Expression and Regulation of Cold Shock Proteins in Inflammatory Kidney and Liver Diseases

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Cheng Zhu

aus

Zhejiang, (V.R.China)

Berichter: Herr Universitätsprofessor
Dr. med. Peter René Mertens

Herr Professor
Dr. med. Oliver Galm

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Contents

List of Abbreviation

aa	Amino acid
APS	Ammonium persulfate
Aqua dest.	Distilled water
bp	Base pair
BSA	Bovine serum albumine
CDK	Cyclin-dependent kinase
CSD	Cold shock domain
CSP	Cold shock protein
CspA/B	Cold shock protein-A/-B
CTCF	CCCTC-binding factor
DAB	Diaminobenzidine
DbpA/B/C	DNA-binding protein-A/-B/-C
DMEM	Dulbeccos Modified Eagle Medium
DMSO	Dimethylsulfoxid
DTT	Dithiothreitol
FCS	Fetal calf serum
EDTA	Ethylenediaminetetraacetic
EGFR	Epidermal growth factor receptor
elF4E	elongation initiation factor-4E
Erk	Extracellular signal-regulated kinases
GEF-H1	Guanine nucleotide exchange factor-H1
GM-CSF	Granulocyte-macrophage colony stimulating factor
GN	Glomerulonephritis
Grp78	Glucose regulated protein-78
HCl	Hydrochloric acid
HEK-293	Human embryonic kidney cells-293
HIV	Human immunodeficiency virus
HK-2	Human kidney cells-2
HKC-8	Human kidney clone-8 cells
HLA	Human leukocyte antigen
HSC	Hepatic stellate cells
IFN	Interferon
IgA	Immunglobulin-A
IGFBP1	Insulin-like growth factor-binding protein-1

IL-13	Interleukin-13
IRP2	Iron regulatory protein-2
kb	Kilobase
kDa	Kilodalton
LPC	Liver progenitor cells
MAPK	Mitogen-activated protein kinase
MDR-1	Multi-drug resistance gene-1
MEK	MAP kinase kinase
MHC	Major histocompability complex
MMP	Matrix-metalloproteinase
MRP1/2	Multidrug resistance-associated protein-1/-2
MsGN	Mesangioproliferative glomerulonephritis
mRNA	Messenger RNA
Notch-3	Notch gene-3
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF-B	Platelet derived growth factor-B
PTB	Polypyrimidine tract binding protein
PTP1B	Protein tyrosine phosphatase-1B
RNP	Ribonucleoprotein particle
rMC	Rat mesangial cell
rpm	Rotations per minute
RANTES	Regulated upon activation normal T cell expressed and secreted
RPMI	Roswell Park Memorial Institute
RSV	Rous Sarkom Virus
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMA	Smooth muscle actin
TEMED	N,N,N',N'-Tetramethylethylendiamine
TF	Transcription factor
TGF-β	Transforming growth factor-beta
Tris	2-Amino-2-(hydroxymethyl)-1,3-propandiol
TTBS	Tris buffered solution/Tween-20
Twist1	Twist gene-1
UTR	Untranslated region
YB-1	Y-box binding protein-1
ZO-1	Zonula occludens-1

1 Introduction

1.1 Cold shock proteins

Adaptation to environmental stress is essential for the survival of cells and living organisms. One of the most archtypical responses to a temperature downshift in microorganisms is the induction of cold shock proteins (CSPs). It was first reported in 1987 that in cultures of E. coli growing at 37 °C a down-shift to 10 °C alters protein synthesis. The synthesis was dramatically reduced except for 15 proteins, the so-called cold shock proteins (CSPs) (Jones, VanBogelen et al. 1987). The major CSPs in E. coli (e.g. CspA and CspB) composites of a protein family showing single nucleic acid-binding cold shock domain (CSD) (Goldstein, Pollitt et al. 1990), in which two consensus RNA binding motifs are found (RNP-1 and RNP-2). CSPs may bind to and stabilize the secondary structures of RNA and DNA molecules, thereby they may reduce efficiency of mRNA translation and transcription. CSPs play important roles in cold shock stress adaptation of microorganisms (Thieringer, Jones et al. 1998). The cold shock domain (CSD) is evolutionarily conserved and is found in prokaryotes as well as eukaryotes, from bacteria to humans, as exemplified in Figure 1.

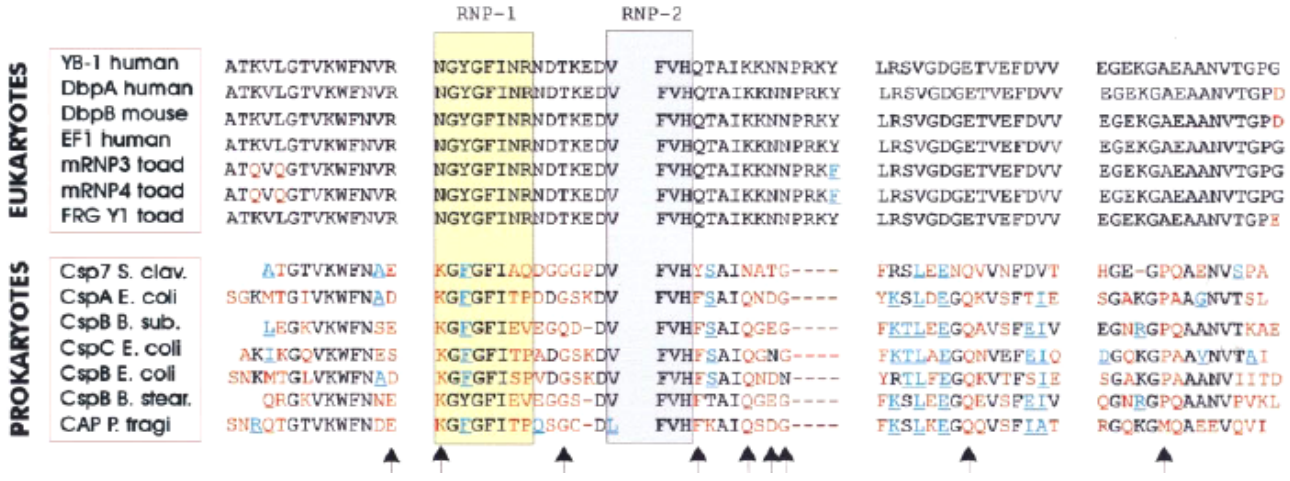


Figure 1. Sequence alignment of cold shock domains (CSDs) of eukaryotic and prokaryotic cold shock proteins. RNA-binding motifs RNP-1 and RNP-2 are highlighted. Identical residues are depicted in black, homologous residues are in blue. Differences in conservation of residues between eukaryotes and prokaryotes are marked by arrows. (Kloks, Spronk et al. 2002)

Unlike bacteria, eukaryotic CSPs contain auxiliary domains. In eukaryotes, three structural domains are disdinguishable: (1) a small domain at the N-terminus, (2) a centrally localized cold shock domain (CSD) and (3) a C-terminal domain. Higher plant CSPs contain only two nucleic acid-binding modules: a single N-terminal CSD and variable numbers of C-terminal retroviral-like CCHC zinc fingers (Matsumoto and Wolffe 1998; Karlson and Imai 2003) (see Figure 2)

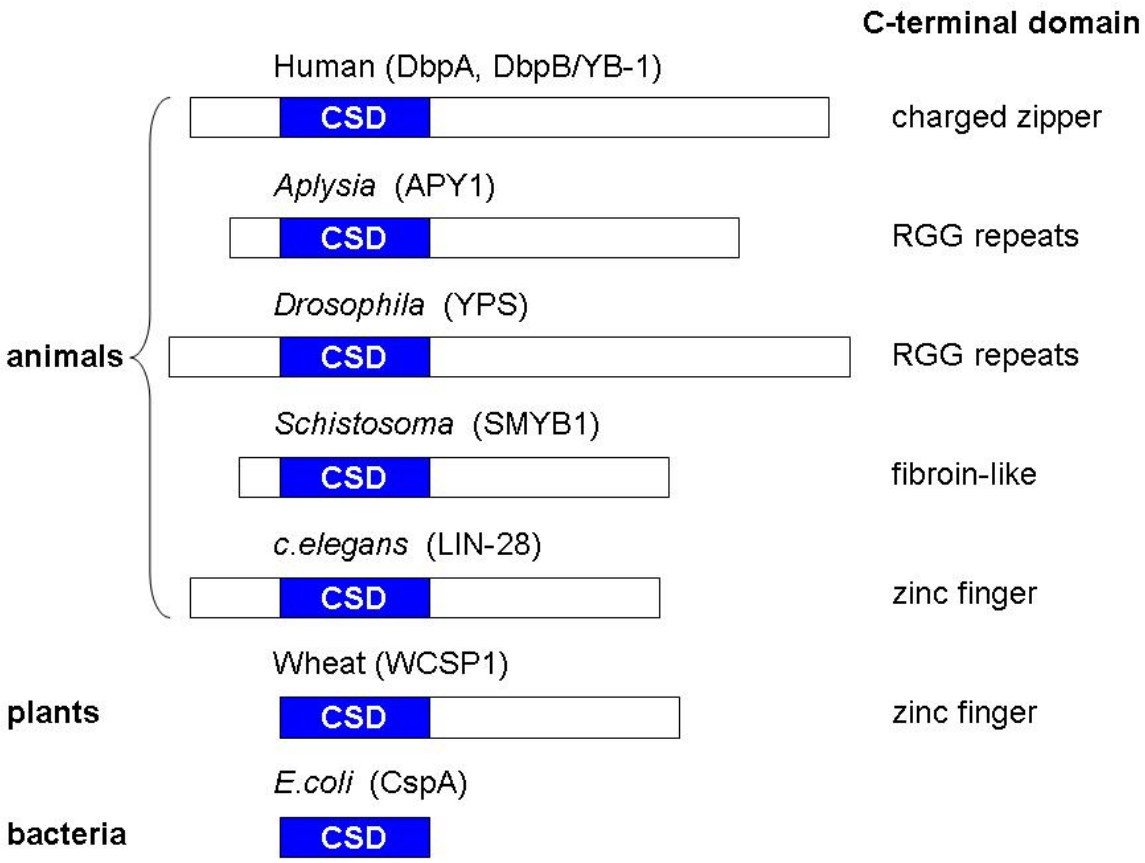


Figure 2. Sequence features of cold shock proteins found in different species. Proteins are aligned at their CSDs. The C-terminal domains significantly differ in their organization.

The cold shock domain may approach RNA, single stranded as well as double stranded DNA (Newkirk, Feng et al. 1994; Schindelin, Jiang et al. 1994) and contributes to both transcriptional and translational functions. The C-terminal domain of CSPs shows significant variability (Figure 2): The C-terminal tail of human cold shock proteins and some other vertebrate CSPs consists of alternating regions of predominantly basic/acidic amino acids and may also bind to nucleic acid residues in isolation (Ladomery and Sommerville 1994). In the marine invertebrate *Aplysia californica* and in *Drosophila melanogaster* C-terminal domains lack acidic amino acids and contain multiple RNA-binding RGG repeats (Skehel and Bartsch 1994; Thieringer, Singh et al. 1997). In *Schistosoma mansoni* the very basic tail domain is proposed to have a structure similar to that of fibroin that is predicted to contubute to nucleic acid binding (Franco, Garratt et al. 1997). In lower animals, such as *Caenorhabditis elegans* and in plants (e.g. wheat), the C-terminal domain contains zinc fingers (Moss, Lee et al. 1997). Protein-protein interactions of the N-terminal domain have been reported. For human CSPs an interaction with cytoskeleton actin is found (Ruzanov, Evdokimova et al. 1999). It is likely that auxiliary domains have been added to the CSD during evolution and that these contribute to nucleic-acid-binding specificities and protein-protein interaction.

1.2 Human cold shock proteins (DbpA, DbpB/YB-1 and DbpC)

1.2.1 Structure analysis of human CSPs

In humans CSPs were first identified as proteins binding to the epithelial growth factor receptor (EGFR) enhancer or c-erb-2 promoter and were denoted DNA-binding protein A (DbpA) and DNA-binding protein B (DbpB) (Sakura, Maekawa et al. 1988). From an independent group, DbpB was cloned as transcription factor that binds to the inverted CCAAT-box (the so-called Y-box) from the HLA-gene promoter (Didier, Schiffenbauer et al. 1988) and was denoted Y-box binding protein-1 (YBX1, YB-1). Thus leads to the names Y-box binding proteins or Y-box proteins.

Later on DbpA was cloned and characterized as two isoforms (DbpA_a and DbpA_b) generated by alternative mRNA splicing (Kudo, Mattei et al. 1995). Given their interaction with tight junction protein zonula occludens-1 (ZO-1), they were also denoted ZO-1-associated nuclear acid binding protein (ZONAB) (Lima, Parreira et al.; Balda and Matter 2000). Another member of the human CSPs is Contrin (DbpC) (Tekur, Pawlak et al. 1999). DbpC is only expressed by germinal cells of the testis (see Figure 3).

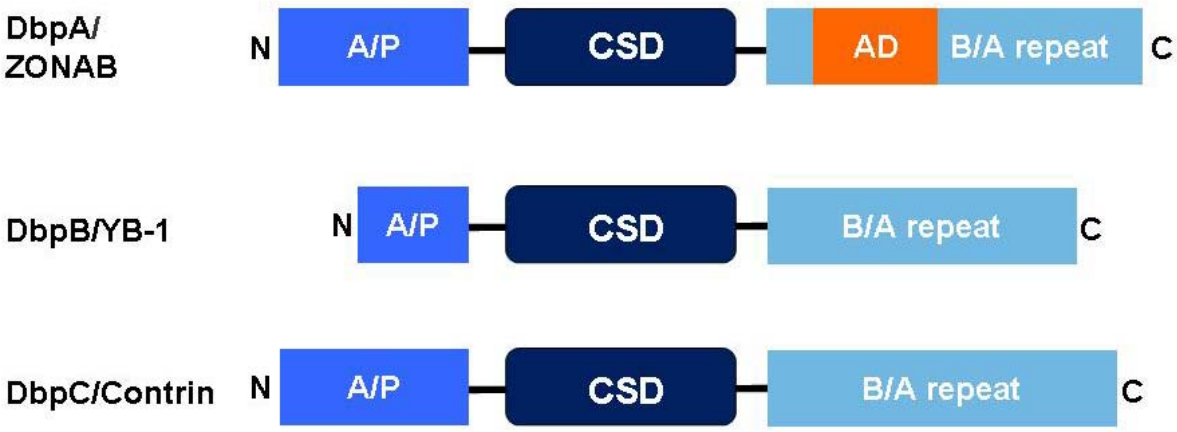


Figure 3. Domain organization of human cold shock protein family members. A/P: alanine- and proline-rich N-terminal domain; CSD: cold shock domain; B/A repeat: basic and acidic amino acid clusters; AD: alternative domain.

Since DbpC/Contrin expression is restricted to germinal cells most of the studies carried out in human cold shock proteins are devoted to DbpA and DbpB/YB-1 expression and functions. Alignment of protein sequences of DbpA and DbpB/YB-1 from different vertebrate species (human, chimp, mouse, rat, cow and dog) shows that the central cold shock domain has close to 100% conservation. The C-terminal domain contains some homologous islands. The N-terminal domains of DbpA and DbpB/YB-1 are quite distinct from each other (Figure 4). These similarities possibly indicate that DbpA and DbpB/YB-1 share some common functions mediated by the central CSD or the C-terminus. Different activities may be contributed by the N-terminal domains. DbpA and DbpB/YB-1 in rat and mouse are highly conserved, 85% and 95% identites respectively.

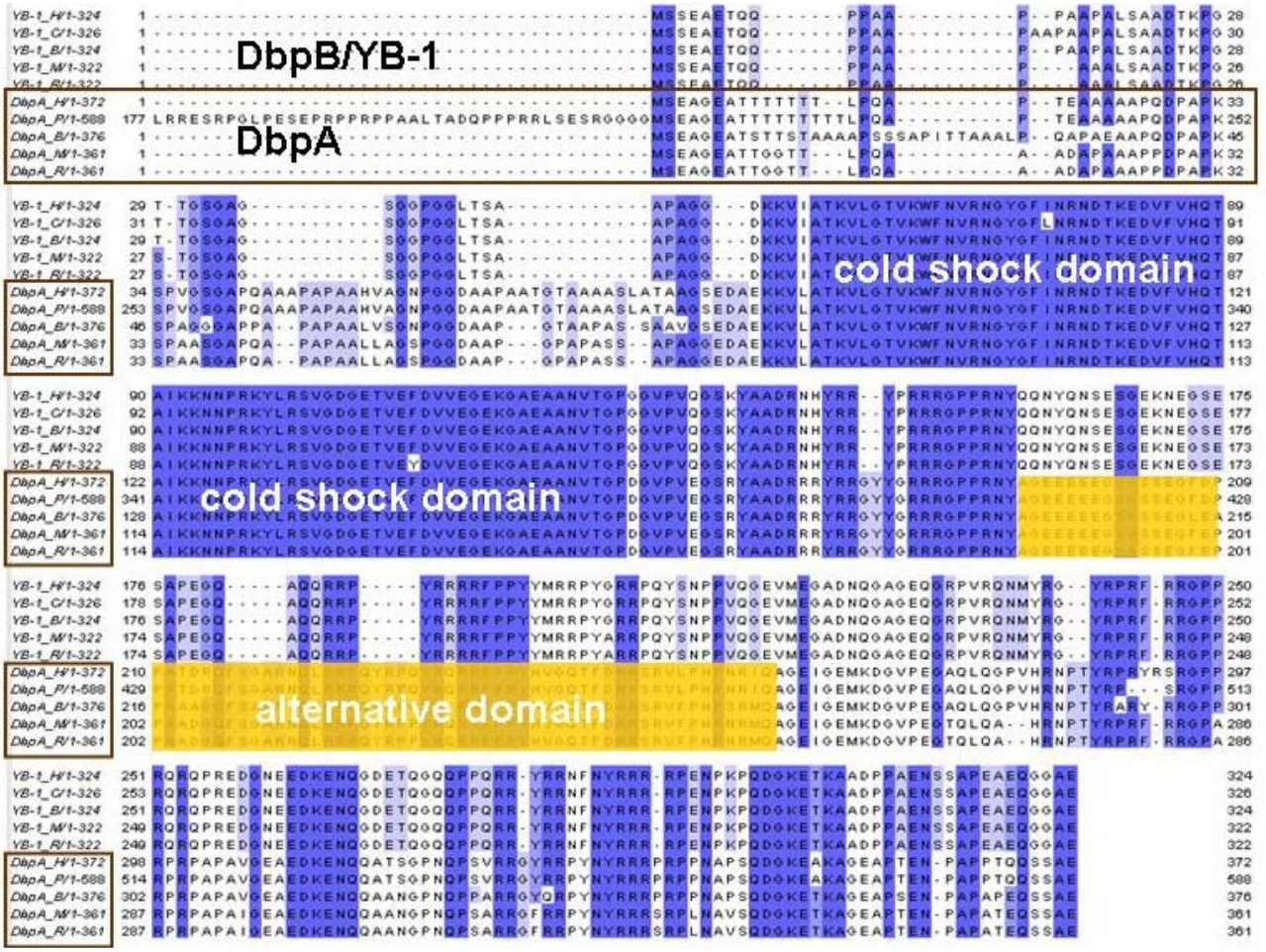


Figure 4. Protein sequence alignment of DbpA and DbpB/YB-1 from different species. Protein homology becomes apparent when human, chimp, cow, mouse and rat proteins are compared;. Identity is indicated by dark blue; homologous residues are in light blue. Alternative domain is boxed in yellow.

1.2.2 Human CSPs as DNA and RNA binding proteins

By virtue of nucleic acid binding activities DbpA and DbpB/YB-1 may alter gene transcription in the nucleus, affect pre-mRNA splicing as well as mRNA translation processes within the nucleus and cytoplasm (Matsumoto and Wolffe 1998). Of importance, the binding motifs that DbpA and YB-1 recognize are not limited to the inverted CCAAT-box (Y-box). Many studies revealed that cold shock proteins rather recognize DNA or RNA structural motifs than a defined nucleotide sequence, and may furthermore exert modifying steps to the nucleic acid backbones. In this regard it is compelling to observe that DbpA/YB-1 protein may propagate separation of double-stranded DNA and stabilize single-stranded loops (MacDonald, Itoh-Lindstrom et al. 1995; Mertens, Alfonso-Jaume et al. 1998; Norman, Lindahl et al. 2001). It has been reported that the C-terminal tail domain is required for homo-multimerization of vertebrate CSPs and this domain facilitates binding to DNA and RNA (Nambiar, Swamynathan et al. 1998; Swamynathan, Nambiar et al. 1998). The alternating blocks of basic and acidic amino acids has led to the interesting proposal that this domain functions as a charged-zipper to mediate protein–protein interactions (Ozer, Faber et al. 1990). This type of sequence is a common feature in proteins that contain nuclear localization sequences (NLS), bind to ribonucleoprotein complexes and shuttle between the nucleus and cytoplasm (Meier and Blobel 1992; Matsumoto and Wolffe 1998). Gene regulation mechanisms of YB-1 at both transcriptional and translational levels are summarized in Figure 5 (Kohno, Izumi et al. 2003).

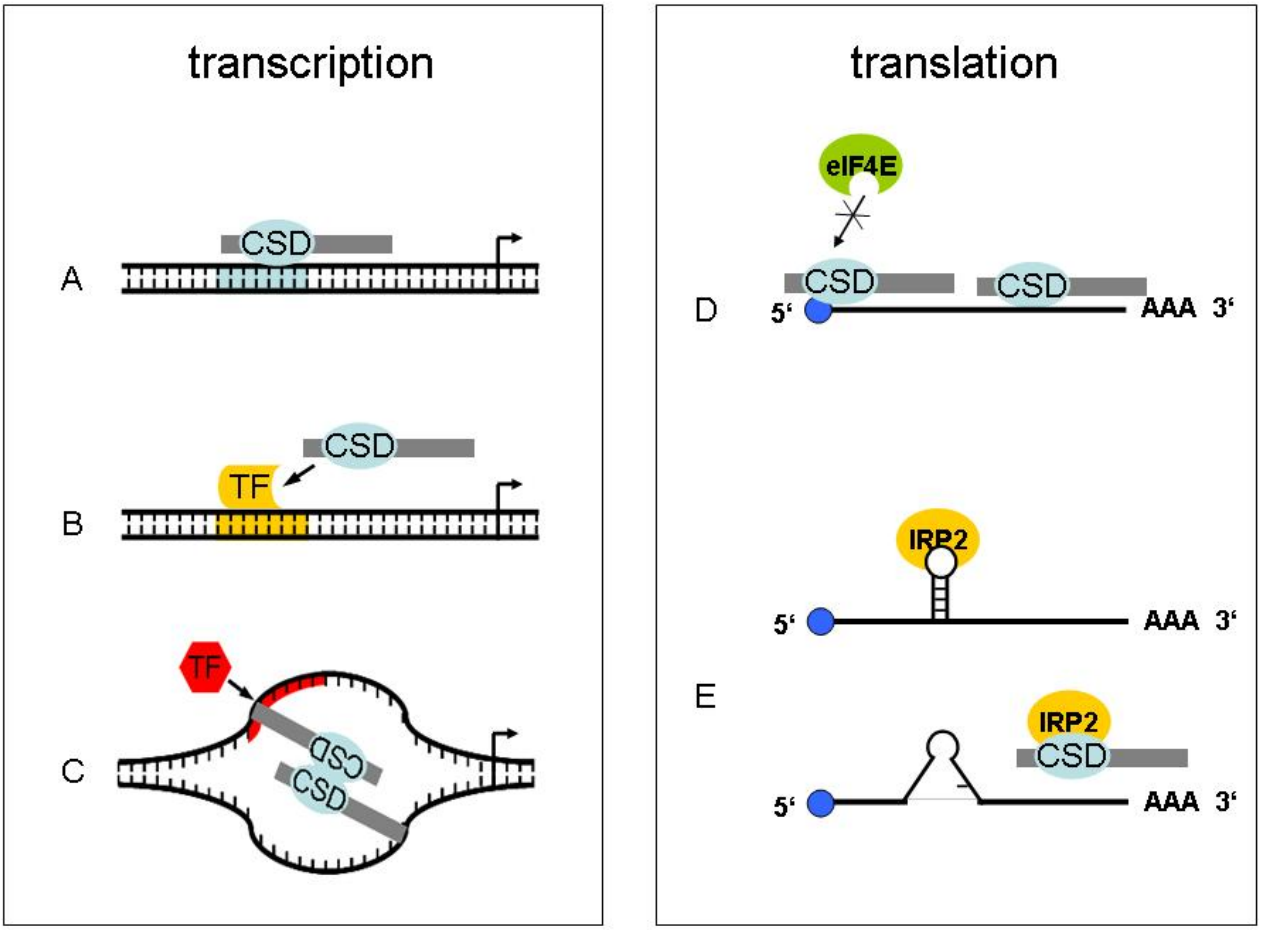


Figure 5. Transcriptional and translational gene regulation by CSPs. A: CSPs directly bind to Y-boxes or related sequences. **B**: CSPs interact with other TFs and functions as either co-activator or co-repressor. **C**: CSPs bind to the single-stranded region of the promoter either to enhance or inhibit the DNA binding of TFs. **D**: CSPs may bind to mRNA to inhibit the access of Cap binding factor (e.g. eIF4E) and repress translation, a process denoted mRNA masking. **E**: A specific model is *via* protein-protein interactions. IRP2 binds to the 5'UTR of ferritin mRNA and inhibits translation. Interaction of CSPs and IRP2 leads to release of these complexes from mRNA. Thereby it becomes accessible for translation. CSD: cold shock domain; TF: transcription factor

Defined target genes of DbpA and DbpB/YB-1 are summarized in Table 1 and Table 2 respectively (▲ indicates up-regulation; ▼ indicates down-regulation). Most studies have focused on DbpB/YB-1, given its conserved amino acids compositions throughout evolution. However, recently efforts are devoted to unravel functions of DbpA (Buchert, Darido et al. 2009; Lima, Parreira et al. 2010).

Table 1. Target genes regulated by DbpA.

Gene	Regulation	References
Cytokines, Chemokines and Receptors		
EGFR/HER2	▲	(Sakura, Maekawa et al. 1988)
IGFBP1	▲	(Tobita, Kajino et al. 2006)
GM-CSF	▼ / ▲	(Coles, Diamond et al. 1996)
Megalin	▼	(Lima, Parreira et al. 2010)
Cubilin	▼	(Lima, Parreira et al. 2010)
Antigen Presentation		
HLA class II	▼	(Didier, Schiffenbauer et al. 1988)
HLA class I	▼	(Lloberas, Maki et al. 1995)
Proliferation		
PCNA	▲	(Sourisseau, Georgiadis et al. 2006)
Cyclin D1	▲	(Sourisseau, Georgiadis et al. 2006)
Viral Promoters		
RSV	▲	(Swamynathan, Nambiar et al. 1998)
Others		
Grp78	▼	(Li, Hsiung et al. 1997)

Table 2: Target genes regulated by DbpB/YB-1.

