 directed against the endoglin N-terminus. B, Following stripping the membrane was reprobed with the antibody sc-6199 directed against the endoglin C-terminus. Equal protein loading in each lane was monitored by staining the membrane with Ponceau S (A, lower). A molecular weight standard is indicated in the right margin (A, B).

For the detection of the extracellular domain and to differentiate between the soluble and the full length endoglin, we successively probed the membrane with the antibody PPabE1 against the N-terminal domain of endoglin (Fig. 28A) and the antibody sc-6199 against the C-terminal domain (Fig. 28B). In the western blot analysis, the sc-6199 antibody detected no protein in the supernatants of empty vector or ECD-Eng transfected cells (Fig. 28B). In contrast, the antibody PPabE1 recognized a protein with an apparent molecular weight of 97 kD. This protein most likely represented the dimeric soluble Endoglin, because the monomer which appeared also in the supernatant shows a relative molecular weight of 64 kD (not shown). Therefore, we could conclude that the soluble endoglin is expressed and secreted by transiently transfected hepatocytes.

In the next step, the activation of the artificial Smad3 reporter was determined. The measurement of the luciferase activities in the corresponding cell lysates clearly demonstrated that ECD-Endoglin has a functional effect on TGF- β 1/Smad3 signaling (Fig. 29).

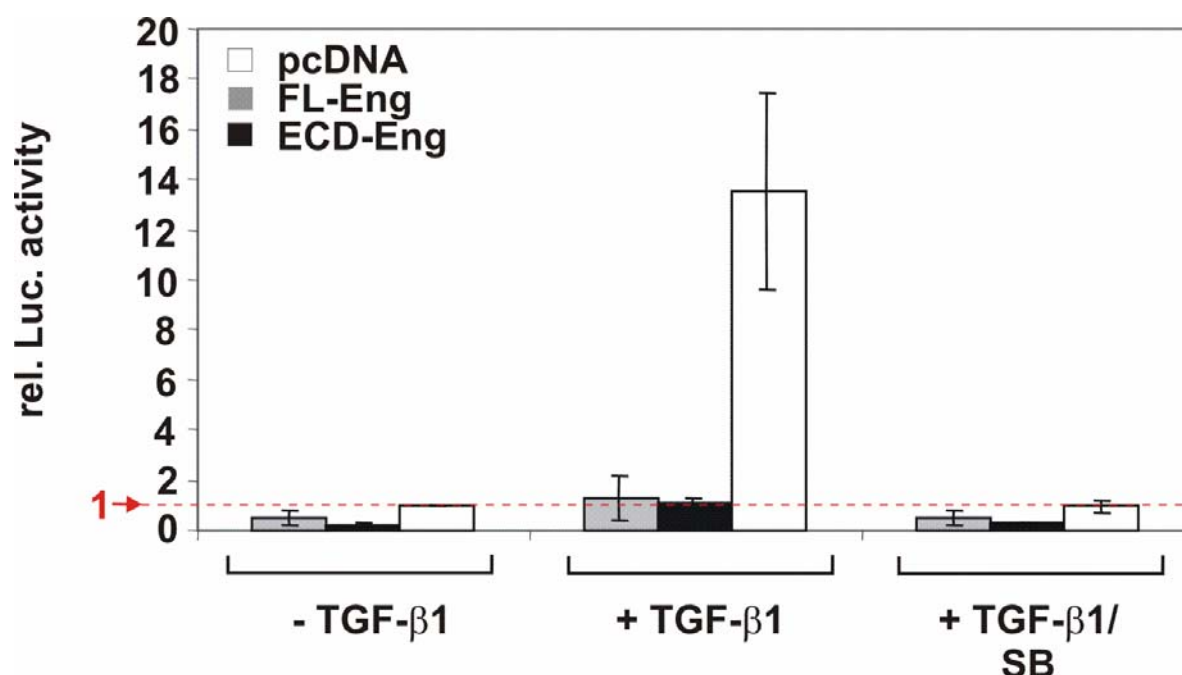


Figure 29: CAGA-luciferase determination of transiently transfected hepatocytes. As mentioned in Fig. 26A and B hepatocytes were transiently transfected with empty vector (pcDNA), full length Endoglin (FL-Eng) or the extracellular domain of Endoglin (ECD-Eng). Cells were incubated either with (+ TGF-β1) or without (- TGF-β1). If indicated (SB), cells received the type I receptor inhibitor SB431542 which inhibits ALK5, ALK4 and ALK7. The red line indicates the relative luciferase activity of 1 (pcDNA without TGF-β1) to which all the other samples were normalized. rel. Luc factor, relative factor of luciferase induction compared to the unstimulated control.

When the cells were left unstimulated the basal luciferase activity was reduced in both samples, the full length and the soluble endoglin transfected cells when compared to the pcDNA control, respectively (Fig. 29, Co.). Application of TGF-β1 led to a strong induction of luciferase activity in the pcDNA control about 14 fold (Fig. 29, white bar, + TGF-β1). In the full length and soluble endoglin transfected cells luciferase activity did not exceed the activity of the untreated control (Fig. 29, grey bar and black bar, - TGF-β1) in response to TGF-β1. To further confirm that the analyzed TGF-β1 signaling, which is modulated by ECD-Eng, is transduced *via* the ALK5 kinase receptor, we stimulated cells in the presence of the type I receptor inhibitor SB431542, which inhibits the receptor-kinases of ALK5, ALK4 and ALK7. Treatment with this inhibitor reduced the signal in all samples to that of the basal luciferase activity seen in the uninduced samples (Fig. 29, cf. panels Co. and panels TGF-β1/SB). Therefore the full length endoglin and ECD-Eng causes a complete block of the TGF-β1 induced ALK5/Smad3 signaling cascade.

Table 9: Dataset of Figures 29. All cells were transiently transfected with the CAGA reporter construct (Reporter). The cells were co-transfected with empty vector (pcDNA) or soluble endoglin (sEng). Each sample was incubated with (+TGF- β 1), without (-TGF- β 1) ligand or with TGF- β 1 in the presence of the SB compound (SB431542; TGF- β 1/SB). The rows represent data corresponding to each experiment. A) The relative induction factors (pb norm Fac) were calculated from the normalized luciferase counts per second (LCPS/Prot) by setting the unstimulated pcDNA LCPS/Prot count as 1 (Normalization first to protein amount, and subsequently normalization of all values to the unstimulated pcDNA control). The mean value of the induction factors of the two experiments is shown in the last column.

Experiments 1+2

Reporter	Ligand	Construct	well	row 1	row 2	Prot mean	Prot Factor	row 1	row 2	LCPS Sum	LCPS/Prot	pb norm Fac	mean	
CAGA	-	FL-Eng		1	0,178	0,181	0,180	0,992	30,700	28,300	29,500	26,640	0,743	0,795
	TGF-β1			2	0,186	0,180	0,183	0,973	81,700	79,200	80,450	73,212	2,042	2,133
	TGF-β1/Inh			3	0,184	0,172	0,178	1,000	34,300	30,800	32,550	31,397	0,876	0,817
	-			4	0,183	0,181	0,182	0,978	34,900	33,900	34,400	32,648	0,847	
	TGF-β1			5	0,190	0,181	0,186	0,960	90,400	81,100	85,750	85,750	2,225	
	TGF-β1/Inh			6	0,187	0,179	0,183	0,973	31,500	29,300	30,400	29,243	0,759	

CAGA	-	s-Eng		1	0,198	0,194	0,196	0,903	7,200	7,600	7,400	6,683	0,186	0,240
	TGF- β 1			2	0,191	0,198	0,195	0,910	40,500	38,400	39,450	35,901	1,001	1,145
	TGF- β 1/Inh			3	0,183	0,184	0,184	0,965	14,000	12,800	13,400	12,925	0,360	0,316
	-			4	0,186	0,187	0,187	0,949	11,900	11,900	11,900	11,294	0,293	
	TGF- β 1			5	0,180	0,174	0,177	1,000	50,200	49,200	49,700	49,700	1,289	
	TGF- β 1/Inh			6	0,187	0,181	0,184	0,962	11,200	10,500	10,850	10,437	0,271	

CAGA	-	pcDNA		1	0,183	0,189	0,186	0,989	36,400	36,100	36,250	35,860	1,000	1,000
	TGF- β 1			2	0,191	0,177	0,184	1,000	645,100	633,000	639,050	639,050	17,821	17,393
	TGF- β 1/Inh			3	0,183	0,215	0,199	0,925	34,100	30,700	32,400	29,958	0,835	0,740
	-			4	0,219	0,192	0,206	0,895	43,100	43,000	43,050	38,546	1,000	
	TGF- β 1			5	0,194	0,191	0,193	0,956	701,700	666,600	684,150	653,941	16,965	
	TGF- β 1/Inh			6	0,201	0,191	0,196	0,939	26,500	26,500	26,500	24,878	0,645	

Experiments 3+4

Reporter	Ligand	Construct	well	row 1	row 2	Prot mean	Prot Factor	row 1	row 2	LCPS Sum	LCPS/Prot	pb norm Fac	mean	
CAGA	-	FL-Eng		1	0,174	0,167	0,171	0,927	12,500	12,400	12,450	11,537	0,144	0,149
	TGF-b1			2	0,167	0,173	0,170	0,929	40,300	39,900	40,100	37,269	0,464	0,436
	TGF-b1/Inh			3	0,166	0,167	0,167	0,949	18,100	19,500	18,800	17,840	0,222	0,195
	-			4	0,172	0,167	0,170	0,932	14,700	14,000	14,350	13,376	0,155	
	TGF-b1			5	0,157	0,158	0,158	1,003	34,400	35,900	35,150	35,262	0,408	
	TGF-b1/Inh			6	0,167	0,157	0,162	0,975	15,300	14,600	14,950	14,581	0,169	

CAGA	-	s-Eng		1	0,158	0,163	0,161	0,984	17,400	16,100	16,750	16,489	0,205	0,188
	TGF-b1			2	0,155	0,158	0,157	1,010	99,800	91,800	95,800	96,718	1,205	1,091
	TGF-b1/Inh			3	0,178	0,166	0,172	0,919	23,400	22,600	23,000	21,128	0,263	0,231
	-			4	0,190	0,173	0,182	0,871	17,500	16,500	17,000	14,799	0,171	
	TGF-b1			5	0,168	0,176	0,172	0,919	95,400	88,300	91,850	84,374	0,976	
	TGF-b1/Inh			6	0,172	0,182	0,177	0,893	19,800	18,700	19,250	17,184	0,199	