Gene	Regulation	References
Cytokines, Chemokines and Receptors		
EGFR/HER2	▲	(Wu, Lee et al. 2006)
PDGF-B	▲	(Stenina, Poptic et al. 2000)
RANTES	▲	(Krohn, Raffetseder et al. 2007)
IFN-alpha & IFN-beta	▲	(Yan and Tamm 1991)
GM-CSF	▼ / ▲	(Coles, Diamond et al. 1996)
Thyrotropin Receptor	▼	(Ohmori, Shimura et al. 1996)
Signal Transduction		
Smad7	▲	(Dooley, Said et al. 2006)
PTP1B	▲	(Fukada and Tonks 2003)
Matrix and Matrix-Degrading Proteins		
Gelatinase A (MMP-2)	▼ / ▲	(Mertens, Harendza et al. 1997)
Collagen Type 1 (alpha1)	▼/ ▲	(Norman, Lindahl et al. 2001; Higashi, Inagaki et al. 2003)
Structural Proteins and others		
Myosin light-chain 2v	▲	(Zou, Evans et al. 1997)
Cyclin A and B1	▲	(Jurchott, Bergmann et al. 2003)
Globin	▼	(Knezetic and Felsenfeld 1993)
α-SMA	▼	(Zhang, Liu et al. 2005)
Antigen Presentation		
HLA class II	▼	(Didier, Schiffenbauer et al. 1988)
HLA class I	▼	(Lloberas, Maki et al. 1995)
Proliferation		
DNA-Polymerase-alpha	▲	(En-Nia, Yilmaz et al. 2005)
c-myc	▼	(Chernukhin, Shamsuddin et al. 2000)

Transport Proteins, Detoxification		
MDR-1	▲	(Bargou, Jurchott et al. 1997)
MRP1	▲	(Chansky, Hu et al. 2001)
MRP2	▼	(Geier, Mertens et al. 2003)
Grp78	▼	(Li, Hsiung et al. 1997)
Viral Promoters		
RSV	▲	(Swamynathan, Nambiar et al. 1998)
HTLV-1	▲	(Kashanchi, Duvall et al. 1994)
HIV	▲	(Ansari, Safak et al. 1999)
JC Polyomavirus	▲	(Safak and Khalili 2001)

1.2.3 Protein-protein interactions of human CSPs

Biological functions of DbpA and DbpB/YB-1 are extended further due to interactions with partner proteins or other co-factors. The following protein-protein interactions of DbpA are summarized in Table 3. Much more studies have focused on the interacting partners of DbpB/YB-1, and known characters are summarized in Table 4.

Table 3: Protein-protein interactions of DbpA

Interacting Protein	Relevance of interaction	References
Phosphorylation		
Erk/RSK	phosphorylates S134	(Sears, Luong et al. 2010)
mRNA Translation		
symplekin	mRNA translation	(Buchert, Darido et al. 2009)
GEF-H1	mRNA translation	(Nie, Aijaz et al. 2009)
PTB	VEGF mRNA stabilization	(Coles, Bartley et al. 2004)
Cytoskeleton		
ZO-1	Tight-junction signaling	(Balda, Garrett et al. 2003)
Cell cycling		
CDK4/CDK5	Cell cycling	(Moorthamer, Zumstein-Mecker et al. 1999)

Table 4: Protein-protein interactions of DbpB/YB-1

Interacting Protein	Relevance of interaction	References
Phosphorylation		
Akt	phosphorylates S102	(Sutherland, Kucab et al. 2005)
Casein kinase II	phosphorylation	(Skabkin, Kiselyova et al. 2004)

Co-Transcription factor		
AP-2	MMP-2 transcription	(Mertens, Alfonso-Jaume et al. 1998)
CTCF	c-myc transcription	(Chernukhin, Shamsuddin et al. 2000)
Agno-protein (JC virus)	viral gene transcription	(Safak, Sadowska et al. 2002)
Nm23-ß	MMP-2 transcription	(Marenstein, Ocampo et al. 2001)
p53	p21 and MMP-2 transcription	(Chen, Gherzi et al. 2000)
Pur α	a-SMA transcription	(Ansari, Safak et al. 1999)
p300/Smad3	collagen gene transcription	(Dooley, Said et al. 2006)
RelA (p65)	viral gene transcription	(Raj, Safak et al. 1996)
T-antigen (JC virus)	viral gene transcription	(Ansari, Safak et al. 1999)
mRNA Transport, stabilization and splicing		
Actin	mRNA transport	(Ruzanov, Evdokimova et al. 1999)
hnRNP K	mRNA transport	(Shnyreva, Schullery et al. 2000)
Nucleolin	IL-2 mRNA stabilization	(Chen, Gherzi et al. 2000)
SRp30c	mRNA splicing	(Raffetseder, Frye et al. 2003)
TLS	mRNA splicing	(Chansky, Hu et al. 2001)
PTB	VEGF mRNA stabilization	(Coles, Bartley et al. 2004)
Microtubulules	mRNA transport	(Chernov, Curmi et al. 2008)
Others		
EGFR	enhance EGFR signaling	(Berquin, Pang et al. 2005)
Endonuclease III	DNA repair	(Marenstein, Ocampo et al. 2001)
Notch-3 receptor	receptor activation	(Rauen, Raffetseder et al. 2009)

1.2.4 Sub-cellular localization of human CSPs

The functions fulfilled by human CSPs take place in different sub-cellular compartments: nucleus, cytoplasm as well as extracellularly. Nuclear translocation of CSPs to activate target genes transcription is found with epithelial cell proliferation. For instance, DbpA releasing from tight junctions and shuttling to the nucleus is observed in proliferating renal tubular cells (Sourisseau, Georgiadis et al. 2006) and intestinal goblet cells (Buchert, Darido et al. 2009). DbpA nuclear accumulation in hepatocellular carcinoma cells is associated with poor outcome (Yasen, Kajino et al. 2005), while similar phenomena of DbpB/YB-1 nuclear localization is also detected in various cancer diseases, e.g. breast cancer (Kashihara, Azuma et al. 2009) and non-small cell lung cancer (Gessner, Woischwill et al. 2004). However, the activities of nuclear CSPs in mesenchymal cells may be distinct from epithelial cells. In mesangial cells, DbpB/YB-1 nuclear accumulation is found in healthy control tissues without cell proliferation (van Roeyen, Eitner et al. 2005). In addition, DbpB/YB-1 nuclear accumulation in hepatic stellate cells functions as an anti-fibrotic factor by stimulation of Smad7 transcription (Dooley, Hamzavi et al. 2008).

On the other hand, human CSPs localized in the cytoplasm regulate translation, e.g. of VEGF (Coles, Bartley et al. 2004), IL-2 (Chen, Gherzi et al. 2000), GM-CSF (Capowski, Esnault et al. 2001) and TGF-β transcripts (Fraser, Phillips et al. 2008) In cancer tissue of different origins, staining of cytoplasmic DbpA and DbpB/YB-1 is often detected (Bergmann, Royer-Pokora et al. 2005; Yasen, Kajino et al. 2005). In addition, cytoplasmic accumulation of DbpB/YB-1 is observed within mesangial cells of animals suffering from mesangioproliferative disease. This suggests that cytoplasmic CSPs may regulate cell proliferation at the post-transcriptional level (van Roeyen, Eitner et al. 2005).

Recently, DbpB/YB-1 has been reported to be released to the extracellular space through a non-classical secretion pathway. Exteracellularly it may interact with the domains of receptor Notch-3 (Frye, Halfter et al. 2009; Rauen, Raffetseder et al. 2009).

Taken together, DbpA and DbpB/YB-1 may function as transcription factors in the nucleus, as translation factors in the cytoplasm, as well as mitogens or chemoattractants in the extracellular compartment (Figure 6).

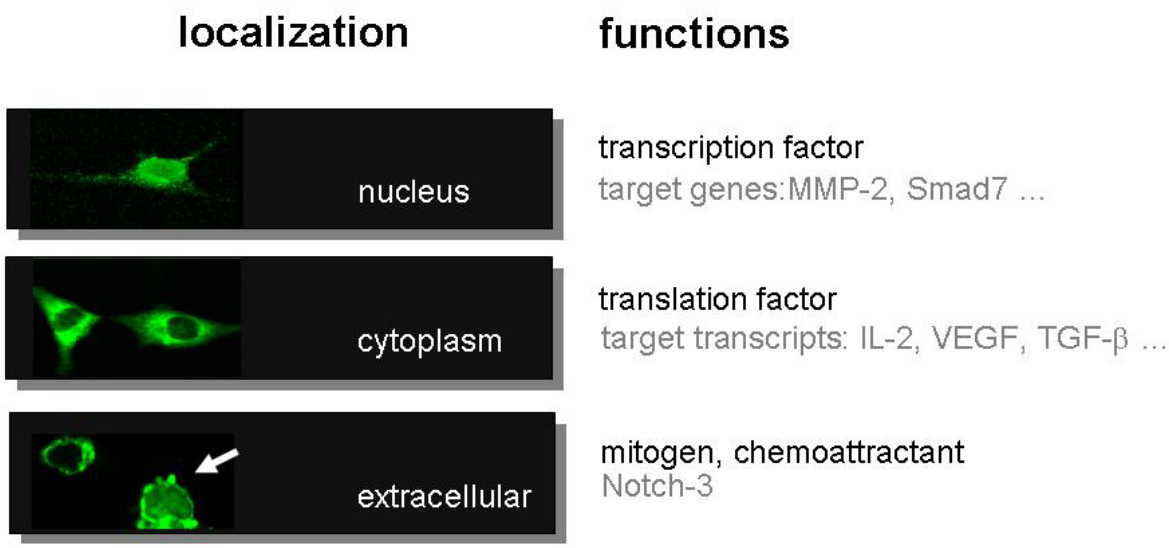


Figure 6. DbpA and DbpB/YB-1 localization and relevant functions.

1.2.5 Regulation of CSP expression, modification and shuttling

The major regulation mechanisms contributing to bacterial CSP CspA accumulation appear to be at the post-transcriptional level (Gualerzi, Giuliodori et al. 2003). There is an unusually long 5'UTR in CspA mRNA, which plays an important role in stabilizing CspA transcripts and enhancing translational activity at low temperature (Mitta, Fang et al. 1997). Human CSPs expression is similarly controlled at the translational level. It has been reported that DbpB/YB-1 auto-regulates its own mRNA translation by binding to a 3'UTR regulatory element (Skabkina, Lyabin et al. 2005). Human CSP genes lack typical eukaryotic regulatory sequences, such as TATA and CCAAT boxes, instead contains E-boxes and GATA motifs. Transcription factors containing the basic-helix-loop-helix structural motif typically approach E-boxes and regulate transcription. Several of them have been reported to be involved in the up-regulation of human CSP transcription, e.g. c-myc (Uramoto, Izumi et al. 2002; Li, Van Calcar et al. 2003), Math2 (Ohashi, Fukumura et al. 2009) and Twist1 (Shiota, Izumi et al. 2008; Shiota, Izumi et al. 2008). Programmed cell death protein 4 (PDCD4) and p53 are reported to regulate DbpB/YB-1 expression by directly interacting with Twist1 (Shiota, Izumi et al. 2008; Shiota, Izumi et al. 2009). Besides E-boxes, a functional GATA motif is found in human CSP promoter sequences. DbpB/YB-1 is transcriptionally regulated by GATA-1, which is crucially involved in erythroid differentiation (Yokoyama, Harigae et al. 2003). In addition, DbpA gene transcription is enhanced by E2F1, which seems to be relevant in hepatocarcinogenesis (Arakawa, Kajino et al. 2004).

Concerning upstream events different growth factors, cytokines and signaling pathways are involved in the regulation of human CSP. DbpB/YB-1 protein expression is induced by various stimuli, e.g. PDGF-B (van Roeyen, Eitner et al. 2005), TGF-β (Feng, Huang et al. 2009) and IL-2 (Sabath, Podolin et al. 1990). The MAPK/ERK pathway mediates PDGF-B effects and thereby up-regulates DbpB/YB-1 expression (van Roeyen, Eitner et al. 2005; Feng, Huang et al. 2009).

Numerous studies show nuclear shuttling of DbpB/YB-1 in the cells that stressed by UV light, hyperthermia (Ohga, Koike et al. 1996) or stimulated with cytokines (like IFN-gamma, IGF-1) and thrombin (Stenina, Poptic et al. 2000; Higashi, Inagaki et al. 2003). Two distinct mechanisms may

be involved: 1. Thrombin induces signals with DbpB/YB-1 cleavage. The resultant protein C-terminal fragment contains a cytoplasmic retention signal (CRS). The N-terminal protein fragment on the other hand shuttles into the nucleus (Stenina, Poptic et al. 2000; Stenina, Shaneyfelt et al. 2001). The 20S proteasome is considered to be a mediator of this cleavage event (Sorokin, Selyutina et al. 2005). Within the highly conserved CSD a phosphorylation site (Ser134 within DbpA and Ser102 within DbpB/YB-1) has been identified as substrate of PI3K/Akt (Bader, Felts et al. 2003; Sutherland, Kucab et al. 2005) or ERK/RSK (Coles, Lambrusco et al. 2005; Stratford, Fry et al. 2008). Studies with breast cancer cells from the group of Prof. Dunn (Vancouver, Canada) show that Ser102 phosphorylation of DbpB/YB-1 mediates nuclear translocation (Sutherland, Kucab et al. 2005; Stratford, Fry et al. 2008; Dhillon, Astanehe et al. 2010). Of note, studies from Evdokimova and colleagues reveal that Akt-mediated phosphorylation of Ser102 within DbpB/YB-1 is critical for its translational activities (Evdokimova, Ovchinnikov et al. 2006; Evdokimova, Ruzanov et al. 2006). Ser102 phosphorylation of DbpB/YB-1 reduces its affinity to capped 5' terminus of mRNA and releases mRNA for translation. The phosphorylated DbpB/YB-1 dissociates from mRNA and may shuttle to nucleus, as observed by Dunn's group.

Besides nuclear translocation, DbpB/YB-1 may also shuttle from the nucleus to the cytoplasm, as observed in a rat model of mesangioproliferative nephritis. PDGF-B with MAPK/ERK signaling are considered to play roles in this nuclear-cytoplasm translocation, but the mechanisms are still unclear (van Roeyen, Eitner et al. 2005). Furthermore PDGF-B may induce DbpB/YB-1 secretion by mesangial and inflammatory cells (Frye, Halfter et al. 2009; Rauen, Raffetseder et al. 2009).

1.3 Biological functions of human CSPs

Human CSPs direct gene transcription (Jurchott, Bergmann et al. 2003), DNA replication (En-Nia, Yilmaz et al. 2005), DNA repair (Das, Chattopadhyay et al. 2007), mRNA stablization and translation (Evdokimova, Ruzanov et al. 2006), drug resistance (Chattopadhyay, Das et al. 2008), cytokines (Chen, Gherzi et al. 2000; Capowski, Esnault et al. 2001) and matrix synthesis (Mertens, Harendza et al. 1997; Norman, Lindahl et al. 2001). CSP DbpA and DbpB/YB-1 share biological roles differences become apparent. The common biological functions are summarized below, difference will be emphasized thereafter.

1.3.1 Promote cell proliferation and carcinogenesis

Most effort of human CSPs studies have been paid to their roles in cell proliferation and carcinogenesis, since over-expression of DbpA or DbpB/YB-1 have be detected in various cancer diseases, e. g. breast cancer (Bargou, Jurchott et al. 1997; Bergmann, Royer-Pokora et al. 2005), non-small cell lung carcinoma (Gessner, Woischwill et al. 2004), ovarian adenocarcinomas (Kamura, Yahata et al. 1999; Oda, Ohishi et al. 2007), human osteosarcomas (Oda, Sakamoto et al. 1998; Oda, Ohishi et al. 2003), colorectal carcinomas (Shibao, Takano et al. 1999; Vaiman, Stromskaya et al. 2007), malignant melanomas (Schittek, Psenner et al. 2007), leukemia (Sears, Luong et al. 2010) and hepatocellular carcinoma (Yasen, Kajino et al. 2005). On the other hand, growth-promoting effects of human CSPs are not restricted to cancer diseases. Recent studies in kidney ontogeny show that DbpA-expressing renal tubulal cells are highly proliferative (Lima, Parreira et al. 2010).

- Underlying mechanisms:

1. Human CSPs promote cell proliferation and carcinogenesis by inducing cell cycle regulatiory proteins, e.g. DbpA induces cyclin D1 and PCNA (Sourisseau, Georgiadis et al. 2006); DbpB/YB-1 activates cyclin A and cyclin B1 (Jurchott, Bergmann et al. 2003). DbpB/YB-1 may

also directly interact with cyclin D1 (Khandelwal, Padala et al. 2009) and PCNA (Ise, Nagatani et al. 1999).

2. Human CSPs may regulate signaling pathways that guide cell proliferation. EGF signaling plays a role in the progression of breast cancer, cell growth EGF receptor (EGFR) and Her-2 have been regarded as important prognostic markers. Human CSP may bind to the EGFR and Her-2 promoter regions and enhance gene expression (Sakura, Maekawa et al. 1988; Stratford, Habibi et al. 2007). Elevated DbpB/YB-1 levels in breast cancer samples correlate with EGFR expression and poor outcome. In addition, EGF signaling may induce DbpB/YB-1 nuclear translocation (Berquin, Pang et al. 2005). The later may indicate a positive feedback loop promoting cell proliferation (Brandt, Raffetseder et al. 2011). Similar regulatory mechanisms were found for PDGF signaling (van Roeyen, Eitner et al. 2005; Zhang, Liu et al. 2005).

3. During the pathogenesis of human hepatocellular carcinoma (HCC), hepatitis virus B and C infection is major contributor. Hino's group reported DbpA and DbpB/YB-1 are binding partners of HBV DNA fragment (nt1855-1914), that human CSPs may contribute to the recombination of HBV and human DNA, cause genomic instability and enhance HCC occurrence (Kajino, Yamamoto et al. 2001).

4. Human CSPs may promote cell proliferation and carcinogenesis by regulating other proliferation-associated genes, e.g. thymidine kinase (Kim, Lau et al. 1997), DNA polymerase α (En-Nia, Yilmaz et al. 2005), c-myc (Kolluri and Kinniburgh 1991) and p53 (Homer, Knight et al. 2005).

1.3.2 Repress cell differentiation

Human CSP may repress cell differentiation and thereby contribute to tumorogenesis and other diseases, e.g. refractory anemia (Yokoyama, Harigae et al. 2003). During kidney ontogeny and polarization of proximal tubular cells, DbpA protein levels decrease in parallel and inversely with progressing apical differentiation. The expression of renal tubular cell specific antigens megalin and cubilin are suppressed by DbpA (Lima, Parreira et al. 2010). In intestinal goblet cells acute myeloid leukemia-1 (AML1) protein has been identified as a promoter of intestinal differentiation mediated by Kruppel-like factor 4 (KLF4) signaling. In cooperation with symplekin, DbpA is found be a transcriptional repressor that directly binds to the promoter of the AML1 gene, thus inhibiting intestinal differentiation as confirmed both *in vitro* and *in vivo* (Buchert, Darido et al. 2009). DbpA also plays an important role during fetal erythropoiesis. Production of fetal haemoglobin is inversely correlated to DbpA expression, since DbpA directly binds to the hemoglobin-2 (HGB2) promoter and suppresses its transcription (Horwitz, Maloney et al. 1994; Petruzzelli, Gaudino et al. 2010). DbpB/YB-1 was reported to function as a negative regulator of myoblast differentiation by suppressing α-SMA gene transcription (Zhang, Liu et al. 2005) or by interacting with Msh homeobox 1 (Msx1) (Song and Lee 2010). Other reports show DbpB/YB-1 is negatively related to neural stem cells (Fotovati, Abu-Ali et al. 2011) and erythroid cell differentiation (Yokoyama, Harigae et al. 2003; Bhullar and Sollars 2011). In addition, DbpA may also regulate muscle cell differentiation. cdk5 kinase, with p35 as its activator, has been demonstrated to be responsible for muscle cells differentiation (Philpott, Porro et al. 1997). Either over-activation or blockage of cdk5/p35 expression may result in muscle disruption, therefore cdk5 kinase activity should be balanced/blocked during terminal differentiation of muscle cells. One possible mechanism would envision DbpA directly interacting with cdk5 and acting as a competitor of p35 (Moorthamer, Zumstein-Mecker et al. 1999). This assumption is partially supported by the fact that DbpA is highly expressed in skeletal and heart muscle cells as compared to other tissues (Kudo, Mattei et al. 1995; Moorthamer, Zumstein-Mecker et al. 1999).

1.3.3 Involved in cell-cell communication and cytoskeleton formation

Human CSPs are involved in the fine tuning of cell-cell communication, early-on recognized given their effects on major histocompatibility complex (MHC) I-A beta (Lloberas, Maki et al. 1995). Human CSPs directly regulate the expression of cytokines, e.g. granulocyte-macrophage colony-stimulating factor GM-CSF (Coles, Diamond et al. 1996; Coles, Diamond et al. 2000; Diamond, Shannon et al. 2001), IL-2 (Chen, Gherzi et al. 2000) and TGF-β (Fraser, Phillips et al. 2008; Jenkins, Bennagi et al. 2010) , as well as receptors, e.g. EGFR (Sakura, Maekawa et al. 1988); Recently, DbpB/YB-1 has reported to be exported to the extracellular space through a non-classical secretion pathway and may interact with the extracellular domain of Notch-3 receptor (Frye, Halfter et al. 2009; Rauen, Raffetseder et al. 2009). It indicates DbpB/YB-1 may also functions as a mitogen or chemoattractant in the extracellular compartment.

Tight and gap junctions, are of great importance for cell-cell communication of neighboring cells. Numerous studies reveal that DbpA is localized within tight junctions (TJ) and regulates TJ-associated activities. Balda and Matter first identified the cooperation of DbpA with zonula occludens 1 (ZO-1) protein, one of the major components of TJ (Balda and Matter 2000); Later on, two additional TJ-associated proteins were identified as partners of DbpA, symplekin (Kavanagh, Buchert et al. 2006) and GEF-H1 (Nie, Aijaz et al. 2009). Several other TJ-associated proteins have been reported to be involved in the orchestration of DbpA activities at TJ, e.g. heat-shock protein Apg-2 (Tsapara, Matter et al. 2006), B-cell lymphoma 2 (Bcl-2) (Li, Backer et al. 2003), Ras-related protein Ral-A (Frankel, Aronheim et al. 2005) and blood vessel epicardial substance (Bves) (Russ, Pino et al. 2011). DbpA-ZO-1 complex at the TJ regulates paracellular permeability. When DbpA is released from the TJ by competitive binding, smodification or TJ disruption, it may form complexes with symplekin and GEF-H1, translocate to the nucleus and thereby affect cell proliferation and differentiation as a transcription factor. This mechanism has been confirmed by several studies *in vivo* (Pannequin, Delaunay et al. 2007; Georgiadis, Tschernutter et al. 2010; Lima, Parreira et al. 2010). DbpA and ZO-1 also colocalize at oligodendrocyte and astrocyte gap junctions in mouse brain (Penes, Li et al. 2005). DbpA was found to be associated with oligodendrocytic connexin Cx47 and Cx32 proteins as well as with astrocytic Cx43. In Cx47 knock-out mice DbpA

was absent in oligodendrocytes (Li, Penes et al. 2008). These data suggest that DbpA may regulate glial gap junction associated signaling and activities.

Hitherto no evidences exist that DbpB/YB-1 is involved in tight or gap junction formation. DbpB/YB-1 promotes microtubule assembly through interaction with tubulin and microtubule (Chernov, Mechulam et al. 2008). In addition, the N-terminal domain of DbpB/YB-1 may directly interact with actin and microfilaments (Ruzanov, Evdokimova et al. 1999). These interactions of DbpB/YB-1 with microtubules and microfilaments may play a role in mRNA transport, anchoring and localization with cytoskeleton elements.

1.3.4 Respond to cell stress and drug-resistance

DbpB/YB-1 was found to be over-expressed in many drug resistant breast cancer patients. Multidrug resistance-1 (MDR-1) gene encodes for P-glycoprotein (an ABC transporter) believed to be responsible for multidrug resistance in cancer cells. DbpB/YB-1 is directly involved in MDR-1 gene transcription in response to genotoxic stress, e.g. cisplatin, mitomycin C and UV radiation (Asakuno, Kohno et al. 1994; Ohga, Uchiumi et al. 1998). Another ABC transporter regulated by DbpB/YB-1 is Mrp2, which is down-regulated at the transcriptional level following stimulation with cytokines (Geier, Mertens et al. 2003). On the other hand, when mammalian cells are subjected to physiological stress targeting the endoplasmic reticulum (ER), cells activate a defense mechanism denoted “unfolded protein response” (UPR), which is evolutionarily conserved from yeast to humans (Foti, Welihinda et al. 1999). The major cellular response protein in UPR is an ER chaperone called Grp78 (Reddy, Mao et al. 2003). Grp78 not only binds to unfolded proteins but also regulates the activation of ER stress signal transducers, that protect stressed cells from apoptosis (Bertolotti, Zhang et al. 2000). DbpA and DbpB/YB-1 have been identified as transcriptional regulators that bind to the stress-inducible change region (SICR) of the Grp78 gene (Li, Hsiung et al. 1997).

1.3.5 Regulate angiogenesis, lymphangiogenesis and metastasis formation

Roles of human CSP in angiogenesis and lymphangiogenesis are easily envisioned given the direct effects on vascular endothelial growth factor (VEGF) expression. In 2002, Coles and the colleagues first reported DbpA as a transcriptional repressor of the VEGF-A gene. DbpA interacts with hypoxia inducible factor-1 (HIF-1) and targets the hypoxia-responsive element (HRE) within the VEGF-A promoter (Coles, Diamond et al. 2002). This mechanism requires DbpA phosphorylation of the N-terminal domains by ERK2 and GSK3b (Coles, Lambrusco et al. 2005). Post-transcriptional regulation of VEGF by DbpA and DbpB/YB-1 occurs given binding to mRNA in cooperation with polypyrimidine tract binding (PTB) RNA proteins (Coles, Bartley et al. 2004). DbpA may represses angiogenesis mediated by VEGF-A, co-confirmed by two groups from Japan (Saito, Nakagami et al. 2008; Matsumoto, Yajima et al. 2010; Saito, Nakagami et al. 2011). In addition, DbpA represses transcription of VEGF-C gene expression *via* targeting to the serum response element (SRE) within the promoter (Saito, Nakagami et al. 2008; Matsumoto, Yajima et al. 2010). Considering VEGF-C is a major growth factor for lymphatic endothelial cells.

These results indicate that CSP repress angiogenesis and lymphangiogenesis. However, various studies indicate that high-expression of DbpA or DbpB/YB-1 in cancer cells goes along with invasive properties (Yasen, Kajino et al. 2005; Wang, Zheng et al. 2009). CSP may promote tumor cell metastasis by binding and modulating the activity of metastasis susceptibility gene ribosomal RNA processing 1 homolog B (Rrp1b) (Crawford, Yang et al. 2009) or by up-regulating expression of metastasis promoting proteins, e.g. gelatinase A (Mertens, Alfonso-Jaume et al. 1998), MUC1 (Shiraga, Winpenny et al. 2005) and snail (Evdokimova, Tognon et al. 2009). Contradictory roles of human CSP with promotion or suppression of metastasis formation may be reconciled by distinct functions in different cell types. Repression of VEGF expression by human CSPs is observed in fibroblasts, while invasive cancer cells with CSP overexpression predominantly represent epithelial cells.

1.4 Human CSPs in inflammatory diseases

Inflammatory diseases are characterized by a complex biological response of tissue to harmful stimuli, such as pathogens, damages and irritants. Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli with increased movement of plasma proteins and leukocytes (e.g. monocytes and granulocytes) from the blood into injured tissue. If the condition causing acute inflammation is not resolved, inflammation may transit into a chronic phase with fibrogenesis. Some pathogens by their enduring and self renewing nature provoke chronic inflammation, e.g. autoimmune diseases. The primary cells of chronic inflammation are monocytes/macrophages and lymphocytes. A cascade of biochemical events mediates the inflammatory response, involving the local vascular system, immune system and various cell-types within the injured tissue. Although studies on human CSP are often focused on malignant diseases, many of the biological functions summarized in the previous paragraphs indicate that CSP also play important roles in inflammatory diseases, e.g. of the kidney and liver.