CAGA	-	pcDNA		1	0,159	0,160	0,160	0,991	79,100	83,000	81,050	80,288	1,000	1,000
	TGF-b1			2	0,171	0,170	0,171	0,927	860,200	844,100	852,150	789,676	9,836	9,606
	TGF-b1/Inh			3	0,180	0,165	0,173	0,916	116,000	117,600	116,800	106,982	1,332	1,196
	-			4	0,163	0,163	0,163	0,969	89,500	88,800	89,150	86,415	1,000	
	TGF-b1			5	0,173	0,162	0,168	0,943	865,800	852,300	859,050	810,328	9,377	
	TGF-b1/Inh			6	0,185	0,177	0,181	0,873	106,000	103,800	104,900	91,570	1,060	

A)

B) The relative normalized factors of all four experiments are included in the mean induction factors shown in B. The standard deviation is calculated from the four values

B)

	Experiments 1+2	Experiments 3+4	Mean	dev
FL-Eng	0,743	0,144	0,472	0,325
	2,042	0,464	1,285	0,851
	0,876	0,222	0,506	0,314
	0,847	0,155		
	2,225	0,408		
	0,759	0,169		
sEng	0,186	0,205	0,214	0,047
	1,001	1,205	1,118	0,133
	0,360	0,263	0,273	0,058
	0,293	0,171		
	1,289	0,976		
	0,271	0,199		
pcDNA	1,000	1,000	1,000	0,000
	17,821	9,836	13,500	3,908
	0,835	1,332	0,968	0,256
	1,000	1,000		
	16,965	9,377		
	0,645	1,060		

3.2.4 Characterization of serum endoglin

It has been mentioned in the introduction, that endoglin is able to bind TGF- β 1 (Cheifetz *et al.*, 1992; Barbara *et al.*, 1999). The binding is restricted to the presence of the type I and type II TGF- β receptors. If these receptors are not present endoglin does not bind TGF- β (Lastres *et al.*, 1996; Barbara *et al.*, 1999). Venkatesha *et al.*, 2006 reported that the soluble human recombinant endoglin was shown to inhibit the binding of TGF- β 1 to the signaling receptor complex by performing cross linking experiments in HUVEC cells.

To determine if endoglin interacts with any other protein in the serum, separation of serum proteins according to their molecular weight was performed by using fast pressure liquid chromatography (FPLC). The presence of endoglin in the eluted fractions was verified by a specific ELISA for the detection of human endoglin. The relative molecular weight was determined according to a panel of proteins included in a calibration standards which were run on the column before the separation of endoglin (Reference Table 2, Fig. 36). The peak fractions of sCD105 for the patients and control samples (patients, P1-P4 upper panels; and controls C1-C2) had a K_{av} value of 0.134. Serum sCD105 showed the same elution profile as the dimeric, recombinant human sCD105 (mixed with and without serum), with a K_{av} value of 0.134. sCD105 was eluted with a single peak for the patients (Fig. 30 P1-P4, upper two

panels), the healthy controls (Fig. 30, middle panel) and the recombinant human protein (Fig. 30, lower panel) the elution volume in which the highest concentration of sCD105 for most of samples was found about 10 ml. This K_{av} represents a molecular weight around 400 kD and does therefore not only display the dimeric protein structure. Because the recombinant human Endoglin ECD showed (when mixed with serum) the same tendency to high molecular weight complexes, it seems to be a characteristic of the soluble endoglin in the serum to aggregate or interact with other serum components.

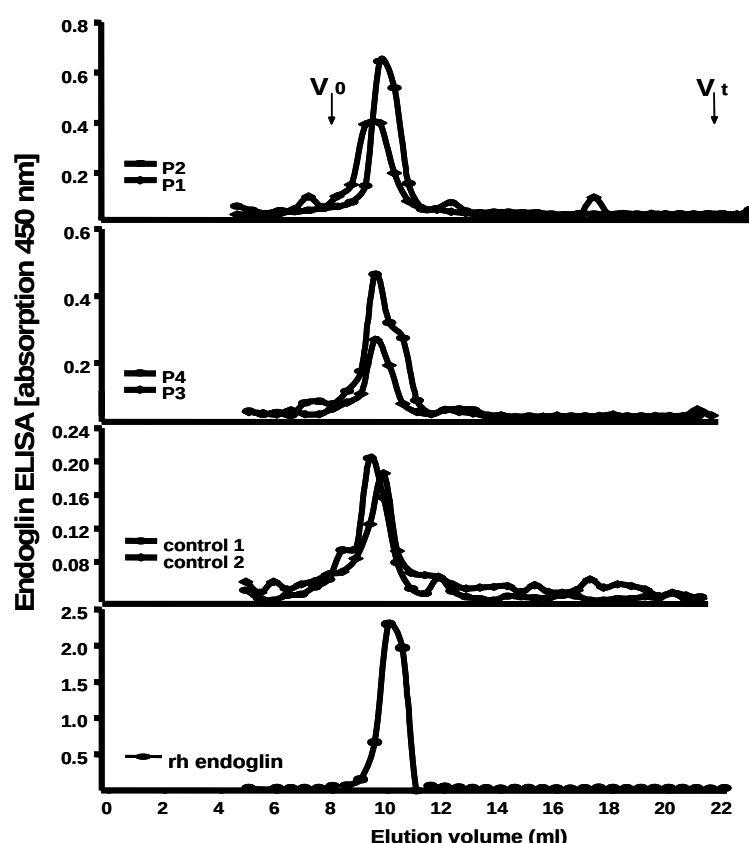


Figure 30: Elution profile of sCD105 using FPLC fractionation of serum proteins. FPLC enrichment of serum sCD105. Serum protein samples (300 μ l) of 4 patients (P1-P4, upper two panels), 2 healthy controls (control 1, 2; middle) and recombinant human sCD105 (rh endoglin lower panel) were fractionated by FPLC. Note that serum sCD105 eluted at the same position as the recombinant human sCD105 and K_{av} was calculated for the peak fractions of each sample according to the equation: $K_{av} = (V_e - V_0) / (V_t - V_0)$ (V_0 = void volume, V_t = total gel bed volume, V_e = elution volume of protein, K_{av} = value of protein of interest).

4 Discussion

4.1 Clinical value of sCD105

Hepatocellular carcinoma is one of the most prevalent solid cancers worldwide, and its incidence is still rising in different countries. It is defined as a malignant tumour derived from hepatocytes, being a primary malignant epithelial tumour of the liver [Harada *et al.*, 1980; Flicker *et al.*, 1997]. Identification of HCC in high-risk patients, who are at a potentially curable stage, is still an unsolved clinical problem and screening procedures for HCC in patients with liver cirrhosis includes like determination of serum AFP, the use of ultrasound and a variety of other radiological techniques. At present, assessment of AFP levels in conjunction with ultrasound is the “standard procedure” for screening for high-risk patients [Pateron *et al.*, 1994]. As elevated AFP is found only in 40%-75% of patients with HCC and the reported diagnostic value of AFP for detecting HCC varies widely (sensitivity of 39-97%, specificity of 76-95%, and a positive predictive value of 9-32%) when used for screening of high-risk populations [DiBisceglie *et al.*, 1988; Sherman *et al.*, 1995; Gebo *et al.*, 2002], there is up till now a strong need to find a complementary or supplementary biochemical marker for screening for HCC in high-risk patients in early stage. Taking advantage of the fact that HCC is a “hypervascular” cancer, we focused on a marker which is associated with angiogenesis, i.e. the soluble TGF- β receptor endoglin (sCD105). It is known that the expression of endoglin is linked to the activation state of the endothelium being higher expressed in activated endothelial cells [Graulich *et al.*, 1998]. Thus, in cases where active angiogenesis takes place, endoglin expression should consistently increase. With respect to tumorigenesis, the diagnostic value of soluble endoglin has been proven in the prediction of the metastasis risk in tumors like breast cancer or colon cancer patients [Li *et al.*, 2000a; Yu *et al.*, 2003; Dales *et al.*, 2004]. From the epidemiological studies, it is well established now that chronic inflammatory liver disease has the potential to promote the development of HCC, and the pathophysiologic process most commonly associated with the disease is cirrhosis, found in up to 80% [Okuda, 2000; Arrieta *et al.*, 2006; ElSaadany and Giulivi, 2006]. In the developed world the most likely causes for the development of cirrhosis are alcohol ingestion and increasingly hepatitis C infection and unfortunately once the cirrhosis is established in these chronic inflammatory liver diseases, the rate of HCC development is 1-4% per year [Johnson and Williams, 1987]. In our patient subgroups the most common causes of liver cirrhosis and HCC were viral hepatitis and alcoholic abuse, followed by cryptogenic cirrhosis (25 %)

Based on the present study sCD105 did not show a significant elevation in our fibrotic liver patients subgroup, who had a persisting high aspartate-aminotransferase and alanine-aminotransferase levels for more than 6 months but were negative for markers of viral hepatitis. More interestingly, a significant elevation in sCD105 occurred with the progression from early fibrosis to cirrhosis followed by an additional offset in values when HCC develops on the basis of cirrhosis.

It was found that in the fibrotic subgroup sCD105 levels (median 3.5 $\mu\text{g/l}$; 95% CI was 3.3 -4.1; $P=0.08$) were similar to the values found in the healthy control subgroup (Table 3 and 4). A significant elevation in the sCD105 concentration was seen in the cirrhotic subgroup (median = 5.8 μg ; 95% CI was 5.0-6.3; and $P = 0.0001$) compared to fibrotic liver subgroup, and even more interestingly that another increment in sCD105 values was seen in HCC with cirrhosis compared to cirrhotic liver patients (median= 7.4 $\mu\text{g/l}$; 95% CI was 6.4-8.8; $P = 0.0006$). The tendency for elevation of sCD105 in the last stage of fibrosis (data not shown due to the their small number) and significant elevation in established cirrhosis may be in part explained by the findings of Torsney *et al.* They found that endoglin receptor expression is upregulated in activated and proliferating endothelial cells. In addition, the expression reaches a peak coinciding with maximal T cell infiltrate. They suggested that angiogenesis is intimately associated with inflammation and endoglin may be a candidate target for anti-inflammation drug delivery [Torsney *et al.*, 2002]. Clemente *et al.*, also found that patients with advanced liver fibrosis, due to chronic hepatitis C infection, showed a significant elevation of sCD105 in comparison with patients displaying early fibrosis. Beside the elevation of sCD105 they also found an increased endoglin expression in the liver tissue itself as well as an increased in expression of the ligand TGF- β 1 in chronic viral hepatitis patients (Clemente *et al.*, 2006). Activation of endothelial cells activation as in inflammatory processes increase the endoglin expression. On the molecular basis hypoxia and TGF- β 1 have been described to have a direct regulatory role in endoglin gene expression in endothelial cells as well as in human and rat mesangial cells [Rodriguez-Barbero *et al.*, 2001; Sanchez-Elsner *et al.*, 2002]. Because the TGF- β is upregulated in the process of fibrotic liver disease this may represent one mechanism which leads under certain conditions to enhancement the expression of the transmembranal endoglin. As our laboratory group and others have published, endoglin in the liver is not only expressed by endothelial cells, but also in hepatic stellate cells (HSC) and portal and septal myofibroblasts [Meurer *et al.*, 2005; Clemente *et al.*, 2006]. HSC and their transdifferentiation is one of the key events in the establishment of liver fibrosis, and this process of transdifferentiation is promoted by TGF- β [Gressner *et al.*, 2006].

However, if transdifferentiating HSC are a potential source for an increased endoglin expression is currently under investigation.

However, we could not find a correlation between the CRP and sCD105 (Spearman rank correlation coefficient $r = 0.118$ and $r = 0.207$) in both the fibrotic and cirrhotic subgroup, respectively. This means that the sCD105 may be related more to the process of angiogenesis than to inflammation.

The state of more significant elevation of soluble endoglin in patients suffered from HCC with cirrhosis in comparison to patients with cirrhosis may be due to neoangiogenesis accompanied to hypoxia associated to neoplastic tissue [Fonsatti *et al.*, 2003; Sanchez-Elsner *et al.*, 2002; Miller *et al.*, 1999], and this also may be related to the elevated of TGF- β in HCC (Ito *et al.*, 1991; Ito *et al.*, 1995; Song *et al.*, 2002).

The wide range of values for AFP concentrations and the high standard deviations of the mean values in the subgroups of patients with chronic liver diseases (fibrotic, cirrhotic with or without HCC) weakens the screening power (sensitivity and specificity) of AFP in general in high risk patients.

The combination of the two parameters [sCD105 and α -fetoprotein], did not show an improvement with respect to sensitivity or specificity at the specific chosen cut-off levels of AFP and sCD105 as depicted in Table 7. In spite of this we suggest that if the cut-off level for α -fetoprotein was increased in this study, the sensitivity of AFP will be more decrease while the specificity will be higher, i.e sCD105 level of determination beside AFP may increase the sensitivity of the test for screening HCC in high risk group [Fig.10 and Table 7].

The logistic regression model identified sCD105 as an independent marker that may have importance in the risk assessment for development of HCC in cirrhotic patients. This study also revealed a mild positive correlation between sCD105 and AFP in patients diagnosed as having liver cirrhosis ($r = 0,358$, $P = 0,007$) and the correlation between both markers in patients group suffering from liver cirrhosis with HCC was $r = 0,479$, $P = 0,001$. This mild to moderate correlation between the sCD105 and α -fetoprotein may be due to the fact that in HCC, synthesis of AFP occurs exclusively in neoplastic hepatocytes [Flicker *et al.*, 1997]. Whereas CD105 is highly expressed in endothelial cells of both peri- and intratumoural blood vessels, and its promoter is strongly active in endothelial cells [Graulich *et al.*, 1999]. It is weakly expressed or absent on neoplastic non-endothelial cells [Fonsatti *et al.*, 2003].

sCD105 expression was also demonstrated in cervical, endometrium, breast and gastric cancer, and seems to be a marker associated with advanced tumour stages, indicating a

poor prognosis in patients with solid tumours [Brewer *et al.*, 2000, Salvesen *et al.*, 2003; Dales *et al.*, 2004; Yu *et al.*, 2003]. The positive correlation between endoglin expression and tumour growth and metastasis was mainly obtained by immunohistochemistry. However, Yang and coworkers [Yang *et al.*, 2006] showed that there is a correlation of the intratumoural microvascular density (IMVD) of CD105 immunostaining with the postoperative recurrence and metastasis of HCC. They could not find an association between liver cirrhosis and microvascular density (MVD-CD105). Moreover, Yang *et al.* [Yang *et al.*, 2006] note that some of the observed patients with a relapse of the tumour had low IMVD. Contrary, Ho and coworkers [Ho *et al.*, 2005] postulated that a tumoural microvessel density (IMVD) by CD105 immunostaining in HCC did not correlate with the prognosis of patients. On the other hand they conclude that the presence of well-diffuse patterns of CD105 expression in the adjacent non-tumorous liver could predict early disease recurrence

4.2 Functions of sCD105

The mechanism by which the soluble endoglin is generated is currently not known. It could be excluded that it is a genetically encoded splice variant of the full length receptor, because no additional splice variant has been described beside the known variants, i.e. L- and S-endoglin. Therefore it is most likely that the soluble endoglin is generated posttranscriptionally by proteolytical cleavage of the membrane receptor. A corresponding protease cleavage site has been postulated [Raab *et al.*, 1999; Llorca *et al.*, 2007]. Surface endoglin receptors might be cleaved in similar way to the shedding process described in detail for betaglycan [Velasco-Loyden *et al.*, 2004] to generate a soluble extracellular domain of the endoglin molecule. However both, the mechanism of CD105 elevation in the circulation and the functional relevance of soluble endoglin in the serum remains to be analysed. To elucidate, if soluble endoglin has any relevant biological function, the rat extracellular part (ECD) of endoglin was heterologously expressed in two cell systems to evaluate the functionality of the construct, i.e. secretion of the corresponding ECD of endoglin. The expression in COS-7 cells resulted obviously only in the appearance of monomeric ECD of endoglin located in the cell lysates (Fig. 19). Only marginal amounts of ECD of endoglin were found in the culture supernatant. On the other hand, when using CHO cells, significant amounts of ECD in dimeric form were found/secreted into the culture supernatant (Fig. 21). We have previously shown that the full length endoglin was highly expressed in COS-7 cells, and a similar high expression was observed for the full length endoglin in CHO cells. Therefore, we are able to exclude that a

higher expression in CHO cells leads to a more effective secretion of the soluble domain. We conclude that the secretion machinery in COS-7 cells is not suited for these purposes. This has been confirmed in another study in our laboratory in an attempt to heterologously express TGF- β . In COS-7 cells secretion was achieved when supplementing the cells with a specific protease, i.e. furin. In CHO cells secretion was facilitated without any modifications. Because secretion is essential for the study of TGF- β function, CHO cells have been the system of choice for these analysis [Saharinen *et al.*, 1996].

The availability of the ECD of endoglin in the cell culture supernatant was a necessary prerequisite for functional analysis. The next question was, if this domain has any impact on TGF- β signaling, which is a critical regulator of the fibrotic response and also is involved in the regulation of the endoglin expression itself. To conduct this analysis, two different TGF- β -sensitive cellular systems were employed s, i.e. L₆E₉ cells and primary hepatocytes. In both cell types Smad3 is potently activated in response to TGF- β . An established reporter system (CAGA)₁₂-MLP Luc (Fig. 24) which contains a Smad3 binding sequence from the TGF- β inducible PAI-1 promoter [Dennler *et al.*, 1998] was used to display the activity of Smad3. Although, it is found that full length endoglin provoked a similar effect in both cell types, i.e. inhibition of the TGF- β 1 signal, the soluble expressed ECD of endoglin led to a strong enhancement of TGF- β /Smad3 signaling in L₆E₉ cells (Fig. 26).

In contrast, the expression of sCD105 in primary hepatocytes caused an inhibition of TGF- β /Smad3 signaling comparable to that seen with the full length receptor (Fig. 29). In a previous study by Venkatesha *et al.*, 2006, in which commercially available ECD recombinant human endoglin was used and this protein represents an identical part of the ECD as used in our experiments. They found that sCD105 blocks several functions of endothelial cell normally mediated by TGF- β , of which one was the (CAGA)₁₂-MLP Luc assay. In a detailed analysis they unravel that the presence of sCD105 reduced the binding of TGF- β to the type II receptor in a dose dependent manner. The underlying mechanism was not analyzed, but may be explained by either of two options in addition to the difference between the two cells types used in the experiment. The first option is the sequestration of TGF- β and thereby reducing the effective ligand concentration present for binding to the type two receptor. In general the binding to the free soluble endoglin in solution is unlikely. It has been shown by several groups that the membrane bound endglin, when expressed in the absence of type I and type II receptors does not bind TGF- β [Barbara *et al.*, 1999]. However, since it was reported that soluble circulating endoglin was detected complexed to TGF- β in

the serum of patients by ELISA, the soluble endoglin shares obviously not the same ligand binding characteristics with the membrane receptor [Li *et al.*, 1998; Li *et al.*, 2000b]. It is not known whether this change in ligand binding ability is induced by loss of the C-terminus (which may impart an inhibitory influence on ligand binding normally released by interaction with the type II receptor). On the other hand this observation may be due to an interaction with another soluble factor present in the serum and/or in the cell culture medium, which may modulate ligand binding to soluble endoglin. The general tendency of the sCD105 for protein interactions in the serum was pointed out in our FPLC analysis, in which the sCD105 eluted at a much higher molecular weight (~400 kDa), as would be expected for the dimeric protein (~130kDa). Albeit these general mechanisms may explain the findings in endothelial cells and hepatocytes, they do not solve the different behaviour of sCD105 in L₆E₉ cells and primary hepatocytes. A sequestration of TGF- β as well as an interaction with a common component would result in a similar response in both cell types. A notable difference in our experiments was the different expression level achieved in both cellular systems. Whereas we could not detect the soluble endoglin in supernatants of L₆E₉ cells, the protein was clearly detectable in the supernatants of expressing primary hepatocytes. This difference in expression was due to the highly variable transfection efficiencies in both cell types, being far lower in L₆E₉ cells compared to primary hepatocytes. If the function of the soluble domain is related to its concentration, the different outcomes could be explained. In addition, this would have consequences for the *in vivo* situation, where the concentration of soluble endoglin is locally high at the site of generation and decreases when it gets diluted in the system.

Another category of proteins able to modulate signaling are soluble factors which regulate ligand binding to their cognate surface receptors. This mechanism facilitates a different response in cells expressing the same subset of receptors [Massague 2000]. One of these regulators, i.e. CTGF, is expressed in both cell types [Obreo *et al.*, 2004; Wickert *et al.*, 2007]. If there are expressed modulatory proteins in either cell type with the potential to provoke a differential responsiveness as seen in our experiments is currently not known.

The inhibitory effect observed for TGF- β signaling in primary hepatocytes may lead in the liver to a protection of hepatocytes from the effects of TGF- β 1, mainly apoptosis or epithelial to mesenchymal transition [Samson *et al.*, 2002; Hayashi *et al.* 2004 ; Kaimori *et al.*, 2007]. We suggest that this elevation of soluble endoglin reflects the over expression of endoglin receptor with the persistent chronic liver inflammation to counteract the effect of elevated TGF- β ligand which acts as potent growth inhibitor for preferentially epithelial cell

types, and plays a key role in the control of parenchymal apoptosis, and stimulates the production of ECM components.

The results of this study led us to conclude that it may be useful to implement sCD105 in future diagnostic algorithms to identify patients with liver cirrhosis at high-risk for HCC. Therefore, further studies using serum samples from larger cohorts of patients are needed to evaluate the diagnostic value of this novel serum marker.