1.4.1 Human CSPs regulate cytokine expression

CSP directly regulate the expression of various inflammatory cytokines. IL-2 is synthesized and secreted by activated T-cells, stimulates the differentiation and survival of antigen-selected cytotoxic T-cells and the maturation of regulatory T-cells. This is of great importance for autoimmune and chronic inflammatory diseases (Taniguchi and Minami 1993). It has been reported that DbpB/YB-1 may directly bind to and stabilize IL-2 mRNA following T-cell activation and thereby translationally regulate IL-2 expression (Chen, Gherzi et al. 2000). Stimulation of T helper lymphocytes with IL-2 induces DbpB/YB-1 expression (Sabath, Podolin et al. 1990). This may indicate the existence of an auto-stimulatory feedback loop of IL-2 and DbpB/YB-1 (Brandt, Raffetseder et al. 2011). Besides IL-2 in T-cells, DbpB/YB-1 may also directly stabilize and up-regulate the translation of GM-CSF transcript in activated eosinophils, that prolongs their survival (Capowski, Esnault et al. 2001). DbpA and DbpB/YB-1 were reported to suppress

GM-CSF gene transcription in embryomic lung fibroblasts (Coles, Diamond et al. 1996). This may indicate a cell-type specific regulatory mechanism. Similar events are reported for monocytes/marcrophages during cell differentiation and RANTES/CCL5 gene transcription (Raffetseder, Rauen et al. 2009). DbpB/YB-1 also controls TGF-β translation in proximal tubule cells (Fraser, Phillips et al. 2008; Jenkins, Bennagi et al. 2010) and PDGF-B transcription in endothelial cells (Stenina, Poptic et al. 2000).

1.4.2 Human CSPs in inflammatory kidney diseases

In human biopsies from kidney transplant patients undergoing a rejection episode, DbpB/YB-1 transcripts were found to be amplified 17-fold in acute and 21-fold in chronic allograft rejection compared to control tissue. This up-regulation closely correlates with RANTES/CCL5 mRNA expression under both conditions (Raffetseder, Rauen et al. 2009). DbpB/YB-1 is a cell-type specific regulator of RANTES/CCL5 expression in infiltrating T-cells and monocytes/macrophages (Raffetseder, Rauen et al. 2009).

DbpB/YB-1 has also been identified as an important mediator/regulator in mesangioproliferative glomerulonephritis. DbpB/YB-1 functions as a downstream signaling target of PDGF-B and mediates a mitogenic effect in mesangioproliferative glomerulonephritis (van Roeyen, Eitner et al. 2005). In healthy kidney cells, DbpB/YB-1 is predominantly localized within the nucleus. Subsequent to PDGF-B infusion and in the course of anti-Thy1.1 model re-localization of DbpB/YB-1 into the cytoplasm is observed and coincides with up-regulation of DbpB/YB-1 protein within the mesangial compartment. These events are dependent on PDGF-B signaling *via* the MAPK pathway because these alterations were prevented by specific PDGF aptamers and the MAPK pathway inhibitor U0126. In experimental models that lack profound mesangial cell proliferation (e.g., Puromycin-nephrosis, passive Heyman nephritis, spontaneous normotensive nephrosclerosis, hyperlipidemic diabetic nephropathy) DbpB/YB-1 localization was nuclear (van Roeyen, Eitner et al. 2005). There is evidence that DbpB/YB-1 is secreted by mesangial cell upon cytokine challenge via a non-classical secretion mode (Frye, Halfter et al. 2009). Secreted DbpB/YB-1 is a non-conventional ligand for receptor Notch-3 (Rauen, Raffetseder et al. 2009). Of interests, extracellular blockade of DbpB/YB-1 by application of an antibody potently induces receptor Notch-3 expression and signaling. This may indicate that DbpB/YB-1 controls Notch-3 expression and signaling *via* an auto-regulatory feedback mechanism in mesangioproliferative diseases (Raffetseder, Rauen et al. 2011).

The immunosuppressive calcineurin inhibitors (CNI) cyclosporine A (CsA) and tacrolimus are widely used in transplant organ recipients. In kidney allografts CNI may cause tubulointerstitial as

well as mesangial fibrosis. Recent study from our group shows that CsA and tacrolimus may induce DbpB/YB-1 expression in mesangial cells. This accumulation results in mRNA stabilization and contributes to renal fibrosis via subsequent generation of interstitial collagen fibers (Hanssen, Frye et al. 2011).

On DbpA few studies have been performed in kidney diseases. A recent report reveals an inverse relationship between DbpA expression and apical proximal epithelial cells differentiation during kidney development (Lima, Parreira et al. 2010). Overexpression of DbpA prevents polarization and differentiation of proximal tubulules cells while promoting a proliferative phenotype. Thus, DbpA may play a role in renal tubular diseases.

1.4.3 Human CSPs in inflammatory liver diseases

A close correlation of human CSP protein expression with hepatocellular carcinoma (HCC) formation is reported for the liver (Yasen, Kajino et al. 2005). Regardless of specific etiologies, most of the HCC patients are suffering from chronic inflammation with ensuing liver fibrosis.

Transforming growth factor-β (TGF-β) is considered to be a major factor contributing to liver fibrogenesis. TGF-β and its intracellular mediators, Smad proteins, orchestrate collagen synthesis in hepatic stellate cells, the major collagen-synthenting celltype in the liver (Massague and Chen 2000). Higashi and colleagues first described that nuclear translocation of DbpB/YB-1 antagonizes TGF-β/Smad3 signaling in hepatic stellate cells *in vitro* (Higashi, Inagaki et al. 2003; Higashi, Inagaki et al. 2003). Lateron, this anti-fibrotic effect was confirmed *in vivo* by adenovirus-mediated overexpression of DbpB/YB-1 driven by a collagen enhancer (Inagaki, Kushida et al. 2005) and by HSc025 stimulated nuclear translocation of DbpB/YB-1 (Higashi, Tomigahara et al. 2011). In addition, Dooley and colleagues revealed that DbpB/YB-1 is a potent inducer of Smad7 expression in activated hepatic stellate cells and that it antagonizes TGF-β signaling during chronic stages of fibro-proliferative diseases. Similar regulatory mechanisms were also observed in the kidney (Dooley, Said et al. 2006).

1.5 Aim of the study

Cold shock proteins fulfill various biological functions considered to be important in inflammatory diseases. Only a few studies have been performed to delineate the expression pattern and underlying regulatory events in inflammatory diseases. In addition, most of the findings relate to DbpB/YB-1, but little is known about another prominent member denoted DbpA. The similarities and differences between these two human CSPs are largely unknown. To provide a more thorough insight into the expression pattern and regulatory mechanisms of cold shcok proteins in inflammatory kidney and liver diseases is the goal of the present work.

Based on the former works from our group, that DbpB/YB-1 mediates PDGF-B effects in the mesangial cells of mesangioproliferative nephritis, the first part of the current study focuses on the expression patern and regulation of DbpA in this disease and to compare the findings with DbpB/YB-1.

The second part of the current study is to elucidate the expression pattern(s) of the cold shock proteins in (chronic) inflammatory liver diseases as well as their regulatory events under the stimulation of pro-inflammatory factor, e.g. TGF-β and IL-13.

In addition, different lengths of DbpA and DbpB/YB-1 promoter regions were cloned in order to reveal transcriptional regulatory mechanisms of human CSPs.

2 Materials and methods

2.1 Reagents

Cell culture reagents

RPMI1640 medium	Invitrogen, Karlsruhe, Germany
F-12/DMEM medium	Invitrogen, Karlsruhe, Germany
DMEM medium	Invitrogen, Karlsruhe, Germany
MCBD_201 medium	Sigma-Aldrich, Munich, Germany
William's E medium	Invitrogen, Karlsruhe, Germany

Cell culture additives

L-Glutamin	Lonza, Verviers, Belgium
HBSS	PAA laboratories, Cölbe, Germany
Fetal calf serum (FCS)	Invitrogen, Karlsruhe, Germany
Insulin-transferrin-selenium (ITS)	Invitrogen, Karlsruhe, Germany
Trypsin. 10X solution (2.5 %)	Invitrogen, Karlsruhe, Germany
Penicillin/Streptomycin	Lonza, Verviers, Belgium

Cytokines

Human transforming growth factor-1 (TGF-β1)	Peprotech, London, UK.
Platelet-derived growth factor-BB (PDGF-BB) Rat	Sigma-Aldrich, Munich, Germany
Platelet-derived growth factor-BB (PDGF-BB) human	Sigma-Aldrich, Munich, Germany
Insulin-like growth factor-II (IGF-II) human	Peprotech, London, UK.
Epidermal growth factor (EGF)	BDBiosciences, Heidelberg, Germany

Chemicals

DMSO. Dimethylsulfoxide	Sigma-Aldrich, Munich, Germany
Triton X-100	Sigma-Aldrich, Munich, Germany

Tris, Tris(hydroximethyl)aminomethane	Bio-Rad Laboratories, Munich, Germany
SDS, Sodium dodecylsulfate	Bio-Rad Laboratories, Munich, Germany
DTT, Dithiothreit	Bio-Rad Laboratories, Munich, Germany
Tween20 solution	Bio-Rad Laboratories, Munich, Germany
β-mercaptoethanol	Merck, Darmstadt, Germany
Hepes	Roth, Karlsruhe, Germany
Complete protease inhibitor cocktail	Roche, Mannheim, Germany
Phosphatase inhibitor cocktail	Sigma-Aldrich, Munich, Germany
PMSF	Roche, Mannheim, Germany
10X transfer buffer solution	Invitrogen, Karlsruhe, Germany
MOPS	Applichem, Darmstadt, Germany
Ponceau S	Sigma-Aldrich, Munich, Germany
TCA, Trichloroacetic acid	Sigma-Aldrich Munich, Germany
PBS. Plus calcium and magnesium	Biochrom AG, Berlin, Germany
Phenol/Chlorophorm/Isoamylalcohol	Roth, Karlsruhe, Germany
Na-acetate	Sigma-Aldrich, Munich, Germany
Isopropanol	Merck, Darmstadt, Germany
Ethanol	Merck, Darmstadt, Germany
Methanol	Merck, Darmstadt, Germany
Hydrogen peroxide. 30 % (w/w)	Sigma-Aldrich, Munich, Germany
Acetone	Merck, Darmstadt, Germany
NaOH	Merck, Darmstadt, Germany
HCl	Merck, Darmstadt, Germany
NaCl	Merck, Darmstadt, Germany
KCl	Merck, Darmstadt, Germany
MgSO4	Merck, Darmstadt, Germany
MgCl2	Merck, Darmstadt, Germany
EDTA	Bio-Rad Laboratories, Munich, Germany
Na-citrate	Merck, Darmstadt, Germany
Paraformadehyde	Sigma-Aldrich, Munich, Germany

Formaldeyde 4%	Applichem, Darmstadt, Germany
RotiClear	Roche, Mannheim, Germany
Rat tail collagen-I	Sigma-Aldrich, Munich, Germany
NP40	Roth, Karlsruhe, Germany
Trypanblue	Sigma-Aldrich, Munich, Germany
Agarose	Biozym, Oldendorf. Germany
LB Agar	Roth, Karlsruhe, Germany
LB medium	Roth, Karlsruhe, Germany
Glycerin	Roth, Karlsruhe, Germany

Antibodies

An overview on antibodies used in current study is provided in Table 5.

Table 5. Antibody list

Target	Species	Company Cat.No.	Dilutions
YB-1_C-term	Rabbit	Eurogentec, EP085177	1:1000 (WB), 1:100 (ICH/IF)
YB-1_Royer	Rabbit	Eurogentec, EP081822	1:1000 (WB), 1:100 (ICH/IF)
DbpA	Rabbit	Eurogentec, EP052151	1:1000 (WB), 1:100 (ICH/IF)
GAPDH	Rabbit	Cell Signaling, 2118L	1:5000 (WB)
β-actin	Mouse	Sigma, A1978	1:10000 (WB)
Twist1	Rabbit	Santa Cruz, sc-15393	1:1000 (WB), 1:50 (ICH/IF)
E-cadherin	Mouse	BD Bioscience, 610181	1:1000 (WB), 1:100 (ICH/IF)
Snail	Rabbit	Abcam, ab85931	1:100 (ICH/IF)
S100A4	Rabbit	Abcam, ab27427	1:50 (ICH/IF)
pSmad2	Rabbit	Cell Signaling, 3101S	1:1000 (WB)
ZO-1	Rabbit	Abcam, ab59720	1:50 (ICH/IF)
Rabbit IgG	Goat	Santa Cruz, sc-2301	1:10000 (WB)
Mouse IgG	Goat	Santa Cruz, sc-2305	1:10000 (WB)
Alexa Fluor 488	Goat	Invitrogen, A-11001	1:200 (IF)
Alexa Fluor 633	Goat	Invitrogen, A-21071	1:200 (IF)

2.2 Buffers and solutions

Cell lysis buffer (RIPA buffer). 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % Nonidet P-40 (NP40), 0.5 % sodium deoxycholate, 0.1 % SDS, Proteases Inhibitor Cocktail (Roche, Mannheim, Germany), Phosphatase Inhibitor Cocktail II, Roche, Mannheim, Germany.

SDS-PAGE

The composition of gels was as follows:

Table 6. SDS-PAGE gels		
Separating gel	**12%**	**10%**
Acrylamid	2.6 ml	2.25 ml
1.5 M Tris pH 8.8	1.6 ml	1.62 ml
ddH2O	2.2 ml	2.61 ml
SDS (10%)	66.7 µl	66.7 µl
TEMED	6.0 µl	6.0 µl
APS (10%)	40.0 µl	40.0 µl
Stacking gel:		
Acrylamid	0.3 ml	
1M Tris pH 6.8	0.5 ml	
ddH2O	1.1 ml	
SDS (10%)	19.1 µl	
TEMED	3.4 µl	
APS (10%)	22.9 µl	

10x Running buffer. 144 g Glycin, 30,34 g Tris (for electrophoresis), 100 ml 10% SDS (for electrophoresis); filled up to 1 L with ddH2O, pH 8.3.

Transfer buffer. 750 ml ddH2O, 200 ml methanol, 50 ml NuPAGE Transfer Buffer (20x) (Invitrogen, Karlsruhe, Germany).

10X TBS (Tris-buffered saline). 12.1 g Tris-base (Merck, Darmstadt, Germany), 87.66 g NaCl (Merck) dissolved in 800 ml ddH2O. pH adjusted to 7.6 with 1 M HCl; filled up with ddH2O to 1L.

TBST. 100 ml of 10 x TBS and 10 ml of 10 % Tween20 solution (Bio-Rad Laboratories, Hercules, CA) adjusted with distilled water to 1 L.

Blocking solutions. 5 g of non-fat milk or BSA dissolved in 100 ml TBST.

Reducing buffer. 1M DTT. 154 mg dithiothreitol (DTT, Sigma) dissolved in 1 ml ddH2O; aliquots were kept at -20°C till use.

5x Tris/Glycin loading buffer. 2.5 ml β-mercaptoethanol (12,5%), 2 g SDS (10%), 10 mg Brom-Phenoblue, 6 ml 1 M Tris HCl pH 6.8 (0.3 M), 200 µl 500 mM EDTA (5 mM), 10 ml 99% glycerin (50%) and 1.3 ml ddH2O.

Stripping buffers

Buffer 1. 62.5 mmol Tris/HCL (7.56 g), 2% SDS (20 g) for 1 L, pH was adjusted to 6.7; working solution as follows: 100 ml buffer, 80 µl β-mercaptoethanol. Membranes were incubated in working solution for 10-30 min at 60-70°C. Washed with TBST and blocked again for re-probing.

Buffer 2. 0.2 M NaOH; procedure: membrane was washed for 5 min in ddH2O, 5 min in 0.2 M NaOH, again 5 min in ddH2O. Then, membranes were washed with TBST and blocked again prior antibody incubation.

MOPS running buffer. Freshly prepared by diluting 25 ml of NuPAGE MOPS SDS Running Buffer (20 x) (Invitrogen GmbH) in 475 ml ddH2O.

Fixation solution

Buffer 1. 10% Formalin. Dilute 37% Formaldehyde 10 time. Do not store longer than 3 months.

Buffer 2. Methacarn. 60% Methanol, 30% Chlorofrom and 10% glacial acetic acid.

Buffer 3. 4 % paraformaldehyde (PFA). Add 4 g paraformaldehyde to 50 ml hot water, add NaOH drop wise under agitation until solution is clear, add 10 ml 10 x PBS, adjust pH 7.4 using phosphoric acid, add water to 100 ml end volume. Store in -80°C or use directly.

Permeabilization buffer. 0.3% Triton X-100 v/v in PBS pH 7.4.

Antigen retrieval buffer

Buffer 1: 10mM Na-Citrate Buffer. To prepare 1 L add 2.94 g Na-Citrate to dH2O and adjust to pH6.0

Buffer 2: 1mM EDTA Buffer. To prepare 1 L add 0.372 g EDTA to dH2O and adjust to pH8.0

Tween washing buffer. add 500 µl 10% Tween 20 solution to 49.5 ml PBS. This results in a final concentration of 0.1% v/v.

Blocking buffer. 1 % BSA in PBS for immunofluorescence; 50% FCS and 0.1%BSA in PBS for immunohistochemistry.

Protein ladder. See Blue plus2 pre-stained (Invitrogen, Karlsruhe, Germany)

TCA solution. add20g TCA to 100 dH2O and store at 4°C

2.3 Cell culture and incubation protocols

Rat mesangial cells were grown in RPMI 1640 medium supplemented with 10% FCS and 100 IU/ml penicillin at 37°C in humidified air containing 5% CO_2.

Primary human mesangial cells (cc-2559, Lonza Group Ltd, Swizerland) were grown in Clonetics™ MsGM™ culture system (CC-3146, Lonza Group Ltd, Swizerland) containing Mesangial Cell Basal Medium 500ml supplemented with FBS25ml, GA-1000 0.5 ml, at 37°C in humidified 5% CO_2 in air.

Human embryonic kidney cells (HEK-293), human proximal tubular epithelial cell lines (HK-2 and HKC-8) were grown in DMEM/F12 medium supplemented with 10% FCS and 100 U/ml penicillin at 37°C in humidified air containing 5% CO_2.

Primary hepatic stellate cells (HSCs) were isolated from livers of Wistar rats and cultured in DMEM medium supplemented with 4 mmol/L-glutamine, 10% FCS and 100 IU/ml penicillin. The first medium change was performed 24 hours after seeding. Cells were maintained at 37°C in humidified air containing 5% CO_2.

Mouse liver progenitor cells (LPC) were grown in Williams'E medium supplied with 2% FCS, 15 ng/ml IGF-II, 10 ng/ml EGF and 5ug/ml ITS.

Cytokine incubation

As TGF-β and IL-13 exhibit cross reactivites between species (human, mouse and rat), human TGF-β (5 ng/ml) and IL-13 (50 ng/ml) were used to perform cytokine incubation studies. 50 ng/ml rat or human PDFG-BB were used to stimulate rat or human mesangial cells, respectively. Before stimulation, mesangial cells were growth-arrested with serum-free MCDB-Medium (Sigma) for 24 hours. FCS was reduced to 0.5% for starvation overnight of primary HSCs and to 0% for other cell lines before cytokine incubation.

2.4 Protein work

Protein lysates of cultured cells

Cell lines and hepatocytes were washed twice with ice cold PBS and lysed with 150 µl ice-cold RIPA buffer in 6 well culture plates. Cells were scraped with a rubber policeman and collected in 1.5 ml tubes. The solution was kept in ice for 45 min and centrifuged for 15 min at 13.000 rpm, 4°C. The supernatant was collected and protein concentrations were determined using the Bio-Rad protein assay. For SDS-PAGE, 10-20 µg/well were supplemented with reducing buffer and loaded onto the gels for electrophoresis.

Protein concentration measurement

Protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories) that is based on the Lowry assay. Each probe was quantified as triplicates in a micro-titer plate and measured. The absorbance was quantified at 690 nm using a spectrophotometer (Tecan). Protein concentrations were calculated as averages and related to the protein standard curve (prepared by a BSA dilution series) and a blank value obtained with RIPA buffer only.

Western blot

For Western bloting 10-20 µg of proteins were pipetted into a 1.5 ml tube. 5 µl of NuPage LDS Sample Buffer (4x) (Invitrogen GmbH) and 1 µl of 1 M DTT were added. Samples were adjusted to 20 µl with sterile water. After 10 min of incubation at 95°C the samples were briefly centrifuged at 14.000 rpm and loaded onto SDS-PAGE gels from Invitrogen. When using self-made gels (Tris/Glycin system), protein samples were added to loading buffer containing β-mercaptoethanol.

SDS-PAGE SDS-polyacrylamide gel electrophoresis under denaturing conditions. NuPAGE 4-12 % Bis-Tris gel (Invitrogen GmbH) and MOPS buffer were used as standard protocol. Electrophoresis was carried out in an XCell II Mini Cell apparatus (Invitrogen GmbH) for about 1 hour at constant voltage of 150 V. Alternative standard protocol: Self-made gels were prepared (10-12 %) and ran in a Bio-Rad chamber for 2 h at constant voltage of 100 V in Laemmli running buffer.

Protein transfer on membranes

Protein transfer onto nitrocellulose membranes was performed using an XCell II blotting apparatus with an XCell II Blot Module (Invitrogen GmbH). Chromatography paper (Whatman, Maidstone, England) and nitrocellulose membrane sheets (0.45 micron, Pierce, Rockford, IL) were used to prepare a gel membrane sandwich. Blotting pads and the gel membrane sandwich were placed in the XCell II Blot module according to the manufacturer's instructions with transfer buffer. Blotting was performed for 1.5 h at constant currency of 250 mA.

Immunodetection of proteins

The blotted nitrocellulose membrane was incubated with Ponceau Red solution to determine equal loading and to allow cutting of membranes. After washing of the membranes with TBST, membranes were blocked for 1 h at room temperature followed by incubation with 1st antibody overnight at 4°C. The membranes were washed 3 times in TBST buffer and incubated with the 2nd antibody conjugated with peroxidase for 1 h at room temperature, then washed 4 times (for 5 min) in TBST. To detect protein on the membranes ECL solution was added. Chemiluminescence was detected using a Fujifilm LAS 1000 image detection system or the Chemi-Smart system from INTAS.

Precipitation of secreted proteins (TCA)

750 ul cell culture medium was added 1:1 to the 20% TCA solution, then mixed and stored in -20°C overnight. Next day, the samples were thawed in ice and centrifuged at 13.000 rpm for 30 min in 4°C. Discard the complete supernatant and re-suspended the pallet with 1ml ice-cold 70% Ethanol, then centrifuged again at 13.000 rpm for 30 min in 4°C and discard the supernatant. When the pellet was dry, dissolved it in ddH2O for immediately using or stored it at -80°C.

2.5 Immunohistochemistry and immunofluorescence staining

Tissue fixation, embedding and sectioning

Tissue samples were fixed for the indicated times (depends on the sample size) with either 10% formalin or methacarn, then dehydrated through a graded ethanol series and embedded with paraffin wax and cast into blocks. Paraffin blocks were cut into 4 um sections and mouted on poly-L-lysine-coated glass slides for further staining processing.

Cells fixation for immunofluorescence

Cells were washed twice with PBS and fixed in 4% PFA/PBS for 10 minutes, followed by permeabilization with permabilization buffer for 5 min and fixed a second time with 2% PFA/PBS for 5 min and washed 3 times in PBS.

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence were performed using following protocols: Paraffin-embedded tissue sections were deparaffinized in RotiClear and rehydrated in graded ethanol. Endogenous peroxidase and nonspecific binding were blocked by incubation with blocking solutions (Dako). The sections were incubated overnight at 4 °C with the primary antibody diluted in PBS plus 1% BSA (Sigma). Following day, after 3 times washed in PBS, the sections were incubated with peroxidase labeled second antibody (Dako) for 1 hour at room temperature. Staining were developed with diaminobenzidine (DAB). For formalin fixed, paraffin-embedded biopsy tissue sections, an additional antigen retrieval step was necessary after the rehydration, that to heat the sections by microwave 600W for 10 min within 10 mM sodium citrate (pH 6.0) or 1 mM EDTA (pH 8.0) buffer. Negative controls for the immunohistochemical procedures consisted of substitution of the primary antibody with nonimmune rabbit IgG (Dako). For immunofluorescence, fluorescencedye-labeled second antibodies were used instead of peroxidase labeled second antibody.

Phase contrast and fluorescent microscopy

Phase contrast and conventional epi-fluorescence images were obtained with the Leica IPB

microscope equipped with a Leica DC500 camera (Leica Microsystems, Wetzlar, Germany). For conventional epi-fluorescent images, excitation was performed with an EQB 100 isolated fluorescent lamp. Image acquisition was done with Leica IM50 software.

Confocal microscopy

Confocal images were obtained by using a Leica laser scanning spectral confocal microscope, DM IRE2, with an HCX PL Apo 40x/1.32 numeric aperture oil objective (Leica Microsystems, Wetzlar, Germany). Excitation was performed with an argon laser emitting at 488 nm, a krypton laser emitting at 568 nm, and a helium/neon laser emitting at 633 nm. Images were acquired with a TCS SP2 scanner and Leica Confocal software, version 2.5 (Leica Microsystems).

2.6 mRNA isolation, cDNA synthesis and real- time PCR

RNA isolation

RNA isolation was performed using RNeasy kit (Qiagen, Hilden, Germany) following the manual. Cells growing in 6 well plates were lysed with 350 µl RLT buffer, homogenized and adjusted with ethanol for proper binding conditions. Samples were pipetted into a spin column for isolation. After centrifugation, followed with 3 time wash; the RNA was eluted with water in a final volume of 40 µl. RNA concentrations were determined using a spectrophotometer. The integrity of the isolated RNA was examined by formaldehyde agarose gel electrophoresis.

Reverse transcription

Reverse transcription reaction to cDNA was performed using Omninscript Reverse Transcription Kit (Qiagen) according to the manual. 1 µg total RNA was reverse transcribed into 20 µl cDNA and used for quantitative real-time PCR and conventional PCR. RT reaction was allowed to proceed at 42°C for 30 min followed by a denaturation step at 94°C for 5 min. For qRT-PCR, samples were diluted 1:10 or 1:100 with ddH2O, respectively.

Quantitative real time PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed with the sequence detection system from Stratagene, Mx3005P using TaqMan Universal PCR Master Mix, No AmpErase UNG (Part No. 4324018) and the following Gene Expression Assays: Rat DbpA (Rn00519737_m1), 18S ribosomal RNA (Hs99999901_s1). All reagents were purchased from Applied Biosystems. Samples were run in triplicate by using an ABI Prism 7700 sequence detector (Applied Biosystems, Weiterstadt, Germany). Levels of gene expression were determined by the comparative CT method for relative quantfication of gene expression. Relative quantification relates the PCR signal of the target transcript in a treatment group to that of another sample such as an untreated control. Real time PCR conditions for analyzed genes: 1 Denaturation 95°C 10 min; 2 Denaturation 95 °C 30 sec; 3 Annealing and amplification 60°C 30 sec; 4 Cycle step 2-3 for 39 times

2.7 DNA work and gene cloning

Design primers for target DNA fragments. Add restriction enzymes target sequence to 5'terminus of forward primers (KpnI) and reverse primers (XhoI) respectively. Primers were designed using online primer designing tool: Primer3. Table 7 shows the primers that have been used for current study.