In addition to the sCD105 value as a complementary biomarker in high risk patients, circulating soluble endoglin could exert a modulatory effect on the TGF- β 1 signaling pathways. This effects may be different with respect to the target cell type encountering this soluble receptor domain, similar to the effects which were observed for the heterologously rat ECD endoglin in primary rat hepatocytes and L₆ E₉ rat skeletal muscle cells, of which both do not express endoglin endogenously. It was obvious that sCD105 inhibited TGF- β 1/Smad3 signaling, measured by the CAGA-luciferase reporter, in primary hepatocytes. In contrast, heterologously soluble endoglin had a stimulatory effect on TGF- β 1/Smad3 signaling in L₆ E₉ cells. From these findings we conclude that the presence of soluble endoglin in the circulation, due to a yet unknown mechanism, is not only a passive event (i.e passive shedding), but could modulate signaling of TGF- β 1.

5 Summary

Liver cirrhosis and chronic viral hepatitis-B and -C infections are well-known risk conditions for the development of HCC, and identification of HCC in high-risk patients, who are at a potentially curable stage, is still an unsolved clinical problem. Therefore, the diagnostic potential and value of soluble endoglin in patients with liver cirrhosis at high-risk of HCC was investigated. In addition, we elucidated the functional impact of heterologously expressed soluble endoglin on TGF- β 1/Smad3 signaling by using an artificial reporter system for the detection of phosphorylated Smad3, i.e. (CAGA)₁₂-MLP Luc. For the evaluation of sCD105 as a marker for the occurrence of HCC in high-risk patients, we measured the concentration of sCD105 in both, 121 patients of chronic liver disease visited Aachen university hospital during one year and 70 healthy individuals, constituting the control group. In parallel, AFP serum concentrations were measured, to compare the results of the sCD105 measurements with an established marker. The 121 patients were classified into 19 patients with liver fibrosis, 57 cirrhotic patients, 45 cirrhotic patients with HCC and 9 patients with HCC only. While sCD105 showed no significant difference in concentration between the healthy group with a median 3.7 μ g/L and patients with first grade of liver fibrosis median 3.5 μ g/L. The most important finding was the discriminatory potential of endoglin with respect to the severity of fibrosis first and malignant transformation as the second, i.e. that first sCD105 concentration showed a significant (P value = 0.0001) higher value in patients with cirrhosis having a median of 5.8 μ g/L compared to the fibrotic group. On the other hand, there is a further significant increase in sCD105 concentration in the cirrhosis with HCC group (median 7.4 μ g/L; P value = 0.0006) compared to the cirrhotic group. Moreover, sCD105 shows higher diagnostic sensitivity when compared to AFP (≥ 90.9 vs. 81.8%) and there was only mild to moderate correlation between these two parameters in the latter groups. For the functional analysis, the rat soluble endoglin was first cloned, followed by the functional expression of the protein in TGF- β sensitive cells, i.e. L₆E₉ cells and primary hepatocytes. The presence of the soluble endoglin in both of these systems modulated the TGF- β 1/Smad3 signaling as evaluated with the phospho-Smad3 specific reporter (CAGA)₁₂-MLP Luc.

From the reported data we conclude that circulating sCD105 may be a complementary marker to identify patients with liver cirrhosis who are at high-risk of HCC. However, further confirmatory studies are needed to evaluate the application potential of this novel serum marker in a large cohort of patients. In addition, we showed that soluble CD105 has a role in TGF- β signaling, as we could clearly show by the heterologous expression of the rat soluble

endoglin. Therefore, it is most likely that the circulating soluble endoglin induces a pathophysiological effect by modulation of TGF- β signaling in responsive cells.

Zusammenfassung

Leberzirrhose und viral bedingte chronische Hepatitis-B und -C Infektionen stellen bekannte Risikofaktoren für das Auftreten von HCC dar. Die Identifizierung von Hoch Risikopatienten, bei denen ein kurativer Eingriff noch möglich ist, stellt derzeit ein ungelöstes Problem dar. In der vorliegenden Arbeit wurde aus diesem Grund das diagnostische Potential von löslichem Endoglin in Hoch Risikopatienten untersucht. Zusätzlich haben wir untersucht, ob lösliches Endoglin einen funktionellen Einfluß auf den profibrogenen TGF- β /Smad3 Signalweg hat. Zur Untersuchung der Relevanz von löslichem Endoglin bei der Identifizierung von HCC Riskopatienten haben wir dessen Konzentration in 121 Patienten mit chronischer Lebererkrankung sowie 70 gesunden Probanden gemessen. Zu Vergleichszwecken haben wir ebenfalls den etablierten Marker AFP bestimmt. Die 121 Patienten wurden unterteilt in Fibrotiker (19 Patienten), Zirrhotiker (57 Patienten), Zirrhotiker mit HCC (45 Patienten) und 9 Patienten mit HCC. Zwischen gesunden Probanden (3,7 $\mu\text{g/L}$) und Fibrotikern konnte kein signifikanter Unterschied im löslichen Endoglin festgestellt werden. Demgegenüber stieg die Konzentration von löslichem Endoglin von Zirrhotikern (5,8 $\mu\text{g/L}$; P Wert 0,0001) im Vergleich zu den Fibrotikern und die Konzentration von löslichem Endoglin von Zirrhotikern mit HCC (7,4 $\mu\text{g/L}$; P Wert 0,0006) im Vergleich zu Zirrhotikern signifikant an. Im Vergleich zu AFP zeigte lösliches Endoglin eine höhere Sensitivität ($\geq 90,9\%$ vs. 81,8%) und es lag nur eine milde bis moderate Korrelation dieser Marker zwischen den beiden letzten Vergleichsgruppen vor. Zur Untersuchung der Funktion des löslichen Endoglins wurde dieses zunächst kloniert und hiernach in TGF- β sensitiven Zellen einer funktionellen Analyse unterzogen. Mit Hilfe des Smad3 spezifischen Reporters konnten wir zeigen, dass lösliches Endoglin TGF- β /Smad3 Signalweg modulieren kann.

Zusammenfassend kann gesagt werden, dass Endoglin einen komplementären Marker darstellen könnte, um HCC in Risikopatienten zu diagnostizieren, dessen klinische Wertigkeit allerdings zunächst an einer größeren Kohorte von Patienten validiert werden müsste. Ebenfalls scheint lösliches Endoglin ein funktionelles Potenzial zu besitzen, indem es zumindest *in vitro* das profibrogene TGF- β /Smad3 Signal moduliert.

6 References

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7 Appendix

Description of cDNAs and vectors used in this thesis

7.1 Sequence of the rat TGF- β receptor endoglin cDNA and corresponding translation.

```
ATG GACCGTAGCATGCTCCCTCTGGTCATTACCTGCTATTGGTCATCTATAGCTTCGTGCCCAAGTCTTGAGAAAGAGTCGGTGT 90
M D R S M L P L V I T L L L V I Y S F V P T S L A E R V G C 30
GATCTACAGCGTGTGGACTCCACAAGGGGTGAGGTGACATACACCACCAGCCAGGTCTCTGAGGGCTGTGTAGCTCAGGTTGCCAACGCT 180
D L Q R V D S T R G E V T Y T T S Q V S E G C V A Q V A N A 60
GCGCATGAAGTCCACGTCTCTTCTTGAATCTTTCCAGAAGGAAGTCCGAGGTGGAGTTGACTCTTCAAGCATCCAAGCAAAATGGCACT 270
A H E V H V L F L N L S R R K S E V E L T L Q A S K Q N G T 90
GAGACTCGGGAGGTGTTTCTGGTCTTCATTTCGAACGAGAATGTCTTGGTGAAGCTCCAGGCCCCAGAAATCCCACTGCACTTGGTCTAC 360
E T R E V F L V F I S N E N V L V K L Q A P E I P L H L V Y 120
AACTCCAGCCTCGAGGTCTTCAAAGGACCAAAAGTCAATAGCACACCACTACCATCTTTACCTCCAAGACACAGATCTCGACTGGGCA 450
N S S L E V F K G P K V N S T P L P S F T S K T Q I L D W A 150
GCCACCAAGGGCACCATTACCTCCATAGCAGCACTGGATGACCCCAAAAGCATTGTCTCCGGCTGGGCCAAGACCCAAAGGCACCACCC 540
A T K G T I T S I A A L D D P K S I V L R L G Q D P K A P P 180
TTCTGCTTCCCAGAAGCTCAGAAGGACATGGGCGTCACACTTGAATGGCAACCACGAACCCAGACACCAAGGCTGCCACTTGGAA 630
F C F P E A Q K D M G V T L E W Q P R T Q T P V Q G C H L E 210
GGTGTGACTGGACACAAGGAGGCTATGTCTGAGGATTTCGATCAGGTTCTGAGGCCGGGCCCGGACAGTGACGGTAACGGTGAACTG 720
G V T G H K E A Y V L R I R S G S E A G P R T V T V T V K L 240
AGTGCACATCTGGGATGCGGTTCTCATCTGCAGGTTCCCGGTACGTCTCTGGCTCATCGATACCAACCACAATATGCAGATCTGG 810
S C T S G D A V L I L Q G P P Y V S W L I D T N H N M Q I W 270
ACCACAGGTGAATACTCCATCAAGATCTTTCCAGAAAACAACATCAAAGGCTTCGAGCTCCAGACACACCCCAAGGCCTGATCGGGGAG 900
T T G E Y S I K I F P E N N I K G F E L P D T P Q G L I G E 300
GCCCGCAAGCTCAATGCCAGCATAGTAACCTTTGTAGAGATCCCTCTGACCAAGTATGCTCTCCCTGACGGTCTCCAGCTGCGGTGGTGGG 990
A R K L N A S I V T F V E I P L T S D V S L T V S S C G G G 330
CTCCAGACCTCCCTGCACCACTTGTGACCACTCCCAAGGACACGTGCAGCCCTGAGCTACTCATGTCCCTGATTGAGCCAAAGTGT 1080
L Q T S P A P V V T T P P K D T C S P E L L M S L I Q P K C 360
GGCAATGACGTCATGACTCTGGCACTCAATAAAAACTCGTGCAGACTCTACAGTGCACCATCACAGGCCTGGCTTTCTGGGACTCCAGC 1170
G N D V M T L A L N K K L V Q T L Q C T I T G L A F W D S S 390
TGCCAGGCTAAAGACCAGGACGCCACCTTGTCTGAGTAGCACTACTCCAGCTGCGGCATGAAAGTGACAGATCATGTGATCAGCAAC 1260
C Q A K D Q D G H L V L S S T Y S S C G M K V T D H V I S N 420
GAGGTGATCATCAATTTGCCATCAGGCTTACCACCACTTCGGAAGAGGTACAGTGCATCGACATGGACAGCCTCTCCTTCCAGCTGGGC 1350
E V I I N L P S G L P P L R K K V Q C I D M D S L S F Q L G 450
CTCTACCTCAGCCGCACTTCTCCAGGCATCCAACACCATAGAGCTAGGCCAGCAGGGCTTCGTACAGGTGAGCATGTCTCCATTGACC 1440
L Y L S P H F L Q A S N T I E L G Q Q G F V Q V S M S P L T 480
TCTGAGGTACAGTCCAGCTAGACAGCTGCCATCTGGATTGGGGCCAGAAGTGACATGGTGGAAGTCACTCCAGAGTCGAGCAGCCAAAG 1530
S E V T V Q L D S C H L D L G P E G D M V E L I Q S R A A K 510
GGCAGCTGTGTGAGCTTGCTGTCCCAAGCCCTGAGGGCGACCCACGTTTCAGCTTTCTCCTCCGTGTCTACATGGTGCCACACCCGCA 1620
G S C V S L L S P S P E G D P R F S F L L R V Y M V P T P A 540
GCTGGCACCTCAGTTGCAACTTAGCCCTGCACCTTAGCACCTTGTCCAGGAAGTCTACAAGACTGTCTCCATGCGCCTGAACATCGTC 1710
A G T L S C N L A L H P S T L S Q E V Y K T V S M R L N I V 570
AGTCTGACCTGTCTGGCAAGGCTCGTCTGCTGCTCTGTACTGGGCATCACCTTTGGTGCCTTCTCATTGGGGCCCTGCTCACAGCT 1800
S P D L S G K G L V L P S V L G I T F G A F L I G A L L T A 600
-----
GCACTCTGGTACATCTATTCTCACACAGTGCCTTAGCAAGCGGGAGCCCGTGGTGGCAGTGGCTGCCCCGGCCTCTCTGAGAGCAGC 1890
A L W Y I Y S H T R A P S K R E P V V A V A A P A S S E S S 630
AGTACCAACCACAGCATCGGGAGCACCCAGAGCACCCCTGCTCCACCAGCAGCATGGCATAGTACCCAGCCTGGAGCCTTGGGGAGCCC 1980
S T N H S I G S T Q S T P C S T S S M A * 650
CCAGCCAGCCCTTGCCCTGCAGGAGAGGCGGAGCAGCCACAAGCTGGGAACCATCGGCCATGAAGTGTCCCCTCAGCCAAGGATGGAGC 2070
GTCTCTCTGTACTGCTCTCATGCCTCCCCGAGAGGCTTGTGCCAGTAGAGACTCCAAGTGGAAACACCTTGGGATCTCTCAACC 2160
CACTTTACCTGCCCAACCCAGGGCCCTGGGATATGGCTGCCTGGGACTACAGAAAGACACCGCACTGCT 2230
```

Figure 31: Structure of rat endoglin cDNA. The nucleotide and deduced amino acid sequence of the rat endoglin cDNA is shown. Nucleotides and amino acids are numbered on the right. The translational initiation

and termination codons are shown in *white* type on *black*. The signal peptide and the transmembrane region are marked by *solid* or *intermitted lines*, respectively (Meurer *et al.*, 2005).

7.2 pGEM®-T Easy sol-Endoglin

RENDOFL sol-Endg	EcoR I GAATTC ACTAGTGATTCTCCCGGGTGGACAGC ATG SM012	ATGACCCTAGCATGCTCCCTCTGGTCATTACCTGCTATTGGTCAT SACCGTAGCATGCTCCCTCTGGTCATTACCTGCTATTGGTCAT	- 47
RENDOFL sol-Endg	CTATAGCTTCGTGCCCAAGTCTTGCAGAAAGAGTCGGTTGTGATCTACAGCGTGTGGACTCCACAAGGGGTGAGGTGA CTATAGCTTCGTGCCCAAGTCTTGCAGAAAGAGTCGGTTGTGATCTACAGCGTGTGGACTCCACAAGGGGTGAGGTGA		- 127
RENDOFL sol-Endg	CATACACCACCGAGGCTCTCTGAGGGCTGTGTAGCTCAGGTTGCCAACGCTGCGCATGAAGTCCACGTTCTCTTCTCTG CATACACCACCGAGGCTCTCTGAGGGCTGTGTAGCTCAGGTTGCCAACGCTGCGCATGAAGTCCACGTTCTCTTCTCTG		- 207
RENDOFL sol-Endg	AATCTTTCCAGAAGGAAGTCCGAGGTGGAGTTGACTCTTCAAGCATCCAAGCAAAATGGCACTGAGACTCGGGAGGTGTT AATCTTTCCAGAAGGAAGTCCGAGGTGGAGTTGACTCTTCAAGCATCCAAGCAAAATGGCACTGAGACTCGGGAGGTGTT		- 287
RENDOFL sol-Endg	TCTGGTCTTCATTTCGAACGAGAATGTCTTGGTGAAGCTCCAGGCCCCAGAAATCCCACTGCACTTGGTCTACAACCTCA TCTGGTCTTCATTTCGAACGAGAATGTCTTGGTGAAGCTCCAGGCCCCAGAAATCCCACTGCACTTGGTCTACAACCTCA		- 367
RENDOFL sol-Endg	GCCTCGAGGTCTTCAAAGGACCAAAAGTCAATAGCACACCACTACCATCTTTACCTCCAGACACAGATCCTCGACTGG GCCTCGAGGTCTTCAAAGGACCAAAAGTCAATAGCACACCACTACCATCTTTACCTCCAGACACAGATCCTCGACTGG		- 447
RENDOFL sol-Endg	GCAGCCACCAAGGGCACCATTACCTCCATAGCAGCACTGGATGACCCCAAAGCATTGTCTCCGGCTGGGCCAAGACCC GCAGCCACCAAGGGCACCATTACCTCCATAGCAGCACTGGATGACCCCAAAGCATTGTCTCCGGCTGGGCCAAGACCC		- 527
RENDOFL sol-Endg	AAAGGCACCAACCTTCTGCTTCCCAGAAGCTCAGAAGGACATGGGCGTCACACTTGAATGGCAACCAGAACCCAGACAC AAAGGCACCAACCTTCTGCTTCCCAGAAGCTCAGAAGGACATGGGCGTCACACTTGAATGGCAACCAGAACCCAGACAC		- 607
RENDOFL sol-Endg	CAGTCCAAGGCTGCCACTTGGAAAGGTGTGACTGGACACAAGGAGGCCTATGTCTGAGGATTTCGATCAGGTTCTGAGGCC CAGTCCAAGGCTGCCACTTGGAAAGGTGTGACTGGACACAAGGAGGCCTATGTCTGAGGATTTCATCA GTCCTGAGGATTTCGATCAGGTTCTGAGGCC		- 687
RENDOFL sol-Endg	GGGCCCCGACAGTGACGGTAACGGTGAAACTGAGTTGCACATCTGGGGATGCCGTTCTCATCTGCAGGGTCCCCCGTA GGGCCCCGACAGTGACGGTAACGGTGAAACTGAGTTGCACATCTGGGGATGCCGTTCTCATCTGCAGGGTCCCCCGTA		- 767
RENDOFL sol-Endg	CGTCTCCTGGCTCATCGATACCAACCACAATATGCAGATCTGGACCACAGGTGAATACTCCATCAAGATCTTTCCAGAAA CGTCTCCTGGCTCATCGATACCAACCACAATATGCAGATCTGGACCACAGGTGAATACTCCATCAAGATCTTTCCAGAAA		- 847
RENDOFL sol-Endg	ACAACATCAAAGGCTTCGAGCTCCAGACACACCCCAAGGCTGATCGGGGAGGCCCGCAAGCTCAATGCCAGCATAGTA ACAACATCAAAGGCTTCGAGCTCCAGACACACCCCAAGGCTGATCGGGGAGGCCCGCAAGCTCAATGCCAGCATAGTA		- 927
RENDOFL sol-Endg	ACCTTTGTAGAGATCCCTCTGACCAGTGATGTCTCCCTGACGGTCTCCAGCTGCGGTGGTGGGCTCCAGACCTCCCTTGC ACCTTTGTAGAGATCCCTCTGACCAGTGATGTCTCCCTGACGGTCTCCAGCTGCGGTGGTGGGCTCCAGACCTCCCTTGC		- 1007
RENDOFL sol-Endg	ACCAGTTGTGACCACACCTCCCAAGGACACGTGCAGCCCTGAGTACTCATGTCCCTGATTAGCCAAAGTGTGGCAATG ACCAGTTGTGACCACACCTCCCAAGGACACGTGCAGCCCTGAGTACTCATGTCCCTGATTAGCCAAAGTGTGGCAATG		- 1087
RENDOFL sol-Endg	ACGTGATGACTCTGGCACTCAATAAAAAAAGTCTGTCAGACTCTACAGTGCACCATCACAGGCCCTGGCTTTCTGGGACTCC ACGTGATGACTCTGGCACTCAATAAAAAAAGTCTGTCAGACTCTACAGTGCACCATCACAGGCCCTGGCTTTCTGGGACTCC		- 1167
RENDOFL sol-Endg	AGCTGCCAGGCTAAAGACCAGGACGCGCCACCTTGTCTCTGAGTAGCACCTACTCCAGCTGCGGCATGAAAGTGACAGATCA AGCTGCCAGGCTAAAGACCAGGACGCGCCACCTTGTCTCTGAGTAGCACCTACTCCAGCTGCGGCATGAAAGTGACAGATCA		- 1247
RENDOFL sol-Endg	TGTGATCAGCAACGAGGTGATCATCAATTGCCATCAGGCTTACCACCATTTCGAAAAAGGTACAGTGCATCGACATGG TGTGATCAGCAACGAGGTGATCATCAATTG AACGAGGTGATCATCAATTGCCATCAGGCTTACCACCATTTCGAAAAAGGTACAGTGCATCGACATGG		- 1327
RENDOFL sol-Endg	ACAGCCTCTCCTTCCAGCTGGGCTCTACCTCAGCCCGCACTTCTCCAGGCATCCACACCATAGAGCTAGGCCAGCAG ACAGCCTCTCCTTCCAGCTGGGCTCTACCTCAGCCCGCACTTCTCCAGGCATCCACACCATAGAGCTAGGCCAGCAG		- 1407
RENDOFL sol-Endg	GGCTTCGTACAGGTGAGCATGTCTCCATTGACCTCTGAGGTCACAGTCCAGCTAGACAGCTGCCATCTGGATTGGGGCC GGCTTCGTACAGGTGAGCATGTCTCCATTGACCTCTGAGGTCACAGTCCAGCTAGACAGCTGCCATCTGGATTGGGGCC		- 1487
RENDOFL sol-Endg	AGAAGGTGACATGGTGGAACTCATCCAGAGTCAGAGCCCAAGGGCAGCTGTGTGAGCTTGCTGTCCCCAAGCCCTGAGG AGAAGGTGACATGGTGGAACTCATCCAGAGTCAGAGCCCAAGGGCAGCTGTGTGAGCTTGCTGTCCCCAAGCCCTGAGG		- 1567
RENDOFL sol-Endg	GCGACCCACGTTTCAGCTTTCTCCTCCGTGTCTACATGGTGCCACACCCGACGTGGCACCCCTCAGTTGCAACTTAGCC GCGACCCACGTTTCAGCTTTCTCCTCCGTGTCTACATGGTGCCACACCCGACGTGGCACCCCTCAGTTGCAACTTAGCC		- 1647
RENDOFL sol-Endg	CTGCACCCCTAGCACCTTGTCCCAGGAAGTCTACAAGACTGTCTCCATGCGCCTGAACATCGTCAGTCTGACCTGTCTGG CTGCACCCCTAGCACCTTGTCCCAGGAAGTCTACAAGACTGTCTCCATGCGCCTGAACATCGTCAGTCTGACCTGTCTGG S P D L S G SM091		- 1727
RENDOFL sol-Endg	K G L V L P S V L G I T F G A F L I G A CAAAGGCTCGTCTGCTGCTCTGTACTGGGCATCACCTTTGGTGCCTTCCTCATTGGGGCCC CTCAAGCGGCCGC Not I		- 1789