Table 7. Primer list

Name	F/R	Sequence	Company
DbpA_P-1633	F	GGT ACC GGT ACC ATG GTC AGT CCT CAC CAG AA	Invitrogen
DbpA_P-1096	F	GGT ACC GGT ACC TGT TTG AGG GAA CAG GCA AT	Invitrogen
DbpA_P-964	F	GGT ACC GGT ACC AAA CCG ACT CTG GGA CAT TG	Invitrogen
DbpA_P-668	F	GGT ACC GGT ACC AGA GGC AAC TGG AGC AGA AG	Invitrogen
DbpA_P-527	F	GGT ACC GGT ACC CAA TTT CCT CAT CTG CGT GTT	Invitrogen
DbpA_P-173	F	GGT ACC GGT ACC GCT TTC CTT GGT TCT GCA AC	Invitrogen
DbpA_P+41	R	CTC GAG CTC GAG CGA TCT TAC TGC CCC AAA AA	Invitrogen
YB-1_P-1393	F	GGT ACC GGT ACC TGG TCG GCC AAG CTT ATA TT	Invitrogen
YB-1_P-1060	F	GGT ACC GGT ACC TCT CAC TCT TCC CCT CAG GA	Invitrogen
YB-1_P-513	F	GGT ACC GGT ACC GCC TGG TAA AAC GGA TCA GA	Invitrogen
YB-1_P-230	F	GGT ACC GGT ACC GGC TAA GGC GTC TTC GAG	Invitrogen
YB-1_P-144	F	GGT ACC GGT ACC GAG ACA CAA CCC TGA ACG TG	Invitrogen
YB-1_P-27	F	GGT ACC GGT ACC TAG TTC CGG TCT CTA TGG CG	Invitrogen
YB-1_P+283	R	CTC GAG CTC GAG CCG GGG TGT GAT GGT AAC TA	Invitrogen

Amplify target DNA fregments with PCR following the protocol from manufacturer, peqGOLD Pwo DNA polymerase from PEQLAB. Run 1% agarose gel with PCR products, check the DNA size and cut the gels with target DNA fragments. Purify the gel extractions with QIAquick Gel Extraction kit from QIAGEN).

Digest insert and vector with restriction enzymes following the protocol from manufacturer, FastDigest® enzymes from Fermentas. Purify the DNA from enzymatic reaction with QIAquick Gel Extraction kit from QIAGEN.

Ligation of vector plasmid and insert performed using T4 DNA ligase from Roche, following the protocol from manufacturer.

Transformation. Ligated plasmids were first mixed with baterial cells and incubated in ice for 30 min. After a heat shock at 42oC for 45 seconds, return it back to ice for 5 min. Then incubate with LB medium in 37 °C for 1 hour. Selection of the taget bacteria with recombinant plasmid was performed spreading the solution onto an LB agar plate containing the designed resistant antibiotic, e.g. Ampicillin.

Culture selected bacteria clones for Miniprep, following the protocol from manufacturer, Miniprep kit from QIAGEN or Fermentas.

Digest the DNA products from Miniprep, run 1% agarose gel and check the DNA size. When the size is correct, send it to sequence.

Maxiprep. When DNA sequence confirmed the cloning was successful, Maxiprep was performed to amplify the plasmids, following the protocol from manufacturer (QIAGEN or Fermentas). The products were used directly for cell transfection or stored at -20 °C for short period or at -80 °C for longer time.

2.8 Luciferase assay

Transient transfection and stimulation

Rat mesangial cells (150,000/well) were seeded in 6-well plate and let them grow overnight to 50-60% confluence. The following day, cells were transfected with pGL4.10-Basic and other construted plasmids (2 μg of each plasmid/well) in triplicate, accompanied with internal control pGL4.74 (0.1 μg/well) using FuGene (Roche) or Tfx50 (Promega). The cells were incubated at 37℃, 5% CO2 for 24 hours, then were growth-arrested with serum free MCDB-Medium (Sigma) for another 24 hours before stimulation. Luciferase assay test was performed after challenge the cells with 50 ng/ml rat PDGF-BB or 5 ng/ml TGF-β for 24 hours.

Luciferase assay test & analysis

After removing the growth medium and washing the wells with PBS, luciferase assay test was performed according to the manufactur's protocol (Promega). Lysate buffer, PLB, was added to each well and incubated at room temperature for ten minutes with agitation. Then the lysate samples were transferred in to a polypropylene tube for testing. Luciferase substract was added and read in a luminometer (Tecan). The reading corresponds to firefly luciferase activity. There after Stop & Glo reagent was added and the reading was taken as the renilla activity. The luciferase activity of each well was normalized by the renilla value using the formula; Ln = F/R (Ln: normalised luciferase activity; F: Firefly luciferase activity reading and R: Renilla activity reading). Transcriptional activity of the control, pGL4.10-Basic, was used to further standardize Ln using the formula; RLU = Ln/pGL4.10-basic (RLU: relative luciferase unit).

2.9 Human biopsies and animal studies

Human biopsies

Human kidney biopsy samples encompassed diagnosis IgA nephropathy (n=8), lupus nephritis type IV (n=5) and interstitial nephritis (n=5). **Human liver biopsy** samples encompassed diagnosis HBV infectd hepatitis (n=22), NASH (n=3) and hepatocellular carcinoma (n=5). Healthy control tissues were sampled from patients undergoing nephrectomy or hepatectomy due to cancer diseases. Macroscopically unaffected tissue was embedded and analyzed. The study protocol involving human samples was approved by the local ethics committees. Patients provided written informed consent. The study protocol adhered to the ethical guidelines of the 1975 declaration of Helsinki.

Mesangioproliferative anti-Thy1.1 nephritis model

Anti-Thy1.1 nephritis was induced in 28 male Wistar rats (Charles River Wiga GmbH; 160 to 180 g) by intravenous injection of 1 mg/kg monoclonal anti-Thy1.1 antibody (clone OX-7; European Collection of Animal Cell Cultures, Salisbury, UK) as described (Floege, Ostendorf et al. 1999). Control and treated animals were sacrificed after 4 hours and days 4, 7, 9, 14, 21 and 28 after disease induction (n=4 at each time point). Tissues were used for protein extracts as well as fixed in Methacarn solution, embedded in paraffin, and sectioned for immunohistochemistry staining.

PDGF-BB infusion

Infusion of PDGF-BB in vivo was performed as described earlier (Floege, Eng et al. 1993). Briefly, five normal male Wistar rats (Simonson, Gilroy, CA) with weights between 180 to 220 g received a 7-d intravenous infusion of 40 ug/d PDGF-BB, and five rats received a vehicle. Infusion was performed continuously through a catheter in the left internal jugular vein with micro-osmotic pumps. Renal biopsies were obtained from each rat at day 4 and day 7.

Treatment of nephritic rats with PDGF-B–specific aptamers

Anti-Thy1.1 mesangioproliferative GN was induced in male Wistar rats as described above. Rats received daily two intravenous injections of 0.33 mg of PDGF-B specific aptamers or control vehicle. starting at day 3 after the onset of anti-Thy1.1 glomerulonephritis (GN) as described

previously (van Roeyen, Eitner et al. 2005; van Roeyen, Eitner et al. 2008). Necropsies were performed in all rats at day 6.

Treatment of nephritic rats with MEK inhibitor U0126

Anti-Thy1.1 mesangioproliferative GN was induced in male Wistar rats as described above. Rats received twice a day intraperitoneal injections of 20 or 200 mg/kg of U0126 dissolved in DMSO or control vehicle, starting at day 3 after the induction of anti-Thy1.1 glomerulonephritis (GN) as described previously (van Roeyen, Eitner et al. 2005; van Roeyen, Eitner et al. 2008). Necropsies were performed in all rats at day 6. The animals received the last dose of U0126 1h before being killed.

3 Results

3.1 DbpA protein expression

Two isoforms of DbpA/ZONAB protein exist in humans that differ from one another by a 69 amino acids (aa) long alternative domain localized within the protein C-terminus (Figure 7A) (Kudo, Mattei et al. 1995; Balda and Matter 2000). The longer isoform is denoted DbpA-a or ZONAB-B and encompasses 372 aa (NP_003642.3), while the shorter isoform, DbpA-b/ZONAB-A consists of 303 aa and lacks the alternative domain (NP_001138898.1). Given the high conservation of the CSD but very low homology within the N-terminal domains, a peptide-derived polyclonal antibody (Ab) was generated that specifically recognizes an epitope within the N-terminus of human DbpA (Figure 7A). With this antibody, both isoforms may be detected by Western blotting and distinguished due to size differences.

DbpA protein expression was first assessed by immunoblotting of whole cell lysates from different cell types, including human monocytic leukemia cell line (THP-1), human proximal tubular epithelial cell line HK-2 and HKC-8, an immortalized rat mesangial cell line (rMC), human primary mesangial cells (p hMC), human embryonic kidney cells (HEK-293), a mouse liver progenitor cell line (LPC), human cholangiocytic cell line (MMNK-1), human hepatocyte cell line (AML-12) and rat hepatic stellate cell line (CFSC) . Differences in expression patterns for DbpA are shown in Figure 7B. Three distinct bands were detected, that correspond to relative MW of ~55, ~50 and ~44 kDa. The two bands at ~55 and ~50 kDa correspond to the reported sizes of DbpA isoforms DbpA_a (~55 kDa) and DbpA_b (~50 kDa) (Kudo, Mattei et al. 1995; Balda, Garrett et al. 2003). The ~44 kDa band has not yet been reported and may be derived by proteolysis or represent an unreported isoform. Specificity of the Ab is confirmed by testing pre-immune serum and by omission of the primary Ab.

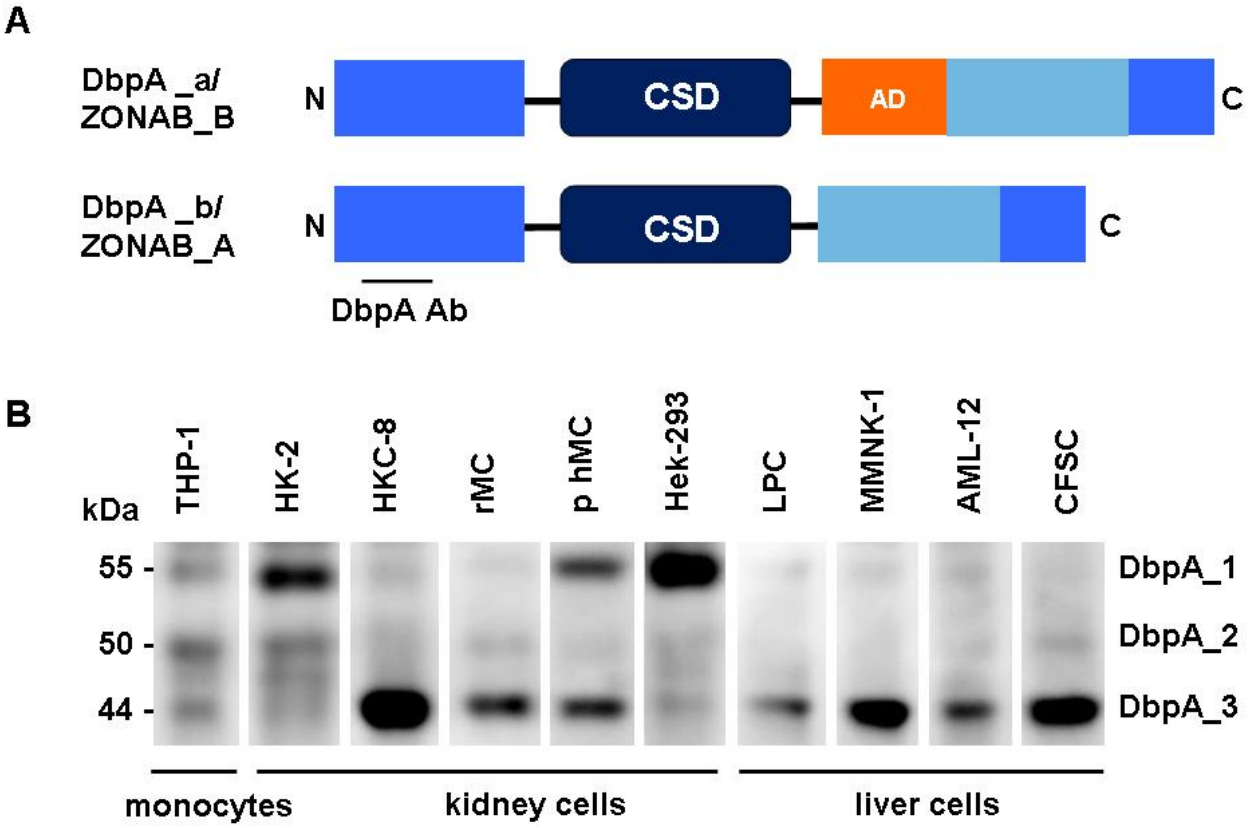


Figure 7. DbpA protein expression in different cell lines. (A) Structural composition of DbpA protein isoforms with alternative domain (AD). DbpA specific antibody (Ab) recognizes an epitope within the N-terminal domain (B) By Western-blot analysis DbpA protein expression of cell lysates from different kidney and liver cell lines: human monocytic cell line (THP-1), human proximal tubular epithelial cells HK-2 and HKC-8, rat mesangial cell line (rMC), human primary mesangial cells (p hMC), human embryonic kidney cells line (HEK-293), mouse liver progenitor cell line (LPC), human cholangiocytic cell line (MMNK-1), human hepatocytic cell line (AML-12) and rat hepatic stellate cells (CFSC) . Three major bands at ~55, ~50 and ~44 kDa are detected and denoted as DbpA_1, _2 and _3 respectively.

3.2 DbpA expression in mesangioproliferative glomerulonephritis (GN)

Mesangial cell (MC) activation and proliferation are prominent features of mesangioproliferative glomerular diseases, in which PDGF-B has been established as a potent mitogen (Floege, Ostendorf et al. 1999; Floege, Eitner et al. 2008). Among the known downstream targets of PDGF-B signaling, cold-shock protein DbpB/YB-1 has been identified previously in mesangioproliferative glomerular disease (van Roeyen, Eitner et al. 2005). Given that DbpA may play a role in cell proliferation and considering the structural and functional similarities between DbpA and DbpB/YB-1 we hypothesized that DbpA may also be a downstream target of PDGF-B signaling.

3.2.1 Elevated DbpA expression in human IgA and lupus (IV) nephritis

IgA nephritis and lupus nephritis (type IV) are often accompanied by MC proliferation and matrix accumulation. Within the glomerulus DbpA protein expression was first assessed in human kidney biopsy samples from IgA and lupus (IV) nephritis patients. Compared to healthy controls a strong induction of DbpA protein expression within the mesangial compartment was detected by immunohistochemistry (Figure 8). The localization of DbpA was mainly within the cytoplasm.

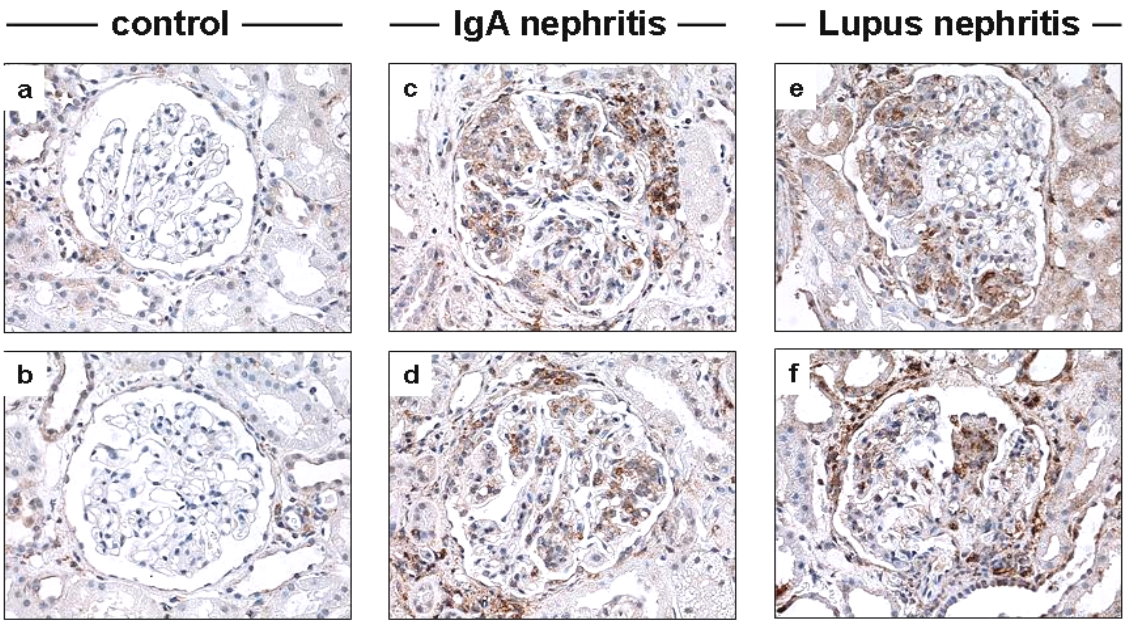


Figure 8. Expression of DbpA in IgA and lupus nephritis patients. By immunohistochemistry, DbpA was hardly detected in healthy controls (a, b). In contrast, DbpA protein was detected within the mesangial compartment of glomeruli from IgA (c, d) and lupus (e, f) nephritis patients.

The analysis was extended to patients suffering from interstitial nephritis (IN). Expression of DbpA was berely detectable within the glomeruli, and in this respect not distinguishable from healthy controls (Figure 9). However tubules and tubulointerstitial cells were immunopositive for DbpA. Notably the staining pattern included unclear and cytoplasmic localization and did not adhere to cell boundaries. These observations provided the first clue that DbpA may be involved in the proliferative mesangial cell response in mesangioproliferative glomerulonephritis.

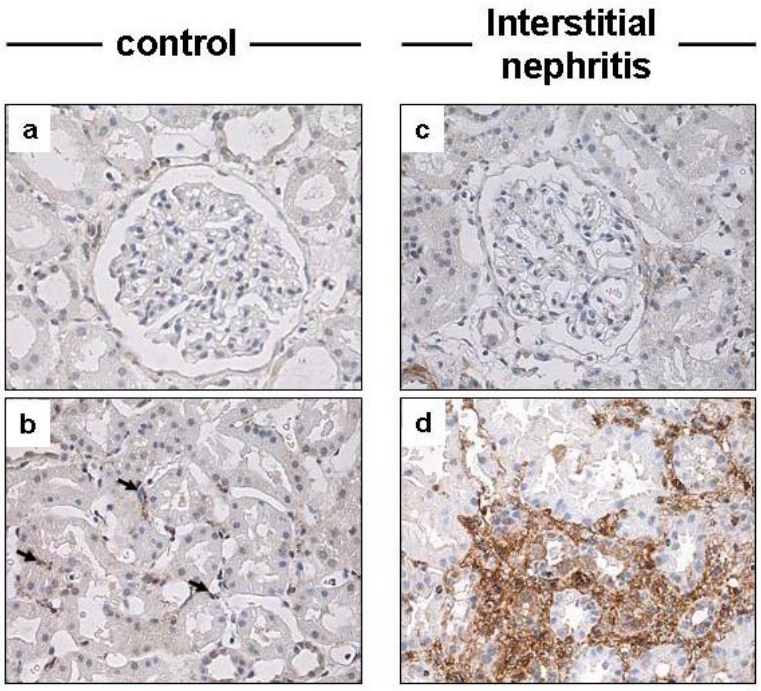
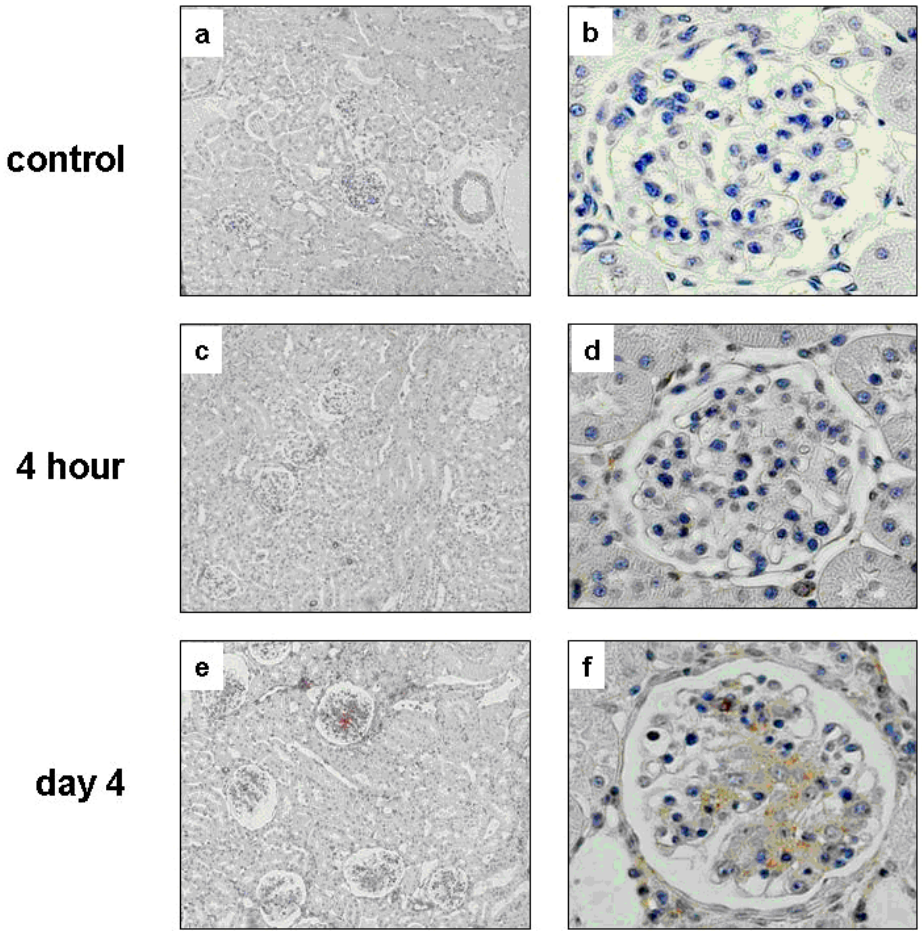


Figure 9. Expression of DbpA in interstitial nephritis patients. DbpA expression was hardly detected in the glomeruli of healthy controls (a) and patients suffering from interstitial nephritis (c). Positive DbpA signal was observed in some cells localized within the interstium of healthy kidney tissue (arrow) within the nuclear compartment (b). Strong induction of DbpA expression was seen within infiltrating cells in interstitial nephritis patients (d).

3.2.2 DbpA expression in the Anti-Thy1.1 mesangioproliferative GN model

The anti-Thy1.1 nephritis model is an antibody-mediated, complement-dependent rodent nephritis model that has ben established as a model for human mesangioproliferative diseases. In healthy rat kidneys DbpA protein expression was hardly detected by immunohistochemistry within the glomeruli. Please note, that smooth muscle cells within blood vessels were immunopositive, yielding an internal positive control (Figure 10, a and b). Induction of the anti-Thy1.1 GN resulted in a marked change of DbpA protein expression within the glomeruli. Immunohistochemistry was performed on tissue samples collected 4 hours, 4, 7 and 21 days after disease induction. In the course of disease, cytoplasmic DbpA protein abundance coincided with mesangial cell activation/proliferation, that reached a plateau at day 7 (Figure 10, g and h) and returned to "background" after 3 weeks (Figure 10, i and j). The localization of DbpA protein remained predominantly cytoplasmic and was prominent within the mesangial compartment (Figure 10, h).



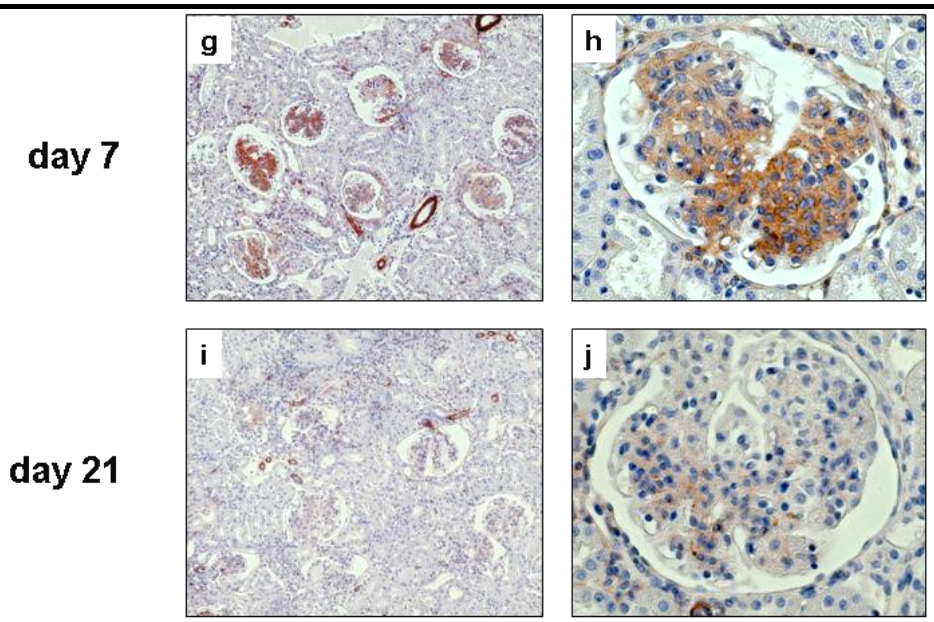


Figure 10. Expression of DbpA protein in healthy rat kidney and following induction of anti-Thy1.1 mesangioproliferative glomerulonephritis. DbpA protein was identified by immunohistochemistry in kidney tissue from healthy rats (a, b) and in the time course of anti-Thy1.1 nephritis (4 hours: c, d; day 4: e, f; day 7: g, h; day 21: i, j). Whereas no DbpA protein was detected in the glomeruli of healthy rats significant DbpA expression within the cytoplasm of mesangial cells was observed following induction of anti-Thy1.1 nephritis from day 4 on, peaking at day 7 and returning to 'background level' after three weeks. (a. c. e. g and i with 100x magnification; b, d, f, h and j with 400x magnification)

Besides immunohistochemistry we performed Western blot analyses of glomerular lysates from animals without and with anti-Thy1.1 nephritis. Here a marked (3.5-fold) up-regulation of DbpA protein expression was apparent within 4 days that peaked at day 7 and subsequently returned to "background level" within two weeks (Figure 11). Of note there was only one form of DbpA protein detected with relative MW of ~44 kDa.