Figure 32: Structure of the cloned rat extracellular domain of endoglin in pGEM. The nucleotide sequence of full length endoglin (7.1, RENDOFL) and soluble endoglin (sol-Endoglin) is shown. The amino acid sequence of the boundary between the extracellular domain and the transmembrane domain is shown on top of the nucleic acid sequence in the one letter code. The primer sequences used to amplify sol-Endg are underlined by an arrow and primer descriptions are indicated. Nucleotides (full length endoglin) are numbered on the right. The translational initiation and termination codons are shown in *white* type on *black*. The nucleic acid sequence corresponding to a part of the transmembrane region is marked by a solid line on the top. Restriction sites, used for successive cloning steps, introduced by the primer (*Not I*) or supplied by the vector (*EcoR I*) are designated and marked in red type. (RENDOFL, rat endoglin full length)

7.3 Map of pGEM®-T Easy Vector

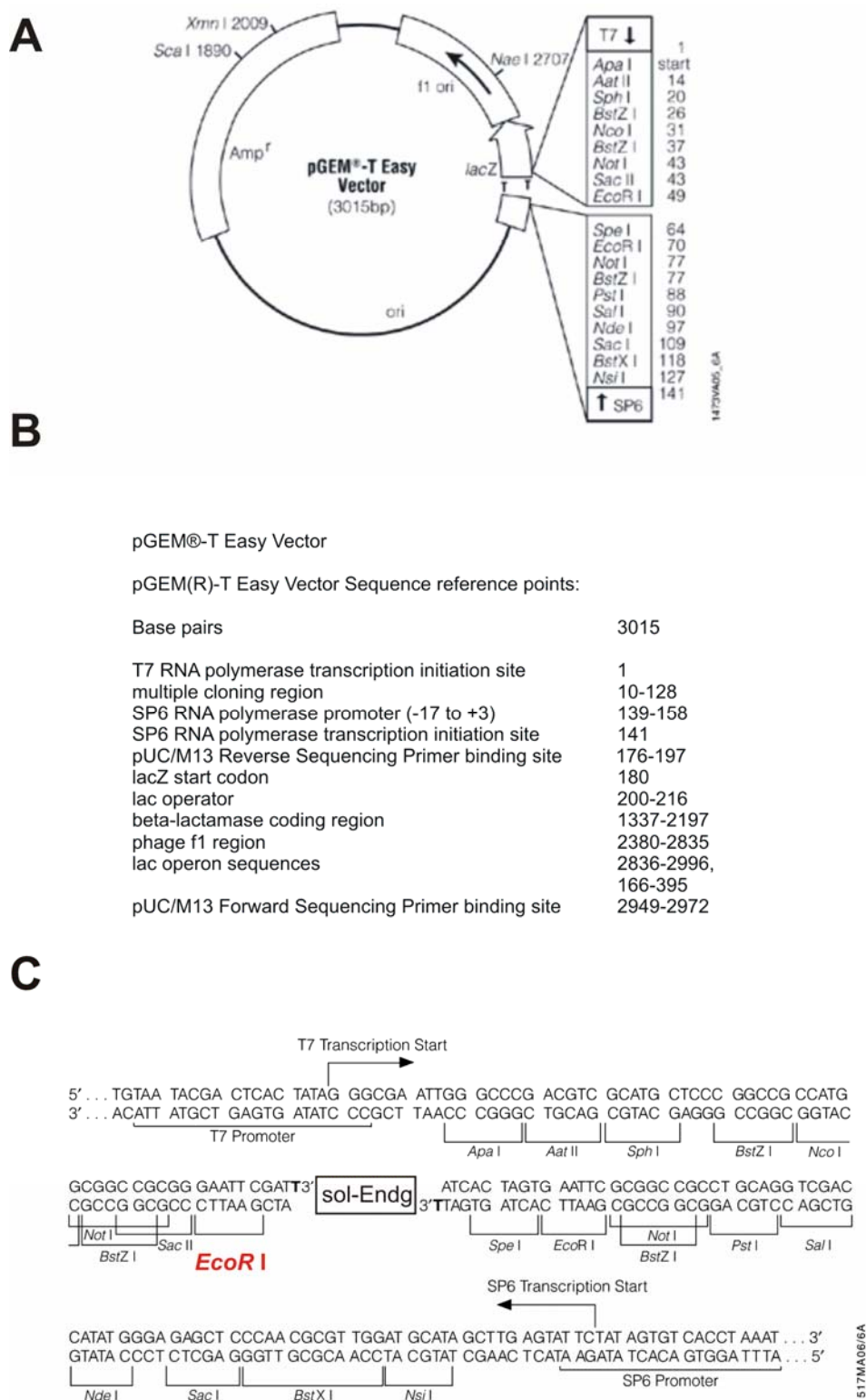
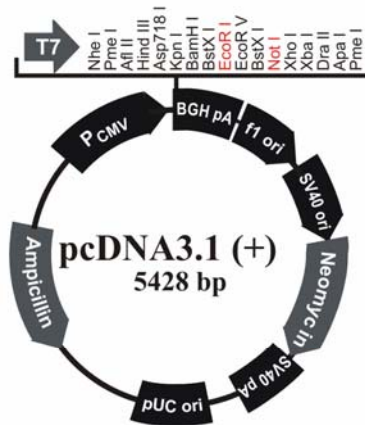


Figure 33: Structure of the pGEM®-T Easy vector (Invitrogen). (A) Schematic representation of the functional elements present in the vector. In the right margin the single cutting restriction enzymes with the respective position in the multiple cloning site is depicted. (B) Sequence positions positions of the elements depicted in (A) are indicated. (C) A detailed representation of the multiple cloning sites is shown. The site where the PCR fragment of soluble Endoglin was introduced and indicated in the figure by the box. The *EcoR* I restriction site (red) was used for further cloning steps.

7.4 Map of pcDNA3.1 Vector

A



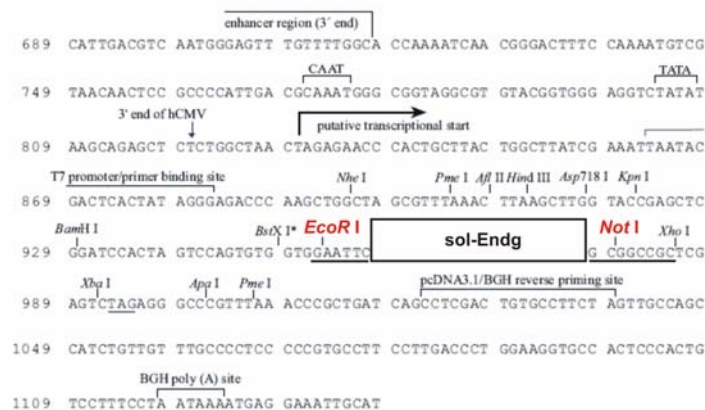
B

Comments for pcDNA3.1 (+) 5428 nucleotides

CMV promoter:	bases 232-819
T7 promoter/priming site:	bases 863-882
Multiple cloning site:	bases 895-1010
pcDNA3.1/BGH reverse priming site:	bases 1022-1039
BGH polyadenylation sequence:	bases 1028-1252
f1 origin:	bases 1298-1726
SV40 early promoter and origin:	bases 1731-2074
Neomycin resistance gene (ORF):	bases 2136-2930
SV40 early polyadenylation signal:	bases 3104-3234
pUC origin: (complementary strand)	bases 3617-4287
Ampicillin resistance gene (bla): (cs)	bases 4432-5428
ORF: (cs)	bases 4432-5292
Ribosome binding site: (cs)	bases 5300-5304
bla promoter (P3): (cs)	bases 5327-5333

(Cs): complementary strand

C



*Please note that there are two *BstX* I sites in the polylinker.

Figure 34: Structure of the pcDNA3.1 vector (Invitrogen). (A) Schematic representation of the functional elements present in the pcDNA3.1 vector. On the top the single cutting restriction enzymes with the respective position in the multiple cloning site is depicted. Enzymes utilized for the cloning are in red type. (B) Sequence positions of the elements depicted in (A) are indicated. (C) A detailed representation of the multiple cloning site is shown. The site where the PCR fragment of soluble Endoglin was introduced is indicated by the box. *EcoR* I and *Not* I restriction sites (red) were used for cloning.