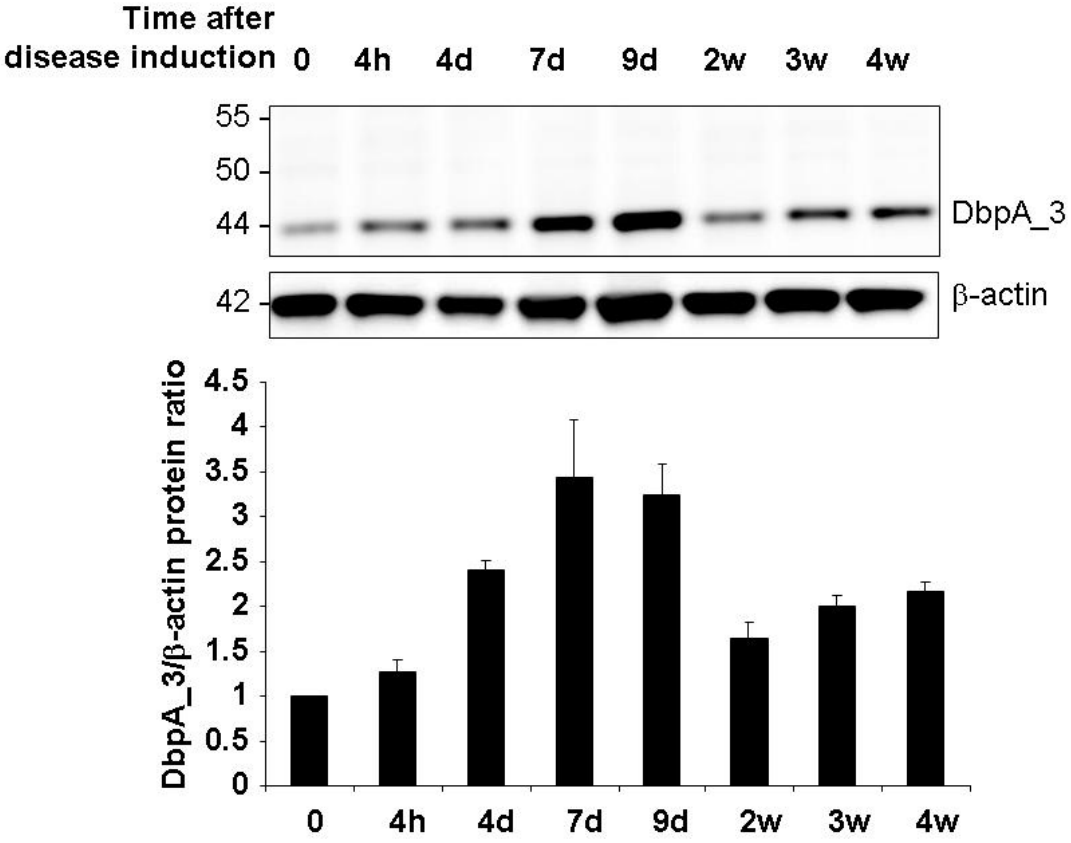


Figure 11. Western blot analysis of DbpA protein expression in the course of anti-Thy1.1 nephritis. Only band corresponding to ~ 44 kDa was detected by Western blot. Quantitative analyses reveal ~ 3.5-fold up-regulation of DbpA protein expression that peaks at day 7 and subsequently returns to background levels.

3.2.3 *In vivo* infusion of PDGF-BB results in stimulated DbpA protein expression

The potent role of PDGF-B in mesangioproliferative glomerular disease is well established, and a strong induction of PDGF-B expression in the anti-Thy1.1 model has been described (Floege, Ostendorf et al. 1999). In order to address the question whether cytokine PDGF-B may induce DbpA expression in mesangioproliferative glomerular disease animals were continuously infused with PDGF-BB (40 ug of PDGF-BB/d *versus* vehicle alone). By immunohistochemistry a marked cytoplasmic expression of DbpA within the mesangial compartment of glomeruli was apparent in the PDGF-BB receiving animals at both examined time points, days 4 and 7. For vehicle-infused animals no DbpA positive staining was observed (Figure 12).

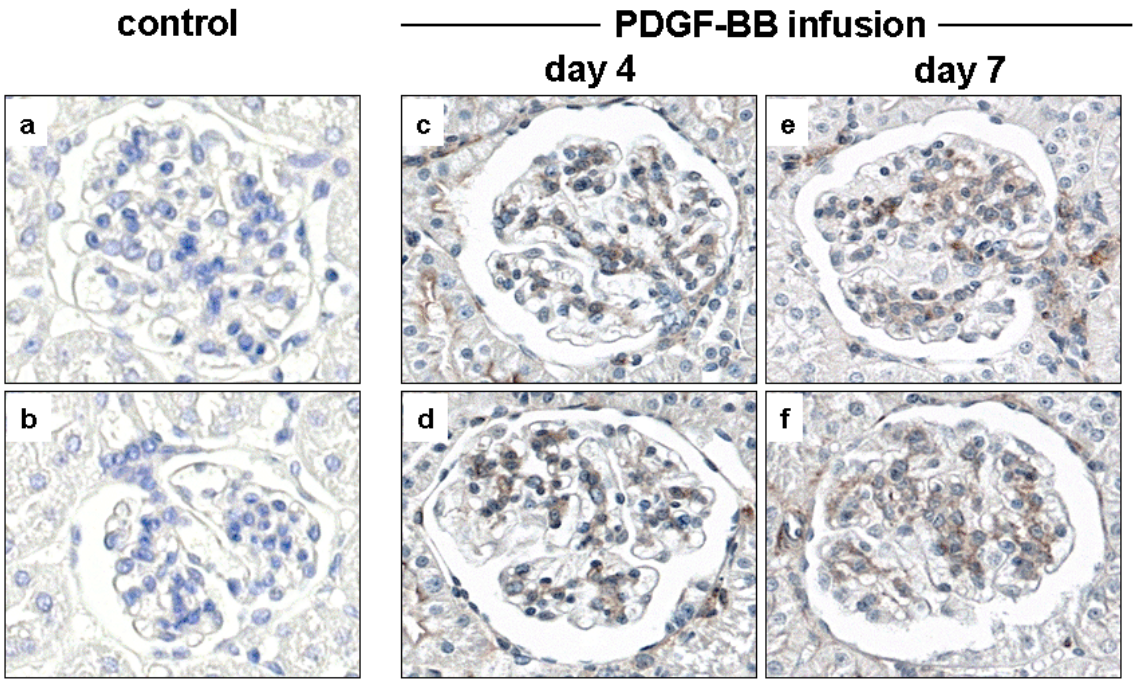


Figure 12. Renal expression of DbpA following *in vivo* infusion of PDGF-BB. Immunohistochemistry for DbpA was performed with kidney biopsies from rats that were infused with vehicle for 7 days (control) or with PDGF-BB for 4 and 7 days respectively. Whereas control tissue was immunonegative for DbpA in the glomeruli DbpA protein' induction was apparent within the mesangium of PDGF-BB infused rats, on days 4 and 7.

3.2.4 PDGF-B blockade inhibits DbpA expression in mesangioproliferative GN

To corroborate PDGF-BB as an endogenous upstream inducer of DbpA expression *in vivo*, in blockade of extracellular PDGF-B activities was sought *via* application of specific neutralizing aptamers in diseased animals. Anti-Thy1.1 nephritis was induced and rats subsequently received twice daily injections of specific PDGF-B aptamers (0.33 mg PDGF-B aptamers *versus* vehicle alone) from days 3 to 6 (van Roeyen, Eitner et al. 2005) (Figure 13A). Application of PDGF-B aptamer antagonists abrogated DbpA protein expression when compared to vehicle-infused diseased animals (Figure 13B). Additionally, DbpA expression closely coincided with mesangial cell activation and proliferation. Taken together these findings indicate that DbpA is a downstream target of PDGF-B.

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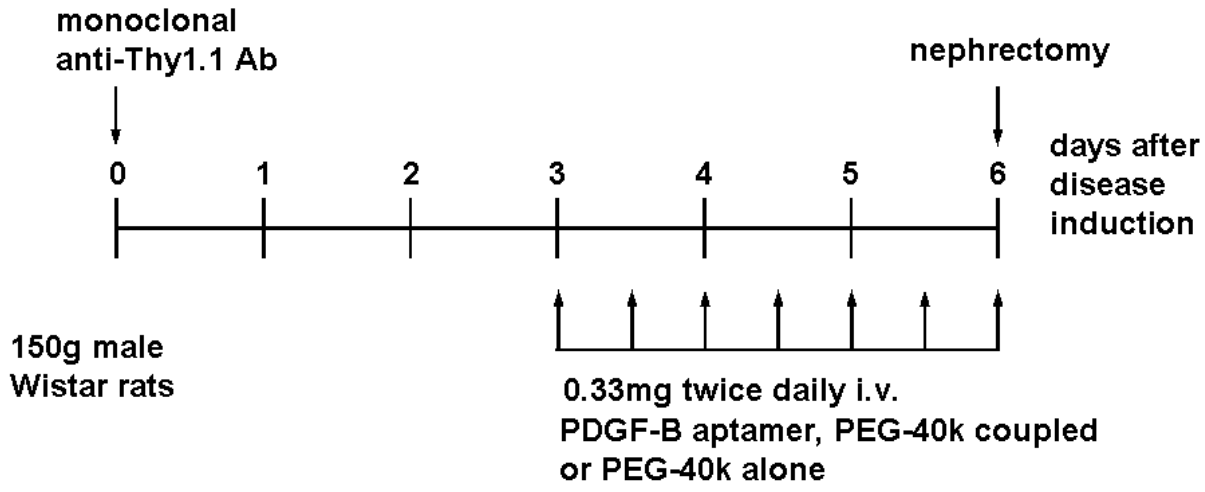


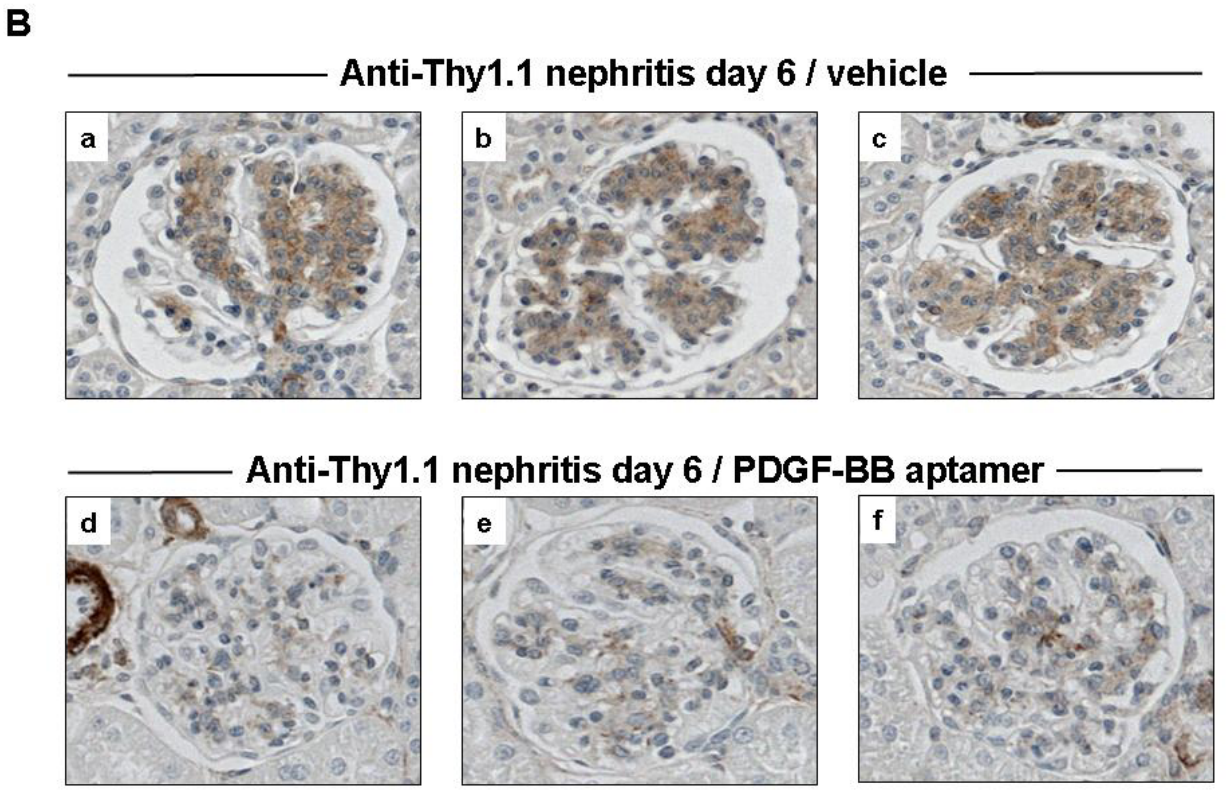
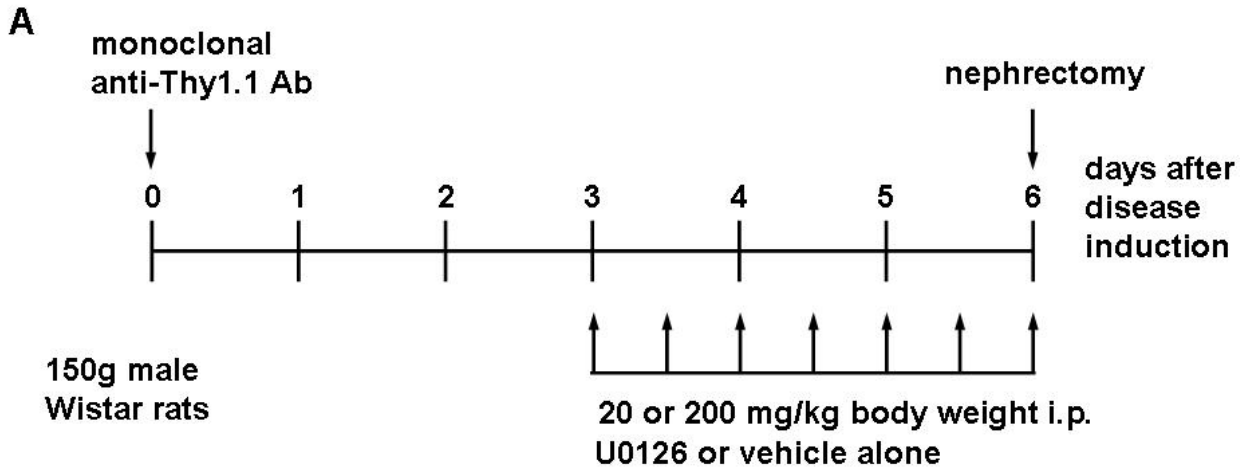


Figure 13. ***In vivo*** **treatment of anti-Thy1.1 nephritis rats with PDGF-B specific aptamers abrogates DbpA up-regulation.** (A) Scheme on experimental protocol. Anti-Thy1.1 nephritis was induced in animals thereafter receiving PDGF-B aptamers or vehicle alone twice daily from days 3 to 6. (B) Immunohistochemistry reveals decreased DbpA expression in PDGF-B aptamer treated rats (d, e and f) compared to vehicle infused animals (a, b and c). Representative results are depicted that were obtained with three different animals in each group.

3.2.5 MEK inhibitor U0126 represses DbpA expression in Anti-Thy1.1 model

Among downstream signaling events activated by PDGF-B the MAPK/ERK pathway seems to be of fundamental important for mesangial cell activation and proliferation. In order to test this hypothesis blockade of MAPK/ERK signaling activities *via* application of MEK inhibitor U0126 in diseased animals was performed. Anti-Thy1.1 nephritis was induced as previously described and the rats subsequently received twice daily injections of U0126 (20 or 200 mg/kg versus vehicle alone) from days 3 to 6 (scheme depicted in Figure 14A). Tissue was processed as described (van Roeyen, Eitner et al. 2005). Following application of U0126 DbpA protein expression was reduced compared to vehicle-infused diseased animals (Figure 14B).



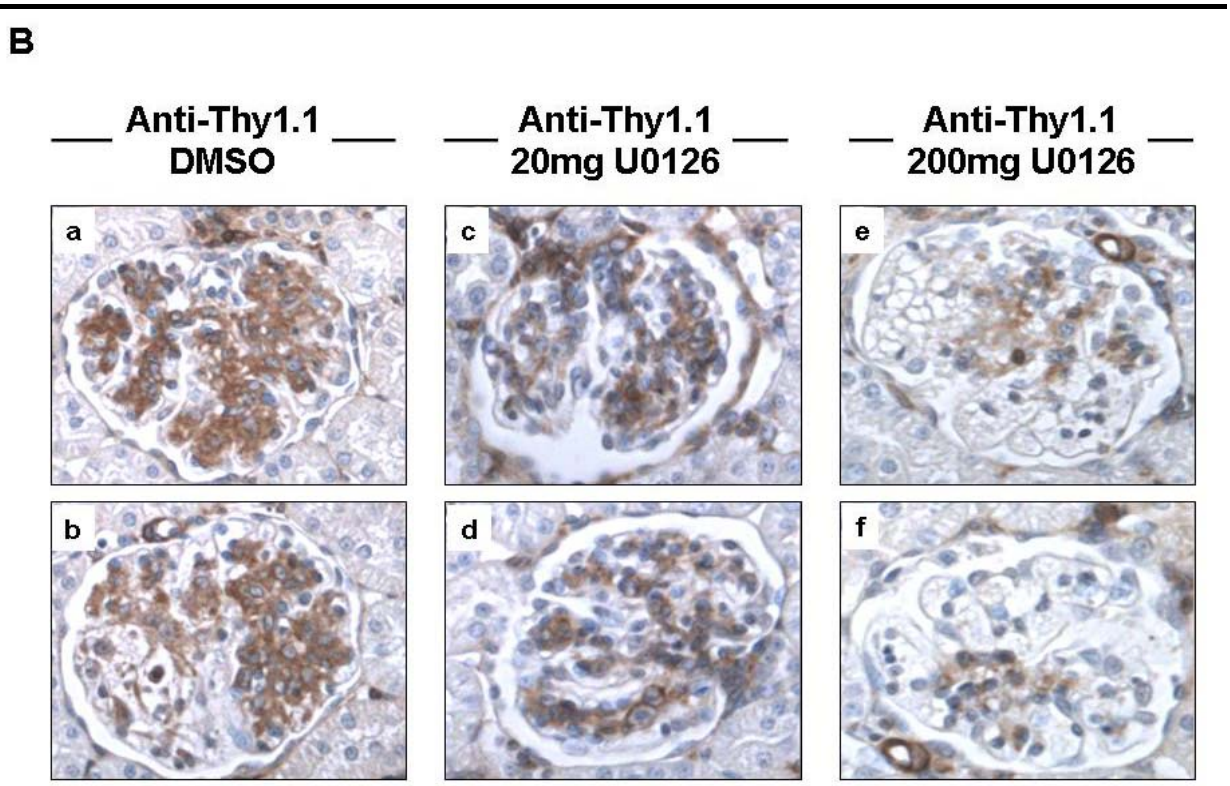


Figure 14. ***In vivo*** **treatment of anti-Thy1.1 nephritis rats with MEK inhibitor U0126 leads to decreased DbpA up-regulation.** (A) Scheme on experimental protocol. Anti-Thy1.1 nephritis was induced in animals, thereafter were injected U0126 or vehicle alone twice daily from days 3 to 6. (B) Immunohistochemistry reveals decreased DbpA expression in U0126 treated rats (c, d, e and f) comparing to vehicle treated (a and b). A concentration-dependent effect becomes apparent. Representative results are depicted that were obtained with three different animals in each group.

3.2.6 PDGF-BB regulates DbpA expression in mesangial cells *in vitro*

Given the above *in vivo* results we next set out to test whether DbpA is a downstream target of PDGF-B signaling in mesangial cells *in vitro*. Following incubation of primary human mesangial cell cultures with PDGF-BB at 50ng/ml for 24 hours morphological changes and increased cell proliferation rates were seen (see Figure 15).

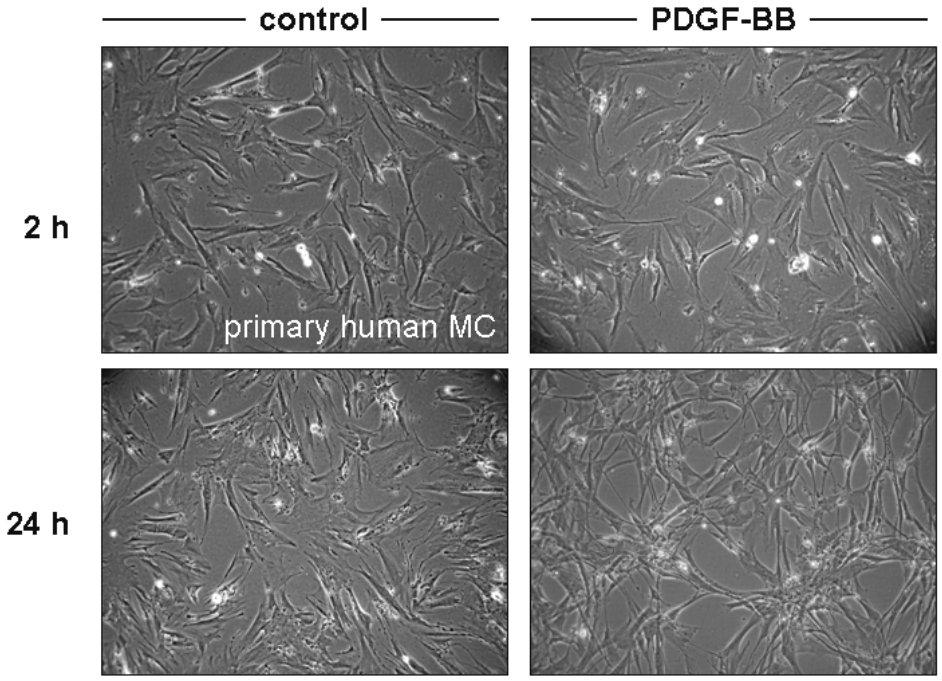


Figure 15. Morphological changes of primary human mesangial cells following PDGF-BB stimulation. Following PDGF-BB stimulation (50ng/ml) changes of mesangial cell morphology and increased cell numbers were apparent within 24 hours.

Immunofluorescence staining for DbpA showed that its expression was increased and the protein predominantly accumulated within the cytoplasm (see Figure 16). These results are in accord with the observations found by immunohistochemistry.

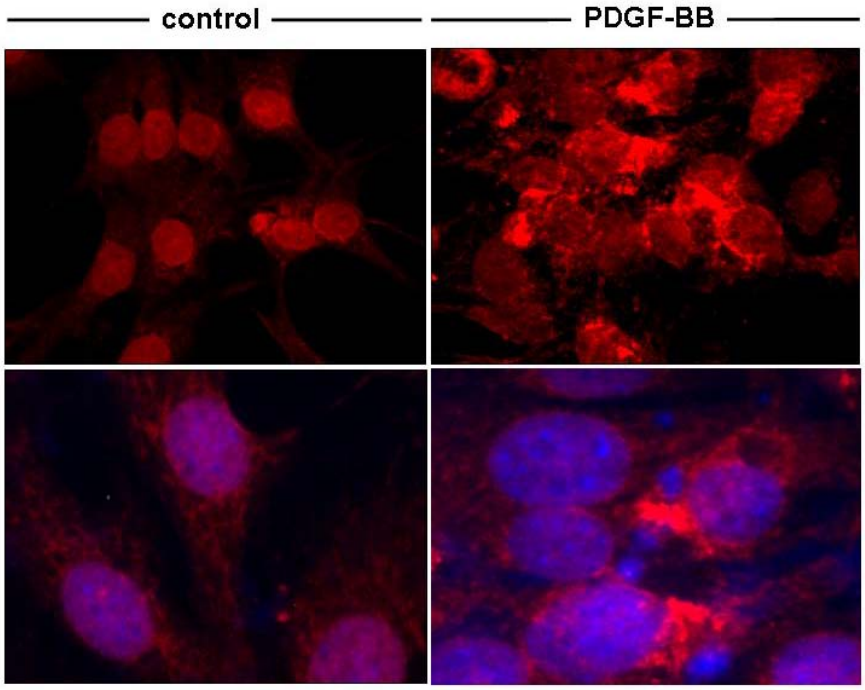
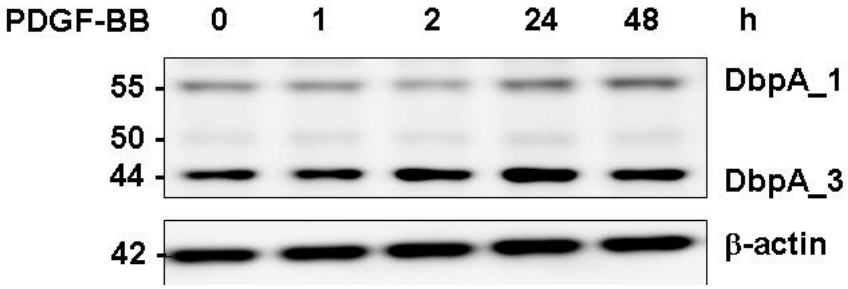


Figure 16. DbpA accumulates within the cytoplasm of rat mesangial cells following incubation with PDGF-BB. Cells incubated with vehicle or PDGF-BB (50ng/ml) for 24 hours reveal a cytoplasmic accumulatetion of DbpA protein.

Western blot analysis performed with cell lysates from primary human mesangial cells incubated with PDGF-BB or vehicle reveals a ~1.7 fold induction of DbpA protein expression. Notably, two isoforms were detected of molecule sizes of ~ 44 and ~ 55 kDa (Figure 17).



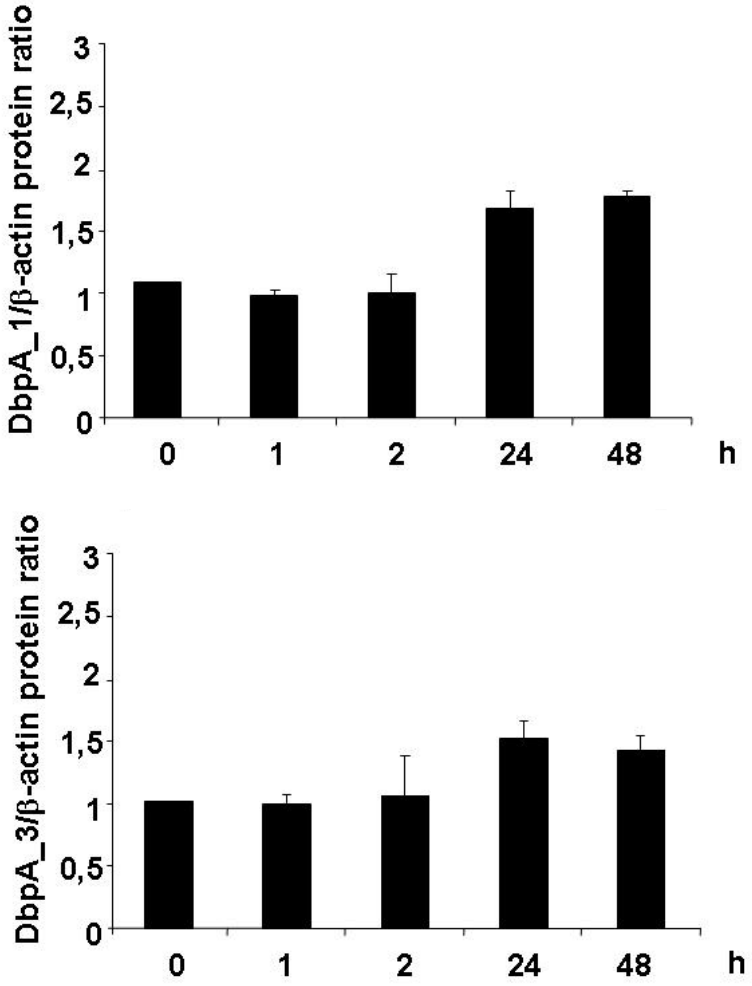


Figure 17. Western blot analysis of DbpA protein expression in PDGF-BB challenged primary human mesangial cells. Quantification of band intensities reveals ~1.5 fold induction of DbpA protein expression (~44 kDa) 24 hours following incubation with human PDGF-BB (50 ng/ml).

In a well characterised rat mesangial cell line (rMC) incubation with PDGF-BB was performed for different time periods (1, 2, 8, 24 and 48 hours). DbpA protein and mRNA levels were determined by Western blot and real-time PCR respectively. A significant 2.5-fold induction of DbpA protein (~ 44 kDa) expression was detected following PDGF-BB stimulation for 24 hours (Figure 18A). DbpA transcripts were 3.5-fold more abundant following 48 hours of PDGF-BB incubation (Figure 18B).