7.5 Functional Map of (CAGA)₁₂-MLP-Luc

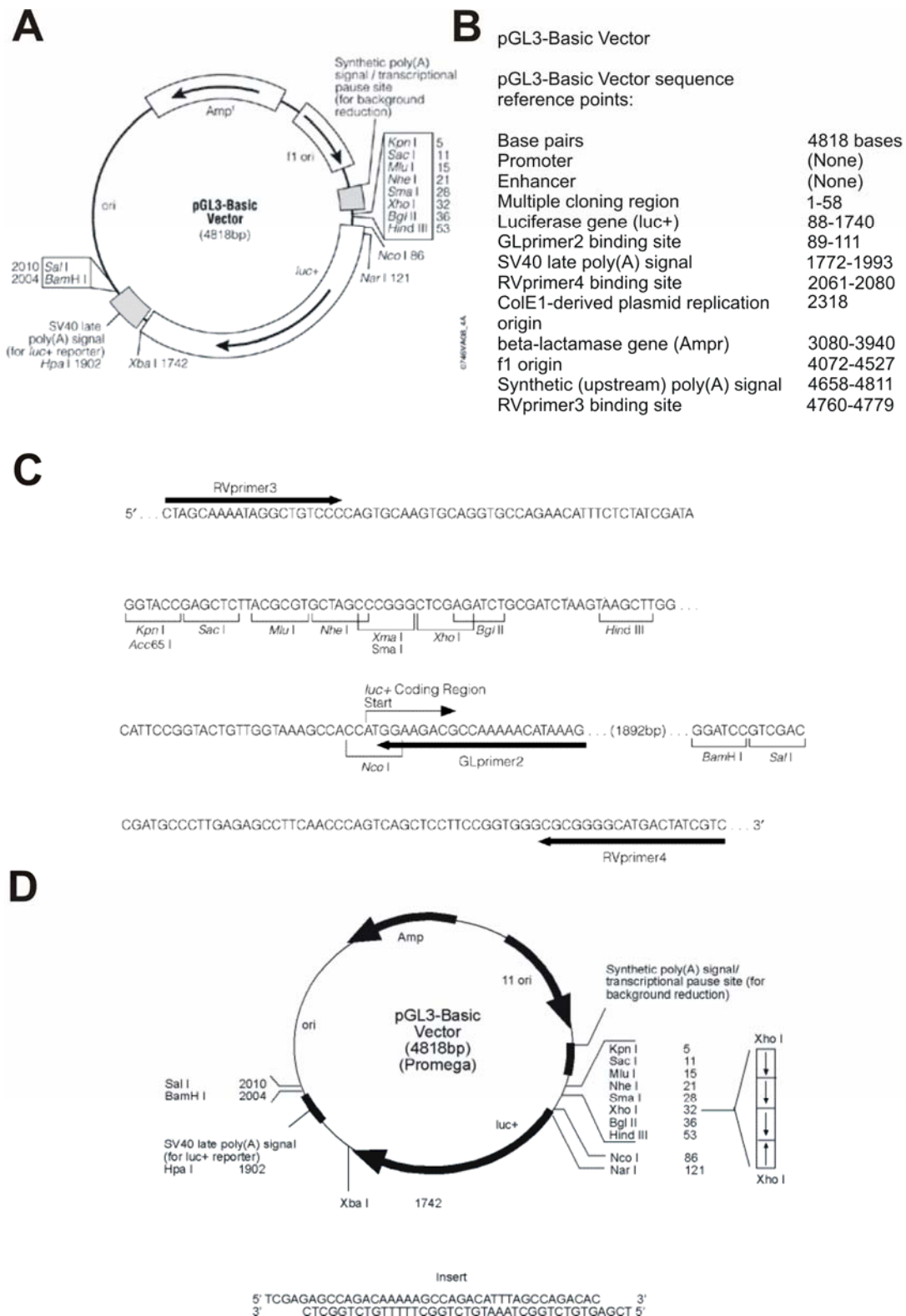


Figure 35: Structure of the pGL3-Basic/(CAGA)₁₂-MLP-Luc vector (Promega). (A) Schematic representation of the functional elements present in the pGL3-Basic vector. In the right margin single cutting restriction enzymes with the respective position in the multiple cloning site are depicted. (B) Sequence positions of the elements depicted in (A) are indicated. (C) A detailed representation of the multiple cloning site is shown. (D) The site where the PCR fragment of soluble Endoglin was introduced is indicated by the box. The *EcoR* I and *Not* I restriction sites (red) were used for cloning.

Reference table (1a): Age, sex (f =2, m =1), sCD105 concentration (ng/ml), and α -fetoprotein concentration (ng/ml) in healthy control group (n = 70).

control healthy group				
No	Age(y)	sex	Endoglin	AFP
1	36	1	4.1	2.9
2	36	1	2.5	3.3
3	48	1	3.9	4
4	55	1	3.8	2.2
5	61	1	3.7	8.4
6	37	1	3.7	2.1
7	57	1	3.4	2
8	37	1	3.3	6.3
9	41	1	4.3	3.3
10	37	1	3.1	2
11	44	1	3.3	4.8
12	38	2	3.1	2.6
13	58	2	3.3	10.1
14	49	2	4	3.2
15	36	1	3.4	5.2
16	46	1	2.9	2.7
17	47	2	3.2	1.7
18	37	2	3.4	1.7
19	38	1	3.7	2.3
20	53	1	3.1	2.6
21	38	1	2.8	3.6
22	36	1	3.5	3.6
23	42	1	3.9	4
24	45	1	3.2	2.6
25	44	1	4	4.3
26	43	1	3.1	14
27	43	1	3.4	4.1
28	58	1	32	3.2
29	36	1	2.5	2
30	45	2	3.8	4
31	36	1	2.7	2
32	46	2	2.5	3.9
33	61	2	3.6	2.4
34	54	1	2.7	7
35	42	1	3.9	2.8
36	50	1	4.1	2.6
37	45	1	3.2	4.5
38	58	2	3.7	98
39	43	1	3.3	2.5
40	51	2	2.9	3.9
41	50	1	2.7	2.9
42	46	2	3.4	2.4
43	49	1	4	22
44	41	2	3.5	3.4
45	61	1	3	2.1
46	37	1	3	1.16
47	63	1	7.8	3.2
48	62	1	5.9	1.3
49	60	1	5	3

50	52	2	5.6	3.8
51	64	1	3.9	3.1
52	54	2	4.3	4.1
53	56	2	3.9	2
54	66	2	4.3	5.8
55	63	1	3.6	4.4
56	67	1	5.9	4
57	59	1	4.2	5.8
58	59	1	5.5	3.9
59	58	1	4.5	6
60	56	1	5.3	1.6
61	59	1	4.1	6.3
62	57	1	5	3.6
63	51	1	4.6	2.3
64	59	1	4.6	2.8
65	51	1	4.5	2
66	56	1	4.3	3
67	52	1	4.6	1.9
68	54	1	4.9	3
69	51	1	4.9	4.7
70	51	1	3.9	1.7

Reference table (1b): Age, sex (f =2, m =1), serum soluble endoglin concentration (ng/ml), and α -fetoprotein concentration (ng/ml) in non cancer non liver diseases as an internal control group.(n = 94).

Non liver patients is as an internal control				
	Age(y)	sex	Endoglin	AFP
1	21	2	3.4	1.44
2	25	2	4.9	0.7
3	62	2	6.4	6.3
4	48	1	3.8	1.79
5	21	1	4.9	1.2
6	39	2	3.1	1
7	78	1	3.7	5.9
8	66	1	3	2.3
9	62	1	4.6	2.4
10	40	2	4.2	2
11	39	1	2.9	4.2
12	68	2	3.7	4.8
13	59	2	2.4	2.3
14	60	2	3.5	2.9
15	79	2	3.8	11.2
16	76	1	4	1.4
17	56	1	3.1	4.7
18	51	2	3.3	4.8
19	78	1	4.4	3.1
20	43	2	4.3	2.1
21	53	2	6	3.6
22	59	2	2.4	3
23	46	2	3.8	8.6
24	73	2	3.6	2.7
25	71	1	3.5	3.9
26	51	2	3.6	2.7

27	78	2	3.9	2.5
28	62	2	3.2	4.6
29	66	1	3.8	2.6
30	41	2	4.8	2.1
31	65	1	3.8	3.9
32	62	2	4.2	1
33	62	1	9.9	1.24
34	38	1	4.3	6.4
35	71	1	3.7	8
36	65	1	4.3	4.1
37	65	1	5.3	2.1
38	78	2	3.8	1.9
39	54	2	3.4	1.4
40	77	1	3.9	5.2
41	37	1	2.8	1.6
42	70	1	3.4	2.2
43	85	2	4.4	3.7
44	76	2	4.8	2.1
45	49	1	3.6	3.5
46	64	1	15.1	2.9
47	76	1	3.1	3
48	73	1	3.4	4
49	41	2	3.8	1.7
50	65	2	3.1	8.5
51	41	2	3.7	2.2
52	60	2	4	3.8
53	63	2	3.4	3.6
54	41	1	4.2	1.2
55	52	1	4.8	5.7
56	58	1	4.3	1.6
57	72	2	4.7	7
58	51	2	6.7	4.2
59	75	2	3.7	9.1
60	68	2	3.8	3.3
61	56	2	3.1	2.7
62	67	2	4.7	7.3
63	51	2	4.5	2.8
64	38	1	3.9	5.9
65	56	2	3.5	4.2
66	54	1	4.9	1.3
67	59	1	3.7	3.5
68	51	1	2.8	3
69	67	2	5.7	1.8
70	62	2	4.9	1.3
71	66	1	5.1	1.2
72	42	1	5	3.5
73	67	1	4.5	3.4
74	75	2	3.4	4.7
75	39	1	4	1.1
76	40	1	4.7	2.1
77	54	1	3.8	3.6
78	51	1	4.1	3.7
79	68	1	6.3	5.24

80	54	1	3.2	4.1
81	83	2	3.5	4.3
82	66	2	5.1	3.2
83	68	1	4.1	5.8
84	42	2	3.6	3
85	51	2	7.5	1.6
86	62	2	4.1	1.4
87	33	1	2.5	2.2
88	48	2	4.7	1.8
89	72	1	4.1	1.8
90	61	2	6.4	4.3
91	60	2	3.9	3.8
92	75	2	3.4	4.1
93	49	2	4.7	3
94	58	2	3.2	3

Reference table (1c): Age, sex (f =2, m =1), sCD105 (ng/ml), and AFP concentration (ng/ml) in fibrotic liver patients subgroup (n = 19).

Fibrotic liver subgroup				
	Age	sex	Endoglin	AFP
1	60	2	5.4	6.5
2	50	2	3.4	8.5
3	66	2	3.8	5.8
4	24	1	3.5	1.8
5	26	2	3.2	1
6	21	1	4.7	4.6
7	51	1	4	2.1
8	40	2	2.8	2.7
9	58	2	3.3	5.4
10	42	1	4.1	4.3
11	53	2	3.4	3.6
12	36	2	3.2	2.7
13	21	2	3.9	4.8
14	40	1	3.4	1.2
15	41	1	7.4	1.4
16	47	2	3.3	4
17	52	1	3.9	1.7
18	72	1	3.4	2.9
19	47	1	5.8	2.5

Reference table (1d): Age, sex (f =2, m =1), sCD105 concentration(ng/ml), and AFP concentration (ng/ml) in cirrhotic liver patients subgroup (n = 57).