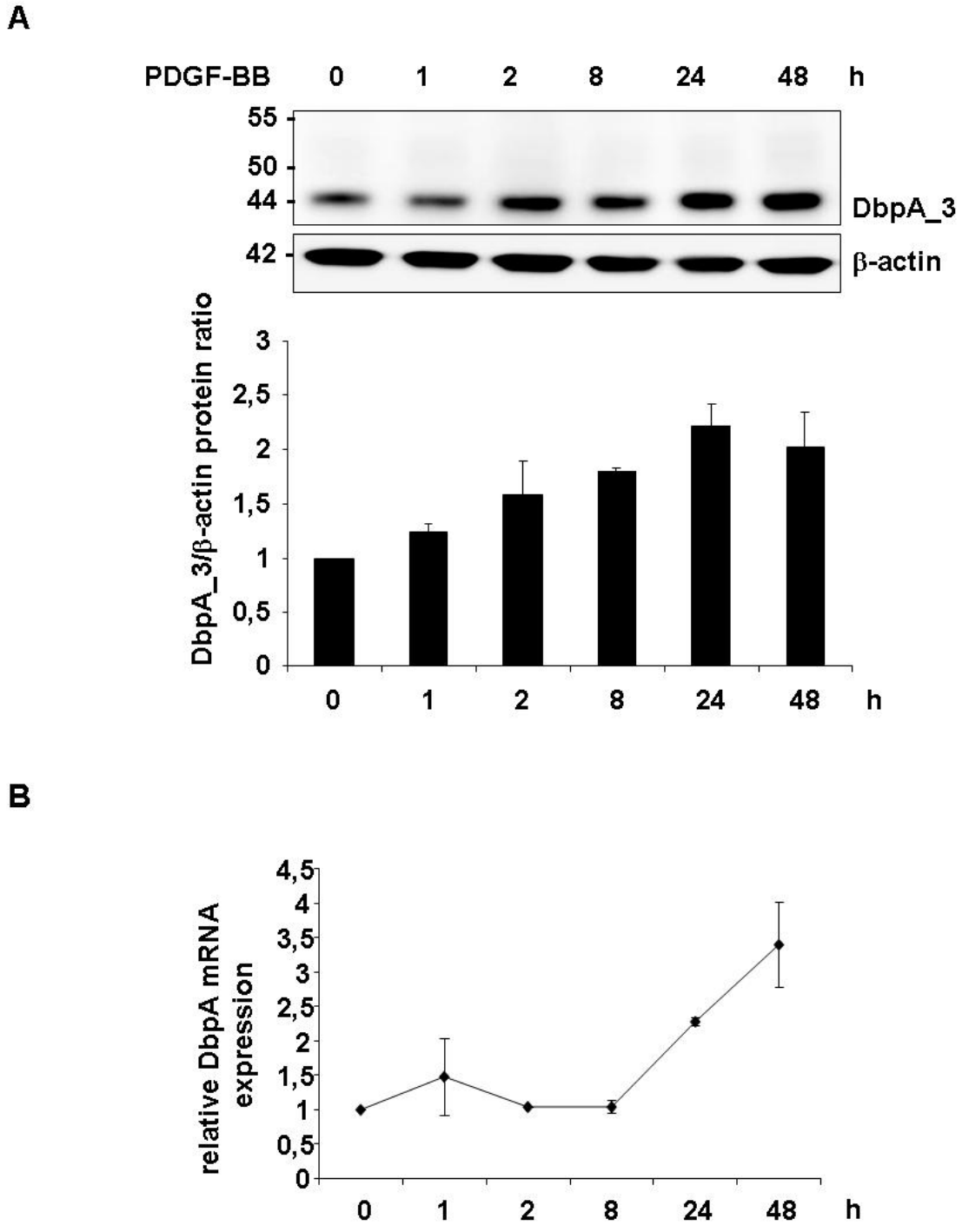
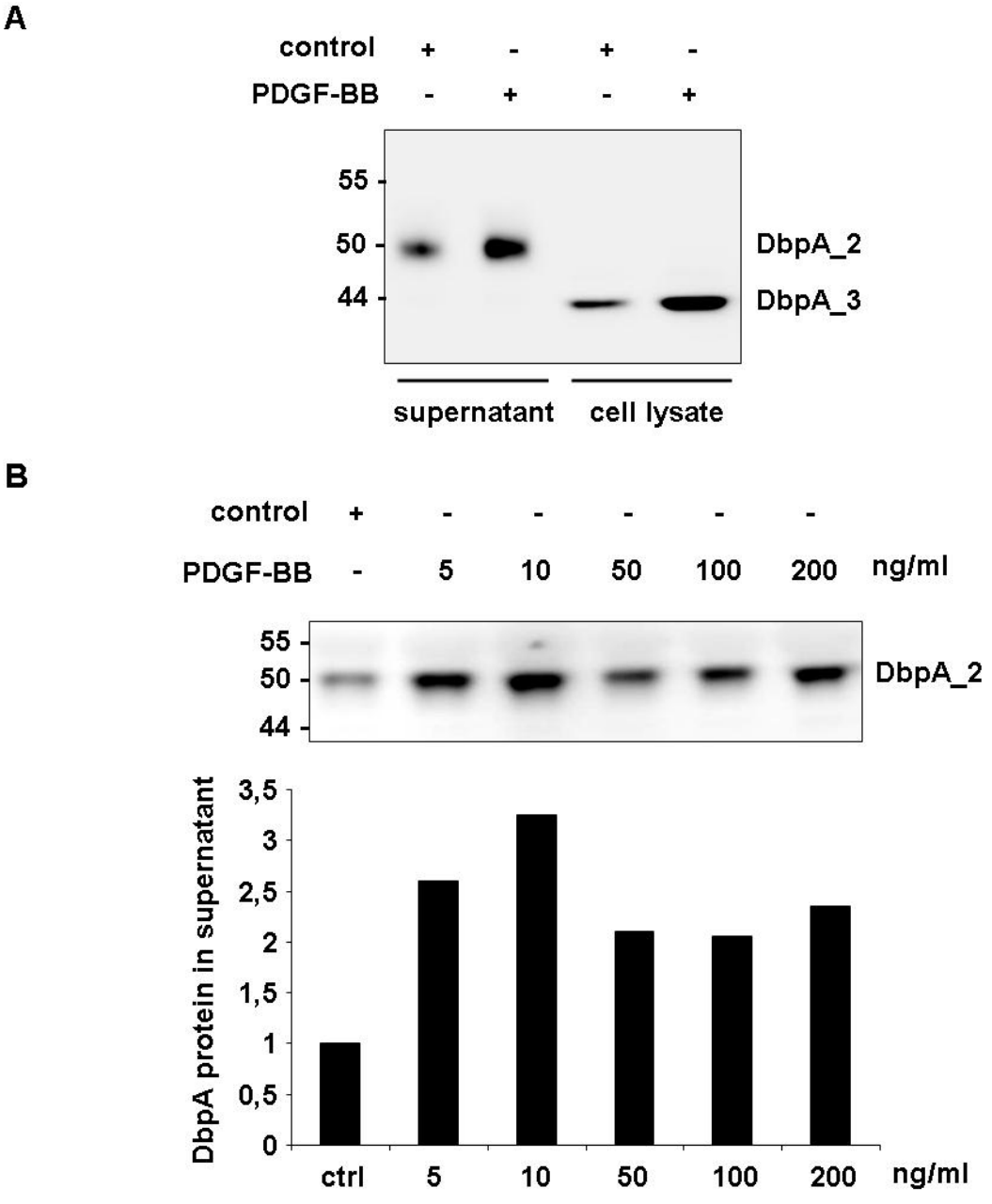


Figure 18. DbpA protein and transcripts are up-regulated following PDGF-BB stimulation of rat mesangial cells. (A) Western blot analysis of DbpA protein expression in rat mesangial cells challenged with rat PDGF-BB for the indicated time periods. A 2.5-fold induction of DbpA protein (~ 44 kDa) was detected after PDGF-BB stimulation for 24 hours. (B) Real-time PCR analysis for DbpA transcripts reveals 3.5-fold up-regulation after 48 hours of PDGF-BB incubation.

3.2.7 PDGF-B stimulates DbpA secretion in rat mesangial cells

We previously identified DbpA/YB-1 as a chemoattractant that is secreted by rat mesangial cells (Frye, Halfter et al. 2009; Rauen, Raffetseder et al. 2009). Here we tested the hypothesis that DbpA may also be secreted following PDGF-BB incubation. Western-blot analysis of conditioned cell culture medium precipitated by trichloroacetic acid (TCA) revealed that DbpA was detected extracellularly with prominent size of 50 kDa (Figure 19A). DbpA abundance in the medium elevated following PDGF-B stimulation in a time-dependent as well as concentration-dependent manner (Figures 19B and 19C).



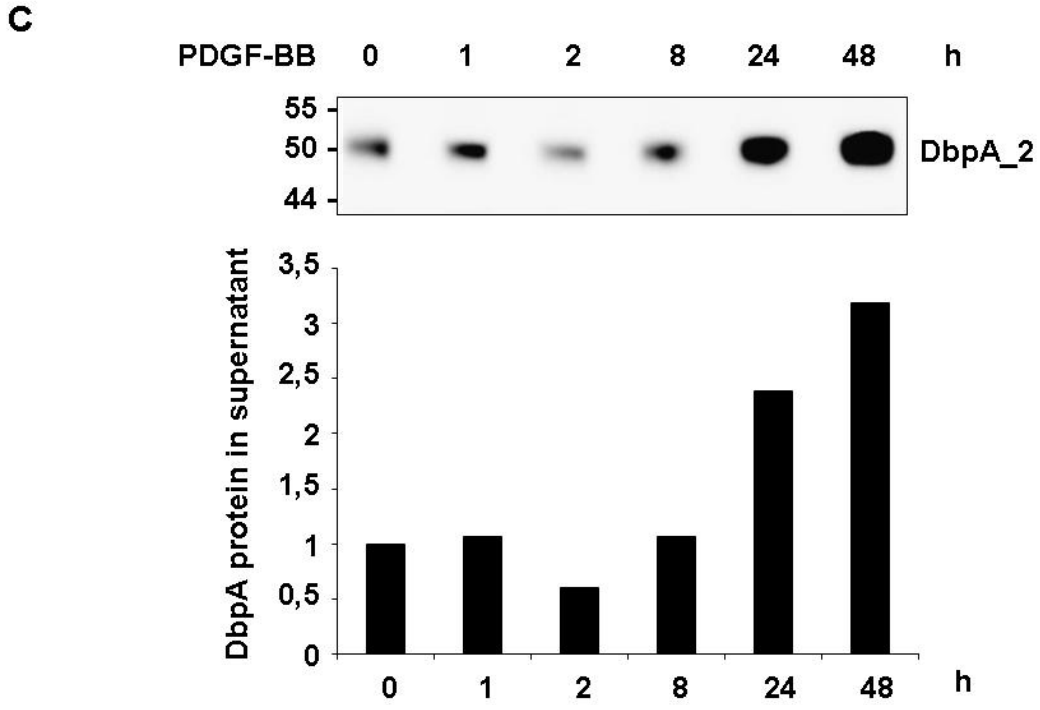


Figure 19. PDGF-B stimulates DbpA extracellular secretion. (A) Western-blot analysis for DbpA expression in conditioned cell culture medium from rat mesangial cells and cell lysate. DbpA with relative MW of 50 kDa was detected within the supernatant, while a 44 kDa protein was detected in cell lysates. (B) Concentration-dependent release of DbpA from mesangial cells following PDGF-BB stimulation. Western blot analysis of DbpA protein in conditioned culture medium of rat mesangial cells challenged with PDGF-BB for 24 hours. Concentration-dependent up-regulation of DbpA (~ 50 kDa) is seen (C) Western blot analysis of DbpA protein abundance in the conditioned medium of rat mesangial cells stimulated with a fixed concentration of PDGF-BB (50 ng/ml) for the indicated time periods. A time-dependent up-regulation of DbpA protein (~ 50 kDa) was apparent after at least PDGF-BB stimulation.

3.3 Expression and regulation of CSP in inflammatory liver diseases

Chronic inflammatory liver diseases may progress to fibrotic and cirrhotic damages of the liver structure, and concomitantly increase the risk for malignant transformation of resident liver cells with advent of hepatocellular and cholangiocellular carcinomas. A correlation of elevated DbpA and DbpB/YB-1 protein expression levels with advanced stages of hepatocellular carcinoma has been reported (Yasen, Kajino et al. 2005). However, little is known about roles that CSP play in chronic inflammatory liver diseases. From *in vitro* studies we know that DbpB/YB-1 activates Smad7 gene expression in hepatic stellate cells (HSC) and thereby attenuates the pro-fibrogenic effects of TGF-β (Dooley, Said et al. 2006). *In vivo* studies confirmed that DbpB/YB-1 has the potential to act protectively in experimental models of liver fibrosis and cirrhosis (Inagaki, Kushida et al. 2005).

3.3.1 DbpA and DbpB/YB-1 expression in HBV-infected cirrhotic livers

Around 80% of hepatocellular carcinomas (HCC) derive from liver fibrosis and cirrhosis, mostly related to viral hepatitis B and C infections (Trevisani, Cantarini et al. 2008). Given that DbpA and DbpB/YB-1 are involved in hepatocarcinogenesis, we first investigated the expression levels of these two proteins in HBV-infected cirrhotic patients. By immunohistochemistry a strong up-regulation of both DbpA and DbpB/YB-1 proteins in HBV infected chronic liver diseases comparing to the healthy liver tissues was apparent, as shown in Figure 20.

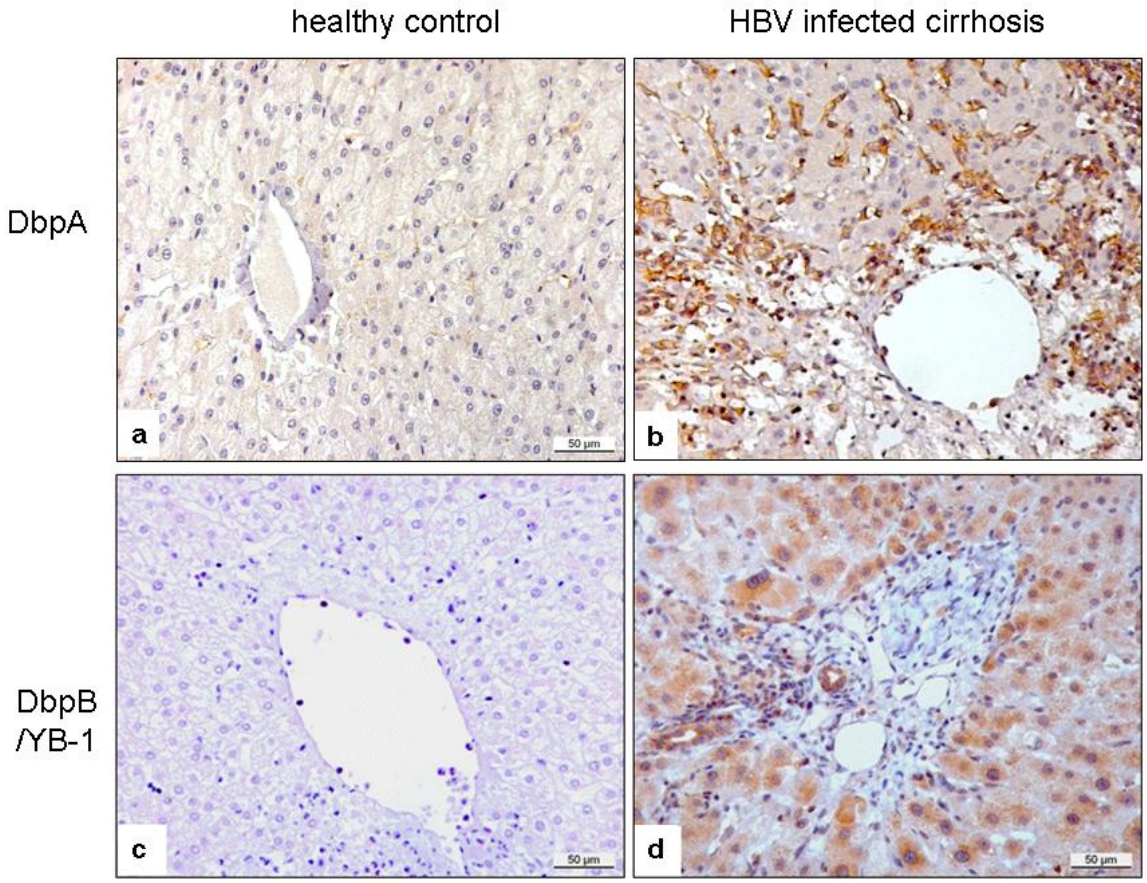


Figure 20. DbpA and DbpB/YB-1 protein expression in HBV infected cirrhotic livers. DbpA and DbpB/YB-1 proteins were investigated by immunohistochemistry in healthy liver tissue (a, c) and in liver tissue from HBV-assosiated cirrhosis patients (b, d).

3.3.2 Distribution of DbpA and DbpB/YB-1 in cirrhotic liver tissue

Both DbpA and DbpB/YB-1 were found to be over-expressed in cirrhotic liver tissue. The expression patterns however differed from each other. DbpA positive cells were mainly localized within the sinusoidal compartment. By immunofluorenscence co-staining with cell-type specific markers CD68 and α-SMA, DbpA positive cells were identified as activated macrophages and activated hepatic stellate cells (HSCs) (Figure 21 and 22). In contrast, DbpB/YB-1 immunopositive cells were mainly hepatic epithelial cells (e.g. hepatocytes, cholangial cells, reactive ductular cells and hepatic progenitor cells) as assessed by morphology and phenotypic appearance (Figure 23).

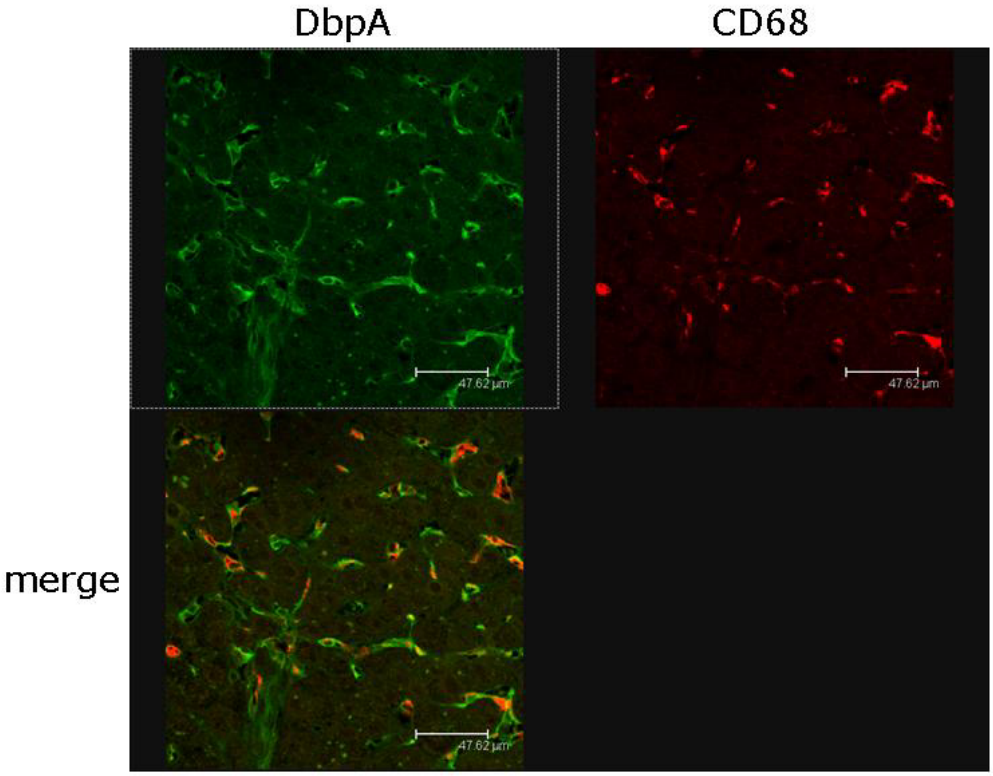


Figure 21. DbpA and CD68 co-expression of cells. By immunofluorescence DbpA (green) and CD68 (red) co-staining of cells (in the overlay appearing as yellow) was visualized predominantly within the sinusoidal compartment of cirrhotic liver tissue.

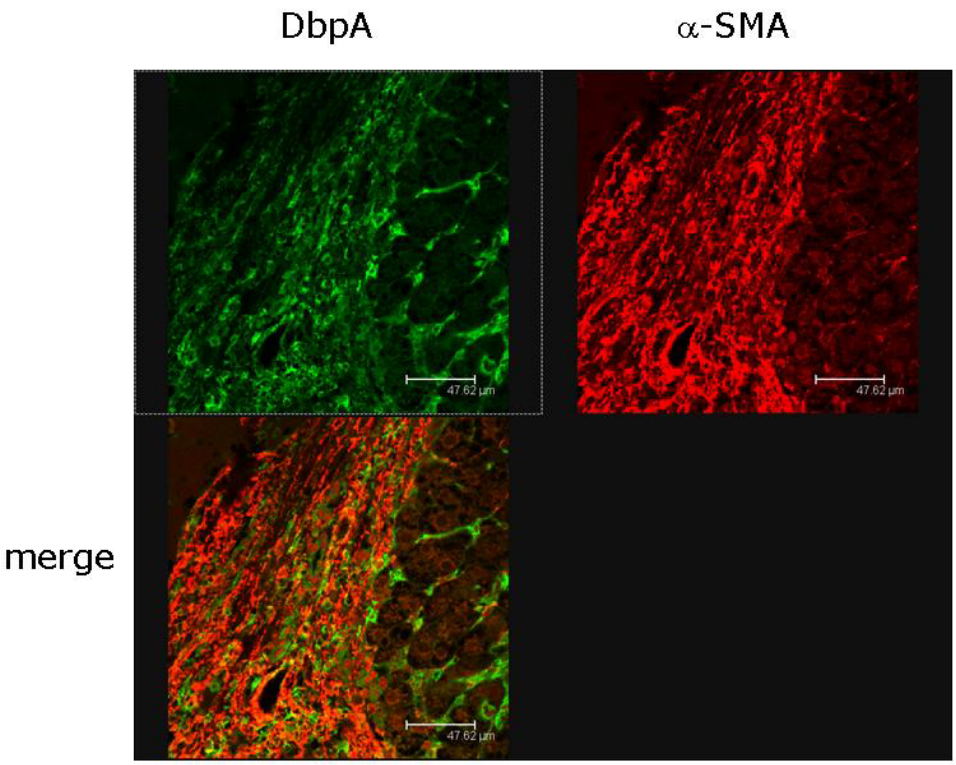


Figure 22. DbpA and α-SMA co-expression of cells. By immunofluorenscence of DbpA (green) and α-SMA (red) co-localization (overlay yellow) was identified within the sinusoidal compartment.

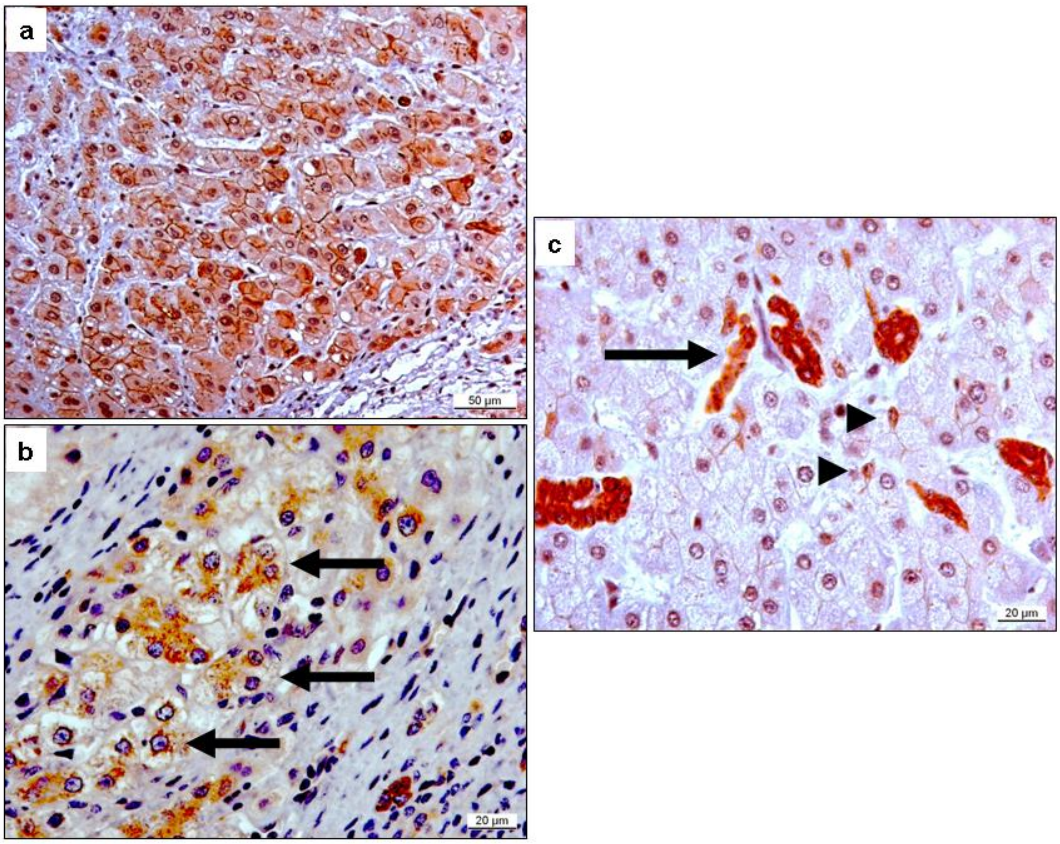


Figure 23. Distribution of DbpB/YB-1 staining within cirrhotic liver tissue. Immunohistochemistry combined with phenotype analysis indicates that DbpB/YB-1 positive cells are mainly intermediated hepatocyte-like cells (a), hepatocytes (arrows, b), hepatic progenitor cells (arrow heads, c) and bile ductular cells (arrow, c).

3.3.3 DbpA is up-regulated in the early phases of hepatic fibrogenesis

Given that DbpA up-regulation predominantly occurs in activated macrophages and activated hepatic stellate cells (HSCs), it may be involved in the fibrogenesis process of inflammatory liver diseases. As shown in Figure 24 DbpA protein expression is up-regulated in cells surrounding and constituting fibrotic tissue (triangles) from cirrhosis livers. However, at the later stages of fibrosis with scar tissue being formed, DbpA protein expression may be hardly detected, as indicated by stars within Figure 24. This distribution indicates that DbpA may be involved in the orchestration of the early phases of hepatic fibrosis.