	Cirrhotic liver subgroup			
	Alter	sex	Endoglin	AFP
1	61	1	5.4	2.1
2	50	1	6.8	6.7
3	51	1	5	4.9
4	39	2	5.8	4
5	78	1	6.3	5.5
6	57	1	8.2	4
7	46	1	4.9	3.3
8	65	2	3.5	3.4
9	41	1	9.5	2
10	54	2	5.6	7.2
11	56	1	7.4	2.5
12	61	1	4.3	5.2
13	66	1	4	3.3
14	55	2	6.5	5
15	56	1	9.4	9
16	43	1	3.4	1.5
17	64	2	5.1	3.58
18	47	1	3.2	4.12
19	50	1	4.7	3.4
20	40	2	9.2	4.9
21	44	2	7.7	3.7
22	75	2	6.1	4.3
23	60	1	3.8	153.9
24	62	1	12.6	4.2
25	63	2	11.7	16.1
26	79	1	6.5	3.9
27	69	2	6.4	2.2
28	68	1	6.2	9.4
29	59	2	6.3	7.4
30	65	1	4.8	4.5
31	62	1	4.4	3.7
32	67	1	5.8	4.3
33	75	1	3.2	0.8
34	54	2	6.9	2.6
35	73	2	2.9	1.3
36	66	1	5.4	13.5
37	64	1	6.5	7.7
38	45	1	7.4	6.5
39	67	1	4.2	1.9
40	73	1	5.9	3.9
41	49	1	2.8	1.9
42	51	2	6	1.6
43	38	1	7.2	6.4
44	58	1	6	17.9
45	54	1	4	3
46	65	2	5.8	6.4
47	78	2	5.1	8.1
48	40	1	3.4	1.2
49	40	1	5.2	1

50	72	1	3.4	2.9
51	60	2	4.2	6.3
52	82	1	9.3	26.8
53	63	1	5.1	43
54	66	1	7.5	2.6
55	66	1	4.4	3.4
56	50	1	6.3	3.8
57	57	2	5	2.7

Reference table (1e): Age, sex (f =2, m =1), sCD105 (ng/ml), and AFP concentration (ng/ml) in cirrhotic liver with HCC patients subgroup (n = 45).

HCC with cirrhosis subgroup				
	Age	sex	Endoglin	AFP
1	43	1	7	2.2
2	38	2	5.6	5
3	44	1	5.2	2.4
4	57	2	3.2	2.5
5	59	1	9.8	2.4
6	49	1	7.8	35.4
7	43	1	10.2	2.8
8	47	1	3.1	3.6
9	63	1	8.8	7.2
10	54	2	3	2.5
11	65	1	7	3.5
12	59	1	6.4	3
13	45	2	7	9.4
14	70	1	2.4	1.4
15	50	1	4.9	2.1
16	70	2	3.7	1.4
17	54	1	11	18.9
18	56	1	5.2	5.2
19	46	1	8	25.9
20	77	2	11.7	8.6
21	51	1	8.2	2.8
22	45	1	9.5	6.2
23	62	2	11.4	8
24	57	1	8.8	14.7
25	39	1	4	2.4
26	46	1	11.5	2.6
27	59	1	5.6	5
28	49	1	6.4	5
29	48	1	6.6	1.8
30	50	2	11.5	3..9
31	58	1	11.4	7.4
32	61	2	12	353000
33	58	1	9.4	24.8
34	63	2	10.4	21946
35	50	1	7.4	1277
36	75	2	7.9	21.4
37	51	1	6.5	388
38	66	1	6.3	1.7
39	60	1	11.5	461100
40	51	2	7.5	8.3

41	67	1	6	129.6
42	72	2	6.1	4.3
43	78	1	8.5	2792
44	61	1	5.4	362
45	76	2	8.8	86.8

Reference table (1f): Age, sex (f =2, m =1), sCD105 concentration (ng/ml), and AFP concentration (ng/ml) in liver patients with HCC patients subgroup (n = 9).

HCC subgroup				
	Age	sex	Endoglin	AFP
1	54	1	6.7	2
2	63	2	8.5	15.1
3	74	1	3.3	2.7
4	63	1	4.6	763
5	70	1	5.1	16.1
6	62	2	5	113.9
7	65	1	6	56
8	59	1	4.6	2.5
9	57	2	4.2	3.9

Reference Table (2): showed the calibration data for the superdex 200 HR 10/30 column by specific standard proteins with their molecular weight and the elution volume at which every standard run through the column. K_{av} is calculated for thr tested protein from special formula.

Protein	MW	V_e (ml)	K_{Av}	MWlog	V_e/V_0
Blue Dextran2000	V_0	8.19			
Thyroglobulin	667000	8.78	0.0437	5.824	1.072
Ferritin	440000	10.07	0.1391	5.643	1.2295
Catalase	232000	11.8	0.2672	5.365	1.4407
Gamma Globulin	158000	11.89	0.2739	5.199	1.4517
BSA	67000	13.06	0.3605	4.826	1.5946
Ovalbumin	44000	14.55	0.4708	4.643	1.7765
Myoglobin	17000	16.84	0.6403	4.23	2.0561
Cyancobalamin	4500	20.52	0.9126	3.653	2.5054
L-Tyrosine	V_t 181	21.7	1	2.258	2.649

$$K_{av} = (V_e - V_0) / (V_t - V_0)$$

(V_0 = void volume, V_t = total gel bed volume, V_e = elution volume of protein of interest, K_{av} = value of protein of interest)

For purification of serum endoglin

$$V_0 = 8.19$$

$$V_e = 10$$

$$V_t = 21.7$$

$$K_{av} \text{ for serum sCD105} = 1.81 / 13.51 = 0.134$$

$$V_e/V_0 \text{ for serum endoglin was } 1.221$$

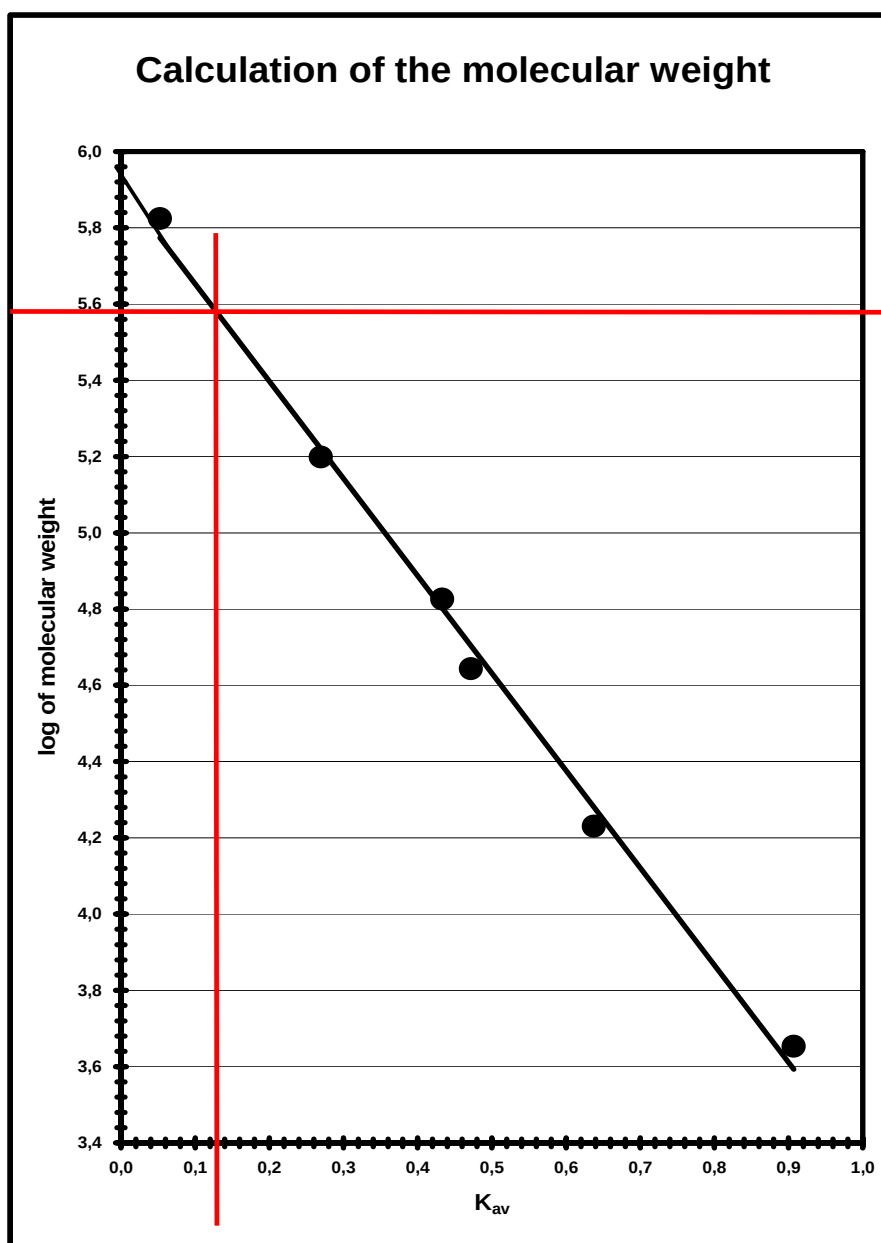


Figure 36: Diagram showing the calibration data of the FPLC (Superdex 200 HR 10/30). To calculate the molecular weight of the soluble endoglin, the data of K_{av} for each standard protein (x-axis) were plotted against the log of the molecular weight of each standard protein (Y-axis). The log of molecular weight for serum endoglin was 5.58.

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9. CURRICULUM VITAE

Name: Mohamed Soliman Rizk
Date of birth: 24 December 1966, El-Menofyia, Egypt
Nationality: Egyptian
E-mail address: drrizk2007@yahoo.de

EDUCATION

School

1972 – 1978 Primary school
1978 – 1981 Prep school
1981 – 1985 Secondary school

University

1985 – 1990 M. B. B.Ch. of Medicine, Faculty of Medicine, El-Menofyia Uni.
1993 – 1994 First part of Master Degree in Clinical Pharmacology
1994 – 1998 Second part of Master Degree in Clinical Biochemistry
Thesis: The value of serum lipids and Lp (a) measurement in ischaemic heart disease.
Faculty of Medicine, El-Menofyia

PROFESSIONS

1991 – 1992 House officer and medical training in Menofyia University Hospital
1992 – 1993 Family doctor and Military service.
1993 – 1997 Demonstrator in Clinical Biochemistry Dep., Faculty of Medicine
1998 – 2002 Assistant Lecture in Cinical Biochemistry department.
2003 Start in the post graduate study (MD) in RWTH- Aachen University Hospital,
2004 Promotional research at the Institute of Clinical Chemistry and Pathobiochemistry, RWTH-Aachen University Hospital, Aachen, Germany

PUBLICATIONS

Eray Yagmur, **Mohamed Rizk**, Sven Stanzel, Claus Hellerbrand, Frank, Lammert, Christian Trautwein, Hermann E Wasmuth, and Axel M Gressner. Elevation of endoglin (CD105) concentrations in serum of patients with liver cirrhosis and carcinoma. Eur J Gastroenterol Hepatol. 2007;9:755-761.

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