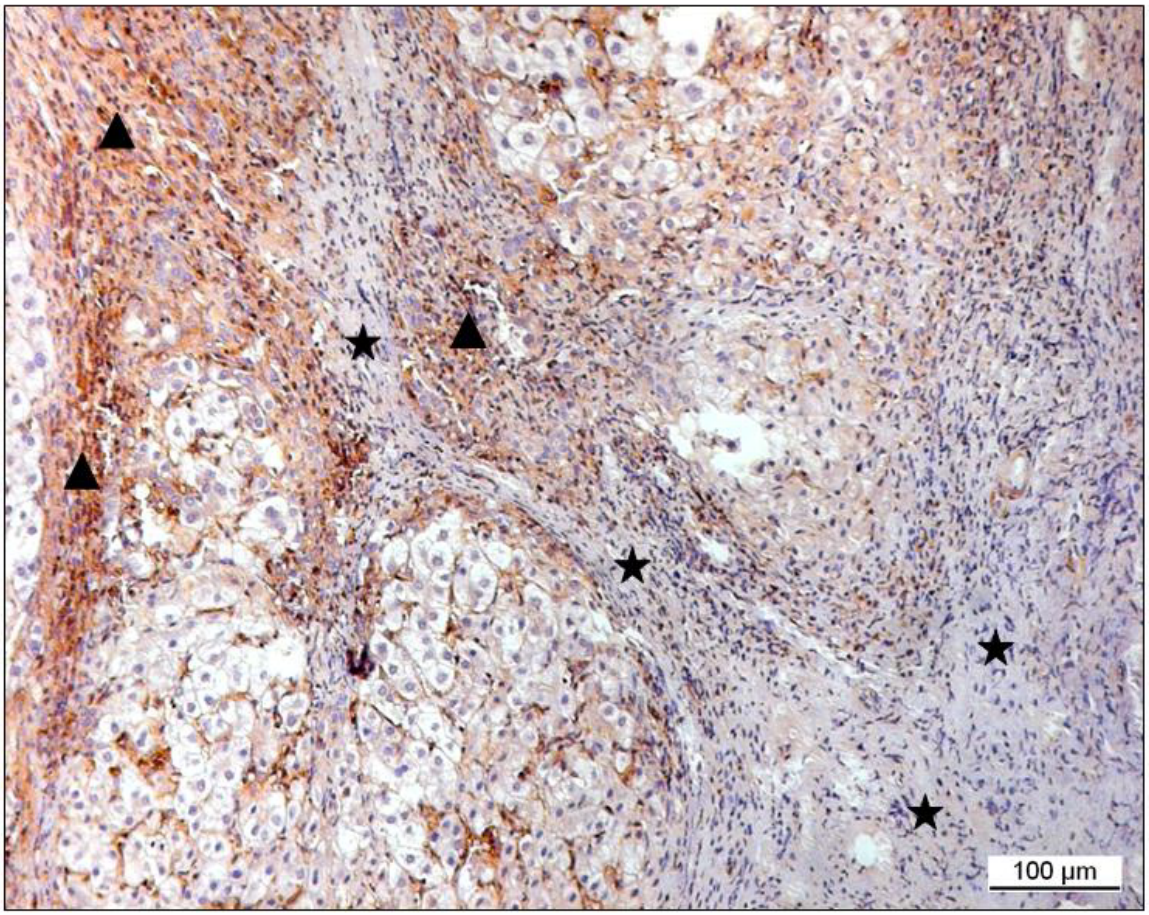


Figure 24. DbpA protein expression in hepatic fibrotic tissue. By immunohistochemisty DbpA was detected in cirrhosis samples, its protein level was found to be strongly up-regulated in active fibrotic areas (triangle). However, DbpA positive cells were scarcely detected in scar tissue (star).

3.3.4 DbpB/YB-1 expression correlates with liver regeneraton and remodeling

As can be seen in Figure 23, reactive ductular cells, hepatic progenitor cells and degenerative hepatocytes, all involved in hepatic regeneration of seriously damaged tissue express DbpB/YB-1. Closer inspection of the DbpB/YB-1staining pattern in patients with HBV-associated cirrhosis revealed that immunopositive cells were predominantly found in areas of tissue regeneration and nodules from formation (triangles in Figure 25). In contrast, DbpB/YB-1 protein was not detected in hepatic tissue without regeneration (stars in Figure 25).

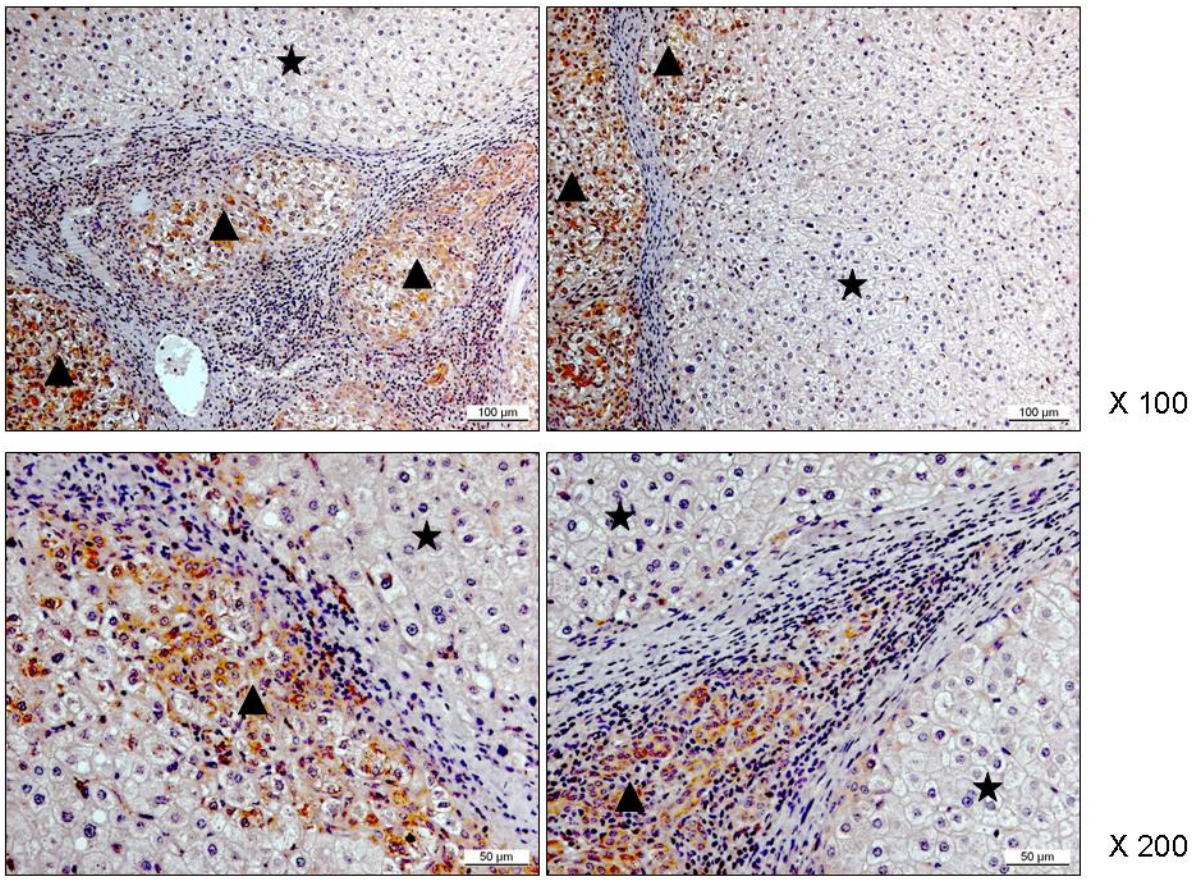


Figure 25. DbpB/YB-1 expression in liver regeneration and tissue remodeling. DbpB/YB-1 IHC staining in patient with HBV infection and associated cirrhosis shows that DbpB/YB-1 is mainly expressed of cells forming regenerative nodules and areas where hepatic tissue architecture is remodeling (triangle), but not in the healthy-appearing tissues (star).

3.3.5 TGF-β and IL-13 up-regulate DbpA expression in HSCs

Transforming growth factor-β (TGF-β) is considered to be a major factor contributing to liver fibrosis (Massague and Chen 2000) by stimulation of collagen synthesis in hepatic stellate cells (HSCs), the major collagen- synthesising cells in the liver. IL-13 also participates in the progress of liver fibrosis as a pro-fibrogenic cytokine, as bystander or cofactor acting together with TGF-β (Wynn 2003; Liu, Meyer et al. 2011). Given that the DbpA expression closely correlates with the early phases of liver fibrogenesis, we stimulate isolated rat primary HSCs with TGF-β and IL-13 *in vitro*. Both cytokines strongly induced DbpA protein expression, as shown in Figure 26. This indicates that DbpA expression is linked to profibrogenic TGF-β and IL-13 activites.

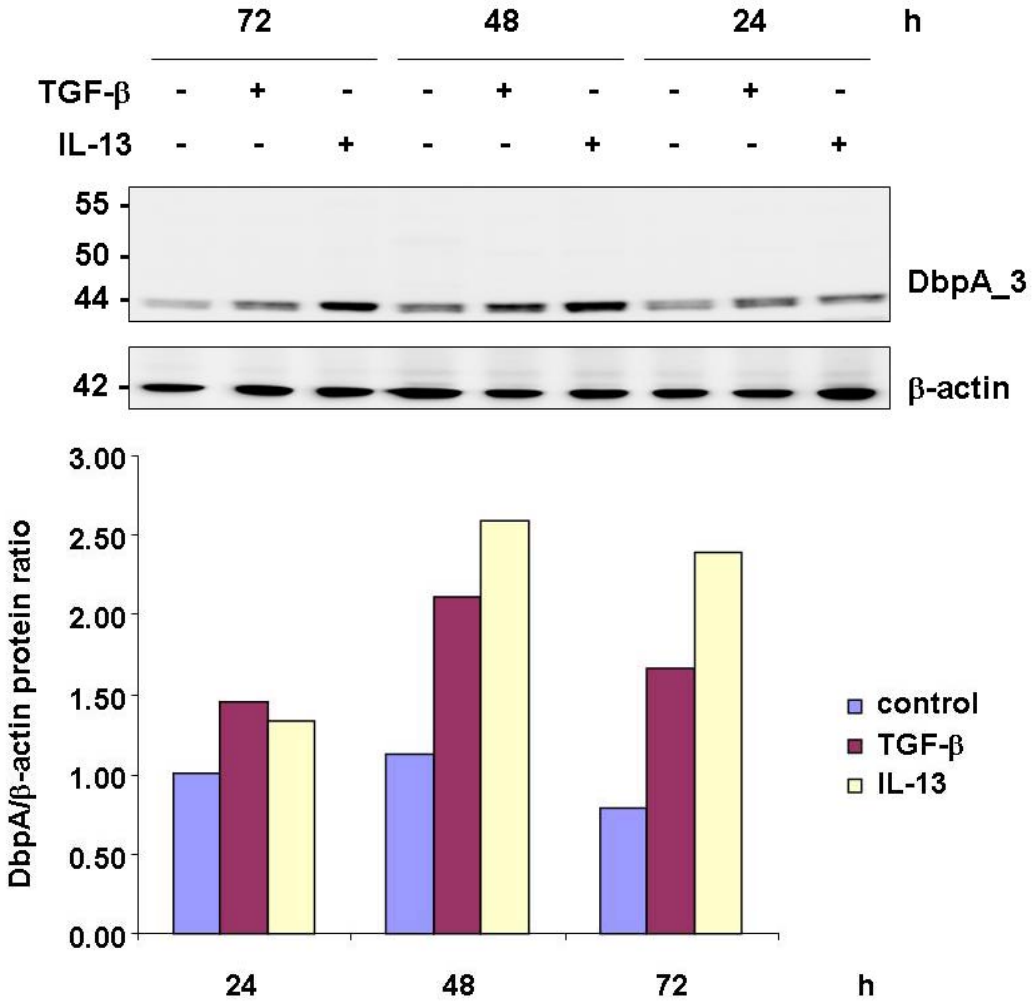


Figure 26. TGF-β and IL-13 stimulate DbpA protein expression in HSCs. Stimulation of isolated rat primary HSCs with TGF-β (5 ng/ml), IL-13 (50 ng/ml) or vehicle for 24, 48 or 72 hours shows up-regulated DbpA protein by expression Western-blot. Actin level was taken as loading control.

3.3.6 TGF-β down-regulates DbpB/YB-1 expression in hepatic epithelial cells

As up-regulated DbpB/YB-1 is present in hepatic epithelial cells, i.e. hepatocytes, bile ductular cells, and hepatic progenitor cells, we stimulated these cells with the major liver fibrogenic factor TGF-β *in vitro*, and checked DbpB/YB-1 protein expression. As shown in Figure 27, DbpB/YB-1 protein expression is suppressed by TGF-β stimulation in all these three cell-types.

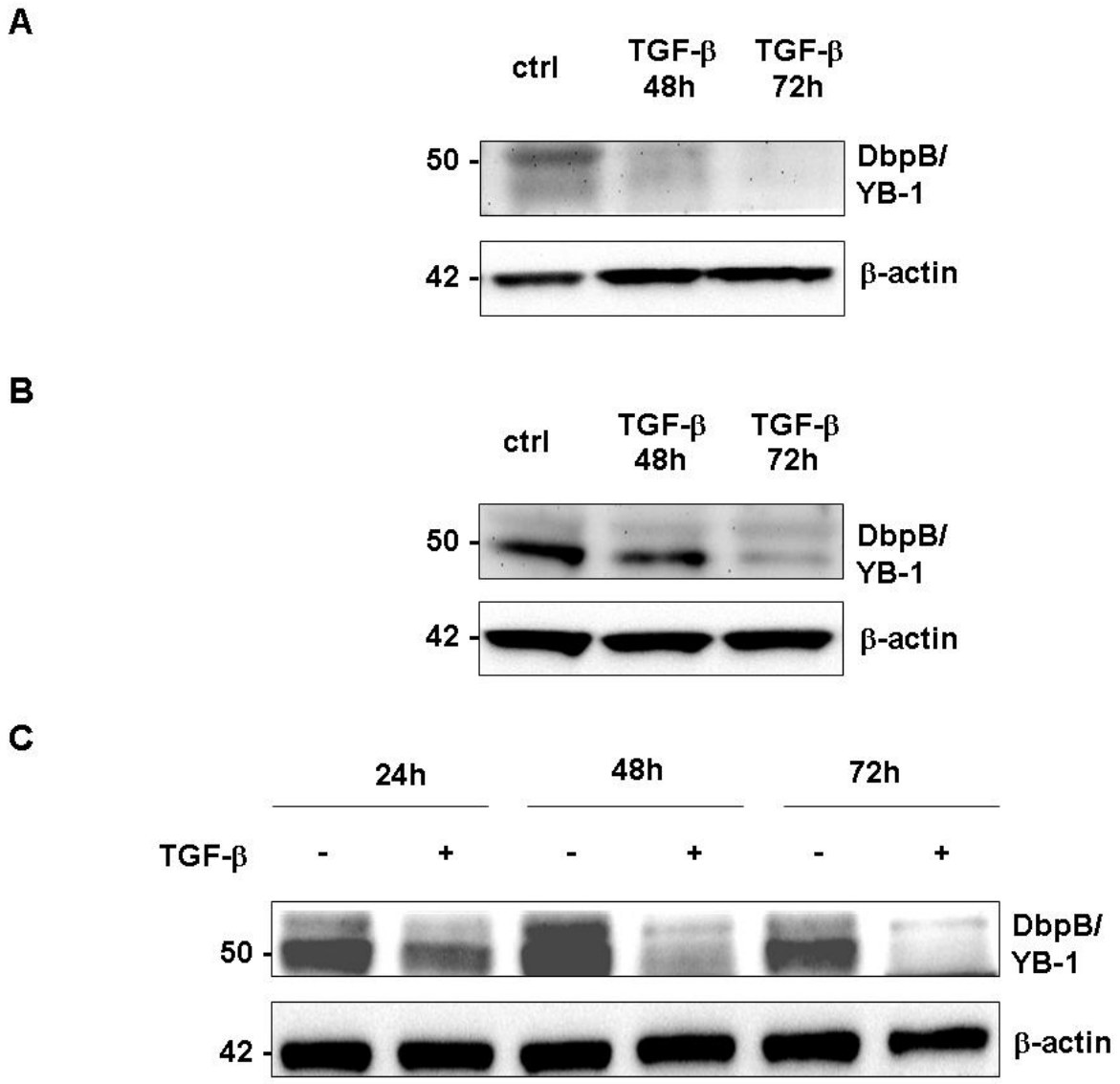


Figure 27. TGF-β suppresses DbpB/YB-1 expression in hepatic epithelial cells. Western-blot analysis of DbpB/YB-1 protein expression in cell lysates from TGF-β (5 ng/ml) stimulated rat primary hepatocytes (A), human cholangiocyte cell line MMNK-1 (B) and mouse liver progenitor cells (C).

3.3.7 DbpB/YB-1 protein expression negatively correlates with EMT

Besides the stimulatory effects on collagen production in HSCs, TGF-β is also considered to be an inducer of epithelial to mesenchymal transition (EMT) in hepatic epithelial cells and plays important roles in liver fibrogenesis, carcinogenesis and tumor metastasis formation. In sequential tissue slices we observed an inverse correlation between DbpB/YB-1 protein expression and number of cells having completed EMT, as assessed by staining for mesenchymal cell-specific marker proteins Snail and S100A4 (Figure 28). *In vitro* incubation of hepatic progenitor cells with TGF-β induced EMT as assessed by reduced E-cadherin level and increased Twist1 expression. At the same time DbpB/YB-1 protein expression was suppressed (Figure 29).

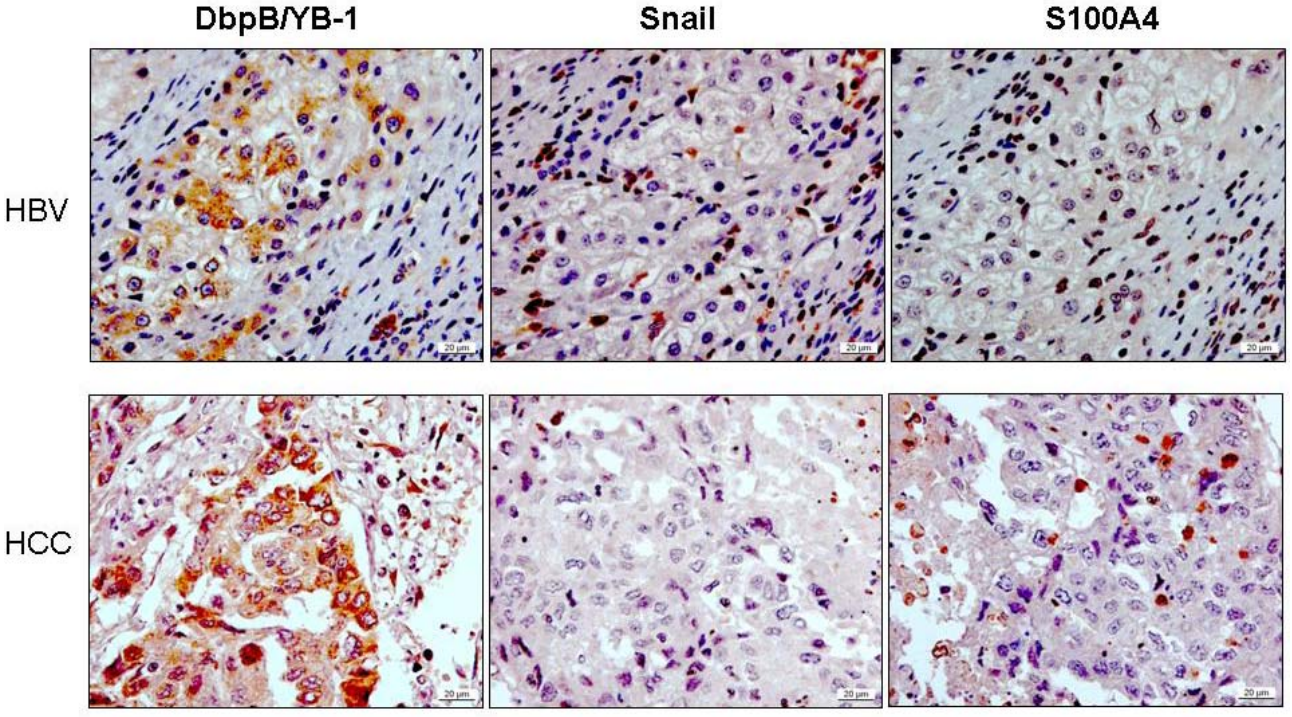


Figure 28. DbpB/YB-1 negatively correlates with EMT *in vivo*. Immunohistochemistry of HBV infected cirrhosis liver as well as HCC tissue. DbpB/YB-1 protein expression does not co-localize with EMT marker proteins Snail and S100A4.

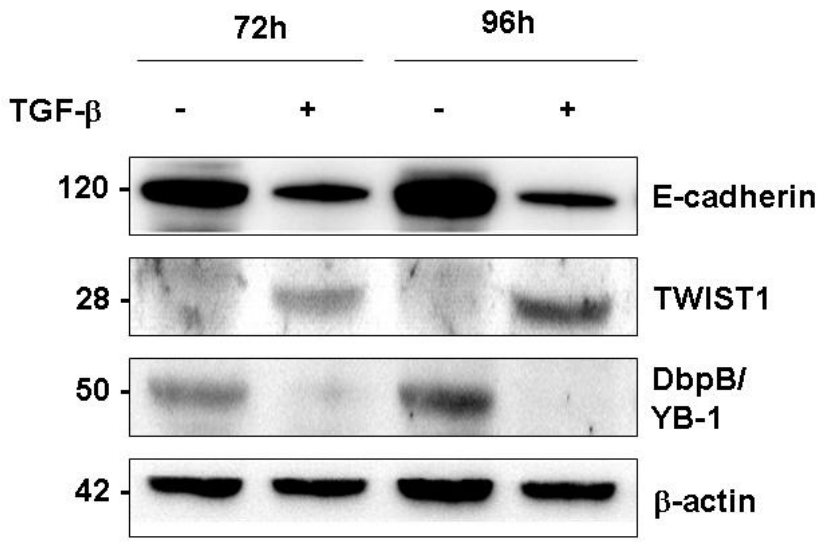


Figure 29. DbpB/YB-1 protein levels negatively correlate with EMT *in vitro*. Western blot analysis of E-cadherin, Twist1 and DbpB/YB-1 protein was performed with cell extracts from liver progenitor cells grown in the absence or presence of TGF-β (5 ng/ml, for 72 or 96 hours). DbpB/YB-1 protein expression was suppressed while EMT marker Twist1 was up-regulated and E-cadherin is down-regulated.

3.3.8 DbpB/YB-1 is up-regulated in liver steatosis

DbpB/YB-1 up-regulation is not restricted to HBV infected fibrotic liver diseases, IHC performed in 3 patients diagnosed with liver steatosis also revealed pronounced YB-1 immunopositivity in all 3 cases (Figure 30). Similar results were obtained with liver tissue from animals that were challenged with alcohol for 4 weeks (Figure 31). Up-regulated DbpB/YB-1 protein expression was localized in hepatic epithelial cells.

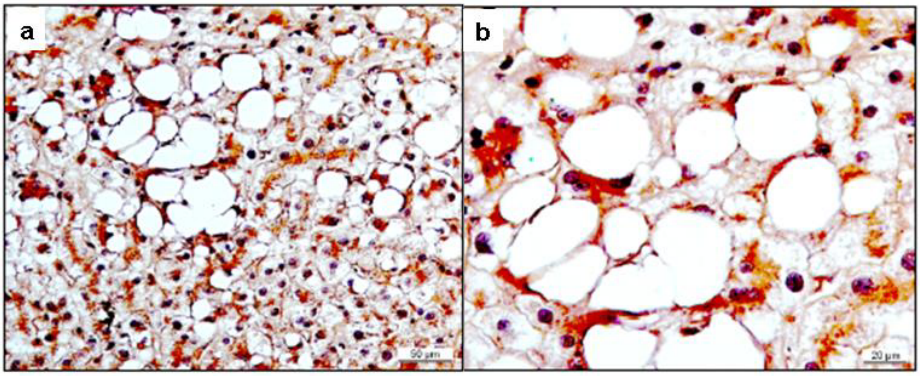


Figure 30. DbpB/YB-1 expression is up-regulated in liver tissue of steatosis patients. IHC staining of DbpB/YB-1 in liver biopsies from patient diagnosed with liver steatosis shown in 100x (a) and 200x (b) magnification.

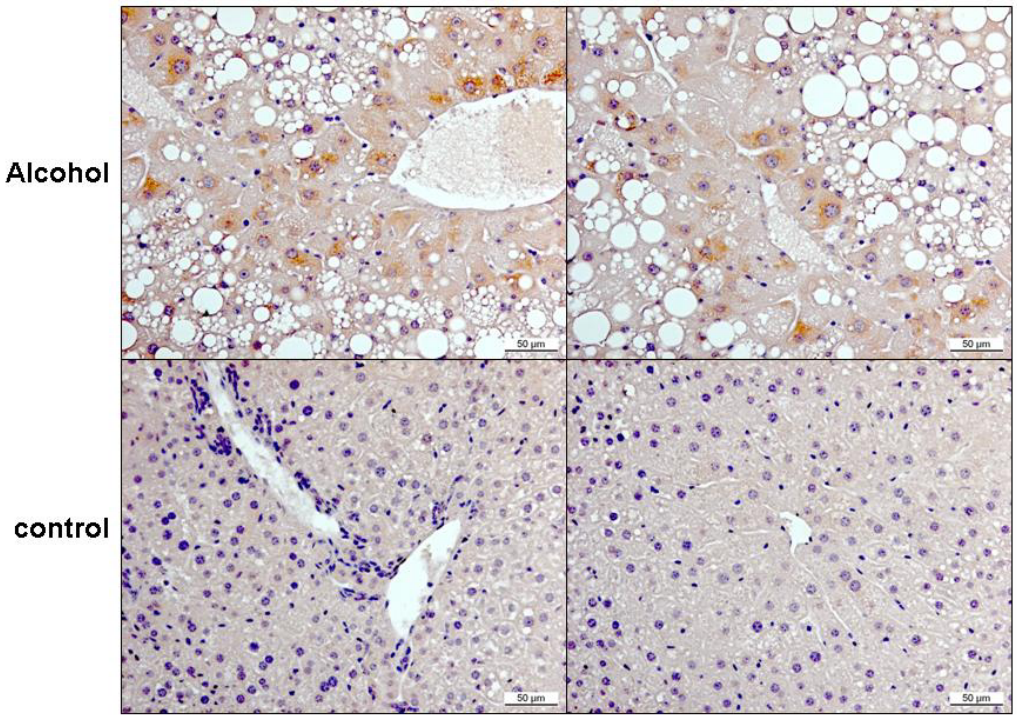


Figure 31. DbpB/YB-1 protein expression is up-regulated in an experimental steatosis model. DbpB/YB-1 was visualized by IHC in liver tissue of mice challenged with alcohol (4 weeks). Diffuse cytoplasmic DbpB/YB-1 staining is detected in hepatocytes of alcohol challenged animals with steatosis, however not in control animals.

3.4 Cloning of DbpA and DbpB/YB-1 gene promoter constructs

The expression of DbpA and DbpB/YB-1 proteins are strongly regulated in inflammatory kidney and liver diseases. In order to unravel transcriptional regulatory mechanisms of human CSPs, different lengths of promoter regions from the human DbpA and DbpB/YB-1 genes were cloned into a luciferase reporter plasmid. According to the homologies of DbpA and DbpB/YB-1 promoter regions between human, mouse and rat, serial 5′-deleted promoter constructs were designed, as shown in Figure 32. Using primers pairs (Table 7) target DNA fragments are amplified by PCR (shown in Figure 33) and cloned into luciferase reporter plasmid pGL4.10.

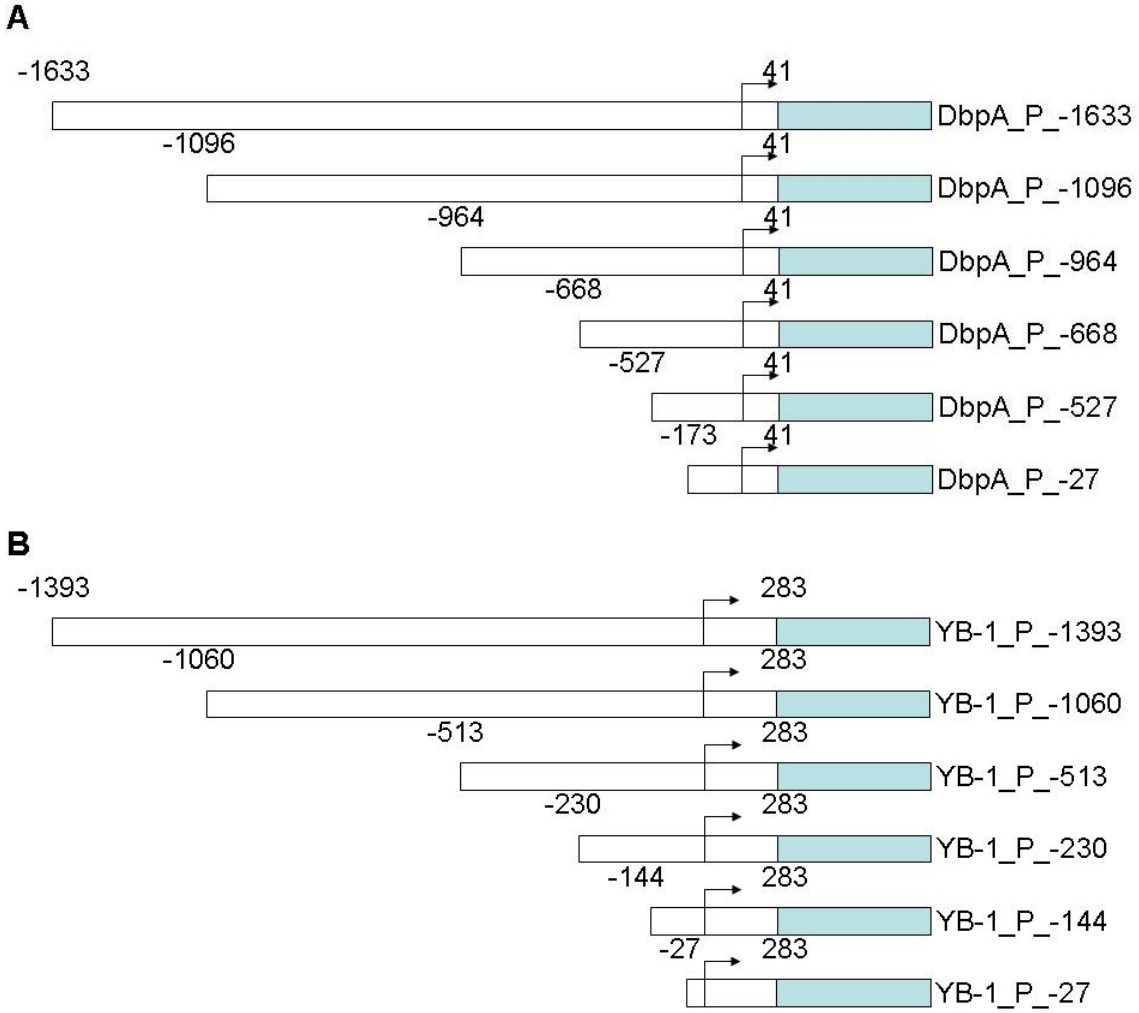


Figure 32. Schematic representation of DbpA and DbpB/YB-1 promoter constructs. Six promoter regions beginning at -1633, -1096, -964, -668, -527, and -173 bp upstream of the translation initiation site of the DbpA gene (A) as well as six regions beginning at -1396, -1060, -513, -230, -144, and -27 bp upstream of the DbpB/YB-1 gene and spanning to bp 284 (B) were cloned into a luciferase reporter plasmid pGL4.10.

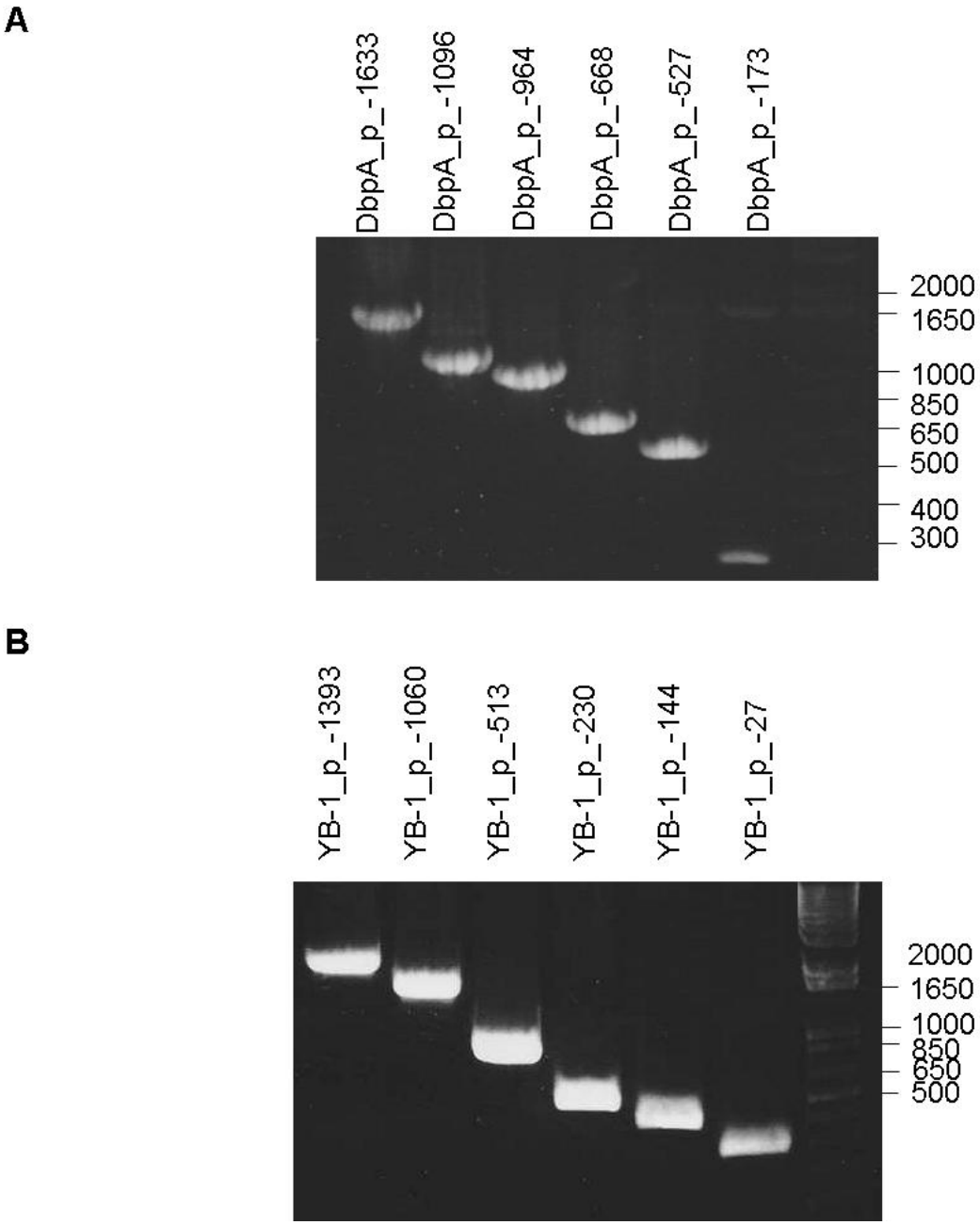


Figure 33. DbpA and DbpB/YB-1 promoter constructs. By means of PCR the target DNA fragments of DbpA and DbpB/YB-1 promoters were amplified. Results were confirmed by electrophoresis.

4 Discussion

The present work sets out to analyze cold shock protein expression and regulation in inflammatory kidney and liver diseases. As major finding we observe a strong up-regulation of cold shock protein DbpA in mesangioproliferative nephritis in a pattern that resembles the one observed with DbpB/YB-1 (van Roeyen, Eitner et al. 2005; Frye, Halfter et al. 2009). There is a strong up-regulation of CSP within the mesangial compartment as down-stream targets of PDGF-B signaling. It indicates that DbpA and DbpB/YB-1 may play similar roles in mesangioproliferative GN. On the other hand, both DbpA and DbpB/YB-1 expression were found to be up-regulated in fibrotic liver tissues, however with different expression patterns. This may indicate different roles that CSPs play in the course of inflammatory liver diseases.

4.1 PDGF-BB as inducer of CSP expression in mesangial cells

PDGF-B is one of the most potent mitogens for mesangial cells and plays a crucial role in mesangioproliferative diseases (Floege, Ostendorf et al. 1999; Floege, Eitner et al. 2008). PDGF-B activates down-stream signaling, including the MAPK pathway. Inhibition of the MAPK/ERK pathway by U0126 leads to significant disease amelioration. Blockade of either PDGF-B by aptamers or MAPK/ERK signaling by U0126 is accompanied by down-regulated DbpA expression. Similar effects have been reported for DbpB/YB-1 (van Roeyen, Eitner et al. 2005).

The MAPK/ERK pathway activates various proto-oncogenes, e.g. c-fos, c-myc and c-jun (Choudhury, Karamitsos et al. 1997; Choudhury, Ghosh-Choudhury et al. 1998), that may regulate human CSP expression *via* the following mechanisms: On the one hand it has been reported that c-myc directly stimulates both DbpA and DbpB/YB-1 transcription (Li, Van Calcar et al. 2003). On the other hand, c-fos dimerizes with c-jun to form AP-1 complexes that may release the E2F transcription factors *via* activation of Cyclin D1/Cdk4 and phosphorylating of pRb (Shen, Uray et al. 2008). Among the various E2F transcription factors E2F1 has been reported to be crucial for mesangial cell proliferation (Inoshita, Terada et al. 1999) and of notably E2F1 is a direct

transcriptional inducer of the DbpA gene (Arakawa, Kajino et al. 2004). The scheme in Figure 36 shows signaling events that may regulate human CSP expression following PDGF-BB stimulation.

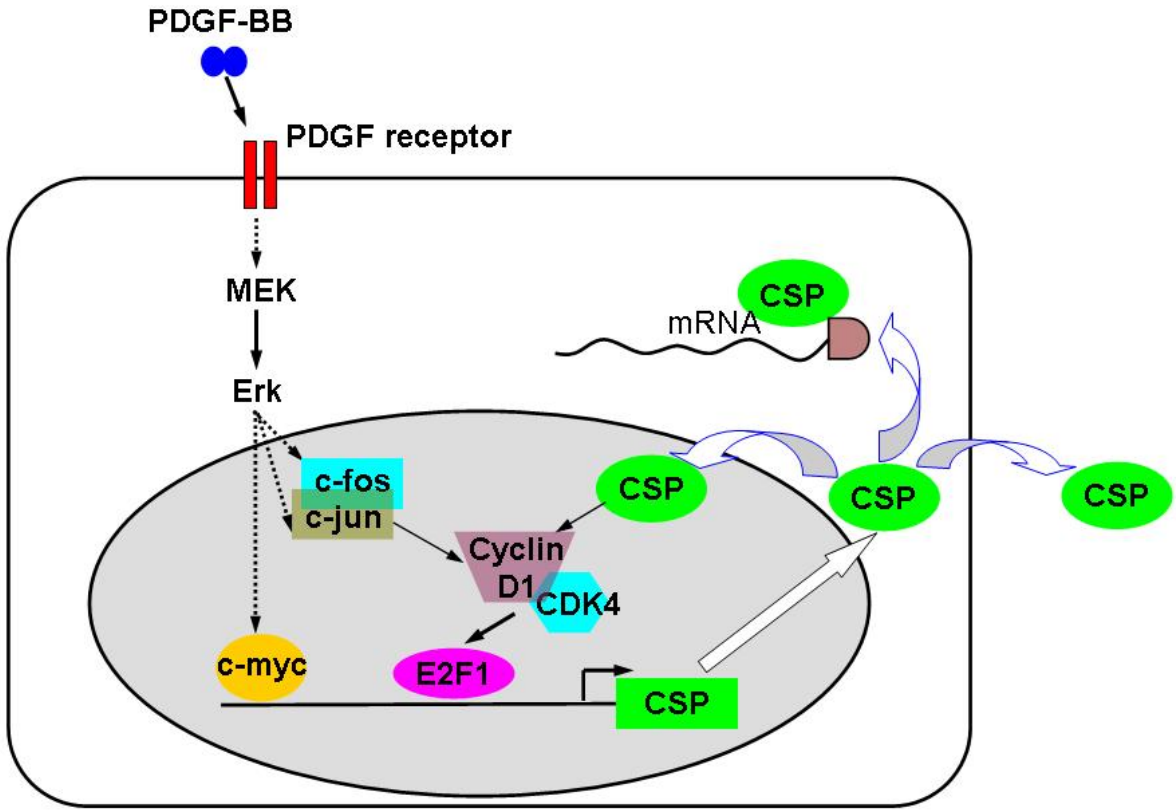


Figure 36. Scheme of signaling events regulating cold shock protein (CSP) expression following PDGF-BB stimulation. PDGF-BB signals *via* binding to its receptor that dimerizes and activates MEK and extracellular receptor kinases (ERK). A known target molecule involves the proto-oncogene c-myc that may up-regulate both DbpA and DbpB/YB-1 transcription. Other target molecules include c-fos and c-jun that may activate E2F1 *via* the Cyclin D1/CDK4 complex. E2F1 may enhance DbpA transcription by binding to its promoter sequences. CSP may function as transcription factors in the nucleus, as translation factors in the cytoplasm, as well as mitogens or chemoattractants in the extracellular compartment.

4.2 Possible roles of DbpA in mesangioproliferative diseases

Mesangial cell proliferation is the key pathological feature in mesangioproliferative diseases. DbpB/YB-1 has been previously reported to promote mesangial cell (MC) proliferation as a mediator of PDGF-B signaling (van Roeyen, Eitner et al. 2005). Considering the similar expression and regulation patterns of both CSP in mesangioproliferative diseases, DbpA may play similar roles as DbpB/YB-1. A pro-mitogenic role of DbpA has been reported in several malignant diseases. The expression of DbpA is up-regulated in hepatocellular carcinoma (HCC) (Hayashi, Kajino et al. 2002; Arakawa, Kajino et al. 2004; Yasen, Kajino et al. 2005) and gastric cancer (Wang, Zheng et al. 2009). Over-expressed DbpA is associated with advanced stages of hepatocellular carcinoma and nuclear translocation of DbpA may indicate poor prognosis (Yasen, Kajino et al. 2005). In chronic myeloid leukemia (CML) phosphorylation of DbpA at Ser134 is reported to be crucial for Bcr-Abl mediated CML-associated cell proliferation and leukemogenesis, though increased DbpA expression is not mentioned (Sears, Luong et al. 2010). Studies also show that DbpA is an important factor in breast cancer (Sharp, Mailer et al. 2008), oveal melanoma (Jayagopal, Yang et al.) and colorectal cancinoma (Buchert, Papin et al. 2010; Dhawan, Ahmad et al. 2011). DbpA-dependent cell proliferation is not only observed in cancer cells. Recent studies show that DbpA expressing and nuclear accumulation is crucial for tubular cell proliferation in the kidney (Lima, Parreira et al. 2010). Several regulation mechanisms may be involved in DbpA-mediated cell proliferation. For instance DbpA is directly regulated by proliferation-associated transcription factors, e.g. c-myc (Li, Van Calcar et al. 2003) and E2F1 (Arakawa, Kajino et al. 2004). c-myc is one of the most studied cancer genes. E2F1 is known to be important for cell proliferation, for the transition of cells from G1 to S phase (Johnson, Schwarz et al. 1993; Cam and Dynlacht 2003). DbpA interacts with guanine nucleotide exchange factor (GEF-H1) and may mediate Rho-regulated G1-S transition in the cell cycle (Nie, Aijaz et al. 2009). By acting as a transcription factor, DbpA may directly bind to the promoter region of several proliferation-associated genes, including epidermal growth factor receptor (EGFR) (Sakura, Maekawa et al. 1988), thymidine kinase (Kim, Lau et al. 1997), cyclin D1 and proliferating cell nuclear antigen (PCNA) (Sourisseau, Georgiadis et al. 2006). Thereby it may enhance target gene expression and promote cell proliferation. On the other hand, DbpA may directly interact with Cdk4 and inhibit the formation of Cdk4/cyclin D1

complexes that inhibit G1/S transition of the cell cycle (Moorthamer, Zumstein-Mecker et al. 1999). DbpA transcriptionally up-regulates cyclin D1 expression, but interestingly inhibits cyclin D1 activity *via* binding to Cdk4. This may indicate the existence of a DbpA mediated auto-regulatory loop that controls cell proliferation (Figure 36). It is possible that when the balance of this loop is disrupted by ectopic over-expression or modification of DbpA abnormal proliferation may occur.

In addition to the effects on cell proliferation, DbpA may also play important roles in cell-cell communication during the course of mesangioproliferative diseases. Tight junctions (TJs) and gap junctions (GJs) are of great importance for cell-cell communication of neighboring cells. Numerous studies revealed that DbpA acts as a regulator of TJ- and GJ-associated activities. DbpA has been identified as interaction partner of several TJ-assosiated proteins, e.g. ZO-1 (Balda and Matter 2000), symplekin (Kavanagh, Buchert et al. 2006) and GEF-H1 (Nie, Aijaz et al. 2009). Although without direct protein interactions several other TJ-associated proteins have been reported to be involved in the regulation of DbpA activities at TJ, e.g. heat-shock protein Apg-2 (Tsapara, Matter et al. 2006), B-cell lymphoma 2 (Bcl-2) (Li, Backer et al. 2003), Ras-related protein Ral-A (Frankel, Aronheim et al. 2005) and blood vessel epicardial substance (Bves) (Russ, Pino et al. 2011). Apart from tight junctions DbpA has been reported to localize at oligodendrocyte and astrocyte gap junctions in the mouse brain (Penes, Li et al. 2005) and was found to be associated with oligodendrocytic connexin Cx47 and Cx32 as well as with astrocytic Cx43. In Cx47 knock-out mice DbpA was absent in oligodendrocytes (Li, Penes et al. 2008). Although mesangial cells do not form TJs one of the striking features of MCs is that these cells possess an extremely high density of GJs (Barajas, Liu et al. 1994). Yao and colleagues reported that disruption of GJ intercellular communication in MCs may be involved in PDGF-B mediated MC proliferation and dedifferentiation (Yao, Morioka et al. 2000; Yao, Kitamura et al. 2006). DbpA may play a role in this GJ-associated MC proliferation and dedifferentiation. A similar role of DbpA has been identified in TJ-associated tubular cell proliferation and dedifferentiation in the kidney (Pannequin, Delaunay et al. 2007; Georgiadis, Tschernutter et al. 2010; Lima, Parreira et al. 2010). Furthermore, we detected a PDGF-B-dependent secretion of DbpA by rat mesangial cells. Similar extracellular release of DbpB/YB-1 protein has been reported. DbpB/YB-1 extracellularly regulates cell-cell communication as a mitogen (Frye, Halfter et al. 2009) as well as a ligand of Notch-3 receptor

signaling (Rauen, Raffetseder et al. 2009). Considering the similarites between DbpA and DbpB/YB-1 in mesangioproliferative GN, secreted DbpA may also act promitogenic, however this has not been addressed in the present study.

Recently experimental evidence suggests a pathophysiologic role of endoplasmic reticulum (ER) stress in mesangioproliferative glomerulonephritis (Inagi, Kumagai et al. 2008). When mammalian cells were subjected to stress that target the endoplasmic reticulum, cells activate a defense mechanism referred to as unfolded protein response (UPR), which is evolutionarily conserved from yeast to humans (Foti, Welihinda et al. 1999). The major cellular respond to the UPR is an ER chaperone, Grp78 (Reddy, Mao et al. 2003). Grp78 not only binds to unfolded proteins but also regulates the activation of ER stress signal transducers that protect stressed cells from apoptosis (Bertolotti, Zhang et al. 2000). Following induction of anti-Thy1.1 nephritis the expression of Grp78 protein in mesangial cells is markedly increased in a time-dependent manner (Inagi, Kumagai et al. 2008). DbpA has been identified as a transcriptional regulator that directly binds to the core promoter region of the Grp78 gene at the stress-inducible change region (SICR) (Li, Hsiung et al. 1997). This assigns DbpA a role in the stress response of mesangial cells in mesangioproliferative GN.

4.3 Differential expression and regulation of CSPs in inflammatory liver diseases

This is the first report that shows human CSPs are up-regulated in inflammatory liver diseases, e.g. HBV associated cirrhosis and steatosis of the liver. However expression patterns of DbpA and DbpB/YB-1 differ from each other. DbpA over-expressing cells are identified as activated macrophages and activated hepatic stellate cells. In contrast, DbpB/YB-1 immunopositive cells are mainly hepatic epithelial cells, i.e. hepatocytes, bile ductular cells and hepatic progenitor cells. DbpB/YB-1 staining results were confirmed by different antibodies targeting both the N- and C-terminus. For DbpA staining, an antibody specific for the N-terminus was used. In a study from Yasen and collegues using an antibody against an epitope within the C-terminus for IHC in HCC tissues yielded similar results (Yasen, Kajino et al. 2005). Concerning the different expression patterns of CSP in inflammatory liver diseases different functions may be postulated.

4.3.1 DbpA expression correlates with liver fibrogenesis

It has been previously reported that DbpB/YB-1 expression may antagonize TGF-β signaling events in activated hepatic stellate cells (HSCs). Thereby it has the propensity to protect the liver from fibrosis (Dooley, Said et al. 2006). In fibrotic liver tissue DbpB/YB-1 protein expression was hardly detected in HSC by immunohistochemistry. It seems that in HSC of fibrotic liver tissue DbpB/YB-1 expression as well as its protective effect is suppressed by certain mechanisms, thereby promoting fibrogenesis. In contrast, DbpA expression positively correlates with liver fibrogenesis. Its protein level is up-regulated in activated HSCs in the early phases of fibrogenesis *in vivo*. This up-regulation was recapitulated *in vitro* by stimulation of isolated primary HSCs with pro-fibrogenic cytokines TGF-β and IL-13. These results indicate that DbpA may be a factor promoting liver fibrogenesis, in contrast to DbpB/YB-1. In addition, epithelial cells undergoing EMT have been considered as another important source of fibrotic cells. DbpB/YB-1 protein expression negatively correlates with EMT in hepatic epithelial cells, which may also indicate a protective role of DbpB/YB-1 in liver fibrogenesis.

TGF-β, mediated by specific Smad signaling, induces the expression of genes involved in extracellular matrix synthesis and plays a major role in liver fibrogenesis (Massague and Chen 2000). Besides the classic Smad signaling, it is known that TGF-β also activates ERK kinase signaling (Lee, Pardoux et al. 2007) and this may contribute to the up-regulation of DbpA expression in HSCs by TGF-β. Most of the biological effects of IL-13 are mediated by the IL-4 receptor and JAK/STAT signaling, particularly STAT6 (Jiang, Harris et al. 2000). However, IL-13 may also bind to the IL-13α(2) receptor and activate its down-stream signaling events, e.g. transcription factor AP-1 (Fichtner-Feigl, Strober et al. 2006), which may up-regulate E2F1 expression and thereby promote DbpA expression (Arakawa, Kajino et al. 2004; Shen, Uray et al. 2008).

4.3.2 DbpB/YB-1 expression in liver regeneration and re-modeling

The presented preliminary data demonstrate that there is a tight regulation of DbpB/YB-1 protein expression in liver cells when they are injured by different challenges, e.g. alcohol or hepatitis B viruses. The pathological sequelae of such challenges are denoted liver steatosis, fibrosis and cirrhosis, which are major causes of malignant liver diseases. The expression pattern indicates that mainly hepatic epithelial cells, but rarely hepatic stellate cells (HSCs), are immunopositive. DbpB/YB-1 expressing cells, intermediate hepatocyte-like cells, ductular reaction and hepatic progenitor cells, are closely related to liver regeneration and re-modeling. It is observed that DbpB/YB-1 expression is elevated in regenerating tissues that forms nodules. In a report from Grant et al. several lines of evidence link DbpB/YB-1 expression in the liver with DNA synthesis and cell proliferation (Grant and Deeley 1993).

DbpB/YB-1 protein levels were strongly reduced following TGF-β incubation in all three hepatic epithelial cells *in vitro*. However, considering the major functions of TGF-β in hepatic epithelial cells (e.g. hepatocytes), that is the suppression of cell proliferation and induction of cell apoptosis and transformation (Massague and Chen 2000) it seems contradictory to the known pro-mitogenic effects of DbpB/YB-1 in these cell types. This gives rise to the hypothesis that TGF-β and DbpB/YB-1 are negatively interrelated. On the other hand, it has been reported that elevated DbpB/YB-1 expression in breast cancer cells induces EMT accompanied by enhanced metastatic potential and reduced proliferation rates (Evdokimova, Tognon et al. 2009). These findings indicate that DbpB/YB-1 may play differential roles in tumor and non-tumor cells. Other mechanisms must exist that up-regulate DbpB/YB-1 expression, apart from TGF-β signaling.

5 Summary

Cold shock proteins have attracted considerable attention in the context of carcinogenesis given their potent promitogenic roles. The participation of cold shock protein DbpB/YB-1 in the course of inflammatory diseases has only recently been described and visualized in experimental models of mesangioproliferative glomerular disease and liver fibrotic diseases. The fundamental question addressed here is whether another prominent member of the cold shock protein family, DbpA, is also regulated in mesangioproliferative disease, although it has been primarily described as protein with activities in epithelial tubular cell proliferation and differentiation. Whereas in healthy kidney tissue DbpA protein was predominantly detected in vascular small muscle cells a profound up-regulation of DbpA protein expression within the mesangial compartment was evidenced in human kidney biopsies diagnosed with mesangioproliferative IgA or lupus nephritis. Such a mesangial up-regulation was recapitulated in an experimental model, the anti-Thy1.1 nephritis. Given the pivotal role of PDGF-B signaling in this disease two interventions were performed, that is the application of PDGF-B neutralizing aptamers and MAPK/Erk signaling inhibitor U0126. Both interventions resulted in markedly decreased DbpA expression. Conversely, continuous PDGF-B infusion induced DbpA expression predominantly within the mesangial compartment in rats. *In vitro* studies of human and rat mesangial cells confirmed the PDGF-B stimulatory effect at transcript and protein levels. Notably, DbpA protein isoforms exhibit sizes of 44, 50 and 55 kDa, with predominance of the 44 kDa isoform. Conditioned cell culture medium of mesangial cells also revealed that PDGF-B induced secretion of DbpA (p50) in a time- and concentration-dependent manner occurs. Taken together, cold shock protein DbpA represents a novel target of PDGF-B signaling in mesangioproliferative glomerular disease.

In inflammatory liver diseases we observed both DbpA and DbpB/YB-1 up-regulation, however with different expression patterns. Whereas DbpA over-expressing cells were identified as activated macrophages and hepatic stellate cells, DbpB/YB-1 immunopositive cells were mainly hepatic epithelial cells. This may indicate differential roles that CSP fulfill in inflammatory liver diseases: DbpA may be functional in the early phases of fibrogenesis while DbpB/YB-1 may be involved in liver regeneration and re-modeling.

6 Reference

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Own publications

Zhu C, van Roeyen C, Schreiter A, Stolze S, Ostendorf T and Mertens PR. Up-regulation of cold shock protein DbpA in mesangioproliferative glomerulonephritis is orchestrated by PDGF-B signaling. [in submission]

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Erklärung zur Datenaufbewahrung

Hiermit erkläre ich, dass die dieser Dissertation zu Grunde liegenden Originaldaten

in der **Klinik für Nieren- und Hochdruckkrankheiten, Diabetologie und Endokrinologie der Otto-von-Guericke Universität Magdeburg** hinterlegt sind.